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RESEARCH ARTICLE

Hornets possess long-lasting olfactory memories

Zhiwen Gong[1,2], Ken Tan[1,2,*] and James C. Nieh[3,*]

ABSTRACT

The ability of animals to learn and remember is an important adaptation for coping with environmental changes. The fitness benefits provided by these cognitive skills, in conjunction with social behaviours, contribute to the success of social insects. How these abilities are shared among the different castes and the long-term persistence of memory are now being elucidated in diverse systems, work that should shed light on general principles underlying cognitive evolution. Here, we provide the first evidence of olfactory learning and long-term olfactory memory in all three castes of an Asian hornet, *Vespa velutina*. Using the first proboscis extension reflex assay developed for hornets or wasps, we found that all hornet castes could learn and remember odours associated with a food reward. Moreover, long-lasting memory was retained without significant decay in gynes (virgin queens) and drones even up to 30 days (workers did not survive for 30 days). Drones learned and remembered simple odorant molecules and gyne sex pheromone with equal facility. These results increase our understanding of the outstanding cognitive abilities of social insects and suggest the likely importance of long-lasting memory in different castes of the same species.

KEY WORDS: Wasp, *Vespa velutina*, Caste, Cognition, Olfactory learning, Memory

INTRODUCTION

Learning and memory are examples of phenotypic plasticity that allow animals to increase their fitness in novel and changing environments (Agrawal, 2001; Tibbetts et al., 2018). Determining the learning skills of different animals is thus useful for understanding general learning mechanisms and the selective pressures that may have shaped such learning (Menzel, 2001). In social insects, most studies on learning and memory have focused on honey bees and have proven quite useful for elucidating mechanisms and understanding general similarities between insect and vertebrate learning (Giurfa, 2007). However, other taxa also possess learning, memory and a diversity of life history traits and social organizations that could enrich our understanding of underlying mechanisms and evolutionary trajectories (Papaj and Alcinda, 2012).

For example, the study of wasp learning and memory has been especially productive, although it has been more extensively explored with vision than with olfaction. *Vespula vulgaris* can learn visual landmarks (Collett, 1995), *Ropalidia marginata* possess spatial memory of their foraging landscapes (Mandal et al., 2017), *Vespula germanica* has long-term spatial memory (Moreyra et al., 2017) and *Polistes fuscatus* can learn conspecific facial features to modulate social interactions (Sheehan and Tibbetts, 2011). Queens are better than workers at learning and remembering conspecific faces (Tibbetts et al., 2018). Female workers have better facial learning than males, although they have similar colour learning (DesJardins and Tibbetts, 2018). The parasitic species *Venturia canescens* and paper wasp *Mischocyttarus flavitarsis* can also learn to associate colour with a food reward (McPheron and Mills, 2007; Lucchetta et al., 2008).

In addition to visual learning, olfactory learning and memory have been demonstrated in multiple wasps. The parasitic species *Aphidius ervi* and *Microplitis croceipes* can associatively learn host odours or plant odours associated with hosts (Lewis and Tumlinson, 1988; Takemoto et al., 2012). Other parasitic wasps can learn odours that subsequently guide their search for appropriate plant hosts (Bleeker et al., 2006; Smid et al., 2007). In some species, larvae can learn before metamorphosis and retain these memories after emergence (Takemoto et al., 2012; Gandolfi et al., 2003). Multiple species (*V. vulgaris*, *V. germanica* and *Vespula maculifrons*) can learn to associate odour with rewarding food sources (El-Sayed et al., 2018; Overmyer and Jeanne, 1998; Jander, 1998). However, many details of learning acquisition and memory, particularly long-lasting memories, remain unexplored in wasps, hornets and other social insects (Gong et al., 2018), although we know that such memories can potentially last >30 days in honey bees (Lindauer, 1960, 1963; Menzel, 1968) and at least 21 days in bumble bees (Chittka, 1998).

We therefore focused on olfactory learning in *Vespa velutina* because odour detection plays an important role in its foraging and intraspecific communication (Ono et al., 1995, 2003; Brodmann et al., 2009; Couto et al., 2014). *Vespa velutina* is strongly attracted to the odours of its common prey, honey bee colonies (Couto et al., 2014). This ability contributes to the detriment that *V. velutina* inflicts in regions where it has invaded, leading to major colony losses and even the abandonment of apiculture in multiple European regions (Villemant et al., 2011; Monceau et al., 2014; Arca et al., 2015).

In addition, there is evidence that multiple *V. velutina* castes (gynes, drones and workers) have olfactory detection pathways and therefore potentially possess olfactory learning (Couto et al., 2016). We thus compared the learning and memory of *V. velutina* gynes, drones and workers. We also tested the ability of drones to learn and remember gyne sex pheromone: 4-oxo-decanoic acid/4-oxo-octanoic acid (4-ODA/4-OOA) (Wen et al., 2017). We used classical conditioning and developed the first proboscis extension reflex (PER) for hornets or wasps. PER is a well-developed paradigm in honey bees and is particularly useful for studying the neural bases of learning and memory because animals can be fully restrained. Finally, we tested for long-lasting memory, 30 days after memory formation.

MATERIALS AND METHODS

Colonies and study sites

We used three *Vespa velutina* Lepeletier 1836 colonies, each maintained in a different wooden nest box (30 cm×20 cm×20 cm),

[1]CAS Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming 650223, China. [2]Southeast Asia Biodiversity Research Institute, Chinese Academy of Science, Menglun 666300, China. [3]Division of Biological Sciences, Section of Ecology, Behavior, and Evolution, University of California, San Diego, La Jolla, CA 92093, USA.

*Authors for correspondence (kentan@xtbg.ac.cn; jnieh@ucsd.edu)

Z.G., 0000-0002-2921-2221; K.T., 0000-0002-0928-1561

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at the apiary of the Eastern Bee Institute of Yunnan, China (GPS coordinates: 25.128849N, 102.752200E). *Vespa velutina* nests are established by an overwintering foundress in the spring (Dazhi and Yunzhen, 1989). Normally, the first comb is built by the queen, with workers taking over after they emerge. The colony reaches its maximum size during the late autumn and produces many gynes (reproductive females that are virgin queens) and males from mid-September to mid-November (Dazhi and Yunzhen, 1989), followed by colony die-off. We conducted our experiments from July to December 2017 and August to September 2018. Colonies were in good condition, as judged by their size and activity, and engaged in natural foraging.

Sample collection for learning and memory

Gynes, workers and drones have distinguishing physical traits (Fig. 1C) (Couto et al., 2016; Perrard et al., 2012). In the afternoon on warm, clear days, we gently captured workers (>15 days old) and gynes (>10 days old) with tweezers from their colony entrances as workers emerged to forage and gynes to mate. We only used workers that were vigorous (based upon their activity level when captured) and had no wing wear, which occurs with ageing. Our age estimates were based upon the ages at which workers typically forage (Dazhi and Yunzhen, 1989). Gynes and drones only mate outside the nest, and mating occurs when they are >10 days old (Dazhi and Yunzhen, 1989; Perrard et al., 2012). Drones were difficult to obtain at the nest entrance. We therefore caught them with a net at a mating congregation area where we observed them flying around looking for mating virgin gynes (Wen et al., 2017). Drones could therefore also have come from wild colonies. To increase drone visitation, we baited this area with 100 µl of synthetic gyne sex pheromone 4-ODA/4-OOA in a ratio of 0.78 (Wen et al., 2017) placed in a clean glass vial attached to a tree.

To assess learning and memory, we used 186 gynes, 357 workers and 423 drones. To examine long-lasting memory (30 days after learning), we used 147 gynes and drones. Detailed sample sizes are given in Table S1.

Classical olfactory conditioning

We immediately placed captured hornets into an incubator (20°C, 65% humidity). At 18:00 h, we removed the hornets from the incubator, fed each with 10 µl sucrose (30% w/v) and then returned them to the incubator. The following day at 09:00 h, we fed each hornet with 5 µl of 30% (w/v) sucrose. We waited 30 min after this feeding and then placed each hornet in a clean glass vial on ice for approximately 7 min until its movements had significantly diminished. We then restrained each hornet in a 1.5 ml centrifuge tube that had a hole cut in its tip. We inserted a straw, cut at an angle and of the same diameter as the tube to further reduce hornet movement. This straw was placed over the abdomen and under the wings (Fig. 1A), following a method developed for honey bees (Gong et al., 2018). Individuals were able to move their heads and proboscises but could not escape (Fig. 1A,B). To allow them to adjust to this restraint, we put them in an incubator (20°C, 65% humidity) for 5 h. Olfactory learning and memory were tested with a PER assay (Bitterman et al., 1983).

During each trial, the hornet was exposed to a continuous air flow of 0.5 l min^{-1} directed through a 60 ml syringe using a tip with an inner diameter of 3 mm. The olfactory conditioned stimulus (CS) consisted of 5 µl of hexane, citral or geraniol (Sigma-Aldrich, St Louis, MO, USA) dispensed onto a filter paper (1 cm×1 cm) inside a syringe. For drones, we also tested two major components of gyne sex pheromone: 4-ODA and 4-OOA (0.78 ratio, which we synthesized to 99.8% purity) (Wen et al., 2017). Each hornet was only trained to a single CS. A fan placed 12 cm behind the hornets exhausted all odours through a window.

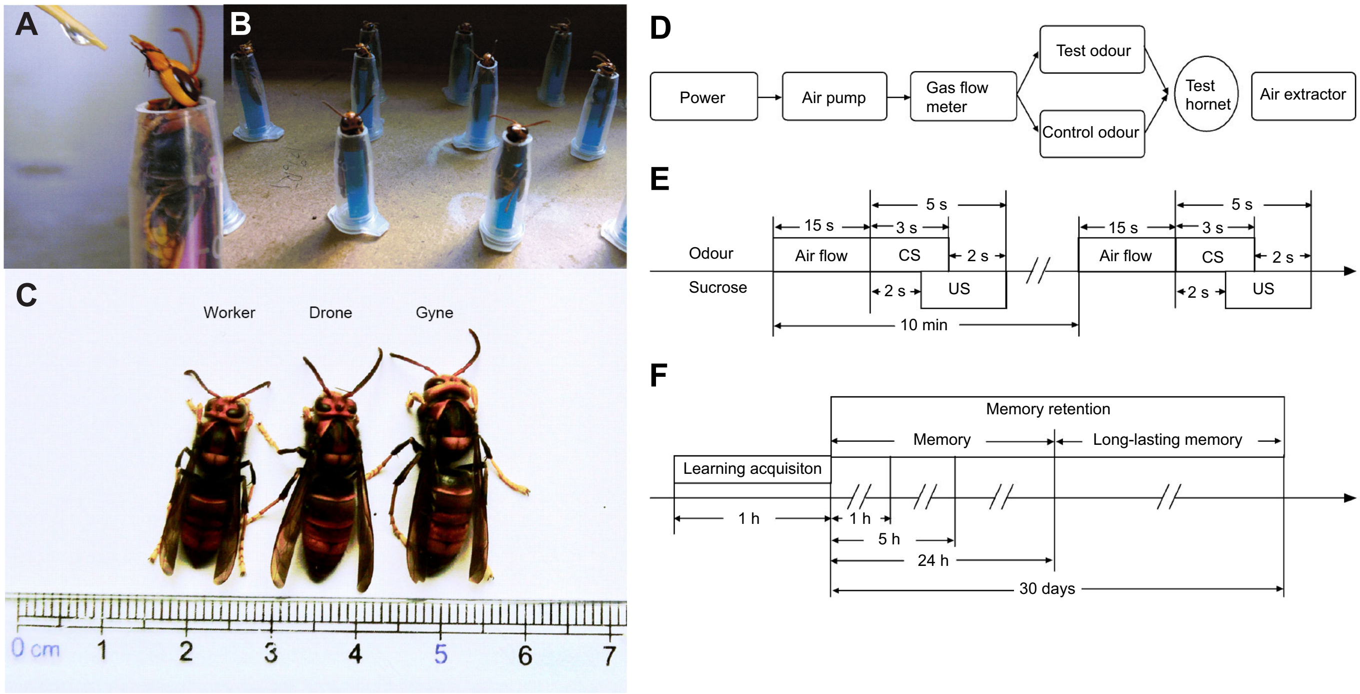

Fig. 1. Experimental apparatus and design. (A) Hornets were restrained in a microcentrifuge tube with the tip removed to allow the hornet to extend its head but not escape. A straw (pink) cut at an angle and placed beneath the wings prevented the hornet from moving backwards. The worker shown is extending its proboscis to feed from sugar solution on the tip of a toothpick. (B) A group of worker hornets being prepared for proboscis extension reflex (PER) testing. (C) The three castes tested: worker, drone and gyne (virgin queen). (D) Schematic diagram of how odours were delivered to each hornet. (E) Learning acquisition procedure for each hornet (6 learning trials per hornet over 1 h). CS, conditioned stimulus; US, unconditioned stimulus. (F) Time intervals for testing memory and long-lasting memory.

During acquisition training, this CS was paired with the unconditioned stimulus (US: 30% w/v pure unscented sucrose solution presented on a wood toothpick) as a reward. We lightly tapped one antenna (left or right, randomly selected) with the US to elicit a proboscis extension response (the unconditioned response) and then briefly allowed the hornet to feed by contacting the proboscis with the US for 3 s. The US was presented 2 s after the CS and overlapped with the CS for 1 s (Fig. 1). If a hornet exhibited learning, it would extend its proboscis during presentation of the CS only (scored as ‘1’). In all experiments, we conditioned each hornet six times with an inter-trial interval of 10 min (Fig. 1D–F), as used by Smid et al. (2007) and Bleeker et al. (2006) for parasitic wasps learning odours associated with oviposition.

To test memory, we exposed each trained hornet to the CS only at 1, 5 and 24 h after the last learning trial. To determine whether the hornets were responding specifically to the CS, we also provided an unrewarded odour (nonanal) 10 min after each of these CS presentations (Fig. 1D–F). These memory test odour presentations were all unrewarded.

To assay long-lasting memory (30 day memory), we removed hornets from their restraints after the 24 h memory test and placed them in cages (30 cm×30 cm×20 cm, 30 hornets per cage) and maintained them in an incubator (20°C, 65% humidity) for 30 days. Each day, we fed hornets *ad libitum* with sucrose solution (30% w/v). After 30 days, we removed the hornets, placed them in the PER harnesses, allowed them to rest for 5 h, and then tested their long-lasting memory by exposing them to the CS alone (Fig. 1D–F). However, none of the caged workers survived to 30 days (Fig. S1). We therefore ran another experiment in which we marked 357 workers from three colonies with enamel paint on their thoraxes after the last memory trial and placed them inside their respective colonies for 30 days. However, we were unable to find any workers inside these nests 30 days later.

Statistics

We included data from all hornets, including those that did not exhibit any learning. We ran separate analyses for learning and memory, using the PER score and the discrimination index (DI, the response to the CS minus the response to the unrewarded, novel control odour) (Biergans et al., 2012). At each test time point per hornet, the DI has a potential value of −1, 0 or 1.

Per odour used, our sample sizes ranged from 48 to 156 hornets (Table S1) and we therefore used a repeated-measures linear mixed model with a REML algorithm to allow between-group and within-group comparisons (Matsumoto et al., 2012). We had two main goals: (1) to compare learning and memory between castes and (2) to test whether drones would have better learning and memory of gyne sex pheromone than other odours. Between castes, we replicated all CS odours except for the sex pheromone, which was only used to test drones. This design led us to test (1) for differences between castes (with odour type as a random effect) and, separately, (2) for the effects of odour (fixed effect) on drones. In all models, caste and trial number (a time variable) were fixed nominal effects.

We used sequential model simplification, first running all interactions and then eliminating them if they were not significant. Tukey’s honestly significant difference (HSD) tests were used to make corrected pairwise comparisons. Where appropriate, we applied the Dunn–Šidák correction (k=2 for learning tests and k=4 for memory tests), and we indicate tests that are significant with ‘DS’. P-values <0.05 for which no correction was necessary are not denoted with DS. We used JMP Pro v13.0.0 (SAS Institute, USA) for all statistical analyses and show means±95% confidence interval (CI) in our plots. Effect sizes are reported as Cohen’s f^2.

RESULTS

Learning

In the overall model (f^2=0.67), there were significant effects of caste ($F_{2,499}$=5.68, P=0.004$^{\mathrm{DS}}$) and trial (indicating learning, $F_{5,4541}$=184.62, P<0.0001$^{\mathrm{DS}}$), but no significant effect of the interaction caste×trial ($F_{10,4531}$=1.70, P=0.08). For trials 1–4, each subsequent trial showed significantly improved learning (Tukey’s HSD test, P<0.05; Fig. 2A). Odour type accounted for only 0.3% of model variance. Gyne PER to rewarded odours (hexane, geraniol or citral) was significantly higher than drone or worker PER responses, which did not differ (Tukey HSD test, P<0.05; Fig. 2A). However, this improved responsiveness was only significant in the fifth and sixth trials (Tukey HSD test, P<0.05). Thus, gynes did not have a significantly higher naive responsiveness to the CS odours.

Although we expected drones to be very sensitive to gyne sex pheromone (4-ODA/4-OOA), they did not exhibit better learning of sex pheromone versus the other odours (overall model f^2=1.43). For drones, there was no significant effect of odour ($F_{3,419}$=1.18,

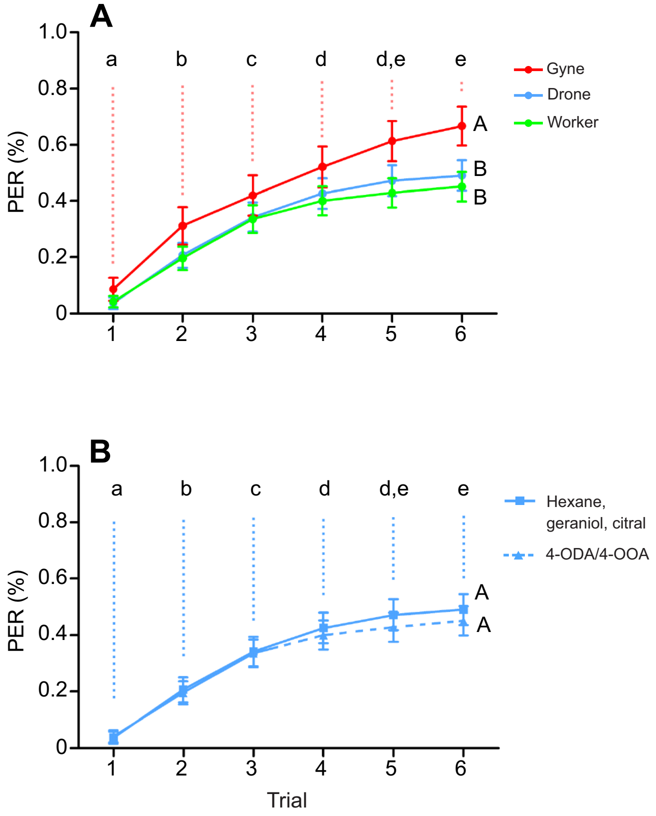

Fig. 2. Comparison of hornet olfactory learning between the three different castes. (A) Individuals were conditioned to associate hexane (gyne: n=60, worker: n=102, drone: n=105), geraniol (gyne: n=60, worker: n=156, drone: n=84) or citral (gyne: n=66, worker: n=99, drone: n=135) with a reward. Gyne learning was significantly better than that of workers or drones, but only for trials 5 and 6 (uppercase letters, Tukey HSD test, P<0.05). (B) Drones were also conditioned (in separate trials) with gyne sex pheromone (4-ODA/4-OOA, n=99), but did not exhibit significantly better learning of sex pheromone than of the other CS odours (hexane, geraniol or citral). The plots show means±95% confidence interval (CI). Different uppercase letters show significant differences between castes, and different lowercase letters indicate significant differences between trials (Tukey HSD tests, P<0.05).

P=0.32) or the interaction trial×odour ($F_{15,2095}$=0.75, P=0.73). Only trial was significant ($F_{5,2110}$=125.82, P<0.0001^{DS}). For trials 1–4, each subsequent trial showed significantly improved learning (Tukey HSD test, P<0.05; Fig. 2B).

Memory up to 24 h

For memory up to 24 h (overall model f^2=0.43), gynes exhibited significantly higher memory retention than drones or workers (Tukey HSD test, P<0.05; Fig. 3A), corresponding to their higher learning (Fig. 2A). Caste ($F_{2,387}$=8.19, P=0.0003^{DS}) and memory trial were significant (corresponding to a decrease in memory retention over time, $F_{2,1907}$=24.18, P<0.0001^{DS}), but the interaction caste×memory trial was not significant ($F_{4,1903}$=1.11, P=0.35). Odour accounted for only 0.6% of model variance. All memory trials were significantly different from each other (Tukey HSD test, P<0.05; Fig. 3A).

In these memory tests, the higher PER scores exhibited by gynes as compared with drones or workers were probably due to differential memory retention (less decay) between castes. PER scores decreased by the same amount in all castes (respective decreases of 10%, 13% and 13% in gynes, workers and drones) when the last learning trial was compared with the first memory test at 1 h (overall model f^2=0.27). There were no significant effects of caste ($F_{2,336}$=0.007, P=0.99), odour ($F_{2,741}$=0.20, P=0.82) or the interaction caste×odour ($F_{4,654}$=1.42, P=0.22).

The higher memory PER scores of gynes were also probably not due to caste-specific differences in naive responsiveness to odours because PER was not significantly elevated in the first learning trial (Fig. 2A). Thus, the caste-specific memory retention is probably a result of the higher learning shown by gynes in the fifth and sixth learning trials (Fig. 2A).

Drones did not show better memory retention for sex pheromone (overall model f^2=1.22). For drones, there was no significant effect of odour ($F_{3,419}$=0.46, P=0.71) or the interaction trial×odour ($F_{6,838}$=0.66, P=0.68). Only trial was significant ($F_{2,844}$=10.70, P<0.0001^{DS}). The 1 and 5 h trials did not significantly differ, but the 24 h trial revealed significantly lower memory retention (Tukey HSD test, P<0.05; Fig. 3B).

Hornets showed memory discrimination (overall model f^2=0.43). Responses to the CS odours (citral, geraniol and hexane) were significantly higher than responses to the unrewarded control odour (nonanal) (Tukey HSD test, P<0.05; Fig. 3A). Caste ($F_{2,447}$=16.43, P<0.0001^{DS}), odour ($F_{3,5062}$=163.34, P<0.0001^{DS}) and trial ($F_{2,4544}$=41.14, P<0.0001^{DS}) were all significant. No interactions were significant ($F_{4,4517}$≤2.31, P≥0.06).

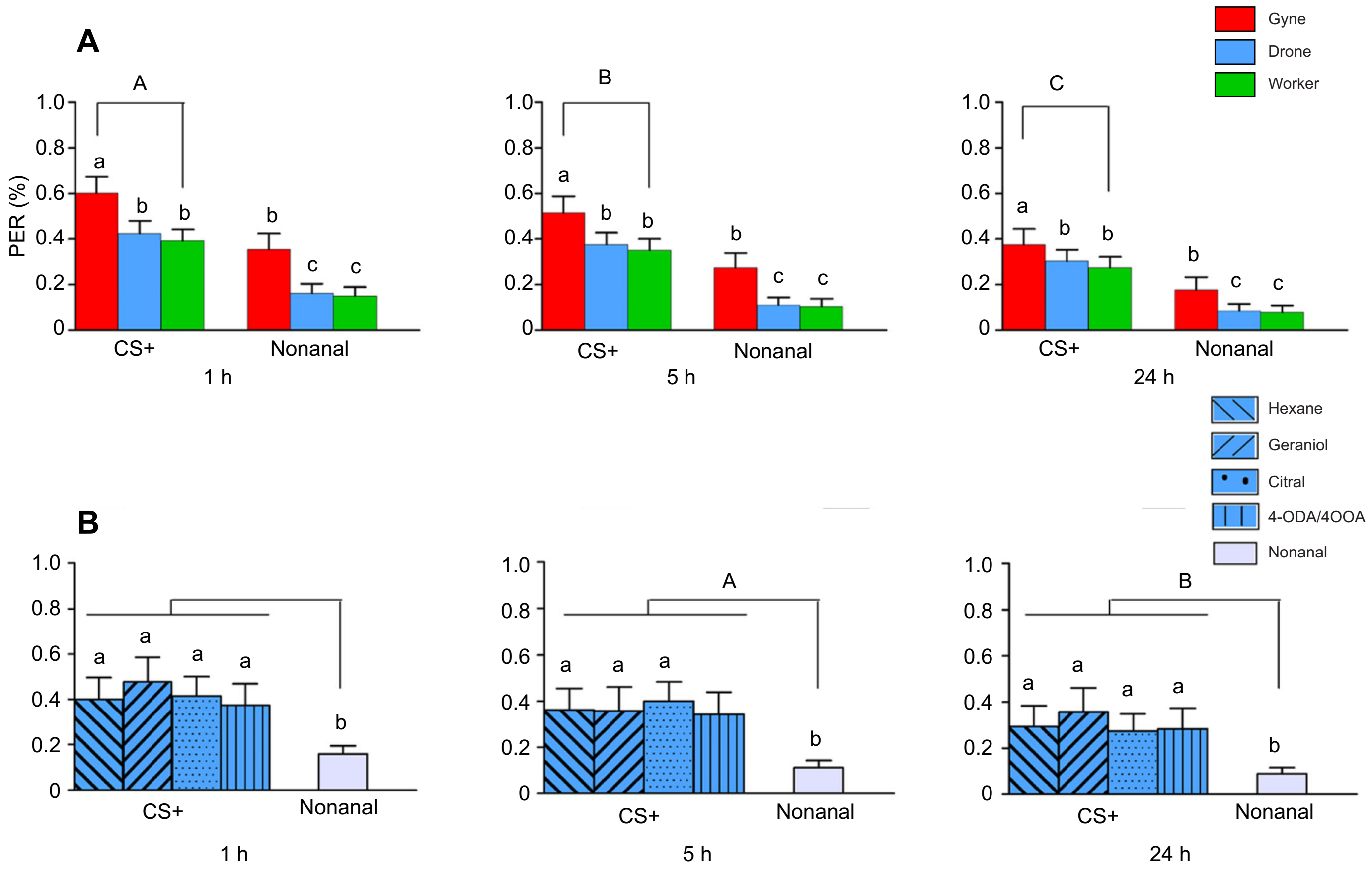

Fig. 3. Comparisons of hornet memory between the three different castes. Hornets were trained to hexane (gyne: n=60, worker: n=102, drone: n=105), geraniol (gyne: n=60, worker: n=156, drone: n=84) or citral (gyne: n=66, worker: n=99, drone: n=135). Drones were also trained, in separate trials, to gyne sex pheromone (4-ODA/4-OOA, n=99). (A) Memory retention was higher at 1 h than at 5 or 24 h (all time points significantly different from each other). At all three memory time points, gyne memory was significantly better than drone or worker memory, and PER for rewarded odours (CS+) was significantly higher than that for the unrewarded odour (nonanal). (B) Drone learning of rewarded odours (CS+), including sex pheromone (4-ODA/4-OOA, to which only drones were tested), likewise showed higher memory retention for rewarded odours than for the unrewarded odour (Tukey HSD tests, P<0.05). Drone memory was significantly better at 1 and 5 h than at 24 h. Plots show means±95% CI. Different uppercase and lowercase letters respectively indicate significant differences between plots and within plots (Tukey HSD tests, P<0.05).

Similarly, drones showed a significantly higher PER response to all CS odours than to the unrewarded nonanal (Tukey HSD test, $P<0.05$; Fig. 3B; overall model $f^2=1.33$). Odour ($F_{4,841}=30.44$, $P<0.0001^{\mathrm{DS}}$) and trial ($F_{2,1690}=18.37$, $P<0.0001^{\mathrm{DS}}$) were significant, but the interaction of odour×trial was not significant ($F_{8,1682}=1.04$, $P=0.40$). At all memory test time points, all CS odours elicited significantly higher PER than the unrewarded nonanal (Tukey HSD test, $P<0.05$).

The DI analysis (Fig. S2) suggests that all castes had the same discrimination ability for all CS odours at all tested time points (overall model $f^2=0.22$). There was no significant effect of caste ($F_{2,1395}=0.18$, $P=0.83$), trial ($F_{2,1900}=2.31$, $P=0.10$), CS odour ($F_{2,2410}=0.73$, $P=0.48$) or any interaction between these factors ($F_{4\text{–}8,1900\text{–}2392}\leq 0.41$, $P\geq 0.80$).

Long-term (24 h) versus very long-lasting memory (30 days)

Only 3% of workers survived for 30 days (in incubated cages provided with food), although 79% of gynes and 59% of drones survived to the 30 day long-lasting memory test time point. We therefore compared only gynes with drones to assess long-lasting memory (overall model $f^2=0.08$). There were no significant interactions between caste, odour and memory type ($F_{1\text{–}3,1834}\leq 1.91$, $P\geq 0.17$; Fig. 4A). Gyne and drone memory did not differ: there was no significant effect of caste ($F_{1,1834}=2.92$, $P=0.09$). However, there was a significant effect of odour ($F_{3,1834}=39.93$, $P<0.0001$), such that the response to the unrewarded nonanal was significantly lower than that to all other odours (Fig. 4A; Tukey HSD test, $P<0.05$) in both gynes and drones. There was no significant difference between 24 h and 30 day memory ($F_{1,1834}=0.001$, $P=0.97$; Fig. 4A), demonstrating that gynes and drones both possessed exceptionally long-term memory that did not significantly decay, even after 30 days, in comparison with their 24 h memory.

For drones (overall model $f^2=0.08$), there was no significant effect of memory type ($F_{4,1326}=1.57$, $P=0.21$) or the interaction odour×memory type ($F_{4,1322}=1.50$, $P=0.20$). There was a significant effect of odour ($F_{4,1326}=23.12$, $P<0.0001$) because responses to the control odour, nonanal, were significantly lower than those to all rewarded odours (Fig. 4A; Tukey HSD test, $P<0.05$). Drones did not have better memory retention of sex pheromone in comparison with other rewarded odours (Fig. 4B; Tukey HSD test, $P<0.05$).

DISCUSSION

All three hornet castes exhibited olfactory learning and memory, and gynes and drones were able to retain this memory for a long period of time, up to 30 days. Because of the evidently shorter life spans of the workers, we could not assess whether they possess such long-lasting memory. However, these results suggest an intriguing persistence of memory that may be a general ability in social insects and point to the need for more study of life-long memories and their

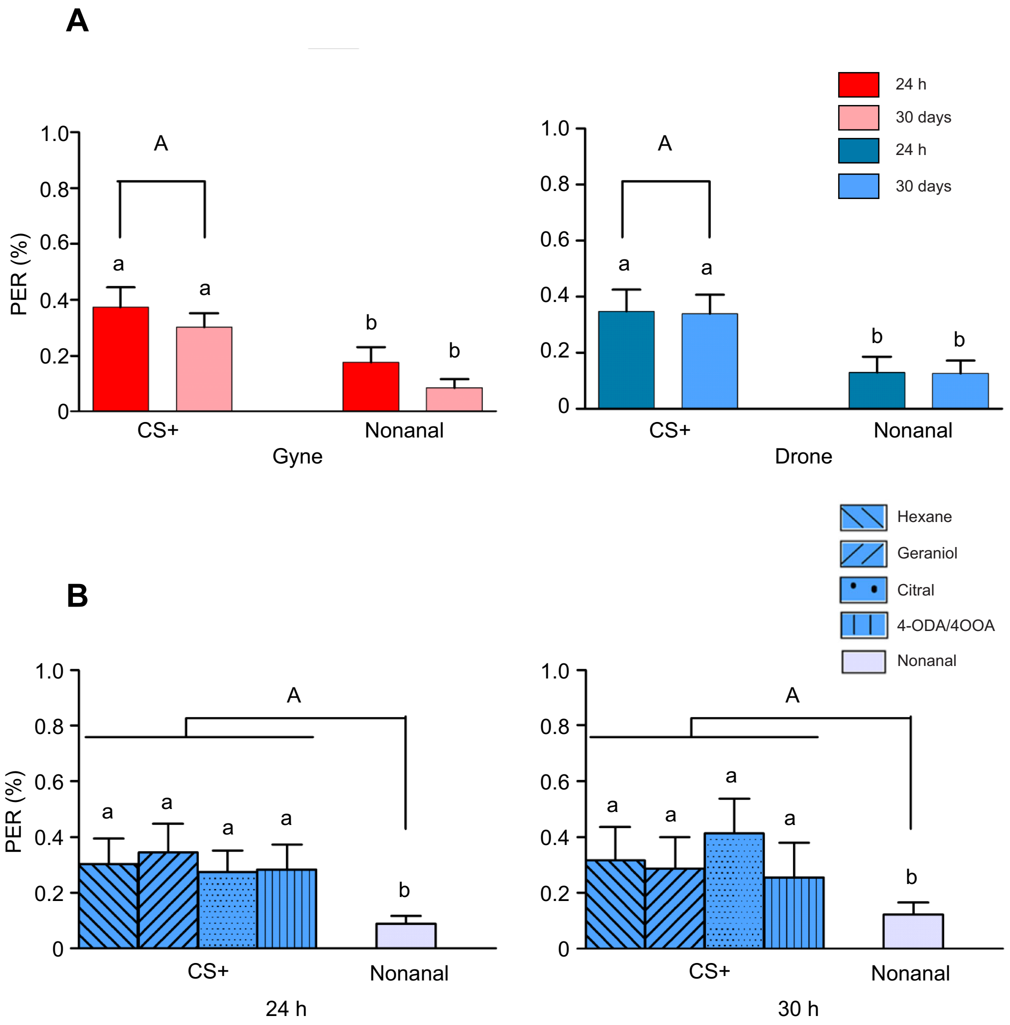

Fig. 4. Comparison of 24 h memory with very long-lasting memory (30 days) in gynes and drones. Hornets were trained to hexane (gyne: *n*=60, drone: *n*=105), geraniol (gyne: *n*=60, drone: *n*=84) and citral (gyne: *n*=66, drone: *n*=135). In separate trials, we trained drones (*n*=99) to gyne sex pheromone (4-ODA/4-OOA). (A) There were no significant differences between 24 h and 30 day memory in gynes or in drones. However, within each caste, responses to rewarded odours (CS+) were significantly higher than responses to the unrewarded control odour, nonanal. (B) Similarly, 24 h memory and long-lasting memory of all rewarded odours (including sex pheromone) in drones did not differ, and responses to all rewarded odours (CS+) were significantly higher than responses to the unrewarded control odour. Plots show means±95% CI. Different uppercase and lowercase letters respectively indicate significant differences between plots and within plots (Tukey HSD tests, $P<0.05$).

adaptive value. The restrained PER assay used in this study, the first to be developed for hornets or wasps, is a technique that should facilitate investigation of the neural bases for their learning and memory, as it has for honey bees (Giurfa and Sandoz, 2012) and bumble bees (Riveros and Gronenberg, 2009).

Differences in learning and memory among castes

An intriguing question is whether gynes exhibited better olfactory learning than drones or workers. Two points support this hypothesis. First, gynes had significantly higher PER scores in the fifth and sixth learning trials, but not at earlier trials. In particular, the naive response to the tested odours (shown in the first trial, Fig. 2A) was not significantly different between any of the castes, and gynes, therefore, did not have higher olfactory responsiveness for any of the tested CS odours. Second, the memory DI was not significantly different for any caste (Fig. S2), suggesting that the ability to generalise odours was the same for all three castes.

However, gynes did have a significantly elevated naive response to nonanal as compared with the other castes (Fig. 3A), suggesting that they had a higher base response to nonanal. Testing the naive responses of all three castes with other olfactory compounds and a set of experiments in which all odours are reciprocally used as control and CS odours would be illuminating.

Although we collected all castes during a similar life cycle point (the transition from intranidal to flight and activity outside the nest), this naturally occurs at different ages (workers were >15 days old and drones and gynes were >10 days old) and these age differences may have contributed to learning and memory differences. It is unclear whether age affects learning ability in hornets and wasps. Tibbetts et al. (2018) showed that foundress wasps (*P. fuscatus*) had better facial learning and memory than workers. These foundresses had overwintered and were thus significantly older than the workers tested. However, in some cases, caste is more important than age in determining learning abilities. DesJardins and Tibbetts (2018) studied *P. fuscatus* workers and males (drones) over a wide age range (5–30 days) and found that workers were consistently better at facial learning, but the two castes were equally good at colour learning. Similarly, Gong et al. (2018) reported that the proportion of honey bee queens (*Apis mellifera*) exhibiting learning was 5-fold higher than that of honey bee workers at every tested age (5–25 days). Finally, it is possible that workers had decreased learning ability as a result of senescence, given that they were 5 days older, on average, than gynes and males. However, workers typically forage when they are 15–30 days old (Dazhi and Yunzhen, 1989), and thus the 5 day age difference is not major. We were also careful to use young foragers that did not show signs of senescence, based upon their activity level when captured or wing wear.

Caste survival

For the 30 day memory test, 79% of gynes survived (a 1.3- and 28.2-fold higher survival rate than drones or workers, respectively; Fig. S1). The higher survival of gynes is not surprising, but the very low survival (3%) of workers prompted us to try maintaining workers in colonies. Unfortunately, none of these workers survived to the 30 day test point, which may approach maximum worker lifespan (Monceau et al., 2014).

Potential mechanisms of olfactory learning and memory in *Vespa*

In *V. velutina*, the antennal lobe contains approximately the same number of olfactory glomeruli in gynes (269) and workers (265), but significantly fewer in males (247) (Couto et al., 2016). However, given that workers and drones had learning curves that were not significantly different (Fig. 2A), glomeruli differences are unlikely to explain our results. Instead, caste-specific differences in how learning and memory are formed may play a role. Smid et al. (2007) demonstrated that transcription and translation inhibitors fed to parasitic wasps (*Cotesia glomerata* and *Cotesia rubecula*) could interfere with long-term memory (LTM) formation of odours associated with oviposition. Protein synthesis is also likely to be important for LTM and long-lasting memory formation in *V. velutina*. DNA methylation may also play a role in wasp and hornet memory, as it does for honey bees (Biergans et al., 2015; Evans and Raine, 2014), but this remains to be determined.

Caste, learning and memory in social insects

Queens in multiple social insects have superior cognitive abilities. Bumble bee queens (*Bombus terrestris*) exhibit significantly better learning of food source colour than workers (Sheehan and Tibbetts, 2008). Honey bee queens (*A. mellifera*) have significantly better memory than workers bees, even up to 7 days (Gong et al., 2018). Queen wasps (*P. fuscatus*) are better than workers at learning and remembering conspecific faces (Tibbetts et al., 2018), and queens can retain these memories for at least 1 week (Biergans et al., 2015). Our results suggest that *V. velutina* gynes may also have better learning than other castes. In the final learning trial, gyne learning was at 70%, worker learning at 58% and drone learning at 51% (Fig. 2A). The memories of the castes corresponded to these learning differences, but when tested at 30 days (long-lasting memory), there were no longer significant differences between gyne and drone memory (Fig. 4).

In comparison, honey bee (*A. mellifera*) worker and queen olfactory learning can reach 80% and 90%, respectively, after six learning trials (Gong et al., 2018). Bumble bees (*Bombus terrestris*) achieved an average of 60% (Riveros and Gronenberg, 2009) or nearly 45% (Stanley et al., 2015) after six learning trials. The stingless bee (*Meliponula ferrugina*) achieved 60% learning after five trials (Henske et al., 2015) and other stingless bee species had even lower learning levels (Henske et al., 2015; Mc Cabe and Farina, 2010). In a restrained olfactory PER assay, hornet learning is therefore like bumble bee or stingless bee learning. However, this similarity may arise from an artefact: how different species handle the stress of being restrained. Honey bees could have higher performance in this assay than other social insects, not because they have inherently superior memory but because they are less stressed by the restrained PER assay, which was first developed for *A. mellifera* (Bitterman et al., 1983). Nonetheless, comparisons between wasp castes, using the same assay, probably reflect caste-specific differences.

With respect to wasps, the parasitic wasp species *C. glomerata* and *C. rubecula* can learn to associate plant odours with oviposition and demonstrate LTM formation that is species specific: 4 h for *C. glomerate* and 3 days for *C. rubecula* after three learning trials (Smid et al., 2007). In *C. glomerata*, a single learning trial was enough to create a LTM that persisted for at least 5 days. In *C. rubecula*, this memory formed by 24 h, but then decreased over subsequent days (Bleeker et al., 2006). Such differences may be adaptive, reflecting the different ecologies and phenotypic plasticity of these two species (Smid et al., 2007). Similarly, the long-lasting memory shown by *V. velutina* suggests that some wasp and hornet species may need long-lasting memories. Because gynes evidently live longer than the other two castes (Monceau et al., 2014), their potentially better memory may be adaptive. For example, queen wasps are better than workers at learning and remembering conspecific faces (Tibbetts et al., 2018), a skill that helps them maintain and negotiate the social hierarchy. For *V. velutina*, it remains unclear why gynes should need better olfactory learning or retain this memory for so long. We speculate that this memory may be useful if gynes must occasionally forage following a major loss of colony foragers or if olfactory recognition plays a role in the colony hierarchy. However, it is also possible for *V. velutina* gynes (and for bumble bee and honey bee queens) that such enhanced learning and memory is a spandrel, an evolutionary by-product of a true adaptation. In this case, the higher fitness, longevity and health of queens as compared with other castes may also be reflected in their improved ability to learn and remember – an interesting question for future research.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Z.G., K.T., J.C.N.; Methodology: Z.G., K.T., J.C.N.; Validation: Z.G., J.C.N.; Formal analysis: Z.G., J.C.N.; Investigation: K.T., J.C.N.; Resources: Z.G., K.T., J.C.N.; Data curation: J.C.N.; Writing - original draft: Z.G., K.T., J.C.N.; Writing - review & editing: Z.G., K.T., J.C.N.; Visualization: Z.G., J.C.N.; Supervision: K.T.; Project administration: K.T.; Funding acquisition: K.T.

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Data availability

All data used in our analyses, figures and table are freely available from the Zenodo repository: doi: 10.5281/zenodo.2555154.

Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.200881.supplemental

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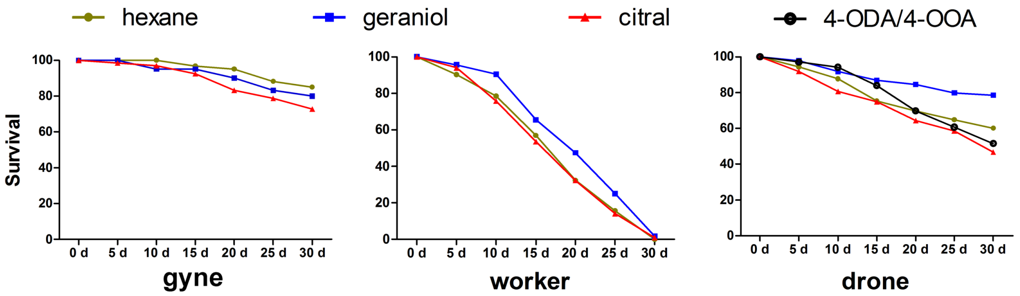

Figure S1. The survival of hornets over 30 d. Survival of the three different castes after conditioning with the different odours. Only drones were conditioned to sex pheromone (4-ODA/4-OOA).

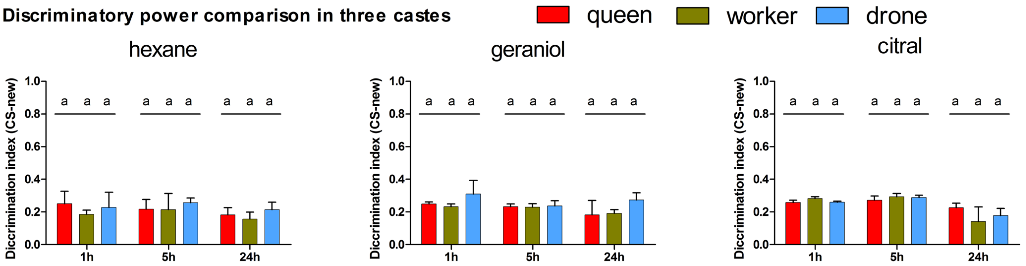

Figure S2. The Discrimination Index in three hornet castes. The Discrimination Index (DI) is the response to the CS minus the response to the unrewarded and novel control odour, nonanal. The DI therefore also compares responses to an odour to which hornets were trained with naïve responses to a novel odour. At each test time point per bee, the DI can have a value of 1, 0, or -1. At the tested memory retention time points, there were no significant differences between the three castes for the tested odours (Tukey HSD test, *P*<0.05). In each plot, the mean±95% CI is shown.

Table S1. The number of hornets used in each experiment to test memory (1 h, 5 h, or 24 h) and very long-lasting memory (30 d). The main components of gyne sex pheromone are 4-ODA and 4-OOA.

					odour	
memory	**Caste**	**Colony**	**hexane**	**geraniol**	**citral**	**4-ODA/4-OOA**
	queen	**C1**	20	20	22	
		C2	20	20	22	
		C3	20	20	22	
LTM	**worker**	**C1**	34	52	33	
		C2	34	52	33	
		C3	34	52	33	
	drone	**C1**	35	28	45	33
		C2	35	28	45	33
		C3	35	28	45	33
	queen	**C1**	17	16	16	
		C2	17	16	16	
LLM		**C3**	17	16	16	
	drone	**C1**	21	22	21	17
		C2	21	22	21	17
		C3	21	22	21	17