

Article type: Original Paper

Tiny but socially valuable: eggs work as sources of communication in *Mischocyttarus* societies

Authors: Rafael Carvalho da Silva^{1*}, Tom Wenseleers², Cintia Akemi Oi^{2,3†}, Fabio Santos Nascimento^{1†}

Affiliations: ¹ Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Departamento de Biologia, Universidade de São Paulo – USP, Avenida Bandeirantes, 3900 – Vila Monte Alegre, Ribeirão Preto, SP 14040-900, Brazil

² Laboratory of Socioecology and Social Evolution, KU Leuven – University of Leuven, Leuven, Belgium

³ University College London, London, United Kingdom

†senior authorship shared

*Corresponding author: rcswasp@gmail.com

ORCID: 0000-0003-2674-8976 (RCS); 0000-0002-3731-7111 (FSN); 0000-0002-1434-861X (TW); 0000-0002-3682-8219 (CAO)

Abstract

Chemical compounds play a major role in the recognition processes in social insects, and one class of compounds namely hydrocarbons cover the adult body, but also the surface of their eggs. The ability to discriminate between friends from foes minimizes the exploitation of resources. We investigated for the first time whether females of *Mischocyttarus cerberus*, which is often attacked by other wasp species, can discriminate their own eggs over the eggs of foes. By using a non-destructive technique, we experimentally collected eggs from post-worker emergent nests and we offered their eggs to other nests to test the policing behavior in *M. cerberus*. Overall, our results show that the females of *M. cerberus* can discriminate eggs according to their origin and most of the removed eggs were policed within the first hour of the experiment, revealing that females have accurate discrimination skills. The discrimination skill allows females to detect eggs. We discuss that chemical cues present over the surface of eggs may be important for them to be accepted or removed, and these cues may be important to avoid parasitism. In this case, eggs represent alternative tools of communication, once they carry chemical compounds linked to their nest. Additionally, dominant females (= queens) are the most likely individuals to remove the eggs. Altogether, our results reinforce that the nestmate recognition ability is not restricted to recognizing adult relatives, but it is also extended to recognizing brood in *Mischocyttarus* societies.

Keywords: egg discrimination; alternative strategies; chemical communication; egg-marking pheromones

Competing Interests: The authors have no relevant financial or non-financial interests to disclose

Significance Statement:

Nestmate recognition is responsible to maintain social integrity in social insect colonies, as individuals can recognize friends from foes. We investigated whether nestmate recognition works on brood recognition in the primitively eusocial wasp *Mischocyttarus cerberus*. The nest of this species is deprived of an envelope and often can be attacked by parasitoids or other wasp species. By transplanting eggs from one colony to another using paper cells, we studied whether females would destroy introduced eggs more often. As a result, we found that *M. cerberus* females are capable to recognize introduced and their own eggs, and remove introduced eggs more often. Hydrocarbons covering the egg surface are the likely chemical cues that allow such recognition to occur. These results suggest that nestmate recognition is not limited to perceiving adult nestmates, but also brood in this wasp species.

Introduction

Social insects have evolved to recognize and accept adult nestmates over non-nestmates (van Zweden and d’Ettorre 2010; d’Ettorre and Lenoir 2010; Mora-Kepfer 2014). Contrarily, whether adult females have the same ability to recognize and discriminate brood remain poorly investigated (Panek and Gamboa 2000; Gamboa 2004). This recognition potentially minimizes the exploitation of resources by unrelated conspecific usurpers or social parasites (Fletcher and Michener 1987; Lorenzi and Filippone 2000). In such a context, the thin layer of chemical compounds that cover the insect body has been proposed to be the main vehicle of information, allowing the communication process to occur (Howard and Blomquist 2005). Cuticular hydrocarbons (CHC) contribute to the recognition process among individuals, being mostly composed by linear alkanes, branched alkanes and unsaturated hydrocarbons (Howard & Blomquist 2005). A higher chemical similarity exists among individuals from the same colony compared with other colonies (Gamboa 2004; Turillazzi et al. 2008), as members of a given colony learn the chemical template early in their adult life (Gamboa et al. 1986a). Evidence of nestmate recognition based on CHCs has been shown in ants (Tannure-Nascimento et al. 2009; Bos et al. 2010; Bos and d’Ettorre 2012; Larsen et al. 2014), bees (Nunes et al. 2008; Nunes et al. 2011; Nascimento and Nascimento 2012; Jones et al. 2012), termites (Bagneres et al. 1991; Yusuf et al. 2010), and wasps (Gamboa et al. 1986b; Cini et al. 2009; Cappa et al. 2020).

Besides covering the insect body surface, hydrocarbons occur over the surface of brood, for example over eggs, larvae, and pupae (in ants: Endler et al. 2004; see Schultner and Pulliainen 2020; in bees: Starkey et

al. 2019; Orlova et al. 2020; in wasps: Cotoneschi et al. 2007; Cervo et al. 2008; Cotoneschi et al. 2009; Oi et al. 2020), suggesting a role in mediating communication between adult-immature individuals. Among social insect species, it is relatively well studied in ants (see review Schultner and Pulliainen 2020), in bees (Free et al. 1983; Starkey et al. 2019; Orlova et al. 2020) and in a few wasp species (Klahn and Gamboa 1983; Panek and Gamboa 2000; Strassmann et al. 2000; Cotoneschi et al. 2009). For example, ants interact with brood, which allow to recognize and discriminate colony origin, maternity, developmental stage, sex, and caste (see Schultner and Pulliainen 2020). In colonies of the bumblebee *Bombus impatiens*, larvae play a role in regulating worker reproduction and behavior, as their presence reduce egg laying rates and worker aggression (Starkey et al. 2019; Orlova et al. 2020). In colonies of social wasps, such as *Vespula vulgaris*, the hydrocarbons covering the eggs' surface mediate policing (= egg destruction behavior), as queen-laid and worker-laid eggs are chemically different (Bonckaert et al. 2012; Oi et al. 2015).

Most of the available literature on policing behavior or brood detection in wasps correspond to social species from temperate areas, with exception of *Parischnogaster mellyi* (Turillazzi et al. 2008) and *Polybia paulista* (Kudô et al. 2016), which are both from tropical areas. *Mischocyttarus* is highly diverse in number of species in the Neotropical area and these wasps are interesting models to investigate policing behavior. *Mischocyttarus* wasps are primitively eusocial species, which means that females from different castes are not easily morphologically distinguished from each other, but instead they behave differently (O'Donnell 2020, Giannotti 1999). The nests start by one or a few females (Miller et al. 2018), and following nest foundation there is the establishment of a linear hierarchy of dominance, which is usually kept along nest ontogeny – nest ontogeny comprises pre-worker emergence, post-worker emergence and decline (Noda et al. 2001). *Mischocyttarus* wasps build stelocytarus gymnodomous nests, sustained by a single pedicel and lack a protective envelope, like *Polistes* paper wasps (Noll et al. 2020). The fact that the nests are deprived of a protective envelope, facilitates unrelated individuals to access their nest content. Nests of *Mischocyttarus* are attacked by parasitoid wasps (Somavilla et al. 2015; da Silva et al. 2019a), usurped by other wasps – *Mischocyttarus* and *Polistes* (Prezoto & Nascimento 1999; Pinto et al. 2004; de Souza et al. 2012; Montagna et al. 2012), attacked by ants (Clouse 1995), and also receive drifting females from other conspecific nests (Giannotti 1999). Given that resident females of *Mischocyttarus* are exposed to such environmental pressures, females that have cognitive abilities to recognize nestmate may help them dealing with nest usurpation. The costs of raising unrelated individuals can be high, if a given resident female is not able to recognize and discriminate its own brood from non-kin brood.

In the present study, we investigate whether females of *Mischocyttarus cerberus* species were able to recognize their own eggs over introduced eggs coming from intraspecific and interspecific nests. We studied wasps that occur in an anthropic area, where in the past, we reported the occurrence of six different species belonging to the *Mischocyttarus* genus (da Silva et al. 2019b). Nests are often found close to each other, which may facilitate parasitism and usurpation to take place (da Silva et al. 2019b). Thus, we hypothesized *M. cerberus* females display egg recognition based on their origin. We predicted that when confronted with options, females would destroy introduced eggs belonging to different colonies or species. In addition, females would destroy more often interim eggs coming from interspecific nests compared with eggs coming from intraspecific introduced nests. We suggest that the recognition process is likely mediated by chemical cues (hydrocarbons) that cover the eggs' surfaces. Thus, the higher accuracy in brood discrimination between species relies on the fact that a higher chemical variation is present between different species, but not within the same species. Lastly, we suggest that dominant females (=queens) may have a key role on such a recognition process. Overall, our results show that interspecific eggs and intraspecific eggs do not go unnoticed in nests of *M. cerberus* and the capacity to recognize and remove undesirable eggs previously reported for *Polistes* is now shown for *Mischocyttarus* wasps.

Material and Methods

Study area and collection

We studied nests in the post-worker emergence phase of *Mischocyttarus cerberus* and *Mischocyttarus montei* in the area of the campus of the University of São Paulo (USP) – Campus of Ribeirão Preto - (21°10'39"S; 47° 48' 37"W, 531 m elevation) between February and May 2021 (da Silva et al. 2019b). The nests lack protective envelope, which facilitates observation and manipulation of the eggs. We used a non-destructive technique to sample and manipulate eggs among different nests, which consisted of sampling eggs in artificial cells.

Experimental setup

In addition of the full description of methods presented below, we also prepared a diagram to summarize the experimental setup (Figure 1).

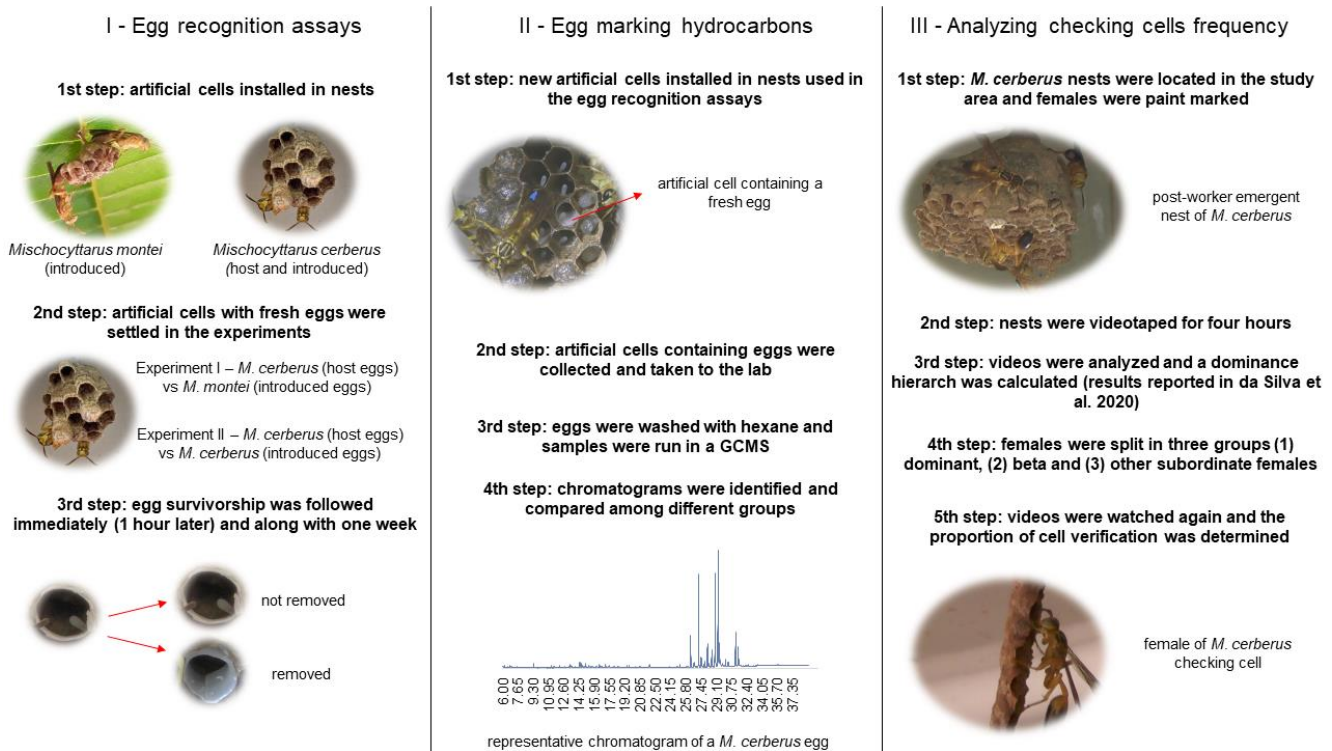


Figure 1: Diagram representing the main steps of experimental setup.

(I) Egg recognition assays

We used artificial paper cells made of waxed paper. We first washed the waxed paper with hexane and then let to dry. We inserted a piece of the waxed paper inside an empty cell from an abandoned nest to shape it, then we used drops of white glue to keep the artificial cell stable, and then they were left to dry. We inserted the paper cells in the natural nests to sample fresh laid eggs. We put artificial cells in either empty cells or in cells that had eggs (the eggs were removed with forceps before the insertion of the artificial cells). We monitored nests daily for the presence of newly laid eggs (eggs laid from 0 to 24 hours). We moved the artificial cells containing a *M. cerberus* egg to a different cell when the experiment was settled to avoid any bias concerning egg habituation (control group or host eggs – artificial cell containing an egg changed to a different cell). Using this control group (host eggs) was key to disregard the possibility of experimental eggs (introduced eggs) being removed because of the paper cells. We selected a random cell containing a *M. cerberus* egg and then the egg was replaced by the artificial cell containing an egg of *M. montei* (Figure S1) (Experiment I - interspecific recognition assay) or an egg of a different *M. cerberus* nest (Experiment II – intraspecific recognition assay). Eggs used in the experiments came from nests that were at least 100 m apart from each other.

We performed the procedure of settling host eggs against introduced eggs at the same time, and we repeated several times in different nests of *M. cerberus* (Experiment I (interspecific assay) n = 34 nests; Experiment II (intraspecific assay) n = 16 nests). Considering that we were interested to investigate egg policing occurring after eggs being placed, after preparing each experiment with both a host and an introduced egg, we waited an hour to evaluate whether egg removal had occurred or not. For eggs that were not removed in the first hour of experiment, we additionally followed them for a week to track eggs' survivorship.

In nests of *Mischocyttarus* wasps, a high rate of females has activated ovaries (not only the dominant responsible for the reproduction) (Murakami et al. 2009; da Silva et al. 2020). At least in *M. cerberus*, when other females lay eggs, the dominant female rapidly remove it (as soon she detects it) (personal observation). In this way, although we are not sure about egg maternity, it is likely that the eggs used in our assays were likely laid by the dominant female.

(II) Egg marking hydrocarbons

After performing the egg discrimination assays, we installed new artificial cells in the nests to collect fresh eggs from *M. cerberus* (either host and invader nests) and *Mischocyttarus montei* (invader nests) for chemical identification. Eggs' chemical profiles were determined via GC/MS analysis. We prepared the samples and analysed data using similar published methods as Oi et al. (2020). We individualized each egg in glass vials to extract their surface hydrocarbons. We washed the eggs with hexane (95% purity, Makron Fine Chemicals) using 100 μ l for 1 minute. We dried the glass vial containing the compounds under a fume hood and we prepared the vials for chemical analyses. The glass vials containing the chemical compounds were resuspended in 50 μ l of hexane (Macron Fine Chemicals 95% purity). We analysed all samples in a system of gas chromatography coupled with a mass spectrometer (GC-MS; Shimadzu, model QP2010 Plus) equipped with a Rxi-1ms column (thickness 0.25 μ m; length 30 m; diameter 0.25 mm) and helium as carrier gas (pressure 57.6 kPa; total flow 50.0 ml/min; column flow 1.11 ml/min; linear velocity 38 cm/sec; purge flow 5.0 ml/min; split ratio -1.0). In the gas chromatograph, we injected 1 μ l of sample using splitless injection. The injector temperature was set to 250 °C and all samples were run in the splitless mode. The oven temperature was initially set to 40 °C and held for 2 minutes. Then the temperature increased by 20 °C/min until 120 °C. In the following step, the temperature was increased by 10 °C/min until 200 °C, and subsequently by 7 °C/min until 250 °C. Lastly, the temperature increased 5 °C/min until 320 °C and it was kept for 4 minutes. A solution of linear alkane leaders (Supelco) from n-C7 to n-C40 was used as references

to calculate cubic spline interpolated retention indexes. Chromatogram peaks were identified based on their mass spectrometric fragmentation patterns and also in previously published references (da Silva et al. 2020b; Ferreira et al. 2022). Total ion chromatogram peaks were integrated using R version 4.0.2.

(III) Analyzing checking cells frequency

We analysed data from 14 post-worker emergent nests of *M. cerberus*. We observed each nest for four hours. Prior videotaping, we marked each female with an ink dot to allow identification during video analysis. We performed video analyses blindly. We divided females into three different groups (I) alpha females (= queens / dominant females), who performs the majority of aggressive acts towards other females, (II) beta females, who received the majority of the aggressive acts coming from the alpha female and (III) other subordinate females (= including all the other females that were not from the first and second groups). In this case, beta individuals represent the females occupying the second position in the dominance hierarchy (first position = alpha or dominant females, second position = beta females, third and so on positions are subordinate females). We assigned females to different groups by studying their dominance hierarchy interactions, and this methodology and results were reported previously by da Silva et al. (2020). After classifying the females, we watched the videos again and the total number of times each female inserted their heads inside a cell was recorded. We calculated the proportion of checking cell behavior and later we used this data in the statistical analysis. We used the checking cell behavior as a proxy to understand which female category would be more likely to be involved in egg detection.

Data analysis

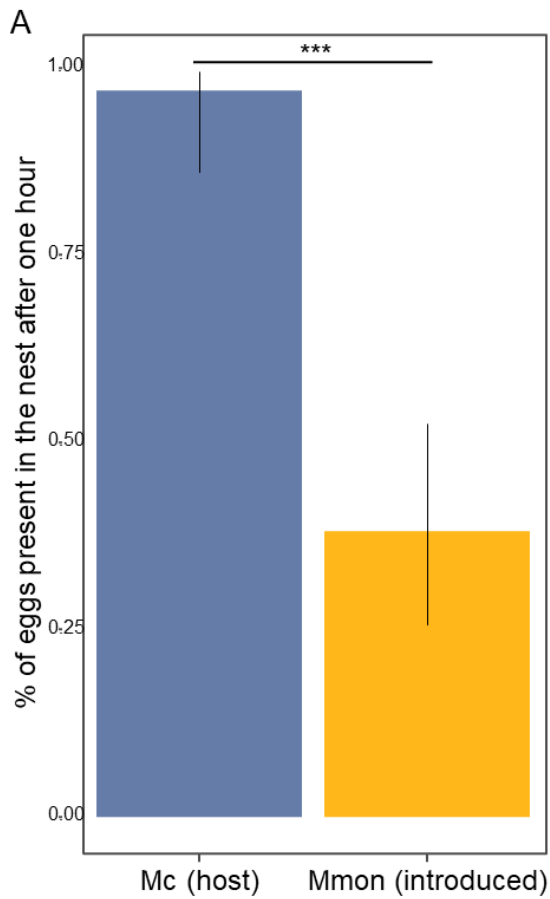
To assess whether the likelihood of eggs being in the nests after an hour of experiment was correlated to their origin, we fitted a generalized linear mixed model with binomial errors and with logit link function using *glmer*. For the chemical analyses, peak areas of each chemical compound were transformed in relative amounts, then we ran a permutation analysis (PERMANOVA) to verify whether eggs from different groups were chemically distinct based on their hydrocarbons. We used the *adonis* function from the *vegan* package and adopted 999 permutations. We performed a principal component analysis (PCA) with the *prcomp* function of the *stats* package (Team RC 2013). We ran a multivariate similarity analysis (SIMPER) using the Bray-Curtis distance and we adopted 999 permutations to check how much each of the components (CHCs) most contributed to the observed variation between eggs from different groups. To uncover whether

the frequency of checking cells varied among females from different groups, we analysed data by using a generalized linear mixed model, fitted by the *glmer* function from the *afex* package (Singmann et al. 2016). All analysis was performed using R (version 4.0.2).

Results

(1) Egg recognition assays

In the first experiment (interspecific recognition), eggs of *M. cerberus* were significantly more tolerated after one hour in the nests than the interspecific introduced ones of *M. montei* (Fig. 2) (ANOVA: LR Chisq = 31.355, $p < 0.001$) (Figure 2). The likelihood of eggs to survive for a week differed significantly according to their origin (Chisq= 36.9; Df = 1; $p < 0.001$) (Figure 2). In the second experiment (intraspecific recognition), host eggs of *M. cerberus* were more tolerated after one hour than the introduced ones from other *M. cerberus* nests (ANOVA: LR Chisq = 13.809; $p < 0.001$) (Figure 3). The likelihood of eggs to survive for a week differed significantly according to their origin in the second experiment as well (Chisq= 14.0; Df = 1; $p < 0.001$) (Figure 3). In both experiments, host eggs survived longer than the introduced ones (see Table S1). Overall, *M. montei* eggs were less likely to survive after one hour of experiment than *M. cerberus* eggs brought from other nests.



Interspecific recognition assay

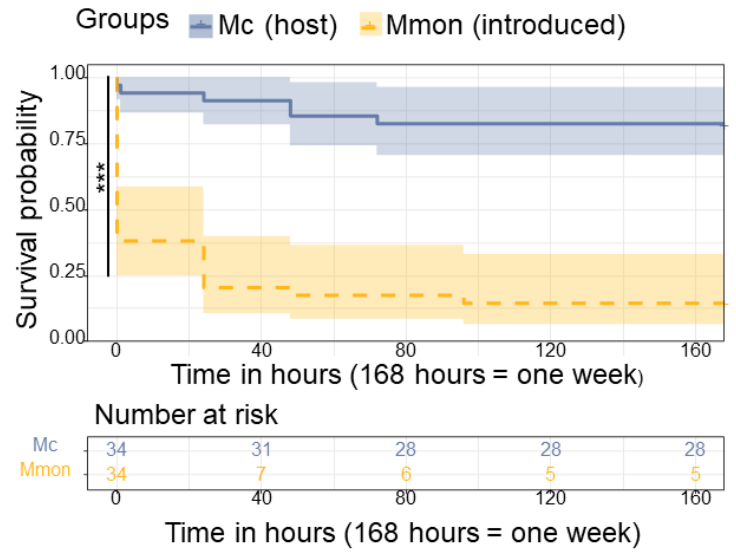
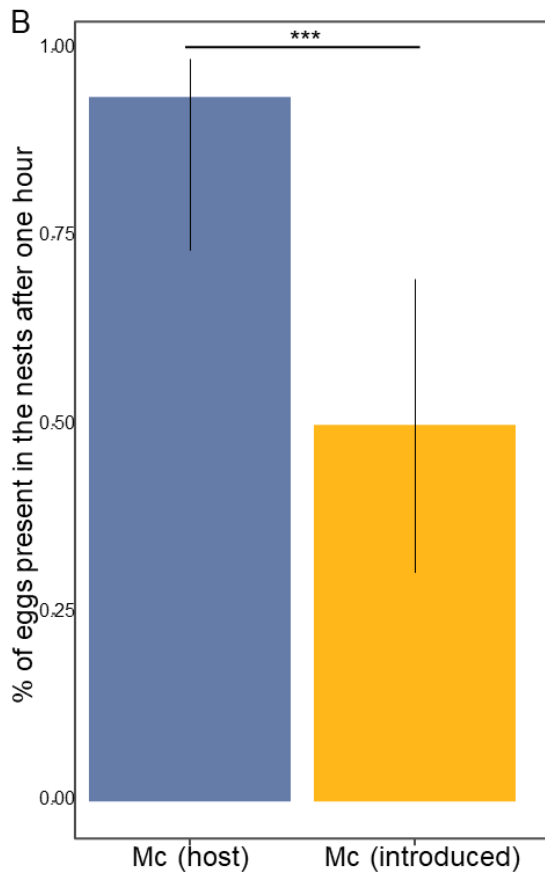


Figure 2: A greater proportion of *Mischocyttarus cerberus* host eggs were present in the nests after an hour.

Survivorship plot of eggs from Experiment 1 (interspecific recognition). The blue line represents the survivorship rate of host eggs, whereas the yellow line represents the survivorship rate of introduced eggs.



Intraspecific recognition assay

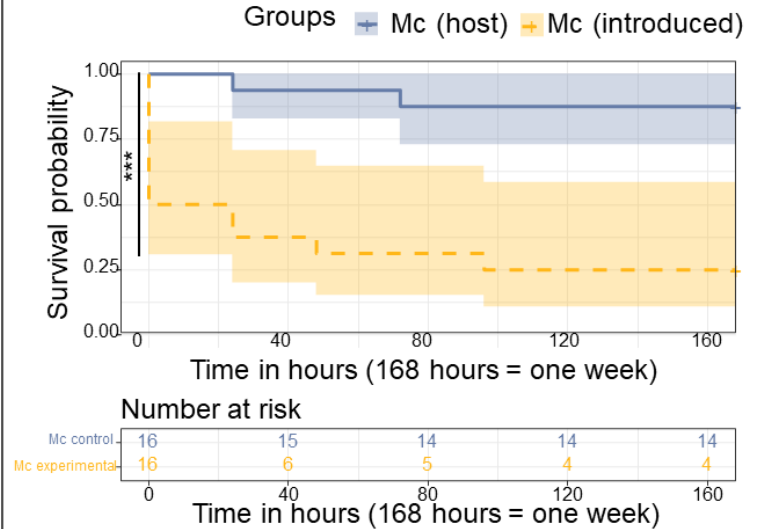


Figure 3: A greater proportion of *Mischocyttarus cerberus* host eggs were present in the nests after an hour. Survivorship plot of eggs from Experiment 2. The blue line represents the survivorship rate of host eggs, whereas the yellow line represents the survivorship rate of introduced eggs.

(II) Egg marking hydrocarbons in *Mischocyttarus cerberus* and *Mischocyttarus montei*

Eggs belonging to different groups (host and introduced nests) differed based on their hydrocarbons (PERMANOVA, $R^2 = 0.455$; $F = 15.889$; $p < 0.001$) (Table 1A). A representative chromatogram of each species is depicted below for reference (Figure 4). We also provide below a list of the compounds identified in the chromatograms (Table 2). The post hoc permutations analysis demonstrated that not all pairs of groups are indeed significantly different (Table 1B). The only pairwise permutations that differed statistically were the ones composed of a combination including introduced eggs from Experiment 1 (*M. montei*) and any other group of eggs (Table 1B). Principal component analysis (PCA) revealed that the introduced eggs from Experiment 1 (*M. montei*) were the most different according to their chemical composition (Figure 5; Table S2). The most important compounds that contributed to group differentiation included linear, methyl, and dimethyl-alkanes (Fig. 5; Table 3).

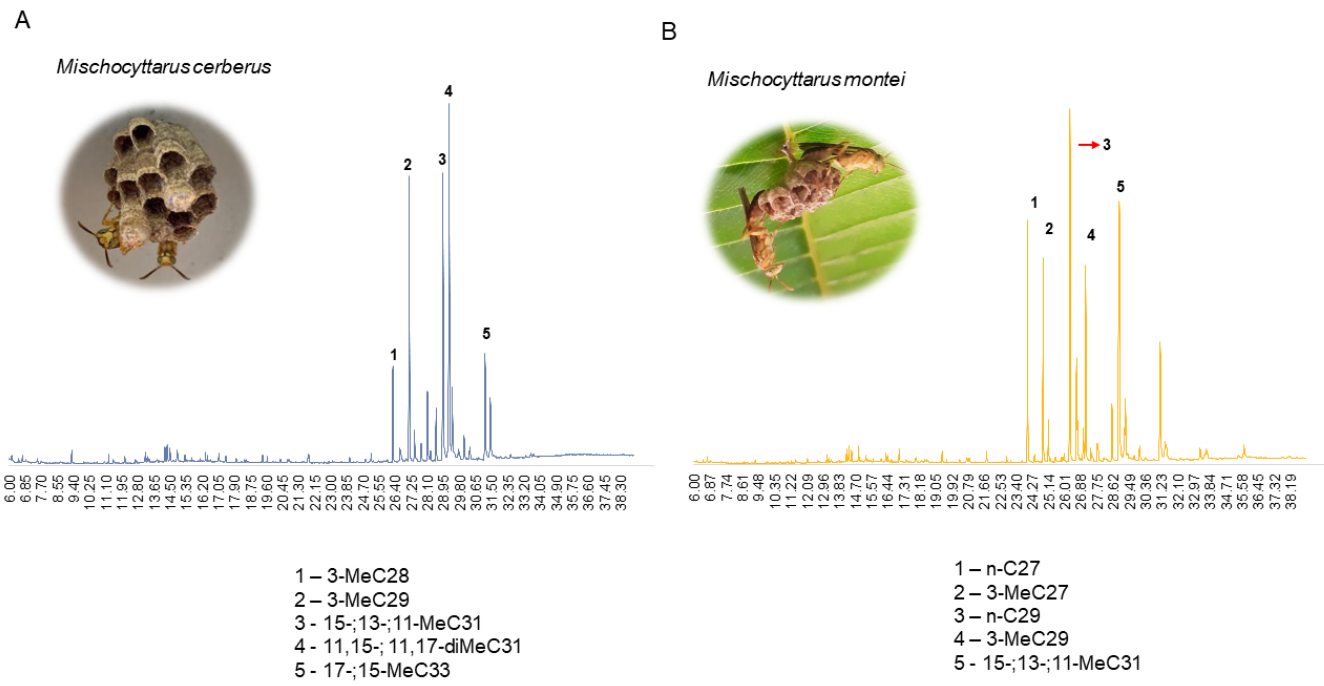


Figure 4: Representative chromatogram obtained from an egg sample a) of *Mischocyttarus cerberus*. b) *Mischocyttarus montei*. In evidence are depicted the major peaks found overall in the samples of both species.

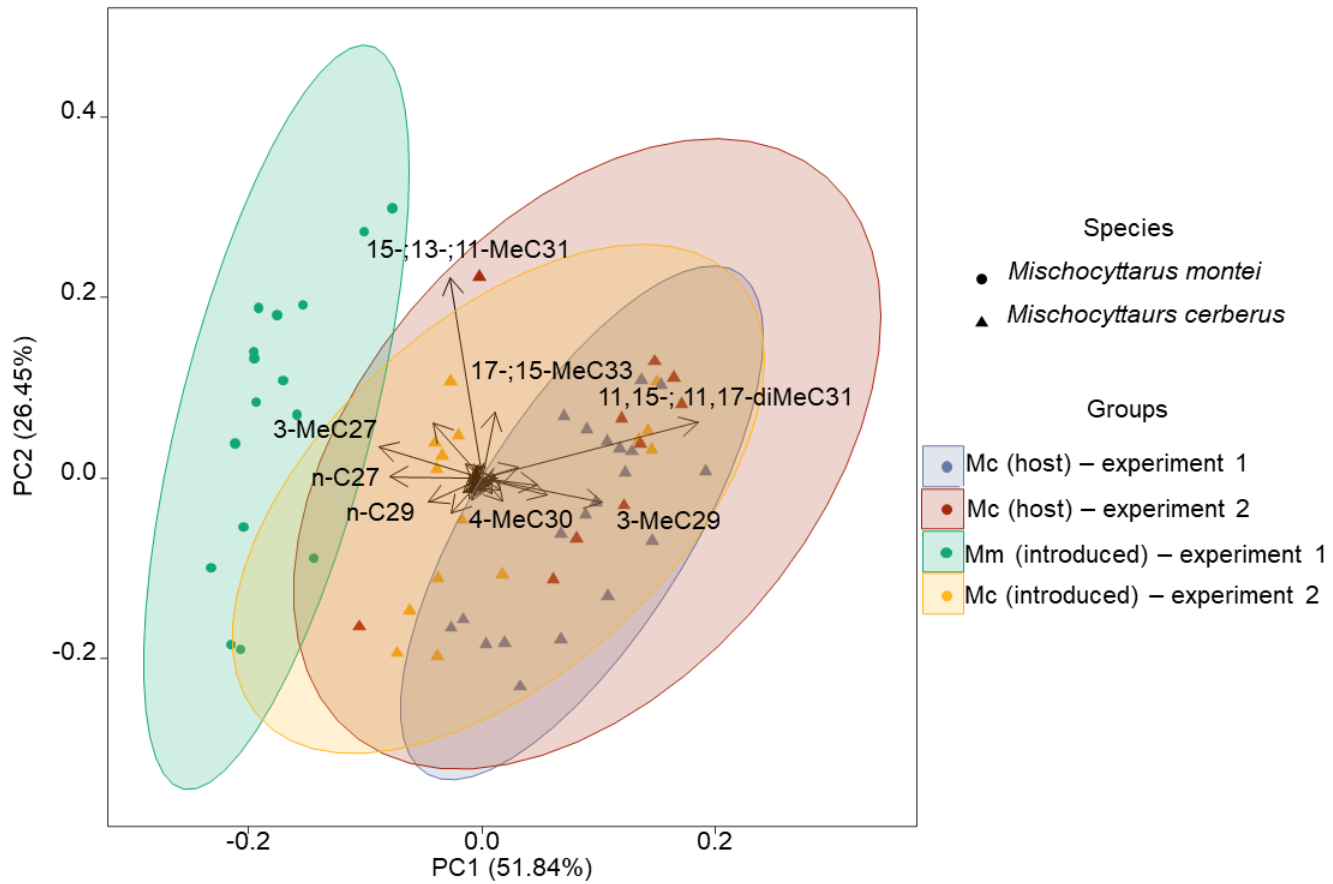


Figure 5: Principal component analysis (PCA) showing the ordination of the four studied groups of eggs based on their hydrocarbons. Chemical compounds in evidence represent the most important to promote group separation according to SIMPER analysis. Mc = *Mischocyttarus cerberus* and Mm = *Mischocyttarus monteii*. Experiment 1 = interspecific recognition and Experiment 2 = intraspecific recognition.

1 **Table 1:** (A) Global permutations (PERMANOVA) using all the groups of eggs collected in Experiment I and Experiment II (host and introduced eggs from both phases). (B)
 2 PERMANOVA pairwise contrasts between the groups of eggs collected in Experiment I and Experiment II. Experiment I = eggs collected from nests used in the interspecific
 3 recognition assays and Experiment II = eggs collected from nests used in the intraspecific recognition assays. Mc: *Mischocyttarus cerberus*; Mmon: *Mischocyttarus montei*.

(A) Global PERMANOVA					
	R2	F value	p value	Significance level	
eggs data\$group	0.455	15.889	< 0.001	***	
Residual	0.544				
Total	1				
(B) Pairwise PERMANOVA					
pairs	R2	F value	p value	Significance level	
Mc host exp. I x Mc introduced exp. II	0.106	3.941	0.186	n.s.	
Mc host exp. I x Mc host exp. II	0.043	1.268	1	n.s.	
Mc host exp. I x Mmon introduced I	0.541	40.171	< 0.01	**	
Mc introduced exp. II x Mc host exp. II	0.119	3.12	0.252	n.s.	
Mc introduced exp. II x Mmon introduced I	0.403	19.64	< 0.01	**	
Mc host exp. II x Mmon introduced I	0.482	22.37	< 0.01	**	

4

5 **Table 2:** List of hydrocarbons identified in the chromatograms of eggs from the four groups of samples, from Experiment 1 and 2. RT = retention time (min). RI = retention
 6 index. sd = standard deviation. Mc: *Mischocyttarus cerberus*; Mmon: *Mischocyttarus montei*.

Compounds	RT	RI	Ions	Experiment 1				Experiment 2			
				Mc host		Mmon introduced		Mc host		Mc introduced	
				mean	sd	mean	sd	mean	sd	mean	sd
n-C16	12.12	1600	226	0.358	0.191	0.380	0.209	0.358	0.255	0.456	0.243
n-C17	13.18	1700	240	0.769	0.438	0.815	0.442	0.704	0.470	0.909	0.427
n-C18	14.23	1800	254	0.875	0.433	0.896	0.451	0.835	0.460	1.054	0.470

n-C19	15.29	1900	268	0.695	0.356	0.730	0.381	0.671	0.398	0.859	0.397
n-C20	16.37	2000	282	0.698	0.437	0.760	0.508	0.617	0.331	0.819	0.409
n-C21	17.46	2100	296	0.481	0.233	0.457	0.259	0.502	0.282	0.599	0.269
n-C22	18.55	2200	310	1.051	0.731	1.010	0.582	0.957	0.557	1.169	0.572
n-C23	19.64	2300	324	0.488	0.183	0.376	0.193	0.496	0.325	0.555	0.200
C25:1	21.57	2475	350	0.369	0.291	0.397	0.247	0.301	0.180	0.416	0.201
n-C25	21.80	2500	352	1.056	0.546	1.241	0.701	0.988	0.471	1.162	0.688
n-C26	22.88	2600	366	0.364	0.189	0.455	0.234	0.339	0.195	0.420	0.235
C27:1	23.71	2669	378	0.422	0.270	0.487	0.245	0.401	0.253	0.497	0.274
n-C27	24.00	2700	380	0.873	0.299	5.707	2.223	0.715	0.273	0.883	0.289
13-;11-;9-;7-MeC27	24.39	2730	169/253/197/225/141/281/113/309	0.519	0.298	0.754	0.227	0.472	0.274	0.563	0.283
5-MeC27	24.55	2744	85/337	0.419	0.254	0.531	0.270	0.375	0.212	0.508	0.282
3-MeC27	24.85	2770	57/365	0.710	0.438	6.362	1.147	0.599	0.345	0.936	0.426
n-C28	25.13	2800	394	1.060	0.372	1.636	0.597	0.911	0.260	1.029	0.343
14-;13-;12-;11-MeC28	25.51	2828	211 / 225 / 197 / 239 / 183 / 253 / 169 / 267 / 393	0.919	0.476	1.093	0.442	0.808	0.486	1.012	0.502
4-MeC28	25.81	2855	71 / 365 / 393	0.386	0.179	0.517	0.144	0.399	0.178	0.450	0.199
3-MeC28	25.98	2869	57/379/393	0.843	0.403	1.155	0.365	0.782	0.353	0.991	0.450
n-C29	26.28	2900	408	7.076	1.944	9.266	2.838	5.446	1.328	7.678	1.503
15-;13-;11-;9-;7-MeC29	26.66	2928	225/197/253/169/281/141/309/113/337/407	2.172	0.635	5.357	1.970	2.595	0.907	2.700	0.698
5-MeC29	26.87	2947	85/365/407	0.324	0.103	0.424	0.113	0.347	0.211	0.409	0.133
11,15-; 9,15-diMeC29	27.02	2960	169 / 239 / 225 / 295 / 141 / 323 / 421	0.726	0.114	1.743	0.895	0.762	0.233	0.841	0.192
3-MeC29	27.15	2971	57/393/407	13.252	2.535	7.072	1.066	12.981	2.748	13.560	2.530
x,y-diMeC29	27.43	2996	-	1.992	0.345	0.913	0.160	1.772	0.357	1.641	0.296
n-C30	27.51	3000	422	0.939	0.248	0.994	0.242	0.916	0.309	0.980	0.291
14-;12-MeC30	27.79	3027	211/253/183/281/421	1.353	0.197	1.743	0.351	1.586	0.297	1.611	0.226
4-MeC30	28.11	3056	71/393/421	3.345	0.601	0.898	0.195	3.575	1.376	2.092	1.203
3-MeC30	28.27	3070	57/407/421	1.150	0.365	0.840	0.315	1.137	0.449	1.465	0.463
4,16-diMeC30	28.45	3085	71/225/253/407/421	0.346	0.120	0.280	0.114	0.349	0.134	0.367	0.132
n-C31	28.57	3100	436	3.922	0.690	2.213	0.686	3.072	0.510	4.472	0.924
15-;13-;11-MeC31	28.94	3128	225/197/281/169/309/435	10.452	3.175	15.333	4.899	13.305	4.106	13.319	3.808
11,15-; 11,17-diMeC31	29.26	3157	169/267/225/323/239/253/435	12.772	3.425	4.493	2.212	13.360	4.802	8.671	4.833
3-MeC31	29.42	3171	57/421/435	5.054	0.738	1.679	0.265	4.604	0.918	4.194	1.029
16-;14-MeC32	30.05	3227	239/253/211/281/449	2.256	0.243	1.691	0.182	2.395	0.447	2.108	0.289
4,14-diMeC32	30.35	3254	71/225/281/435/449	2.362	0.513	1.472	0.406	2.151	0.353	2.045	0.443
n-C33	30.82	3300	464	1.025	0.279	0.781	0.285	0.837	0.323	1.055	0.351
17-;15-MeC33	31.16	3328	253/225/281/463	5.828	1.295	6.554	2.072	5.993	1.477	4.835	1.266

15,19-; 13,17-; 13,19-diMeC33	31.44	3353	225/295/197/267/253/323/295/477	5.245	0.763	3.911	0.920	5.438	1.133	4.342	0.769
17-;15-;13-;11-MeC35	33.32	3528	281/253/225/309/197/337/169/365/491	1.492	0.546	2.016	0.365	1.493	0.708	1.845	0.712
17,21-diMeC35	33.58	3552	253/323/225/295/505	1.133	0.330	1.477	0.256	1.130	0.391	1.279	0.452
x,y-diMeC37	35.70	3755	-	2.429	1.104	3.089	0.729	2.527	1.578	3.203	1.577

7

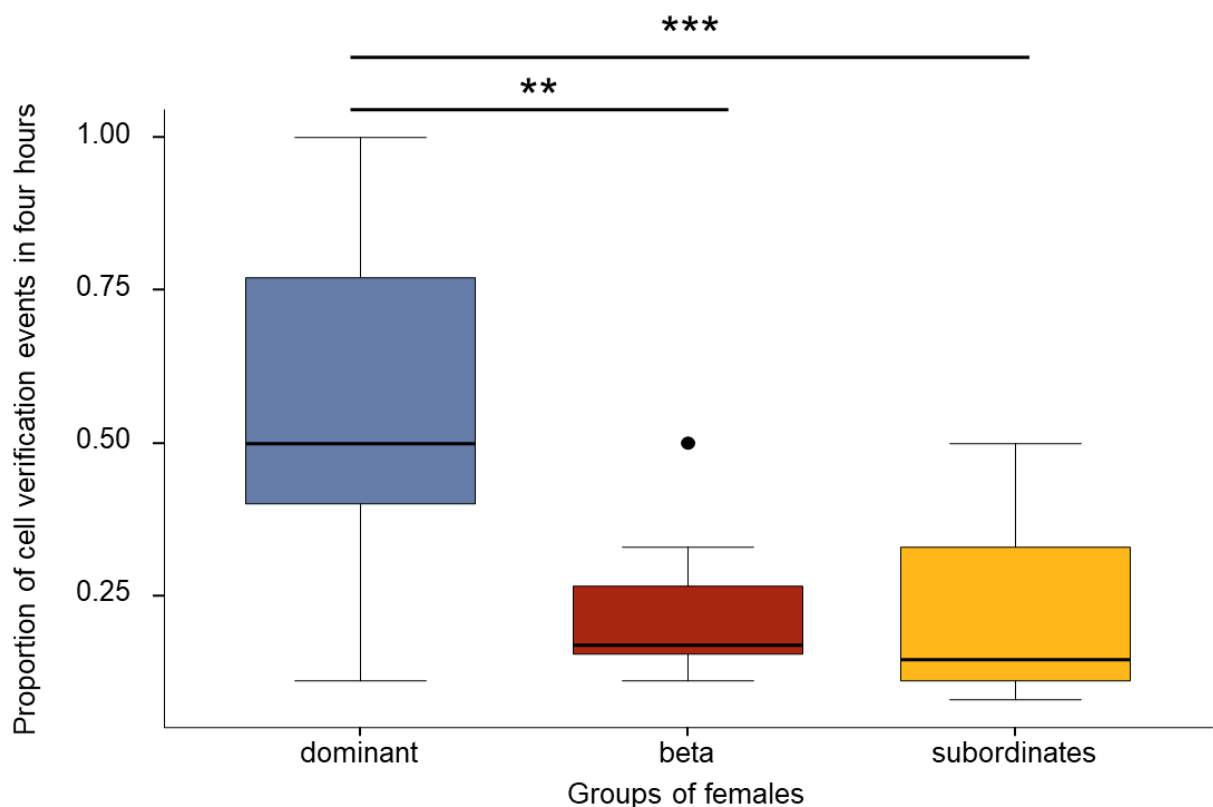
8 **Table 3:** SIMPER analysis showing the five most important compounds that contributed for group separation (pairs of groups). Average group 1 = *Mischocyttarus cerberus*
9 host from Experiment 1 (n = 20 eggs); Average group 2 = *Mischocyttarus montei* introduced from Experiment 1 (n = 16 eggs); Average group 3 = *Mischocyttarus cerberus* host
10 from Experiment 2 (n = 10 eggs); Average group 4 = *Mischocyttarus cerberus* introduced from Experiment 2 (n = 15 eggs). Sig. level = significance level. SD = standard
11 deviation.

Groups	Compound	Average	SD	Ratio	Average group 1	Average group 2	p value	Sig. level
Mc host exp. 1 x Mmon introduced exp. 1	11,15-; 11,17-diMeC31	0.042	0.019	2.246	12.772	4.493	0.001	***
	15-; 13-; 11-MeC31	0.032	0.020	1.625	10.451	15.333	0.001	***
	3-MeC29	0.031	0.013	2.368	13.253	7.072	0.001	***
	3-MeC27	0.028	0.006	4.755	0.711	6.361	0.001	***
	n-C27	0.024	0.011	2.223	0.873	5.707	0.001	***
Groups	Compound	Average	SD	Ratio	Average group 1	Average group 3	p value	Sig. level
Mc host exp. 1 x Mc host exp. 2	11,15-; 11,17-diMeC31	0.023	0.017	1.325	12.772	13.361	1.000	n.s.
	15-; 13-; 11-MeC31	0.022	0.019	1.179	10.451	13.304	0.888	n.s.
	3-MeC29	0.014	0.011	1.339	13.252	12.979	0.999	n.s.
	n-C29	0.011	0.008	1.302	7.076	5.445	0.918	n.s.
	17-; 15-MeC33	0.008	0.006	1.380	5.829	5.994	0.960	n.s.
Groups	Compound	Average	SD	Ratio	Average group 1	Average group 4	p value	Sig. level
Mc host exp. 1 x Mc introduced exp. 2	11,15-; 11,17-diMeC31	0.030	0.019	1.616	12.772	8.671	0.480	n.s.
	15-; 13-; 11-MeC31	0.023	0.016	1.416	10.451	13.321	0.860	n.s.
	3-MeC29	0.014	0.010	1.351	13.253	13.560	1.000	n.s.
	n-C29	0.010	0.007	1.443	7.077	7.679	0.995	n.s.

Groups	Compound	Average	SD	Ratio	Average group 2	Average group 3	<i>p</i> value	Sig. level
	17-; 15-MeC33	0.008	0.006	1.408	5.830	4.835	0.935	n.s.
Mmon introduced1 x Mc host exp. 2	11,15-; 11,17-diMeC31	0.046	0.022	2.066	13.361	4.493	0.001	***
	3-MeC29	0.030	0.014	2.174	12.979	7.072	0.001	***
	3-MeC27	0.029	0.006	4.958	0.600	6.361	0.001	***
	15-; 13-; 11-MeC31	0.028	0.017	1.663	13.304	15.333	0.152	n.s.
	n-C27	0.025	0.011	2.296	0.715	5.707	0.001	***
Groups	Compound	Average	SD	Ratio	Average group 1	Average group 4	<i>p</i> value	Sig. level
Mmon introduced 1 x Mc invader exp. 2	3-MeC29	0.033	0.013	2.553	13.560	7.072	0.001	***
	3-MeC27	0.027	0.006	4.568	0.937	6.361	0.001	***
	15-; 13-; 11-MeC31	0.026	0.018	1.481	13.321	15.333	0.262	n.s.
	11,15-; 11,17-diMeC31	0.025	0.022	1.142	8.671	4.493	0.997	n.s.
	n-C27	0.024	0.011	2.218	0.883	5.707	0.001	***
Groups	Compound	Average	SD	Ratio	Average group 3	Average group 4	<i>p</i> value	Sig. level
Mc host exp. 2 x Mc introduced exp. 2	11,15-; 11,17-diMeC31	0.034	0.021	1.624	8.671	13.361	0.031	*
	15-; 13-; 11-MeC31	0.021	0.016	1.294	13.321	13.304	0.932	n.s.
	3-MeC29	0.014	0.011	1.328	13.560	12.979	0.998	n.s.
	n-C29	0.013	0.008	1.634	7.679	5.445	0.627	n.s.
	4-MeC30	0.010	0.006	1.490	2.093	3.573	0.009	**

13 (III) Frequency of checking cells

14 The proportion of events in which females checked cells varied significantly among the groups ($\chi^2 = 25.378$,
15 $Df = 2$, $p < 0.001$). The post hoc pairwise tests revealed that indeed groups were significantly different from
16 each other (dominant x beta: $t.ratio = 3.407$, $p < 0.01$; dominant x subordinates: $t.ratio = 4.760$, $p < 0.001$),
17 with exception for the comparison of beta x subordinates ($t.ratio = 0.082$, $p > 0.05$) (Figure 6). Dominant
18 females checked cells more frequently than the other two groups of females.



19

20 **Figure 6:** Proportion of cell verification events performed by females during four hours of observation.
21 Alpha = 13 females, beta = 7 females, subordinates = 26 females. The proportion of cell verification varied
22 significantly among groups of females ($\chi^2 = 25.378$, $Df = 2$, $p < 0.001$). The post hoc pairwise tests revealed
23 that indeed groups were significantly different from each other (dominant x beta: $t.ratio = 3.407$, $p < 0.01$;
24 dominant x subordinates: $t.ratio = 4.760$, $p < 0.001$), with exception for the pair beta x subordinates ($t.ratio$
25 $= 0.082$, $p > 0.05$).

26 **Discussion**

27 The ability to correctly recognize a nestmate over a non-nestmate is important to maintain the social
28 integrity in social insects, once it prevents their nests to be usurped by other individuals (adults or brood).
29 Here, by using a non-destructive technique to sample eggs, we empirically demonstrated that females of
30 *Mischocyttarus* wasps have the capacity to recognize and destroy eggs at two different levels, intra and
31 interspecifically. We confirmed our initial hypothesis that *M. cerberus* females would correctly recognize
32 eggs based on their origin. We found that females destroy introduced eggs more frequently than their own.
33 The recognition abilities worked similarly when they were confronted either with interspecific and
34 intraspecific eggs (although *M. montei* eggs were less likely to survive after one hour of experiment than
35 *M. cerberus* eggs coming from other nests). Furthermore, we detected quantitative variations on the
36 hydrocarbons covering the eggs, suggesting that females likely rely on the chemical cues to make decisions.
37 A more pronounced chemical variation was detected between eggs from different species (*M. cerberus* x
38 *M. montei*), although a slight dissimilarity was also detected among eggs from different *M. cerberus* nests.
39 Lastly, we demonstrated that dominant females (= queens or alpha females) are the ones that check cells
40 more often within their nests, indicating that they are likely responsible for removing eggs. Taking into
41 consideration that *M. cerberus* can have their nests usurped by other *Mischocyttarus* or *Polistes* species,
42 egg recognition skills potentially help females to remove unrelated brood from their nests.

43 The survivorship results of the eggs suggest that females perform eggs' destruction shortly after being
44 placed (= laid), and this is true for intra and interspecific eggs. However, some introduced eggs escaped
45 policing and were reported to be in the nests after a week. We believe that there are two possible
46 explanations, firstly, eggs may escape being removed because after a while in the nest, they indirectly
47 acquire some chemical cues throughout the interaction that females maintain with them (grooming) or from
48 the nest material; or alternatively, it is possible that not all the females have accurate discrimination skills,
49 then these nests would be more susceptible to usurpers. Although these two explanations are speculative in
50 our case, previously published studies demonstrated that these may be the case for other species of social
51 insects. For example, as previously demonstrated for two termite species (*Reticulitermes santonensis* and
52 *R. lucifugus grassei*) allogrooming behavior (grooming towards another individual) is a way in which
53 cuticular compounds can be transferred passively among individuals (Vauchot et al. 1998). In ant species,
54 allogrooming helps them to spread CHCs stored in their glands that they received from different individuals
55 (Lenoir et al. 2001). Trophallaxis is also a route of disseminating hydrocarbons among nestmates (LeBoeuf
56 et al. 2016). In *Polistes metricus*, females do not perform a high frequency of egg removal when confronted

57 with their own and alien eggs (Wright et al. 2019). In this case, there seems to be an effect of olfactory
58 responsiveness of each female, the most responsive females are more likely to reject alien eggs, whereas
59 the contrary is true for the less responsive ones (Wright et al. 2019). Future research should address
60 questions related to chemical cues transference between adult-immature individuals, and also check
61 whether females of *M. cerberus* (especially queens) have a similar olfactory responsiveness, which
62 probably would help us understand whether all females have similar discriminatory skills.

63 As demonstrated previously for other social insects, the hydrocarbons covering eggs' surfaces are likely
64 the proximal factors that allow *M. cerberus* females to recognize their eggs over alien ones. Here we could
65 not explore how *M. cerberus* females would behave if confronted with eggs of *Mischocyttarus consimilis*
66 or *Mischocyttarus cassununga*, however the two species that were already reported to interact with *M.*
67 *cerberus* (usurpation interactions) (Pinto et al. 2004; Montagna et al. 2012; personal observation). The
68 accurate ability to eliminate eggs from other *M. cerberus* and *M. montei* nests suggest that they may be able
69 to associate specific chemical cues of their brood and react properly. Egg marking chemical cues are useful
70 not only to prevent nests from being usurped in social wasps (Lorenzi and Filippone 2000), but they are
71 also the main route to resolve internal queen-worker reproductive conflicts (see Wenseleers et al. 2020a;
72 Wenseleers et al. 2020b; Oi et al. 2020). Although the queen is the main responsible for reproduction in the
73 colony, workers in some cases are not fully sterile and try to invest in direct fitness by laying their own
74 eggs (male-destined eggs), however, reproductive workers can represent a cost for the whole colony since
75 they do not perform the same amount of work as the remaining sterile ones (Wenseleers et al. 2004). Under
76 such circumstances even though workers can activate their ovaries and even lay some eggs, these eggs are
77 effectively removed by their nestmates (Foster and Ratnieks 2001; Wenseleers et al. 2005; Liebig et al.
78 2005). The cues that allow such recognition are the chemical compounds that cover them (Oi et al. 2015;
79 Oi et al. 2020), as queen-laid and worker-laid eggs are chemically different. For example, in the social wasp
80 *V. vulgaris* a methylated hydrocarbon (3-MeC29) is more abundant over queen-laid eggs, and experiments
81 have shown that the topical application of these hydrocarbon over worker-laid eggs let them to be less
82 removed (Oi et al. 2015). In *Polistes dominula* the chemical composition of subordinate eggs does not
83 match the same that are laid by queens, then this variation may help queens to recognize and destroy
84 undesirable eggs in order to increase their fitness (Dapporto et al. 2007). The fact that not only queens have
85 activated ovaries and are inseminated in colonies of *M. cerberus* (da Silva et al. 2020b) raise the possibility
86 that reproductive conflicts may exist among females. Beta females and the other subordinate females may

87 attempt to invest in their own reproduction. If *M. cerberus* females rely on such egg marking chemical cues,
88 their recognition abilities can help reproductive conflicts to be solved.

89 In *Polistes biglumis*, females have the same ability to recognize their own eggs when confronted with other
90 from unrelated females. However, they behave differently depending on the eggs' fate, reproductive-
91 destined eggs are removed whereas worker-destined eggs receive care (Lorenzi and Filippone 2000). In
92 this case, the differential responses exhibited by *P. biglumis* females seem to be a result of the different
93 fitness return that females may have from raising alien brood. Caring for worker-destined eggs means
94 receiving help with nest maintenance when these adult females emerge, while caring for reproductive-
95 destined eggs does not mean receiving any help (Lorenzi and Filippone 2000). We cannot assure that eggs'
96 fate was a determinant factor that contributed for some introduced eggs to be tolerated for a longer time in
97 our experiments. Future experiments should address if worker-destined eggs or male-destined eggs would
98 be removed at different rates in *M. cerberus* colonies.

99 Although we were not able to correctly assign which female is responsible for scanning the nest to remove
100 introduced eggs, we believe that this task is probably performed more often by females responsible for
101 reproduction, the dominant ones (= queens) in *M. cerberus*. Our data demonstrate the proportion of
102 checking cell events from different groups in a limited time (four hours of observation), however, we were
103 already able to see a trend for dominant females to check cells more often. Females of *M. cerberus* probably
104 check cells for different reasons. Dominant females stay on the nest and dedicate their time to reproductive
105 tasks, which includes laying eggs and checking them right after oviposition (personal observation / see
106 Figure S2), additionally, they also display the brood attending behavior, and they frequently perform
107 aggressive acts toward other females (da Silva et al. 2020b). The remaining females however, often put
108 their heads inside a cell when they are feeding larvae, for example forager females perform this right after
109 returning from the field (personal observation). In social wasps belonging to different social levels, from
110 primitively to highly eusocial, it is well established that policing behavior is performed not only by the
111 queen, but also by other workers (Saigo and Tsuchida 2004; Wenseleers et al. 2005). Thus, even though
112 we believe that dominant females are the ones responsible to realize such a task, the likely involvement of
113 other females in egg screening behavior is not dismissible. The successful use of artificial cells to collect
114 eggs used here will allow more studies addressing questions related to policing behavior in *Polistine* wasps.

115 The hydrocarbons found covering the egg's surface likely work as the proximal cues that allow
116 discrimination of brood. The majority of the hydrocarbons covering the eggs originate from the *Dufour's*
117 gland (Ferreira et al. 2022), but secretions released by cephalic glands during the egg licking behavior may
118 also exist. Thus, whether parasitism is costly and frequent in *M. cerberus* nests, it is possible that
119 hydrocarbons of these glands may have been selected to carry information that match specifically species
120 and nest origin. Our results demonstrate the ability to recognize brood in *M. cerberus*. Future experiments
121 should also address whether the removal rates of eggs coming from *M. cassununga* and *M. consimilis* nests
122 are consistent with the data that we present here for *M. montei* and also, whether the position of cell or eggs
123 make a difference for removal rates.

124 In conclusion, the results of our work reinforce that in primitively eusocial wasps, nestmate recognition
125 skills are not limited to interaction established with adult individuals, but also brood (e.g. eggs).
126 Additionally, we conclude that hydrocarbons are likely the proximate cues that allow egg recognition to
127 occur in *M. cerberus*. Finally, we report that the process of egg recognition and hence removal may be
128 performed by the dominant females, since they are the ones that seem to be more engaged in checking cell
129 behavior.

130 **Competing Interests**

131 The authors have no relevant financial or non-financial interests to disclose.

132 **Acknowledgements**

133 This study was financially supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior –
134 Brasil (CAPES) – Finance Code 001 and grant 2018/22461-3 São Paulo Research Foundation (FAPESP)
135 to RCS. Funding was provided from Bilateral grant FWO-FAPESP to CAO, FSN and TW (process:
136 2018/10996-0 FAPESP and FWO: GOF8319N), Research Foundation Flanders to CAO (postdoctoral
137 fellowship FWO-12V6318N and research grant FWO-1513219N) and Conselho Nacional de
138 Desenvolvimento Científico e Tecnológico to FSN (307702/2018-9).

139 **Author contribution**

140 Conceptualization: RCS and CAO. Methodology: RCS. Formal analysis and investigation: RCS and CAO.
141 Writing original draft: RCS and CAO. Writing review and editing: all authors. Funding acquisition: all
142 authors.

143 **Data availability**

144 The data that supports the findings of this study are available in the supplementary material of this
145 manuscript.

146 **References**

- 147 Bagneres AG, Killian A, Clement JL, Lange C (1991) Interspecific recognition among termites of the genus
148 *Reticulitermes*: evidence for a role for the cuticular hydrocarbons. *J Chem Ecol* 17: 2397-2420.
149 <https://doi.org/10.1007/BF00994590>
- 150 Bates D, Mächler M, Bolker B, Walker S (2014) Fitting linear mixed-effects models using lme4. arXiv preprint
151 arXiv:1406.5823.
- 152 Bonckaert W, Drijfhout FP, d’Ettorre P, Billen J, Wenseleers T (2012) Hydrocarbon signatures of egg maternity,
153 caste membership and reproductive status in the common wasp. *J Chem Ecol* 38: 42-51.
154 <https://doi.org/10.1007/s10886-011-0055-9>
- 155 Bos N, d’Ettorre P (2012) Recognition of social identity in ants. *Front Psychol* 3: 83.
156 <https://doi.org/10.3389/fpsyg.2012.00083>
- 157 Bos N, Guerrieri FJ, d’Ettorre P (2010) Significance of chemical recognition cues is context dependent in ants.
158 *Anim Behav* 80: 839-844. <https://doi.org/10.1016/j.anbehav.2010.08.001>
- 159 Cappa F, Cini A, Signorotti L, Cervo R (2020). Rethinking recognition: social context in adult life rather than
160 early experience shapes recognition in a social wasp. *Philos Trans R Soc Lond B Biol Sci* 375: 20190468.
161 <https://doi.org/10.1098/rstb.2019.0468>
- 162 Carlson DA, Bernier UR, Sutton BD (1998) Nov. Elution patterns from capillary GC for methyl branched alkanes.
163 *J Chem Ecol* 24: 1845-1865. [https://doi: 10.1023/a:1022311701355](https://doi:10.1023/a:1022311701355).
- 164 Cervo R, Dani FR, Cotoneschi C, Scala C, Lotti I, Strassmann JE, Queller DC, Turillazzi S (2008) Why are larvae
165 of the social parasite wasp *Polistes sulcifer* not removed from the host nest?. *Behav Ecol Sociobiol* 62: 1319-1331.
166 <https://doi.org/10.1007/s00265-008-0560-1>
- 167 Cini A, Gioli L, Cervo R (2009) A quantitative threshold for nest-mate recognition in a paper social wasp. *Biol*
168 *Lett* 5: 459-461. <https://doi.org/10.1098/rsbl.2009.0140>
- 169 Clouse RM (1995) Nest usurpation and intercolonial cannibalism in *Mischocyttarus mexicanus* (Hymenoptera:
170 Vespidae). *J Kansas Entomol Soc* 67-73.

171 Cotoneschi C, Dani FR, Cervo R, Scala C, Strassmann JE, Queller DC, Turillazzi S (2009) *Polistes dominulus*
172 (Hymenoptera, Vespidae) larvae show different cuticular patterns according to their sex: workers seem not use
173 this chemical information. *Chem Senses* 34:195-202. <https://doi.org/10.1093/chemse/bjn079>

174 Cotoneschi C, Dani FR, Cervo R, Sledge MF, Turillazzi S (2007) *Polistes dominulus* (Hymenoptera: Vespidae)
175 larvae possess their own chemical signatures. *J Insect Physiol* 53: 954-963.
176 <https://doi.org/10.1016/j.jinsphys.2006.12.016>

177 da Silva RC, Assis DS, de Souza AR, Nascimento FS, Giannotti E (2020a) Notes on brood morphology and the
178 development of the Neotropical eusocial wasp *Mischocyttarus cerberus* (Hymenoptera, Vespidae, Polistinae).
179 *Sociobiology* 67: 301-307. <http://dx.doi.org/10.13102/sociobiology.v67i2.4562>

180 da Silva RC, Prato A, Oi CA, Turatti ICC, Do Nascimento FS (2020b) Dominance hierarchy, ovarian activity and
181 cuticular hydrocarbons in the primitively eusocial wasp *Mischocyttarus cerberus* (Vespidae, Polistinae,
182 *Mischocyttarini*). *J Chem Ecol* 46: 835-844. <https://doi.org/10.1007/s10886-020-01206-1>

183 da Silva RC, Silva AP, Assis DS, Nascimento FS (2019a) The first record of the parasitoid wasp *Pachysomoides*,
184 associated with *Mischocyttarus cerberus* nests and some insights about the parasitoid wasps behavior. *R Bras Zoo*
185 20: 1-7. <https://doi.org/10.34019/2596-3325.2019.v20.24794>

186 da Silva RC, da Silva AP, Assis DS, Nascimento FS (2019b) Occurrence and nesting behavior of social wasps in
187 an anthropized environment. *Sociobiology* 66: 381-388. <http://dx.doi.org/10.13102/sociobiology.v66i2.4303>

188 Dapporto L, Dani FR, Turillazzi S (2007) Social dominance molds cuticular and egg chemical blends in a paper
189 wasp. *Curr Biol* 17: R504-R505. <https://doi.org/10.1016/j.cub.2007.05.002>

190 De Souza AR, da Silva RC, Ferreira RP, Mateus S, Prato A, Oi CA, Noll FB, Lino-Neto J (2020) Larval sex
191 identification of in vivo and long-term ethanol-preserved social wasps. *Entomol Exp Appl* 168: 710-714.
192 <https://doi.org/10.1111/eea.12960>

193 De Souza AR, Silva NJ, Prezoto F (2012) A rare but successful reproductive tactic in a social wasp (Hymenoptera:
194 Vespidae): Use of heterospecific nests. *Rev Chil de Hist Nat* 85: 351-355.

195 d’Ettorre P, Lenoir A (2010) Nestmate recognition. *Ant ecology*, 194-209.

196 El-Sayed AM. 2016. The Pherobase: Database of insect pheromones and semiochemicals [Internet]. Available
197 from <http://www.pherobase.com>

198 Endler A, Liebig J, Schmitt T, Parker JE, Jones GR, Schreier P, Hölldobler B (2004) Surface hydrocarbons of
199 queen eggs regulate worker reproduction in a social insect. *PNAS* 101: 2945-2950.
200 <https://doi.org/10.1073/pnas.0308447101>

201 Fletcher DJ, Michener CD (1987) Kin recognition in animals. John Wiley & Sons.

202 Foster KR, Ratnieks FL (2001) Convergent evolution of worker policing by egg eating in the honeybee and
203 common wasp. *Proc Biol Sci* 268(1463), 169-174. <https://doi.org/10.1098/rspb.2000.1346>

204 Free JB, Winder ME (1983) Brood recognition by honeybee (*Apis mellifera*) workers. *Anim Behav* 31: 539-545.
205 [https://doi.org/10.1016/S0003-3472\(83\)80077-3](https://doi.org/10.1016/S0003-3472(83)80077-3)

206 Gamboa GJ (2004) Kin recognition in eusocial wasps. In *Annales Zoologici Fennici* (pp. 789-808). Finnish
207 Zoological and Botanical Publishing Board.

208 Gamboa GJ, Reeve HK, Ferguson ID, Wacker TL (1986a) Nestmate recognition in social wasps: the origin and
209 acquisition of recognition odours. *Anim Behav* 34: 685-695. [https://doi.org/10.1016/S0003-3472\(86\)80053-7](https://doi.org/10.1016/S0003-3472(86)80053-7)

210 Gamboa GJ, Reeve HK, Pfennig DW (1986b) The evolution and ontogeny of nestmate recognition in social wasps.
211 *Annu Rev Entomol* 31: 431-454.

212 Giannotti E (1999) Social organization of the eusocial wasp *Mischocyttarus cerberus styx* (Hymenoptera:
213 Vespidae). *Sociobiology* 33:325-338

214 Guerrieri FJ, d'Ettorre P (2010) Associative learning in ants: conditioning of the maxilla-labium extension
215 response in *Camponotus aethiops*. *J Insect Physiol* 56: 88-92. <https://doi.org/10.1016/j.jinsphys.2009.09.007>

216 Howard RW, Blomquist GJ (2005) Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annu*
217 *Rev Entomol* 50: 371-393. <https://doi.org/10.1146/annurev.ento.50.071803.130359>

218 Jones SM, van Zweden JS, Grüter C, Menezes C, Alves DA, Nunes-Silva P, Czaczkes T, Imperatriz-Fonseca VL,
219 Ratnieks, F. L. (2012). The role of wax and resin in the nestmate recognition system of a stingless bee,
220 *Tetragonisca angustula*. *Behav Ecol Sociobiol* 66: 1-12. <https://doi.org/10.1007/s00265-011-1246-7>

221 Klahn JE, Gamboa GJ (1983) Social wasps: discrimination between kin and nonkin brood. *Science* 22: 482-484.
222 <https://doi.org/10.1126/science.221.4609.482>

223 Kudô K, Oliveira LA, Mateus S, Zucchi R, Nascimento FS (2017) Nestmate larval discrimination by workers in
224 the swarm-founding wasp *Polybia paulista*. *Ethol Ecol Evol* 29: 170-180.
225 <https://doi.org/10.1080/03949370.2015.1129363>

226 Laloi D, Sandoz JC, Picard-Nizou AL, Marchesi A, Pouvreau A, Taséi JN, Poppy G, Pham-delègue MH (1999)
227 Olfactory conditioning of the proboscis extension in bumble bees. *Entomol Exp Appl* 90: 123-129.
228 <https://doi.org/10.1046/j.1570-7458.1999.00430.x>

229 Larsen J, Fouks B, Bos N, d'Ettorre P, Nehring V (2014) Variation in nestmate recognition ability among
230 polymorphic leaf-cutting ant workers. *J Insect Physiol* 70: 59-66. <https://doi.org/10.1016/j.jinsphys.2014.09.002>

231 LeBoeuf AC, Waridel P, Brent CS, Gonçalves AN, Menin L, Ortiz D, Riba-Grognuz O, Koto A, Soares ZG, Privman
232 E, Miska EA, Benton R, Keller, L. (2016). Oral transfer of chemical cues, growth proteins and hormones in social
233 insects. eLife 5: e20375. <https://doi.org/10.7554/eLife.20375>

234 Lenoir A, Hefetz A, Simon T, Soroker V (2001). Comparative dynamics of gestalt odour formation in two ant
235 species *Camponotus b fellah* and *Aphaenogaster senilis* (Hymenoptera: Formicidae). *Physiol Entomol* 26: 275-
236 283. <https://doi.org/10.1046/j.0307-6962.2001.00244.x>

237 Liebig J, Monnin T, Turillazzi S (2005) Direct assessment of queen quality and lack of worker suppression in a
238 paper wasp. *Proc R Soc B: Biol Sci* 272: 1339-1344. <https://doi.org/10.1098/rspb.2005.3073>

239 Linstrom PJ, Mallard WG. 2016. NIST chemistry webbook, NIST standard reference database number 69
240 [Internet]. Gaithersburg, MD: National Institute of Standards and Technology. Available from
241 <http://webbook.Nist.Gov>.

242 Lorenzi MC, Filippone F (2000) Opportunistic discrimination of alien eggs by social wasps (*Polistes biglumis*,
243 Hymenoptera Vespidae): a defense against social parasitism?. *Behav Ecol Sociobiol* 48: 402-40.
244 <https://doi.org/10.1007/s002650000251>

245 Masterman R, Smith BH, Spivak M (2000) Brood odor discrimination abilities in hygienic honey bees (*Apis*
246 *mellifera* L.) using proboscis extension reflex conditioning. *J Insect Behav* 13: 87-101.
247 <https://doi.org/10.1023/A:1007767626594>

248 Messadi D, Helaimia F, Ali-Mokhnache S, Boumahraz M (1990) Accurate determination of retention indices in
249 programmed temperature gas chromatography. *Chromatographia* 29:429-578. <https://doi: 10.1007/bf02261389>

250 Miller SE, Bluher SE, Bell E, Cini A, da Silva RC, de Souza AR, Gandia KM, Jandt J, Loope K, Prato A, Pruitt
251 JN, Rankin D, Rankin E, Southon RJ, Uy FMK, Weiner S, Wright CM, Downing H, Gadagkar R, Cristina Lorenzi
252 M, Rusina L, Sumner S, Tibbetts EA, Toth A, Sheehan MJ (2018) WASPnest: a worldwide assessment of social
253 Polistine nesting behavior. *Ecology* 99:2405. <https://doi.org/10.1002/ecy.2448>

254 Montagna TS, Neves ÉF, Antonialli-Junior WF (2012) First report of interspecific facultative social parasitism in
255 the paper wasp genus *Mischocyttarus* Saussure (Hymenoptera, Vespidae). *Rev Bras Entomol* 56: 263-265.
256 <https://doi.org/10.1590/S0085-56262012005000025>

257 Mora-Kepfer F (2014). Context-dependent acceptance of non-nestmates in a primitively eusocial insect. *Behav*
258 *Ecol Sociobiol* 68: 363-371. <https://doi.org/10.1007/s00265-013-1650-2>

259 Murakami AS, Shima SN, Desuó IC (2009) More than one inseminated female in colonies of the independent-
260 founding wasp *Mischocyttarus cassununga* von Ihering (Hymenoptera, Vespidae). Rev Bras Entomol 53: 653-
261 662. <https://doi.org/10.1590/S0085-56262009000400017>

262 Nascimento DL, Nascimento FS (2012) Acceptance threshold hypothesis is supported by chemical similarity of
263 cuticular hydrocarbons in a stingless bee, *Melipona asilvai*. J Chem Ecol 38: 1432-1440.
264 <https://doi.org/10.1007/s10886-012-0194-7>

265 Noda SCM, Silva ERD, Giannotti E (2001) Dominance hierarchy in different stages of development in colonies
266 of the primitively eusocial wasp *Mischocyttarus cerberus styx* (Hymenoptera, Vespidae). Sociobiology 38:603-
267 614.

268 Noll FB, da Silva M, Soleman RA, Lopes RB, Grandinete YC, Almeida EA, Wenzel JW, Carpenter JM (2020)
269 Marimbondos: systematics, biogeography, and evolution of social behavior of neotropical swarm-founding wasps
270 (Hymenoptera: Vespidae: Epiponini). Cladistics. <https://doi.org/10.1111/cla.12446>

271 Nunes TM, Mateus S, Turatti IC, Morgan ED, Zucchi R (2011) Nestmate recognition in the stingless bee
272 *Frieseomelitta varia* (Hymenoptera, Apidae, Meliponini): sources of chemical signals. Anim Behav 81: 463-467.
273 <https://doi.org/10.1016/j.anbehav.2010.11.020>

274 Nunes TM, Nascimento FS, Turatti IC, Lopes NP, Zucchi R (2008) Nestmate recognition in a stingless bee: does
275 the similarity of chemical cues determine guard acceptance?. Anim Behav 75: 1165-1171.
276 <https://doi.org/10.1016/j.anbehav.2007.08.028>

277 Oi CA, Brown RL, da Silva RC, Wenseleers T (2020) Reproduction and signals regulating worker policing under
278 identical hormonal control in social wasps. Sci Rep 10: 1-10. <https://doi.org/10.1038/s41598-020-76084-4>

279 Oi CA, Wenseleers T, Oliveira RC (2021) Causes and consequences of reproductive conflicts in wasp societies.
280 In *Neotropical Social Wasps* (pp. 147-178). Springer, Cham.

281 Oi CA, Van Oystaeyen A, Oliveira RC, Millar JG, Verstrepen KJ, van Zweden JS, Wenseleers T (2015) Dual
282 effect of wasp queen pheromone in regulating insect sociality. Curr Biol 25: 1638-1640.
283 <https://doi.org/10.1016/j.cub.2015.04.040>

284 Oliveira VC, Desuó IC, Murakami ASN, Shima SN (2012) Dominance and Subordination Interactions Among
285 Nestmates in Pre and Post-Emergence Phases of the Basal Eusocial Wasp *Mischocyttarus* (Monogynoecus) *montei*
286 (Hymenoptera, Vespidae). Sociobiology 999-1013. <https://doi.org/10.13102/sociobiology.v59i3.562>

287 Oliveira VC 2007 Diferenciação etológica e morfofisiológica das castas de *Mischocyttarus* (Monogynoecus)
288 *montei*, Zikán 1903 (Hymenoptera, Vespidae, Mischocyttarini), com especial referência à regulação social das
289 colônias. Master thesis, UNESP—Campus de Rio Claro; Rio Claro; 159 pp.

290 Orlova M, Starkey J, Amsalem E (2020) A small family business: synergistic and additive effects of the queen and
291 the brood on worker reproduction in a primitively eusocial bee. *J Exp Biol* 223: jeb217547
292 <https://doi.org/10.1242/jeb.217547>

293 Panek LM, Gamboa GJ (2000) Queens of the paper wasp *Polistes fuscatus* (Hymenoptera: Vespidae) discriminate
294 among larvae on the basis of relatedness. *Ethology* 106: 159-170. [https://doi.org/10.1046/j.1439-](https://doi.org/10.1046/j.1439-0310.2000.00502.x)
295 [0310.2000.00502.x](https://doi.org/10.1046/j.1439-0310.2000.00502.x)

296 Pinto NPO, Gobbi N, Noll FB, Penna MAH, Nazareth SL (2004) Coexistência interespecífica em *Mischocyttarus*
297 Saussure (Hymenoptera: Vespidae, Mischocyttarini) durante a fase inicial de desenvolvimento de um ninho. *Ver*
298 *Etol* 6: 95-100.

299 Prezoto F, Nascimento FS (1999) Nota sobre a ocorrência de usurpação de um ninho de *Mischocyttarus*
300 *cassununga* por *Polistes versicolor* (Hymenoptera, Vespidae). *Rev Etol* 1: 69-71.

301 R Core Team. (2018). R: a language and environment for statistical computing. Vienna: R Foundation for
302 Statistical Computing. Available at: <http://www.R-project.org>

303 Riveros AJ, Gronenberg W (2009) Olfactory learning and memory in the bumblebee *Bombus occidentalis*.
304 *Naturwissenschaften* 96: 851-856. <https://doi.org/10.1007/s00114-009-0532-y>

305 Schultner E, Pulliainen U (2020) Brood recognition and discrimination in ants. *Insectes Soc* 67: 11-34.
306 <https://doi.org/10.1007/s00040-019-00747-3>

307 Singmann H, Bolker B, Westfall J, Aust F, Ben-Shachar MS (2015) afex: Analysis of factorial experiments. R
308 package version 0.13–145.

309 Somavilla A, Schoeninger K, Carvalho AF, Menezes RS, Del Lama MA, Costa MA, Oliveira ML (2015) Record
310 of Parasitoids in nests of social wasps (Hymenoptera: Vespidae: Polistinae). *Sociobiology* 62: 92-98.
311 <https://doi.org/10.13102/sociobiology.v62i1.92-98>

312 Starkey J, Brown A, Amsalem E (2019) The road to sociality: brood regulation of worker reproduction in the
313 simple eusocial bee *Bombus impatiens*. *Anim Behav* 154: 57-65. <https://doi.org/10.1016/j.anbehav.2019.06.004>

314 Strassmann JE, Seppä P, Queller DC (2000) Absence of within-colony kin discrimination: foundresses of the social
315 wasp, *Polistes carolina*, do not prefer their own larvae. *Naturwissenschaften* 87: 266-269.
316 <https://doi.org/10.1007/s001140050718>

317 Tannure-Nascimento IC, Nascimento FS, Dantas JO, Zucchi R (2009) Decision rules for egg recognition are
318 related to functional roles and chemical cues in the queenless ant *Dinoponera quadriceps*. *Naturwissenschaften*,
319 96: 857-861. <http://doi.org/10.1007/s00114-009-0535-8>

320 Team RC, Team MRC, Suggests MASS, Matrix S (2013) Package “Stats.”. *RA Lang. Environment Stat. Comput.*
321 Vienna, Austria: R Foundation for Statistical Computing. Turillazzi S, Fanelli D, Theodora P, Lambardi D, Ortolani
322 I, Hashim R, Baracchi D (2008) Determinants of immature brood and nest recognition in a stenogastrine wasp
323 (Hymenoptera Vespidae). *Ethol Ecol Evol* 20: 17-33. <https://doi.org/10.1080/08927014.2008.9522540>

324 van Zweden JS, d’Ettorre P (2010) Nestmate recognition in social insects and the role of hydrocarbons. *Insect*
325 *hydrocarbons: biology, biochemistry and chemical ecology*, 11, 222-243.

326 Wenseleers T, Princen S, Oliveira RC, Oi CA (2020) Conflicts of interest within colonies.
327 https://doi.org/10.1007/978-3-319-90306-4_28-1

328 Wenseleers T, Oi CA, Caliani Oliveira R (2020) Worker Policing.

329 Wenseleers T, Tofilski A, Ratnieks FL (2005) Queen and worker policing in the tree wasp *Dolichovespula*
330 *sylvestris*. *Behav Ecol Sociobiol* 58: 80-86. <https://doi.org/10.1007/s00265-004-0892-4>

331 Wenseleers T, Helanterä H, Hart A, Ratnieks FL (2004) Worker reproduction and policing in insect societies: an
332 ESS analysis. *J Evol Biol* 17: 1035-1047. <https://doi.org/10.1111/j.1420-9101.2004.00751.x>

333 Wright CM, McEwen BL, Fisher DN, Lichtenstein JL, Antoun A, Tibbetts EA, Pruitt JN (2019) Egg
334 discrimination is mediated by individual differences in queen olfactory responsiveness and boldness. *Behav Ecol*
335 30: 1306-1313. <https://doi.org/10.1093/beheco/arz078>

336 Yusuf AA, Pirk CW, Crewe RM, Njagi PG, Gordon I, Torto B (2010) Nestmate recognition and the role of
337 cuticular hydrocarbons in the African termite raiding ant *Pachycondyla analis*. *J Chem Ecol* 36: 441-448.
338 <https://doi.org/10.1007/s10886-010-9774-6>

339