Original paper

Histological aspects and protein content of *Apis mellifera* L. Worker venom glands: the effect of electrical shocks in summer and winter

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ABSTRACT. This paper analyzes the summer and winter total protein content of 7, 14, 21, 28, 35, and 40-day old *Apis mellifera* L. worker venom glands before (control) and 24 and 96 hours after applying electrical shocks for venom extraction (experimental). During venom extraction, 7-day old workers responded more slowly and weakly to electrical shocks. This response intensifies with age, so that the workers approaching 30 days old respond faster and more aggressively to the shocks. Statistical analysis, using the non-parametric Wilcoxon and Kruskall-Wallis tests and complemented by the Jonckheere test, showed that the protein content varied from one age to another in the experimental group, which was well distinguishable from the values in the control Group in summer and winter. Summer values at all ages were always higher than those detected in winter in both groups. This variation seems to indicate the occurrence of more than one winter glandular development cycle. Histological studies showed secretion in the lumen of the control Group secretory tubes and reservoirs. The experimental group only showed vestigial secretion in the collapsed reservoirs at all ages, except at 7 days. These workers, which reacted less efficiently to electrical shocks, showed secretion in the lumen, reservoir, and tubes, even after the application of electrical shocks. During the 96 hours following the electrical shocks, a slight protein replacement was seen at some ages. This, although higher in summer than in winter, was much lower than the level detected in the control group at all ages. The significantly lower values were frequent in the older workers 96 hours after extraction and could reflect reabsorption or degradation of proteins from glandular secretion due to aging. Our results show that venom extraction is more productive in summer using older workers. However, their capacity of replacing protein eliminated during stinging of the substrate, in response to shocks is shown to be low, as demonstrated for other analyzed bees.

KEY WORDS: bees, histology, protein content, summer, winter, electrical shock, venom gland, *Apis mellifera.*
INTRODUCTION

Many reports on venom glands of *Apis mellifera* workers are found in literature (2,7-9,14). These structures are located in the posterior portion of the abdomen, between the workers’ rectum and ovaries. They consist of a secretory filamentous region, connected to a reservoir at its proximal portion, in which the venom is stored (10,13). The cells of the secretory region bearing canaliculi are tall and form an epithelium that defines a lumen, in which the secretion is eliminated through the canaliculi, reaching the reservoir cavity. Small flat cells also bearing canaliculi form the distal region of the reservoir, where their products contribute to venom composition (5,7).

Venom elimination is part of workers’ defense mechanism, which is probably very important when they begin their activities outside the colony, where they become more exposed to hazardous situations. This mechanism is also important for the colony defense and can be used by bees living inside the hive.

Workers sting a single time, which leads to their death. Thus, the occurrence in nature of a single development cycle of the venom glands can be justified, characterized by a secretory phase in young workers and a degeneration phase in old workers (7,8,16). However, in temperate climates, Owen (15), studying the variations in histamine content present in *Apis* venom at different ages, observed a re-synthesis of this compound after extracting the venom by electrical shocks.

It should be noted that the venom is essentially protein (3) and has been extensively used in medical research mainly for the treatment of rheumatic diseases (11,12). The objective of this study was to assess the venom-eliminating capacity and the chronology of venom replacement in *A. mellifera* workers, using electrical shock extraction, by the workers' age and season of the year. The results can be of great help in research on venom production for commercial purposes, providing funds to improve exploitation of the colonies.

MATERIALS AND METHODS

The experiments were performed during three consecutive summers and two winters. For each experiment, about 600 bees were removed from different colonies, marked, returned, and then collected 7, 14, 21, 28, 35, and 40 days after marking to form the study groups: control (C) and experimental (E).

The workers of Group C were dissected immediately after collection, while those of Group E were submitted to electrical shocks for venom extraction. These were confined, and then collected for dissection 24 and 96 hours post-shock. Ten bees from each group were studied.

Venom was extracted using an extracting apparatus, based on the Brandeburgo (4) model, modified for use with a single specimen at a time. Each bee
was submitted to shock application every ten seconds for five minutes, with an electrical current of 29mA, inducing the bee to prick an appropriately prepared substrate (PVC film), allowing venom elimination without loss of the sting.

After dissection in saline solution for insects (7.5g NaCl+2.72gKH2PO4+2.38g Na2HPO4), the venom glands were processed using the modified Bradford (17) method for determination of total protein content and the routine technique for histological sections stained with hematoxilyn and eosin. The material was then analyzed and photographed using a Zeiss photomicroscope.

The non-parametric Wilcoxon test was used to analyze the protein content variation of control and experimental workers of the same age during summer and winter. Variations between ages and variations between the same ages in both control and experimental groups during summer and winter were analyzed, using the non-parametric Kruskall-Wallis test, complemented by the Jonckheere test (1954 apud Campos) (6).

RESULTS

During venom extraction, 7-day old workers responded more slowly and weakly to electrical shocks. This response intensifies with age, so that the workers approaching 20 days old responded faster and more aggressively to shocks.

The values of total protein content (μg/gland) are shown in Table 1 and were used in Figure 1 and Figure 2, corresponding to summer and winter, respectively. The results of the Wilcoxon test showed that variations in the protein content were significant between summer and winter workers of corresponding ages and groups (Table 2).
TABLE 1. Means and standard deviations of protein content values (mg/gland) in summer (S) and winter (W) before venom extraction (C) and 24 and 96 hours after extraction (E).

| Ages/days | Intervals | X ± SD _S_ | X ± SD _W_ |
|-----------|-----------|------------|------------|
| 7         | Control   | 577 ± 31.9 | 10.6 ± 1.5 |
| 7         | 24h       | 18.9 ± 5.9 | 3.4 ± 1.0  |
| 7         | 96h       | 19.6 ± 10.7| 4.9 ± 2.7  |
| 14        | Control   | 94.1 ± 33.3| 35.3 ± 7.2 |
| 14        | 24h       | 12.5 ± 1.4 | 12.4 ± 9.1 |
| 14        | 96h       | 22.9 ± 12.3| 17.6 ± 19.7|
| 21        | Control   | 104.4 ± 31.0| 87.4 ± 5.2 |
| 21        | 24h       | 30.2 ± 10.2| 12.9 ± 9.1 |
| 21        | 96h       | 13.7 ± 15.2| 19.4 ± 15.6|
| 28        | Control   | 126.2 ± 43.7| 58.5 ± 8.4 |
| 28        | 24h       | 23.9 ± 11.9| 18.2 ± 10.8|
| 28        | 96h       | 27.2 ± 6.6 | 9.4 ± 8.0  |
| 35        | Control   | 114.0 ± 45.9| 65.1 ± 5.5 |
| 35        | 24h       | 6.1 ± 1.6  | 6.9 ± 7.0  |
| 35        | 96h       | 5.5 ± 1.2  | 39.4 ± 11.0|
| 40        | Control   | 85.7 ± 61.7| 80.8 ± 8.2 |
| 40        | 24h       | 29.0 ± 19.9| 30.6 ± 25.0|
| 40        | 96h       | 4.0 ± 1.9  | 19.7 ± 20.0|

FIGURE 1. Mean values of the total protein content in (mg/gland) of Apis mellifera L. workers venom glands at different ages in summer before venom extraction (control Group C) and 24 and 96 hours after extraction (experimental Group E).

![Graph showing protein content over ages](image)
FIGURE 2. Mean values of the total protein content in (mg/gland) of *Apis mellifera* workers venom glands at different ages in winter before venom extraction (control Group C) and 24 and 96 hours after extraction (experimental Group E).

TABLE 2. Results of the Wilcoxon's non-parametric test for protein content values in summer and winter workers of corresponding ages and Groups (C and E).

| Ages/days | Control Group (C) | Experimental Group (E) |
|-----------|-------------------|------------------------|
| 7         | 0.0410*           | 0.0048* 0.0289*        |
| 14        | 0.0042*           | 0.3743* 0.3751*        |
| 21        | 0.3751*           | 0.0301* 0.1255*        |
| 28        | 0.0292*           | 0.1262* 0.0181*        |
| 35        | 0.2911*           | 0.9656* 0.0046*        |
| 40        | 0.3751*           | 0.8095  0.0112*        |

Table 3 shows that in Groups C and E the venom glands of winter workers present significantly lower levels of protein content at all ages than those of summer workers. Table 3 also shows a difference in behavior for all ages between winter and summer (Figure 1 and Figure 2). The total protein content of the venom glands from the control group during the summer practically doubled from days 7 to 28, and then decreased slightly (Table 1 and Table 3, Figure 1). In winter, the venom glands of Group C workers showed the lowest protein content at day 7, with a significant increase up to day 21, followed by a decrease at day 28. After this, the protein content undergoes a slight increase (Table 1 and Table 3, Figure 2).

TABLE 3. Results of the Kruskal-Wallis non-parametric test for protein content values at all ages in summer (S) and winter (W).
After venom extraction, there is a significant fall in the amount of total glandular protein at all ages, both in summer and winter (Table 1 and Table 3). Variations in Group E for all ages follow practically the same pattern as in summer (Figure 1) and winter (Figure 2), except at day 35, when 96 hours after extraction, the protein content increases significantly in the winter workers (Figure 2) in comparison to summer workers of the same age (Figure 1). At some ages, the values of protein content differ significantly 24 and 96 hours after venom extraction (Table 4), being higher either 24 or 96 hours after extraction, as shown in Table 1 and Figure 1 and Figure 2.

**Table 4.** Results of the Kruskal-Wallis non-parametric test for protein content values in the control (C) and experimental (E) Groups (24 and 96 hours) at each age in summer (S) and winter (W).

| Groups | 7 days | 14 days | 21 days | 28 days | 35 days | 40 days |
|--------|--------|---------|---------|---------|---------|---------|
|        | S      | S       | S       | S       | S       | S       |
| C - 24h | 43*    | 60.5*   | 61*     | 40*     | 44*     | 60*     | 55*     | 42*     | 52*     | 52*     | 30*     | 32*     |
| C - 96h | 42*    | 47.5*   | 47*     | 44*     | 64*     | 48*     | 53*     | 66*     | 56*     | 56*     | 64*     | 64*     |
| E - 24h-96h | 6     | 13*     | 14*     | 4       | 20*     | 12*     | 2       | 24*     | 4       | 4       | 32*     | 32*     |

* Significance 5%.

Histologically, in Group C workers, the presence of secretion in the lumen of the secretory filament (Figure 3A) and in the reservoir (Figure 3B) was always observed at all ages in summer and winter. After venom extraction, the lumen of the glandular filament lacked any secretion (Figure 3C), and the reservoir was collapsed and almost completely empty (Figure 3D). For 7-day old workers, the reservoir wall did not collapse (Figure 3E), retaining a substantial amount of secretion after shock, as did the glandular tubules (Figure 3F). The secretory cells remained histologically unaffected (Figures 3A, 3C and 3F), independent of age, treatment, or season.
FIGURE 3. Venom gland of *Apis mellifera* L. workers. A= Secretory filament before shock, 35-day old (X530); B= Venom reservoir before shock, 35-day old (X70); C= Secretory filament after shock, 35-day old (X480); D= Venom reservoir after shock, 35-day old (X190); E= Venom reservoir after shock, 7-day old (X60); and F= Secretory filament after shock, 7-day old (X530). c= cuticle; l= lumen; n= nucleus; and s= secretion.

DISCUSSION

The speed at which the workers responded to the electrical shocks increased with age. The fact that 7-day old workers responded less efficiently to these stimuli may be related to the immaturity of these bees. At this age, they only work within the colony, and sting use is not required. This immaturity is also reflected by the low protein content found in 7-day old workers of the control group and indicates that these bees are not able to defend the colony. On the other hand, the older workers are more efficient in defending themselves and the colony when they leave, mainly in search of food, since they are more exposed to actual dangers and respond faster to environmental stimuli. This behavior requires the sting mechanism to mature and produce a higher amount of venom. An increase in the amount of venom can be inferred from the increase in total protein content and from the histology of the glandular cells, which is common for workers of all ages both in summer and winter.
The cycle of the venom glands, identified by the total protein content, was different in summer and winter bees. The decrease in protein content of summer bees begins at the age of 35 days and appears to be continuous, indicating the onset of the regression phase of a single glandular cycle. There are indications that more than one cycle occurs in winter bees, since the protein content increases up to 21 days and then decreases, increasing again at 40 days. The higher longevity of winter workers (2,8,14) may justify this. The cells of the distal portion of the secretory tubule in old summer and winter workers are still histologically intact (1), which enables them to produce secretion.

The fact that the protein content in the venom glands of winter workers is less than in summer workers at all ages shows a lower physiological activity of winter bees, which is in agreement with the observations of Autrum & Kneitz (2) and Cruz-Landim et al. (8). They suggested a period of quiescence in the life of winter bees. In the control group, the highest protein content was detected 21 days of age and corresponded to 70% of the highest value of summer workers that was measured at 28 days (126.20±g/gland). In addition to this, the lowest protein content found was about 50% in 7-day old summer workers and 10% in 7-day old winter workers. These data indicate that venom extraction is more productive in summer using older workers.

In the glands of all workers analyzed 24 and 96 hours after shock application, the protein content seems to show a capacity of replacing a reasonable amount of the protein eliminated during stinging of the substrate. However, while considering that the glands are capable of synthesizing proteins after venom extraction, the levels obtained were well below those detected in Group C. This results in unproductive reuse of these workers for venom extraction. Both the highest levels of venom replacement at each age and the necessary time for replacement varied in summer and winter.

The results of this study differed considerably from those obtained by Owen (15), which showed a level of histamine replacement of up to 87.5% of the initial value obtained before extraction. Moreover, as only about 65-75% of the histamine was removed from the workers’ reservoirs by electrical shocks, this would result from the rigidity of the endocuticular layer that covers and maintains the form of the reservoir wall. However, this and other recent studies using optical and scanning electron microscopy showed reservoirs with totally collapsed walls and only traces of secretion after electrical shock, indicating venom removal (1). The secretion measured by histological and protein content analyses of glands of 7-day old workers may result from a less efficient response to electrical shocks, with partial retention of venom in the gland. Therefore, the replacement levels of about 50% found in 7-day old workers in winter and summer may be due to protein re-synthesis or to proteins present in the venom retained by the inferior stinging ability of young workers.

Variation in the protein content values of Group E between 24 and 96 hours after extraction at a similar age was significant. The values of protein content at 96 hours after extraction were significantly lower than those at 24 hours at several ages, although they were expected to be either higher or at least equal to the content at 24 hours both in summer and winter. This finding was more frequent in older workers, reflecting both individual differences to the response of the extraction and re-
absorption or degradation of proteins from the glandular secretion with aging, indicating that very old workers are less appropriate for obtaining venom.

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