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# Electrophysiological studies of olfaction in the whip spider *Phrynos parvulus* (Arachnida, Amblypygi)

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## Abstract

The olfactory response of the whip spider *Phrynos parvulus* from Costa Rica was examined using a technique analogous to that used for insect electroantennograms on the tarsi of the antenniform legs which bear multiporous sensilla. Responses to 42 chemicals representing different chain lengths of alkanes, carboxylic acids, alcohols, aldehydes, and ketones, as well as some esters, monoterpenes, and phenolics were examined. Fifty-four percent of the chemicals tested elicited responses. Concentration–response curves were generated for guaiacol, hexanal, methyl salicylate, benzaldehyde, octanoic acid, and linalool. Guaiacol, benzaldehyde, and hexanol elicited the greatest responses and no differences were detected between the sexes. Compounds with chain lengths of six carbon atoms generated strong responses and most monocarboxylic acids and ring compounds elicited responses. Some compounds produced increases in potential believed to arise from a hyperpolarizing effect on the neurons. The broad spectrum of chemicals to which these animals respond is similar to results of other studies examining the general olfactory sense of insects. It is possible that odor learning plays a significant role in the behavior of amblypygids.

**Keywords:** amblypygid, arachnid, *Phrynos*, olfaction, electrophysiology

## 1. Introduction

The use of chemicals for information transfer is thought to be the oldest form of animal communication and the reliance on chemical cues for intra- and inter-specific signaling and information gathering plays an important role in the lives of many organisms (Bradbury and Vehrencamp, 1998). Most studies of insect olfaction have focused on responses to highly specific stimuli such as pheromones or host odors (Dickens *et al.*, 1993; Bartelt *et al.*, 1995; Chambers *et al.*, 1996; Raguso *et al.*, 1996; Honda *et al.*, 1998; Zhu *et al.*, 1999). The specificity of responses to particular odors such as pheromones has been well documented in insects and has led to a more general understanding of sensory detection and processing. However, when a broad range of chemicals is used to test olfactory responses, insects seem to respond to a wide variety of compounds, suggesting the presence of a

more general olfactory capability in addition to specializations for certain chemicals (Topazzini *et al.*, 1990; Raguso *et al.*, 1996; Sant'Ana and Dickens, 1998).

The central processing of olfactory inputs via glomeruli to the mushroom bodies of the brain (which are thought to function in learning and memory) suggests that behavioral responses to odors may arise from learned associations. The relative sizes of arthropod mushroom bodies vary tremendously and are thought to be correlated to a certain degree with olfactory capability as well as learning and memory. Most arthropods possess mushroom bodies but in the arachnid order Amblypygi the mushroom bodies are so large and convoluted that “they appear to have miniaturized other brain neuropils” (Strausfeld *et al.*, 1998). The enlarged size of amblypygid mushroom bodies suggests that odor recognition and associated learning may be very important in the lives of these nocturnal animals.

The importance of olfaction for arachnids in general has not been well studied and, thus, is not well understood. Although it is generally accepted that spiders possess the ability to smell, or to detect airborne chemicals, there is still debate as to the location and form of a spider's odor receptors and most of the studies indicating the olfactory abilities of spiders are based upon behavioral observations alone (Foelix, 1996). According to both the structural morphology of particular sensillum types and studies of the neuroanatomy of different arachnid groups, it seems likely that there are other orders in which the ability to smell is important, but the degree to which their olfactory capabilities are developed remains unknown. Members of the order Acari, mites and ticks, possess multiporous sensilla with a demonstrated olfactory function (Waladde, 1982) and it has been well documented that pheromones and semiochemicals play important roles in mating behavior and host finding of these animals (Sonenshine, 1985; Hamilton *et al.*, 1989; De Bruyne and Guerin 1994, 1998). Electrophysiological studies have also been conducted on the pectines of scorpions demonstrating that these animals can perceive a wide range of olfactory stimulants (Gaffin and Brownell, 1997).

Since no chelicerates possess antennae, their olfactory glomeruli instead reside in segmental neuromeres associated with other appendages (Strausfeld *et al.*, 1998). In both amblypygids (whip spiders) and uropygids (vinegaroons or whip scorpions) the first pair of legs are elongated into antenniform appendages. The antenniform legs of amblypygids possess hundreds of sensilla encompassing a variety of receptor types including two different types of multiporous sensilla that resemble the olfactory sensilla of insects. The structure and arrangement of the associated sensory neurons are basically the same as those occurring in insect olfactory sensilla (Altner and Prillinger, 1980) and the presumed function of these sensilla is olfactory but this has never been demonstrated experimentally (Foelix *et al.*, 1975; Igelmund, 1987). Due to both the presence of large numbers of presumed olfactory sensilla and the fact that the amblypygid mushroom body is extremely large and well developed, one might predict that the olfactory capability of amblypygids is also extremely well developed.

This study examines the olfactory capabilities of the amblypygid *Phrynus parvulus* using an electrophysiological technique analogous to the electroantennogram (EAG) of insects. Because the experiments involve the modified first walking legs of the animals, the records are referred to as electrolegograms (ELGs). The responses of individuals of both males and females were recorded to a series of compounds representing different chemical classes.

## 2. Materials and methods

### 2.1. Animals

Individuals of *Phrynus parvulus* were collected at night from the arboretum at La Selva Biological Station, Costa Rica, and brought to the laboratory at the University of Arizona where they were housed individually in plastic containers, width 9 cm and height 11 cm. Inside the containers, pieces of screening were taped to two of the walls to provide a vertical surface upon which the amblypygids could climb. Holes were burned into the bottom of the containers through which cotton wicks were placed. The cages were placed on top of reservoirs of water so that half of the cotton wicks sat in the water and the other half remained inside the cage. This design allowed a constant source of water and increased humidity inside the cages. Individuals were kept in an environment chamber on a L12:D12 light cycle at a temperature between 27 and 30°C. The light cycle was reversed so that the lights were out during the day and on throughout the night. Individuals were fed 2–3 live crickets once a week.

### 2.2. Chemicals

All chemicals were obtained from commercial sources (Aldrich, Sigma). The 42 chemicals chosen as olfactory stimuli are shown in Table 1 and included varying chain lengths of monocarboxylic acids, aldehydes, alcohols, and acetates, along with some monoterpenes, and phenolic compounds. All chemicals were dissolved in hexane to give loadings on the delivery filter paper of  $10^{-3}$  g. Concentration series were also prepared for guaiacol, hexanal, benzaldehyde, octanoic acid, linalool, and methyl salicylate to give loadings upon delivery of  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ , and  $10^{-7}$  g.

### 2.3. Electrophysiology

All recordings were made in the first four hours of the animal's scotophase starting at 0800 hours local time. ELGs were measured and recorded using a Syntech INR-2 portable recording unit in the laboratory. Approximately 15 mm of the distal portion of the antenniform leg of a test animal comprising the distal 27 tarsal annuli was cut off and then the most distal annulus of this cut piece was removed. The two ends of the excised piece of antenniform leg were inserted into microcapillaries on the recording unit, with the distal end in the different electrode, so that approximately 9 mm of tarsus remained exposed. This is the portion of the leg bearing the putative olfactory receptors. The hemolymph in the leg was in continuity with the saline solution that filled the microcapillaries. Because there is no information on the ionic composition of amblypygid hemolymph, the

**Table 1.** Chemicals used in the experiments, the numbers of animals tested, electrolegogram responses at a loading of  $10^{-3}$  g relative to the response to guaiacol, and vapor pressures at 24°C.

Chemical class	No.	Chemical	Number		Electrolegogram (%)		Vapor pressure (kPa)
			Males	Females	Mean	SE	
Alkane	1	hexane (10 $\mu$ l)	4	8	6	2	13.79
Carboxylic acids	2	formic acid	4	5	32	7	5.516
	3	acetic acid	5	5	2	6	1.379
	4	propionic acid		4	17	5	0.483
	5	butyric acid		4	18	5	0.138
	6	valeric acid	4	3	6	9	0.034
	7	hexanoic acid	4	5	37	3	0.006
	8	heptanoic acid	4	4	26	8	0.0007
	9	octanoic acid	5	5	44	5	0.0005
	10	ethanol		8	4	8	6.895
Alcohols	11	butanol	2	8	2	2	0.758
	12	pentanol	2	9	0.8	5	0.345
	13	hexanol	6	8	56	9	0.138
	14	heptanol	6	9	9	5	0.034
	15	octanol		7	-27	8	0.010
	16	nonanol		4	-21	8	0.005
	17	decanol		5	-8	4	0.002
	18	undecanol		4	30	36	0.0006
	19	hexenol		1	78	0	?
Aldehydes	20	valeraldehyde	6	5	4	5	4.826
	21	hexanal	5	4	17	4	1.379
	22	heptaldehyde	3	9	0	2	0.483
Ketone	23	cyclohexanone	6	5	25	3	0.552
Esters	24	propyl acetate	1	5	-4	5	4.137
	25	butyl acetate	1	5	-3	2	1.517
	26	pentyl acetate	6	8	2	1	0.483
	27	hexyl acetate		4	-3	6	0.103
	28	amyl acetate	5	5	3	3	0.483
	29	isoamyl acetate	5	5	7	3	0.552
	30	methyl butyrate	1	5	-2	3	4.137
	31	carvone	6	5	21	5	0.014
Monoterpenes	32	citral	2	8	-25	6	0.014
	33	linalool	1	9	-55	9	0.069
	34	$\alpha$ -pinene	1	8	4	2	0.689
Phenolics	35	benzyl alcohol	5	3	56	8	0.014
	36	benzaldehyde	6	9	116	12	0.138
	37	benzyl acetate	1	5	-44	10	0.021
	38	benzyl benzoate	5	5	5	3	$3.45 \times 10^{-5}$
	39	methyl salicylate	6	4	73	6	0.006
	40	coumarin	6	4	63	7	0.006
	41	eugenol		4	39	9	0.0007
	42	guaiacol	6	9	100	0	0.028

saline was one commonly used for physiological experiments with *Manduca sexta* (150 mM NaCl, 3 mM  $\text{CaCl}_2$ , 3 mM KCl, 10 mM TES (N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid) buffer, pH 6.9; modified from Pichon *et al.*, 1972). The saline was connected via silver wire to the amplifier and computer.

The part of the leg between the microcapillaries was bathed continuously in an airflow of 650–700 ml/min delivered through an 8-mm diameter glass tube with a side port through which odors were injected into the air

stream. At this airspeed, an injection of air alone produced little or no signal after the mechanoreceptors of the leg had adapted (see Figure 2a). The air was charcoal-filtered and humidified and its temperature was approximately 24°C. Immediately prior to testing, 10  $\mu$ l of the test chemical solution were pipetted on to a piece of Whatman No. 1 filter paper ( $\sim 3$  mm  $\times$  14 mm). The filter paper was exposed to the air for 30 s to allow the hexane to evaporate, then placed inside a Pasteur pipette and attached to a syringe with an automated, motor-driven de-

livery system. Before the pipette was connected to the airflow over the leg, 5 ml of air were expressed in 1 s to evacuate the odor-laden air in the pipette. After a 30 s pause, 5 ml was injected in 1 s into the airflow to stimulate the leg. In this way, the time for evaporation of each test chemical in the pipette was standardized. Each test animal only received one 5-ml dose of each chemical. The continuous flow of clean air over the preparation ensured that odors were immediately removed from its vicinity.

Due to the diminished responsiveness of the antenniform leg over time, standards ( $10^{-3}$  g guaiacol) and blanks (air) were included after every fifth or sixth chemical tested. The response to hexane was also tested every twelfth stimulus. The time interval between chemical stimuli was approximately 2 min. No preparations were used for more than 2 h. During this time the response to the guaiacol standard declined on average to about 50% of its original value.

Data were recorded directly on to a computer from which it was possible to read off the voltage of the signal. The decline in response to the guaiacol standard was approximated to a straight line and the responses to other chemicals or concentrations were expressed as a percentage of the response to the standard after subtracting any mechanosensory signal produced by air alone. A total of six males and nine females were tested but all 42 chemicals, along with all six concentration series, were not tested on each individual and thus the sample sizes for each chemical stimulus vary. Attempts were also made to record electrolegograms from the basal half of the tarsus of the antenniform leg of three individuals. There are no multiporous sensilla on this part of the leg.

Statistical procedures were carried out with the JMP® Start statistics program.

### 3. Results

The magnitude of the response to guaiacol at a loading of  $10^{-3}$  g on the filter paper was similar in the two sexes [males,  $0.78 \pm 0.09$  mV (mean  $\pm$  standard error); females,  $0.59 \pm 0.08$  mV;  $t=1.569$ , 13 df,  $P=0.141$ ]. With 22 other chemicals, three or more animals of each sex were tested. Analysis of variance on transformed data to account for lack of normality showed no difference in the relative responses of the sexes to any of the chemicals (Compound,  $F=15.3243$ ,  $P<0.0001$ ; Sex,  $F=0.1978$ ,  $P=0.657$ ; Sex  $\times$  Compound,  $F=1.1609$ ,  $P=0.290$ ) and data for both sexes are combined in the following analyses. Responses to odor were only obtained with distal parts of the tarsus; experiments with proximal regions gave no evidence of olfactory sensitivity.

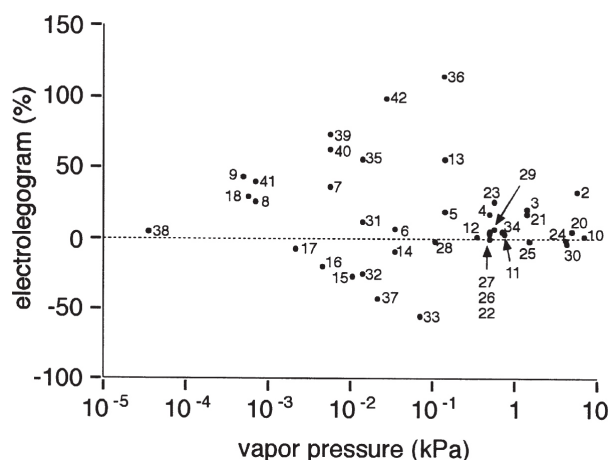
Three types of response are separated. Chemicals which gave mean ELGs within  $\pm 10\%$  of zero often had standard errors which overlapped zero and these are considered to have produced no signal. Based on this

criterion, 19 of the chemicals failed to stimulate the sensilla (Table 1). Eighteen of the chemicals produced a significant downward deflection indicative of a reduction in potential and comparable with that produced in a typical insect EAG (Figure 2b). By analogy with insect studies, we infer that such a response by the sensory neurons would lead to the production of action potentials and we refer to it as "excitatory". Five chemicals produced an upward deflection (Figure 2c,d), an increase in potential, and such deflections we refer to as "inhibitory".

The magnitudes of ELG responses relative to the guaiacol standard for all the compounds tested at a loading on the filter paper of  $10^{-3}$  g are given in Table 1 and Figure 1 in relation to vapor pressure of the compounds at 24°C. Excitatory responses were obtained to compounds of vapor pressures between 0.0005 and 5.516 kPa and the signals produced were usually of the type shown in Figure 2b. Within this same range of vapor pressures and chemical loading, other compounds produced no signal and some consistently gave inhibitory responses (Figure 2c).

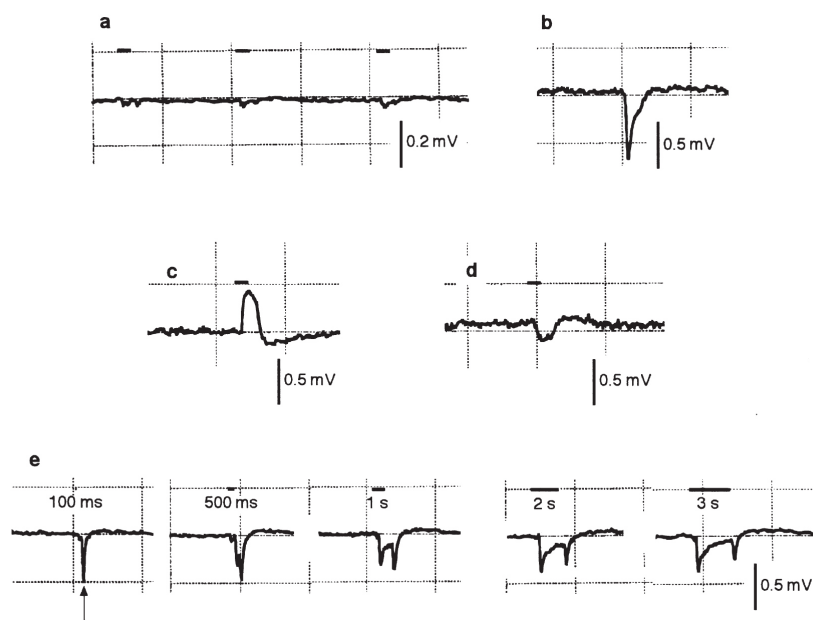
The compounds with inhibitory ELG responses were octanol, nonanol, citral, linalool, and benzyl acetate. When the orientation of the leg between the electrodes was reversed, the signal was also reversed for all these compounds (Figure 2c,d). No response was elicited by these compounds when the leg was allowed to dry. Figure 3 shows the dose response for linalool.

All the monocarboxylic acids tested produced positive ELGs with octanoic acid producing the greatest response (Figure 4a). A loading of  $10^{-7}$  g failed to elicit a response but above this the magnitude of the ELG reached a plateau above  $10^{-5}$  g (Figure 3). Of the primary

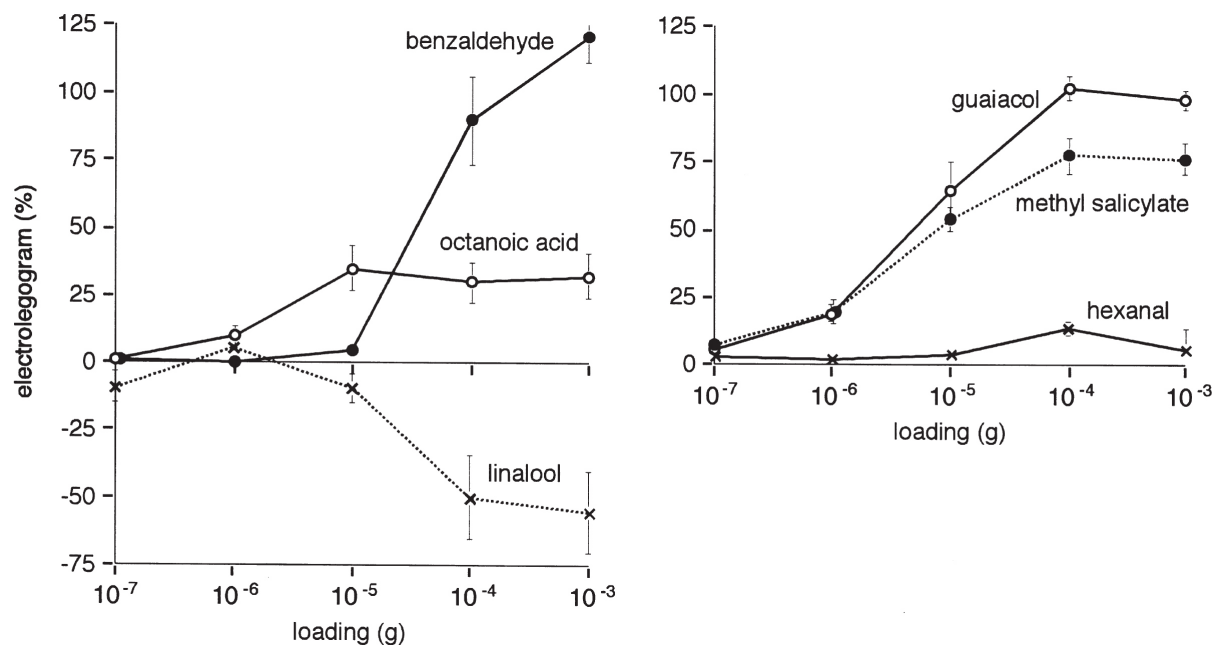


**Figure 1.** Electrolegogram responses (relative to guaiacol standard) to different compounds at loadings of  $10^{-3}$  g on the filter paper in relation to their vapor pressure at 24°C. Compounds are numbered as in Table 1 and each point is the mean response of the sum of males and females given in the table. In Figure 1, Figure 3 and Figure 4, positive values represent downward deflections of the ELG (excitation), negative values represent upward deflections as in Figure 2c (inhibition).





**Figure 2.** Examples of electrogogram responses all at a loading of  $10^{-3}$  g on the filter paper. Upper bars show stimulus duration. Except in (e), this was 1 s. **(a)** Responses to three air puffs. **(b)** Response to benzaldehyde. Most responses were of this type. **(c)** Inhibitory signal produced by linalool. **(d)** Reversal of response to linalool when the position of the leg was reversed between the electrodes. **(e)** Bimodal response to guaiacol. The timing of the off-response was dependent on stimulus duration (upper bars). Note that at very short durations the on- and off-responses were additive (arrow).



**Figure 3.** Concentration-response curves of electrogograms (relative to guaiacol standard) for six chemicals (mean  $\pm$  standard error). For guaiacol and benzaldehyde,  $n=8$ ; all others,  $n=4$ .

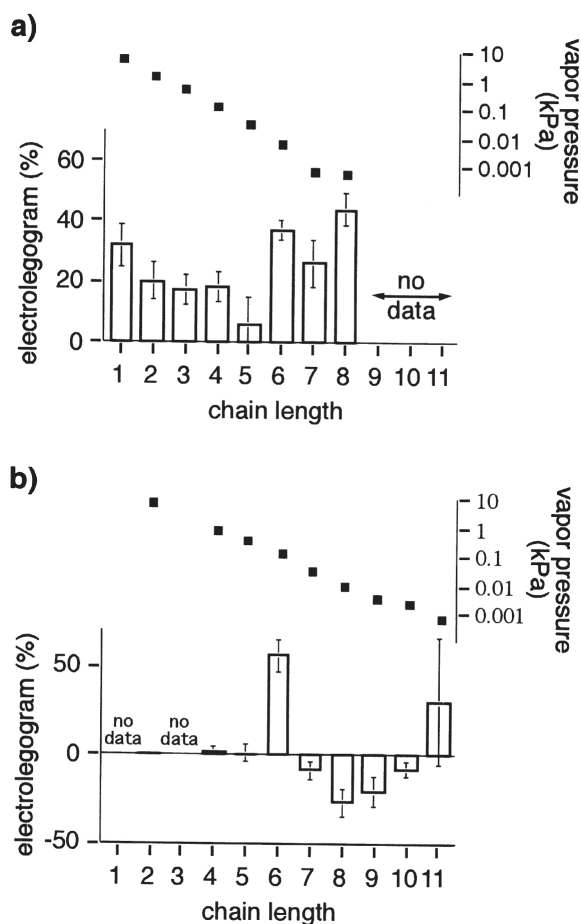
alcohols tested, only hexanol gave a consistent excitatory ELG while both octanol and nonanol gave inhibitory signals (Figure 4b). The response to undecanol was variable. A single animal tested with hexenol gave a strong excitatory signal.

Three straight-chain aldehydes were tested (Table 1) but only hexanal produced a signal. The response had a threshold close to  $10^{-5}$  g and reached a maximum at  $10^{-4}$  g (Figure 3). The only ketone tested was cyclohexanone; it produced a moderate signal. None of the acetates ex-

cept benzyl acetate (see below) produced a response, nor did methyl butyrate.

Four monoterpenes were tested. Carvone produced a small excitatory signal,  $\alpha$ -pinene had no effect, while citral and linalool both elicited inhibitory signals. The threshold of response to linalool was close to  $10^{-5}$  g loading, with a plateau above  $10^{-4}$  g (Figure 3).

All the phenolic compounds except benzyl benzoate produced moderate to strong responses, all of which were excitatory except for benzyl acetate. The magnitude of response to benzaldehyde was similar to that to guaiacol. The latter always produced a bimodal signal with a second sharp downward deflection (off effect) when the stimulus was removed (Figure 2e). The size of the second peak varied independently of the first peak. The threshold of response to benzaldehyde occurred at a loading close to  $10^{-5}$  g, but sensitivity to guaiacol and methyl salicylate was much greater with thresholds at or below  $10^{-7}$ . The response to these two compounds plateaued at loadings above  $10^{-4}$  g (Figure 3).



**Figure 4.** Electrophysiological responses (mean  $\pm$  standard error, relative to guaiacol standard) for (a) monocarboxylic acids and (b) primary alcohols of varying chain lengths and the approximate vapor pressures at 24°C. Values for  $n$  are the sums of males and females in Table 1.

## 4. Discussion

Our results show that *P. parvulus* is sensitive to odors in a number of chemical classes and strongly support the putative olfactory function of multiporous sensilla on the distal annuli of the antenniform legs. No evidence of an olfactory response was obtained from parts of the leg where these sensilla were absent.

### 4.1. Vapor pressure

The chemicals used in this study vary greatly in their volatility. Based upon our experimental methodology, differences in chemical volatilities could have greatly influenced our results. In order to take some account of differences in volatility, we obtained vapor pressures for most of our test chemicals from Yaws (1994) (see Table 1). Hexane, which was used as our solvent, has a vapor pressure at 24°C of 13.79 kPa and Brockenhoff and Grant (1999) found that it had completely evaporated from similar-sized pieces of filter paper after 30 s. Thus, based upon our protocol, hexane should never have been present at the time of test odor delivery and when hexane alone was tested no response was elicited.

In examining Figure 1, it is clear that most of our results are not likely to be confounded by differences in vapor pressure. None of the chemicals used in our study had a higher volatility than hexane. Formic acid has a relatively high vapor pressure, yet it elicited a fairly strong response, indicating that it was still present in the syringe upon odor delivery (Figure 1, chemical no. 2). It seems likely that non-responses in the vapor pressure range between 0.001 and 10 kPa are true non-responses and not an artifact of the chemical being evaporated off. Most of the chemicals were only tested at one loading,  $10^{-3}$  g, and it is possible that a response would have been obtained at higher concentrations. However, the six chemicals for which dose responses were obtained had thresholds at loadings well below  $10^{-3}$  g, and concentrations above this may not be biologically relevant.

Another concern is that chemicals with low volatility may not have volatilized enough in the syringe at the time of odor delivery to produce a response. However, this seems unlikely since there are four chemicals with vapor pressures between 0.0001 and 0.001 kPa that do elicit strong responses. Except for benzyl benzoate (Figure 1, no. 38), we feel confident that all of our non-responses are real and not artifacts of vapor pressure differences. The magnitudes of responses, however, will have been affected because numbers of molecules delivered over the legs will have varied between compounds in a manner which we cannot predict.

#### 4.2. Putative inhibitory signals

As far as we are aware, inhibitory responses have not been documented in insect electroantennograms but there are numerous examples of inhibition of the spontaneous activity of olfactory receptor neurons (for example, Anderson *et al.*, 1995; Den Otter and Van der Goes van Naters, 1993). In a few cases, an accompanying hyperpolarization of the neuronal membrane has also been demonstrated (Den Otter *et al.*, 1980; Pophof, 1997). If a sufficient number of neurons responded in this way they would be expected to produce an increase in the potential recorded by an electroantennogram. We believe that the increases in potential we have observed in ELGs of *P. parvulus* are a result of such hyperpolarizations of cells in the leg and are not artifacts. Linalool produced a typical but inverted increase in response as concentration was increased; reversing the orientation of the leg between the electrodes produced a reversal of the signal; and the signal was lost once the leg died. These results are consistent with a real biological phenomenon and would not be expected if the effect was simply some physical artifact.

#### 4.3. Guaiacol off-effect

The off-effect observed in response to guaiacol is also a genuine biological effect. None of the other chemicals, nor air alone, produced an off-effect so it is not simply an electrical artifact of the switching system. Den Otter *et al.* (1980) depict an off-effect (which they call a rebound) in a single olfactory receptor cell after inhibition by pure butyric acid and off-effects in interneurons are common (see, for example, Christensen and Hildebrand, 1987). Since the antenniform legs of amblypygids contain the cell bodies of giant interneurons, some of which may have connections with olfactory neurons (Igelmund and Wendler, 1991), it is possible that the effect we observed derives from the activity of these cells rather than the sensory neurons themselves.

#### 4.4. Ecological significance

The chemicals to which *P. parvulus* exhibited responses are all commonly occurring in the environment, most of them being produced by plants (see, for example, Gibbs, 1974) and some possibly by animals. As with many insects (for example, Dickens *et al.*, 1993; Chinta *et al.*, 1994; Thiery and Marion-Poll, 1998), *P. parvulus* responds to a number of six-carbon-atom compounds which are common components of green leaf odor, but the strongest responses were those to a number of phenolic compounds. The amblypygids are generalist predators and are found in association with a wide variety of trees, and we believe that none of the compounds producing large ELG responses is likely to have specific importance to the animals in the field (E. A. Hebets,

unpublished observations). Based upon the olfactory capabilities demonstrated in this study, as well as the extensive development of the mushroom bodies of amblypygids, it seems possible that olfactory learning plays a major role in the biology of these animals.

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