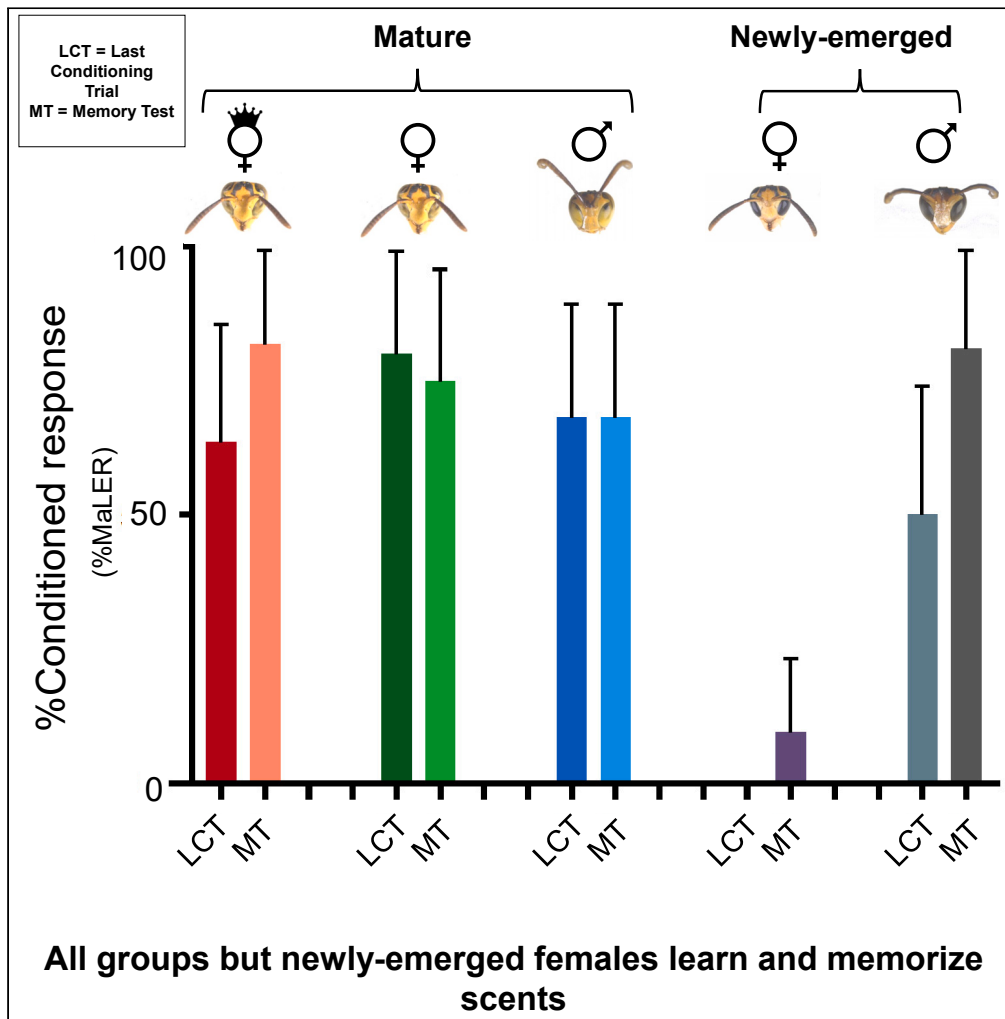


Article

Sex and lifestyle dictate learning performance in a neotropical wasp



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Highlights

A Pavlovian laboratory protocol was set to study olfactory cognition in wasps

The neotropical wasp *Mischocyttarus cerberus* learns and memorizes rewarded odorants

Learning and memory are affected by sex and age in accordance with lifestyle

In castes with efficient learning, performance is independent of the odor trained

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Article

Sex and lifestyle dictate learning performance in a neotropical wasp

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SUMMARY

In contrast to extensive investigations on bee cognition, the cognitive capacities of wasps remain largely unexplored despite their key role as pollinators and predators of insect pests. Here we studied learning and memory in the neotropical wasp *Mischocyttarus cerberus* using a Pavlovian conditioning in which harnessed wasps respond with conditioned movements of their mouthparts to a learned odorant. We focused on the different castes, sexes, and ages coexisting within a nest and found that adults of *M. cerberus* learned and memorized efficiently the odor-sugar associations. In contrast, newly emerged females, but not males, were unable to learn odorants. This difference concurs with their different lifestyle as young males perform regular excursions outside the nest while young females remain in it until older age. Our results thus highlight the importance of socio-ecological constraints on wasp cognition and set the basis for mechanistic studies on learning differences across ages and castes.

INTRODUCTION

Insects are valuable models for the study of cognitive phenomena because some species are efficient learners and provide simultaneous access to the neural and molecular underpinnings of these performances.¹ The fact that insects possess small brains, with a reduced number of neurons, does not represent a limitation for learning multiple associations and solving multiple problems, some of them being highly sophisticated.^{2,3} Research on insect cognition has mainly focused on simple forms of associative learning as they are a necessary first step for further explorations of higher-order learning phenomena. Thus, various Pavlovian and operant protocols using different forms of reward^{4–7} or punishment^{8–12} have been conceived to study the insects' capacity to acquire and memorize new information.¹ Yet, the number of species for which accurate characterizations of learning and memory are available is relatively small. This limitation is due to the double difficulty of 1) developing appropriate laboratory protocols to study these phenomena under controlled conditions and 2) establishing appropriate raising methods ensuring regular supply of individuals for the experiments. Insects such as the fruit fly *Drosophila melanogaster*,^{9,13,14} the honeybee *Apis mellifera*⁶ (Menzel, 1999), the cricket *Gryllus bimaculatus*,⁴ and some bumble bee^{15–17} and ant species¹⁸ fulfill these conditions and are among the species most studied. Yet, more efforts are needed to increase the number and diversity of species studied to improve comparative and evolutionary analyses of learning and memory.

Among social insects, the champion role in the field of investigations on learning and memory goes back to the honeybee *Apis mellifera*.^{2,5} This is due, to a large extent, to the establishment in the sixties of a Pavlovian olfactory conditioning protocol,¹⁹ which was improved later^{20,21} and used in numerous research domains to quantify learning, memory, and odor perception in honeybees, from the behavioral to the molecular levels.²² In this improved protocol, termed the olfactory conditioning of the proboscis extension response (PER), harnessed hungry bees exhibit the appetitive PER upon contact of sucrose solution (the unconditioned stimulus (US) or US) with their antennal taste receptors. Pairing a neutral odorant (the conditioned stimulus or CS) with antennal sucrose stimulation results in learning the CS-US association, so that successful bees show PER to the odorant that anticipates the sucrose reward.^{19,20,22} This protocol has been adapted to insect species that lack a proboscis such as ants and wasps.^{18,23–26} In this case, the conditioned response was an extension of the maxilla-labium mouthparts (the so-called MaLER¹⁸ in response to an odorant previously paired with sucrose solution [Video S1].^{18,24,25,27}

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Compared to bees and ants, less is known about the learning and memory capacities of wasps. Filling this void is important, given the key environmental role of wasps, both as pollinators and as predators of insect pests.²⁸ Characterizing their cognitive capacities is relevant not only for implementing applications beneficial to humans but also for conservation purposes, given the observed decline of natural wasp populations in the past years.^{29,30} Here, we focused on a neotropical primitively eusocial wasp, *Mischocyttarus cerberus*, in which females belonging to different castes do not differ morphologically but behaviorally and chemically.^{31,32} Newly emerged individuals of this species have black eyes that become green through a process of depigmentation after about a week of life.³³ Males are easily distinguished from females because they have an additional bent antennomere and because of their lighter colored faces.³³ During the first week, newly emerged females remain mostly immobile in the nest without performing any specific task behavior. By the second week, they start leaving the nest and foraging for resources.³¹ On the contrary, while newly emerged males remain in the nest for approximately 7–10 days,^{31,34} they tend to perform regular excursions to search for mating partners, leaving afterward the nest for once and start to forage for themselves and possibly continue to look for female partners.^{31,34} This behavior is well studied for some *Mischocyttarus* species, where newly emerged males leave the nests earlier than females, after around 4 to 9 days of emergence.^{35–37} Considering that *M. cerberus* experiences differ according to their age, caste, and sex, we studied whether individuals belonging to these groups differ in their learning and memory skills. By using a Pavlovian conditioning protocol adapted from the PER protocol of honeybees, we tested the ability of wasps to learn and memorize odors. We hypothesized that older individuals would learn and memorize odors more successfully than younger ones, irrespective of their sex, because of cumulative foraging experience and neural maturation.³⁸ Our results show remarkable learning and memory capacities in the different categories of *M. cerberus* studied except in newly emerged females, in contrast to newly emerged males. We proposed explanations for this difference and future research avenues for the study of wasp cognition with controlled conditions.

RESULTS

Experiment 1—Olfactory learning of female adult foragers

We first studied the learning and memory capacities of foragers (green-eyed females returning from the field with resources; see inset [Figure 1](#)) of *M. cerberus* obtained from nests located in the area of the University of São Paulo (University of São Paulo (USP)) – Campus of Ribeirão Preto (see [Figure 4](#) for the experimental setup). We focused on these individuals given their extensive foraging experience in the field. We hypothesized that learning and memory should be present in these animals as an important component of their foraging activities. Collected wasps were individually harnessed within Eppendorf plastic tubes from which only the head protruded. Wasps were kept in a dark, climate room during 2 h before the start of the experiments, a period that ensured high appetitive motivation required for appetitive learning.²¹ In honeybees, for instance, sucrose responsiveness is higher after this period and dopamine levels in the brain are increased in accordance with an enhanced appetitive motivation.³⁹

Wasps were conditioned using an absolute conditioning regime in which a single odorant (the CS), linalool, was delivered six times (6 conditioning trials) by means of an automated odor-releasing machine controlled by a microcomputer. In the *paired group* ($n = 22$), each CS stimulation was paired with a 50% (v/v) sucrose solution (the US) delivered to the antennae and mouthparts. In the *unpaired group* ($n = 20$), CS and US stimuli were delivered in different trials following a pseudorandom sequence. We trained wasps with six conditioning trials as in other social Hymenoptera (ants, bees) robust olfactory learning and long-term memories are obtained after various spaced trials. In honeybees, for instance, three- to five-spaced trials (i.e., separated by more than 5 min) are typically used to generate robust olfactory learning and long-term memories.^{6,21,22} We thus reasoned that six trials would suffice to the same end if our wasps were capable of olfactory learning in our experimental conditions.

[Figure 1A](#) shows the population responses of the paired and the unpaired groups. The paired group learned the association between the CS and the US (ANOVA – $\chi^2 = 21.656$, $df = 1$, $p < 0.001$) and exhibited, therefore, an increase of conditioned maxilla-labium extension response (MaLER) to linalool. On the contrary, the unpaired group did not exhibit changes in the proportion of MaLER across trials ($\chi^2 = 0.232$, $df = 1$, $p = 0.629$), which remained low. As a consequence, the learning curves of the paired and the unpaired groups differed significantly ($\chi^2 = 20.358$, $df = 1$, $p < 0.001$). This between-group difference shows that restrained forager wasps can learn rewarded odorants under harnessing conditions and that this learning is truly associative given the absence of conditioning in the *unpaired group*.

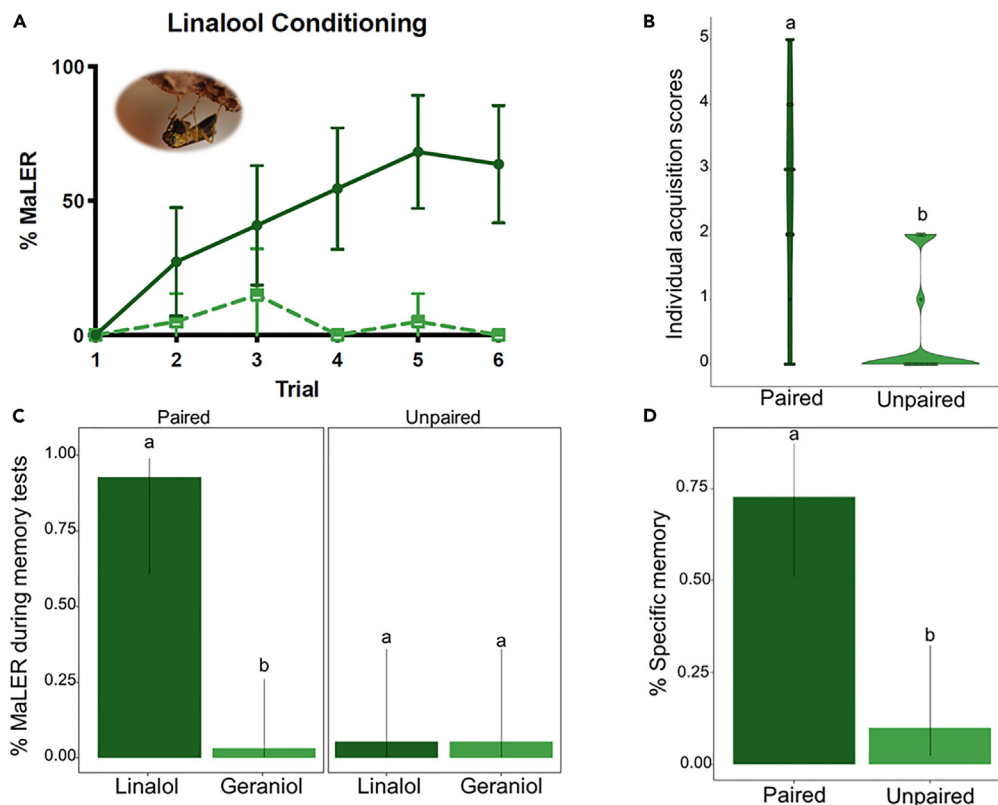


Figure 1. Olfactory learning and memory in female adult foragers of *Mischocyttarus cerberus*

(A) Population acquisition performances of a paired ($n = 22$) and an unpaired group ($n = 20$) of wasps conditioned with linalool as the CS during 6 trials. The graph shows the % of wasps exhibiting MaLER to the conditioned odorant (CS). For each percentage, the 95% confidence interval is shown.

(B) Individual acquisition scores of wasps of the paired and unpaired group. The graph shows the average number of correct responses for individuals of the groups paired and unpaired in the last 5 conditioning trials.

(C) Population retention performances of both groups of wasps 1 h after conditioning. The graph shows the % of wasps exhibiting MaLER to the CS (linalool) and to a novel odorant (Nod: geraniol). In each case, the 95% confidence interval is shown. Only wasps of the paired group exhibited significant memory retention.

(D) Percentage of wasps expressing specific memory in the retention test (i.e., wasps responding to the CS and not to the NOd) in the paired and the unpaired group. In each case, the 95% confidence interval is shown. In panels B), C), and D), different letters above bars indicate significant differences ($p < 0.05$).

Population responses in the form of percentage of individual responding per conditioning trial may hide individual learning dynamics in the case of a binomial response (MaLER or no MaLER).⁴⁰ Thus, to verify the previous conclusions, we quantified individual learning scores as the number of conditioned responses during the last 5 conditioning trials. The scores varied therefore between 0 (no learning) and 5 (maximal learning). The first trial was excluded as the wasp response may be random upon the first odor presentation. Figure 1B shows that the learning scores of the paired and the unpaired group differed ($\chi^2 = 21.437$, $df = 1$, $p < 0.001$) as the majority of scores of the paired group were higher than 1 while they were mostly 0 for the unpaired group.

Memory retention was assessed 1 h after conditioning by presenting to each conditioned wasp the CS (linalool) and a new odor (NOd; geraniol), which allowed to determine the specificity of retention. Both odorants were delivered in a sequence that varied from wasp to wasp and in the absence of reward (extinction conditions). Figure 1C shows that while wasps of the *paired* group responded significantly more to the CS than to the NOd (odds.ratio = 387.195; $p = 0.013$; Figure 1), wasps of the *unpaired* group did practically not respond to either odorant (odds.ratio = 1.000; $p = 1.000$), consistently with the learning performances observed during training (Figures 1A and 1B). In consequence, a significant difference was found between groups ($\chi^2 = 7.559$; $df = 1$; $p = 0.005$) and odorants ($\chi^2 = 9.140$; $df = 1$; $p = 0.002$), as well as a significant

interaction group x odorant ($\chi^2 = 6.537$; $df = 1$; $p = 0.010$). These results indicate the presence of a robust, specific olfactory memory 1 h after conditioning in female foragers. To focus on individual retention performances, we quantified for each group the proportion of wasps with perfect memory (i.e., responding to the CS and not to the NOd). This parameter shows the specificity of the memories established in the groups under study^{21,41} and is termed CS-specific memory. Figure 1D shows that a large % of wasps of the paired group (72.72%) established a CS-specific memory contrary to wasps of the unpaired group ($\chi^2 = 18.579$, $df = 1$, $p < 0.001$), which was expected as the unpaired group was unable to learn and thus to form memories.

Experiment 2—Comparing learning and memory retention across wasp classes

Having established the conditioning procedure for harnessed forager wasps, we aimed at determining if distinct groups differing in age, sex, and habits learned and retained memory similarly. We established five groups of at least 20 wasps each, which were trained using the same absolute conditioning protocol employed above: 1) foragers (adult females, as the positive control group) ($n = 22$), 2) queens ($n = 20$), 3) newly emerged females ($n = 22$), 4) newly emerged males ($n = 22$) and 5) adult males ($n = 21$). As the associative nature of learning had already been shown in the previous experiments, *unpaired groups* were not included in this section.

Figure 2A shows the group responses of these five wasp categories during the 6 conditioning trials. Four of the five groups learned efficiently the association between linalool and sucrose reward (Factor Trial; queens: $\chi^2 = 13.1436$, $Df = 5$, $p = 0.022$; foragers: $\chi^2 = 19.611$, $df = 5$, $p = 0.001$; newly emerged males: $\chi^2 = 20.649$, $df = 5$, $p < 0.001$; and males: $\chi^2 = 19.368$, $Df = 5$, $p = 0.001$) and did not differ in their MaLER performances (Factor Group: $\chi^2 = 2.708$, $df = 3$, $p = 0.438$). Remarkably, the newly emerged females, which did not differ in age from the newly emerged males, did not show any sign of learning across conditioning trials (Factor Trial: $\chi^2 = -3.552$, $df = 1$, $p = 1.000$). This result shows a clear sex-dependent cognitive difference between newly emerged individuals. In addition, the fact that newly emerged males learned as well as experienced foragers, queens, and adult males shows that learning success is not primarily constrained by age. As in the previous experiment, we quantified the individual learning scores of the five wasp categories. Figure 2B confirmed that all groups but the newly emerged females had learning scores higher than 1 while the majority of newly emerged females had a score of 0 ($\chi^2 = 49.352$, $df = 4$, $p < 0.001$).

Similar conclusions were obtained after performing the memory retention test 1 h after conditioning (Figure 2C). Retention was robust and specific in three of the four categories that learned the CS-US association (foragers: odds.ratio = 45.000; $p = 0.001$; newly emerged males: odds.ratio = 21.533; $p = 0.005$; and males: odds.ratio = 10.625; $p = 0.043$) while responses to both the CS and the NOd were low and similar in the case of the newly emerged females, which had not learned to respond to the rewarded odorant (odds.ratio = 2.100, $p = 0.999$). The case of queens was intermediate as they clearly remembered the learned CS but their level of responses to the NOd was slightly higher than that of the other groups, thus leading to a non-significant differentiation between CS and NOd despite the different response levels induced by these stimuli (odds.ratio = 9.000; $p = 0.078$). Overall, the ANOVA test revealed significant Group ($\chi^2 = 35.977$; $df = 4$; $p < 0.001$) and Odorant effects ($\chi^2 = 26.367$; $df = 1$; $p < 0.001$). Lastly, we estimated the proportion of wasps expressing specific memory (Figure 2D). We found a significant difference between the five wasp categories ($\chi^2 = 31.174$, $df = 4$, $p < 0.001$) as the majority of individuals belonging to the four categories that learned demonstrated specific memory (Figure 2D) with the exception of newly emerged females. Interestingly, although the percentages of wasps with specific memory did not differ between the four categories that learned the CS-US association, queens tended to have a lower % of individuals with specific memory (50%). This indicates that queens learned the CS odorant in association with sucrose reward but failed to demonstrate CS vs. NOd differentiation in the population responses due to a higher tendency to generalize to the NOd.

Experiment 3—Learning and memory retention of a neutral odorant

In the previous experiments, the CS used was linalool, a floral scent that elicits spontaneous appetitive responses and attraction in other social insects such as honeybees⁴² and that is associated with enhanced neural and behavioral sensitivity in many pollinator species.⁴³ Although we took care of discarding spontaneous linalool responders at the beginning of conditioning (see STAR Methods), performances recorded may have been enhanced artificially by this appetitive odorant. To control for this possibility, and confirm

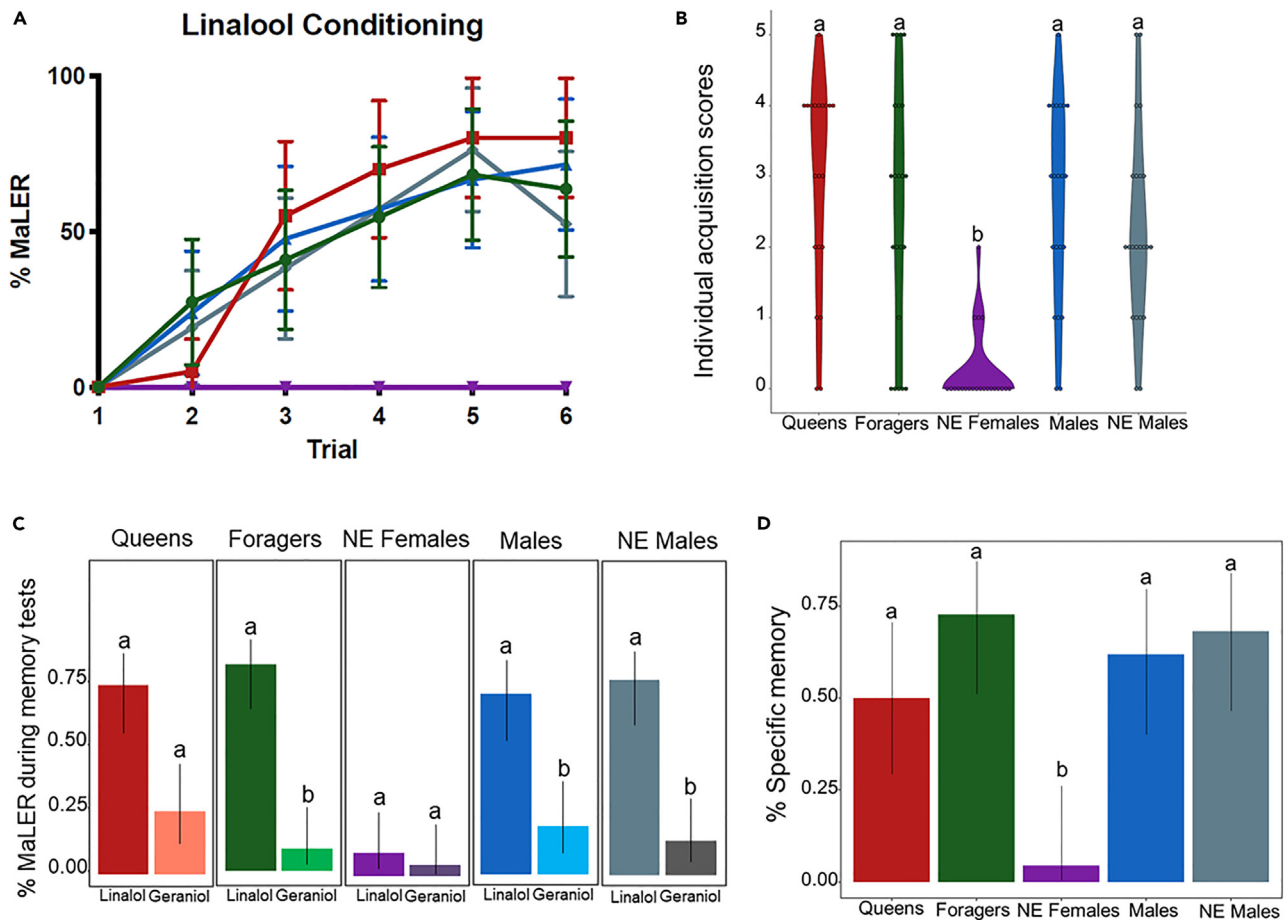


Figure 2. Learning and memory in five groups of wasps *Mischocyttarus cerberus* differing in sex, age and habits: queens (n = 20), newly emerged (NE) females (n = 22), adult females (i.e., foragers) (n = 22), NE males (n = 22), and adult males (n = 21)

(A) Population acquisition performances of the five groups of wasps conditioned with linalool as the CS during 6 trials. The graph shows the % of wasps exhibiting MaLER to the conditioned odorant (CS). For each percentage, the 95% confidence interval is shown.

(B) Individual acquisition of wasps of the five conditioned groups. The graph shows the average number of correct responses for individuals of the 5 conditioned groups in the last 5 conditioning trials.

(C) Population retention performances of the five groups of wasps 1 h after conditioning. Retention responses shown are the % of wasps exhibiting MaLER to the CS (linalool) and to a novel odorant (Nod: geraniol). In each case, the 95% confidence interval is shown. All groups, except that of the NE females exhibited significant retention. Queens generalized more than the other groups their response to the NOd thus leading to non-significant differentiation between CS and NOd in the memory test.

(D) Percentage of wasps expressing specific memory in the retention test (i.e., wasps responding to the CS and not to the NOd). In each case, the 95% confidence interval is shown. NE = newly emerged. In panels B), C) and D), different letters above bars indicate significant differences ($p < 0.05$).

that irrespective of the odorant used, the performances observed in the previous experiments are reproducible, we trained *M. cerberus* foragers (n = 20), newly emerged females (n = 22), and newly emerged males (n = 20) using the same protocol as before but with nonanal as the CS. This aldehyde is neutral for these wasps as shown by the total absence of spontaneous responses in all three wasp categories upon its first presentation. Queens and males were not used because they were not abundant in the study area at the time of the experiment.

Figure 3A shows the learning performances of the three groups of wasps studied. As shown in the previous experiment, significant differences were found between groups as foragers and newly emerged males learned efficiently the CS-US association during the six conditioning trials (Factor Trial; foragers: $\chi^2 = 21.805$, $df = 5$, $p < 0.001$; newly emerged males: $\chi^2 = 17.904$, $df = 5$, $p = 0.003$). As in the previous experiment, the responses of foragers and newly emerged males did not differ significantly (Factor Group; $\chi^2 = 1.439$, $df = 1$, $p = 0.230$). The newly emerged females failed again to learn the association

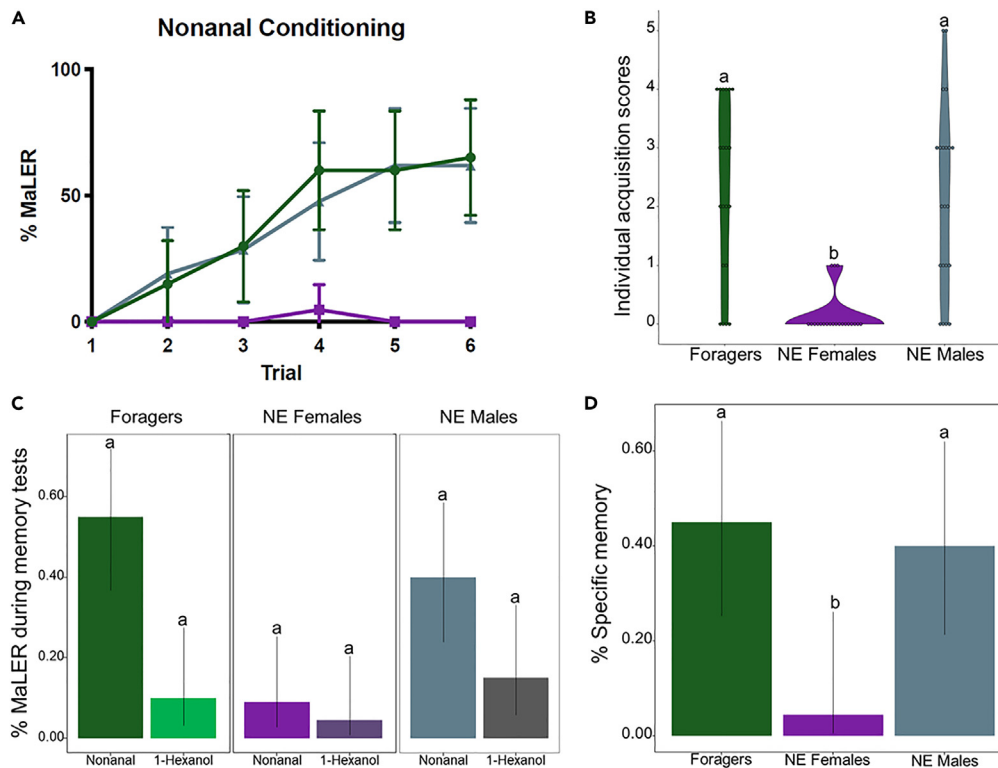


Figure 3. Learning and memory in three groups of wasps *Mischocyttarus cerberus* differing in sex and age and trained with a neutral odorant: NE females (n = 22), adult females (i.e., foragers) (n = 20) and NE males (n = 20)

(A) Population acquisition performances of the three groups of wasps conditioned with nonanal as the CS during 6 trials. The graph shows the % of wasps exhibiting MaLER to the conditioned odorant (CS). For each percentage, the 95% confidence interval is shown.

(B) Individual acquisition scores of wasps of the three conditioned groups. The graph shows the average number of correct responses for individuals of the three groups in the last 5 conditioning trials.

(C) Population retention performances of the three groups of wasps 1 h after conditioning. Retention responses shown are the % of wasps exhibiting MaLER to the CS (nonanal) and to a novel odorant (Nod: 1-hexanol). In each case, the 95% confidence interval is shown. Foragers and NE males but not NE females responded more to the CS than to the NOD yet without reaching significant differentiation.

(D) Percentage of wasps expressing specific memory in the retention test (i.e., wasps responding to the CS and not to the NOD). In each case, the 95% confidence interval is shown. Foragers and NE males had significantly more individuals with specific memory than NE females. NE = newly emerged. In panels (B), (C), and (D), different letters above bars indicate significant differences ($p < 0.05$).

(Factor Trial - $\chi^2 = 0.000$, $df = 1$, $p = 0.992$). This result confirms the incapacity of newly emerged females to learn odorants in an appetitive associative context, which is independent of the odorant used. On the other hand, the learning curves of foragers and newly emerged males reached levels that were similar to those observed in the prior experiments in which linalool was used as CS, thus excluding an enhancing effect of this natural olfactory attractant on previous performances (foragers, Exp 2 vs. Exp 3: - $\chi^2 = 0.1344$, $df = 1$, $p = 0.7139$; newly emerged males, Exp 2 vs. Exp 3: - $\chi^2 = 0.026$, $df = 1$, $p = 0.8712$). As reported in Experiment 2, the individual learning scores (Figure 3B) of foragers and newly emerged males were consistently higher than 1, whereas those of newly emerged females were 0 in practically all cases. In consequence, the learning scores varied significantly between the three groups of wasps ($\chi^2 = 36.977$, $df = 2$, $p < 0.001$).

One hour after conditioning, the three groups were tested for memory retention using the CS and a different neutral odorant, 1-hexanol, as NOD (Figure 3C). Foragers and newly emerged males exhibited higher responding to the CS than to the NOD yet without reaching significance (foragers: odds.ratio = 0.090, $p = 0.064$; newly emerged males: odds.ratio = 0.264, $p = 0.521$) while newly emerged females did neither respond to the CS nor to the NOD (odds.ratio = 0.476, $p = 0.991$). These results indicate that although foragers and newly emerged males were able to learn the CS-US association during the

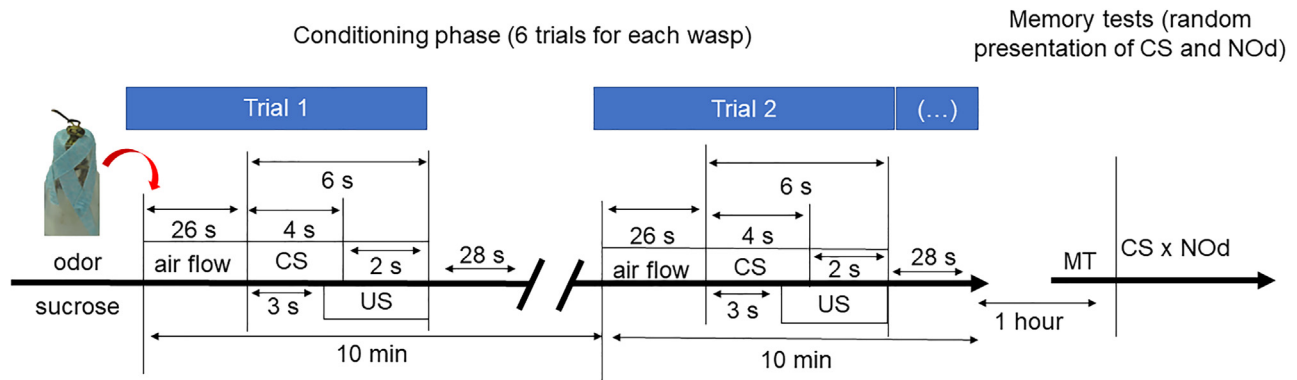


Figure 4. Olfactory conditioning procedure

Two consecutive conditioning trials are shown as example, followed by the retention tests. Each trial lasted 1 min and consisted of a familiarization phase before (26 s) and after stimulation (28 s) with the conditioned odorant (CS, 4 s) and sucrose solution as the US (3s). The interstimulus interval was 3s. In the retention test, the CS and a novel odorant (NOd) were presented one after the other in the absence of US. Their sequence of presentation was randomized from wasp to wasp.

conditioning phase, they were less specific in the memory tests as they generalized more their conditioned response to the NOd. This conclusion was confirmed by the analysis of individuals with specific memory (Figure 3D), which showed that the categories of foragers and newly emerged males included individuals that exhibited specific memory while newly emerged females did not ($\chi^2 = 12.121$, $df = 2$, $p < 0.01$). Thus, the absence of differentiation evinced by population responses (Figure 3C) was due to the presence of individuals that generalized to the NOd in the two categories that learned rather than to an absence of memory. We conducted an ANOVA followed by posthoc Tukey tests, and we found that generalization levels between wasps in this experiment and in the previous Experiment 2 did not differ significantly ($p > 0.05$ for all comparisons).

Unconditioned stimulus responsiveness across experiments

The absence of learning in newly emerged females was not due to an absence of sucrose sensitivity as shown by the analysis of sucrose responsiveness across experiments 2 and 3 where this category was included. In experiment 2, we excluded five individuals because they did not respond consistently to US (1 queen, 1 male, 1 newly emerged male and 2 newly emerged females). In experiment 3, we excluded three individuals (2 newly emerged females and 1 forager). In all experiments, all individuals that were conditioned responded to the US (100%). Thus, the absence of learning in newly emerged bees cannot be ascribed to differences in US responsiveness.

DISCUSSION

Our work studied the cognitive skills of *M. cerberus* wasps using an adaptation of the well-established protocol of olfactory PER conditioning available for honeybees.^{20,21} In the absence of proboscis, we focused on the MaLER, which is an appetitive response exhibited by these wasps upon antennal stimulation with sucrose solution.¹⁸ We showed that foragers (adult females), adult males, newly emerged males, and queens are able to learn and memorize efficiently olfactory stimuli paired with a reward of sucrose solution, irrespective of the nature of the odorant used as conditioned stimulus. On the contrary, newly emerged females were unable to learn appetitive associations between an odorant and sucrose reward, thus revealing a sex and age dependent effect on learning and memory in these wasps.

Our analyses integrated the traditional population responses (% of individuals responding to the conditioned stimulus) that are characteristic of learning studies and individual-based analyses in terms of learning scores and individuals with specific memory. This dual analysis is important in the case of binary responses such as MaLER or PER as the progressive variation described by learning curves is only apparent and does not reflect the dynamic of learning.^{40,44} For instance, while learning curves based on percentages of individuals responding to the CS increase gradually, the response of each single individual is either 0 (no response) or 1 (CS response). In our experiments, although population retention performances were unable to detect differentiation between the CS and the NOd used to assess memory specificity,

individual-based analyses of specific memory revealed that a large percentage of wasps had indeed established a specific memory and that the absence of significance at the population level was due to the presence of individuals with a tendency to generalize their conditioned response to the NOd. This was, for instance, the case of queens conditioned with linalool (Figures 2C and 2D) and of foragers and newly emerged males conditioned with the neutral odorant nonanal (Figures 3C and 3D). The case of the newly emerged males trained with nonanal (Figures 3C and 3D) clearly shows the importance of using analyses based on individuals with specific memory (i.e., wasps that responded correctly both to the CS and to the Nod in the retention test). While the population analysis of retention performances (Figure 3C) was unable to detect significant differences in the responses of these wasps to the conditioned nonanal and the Nod 1-hexanol despite a tendency toward differentiation, the critical variable of the percentage of individuals with specific memory revealed that the newly emerged males were as efficient as the foragers in forming olfactory memories (Figure 3D).

In the case of linalool, the group of queens exhibited a higher tendency to generalize than the other three categories of wasps that learned the odor-sugar association (adult female foragers, adult males, and newly emerged males) and that tended to have, therefore, a lower proportion of individuals with specific memory (Figure 2D). The causes for this high generalization remain unknown but could be related to the older age of queens compared to the other wasp categories. The average life expectancy of queens is 190 days, whereas workers live ca. 14 days, and males stay in the nests for about 11 days.³¹ Through their longer life, queens may increase their olfactory experience and thus enhance the probability of experiencing odorants other than linalool in an appetitive context.

A remarkable difference found in our study refers to the incapacity of newly emerged wasps to learn an odor-sucrose association compared to the other wasp categories, including that of newly emerged males which were matched in age. In the visual-learning domain, older females (foundresses) of the wasp *Polistes fuscatus* have better learning and memory skills for the facial traits that are characteristic of each individual in the colony,⁴⁵ a comparison that is consistent with the fact that foragers, i.e., older females of *M. cerberus*, learned and memorized efficiently the odorants paired with sucrose while the newly emerged females did not. In honeybees, newly emerged workers are able to learn odor-sugar associations if they exhibit a higher responsiveness to sucrose.⁴⁶ However, their level of acquisition is lower than that of older foragers. This factor could be excluded in our work, as all individuals selected for conditioning exhibited a similar sucrose responsiveness and responded always to the US in conditioning trials. Moreover, the fact that all wasps responded equally to the sucrose reward excludes differential effects of harnessing, in terms of subjective stress, between categories. In *Vespula germanica*, pre-forager and forager workers kept under lab conditions have similar levels of responsiveness toward carbohydrate and protein resources, irrespective of their age and task specialization,²⁶ which is consistent with the homogeneous sucrose responsiveness observed in our work for *M. cerberus*.

The cause for the inability of newly emerged females to learn and memorize odorants in an appetitive context could, thus, be searched in their particular lifestyle and the consequences that such lifestyle might have. Newly emerged females do not leave the nest during a period of approximately 10 days, while observations on newly emerged males indicate that they leave their nests earlier than females, in order to search precociously for food and partners.³¹ This behavior is conserved across *Mischocyttarus* as not only males of *M. cerberus* but also male individuals of other species of the genus, leave their nests earlier than females.^{35–37} This different ecological context provides therefore opportunities for males to learn odorants appetitively, while it restricts them for newly emerged females. This may lead to differences in brain and/or olfactory-circuit development and signaling between newly emerged males and newly emerged females. In honeybees, age-matched individuals subjected to different appetitive experiences (natural foraging in a diverse landscape vs. forced foraging within a tent in which a single odorant was provided with food) exhibit different development of the antennal lobes, the primary olfactory centers.⁴⁷ Bees with enriched foraging experience had more variable odor responses at the level of the antennal lobe while bees with impoverished odor experience were less efficient at distinguishing between components of an odor mixture in a behavioral assay. The influence of odorants during early phases of development is further highlighted by results showing that early odor learning in honeybees (i.e., 5–8 days after emergence) induces selective increases in volume of glomeruli (the functional units of antennal lobes) that are specific to the learned odor and that remain present at the adult stage (i.e., when bees are 17-day-old foragers).⁴⁸ Such a precocious olfactory learning also results in increased general odor-induced activity in the adult

antennal lobe not only for the experienced odorant but also for other perceptually similar odorants, thus highlighting the profound impact of odor experiences during early phases of development.⁴⁹ It is thus conceivable that the different lifestyle of newly emerged wasps restricted and expanded odor experiences in females and males, respectively, leading to changes in their olfactory system and to different learning performances. Accordingly, newly emerged females of *P. fuscatus* have significantly smaller brain volumes than older females.³⁸ Yet, volume *per se* is not necessarily indicative of poorer learning. The critical feature to be determined is if the connectivity of the olfactory circuit is finalized and functional in newly emerged females or if it requires further maturation to allow olfactory learning at a later stage. A comparative study looking at the connectivity and responses of olfactory pathways in females and males of different ages is necessary to test this hypothesis.

Overall, our results reveal remarkable learning and memory capacities in wasps, which open new perspectives for further studies addressing questions such as the neural bases of sex and age dependent olfactory learning performances, the duration of appetitive memories and their molecular substrates, the capacity to learn odorants in an aversive context, and the existence of higher-order phenomena beyond simple associative learning, among others. In particular, the age- and sex-dependent influence on learning uncovered by our work deserves to be explored at the neural level to determine if there is a tight connection between experience, brain maturation, and the development of learning and memory abilities in females and males. Additionally, it would be interesting to investigate how learning and memory abilities affect social interactions, for instance, regulating nestmate recognition toward adult and immature individuals.

Limitations of the study

The present study aimed at investigating learning and memory in primitively eusocial wasps and testing if they vary according to sex and lifestyle. A limitation of this study refers to the fact that we did not explore the neural mechanisms underlying the difference between newly emerged males and females. Neural differences may exist at the level of olfactory processing centers in the brain given that sucrose responsiveness was the same for both groups. While exploring these differences may be possible (e.g., via calcium imaging of antennal lobe activity) it was technically out of reach of our Brazilian group and wasps cannot be imported to France to be studied applying this methodology. Future studies should unravel the mechanistic nature of this difference between newly emerged males and females.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2023.106469>.

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AUTHOR CONTRIBUTIONS

The study was conceptualized by R.C.S., J.M.R.B.V.A., and M.G. R.C.S. and J.E.B. sampled wasps. R.C.S., J.M.R.B.V.A. and J.E.B. conducted the behavioral experiments. Statistical analyses were performed by R.C.S. and J.M.R.B.V.A. Results were discussed by all authors. The first version of the manuscript was written by R.C.S., J.M.R.B.V.A., and M.G. All experiments were supervised by C.A.O., M.G., and F.S.N. All authors reviewed and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in their field of research or within their geographical location. One or more of the authors of this paper self-identifies as a gender minority in their field of research. One or more of the authors of this paper self-identifies as a member of the LGBTQIA+ community.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Linalool 97%	Sigma-Aldrich	L2602-500G
Geraniol 98%	Sigma-Aldrich	163333-25G
Nonanal 95%	Sigma-Aldrich	N30803-25G
1-Hexanol 98%	Sigma-Aldrich	H13303-100ML
Deposited data		
Repository data	Figshare	https://doi.org/10.6084/m9.figshare.21085231.v1 https://doi.org/10.6084/m9.figshare.21085189.v1 https://doi.org/10.6084/m9.figshare.22153616
Experimental models: Organisms/strains		
<i>Mischocyttarus cerberus</i> wasp individuals (foragers, queens, adult males, newly emerged males, newly emerged females)		N/A
Software and algorithms		
RStudio Version 1.4.1717	R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/	N/A
GraphPad version 6.0e	Prism	www.graphpad.com
Custom R scripts	This paper	https://doi.org/10.6084/m9.figshare.21085231.v1

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rafael Carvalho da Silva (rscwasp@gmail.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data have been deposited at <https://doi.org/10.6084/m9.figshare.21085189.v1> and are publicly available as of the date of publication. DOIs are listed in the [key resources table](#).
- Original codes have been deposited at <https://doi.org/10.6084/m9.figshare.21085231.v1> and are publicly available as of the date of publication. DOIs are listed in the [key resources table](#).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Insects

Mischocyttarus cerberus individuals used in the experiments were collected from nests (n = 48) located in the area of the Universidade de São Paulo (USP) – Campus of Ribeirão Preto - (21°10'39"S; 47° 48' 37"W, 531 m elevation). In this location, nests of different ontogenetic phases are found at the same time across the whole year.⁵⁰ Only post-emergence phase nests were used. Observation of individuals present in the nests prior to the experiments allowed assigning them to the different categories studied.^{31,32,51} We identified the different castes based on their morphological differences and/or behaviour. We used queens (the most aggressive females),³² which have a lifespan that can reach up to 190 days from their emergence until the end of the nests they establish as foundresses,³¹ foragers (females returning from the field with

resources), whose age varies between 7 and 14 days,³¹ old males (with green eyes), whose age varies between 7 and 10 days and newly emerged males and females (with black eyes), which were individuals between 0 (just emerged) and 3 days of age. A total of 180 individuals were used in our experiments. Individuals without proper appetitive motivation, i.e., not responding to the US, were discarded (*Experiment 2*: 1 queen, 2 foragers, 2 males, 2 newly-emerged females and 1 newly-emerged male; *Experiment 3*: 2 foragers, 2 newly-emerged females and 1 newly-emerged male). No females were discarded in *Experiment 1*.

METHOD DETAILS

Olfactory conditioning

Collected wasps were placed in a plastic box and moved to a freezer for 2 minutes. After that, each wasp could be individually mounted in Eppendorf plastic tubes (0.5ml) with the lower part removed. In this way, wasps were restrained and only their head protruded through the hole. Two strips of tape were placed around the wasps' head to prevent them from escaping from the plastic tube. Finally, a piece of cotton was placed at the bottom of the plastic tube to provide support for the wasps' body. After being harnessed, wasps were kept in a controlled dark climate room at ca. 28°C and 60% of relative humidity (RH) during two hours. After this resting time, which is important to keep individuals hungry and motivated for appetitive rewards,²¹ experiments were initiated.

Before starting the conditioning assays, all the wasps were stimulated with a toothpick embedded with 50% (w/w) sucrose solution delivered to their antennae. This appetitive unconditioned stimulus (US) triggers MaLER as the unconditioned response. MaLER clearly differs from a normal mandible opening or biting response as wasps not only open their mandibles but also extend their tongue (maxilla-labium; check video uploaded as Supplementary Material). We thus checked if wasps were properly motivated to respond to the US prior conditioning. Wasps that did not respond to the US were removed from the experiment.

The olfactory stimuli (conditioned stimuli or CS) used during the experiments were placed inside glass vials and were presented to the wasps by means of an automated odor-releasing machine controlled by a micro-computer (Arduino®Uno, Tilburg, North Brabant, The Netherlands).^{52,53} The harnessed wasp was placed in front of the machine. In this system, a continuous flow of clean air is released towards the wasp's head. An air extractor placed behind the wasp prevented odorant accumulation. The air flow could be diverged to the glass vials containing the olfactory stimuli when they were presented to the tested wasps.

We used an absolute conditioning protocol, in which a single odorant is paired with sucrose reward during six conditioning trials spaced by 10 min. Each trial consisted of a resting phase of 26 s during which the wasp was placed in front of the olfactometer for context familiarization. Then, the CS was delivered for 4 s, followed by the US, which lasted 3 s and had a 1-s overlap with the CS. The US was delivered to the antennae and mouthparts. Thereafter, the wasp remained in front of the setup for 28 s until completing 1 min, to render contextual information non-predictive of the US. The inter trial interval (ITI) was 10 minutes, which allowed to condition 10 wasps simultaneously. The procedure was repeated until all wasps experienced the 6 conditioning trials (Figure 4). Wasps that responded to the CS before the US in the first trial were removed from the experiment. One hour after conditioning, a retention test was performed in which the CS was presented without reward. In this test, a new odorant (NOd) was also presented in the absence of reward to determine the specificity of the memory retrieved (Figure 4). The sequence of CS and NOd testing varied randomly from wasp to wasp. At the end of the memory test, the MaLER to sucrose stimulation of conditioned wasps was checked. Non-responding wasps were excluded from the experiments.

Experiment 1 – Olfactory learning of female adult foragers

In this experiment, adult female foragers were used as their status guaranteed the proper appetitive motivation for conditioning. Wasps were conditioned with linalool (CS) and 50% (w/w) sucrose solution (US). For the retention tests, Geraniol was used as the new odorant (NOd). Both olfactory stimuli were chosen because they are likely to have a biological relevance for wasps, as they are naturally found in flowers, where these wasps can collect food resources, such as nectar.⁴³ It was, therefore, expected that wasps would be capable of associating the conditioned linalool with sucrose solution, in particular, because this odorant was previously used to condition appetitively two wasp species, *Polistes metricus* and *Polistes dominula*.²⁴ In this experiment, 20 foragers were conditioned using a paired procedure (6 trials of CS-US presentations, with an inter-trial interval – ITI - of 10 min) and 20 other foragers were conditioned using an unpaired procedure. The unpaired group experienced 12 trials, with an ITI of 5 min. Thus, the mean ITI between two CS

or two US presentation was 10 min as for the paired group. In unpaired trials, only the CS or the US was presented, so that each individual received the same number (six) of CS and US presentations as individuals in the paired group. The duration of conditioning was the same for both the paired and the unpaired group. A pseudo randomized order was used for CS and US presentations. To match the experience of the paired and the unpaired group as much as possible, in US trials wasps were presented with the airflow diverging from an empty glass vial (blank) before the US.

Wasps of both groups were subjected to a memory retention test during which individuals were presented with the CS (Linalool) and the NOd (Geraniol) in the absence of reward. In the case of the unpaired group, the response to the air from the empty vial (blank) was also recorded, but no wasp responded to this blank stimulus during the memory retention tests. This step was essential to demonstrate that the lack of pairing between the CS and US would result in individuals not learning to associate both stimuli across the six trials, and hence not responding to the CS in the memory assays.

Experiment 2 – Comparing learning and memory retention across wasp classes

In this experiment, five groups of at least 20 individuals were used: (I) queens, (II) foragers, (III) newly-emerged females, (IV) males, and (V) newly-emerged males. All five groups were subjected to the same conditioning protocol described above for the paired group (six trials of absolute conditioning spaced by an ITI of 10 min; see also [Figure 4](#)). Linalool was also used as the CS during conditioning and Geraniol as the NOd in the retention tests.

Experiment 3 – Learning and memory retention of a neutral odorant

This experiment was performed to check whether *M. cerberus* individuals were able to learn and memorize neutral odorants besides the biologically relevant odorants used in the previous experiments. During conditioning, nonanal was used as CS after verifying that no wasp exhibited appetitive MaLER to it. In this experiment, three groups of at least 20 individuals each were used: (I) foragers, (II) newly-emerged females, and (III) newly-emerged males. Queens and males were not used because they were not abundant in the study area at the time of this experiment. Individuals of the three groups were subjected to the same conditioning protocol described above for the paired group (six trials of absolute conditioning spaced by an ITI of 10 min; see also [Figure 4](#)). In the memory retention test performed one hour after conditioning, wasps were presented with the CS (Nonanal) and 1-Hexanol as the NOd.

QUANTIFICATION AND STATISTICAL ANALYSIS

The responses of wasps to the olfactory stimuli (MaLER) were coded in a binomial fashion (0 = absence of MaLER and 1 = presence of MaLER). The percentage of wasps responding to an odorant with MaLER was quantified and represented as a population response (i.e., percentage of wasps responding correctly to the conditioned odor along consecutive trials). For each percentage, the 95% confidence interval is shown. In addition, we quantified individual learning scores as the number of responses to a conditioned odorant during the last 5 trials. Responses in the first trial were not included as in this case, in the absence of training, they were necessarily random. The learning score could thus vary between 0 (no learning) and 5 (highest learning performance). As an example, if a wasp responded 2 times and another bee responded 4 times to the conditioned odor, the average correct response (i.e., the average learning score) would be 3.

In retention tests, we also determined the proportion of individuals exhibiting specific memory, i.e., responding to the CS and not to the NOd. These two variables (learning scores and individuals with specific memory) complemented the population analyses with an individual-based analysis of performances.

The wasps' performance was analyzed by using generalized linear mixed models (GLMM). Data concerning MaLER, trial number, condition (paired or unpaired), and group (queen, forager, newly-emerged female, male, and newly-emerged male) were introduced as fixed factors, whereas information concerning the date, nest origin, and wasp code were entered as random factors. For the learning assays, we tested whether MaLER would vary according to the interaction between trial and condition (unpaired experiment) and trial and group (Experiment 1 - Linalol / Experiment 2 - Nonanal). A posthoc Tukey test was performed to detect specific differences between trials and groups. In this analysis, we found no differences in learning for queens, foragers, males and newly emerged males in Experiment I, so these data are presented in a single analysis at the [results](#) section. In a similar way, no difference was found in learning between foragers

and newly emerged males in Experiment 2, and these data are also presented in a single analysis. To calculate the learning scores, we first sum up the number of positive responses to the CS during the learning phase (excluding the responses for the first trials, where no association was expected to exist between US and CS). Individual learning scores were compared between groups by means of ANOVA, followed by pairwise comparisons adopting *fdr* correction. For the memory assays, we tested whether the MaLER results would vary according to the interaction between odor (CS = Linalol / NOd = Geraniol) and condition (unpaired experiment), and odor (Experiment 1 – CS+ = Linalol / NOd = Geraniol; Experiment 2 – CS+ = Nonanal / NOd = 1-Hexanol) and group. An ANOVA was performed in order to check overall statistical differences among different trials and individuals belonging to different groups. Finally, to analyze whether the proportion of individuals with specific memory varied according to the experimental condition, we conducted an ANOVA followed by pairwise tests when appropriate. All analyses were performed using the software R Studio (version 1.4.1717) and the packages *afex*⁵⁴ and *lsmeans*.⁵⁵