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Characterization of the Antennal Olfactory System of the Bed Bug (*Cimex lectularius*)

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Abstract

The common bed bug *Cimex lectularius* (Hemiptera; Cimicidae) is a temporary ectoparasite on humans that is currently reinvading the developed countries. Like other haematophagous arthropods, host seeking and orientation in *C. lectularius* is partially mediated by olfaction. In this study, we reconfirmed the distribution of the 44 olfactory sensilla and identified 3 different sensillum types located at the distal tip of *C. lectularius* antenna by external morphology mapping. Using a panel of relevant odorants previously reported to be bioactive in various haematophagous arthropods, we correlated the morphological mapping with an electrophysiological characterization of the olfactory receptor neurons housed in each specific sensillum. We found that all 9 grooved peg sensilla responded specifically in a dose-dependent manner to ammonia, whereas (*E*)-2-hexenal, (*E*)-2-octenal, dimethyl trisulfide, 6-methyl-5-hepten-2-one, α-pinene, indole, and ethyl butyrate evoked dose-dependent responses within the 6 smooth peg sensilla. Based on the pattern of response to the tested compounds, we were able to separate the 6 smooth peg sensilla of the bed bug into 3 distinct functional classes. We compare our results with previous electrophysiological recordings made with these compounds on other haematophagous arthropods.

Key words: aggregation cues, chemoreception, electrophysiology, host cues, olfactory receptor neurons, single sensillum recording

Introduction

The family Cimicidae (Hemiptera) comprises 74 species (Usinger 1966). Among them, the common bed bug, *Cimex lectularius* L., is to one best adapted to human environments worldwide. All developmental stages and both sexes of this species are obligate blood-feeders. After their nocturnal blood meal on the resting host, they return to their refuge where they mate, molt, oviposit, and remain hidden until their next meal (Usinger 1966). Prior to the mid-20th century, infestations of common bed bugs were very common but were then successfully controlled using residual insecticides (Usinger 1966). Since the early 21st century we have seen a resurgence of the common bed bug in the developed world, where reduced use of organochlorine insecticides as well as increased international travel are believed to be the major causes (Reinhardt and Siva-Jothy 2007). Increased resistance to insecticides has been reported in UK (Boase et al. 2006) and Denmark (Kilpinen et al. 2008) as has also been observed in its tropical counterpart *Cimex hemipterus* (Myamba et al. 2002). Therefore, the need for a better understanding of the ecology and behavior of the common bed bug is required in order to develop novel strategies to monitor and control this reinvading urban pest.

Bed bugs locate their hosts through thermal and olfactory cues (Johnson 1941; Usinger 1966; Aboul-Nasr and Erakey 1967; Aboul-Nasr and Erakey 1968) and removal of the terminal segment of the antennae leads to the loss of behavioral responses driven by olfactory cues such as the alarm pheromone and assembling scent (Levinson et al. 1974). Structural analysis of the terminal antennal segment of *C. lectularius* has revealed a low number of olfactory sensilla: 9 grooved peg sensilla (C sensilla), housing 4–5 olfactory receptor neurons (ORNs); 6 smooth peg sensilla (D sensilla), housing 8–19 ORNs; and 29 hairs (i.e., trichoid sensilla; E sensilla), housing 1–3 ORNs (Steinbrecht and Müller 1976). Until

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now, electrophysiological analysis of these sensilla is restricted to a single study by Levinson et al. (1974), showing responses to the alarm pheromone cues, (*E*)-2-hexenal and (*E*)-2-octenal (hereafter referred to as hexenal and octenal).

Electrophysiological studies, along with behavioral studies, have been performed in other blood-feeding insects to achieve a better understanding of their peripheral olfactory interface and the chemical cues responsible for driving their various behaviors (reviewed by Lehane 2005). Some of this information has subsequently been used when establishing novel control methods for these insects. In an effort to increase our understanding of the olfactory system of *C. lectularius*, we here report a functional analysis of their antennal olfactory sensilla using a panel of potential kairomones, including those shown to be behaviorally active for *C. lectularius*, as well as volatiles identified as active for other haematophagous insects.

Materials and methods

Insects

Bed bugs were reared for several generations in an incubator (KB8400 FL, Termaks, Norway) under a 12:12h light:dark cycle at 25°C, 70% room temperature. The colonies were kept in plastic jars (40 mm, 30 mm internal diameter [i.d.]), covered by nylon netting (0.5 mm), which contained a folded filter paper to allow the insects to walk and lay eggs. Bed bugs were fed every 1–2 weeks on defibrinated chicken or sheep blood using an in vitro feeding system, as described by Montes et al. (2002). After a blood meal, last instar nymphs were isolated individually until they molted as adults. One- to five-weeks-old unmated males and females were used for electrophysiological experiments at least 1 week after their last meal.

Scanning electron microscopy

Whole animals were dehydrated in an ethanol series (70%, 96%, 99.5%, 99.5%, 99.5%; 24 h in each bath) and then air-dried. Specimens were then mounted on scanning electron microscope stubs with the antennae fixed by double-sided tape and coated with gold/palladium (80:20, Polaron SC7640) prior to visualization using a JEOL (5600LV) scanning electron microscope.

Single sensillum recordings

For single sensillum recordings, a bed bug was dorsally restrained on a microscope slide (76 × 26 mm) using double-sided sticky tape. The bed bug was secured by covering the ventral part of the thorax and the abdomen with a second piece of tape. One antenna was then placed on a coverslip (18 × 18 mm) bearing a strip of double-sided sticky tape and viewed through a Nikon Eclipse (E600-FN) microscope, which allowed us to identify the morphology and map the distribution of individual sensilla at high magnification (×750) (see Figure 1 and Steinbrecht and Müller [1976] for more details).

For electrophysiological recordings, 2 tungsten microelectrodes were electrolytically sharpened to a tip size of ~1 μm by repeated dipping in saturated KNO_2 solution while passing 2–10 V through the solution. A reference electrode was inserted into the neck region with the tip of the electrode reaching inside the bed bug's head capsule. The recording electrode was inserted into the shaft or base of a sensillum using a piezoelectric micromanipulator (DC-3K, Märzhauser Wetzlar, Germany). The recording electrode was connected to a preamplifier (×10, Syntech, Kirchzarten, Germany), and the electrical signals were fed through an analogue-digital signal converter (IDAC-4, Syntech, Germany) and then visualized and recorded on a computer using the software Autospike (v3.3, Syntech, Germany).

Stimuli and stimulus delivery

The panel of compounds used in the present study was selected based on their putative role as semiochemical cues for bed bugs (Levinson et al. 1974; Siljander et al. 2008; Gallagher et al. 2008). In addition, we selected compounds that previously have been shown to be electrophysiologically active in other haematophagous arthropods (Table 1). Depending on their nature, the chemicals were either dissolved in paraffin oil (Merck) or nano-pure water to reach logarithmic dilutions of 10^{-1}–10^{-6} steps (v/v for liquid or w/v for solid), with the 10^{-3} dilution used during the screening stage (Table 1). Compounds diluted in water evaporated at a higher rate compared with those diluted in paraffin oil, and therefore, water diluted stimuli were changed every 5 injections. In contrast, paraffin oil retards the release of test chemical, and hence, test stimuli diluted in this way were only changed every 10–15 injections. Even if we cannot assess the exact amount of odorants reaching the bed bug's antenna, we expect that the quantity decreases proportionally to the dilutions used. During dose–response experiments, all compounds were tested in a series from the lower to the higher dilution. Ammonia was tested at a range from 10^{-4} to 10^{-1}, with corresponding half-dilution steps to increase the number of points. Nano-pure water and pure paraffin oil were used as controls.

Aliquots of 10 μL of each compound were deposited onto a filter paper strip (~5×15 mm) placed inside a Pasteur pipette. The insect antenna was placed in a humidified charcoal-filtered air stream delivered at 1 m/s via a glass tube (6 mm i.d.), and the stimulus controller (CS-02, Syntech, Germany) permitted us to divert during 500 ms, a 2 mL/s airflow through the Pasteur pipette containing the stimulus. The interval between each stimulation lasted until the neuronal activity reached the prestimulus frequency level.

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Table 1 Compounds used to characterize the antennal olfactory system of *Cimex lectularius*

Compounds[a]	CAS number	Purity (%)	Company	References[b] species studied			References[b] techniques used	
				Mosquitoes spp.	Blood-feeding flies	Kissing bug bugs	EAG	SSR
(*E*)-2-hexenal	1335-39-3	98	Aldrich		11,24		24	11
(*E*)-2-octenal	2363-89-5	≥94	Sigma					
Benzaldehyde	100-52-7	99	Merck	3,19,21	10,11,15		15,19,21	3,10,11
1-Octen-3-ol	3391-86-4	≥99	Acros	3,5,6,16,22,27	9,11,17,18,23,24		5,6,17,18,23,24	3,9,11,16,22,25,27
Lactic acid*	50-21-5	90	Fluka	3,4,5,6,7,8,16,22,25	11,23	1,14	5,6,23	1,3,4,7,8,11,14,16,22,25
Butanoic acid	107-92-6	≥99	Aldrich	4,5,8,16,22,27	10,15,17,18,23	1,14	5,15,17,18,23	1,4,8,10,14,16,22,27
Hexanoic acid	142-62-1	99.5	Aldrich	12,16,21,22,25	13,15,18		13,15,18,21	12,16,22,25
(-)-Ethyl-L-lactate	687-47-8	≥99	Fluka	7,12,16				7,12,16
Ethyl butyrate	105-54-4	99	Aldrich	12,16				12,16
Acetone*	67-64-1	99.5	LabScan	3,7	9,23		23	3,7,9
Butanone	78-93-3	99	Aldrich	22	13		13	22
2-Undecanone	112-12-9	99	Aldrich	16	13,18		13,18	16
6-Methyl-5-hepten-2-one	110-93-0	99	Aldrich	19,20,22,25	18		18,19	20,22,25
Geranyl acetone	3796-70-1	99	Aldrich	16,19,20,22,25	18		18,19,25	16,20,22,25
Phenol	108-95-2	99	Aldrich	16,21,22,25	9,11,13,17		13,17,21	9,11,16,22,25
4-Methyl phenol	106-44-5	99	Aldrich	2,5,6,16,27	9,13,15,17,18,23		2,5,6,13,15,17,18,23	9,16,27
4-Ethyl phenol	123-07-9	99	Aldrich	16,22	24		24	16,22
Indole	120-72-9	99	Aldrich	2,3,12,16,19,20,22,25	17		2,17,19,25	3,12,16,20,22,25
Benzothiazole	95-16-9	96	SMA					
Dimethyl disulfide	624-92-0	99	Fluka	22	15		15	22
Dimethyl trisulfide	3658-80-8	98	Aldrich	16	15,17,18		15,17,18	16
Linalool	708-70-6	97	Aldrich	16,19,25	11		19	11,16,25
(±)-Limonene	5989-27-5	≥99	Fluka	3,16,19	11,15,17,18,24		3,15,17,18,19,24	3,11,16
(±)-α-Pinene	80-65-8	98	Aldrich	3,7,12,16	11,15,24		3,15,24	3,7,11,12,16
α-Thujone	546-80-5	≥96	Fluka	3,12,16,25			3	3,12,16,25
β-Caryophyllene	87-44-5	98.5	Aldrich		11,15,17,18,24		15,17,18,24	11
Ammonia*	7664-41-7		Ekstrands	3,7,20,22		1,26	3,26	1,3,7,20,22,26
Diethyl amine*	109-89-7	98	Aldrich					
Trimethyl amine*	75-50-3	99	Aldrich	25				25

[a]Compounds with asterisks were diluted in nano-pure water, whereas all the other were diluted in paraffin oil (see Materials and Methods for details).
[b]Numbers refer to published studies in which a certain compound has been tested electrophysiologically on other haematophagous arthropods: 1) Bernard (1974); 2) Blackwell et al. (1993); 3) Bowen (1992); 4) Bowen (1995); 5) Cork and Park (1996); 6) Costantini et al. (2001); 7) Davis (1976); 8) Davis (1988); 9) den Otter and van der Goes van Naters (1993); 10) Dougherty et al. (1995); 11) Dougherty et al. (1999); 12) Ghaninia et al. (2007); 13) Gikonyo et al. (2002); 14) Guerenstein and Guerin (2001); 15) Harraca et al. (2009); 16) Hill et al. (2009); 17) Jeanbourquin and Guerin (2007a); 18) Jeanbourquin and Guerin (2007b); 19) Logan et al. (2008); 20) Meijerink et al. (2001); 21) Puri et al. (2006); 22) Qiu et al. (2006); 23) Schofield et al. (1995); 24) Syed and Guerin (2004); 25) Syed and Leal (2009); 26) Taneja and Guerin (1997); 27) van den Broek and den Otter (1999). In the text, (*E*)-2-hexenal is referred to as hexenal, (*E*)-2-octenal as octenal, (-)-ethyl-L-lactate as ethyl lactate, and 6-methyl-5-hepten-2-one as sulcatone.

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Analysis

The total number of recordings for each sensillum type was 30 for the 6 D sensilla, 40 for the 9 C sensilla, and 40 for the 21 E sensilla. For each of the 6 D sensilla and the 9 C sensilla, at least 5 and 2 replicates were made, respectively, on different individuals. The response spectrum of each specific sensillum was then estimated as the mean electrical response recorded from the different bed bugs. The responses of both sexes antenna were pooled, as there was no difference between males and females, at any dose, of the 31 tested compounds.

As a high number of ORNs are present in each sensillum type, we were unable to differentiate single ORN classes using the shape and amplitude of their action potentials (Figure 2). For this reason, the total number of all spikes (action potentials) was manually counted 500 ms before and 500 ms after stimulation for each recording allowing us to assess the overall ORN activity within a specific sensillum elicited by a single odorant. The number of action potentials after stimulation was subtracted from the number of action potentials before stimulation and multiplied by 2 in order to quantify the firing rate change in one sensillum in spikes per second. However, this general electrical activity measurement may lead to an under estimation of the sensitivity of the ORN, as small variations in action potential firing may be masked by the spontaneous activity of other neurons (see Figures 3 and 6).

Each recording was positioned as a point in a 31 dimensional space using its response spectra. The hierarchical analysis using Ward's method allowed us to measure the distance between each of these points with the aim to build a dendrogramme grouping the most similar recordings (SPSS software, release 17.0, SPSS, US). This hierarchical cluster analysis has previously been used in similar electrophysiological studies (e.g., de Bruyne et al. 1999; Ghaninia et al. 2007) and objectively classifies the sensilla using their response spectra.

Results

Distribution of antennal sensilla

Olfactory sensilla are differentially distributed on the inner (olfactory region O_1) and outer (olfactory region O_2) sides of the distal tip of the second (terminal) antennal flagellum (Steinbrecht and Müller 1976). Six smooth peg sensilla (D sensilla) are located on O_1 and can easily be distinguished based on their localization (Figure 1, Steinbrecht and Müller 1976). Nine grooved peg sensilla (C sensilla) are located on both O_1 and O_2 (4 on O_1 and 5 on O_2) (Figure 1, Steinbrecht and Müller 1976). Approximately 29 hairs (E sensilla) are distributed all over the terminal antennal flagellum, primarily on O_1 and O_2 (Figure 1, Steinbrecht and Müller 1976). The identity and topographical distribution of all sensillum types could easily be distinguished under a light microscope (Figure 1), and we only observed slight variations in position of single sensilla between individuals.

Three functional pairs of D sensilla

D sensilla house 8–19 ORNs (Steinbrecht and Müller 1976), which do not allow us to fully separate the response of single

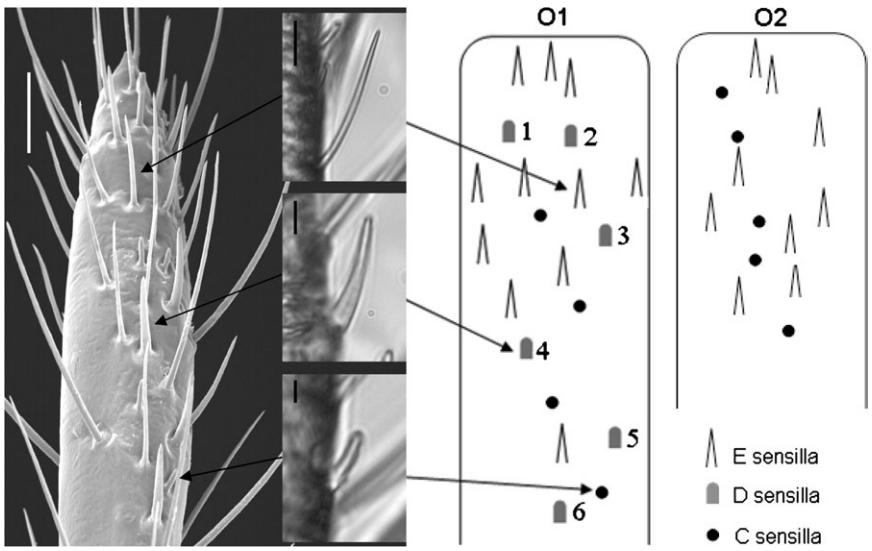

Figure 1 A scanning electron micrograph showing the inner side (O_1) of the distal tip of the second antennal flagellum of *Cimex lectularius*. This region is covered by long bristles (nonolfactory sensilla) as well as grooved peg sensilla (C sensilla), smooth peg sensilla (D sensilla), and hairs (E sensilla). Scale bar indicates 200 μm. The light micrographs (from top to bottom: E, D, and C sensilla) illustrate the view of the different sensillum types during single sensillum recordings. Scale bars indicate 10 μm (E sensillum) and 5 μm (D and C sensilla). The schematic maps of O_1 and O_2 are based on observations of light and scanning electron micrographs and the map proposed by Steinbrecht and Müller (1976).

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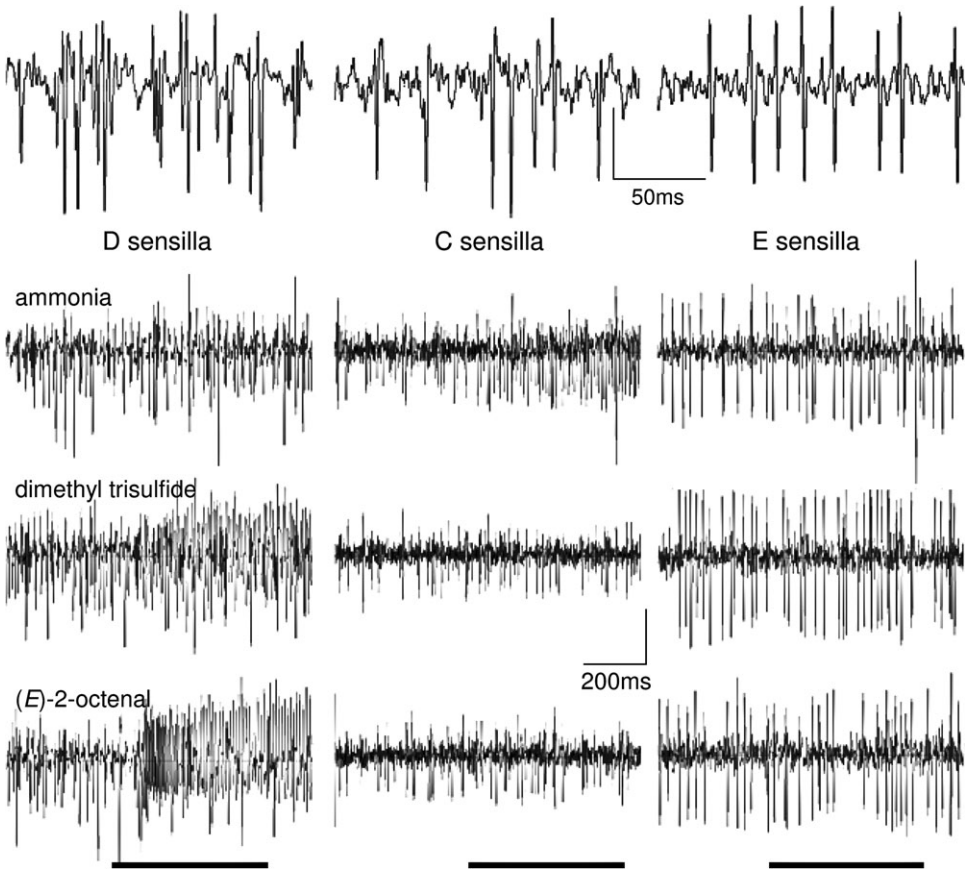

Figure 2 Representative examples of single sensillum recordings from smooth peg sensilla (sensilla D), grooved peg sensilla (C sensilla), and hairs (E sensilla) after stimulation with ammonia, dimethyl trisulfide, and (*E*)-2-octenal at 10^{-3} dilution. The 2 vertical scale bars represent 100 μV for C and D sensilla and 200 μV for E sensilla.

ORNs colocalized in this sensillum type (Figure 2). That said, in some recordings, we observed an increased firing rate of spikes with distinct spike amplitude after stimulating with specific compounds. For instance, D3 and D4 sensilla, stimulation with dimethyl trisulfide elicited an increased firing rate of small amplitude spikes, whereas octenal triggered an increased firing rate of large amplitude spikes (Figure 2). However, this difference in action potential amplitudes was only distinguishable at high spike frequency, that is, when the ORN was responding, and not during spontaneous activity; for this reason, we only consider the overall response of the ORNs. Seven of the 31 compounds, at a dilution of 10^{-3}, elicited a response in the ORNs housed in the D sensilla, but the response levels were different between sensilla. ORNs housed in D1 and D2 sensilla (Figure 1) responded specifically to hexenal (Figure 3). ORNs of D3 and D4 sensilla (Figure 1), on the other hand, responded strongly to octenal, dimethyl trisulfide and ethyl butyrate. Lower responses were also observed to hexenal, dimethyl disulfide and benzaldehyde (Figure 3). ORNs housed in D5 and D6 sensilla (Figure 1) responded to 6-methyl-5-hepten-2-one (hereafter referred to as sulcatone) and to indole and α-pinene. D sensilla could be grouped into functional pairs based on their response spectra to our panel of 31 odors. To test this, we performed a cluster analysis of the overall responses from the 30 recordings of the 6 D sensilla to the 31 tested compounds. This clearly revealed 3 clusters: the Dα cluster grouped the D1 and D2 sensilla; the Dβ cluster grouped the D3 and D4 sensilla; and the Dγ cluster grouped the D5 and D6 sensilla (Figure 4).

Dose-reponse curves were generated for each of the 3 sensillum types, using relevant compounds, hexenal, octenal, dimethyl trisulphide, ethyl butyrate, indole, and α-thujone. These compounds were selected as they elicited a strong, constant response at a dilution of 10^{-3} in at least one pair the D sensilla (Figure 3). The overall response of the ORNs in all 3 sensillum types was generally sigmoid, often spanning 2 or more orders of magnitude (Figure 5). Olfactory receptor neurons housed in Dβ and Dγ sensilla responded to most of the tested compounds at high concentrations, in contrast to ORNs housed in Dα sensilla. These high concentration responses are clearly visible with α-thujone, as this compound elicits a high firing rate increase in all of the 6 sensilla at 10^{-1} and 10^{-2} (Figure 5). In contrast, only hexenal in the Dα sensilla, dimethyl trisulfide and octenal in the Dβ sensilla, and sulcatone in the Dγ sensilla gave rise to electrophysiological

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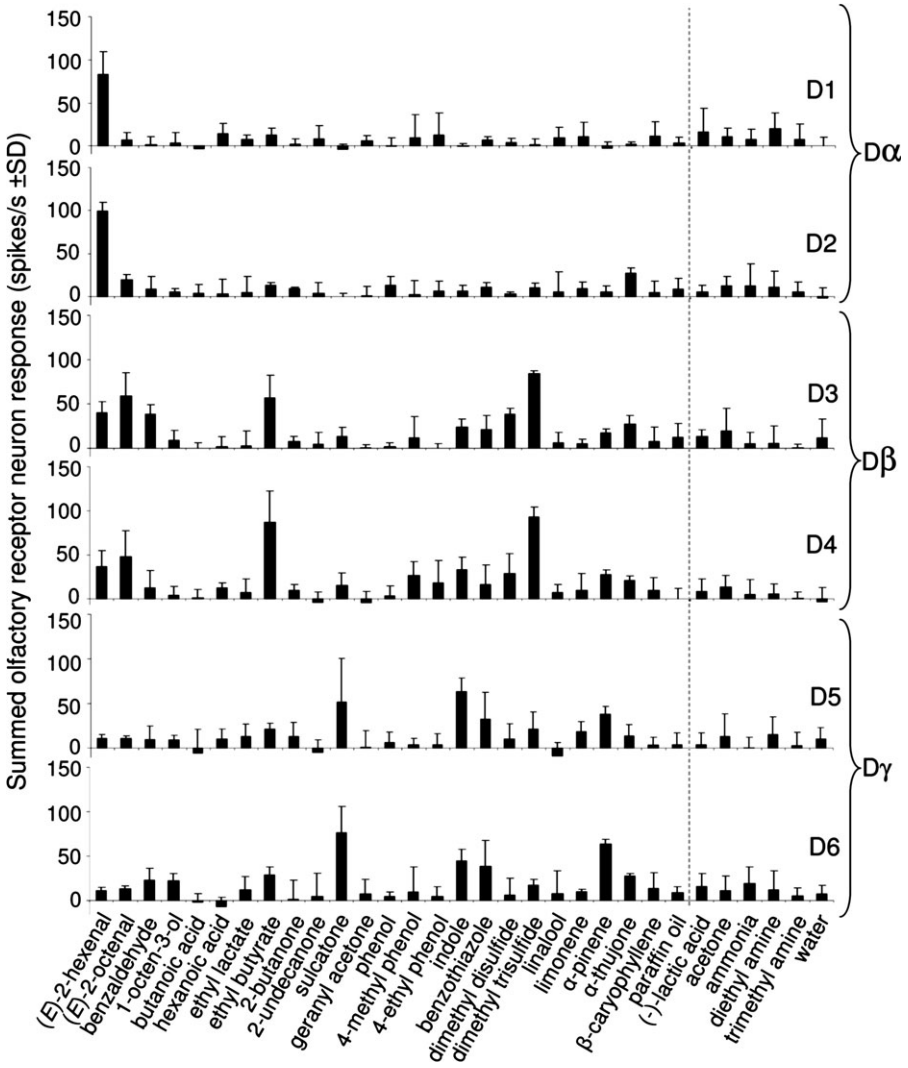

Figure 3 Response spectra of the olfactory receptor neurons housed in the smooth peg sensilla (i.e., 6 D sensilla with $n = 5$) after injection of 31 compounds at a 10^{-3} dilution.

responses when low doses passed over the sensilla (Figures 3 and 5). The threshold level was not clearly reached with 10^{-6} dilution for some of the compounds such as sulcatone and indole. However, as the response show a large standard error, we may venture the hypothesis that they may be different ORNs within this sensillum type exhibiting different sensitivities to these compounds.

ORNs in C sensilla detect ammonia

A cluster analysis on all the 18 recordings on C sensilla (not shown) gave rise to a single sensillum type among these sensilla (Figures 1 and 2). This sensillum type consistently responded to ammonia (Figure 6), and a dose–response test with this compound revealed a sigmoid curve with a threshold at 5×10^{-4}, reaching saturation at 5×10^{-2}. Large variations of responses were recorded with this compound, and even if we may hypothesize that C sensilla house ORNs exhibiting different sensitivities, we were not able to differentiate functional subtypes. Butanoic acid was also tested as it elicited a low response at 10^{-3}, but consistent responses were only observed at higher concentrations (Figure 7).

Two physiological types of E sensilla

The E sensilla house 1 or 2 ORNs, characterized by an irregular spontaneous activity (Figure 2). Fourteen E sensilla of the 21 tested had a very irregular and low spontaneous firing rate (11 ± 3 spikes/s; $n = 796$ recordings), whereas the remaining sensilla had a higher and more regular spontaneous spike frequency (31 ± 6 spikes/s; $n = 355$ recordings). There was no correlation between the number of neurons and spontaneous activity. None of the 31 tested compounds of our panel elicited any response from any of the 14 E sensilla recorded (Figure 6).

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Discussion

Most insects, including bed bugs (Usinger 1966; Aboul-Nasr and Erakey 1968), are dependent on their olfactory system to guide them to resources in their environment. In order to elucidate the mechanisms by which olfactory information is encoded, and ultimately how this information is guiding the insect behavior, an understanding of the functional characteristics of the peripheral olfactory organ is required. Most peripheral olfactory organs are too complex to fully characterize as they include a high number of sensilla (McIver 1987). However, the common bed bug's major olfactory organ only contains 44 olfactory sensilla per antenna (Steinbrecht and Müller 1976). These sensilla can be individually recognized and are easily accessible with single sensillum recording electrodes, which allowed us to functionally characterize the antennal olfactory organ in quite some detail. Seven compounds were specifically detected by the ORNs housed in the 6 smooth pegs, and by using their response pattern to all the tested compounds, we were able to cluster them in functional pairs. The ORNs of the grooved pegs responded specifically to ammonia, whereas among the 31 compounds tested in this study, none elicited any responses in the E sensilla's ORNs.

The smooth peg sensilla, named D sensilla by Steinbrecht and Müller (1976), are structurally similar to the E sensilla of the kissing bug *Triatoma infestans* (Bernard 1974) and the blunt-tipped sensilla trichodea of mosquitoes (McIver 1982). This sensillum type houses a large number of ORNs in bed bugs and kissing bugs, but the morphological equivalents among mosquitoes' sensilla house only 1 or 2 ORNs. The large number of ORNs lead Bernard (1974) to declare that, for kissing bugs, this type of sensilla are not favorable for precise SSR. However, this electrophysiological technique permits a precise measurement of the neuronal activity during stimulation, contrary to electroantennogram recordings, which give general information on the olfactory perception. Our single sensillum study thus gave information on the bed bug's olfaction capability, sensillum by sensillum, even if the threshold of odor detection was probably underestimated due to electrical activity from several ORNs. However, the dose–response measurements enabled us to select the most adequate and efficient binding compounds. Thus, 7 of the 31 compounds tested at and below 10^{-3} dilution elicited replicated electrophysiological responses of the ORNs.

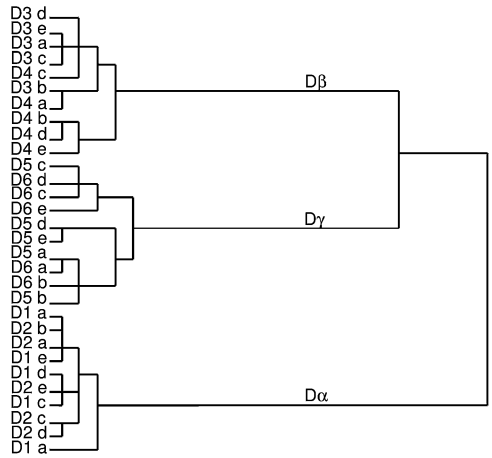

Figure 4 Dendrogram of a Ward cluster analysis of ORNs housed in D sensilla, comparing their overall response to 29 compounds diluted at 10^{-3}. Branch lengths are proportional to distance and permit the division into 3 clusters, Dα, Dβ, and Dγ, thus grouping the smooth peg sensilla into pairs: D1-D2, D3-D4, and D5-D6. Letters (a–e) correspond to the 5 replicate recordings made for each D sensilla.

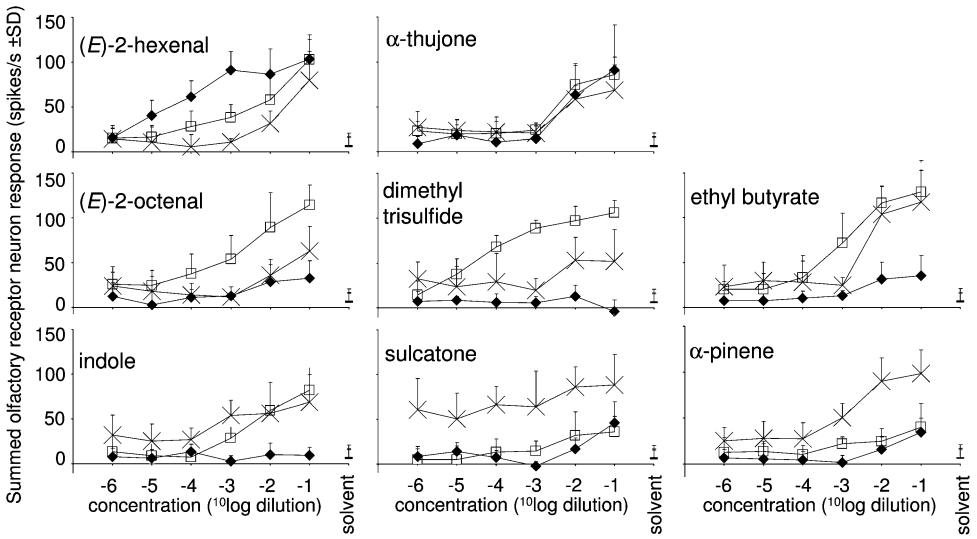

Figure 5 Firing rate increase of the olfactory receptor neurons housed in the 3 functional classes found among the 6 smooth peg sensilla (i.e., Dα [filled diamond], Dβ [open square], and Dγ [cross] with $n = 10$ for each) after injection of hexenal, octenal, ethyl butyrate, sulcatone, indole, dimethyl trisulfide, α-pinene, and α-thujone at 6 different doses ranging from 10^{-6} to 10^{-1} dilution steps.

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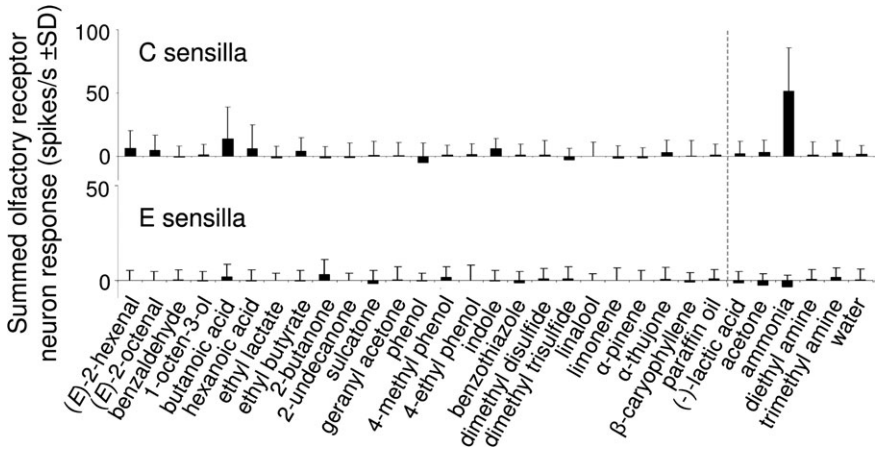

Figure 6 Response pattern of the olfactory receptor neurons housed in grooved peg sensilla (i.e., 9 C sensilla with $n \geq 18$) and in hair sensilla (i.e., 21 E sensilla with $n \geq 21$) after injection of 31 compounds at a 10^{-3} dilution.

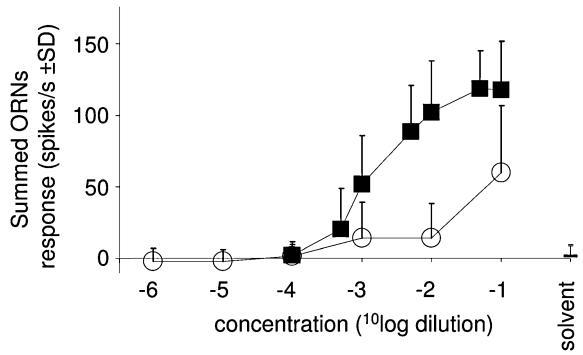

Figure 7 Firing rate increase of the olfactory receptor neurons housed in the grooved peg sensilla (i.e., the 9 C sensilla with $n \geq 18$) after injection of ammonia (filled square) at 7 doses (from 10^{-4} to 10^{-1} with corresponding half-dilution steps) and butanoic acid (open circle) at 6 doses (from 10^{-6} to 10^{-1} dilution steps).

The 7 electrophysiologically active compounds identified in our study belong to several chemical classes and have previously been found to be used as signals for different purposes such as host-, refuge-, or oviposition-site localization. Despite the fact that haematophagous arthropods have very different life histories, some compounds are commonly present in specific places of interest, and they have been found to be perceived communally and trigger similar behaviors. For example, compounds produced from biological degradation of amino acids, such as indole from tryptophane, phenols from tyrosine, or sulfur compounds from methionine and cysteine, may indicate suitable oviposition sites (Davis 1976; Blackwell et al. 1993; Dougherty et al. 1995; Jeanbourquin and Guerin 2007a) as well as hosts (Costantini et al. 2001; Meijerink et al. 2001; Puri et al. 2006; Jeanbourquin and Guerin 2007b; Gallagher et al. 2008; Harraca et al. 2009) for many haematophagous insects (Table 1). Among the aromatic compounds tested on the antennal olfactory organ of *C. lectularius*, indole evoked an increase in the firing rate, whereas phenolic compounds did not. Among sulfides, dimethyl trisulfide triggered a dose-dependent increase of electrical activity in the ORNs housed in the Dβ sensilla. Several terpenes and ketones are reported to be perceived by haematophagous insects (Table 1), and these compounds have been reported in host emanations (Gikonyo et al. 2002; Gallagher et al. 2008; Logan et al. 2008; Syed and Leal 2009) as well as in plants (Bowen 1992; Syed and Guerin 2004), which provide nectar feeding and refuge. Among the 5 terpenes tested in this study, only α-pinene elicited a response at 10^{-3} dilution in Dγ sensilla. Limonene and geranyl acetone have been identified as being behaviorally active components of the aggregation pheromones of *C. lectularius* (Siljander et al. 2008), but we did not find any ORNs responding to these compounds in the antennal olfactory sensilla of the bed bug. Siljander et al. (2008) also pointed out sulcatone as one essential cue of the odor of bed bug's refuge, and this ketone gave significant responses in the Dγ sensilla.

We were able to distinguish 3 functional types of sensilla, Dα, Dβ, and Dγ, based on the electrophysiological responses of the ORNs housed in these sensilla. These types are distributed pair-wise on O_1, where the Dα sensilla, D1 and D2, are located at the distal tip of the flagellum; the Dγ sensilla, D5 and D6, at the proximal end; and the Dβ sensilla, D3 and D4, are distributed in between Dα and Dβ. The functional characterization resulting from the hierarchical cluster analysis corresponds well with the described morphological characterization of these sensilla (Steinbrecht and Müller 1976). They morphologically differentiated D1 and D2 (i.e., Dα) sensilla from D3, D4, D5, and D6 (i.e., Dβ and Dγ) sensilla based on the number of ORNs' bundles they housed (Steinbrecht and Müller 1976). Our cluster analysis showed that Dβ and Dγ sensilla are functionally more related to each other than to Dα sensilla (Figure 4).

Compared with the 2 other olfactory sensillum types, the grooved peg sensilla, named C sensilla by Steinbrecht and

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Müller (1976), have a thicker wall structure (Steinbrecht and Müller 1976), which suggests that they house ORNs detecting other compound classes. Indeed in other haematophagous insects, the ORNs housed in the grooved pegs did not respond to compounds such as 1-octen-3-ol, 3-methyl phenol, limonene, sulcatone, indole, or α-pinene, which are instead detected by ORNs housed in other sensillum types (Davis 1976; Davis 1977; Guerenstein and Guerin 2001; Qiu et al. 2006; Syed and Leal 2009). However, electrophysiological responses to ammonia have been shown in grooved pegs of kissing bugs (Bernard 1974; Taneja and Guerin 1997; Guerenstein and Guerin 2001) and different mosquito species (Davis 1976; Davis 1988; Bowen 1995; Meijerink et al. 2001; Syed and Leal 2009). Ammonia is the final degradation step of proteins and indicates various resources such as entry of the refuge and presence of a host or an oviposition site (Table 1), and we found in concordance that ORNs housed in C sensilla of bed bugs respond to ammonia in a dose-dependent manner.

The hairs, E sensilla according to Steinbrecht and Müller (1976), are structurally similar to type D sensilla of kissing bugs (Bernard 1974) and long point-tipped sensilla trichodea of mosquitoes (McIver 1982). We noticed 2 rhythms of spontaneous firing rates in these E sensilla, which might correspond to the 2 morphological E-types only distinguishable by cross-sectioning (Steinbrecht and Müller 1976). Similar to that reported from other haematophagous insects, we were unable to observe any responses to any of the tested odorants (Bernard 1974, Ghaninia et al. 2007; Hill et al. 2009; Syed and Leal 2009), but this sensillum type is believed to be the site of pheromone detection. The bed bug E sensilla were previously reported to house ORNs responding to alarm pheromone (i.e., hexenal and octenal), as Levinson et al. (1974) measured electrophysiological responses to these self-produced compounds. However, these authors also mentioned that other receptor cells of adjacent sensilla may have contributed to the recorded receptor potentials. Thus, our study concludes that ORNs responding to these alarm pheromones are exclusively housed in the D sensilla. Hexenal is perceived at low concentration in the Dα sensilla and have a much lower threshold than octenal perceived in the Dβ sensilla. This result supports the behavioral experiments of Levinson et al. (1974), which revealed a stronger reaction in bed bugs stimulated by hexenal than in individuals stimulated by octenal.

As suggested by Steinbrecht and Müller (1976) and McIver (1987), a close association between the obligatory blood-feeding bed bugs and their host may have led to a decrease and a conservation of the most fundamental ORNs. Therefore, the few compounds for which *C. lectularius* still possesses receptors are essential for its sensory ecology, as well as remains from its primary habitats in caves and its hosts. Even if other ligands for the remaining ORNs housed in the bed bugs olfactory sensilla still need to be identified, this study permits to refine our understanding about the olfactory capabilities of *C. lectularius* compared with other haematophagous insects. Thus, we found that all the 9 grooved pegs housed ORNs detected ammonia and that 3 different classes of paired smooth pegs housed ORNs sensitive to low amounts of hexenal, octenal, ethyl butyrate, sulcatone, dimethyl trisulfide, and α-pinene.

This is the first single sensillum electrophysiological study conducted on bed bugs, *C. lectularius* and it comprises of recordings from all olfactory sensilla in the olfactory region of the antennae. Behavioral experiments will further evaluate the biological effect of these compounds detected and could eventually lead to an environmentally sustainable kairomone- and/or pheromone-based control against this reinvading blood-feeding pest.

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