

# Heterochrony of cuticular differentiation in eusocial corbiculate bees

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**Abstract** – The exoskeleton (cuticle) of insects varies widely in shape, biomechanical properties, and functions, which are inherent to the biological species and developmental stage, besides showing a wealth of architectural specializations and nuances in the different body regions. The morphological pattern of exoskeleton maturation was studied in eusocial and solitary bees, including species of all three eusocial tribes of corbiculate bees as follows: Apini, Meliponini, and Bombini. The results showed striking differences in the rate of cuticle maturation between the solitary bees that leave the nest soon after the adult ecdysis, and the eusocial bees that take longer to leave the colony for foraging activities. There was a clear delay in the post-ecdysial cuticle tanning (melanization and sclerotization) in the eusocial bee species in comparison to the solitary species, suggesting adaptation to sociality and to the protective environment of the colony. Such heterochrony of cuticle maturation seems a conserved ontogenetic trait related to the way of life in corbiculate bees. The data generated a basic framework of exoskeleton maturation in corbiculate bees, under ontogenetic and evolutionary approaches, and give experimental support for further research on adaptation to the colony environment.

**exoskeleton / tanning / eusociality / Apinae / solitary bees**

## 1. INTRODUCTION

The cuticular exoskeleton is a multifunctional structure because in addition to the support it provides the insects, it allows locomotion and movements, protects against predators, and prevents desiccation, besides functioning in the mechanical digestion carried out by mouthparts,

and in the sensorial perception via antennae and sensorial setae (Hepburn 1985).

Exoskeleton differentiation is a remarkable event of insect ontogenesis and occurs through a process known as tanning, meaning the progressive darkening (melanization) and hardening (sclerotization) of the newly-secreted cuticle (Andersen 2005). Ontogenetically, two basic types of exoskeleton can be identified in the honey bees, the flexible and colorless (unpigmented) larval and pupal exoskeletons, and the rigid (highly sclerotized) and pigmented adult exoskeleton. Melanin pigments are deposited into the adult cuticle, which becomes increasingly sclerotized, as it differentiates

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underneath the pupal cuticle. In the honey bee, melanization starts in the developing adult cuticle 80 h after pupal cuticle apolysis, approximately (Michelette and Soares 1993). Apolysis progressively occurs along the anterior–posterior body axis, and adult cuticle deposition and tanning follow this pattern. Thus, cuticle tanning is first seen in the head and thorax. The abdomen is weakly pigmented and sclerotized until close to the adult ecdysis.

Cuticular melanization and sclerotization occur simultaneously through the same biosynthetic pathway starting with hydroxylation of tyrosine to dopa, followed by a step requiring the catalytic activity of dopa-decarboxylase for dopamine production. Dopamine is a precursor in the synthesis of melanin, catalyzed by phenoloxidases, and in the synthesis of sclerotizing agents. Sclerotization starts when *N*-acetyldopamine and *N*- $\beta$ -alanyldopamine are oxidized to quinones by the action of phenoloxidases and laccases. Quinones then react with cuticular proteins, thus stabilizing cuticle structure. These reactions can occur before the ecdysis (pre-ecdysial sclerotization) or after ecdysis (post-ecdysial sclerotization) (review in Andersen 2010). In the honeybee, the cuticle deposited before the adult ecdysis is less sclerotized than the post-ecdysial cuticle (Andersen et al. 1981) thus indicating that sclerotization proceeds throughout part of the adult stage. A detailed description of the morphogenesis of the adult integument (epidermis and its associated cuticle) in the honeybee, using conventional histology and light microscopy, evidenced that exoskeleton maturation continues and is intensified after ecdysis to the adult stage (Elias-Neto et al. 2009).

We have observed that the rate of post-ecdysial cuticle tanning differs among bee species. This variation in the temporal pattern of exoskeleton tanning, or heterochrony, may represent an adaptive mechanism related to the bee lifestyle. Heterochrony is defined as any evolutionary change in the temporal pattern of expression of a certain characteristic (West-Eberhard 2003), meaning the shift (acceleration

or delay) in the relative timing of developmental events. Heterochrony is a phenomenon of great importance in the evolution, both in the frequency of occurrence and the origin of significant evolutionary changes (Gould 1977). New approaches and tools for the study of heterochrony at cellular and molecular levels have provided opportunities to a deeper understanding on the basis of the shifts in the timing of ontogenetic events (Smith 2003).

In this context, the evolution of insect sociality represents remarkable scenery for testing hypotheses related to adaptive mechanisms of exoskeleton maturation. The protective environment of the colony possibly has led to a loosening of the selective pressures over the cuticular differentiation, which enables, for instance, the emergence of adults with a relatively immature exoskeleton. Complete maturation is only attained several days later, when the eusocial bees become foragers. In contrast, the solitary bees leave the nest soon after the emergence from the brood cells, and this evidently demands a fully mature exoskeleton. Therefore, morphological markers of exoskeleton tanning, such as the dynamics of melanization and sclerotization, are useful to investigate whether the complexity of the evolution of eusociality included the slowdown in the rate of exoskeleton maturation.

Corbiculate bees (Holometabola, Hymenoptera, Apidae) are a monophyletic group (Michener 2007), supported by a series of synapomorphies, including the presence of a corbícula (basket-shaped structure in the female tibia which is used for pollen transport). Such bee group forms a distinct clade within Apinae (Apidae). It includes four tribes as follows: Euglossini (“orchid bees”), Bombini (“bumblebees”), Apini (“honey bees”), and Meliponini (“stingless bees”). Euglossini species are solitary, communal or primitively social; Bombini species are primitively eusocial (except for the Psithyrus, which comprises social parasites) (Silveira et al. 2002); Meliponini and Apini show true sociality (eusociality) (Michener 2007).

Except for a few studies in *Apis mellifera* (Thompson 1978; Elias-Neto et al. 2009), other corbiculate bee species have not been studied as regards to developmental morphology of the

exoskeleton, despite a series of reports (compiled in Cruz-Landim and Abdalla 2002) on characterization of exocrine glands associated with the epidermis and cuticle.

Species of the bee tribes above mentioned were used to investigate whether the temporal shift in cuticle tanning reflects the evolutionary transition to eusociality. In a comparative approach, the purpose of the present work was to investigate the hypothesis that the heterochrony of cuticular maturation is a conserved ontogenetic phenomenon related to the social environment in corbiculate bees.

## 2. MATERIALS AND METHODS

### 2.1. Bees

This study included species of all three eusocial tribes of corbiculate bees as follows: Apini (*A. mellifera*), Meliponini (*Frieseomelitta varia*, *Tetragonisca angustula*, *Scaptotrigona* aff. *depilis*, *Melipona scutellaris*), and Bombini (*Bombus morio*). The outgroup consisted of the solitary bees *Centris analis* (Centridini) and *Tetrapedia diversipes* (Tetrapediini). Females (workers) of these species were collected soon after the adult ecdysis (newly-emerged bees) and when they were foraging.

The bees were collected at the Campus of the São Paulo University in Ribeirão Preto. The species *A. mellifera*, *F. varia*, *T. angustula*, *S.* aff. *depilis*, and *M. scutellaris* were obtained in the experimental apiary/meliponary of the Department of Genetics. Specimens of *B. morio* were collected from a colony at the Laboratory of Ecology, Department of Biology, where trap-nests of *C. analis* and *T. diversipes* are also maintained.

The criteria for the selection of the species were based on the representativeness of different evolutionary lineages. Previous studies on the biology of Meliponini, Bombini, and solitary species, which enabled their maintenance and collection (Camillo et al. 1995; Nogueira-Neto 1997; Jesus and Garófalo 2000; Goulson 2006), also influenced our choice.

### 2.2. Integument sections

Histological sections were prepared using the integument dissected from the dorsal region of the thorax of newly-emerged and forager bees. The

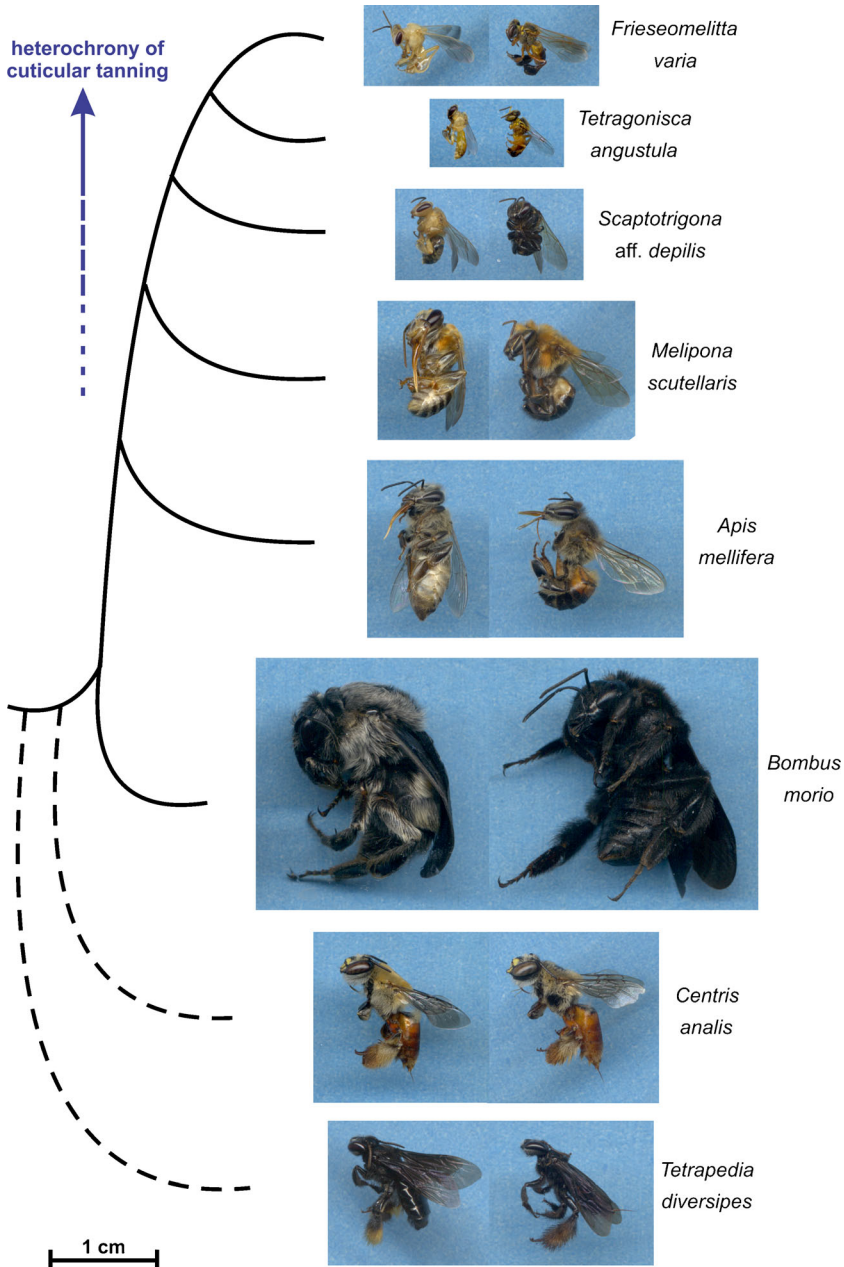
thoracic sections were made in a longitudinal plane, cutting the dorsal portion of the thorax (including pronotum, mesonotum, and metanotum) at the midline. The cuticle pieces were fixed for 24 h at 4 °C in 4 % paraformaldehyde in phosphate buffer (0.1 M, pH 7.3). This was followed by dehydration in successively higher concentrations (70, 80, 90, and 95 %) of ethanol in water (v/v) for 30 min in each solution. The cuticles were then embedded in methacrylate resin. Sections of 5 µm were obtained with the Microm HM200 Ergostar Microtome, and were stained with methylene blue and basic fuchsin for 3 min, followed by a rapid washing in distilled water. Sections were mounted in Entellan (Merck) and examined and photographed using an optical microscope (Axioskop II photomicroscope, Zeiss). At least three samples of each developmental stage were used in the histological preparations. The best-quality photographs (mesonotum) were presented.

### 2.3. Whole mounts

Whole mounts were also prepared with the dorsal portion of the thorax (pronotum, mesonotum, and metanotum). The thoracic integument samples were gently rinsed in Ringer saline, cleaned of excess fat body and muscles, and mounted on slides using pure glycerol. The whole mounts were immediately examined in the Axioskop II photomicroscope and photographed. At least three samples were obtained for each stage of development. The best-quality photographs (mesonotum) were shown.

## 3. RESULTS

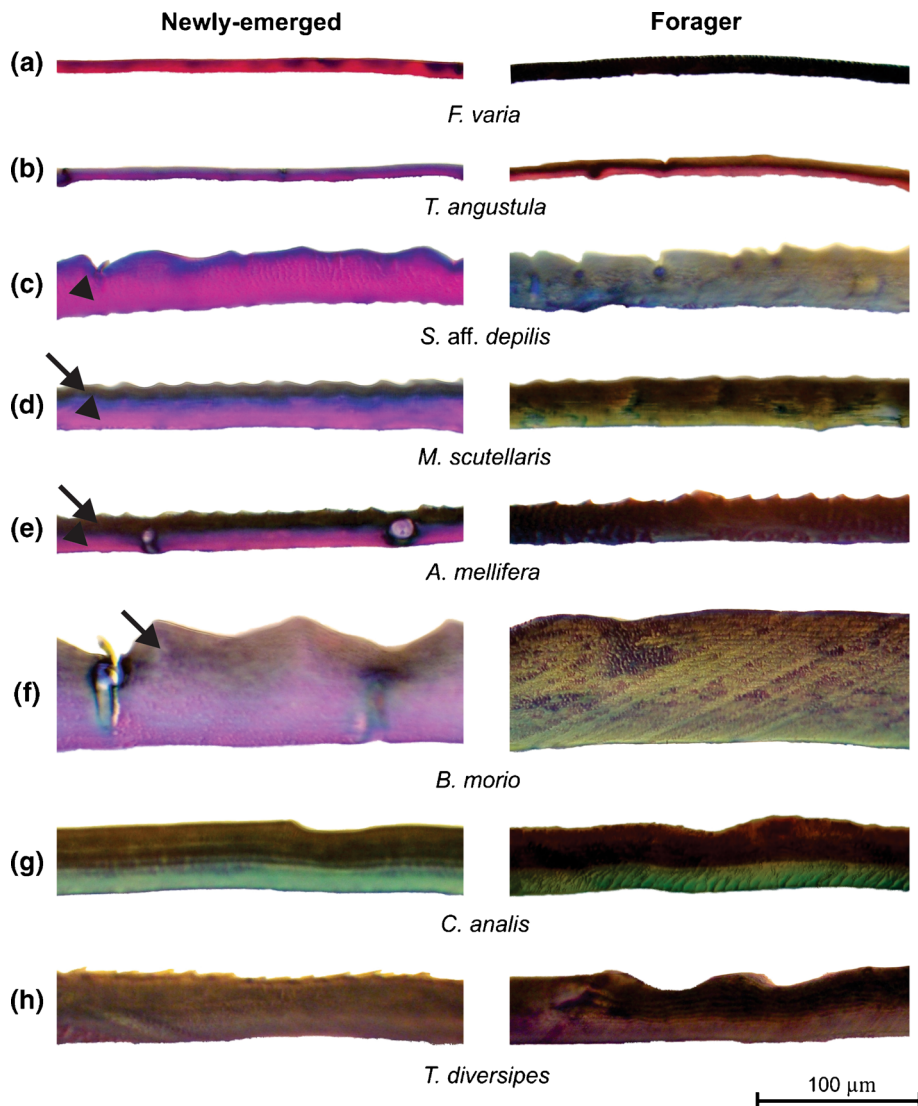
Forager bees from the eusocial species *A. mellifera*, *F. varia*, *T. angustula*, *S.* aff. *depilis*, *M. scutellaris*, and *B. morio* showed in a lesser or greater degree, depending on the species, a darker and stiffer cuticular exoskeleton than newly-emerged bees. Such difference was not observed in the solitary bee species (*C. analis* and *T. diversipes*) (Figure 1). These intra- and interspecific comparisons highlighted that in contrast to the solitary bees, the exoskeleton of eusocial bees takes longer to differentiate as a completely mature structure.



**Figure 1.** Evolutionary relationships of eusocial corbiculate bees (*continuous line*) and solitary bees (*dotted line*) according to strongly corroborated phylogenetic hypotheses (Michener 2007). Side-views of newly-emerged (*left*) and forager (*right*) females. Newly-emerged and forager bees in each eusocial species show contrasting exoskeleton differentiation, a phenomenon which was not observed in solitary bees.

To a deeper approach on this issue, the thoracic cuticle was then dissected from newly emerged

and forager bees of the eusocial and solitary species, and prepared for microscopy analysis



**Figure 2.** Sagittal sections of the dorsal thoracic exoskeleton of newly-emerged (*left*) and forager (*right*) bees of eusocial and solitary species. The distinct morphological patterns of the newly-emerged and forager eusocial bees (**a–f**) were not observed in the solitary bees (**g, h**). Fuchicine and methylene blue highlighted the degrees of cuticle differentiation, the *reddish pink* color (*arrowheads*) evidencing the non-differentiated cuticle. The *black* (*melanized*) layers (*arrows*) indicate the intense sclerotization of the mature cuticle.

(Figures 2 and 3). Both preparations—sagittal sections (Figure 2) and whole mounts (Figure 3)—emphasized the morphological differences between the cuticle of the newly-emerged and forager bees in the eusocial species,

both in the intensity of pigmentation and in the process of sclerotization. The cuticle proved to be always much more melanized and rigid in the foragers (Figure 2a–f). It is lacking in the newly-emerged *F. varia*, *T. angustula*, and *S. aff. depilis*,

the melanized superficial layer, which is only evident in the foragers. In spite of the presence of such a layer in the cuticle of newly-emerged bees of the other eusocial species, *M. scutellaris*, *A. mellifera*, and *B. morio* (Figure 2d–f, arrows), it is comparatively thicker in the foragers of these species. The melanized surface of the cuticle is not always well-delimited in the stained cross sections, thus making the comparison less obvious in some species, like *B. morio* (Figure 2f). Even so, it is clear that a completely differentiated (mature) cuticle occurs in the eusocial bees only several days after the emergence. In contrast, the solitary species, *C. analis* and *T. diversipes*, collected at the emergence or when they were foraging, showed very similar cuticle patterns, which are characterized by a thick melanized layer (Figure 2g, h).

In addition to the differences in melanization, cuticle sections of the newly-emerged eusocial bees showed a pink-reddish layer that varies in thickness among the different species (Figure 2a–f, arrowheads). This layer undergoes intense modifications in its chemical properties during cuticular maturation as deduced by the change in its color in foragers of the eusocial species. The pink-reddish color thus marks the yet undifferentiated layer of the cuticle.

In the whole mount preparations (Figure 3), there was also a clear distinction between the cuticular patterns of the newly-emerged and forager bees of the eusocial species (Figure 3a–f), which was not observed in the solitary species (Figure 3g, h). *F. varia*, *T. angustula*, and *S. aff. depilis* showed the highest degree of cuticular maturation heterochrony (Figure 3a–c). Foragers of these species have a brownish cuticle with a homogeneous distribution of pigments, whereas in the newly-emerged bees, pigmentation was concentrated in the setae and setal sockets.

Compared to *F. varia*, *T. angustula*, and *S. aff. depilis*, the eusocial species *M. scutellaris*, *A. mellifera*, and *B. morio* showed a moderate degree of cuticular maturation heterochrony, although indubitably pigmentation is much more intense in the foragers (Figure 3d–f).

The newly-emerged and forager bees of the solitary species, *C. analis* and *T. diversipes*,

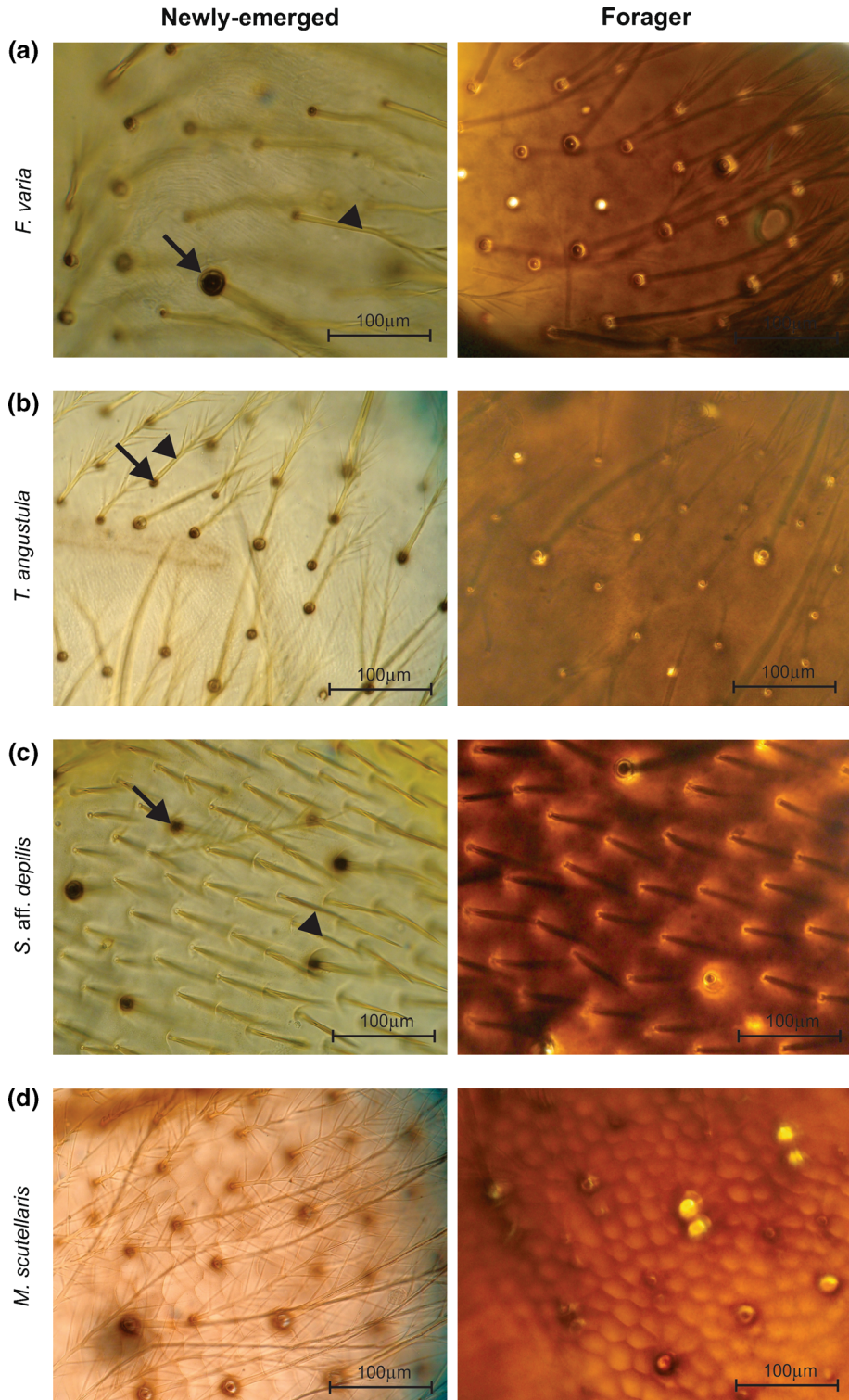
**Figure 3.** Whole preparations of the dorsal thoracic integument of newly-emerged (*left*) and forager (*right*) bees of the eusocial (*F. varia*, *T. angustula*, *S. aff. depilis*, *M. scutellaris*, *A. mellifera*, *B. morio*) and solitary (*C. analis*, *T. diversipes*) species. The cuticle is much more intensely pigmented in foragers than in the newly-emerged of each eusocial species (a–f); newly-emerged and foragers of the solitary species showed a similar degree of cuticle pigmentation (g, h). Setae (*arrowheads*) and setal sockets (*arrows*).

showed cuticles morphologically very similar, without apparent differences in the pigmentation pattern (Figure 3g, h).

#### 4. DISCUSSION

The present study included six species of Bombini, Meliponini, and Apini, which represent branches of the corbiculate phylogenetic tree. Although there is some controversy about the phylogeny of corbiculate bees (Lockhart and Cameron 2001; Danforth et al. 2013), a strongly corroborated grouping, both by the combined use of morphological and molecular characters (Chavarría and Carpenter 1994) and also by behavioral evidence (Noll 2002), consists of Euglossini + (Bombini + (Meliponini + Apini)). This classification was already seen in previous classical reviews (Michener 1944; Kerr and Esch 1965).

In addition to the six social species, our study included two solitary species, which comprise the majority of bee species (Batra 1984; Silveira et al. 2002), and were characterized by the female independence during nest construction. There is no cooperation or division of labor among the females of a same generation and, usually, there is no overlap in generations between parents and offspring. The massive provisioning of food ensures the complete development of the larvae toward the adult stage (Michener 1974). In general, the solitary bees dig their nests mainly in the ground or in tree trunks, although about 5 % of the species nest in preexisting cavities (Krombein 1967). Such feature has benefited bee biology studies, since females are drawn to nesting in artificially



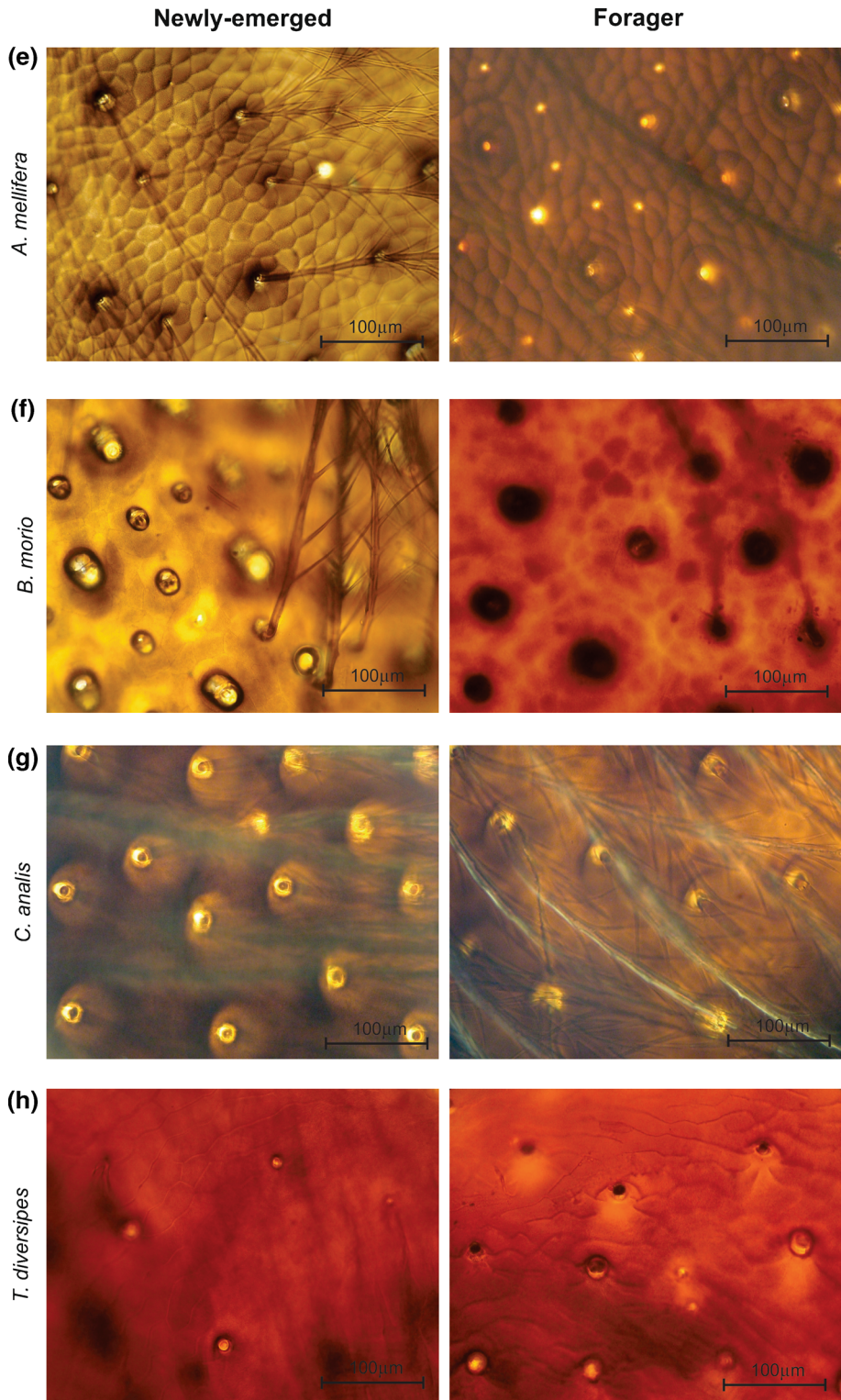


Figure 3. continued.



built cavities, the trap-nests (Camillo et al. 1995). More important in the context of the present study, and in contrast to the eusocial bees, the solitary bees leave the nest immediately after emerging as adults. Their cuticular exoskeleton, therefore, is ready to face the external environmental conditions.

The delay in exoskeleton tanning, or maturation, is apparently a conserved ontogenetic phenomenon among the eusocial corbiculate bees. We noticed distinct degrees of cuticular maturation heterochrony between the adults of the phylogenetically related eusocial species included in this study. As an example, in *F. varia*, which shows the greater degree of cuticular heterochrony, an extreme eusocial trait is also observed, such as the permanent sterility of the workers (Da Cunha and Campos 1993; Boleli et al. 1999). Conversely, the primitively eusocial bee *B. morio* shows a more subtle cuticular heterochrony. The existence of such gradient is hypothetical and new studies involving other species are needed to obtain a more elucidative scenario on this subject.

There is a straight association between social life and the nest, and any theory that addresses sociality evolution must consider some previous adaptations which favor gregarious behavior and inhibit dispersion behaviors (Nowak et al. 2010). The immature cuticle at the moment of adult emergence acts in both ways. The fragile and slightly pigmented exoskeleton keeps the bees from leaving the nest because it does not provide enough body protection against the damaging effects of the external environment (*developmental constraint*). At the same time, the plastic cuticle, from the ontogenetic point of view, possibly allows for the modeling of cuticular communication systems between the colony members (*developmental plasticity*).

An incipient and plastic cuticle seems an advantage during the time window that goes from emergence (brood cell-to-indoor nest transition) to the age at onset of foraging (nest-to-field transition). It is exactly during this time interval that the social life in the colony manifests more intensely. As an organ that makes the interface between the organism and

the environment, the cuticle is an important source of semiochemicals, such as hydrocarbons, for social communication. The cuticular hydrocarbons show discriminated abilities for recognition of nestmates, and cuticle from young and forager bees of social species differ in the profiles of these compounds (Kather et al. 2011; Falcon 2013). Therefore, the relatively immature cuticle of the newly-emerged and young bees is possibly advantageous for their recognition and acceptance by the nestmates, and integration in the colony. Like melanization and sclerotization, the blends of hydrocarbons are markers of exoskeleton maturation, since it can distinguish the immature from the mature cuticle. In this sense, variations in hydrocarbon profiles are consistent with the hypothesis that the cuticle of eusocial bees only becomes completely mature when they start foraging activities.

The lateness in exoskeleton maturation is here tentatively assigned to the way of life within a protective nest with a humid environment and controlled temperature (Winston 1991), both certainly contributing for the cuticle waterproofing function. In addition, honeybees show mechanisms for protection against diseases, such as hygienic behavior, that confer a degree of social immunity (Evans and Spivak 2010) and protect colony members (including those with an immature cuticle) against parasites and microbial pathogens. Therefore, an immature exoskeleton at the moment of emergence from the brood cell does not appear to be a disadvantage to the eusocial bee as to its protection.

Thus, the cuticle of young eusocial bees is evidently adapted to the within-hive environment where they live and exert tasks as cooperative care of the brood during the first half of adulthood. At the right time, it achieves the structural complexity needed for the intense foraging activities usually carried out during the second half of adulthood.

Changes in bee behavior and physiology during age-related task transition from inside to outside the nest have been linked to extensive changes in gene expression in the brain (Ament et al. 2008, 2011). However, there are no studies

on global gene expression in the cuticle-producing epidermis during transition for foraging. Honeybee genes involved in adult cuticle formation and maturation, such as the genes encoding a structural cuticular protein (*AmelCPR14*) (Soares et al. 2007), a laccase enzyme (*Amlac2*) (Elias-Neto et al. 2010), and a subunit of the neurohormone bursicon (unpublished data), showed expression profiles shifted toward the adult stage. This suggests that these genes are involved in the post-ecdysial maturation of the exoskeleton.

Cuticular maturation heterochrony in bees possibly has arisen by the combined influences of environmental and genetic factors. Heterochrony can be caused by environmental stimuli, which may generate intraspecific alterations in the life cycle, caste, or gender (Miyazaki et al. 2010) and even in speciation events (Gould 1977). The environment does not act in the evolutionary process solely through natural selection, but also as an active agent in the development (West-Eberhard 1989, 2005). Therefore, cuticular maturation postponement in eusocial bees may have been induced by colony environment, and possibly is associated with delayed activation of genes involved in cuticle formation and maturation. Further transcriptome-wide analysis using integuments of newly-emerged and forager bees from solitary and eusocial species has the potential to determine the extension of expression of genes involved in exoskeleton maturation, and whether this expression differs dependently on the bee lifestyle. In addition to this approach, other question on this matter is whether the cuticle reaches the complete maturity even in bee workers that permanently remain into the hive without ever leaving for foraging, as exceptionally verified (Seehuus et al. 2006; Amdam 2011). Such knowledge may help address whether cuticular maturation is dependent upon the social context.

The acquisition of eusociality is a great evolutionary transition, in which complex interaction systems resulted in the emergence of new properties, such as social homeostasis (Maynard Smith and Szathmáry 1995). The origin and fixation of heterochrony of cutic-

ular maturation in eusocial bee species perhaps may be understandable if we look for the concept of colony as an adaptive unity (Seeley 1989) subject to natural selection acting in different levels of biological organization—*multilevel selection* (Krause and Ruxton 2002; Korb and Heinze 2004).

Certainly, comparative studies along a gradient of sociality to include solitary, semi-social, and eusocial bee species can actually reveal unique aspects of exoskeleton maturation in the context of evolution of sociality.

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### Hétérochronie de la différenciation cuticulaire chez les abeilles corbiculées eusociales

Exosquelette / tannage / eusocialité / Apinae / abeilles solitaires

### Heterochronie in der Differenzierung der Kutikula bei eusozialen corbiculaten Bienen

Exoskelett / Aushärtung / Eusozialität / Apinae / solitäre Bienen

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