Identification of Nasonov Pheromones and the Effects of Synthetic Pheromones on the Clustering Activity of the Asiatic Honeybee (*Apis Cerana*)

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**ABSTRACT**

Worker bees from several *Apis cerana* colonies were sampled for identification of Nasonov secretions and their clustering activity in response to different synthetic components of the swarming pheromones. Extraction of Nasonov gland secretion was carried out by three methods – Abdomen Dipping method, Excised Gland method and Syringe Extraction method – and its analysis was carried out by Gas Liquid Chromatography. Nasonov components detected were (E)-citral, (Z)-citral and geraniol. However, only (E)-citral and geraniol were quantified. The Abdomen Dipping method detected 20.0 ug per bee of (E)-citral, whereas the Excised Gland method detected only 3.8 ug. Geraniol was only detected by the latter technique at 2.5 ug per bee. (Z)-citral was detected in trace amounts. The clustering experiments were conducted in a confined darkened room at room temperature. Chemical components, consisting of 9-oxo-2-decenoic acid designated as 9ODA, (E), (Z)-citral, geraniol, nerol and farnesol, were tested either singly or in various combinations. Each component or mixtures of these components were adsorbed on a filter paper placed in a lighted perforated cardboard cannister. Five cannisters were used in each trial. Among the synthetic components tested, the (E), (Z)-citral did not form a cluster in attracting worker bees to form a cluster.
INTRODUCTION

The Nasonov gland, situated between the tergites of the 6th and 7th abdominal segments of the honeybee, secretes Nasonov pheromones. In the Nasonov gland of the honeybee Apis mellifera, the Nasonov pheromone components were identified as (E) and (Z)-citral, nerol, geraniol, nerolic acid, geranic acid and (E,E)-farnesol, in 1:1:1:1:100:75:12:50 proportions, respectively (Free et al. 1981). It was found that the Nasonov pheromone secretions of A. mellifera elicited responses such as clustering activity during swarming and foraging at artificial feeding of sugar syrup (Morse and Boch 1971; Mautz et al. 1972; Free and Williams 1970). It was also shown by Free et al. (1983) that Nasonov pheromone lures, singly, in concert or with the queen substance (9-oxo-2-decenoic acid, designated as 90DA) could result in clustering activities.

Apis cerana, a common species in the Indo-Malayan region, has not been studied in terms of the components of the Nasonov gland secretions and their clustering or swarming effects. This study was intended to identify the natural components of Nasonov gland secretions and the effects of synthetic chemicals consisting of 9-oxo-2-decenoic acid, (E), (Z)-citral, geraniol, nerol and farnesol on the swarming activity of A. cerana.

MATERIALS AND METHODS

Chemical components used to resemble naturally occurring pheromones were pure (E), (Z)-citral (Koch Light), 95% farnesol (mixture of isomers, Aldrich), 95% geraniol (Sigma), 95% nerol (Tokyo Kasei) and 90DA (from J.A. Pickett). These chemical components were used without further purification.

Capturing of Worker Bees

About 700-1000 bees were captured from a number of wild bee colonies in the vicinity of coconut holdings at Universiti Pertanian Malaysia. Bees from one colony were used for one trial. They were captured directly from the hives and placed in a box (40x40x40 cm) fitted with a wire gauze for ventilation. All experiments on clustering behaviour were done after the captured bees were settled and calmed inside the box for approximately two hours. Some worker bees were kept aside for the extraction of the Nasonov pheromones. The extraction of gland secretions was carried out on the same day.

Analyses of Nasonov Gland Secretions

1. Extraction of Nasonov Gland Secretion

The following methods were employed for extraction of secretions of the Nasonov gland between the 6th and 7th abdominal tergites of the honey bee.

a. Syringe Extraction Method

To obtain a sample of the Nasonov gland, the live bee (immobilized by placing it in the cold at 4°C for 3-5 min) was held between the forefinger and thumb with the Nasonov groove exposed. The tip of the needle of a 10 μl syringe containing 2 μl hexane was placed between the 6th and 7th dorsal abdominal tergites and the hexane was expelled from the syringe onto the Nasonov gland groove. The hexane expelled was immediately withdrawn back into the syringe. About 0.4 μl of hexane solution was recovered and analysed by gas liquid chromatography (GC).

b. Abdomen Dipping Method

When the A. cerana workers were captured and released in the experimental room, it was observed that many of them raised their abdomen and exposed their Nasonov glands and fanned them with their wings. Such workers were caught by the abdomen using a forcep and the lower part that contained the Nasonov gland was shaken in 0.5 ml hexane. Ten bees were used for each 0.5 ml hexane and 1.5 μl was directly analysed by GC.

c. Dispersing Excised Gland Method

For determining the absolute amounts of pheromones present in the Nasonov gland, the bees were killed and the 6th and 7th tergites of the abdomen containing the Nasonov gland was dissected and placed in 0.5 ml hexane. Ten glands were isolated and shaken in 1 ml hexane, and 1.5 μl solution was immediately analysed by GC.
2. Identification of Pheromone by Gas Chromatography

Nasonov pheromones of A. cerana were analysed by Gas Chromatograph (HP 5840 fitted with an FID detector). Two prepacked glass columns (Supelco, 2.5% Carbowax 20M on Chromosorb G-HP 80-100 mesh, 6' x 1/4") were used for the analyses with nitrogen as the carrier gas with a flow rate of 20 ml/min. The oven temperatures were programmed to change from 50 to 200°C at 10°C/min. A standard mixture of 8 mg (E), (Z)-citral, 4 mg nerol, 4 mg geraniol and 4 mg farnesol per ml hexane was used as the reference.

Effects of Synthetic Pheromones on the Clustering Activity of A. cerana

Clustering response to the synthetic pheromone components were conducted in an experimental room measuring 3.3x2.7x3.1 m. The room was darkened by plastering black paper over the glass windows as preliminary experiments showed that bees were attracted to the bright daylight. Cardboard containers in the form of cannisters measuring 24 cm in length and 8 cm in diameter, perforated with holes (3 mm wide and 1.5 cm apart) were used to hold the synthetic pheromone components. They were hung in a straight line on a wire at about 50 cm apart. The cannisters were lighted with 5 watt bulbs (which was placed in each cannister) while the rest of the room was in darkness. This was necessary because in earlier experiments it was observed that bees would converge for light at the glass windows. The lighted cannisters would attract bees equally and any differences would be the results of the various chemicals used. The lighted cannisters would also facilitate observation of the bees in the darkroom. The temperature and relative humidity of the room was about 28°C and 60%, respectively. The positions of each cannister containing synthetic components of the Nasonov pheromone was randomly changed for every experiment and its replicate. The same cannisters were used for all trials after a week of aeration.

Each pheromone was tested singly or in concert and the amount used for studying the clustering activity was 60 µg, 30 µg, 1.5 mg, 3 mg and 1.5 mg for (E), (Z)-citral, nerol, farnesol, geraniol and 90DA, respectively. These levels were 15 times higher than those used by Ferguson et al. (1979) in their study on the clustering activity of A. mellifera. Higher quantity was necessary since preliminary experiments showed that lower levels were not effective with A. cerana.

The synthetic pheromone components dissolved in hexane was adsorbed onto filter paper (Whatman No.1) and placed in each cannister. The cannisters were then covered by wrapping a piece of paper around them. The covered cannisters were then hung in the room. After 30 min the cover over each cannister was removed and 700-1000 worker bees were immediately released into the room. One cannister was always left untreated (control) for each experiment.

Each experiment was repeated at least twice on different days using a fresh batch of worker bees from a different colony. The number of bees which had clustered on each cannister was counted at 5 min intervals for 1 hour.

RESULTS AND DISCUSSION

Detection of Pheromones

Table 1 shows the Nasonov pheromones detected and their concentrations according to the methods used to extract them from the worker bees. Gas chromatograph peaks corresponding to (E)-citral, (Z)-citral and geraniol were detected from samples obtained by the Syringe Extraction procedure. Other extraction procedures showed the presence of only (E)-citral and geraniol. For (E)-citral, the amount detected in the glands by the Abdomen Dipping method gave a higher value (20 µg per bee) than the Excised Gland method (3.8 µg per bee). The amount of geraniol detected by the Excised Gland method was 2.5 µg per bee. Pickett et al. (1980), using the 'Dispersing Excised Gland' method, reported that A. mellifera excreted 0.02 and 1.80 µg per bee of (E)-citral and geraniol, respectively. In the present study, the other components of Nasonov gland produced by A. mellifera (Free et al. 1981) were not detected in A. cerana. The failure to detect their presence could possibly be due to their minute amounts and also to
TABLE 1
Components of Nasonov gland secretions and their concentration

<table>
<thead>
<tr>
<th>Extracting Methods</th>
<th>Pheromones detected</th>
<th>µg per bee</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A. cerana</td>
</tr>
<tr>
<td>Syringe Extraction</td>
<td>(E)-citral</td>
<td>not quantified</td>
</tr>
<tr>
<td></td>
<td>(Z)-citral</td>
<td>not quantified</td>
</tr>
<tr>
<td></td>
<td>Geraniol</td>
<td>20.0</td>
</tr>
<tr>
<td>Abdomen Dipping</td>
<td>(E)-citral</td>
<td>3.8</td>
</tr>
<tr>
<td>Dispersing Excised Gland</td>
<td>(E)-citral</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Geraniol</td>
<td></td>
</tr>
</tbody>
</table>

*Pickett et al. 1980.

the fact that they are only produced when required (Pickett et al. 1981).

It would have been better if the two honey bee species could be studied at the same time. However, this is not possible since *A. Mellifera* is not available in this country.

**Clustering Activity of A. cerana**

The amount of pheromones used was 15 x higher than that used for *A. mellifera* (Ferguson et al. 1979) as lower concentrations were found ineffective.

Since the number of bees collected for each trial varied within the range of 700 to 1000, the number of bees attracted to each cannister at each observation was normalized before the data could be presented. This was done by giving to the maximum number of bees attracted to a particular cannister for each trial a value of one. All other counts of bees attracted to each cannister in the trial were compared to this maximum number and calculated as a proportion of the maximum. In this clustering activity study, up to 30% difference in response to a particular treatment was observed between duplicates.

**Fig. 1** shows the relative number of bees that landed on the cannister containing either (E), (Z)-citral, geraniol, nerol or 90DA. The first 15 min after their release could be considered as an adaptation period where the bees just crawled around and made short flights. The bees then began to fly and clustered on the cannisters. After 30 min, the number of bees on the cannister containing (E), (Z)-citral was the highest. However the cluster was not stable after 40 min. The other Nasonov pheromones viz, nerol, geraniol and farnesol were not effective when compared to (E), (Z)-citral. Geraniol and 90DA were able to attract only a few number of bees.

When the cannisters were treated with 90DA in combination with either geraniol, nerol, farnesol or (E), (Z)-citral, bees were found to be attracted to all these mixtures (Fig. 2). Again, the clusters did not seem to be stable after 40 min. When 90DA was tested in concert with (E), (Z)-citral, geraniol, nerol and farnesol, its presence was not essential (Fig. 3), but the cluster on the cannister containing CGFN +
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90DA seemed to be more stable. However, (E), (Z)-citral was observed to be more effective when tested against this combination (Fig. 4), but as observed earlier the cluster was not stable after 40 min. Experiments with different levels (20-60 μg) of (E), (Z)-citral showed no effects of citral concentrations on the number of bees settling on the cannisters.

The results indicate that (E), (Z)-citral plays an important role in attracting bees, but the other components (nerol, geraniol, farnesol and 90DA) are also required for the cluster to be more stable. With A. mellifera, Free et al. (1981) also observed the effectiveness of citral in clustering activity when compared to geraniol or farnesol. 90DA was also not effective in clustering activity of A. cerana. It could be possible that other factors are required for cluster formation as Ferguson et al. (1979) observed pure queen factor was rather ineffective compared to a crushed queen’s head. Nerol, as similarly observed in A. mellifera (Free et al. 1981), was also not effective.

The effectiveness of (E),(Z)-citral against a combination of 90DA, geraniol, nerol and farnesol needs to be mentioned again as the results seemed to be in contrast to the findings of Ferguson et al. (1979) where they observed a combination of 90DA and the other components to be more effective than the individual component. Since (E)-citral was detected in substantial amounts, (up to 20 μg/bee) it could be the key factor in initiating cluster formation in A. cerana. The fact that higher concentration was required for its activity was congruous to its high production in the gland. However, the
clustering activity was tested in a confined space and the results may or may not be the same under field conditions.

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REFERENCES


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