Insectes soc. 49 (2002) 229–233
0020-1812/02/030229-05


Insectes Sociaux

Research article

# Production of wax by virgin queens of the stingless bee *Melipona bicolor* (Apidae, Meliponinae)

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Received 29 August 2001; revised 25 March 2002; accepted 28 March 2002.

**Summary.** Young virgin queens of the stingless bee *Melipona bicolor* have been shown to produce wax like workers. The small, white to transparent flakes of wax of the queens protrude from the intersegmental space and cover the anterior part of the tergite cuticle, in a way similar to that in workers. This points to the presence of wax glands in the queens. However, workers produce wax from glands located at the fourth to seventh tergites whereas queens secrete wax from the epidermal gland at the third tergite only. Analysis of the queen-produced wax showed that it contains the same substances as the worker-produced wax with minor differences in composition. The wax consists chiefly of the long-chain esters triacontanyl acetate and octacosanyl acetate, smaller amounts of linear $C_{21}$ to $C_{31}$ alkanes and alkenes, and still smaller quantities of linear aldehydes and isobutyrate esters. Analysis of wax from wax deposits and wax constructs showed the same composition. Wax from *M. bicolor* is similar to that of other stingless bees in containing the range of linear long-chain alkenes and alkanes and different from that of *Apis* bees which contains a more complex mixture, less hydrocarbons and more long chain esters.

*Key words:* *Melipona,* wax production, virgin queens, long-chain acetates, hydrocarbons.

## Introduction

*Melipona bicolor* is a stingless bee occuring in the more southern parts of the Brazilian Atlantic rain forest. It is unusual in that its colonies can have more than one physogastric queen active in egg laying (Bego, 1989). Workers in stingless bees are known to produce wax with epidermal glands located at the fourth to seventh tergites (Cruz-Landim, 1967). Wax is produced by workers in *M. bicolor* predominantly at about 6–30 days of age (Bego, 1983). The workers first gather it at specific places in the nest, so-called wax deposits (Hebling et al., 1964), and then wax nest structures are made using the wax from these deposits, mixing it with propolis, or plant resin, to increase its rigidity.

There are a few recorded cases of apparent wax production in stingless bees by individuals other than workers. Da Silva (1977; reviewed by Kerr, 1997) reported the production by males, there is a notice about wax secretion by a virgin queen of *Trigona (Scaptotrigona) bipunctata* (Kerr and de Lello, 1962), and in the case of *Melipona marginata,* wax glands were encountered in males as well as in queens (Cruz-Landim, 1967). In none of these cases was the original wax ever collected nor was the secreted substance tested for its characteristics. We have occasionally seen virgin queens of *M. bicolor* secreting small amounts of wax-like structures. We have collected and examined wax produced by the two castes of *Melipona bicolor* by picking the secreted scales directly from the abdomen of living individuals. We also show that the queen-produced wax and worker wax have essentially the same composition, and that the wax deposit and involucrum wax of the nest are very similar with the addition of propolis.

## Materials and methods

Wax scales were collected directly from the abdomen of workers and virgin queens of *M. bicolor* Lepeletier by carefully peeling them off the cuticle with the help of an insect pin. Since individual virgin queens always produced only very tiny bits of wax, five queens were used for the collection of the wax scales. These were immediately placed in soft glass capillaries and sealed as described by Morgan (1990). The wax scales of single workers were placed in several capillaries using in total three workers. About 100 mg of wax from wax deposits and a similar quantity of wax constructs were collected in small plastic vials (Low Temperature Freezer Vials; 1.2 ml; VWR, Scientific Products).

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The sealed capillaries and plastic vials were transported to Keele where they were analysed by gas chromatography using the solid-sampling method described by Morgan (1990). Chromatography was carried out with a Hewlett-Packard 5890 gas chromatograph directly coupled to a 5970B Mass Selective Detector (quadrupole mass spectrometer using 70 eV electron impact ionisation). The system was controlled and data accumulated on a Hewlett-Packard series 300 computer with HP 5970C Chemstation. Mass spectra were scanned from m/z 35 to m/z 550. Scan time was about 2.4 sec. Chromatography was performed on polydimethylsiloxane phase in a fused silica column (15 m × 0.2 mm, 0.33 µm film thickness) (SGE, Milton Keynes, UK). Helium was used as carrier gas at 1 ml/min. The sample in the glass capillary was heated in the injection port (temp 250 °C) for 2 min before crushing and starting the chromatography. The oven was programmed from 60 °C (3 min) at 5 °C/min to 320 °C. The split valve was closed before crushing the sample and reopened 30 s later. Deposit wax and construct wax were analysed both by solid injection and dissolved in hexane for injection (1 µl) in the usual way. The identification of compounds was confirmed by comparison of their mass spectra and retention times with those of standard alkanes and using the Wiley-NIST database, version 1994.

Double-bond positions in alkenes were determined in a sample of wax by the dimethyldisulphide method (Billen et al., 1986), except that the reaction was carried out at 46 °C for 12 hours under an atmosphere of argon. The dimethyldisulphide adducts were identified in the gas chromatogram of the product mixture and the positions of attachment were deduced from the fragmentation pattern.

Transesterification experiments were performed with boron trifluoride-methanol complex and sodium methoxide in methanol. Portions of wax from the involucrum and wax deposits in the nest (100–200 mg) were added to boron trifluoride-methanol (100 ml of 14% $BF_3$ in methanol) and the mixture heated at 60 °C for 1 h. Reaction was stopped by adding water (100 ml). The wax compounds were extracted twice with chloroform (200 ml), which was evaporated to a small volume and used for gas chromatography. Basic transesterification was performed by dissolving sodium (100 mg) in dry methanol (1 ml). When solution was complete, the methanol was cooled and a sample of wax from the involucrum or wax deposit was added. The mixture was then heated at 50 °C for 6 h, the reaction was stopped by adding dilute aqueous hydrochloric acid and extracting the wax compounds with chloroform for gas chromatography as above.

## Results

Occasionally, young virgin queens of *M. bicolor* were seen walking around the nest having scales of wax on the abdomen. In the queens the positioning of the wax flakes, being small, white to transparent bands protruding from the intersegmental space and covering the anterior part of the tergite cuticle, was similar to that for worker-produced wax. However, virgin queens were seen to produce wax scales from their epidermal glands at the third tergite only whereas workers produce them from the glands at the fourth to the seventh tergites.

Analysis of the white translucent scales of wax by gas chromatography and mass spectrometry showed, as the principal component, the ester triacontanyl acetate, with small amounts of acetates of other long-chain alcohols. There were still smaller amounts of the isobutyrates of the same alcohols, the corresponding aldehydes and a series of linear alkenes and alkanes from $C_{20}$ to $C_{31}$ (Fig. 1). It is clear from the relative retention times that the alkenes are mono-enes. The constituents were the same for both queen and worker wax, but there were differences in the proportions of alkenes to alkanes between them and while the quantity of hydrocarbons peaked at $C_{25}$ for the queen wax, it peaked at $C_{23}$ for the workers.

Formation of the dimethyldisulphide derivatives of the alkenes revealed that they had the double bond either at Δ7 or Δ9, with some contribution from Δ8 (Table 1). Because the proportions of these isomers could not be detected from the gas chromatograms, they were estimated from the single ion chromatograms for the specific ions m/z 145 (characteristic of the derivatives of Δ7 alkenes) and m/z 159 and 173 (for Δ8 and Δ9 alkene derivatives respectively). In the case of the minor components with even-numbered chain length

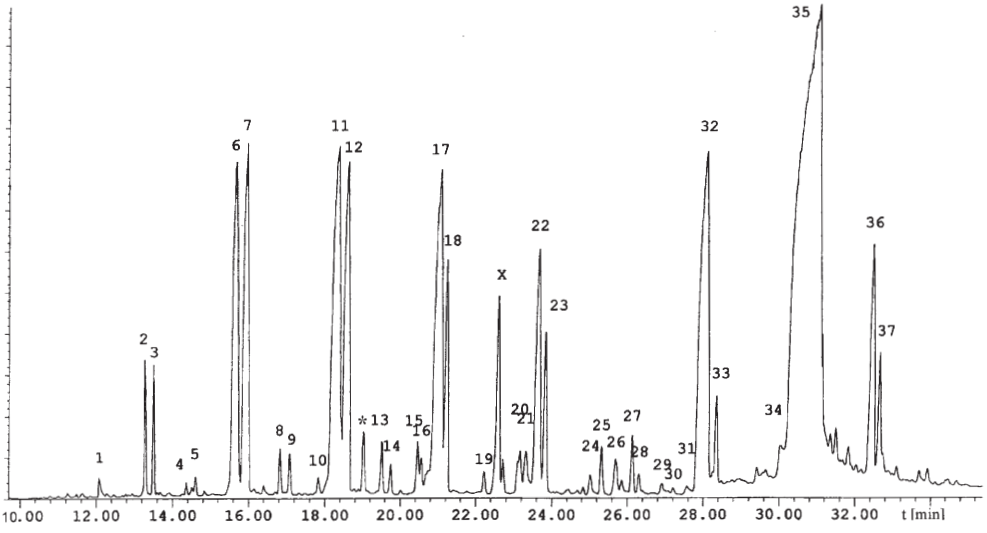


**Figure 1.** Total ion chromatogram of a tiny flake of wax taken from the third abdominal tergite of a virgin queen of *Melipona bicolor*. Numbered peaks correspond to the numbers in Table 1. The peak marked with * is phthalate plasticizer, a universal contaminant, and that marked X is squalene, a contaminant from human hands

**Table 1.** Quantification of compounds identified in the wax of queens ($n = 5$) and workers ($n = 3$) of *Melipona bicolor* (ratios in parentheses are the proportions of the positional isomers of the alkenes)

| Peak Number in Figure 1 | Compound | Queens Mean (%) ± SD | | Workers Mean (%) ± SD | |
|---|---|---|---|---|---|
| 1 | Unknown | 0.14 | 0.09 | 0.11 | 0.09 |
| 2 | 7-Heneicosene | 0.15 | 0.27 | 0.28 | 0.31 |
| 3 | Heneicosane | 0.17 | 0.24 | 0.39 | 0.25 |
| 4 | 7-Docosene | 0.05 | 0.10 | 0.02 | 0.03 |
| 5 | Docosane | 0.24 | 0.46 | 0.12 | 0.08 |
| 6 | 7-Tricosene and 8-tricosene (80:1) | 0.96 | 2.10 | 3.06 | 2.50 |
| 7 | Tricosane | 1.76 | 1.90 | 4.95 | 1.81 |
| 8 | 7-Tetracosene | 0.11 | 0.17 | 0.16 | 0.10 |
| 9 | Tetracosane | 0.20 | 0.10 | 0.32 | 0.06 |
| 10 | Docosanal | 0.14 | 0.11 | 0.10 | 0.07 |
| 11 | 7-Pentacosene, trace of 8-pentacosene and 9-pentacosene (110:1) | 2.73 | 2.97 | 6.25 | 4.44 |
| 12 | Pentacosane | 6.67 | 1.51 | 6.81 | 1.21 |
| 13 | 7-Hexacosene | 0.07 | 0.14 | 0.35 | 0.07 |
| 14 | Hexacosane | 0.51 | 0.31 | 0.38 | 0.23 |
| 15 | Tetracosenal | 0.36 | 0.57 | 0.29 | 0.17 |
| 16 | Tetracosanal | 0.62 | 1.36 | 0.23 | 0.13 |
| 17 | 7-Heptacosene and 9-heptacosene (2.3:1) | 5.82 | 3.40 | 7.37 | 1.77 |
| 18 | Heptacosane | 4.05 | 2.87 | 3.01 | 0.83 |
| 19 | 7-Octacosene | 0.18 | 0.12 | 0.23 | 0.11 |
| 20 | Tetracosanyl acetate | 1.31 | 0.82 | 0.56 | 0.23 |
| 21 | Hexacosanal | 0.84 | 0.81 | 0.40 | 0.14 |
| 22 | 7-Nonacosene and 9-nonacosene (2.3:1) | 0.70 | 0.76 | 0.45 | 0.18 |
| 23 | Nonacosane | 2.81 | 2.44 | 2.70 | 1.68 |
| 24 | 7- and 9-Triacontene | 3.97 | 2.61 | 2.48 | 1.10 |
| 25 | Triacontane | 0.69 | 1.75 | 0.19 | 0.21 |
| 26 | Tetracosanyl isobutyrate | 0.90 | 1.41 | t | – |
| 27 | Hexacosanyl acetate | 1.19 | 0.98 | 0.83 | 0.35 |
| 28 | Octacosanal | 0.64 | 0.70 | 0.34 | 0.04 |
| 29 | 7-Hentriacontene and 9-hentriacontene (0.8:1) | 0.51 | 0.50 | 0.33 | 0.13 |
| 30 | Hentriacontane | 0.47 | 0.36 | 0.44 | 0.39 |
| 31 | Hexacosyl isobutyrate | 0.80 | 0.49 | t | – |
| 32 | Octacosanyl acetate | 18.08 | 5.34 | 12.01 | 2.23 |
| 33 | Triacontanal | 0.20 | 0.48 | 0.95 | 0.57 |
| 34 | Octacosanyl isobutyrate | 0.57 | 0.26 | 0.09 | 0.05 |
| 35 | Triacontanyl acetate | 38.84 | 7.68 | 40.50 | 10.16 |
| 36 | Triacontanyl isobutyrate | 0.63 | 1.41 | 2.24 | 1.14 |
| 37 | Dotriacontanyl acetate | 1.94 | 1.22 | 1.03 | 0.38 |

t = trace, less than 0.01%.

(docosene, tetracosene, etc.) while Δ7 could be detected for all of them, the weakness of their spectra did not permit ruling out smaller amounts of the other isomers (Table 1).

To check if there were any higher mass esters too involatile for gas chromatography, samples of deposit wax and wax from the involucrum were subjected to acidic and basic hydrolysis. In both cases essentially only a spectrum of the hydrocarbons remained. The amounts of long-chain alcohols (tetracosanol to triacontanol) released from their esters were smaller than expected, as these substances were partially adsorbed on the column during chromatography. Traces of $C_{14}$ to $C_{18}$ methyl esters were seen, but these may have come from surface triglycerides from the bodies of worker bees or from plant substances. No free fatty acids were found in the wax. It was noticed that involucrum wax, construct wax from the walls of the hive, whether subjected to hydrolysis or simply making a solution injection showed amounts of terpenes (chiefly monoterpenes and sesquiterpenes) comparable with the amounts of wax hydrocarbons. Deposit wax contained only a little contamination with terpenes. *Trans-β*-caryophyllene was a major component of the terpenes, but this only reflects the plant from which the resin was collected (Patricio et al., 2002).

## Discussion

Comparison of chromatograms of the waxes of queens and workers of *M. bicolor* showed the same pattern of composition, the differences were found chiefly in the proportions of the alkenes and alkanes (Table 1). These differences would have very little effect upon the physical properties of the wax. When virgin beeswax scales were compared with wax from wax deposits and involucrum wax (i.e. the inner lining of the nest), the pattern was the same, except that the involucrum had been strengthened by the addition of propolis, which was evident in the large amount of monoterpenes, sesquiterpenes and even triterpenes found in the chromatogram. The range of hydrocarbons and esters and their proportions were the same as in the pure wax flakes from workers and queens.

It is noteworthy that Δ7-alkenes predominated in the shorter carbon chains, and tended towards more of the Δ9-alkenes at longer chains. This suggests the shorter carbon chains are built up from palmitoleic acid by addition of 3, 4 or 5 acetate units before decarboxylation and the longer ones from oleic acid by similar chain lengthening (Tillman et al., 1999).

Up to now, the only wax from stingless bees that has been analysed has been from nest material, and that only from trigonine bees (Table 2) (Blomquist et al., 1985; Milborrow et al., 1987). The composition of *Melipona* wax found here is quite different from that of honeybee wax, and much closer to that of the trigonine wax, but still different from that. The wax of the Australian stingless bee *Trigona australis* consisted almost entirely of hydrocarbons (90%) in the range $C_{23}$ to $C_{35}$, with odd chain lengths predominating (Milborrow et al., 1987). There was only 6% of esters compared with 64% esters in queen wax and 57% esters in pure worker wax found here for *M. bicolor*. The American *Trigona (Trigonisca) buyssoni* and *T. atomaria* waxes were much closer to those we found in *Melipona*. There was 26.8% and 25.5% esters in *T. buyssoni* and *T. atomaria* respectively, with the alcohols in the $C_{24}$ to $C_{30}$ range as found here (Blomquist et al., 1985). Of the 59.3% hydrocarbons in *T. buyssoni*, 90.2% were alkanes, 8.4% alkenes and 1.4% methyl-branched alkanes, while in *T. atomaria* of the 70.9% hydrocarbons, 87.1% were normal alkanes, 12.4% were alkenes and no branched chain hydro-

carbons were found (Blomquist et al., 1985). The hydrocarbons in *T. buyssoni* and *T. atomaria* are mostly saturated (about 90%) whereas in *T. australis* they are in general unsaturated. The hydrocarbons were in the range $C_{21}$–$C_{37}$. Our results showed approximately equal quantities of alkanes and alkenes with no evidence of branched chains. Free acids have a chain length between $C_{12}$–$C_{30}$+ in *T. buyssoni* and *T. atomaria,* and between $C_{10}$–$C_{20}$ in *T. australis* (Table 2). We found no free acids but should note that squalene was also found in the samples we analysed, but as this is an ubiquitous compound, found in human skin, and difficult to avoid transferring to instruments and samples, we discount its presence.

The chemistry of *Apis* wax has already been investigated in detail revealing more than 300 individual components (Tulloch, 1971, 1972, 1980). Hydrocarbons (14%), monoesters (35%), diesters (14%), free acids (12%) and hydroxy polyesters (8%) form the major part (Table 2). Three long-chain esters, with total carbon numbers $C_{40}$, $C_{46}$ and $C_{48}$, and one $C_{24}$ acid make up over 20% of the total. Because of their high molecular mass, not all these materials are directly analysable by gas chromatography. Analysis of honeybee wax under the same conditions as used here showed it to contain a mixture of $C_{16}$ to $C_{22}$ fatty acids, $C_{24}$ to $C_{32}$ linear alcohols and $C_{23}$ to $C_{27}$ alkanes and alkenes. A summary of an analysis of honeybee wax is given in Table 2. While both honeybee and stingless bee waxes contain high proportions of monoesters and hydrocarbons, the appearance of the chromatograms of the two types, under the same conditions look very different.

Because some high-mass components are not sufficiently volatile to be detected in gas chromatographic analysis of honeybee wax, we considered the possibility that similar high-mass components could be present in stingless bee wax also. We therefore carried out acidic and alkaline hydrolysis experiments on the wax from the nest, but found no evidence of high mass esters.

The great diversity in composition of *Apis* beeswax is most probably the principal reason for its plasticity and relatively low melting point (Tulloch, 1980). A study of the conversion of virgin beeswax into finished comb showed that the crystalline virgin beeswax scales are masticated giving the wax a random crystallographic arrangement which lessens its stiffness (Kurstjens et al., 1985). However, the addition of protein during worker mastication converts it into a final product having twice the stiffness of the starting material.

The beeswax of stingless bees is simpler in composition than *Apis* wax, has a lower melting point and has lipids with average shorter chain lengths (Blomquist et al., 1985; Milborrow et al., 1987). Meliponine wax contains a higher percentage of hydrocarbons and a lower percentage of monoesters. Di- and tri-esters were not found here or in the *Trigona* species. With the exception of *T. buyssoni* and *T. atomaria* (Blomquist et al., 1985), meliponines generally use wax mixed with a variety of plant resins (Roubik, 1989; Patricio et al., 2002), probably to increase its strength. *T. australis* workers mix wax mainly with pollen of *Eucalyptus* sp. causing the solid residue to comprise between 12% and 30% by weight (Milborrow et al., 1987).

In the few recorded cases of apparent wax production by individuals, other than workers, mentioned for stingless bees (Kerr and de Lello, 1962; Cruz-Landim, 1967; da Silva, 1977; reviewed by Kerr, 1997), wax samples were never taken from the bees nor was the secreted material verified for its properties. Wax production by stingless bee males is one of the arguments which Kerr and da Cunha (1990) use to support their concept that in stingless bees the overall similarity

**Table 2.** Summary of analysis of comb wax from *Apis mellifera* colonies (Tulloch, 1971), and three species of *Trigona* stingless bees (Milborrow et al., 1987; Blomquist et al., 1985)

| Component | *Apis mellifera* | | *T. australis* | | *T. atomaria* | | *T. buyssoni* | |
|---|---|---|---|---|---|---|---|---|
| | Chain length | % of total | Chain length | % of total | Chain length | % of total | Chain length | % of total |
| Free fatty acids | 24–34 | 12 | 10–20 | – | 12–30+ | 1.7 | 12–30+ | 5.4 |
| Primary alcohols | – | – | – | – | 16–32 | 1.0 | 16–32 | 7.0 |
| Long chain esters | | | | | | | | |
| Monoesters | 38–52 | 36 | [a] | 6 | [b] | 25.5 | – | 27 |
| Diesters | 56–64 | 14 | – | – | – | – | – | – |
| Triesters | 48–54 | 3 | – | – | – | – | – | – |
| Hydroxyesters | 40–50 | 12 | – | – | – | – | – | – |
| Polyesters | 69–75 | 2 | – | – | – | – | – | – |
| Hydrocarbons | | | | | | | | |
| Saturated | 19–31 | 14 | 23–35 | 90[c] | 23–37 | 62 | 23–37 | 59 |
| Unsaturated | 31–33 | | | | 27–37 | 9 | 27–37 | 5 |
| Branched | – | – | – | – | – | 0 | 30 & 32 | 1.4 |
| Unidentified (pigments, propolis, etc.) | – | 7 | | 16–30 | – | 0.3[d] | – | 1.5[d] |

[a] Higher molecular mass esters beyond $C_{35}$ were found, but after hydrolysis, no acids higher than $C_{20}$ were present.
[b] Fatty acids $C_{12}$ to $C_{30}$+, alcohols $C_{16}$ to $C_{30}$ with $C_{24}$ to $C_{28}$ the major portion.
[c] Unstaurated and saturated hydrocarbons were not resolved in chromatography, but unsaturates represented a greater proportion of the $C_{31}$ to $C_{35}$ hydrocarbons.
[d] These bees do not add plant resins to their wax.

between workers and males is higher than that between workers and queens when compared to *Apis*.

The resemblance in the positioning of the wax flakes on the abdomen of both virgin queens and workers in *M. bicolor* points to the presence of wax glands in the queens as well. Virgin queens were seen walking around the nest on a regular basis. However, the occasional presence of these queens with wax scales shows that they synthesize wax during only a very short period.

Here we report the first analysis of virgin beeswax scales in stingless bees and of queen-produced wax in particular. The wax production by virgin queens in *M. bicolor* does not tell us whether queens really deposit their wax scales at deposits as do the workers or use them in another way. It does reveal however that in as far as the production of wax in *M. bicolor* is concerned, workers are more similar to queens than to males, a view to some extent opposite to the idea proposed by Kerr and da Cunha (1990).

## Acknowledgements

We thank the research network „Social Evolution“ financed by the European Commission via the Training and Mobility of Researchers (TMR) programme for support for E.D.M., H.J., J.T., and G.R. Jones. E.D.M. also thanks São Paulo University for supporting his visit to São Paulo. D.K. is grateful to Dr. H.H.W. Velthuis for practical and scientific support and is indebted to the bee laboratory of Prof. V.L. Imperatriz-Fonseca for providing research possibilities. D.K. was financially supported by FAPESP (grant no. 98/14231–2).

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