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COMPARATIVE STUDY BY ELECTROPHYSIOLOGY OF OLFACTORY RESPONSES IN BUMBLEBEES (*Bombus hypnorum* and *Bombus terrestris*)

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Abstract—Electrophysiological data (EAG) were recorded on adult bumblebees stimulated with floral and/or pheromonal pure odorants at different concentrations. The responses of queen, worker, and male bees are compared and the sensitivities of these insects to the pure odorants tested are discussed.

Key words—Apoidea, *Bombus hypnorum, Bombus terrestris,* Hymenoptera, sex, caste, floral odorants, pheromones, electroantennography, olfactory equipment.

INTRODUCTION

Bumblebees (Apoidea, Hymenoptera) that live in groups grow up in a complex chemical environment, whether inside or outside their nests. Intraspecific relations seem to be determined, to a great extent, by chemical cues: brood pheromone (Heinrich, 1974), queen pheromone (Röseler and Röseler, 1974; Röseler, 1975, 1977; Plowright and Pendrel, 1977; Van Honk et al., 1980; Röseler et al., 1981), sexual pheromones (Kullenberg et al., 1970, 1973; Svensson and Bergström, 1977; Van Honk et al., 1978; Ågren et al., 1979; Svensson, 1979), trail pheromone (Cederberg, 1977b), and alarm pheromone (Cederberg, 1977a). Chemical volatiles cooperate with other mechanisms (spatial, temporal, mechanical) as premating isolating mechanisms and so prevent inbreeding (Svensson, 1980; Bergström et al., 1981).

On the other hand, these insects, which are active foragers, learn to

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recognize the visual, chemical, and morphological signals of nectariferous plants. For the honeybee (*Apis mellifica*), another pollinator, chemical signals are the most effective signals (von Frisch, 1923; Kriston, 1973; Koltermann, 1973). Comparative behavioral experiments, applied to whole colonies of honeybees and bumblebees, have shown that conditioning to pure odorants (geraniol, limonene) is similar for both Hymenoptera (Pham-Delegue et al., 1983). Therefore it appears that volatile pheromones and plant aromas are essential sensory criteria for bumblebees to ensure the survival of the species.

It was then interesting to analyze, at different levels (perception, identification, and message integration), the nervous mechanisms which represent the key to the behavioral responses induced by chemical stimuli. We first studied the antennal olfactory equipment of *Bombus hypnorum* (Fonta and Masson, 1982) and quantified, by means of scanning electronic microscopy, sensilla placodea distribution on adult insect antennae. As has been well demonstrated in honeybees, plate organs are sensory structures whose nervous cells are specially adapted to detect volatile compounds (Lacher and Schneider, 1963; Kaissling and Renner, 1968; Esslen and Kaissling, 1976). For each antennal segment, the average density of the sensilla placodea is similar for male, worker, and queen bumblebees, yet the male antenna flagellum is one segment longer and the s. placodea population is denser on the distal segment (Fonta and Masson, 1982). However, differences in the number of sensory neurons, as has been found between male and worker of *Apis* (Esslen and Kaissling, 1976), or in the function of the placodea neuroreceptors (discrimination ranges and sensitivities) might exist between sexes and/or castes at this peripheral level.

The present work aims to conduct further studies on the electrophysiological recordings from single cells to analyze the insect olfactory detection level and perception characteristics. The antennal electric activity was measured by electroantennography recordings (EAG). Neurophysiological studies on bumblebees which have been published previously deal only with the visual system (Meyer-Rochow, 1980).

METHODS AND MATERIALS

Biological Materials. Two species (*Bombus hypnorum* L. and *Bombus terrestris* L.) of adult bees of diverse ages were studied. The experimental animals belonged to different colonies that were reared in the laboratory. The individual size variation inside each caste was kept narrow. The worker bees are foragers; the males have reached their sexual maturity whereas the queens are potential foundresses which have not yet hibernated.

Odorants. Eight pure substances (purity = 99.9%) were tested: butanol (BUT), the usual reference compound for functional studies in olfaction, limonene (LIM), geraniol (GER), nerol (NER), citral (CIT), eugenol (EUG), vanillin (VAN), and isoamylacetate (ISO), floral aroma constituents (Loper, 1972; Lawrence, 1978; Etievant et al., 1984) and/or pheromonal elements of various Hymenoptera (e.g.: *Apis:* Boch and Shearer, 1962; Pickett et al., 1980, 1981; *Bombus:* Kullenberg et al., 1970; Svensson and Bergström, 1977; Bergström et al., 1981; *Andrena:* Bergström and Tengö, 1974; Tengö and Bergström, 1976, 1977; Francke et al., 1981; Bergström et al., 1982; *Anthophoridea:* Vinson et al., 1982).

Methods. Experiments were carried out on live insects, immobilized in a special restraining device.

The recording electrode (a drawn out glass microcapillary, 1 mm inner diameter) was set up on the left antenna tip when the distal part of the last segment had been removed. The reference electrode (inner diameter: 0.5 mm) covered the right antenna. The two electrodes were filled with 2 M NaCl solution (116.88 g/liter). They were connected to an impedance adapter, which was itself connected to a storage cathodic oscilloscope.

The stimulation device was an olfactometer with dynamic and controlled gas dilution (Masson and Friggi, 1974; Masson et al., in preparation). The head of the insect was swept by pure nitrogen between the stimulations or by odoriferous nitrogen during the stimulations. The carrier air was scented when passed through the enclosed spaces containing the odorants and delivered at about 4 mm from the longitudinal axis of the left antenna. This flow is associated with a constant nitrogen flow (7.3 liter/hr) which is necessary for the antennal mechanoreceptors to be habituated and which cleans the circuits, followed by the scented air flows. The carrier air pressure and thus the concentration of the stimulating odorous molecules were controlled by a water manometer.

The following equation gives the number of molecules in each milliliter of carrier air (Masson, 1973; Masson and Friggi, 1974):

$$ni = \frac{(6 \times 10^{23}) \times 273\ Pi}{22400 \quad T \times 760} \sim 0.96 \times 10^{19} \frac{Pi}{T}$$

where ni = number of molecules/ml of carrier gas; Pi = vapor pressure in mm Hg at 20°C; and T = temperature in degrees Kelvin.

Protocol. Stimulation with pure odorants used alone lasted 2 sec, which is the time required for the stimulus to reach the neighborhood of the antenna. Between two successive stimulations, a 2-min interval ensured desorption of the membrane neuroreceptors.

Each of the eight odorants was tested at three concentrations (Table 1) which were linked to the three nitrogen flows: D_1 = 1.2 liter/hr, D_2 = 3.6

TABLE 1. NUMBER OF MOLECULES (*Ni*) OF A 2-SEC STIMULATION[a]

	Stimulus Quantity					
	D_1 = 1.2 liter/hr		D_2 = 3.6 liter/hr		D_3 = 6.05 liter/hr	
Odorants	*Ni*	log *Ni*	*Ni*	log *Ni*	*Ni*	log *Ni*
Butanol (BUT)	1.40×10^{17}	17.146	4.22×10^{17}	17.625	7.10×10^{17}	17.851
Geraniol (GER)	4.45×10^{14}	14.648	1.34×10^{15}	15.127	2.26×10^{15}	15.354
Nerol (NER)	1.53×10^{15}	15.185	4.62×10^{15}	15.664	7.77×10^{15}	15.890
Citral (CIT)	1.26×10^{15}	15.100	3.80×10^{15}	15.580	6.39×10^{15}	15.806
Isoamylacetate (ISO)	1.21×10^{17}	17.083	3.66×10^{17}	17.563	6.16×10^{17}	17.790
Limonene (LIM)	3.04×10^{16}	16.483	9.17×10^{16}	16.962	1.54×10^{17}	17.188
Eugenol (EUG)	3.00×10^{14}	14.477	9.05×10^{14}	14.957	1.52×10^{15}	15.182
Vanillin (VAN)	3.69×10^{12}	12.567	1.11×10^{13}	13.045	1.87×10^{13}	13.272

[a] D_1, D_2, D_3: the three flow rates of the carrier air.

liter/hr, D^3 = 6.05 liter/hr. For each flow rate, stimulations were randomized. The maximal amplitude of depolarization induced by the stimulation was recorded.

The results reported and discussed here represent the mean of the responses of 10 insects; the substances were tested on each animal and 1–3 trials were conducted with each experimental insect.

Average amplitude of responses is figured by a function of decimal logarithm of the odorant molecules concentration.

RESULTS

The odorous chemicals used in this work stimulate the neuronal activity of the insect peripheral olfactory system.

The EAG responses, which are functions of the stimulating molecule concentrations, are represented by linear functions in the range of chosen concentrations.

In *Bombus hypnorum* (Figure 1), the EAG responses of workers and males were similar for each stimulus, response amplitudes increasing in the same way for both. The future foundresses of this species gave, in general, slightly higher responses. The largest difference was shown to be between queens and workers and males for the highest concentrations of butanol used in our experimental situation (2:1 quotient). The responses of queens and workers increased in parallel, except for butanol and eugenol. In comparing males and workers, a more irregular evolution was found in the response amplitude ratios.

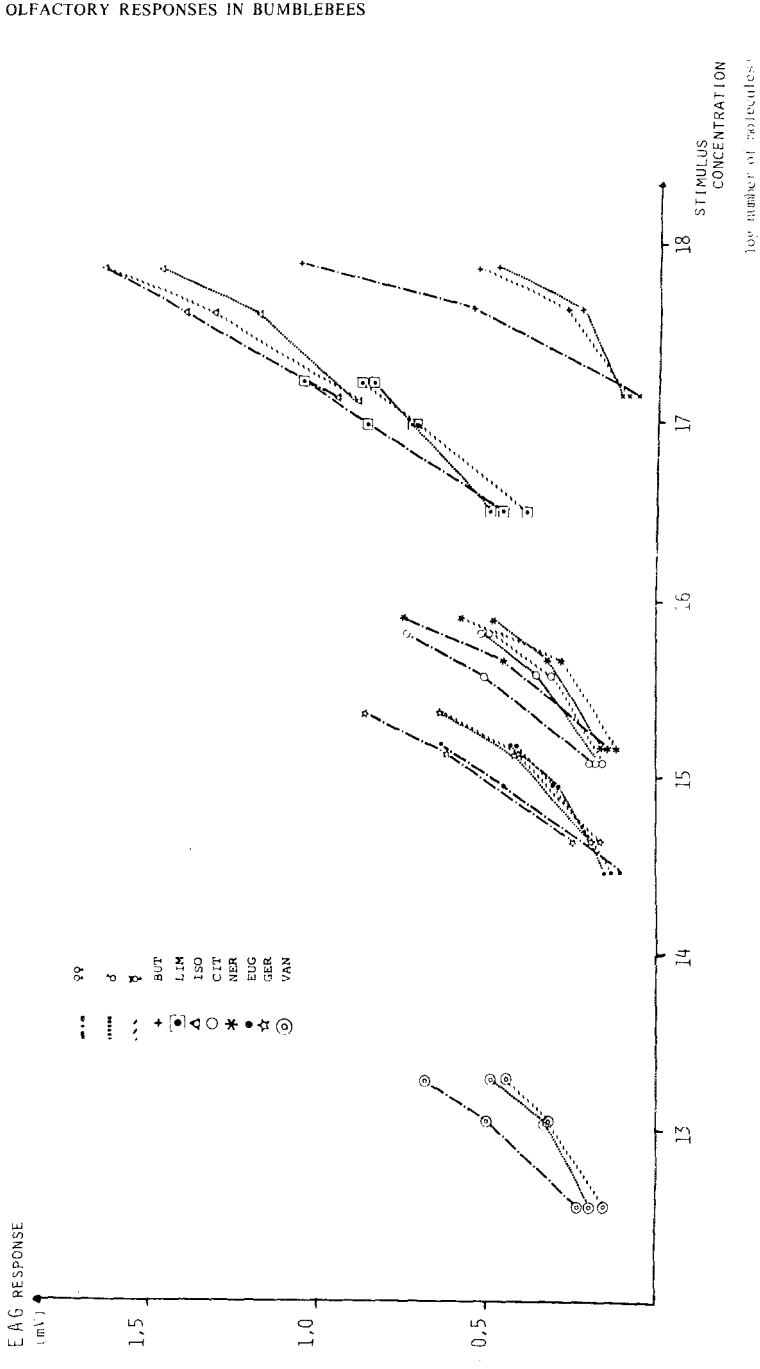

FIG. 1. EAG responses of *Bombus hypnorum* (in millivolts) to odoriferous stimulations at different concentrations (in decimal logarithm of the number of molecules). BUT: butanol; CIT: citral; EUG: eugenol; GER: geraniol; ISO: isoamylacetate; LIM: limonene; NER: nerol; VAN: vanillin.

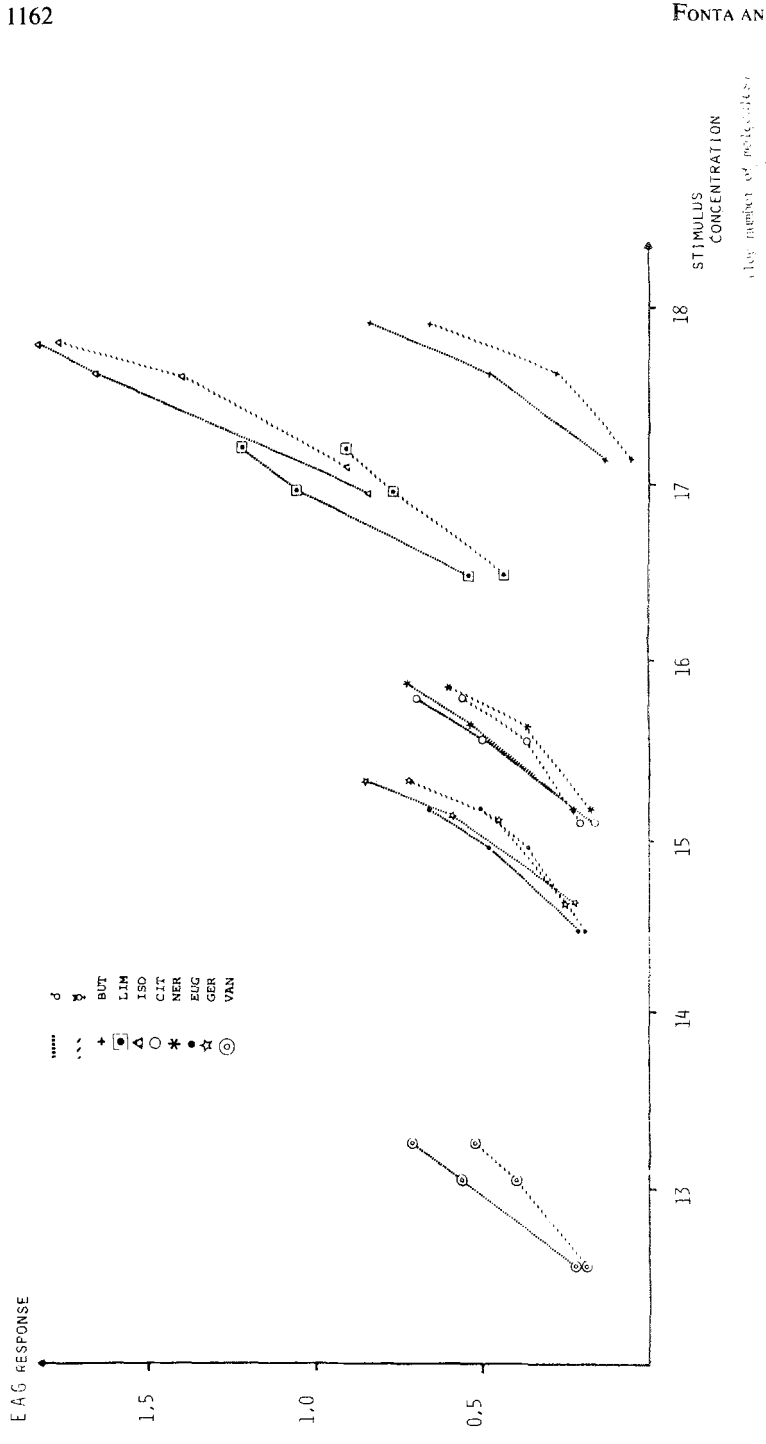

FIG. 2. EAG responses of *Bombus terrestris* (in millivolts) to odoriferous stimulations at different concentrations (in decimal logarithm of the number of molecules). BUT: butanol; CIT: citral; EUG: eugenol; GER: geraniol; ISO: isoamylacetate; LIM: limonene; NER: nerol; VAN: vanillin.

In *Bombus terrestris* (Figure 2), the responses of workers and males varied in an analogous way with the stimulus quantity. The depolarization amplitudes were higher for males than for females; this difference was emphasized with the upper concentration of limonene. Generally, the curves of stimulus concentration–response are not parallel for the two sexes for each odorant; amplitude ratio variation is different for substances tested in comparable concentration ranges (for example, limonene and butanol).

A linear regression ($y = mx + b$) has been calculated with three coordinates for each function derived experimentally. Correlation coefficient values range from 0.92 to 0.99. Consequently, the stimulus concentration values (x) obtained from the linear regressions can be relied on for a response amplitude (y) of 0.5 mV.

The results can be analyzed for the two species together. The eight pure odorants tested can be classified in five groups if the stimulus intensity required to obtain a 0.5-mV amplitude detection response (Table 2) is used as the classification criterion: (1) VAN ($\sim 10^{13}$ molecules), (2) GER-EUG ($\sim 10^{15}$ molecules), (3) CIT-NER ($\sim 3 \times 10^{15}$ to 10^{16} molecules), (4) LIM-ISO (~ 3–6×10^{16} molecules), and (5) BUT ($\sim 3 \times 10^{17}$ to 10^{18} molecules).

There is a 10^4 factor between groups 1 and 5, the first compound (VAN) being clearly distinct from the second class of compounds (GER-EUG). This hierarchy in sensitivity and detection method for the eight substances is similar within each species, each caste and each sex.

DISCUSSION

All the EAG responses obtained in these experiments are expressed as depolarizations. The results, by quantitative analysis, do not show strong differences in the responses of the three kinds of individuals except for butanol; this odorant molecule induces an antennal electrical activity higher for the young queens than for workers and males of *Bombus hypnorum.*

As demonstrated here all the responses of the queens are more pronounced, and the response amplitudes of males are superior to those of the workers. Two alternative explanations could account for these observations: (1) A first-order sensory fibers equipment difference for the three types: various numbers of neuroreceptors in the sensilla placodea and/or a different s. placodea population, in absolute value (on the one hand the body size and therefore the antennal area of queens is bigger than workers and males; on the other hand, the antennal flagellum of the male has one additional segment). (2) Sensitivity and discrimination power of the peripheral sensory neurons may show characteristics for each caste and/or each sex. Until now, no study has been undertaken at the unitary level with bumblebees.

TABLE 2. NUMBER OF ODOR MOLECULES (N) INDUCING A 0.5-mV EAG AMPLITUDE (y)[a]

Insects	Stimulation	Pure Odorants							
		BUT	GER	NER	CIT	ISO	LIM	EUG	VAN
hypnorum									
♀♀	x	17.501	14.963	15.645	15.522	16.618	16.496	15.012	13.010
	N	3.170×10^{17}	9.183×10^{14}	4.416×10^{15}	3.327×10^{15}	4.150×10^{16}	3.133×10^{16}	1.028×10^{15}	1.023×10^{13}
♂	x	18.031	15.198	15.993	15.816	16.608	16.530	15.424	13.389
	N	1.047×10^{18}	1.578×10^{15}	9.840×10^{15}	6.546×10^{15}	4.055×10^{16}	3.388×10^{16}	2.655×10^{15}	2.449×10^{13}
☿	x	17.886	15.210	15.878	15.906	16.733	16.644	15.418	13.474
	N	7.691×10^{17}	1.622×10^{15}	7.551×10^{15}	8.054×10^{15}	5.408×10^{16}	4.406×10^{16}	2.618×10^{15}	2.979×10^{13}
terrestris									
♂	x	17.560	14.974	15.592	15.567	16.672	16.434	14.958	12.964
	N	3.631×10^{17}	9.419×10^{14}	3.908×10^{15}	3.690×10^{15}	4.698×10^{16}	2.716×10^{16}	9.078×10^{14}	9.207×10^{12}
☿	x	17.764	15.094	15.786	15.764	16.788	16.581	15.224	13.228
	N	5.808×10^{17}	1.242×10^{15}	6.109×10^{15}	5.808×10^{15}	6.138×10^{16}	3.811×10^{16}	1.675×10^{15}	1.690×10^{13}

[a]The term x determined by linear regression $y = mx + b$; $x = \log N$.

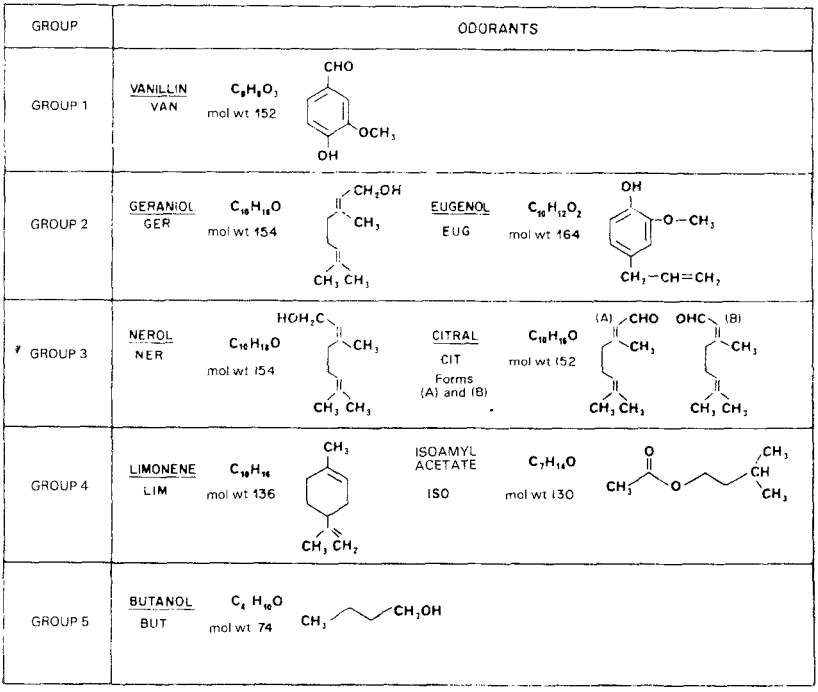

FIG. 3. An attempt to classify the stimuli tested according to the biological responses of bumblebees. MOL WT: molecular weight.

These explanations are necessary to account for the range of olfactory sensitivities expressed by the bumblebees for the tested odorants. The classification in five groups that was hypothesized is now considered to be temporary and arbitrary; the substances studied in this work may be representative of molecular groups whose diverse structural parameters should be decisive in the release (potential receptor) and transmission (generator potential) of the nervous influx along the sensory fiber. We emphasize here that the stereoisomers geraniol and nerol tested at the same concentration induce different EAG responses.

Although fine relationships between the structure and function of odorants cannot be studied with EAG recordings, this work shows that butanol, with the lowest molecular weight (74) and the poorest "response-inducing structure" in terms of chemical functions (one alcohol function only), is not as well perceived as the other stimuli tested (Figure 3). The sensitivity of the receptor cells is more or less linked to the molecular weight (Figure 3); as the number of carbon atoms does not seem to be directly concerned, our results agree with the general hypothesis that molecular

structure and chemical functions are determining factors in the interactions between odorant molecules and neuronal membrane receptors of generalist cells.

Moreover, studies of discriminatory abilities of vertebrate olfactory neuroreceptors (Sicard, 1980) have shown that *d*-limonene is representative of a "terpene group" which does not include isoamylacetate, and butanol appears to be independent of the other odor molecules tested in this work.

The data described suggest that the method used in this work is suitable for the study of the EAG recordings of bumblebees stimulated with odorants belonging to their floral and pheromonal language. These studies have two principal aims. The first is to help, by biological assay, with the chemical identification (by gas chromatography and mass spectrometry) of gland and cuticle extractions, generators of odor substances which are thought to have intra- or interspecific actions (chemical analysis of such odors is now in progress and the results will soon be published). The second is to study the peripheral olfactory nervous system function of a "generalist insect" (Masson and Brossut, 1981) and its part in the identification processing of a pheromonal blend which produces (after the integration of messages in the central nervous system) behavioral or physiological responses from the insect stimulated. Further development of this work requires the measurement of recording at the cellular level.

In *Apis*, wide-spectrum neuroreceptors (Lacher, 1964) and cells responsible for the detection of pheromones (Kaissling and Renner, 1968) have been demonstrated. Are the same features present in bumblebees? In addition, is the sensory olfactory equipment of bumblebees organized in specific cell groups as has been demonstrated in *Apis* (Vareschi, 1971)?

If the inherent potentialities of the olfactory sense of bumblebees were known, then one can determine the complexity and elaboration level of chemical communication and elucidate the actual importance of olfaction to the sensory spectrum of these insects.

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