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Identification of olfactory receptor neurons in two Species of scarab beetles: a comparative study by means of single sensillum recording

HAMIDA KHBAISH

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Identification of olfactory receptor neurons in two species of scarab beetles: a comparative study by means of single sensillum recording

Hamida Khbaish, for information, hamida.khbaish@ltj.slu.se

Supervisor: Mattias Larsson, Swedish University of Agricultural sciences, Plant Protection Biology

Assistant supervisors: Ylva Hillbur, Swedish University of Agricultural Sciences, Plant Protection Biology
Jonas Bengtsson, Swedish University of Agricultural Sciences, Plant Protection Biology

Examiner: Peter Anderson, Swedish University of Agricultural sciences, Plant Protection Biology

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3) group of *Pachnoda marginata* beetles eating banana fruit (Photo: Hamida Khbaish)

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Swedish University of Agricultural Sciences
Faculty of Landscape Planning, Horticulture and Agricultural Science
Department of Plant Protection Biology
Division of Insect Chemical Ecology

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Abstract

Few studies have addressed how olfactory systems may be adapted to different odour environments. I have performed the largest study to date, comparing olfactory receptor neurons in the two congeneric species of scarab beetle, *P. marginata* and *P. interrupta.* Both species are fruit- and flower-eaters but geographically separated (equatorial versus sub-Saharan Africa, resp.). They have similar lamellate antennae covered mostly with numerous olfactory sensilla placodea (plate sensilla) and a lesser number of other types, mainly sensilla coeloconica (grooved peg), and smooth peg sensilla of unknown function. By means of single sensillum recordings with tungsten microelectrodes I screened a great number of olfactory sensilla with a large array of odorants. I compared 456 sensilla placodea, containing 212 responding cells, over the whole antennae in both species. The olfactory systems of these two species displayed an amazing degree of conservation, with 20 identified olfactory neuron classes, all except two of which were found in both species with no detectable difference in response profiles. The exceptions were two olfactory receptor neuron classes: methyl benzoate and gamma-nonalactone, which were only found in *Pachnoda marginata*. In general, the two species showed an almost total overlap in their receptor neuron assemblies. One aim of the study was also to test whether the arrangement of neurons within sensilla was conserved between the species, but this could not be tested as very few combinations of characterized neurons were encountered during the study.

Key words: olfaction, electrophysiology, scarab beetles, *Pachnoda marginata*, *Pachnoda interrupta*, single sensillum recording, olfactory receptor neurons

Author's address: Hamida Khbaish, Department of Plant Protection Biology, Division of Chemical Ecology, SLU, P.O. Box.102, SE-230 53 Alnarp, Sweden. E-mail, hamida.khbaish@ltj.slu.se

1 Introduction

Most insects are highly dependent on their olfactory system to detect and recognize suitable food sources, mating partners, host plants for oviposition, and to escape from predators and other natural enemies (Visser, 1986).
Olfactory systems of different insect species are equipped with a multitude of olfactory receptor neurons enabling them to detect and perceive different odors from their surrounding environment (de Bruyne et al., 2001; Hansson, 1995). Early investigations of extracellular recording techniques (Schneider and Boeckh, 1962) from individual olfactory receptor neurons allowed identification of the functional specialization of these olfactory receptor neurons in many insect species (Hansson, 2002; Mustaparta, 2002). Olfactory receptor neurons are classified according to their individual response profiles into a spectrum of selectivity, ranging from specialist olfactory receptor neurons which respond to a narrow spectrum of related compounds with high sensitivity and selectivity, such as a pheromone component, and the generalist olfactory receptor neurons which respond to a broad range of odorants (Anderson et al., 1995; Schneider et al., 1964; Shields and Hildebrand, 2001; Visser, 1986). These neurons are important for the insects and play role in discriminating their hosts from the surrounding environment (de Bruyne et al., 1999). But this classification does not always apply to all insect species, however: the pine weevil *Hylobius abietis*, the Douglas fir beetle *Dendroctonus pseudotsugae* and *Dendroctonus frontalis* beetles have pheromone receptor cells that respond to stimulation with host-tree odors (Dickens and Payne, 1977; Dickens et al., 1984; Mustaparta, 1975; Visser, 1986). Furthermore, several studies have demonstrated that plant-odour detecting neurons show high sensitivity to only one or few compounds (Anderson et al., 1995; Anderson et al., 1993; Todd and Baker, 1993).
Many insects are specialized to a particular plant, as well as parasitoids to their host; other insects are generalists feeding on a wide variety of resources, such as the honeybees which feed on nectar and pollen of many flowering plants (Galizia and Szyszka, 2008). On the other hand scarab beetles of the superfamily Scarabaeoidea display a wide range of feeding habits: some are coprophagous, feeding only on dung, while most scarab groups are highly polyphagous herbivores feeding on a wide variety of plants, fruits and flowers (Larsson et al., 2001; Scholtz and Chown, 1995).
Previous studies of a closely related group of insects, the *Drosophila* subgroup, demonstrated that a *Drosophila melanogaster* sibling species, *D. sechellia* is attracted to Noni (*Morinda citrifolia*) fruit as its only host plant. *M. citrifolia* contains high levels of acids that are toxic for other sibling species (Dekker et al., 2006; Rkha et al., 1991; Stensmyr et al., 2003). In behavioral bioassays, all *Drosophila* subgroup species avoid *Morinda* fruit except *D. sechellia*, which displayed qualitative and quantitative shifts of the olfactory receptors that respond to typical *Morinda* fruit volatiles (Dekker et al., 2006).
Pachnoda species of the sub family Cetoniinae are highly similar in their food preferences as well as in many of their general characters e.g. the morphology of their antennae (Bengtsson et al., manuscript in Bengtsson (2010); (Bengtsson, 2010; Larsson et al., 2001). However at the odour detection level it is still unknown whether the olfactory receptor neurons of their olfactory system are tuned to the same ligands for their discrimination of different host odours.
P. marginata and *P. interrupta* are two closely related species of scarab

beetles (Coleoptera: Scarabaeidae, subfamily: Cetoniinae) that are widely distributed in central and northern sub-Sarahan Africa, respectively (Larsson et al., 2001; Rigout, 1989).

P. interrupta has been recorded as a major pest insect to sorghum in Ethiopia because of their damage to the seeds and flowers. Average yield reduction of 70% has been recorded in severely infested areas (Yitbarek and Hiwot, 2000). Besides sorghum, *P. interrupta* feeds on different plants like sunflowers, henna, cucumber, cassia, roses and the capsule of okra (Grunshaw, 1992; Schmutterer, 1969). On the other hand, *P. marginata* is not a major pest of any crop. The kinds of plants on which it feeds are e.g. ripening guavas and mangoes, mango flowers, garden roses, henna, green cotton bolls and durra heads in which it feeds on the milk-ripe grains (Schmutterer, 1969). In laboratory cultures, both species showed high attraction to banana and apple which were the main food for the adults and larvae.

A lot of research has been performed on scarab beetles, mainly focusing on pest species and how to apply management tools for controlling damage (Larsson et al., 1999; Leal, 1991; Leal et al., 1993; Potter and Held, 2002). Nevertheless, the background knowledge about food odour perception in scarab beetles sets the stage for a comparative study between the olfactory systems of *P. marginata* and *P. interrupta*, in order to characterize the degree of conservatism and change between their olfactory systems.

The objective of this research:

(1) Characterization of morphological types of olfactory sensilla of *P. marginata* by means of scanning electron microscopy, as an extension of previous studies in this species (Stensmyr et al., 2001) and for comparison with previous studies in *P. interrupta* in (Bengtsson et al., manuscript in Bengtsson (2010))

(2) Characterize olfactory receptor neurons in an important African agricultural pest insect, the sorghum chafer *P. interrupta* and its relative *P. marginata* by using the electrophysiological method single sensillum recording.

(3) Determine the degree of similarity between the olfactory systems of the two species, in order to study how the two species have adapted to their respective olfactory environment.

2-Brief background: biology of Scarab beetles and olfactory systems

The superfamily Scarabaeoidea, commonly called scarabs, are a large distinct group of highly specialized beetles, also called Lamellicornia due to their lamellate antennae (Sawada, 1991). The superfamily Scarabaeoidea consists of eight families (Leal, 1998), one of which is the Scarabaeidae, which contains about 2,000 genera and 25,000 species, divided into subfamilies and numerous tribes (Parker., 1982). According to the classification by Lawrence and Newton (1995), the family Scarabaeidae includes the subfamilies Aclopinae, Aphodiinae, Scarabaeinae, Melolonthinae, Dynastinae, Orphninae, Allidiostomatinae, Rutelinae, Cetoniinae, Trichiinae, and Valginae. Scarab beetles of the family scarabaeidae are divided into two groups: dung beetles or Laparosticti and chafers or Pleurosticti (Sawada, 1991). Adults of scarab beetle are diverse in their diet, some being attracted to dung (dung beetles), carrion, and fungi, and others highly attracted to fruits, flowers, and pollen (beetles of subfamily Cetoniinae), and yet others which feed on roots. Others are pest insects that cause huge damage in gardens such as the Japanese beetle *Popillia japonica* which feeds on leaves, flowers and fruits of more than 300 plant species. The larvae of this species are root-feeding and economically recorded as a pest as well as the adults beetles (Potter and Held, 2002; Ratcliffe, 1991; Ritcher, 1958; Woodruff, 1973).

Fig. 1. The flower chafer *Cetonia aurata aurata* feeds on different flowers of many plant species.

The subfamily Cetoniinae was redefined by Krikken (1984). Flower beetles or chafers are used as a common name for this group due to the poorly developed mouth parts (Grunshaw, 1992). The Cetoniini are a large tribe in the subfamily Cetoniinae and consist of approximately 107 genera. They are widely distributed around the world (Krikken, 1984). Adult chafers of subfamily Cetoniinae prefer nectar, sap, or juice of ripening fruits and vegetables and some are common visitors to flowers to feed on nectar and pollen (Sawada, 1991). The antenna is 10-segmented, with a 3-segmented club at the distal end (Krikken, 1984).

The Pachnoda genus in the subfamily Cetoniinae is distributed over the African continent and contains about 100 species (Rigout, 1989). The Pachnoda are polyphagous and feed on a variety of fruits, nectar and pollen (Rigout, 1989; Stensmyr et al., 2001). Some Pachnoda species have been recorded as pest insects. In Ethiopia and Mali, some species attack important crop plants, e.g. sorghum and pearl millet (Bengtsson et al., 2009; Grunshaw, 1992; Schmutterer, 1969; Stensmyr et al., 2001; Wolde-Hawariat, 2007; Yitbarek and Hiwot, 2000).

Fig. 2. Adults *Pachnoda interrupta* feeding on the milky-ripe seeds of a sorghum plant.

2.1. The antennae of scarab beetles

Scarab beetles have lamellate antennae consisting of eight to eleven segments (usually ten segments), with the last 3 to 7 segments forming a club (Cooper, 1983; Leal, 1998; Scholtz, 1990). In the subfamily Cetoniinae, the club contains three lamellae (Krikken 1984). The inner surfaces of the lamellate club segments carry various morphological types of olfactory sensilla (Leal 1998). Olfactory sensilla of scarab beetles have been described for many species by scanning electron microscopy, e.g. *Adoryphorus couloni*, *Anomala cuprea*, *Oryctes rhinoceros* (L.), *Popillia japonica*, *Phyllophaga anxia*, and *Phyllophaga obsoleta* (Kim and Leal, 2000; Leal and Mochizuki, 1993; Mcquillan and Semmens, 1990; Ochieng et al., 2002; Renou et al., 1998; Romero-Lopez et al., 2004). Most morphological types of scarab olfactory sensilla are modified from three basic types: recessed pore plates, pore plates on sockets, and hair-like sensilla (Leal, 1998). In species studied so far, pheromone-sensitive receptor neurons have been found in sensilla placodea variously described as sensilla placodea without pits and smooth placodea (the latter description will be used in this thesis) (Leal and Mochizuki, 1993; (Larsson et al., 1999).

2.2. The detection of odors

Odors are mainly perceived and detected by sensory organs called sensilla on the insect antennae. Each sensillum houses one to several olfactory receptor neurons (ORNs). In wasps, placoid olfactory sensilla are innervated by up to 140 olfactory receptor neurons (ORNs) (Keil, 1999). The cell bodies of olfactory receptor neurons are located in the tissue under the cuticle (Keil, 1997; Keil, 1999). Each ORN sends a dendrite into the sensillum. The dendrite can be more or less branched. In the lymph between the sensillum wall and the neuron dendrite, there are special odorant binding proteins (OBPs). These binding proteins are thought to transport odour molecules from the inner openings of the sensillum wall, through the lymph, to the dendritic membrane where receptor proteins receive the odour stimuli and determine the physiological specificity of the ORN (Kaissling, 2001; Vogt, 2002; Vogt et al., 2002; Vosshall, 2000; Vosshall et al., 2000; Vosshall et al., 1999). There are two classes of proteins involved in odour transduction with odorant binding proteins in insects: odorant receptors placed on the membrane of olfactory neurons, and degrading enzymes present in the sensillar lymph surrounding these neuronal cells (Pelosi, 1996).

2.3 Olfactory transduction and electrophysiological recordings

Insect ORNs are able to detect volatile chemical compounds with high specificity and sensitivity, and respond to these chemical signals with action potentials (Huotari and Lantto, 2007). The action potential is a wave of ions, which travels along the membrane of the cell (ORN), and it carries and transmits the information from neuron to another neuron or other body tissues such as muscles (Huotari and Lantto, 2007). The electrical signals of ORNs have been measured with different electrophysiological methods, e.g. the electroanntenogram technique (EAG), which is used for recording from the whole insect antenna. This method was first used in 1957 by Schneider (Schneider, 1957) for recording from antennae of male *Bombyx mori*, for screening compounds which could be the sex pheromones of this species (Ignell and Hansson, 2005).

At the single cell level, the single sensillum recording technique (SSR) was investigated by Boeckh in 1962 using tungsten microelectrodes.
SSR allows measurements of the activity and excitation or inhibition of an olfactory receptor neuron by specific compounds. When several olfactory receptor neurons are housed in the same sensillum, they can often be discriminated by their action potential (spike) shapes and/or amplitudes (de Bruyne et al., 2001). Action potential frequencies can be calculated over discrete time intervals either automatically or manually (Bjostad, 1998).

3. Materials and Methods

3.1 Insect rearing

Pachnoda marginata

We obtained beetles from different suppliers in Sweden and combined these into an aggregate culture. Beetles were reared in plastic boxes (30 x 12 x 22 cm, Cofa Plastics AB, Stockholm, Sweden) containing a mixture of planting soil (Yrkesplantjord, Weibull Trädgård AB, Hammenhög, Sweden), composted cow dung (Simontorps Bas, Weibull Trädgård AB) and dried leaves of different plants collected from leaf litter in deciduous forest. The insect boxes were kept moist but not wet at 25° C, 70% relative humidity, and a L16:D8 h cycle. Beetles were fed organic banana and apple (KRAV) *ad libitum*.

Pachnoda interrupta

Adult beetles were obtained from the field in Ethiopia, by Dr Jonas Bengtsson (Bengtsson et al., 2009).

Both beetle species were sexed according to the presence of a ventral abdominal groove in males (Rigout, 1989).

3.2. Laboratory experiments

3.2.1. Scanning electron microscopy:

The antenna of *P. marginata* were collected from both sexes and kept overnight in 70% of ethanol at 4°C. The samples were transferred into 80%, 90%, and 100% of ethanol for dehydration and mounted on the microscope holders and coated with gold/palladium (3:2) with ion sputter (JEOL JFC-1100) and scanned by scanning electron microscope (LEO 435 VP, UK).

3.2.2. Electrophysiology

3.2.2.1. Chemicals and stimuli

-First screening

To characterize different olfactory receptor neuron classes in the two beetle species, we started a broad screening by using a total of 85 synthetic compounds. These compounds have been identified previously as active compounds in GC-MS for these beetles. Some compounds have been found in the preferred food of these species like banana and abutilon, or volatiles from flowers, fruits and fermentation processes, while others were electrophysiologically or behaviorally active compounds for *P. marginata* (Larsson et al., 2003) or *P. interrupta* (Bengtsson et al., 2009).

Most compounds had a minimum purity of 99%, but all have a minimum purity of at least 95% (Table 1). Neat compounds were diluted in hexane, acetone or paraffin oil to a concentration of 1µg/µl and were used as single stimuli. They were also combined into thirteen blends (mixtures) grouped by chemical classes: phenolic (P3), solvents (SLV), green leaf volatiles (GLVs3, GLVs4), aromatic (ARM1, ARM2), acid (ACD), terpenes (TRP), lactones and relatives (LAC), esters (EST1, EST2, EST3), and “other compounds” (OTH).

Table 1. Synthetic compounds used in the first screening of single sensillum recording; all chemicals are used at concentration of 1µg/µl.

compounds	Blend group	solvent	CAS	Purity %
1,4-Benzoquinone	P3	A	106-51-4	99
Toluquinone	P3	A	553-97-9	98
Phenol	P3	A	108-95-2	99
4-Ethyl phenol	P3	A	123-07-9	99
4-Methyl phenol	P3	A	106-44-5	99
Ethanol	SLV	P	64-17-5	99
Acetone	SLV	P	67-64-1	99,9
Ethyl acetate	SLV	P	141-78-6	99,5
Acetic acid	SLV	P	64-19-7	99
Propionic acid	SLV	P	79-09-4	99,5
(*e*)-2-Hexenal	GLV	H	6728-26-3	98
(*e*)-2-Hexen-1-ol	GLV	H	928-95-0	96
(*e*)-Hexenyl acetate	GLV	H	2497-18-9	98
(*e*)-3-Hexen-1-ol	GLV	H	928-97-2	98
(*z*)-3-hexen-1-ol	GLV	H	928-96-1	98
(*z*)-3-hexenyl acetate	GLV	H	3681-71-8	98
Hexanal	GLV	H	66-25-1	98
1-Hexanol	GLV	H	111-27-3	98
Hexyl acetate	GLV	H	142-92-7	98
Nonanal	GLV	H	124-19-6	95
1-Nonanol	GLV	H	143-08-8	99,5
1-Octanol	GLV	H	111-87-5	99,5
3-Octanol	GLV	H	589-98-0	99
1-Octen-3-ol	GLV	H	3391-86-4	98
Anethole	ARM	H	4180-23-8	99
Benzaldehyde	ARM	H	100-52-7	99.5
Benzyl alcohol	ARM	H	100-51-6	99
Eugenol	ARM	H	97-53-0	98
Methyl benzoate	ARM	H	93-58-3	99
Methyl anthranilate	ARM	H	134-20-3	99
2-Phenyl ethanol	ARM	H	60-12-8	98
2-Phenylethyl propionate	ARM	H	122-70-3	98
Acetoin	ARM	A	513-86-0	97
2,3-Butane diol (racemic)	ARM	A	513-85-9	99
Carvacrol	ARM	A	499-75-2	98
Cinnamic aldehyde	ARM	A	104-55-2	98
Methyl cinnamate	ARM	A	103-26-4	99
Methyl salicylate	ARM	A	119-36-8	99
Phenyl acetaldehyde	ARM	A	122-78-1	90
Phenylacetonitrile	ARM	A	140-29-4	99
Thymol	ARM	A	89-83-8	99,5
Butyric acid	ACD	H	107-92-6	99
N-Caproic acid	ACD	H	142-62-1	99,5
Isovaleric acid	ACD	H	503-74-2	98
Valeric acid	ACD	H	109-52-4	99,8
Isoamyl alcohol	OTH	H	123-51-3	98
6-Methyl-5-hepten-2-one	OTH	H	78-70-6	99
Tetradecane	OTH	H	629-59-4	99,5
Tridecane	OTH	H	629-50-5	99,5
beta-Caryophyllene	TRP	H	87-44-5	98,5
beta-Citronellol	TRP	H	106-22-9	95
Geraniol	TRP	H	106-24-1	98
Geranyl acetate	TRP	H	105-87-3	98

(±)-Linalool	TRP	H	78-70-6	97
Linalool oxides	TRP	H	Mixes of isomers	97
Methyl jasmonate	TRP	H	1211-29-6	95
Nerolidol	TRP	H	7212-44-4	98
(±)-delta-Decalactone	LAC	H	705-86-2	98
(±)-gamma-Decalactone	LAC	H	706-14-9	97
gamma-Hexalactone	LAC	H	695-06-7	98
gamma-Nonalactone	LAC	H	104-61-0	97
gamma-Octalactone	LAC	H	104-50-7	97
gamma-Undecalactone	LAC	H	104-67-6	99
Ethyl-3-hydroxy-butyrate	EST	H	5405-41-4	97
(*z*)-3-Hexenyl butyrate	EST	H	16491-36-4	98
(*z*)-3-Hexenyl isobutyrate	EST	H	41519-23-7	98
(*z*)-3-Hexenyl tiglate	EST	H	67883-79-8	97
Butyl butyrate	EST	H	109-21-7	98
Ethyl butyrate	EST	H	105-54-4	99
Ethyl hexanoate	EST	H	123-66-0	99
Ethyl propionate	EST	H	105-37-3	99
Hexyl butyrate	EST	H	2639-63-6	98
Methyl butyrate	EST	H	623-42-7	99
Methyl hexanoate	EST	H	106-70-7	99
Methyl octanoate	EST	H	111-11-5	99
Methyl propionate	EST	H	554-12-1	99
Propyl butyrate	EST	H	105-66-8	99
Butyl isobutyrate	EST	H	97-87-0	97
Hexyl hexanoate	EST	H	6378-65-0	97
Isoamyl acetate	EST	H	123-92-2	98
Isoamyl butyrate	EST	H	106-27-4	98
Isobutyl acetate	EST	H	110-19-0	99,8
Isobutyl isobutyrate	EST	H	97-85-8	99
Isopentyl isobutyrate	EST	H	2050-01-3	98
Isopropyl acetate	EST	H	108-21-4	99,8

A= Acetone
P= Paraffin oil
H= Hexane

For single sensillum recordings, a volume of 10µl from either a mixture or a single compound was applied on a filter paper inside Pasteur pipettes capped with 1000µl Finnpipette tips. Stimuli were kept at -18 ºC until used in experiments. Screening pipettes were prepared daily, and pipettes for single compound at first screening renewed every week.

-Second screening

37 compounds, judged to be the most active and/or provide the most diagnostic information, were chosen for a comparative classification of ORNs in both species. In the second screening, all compounds were diluted in paraffin oil to the concentration of 100ng/µl except acetoin, which was diluted in distilled water. These compounds are combined as well into five blends, DARM1, DGLV, DEST, DTRP and DJNK. These blends are grouped based on the chemical similarity between the compounds. For chemical purity and CAS number see (table 2).

Table 2. Synthetic compounds used in second screening of single sensillum recording; each compound was used at concentration of 100ng/µl.

compounds	Blend group	solvent	CAS	Purity %
2-Phenylethyl propionate	DARM	P	60-12-8	98
Anethole	DARM	P	4180-23-8	99
Benzaldehyde	DARM	P	100-52-7	99.5
Benzyl alcohol	DARM	P	100-51-6	99
Eugenol	DARM	P	97-53-0	98
Methyl benzoate	DARM	P	93-58-3	99
Methyl anthranilate	DARM	P	134-20-3	99
Methyl salicylate	DARM	P	119-36-8	99
Phenyl acetaldehyde	DARM	P	122-78-1	90
Phenylacetonitrile	DARM	P	140-29-4	99
Butyl butyrate	DEST	P	109-21-7	98
Butyl isobutyrate	DEST	P	97-87-0	97
Hexyl acetate	DEST	P	142-92-7	98
Methyl hexanoate	DEST	P	106-70-7	99
Methyl octanoate	DEST	P	111-11-5	99
(*z*)-3-Hexenyl butyrate	DEST	P	16491-36-4	98
3-Octanol	DGLV	P	589-98-0	99
(*e*)-2-Hexenal	DGLV	P	6728-26-3	98
(*e*)-2-Hexen-1-ol	DGLV	P	928-95-0	96
(*e*)-3-Hexen-1-ol	DGLV	P	928-97-2	98
(*z*)-3-Hexen-1-ol	DGLV	P	928-96-1	98
(*z*)-3-Hexenyl acetate	DGLV	P	3681-71-8	98
Nonanal	DGLV	P	124-19-6	95
Acetoin	No group	W	513-86-0	97
1,4-Benzoquinone	DJNK	P	106-51-4	99
4-Methyl phenol	DJNK	P	106-44-5	99
6-Methyl-5-hepten-2-one	DJNK	P	78-70-6	99
(±)-*gamma*-Decalactone	DJNK	P	706-14-9	97
Isovaleric acid	DJNK	P	503-74-2	98
Toluquinone	DJNK	P	553-97-9	98
gamma-Nonanlactone	DJNK	P	104-61-0	97
beta-Caryophyllene	DTRP	P	87-44-5	98,5
Geraniol	DTRP	P	106-24-1	98
Geranyl acetate	DTRP	P	105-87-3	98
(±)-Linalool	DTRP	P	78-70-6	97
Linalool oxides	DTRP	P	Mixes of isomers	97
2,3-Butane diol (racemic)	No group	P	513-85-9	99

P= Paraffin oil
W= Water

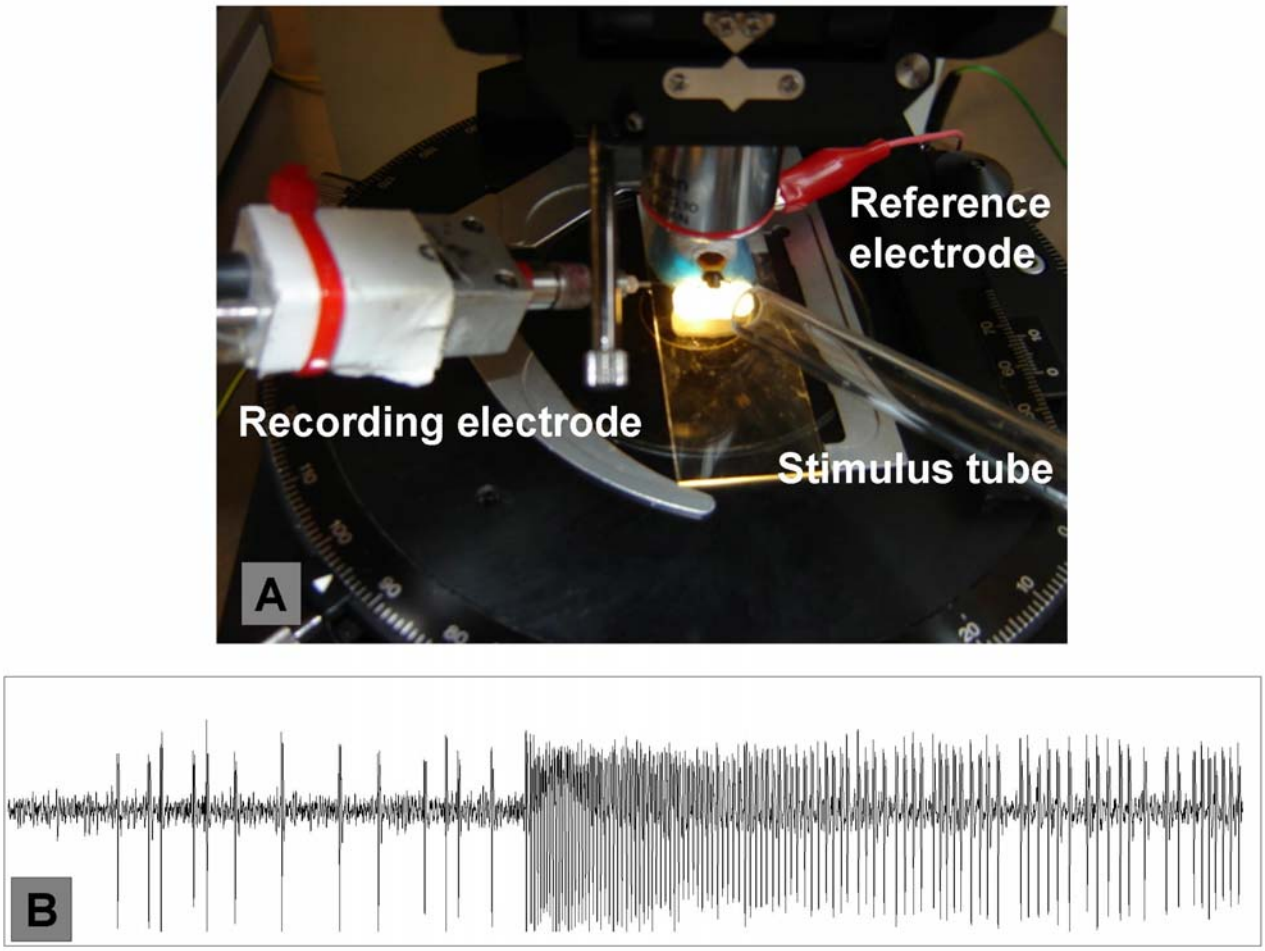


Fig. 3.(A) Single sensillum recording (SSR) setup, showing the position of the beetle *(P. marginata)* during recording. The recording electrode is inserted in a single sensillum on the antennae of the beetle and a reference electrode is inserted in the abdomen. At the lower left is a glass tube through which the air stream flows, and odour stimuli are introduced. Photo: Hamida Khbaish
(B) Single sensillum recording showing action potentials from an olfactory receptor neuron.

3.2.2.2. Single sensillum recordings (SSR)

-Insect preparation
For single sensillum recordings, a preparation with a whole, live adult was used. The insect was immobilized by wrapping it in parafilm (PM-992, Pecheney plastic packaging, Menasha, WI, USA), and was mounted on a glass slide using dental wax (Surgident periphery wax, Heraeus Kulzer GmbH, Hanau, Germany). The antennae were fixed on a layer of wax, and the three lamellae of the antennal club were kept open using thin tungsten pins. The antennae was viewed under a light microscope (Olympus BX51WI) at 500x magnification to identify morphological types of sensilla and to position the recording electrode (Fig 3).

-Single Sensillum Recordings procedure

A silver wire was inserted in the insect abdomen, between the elytra, as a reference electrode. The recording electrode, which was inserted into individual sensilla on the antennae, was made from thin tungsten wire that was sharpened electrolytically in a KNO_2-solution (Hubel 1957).
After contact was established with an ORN, the antenna was exposed to the constant flow of 0.5 m/s of charcoal-filtered and humidified air from glass tube with it's outlet about 15 mm away from the antennae. Odour stimuli were introduced to the insect by inserting the stimulus pipette through a hole in the stimulus tube, and blowing an air puff of 2.5 ml during 0.5 s through the pipette into the air stream, by using a stimulus controller (Syntech CS-02, Hilversum).
The signal from ORNs was transferred to a computer with the software Auto spike v. 2.2 where the action potential (spikes) of the neuron action potentials were analyzed. The total number of spikes of each individual olfactory receptor neuron were counted manually 500 ms before stimulation and 500 ms after stimulation, and subtracted to give the net response. Then net response number was subtracted from the blank and multiplied by 2 to give the net Hz of this neuron (net increase in firing rate of the spikes of the olfactory receptor neuron per second).

3.3. Statistics, spike analysis and cell classification

ORNs were classified according to which odorant elicited the strongest response (primary odorant, secondary odorant), and by cluster analysis using average linkage and Euclidean distance (SPSS 13.0 for windows, SPSS Inc., Chicago, IL, USA). Separation of the cell types in one recording was based on differences in spike amplitudes and waveforms. The cell with the largest amplitude was denoted A and the cell with the smaller amplitude denoted B.
After clustering the results to the cells group, the mean response and standard error was calculated for each cell type.

4 Results

4.1 Antennal morphology

P. marginata has a typical lamellate scarab antenna, with club consisting of three lamellae (Fig 4-A). Olfactory sensilla were present on the four inner surfaces of the lamellae. Most sensilla could be grouped into two morphological classes typically associated with olfactory sensilla: sensilla placodea and sensilla coeloconica (grooved pegs), and a third class of unknown modality: smooth pegs (Fig 4). Placiode sensilla were by far the most abundant morphological type, and could be found in two categories: smooth (in line with the surrounding antennal surface) and grooved (surrounded by a groove separating them from the surrounding antennal cuticle). Coeloconic and smooth peg sensilla were present in much lower numbers. All types of sensilla were found on the inner lamellum surface, which was divided into two zones: a dorsally situated area with a rough, heterogeneous appearance, consisting of grooved placodea, coeloconic, and smooth peg sensilla, and a ventrally situated smooth area consisting only of smooth placoid sensilla (Fig 4-B). *P. interrupta* has identical morphological type of antennae, described previously by Bengtsson et al. (manuscript).

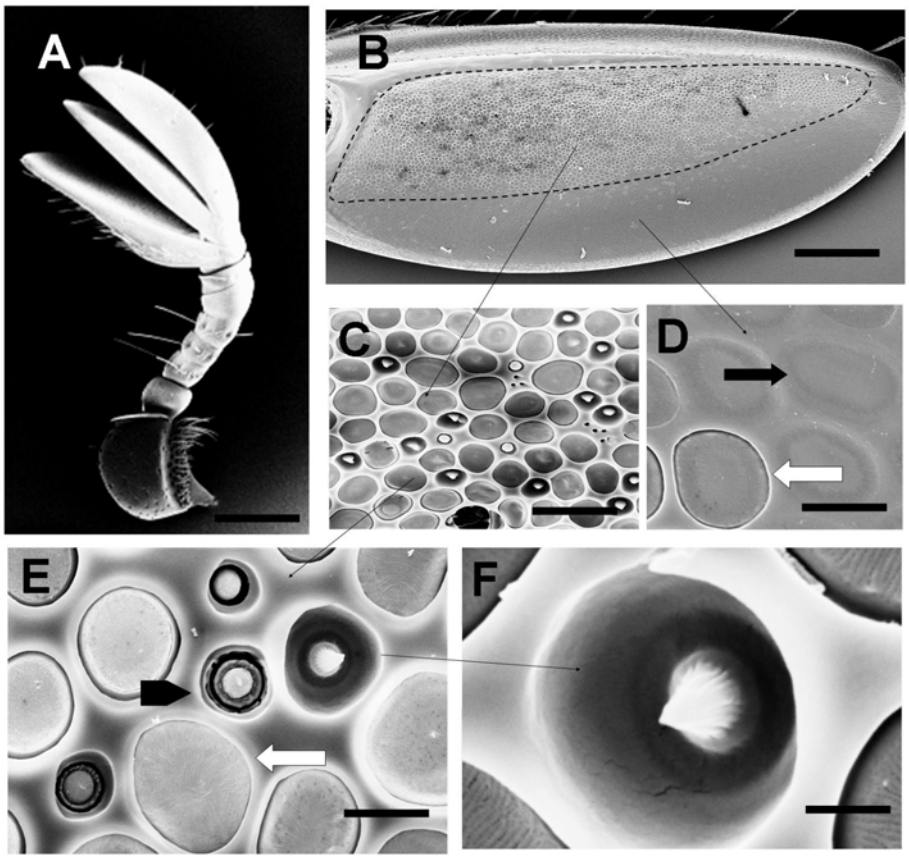


Fig. 4. Scanning electron micrograph of *P. marginata* antennae. (A) The antennae has a lamellate shape, and olfactory sensialla are located on the four inner surfaces of the three apical lamellae. Scale bar is 300µm. (B) An antennal lamella showing two zones, (C) a heterogeneous area consisting of different morphological types of sensilla, (D) a smooth area, mainly placod sensilla. Legend: smooth placodea (black arrow), grooved placodea (white arrow), smooth peg (black arrow head), (F) coeloconic sensilla. Scale bars are 100 µm (B), 2 µm (C, D), 1 µm (E), 300 nm (F).

4.2 Electrophysiology

A total of 456 single sensillum recordings were performed from sensilla placodea (see table 3). I was unable to record from grooved peg (coeloconic) and smooth peg sensilla, due to the difficulty in establishing stable contacts. In general, both smooth and grooved placoid sensilla appeared to be innervated by two olfactory receptor neurons, which could typically be distinguished based on their relative spike amplitudes (Fig 5). In some cases it was difficult to distinguish whether one or both neurons are responding based on their amplitude sizes (Fig 6), but in these cases stimulation with combinations of compounds confirmed the identity of each neuron (Fig 5).

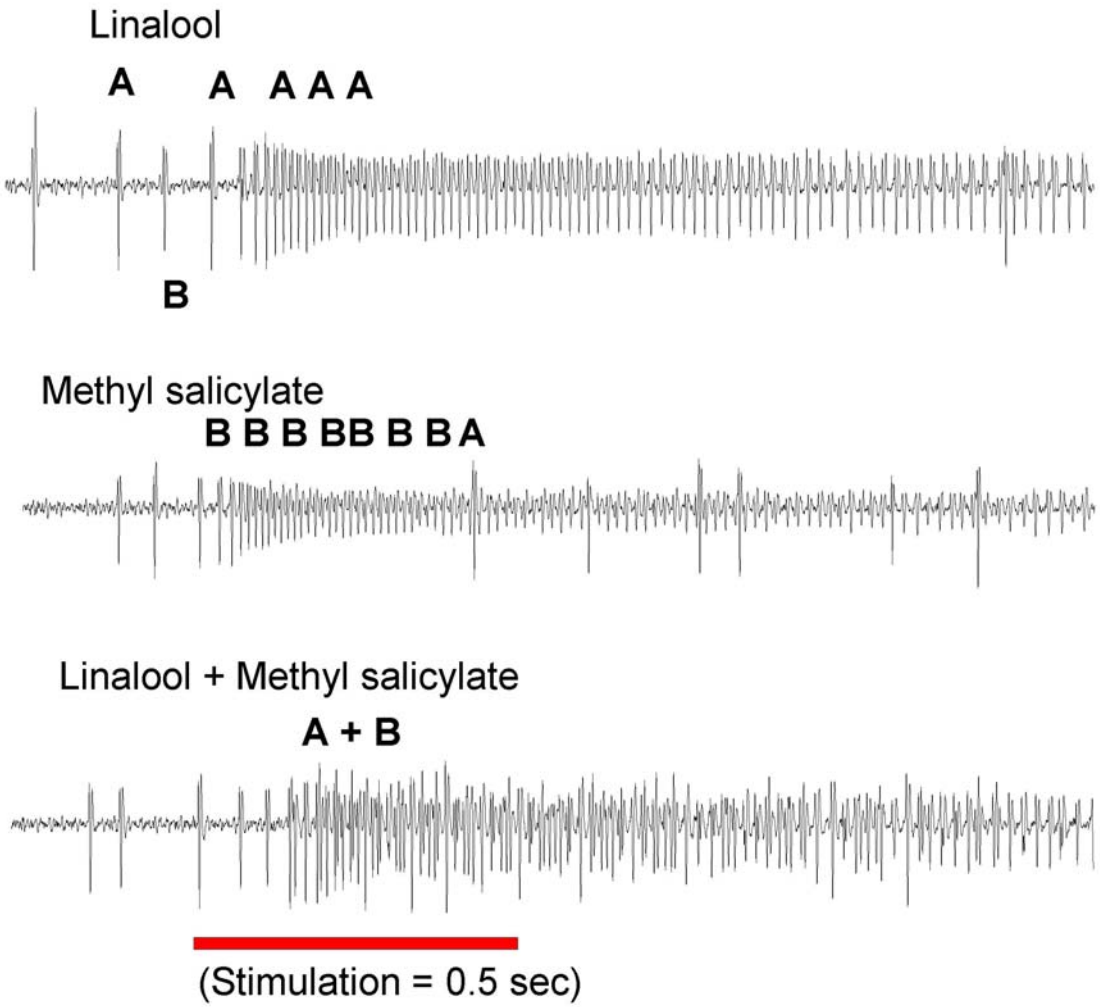


Fig. 5. Electrophysiological recordings from one sensillum showing the responses of two ORNs A and B which housed at the same sensillum. Neuron A responded to linalool whereas neuron B responded to methyl salicylate.

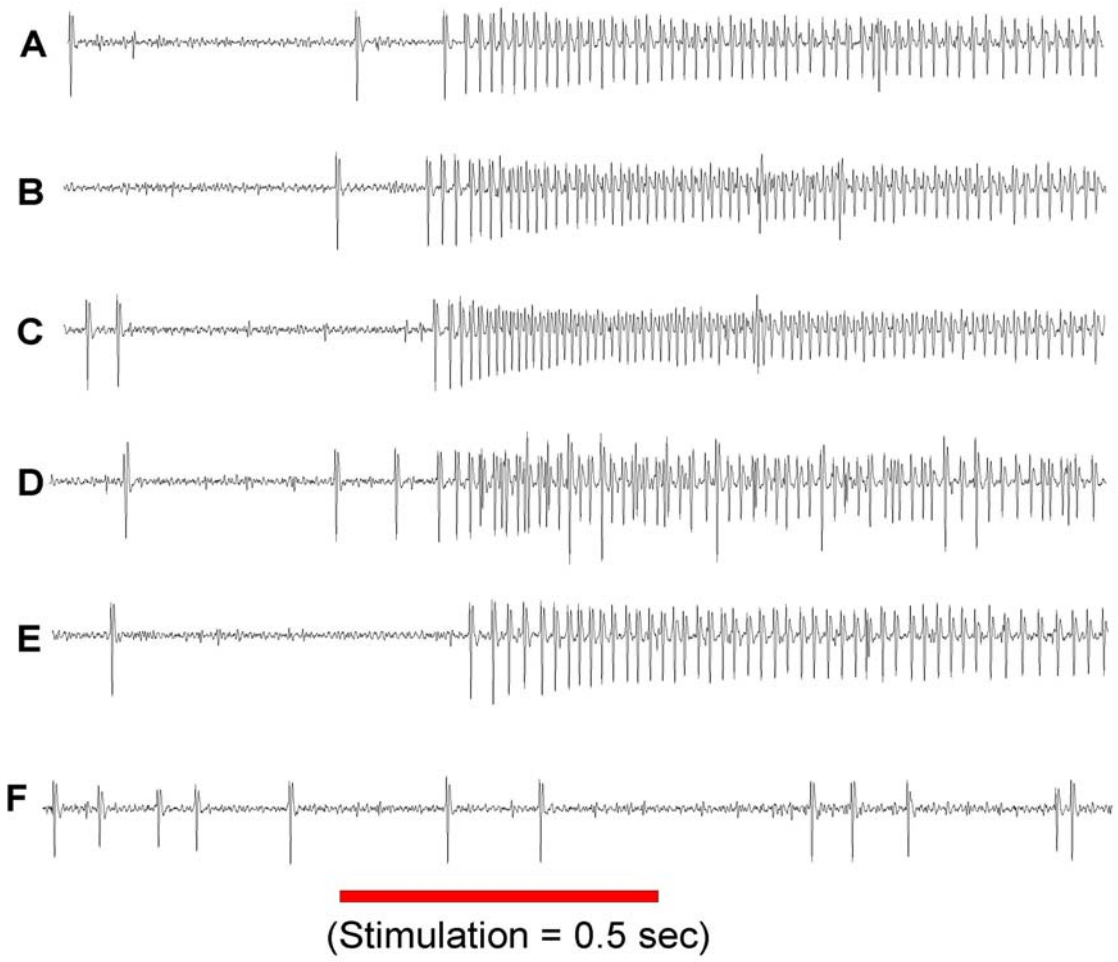


Fig. 6. Response of a single ORN to stimulation with multiple volatile compounds: (A) Butyl isobutyrate, (B) 3-Octanol, (C) Butyl butyrate, (D) Methyl hexanoate, (E) Sulcatone and (F) Paraffin oil (Blank).

In the first screening, recordings were obtained from 65 olfactory sensilla (53 grooved and 12 smooth) in *P. interrupta* and 75 olfactory sensilla (70 grooved and 5 smooth) from *P. marginata*. Among these olfactory sensilla, 71% responded with a net frequency above 40 Hz to at least one screening blend followed by at least one single compound. The remaining 29% of olfactory sensilla were nonresponding, defined as a <40 Hz net increase in their spike frequency after stimulation with screening blends or (occasionally) with single compounds following a response to a screening blend. ORNs responding to test compounds were rarely found in smooth placodea. The ORNs that responded to stimulation did so to a varying number of compounds, ranging from single compounds to several. When ORNs responded to multiple compounds, they were usually structurally similar. Based on all these results from the first study I proceeded with a second screening comprising 37 chemical compounds out of the 85 used in the first screening. Compounds were chosen for the second

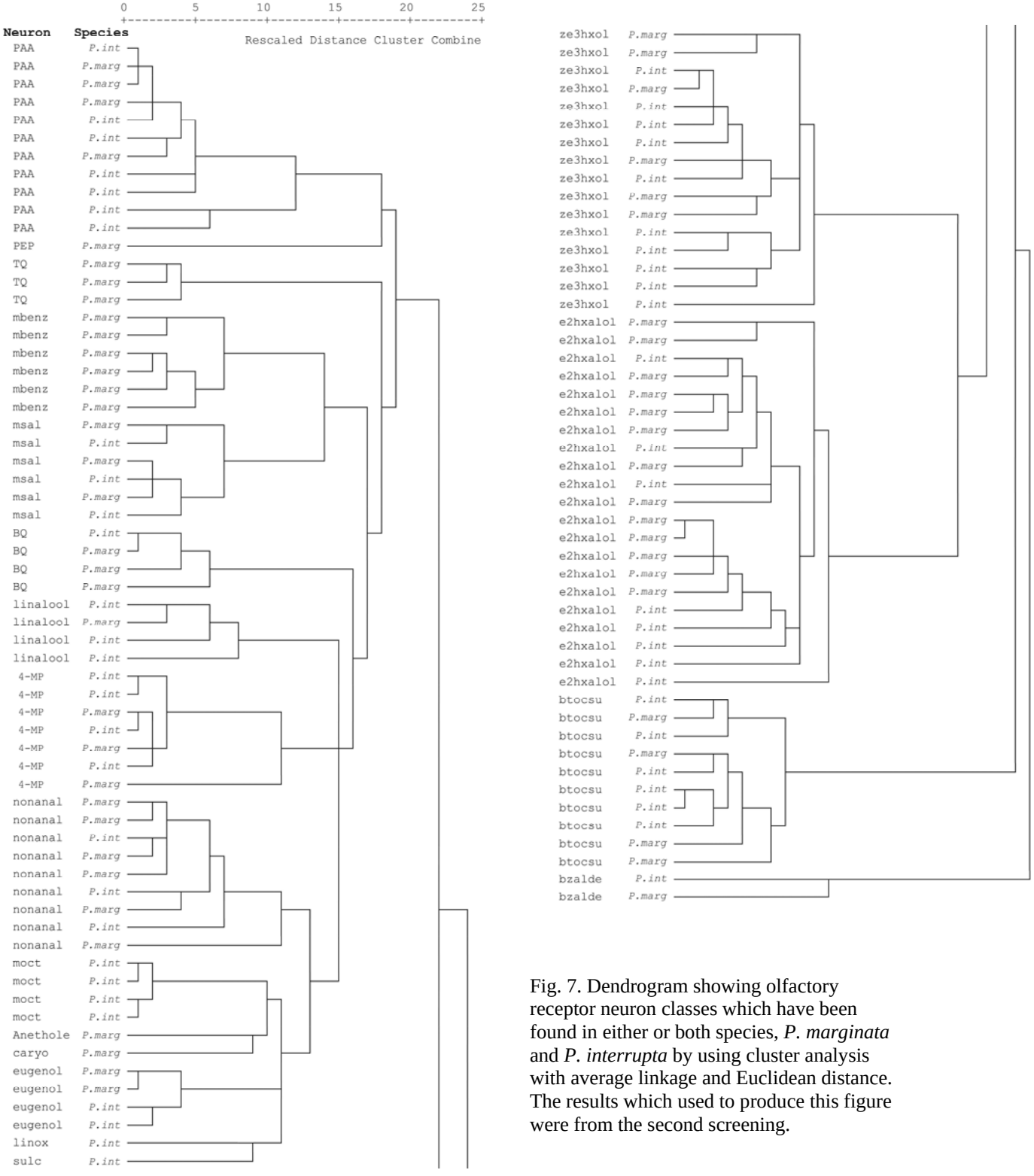


Fig. 7. Dendrogram showing olfactory receptor neuron classes which have been found in either or both species, *P. marginata* and *P. interrupta* by using cluster analysis with average linkage and Euclidean distance. The results which used to produce this figure were from the second screening.

Screening based on being best available ligands for specific types of neurons, and/or Segregating different neuron types. In the second screening I obtained 157 recordings from *P. marginata* and 157 recordings from *P. interrupta*. An overall percentage of 39% constituted responding sensilla, whereas 61% were nonresponding. From the second screening phase I identified 19 ORN classes, classified according to the compounds influencing their activity, by means of cluster analysis based on net responses to all 37 diagnostic compounds (using average linkage and Euclidean distance) (Figure 7).

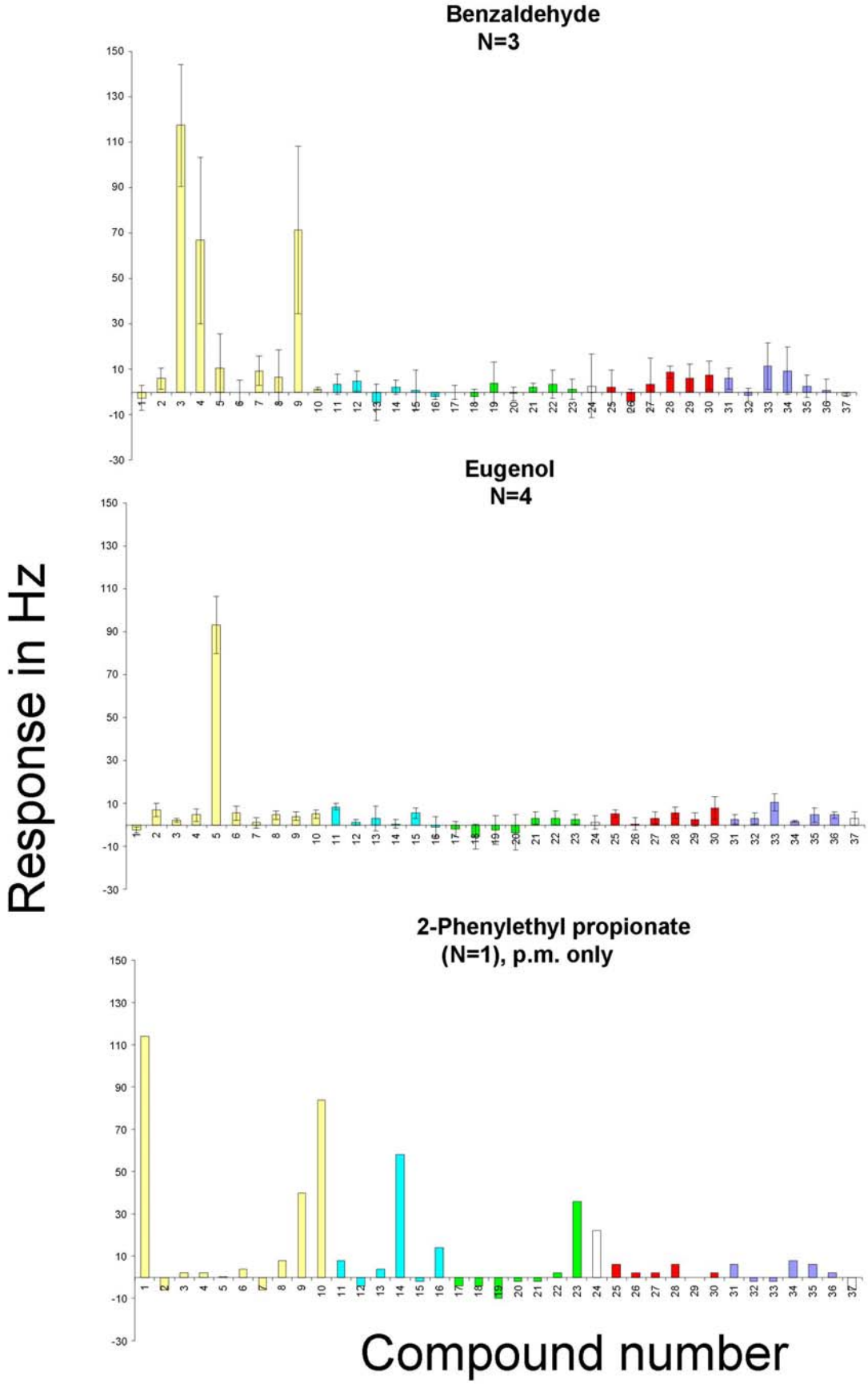
Most responding neurons were found in grooved placodea, with responding neurons rarely found in smooth placodea, only 6% of all responding neurons. I observed no apparent differences in responses of female or male beetles of either species to the range of compounds tested. Response spectra of all responding classes from the second phase are presented in Figure 8.

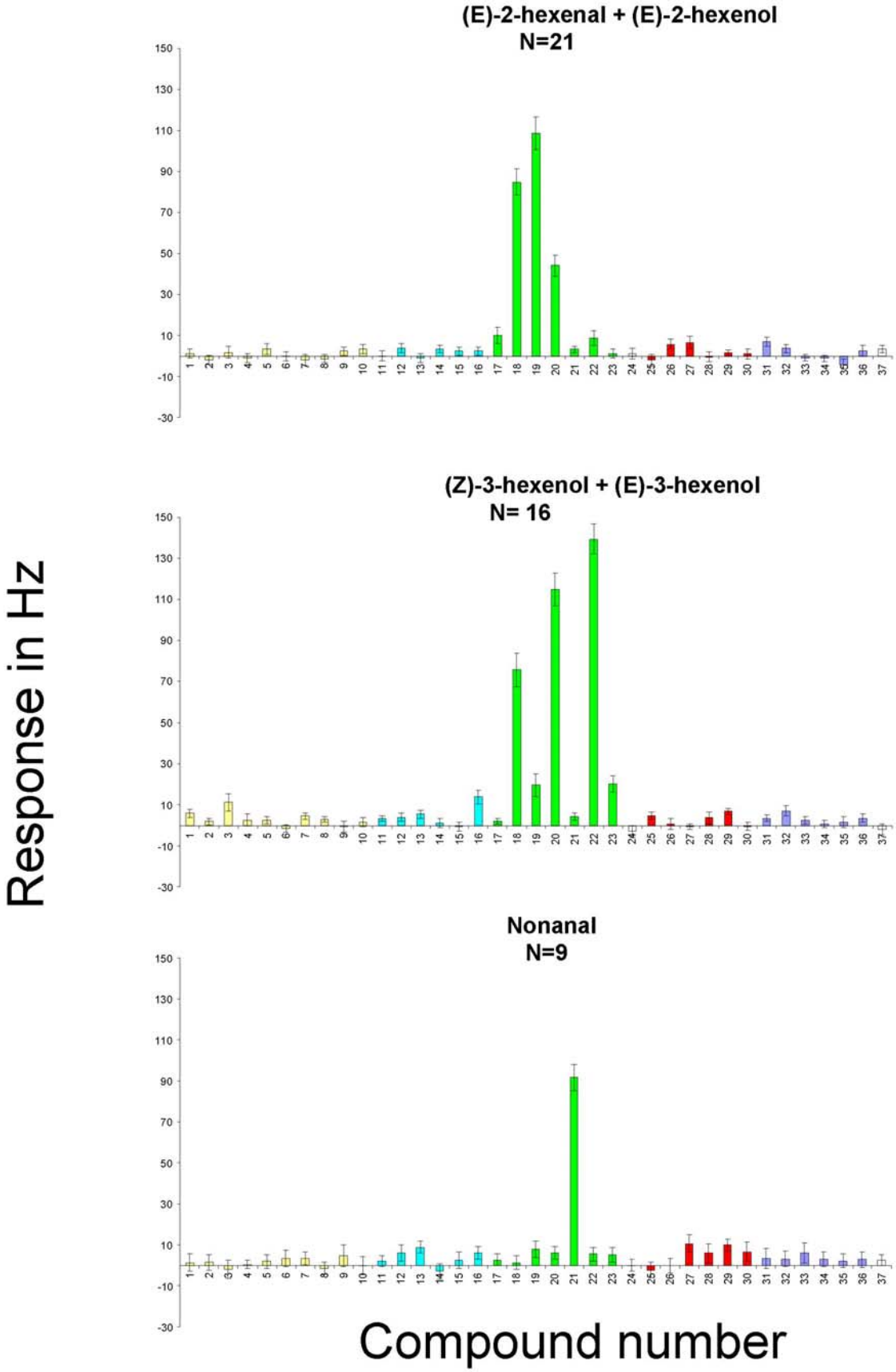
Based on the analysis of results from the second screening, 63 responding receptor neurons from the first screening could be classified into one of 19 classes found in the second screening. An additional class defined only from the first screen contained neurons responding to isovaleric acid. These ORNs could be unambiguously identified based on their specific responses to isovaleric acid and their co-compartmentalization with nonanal neurons (see below). The remaining 27 responding neurons from the first stage were left as “unclassified”. All responses to apparent key ligands from the first screening were also found at the second screening, with the exception of isovaleric acid neurons, which were never found in the second screening.

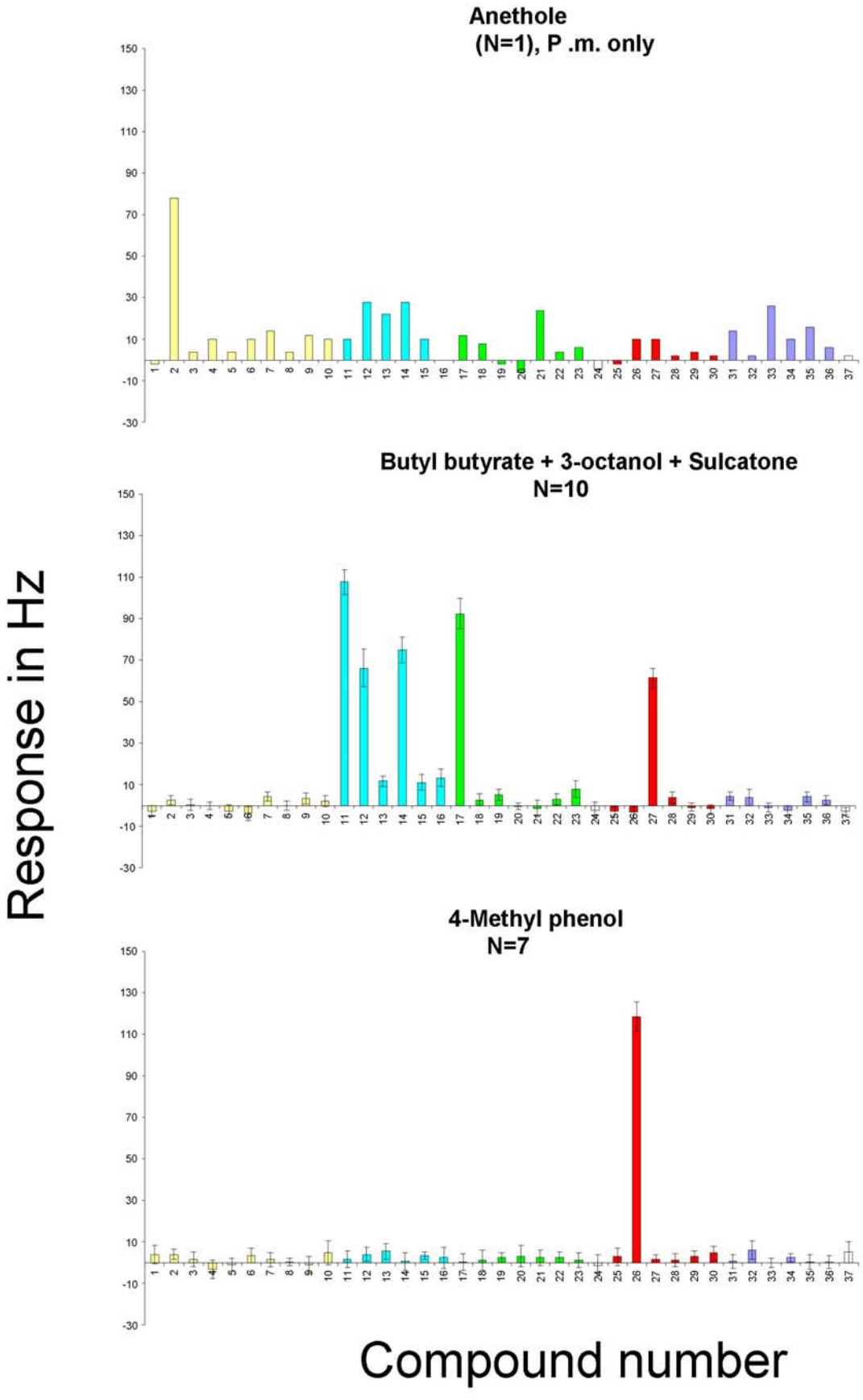
After integrating the results from the first screening and second screening, some cells appeared to be unique for one species based on our data set.

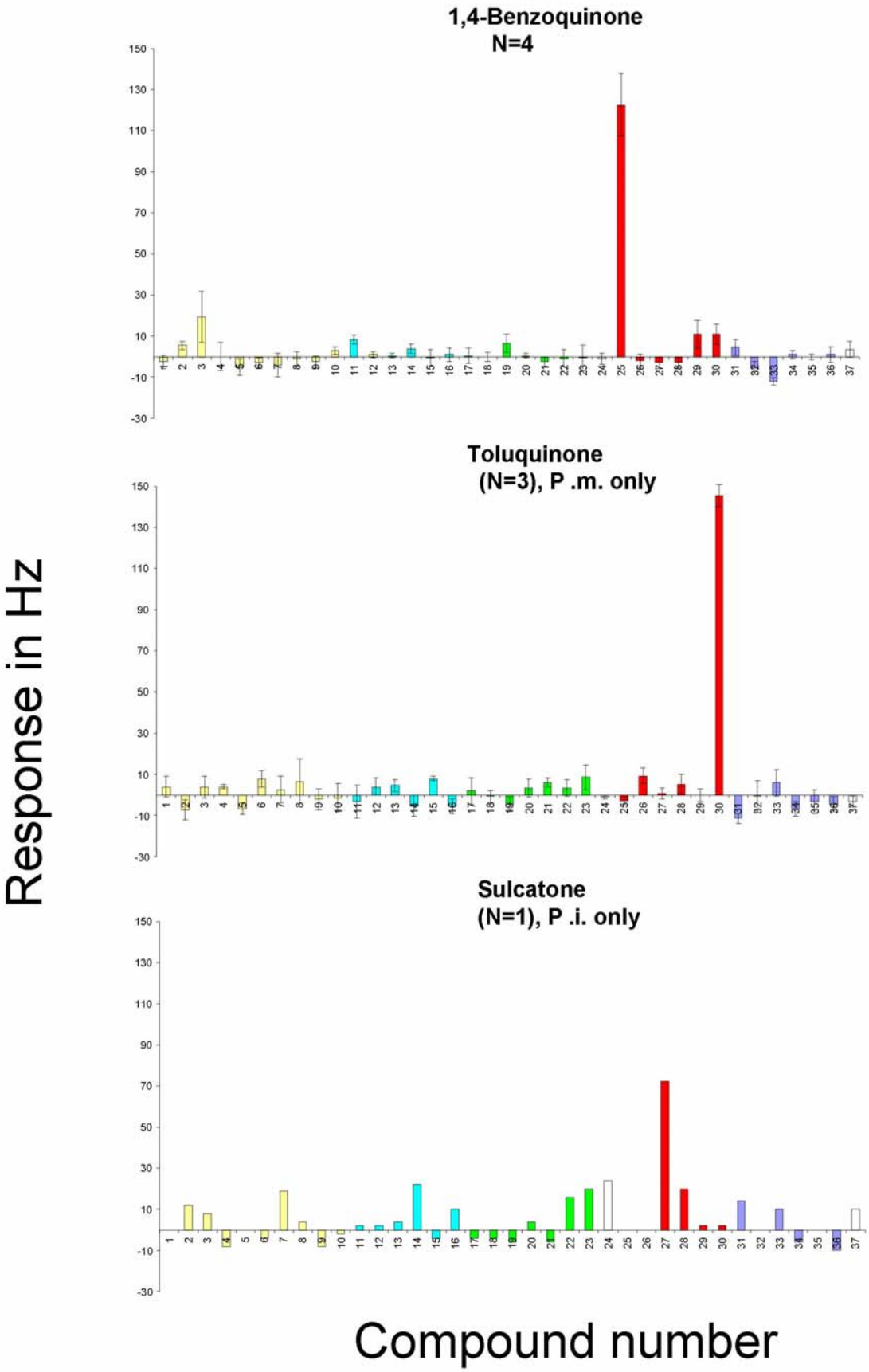
Gamma-nonalactone neurons (results not shown in the figures 7 and 8) and methyl benzoate neurons appeared to be unique cells classes for *P. marginata*, whereas 2,3-butane diol neurons or methyl anthranilate neurons appeared to be unique cell classes for *P. interrupta* (results are not present in the figures 7 and 8). The most common olfactory receptor neurons classes occurred in both species, such as nonanal, benzaldehyde, phenylacetaldehyde, butyl butyrate + butyl isobutyrate+ methyl hexanoate+ 3-octanol+ sulcatone. Two ORN classes responding to green leaf volatiles were the most common in both species: (*z*)-3-hexenol + (*e*)-3-hexenol and (*e*)-2-hexenal+ (e)-2-hexenol.

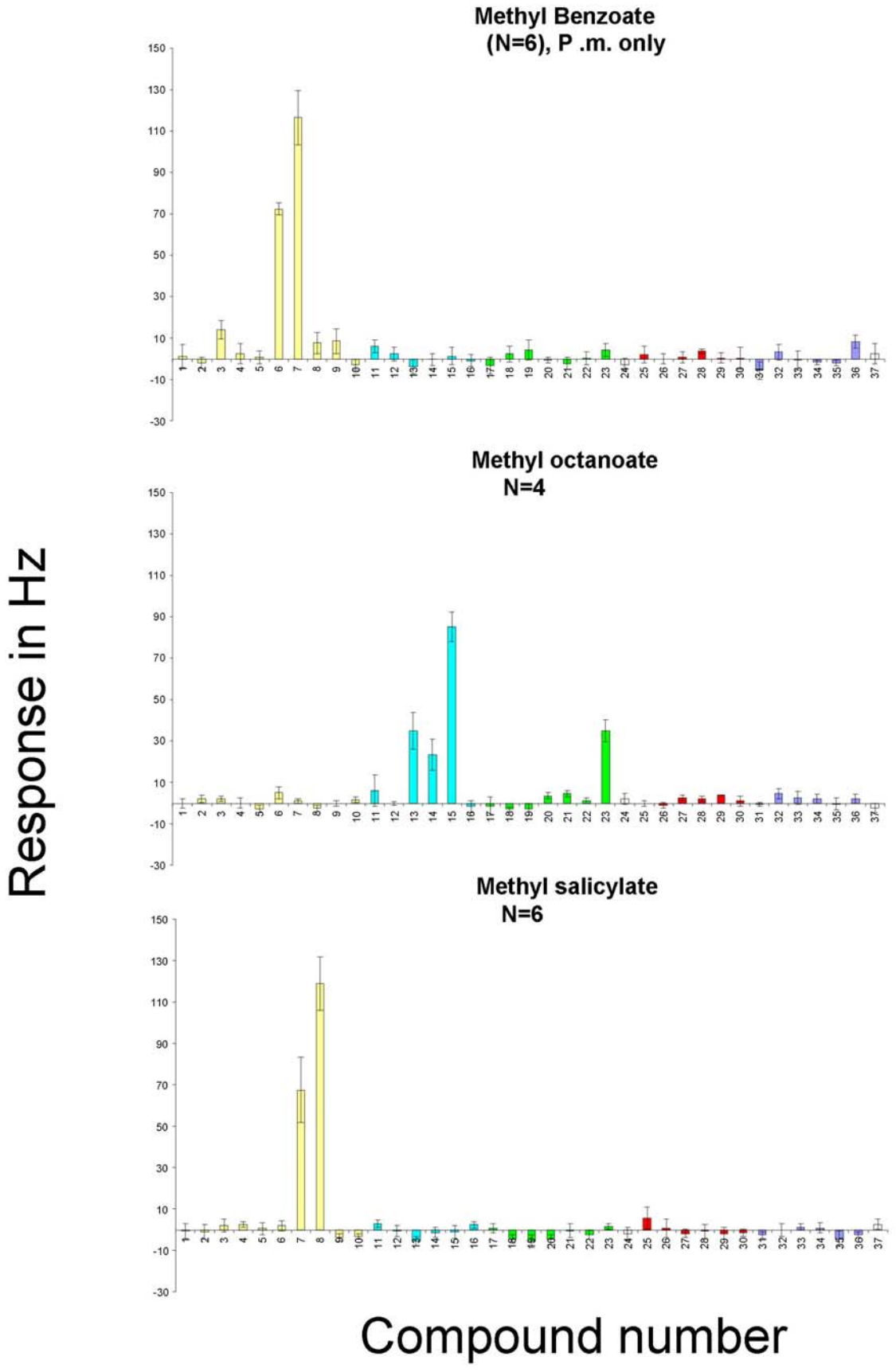
Only in a few cases was more than one responding neuron found in one sensillum. In all these cases, both responding neurons responded to different compounds. The most common combination had the A neuron resonding to (*z*)-3-hexen-1-ol and (*e*)-3-hexen-1-ol, and the B neuron responding to phenylacetaldehyde. A total of nine sensilla in both species had this combination of ORNS. Other pairings of ORNs occurred only once or twice. Some combinations were found in the first screening, e.g. Linalool with methyl salicylate, and benzaldehyde with eugenol. Other combinations occurred only in the first screening and were absent in the second screening, e.g. nonanal with isovaleric acid (the isovaleric acid ORN class is not present in Figures 7 or 8). We found no or very few responses to many typical degradation and fermentation compounds. No ORNs responding to propionic acid, acetic acid, ethanol or acetone were found, even though both species are highly attracted to rotten fruits that are rich in these compounds.

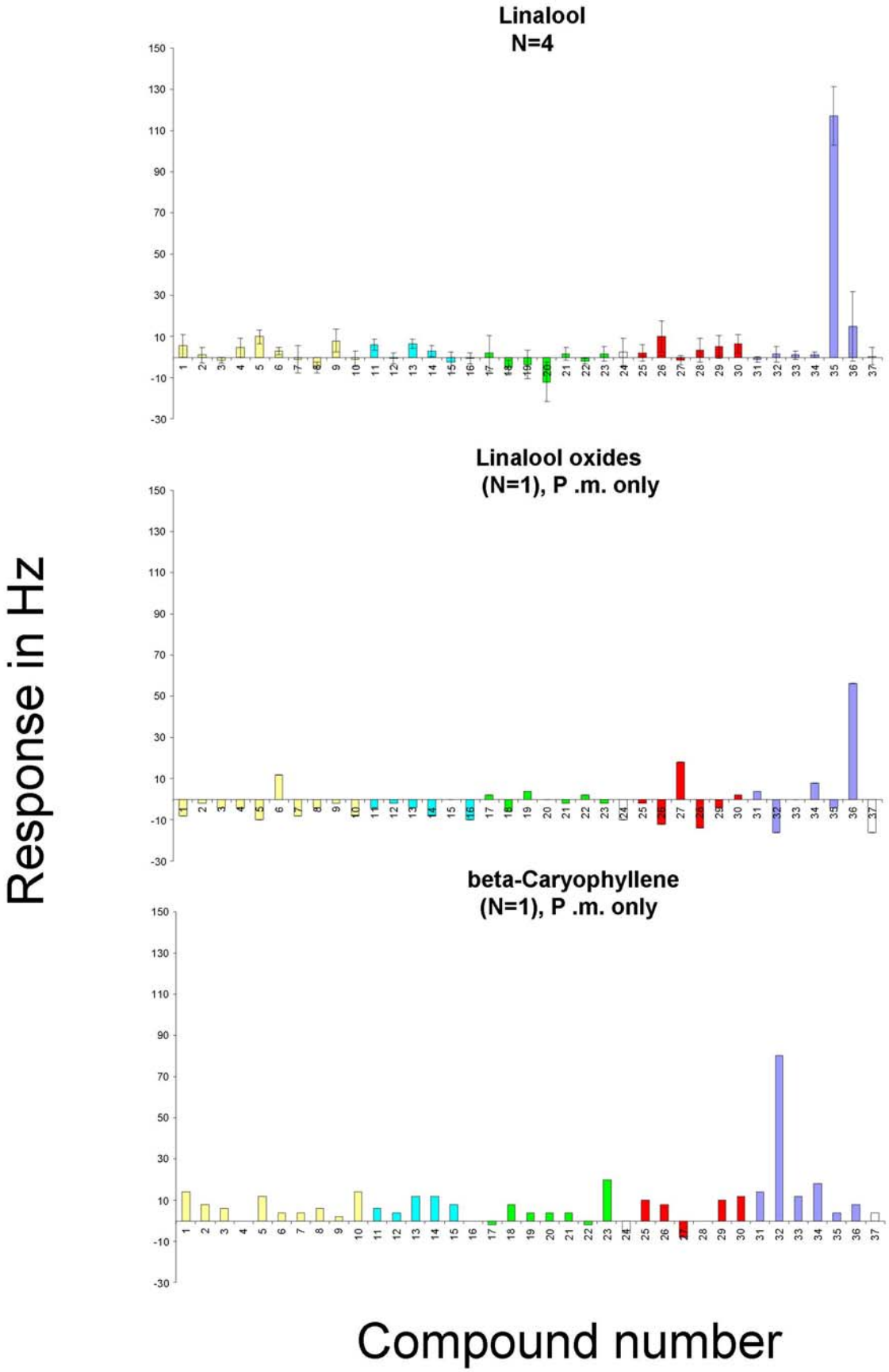

Benzaldehyde
N=3
Eugenol
N=4
2-Phenylethyl propionate
(N=1), p.m. only
Response in Hz
Compound number


(E)-2-hexenal + (E)-2-hexenol
N=21
(Z)-3-hexenol + (E)-3-hexenol
N= 16
Nonanal
N=9
Response in Hz
Compound number


Anethole
(N=1), P .m. only
Butyl butyrate + 3-octanol + Sulcatone
N=10
4-Methyl phenol
N=7
Response in Hz
Compound number


1,4-Benzoquinone
N=4
Toluquinone
(N=3), P .m. only
Sulcatone
(N=1), P .i. only
Response in Hz
Compound number


Methyl Benzoate
(N=6), P .m. only
Methyl octanoate
N=4
Methyl salicylate
N=6
Response in Hz
Compound number


Linalool
N=4
Linalool oxides
(N=1), P .m. only
beta-Caryophyllene
(N=1), P .m. only
Response in Hz
Compound number

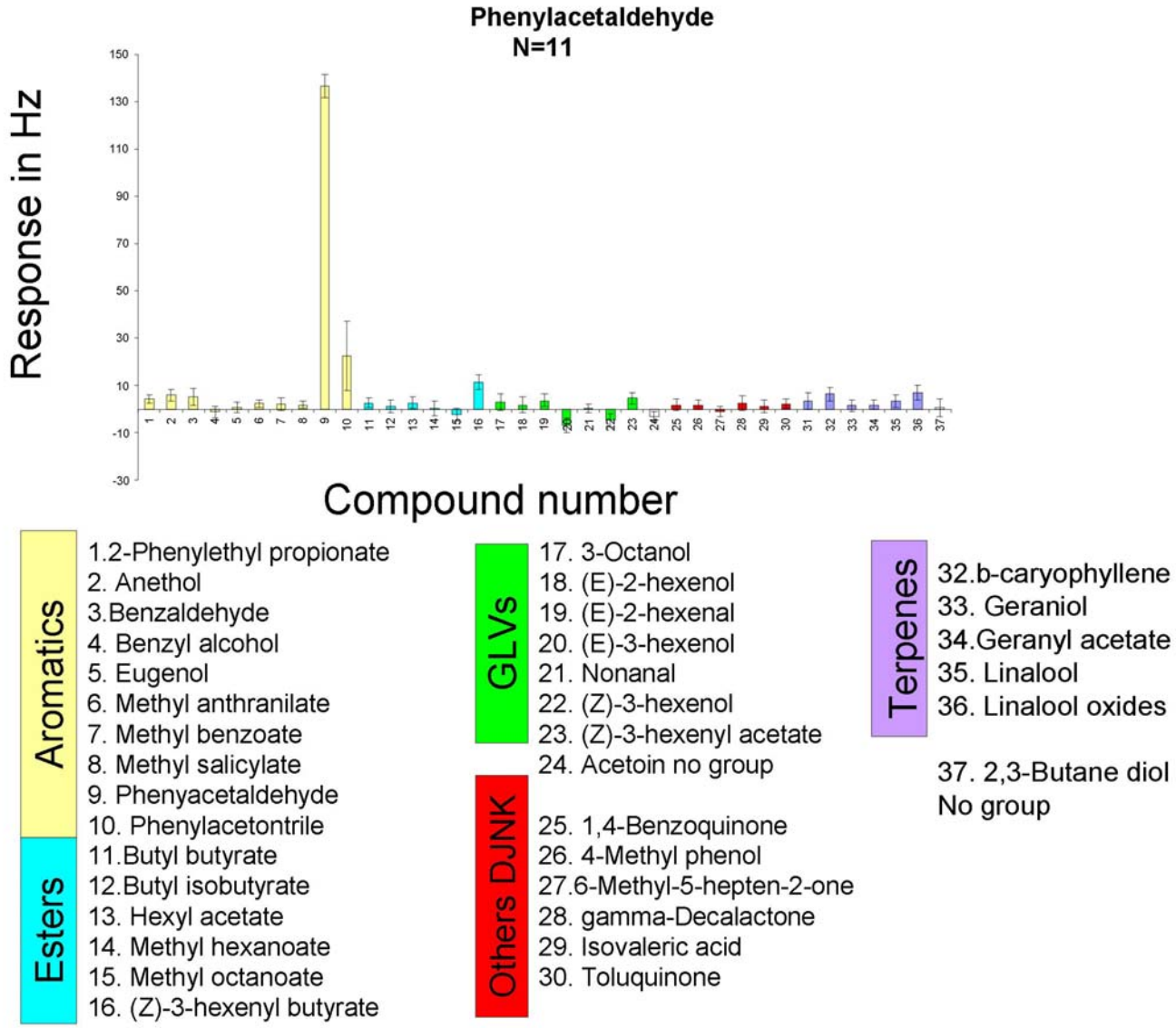


Fig. 8. Response patterns of 19 classes of ORNs housed in sensilla placodea of the two Scarabs *P. marginata* and *P. interrupta*. The classification is based on a cluster analysis of the ORNs to a set of odorants presented in the graphs as numbers (1-37) at a concentration of 100ng/µl (see table 2). The neuronal responses are shown as average response of ORN (means ± S.E). (Modified from Bengtsson, (2010))

Table 3. ORN classes identified in the first and second screening in *P. interrupta* and *P. marginata*

Grooved placodea	**First screening**		**Second screening**	
Classes	***P. int***	***P. marg***	***P. int***	***P. marg***
1,4-Benzoquinone	1	0	1	3
Toluquinone	1	0	0	3
4-Methyl phenol	4	1	4	3
(*e*)-2-Hexenal+ (e)-2-Hexenol	0	2	8	13
(z)-3-Hexenol+(e)-3-Hexenol	3	0	10	6
Nonanal	3	3	3	3
Anethole	2	2	0	1
Benzaldehyde	3	4	1	2
Eugenol	0	3	2	2
Methyl benzoate	0	2	0	4
2-Phenylethyl propionate	0	0	0	1
2,3-Butane diol	3	0	0	0
Methyl salicylate	5	2	3	3
Phenylacetaldehyde	1	0	7	4
Isovaleric acid	1	6	0	0
Sulcatone	1	2	1	0
beta-Caryophyllene	1	0	0	1
Linalool	2	2	3	1
Linalool oxides	0	1	1	0
gamma-Nonalactone	0	6	0	0
Butyl butyrate+3-Octanol+Sulcatone	4	0	6	4
Methyl octanoate	0	1	4	0
Unclassified neurons	12	15	0	0
Total responding neurons	47	52	54	54
Nonresponding sensilla	10	18	57	39
Total contacted sensilla	57	70	111	93
Smooth placodea	**First screening**		**Second screening**	
Classes	***P. int***	***P. marg***	***P. int***	***P. marg***
1,4-Benzoquinone	0	0	0	1
Nonanal	0	0	0	3
Methyl benzoate	0	0	0	3
Total responding neurons	0	0	0	7
Nonresponding sensilla	12	5	46	56
Total contacted sensilla	12	5	46	63
Sensilla with 1+ responding cells (both species) = 217				
Non-responding sensilla total (both species) = 239				
Total recordings sensilla (both species) = 456				

5. Discussion

This study demonstrates that the two species studied have a high level of morphological similarity in their respective olfactory physiology. *P. marginata* and *P. interrupta* have lamellate antenna, with and four morphological types of sensilla found on the inner sides of the three apical lamellae. In both species the most abundant antennal sensillum types were the two types of placoid sensilla: grooved and smooth sensilla placodea, which together constitute about 95% of all sensilla (Bengtsson et al., manuscript). By using extracellular single sensillum recordings, we characterized and compared a large number of olfactory receptor neuron classes that are housed in sensilla placodea (smooth, grooved). A broad array of odors stimuli was used in the characterization, including compounds previously shown to be behaviorally and/or electrophysiologically relevant for these species or other chafers (Bengtsson et al., 2009). In the present study I identified 20 olfactory receptor neurons classes in these two species. Most responding ORNs were found in grooved placodea, whereas responding ORNs in smooth placodea were very few. In general, the results showed that these insects use the same set of key ligands to discriminate different odours; therefore it is hardly surprising that the two species are attracted to the same food sources. In my study, 16 ORNs classes were present in both species, with no apparent significant differences in relative frequencies either between species or sexes. Among the common classes were nonanal, 1,4-benzoquinone and toluquinone, which have been identified as potential pheromone compounds for a scarab species: the European cockchafer *Melolontha melolontha* (Reinecke et al., 2002). These compounds were identified in extracts of both sexes, and elicited comparable electrophysiological responses on antennae from both sexes (Reinecke et al., 2002). *M. melolontha* and *M. hippocastani* have been recorded as two of the most abundant coleopteran pests in central Europe (Ruther et al., 2002b). Both species showed strong attraction to the green leaf volatile (GLV) (*z*)-3-hexenol. Traps baited with phenol, (*z*)-3-hexenol, and the sex pheromone of each species attracted high numbers of males of both species in the field (Ruther et al., 2002a). In our study, the most common ORNs in both species detected GLVs. Similar ORN classes have been found previously in other species, e.g. the Japanese scarab beetles *Phyllopertha diversa* (Hansson et al., 1999) and *Anomala cuprea* (Larsson et al., 2001), but with a slightly different set of key ligands. These olfactory receptor neurons showed a strong sensitivity to (*z*)-3-hexenyl acetate, (*e*)-2-hexenal and (*z*)-3-hexenol, respectively. Dose-response experiments showed them to have very low response thresholds to the compounds. In our model species two common types of GLV neurons responding to (*e*)-2-hexenal + (*e*)-2-hexenol and (*z*)-3-hexenol + (*e*)-3-hexenol, were found. As dose-response tests on these two types of receptor neurons were not performed, this not possible to determine the absolute degree of sensitivity or specificity of these neurons. Phenylacetaldehyde was a common olfactory receptor neuron in both *Pachnoda* species. This compound has been extracted from the abdomen tip of female *P. interrupta* and has been indicated as a pheromone component for this species (Bengtsson et al., in press). In field experiments, phenylacetaldehyde has been shown to be one of the most attractive compounds tested for this species, capturing high numbers of both sexes of *P. interrupta* (Bengtsson et al., in press). Phenylacetaldehyde has previously been tested in laboratory bioassays with *P. marginata* and was found to be highly attractive (Larsson et al., 2003).

ORNs detecting floral compounds such as geraniol (found in the first screening and not included in figures), methyl salicylate and methyl anthranilate have been found in both species. Geraniol and methyl salicylate have been found to be attractive to other scarab species, e.g. *Cetonia aurata aurata* and *Potosia cuprea* (Vuts et al., 2010). In tests with the electroantennogram (EAG) technique, they elicited the strongest responses among all compounds tested. In field studies, methyl salicylate caught high numbers of *P. interrupta* (Bengtsson et al., 2009; Wolde-Hawariat, 2007). A previous behavioral study demonstrated high attraction of *P. marginata* to methyl salicylate (Larsson et al., 2003). In an extended study of the two model scarab species, methyl anthranilate neurons were found only in *P. interrupta* (data not included, for more details see Bengtsson et al., manuscript, in Bengtsson, 2010).

Though *P. interrupta* and *P. marginata* are highly polyphagous and feed on many different fruits (Larsson et al. 2003; Wolde-Hawariat, 2007), their ORNs showed very high sensitivity (data not included, for more details see Bengtsson et al., manuscript, in Bengtsson 2010) and specificity to GLVs. Other compounds that elicited response in ORNs were mostly fruit and floral compounds. Conversely, olfactory receptor neurons responding to the fermenting plant material, such as acetoin, 2,3-butanediol and isovaleric acid were comparatively few; only one neuron in *P. marginata* responded to acetoin (data not shown), and three neurons in *P. interrupta* responded to 2,3-butanediol (data not shown). Interestingly these two chemical compounds have been tested electrophysiologically and behaviorally for *P. marginata* (Larsson et al. 2003), and only acetoin was attractive to this species. For *P. interrupta*, 2,3-butanediol was highly attractive in the field, while acetoin has not been tested behaviourally (Bengtsson et al., 2009). Seven ORNs responding to isovaleric acid were found from both species during the first screening, but no ORNs of this type were found in the second screening. Isovaleric acid has been demonstrated to be an attractant for *P. marginata* (Larsson et al., 2003). For fermentation and degradation compounds such as acetic acid, propionic acid, acetone and ethanol, no responding ORNs were found in either species. It is conceivable that these compounds could be detected by receptor neurons in coeloconic sensilla, which were not tested in the present study, especially since coeloconic sensilla have been shown to detect similar compounds in other species (Yao et al., 2005).

Regarding my comparisons between the olfactory systems, there were overall rather few differences observed between the two species. Most ORNs had virtually identical ligand specificity based on the similarity of their response spectra, suggesting that their olfactory receptor proteins (Vosshall et al., 1999) are functionally highly conserved. Two possible exceptions from this rule may be represented by the 2,3-butane diol/acetoin neurons and methyl benzoate/methyl anthranilate neurons, respectively. The high similarity in their ligand specificities suggest that these neurons may represent pairs of ancestral neurons whose respective ligand specificity has diverged in the two species. In the absence of known host plant differences between the two species it is not clear whether these differences in ORN specificity are a result of adaptive selection or genetic drift. In addition to slight shifts in ligand specificity, some differences in relative frequency of ORN types suggest that their expression patterns may differ quite extensively between the species, however. The low frequency of paired, known ORN types precludes a meaningful comparison of conserved pairing rules between the two species.

6. Conclusion and Future Research

In this study, olfactory receptor neurons classes of two related scarab species, the sorghum chafer *P. interrupta* and the fruit chafer *P. marginata*, have been identified. The aim of this project was to study how insect species of the same group employ the olfactory sense to distinguish different odors sources. As *P. interrupta* is a pest insect, another reason for studying olfaction in this species was to identify new active compounds which can be tested in the field for possible future use in control. The results of this study demonstrate that the olfactory receptor neurons classes of the two species show a high level of overlap, with only two classes unique to *P. marginata*, and one class unique to *P. interrupta*. This means that the two species are likely to use a highly similar strategy for host plants search. Further work could clarify the details of this system, e.g. by recording from other morphological types of olfactory sensilla in both species, and by studying higher brain centers such as the antennal lobes or mushroom bodies.

7. Acknowledgments

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