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**Original article**

# Setosa membrane structure and occurrence of eicosenol in honeybees (*Apis* sp.)*

Stephen J. MARTIN[a], Junichi TAKAHASHI[b], Falko P. DRIJFHOUT[c]

[a] Laboratory of Apiculture and Social Insects, Department of Animal and Plant Sciences, University of Sheffield, Western Bank, Sheffield, S10 2TN, UK
[b] Department of Ecology and Systematics, Graduate School of Agriculture, Tamagawa University, Tokyo, Japan
[c] Chemical Ecology Group, School of Chemistry and Physics, Lennard Jones Laboratories, Keele University, Staffordshire, ST5 5BG, UK

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**Abstract** – Morphological analysis showed that the setosa membrane of workers is reduced in *Apis cerana* and *A. koschevnikovi* in comparison to that of *A. mellifera*. This may be due to different predator pressures among these species. This finding is supported by the marked reduction in the amount of the alarm pheromone eicosenol detected on the sting apparatus (setose area) in *A. cerana* workers in relation to that found in *A. mellifera* workers. The setosa membranes of the three open nesting species, *A. andreniformis*, *A. florae* and *A. dorsata* are well developed despite eicosenol being completely absent in *A. dorsata* and present in only trace amounts in *A. florea* and *A. andreniformis* workers. We also confirm that during dissection the internal glands of the sting apparatus do not become contaminated with compounds such as eicosenol, which can be present on the sting surface in high concentrations.

**honeybee / eicosenol / Dufour gland / defence / alarm pheromone**

## 1. INTRODUCTION

The setosa membrane (a setaceous membrane) is a hairy region of cuticle formed from the median part of the ninth sternum which surrounds the bulb of the sting shaft and may act as a platform for the release of alarm pheromones (Lensky et al., 1995). In *Apis mellifera* workers, the surface area of the setosa membrane is greatly increased by numerous setae which assist in the release of alarm pheromones. These are primarily the highly volatile alarm pheromone isopentyl acetate (Gunnison and Morse, 1968) and longer lasting [*Z*]-11-eicosenol (Pickett et al., 1982). The role of eicosenol in *A. mellifera* seems to extend the activity of isopentyl acetate and increase the speed of a defensive response (Koeniger et al., 1979). However, in *A. florea* and *A. dorsata* a different low-volatile substance 2-decen-1-yl-acetate appears to perform a similar function to eicosenol (Veith et al., 1978; Koeniger et al., 1979).

Isopentyl acetate probably is secreted by the sting sheath (Cassier et al., 1994) and the Koschewnikow gland (Mauchamp and Grandperrin, 1982), whereas eicosenol was shown to be secreted by the Dufour gland (Martin and Jones, 2004). However, due to the high eicosenol concentrations found on the setosa membrane of *A. mellifera* workers (Pickett et al., 1982; Schmidt et al., 1997; Martin and Jones, 2004) concerns have been raised that the presence of eicosenol in the Dufour gland may be due to contamination during dissection. Thus the glandular source of eicosenol remains questionable.

Corresponding author: S.J. Martin,
s.j.martin@sheffield.ac.uk
* Manuscript editor: Klaus Hartfelder

This study investigates the morphology of the setosa membrane within the *Apis* genus and its association with eicosenol. It also addresses the concerns that chemical contamination of the Dufour and venom gland may occur during dissection.

## 2. MATERIALS AND METHODS

### 2.1. Collection of individuals

The sting apparatus from foraging workers of six species of *Apis* were collected from various locations; *A. mellifera* (UK), *A. cerana* (Japan, Thailand), *A. koschevnikovi* (Sabah), *A. florea* (Philippines, India, Myanmar, Malaysia, Thailand), *A. andreniformis* (Sabah, Malaysia), and *A. dorsata* (Sabah, Philippines). All stings and their attached glands were air dried before being analysed in the UK. Additional stings from workers of *A. mellifera* (UK) and *A. cerana* (Japan) were collected and the setosa membrane and venom sac removed and air dried before being separately analysed.

### 2.2. Setosa membrane morphology

Two or three stings of each species were coated with 25−30 nm of gold using a Edwards S150B sputter coater and studied using a Philips PSEM501B scanning electron microscope set at an accelerating voltage of 30 KV. The length of the setae were measured using a graticule installed in a Leica (× 60) binocular microscope.

### 2.3. Presence of eicosenol

The entire sting apparatus, venom sac or setosa membrane were air-dried before being sealed separately into small glass tubes which were analysed by crushing them inside a GC-MS (gas chromatography – mass spectrometry) injector port using a Keele injector (Morgan, 1990). GC-MS analyses were performed on a 5890 Hewlett Packard GC coupled as previously described (Martin and Jones, 2004) with compounds being identified by comparison of retention times and mass spectra with synthetic standards. Compounds were quantified by measuring chromatogram peak areas. The *t*-test was used to compare if differences in the mean amounts of eicosenol among the different species.

### 2.4. Contamination study

To determine whether the Dufour or venom glands become contaminated with eicosenol from the external surface of the sting during dissection we spiked the setosa region of stings of seven freshly killed *A. mellifera* workers with 0.5 μL of a 10 mg/mL solution of 1-docosanol in chloroform. Docosanol is not normally found in the sting region of *A. mellifera* workers and is similar to eicosenol in both structure and size, thus ensuring it has similar chemical properties. The stings were left for 15 minutes before the Dufour gland, venom gland and setosa membrane were dissected as previously described (Martin and Jones, 2004) and analysed by GC-MS for the presence of eicosenol and docosanol.

## 3. RESULTS

### 3.1. Morphology of the setosa membrane

The SEM images showed that the workers of *A. mellifera* have the most heavily sclerotized setosa membrane which is covered with the longest (c. 120 μm) hairs of any honeybee species (Fig. 1a). In contrast, workers of *A. cerana* (Fig. 1b) and its sister species *A. koschevnikovi* (Fig. 1d) have the shortest hairs (c. 20−25 μm) and the level of chitinisation is markedly reduced, i.e., in *A cerana* the setose area is pale, almost transparent, while in *A. mellifera* and other species the setosa membrane is much darker indicating a higher degree of sclerotization. The setosa membranes of workers of *A. dorsata* (Fig. 1c), *A. florae* (Fig. 1e) and *A. andreniformis* (Fig. 1f) are well defined and covered with 40−60 μm long hairs.

### 3.2. Occurrence of eicosenol in *Apis*

Eicosenol was detected in large amounts (> 50% of all non-polar compounds) in the sting apparatus of all foraging workers of *A. mellifera* (n = 29), *A. cerana* (n = 13) and *A. koschevnikovi* (n = 23). Only very small amounts (<< 1%) of eicosenol were detected in *A. andreniformis* (n = 22) and A. *florea*

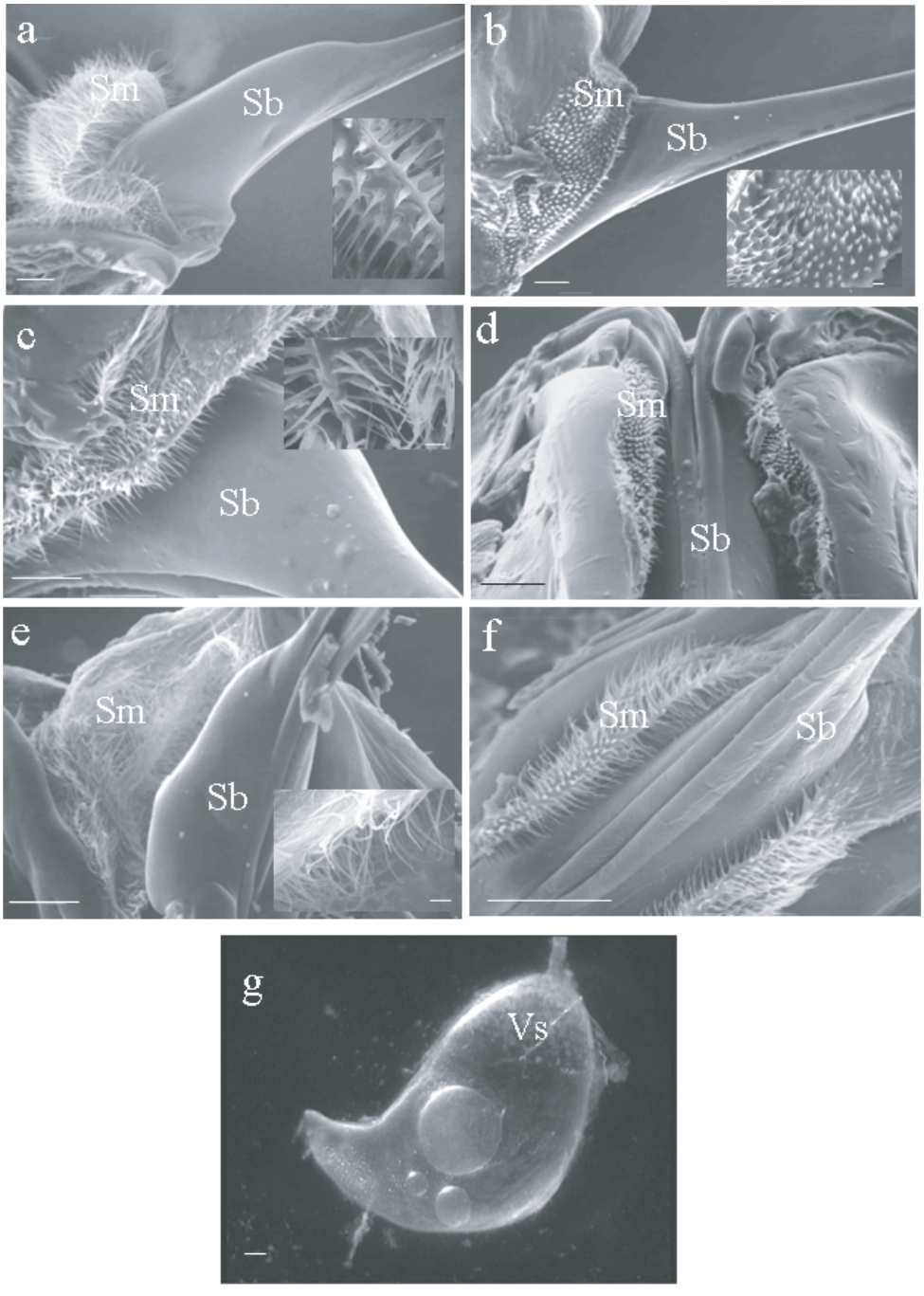


**Figure 1.** SEM images (a–f) of the sting apparatus of workers of *A. mellifera* (a), *A. cerana* (b), *A. dorsata* (c), *A. koschevnikovi* (d), *A. florea* (e) and *A. andreniformis* (f) showing the position of the sting bulb (Sb) and variation in hairiness of the setosa membrane (Sm). The light microscope image (g) shows the venom sac (Vs) of an *A. cerana* worker and clearly illustrate the conspicuous droplets that contain predominately eicosenol. Scale bars are 100 μm and 10 μm in the inserts.

**Table I.** The distribution of naturally occurring eicosenol in seven *A. mellifera* workers whose stings were spiked with 1-docosanol. Mean ion counts ($\times 10^6$) of the two compounds are given ± SD.

| | Sting remains | Setosa membrane | Dufour gland | Venom gland |
|---|---|---|---|---|
| Eicosenol | 3685 ± 1922 | 634 ± 545 | 251 ± 328 | 24 ± 23 |
| Docosanol | 2093 ± 1547 | 129 ± 85 | 2 ± 4 | 2 ± 3 |
| Docosanol:Eicosenol ratio | 1:2 | 1:5 | 1:126 | 1:12 |

(n = 22) workers, and the compound was completely absent in *A. dorsata* (n = 23) workers. We found no geographical variation in eicosenol production within the various *Apis* species.

The average amount of eicosenol (ions/sting) detected on the sting surface (setose area) of *A. cerana* workers (x = $135 \times 10^6$, s.d. = 67, n = 8) was significantly (*t*-test, $t = 7.3$, d.f. = 14, $P < 0.0001$) lower than in *A. mellifera* (x = $523 \times 10^6$, s.d. = $134 \times 10^6$, n = 8) workers. Furthermore, in all six *A. cerana* workers studied > 90% of the eicosenol detected in the sting apparatus was contained in large oily droplets in the venom sac (Fig. 1g), whereas no eicosenol was detected in the venom sac of *A. mellifera* (n = 6) workers.

### 3.3. Cross-contamination study

The spiking of the setosa region of *A. mellifera* workers resulted in high levels of docosanol ($2222 \times 10^6$ ions) on the external surface of the sting in all seven *A. mellifera* workers. Despite this, no detectable or only small amounts of docosanol were detected in either the Dufour or venom gland (Tab. I). However, eicosenol was present in all seven of the Dufour gland samples ($\bar{X} = 251 \times 10^6$ ions) but present only in very small amounts ($\bar{X} = 24 \times 10^6$ ions) on the nearby venom gland.

## 4. DISCUSSION

### 4.1. Morphology and chemical ecology of the setosa membrane

The morphological differences between the setosa membranes of *A. mellifera*, *A. cerana* and *A. koschevnikovi* workers (Fig. 1) suggest a reduction in the functionality of the setosa membrane in *A. cerana* and *A. koschevnikovi* workers. According to Morse and Benton (1967) the amount of the main alarm pheromone 'isopentyl acetate' in *A. cerana* workers is only half of that found in *A. mellifera* workers, and we found a similar reduction in the amount of eicosenol on the sting surface of *A. cerana*. Schmidt et al. (1997) also found that in *A. cerana* eicosenol is absent or present in only small quantities on the surface of the sting, whereas in *A. mellifera* workers eicosenol was associated with the setose area. These reductions in the surface area of the setosa membrane and in the amount of alarm pheromones on the sting surface may help explain the differences in defensive behaviour between these two species. *A. cerana* workers are not fiercely aggressive and mount only a moderately powerful stinging defence (Seeley et al., 1982) compared with *A. mellifera*. The other unusual feature of *A. cerana* is the presence of conspicuous oily droplets containing eicosenol in the venom sac of all *A. cerana* workers so far studied, e.g., from Japan and Philippines (this study), Hong Kong and Malaysia (Schmidt et al., 1997), as well as in its sister species *A. koschevnikovi* from Borneo (this study). This trait may also occur in *A. nigrocincta* and *A. nuluensis* which are part of the *A. cerana* species complex (Tanaka et al., 2001). It remains unclear how and why eicosenol accumulates within the venom sac in *A. cerana* workers. It is uncertain how these very large oily droplets could be expelled along with the venom and what role, if any, they play, since if injected into the victim along with the venom their role as a pheromone may be very limited.

The more vulnerable open nesting species *A. dorsata*, *A. florea*, and *A. andreniformis* need to and can mount aggressive stinging

attacks and all have a well developed setosa membrane. However, the production of eicosenol is vastly reduced (*A. florae* and *A. andreniformis*) or completely absent (*A. dorsata*) in these species. It appears that another compound decenyl-acetate has replaced the function of eicosenol in these species as it has been found in the stings of *A. dorsata* and *A. florae* (Veith et al., 1978). We did detect 2-decen-1-yl-acetate in the dried stings of *A. andreniformis*, *A. dorsata* and *A. florae* workers but not in *A. mellifera*, *A. cerana* or *A. koschevnikovi*. However, probably due to the collection method the amounts detected were very low and too inconsistent to be quantified. However, we would predict that 2-decen-1-yl-acetate, like eicosenol will be secreted by the Dufour gland.

### 4.2. Cross-contamination

The almost complete lack of docosanol in the Dufour and venom gland samples, despite the presence of large amounts on the sting surface, shows that the level of contamination of the internal glands during dissection was negligible. This confirms that the Dufour gland can be considered as being the source for eicosenol (Martin and Jones, 2004), a view that is further supported by the location of the gland's exit in relation to the setosa membrane (Martin et al., 2005).

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**Structure de la membrane sétacée qui entoure la gaine de l'aiguillon et présence d'eicosénol chez les abeilles du genre *Apis*.**

***Apis* / glande de Dufour / phéromone d'alarme / eicosénol / appareil venimeux / morphologie fonctionnelle / comportement de défense**

**Zusammenfassung – Struktur des Stachelrinnenpolsters und Vorkommen von Eicosenol bei Honigbienen (*Apis* sp.).** Das Stachelrinnenpolster ist ein Haarfeld der Kutikula, das die verdickte Basis des Stachelschafts umgibt. Es wird angenommen, dass die Alarmpheromone von dieser Fläche abgegebenen werden. Bei *Apis mellifera* Arbeiterinnen ist die Oberfläche durch unzählige kleine Haare vergrößert, um die Freisetzung der 2 Hauptkomponenten des Alarmpheromons (Isopentylazetat und das weniger flüchtige Eicosenol) zu unterstützen. Um die Morphologie und Chemie des Stachelrinnenpolsters zu untersuchen, wurde der Stachelapparat bei Sammelbienen von 6 *Apis* Arten an verschiedenen Orten gesammelt; *A. mellifera* (England), *A. cerana* (Japan, Thailand), *A. koschevnikovi* (Sabah), *A. florea* (Philippinen, Indien, Myanmar, Malaysia, Thailand), *A. andreniformis* (Sabah, Malaysia), *A. dorsata* (Sabah, Philippinen). Die Stachel und ihre anhängenden Drüsen wurden an der Luft getrocknet, bevor sie nach England geschickt wurden. Die Struktur des Stachelrinnenpolsters wurde sowohl mit dem Rasterelektronen- als auch mit dem Lichtmikroskop untersucht. Das Vorkommen bzw. Nicht-Vorkommen von Eicosenol wurde mit einem Gaschromatographen gekoppelt mit Massenspektrometrie bestimmt. Um sicher zu stellen, dass die inneren Stacheldrüsen bei der Präparation nicht mit Verbindungen der Außenfläche verunreinigt wurden, versahen wir das Haarpolster mit Docosanol, einem Stoff, der sonst nicht bei *Apis* Arbeiterinnen nachgewiesen wurden. Die REM Bilder zeigten, dass Stachelrinnenpolster der Arbeiterinnen von *A. mellifera* von allen Honigbienen am stärksten chitinisiert ist und die längsten Haaren (c. 120 μm) hat (Abb. 1a). Arbeiterinnen von *A. cerana* (Abb. 1b) und ihre Schwesterart *A. koschevnikovi* (Abb. 1d) haben die kürzesten Haare (c. 20−25 μm) und der Grad der Chitinisierung ist deutlich geringer. Das Stachelrinnenpolster der *A. dorsata*, *A. florae* and *A. andreniformis* Arbeiterinnen ist gut ausgebildet und mit 40−60 μm langen Haaren bedeckt. Große Mengen von Eicosenol (> 50 % von allen nicht polaren Substanzen) wurden im Stachelapprat bei allen *A. mellifera* (n = 29), *A. cerana* (n = 13) and *A. koschevnikovi* (n = 23) Arbeiterinnen nachgewiesen. Dagegen wurden nur geringe Mengen Eicosenol (< 1 %) bei allen *A. andreniformis* (n = 22) und A . *florea* (n = 22) Arbeiterinnen gefunden, bei *A. dorsata* (n = 23) Arbeiterinnen konnte nichts nachgewiesen werden. Die Menge an Eicosenol bei *A. cerana* Arbeiterinnen war signifikant (*t*-test, $P < 0,0001$) niedriger als bei *A. mellifera* Arbeiterinnen. Außerdem befand sich das meiste Eicosenol der *A. cerana* Arbeiterinnen in großen öligen Tropfen in der Giftblase (Abb. 1g). Durch die experimentelle Verunreinigung des Stachelrinnenpolsters von *A. mellifera* Arbeiterinnen konnte gezeigt werden, dass die inneren Organe sauber präpariert wurden (Tab. I). Die morphologischen Unterschiede der Stachelrinnenpolster lässt

seine funktionelle Reduktion bei *A. cerana* und *A. koschevnikovi* Arbeiterinnen vermuten und könnte die Unterschiede im Verteidigungsverhalten zwischen den Arten erklären, denn Arbeiterinnen der beiden letztgenannten Arten sind nicht sehr verteidigungsbereit. Dagegen haben *A. dorsata*, *A. florea*, and *A. andreniformis* sehr gut entwickelte Stachelrinnenpolster. Bei ihnen wurde die Rolle von Eicosenol anscheinend durch Dezenylazetat ersetzt.

**Honigbienen / Eicosenol / Dufour Drüse / Verteidigung / Alarmpheromon**

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