Is the number of antennal plate organs (sensilla placodea) greater in hygienic than in non-hygienic Africanized honey bees?

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ABSTRACT. Hygienic behavior is a desirable trait in honey bees (*Apis mellifera* L.), as hygienic bees quickly remove diseased brood, interrupting the infectious cycle. Hygienic lines of honey bees appear to be more sensitive to the odors of dead and diseased honey bee brood, and Africanized honey bees are generally more hygienic than are European honey bees. We compared the number of sensilla placodea, antennal sensory structures involved in the perception of odor, in 10 bees from each of six hygienic and four non-hygienic colonies of Africanized honey bees. The sensilla placodea of three of the terminal segments (flagellomeres) of the right antenna of each bee were counted with a scanning electron microscope. There were no significant differences in the mean numbers of sensilla placodea between the hygienic and non-hygienic bees, though the variance was higher in the hygienic group. Flagellomere 4 had significantly more sensilla placodea than flagellomeres 6 and 8. However, there was no significant difference between the other two flagellomeres. As hygienic bees are capable of identifying dead, injured, or infested brood inside a capped brood cell, sensilla placodea

probably have an important role in enabling worker bees to sense sick brood. However, we did not find greater numbers of this sensory structure in the antennae of hygienic, compared to non-hygienic Africanized honey bees.

Key words: Hygienic behavior, Honey bee, Africanized honey bee, Antennae, Flagellomere, Plate organs, Sensilla placodea

INTRODUCTION

Some worker bees (*Apis mellifera*) are capable of recognizing and removing diseased, damaged, or dead brood in capped brood cells. This uncapping and removal behavior is termed hygienic behavior (Rothenbuhler, 1964a). Despite the unquestionable relevance of the work of the genetic mechanisms studied by Rothenbuhler, it is not clear which mechanisms are used by the bees to identify and recognize the cells that contain dead brood, nor do we know the stimuli involved in this process. It has been demonstrated that this behavior is controlled by two pairs of recessive genes, u (uncapper) and r (remover) (Rothenbuhler, 1964b). Moritz (1998) made a reevaluation of the two-locus model for hygienic behavior in honey bees and proposed a three-locus model, but did test his hypothesis experimentally. Based on experimental work, Gramacho (1999) proposed that the two-locus model should be amplified to three pairs of genes, two pairs of which (u1 and u2) would be responsible for initially making a hole in the cell capping and uncapping, respectively, and one pair for the phase of removal (r) of the brood.

Hygienic behavior seems to be a generalized response of the bees towards pathogens and parasites in the colony. Many cues are probably used by honey bees to identify if the larvae or pupae inside a cell are healthy, sick, damaged or infested with mites. The sensory structures in the antennae are likely used by nurse bees to distinguish healthy from "abnormal" brood.

The smell of mites inside the cell with the brood, for instance the mite *Varroa destructor* (Anderson and Trueman, 2000), may stimulate the bees to take action and remove infested brood (Rosenkranz et al., 1993; Boecking and Drescher, 1994; Aumeier, 1995; Aumeier et al., 1996; Sasagawa et al., 1999).

Africanized honey bees have been found to be more resistant towards *V. destructor* than European honey bees (Moretto et al., 1991; De Jong and Gonçalves, 1998; Guerra Jr. et al., 2000). The levels of infection by this mite vary from one region to another, as well as with climate and bee race (De Jong et al., 1984; Kulincevic et al., 1988; Moretto et al., 1991). Gramacho (1999) found differences in hygienic behavior between Africanized honey bees and Carniolan bees.

It is also known that worker bees are able to identify and uncap cells infested by *V. destructor*, removing the infested brood (Peng et al., 1987; Boecking and Drescher, 1992, 1994; Spivak, 1996; Corrêa-Marques and De Jong, 1998), which is hygienic behavior. However, not much is known about the mechanisms that allow the worker bees to recognize brood infested by the mites before they remove them.

Factors or stimuli that initiate the hygienic or cleaning behaviors may, initially, stimulate the process of recognition of the dead, sick, or infested brood by the hygienic bees. These factors could include: a) physical: vibrations provoked by the brood or by the mites inside the cell that are recognized by the hygienic workers (Schultz-Langner, 1960), temperature differences

between dead and live brood (Gramacho et al., 1998); b) chemicals: volatile substances released by the dead, sick, or damaged brood (e.g., substances in decomposition) or even a lack of production of specific substances that act as "chemical signals" to activate hygienic behavior (Titera and Kokoris, 1994; Spivak and Downey, 1998), and c) an interaction between chemical and physical factors, which may also act together to initiate or inhibit hygienic behavior.

The behavior of the bees is influenced by external stimuli that can be detected by sensory organs (Warnke, 1976). One of these organs, the antenna, is highly complex, with a series of sensory components, including the plate organs or sensilla placodea, which are used for the perception of odors (McIndoo, 1914; Frisch, 1921; Kaissling and Renner, 1968), sensilla trichodea and sensilla basiconica, which are touch (Frisch, 1921), and chemical receptors, respectively (Snodgrass, 1935, 1956); the sensilla ampullacea and sensilla coeloconica, which are hygroreceptors (Kuwabara and Takeda, 1956) and the sensilla campaniformia, which allow the perception of temperature, CO_2, and humidity (Dietz and Humphreys, 1971).

Although some researchers have found correlations between the number of sensory structures and some types of behavior, such as defensive behavior (Stort, 1979; Stort and Rebustini, 1998), there have been no investigations of antennal sensory structures and hygienic behavior of honey bees. We examined the number of sensilla placodea in the terminal segments of the antennae of hygienic and non-hygienic Africanized honey bees.

MATERIAL AND METHODS

Measurement of hygienic behavior

The pin-killed brood assay, developed by Newton and Ostasiewski Jr. (1986) and modified by Gramacho and Gonçalves (1994), was used to measure hygienic behavior. This assay consists of piercing the sealed worker brood of the same age (corresponding to the pink eye pupal phase), using a number 1 entomological pin. A test comb with capped brood cells was removed from each colony; 100 brood cells in one area of the comb were perforated, and another 100 cells in the same comb were used as controls. After perforating the brood, the combs were returned to the colonies. After 24 h, the test combs were taken to the laboratory to count the cells that had been cleaned by the bees. The degree of hygienic behavior (HB) was determined as a percentage of cleaned cells 24 h after piercing the brood:

$$HB = \frac{EC\ (24\ h) - EC\ (0\ h)}{CC\ (0\ h)} \times 100 - Z$$

where, EC (24 h) = number of empty cells 24 h after perforation; EC (0 h) = number of empty cells before perforation; CC (0 h) = number of capped cells before perforation, and Z = control correction factor

$$Z = \frac{Y}{A} \times 100$$

where, A = number of capped cells in the control before placing the comb into the colony for the cleaning test; Y = number of new empty cells, calculated as Y = C – B; C = number of empty cells after the cleaning test, and B = number of empty cells before the cleaning test.

Twenty colonies of wild-type Africanized honey bees *Apis mellifera* L., in an apiary in Ribeirão Preto, SP, were tested for their hygienic behavior. Each of the colonies was tested five times, during a 10-15-day period. A colony was considered hygienic if it removed a mean of 80% or more of the dead brood. Six hygienic and four non-hygienic colonies were selected for the study of sensory structures (Table 1).

Table 1. Colonies of Africanized honey bees classified into two groups (H = hygienic, NH = non-hygienic) according to the hygienic behavior test (mean and standard deviation (SD) in %). (Colonies with percentage ≥80% were considered hygienic and less than 80% were non-hygienic).

Colony	Hygienic behavior	
	Mean	SD
Hygienic colonies		
1	90.56	6.75
2	87.52	9.94
3	85.85	12.57
4	91.03	7.37
5	98.14	1.82
6	91.12	10.21
Non hygienic colonies		
7	43.42	14.00
8	65.52	18.01
9	34.27	11.47
10	41.14	15.24

Counting the number of sensilla placodea

Ten bees were obtained from each of the colonies and killed by asphyxiation with ether, fixed with Dietrich's. Subsequently, the right antennae were withdrawn and three segments of the flagellum, or flagellomeres, classified from the base of the antennae as numbers 4, 6 and 8, were separated.

Each antennal segment (Figures 1 and 2) was opened longitudinally with two entomological pins and stretched on a histological glass slide, sealed with Canada balsam, and covered with a cover slip. They were photographed with a Zeiss II photo microscope. The film was placed in slide mounts and projected onto a screen, where the plate organs were counted (Stort, 1979). The plate organs were counted "blind", without knowing if the colonies were hygienic or non-hygienic (Table 2).

Statistical analysis

The data were submitted to an analysis of variance for one dependent variable (number of sensilla placodea) by two factors (H = hygienic x NH = non-hygienic groups of bees and flagellomeres) and another variance analysis was made without grouping the colonies as hygienic or non-hygienic. Using the general linear model procedure, we tested the null hypotheses of the effect of the variables on the means of the groups of the single dependent variable (number of sensilla placodea). The interactions between the factors as well as the effects on
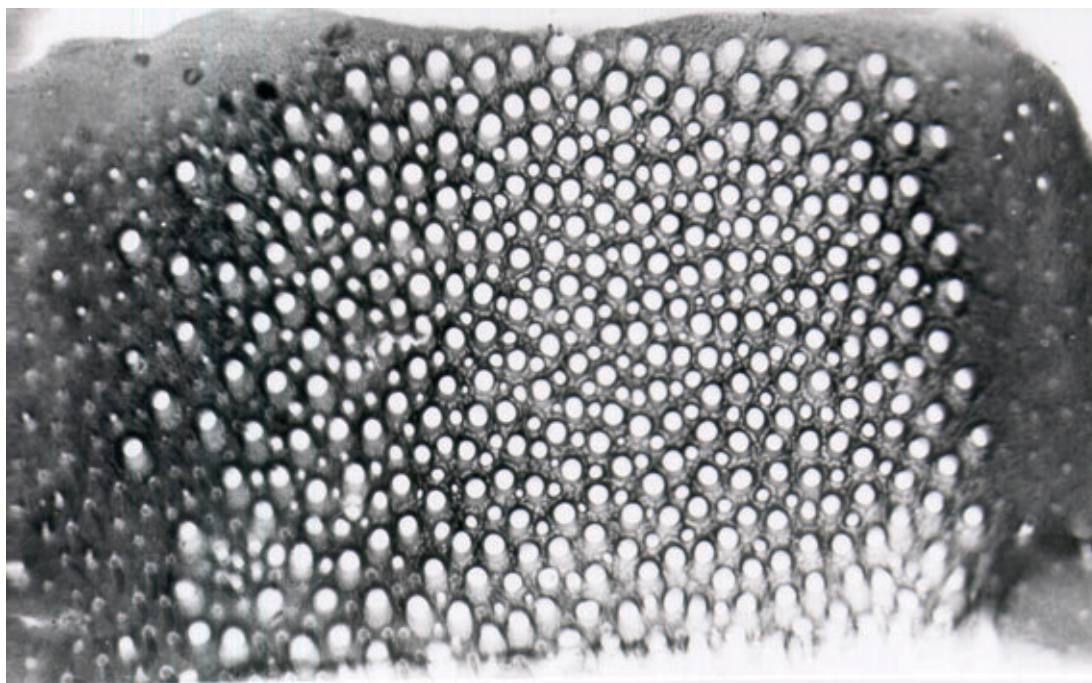
Figure 1. Flagellomere number 8 of the antennae of an Africanized worker honey bee that was opened and photographed with a photomicroscope. The larger spherical structures found throughout the structure are sensilla placodea (plate organs).

individual factors were examined. The means of the number of sensilla placodea of flagellomeres 4, 6 and 8 were also compared with the Duncan test.

RESULTS

The mean number of plate organs was similar in the hygienic and non-hygienic colonies ($P > 0.5$, Table 2), though the variance was greater in the hygienic colonies. Colony number 5, with the highest mean, also had the highest rate of hygienic behavior (Table 2). There were significant differences in the number of sensilla placodea among the three flagellomeres (ANOVA, $P < 0.05$). Flagellomere number 4 had more plate organs than flagellomeres 6 and 8 (Duncan test, $P < 0.05$).

DISCUSSION AND CONCLUSIONS

In *Apis mellifera* (Stort and Rebustini, 1998), and in stingless bees (Meliponinae) (Stort and Moraes-Alves, 1998), there are greater numbers of sensory sensilla in the terminal segments or distal flagellomeres of the antennae (mainly number 10), decreasing in number in the proximal flagellomeres. In bees more primitive than *Apis mellifera*, such as the genus *Ceratina*, the differences are much more pronounced, as they have these sensilla structures only in the five distal flagellomeres (Stort, A.C., unpublished data). However, in our sample, flagellomere 4 had significantly more sensilla placodea than flagellomeres 6 and 8.

We did not find significant differences in the number of sensilla placodea between the
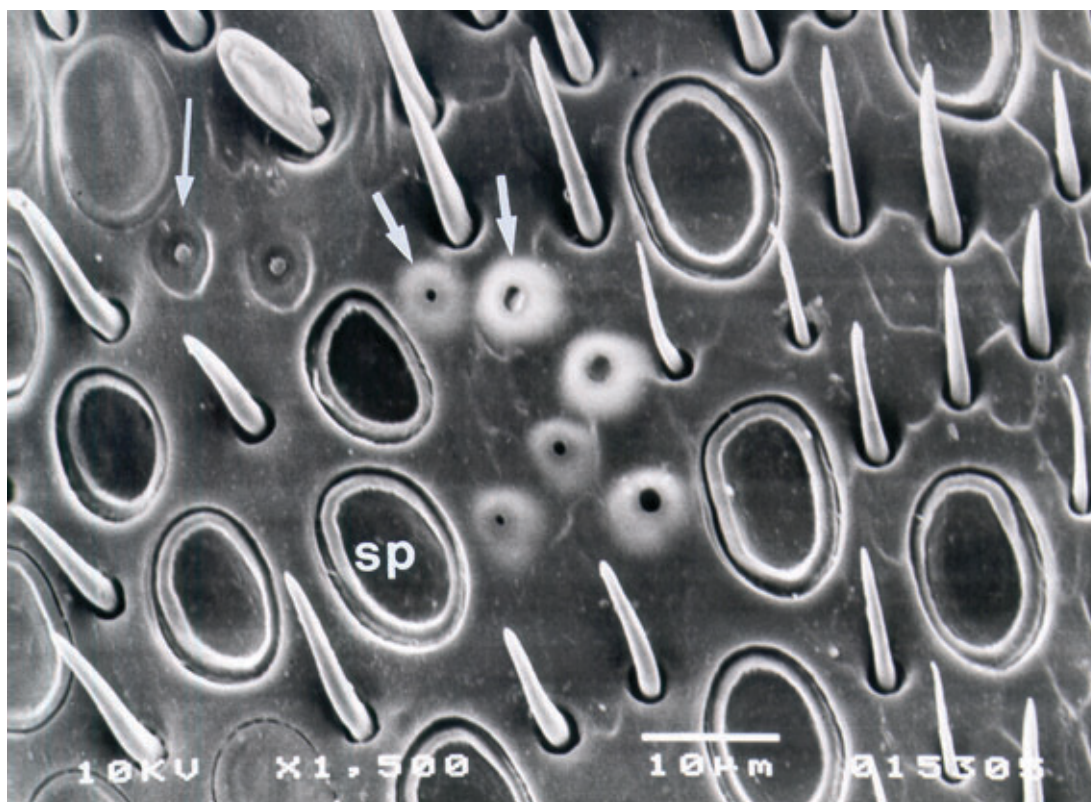
Figure 2. Scanning electron micrograph of antennal flagellomere number 6 of an Africanized worker honeybee. Details of sensilla coeloconica with a larger opening and sensilla ampullacea with a smaller opening (short arrows) and of sensilla campaniformia (long arrow). SP = sensilla placodea (plate organs).

Table 2. Mean and standard deviation (SD) of the number of plate organs (sensilla placodea) of the antennae of hygienic and non-hygienic workers of Africanized honey bees.(N = 30 flagellomeres/colony; segments 4, 6 and 8, summed together).

Group	Colony	Mean	SD
Hygienic bees	1	329.6	20.1
	2	344.9	20.6
	3	344.2	19.5
	4	338.9	20.7
	5	367.7	19.0
	6	323.3	22.3
Overall mean		341.4	20.3
Non-hygienic bees	7	339.9	16.8
	8	332.4	17.7
	9	339.7	16.4
	10	339.3	14.0
Overall mean		337.8	16.2

hygienic and non-hygienic bees (P > 0.5). However, the colonies of the hygienic group had a higher variability.

Masterman et al. (2000) examined whether bees can differentiate natural from "abnormal" brood odors, as a mechanism involved in hygienic behavior. Using the proboscis extension reflex conditioning technique, they found that bees from hygienic and non-hygienic colonies are equally capable of differentiating flower odors. However, hygienic honey bees were significantly more efficient in distinguishing healthy from sick pupae infected with the fungus *Ascosphaera apis*.

Although the most hygienic colony had the largest number of sensilla placodea and the three colonies with the largest number of these sensory organs were all in the hygienic colony group, the mean number of sensilla placodea was similar in the hygienic and non-hygienic colonies (Table 1). If these hygienic bees are more efficient at recognizing the odor of sick or damaged brood, then factors other than the number of sensilla placodea are probably involved. Possibly, hygienic bees are differentially more sensitive to odors associated with dead brood. Since hygienic and non-hygienic bees have been found to be equally sensitive to flower odors (Masterman et al., 2000), and we also found no significant differences in the number of antennal sensory organs, our original (alternative) hypothesis that hygienic bees have more sensilla placodea is probably incorrect.

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