

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

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### ABSTRAK

*Lebah madu Apis cerana jenis pekerja telah diambil daripada beberapa koloni untuk kajian pencirian rembesan Nasonov dan aktiviti berkelompokan yang diuji dengan beberapa jenis komponen feromon berkelompokan sintetik. Ekstraksi rembesan kelenjar Nasonov dilakukan dengan tiga kaedah iaitu – kaedah pencelupan abdomen, kaedah pengasingan kelenjar dan kaedah ekstraksi menggunakan pecagari. Analisis feromon dilakukan dengan Kromatografi Cecair Gas. Komponen-komponen Nasonov yang dikesan ialah (E)-sitral, (Z)-sitral dan geraniol. Bagaimanapun hanya (E)-sitral dan geraniol dapat dinilai kepekatan. Kaedah pencelupan abdomen mengesan 20.0 ug (E)-sitral per lebah manakala kaedah pengasingan kelenjar hanya mengesan 3.8 ug per lebah. Geraniol pula hanya dapat dikesan dengan kaedah pengasingan kelenjar dengan paras 2.5 ug per lebah. (Z)-sitral hanya dapat dikesan, tetapi kepekatan sangat rendah untuk dianggarkan. Kajian berkelompokan pula dijalankan dalam sebuah bilik tertutup pada suhu bilik. Komponen sintetik seperti asid 9-okso-2-desenoik (9ODA), campuran (E), (Z)-sitral, geraniol, nerol dan farnesol diuji secara berasingan atau secara berkombinasi. Setiap komponen atau kombinasi campuran dijerap pada kertas turas dan digantung di dalam silinder bertebuk yang dipasang lampu di dalamnya. Lima silinder digunakan untuk setiap ujian. Di antara komponen sintetik yang diuji, campuran (E), (Z)-sitral didapati paling berkesan untuk menarik lebah pekerja supaya membentuk kelompok.*

### 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 was found to be most effective 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 1:1:1:100:75:12:50 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 pheromonas 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

Extracting Methods	Pheromones detected	$\mu\text{g}$ per bee	
		<i>A. cerana</i>	<i>A. mellifera</i> <sup>a</sup>
Syringe Extraction	( <i>E</i> )-citral	not quantified	-
	( <i>Z</i> )-citral	not quantified	-
	Geraniol	not quantified	-
Abdomen Dipping	( <i>E</i> )-citral	20.0	-
Dispersing Excised Gland	( <i>E</i> )-citral	3.8	0.02
	Geraniol	2.5	1.80

<sup>a</sup>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

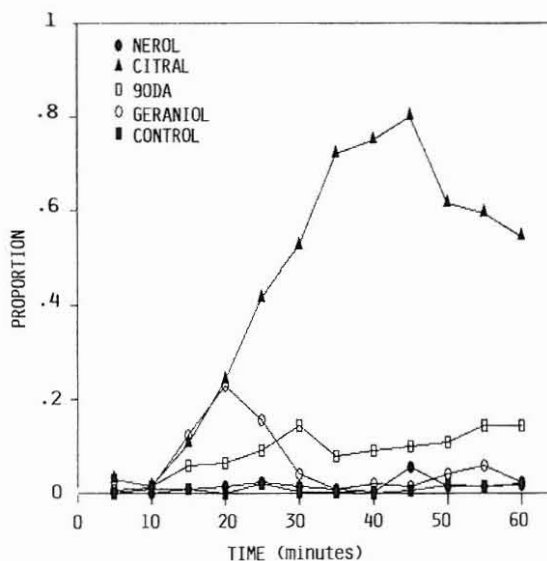


Fig. 1: Clustering activity of *A. cerana* worker bees on pheromones tested singly.

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 +

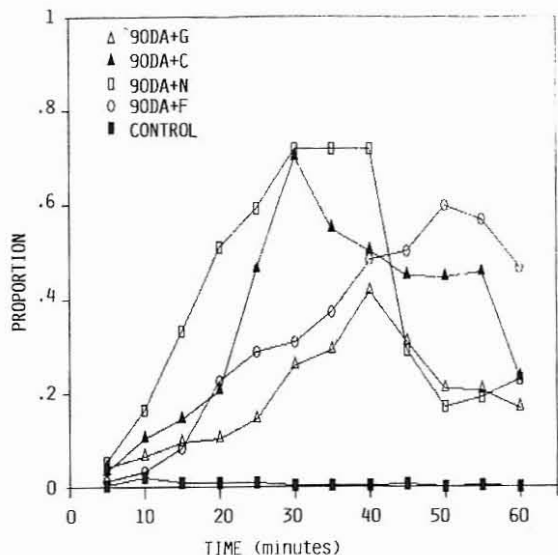


Fig. 2: Clustering activity of *A. cerana* worker bees on various combinations of pheromones.

Legend: 90DA - 9-oxo-2-decenoic acid

C - (E), (Z)-citril

N - nerol

G - geraniol

F - farnesol

(as in subsequent figures)

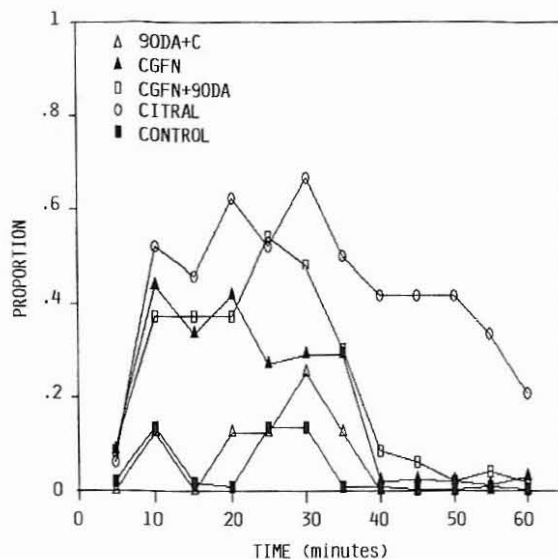


Fig. 4: The effectiveness of (E), (Z)-citril in clustering activity of *A. cerana* worker bees.

(20-60 μg) of (E), (Z)-citril showed no effects of citral concentrations on the number of bees settling on the cannisters.

The results indicate that (E), (Z)-citril 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)-citril 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)-citril 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

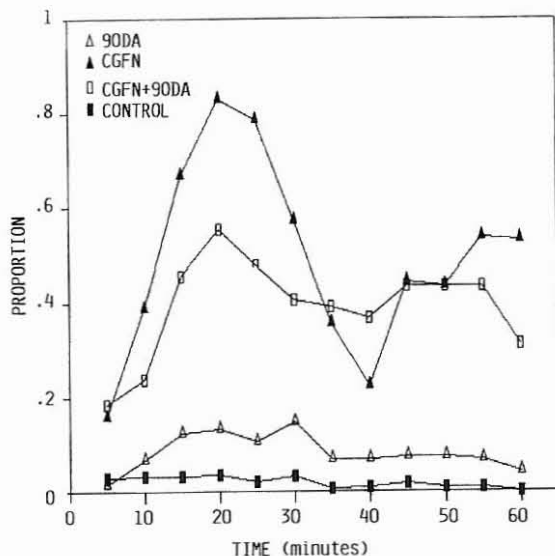


Fig. 3: The effect of 90DA on clustering activity of *A. cerana* worker bees.

90DA seemed to be more stable. However, (E), (Z)-citril 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

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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