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Chemicals in Laboratory Room Air Stimulate Olfactory Neurons of Female *Bombyx mori*

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Abstract

Laboratory air contained odorants that elicited electrophysiological responses in female *Bombyx mori* antennae. Air entrainments on charcoal filters, extracted with CS_2 and subsequently with acetone, were analyzed by coupled gas chromatography (GC)–electroantennogram (EAG) as well as by GC–mass spectrometry. The CS_2 extract contained 12 EAG-active peaks from which benzaldehyde, octanal, limonene, 1,8-cineol, methyl benzoate, nonanal, decanal and geranyl acetone were identified. In the acetone extract we identified eight EAG-active peaks as phenol, nonanal, 2-ethylhexanoic acid, octanoic acid, benzoic acid, nonanoic acid, decanoic acid and dimethyl phthalate. The concentrations of benzoic acid and benzaldehyde present in laboratory air were determined. The origin of the substances and importance of such odorants in laboratory air for the interpretation of physiological experiments on the olfactory system is discussed.

Introduction

The sensilla trichodea on the female antenna of *Bombyx mori* are innervated by two sensory neurons. One of these neurons is called 'terpene cell' (firing larger nerve impulses) since it responds to many terpenes, including (±)-linalool (3,7-dimethyl-1,6-octadien-3-ol), which is used here as a standard stimulus. The most effective compound known so far is a compound related to linalool, 2,6-dimethyl-5-hepten-2-ol. The second sensory neuron in the sensilla trichodea is called 'benzoic acid cell'. It fires smaller nerve impulses and responds best to benzoic acid, and with less sensitivity to benzaldehyde and various other chemicals (Priesner 1979). Since both types of neurons in the sensilla trichodea respond to secondary plant compounds the sensilla might be involved in host-plant selection (Bernays and Chapman, 1994). However, the only natural source known to stimulate the benzoic acid cell was the meconium, the intestinal waste products of the pupae, excreted by the adult upon disturbance a short time after eclosion (Heinbockel and Kaissling, 1990, 1996). No behavioral response of females to meconium or benzoic acid was observed.

While most terpene cells did not show any background activity of impulse firing, the background discharge rate of the benzoic acid cell in air was high, in the range of 3–10 impulses/s (Heinbockel and Kaissling, 1990, 1996). Previous experiments (Stange and Kaissling, 1995) indicated that there were chemicals in the laboratory room air responsible for the background activity. In the tip recording technique, routinely used in our laboratory for recordings on moths, normally only the tip of the hair is covered by the recording electrode. After covering the whole hair by pushing the electrode down to the hair base, it is still possible to record impulses of the cells, but no airborne stimuli may reach the hair and elicit impulse firing. With the hair covered, the background impulse frequency of the benzoic acid cell dropped considerably. The same reduction of background activity was obtained by blowing air over the preparation that was rigorously filtered with activated charcoal. These observations led us to search for chemicals present in room air that caused the permanent background activity of the benzoic acid receptor neuron. For two of the compounds identified, benzoic acid and benzaldehyde, we estimated the concentrations found in laboratory room air.

Material and methods

Insects

Adult female *B. mori* were obtained from pupae bred at the Instituto Sperimentale per la Zoologia Agraria, Padova, Italy; at the INRA Unité nationale séricole, La Mulatiére-France; and at Worldwide Butterflies, Sherborne, UK. Pupae were maintained at room temperature. Adult females

were kept at 12°C and used for the experiments within 10 days.

Air extracts

The volatile constituents in air were adsorbed on an activated charcoal trap (5 mg of carbon in a glass tube, Fisons Instruments, Mainz, Germany) connected with a suction pump (flow rate 1.7 l/min). The sampling time ranged from 2 to 50 h in the years 1996 to 1998. The samples further analyzed by coupled GC–MS were drawn in August 1997 and September 1998 for 8–10 h overnight with no persons present in the laboratory. The adsorbed volatiles were first eluted with 200 µl CS2 (Fluka, Buchs, Switzerland) as solvent (primary extract). After washing with 1 ml of CS_2 the filter was extracted with 200 µl of acetone (Roth, Karlsruhe, Germany), distilled over a Vigreux column (secondary extract). The extracts were stored in glass vials at –20°C. In addition to room air, compressed air from the local pressurized air system and outdoor air were also sampled.

Gas chromatography (GC)

GC analyses of the air extracts were conducted on a Shimadzu GC–17A instrument fitted with a split/splitless injector (220°C) and a flame ionization detector (FID, 260°C). The compounds were separated on a fused silica capillary column (25 m × 0.25 mm i.d., PERMABOND® SE-54, Macherey-Nagel, Düren, Germany). The carrier gas was nitrogen (1.3 ml/min). At the end of the column the flow was split, one half going to the FID, the other half to the EAG recording system. Injections onto the analytical column were made with the split valve closed for 30 s; the temperature was held at 50°C for 4 min, then increased by 5°C/min to 260°C and held at 260°C for 5 min.

Electrophysiology

Electroantennograms (EAG) were recorded using Ag–AgCl glass electrodes filled with hemolymph Ringer (Kaissling, 1995). Antennae from unmated females were cut off at the base and at the tip, and electrodes were inserted in the antennal stem. Thus the excised antenna was suspended between the two electrodes. The proximal electrode was connected to ground, the distal one to a high impedance amplifier (Syntech AM-92). Signals generated by the antennae were displayed on a computer screen (Syntech EAG software and hardware). For EAG tests, pure chemicals were applied to filter paper (1 cm^2) and placed into Pasteur pipettes. Each stimulus (1 s) was delivered into a purified and humidified airstream (700 ml/min) flowing continuously over the preparation via a Teflon-coated metal tube. In the coupled GC–EAG, the outlet of the column (~14 cm from the preparation) delivered the separated chemicals into the continous air stream blown over the preparation.

Electrophysiological recordings from single sensilla trichodea were done as described previously (Kaissling, 1995). In short, we used the cut tip recording technique on isolated antennae with glass capillary Ag–AgCl electrodes (tip diameter: recording electrode 10–15 µm; DMZ-Universal Microelectrode Puller, Zeitz Instruments, Augsburg, Germany). The recording electrode was filled with sensillum lymph Ringer; the reference electrode filled with hemolymph Ringer (Kaissling, 1995) was inserted in the antennal stem. The preparation was constantly emersed in a humidified airstream. During stimulation this continuous air stream was switched off and the air was led through a glass cartridge with the filter paper loaded with odorants, and over the antenna.

Coupled GC–mass spectrometry (MS)

Identifications of the compounds were done on a gas chromatograph with a splitless injector (200°C) and a mass detector (Fisons MD 800), working in electron impact ionization mode (ion source temperature 200°C). A BPX5 column (SGE, 30 m × 0.22 mm, film thickness 0.25 mm) and helium as carrier gas (flow 0.55 ml/min at 50°C) were used for the separations. The temperature program was identical to that used for GC–EAG. The identification of compounds was based on their mass spectra compared with those in the National Institute of Standards and Technology Library (NIST, USA) and those in the *Wiley Registry of Mass Spectral Data* (6th edn), and on the co-chromatography with synthetic or commercially available standards. A mixture of *n*-alkanes (C9–C25) was used to correlate the corresponding peaks in GC–EAG and GC–MS runs. Tentative identifications by GC–MS were confirmed by comparison with authentic samples in relation to their electrophysiological activity as well as to their retention times in the GC–EAG system.

Stimulus compounds

All chemicals were purchased from Fluka except benzaldehyde (Sigma-Aldrich, Munich, Germany) and limonene (Roth). Purity of the substances was >95% for nonanoic acid and limonene, and >98% for all other substances. Dilutions for the electrophysiological recordings were made in double-distilled acetone. Liquid stimulus compounds were used as pure compounds or diluted in paraffin oil (Roth), 50 µl of the stimulus solution being loaded onto filter papers (1 cm^2).

Results

Air extracts excite single receptor neurons

Our first question was whether extracts of room air elicited responses of the benzoic acid cell, the sensory neuron responding to air contaminants (see introduction). Significant nerve impulse responses were obtained with extracts of room air as well as with air from the local pressurized system, but not with outdoor air (Figure 1). Surprisingly

the primary CS_2-extracts of room air and compressed air were inactive in single sensillum recordings, whereas the secondary acetone extract excited the benzoic acid cell. The activities of the secondary (acetone) extracts of room air and compressed air were equivalent to ~2 and 5 µg of benzoic acid respectively. On the other hand, the terpene cell did not respond to any of the extracts (Figure 1).

Identification of active chemicals in laboratory air

The next step of the study was to run gas chromatograms of the air extracts. The primary CS_2 extracts from indoor air and from compressed air gave similar GC spectra, with >100 peaks each. The secondary acetone extracts showed ~20 peaks. The extracts made from outdoor air showed much fewer and mostly smaller peaks than indoor extracts. The overall peak area of outdoor air was only 5–10% for the CS_2 extract and ~10–25% for the acetone extract of the indoor air values respectively. While indoor and compressed air showed numerous higher-molecular-weight substances eluting after 20 min, these were almost completely missing in outdoor air.

Coupled GC–EAG of the CS_2 extract revealed 12 peaks that led to EAG responses (Figure 2, Table 1). Among these we could identify the following compounds by comparison with known mass spectra (arranged according to increasing retention time): benzaldehyde, octanal, limonene, 1,8-cineol, methyl benzoate, nonanal, decanal and geranyl acetone. In the acetone extract we could identify eight EAG-active peaks (Figure 3, Table 2): phenol, nonanal (again), 2-ethyl-

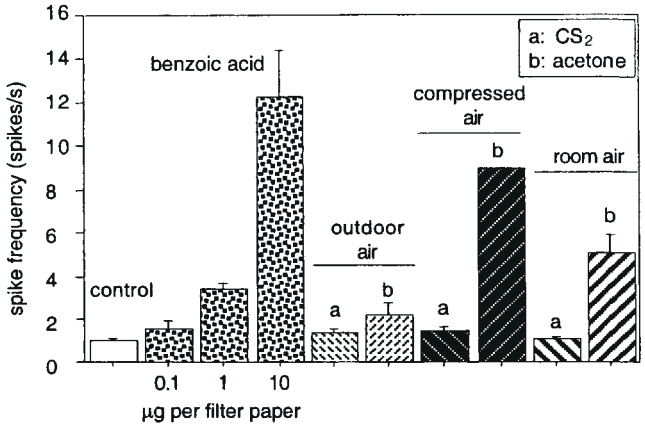

Figure 1 Responses of the benzoic acid receptor neuron to 1 s stimuli of three concentrations of benzoic acid and of three types of air extracts (collected by charcoal filters for 50 h with 1.7 l/min flow rate). The primary 200 µl CS_2 extracts **(a)** elicited reactions not significantly different from control (purified air). The secondary acetone extract **(b)** excited the benzoic acid receptor neuron. Averages (± SE) from 1–40 sensilla.

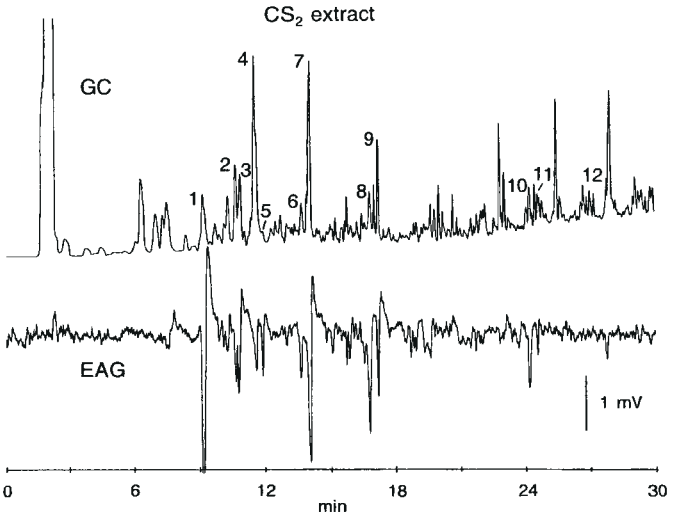

Figure 2 GC–EAG analysis of the primary CS_2 extract of room air. Numbers denote compounds eliciting EAGs. Some of the compounds were identified using MS: (1) benzaldehyde, (2) octanal, (4) limonene, (5) 1,8-cineol, (6) methyl benzoate, (7) nonanal, (9) decanal, (10) geranyl acetone. Compared with Figure 3, the GC signals are reduced to 40%. The EAG signals were AC-filtered ($t_{½}$ = 10 s).

Table 1 Active peaks from the CS_2 extract and the effects of the identified compounds on single receptor neurons

Peak number in Figure 2	Chemical	Retention time (min)	Effect on single benzoic acid cells	Effect on single terpene cells
1	benzaldehyde	9.43	+	0
2	octanal	10.85	+	+
3	unidentified	11.06		
4	limonene	11.74	0	+
5	1,8-cineol	12.13		
6	methyl benzoate	14.17		
7	nonanal	14.26	+	+
8	unidentified	17.18		
9	decanal	17.51	+	+
10	geranyl acetone	24.63	–	?
11	unidentified	25.40		
12	unidentified	28.24		

+ = excitation, – = inhibition, 0 = no effect.

hexanoic acid, octanoic acid, benzoic acid, nonanoic acid, decanoic acid and dimethyl phthalate.

Electrophysiological responses to room air chemicals

The identified chemicals were tested as pure substances for their electrophysiological activity. All of them led to dose-dependent EAGs. Single sensillum recordings with the pure compounds showed that most of the identified chemicals act on one or both of the sensory neurons within the sensilla trichodea (Figures 4 and 5). Interestingly, the fatty acids with 8–10 carbon atoms (Figure 5) as well as the corresponding aldehydes (Figure 4) activated both of the neurons. The effects of these compounds were weaker with increased chain length.

Not only the response strength but also the time course of the responses varied for different compounds. The relatively fast decline (repolarization) of the receptor potential after the end of stimulation is characteristic of the most potent compounds, linalool and benzoic acid. In contrast, most of the other excitatory compounds showed a slower decline and sometimes also a slower rise of the receptor potential at the onset of the stimulus. Furthermore, the action potentials of the terpene cell started with a delay upon stimulation with octanoic and nonanoic acid (Figure 5), in contrast to the immediate onset of spiking upon octanal and nonanal stimuli (Figure 4).

Geranyl acetone (Figure 4) and phenol (Figure 5) regularly led to hyperpolarization, both inhibiting the background activity of the benzoic acid cell. Finally, dimethyl phthalate (Figure 5) elicited nerve impulses from the terpene cell. In spite of a pronounced increase in spike frequency, the receptor potential was relatively small or even absent. This effect could indicate that, together with a depolarization of the terpene cell, the benzoic acid cell within the same hair was hyperpolarized, both effects cancelling each other out.

It should be noted that the compounds inhibiting (i.e. hyperpolarizing) neurons of the sensilla trichodea still elicited negative EAGs, indicating excitation of receptor neurons (Figures 2 and 3). This suggests that sensory neurons of other types of sensilla must have been depolarized and governed the EAGs.

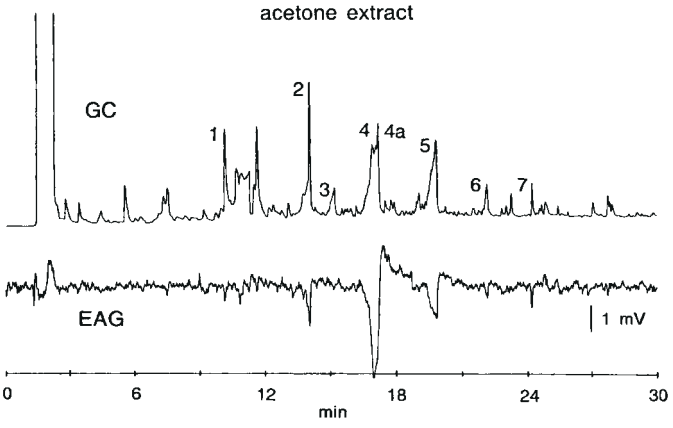

Figure 3 GC–EAG analysis of the secondary acetone extract (cf. Figure 2). EAG-active compounds: (1) phenol, (2) nonanal, (3) 2-ethylhexanoic acid, (4) octanoic acid, (4a) benzoic acid, (5) nonanoic acid, (6) decanoic acid, (7) dimethyl phthalate. Compared with Figure 2, the GC signals are amplified by a factor of 2.5. The EAG signals were AC-filtered ($t_{½}$ = 10 s).

Quantitative determination of benzoic acid and benzaldehyde in room air

Since benzoic acid and benzaldehyde strongly activate the benzoic acid cell (De Brito Sanchez, 1996), we focused on these chemicals for quantitative determination of their concentrations in air. For calibration known concentrations of benzaldehyde and benzoic acid were injected into the GC. The room air was then sampled for various times (2, 4 and 12 h), and the extracts were investigated for their amounts of benzoic acid and benzaldehyde. The amount of chemicals extracted increased linearly with the sampling time, indicating that the charcoal filters were not saturated after 12 h. Comparing the peak areas of the chemicals sampled from room air with the calibration curve, and considering the flow rate of the suction pump, we obtain apparent concentrations of benzaldehyde and benzoic

Table 2 Active peaks from acetone extract and the effects of the identified compounds on single receptor neurons

Peak number in Figure 3	Chemical	Retention time (min)	Effect on single benzoic acid cells	Effect on single terpene cells
1	phenol	11.04	–	?
2	nonanal	14.00	+	+
3	2-ethyl-hexanoic acid	15.22	+	0
4	octanoic acid	17.07	+	+
4a	benzoic acid	17.19	+	0
5	nonanoic acid	19.93	+	+
6	decanoic acid	22.45	+	(+)
7	dimethyl phthalate	24.64	–	+

+ = excitation, – = inhibition, 0 = no effect.

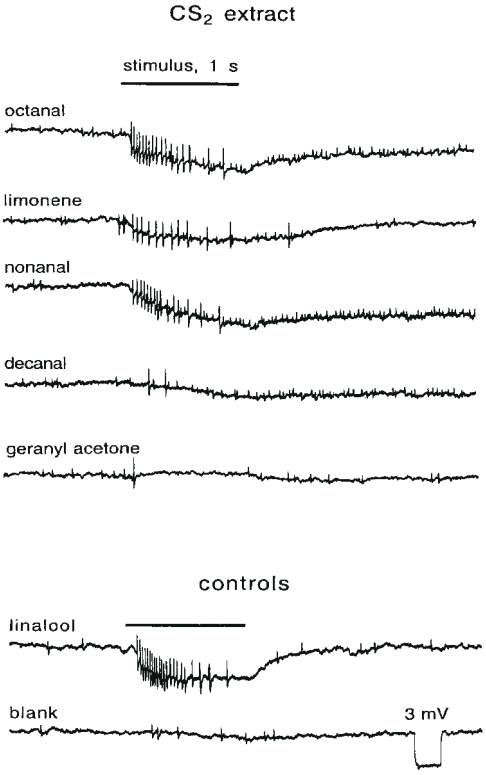

Figure 4 DC recordings from a single sensillum trichodeum. **Top:** Responses to selected compounds (50 µl per filter paper) identified from the CS_2 extract of room air. Large nerve impulses: terpene receptor neuron. Small nerve impulses: benzoic acid receptor neuron. **Bottom:** Controls show a typical response to the standard ± linalool (50 nl in 50 µl paraffin oil per filter paper) and a response to humidified but unfiltered compressed air.

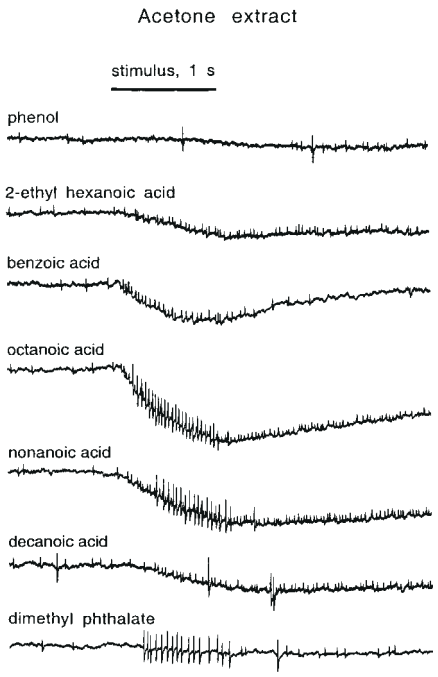

Figure 5 DC recordings from the same sensillum trichodeum as in Figure 4 (see legend to Figure 4). Responses to compounds found in the acetone extract of room air. 50 µl of each compound per filter paper, except for phenol (1 mg per filter paper), benzoic acid (1 µg per filter paper), and decanoic acid (100 µg per filter paper).

acid in room air as 0.07 ± 0.01 and 0.03 ± 0.005 p.p.b. respectively.

These values need to be corrected since benzoic acid may be formed as an oxidation product of benzaldehyde during the filtering of air by the charcoal trap. In order to test this we blew air for 90 min over a filter paper soaked with 50 µl of paraffin oil mixed with 500 µg of freshly distilled benzaldehyde and filtered it by a charcoal trap. In addition to 35 µg of benzaldehyde in the CS_2 extract of this trap we found 2 µg of benzoic acid in the acetone extract. Thus 5.4% of the trapped benzaldehyde must have been oxidized to benzoic acid. From this value the 'true' concentrations in air for benzaldehyde and benzoic acid can be calculated as 0.074 and 0.026 p.p.b. respectively.

Discussion

The idea of investigating room air volatiles that stimulate olfactory neurons of insects seems quite artificial in the first place. However, since many experiments on the olfactory system are conducted in exactly such an environment, any influence of chemicals in the laboratory air on the olfactory neurons must be of interest. Often experimental animals are exposed to room air volatiles for many days before they are tested. For instance, room air volatiles could influence behavioral responses of insects to odorants. Thus it may be possible that the lack of behavioral responses of female *B. mori* to meconium and benzoic acid was due to contaminations in room air.

It cannot be excluded that cells adapt to a permanent low-level stimulus in the room air and respond differently than non-adapted ones to further olfactory stimulation. Stange and Kaissling (Stange and Kaissling, 1995) showed that the benzoic acid cell in the trichoid sensilla of female *B. mori* was permanently stimulated by chemicals in the laboratory air and fired nerve impulses without measurable adaptation, even if exposed for hours. However, a lack of receptor cell adaptation does not exclude adaptation of cells in the CNS controlling behavioral reactions (habituation). Examples of permanent stimulation and non-adaptive responses were reported from the carbon dioxide receptors of several species. These neurons were constantly stimulated by the normal CO_2 content of 0.04% in air and their responses to odorants were influenced by the background concentration of CO_2 (Bogner, 1990; Ziesmann, 1996).

Identification of electrophysiologically active compounds

CS_2 is described as the best solvent to desorb organic volatiles from a charcoal trap (Habich and Grob, 1984; Stein and Narang, 1996). The unexpected responses to the secondary (acetone) extract in the electrophysiological

experiments together with the high number of peaks detected in the GC experiments revealed that the secondary acetone extract contained a number of active volatile constituents which could not be eluted with CS_2, obviously chemicals of higher polarity, such as carbonic acids including benzoic acid. The latter compound is known as the most potent stimulant for the benzoic acid receptor neuron of the sensilla trichodea (Priesner, 1979).

Coupled GC–electrophysiology techniques have been employed to identify a wide range of insect and plant semiochemicals (Wadhams, 1990). Using GC–MS techniques we could identify a total of 16 chemicals from room air that excited or inhibited the sensory neurons innervating the sensilla trichodea of female *B. mori*, the benzoic acid cell and the terpene cell. A number of compounds excited both types of neurons. This is surprising since the most effective compounds of the two types of neurons appear to be chemically quite different. However, it should be noted that the compounds acting on both neuron types had to be applied in concentrations of several orders of magnitudes higher than those necessary for the most potent compounds benzoic acid, 2,6-dimethyl-5-hepten-2-ol and linalool (Figures 4 and 5).

Interestingly, phenol and geranyl acetone produced hyperpolarization and inhibition of impulse firing of the benzoic acid cell. This was not a general anesthetic effect of these substances as they elicited normal (negative) EAGs, indicating that there were other olfactory neurons on the antenna that responded with depolarization. The neurons producing the observed EAG responses may belong to the sensilla basiconica and sensilla coeloconica on the antennae. The latter are known to respond to a variety of chemicals, including octanal, nonanal, octanoic acid, nonanoic acid and decanoic acid (Pophof, 1997), that were found in the laboratory air. High background activities with an average of 17 impulses/s were reported for neurons innervating sensilla coeloconica. It has been tested that this background activity was not elicited by room air volatiles (Pophof, 1997). The sensory neurons within sensilla basiconica of *B. mori* respond to terpenes (eugenol, nerolidol, terpineol) and also to kresol, creosol and ethyl phenyl ether; often they are inhibited by linalool (B. Pophof, personal communication).

The variety of cell responses observed of the olfactory neurons, such as excitation and inhibition, fast and slow time courses of receptor potentials, and nerve impulse responses without and with delay, needs further investigation. One goal would be to find or confirm the most effective compounds for the two types of receptor neurons, especially for the terpene cell. While a natural stimulus for the benzoic acid cell is the meconium (containing benzoic acid), the biological function of the terpene cell of the female *B. mori* has not been investigated yet.

Benzaldehyde and benzoic acid in laboratory air

So far it seems clear that most of the compounds in our air extract elicited EAGs but were not concentrated enough to elicit single cell responses. Benzaldehyde gave a substantial peak in the GC of the CS_2 extract (Figure 2). Nevertheless the CS_2 extract produced practically no response of the benzoic acid cell in the single cell recordings, although this type of neuron is sensitive to benzaldehyde (De Brito Sanchez, 1996). The relatively large EAG produced by the benzaldehyde peak of the CS_2 extract (Figure 2) might indicate that receptor neurons other than the benzoic acid cell respond to benzaldehyde and contribute to the EAG. The only single cell response observed of the benzoic acid cell was the response to the acetone extract of room air (and compressed air) containing benzoic acid.

The permanent background activity of the benzoic acid cell observed in room air could, in principle, be due to benzaldehyde and benzoic acid found in the room air extracts, with concentrations of 0.074 and 0.026 p.p.b. respectively. However, since the sensitivity of the benzoic acid cell is more than 10 times higher for benzoic acid than for benzaldehyde (De Brito Sanchez, 1996), the background activity of the cell must be mainly due to the benzoic acid in the room air.

A concentration of 0.026 p.p.b. of benzoic acid in the room air eliciting impulse firing corresponds to 7×10^8 molecules/cm^3 of air. This concentration is in the range of the detection threshold of human noses for a few most powerful odorants (Devos *et al.*, 1990); obviously this concentration is already detected by single benzoic acid cells of *Bombyx mori* females. The human threshold for benzoic acid has not been found in the literature. For comparison, the human threshold for benzaldehyde is ~10^{12} molecules/cm^3 of air, which is ~100-fold higher than the threshold of the benzoic acid cell for benzaldehyde.

Possible sources for the detected chemicals

In principle the chemicals in the laboratory air might have originated from (i) natural sources such as microorganisms, plants, animals or humans within the building; (ii) natural sources outdoors; (iii) former experiments with these substances; or (iv) artificial sources in the building such as motors, paint, furniture, plastics or cleaning material.

It would be highly speculative to name the actual source for one specific chemical. Anyway, as outdoor air contained much less odorants than indoor air, it is unlikely that this was an important source. On the other hand, the compressed air, which was drawn from the outside, contained numerous impurities and elicited olfactory responses. Therefore motors such as those in pumps or compressors might have been a considerable source. Additionally a number of the substances found in laboratory air are emitted by humans. For example, geranyl acetone as well as the C8–C10 acids and aldehydes are all regularly found in human emanations (Dravnieks, 1975; Labow *et al.*, 1979; Sastry *et al.*, 1980; Kanda *et al.*, 1990). Benzaldehyde is often found in carpets, furniture and paint, while dimethyl

phthalate and other phthalates can be found as softeners in nearly all plastic products (Dietert, 1996). Additionally micro-organisms may play an important role (Wessén and Schoeps, 1996). Benzaldehyde is used as an odorant and flavouring chemical, as well as an ingredient in dyes. Benzoic acid has not been described in room air so far; it is used to preserve foods, fats and fruit juices.

Conclusion

While it is unlikely that one of the highly specialized pheromone receptor cells of male *B. mori* can ever be affected by room volatiles, our investigation showed that researchers have to be very careful when olfactory cells with broader reaction spectra are investigated in the laboratory. Olfactory cells that are involved in insect–plant or insect–host interactions usually respond to a variety of odorants in the environment. In particular, in these cases it might be important to exclude possible interactions with room air volatiles in future experiments.

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