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Nest-mate recognition in *Manuelia postica* (Apidae: Xylocopinae): an eusocial trait is present in a solitary bee

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In eusocial Hymenoptera, females are more tolerant towards nest-mate than towards non-nest-mate females. In solitary Hymenoptera, females are generally aggressive towards any conspecific female. Field observations of the nest biology of *Manuelia postica* suggested nest-mate recognition. Experiments were performed involving two live interacting females or one live female interacting with a dead female. Live females from different nests were more intolerant to each other than females from the same nest. Females were more intolerant towards non-nest-mate than towards nest-mate dead females. When dead females were washed with pentane, no differences in tolerant and intolerant behaviours were detected between non-nest-mate and nest-mate females. Females were more intolerant towards nest-mate female carcasses coated with the cuticular extract from a non-nest-mate than towards non-nest-mate female carcasses coated with the cuticular extract from a nest-mate. The compositions of the cuticular extracts was more similar between females from the same nest than between females from different nests. The results demonstrate for the first time nest-mate recognition mediated by cuticular chemicals in a largely solitary species of Apidae. The position of *Manuelia* at the base of the Apidae phylogeny suggests that nest-mate recognition in eusocial species apical to *Manuelia* represents the retention of a primitive capacity in Apidae.

Keywords: nest-mate recognition; Hymenoptera; Apidae; solitary bee; cuticular compounds

1. INTRODUCTION

Conspecific recognition plays a key role in the evolution of cooperative behaviour between related (e.g. Hamilton 1964*a*) and unrelated animals (e.g. Trivers 1971). It is based on the traits related to the identity of individuals, kinship or membership to categories such as nest-mate or non-nest-mate (review: Wilson 1975). Nest-mate recognition is widespread among eusocial insects (Smith & Breed 1995; Breed 1998; Breed *et al.* 2004*a*), and can be inferred from the outcome of the interaction between conspecifics from the same or different nests (or colonies; Gamboa 2004). For instance, females of social species of Hymenoptera attack non-nest-mates more often than nest-mates, and nest-mates are mutually tolerant, i.e. they exhibit little mutual aggression (Buckle & Greenberg 1981; Michener & Smith 1987; Inoue *et al.* 1999). By contrast, high levels of aggressive behaviours towards conspecifics have been observed in solitary species (Hölldobler & Michener 1980; Field 1992; Kukuk 1992). From an evolutionary point of view, the transition from solitary to social life requires a regulation of such aggressiveness and therefore intraspecific recognition, because individuals within a group must develop reciprocal tolerance to maintain cohesion (Lin & Michener 1972; Michener & Smith 1987; Jeanson *et al.* 2005).

Most research on discrimination between hymenopteran conspecifics from the same and different nests or colonies, and the associated recognition cues have been performed in social species (reviews: Gadagkar 1985; Smith & Breed 1995; Singer 1998; Gamboa 2004; Howard & Blomquist 2005), probably due to the importance of this process for such species, i.e. to support the social structure of a colony (Hölldobler & Wilson 1990), to maintain high cooperation and low aggression necessary for communal life (Paxton *et al.* 1999) and, from an evolutionary point of view, to maximize inclusive fitness (Hamilton 1964*a*,*b*). Nevertheless, some evidence has been presented, which suggests that solitary species may also exhibit mutual tolerance and nest-mate recognition abilities. Thus, Wcislo (1997) reported that reproductively inactive females of *Lasioglossum* (*D.*) *figueresi*, a largely solitary species belonging to a basal branch in the phylogeny of Halictidae (Danforth *et al.* 2003), recognize familiar females and consequently modify their agonistic behaviour.

The family Apidae includes numerous solitary species, particularly among the Xylocopinae, its most basal subfamily (Michener 2000). However, no studies in this subfamily have demonstrated nest-mate recognition through behavioural assays comparing conspecifics from the same or different nests. A particularly interesting solitary taxon in Xylocopinae is the genus *Manuelia*, which has been proposed as the most basal taxon in the phylogeny of Xylocopinae (Sakagami & Michener 1987), which in turn is the most basal subfamily of Apidae (Michener 2000). Moreover, none of the known species of the family Megachilidae, the sister group of Apidae, exhibit eusocial behaviour (Michener 1974), suggesting that the solitary behaviour in *Manuelia* derives from a common ancestor to Apidae and Megachilidae without eusocial behaviour. Recently, L. Flores-Prado, E. Chiappa & H. M.

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Niemeyer (2007, unpublished results) described the occasional presence of two females inside the nests of *Manuelia postica*, suggesting a certain degree of tolerance between nest-mate conspecifics, and the agonistic interactions between a guarding female and an intruder, suggesting rejection between non-nest-mate conspecifics. These observations support the idea that nest-mate recognition is a capacity present in a natural context in *M. postica*. In this paper, we describe experiments with paired females from the same or different nests, which show the occurrence of nest-mate discrimination. Finally, we demonstrate the role that cuticular compounds play in nest-mate recognition.

2. MATERIAL AND METHODS

(a) *Females of* M. postica

Females of *M. postica* were extracted from 54 nests constructed inside the stems of *Chusquea quila* (Poaceae: Bambuseae) at the Altos de Lircay National Park, east of Talca, Chile (35°29′ S, 70°58′ W). These females were used to perform the experiments with live females in the field. Another group of 73 nests, which were judged to contain young adults before they destroy the partitions separating individual cells (L. Flores-Prado, E. Chiappa & H. M. Niemeyer 2007, unpublished results), was collected and transported to the laboratory in Santiago. These nests were kept at low temperature (10–15°C) and females were withdrawn from them as needed to conduct the experiments.

(b) *Recognition between two live females*

The following experiments were performed in the field. Two live females (from the same generation) were placed at the extremes of 20 cm long Tygon tubing whose 5 mm internal diameter was similar to that of the galleries where the bees live in nature; thereafter, the two extremes of the tubing were joined together forming a 'circular tube' arena (Breed *et al.* 1978). The agonistic and tolerant behaviours of both the females were recorded using an 'instantaneous sampling' method (Altmann 1974), in which the activity of the bees was observed for 5 s at 1 min intervals during 20 min. The behaviours were defined on the basis of those reported for other species of Apidae (e.g. Michener 1969, 1974), and were classified as tolerant or intolerant according to the patterns described by several authors for the species of Apoidea (e.g. Smith & Weller 1989; Breed & Julian 1992; Kukuk 1992; Wcislo 1997; Paxton *et al.* 1999; Pabalan *et al.* 2000; Packer 2000). Thus, the behaviours were scored as tolerant if (i) the females were near each other (less than one body length, tolerant approach), (ii) the females were in contact with each other with no signs of mutual aggression (tolerant contact) or (iii) one female passed by the other venter to venter (pass). If a female exhibited a C-posture (she curls her abdomen while her mandibles and sting are pointed towards the other female), or if she was observed pushing, biting, stinging or touching the other female with her legs, the behaviour was classified as intolerant. If females remained far away (less than one body length) from each other (intolerant spacing), or if one female facing the other moved back, the behaviours were also classified as intolerant. An agonistic index for the interaction between two females was modified from Barki *et al.* (1992) and Lehner (1996) as

$$\mathrm{AI}_{\mathrm{A+B}} = \frac{1}{20}\left[\sum B_{\mathrm{A}} + \sum B_{\mathrm{B}} + \sum B_{\mathrm{A+B}}\right],$$

where B_{A} and B_{B} are the number of intolerant behaviours performed only by female A or B, respectively, while the other one is at rest; $B_{\mathrm{A+B}}$ is the number of intolerant behaviours performed simultaneously by both females; and 20 is the maximum potential number of behaviours observed during the 20 min period.

Two treatments were performed: (i) an intra-nest treatment, consisting of two females from the same generation taken from the same nest and (ii) an inter-nest treatment with two females from different nests. Each treatment was replicated 31 times, using a new circular tube in each trial. Since results from similar experiments have indicated a relationship between aggressive behaviour and body size (Smith & Weller 1989; Hogendoorn & Velthuis 1999), the length of the forewing from its base to the tip of each female was measured as an estimation of body size (Breed *et al.* 1978; Smith & Weller 1989).

(c) *Recognition between a live and dead female*

The following experiments were performed in the laboratory. The recognition bioassay (adapted from Ruther *et al.* 1998, 2002) involved two females (from the same generation): a 'treated' dead female and a 'test' living female. The treated female was placed at one end of a 7 cm long glass tube whose 5 mm internal diameter was similar to that of the galleries where the bees live in nature, and the test female was placed at the opposite end. After placing the two bees inside the tube, the ends were sealed with Teflon stoppers.

The behaviour of the test female was video recorded during 20 min. Behavioural events and states were determined during tape playback and were analysed using the software The Observer v. 3.0 (Noldus). The behavioural events were the same as described for the experiment performed in the field, i.e. passing, pushing, biting, stinging, touching with the legs, C-posture, moving backwards. In addition, the duration of the following behavioural states was determined: (i) a tolerant approach, if the test female remained near the dead female (less than one body length), (ii) a tolerant contact, if the test female was in contact with the dead female but did not exhibit aggressive behaviours, (iii) an intolerant spacing, if the test female remained far away from the dead female (more than one body length), (iv) an intolerant contact, if the test female was in contact with the dead female and exhibited aggressive behaviours and (v) an intolerant escape, if the live female moved away from the dead female, and bit or pushed the stopper at the end of the glass tube. The total number of tolerant and intolerant events was counted, and the duration of tolerant and intolerant states was assessed.

Three bioassays were performed with two treatments in which the treated female was a nest-mate or a non-nest-mate of the test female. The treated female was (i) a dead female, killed by exposure to pentane vapours, (ii) a pentane-washed air-dried carcass or (iii) a pentane-washed carcass that had previously been coated with the extract made from another individual female. In the latter case, if the dead female was a nest-mate of the test female, it was coated with the extract of a non-nest-mate of the test female; if the dead female was a non-nest-mate of the test female, then it was coated with the extract of a nest-mate of the test female. Each treatment in each bioassay was replicated 15 times. Each replicate was performed with a different pair of females and glass tube. Temperature was maintained between 23 and 25°C during the experiments.

The use of dead females in these bioassays not only allows the manipulation of cuticular compounds but also avoids the potential problem that females can adjust or modify their agonistic behaviour in response to the behaviour of the opponent (Smith & Weller 1989; Schneider *et al.* 2001; Breed *et al.* 2004*b*).

(d) *Extraction of cuticular compounds*

The cuticular compounds were extracted by immersing females individually in glass vials containing 250 µl pentane, enough to cover their body completely, for 45 min; extracts were maintained at −18°C until they were analysed (Salvy *et al.* 2001; Saul-Gershenz & Millar 2006).

(e) *Chemical analysis*

The cuticular extracts were concentrated to 30 µl with a flow of pure nitrogen. Ten microlitres of the concentrated extract were injected in splitless mode into a gas chromatograph (GC model HP-5890; Hewlett-Packard, Palo Alto, CA) fitted with a capillary column (SPB5, 30 m×0.25 mm ID; Supelco, Deerfield, IL, USA) and directly coupled to a mass detector (MD model HP-5972; Hewlett-Packard) with an integrated data system. Helium was used as the carrier gas at 2 ml min^{-1}. The GC oven was programmed to remain at 150°C for 5 min, then to increase to 260°C at a rate of 5°C min^{-1} and finally to remain at 260°C for 20 min. Ionization by electron impact (70 eV) was carried out at 280°C. The presence or absence of a given compound in the chromatographic profile of each individual extract was determined by comparison of mass spectra with a library database, and comparison of retention indexes with those of authentic standards—when available—or with data from the literature. Identifications were considered positive if coincidence between experimental and library mass spectra was higher than 90% (values were typically higher than 95%), and if retention indexes did not differ by more than 5 units (differences were typically less than 3 units).

The extracts from 17 females from 8 different nests with 2 or 3 females each were analysed. This allowed the setting up of 10 intra-nest comparisons and 126 inter-nest comparisons. The composition of cuticular extracts was compared between females from the same nest and from different nests using the simple matching coefficient (Krebs 1989), calculated as the number of positive and negative matches (in terms of the presence or absence of a given compound) between female pairs divided by 28, the total number of compounds identified. Thus, the simple matching coefficient for each comparison varied from 0 to 1.

(f) *Statistical analyses*

The agonistic index for the interaction between two live females obtained in intra and inter-nest treatments were compared using one-way ANOVA (Sokal & Rohlf 1995). The agonistic index for the interaction between two live females from either the intra-nest or inter-nest treatments was correlated with the differences in forewing length using Pearson product–moment correlation (Sokal & Rohlf 1995). The number of tolerant and intolerant behavioural events and the duration of tolerant and intolerant behavioural states in bioassays involving dead females were compared between treatments (intra- and inter-nest) using the Mann–Whitney *U* test because data did not show a normal distribution (Siegel & Castellan 1988). Simple matching coefficients for females from the same or different nests were compared using the Mann–Whitney *U* test because data did not show a normal distribution (Siegel & Castellan 1988).

3. RESULTS

(a) *Recognition between two live females*

The experiments in the field involving two live females demonstrated that females were more aggressive towards non-nest-mates than towards nest-mates. Thus, the agonistic index in the inter-nest treatment (two females from different nests) was higher than that in the intra-nest treatment (two females from different nests; mean ± s.d. = 1.39 ± 0.49 and 0.99 ± 0.75, for inter-nest and intra-nest treatments, respectively; one-way ANOVA: $F_{1,60} = 6.05$, $p < 0.05$). Furthermore, the agonistic index was not correlated with differences in body size (forewing length) of females used either in the intra-nest treatment ($n = 31$, $r = 0.24$, $p = 0.19$) or in the inter-nest treatment ($n = 31$, $r = -0.26$, $p = 0.14$).

(b) *Recognition between a live and dead female*

The results of the laboratory bioassays involving a dead and live (test) female showed that test females were more intolerant towards non-nest-mate dead females than towards nest-mate dead females, as demonstrated by an increased number of intolerant behavioural events (figure 1*a*) and the increased duration of behavioural states (figure 1*d*). When the treated dead female was washed with solvent, no differences were apparent between non-nest-mates and nest-mates for any of the parameters analysed (figure 1*b*,*e*). Nevertheless, when the pentane-washed carcasses were coated with the cuticular extracts, the test females were more intolerant towards nest-mate female carcasses coated with the cuticular extract from a non-nest-mate than towards non-nest-mate female carcasses coated with the cuticular extract from a nest-mate (figure 1*c*). This reversal of the behavioural pattern by the application of cuticular extracts also occurred in the duration of behavioural states (figure 1*f*). Opposite patterns were found when the duration of tolerant behavioural states were considered (figure 1*d*,*f*), but not when the mean number of tolerant behavioural events was considered (figure 1*a*,*c*). On the other hand, test females were more tolerant towards dead nest-mate females than towards dead non-nest-mate females in terms of duration of behavioural states (figure 1*d*), and when the solvent-washed carcasses were coated with the cuticular extracts, the test females were more tolerant towards non-nest-mate female carcasses coated with the cuticular extract from a nest-mate than towards nest-mate female carcasses coated with the cuticular extract from a non-nest-mate (figure 1*f*).

(c) *Cuticular compounds*

The cuticular compounds were extracted from 17 females of *M. postica* from eight different nests. Table 1 shows the compounds identified in the extracts and their abundance. They were carboxylic acids, esters and hydrocarbons (alkanes and alkenes). The most abundant compounds were hydrocarbons, particularly C23 and C25 saturated hydrocarbons, as found in the solitary bee *Habropoda pallida* (Apidae: Apinae; Saul-Gershenz &

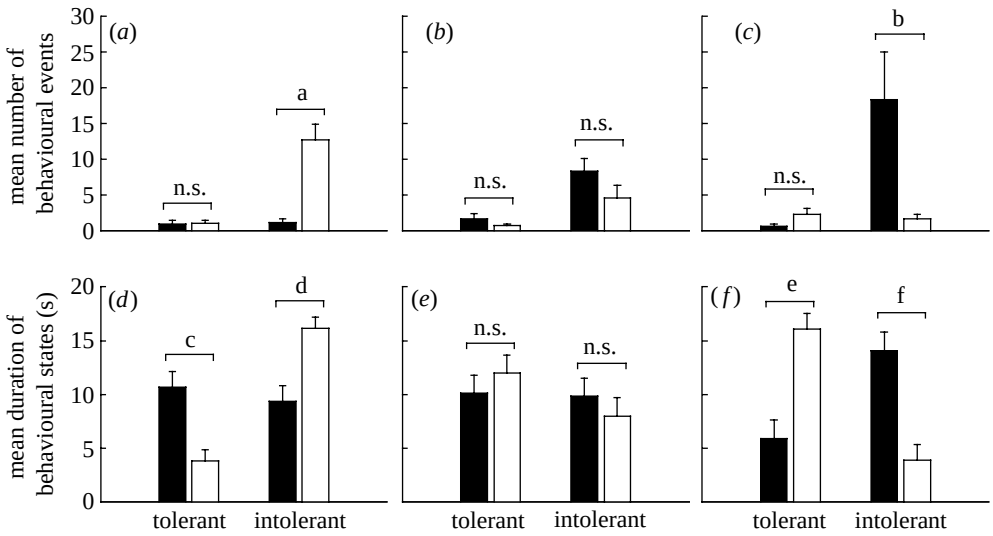

Figure 1. Mean number of tolerant and intolerant behavioural events and mean duration of behavioural states of *Manuelia postica* females in the presence of a dead female from the same nest (black bars, intra-nest treatment) or from a different nest (white bars, inter-nest treatment). Behaviours were recorded from bioassays involving: (*a*,*d*) dead females, (*b*,*e*) washed dead females and (*c*,*f*) washed and coated dead females (see the §2). The observation period was 20 min. Statistics for significant comparisons (Mann–Whitney *U* test): a ($Z=-4.33$, $p<0.001$); b ($Z=2.34$, $p=0.019$); c ($Z=2.92$, $p<0.01$); d ($Z=-2.92$, $p<0.01$); e ($Z=-3.75$, $p<0.001$); f ($Z=3.75$, $p<0.001$).

Table 1. Cuticular compounds identified in the extracts of females of *M. postica*.

compound	retention index	no. of females[a]	abundance (% ± s.d.)[b]	identification method[c]
hexadecanoic acid, ethyl ester	1991	7	0.21 ± 0.41	MS, RI, ST
hexadecanoic acid, methyl ester	2010	3	0.05 ± 0.12	MS, RI, ST
10-heneicosene[d]	2076	13	1.44 ± 2.32	MS, RI
heneicosane	2095	17	3.91 ± 4.77	MS, RI, ST
octadecanoic acid	2164	6	0.65 ± 1.27	MS, RI, ST
(*Z*)-9-octadecenoic acid, ethyl ester	2175	9	0.88 ± 1.61	MS, RI, ST
octadecanoic acid, ethyl ester	2190	6	0.41 ± 1.01	MS, RI, ST
docosane	2201	15	0.67 ± 0.45	MS, RI, ST
acetic acid, octadecyl ester	2212	10	0.35 ± 0.57	MS, RI, ST
(*Z*)-9-tricosene	2278	16	15.97 ± 13.70	MS, RI, ST
tricosane	2299	17	26.86 ± 10.92	MS, RI, ST
tetracosene[d]	2370	12	0.45 ± 0.51	MS, RI
1-tetracosene	2392	7	1.07 ± 2.53	MS, RI
tetracosane	2400	13	2.58 ± 4.14	MS, RI, ST
pentacosene[d]	2465	9	1.29 ± 1.77	MS, RI
4-pentacosene	2469	13	3.92 ± 3.22	MS, RI
pentacosane	2498	17	14.98 ± 10.71	MS, RI, ST
hexacosane	2590	16	0.69 ± 0.43	MS, RI, ST
heptacosene[d]	2663	10	1.19 ± 1.29	MS, RI
13-heptacosene[d]	2670	15	3.63 ± 3.51	MS, RI
heptacosane	2699	17	4.50 ± 2.42	MS, RI, ST
octacosane	2795	15	0.43 ± 0.35	MS, RI, ST
nonacosadiene[d]	2848	16	4.30 ± 2.61	MS, RI
nonacosadiene[d]	2855	15	1.45 ± 1.38	MS, RI
nonacosene[d]	2866	9	1.20 ± 1.97	MS, RI
nonacosene[d]	2872	12	2.13 ± 1.94	MS, RI
nonacosene[d]	2877	9	1.66 ± 1.82	MS, RI
nonacosane	2898	17	1.17 ± 0.39	MS, RI, ST

[a] Number of females in which the compound was found.
[b] Percentage of the chromatographic area of the compound in relation to the total area of the chromatogram.
[c] MS, mass spectrum; RI, retention index in relation to *n*-alkanes; ST, standard compound.
[d] Isomer undetermined.

Millar 2006). With the exception of 10-heneicosane, all compounds have been described previously as the constituents of the cuticular extracts of Hymenoptera (El-Sayed 2007). The number of compounds detected in any given female ranged from 10 to 24 (mean ± s.d. = 20.1 ± 4.18). The number of females in which a given compound occurred ranged from 3 to 17 (mean ± s.d. = 12.1 ± 4.07). The mean area of chromatographic peaks,

which could not be identified, corresponded to 2.0±2.6% (mean±s.d.) of the total chromatographic area.

Females from the same nest were more similar between each other than females from different nests (simple matching coefficient, mean±s.e.=0.764±0.026 and 0.683±0.011, for comparisons of females from the same or different nests, respectively; Mann–Whitney *U* test: $Z=2.25$, $p=0.024$).

4. DISCUSSION

(a) *Recognition between two live females*

L. Flores-Prado, E. Chiappa & H. M. Niemeyer (2007, unpublished results) described the occasional presence of two females inside the nests of *M. postica*, and the agonistic interactions between a guarding female and an intruder, suggesting nest-mate recognition capacity. Moreover, these observations support the idea that some components of kin discrimination are already present in *M. postica* in a natural context, as shown earlier for other solitary species (Wcislo 2000). Nest-mate recognition ability was tested in experiments, which demonstrated that females were more aggressive towards non-nest-mates than towards nest-mates, as in the eusocial species of bees (Buckle & Greenberg 1981; Michener & Smith 1987; Inoue *et al.* 1999).

Body size has been shown to affect the outcome of intraspecific interactions between hymenopterans (Gamboa & Dropkin 1979; Sullivan & Strassmann 1984). For example, laboratory observations of the interaction between nest-mates and non-nest-mates of the same generation have demonstrated that larger female bees are more aggressive than smaller ones (Smith & Weller 1989; Hogendoorn & Velthuis 1999; Arneson & Wcislo 2003). The differential agonistic responses exhibited by the females of *M. postica* were not correlated with the differences in body size, suggesting that in this experimental context visual stimuli are not particularly important in conspecific interactions and that other stimuli, such as chemical cues, affect the outcome of encounters between nest-mates and non-nest-mate females.

(b) *Recognition between a live and a dead female*

The perception of chemical cues as signals for nest-mate recognition has been demonstrated in the laboratory bioassays measuring agonistic responses in social Hymenoptera (Roulston *et al.* 2003). Bioassays developed in eusocial hymenopterans in which cuticular compounds have been removed and reapplied, or have been modified by the addition of compounds from external sources, have pointed to cuticular hydrocarbons as nest-mate recognition pheromones (Dani *et al.* 2001, 2005; Ruther *et al.* 2002). Our results are unequivocal about the central role that cuticular compounds play in nest-mate recognition in *M. postica*. Firstly, test females of *M. postica* discriminated dead females depending on whether they were nest-mates or non-nest-mates. Secondly, extraction of the potential recognition signal led to the disappearance of the associated discrimination patterns. Thirdly, the pattern of discrimination observed towards normal dead females was changed by coating the solvent-washed carcasses with the cuticular extracts. Finally, the composition of cuticular extracts was more similar between females from the same nest than between females from different nests. Hence, this series of bioassays unequivocally demonstrate that cuticular compounds are the cues employed by *M. postica* females in nest-mate recognition.

The importance of cuticular hydrocarbons in intraspecific communication has been demonstrated in a wide diversity of social Hymenoptera (Singer 1998; Tsutsui 2004; Howard & Blomquist 2005). The present report is the first to demonstrate nest-mate recognition and show the role of cuticular chemicals in nest-mate recognition in a largely solitary species of Apidae, *M. postica*. Nest-mate recognition has been suggested as one of the characteristic attributes of eusocial species (Breed *et al.* 2004*a*); the present results show that it is not an exclusive ability of eusocial insects. Furthermore, since *Manuelia* is at the base of the Apidae phylogeny and possesses several ancestral features (Michener 2000), nest-mate recognition exhibited by more apical eusocial species may represent the retention of a primitive capacity in Apidae.

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