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A SIMPLE *IN VIVO* ESTIMATION OF HYPOPHARYNGEAL GLAND ACTIVITY IN HONEYBEES (*APIS MELLIFERA* L., APIDAE, HYMENOPTERA)

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Summary

The hypopharyngeal glands (HGs) are important protein-producing food glands in worker honeybees (*Apis mellifera* L.). A simple *in vivo* method is described for estimating HG activity. Radioactive ^{14}C-L-leucine (0·1 μCi in 1 μl) is injected directly into the haemocoele of honeybees, and is subsequently incorporated into the HGs during protein synthesis. After 2 h incubation, the amount of radioactive leucine incorporated by the gland is measured and serves as an indicator of synthetic activity in the glands. The technique has been used successfully to reveal gland activity differences in bees from hives with and without brood. This technique is simple, fast, and reliable. Its advantages and disadvantages compared with the *in vitro* assay method of Brouwers (1982) are also discussed.

Introduction

The hypopharyngeal glands (HGs) are pairs of food glands located in the heads of worker honeybees (*Apis mellifera* L.). Consisting of many acini attached to a central duct, the glands produce proteinaceous secretions which are fed to the larvae, queens, and drones (Ribbands, 1953; Snodgrass, 1956). These glands also produce digestive enzymes for honey processing (Simpson et al., 1968). The development and activity of these glands have been subjects of study since early this century because the glands are intimately involved in many important aspects of honeybee biology, including the division-of-labour (Free, 1961; Fluri et al., 1977); differentiation of summer and winter bees (Halberstadt, 1966); queen–worker differentiation (Jung-Hoffman, 1966); and worker–brood interactions (Halberstadt, 1967; Buhler et al., 1983; Huang & Otis, 1989; Huang et al., 1989).

Several different methods have been developed to estimate HG activity. The earlier methods mostly relied on morphology and/or sizes of acini, and implicitly assumed that larger glands were always more active. For example, Maurizio (1954) used four arbitrary categories based on glandular morphology; Hassanein (1952) used the size of globules of the glands; Rosca et al. (1972, cited by Brouwers, 1982) used the total protein content of the glands; and Fluri et al. (1982) used the dry weight of the glands. Brouwers (1982) developed a novel method to measure gland activity. Strictly speaking, only this method measures the actual ‘glandular activity’, because the rate of protein synthesis was measured by incubating isolated glands *in vitro*. Brouwers (1982) showed that the correlation between size and glandular activity is not straight-forward, because broodless bees (such as winter bees or bees in a swarm) usually have hypertrophied glands and a low rate of protein synthesis. Huang et al. (1989) also observed that medium-sized glands were more active in broodfood production.

In a series of experiments to investigate the brood–worker interactions (Huang, 1988), it became necessary to use a simple and accurate technique to estimate the HG activity due to the large numbers of bees being sampled. In this paper, an *in vivo* method is described, in which the gland activity is estimated by using a ^{14}C-labelled amino acid. This new method has several advantages over previous techniques.

Materials and Methods

Honeybees (*Apis mellifera* L.) from the University of Guelph apiaries were used throughout the experiment. These bees represent a mixture of European races of bees. Capped brood was kept in incubators (34–35°C, 50% RH) to obtain newly emerged bees which were either used

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directly in tests or colour marked and introduced into single broodbox colonies within 24 h of emergence. These bees were sampled and tested in the laboratory when they reached the required age.

Measurement of radioactivity

The radioactive amino acid was [U-^{14}C] L-leucine (337 mCi*/mmol, New England Nuclear). The radioactivity was 0·25 mCi in 2·5 ml of 0·01-N HCl. Thus 1 μl of the original solution contains 0·1 μCi radioactivity. *Apis* saline, made according to Brouwers (1982), was used to dilute the leucine as required.

Radioactive L-leucine was injected into bees which were left alive for a period of time to allow the glands to take up part of the leucine. The details of that part of the technique varied with different experiments and are described separately below in three sections. At the end of the incubation time, HGs of individual bees were removed and washed four times in 7% trichloroacetic acid (TCA), and put into scintillation vials which contained 0·2 ml of 1-N NaOH for at least an hour to dissolve the protein. Then 10 ml of Aqueous Counting Scintillant (ACS, Amersham) was added to each vial. Radioactivity was measured by a Packard Scintillation Counter 460C. The counter gave direct readings of disintegrations per minute (DPM) which were obtained by correcting the counts per minute (CPM) with a 'quench curve' stored in the memory of the scintillation counter. The counting efficiencies of this model of scintillation counter are 92–95% for ^{14}C.

Dose-response experiment

Three different doses of ^{14}C-leucine (0·005, 0·05, 0·1 μCi in 1 μl) were injected into the abdomens of bees just emerged (0–24 h old) or bees 8 days old. The latter were colour-marked bees of known age, sampled from a colony with queen and brood. Because it was observed that injected bees had a slightly higher mortality at 35°C than at room temperature (29–31°C), injected bees were 'incubated' for 4 h at room temperature. They were then killed by 20% TCA, their glands were dissected in cold *Apis* saline, and the amount of radioactivity in the glands was measured as described. Ten bees were used at each concentration for newly emerged and 8-day-old bees, so altogether, 60 bees were used.

Determination of estimation parameters

In the dose–response test, bees were injected in the abdomen and incubated for 4 h. However, it was not clear if this represented the best incubation interval or site of injection. Furthermore, it was of practical interest to learn whether the bees killed with TCA could be left for several hours before proceeding with the determination of HG activity without affecting the amounts of radioactivity measured in the glands. A factorial experiment was designed to test all these variables in order to maximise the recovery of radioactivity. Bees of age 0 (newly emerged) and 4 days were used with bee ages serving as 'blocks'. Variables tested were the duration of incubation, the interval before dissection of the glands, and the site of injection. A detailed description of the factorial experiment is given in Table 1. Thirty two bees were used in the test, and the test was replicated two times. For subsequent experiments, a standardized procedure was adopted based on the outcome of this experiment.

Table 1. Description of a 4 × 2 × 2 factorial experiment to determine the effects of duration of incubation, time of dissection, and site of injection on uptake of radioactive leucine by HGs.

Factor	*Level*	*Description*
Age (block)	2	Bees 0 and 4 days old
Duration	4	Bees incubated for 0, 1, 2, and 3 h after injection
Time	2	Dead bees held for 0 and 15 h before dissection
Site	2	^{14}C injected into thorax or abdomen

* 1Curie = $3{\cdot}7 \times 10^{10}$ Becquerels.

Evaluation of the estimation

To evaluate the estimation, bees were sampled from 2 colonies with brood and 2 colonies without brood. Broodless colonies were created by placing the queen in a wire cage (8 mesh), which allowed the worker bees to contact the queen and her pheromones, but prevented her from laying eggs. After the broodless colonies had been without brood for 20 days, 20 newly emerged bees (0–24 h old) were marked with coloured Testor's paint and introduced to the 4 colonies. This was repeated for 3 consecutive days, each day with a different colour mark. When the first colour-marked cohort was 8 days old, 5 bees were sampled from each colony. This was repeated for 8-day-old bees on 3 consecutive days, resulting in 15 bees being studied from each colony. Altogether, 60 bees were sampled, of which 2 died after injection. The standardized procedure was used to measure the radioactivity incorporated into the glands of these bees.

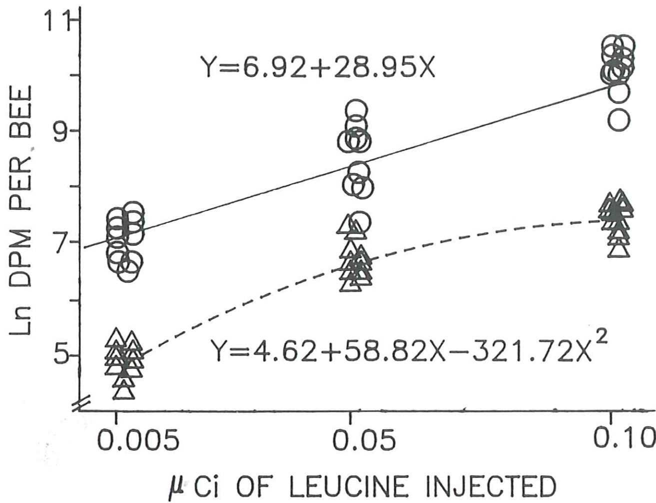

FIG. 1. Dose–response curves for the *in vivo* method.
For bees 8 days old (circles), a linear relationship existed between the Ln DPM per bee and dosage of leucine injected (solid line); for bees 0 days old (triangles), a quadratic curve was the best fit (dotted line).

Statistical analysis

Statistical analyses were all performed after data transformation by taking the natural logarithm (Ln) of DPM, since it was found that DPM was not normally distributed after residual analysis. Linear and quadratic regression and variance analysis of the factorial experiment were performed using the Statistical Analysis System (SAS version 5·16 ETS 1984, 1985) accessed through the mainframe computer (IBM 4381-3, VM/SP 4·11) at the University of Guelph. The t-test comparing the two means was performed according to Steel and Torrie (1980).

TABLE 2. ANOVA table for the factorial experiment.

Source	*df*	*Sum of squares*	*F value*	*PR*
Age (block)	1	114·67	79·18	0·0001
Duration (A)	3	204·25	47·01	0·0001
Time (B)	1	0·15	0·10	0·75
Position (C)	1	1·87	1·29	0·26
A × B	1	74	0·86	0·47
A × C	3	1·00	0·23	0·88
B × C	1	0·29	0·20	0·66
A × B × C	3	2·26	0·52	0·67
Error	47	68·07		

Results

Dose-response experiment

For newly emerged bees, a quadratic relationship was found between the amount of leucine injected and the radioactivity recovered from the glands, while for bees 8 days old, there was a linear relationship (Fig. 1). The greatest differentiation between bees with active glands (8-day-old bees) and bees with inactive glands (bees newly emerged) occurred at a concentration of 0·1 μCi. This concentration was adopted in subsequent experiments.

Determination of estimation parameters

The results of the factorial experiment are presented in Table 2. The block effect (age of bees) was found to be highly significant ($P = 0{\cdot}0001$). Four-day-old bees sampled from a colony with brood had a much higher HG activity than recently emerged bees (Fig. 2). The incubation duration (the time during which active incorporation of ^{14}C-leucine could occur) also had a significant influence on the amount of ^{14}C incorporated into the glands ($P = 0{\cdot}0001$). A comparison between different incubation periods for both age groups showed that, at 2 h after injection, the glands had attained the maximum incorporation of ^{14}C-leucine (Fig. 2). Dissection of bees immediately after killing or 15 h later, did not affect the recovery of radioactivity ($P = 0{\cdot}75$). Nor did injection into the thorax or abdomen make any difference ($P = 0{\cdot}26$). All interactions between these factors were not significant.

Taking into consideration the above information, the procedure for measuring the HG activity was 'standardized' by selecting those conditions that maximized the recovery of radioactivity. The standardized *in vivo* technique is outlined below.

1. Inject bees in the thorax with 1 μl ^{14}C-leucine (0·1 μCi).
2. Leave bees at room temperature for 2 h in glass vials.
3. Pipette 3 ml 20% TCA into the vials to kill the bees.
4. Dissect the glands from the bees 0–15 h later.
5. Rinse the glands 4 times with 7% TCA.
6. Dissolve the glands in 0·2 ml NaOH (1 N).
7. Measure ^{14}C incorporated by the glands with a liquid scintillation counter.

The thorax was chosen as the preferred site of injection, because less bleeding occurred with injection into the thorax than into the abdomen.

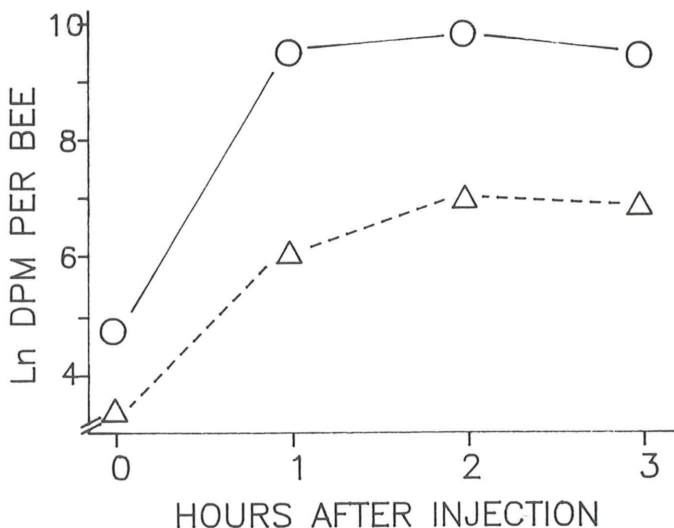

FIG. 2. The amount of radioactivity recovered in HGs after different incubation periods. The two sets of data are for: newly emerged bees (triangles) and 4-day-old bees (circles). Each point represents a mean of 6 bees.

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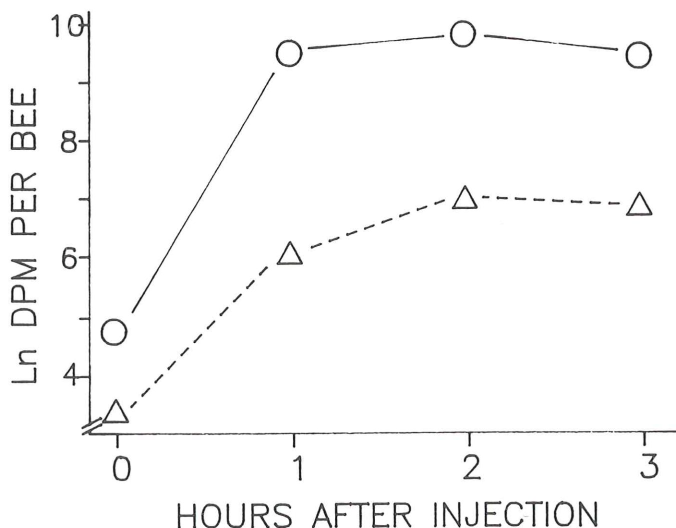

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Evaluation of the technique

This standardized method was evaluated by testing gland activity of bees from hives with and without brood. The results are presented in Table 3. No difference was found between the two hives within each treatment. Consequently, bees from different hives were pooled together for statistical testing. A *t*-test showed that there was a highly significant difference between bees from broodright and broodless hives ($t = 5{\cdot}37$, $df = 56$, $P<0{\cdot}001$).

TABLE 3. Means and their standard errors for HG activity of worker bees in hives with and without brood.

Hive	*Brood*	*No. bees*	*Mean DPM*	*Ln DPM*
1, 2	present	30	18554·60 ± 2234·29*	9·57 ± 0·14
3, 4	absent	28	6133·25 ± 502·95	8·60 ± 0·11

* The gland activity (DPM) of bees from broodless and broodright colonies are significantly different according to Student's *t*-Test. Test done on the Ln DPM: $t = 5{\cdot}37$, $df = 56$, $P<0{\cdot}0001$.

Discussion

This study demonstrates that the HG activity of honeybees can be satisfactorily estimated by an *in vivo* method. The principle behind the method is very similar to that of Brouwers (1982), but the incorporation of the radioactive amino acid is *in vivo* rather than *in vitro*. This method does not distinguish labelled free amino acids from those actually incorporated into the proteinaceous glandular secretions, since the glands are not homogenized with subsequent filtration of precipitated protein. Rinse in TCA would not wash away free amino acids, since the glands are globulous. However, the amount of radioactivity recovered from the glands is undoubtedly correlated with the rate of protein production by these glands, since newly emerged bees which still had undeveloped HG glands always showed substantially lower amounts of ^{14}C-leucine in their glands compared with bees 8 days old, which had fully developed glands and should have been nurse bees (Free, 1965). Differences of the same relative magnitude in HG activity of bees from hives with and without brood, as measured by both this *in vivo* method and the *in vitro* method of Brouwers (1982), further indicated the reliability of the *in vivo* method.

The main purpose of this study was to develop a simple method for differentiating bees with HGs actively producing broodfood from those with inactive glands. The *in vivo* method described herein accomplishes this. Several physiological factors which could have produced the observed difference in gland activity between broodless and broodright bees were not measured. For example, broodless bees may have a considerably larger endogenous free leucine pool, so that injected radioactive leucine would be more diluted in those bees, resulting in lower observed activity. This could not have happened for the reasons presented below. It has been shown that nurse bees have a high leucine concentration (Snitsky & Levchenko, 1971; Crailsheim, 1986). In contrast, winter bees as well as foragers, have low leucine concentrations (Crailsheim, 1986). It has been shown repeatedly, that winter bees differ physiologically from summer bees, not because of the seasonal difference, but because of the absence of brood; 'winter' bees can be produced in summer by caging the queen and creating broodless colonies (Maurizio, 1950; Halberstadt, 1967; Fluri et al., 1982). Therefore, broodless bees in this experiment, physiologically similar to those of winter bees as shown by their low HG activity, at best should have lower leucine concentrations than the nurse bees from the broodright colonies. Consequently, the gland activity of the broodless and broodright bees must be regarded as different *despite* any possible leucine pool difference, not because of it.

Difference in total haemolymph volume would also affect the concentration of injected ^{14}C-leucine in the experimental bees. The method would be inadequate if for some reason nurse bees have a smaller haemolymph volume. However, nurse bees actually have larger volumes of haemolymph (18·3 μl) than either winter bees (15·9 μl) or foragers (14·9 μl) (Crailsheim, 1985). Other fine-level differences, such as the difference in intracellular compartmentalization of endogenous leucine, or in utilization of intra- *vs.* intercellular

leucine, if they exist, should not have created a difference of the magnitude shown in Table 3. A similar *in vivo* technique for measuring the protein synthesis of the whole bee body tissues, which include the HGs has been developed and successfully used to study the dependence of honeybee protein metabolism on worker age and season (Crailsheim, 1986).

Because the HGs consist of a thousand or more, pear-shaped acini attached to a central duct (Ribbands, 1953) tightly coiled together in the head, it is almost impossible to remove the glands without physical damage. It is not known how this damage might influence the protein synthesis *in vitro* or leakage of synthesized proteins from the glands. The *in vivo* method avoids this problem by 'incubating' the glands *in situ* in the head of the live bee. Only after the incorporation period is completed are the glands taken out. At this time, the glands are less fragile, possibly due to the effects of TCA. Injections into the thorax, which the *in vivo* method involve, caused no apparent damage to the bees. Injection of exogenous materials into bee haemolymph is a common practice employed for a variety of experimental purposes, such as studying haemolymph volume (Crailsheim, 1985; Weinberg & Madel, 1985); the effect of juvenile hormone on bee division-of-labour (Jaycox et al., 1974; Rutz et al., 1974; Jaycox, 1976; Rutz et al., 1976) and on HG activation (Brouwers, 1983); and protein, lipid and carbohydrate metabolism in honeybees (Bounias et al., 1986; Crailsheim, 1986). In some of these studies, injected bees were returned to their original hives for extended periods of behavioural observation, and no abnormal behaviour caused by injections was reported by these authors.

The technique described here is sensitive enough to be used in the investigations of changes in HG activity (Huang & Otis, 1989). The DPMs per bee (broodright or broodless) obtained by this method, are in the same range as those obtained with the *in vitro* method (Brouwers, personal communication), even though the *in vivo* method uses only one fifth as much radioactivity, and the period of incubation is shorter (2 *vs.* 4 h). Compared with Brouwers' (1982) method, the *in vivo* method described here is simpler (no incubation medium is needed), less time-consuming, and more economical (less radioactive leucine used for the same result). One characteristic is that, by 'incubating' the glands in living bees, the physiological systems of the bees being tested are minimally disturbed, and the rate of uptake by the glands may more accurately reflect the physiological state of the bees being tested. Therefore, this method could be employed when the purpose of the experiment is to focus on the response of individual bees. One disadvantage is that because of its simplicity, the method cannot control other possible factors affecting the rate of incorporation of labelled amino acid. The *in vitro* method by Brouwers (1982) is more refined, in that it can eliminate influences by other uncontrollable factors such as the differences in leucine pool and haemolymph volume. Another advantage of the *in vitro* method is manifested when one aims to study the response of isolated glands, rather than individual honeybees, to various physiological conditions which can be stimulated through changes in the incubating medium.

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