

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

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## **Abstract:**

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 lack of ability to transfer olfactory associative memory, in a 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. Upon recreating the setup using which the memory on the contralateral side has been reported at three hours 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 block of odor stimuli in this setup. Moreover the behaviour 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.

## **Introduction:**

Lateral transfer of information helps environmental stimuli acquired, and learnt on one side to become accessible to both lobes of a bilateral brain (Aboitiz and Montiel, 2003; Gazzaniga, 2000). This aids maximizing 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; Gazzaniga, 2014). Information transfer across the midline has also been theorised 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; Gazzaniga, 2014; Suarez 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 (ESS). Does this evolutionary jump have correlates in invertebrates such as insects? Insects despite their primitive nature, are known to be able to perform complex tasks with their rather simple brains consisting of a few 100,000 neurons. Insect such as ants, wasps, honeybees, especially those 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 et al., 2010; Su et al., 2009).

In free-flying bees (Masuhr et al., 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 proboscis extension response (PER) to associate an odor with reward when stimuli is applied to only one antenna, it can be retrieved by applying trained odor to the untrained contralateral antenna, three hours 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 three hours post training, up to 50% of the bees responded by extending 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. In 2016, Guo. Y et.al reported the changes on a molecular level in the contralateral side after training even if the contralateral side was isolated by coating the antenna (Guo et al., 2016). This study, however, did not show transfer using behaviour, compared to 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 hours transcriptomic analysis was carried out and the results showed an up-regulation 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 us 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 (MB) in a species of grasshopper, *Hieroglyphus banian* (Singh and Joseph, 2018). In addition, a cluster of lateral horn (LH) 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 lab, olfactory pathway and PER conditioning in *Apis dorsata* has been shown to be very similar to *Apis mellifera* (Mogily et al., 2018). We trained *Apis*

*dorsata* in PER conditioning to, 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 hrs 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 *Apis mellifera* the results were consistent with our results in *Apis dorsata*. The learning rate and retention rate when both antennae are open is predictable by a model in which the bee decides to extend proboscis if either of the two sides decides to extend proboscis independently. To explain the discrepancy between these results and those from Sandoz and Menzel (2001) we repeated the procedure by Sandoz and Menzel (2001), using a partition of the kind used in that work to prevent odor from reaching the untrained antenna and carried out two control experiments. One where we tested memory on the contralateral side immediately after training itself without a 3 hour delay and found it to be present. Second, 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 wall is not an effective way for isolating one antenna from the other in our hands. These results from learning assays together with the absence of visible bilateral tracts between the olfactory pathways tract-tracing experiments (Mogily et al., 2018) lead us to conclude that the olfactory pathways on the two sides of the brain learn independently and decide on the olfactory associated PER behaviour independently.

## **Materials and methods:**

### **Bee collection:**

*Apis dorsata* foragers were collected at 9 am from the flower sources such as *Turnura subtula*, *Tecoma stans*, *Eucalyptus globulus*. The bees were immobilized by cooling at 4°C for ten minutes followed by mounting and tethering them in plastic holders using insulation tape. The bees were allowed to familiarize with this situation for two hours and then training was carried out. 15 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 antenna 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 was used as baseline for comparisons. *Apis mellifera* foragers were collected at the entrance of the hive box at 9 AM and the same mounting and acclimatization procedure as that for *Apis dorsata* was maintained.

### **Side-Specific Training for *Apis dorsata* and *Apis mellifera*:**

1-hexanol (Sigma Aldrich) was used to train the bees. Geraniol (Sigma Aldrich) was used to check for discrimination at 3 hours in *Apis mellifera*. A total of 149 *Apis dorsata* bees 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 placed on the pedestal for 14 seconds followed by the onset of the odor for 4 seconds (Conditioned stimulus-CS), the 30% sucrose reward (Unconditioned stimulus-US) was presented to the bee at the 3<sup>rd</sup> second of odor onset and held for 3 seconds. (A 4 second CS and a 3 second US with 2 second overlap) Odor was delivered as a constant flow of air applied to the antenna via a 5mm diameter tube placed 4cm away from the antennae. Odor was driven into the airstream from a 30ml glass bottle by pressurised air controlled by a valve. Glass bottles containing the aromatic liquid odors, were vacuum sealed and an odor delivery was carried out by a Teflon tubing connected to the glass bottle. A computer program controlled the valve and light emitting diodes that signalled 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 minute inter-trial interval (ITI) was maintained between CS-US pairings and 5 trials were carried out with the entire

training procedure lasting for one hour. The bees that spontaneously exhibited proboscis extensions were eliminated from the study. During the training if the bees extended their proboscis within 3 seconds of the odor onset (CS) they were counted as having odor evoked PER. For *Apis 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 minutes before testing. The schematic of the set of experiments is given in (Fig. 1 A, and Fig. 2 A).

To test that the acrylic paint was not causing damage to the antenna, in a group of *Apis dorsata* bees (n=17), the acrylic paint was applied to both the antennae and left for an hour (similar to the training period) (Supplementary Fig 2). 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) were first trained as per the one antenna blocked training protocol and 15 minutes 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 *Apis mellifera* bees in identical experiments where n= 59 bees were used for the untrained antenna test and of the 59 once tested for retention at the 3<sup>rd</sup> 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, n=45 bees were used for the closed antennae control group. A schematic representation of the experiments are given in (Fig. 1 B and Fig. 2, B)

### **Checking for Contextual Stimulus:**

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

### **Side-specific training with wall partition:**

We carried out the side-specific training for n=29 *Apis mellifera* bees using a wall barrier as specified in protocol in (Sandoz and Menzel, 2001). A plastic wall (40mm x 50mm) cut in the shape 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 bee's head were sealed 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 (Supplementary Fig.1).

### **Statistical Analysis:**

Statistical analysis for the experiments was done using Cochran's q test a non-parametric test for dichotomous values, we corrected the value of  $\alpha$  using Bonferroni correction. MATLAB and Microsoft Excel were used to create the graphs and figures. The xlsheets for the behavioural experiments were uploaded to the Dryad Digital Repository, and the data is available from the repository by using the following link <https://doi.org/10.5061/dryad.b9629q7>. The sheets are named corresponding to the figure numbers.



## Results:

### Evidence for absence of lateral transfer of memory in *Apis dorsata* and in *Apis mellifera* (A+/0) :

The learning rate for *A. dorsata* bees with one antenna blocked at the end of the 5<sup>th</sup> trial reached 37.7% (n=51) (Fig. 1 A, C and Fig. 2 A, 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 to the condition where both the antenna were closed ( $p=0.045$  Cochran's  $q=4$ ) consistent with the absence of lateral transfer of memory.

For *Apis mellifera* bees the acquisition reached 95% (n = 59) at the end of the 5<sup>th</sup> trial (Fig. 1 B, D). Given the high learning and acquisition rate, only the, both antennae closed control group (n=45) was maintained through the training procedures. The retention rate in the contra test group was 10.1% and not different from the retention of the both antenna closed group 6.8% ( $p=0.22$  Cochran's  $q=1.5$ ). There was also no odor discrimination exhibited by the bees which had contra retention and the bees which responded to 1-Hexanol also responded to 1-Geraniol (Fig. 1 B, D).

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

If 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 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  $(2*0.38-0.38*0.38=0.62)$ , approximately equals 0.64, the observed learning rate in *Apis dorsata*. Same should follow for retention  $(2*0.35-0.35*0.35=0.57)$  approximately equal 0.56, the observed retention rate in *Apis dorsata*. These predictions match, indicating that the two sides make decisions independently and indicates no lateral transfer of olfactory learnt memory, while

learning or after 3 hours. Thus performance in learning and retention were consistent with the olfactory pathways in the two sides acting independently

***Apis dorsata* showed high memory retention on trained side, be it left or right, (A+/0):**

The learning rate reached 38% at the end of the 5<sup>th</sup> trial rate for the bees (n=34) with one antenna blocked (Figure 2 A, C). The learning rate of the bees with both antenna open reached 64% (n=25) at the end of the 5<sup>th</sup> trial and bees with both antennae closed (n=39) showed 0% learning. 92% of the one antenna trained bees that learned, retained information on the trained side at 3 hours. 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, tested and the group of closed antennae bees ( $p=8 \times 10^{-4}$ , Cochran's  $q= 11.15$ ) (Figure 2 A). 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 3hours on the trained side in *Apis mellifera* bees 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 *Apis 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 contra (untrained) antenna were checked for trained antenna retention after removing the cover from the trained antenna (Figure 2 B, D). Retention upon carrying out this paradigm was 82% (n= 53) and significantly above both antenna closed group ( $p=1.8 \times 10^{-9}$ , Cochran's  $q=36.1$ ) (Figure 2 B). Retention was also significantly higher compared to the same bees checked for

untrained antenna retention ( $p=8.7 \times 10^{-9}$ , Cochran's  $q=33.1$ ). Moreover discrimination between Hexanol and novel odor Geraniol was also observed in the bees checked with their training antenna reopened ( $p=1.4 \times 10^{-8}$ , Cochran's  $q=32.1$ ). Thus the bees that don't show retention at 3 hours 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 in contra side when using a wall to separate the antennae:**

*Apis mellifera* bees ( $n=29$ ) were trained with a wall separating the antenna. Learning rate reached 90% for ( $n=10$ ) bees progressively over the training. When tested, 50% learning was seen on the antenna on the other side of the wall in the 6<sup>th</sup> trial itself (Fig. 3 A). 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 5 trials (Fig. 3 B). They attained a learning percentage of 65% ( $n=19$ ) by the 5<sup>th</sup> trial despite having the training antenna blocked. When the untrained antenna was tested in the 6<sup>th</sup> trial the 65% learning was maintained. This fraction of bees retained the memory for 3 hours. 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* bees ( $n=37$ ) were trained with one antenna covered and split into two groups. One was tested without removing the coating ( $n=19$ ) and the other was tested with the coating removed ( $n=18$ ). There is 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 is covered with acrylic as a different stimulus than odor received on both the 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. 15 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 learnt information and memory retention was 99%.

**Effect of acrylic paint coat to the antenna on learning:**

The antennae of *Apis dorsata* (n=19) was covered with acrylic paint and left for an hour. The paint was peeled off after one hour and the bees trained. Over the 5 trials the bees learnt equally well as bees with both antennae open. The learning rate at the 5<sup>th</sup> trial was 76% for these bees (Supplementary Figure 2). 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 (Suarez 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 behaviour, 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 like olfaction. 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; Menzel and Benjamin, 2012). This neuron has its soma located ventro-medial to the  $\alpha$ -lobe and arborizes adjacent to the contralateral  $\alpha$ -lobe (Mauelshagen, 1993; Menzel and Muller, 1996; Okada et al., 2007). Given its anatomic positioning and learning dependant functional plasticity, the question about whether olfactory learned information with one antenna in honey bees can be retrieved from the contralateral side seemed a possibility. The ventral unpaired median neuron of the maxillary neuromere (VuMmx1) has its soma located at the subesophageal ganglion, its branches innervate, the basal lip of the MB, lateral horns, and antennal lobes, bilaterally (Hammer, 1993; Hammer, 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 above mentioned 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 (MBON) population that codes for the rewarded stimulus has a different representation on the untrained side after training on the trained side.

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

### **The learning and decision making in the two sides of in *Apis dorsata* are independent**

We tested the hypothesis of whether the phenomenon of lateral transfer of olfactory information exists in *Apis dorsata*. Our result in *A. dorsata*, however, was quite contrary to the results from previous works on *A. mellifera* and not only did we see negligible transfer of olfactory learnt information, but it was also observed that learning rate with one antenna in use, 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 *Apis mellifera* learning rate with one antenna reached up to 95% nearly saturating. In addition neuron tract tracing experiments from our lab using *Apis 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 contralateral antennal lobes (Mogily et al., 2018). These tract tracing experiments further

strengthened the possibility of each lobe processing olfactory information independently. This is consistent 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 the 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 1972 by Mahsur et al., that with respect to olfaction the honey bee seems to use each lobe independently. It is not clear if there is a set of non-motor, decision neurons, that receive input from both the 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 higher level in honeybees.

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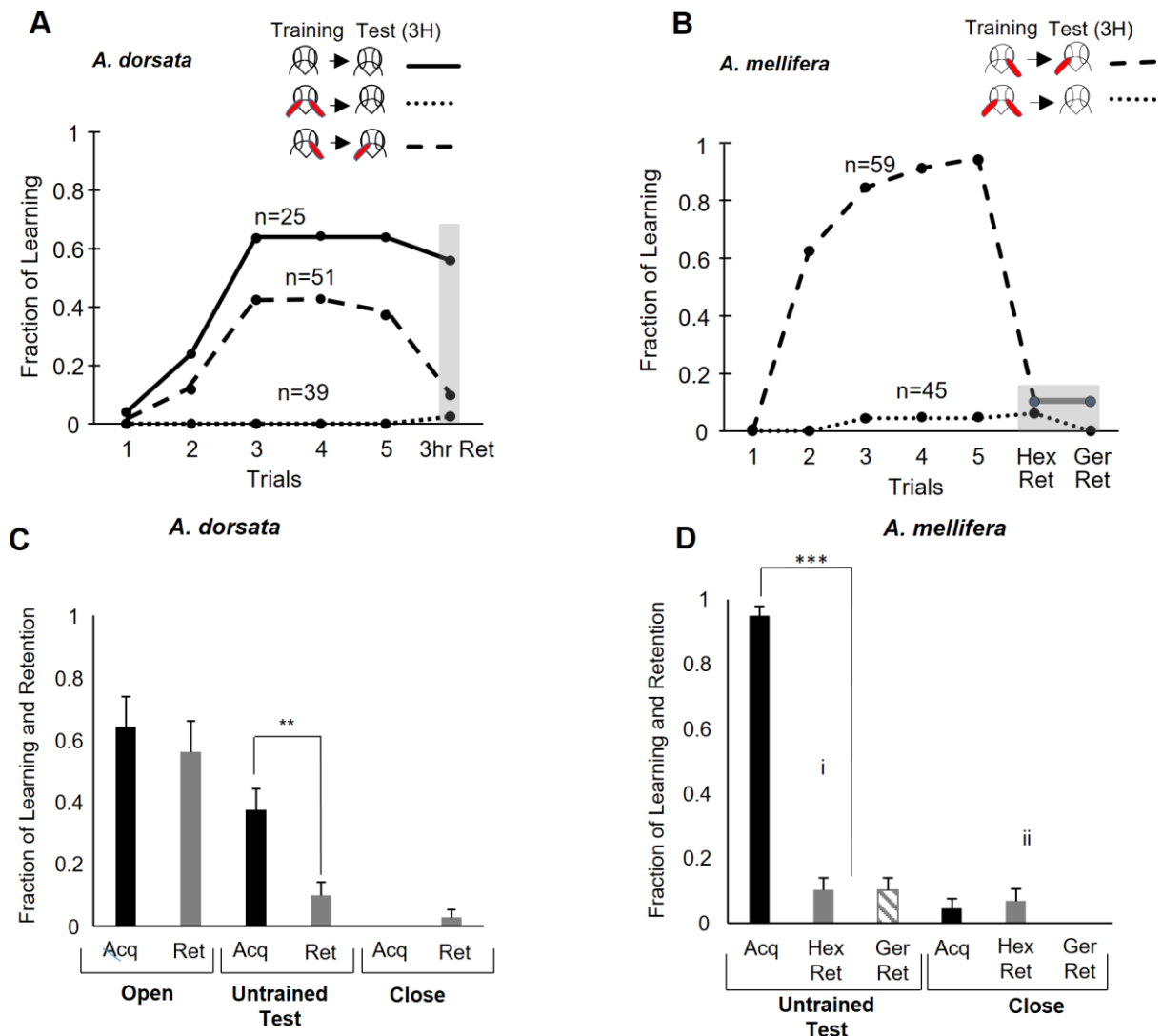
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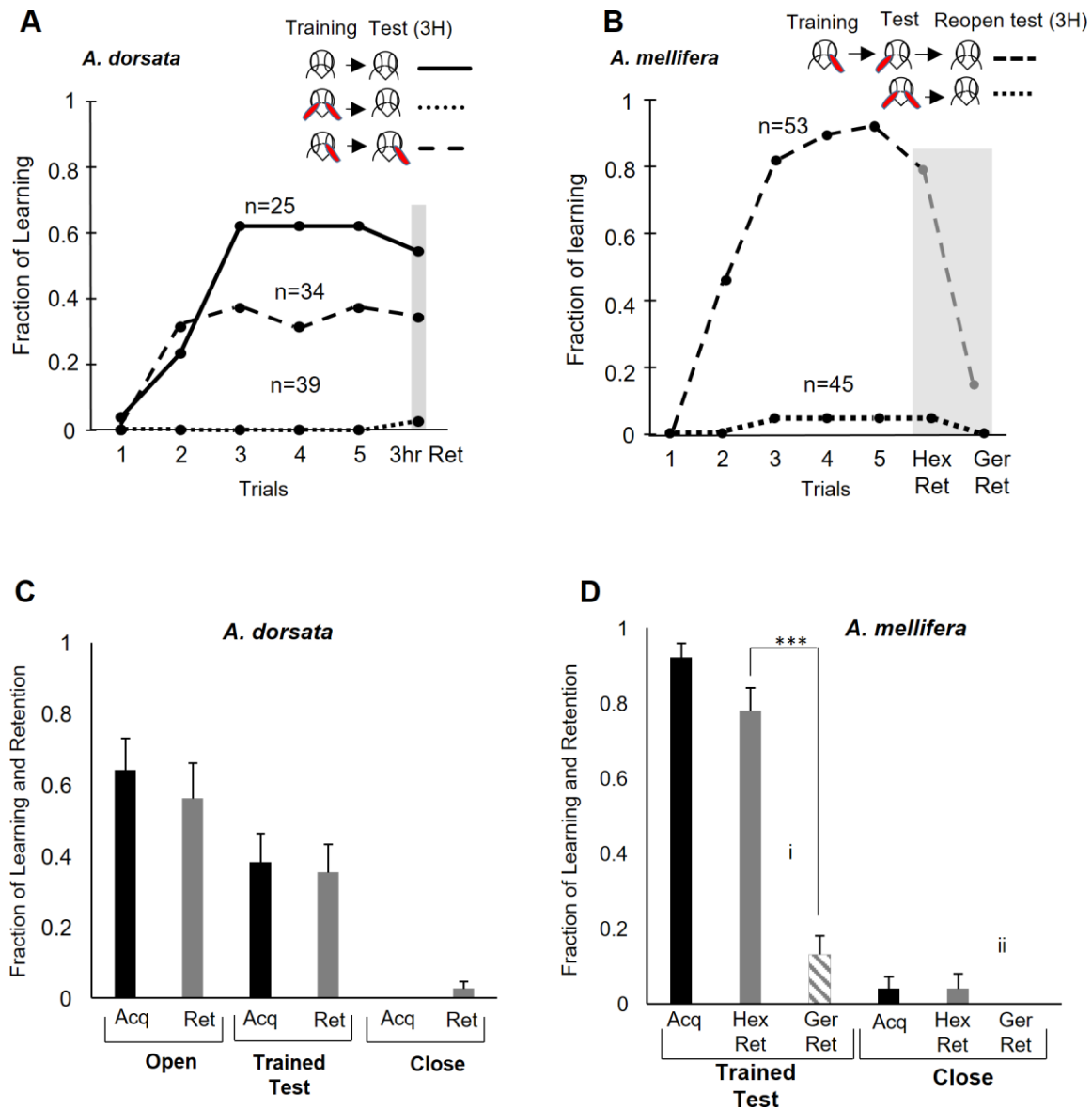
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## Figures



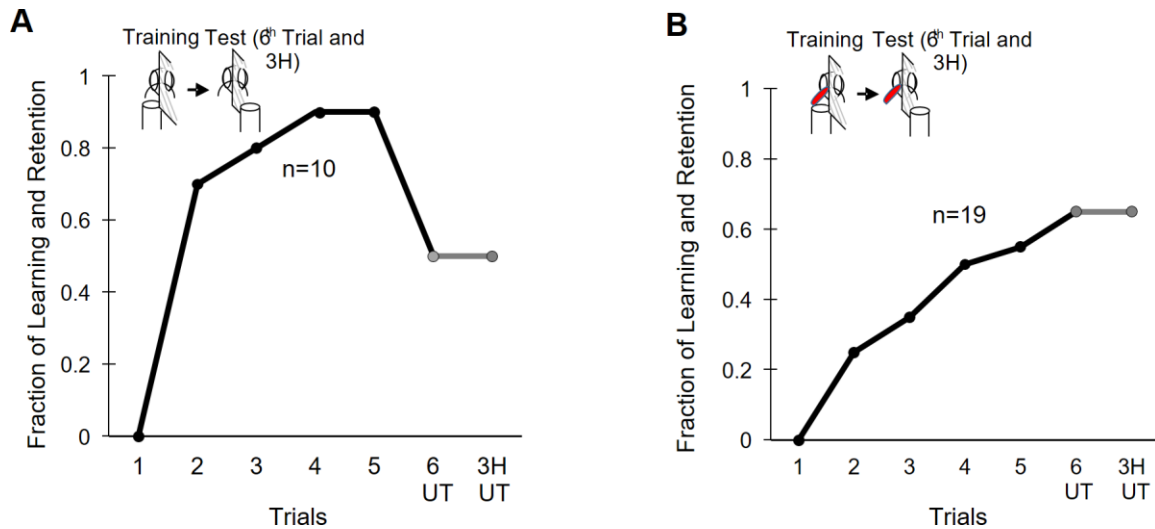
**Fig 1: Testing for lateral transfer of olfactory memory in *Apis dorsata* and *Apis mellifera*** : The schematic representation of the procedure is shown above the plots. The red cover on the antennae represents the acrylic coat. **A)** Testing for lateral transfer at 3 hours (3hr Ret) in *Apis dorsata* (n=25 open antenna control, n=25 untrained antenna test, n=39 closed antenna control) shows that memory on the untrained side at 3 hours (Grey box) is nearly zero ( $p=0.045$  Cochran's  $q=4$ ). **B)** *Apis mellifera* bees (n=59 untrained antenna test, n=45 closed antenna control) also did not show any significant lateral transfer at 3 hours (Grey box) ( $p=0.22$  Cochran's  $q=1.5$ ) though they had 95% acquisition. All the bees that responded to 1-hexanol (Hex Ret) responded to 1-geraniol (Ger Ret) as denoted by the grey line, on the transferred side at three hours indicating no discrimination ( $p=1$ ). **C)** *Apis dorsata* showed significant difference between the learning (Acq) and retention (Ret) in the side contralateral to the trained, 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) contralateral side was similar to the group with (ii) both antenna closed. Bar graphs are a representation of mean $\pm$ SEM



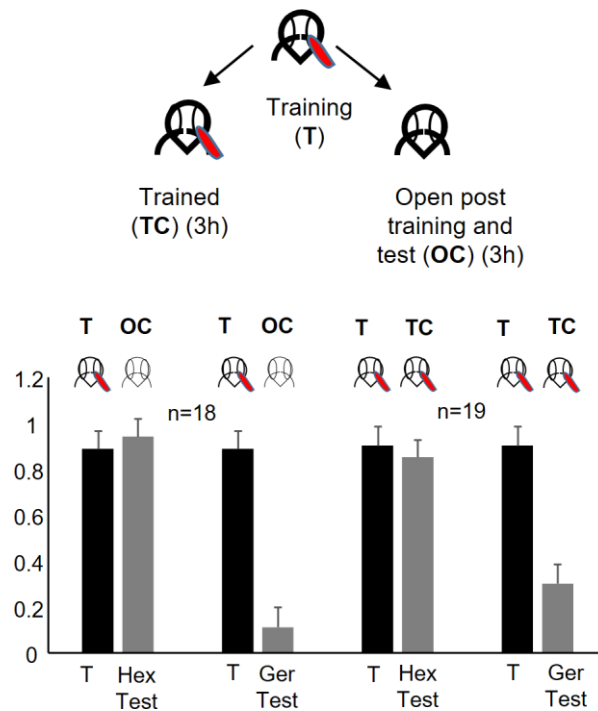
**Fig 2 : Memory is retained on the trained side in *Apis dorsata* and *Apis mellifera*:** **A)** The schematic representation of the procedure is shown above the plots. The red cover on the antennae represent the acrylic coat. **A)** *A. dorsata* (n= 25 open antenna control, n=34 trained antenna test, n=39 closed antenna control) learned and retained memory after 3 hours (3hr Ret, Grey box) with one antenna if tested with the same antenna and retention was found to be significantly higher than 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 to two antenna training but wasn't 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 does not affect the acquired memory on the trained side, (Grey box) 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 hours ( $p=1.8 \times 10^{-9}$ , Cochran's

q=36.1). They also showed clear discrimination between 1-hexanol (Hex Ret) and geraniol (Ger Ret) as denoted by the grey dotted line ( $p=1.4 \times 10^{-8}$ , Cochran's  $q=32.1$ ) showing that the covering and uncovering does not stress the bee and cause memory loss. **C)** *A dorsata* learned and retained memory after 3 hours with one antenna and two antenna. The acquisition and retention with two antenna were approximately 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 antennae once reopened post untrained antenna check showed significant discrimination between the trained odor (i) Hex Ret and Untrained odor, Ger Ret. There was no discrimination (ii) between the two odors by the bees trained with both antenna closed. Bar graphs are a representation of mean $\pm$ SEM.

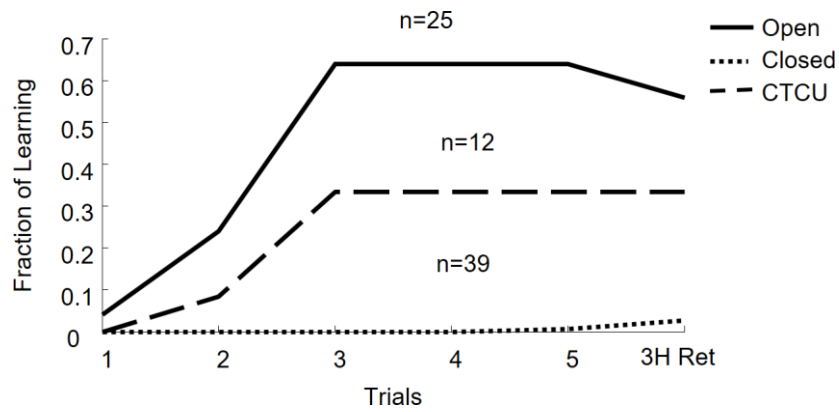


**Fig 3: Training using a plastic wall to separate the two antennae shows learning in the contralateral side even as training is taking place. A)** *Apis mellifera* exhibited PER to the trained odor on the untrained (UT) side at the 6<sup>th</sup> trial itself when trained with plastic partition for isolation. This memory was retained on the untrained side denoted by the grey line at 3hrs post training (3 UT) (n=10). **B)** *Apis mellifera* exhibited learning on the trained side even when the trained antenna is insulated with acrylic and with plastic partition used for isolation. 65% retention was seen by the untrained antenna at the 6<sup>th</sup> trial itself (6 UT), this memory was retained on the untrained side at 3hrs post training denoted by the grey line (3H UT) (n=19).

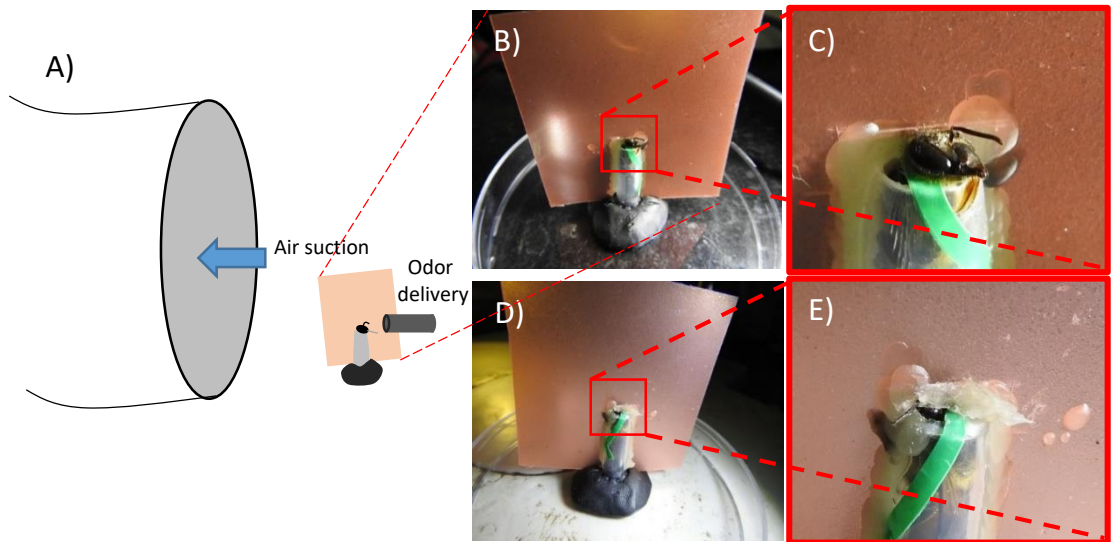




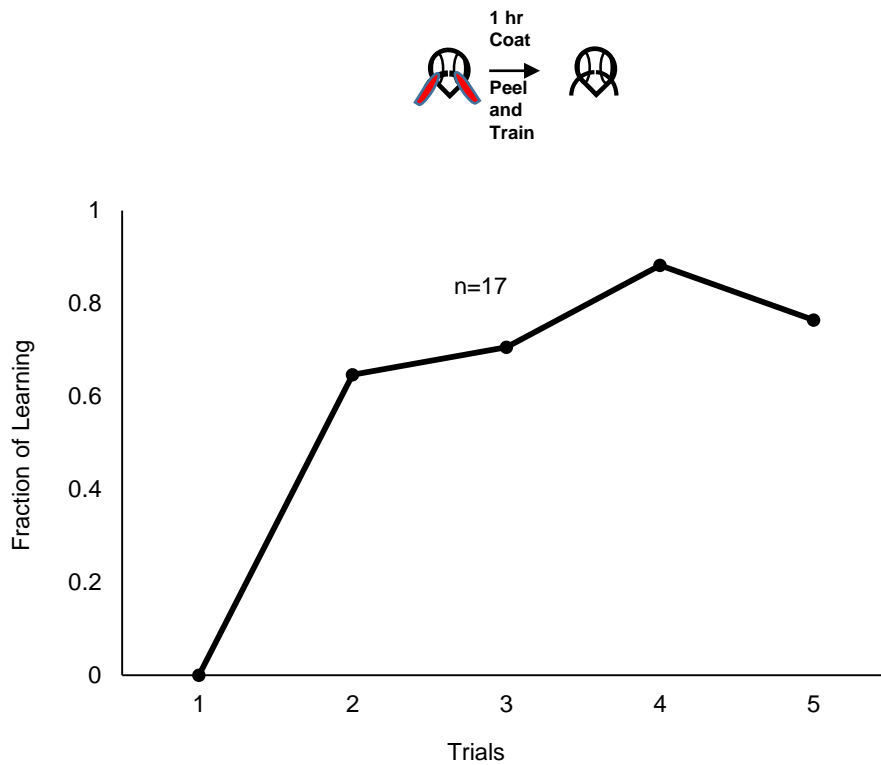
**Fig 4: To check for learning of contextual stimuli, *Apis mellifera* bees were trained (T) with one antenna covered (n=37). At 3 hours the bees were divided randomly, in to two sets, a set of bees were checked for retention with the block opened (OC) (n=18) and the other set of bees were checked for retention in the trained condition (TC) (n=19). The bees with both antenna opened (OC) performed equally with a 100% and 94% (TC) retention in each case. The bees with both antenna open during retention test showed only marginally better discrimination (Ger Test OC and Ger Test TC) compared to the bees with one antenna covered. Bar graphs are a representation of mean±SEM.**



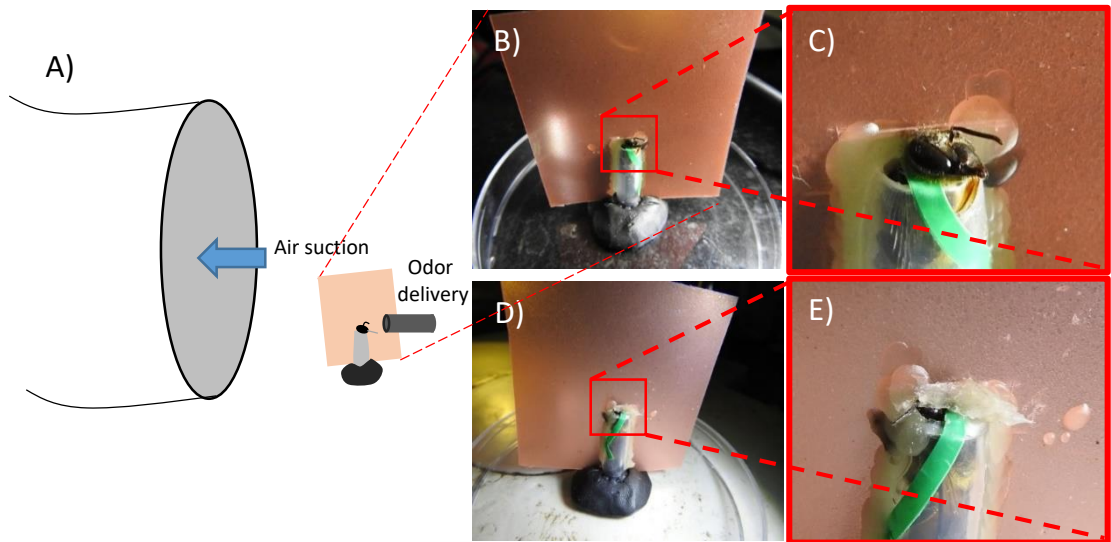
**Fig 5: The process of coating and un-coating does not shock the bee into forgetting or harm the antenna.** In *Apis dorsata* bees, the training antenna was coated with paint just prior to the 3 hour retention test (3h Ret) (n=12). The removing of the coat does not shock the bees into forgetting the acquired memory as indicated by the 100% retention in the learnt bees (Close Test Cover Uncover test – CTCU).



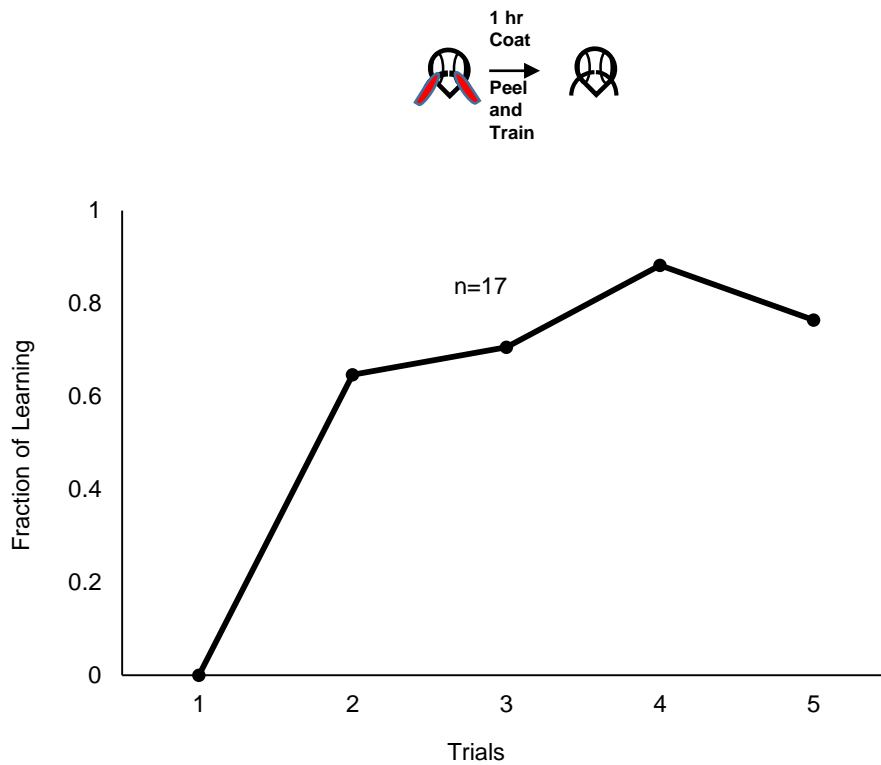
**Figure S1. Setup for training with partition,** A) Schematic representation of the setup with a wall B) The view of the training side of the bee with the animal's mandible, proboscis and one antenna to one side. C) A magnified view of the training side. D) The view of the untrained side, with the untrained antenna separated from the training side. E) A magnified view of the untrained side.



**Figure S2. Acrylic paint coat does not cause damage to the antennae:** In *Apis dorsata* bees (n=17) the coating with acrylic paint and peeling does not harm the antennae and the bees learnt as well as their open antennae counterparts. Final learning rate achieved in these bees was 76% and was not significantly different from the learning rate of the both antenna open bees (Cochran's  $q=0.8$ ,  $p=0.3$ ).



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