Larval Anatomy of the Bee *Tetragonula sapiens* (Cockerell) (Meliponini); Further on the Larva of *Melipona fallax* Camargo and Pedro, with a Preliminary Characterization of Mature Larval Meliponini; and Analysis of Multipronged Spicules (Apoidea: Apidae)

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ABSTRACT

The small (length less than 6 mm) postdefecating larva of *Tetragonula* (*Tetragonula*) *sapiens* (Cockerell) from the Huon Peninsula, Papua New Guinea is described and illustrated, the first representation of the immature stage for any member of this genus of stingless bees. A reexamination of the recently described larva of *Melipona fallax* Camargo and Pedro using a new technique reveals valuable insights regarding its integumental microstructures. On the basis of these findings and previously published accounts of larval representatives of five other genera (*Melipona*, *Nogueirapis*, *Partamona*, *Plebeia*, and *Trigonisca*), a preliminary description of the mature larva of the tribe Meliponini is presented. Multipronged spicules, recently discovered in meliponines, are investigated.

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INTRODUCTION

The first part of this study is based on a massive collection of specimens from a single nest collected by Hobart M. van Deusen on 12 May 1964 on the seventh Archbold Expedition of the American Museum of Natural History. The collection was preserved in alcohol and labeled: “1964 Archbold Exp. … Terr. of New Guinea, Huon Peninsula, Masba Creek.” It has been stored since then in a single Atlas Whole Fruit Jar (figs. 1, 2) in the collections of the museum. It contains, roughly calculated, 1632 cocoons (enclosing pupae and late-stage mature larvae) and a good number of free-living teneral as well as fully pigmented adult bees. In addition, the liquid preservative incorporates an abundance of thick waxy nest material including fragments of nest cells and abundant pollen from stored provisions. Although labeled “Sweat Bee Nest” (the common name for Halictidae), the adults are obviously not of this family of bees, but of a stingless bee (Apidae: Meliponini). The bee has now been identified to species for this study by Michael S. Engel as *Tetragonula (Tetragonula) sapiens* (Cockerell) based upon examination of the genitalia of the single male among a total of 20–25 adults randomly extracted from the jar.

One aspect of the contents of this jar is difficult to understand and perhaps important to note. Although there are a good many adult specimens scattered among cocoons containing mature larvae and pupae, not a single immature larva, mature larva without a cocoon, or egg was observed at any time during numerous searches for them. In studies of nests of other stingless bees, eggs and immature feeding larvae as well as pupae and larvae in cocoons are normally encountered. The most obvious explanation that comes to mind is that the liquid preservative was inadequate in preserving tissue of the young larvae over a storage period of 55 years whereas the presence of a cocoon somehow affords such protection. Further study and analysis are required.

The study of *Tetragonula sapiens* augments a number of current and recent studies treating the late-stage larvae of the Meliponini (Engel et al., in prep.; Rozen et al., 2019a; 2019b). Thus, there is first-hand comparative information regarding the following taxa: *Melipona fallax* Camargo and Pedro, *Melipona trinitatis* Cockerell, *Nogueirapis mirandula* (Cockerell), *Partamona musarum* (Cockerell), *Plebeia “nan1,” Tetragonula sapiens* (Cockerell), *Tetragonisca angustula* Latreille, and a currently unnamed species of *Trigona* “nan1,” augmented by earlier descriptions of larval *Meli-pona quadrifasciata quadrifasciata* Lepeletier, *M. variegatipes* Gribodo, *M. marginata* Lepeletier, *Partamona peckolti* (Friese) (as *Trigona (Partamona) cupira* Smith), and *Trigona corvina* Cockerell (Michener, 1953).

The second part of this paper provides new details about the mature larva of *Melipona (Mich-melia) fallax* that has recently been examined by Rozen et al. (2019a). However, the specimens examined in that study failed to reveal important details concerning the spicules and sensilla on the integument. Submerging specimens for a few seconds in an ultrasonic cleaner (Cole-Parmer, model no. B200) removed considerable surface debris, so that details of surface structure were revealed with an SEM, providing a more precise understanding of the anatomical diversity, function, and distribution of the sensilla and spicules.

Information from previous studies as well as the current one, when synthesized, permits a preliminary larval characterization of the tribe Meliponini, see below. At the end, the paper draws
attention to an unreported integumental microstructure, here termed a multiprongued spicule, that has been detected on the mature larvae of all meliponine taxa examined.

METHODS

In preparation for study, several larval specimens of *Tetragonula sapiens* had their heads separated from their bodies, and the both parts were cleared by boiling in an aqueous solution of sodium hydroxide. Removed to ethanol and stained with Chlorazol Black E, the specimens were then placed in glycerin on well slides for light microscopic examination. For SEM examination, entire specimens were critical point dried and carefully positioned on SEM stubs for examination with a Hitachi S5700 SEM in the Microscopy and Imaging Facility of the American Museum of Natural History.

Although in the earlier study of *M. fallax*, specimens were prepared as described above, for this study clearing and staining were unnecessary. Heads of two specimens were simply removed from their bodies, sonicated briefly, critical point dried, and then placed on an SEM stub to be coated and then examined.

When comparing the mature larva of *T. sapiens* with available mature larvae of other Meliponini, vast differences in body sizes among taxa within the tribe were immediately obvious. How could they be conveniently measured so that one taxon could be concisely compared with another? If bodies died straight in lateral view, then a simple measurement of length from front of head to apex of the 10th abdominal segment would be satisfactory. However, on preservation, larvae always more or less curl. Here a new method to standardize and compare body lengths is proposed:

For each species, select a mature larva, and then measure and record the distance between each of the 10 spiracles along one side of the body in lateral view to yield nine data. Then record the distance in lateral view between the first thoracic spiracle and the farthest point...
on the front of the head. Finally, add the distance in lateral view between the spiracle on abdominal segment 8 and the most posterior surface of abdominal segment 10. Summation of all 11 measurements provides a reliable measure of the length of that specimen representing that species that then can be compared with those of other species. Furthermore, measurement can be obtained: (1) from actual specimens, (2) from SEM images that include scales, (3) and/or from carefully prepared camera lucida illustrations with bar scales. This method of measuring larval length should be referred to as an estimate of larval length based on interspiracular distances. Table 1 provides the comparative lengths measured this way along the spiracular lines of mature postdefecating larvae of the Meliponini recently studied. Table 2 presents the same end data but omits interspiracular data, thereby demonstrating a simpler presentation. In tables 1 and 2 some species are identified by a generic name followed by “nan1.” These refer to species that have yet to receive a nomenclatorial valid name that is based on the description of an adult as is explained in Rozen et al. (2019b).

MATURE LARVA OF TETRAGONULA (TETRAGONULA) SAPIENS (COCKERELL)

Figures 3–16

**Diagnosis:** Although it was hoped that anatomical features of the mature larva of this bee would be found to distinguish it from other stingless bees of comparable body sizes, none was identified. This perhaps is not surprising since recent studies of the immature stages of stingless bees (Rozen et al., 2019a; 2019b; Engel et al., in prep.) have demonstrated little diversity in their mature larvae. However, among the variable features found within the tribe, the following are characteristic of this species: front of head capsule above each antenna and mesad of each parietal band normally smooth, without unusual distortion; parietal band strongly impressed; antennal papilla (figs. 4, 12, 13) surrounded by large membranous ring with a radius about equal to the basal diameter of the papilla.
toclypeal area not projecting beyond labrum (fig. 5); apical surface of labrum (figs. 11, 13–15) bearing band of large hemispherical sensilla each often with single elongate, pointed apex; these sensilla intermixed with elongate, multipronged spicules (figs. 11, 13–15).

Mandibular apex pigmented; mandible (figs. 11, 16) slender, curved, elongate, narrowing evenly from base to pointed apex; apical concavity narrow, considerably longer than distance from mandibular base at base of cusp; dorsal edge of apical concavity with numerous long slender sharp teeth (fig. 13); ventral edge nearly smooth; mandibular apex a simple pointed tooth (figs. 13, 16).

Labium and maxilla extending more or less equally in lateral view (fig. 13). Maxillary apex not bent mesad; palpus apical in position, more than twice as long as basal diameter; galea projecting at maxillary apex; articulating arm of stipes present; dorsal surface of maxilla spiculate. Labium divided into prementum and postmentum, bearing apically projecting broad lips of slitlike salivary opening, its width less than distance between bases of labial palpi; length of labial palpus about twice basal diameter.

**Body:** Middorsal integument of body from posterior margin of head toward posterior end covered with fine sharp spicules, with those of thoracic segments most dense; spiculation extending laterally along dorsal surface of each caudal swelling to paired small, elevated sublateral body tubercle, below which spiculation ends. Spiculate areas with widely scattered sensilla approximately equal in size to spicules; abdominal segment 10 with broad cluster of

| Taxon                      | Distances   | Total | Scale | Length |
|----------------------------|-------------|-------|-------|--------|
| Melipona fallax            | 35 18 15 18 18 20 16 18 17 13 26 214 /12 17.83 |
| Melipona trinitatis        | 35 17 15 17 23 17 19 16 16 13 27 215 /12 17.92 |
| Nogueirapis mirandula      | 35 24 19 16 20 21 18 15 16 10 16 210 /12 17.50 |
| Partamona musarum          | 36 19 19 18 19 21 18 20 19 19 25 243 /15 16.20 |
| Plebeia "nan1"             | 37 17 13 13 14 18 13 13 13 15 21 187 /30 6.23 |
| Tetragonula sapiens        | 37 18 14 14 12 12 12 13 13 15 25 185 /23 8.04 |
| Trigonisca "nan1"          | 40 20 20 14 15 17 13 15 12 10 25 225 /35 6.43 |
|                             | 50 27 28 23 23 27 27 21 22 18 31 297 /50 5.94 |
|                             | 52 22 21 16 18 15 18 17 16 12 23 230 /60 3.83 |
8–10 setiform sensilla on each side of anus below spiculation. Each thoracic segment with pair of small elevated dorsolateral tubercles. Most abdominal segments each with pair of similar elevated tubercles. When larva cleared and stained with Chlorazol Black E, transversely oblong paired dorsolateral swelling of caudal annulets on three thoracic segments and on paired abdominal caudal swellings of segments 1–8, and perhaps 9, more stained in contrast to surrounding integument. Spiracle with radial width of peritreme about one-half diameter of atrial opening so that atrial opening moderately large; atrial wall mostly smooth, with few indistinct concentric ridges; primary tracheal opening a simple rim; subatrium shallow, consisting of only several annulations; flexure collapsed into single long, narrow, collapsed tube.

Material Studied: 20+ postdefecating larvae, all bearing internal developing pupal anatomy.

Discussion: The strongly curved dorsal silhouette of the entire larva (figs. 1, 6) is almost certainly an artifact created by the larva being preserved while in its cocoon. Since most of the specimens of *T. sapiens* had already well-developed pupal tissue, it can be assumed that the strongly robust anterior part of the larva was an accommodation for developing legs and other pupal features and therefore earlier last larval instars are somewhat slimmer.

In addition to the exaggerated curvature to the dorsal silhouette of the body in lateral view, it is likely that constraint imposed by the cocoon fabric may also play a role in the apparent extreme narrowness of the larval vertex in lateral view (fig. 6). Without larvae living free from cocoons, it is not possible to test this hypothesis or to estimate the extent of its possible effect if true.

Prepared specimens did not stain well with Chlorazol Black E. This might have been a result of their normal condition or because they were preparing for ecdysis.

Based on the representatives of the six genera whose mature larvae are known, they appeared to be closely related in that their mature larvae share the following:

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TABLE 2. The same information as provided in table 1 exclusive of the interspiracular data. This exclusion should be evaluated in future publications.

| Taxon                  | Total length | Scale (denominators) | Length (mm) |
|------------------------|--------------|----------------------|--------------|
| *Melipona fallax*      | 214          | /12                  | 17.83        |
|                        | 215          | /12                  | 17.92        |
|                        | 210          | /12                  | 17.50        |
| *Melipona trinitatis*  | 243          | /15                  | 16.20        |
| *Nogueirapis mirandula*| 187          | /30                  | 6.23         |
| *Partamona musarum*    | 185          | /23                  | 8.04         |
| *Plebeia “nan1”*       | 225          | /35                  | 6.43         |
| *Tetragonula sapiens*  | 297          | /50                  | 5.94         |
| *Trigoniisca “nan1”*   | 230          | /60                  | 3.83         |
Body form thick with most caudal annulets bearing paired dorsolateral mounds contrasting with lower cephalic annulets in lateral view.

Thoracic segments bearing small elevated tubercle on each caudal swelling. Either first abdominal segment alone or most abdominal segments with single elevated tubercle on each paired caudal swelling.

Body vestiture of fine spicules restricted to dorsal surfaces. This vestiture densest anteriorly on body and thinning toward posterior end, laterally ending just below tubercles.

FURTHER ON LARVA OF *MELIPONA FALLAX* CAMARGO AND PEDRO

Figures 17–22

By treating the two severed heads of *M. fallax* using an ultrasonic cleaner, vestiture details became clearly visible. The labrum is distinctly divided into an upper, nearly smooth, curved surface and a lower heavily spiculate surface also bearing numerous sensilla (fig. 17). For the first time, the weakly bituberculate format of the labrum is detected by identifying the two clusters of sensilla identified by rectangles in fig. 17. Close-ups of the hemispherical sensilla (figs. 19, 20) reveal two types, those with elongate, pointed apices or with buttonlike apices, almost certainly reflecting two different phenomena. Now that the anatomy of the labrum is better understood, observations of live larvae while they are feeding may explain how the labrum and mandibles interact to force the pollen-laden food into the bee’s esophagus.

Figure 21 shows a close-up of the labral apex above, mandibular apices on the sides, and the slitlike horizontal salivary opening at the bottom. In the middle is the finely spiculate surface of the hypopharynx. Figure 22 is a close-up of the rectangle in figure 21, and figure 23 is a close-up of the rectangle in figure 22, both of which clearly demonstrate a mixture of simple setiform spicules and multipronged spicules. Figure 24 is the outer surface of the left maxilla demonstrating that multipronged spicules occur on other mouthparts. These are structures that were not understood by Rozen et al., 2019a.
FIGURES 6–16. SEM micrographs of mature larva of *Tetragonula sapiens*. 6. Entire larva, approximate lateral view. 7. Dorsal surface of three thoracic segments lateral view showing spiculate dorsal surface and dorsolateral mounds of pro-, meso- and metathorax, each with elevated, nipplelike tubercle. 8–10. Close-up of tubercles (note small setiform sensilla, arrows).
FIGURES 11–16. 11. Head, frontal view. 12. Left antenna showing central conical papilla surrounded by large ring of membrane with radial width about equal to basal diameter of papilla. 13. Labrum and mandibular apices. 14. Close-up of central part of labrum showing complex of multipronged spicules and sensilla, each with hemispherical base and thin, tapering apex. 15. Mouthparts mostly of left side, showing labral apex, mandibles, labial apex with horizontal transverse salivary opening (arrow) below which are paired labial tubercles and left maxillary apex. 16. Multipronged spicule from labral apex of another specimen showing transverse linear base (arrow) bearing linear series of seven prongs. When bases are close together, they may form linear lamella of prongs.
PRELIMINARY DESCRIPTION OF THE MELIPONINI BASED ON THEIR LAST LARVAL INSTARS

Figures 3–16

Because of a number of recent studies of the mature larvae of the Meliponini (Rozen et al., 2019a; 2019b), a preliminary characterization of the last larval instar of the tribe is presented.

Diagnosis: Rozen et al. (submitted) presents a short key distinguishing the mature larvae of the four tribes (Apini, Bombini, Euglossini, and Meliponini) of the corbiculate Apinae.

Description: Body form: Form extremely robust in lateral view (figs. 1, 6) with small to very small head attached to large body.

Head: With large bodied species (i.e., Melipona) sclerotized areas of head and appendages somewhat darkly pigmented, but with all other taxa pigmentation restricted to mandibular apices. Front of head in lateral view flat below very narrow vertex, so that frons, clypeus, and
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Description: **Body form:** Form extremely robust in lateral view (figs. 1, 6) with small to very small head attached to large body.

**Head:** With large bodied species (i.e., *Melipona*) sclerotized areas of head and appendages somewhat darkly pigmented, but with all other taxa pigmentation restricted to mandibular apices. Front of head in lateral view flat below very narrow vertex, so that frons, clypeus, and labrum closely aligned and angling sharply with strongly projecting labiomaxillary region in lateral profile (fig. 5); head capsule usually broad to very broad in frontal view (fig. 4); top of head in frontal view straight, except bilobed in predefecating larva of *Tetragonisca angustula* (Rozen et al., 2019: fig. 51), without paired tubercles. Condition of tentorium unknown. Posterior tentorial pits normal in position; posterior thickening of head capsule narrow, not bending forward medially as seen in dorsal view; internal coronal ridge absent; internal epistomal ridge on cleared head capsule (figs. 4, 6) evident from anterior mandibular articulation to anterior tentorial pit but absent immediately mesad of...
labrum closely aligned and angling sharply with strongly projecting labiomaxillary region in lateral profile (fig. 5); head capsule usually broad to very broad in frontal view (fig. 4); top of head in frontal view straight, except bilobed in predefecating larva of Tetragonisca angustula (Rozen et al., 2019a: fig. 51), without paired tubercles. Condition of tentorium unknown. Posterior tentorial pits normal in position; posterior thickening of head capsule narrow, not bending forward medially as seen in dorsal view; internal coronal ridge absent; internal epistomal ridge on cleared head capsule (figs. 4, 6) evident from anterior mandibular articulation to anterior tentorial pit but absent immediately mesad of anterior tentorial pits; front of head capsule above each antenna and mesad of each parietal band varying from normally smooth, without unusual distortion to collapsed, extensively wrinkled depending on taxon. Antennal papilla a more or less conical projection surrounded by membranous ring of varying dimensions depending on taxon; papilla bearing perhaps 3–5 sensilla. Vertex narrow in lateral view (figs. 4, 6); frontoclypeal area not projecting beyond labrum (figs. 5, 6); lower apical surface of labrum bearing band of large hemispherical sensilla each with single elongate, pointed apex intermixed with elongate, multipronged spicules (fig. 13), in Melipona fallax hemispherical sensilla forming two sublateral clusters indicating paired but weak labral tubercles (fig. 17).

Mandibular apex pigmented; mandible seen in inner view more or less slender depending on taxon, curved, elongate, narrowing evenly from base to apex; apical concavity narrow, considerably longer than distance from mandibular base at base of cusp; dorsal edge of apical concavity with numerous long slender sharp teeth (figs. 13, 16); ventral edge with or without teeth depending on taxon; mandibular apex variable depending on taxon.

Labium and maxilla extending, more or less equally in lateral view (fig. 4, 16). Maxillary apex not bent mesad; palpus apical in position, more than twice as long as basal diameter; galea projecting at maxillary apex; articulating arm of stipes usually evident; dorsal surface of maxilla spiculate. Labium divided into prementum and postmentum, bearing apically projecting broad lips of slitlike salivary opening, its width less than distance between bases of labial palpi; length of labial palpus about twice basal diameter.

Body: Prothorax (figs. 3, 6) not greatly extending, unlike in Euglossini (Rozen, 2018); most body caudal annulets with more or less evident paired dorsolateral swellings, with those of thoracic segments each bearing small elevated tubercle that is not obviously sclerotized; first abdominal caudal annulet also with paired swellings, each bearing small tubercle; abdominal caudal annulets 2 to at least 7, with paired swellings with or without elevated tubercles depending on taxon. Middorsal integument of body from posterior margin of head toward posterior end covered with fine sharp spicules, with those of thoracic segments often most dense; spiculation extending laterally along dorsal surface of each body segment to small but elevated paired sublateral body tubercles, below which spiculation ends. Spiculate areas with widely scattered setiform sensilla approximately equal in size to spicules. Spiracles moderately large to small; atrial rim well identified; most if not all with peritreme; atrium moderate to shallow; atrial wall without spines, mostly smooth or nearly so, primary tracheal opening without spines or ornamentation; subatrial length short to moderately long.

Conclusion: Although our understanding of the anatomy of the mature larvae of the Meliponini is based on specimens representing only six of the 24 genera assigned to the
tribe in Michener's treatment of the tribe (Michener, 2007), the mature larvae of those six genera are remarkably similar to one another though they vary considerably in body length, as demonstrated in tables 1 and 2.

ANALYSIS OF MULTIPRONGED SPICULES

Although earlier studies of meliponine larvae consistently reported that the labral apex was densely spiculate (Rozen et al., 2019a), the study of Plebeia (2019b: figs. 13, 14) was the first to indicate that the labral spicules were multipronged, that is, instead of consisting of a single long, tapering setiform apex, it consisted of a single base that immediately branches into two to six, or sometimes more, long, tapering branches that often are arranged as a linear series. When vestiture is very dense, linear series may seem to coalesce into lamellate series of branches. These spicules occur at the labral apices of most, if not all, known meliponine taxa and they are also found on other surfaces, as exemplified by M. fallax herein, including: lateral surfaces of labrum, surfaces of maxilla, labium, and hypopharynx. This seems to suggest that their function pertains to food ingestion, such as the movement of the dorsal serrated mandibular edge over the labral apex during feeding. Do the mandibles comb or rake food from the spicules or create a flow of semiliquid food toward the esophagus? Microscopic observations of feeding larvae of large bodied species like those of Melipona might explain the exact functional mechanics of ingestion by melipoinines.

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