

RESEARCH ARTICLE

Evidence for absence of bilateral transfer of olfactory learned information in *Apis dorsata* and *Apis mellifera*

Meenakshi Vijaykumar^{1,2}, Sandhya Mogily¹, Aparna Dutta-Gupta² and Joby Joseph^{1,*}

ABSTRACT

The capacity and condition under which the lateral transfer of olfactory memory is possible in insects is still debated. Here, we present evidence in two species of honeybees, *Apis mellifera* and *Apis dorsata*, consistent with the lack of ability to transfer olfactory associative memory in a proboscis extension response (PER) associative conditioning paradigm, where the untrained antenna is blocked by an insulating coat. We show that the olfactory system on each side of the bee can learn and retrieve information independently and the retrieval using the antenna on the side contralateral to the trained one is not affected by the training. Using the setup in which the memory on the contralateral side has been reported at 3 h after training, we see that the memory is available on the contralateral side immediately after training. In the same setup, coating the antenna with an insulator on the training side does not prevent learning, pointing to a possible insufficiency of the block of odor stimuli in this setup. Moreover, the behavior of the bee as a whole can be predicted if the sides are assumed to learn and store independently, and the organism as a whole is able to retrieve the memory if either of the sides have the memory.

KEY WORDS: PER conditioning, Mushroom body, Olfactory coding, Insect olfaction, Learning and memory

INTRODUCTION

Lateral transfer of information helps environmental stimuli acquired and learned on one side to become accessible to both lobes of a bilateral brain (Aboitiz and Montiel, 2003; Gazzaniga, 2000). This helps to maximize the computational ability of the brain by allowing each side of the brain to co-opt the other for joint decision-making or to avoid duplicity of storage for efficient use of the substrate (Aboitiz and Montiel, 2003; Gazzaniga, 2000, 2014). Information transfer across the midline has also been theorized to be the basis of unified consciousness (Barron and Klein, 2016) and its importance has been highlighted in split-brain patients (Gazzaniga, 2014). In higher mammals, this crucial function is carried out by the corpus callosum, a tissue present in eutherian mammals alone (Aboitiz and Montiel, 2003; Gazzaniga, 2000, 2014; Suárez et al., 2014). The formation of the corpus callosum has been suggested to be an evolutionary innovation (Mihirshahi, 2006), highlighting the importance of developing and evolving the process of transfer of information as an evolutionarily stable strategy. Does this

evolutionary jump have correlates in invertebrates such as insects? Despite their primitive nature, insects are known to be able to perform complex tasks with their rather simple brains consisting of a few 100,000 neurons. Insects such as ants, wasps and honeybees, belonging to the order Hymenoptera, can perform complex tasks involving locating food sources, nesting sites and foraging back and forth between the food source and nest, which would require coordination of a range of modalities (Hansson and Stensmyr, 2011; Kaupp, 2010; Matsumoto et al., 2012; Roper et al., 2017; Sanes and Zipursky, 2010; Su et al., 2009).

In free-flying bees (Masuhr and Menzel, 1972), it was reported that side-specific olfactory conditioning does not transfer to the contralateral side. It was later reported that in *Apis mellifera*, if the bee is trained in the proboscis extension response (PER) to associate an odor with reward when a stimulus is applied to only one antenna, the odor memory can be retrieved by applying trained odor to the untrained contralateral antenna, 3 h after training (Sandoz and Menzel, 2001). In these experiments, a wall was used to separate the two antennae and deliver the odor in a side-specific manner, arguing that blocking the antenna using a coating influences the context of training and impairs transfer. In their study, 3 h post training, up to 50% of the bees responded by extending their proboscis when the learned odor and not a novel odor was applied only to the contralateral antenna, suggesting the presence of a commissure relaying encoded odor-specific memory between sides. Guo et al. (2016) reported changes on a molecular level in the contralateral side of *A. mellifera* after training even if the contralateral side was isolated by coating the antenna. That study, however, did not show transfer using behavior, compared with controls trained with both sides closed. This group used silicon paste to block one antenna while training the exposed antenna to an odor. Post 24 h transcriptomic analysis was carried out and the results showed an upregulation in memory and learning-related genes on the untrained side of the brain, indicating a possible lateral transfer of this learned information and memory. The above experiments and works pointed to the possible presence of a commissure dedicated to the relaying of olfactory learned information from one brain lobe to the other. If this is true, then recording the activity of the neurons in this commissure would also provide insight into the nature of olfactory code, an exciting prospect.

Work in our laboratory recently showed the presence of bilateral extrinsic neurons of the mushroom body calyx in a species of grasshopper, *Hieroglyphus banian* (Singh and Joseph, 2018 preprint). In addition, a cluster of lateral horn neurons in *Schistocerca americana* have been shown to have a bilateral innervation (Gupta and Stopfer, 2012). Thus, there are very few possible substrates for lateral transfer of olfactory memory in insects and none reported in Hymenoptera. We attempted to look for the neuronal basis of the phenomenon of bilateral transfer of information in a species of honey bee native to South East Asia, *Apis dorsata*, also referred to as the giant honey bee or the rock bee, which is one of the crucial pollinators in the region. In our

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laboratory, the olfactory pathway and PER conditioning in *A. dorsata* have been shown to be very similar to those of *Apis mellifera* (Mogily et al., 2018 preprint). We trained *A. dorsata* in PER conditioning, pairing odor on one side with reward while the contralateral side was closed with acrylic paint (Letzkus et al., 2006), and tested for retention on the contralateral side at 3 h post training and found no transfer. While testing, the trained side antenna was closed with acrylic paint and the untrained side was left open. Upon repeating this with *A. mellifera*, the results were consistent with our results in *A. dorsata*. The learning rate and retention rate when both antennae are open is predictable by a model in which the bee decides to extend the proboscis if either of the two sides decides to extend the proboscis independently. To explain the discrepancy between these results and those from Sandoz and Menzel (2001), in the present study, we repeated the procedure by Sandoz and Menzel (2001) using a partition of the kind used in that study to prevent odor from reaching the untrained antenna, and carried out two control experiments. In the first experiment, we tested memory on the contralateral side immediately after training itself without a 3 h delay, and found it to be present. In the second experiment, even when the antenna on the side being trained was covered with acrylic, the bees learned when the isolation was attempted using the wall partition, indicating that the wall is not an effective way to isolate one antenna from the other in an experimental setting. These results from learning assays together with the absence of visible bilateral tracts between the olfactory pathway tract-tracing experiments (Mogily et al., 2018 preprint) lead us to conclude that the olfactory pathways on the two sides of the brain learn independently and decide on the olfactory-associated PER behavior independently.

MATERIALS AND METHODS

Bee collection

Apis dorsata Fabricius 1793 foragers were collected at 09:00 h from the flower sources such as *Turnura subtula*, *Tecoma stans* and *Eucalyptus globulus*. The bees were immobilized by cooling at 4°C for 10 min followed by mounting and tethering them in plastic holders using insulation tape. The bees were allowed to familiarize with this situation for 2 h and then training was carried out. Fifteen minutes before training, generic acrylic paint (Pebeo Studio Acrylics) was gently applied to one of the two antennae. Two control groups were always maintained during the training procedure, namely groups with both antennae open and both antennae blocked. Efficiency of the block was confirmed by the absence of learning in the group with antennae blocked, and PER rates of this group were used as baseline values for comparisons. *Apis mellifera* Linnaeus 1758 foragers were collected at the entrance of the hive box at 09:00 h and the same mounting and acclimatization procedure as that for *A. dorsata* was maintained.

Side-specific training for *A. dorsata* and *A. mellifera*

1-Hexanol (Sigma Aldrich) was used to train the bees. 1-Geraniol (Sigma Aldrich) was used to check for discrimination at 3 h in *A. mellifera*. A total of 149 *A. dorsata* were used for the side-specific training: $n=51$ for the untrained antenna test, $n=34$ for the trained antenna test, $n=25$ for the open antenna control group and $n=39$ for the closed antenna control group. Once the acrylic paint was coated and dried, the bees were divided into the three groups: one experimental, either trained antenna test or untrained antenna test, and the two control groups. Each bee was placed on the pedestal for 14 s followed by the onset of the odor for 4 s (conditioned stimulus, CS); the 30% sucrose reward (unconditioned stimulus, US) was presented to the bee at the third second of odor onset and held for 3 s

(a 4 s CS and a 3 s US with 2 s overlap). Odor was delivered as a constant flow of air applied to the antenna via a 5 mm diameter tube placed 4 cm away from the antennae. Odor was driven into the airstream from a 30 ml glass bottle by pressurized air controlled by a valve. Glass bottles containing the aromatic liquid odors were vacuum sealed and odor delivery was carried out via Teflon tubing connected to the glass bottle. A computer program controlled the valve and light-emitting diodes that signaled the experimenter. In all the experiments, an air suction exhaust was placed behind the animal so as to remove the odor after it had blown over the antennae. The bee would respond to the presence of the US by exhibiting PER (Bitterman et al., 1983; Matsumoto et al., 2012). A 10 min inter-trial interval was maintained between CS–US pairings and five trials were carried out with the entire training procedure lasting for 1 h. The bees that spontaneously exhibited proboscis extensions were eliminated from the study. During the training, if the bees extended their proboscis within 3 s of the odor onset (CS) they were counted as having odor-evoked PER. For *A. dorsata* bees that were trained with one antenna and checked with the same antenna (trained check), the acrylic coat was left intact on the untrained antenna. For the untrained test bees, the block was removed gently post training and the trained side was coated 15 min before testing. The schematic of the set of experiments is given in Figs 1A and 2A.

To test that the acrylic paint was not causing damage to the antenna, in a group of *A. dorsata* bees ($n=17$), the acrylic paint was applied to both the antennae and left for 1 h (similar to the training period) (Fig. S2). The coat was then peeled off and the bees were trained and tested for PER conditioning (Bitterman et al., 1983). To confirm that the procedure of removing the paint was not causing a loss of memory by stress, a set of bees ($n=12$) was first trained as per the one antenna blocked training protocol, and 15 min before the retention test, a coat of acrylic paint was applied on both the antennae. Once dried, the coat was peeled off from the trained side antenna. The bees were then tested for retention of the odor memory. In all cases, identical protocols were followed for a total of $n=104$ *A. mellifera* in identical experiments, where $n=59$ bees were used for the untrained antenna test and of the 59 once tested for retention at the third hour, $n=52$ bees were further tested with both their antennae open after peeling off the paint coat from the trained antenna (trained antenna test), those bees whose antenna were damaged during the process of uncoating were eliminated from the test, and $n=45$ bees were used for the closed antennae control group. A schematic representation of the experiments is given in Figs 1B and 2B.

Checking for contextual stimulus

In order to confirm that coating with paint does not act as a contextual stimulus, $n=37$ *A. mellifera* bees were first trained with either antenna covered with a coat of acrylic paint. The trained bees were then divided equally into two groups: one set of bees ($n=19$) was tested for retention at 3 h with the coat on, and the other set ($n=18$) was tested for retention with the coat removed. The experimental procedure is represented in Fig. 4.

Side-specific training with wall partition

We carried out the side-specific training for $n=29$ *A. mellifera* using a wall barrier as specified in the protocol in Sandoz and Menzel (2001). A plastic wall (40×50 mm) with a cut in the shape of the side profile of the bee in its holder was used to separate the two antennae. The wall was placed such that the mandible and proboscis were adjusted slightly to one side depending on which antenna was to be trained. The spaces between the wall and the bee's head were sealed

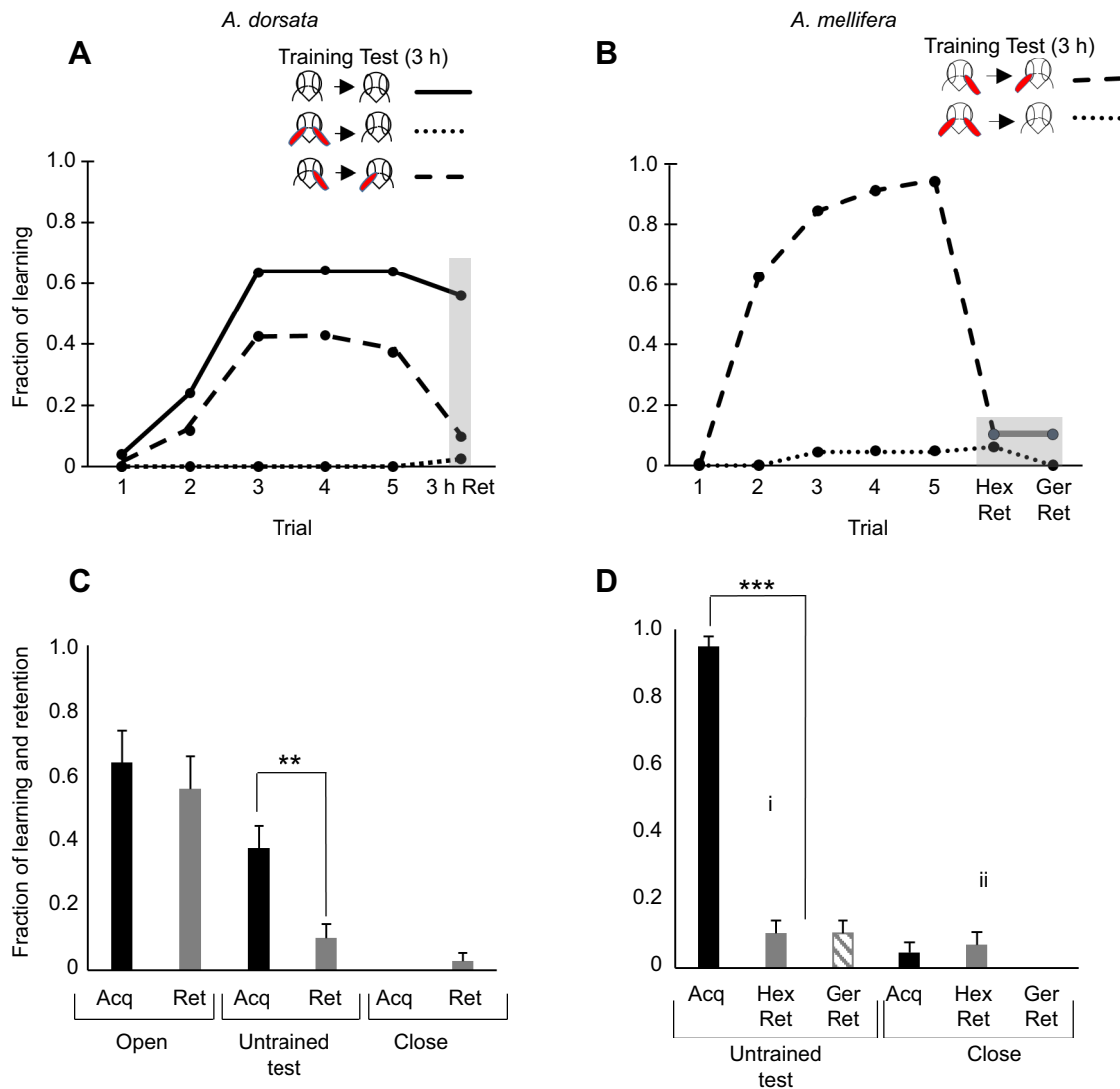


Fig. 1. Testing for lateral transfer of olfactory memory in *Apis dorsata* and *Apis mellifera*. (A,B) The schematic representation of the procedure is shown above the plots. The red cover on the antennae represents the acrylic coat. (A) Testing for lateral transfer at 3 h (3 h Ret) in *A. dorsata* ($n=25$ open antenna control, $n=25$ untrained antenna test, $n=39$ closed antenna control) shows that memory on the untrained side at 3 h (gray box) is nearly zero ($P=0.045$, Cochran's $q=4$). (B) *Apis mellifera* ($n=59$ untrained antenna test, $n=45$ closed antenna control) also did not show any significant lateral transfer at 3 h (gray box) ($P=0.22$, Cochran's $q=1.5$), though they had 95% acquisition. All bees that responded to 1-hexanol (Hex Ret) responded to 1-geraniol (Ger Ret), as denoted by the gray line, on the transferred side at 3 h indicating no discrimination ($P=1$). (C) *Apis dorsata* showed a significant difference between the learning (Acq) and retention (Ret) in the side contralateral to the trained side, and no significant difference between the (i) untrained retention and (ii) closed antenna control group. The closed antenna group showed 0% learning and 2% retention which may indicate the success rate of our method of coating the antenna for blocking. (D) *Apis mellifera* showed 95% acquisition but the transfer of memory to (i) the contralateral side was similar to that of the group with (ii) both antenna closed. Bar graphs are means \pm s.e.m. ** $P<0.01$; *** $P<0.001$.

with wax. For $n=19$ bees, the training antenna was covered with acrylic paint and for $n=10$ bees the training antenna was left open. An exhaust vent behind the setup constantly drew the air with the applied odor away from the preparation (Fig. S1).

Statistical analysis

Statistical analysis for the experiments was performed using Cochran's q -test, a non-parametric test for dichotomous values; we corrected the value of α using Bonferroni correction. MATLAB and Microsoft Excel were used to create the graphs and figures. The datasheets for the behavioral experiments are available from the Dryad Digital Repository (<https://doi.org/10.5061/dryad.b9629q7>; Vijaykumar et al., 2019). The sheets are named corresponding to the figure numbers.

RESULTS

Evidence for absence of lateral transfer of memory in *A. dorsata* and *A. mellifera*

The learning rate for *A. dorsata* with one antenna blocked at the end of the fifth trial reached 37.7% ($n=51$) (Figs 1A,C and 2A,C). The learning and retention with both antennae closed ($n=39$) was negligible (2%), as expected. The retention test with the untrained antenna was not significant compared with the condition where both the antennae were closed ($P=0.045$, Cochran's $q=4$), consistent with the absence of lateral transfer of memory.

For *A. mellifera*, the acquisition reached 95% ($n=59$) at the end of the fifth trial (Fig. 1B,D). Given the high learning and acquisition rate, only the both antennae closed control group ($n=45$) was maintained through the training procedures. The

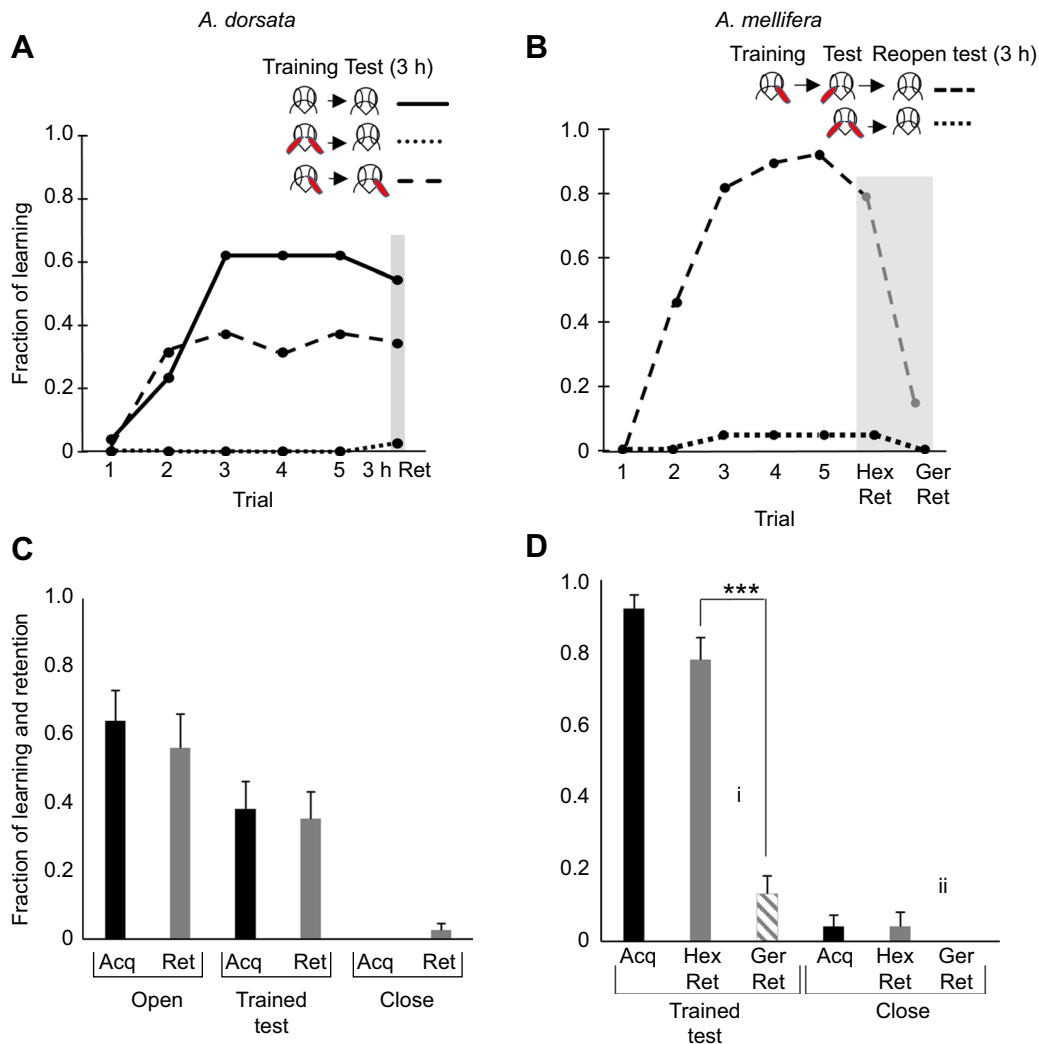


Fig. 2. Memory is retained on the trained side in *Apis dorsata* and *Apis mellifera*. (A,B) The schematic representation of the procedure is shown above the plots. The red cover on the antennae represents the acrylic coat. (A) *Apis dorsata* ($n=25$ open antenna control, $n=34$ trained antenna test, $n=39$ closed antenna control) learned and retained memory after 3 h (3 h Ret, gray box) with one antenna if tested with the same antenna, and retention was found to be significantly higher than that of the closed antenna group ($P=8 \times 10^{-4}$, Cochran's $q=11.15$). The bees also showed lower acquisition and retention with one antenna compared with two antennae training, but this was not significant ($P=0.13$, Cochran's $q=2.22$). (B) For *Apis mellifera* ($n=52$ trained antenna reopened test, $n=45$ closed antenna control), coating and removing the coat did not affect the acquired memory on the trained side (gray box); the difference between the retention of trained tested and closed control group was significant. *Apis mellifera* showed 95% acquisition, and retention to 1-hexanol was seen to be stable once the trained ipsilateral antenna was unblocked at 3 h ($P=1.8 \times 10^{-9}$, Cochran's $q=36.1$). They also showed clear discrimination between 1-hexanol (Hex Ret) and geraniol (Ger Ret), as denoted by the gray dashed line ($P=1.4 \times 10^{-6}$, Cochran's $q=32.1$), showing that the covering and uncovering did not stress the bee and cause memory loss. (C) *Apis dorsata* learned and retained memory after 3 h with one antenna and two antennae. The acquisition and retention with two antennae were approximately the same, as would be predicted from the rates with one antenna if the olfactory pathway of each side learned and retained memory independently. (D) *Apis mellifera* showed 95% learning. The trained antenna, once reopened after the untrained antenna check, showed significant discrimination (i) between the trained odor Hex Ret and the untrained odor, Ger Ret. There was no discrimination (ii) between the two odors by the bees trained with both antennae closed. Bar graphs are means \pm s.e.m. *** $P < 0.001$.

retention rate in the contralateral test group was 10.1% and not different from the retention of the both antennae closed group 6.8% ($P=0.22$, Cochran's $q=1.5$). There was also no odor discrimination exhibited by the bees that had contralateral retention, and the bees that responded to 1-hexanol also responded to 1-geraniol (Fig. 1B,D).

Is there interaction of PER conditioned memories on the two sides in decision-making of the bees?

If a decision by any of the two sides can cause PER, then one would expect that the probability of evoking PER should be predictable from the learning rate and retention rates of one side alone, which

would be the sum of the probabilities of either of them deciding to evoke PER minus the probability that both of them would. For the learning rate, the prediction would be $2 \times 0.38 - 0.38 \times 0.38 = 0.62$, which is approximately equal to 0.64, the observed learning rate in *A. dorsata*. The same should follow for retention ($2 \times 0.35 - 0.35 \times 0.35 = 0.57$), which is approximately equal to 0.56, the observed retention rate in *A. dorsata*. These predictions match, indicating that the two sides make decisions independently and that there is no lateral transfer of olfactory learnt memory, either while learning or after 3 h. Thus, performance in learning and retention were consistent with the olfactory pathways in the two sides acting independently.

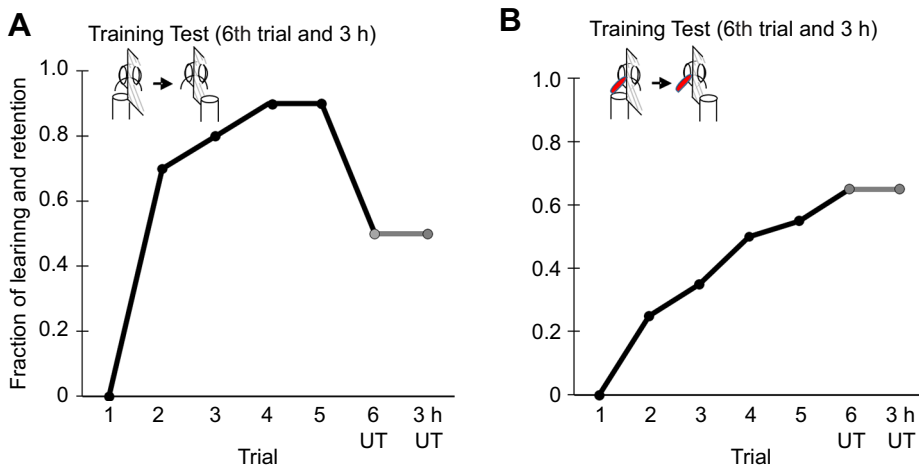


Fig. 3. Training using a plastic wall to separate the two antennae shows learning in the contralateral side even as training is taking place. (A) *Apis mellifera* exhibited a proboscis extension response to the trained odor on the untrained (UT) side at the sixth trial when trained with the plastic partition for isolation. This memory was retained on the untrained side, denoted by the gray line, at 3 h post training (3 UT; $n=10$). (B) *Apis mellifera* exhibited learning on the trained side even when the trained antenna was insulated with acrylic and with a plastic partition used for isolation. A retention of 65% was seen by the untrained antenna at the sixth trial (6 UT); this memory was retained on the untrained side at 3 h post training, denoted by the gray line (3 h UT; $n=19$).

***Apis dorsata* showed high memory retention on the trained side, be it left or right**

The learning rate reached 38% at the end of the fifth trial rate for the bees ($n=34$) with one antenna blocked (Fig. 2A,C). The learning rate of the bees with both antenna open reached 64% ($n=25$) at the end of the fifth trial, and bees with both antennae closed ($n=39$) showed 0% learning. Of the one antenna trained bees that learned, 92% retained information on the trained side at 3 h. Open antenna trained bees exhibited 87% retention, and a 2% retention was seen in bees with both the antennae closed. The difference in retention rate between the one antenna trained and tested group and both antennae trained and tested control group was found to be insignificant ($P=0.13$, Cochran's $q=2.22$). A significant difference was found between the retention of the one antenna trained and tested group and the group of closed antennae bees ($P=8\times 10^{-4}$, Cochran's $q=11.15$; Fig. 2A). No significant difference was seen between the acquisition and retention. Further, there was no significant difference in learning and retention observed between left antenna or right antenna trained bees.

Retention at 3 h on the trained side in *A. mellifera* that do not have the memory on the untrained side

To further confirm that lack of memory on the untrained side seen in the trained *A. mellifera* honey bees ($n=52$) is not because of the loss of memory on the trained side, the same bees that were trained with one antenna and tested with the contralateral (untrained) antenna were checked for trained antenna retention after removing the cover from the trained antenna (Fig. 2B,D). Retention upon carrying out this paradigm was 82% ($n=53$) and significantly above that of the both antenna closed group ($P=1.8\times 10^{-9}$, Cochran's $q=36.1$; Fig. 2B). Retention was also significantly higher compared with the same bees checked for untrained antenna retention ($P=8.7\times 10^{-9}$, Cochran's $q=33.1$). Moreover, discrimination between hexanol and the novel odor geraniol was also observed in the bees checked for discrimination with their training antenna reopened ($P=1.4\times 10^{-8}$, Cochran's $q=32.1$). Thus the bees that do not show retention at 3 h on the untrained side do preserve it on the trained side. No significant difference in learning and retention was seen between the left antenna or right antenna trained bees.

Learning rate on the contralateral side when using a wall to separate the antennae

Apis mellifera ($n=29$) were trained with a wall separating the antenna. The learning rate reached 90% for bees ($n=10$) progressively over the training. When tested, 50% learning was seen on the antenna on the

other side of the wall in the sixth trial itself (Fig. 3A). In the same setup with a wall, even when the trained antenna was blocked with acrylic paint while being trained, the bees learned gradually over the five trials (Fig. 3B). They attained a learning percentage of 65% ($n=19$) by the fifth trial despite having the training antenna blocked. When the untrained antenna was tested in the sixth trial, the 65% learning was maintained. This percentage of bees retained the memory for 3 h. Despite our best efforts, it seemed impossible to robustly separate the two antennae with a wall.

The acrylic paint block does not act as a contextual stimulus

Apis mellifera ($n=37$) were trained with one antenna covered and split into two groups. One was tested without removing the coating

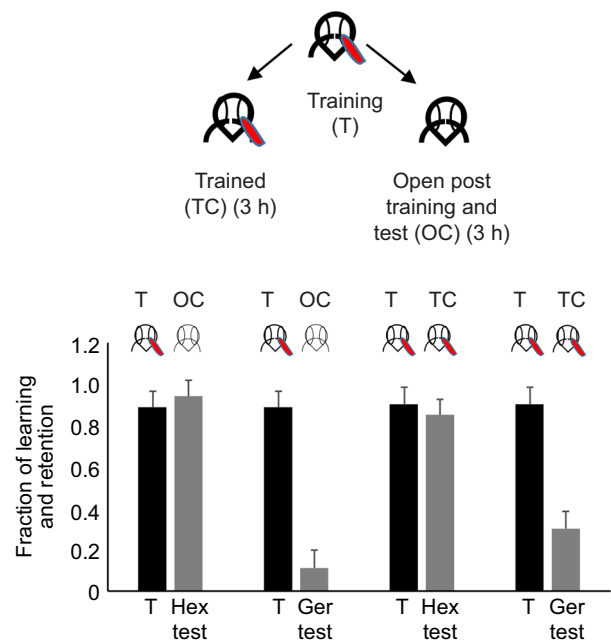


Fig. 4. Learning of contextual stimuli. *Apis mellifera* bees were trained with one antenna covered (T; $n=37$). At 3 h, the bees were divided randomly into two sets: one set of bees was checked for retention with the block opened (OC; $n=18$) and the other was checked for retention in the trained condition (TC; $n=19$). Bees with their antenna opened (OC) showed 100% retention while the bees with one antenna covered (TC) and checked showed 94% retention. The bees with both antennae open during the retention test showed only marginally better discrimination (Ger test OC and Ger test TC) compared with the bees with one antenna covered. Bar graphs are means \pm s.e.m.

($n=19$) and the other was tested with the coating removed ($n=18$). There was no observable difference in the percentage of retention between the bees with one antenna covered and the bees with the antennae uncovered at the time of testing ($P=0.8$, Cochran's $q=0.05$). No significant difference was seen in the discrimination either ($P=0.1$, Cochran's $q=2.6$). The bees with their antenna uncovered discriminated marginally better than the bees with the one antenna covered (Fig. 4). Thus the bees did not learn the stimulus to one antenna alone while the other was covered with acrylic as a different stimulus than odor received on both antennae simultaneously.

Does the process of peeling away the paint shock the bee into forgetting?

For *Apis dorsata* ($n=12$), we tested whether the coating and uncoating of the acrylic paint shocked the bees into forgetting (Fig. 5). To check this, we first trained the bees with either one of the antenna blocked. Fifteen minutes before the retention test, we coated the trained antenna with the paint, waited for it to dry, and then uncovered the coat before testing for retention. The process of removing the coating did not cause the bees to forget the learned information, and memory retention was 99%.

Effect of acrylic paint coat on learning

The antennae of *A. dorsata* ($n=19$) were covered with acrylic paint and left for 1 h. The paint was peeled off after 1 h and the bees were then trained. Over the five trials, the bees learned equally as well as bees with both antennae open. The learning rate at the fifth trial was 76% for these bees (Fig. S2). There was no significant difference in the learning rate between bees trained with both antennae open and bees coated with the paint ($P=0.3$, Cochran's $q=0.8$).

DISCUSSION

Connections between the olfactory pathways of the two sides of the brain are prominent in lower and higher complex vertebrates in the Kingdom Animalia (Suárez et al., 2014). Bilateral connections have been shown in the visual system of insects (de Lussanet and Osse, 2012; Roper et al., 2017; Sanes and Zipursky, 2010). From the point of fundamental behavior, the bilateral integration of vision would be advantageous especially for optimal orientation and direction alignment. For insects such as hymenopterans, olfaction is a dominant sense, imperative for the animal's survival. However, the question remains of how pivotal this bilateral integration and transfer of information is in other modalities such as olfaction. The

presence of a number of known bilateral neurons involved in PER associative conditioning with odor supports the possibility of transfer of association from one side of the brain to the other in the olfactory pathway. One multisensory mushroom body extrinsic neuron, the PE1, has been shown to display learning-related plasticity with respect to olfactory information in a time-dependent manner (Mauelshagen, 1993; Menzel, 2012). This neuron has its soma located ventro-medial to the α -lobe and arborizes adjacent to the contralateral α -lobe (Mauelshagen, 1993; Menzel and Muller, 1996; Okada et al., 2007). Given its anatomic positioning and learning-dependent functional plasticity, the question about whether olfactory learned information with one antenna in honey bees can be retrieved from the contralateral side seems a possibility. The ventral unpaired median neuron of the maxillary neuromere (VuMmx1) has its soma located at the subesophageal ganglion, its branches innervate and the basal lip of the mushroom body, lateral horns and antennal lobes bilaterally (Hammer, 1993, 1997; Hammer and Menzel, 1995). This bilateral neuron has also been shown to be octopaminergic positive and, more crucially, it displays plasticity upon olfactory learning (Hammer, 1993; Rein et al., 2013). This neuron can possibly act as the placeholder for reward bilaterally. However, the bilateral transfer of olfactory memory is claimed to be odor specific, and this would require either many neurons to use a population code or a very few neurons to use a complex temporal code. The evidence for using either of these by PE1 and VUM neurons is minimal. Thus, it is not clear how the abovementioned neurons can be used to associate reward bilaterally in an odor-specific way. Using the wall setup, Strube-Bloss et al. (2016) showed that the mushroom body output neuron population that codes for the rewarded stimulus has a different representation on the untrained side after training on the trained side.

To investigate the existence of lateral tracts in the olfactory pathway and the mechanism of lateral transfer of olfactory information and memory, we needed to first validate the existence of lateral transfer using a robust behavioral protocol. Given that this behavioral phenomena was said to have been observed in *A. mellifera* (Sandoz and Menzel, 2001), we attempted to observe the same behavioral output in a native Asian honey bee, *A. dorsata*. However, over the course of our behavioral study, we did not observe lateral memory transfer, thus diminishing the possibility of finding such tracts in *A. dorsata*. These results remained consistent when we repeated the experiments using *A. mellifera*.

Learning and decision-making on the two sides of *A. dorsata* are independent

We tested the hypothesis of whether the phenomenon of lateral transfer of olfactory information exists in *A. dorsata*. Our results in *A. dorsata*, however, were quite contrary to the results from previous works on *A. mellifera*, and we not only found negligible transfer of olfactory learnt information, but it was also observed that the learning rate with one antenna in use was reduced significantly. We surmise that for this species of honey bee the learning is independent, implying the parallel working of both antennal lobes and olfactory pathways. In the same protocol, the *A. mellifera* learning rate with one antenna reached up to 95%, nearly saturating. In addition, neuron tract-tracing experiments from our laboratory using *A. dorsata* showed no bilateral connections between the mushroom body calyx and the contralateral alpha lobe. Further, no connections were seen between the alpha lobe and the contralateral antennal lobes (Mogily et al., 2018 preprint). These tract-tracing experiments further strengthened the possibility of each lobe processing olfactory information independently. This is consistent

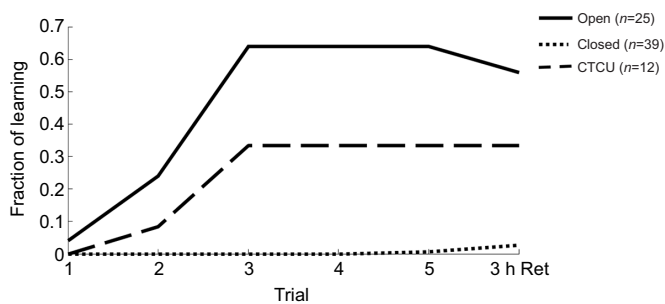


Fig. 5. The process of coating and un-coating does not shock the bee into forgetting or harm the antenna. In *Apis dorsata* bees, the training antenna was coated with paint just prior to the 3 h retention test (3 h Ret). The removing of the coat did not shock the bees into forgetting the acquired memory, as indicated by the 100% retention in the learnt bees (close test cover uncover test, CTCU).

with our result that the performance of bees with both the antenna open can be predicted using performance with one antenna, if independence of decision-making on the two sides is assumed.

Probable ecological significance of parallel pathways

The ecological importance of having independent parallel olfactory pathways in honey bees is still an enigma. Our results in both species were consistent with no transfer of memory from the trained side to the untrained side and each side learning and retrieving independently. Our results remain consistent with the finding in Masuhr and Menzel (1972) that with respect to olfaction, the honey bee seems to use each lobe independently. It is not clear whether there is a set of non-motor, decision neurons that receives input from both sides, or whether the two sides drive the motor neurons and thus the muscles independently, and this requires further investigation. Our results question the possibility of finding robust odor-coding bilateral tracts at a higher level in honeybees.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.V., J.J.; Methodology: M.V., S.M., J.J.; Software: J.J.; Validation: M.V., J.J.; Formal analysis: S.M., J.J.; Investigation: M.V.; Data curation: M.V.; Writing - original draft: M.V., J.J.; Writing - review & editing: M.V., S.M., A.D.-G., J.J.; Visualization: M.V., J.J.; Supervision: A.D.-G., J.J.; Funding acquisition: M.V., A.D.-G., J.J.

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Data availability

The data for the behavioral experiments are available from the Dryad Digital Repository (Vijaykumar et al., 2019): dryad.b9629q7. The sheets are named corresponding to the figure numbers.

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.196584.supplemental>

References

- Aboitiz, F. and Montiel, J.** (2003). One hundred million years of interhemispheric communication: the history of the corpus callosum. *Braz. J. Med. Biol. Res.* **36**, 409-420. doi:10.1590/S0100-879X2003000400002
- Barron, A. B. and Klein, C.** (2016). What insects can tell us about the origins of consciousness. *Proc. Natl. Acad. Sci. USA* **113**, 4900-4908. doi:10.1073/pnas.1520084113
- Bitterman, M. E., Menzel, R., Fietz, A. and Schäfer, S.** (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Psychol.* **97**, 107-119. doi:10.1037/0735-7036.97.2.107
- de Lussanet, M. H. E. and Osse, J. W. M.** (2012). An ancestral axial twist explains the contralateral forebrain and the optic chiasm in vertebrates. *Animal. Biol.* **62**, 193-216. doi:10.1163/157075611X617102
- Gazzaniga, M. S.** (2000). Cerebral specialization and interhemispheric communication: does the corpus callosum enable the human condition? *Brain* **123**, 1293-1326. doi:10.1093/brain/123.7.1293
- Gazzaniga, M. S.** (2014). The split-brain: rooting consciousness in biology. *Proc. Natl. Acad. Sci. USA* **111**, 18093-18094. doi:10.1073/pnas.1417892111
- Guo, Y., Wang, Z., Li, Y., Wei, G., Yuan, J., Sun, Y., Wang, H., Quin, Q., Zeng, Z., Zhang, S. et al.** (2016). Lateralization of gene expression in the honeybee brain during olfactory learning. *Sci. Rep.* **6**, 34727. doi:10.1038/srep34727
- Gupta, N. and Stopfer, M.** (2012). Functional analysis of a higher olfactory center, the lateral horn. *J. Neurosci.* **32**, 8138-8148. doi:10.1523/JNEUROSCI.1066-12.2012
- Hammer, M.** (1993). An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature* **366**, 59. doi:10.1038/366059a0
- Hammer, M.** (1997). Neural basis of associative reward learning in honeybees. *Trends. Neurosci.* **20**, 245-252. doi:10.1016/S0166-2236(96)01019-3
- Hammer, M. and Menzel, R.** (1995). Learning and memory in the honeybee. *J. Neurosci.* **15**, 1617-1630. doi:10.1523/JNEUROSCI.15-03-01617.1995
- Hansson, B. S. and Stensmyr, M. C.** (2011). Evolution of insect olfaction. *Neuron* **72**, 698-711. doi:10.1016/j.neuron.2011.11.003
- Kaupp, U. B.** (2010). Olfactory signalling in vertebrates and insects: differences and commonalities. *Nat. Rev. Neurosci.* **11**, 188-200. doi:10.1038/nrn2789
- Letzkus, P., Ribi, W. A., Wood, J. T., Zhu, H., Zhang, S.-W. and Srinivasan, M. V.** (2006). Lateralization of olfaction in the honeybee *Apis mellifera*. *Curr. Biol.* **16**, 1471-1476. doi:10.1016/j.cub.2006.05.060
- Masuhr, T. and Menzel, R.** (1972). Learning experiments on the use of side-specific information in the olfactory and visual system in the honey bee (*Apis mellifera*). In *Information Processing in the Visual Systems of Anthropods* (ed. R. Wehner), pp. 315-321. Springer Berlin Heidelberg.
- Matsumoto, Y., Menzel, R., Sandoz, J.-C. and Giurfa, M.** (2012). Revisiting olfactory classical conditioning of the proboscis extension response in honey bees: a step toward standardized procedures. *J. Neurosci. Method.* **211**, 159-167. doi:10.1016/j.jneumeth.2012.08.018
- Mauelshagen, J.** (1993). Neural correlates of olfactory learning in an identified neuron in the honey bee brain. *J. Neurophysiol.* **69**, 609-625. doi:10.1152/jn.1993.69.2.609
- Menzel, R.** (2012). The honeybee as a model for understanding the basis of cognition. *Nat. Rev. Neurosci.* **13**, 758-768. doi:10.1038/nrn3357
- Menzel, R. and Muller, U.** (1996). Learning and memory in honeybees: from behaviour to neural substrates. *Annu. Rev. Neurosci.* **19**, 379-404. doi:10.1146/annurev.ne.19.030196.002115
- Mihirshahi, R.** (2006). The corpus callosum as an evolutionary innovation. *J. Exp. Zool. B. Mol. Dev. Evol.* **306**, 8-17. doi:10.1002/jez.b.21067
- Mogily, S., Vijaykumar, M., Sethy, S. K. and Joseph, J.** (2018). Characterization of the olfactory system in *Apis dorsata*, an Asian honey bee. *bioRxiv*. doi: 10.1101/420968
- Okada, R., Rybak, J., Manz, G. and Menzel, R.** (2007). Learning-related plasticity in PE1 and other mushroom body-extrinsic neurons in the honeybee brain. *J. Neurosci.* **27**, 11736-11747. doi:10.1523/JNEUROSCI.2216-07.2007
- Rein, J., Mustard, J. A., Strauch, M., Smith, B. H. and Galizia, C. G.** (2013). Octopamine modulates activity of neural networks in the honey bee antennal lobe. *J. Comp. Physiol. A. Neuroethol. Sens. Neural. Behav. Physiol.* **199**, 947-962. doi:10.1007/s00359-013-0805-y
- Roper, M., Fernando, C. and Chittka, L.** (2017). Insect bio-inspired neural network provides new evidence on how simple feature detectors can enable complex visual generalization and stimulus location invariance in the miniature brain of honeybees. *PLoS. Comput. Biol.* **13**, e1005333. doi:10.1371/journal.pcbi.1005333
- Sandoz, J.-C. and Menzel, R.** (2001). Side-specificity of olfactory learning in the honeybee: generalization between odors and sides. *Learn. Mem.* **8**, 286-294. doi:10.1101/lm.41401
- Sanes, J. R. and Zipursky, S. L.** (2010). Design principles of insect and vertebrate visual systems. *Neuron* **66**, 15-36. doi:10.1016/j.neuron.2010.01.018
- Singh, S. and Joseph, J.** (2018). Evolutionarily conserved anatomical and physiological properties of olfactory pathway till fourth order neurons in a species of grasshopper (*Hieroglyphus banian*). *bioRxiv*. doi: 10.1101/436626
- Strube-Bloss, M., Nawrot, M., P. and Menzel, R.** (2016). Neural correlates of side-specific odour memory in mushroom body output neurons. *Proc. R. Soc. B Biol. Sci.* **283**, 20161270. doi:10.1098/rspb.2016.1270
- Su, C.-Y., Menz, K. and Carlson, J. R.** (2009). Olfactory perception: receptors, cells, and circuits. *Cell.* **139**, 45-59. doi:10.1016/j.cell.2009.09.015
- Suárez, R., Gobius, I. and Richards, L. J.** (2014). Evolution and development of interhemispheric connections in the vertebrate forebrain. *Front. Hum. Neurosci.* **8**, 497. doi:10.3389/fnhum.2014.00497
- Vijaykumar, M., Mogily, S., Dutta-Gupta, A. and Joseph, J.** (2019). Data from: Evidence for absence of bilateral transfer of olfactory learned information in *Apis dorsata* and *Apis mellifera*. Dryad Digital Repository. <https://doi.org/10.5061/dryad.b9629q7>