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Comparison olfactory learning three races of bees (*Apis mellifera meda, Apis mellifera carnica* and *Apis cerana* F.) under laboratory conditions and memory recall in drones of hive honeybee species in Iran

Vaziritabar Shakib and Esmaeilzade Sayed Mehdi

Abstract
The proboscis extension reflex is a classical behavioral trait used to determine the learning behavior in honeybees to odors. The experiments on olfactory learning and memory recall in 3 drone bees of (*A.m. meda*), (*A.m. carnica*) and (*A.c. indica*) were conducted by conditioning to nine odors (10-hydroxy (E)-2 decanoic acid (10-HDA), Isopentyl Acetate (IPA), Nerol, Citral, Geraniol and Aldehyde groups such as (Hexanal, Heptanal, Octanal and Nonanal). The observations revealed that the drones of three species responded to the test odors with varied degrees of learning and memory recall. The level of memory recall was high for 10-HDA followed by Nerol, Octanal, Nonanal, Heptanal, Hexanal, Geraniol. However, the low response was recorded for Citral and IPA. The level of memory recall was greater in drones of (*A.m. meda*), than two races of bees (*A.c. indica*) and (*A.m. carnica*) and the highest level of learning and memory recall was recorded at 3 hrs.

Keywords: Olfactory learning, Drone bees, PER conditioning, memory recall, laboratory

1. Introduction
Animals of most species are capable of discriminating between varieties of odors. This ability is often crucial for the organization of feeding, mating, and social communication, as well as for the processes of learning and memory that are associated with these behaviors. Thus, in order to understand these behaviors and processes, it is important to gain insight into the neural mechanisms underlying the discrimination of odors. Honeybees (*Apis mellifera*) are generalist foragers of floral resources. The distribution of nectar and pollen in the environment is highly variable and hard to predict, and to aid their efficient foraging, bees rapidly learn complex multimodal stimuli associated with profitable flowers [52]. Choosing flowers often involve making decisions based on incomplete information. One behavioral strategy that can aid animals in making decisions when uncertain is the formation of learned biases in response to novel or confusing stimuli. An example of such a strategy is peak shift [37]. Animals can learn that an originally neutral stimulus can act as a predictor (conditioned stimulus; CS) for a biologically significant stimulus (unconditioned stimulus; US). Such a basic association between single stimuli can be acquired by a great variety of animals through classical conditioning [88]. For a century now, the honeybee (*Apis mellifera* L.) has been a key insect model in which behavioral, neuro-anatomical, and neuro- physiological approaches have been performed to unravel the basis of olfaction and olfactory learning. Honeybees are social insects which present a wide range of behaviors relying on olfaction both within and outside of the colony [107, 122]. Moreover, the study of olfaction is easily amenable to the laboratory, since dedicated protocols have been developed in which bees show rapid and robust odor learning abilities [35, 73]. In addition, the olfactory pathway of the honey-bee brain has been extensively described [48, 51, 81, 102]. The role of the olfactory system is to decode the complex eddies of molecules in the environment and shape them into pieces of relevant information that will allow the animal to make decisions and engage in adapted behaviors. Major tasks of the olfactory system are for instance the identification of food sources, the detection of possible dangers (such as fire or predators), there cognition of potential mates as well as allowing social interactions.

How the nervous system operates this transformation from the detection of chemical molecules via the formation of neural representations until the creation of percepts has been the focus of intense research especially in vertebrates [53, 56, 63, 66] and insects [33, 40, 59, 64].

Any study of memory must carefully separate the effect of storage and recall mechanisms [109]. The results reported here indicate a complex relationship between olfactory storage and recall mechanisms in the honeybee. That is, what is recalled after memory consolidation [19] can be slightly different from the conditioning event, and a lack of a response does not necessarily imply forgetting [109]. Associative learning is an essential component of the bee's central place foraging behavior and dance communication. Hive mates attending a dance performance learn the odor emanating from the dancing bee and seek it at the indicated food site. The odor, color, and shape of flowers are learned when the bee experiences these stimuli shortly before it finds food (nectar, pollen). This appetitive learning in bees has many characteristics of associative learning well known from mammalian learning studies [9, 79, 80]. It follows the rules of classical and operant conditioning, respectively, so that stimuli or behavioral acts are associated with evaluating stimuli. Since associative learning, especially of the classical type, is well described at the phenomenological and operational level [95], it provides a favorable approach in the search for the neural substrate underlying learning and memory. Insects are important model organisms for studying the neural basis of learning and memory [31, 35, 70]. Honeybees rapidly learn to associate the scent of flowers with food, and bees' odor–reward learning can easily be accessed by classical delay and trace conditioning of the appetitive proboscis extension reflex [10, 50]. Physiological measurements of brain activity allow us to relate behavior to brain activity [36]. In the insect brain, the primary olfactory areas are the antennal lobes, which are structurally and functionally similar to the mammalian olfactory bulbs [36]; both are made up of glomeruli [72].

1.1. Honey bee behavior in a natural context

The honeybee (*Apis. mellifera*) has been a central insect model in the study of olfactory perception and learning for more than a century, starting with pioneer work by Karlvon Frisch. Research on olfaction in honeybees has greatly benefited from the advent of a range of behavioral and neurophysiological paradigms in the Lab. Several reasons justify the use of the honey bee as a model for the study of complex learning abilities. In a natural context and despite their small size, honey bees exhibit an extremely rich behavioral repertoire [25]. A social lifestyle is obligatory, and a single bee cannot survive very long independent of its nest mates. Outside of the hive, a bee travels over a distance of several kilometers and visits hundreds of flowers in quick and efficient succession for gathering food (nectar or pollen). It also collects resin and water and roams for information-gathering purposes. Sensory capacities and motor performances are highly developed. Bees see the world in color [76]. Perceive shapes and patterns [39, 108,121] and resolve movements with a high temporal resolution [60].

Insects constitute successful models for the study of learning and memory due to their remarkable learning abilities mediated by relatively simple neural systems containing lower numbers of neurons compared to vertebrates [17, 35, 67, 73]. Among insects, honey bees (*Apis mellifera*) are reported to have the highest and broadest range of learning abilities [30, 35, 38, 68, 73, 97]. Honey bees are able to associate a food reward with different sensory stimuli such as odors, colors and visual patterns, tactile or thermal stimuli [35, 73]. However, studies on learning and memory in honey bees have mostly used harnessed individuals and olfactory learning protocols when the goal was to achieve a full control of behavior by the experimenter. The most popular protocol used to this end is the olfactory conditioning of the proboscis extension response (PER), which is a case of classical (Pavlovian) conditioning [10, 30, 115, 117]. The PER is a reflexive response of hungry bees which is part of their feeding behavior while foraging or within the hive [27, 28]. It occurs when the antennae, tarsi or mouth parts come in contact with sucrose solution; the bee then reflexively extends its proboscis (PER) to reach the sucrose solution and drink it. Odors generally do not evoke the PER in bees naïve to the experimental conditions. During conditioning, an odor (CS) is presented in close temporal association with sucrose solution (US). At the end of training, the odor alone elicits the PER, indicating that the bee has taught the odor-sucrose association [10, 115]. PER is usually recorded as a dichotomous response (1 or 0), which can thus be used as an index for learning and memory performances. The protocol of olfactory PER conditioning, first established by Takeda (1961), was later standardized by Bitterman *et al*. (1983). Since then, numerous studies have used it to study the behavioral, neural and molecular bases of olfaction, learning and memory formation [30]. Yet, throughout the years, a series of procedural variants and deviations from the original procedure have arisen, which render comparative analyses of behavioral performances difficult. Moreover, because even slight variations in conditioning procedures may introduce significant differences in acquisition and retention performances, such procedural variations are not trivial and need to be considered carefully. In many cases, researchers willing to use olfactory PER conditioning for the first time complain that section two is usually not detailed enough to achieve successful conditioning in a straightforward way. A first attempt to overcome these problems has focused on neuro-pharmacological experiments which address the molecular bases of olfactory PER conditioning [21]. Here we provide a broader approach and we detail and discuss all the procedural steps required for efficient olfactory PER conditioning. We conditioned bees to odors and tested generalization responses to different odors.

1.2 Neuro-anatomy of the honeybee olfactory system

An advantage of the bee model for understanding olfaction and olfactory learning is that the neuro-anatomy of its olfactory pathway is known in great detail [5, 48, 51, 81, 87, 102, 111]. Olfactory processing follows different steps, from the detection of molecules at the periphery, via primary processing by antennal lobe (AL) networks, until the establishment of olfactory representations in higher-order brain centers (Figure1), the bee brain is thought to contain multiple odor representations, which can be characterized by different odor-similarity matrices. Sequential and/or parallel transformation of olfactory information shapes odor representations in higher-order centers that would eventually determine olfactory behavior [97]. Chemical molecules, especially volatile ones, are the vessel of crucial information that may determine an animal's eventual survival and reproductive success. Perhaps for this reason, the sense of chemoreception is ubiquitously represented in the animal kingdom [3]. The role of the olfactory system is to decode the complex eddies of molecules in the environment and shape them into pieces of relevant information that will allow the

animal to make decisions and engage in adapted behaviors. Major tasks of the olfactory system are for instance the identification of food sources, the detection of possible dangers (such as fire or predators), the recognition of potential mates as well as allowing social interactions. How the nervous system operates this transformation from the detection of chemical molecules via the formation of neural representations until the creation of percepts has been the focus of intense research especially in vertebrates [53, 56, 63, 66] and in insects [33, 40, 59, 64]. A general finding of these studies is that the basic rules underlying olfactory processing in these different classes of animals are highly similar [3, 42]. For the most part, this resemblance is thought to result from evolutionary convergence due to similar constraints [18].

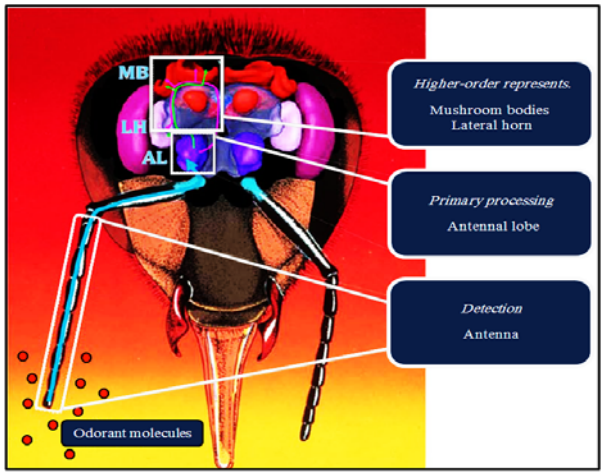

Fig 1: Frontal view of the bee head showing a three-dimensional model (from Brandt *et al*., 2005) of the brain. Olfactory processing follows three main steps. First, odors are detected at the level of the antenna. Information is conveyed to the antennal lobe (AL) for primary processing. Processed information is then relayed by different pathways to higher-order centers, the mushroom bodies (MB), and the lateral horn (LH), creating multiple olfactory representations in the bee brain. (Photograph by Sandoz, 2011; Evolution, Genomes and Speciation Lab, Centre National de la Recherché Scientifique, Gif-sur-Yvette, France).

The Western honeybee, *Apis mellifera*, is well established as a model for studying olfactory learning using a classical association assay. This assay is based on the powerful Pavlovian conditioning protocol, the proboscis extension response (PER), [10, 30, 61]. In PER conditioning, a hungry bee is first exposed to a conditioned stimulus (CS; such as an odor, color, or texture) followed by gently touching the bee's antennae with the unconditioned stimulus (US) that elicits proboscis extension response; the US was then delivered to the proboscis. If the bee responds with proboscis extension when presented with CS, this suggests that the bee has learned of the association [10]. Recently, proboscis extension response (PER) assays has been used to investigate the learning ability of other bee species. Some have proved amenable to the use of PER, such as *Vespula* wasps [116] and the red dwarf honey bee *A. florea* [46]. *A. mellifera* and *Apis cerana* have evolved in distinct ecologies; their social organization as well as mating behavior has been successfully shaped by their respective ecosystems. *A. mellifera* was introduced in China in the 1920s for its high productivity [123] and now live sympatric with *A. cerana* in Asia. *A. cerana* was smaller in body size than *A. mellifera* and also showed stronger merits in resisting bee mites [86], hornets [112, 113], and extreme climates [112, 113]. Studies utilizing color and grating patterns to investigate learning differences between these two species suggested that *A. cerana* perform as well as *A. mellifera* [89]. However, the role of olfaction in learning two species *A. mellifera meda* and *A. cerana* indicaare still unknown. Here, we aim to (a) determine whether *A. mellifera meda* and *A. cerana indica* workers can be used to study learning applying the classic proboscis extension response (PER) conditioning paradigm, (b) investigate the olfactory learning ability of *A. mellifera meda* and *A. cerana indica*, and (c) compare this ability with olfactory associative learning of three races of honeybees (*Apis mellifera meda, Apis mellifera carnica and Apis cerana indica*) under laboratory conditions. The present study investigates the response of drone honeybees to different pheromonal and non-pheromonal odors under laboratory conditions.

The honeybee (*Apis mellifera* L.) offers an excellent opportunity to study the mechanisms of configurable learning. In a natural context, honeybees learn a great variety of sensory cues usually presented as compounds and primarily associated with their nest and/or food sources, flowers [68, 74, 75, 79]. Olfactory learning in the honeybee has been extensively characterized [43, 44, 74] and offers an appropriate preparation for studying configural learning in a controlled way. Harnessed honeybees can be conditioned to olfactory stimuli, either presented alone or as a compound [10]. When the antennae of a hungry bee are touched with sucrose solution, the animal reflexively extends its proboscis to reach out to and suck the sucrose. Odors to the antennae do not release such a reflex in naive animals. The structure of an animal's environment can modulate memory storage and retrieval of associations among stimuli relevant for survival and reproduction. Reliable positive correlations among stimuli over evolutionary time may lead to the evolution of predispositions to learn specific associations of cues with motor patterns [41]. In the honeybee, for example, floral odors in general serve to indicate resources such as pollen and nectar [25], but the reliability of any specific odor with floral resources may change rapidly within a bee's lifetime. Therefore, the learning task for a foraging bee is to track quickly which odors indicate presence or absence of resources at any given time. This ecological background can help to interpret olfactory memory consolidation after proboscis extension conditioning of restrained honeybees [50, 78]. After a single conditioning trial during which presentation of an odorant precedes the sucrose unconditioned stimulus (US) by 1-5 s, 30-50% of restrained worker bees will extend their proboscides upon further presentation of the odorant alone [83]. Asymptotic responses to olfactory conditioning are typically attained after only two or three such trials. The evolution of such rapid acquisition, as well as temporal phases associated with olfactory memory processing, can be understood in terms of the importance of olfactory cues for the identification of floral resources, the average flight times between flowers, and trips between flower patches and the colony [79, 80]. Further work has shown that the conditioned response (CR) to an odorant depends on the odorant used for conditioning. Certain odorants elicit much stronger appetitive responses than others after the same conditioning procedure [110]. Thus, the olfactory memory of the honeybee is biased, probably through experience and innate mechanisms, towards making an association of certain odorants with a sucrose reward. The expression of learning performance within 10-15 min of a learning trial reflects at least two physiological phases as the memory is being consolidated. Each phase is characterized by a specific treatment, or lack of one, that causes retrograde

amnesia [19]. Thus, the consolidation of olfactory memory from a more labile phase to a more permanent one can be established [80, 82]. The associative olfactory learning ability of the Western honey bee (*Apis mellifera*) has been widely studied using conditioning of the proboscis extension response (PER; also known as "proboscis extension reflex") under laboratory conditions [8, 10, 35, 74, 115]. In proboscis extension response (PER) conditioning, the measured response is whether or not a subject bee extends its proboscis to a conditioned stimulus (CS). The CS is presented to the subject followed by gently touching the bee antennae with sugar solution representing the unconditioned stimulus (US). If on a subsequent presentation of the CS, the bee responds with proboscis extension, it is interpreted as an indication of the bee having learned the association. The success of proboscis extension response (PER) conditioning in advancing the study of learning and memory in *A. mellifera* has prompted attempts to use this technique for the comparative study of other bee species. Among the few species studied, the success of proboscis extension response (PER) olfactory conditioning tends to increase with the level of sociality and to be influenced by the foraging system [62, 116]. Solitary species have generally not been amenable to PER conditioning. For example, three species of megachilid (*Osmia lignaria*, *Megachile rotundata*, and *Megachile pugnata*) tested in classic proboscis extension response (PER) conditioning procedure were found to not extend their proboscis to sugar stimulation [116].

The olfactory signals play an important role in survival and reproduction of most animal groups. Honeybees exhibit an extremely rich and interesting behavioral repertoire. The social organization of a honeybee colony is widely determined by chemical signals that are actively produced and transmitted by the queen, the workers brood and possibly drones. The ability of worker bees to learn and recognize olfactory cues has been widely studied since the founding work by Frisch (1967) [25]. Bees are also able to memorize the cues associated with the hive and food sources [75]. The good learning abilities of bees made it possible to address higher order cognitive capacities which were long considered to be the exclusive patrimony of some vertebrates [35]. It has been documented that the olfactory sense is able to distinguish a large range of odors in honeybees [117]. Pheromonal odors play a vital role in sexual attraction, social behavior and location of profitable food sources [16]. The importance of different pheromones produced by queens [13, 34] as well as worker bees [11, 114] in bee colonies has been well documented. Koeniger *et al*. (2000) [49] reported the mating behavior of drones of different honeybee species. By contrast, little is known about the influence of odors produced in the bee colonies on drone bees [98]. The present study investigates the response of drone honeybees to different pheromonal and nonpheromonal odors under laboratory conditions.

1.3 Summary of the behavioral studies reported

Bee species	Lateralized behavior	References
Apis mellifera	Right-antenna advantage in olfactory performance.	[54]
Apis mellifera	Right-eye advantage in visual performance.	[55]
Apis mellifera	Use of the right antenna in short-term memory recall and of the left antenna in long-term memory recall: a functional lateral shift.	[90]
Apis mellifera	Response competition associated with right-left antennal asymmetries of new and old olfactory memory traces.	[24]
Apis mellifera	Short-term memory lateralization may be odor-specific, or the lateral shift in the transition from short-term to long-term memory occurs at different time scales for different types of odors.	[91]
Apis mellifera	Right-antenna in social interactions.	[90]
Trigona carbonaria *Trigona hockingsi* *Austroplebeia australis*	Use of the right antenna in short-term memory recall and of the left antenna in long-term memory recall.	[23]
Osmia cornuta	No right-antenna advantage in olfactory performance.	[2]
Bombus terrestris	Right-antenna advantage in olfactory performance.	[1]
Bombus spp.	Asymmetry in circling around a vertical inflorescence.	[47]
	Social learning drives handedness in nectar-robbing bumblebees.	[29]
Apis mellifera	In some combinations, responses to a novel odorant are significantly stronger than responses to the conditioning odorant after memory consolidation.	[110]
Apis mellifera	Results show that stimulation of the sensory receptors on the proboscis and/or ingestion of the sucrose reward during appetitive olfactory conditioning are necessary for longterm memory formation.	[120]
(*Apis m. carnica*) and (*Apis c. indica*)	The observations revealed that, the drones of both species responded to the test odors with varied degrees of learning and memory recall. The level of memory recall was high for 10-HDA followed by Nerol, Octanol and Citral. However, low response was recorded for IPA. The level of memory recall was greater in drones of *A.c. indica* than *A.m. carnica*.	[84]
Apis mellifera	Electroantenogram (EAG) response of drones to isopentyl acetate, a component of the alarm pheromone of worker bees, and it is found to be surprisingly high, almost in the same range as in that of workers.	[118]
Apis mellifera	Higher generalization was found between long-chain than between short-chain molecules and between groups such as primary and secondary alcohols Ketones, on the other hand, showed a heterogeneous effect, as 2-hexanone seemed to have a low salience (it was not well learnt) and induced a high generalization to other odors, while 2-nonanone consistently reduced generalization to other odors.	[36]
Apis mellifera	Two groups of bees were conditioned in an explicitly paired manner, either with 1-nonanol (n = 46) or with 2-hexanol as CS (n = 46) and There was a significant difference in acquisition between both groups (repeated measure ANOVA group × trial, group effect, $F_{1,188} = 449.13$, $P<0.0001$) and the group × trial interaction was also significant, thus underlining the different response patterns of both groups during conditioning (repeated measure ANOVA group × trial, interaction effect $F_{4,752} = 82.47$, $P<0.0001$).	[61]
(*Apis cerana*) and (*Apis mellifera*)	*A. cerana* associative olfactory learning with three different odors (hexanal, nonanal, and geraniol) and compared it with the learning performances of *A. mellifera*. (*A. mellifera*) showed higher learning scores than (*A. cerana*). However, there was no statistical difference between the two species in the retention phase.	[119]
Apis florea	*A. florea* levels of acquisition in olfactory associative learning were higher than those of *A. mellifera*, and they showed better retention of learning after 24 h than *A. mellifera*. They results showed that *A. florea* can be studied using PER procedures, enlarging the scope for future comparative studies of associative olfactory learning ability in bees.	[46]

2. Materials and Methods

Three races of honey bees (*Apis mellifera meda, Apis mellifera carnica and Apis cerana indica*), were caught at the entrance of outdoor hives at the beginning of each experimental day. Adult foraging bees, (*Apis mellifera meda, Apis mellifera carnica and Apis cerana indica*), were caught at the hive in the afternoon 1 day before the experiments. Each bee was placed in a small glass vial and cooled in a freezer until it ceased movement (Figure 2). Individuals were mounted into restraining harnesses so they could only move their antennae and mouthparts, including the proboscis [10, 115]. The Eastern honeybee, (*Apis. cerana* F), is indigenous to Asia and is an important pollinator for Asian ecosystems; the Western honeybee, (*Apis. mellifera carnica*), has been introduced to Asia because of its high honey yields. These two exotic species are now sympatric and share a similar environment. Western honeybee or European hybrid honeybee, (*Apis. mellifera carnica*) is native to the Iranian continent and is adopting apiaries by the beekeepers in Iran. We aim to study investigate the response of three drone honeybees to different pheromonal and non-pheromonal odors under laboratory conditions.

2.1 Experimental design

Our work was designed to obtain a generalization matrix with 9 different odors. The first experimental procedure the effects of different odorants on recall. Each subject received one conditioning trial (i.e. odorant-sucrose pairing) in a trace-conditioning procedure. During both trials the response in terms of extension of the proboscis to the odorant alone was categorized as either extended or not (if extension occurred on the first trial prior to US stimulation then the subject was scored as a spontaneous responder and tested normally in the second trial). After two trials the subject was never used in another test. Through a comparison of the responses at the various post-conditioning times, the second trial provides a measure of the ability of memory to release behavior over time. The nine odorants were tested in different groups of subjects. Ideally, after conditioning each of the 9 odors as CS, the response to each odor (including the CS) should be measured (i.e., 9 odors such as; (10- hydroxy (E)-2 decanoic acid (10-HDA), Isopentyl Acetate (IPA), Nerol, Citral, Geraniol and Aldehyde groups such as (Hexanal, Heptanal, Octanal and Nonanal). The observations revealed that the drones of three species responded to the test odors with varied degrees of learning and memory recall). This work laid the compare this ability with olfactory associative learning of three races of honeybees (*Apis mellifera meda, Apis mellifera carnica and Apis cerana indica*) under laboratory conditions. Three sec after onset of the CS (conditioned stimulus) the antennae were stimulated with the US (unconditioned stimulus) leading to a proboscis extension. The purpose of the present study is to investigate the extent to which the reward pathway experienced during olfactory learning affects the formation of memory in honey bees. In our experiments, we examined in detail whether stimulation of a honey bees antennae is sufficient to allow robust associative conditioning, short-term memory formation and long-term memory formation.

2.2 Test trials

The procedure was similar to that for conditioning trials but no US (unconditioned stimulus) was given after odor delivery. After the four test trials, PER (proboscis extension response) to the US (unconditioned stimulus) was checked once again. Animals unable to show PER (proboscis extension response) at this point were not considered for the analyses. Overall, less than 1% of the bees died during the experiment, and less than 1% of the survivors showed no US (unconditioned stimulus) reaction at the end of the tests.

Fig 2: Illustrations are shown three races of honey bees (*Apis mellifera meda, Apis mellifera carnica* and *Apis cerana indica*), were caught at the entrance of outdoor hives at the beginning of each experimental day and tethered bees (three honeybee races) in plastic containers (cylinders of 8 cm diameter × 20 cm height) covered with voile cloth secured with rubber bands and so that their heads and proboscises could move freely.

2.3 Study areas

The studies on olfactory learning on Iranian honeybee (*Apis. mellifera meda*) and two exotic species (*Apis. cerana* F.) and hybrid European honeybee (*Apis. mellifera carnica*) were conducted at Varamin-Pishva University and also at Animal Sciences Research Institute Karaj of Iran (ASRI) under laboratory conditions. (Figure 4 and 6). The study was carried out in (2015-2016) in the Karaj state, located between (35° 50′ 8″ North latitude and 51° 0′ 37″ E East longitude) in the Alborz province of Iran (Figure 3).

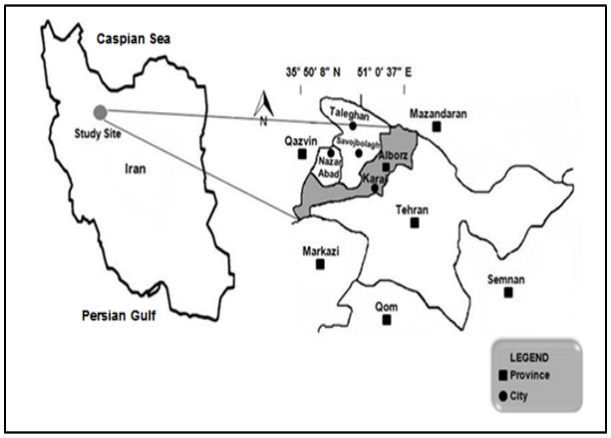

Fig 3: The locations of Karaj and Varamin-Pishva in the west and southeast of Tehran.

2.4 Insect

An explicit control of the bee caste used for conditioning is recommended because foragers are the individuals that exhibit the highest appetitive motivation for sucrose within the hive [104] and that are, therefore, more appropriate for appetitive olfactory conditioning. Capture of bees departing from the hive in the morning or late afternoon (avoiding mid-day times when young bees perform their first orientation flights) enhances the probability of obtaining empty foragers for experiments. Empty foragers (i.e. with empty crop) are necessary to ensure highest appetitive motivation for the

experiments. All colonies were queen right and housed in standard Langstroth hives. Before we started our experiments, we equalized the colonies such that each contained six frames covered with adult workers and at least three frames of brood and three frames of honey and pollen. The studies on olfactory learning on Iranian honeybee (*Apis. mellifera meda*) and two exotic species (*Apis. cerana* F.) and hybrid European honeybee (*Apis. mellifera carnica*) were conducted at Varamin-Pishva University and also at Animal Sciences Research Institute Karaj of Iran (ASRI) under laboratory conditions (Figure 4).

Fig 4: The studies on olfactory learning three races of bees under laboratory conditions.

2.4.1 Location and categorizing natural distribution of three honey bee races

The natural distribution of honeybee populations in the study region (Figure 5) has been affected seriously by human activity. Humans have transported and propagated non-native races of honey bees throughout Asia. In many parts of Asia the native honeybee populations and the local ecotypes are considered to be extinct. In most countries the importation of non-indigenous races was carried out with the aim of hybrid production, especially in Northern Europe. Over the past century different breeding policies have developed causing various kinds of impacts on the native honeybee populations. In many countries races different from the native one are imported to produce hybrid queens that will from colonies giving high honey yields, fv (hybrid vigor).

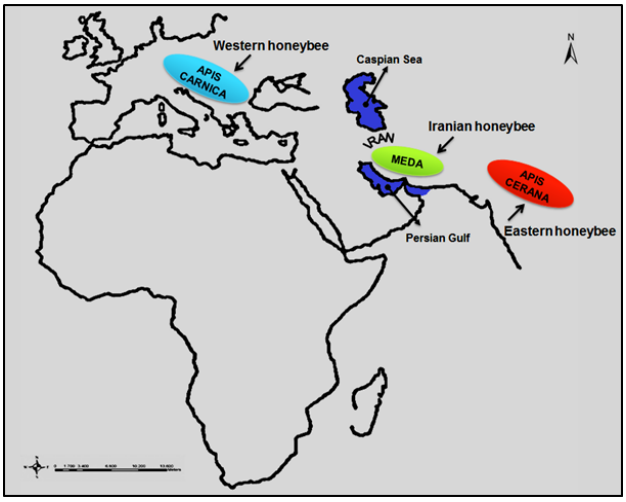

Fig 5: Location and natural distribution of three honeybee races in study zone (*Apis. mellifera carnica* hybrid, *Apis. mellifera meda* and *Apis. cerana* F).

The Eastern honeybee, *Apis cerana*, is indigenous to Asia and is an important pollinator for Asian ecosystems; the Western honeybee, *Apis mellifera carnica* hybrid, has been introduced to Asia because of its high honey yields. These two species are now sympatric and share a similar environment. In order to efficiently obtain food from flowers, nectar-collecting insects learn the association between the food, which serves as a positive reinforcement, and several floral cues, such as the floral odor (for review see Menzel, 1999). Learning the food odor during foraging facilitates the return to a profitable flower patch [26]. The Western honeybee, *Apis mellifera carnica*, is well established as a model for studying olfactory learning using a classical association assay. This assay is based on the powerful Pavlovian conditioning protocol, the proboscis extension response (PER), [10, 30, 61]. In PER conditioning, a hungry bee is first exposed to a conditioned stimulus (CS; such as an odor, color, or texture) followed by gently touching the bee's antennae with the unconditioned stimulus (US) that elicits proboscis extension response; the US was then delivered to the proboscis. If the bee responds with proboscis extension when presented with CS, this suggests that the bee has learned of the association [10]. Recently, proboscis extension responses (PER) assays have been used to investigate the learning ability of other bee species. Some have proved amenable to the use of proboscis extension response (PER), such as *Vespula* wasps [116] and the red dwarf honey bee *A. florea* [46]. Conversely, PER has proved to be an ineffective tool for some species such as the stingless bee Scaptotrigona deplis [64]. *A. mellifera* and *Apis cerana* have evolved in distinct ecologies; their social organization, as well as mating behavior, has been successfully shaped by their respective ecosystems. *A. mellifera* is native to Europe and Africa, while the rest are native to the Asian continent and *A. mellifera* was introduced in Asian country for its high productivity [4] and now live sympatric with *A. cerana* in Asia. *A. cerana* was smaller in body size than *A. mellifera* and also showed stronger merits in resisting bee mites [86], hornets [112, 113].

2.5 Catching and harnessing bees

These bees were transferred to the laboratory and narcotized on ice for 5 min until they stopped moving. Vials are then placed in crushed ice as long as it is necessary to render the bees motionless (usually between 3 and 5 min) so that they can be harnessed individually. Cooling time should be kept to a minimum as extended cooling could impair learning performances [22] and survival in the harness. Tethered bees in plastic containers (cylinders of 8 cm diameter × 20 cm height) covered with voile cloth secured with rubber bands and so that their heads and proboscises could move freely (Figure 6). Thus, the bees were restrained in such a way that they were still able to move their heads and proboscis. The bees were fed 12 µL of 2.5 M sucrose solution. The tests were carried out in a climate controlled growth chamber at (25±2 °C and 50±10% RH) overnight. The next morning, we trained all surviving bees to associate an odor with a sucrose reward using the proboscis extension response (PER) procedure [10]. Prior to training, we tested the bees and discarded any that showed no PER to sugar solution (unconditioned stimulus) or showed proboscis extension response (PER) to air flow carrying a test odor. The drone bees collected on the combs as well as at the hive entrance were immobilized by cooling in glass vials with ice packs. Drones were placed in well-fitted small metal holders, with strips of adhesive tape attached between head and thorax and over the abdomen. The test drones were starved for 2-3 hrs prior to conditioning. Ten

minutes before starting the experiments, each subject was checked for intact proboscis extension response (PER) by lightly touching one antenna with a toothpick imbibed with sucrose solution without subsequent feeding. The antennae of each drone were stimulated with honey by a micropipette. Drones that showed an immediate proboscis extension response to antennal stimulation with honey were taken for the tests (Figure 6). Those drone bees showing a spontaneous response to one of the odors were not used in the experiment.

2.6 Odorants and stimulation apparatus

The conditioning experiments were performed in a classical setup after [50] and [10]. Nine different odors such as 10-hydroxy (E)-2 decanoic acid (10-HDA), Iropentyl Acetate (IPA), Nerol, Citral, Geraniol and Aldehyde groups such as (Hexanal, Heptanal, Octanal, and Nonanal). were used in the experiments. In each trial a piece of filter paper (5mm diameter) soaked with respective test odor (2µl) was placed in a syringe (20 ml) and a volume of 10 ml of air was gently blown on the antennae as conditioned stimulus (CS) followed by stimulation with honey as an unconditioned stimulus (US). The drones which showed proboscis extension response were rewarded with a small amount of honey. The observations were recorded from 20 sec, 1 min, 3 min and from 1 h to 6 h and 1-day conditioning.

2.7 Odorants

A total of 9 odorants, including homologous series of aldehydes (C6-C9 respectively), terpenes (geraniol and citral), 10-hydroxy (E)-2 decanoic acid (10-HDA), Iropentyl Acetate (IPA) and Nerol was used (Table 1).

Table 1: Chemical and Substance characteristic of the odors used.

No.	Functional Groups	Odor	Chemical structure-odor
1	Aldehydes	*n*-hexanal	C6 H12 O
		n-heptanal	C7 H14 O
		n-octanal	C8 H16 O
		n-nonanal	C9 H18 O
2	Terpenes	geraniol	C10 H18 O
		citral	C10 H16 O
3	Other	10-hydroxy (E)-2 decanoic acid	(10-HDA)
		Nerol	-
		Iropentyl Acetate	-

2.8 Data analysis

The data were analyzed by using a one-way ANOVA. The mean, SD and SEM were calculated for each odor.

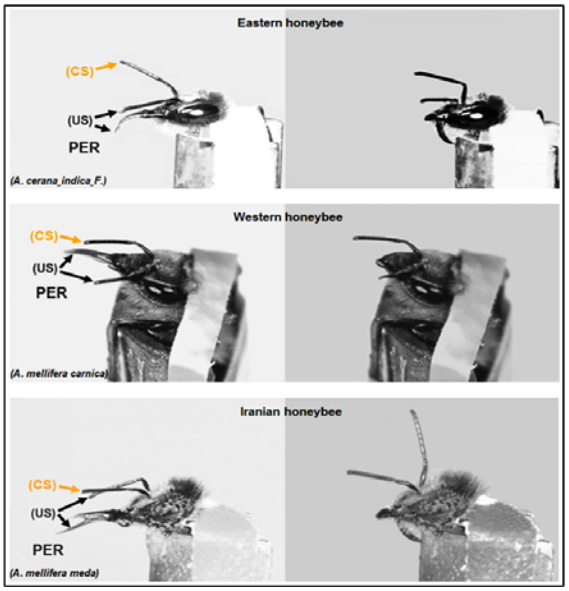

Fig 6: Olfactory conditioning of the proboscis extension response (PER) on restrained bees. The proboscis extension reflex (PER) is a response shown by bees when their antennae, tarsi, or mouthparts are contacted with sucrose solution. During conditioning, an odor (conditioned stimulus) is presented in temporal association with sucrose solution to the antennae and to the proboscis (unconditioned stimulus). This allows free movement of the antennae and the mouth parts. Contact of the antennae and/or the proboscis (enlarged inset) with sucrose solution elicits the PER. Bees are conditioned to extend their proboscis in response to an odor (conditioned response) when the odor (CS) is presented contingent upon a sucrose stimulus (US). After conditioning, the odor CS, which initially did not evoke any response, triggers the proboscis extension response (PER). (Photograph by Shakib Vaziritabar, Department of Animal Science, Islamic Azad University Varamin, Pishva Branch, Tehran, Iran).

2.9 Dissection of honeybees' olfactory memory phases

One of the crucial contributions of proboscis extension reflex (PER) conditioning to invertebrate neuroscience was that it permitted a careful dissection of appetitive olfactory memories in bees, as well as the elucidation of some of their key molecular actors, thanks to the fact that pharmacological injections and un-caging experiments could be coupled with controlled PER conditioning procedures [70, 74, 77]. As for other classical (Pavlovian) protocols, olfactory memory acquired after PER conditioning is dependent on variables such as the kind of CS, US intensity (i.e., the amount and/or quality of sucrose solution received during conditioning), the number of conditioning trials, and the inter-trial interval [65, 69]. Trial spacing is the dominant factor both for acquisition and retention performance. Generally, massed trials (i.e., trials succeeding each other in a fast sequence) lead to lower memory performances compared to spaced trials (i.e., trials separated in time). Longer inter-trial intervals lead to better acquisition and higher retention. Several studies on olfactory memory dynamics [73] showed that memories in bees pass through an early consolidation phase and are fragile before consolidation is completed. Transfer from short-term memory (STM) to long-term memory (LTM) via mid-term memory (MTM) is not a purely sequential process but also includes parallel processes [73].

At least four types of olfactory memory phases were identified for three races of bees (Figures 6-8). After a single conditioning trial, responses to the CS are high shortly (1–2 min) after conditioning, then decrease, showing a "dip" ~ 3 min, and are high again after 4 min, until ~ 1 d, when performance definitively decays (Figures 6-8). Two different memory phases are thought to underlie this performance: In the first minutes after conditioning, performance depends on STM, which is mostly nonassociative (because of sensitization from the US). While STM decays after 2–3 min, a consolidation process leads to a highly odor-specific MTM, which lasts ~ 1 d. This consolidation process is characterized

by a prolonged activity of the cAMP-dependent protein kinase A (PKA) [71]. Different memory phases, which rely on different cellular actors, are established after multiple conditioning trials (Figures 6-8). The early phase (e-LTM) depends on the translation of already existing mRNA and is predominantly induced by massed training (short inter-trial intervals, usually 1 minute).Olfactory memory phases were found to correspond to the temporal dynamics of foraging activities in the field [73] so that early components of memory can be related to the fast succession of experiences that a bee gathers while foraging within a patch or when moving between close patches. In the same way, mid-term memory corresponds, because of its intrinsic dynamics, to the intervals occurring between foraging bouts. Finally, long-term memory relates to foraging bouts that are spaced in time and which may occur on different days [73].

3. Results

The observations indicated that the drones of Indian honeybee, (*A. mellifera. meda*) exhibited greater memory recall to most of the test odors than Eastern honeybee, (*A. cerana.* F) and western honeybee, (*A. mellifera. carnica*). However, in three species, the level of memory recall was highest for 10-HDA followed by Nerol and Octanal. The drones of *A.m. carnica* responded to all the test odors with varied levels of memory recall (Figure 7). The drone bees showed greater response to 10-HDA and low response to IPA. However, an intermediate response was recorded with the odors, Nerol, Octanal, Nonanal, Heptanal, Hexanal, geraniol, and Citral. The highest response was recorded at 3 hrs of conditioning and the lowest response at 1min of conditioning for all the odors. A minimum and maximum mean response of 20.22% and 61.70% were recorded with IPA and 10-HDA respectively (Table 2).

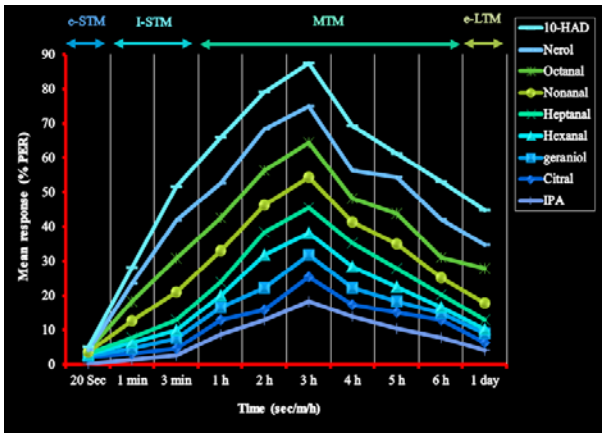

Fig 7: Memory recall of *Apis mellifera carnica* drones to different odors during classical conditioning (N=80).

Table 2: Memory recall of *Apis mellifera carnica* drones (Mean, SD and SEM) to different odors.

Odor	Mean	SD	SEM
10-HDA	61.70	36.93	10.05
Nerol	40.88	21.96	8.48
Octanal	40.38	21.36	8.01
Nonanal	32.42	18.31	7.28
Heptanal	26.16	15.09	5.40
Hexanal	29.02	17.84	6.50
Geraniol	25.34	14.66	5.21
Citral	24.64	14.52	5.19
IPA	20.22	10.16	4.32

Similar to (*A. mellifera. carnica*), the drones of (*A. cerana. indica*) showed a response to all the test odors. However, the low response was recorded towards IPA and greater response towards 10-HDA (Figure 8). The high response was recorded at 3hrs and lowest memory recall at 1 m of conditioning. However, an intermediate response was recorded towards Nerol, Octanal, Nonanal, Heptanal, Hexanal, Geraniol and Citral. The minimum and maximum mean numbers of drones of (*A. cerana.* F) responded towards IPA and 10-HDA was 24.64% and 74.26% respectively (Table 3). (*Apis. cerana.* F) drones responded to all the test odors in greater numbers than (*Apis. mellifera. carnica*) drones. The response of (*Apis. mellifera. carnica*) drones towards 10-HDA was significantly higher than those drones responding to Citral and IPA ($P<0.05$). Similarly the drones response of (*A. cerana.* F) to 10- HDA was significantly higher than that of IPA ($P<0.001$) and (*A. mellifera. carnica*) drones responding to Geraniol, Citral and IPA ($P<0.001$).

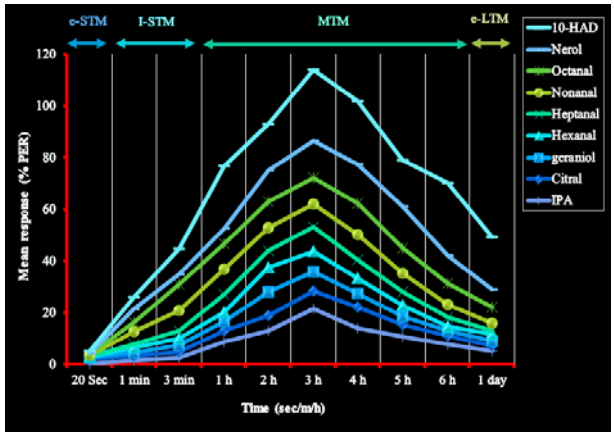

Fig 8: Memory recall of *Apis cerana indica* drones to different odors during classical conditioning (N=100).

Table 3: Memory recall of *Apis cerana indica* drones (Mean, SD and SEM) to different odors.

Odor	Mean	SD	SEM
10-HDA	74.26	43.69	10.36
Nerol	52.88	26.96	9.48
Octanal	44.30	22.46	8.18
Nonanal	34.42	18.41	7.30
Heptanal	28.16	17.09	6.33
Hexanal	30.02	18.24	7.14
Geraniol	27.64	16.66	6.24
Citral	28.73	17.83	6.30
IPA	24.64	14.42	5.09

The drone bees of three races (*Apis. mellifera. carnica*), (*Apis. mellifera. meda*) and (*Apis. cerana. indica*) responded to all the test odors. This is attributed to this ability of honeybee species to distinguish a large range of odors through olfactory sense [36]. The greater memory recall towards 10-HAD could confirm the possible role of this pheromone in communicating the food reward to drone bees. In addition, this pheromone would also act as sex cue for matured drones in mating. The flagellar surface of the drones is twice as large as that of worker bees with many sensory cells [12, 20]. This leads to greater odor perception in drone bees. In honeybees, the behavioral performance reflecting memory storage and retrieval is guided by multiple and discrete memory traces rather than by a single continuously decaying memory trace [70]. Finally, similar to (*A. mellifera. meda*), the drones of (*A. cerana.* F) Showed a response to all the test odors. However,

the low response was recorded towards IPA greater response towards 10- HDA (Figure 9). The high response was recorded at 3hrs and lowest memory recall at 1 m of conditioning. However, an intermediate response was recorded towards Nerol, Octanal, Nonanal, Heptanal, Hexanal, Geraniol and Citral. The minimum and maximum mean numbers of drones of (*A. mellifera. meda*) responded towards IPA and 10-HDA was 27.73% and 82.06% respectively (Table 4). (*Apis. mellifera. meda*) drones responded to all the test odors in greater numbers than (*Apis. cerana. Indica*) drones. The response of (*Apis. mellifera. meda*) drones towards 10-HDA was significantly higher than those drones responding to IPA and Citral ($P<0.05$). Similarly, the response of drones of (*Apis. mellifera. meda*) to 10-HDA was significantly higher than that of IPA ($P<0.001$) and (*Apis. cerana.* Indica) drones responding to Geraniol, Citral and (Isopentyl Acetate) IPA ($P<0.001$).The level of memory recall was greater in drones of (*A. mellifera. meda*), than two races of bees (*A. cerana.* F) and (*A. mellifera. carnica*) and the highest level of learning and memory recall was recorded at 3 hrs.

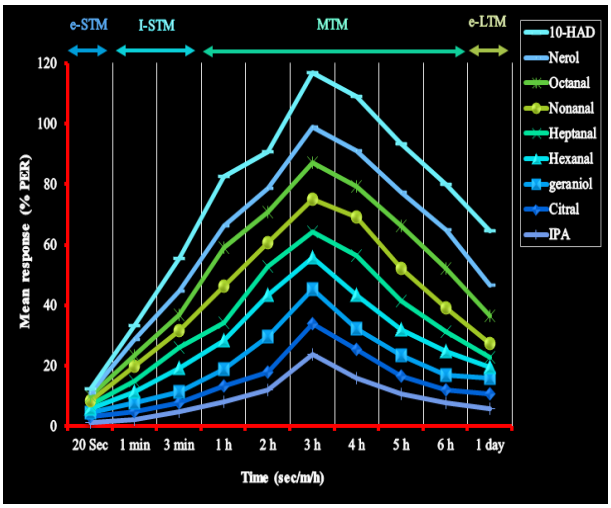

Fig 9: Memory recall of *Apis mellifera meda* drones to different odors during classical conditioning (N=100).

Table 4: Memory recall of *Apis mellifera meda* drones (Mean, SD, and SEM) to different odors.

Odor	Mean	SD	SEM
10-HDA	82.06	43.03	12.43
Nerol	58.61	28.32	9.52
Octanal	44.61	22.86	8.20
Nonanal	36.05	18.44	7.07
Heptanal	30.16	19.03	7.21
Hexanal	29.76	18.04	7.05
Geraniol	38.34	20.56	8.38
Citral	28.22	17.02	6.18
IPA	27.73	16.80	5.65

4. Discussion

The intermediate response of drones to the odors such as Nerol, Octonal and Citral would explore the possible availability of components of these odors in the bee hive, which may be used by drones in orientation and food reward. The reward quality, internal sucrose threshold Scheiner *et al.* (1999) [105, 106], and the concentration of the odor [85] are known to influence the learning ability. In addition, these pheromones may be co-attractants with 9- ODA in a short range [14] and are detected and processed in drone honeybees by the macroglomeruli [6, 77]. Similarly, the low response towards Citral and IPA may be a mechanism of protection against the intruders, where the workers generally release this pheromone for defensive purpose in bee colonies. Vetter and Visscher (1997) studied an electroantennogram (EAG) response of drones to isopentyl acetate, a component of the alarm pheromone of worker bees, and it is found to be surprisingly high, almost in the same range as in that of workers. This high sensitivity suggests that drones perceive IPA pheromone and that it might be important for their behavioral reactions. The results of the study are in conformity with the findings of [7] who found that the learning success of the drones was medium for Heptanal and low for Citral and Isopentyl Acetate (IPA).

5. Conclusion

The drones of (*A. mellifera. carnica*), (*A. mellifera. meda*) and (*A. cerana. indica*) responded to all test odors with varied rates of response. However, the former responded in low numbers compared to the latter. The results of the study confirm that the test odors may be available in bee colonies and are used for various activities of colony members including drone bees. The electrophysiological studies show that an important part of the drone peripheral olfactory system is dedicated to the detection of 9-ODA [118]. The memory recall was gradually decreased with increased duration of conditioning. It is probable that short term memories which allow keeping memory active during shorter periods of time are dominated by non-associative processes such as sensitization. Olfactory memories that are older than four days are indeed known to depend on protein synthesis and gene transcription [100]. In addition, single learning trial leads to a memory trace that fades after a few days while three learning trials lead to a lifelong memory. The greater response of (*A. mellifera. meda*) *and* (*A. cerana. indica*) to the test odors also demonstrate that they have a faster learning and memory recall than (*A.m. carnica*) drone bees. These three species are now sympatric and share a similar environment. Whether learning in *A. cerana* can be studied using the proboscis extension response paradigm, as developed for (*A. mellifera*), is still unexplored. Here, we investigate (*A. mellifera meda's*) associative olfactory learning with nine different odors [(10-hydroxy (E)-2 decenoic acid (10-HDA), Isopentyl Acetate (IPA), Nerol, Citral, Geraniol and Aldehyde groups such as (Hexanal, Heptanal, Octanal and Nonanal)] and compared it with the learning performances of (*A. cerana* F) and (*A. mellifera carnica*). Finally, we conclude that (*A. mellifera meda*) higher learning scores than *A. cerana* F. (Eastern honeybee) and *A. mellifera carnica* hybrid (Western honeybee). Our results show that *A. mellifera meda* (Iranian honeybee) can be studied using proboscis extension reflex (PER) procedures, enlarging the scope for future comparative studies of associative olfactory learning ability in bees. Studies on honeybee behavior allow researcher to be optimistic in their attitude in facing these questions. Moreover, as learning in honeybees can be compared to that of vertebrate in many senses, the honeybee may serve as model system for understanding intermediate levels of complexity of cognitive functionsand their neural substrates [68].

We found that in addition to being amenable to being harnessed and eliciting the proboscis extension reflex (PER), *A. mellifera. meda* drones also performed well in an olfactory conditioning task. A group that was presented with odors in association with a sugar reward quickly learned to respond to the odors, whereas a group that was presented with odors that

was not in association with a sugar reward did not respond to these odors. For example, in a comparison between *A. mellifera* drones and workers, Shafir *et al*. (2005) [101] found that drones generally responded more with PER (unpublished data), but that workers showed better discrimination and greater sensitivity to reward variability. Also, workers tested in spring were more responsive than those tested in winter, but their sensitivity to reward variability was similar Riveros and Gronenberg (2009) [92]. Such olfactory behavior is not observed in all bees studied. For example, Vorel and Pitts-Singer (2010) [116] failed to elicit proboscis extension reflex (PER) in three megachilid bees tested, even after maintaining the bees in less restrictive harnesses and for prolonged times, which may facilitate proboscis extension reflex (PER). Such modifications are often necessary in order to elicit proboscis extension reflex (PER) in bumblebees, but even then response proportions are lower than those typically found in *A. mellifera*. Within bumblebees, *B. terrestris* [1, 57, 58, 94] and *B. occidentalis* [92] seem most amenable to proboscis extension reflex (PER) and limited success has been reported for another eight species studied [116].

The Proboscis Extension Reflex (PER) paradigm [9] is a well-known classical conditioning paradigm widely used with honeybees. Restrained bees (Figure 2) are conditioned to extend their proboscis in anticipation of a food (sugar) reward when they perceive an odor stimulus. The first study that reported evidence of lateralization of olfaction in the honeybee *A. mellifera* was conducted by Letzkus *et al.* (2006) [54] who investigated asymmetries in olfactory learning performance using two different versions of the PER paradigm. In the first version, honeybees were conditioned to extend their proboscis to a scented drop of sugar water but not to an unscented drop of salt water; in the second version, honeybees were conditioned to extend their proboscis to one odor (lemon essence dissolved in a sugar solution-reward) but not to another odor (vanilla essence dissolved in a salt solution- punishment). Results of tests, performed the morning after the training and with any covering of antennae still in place, revealed that the bees with the right antenna covered had learnt less well than the bees with their left antenna covered and bees with both antenna uncovered. In a series of experiments on the laterality of response to a conditioned stimulus (CS), *i.e.*, odor stimuli, Sandoz and Menzel (2001) [99] shown that honeybees could be trained to produce side- specific responses. Bees conditioned to two different odorants, one being learned on each side, produced rather nonspecific response patterns, responding to both odorants on both sides. When conditioned to an odor on one side only, bees responded after a retention period of three hours to this odor on both sides, suggesting that the learned information is indeed transferred between sides. However, Sandoz and Menzel (2001), [99] did not show any asymmetry in the recall of memory. This is maybe due to the retention period of three hours that Sandoz and Menzel (2001) [99], used in their experiments. In fact, as Rogers and Vallortigara (2008) [93] showed, time is a fundamental factor in the recall of olfactory memories and, as a consequence, also in the antennal asymmetries connected with these processes that the 3 h point is the cross-over time when no laterality is apparent. Sandoz *et al.* [99] investigated whether bees can learn a CS when associated with any US components, and what effect such learning has on the opposite brain side. They observed that the antenna-US induces both unilateral and bilateral reinforcement processes, whereas the proboscis-US produces only bilateral effects. In particular, in one of the experiments conducted, bees were subjected to two conditioning phases, the side of CS input changing from one phase to the other. For both antenna-US and proboscis-US, after this change of CS input, a drop in responses was observed, indicating that the CS-US association was not yet retrievable on the other brain side. Interestingly, the main difference between the two studies by Sandoz *et al.* (2001, 2006) [98, 99] was the amount of time after which transfer was tested. Sandoz (2011) [97] tested transfer after a 9-min inter-trial interval, whereas [99] tested for bilateral transfer at 3 h and 24 h after conditioning. To make a comparison between the study by Rogers and Vallortigara (2008) [93] and the previous findings, it is important to emphasize the different experimental procedures. All the bees in the study performed by Rogers and Vallortigara (2008) [93] were trained (learning phase) using both antennae and only after the training (before the recall test) the left or the right antenna was coated with the silicone compound; whereas the bees tested by Letzkus *et al.* (2006) [54] used only one antenna in both the learning and testing phases, and so also did bees tested by Anfora *et al.* [20] to be discussed later. Given that Letzkus *et al.* (2006) [54] tested the bees on the day after the training phase, the differences they revealed to some extent confounded learning with long-term memory recall. In their experiment, bees forced to use only their right antenna both learnt and recalled the task a day later. A similar pattern of lateralized recall of short-term and long-term memory has been shown in three species of Australian stingless bees: (*Trigona carbonaria*), (*Trigona hockingsi*) and (*Austroplebeia australis*) [23]. Bees were trained using both antennae in the PER paradigm to discriminate lemon plus sucrose solution as the positive stimulus and vanilla plus saturated saline as the negative stimulus. Recall of the olfactory memory at 1 h after training was better when the odor was presented to the right than to the left side of the bees. In contrast, recall at 5 h after training was better when the odor was presented to the left than to the right side of the bees. Frasnelli *et al.* (2010) [24] extended the study by Rogers and Vallortigara (2008) [93] by testing lateralized recall of olfactory memory in honeybees at 1 or 6 h after training using different odours. After training with lemon (+) / vanilla (-) or cineol (+) / eugenol (-) recall at 1 h was better when the odor was presented to the right side of the bee than when it was presented to the left side. In contrast, recall at 6 h was better when the odor was presented to the left than to the right side. However, when trained with either a familiar appetitive odor (rose) as a negative stimulus, or with a naturally aversive odor (isoamyl-acetate, IAA-alarm pheromone) as a positive stimulus, bees showed suppression of the response to odors presented on both the right and the left sides at 1 h after training (likely due to retroactive inhibition) [15] and at 6 h after training proboscis protrusion occurred to both odors on both sides. A possible explanation for this behavior is that at 6 h, when access to memory has shifted to the left antenna, memory of these familiar odors in the left side of the brain would be present as two mutually exclusive forms. As a result of very long-term memory either biologically encoded or acquired before testing, the memory would be present as rose positive and IAA negative and, as a result of the long-term memory of training, it would be present as rose negative and IAA positive. A strong effect of the odor used in the Proboscis Extension Reflex (PER) task on the lateralization of short-term memory recall has been reported in honeybees [91]. A series of behavioral experiments assessed response asymmetry of odor recall following proboscis extension reflex conditioning at 1 h after training using three different plant odours (1-octanol, 2-octanone and

1-linalool). The training and the following test were performed on three groups of bees. Hammer *et al.* (2009) [45] showed that the PER paradigm may be used with honeybees not only to investigate olfactory learning but also thermal learning. They showed that honeybees can learn to associate a nectar reward with a heated stimulus applied to the antenna to mimic natural contact with a warm flower or nectar-offering forager. Hammer *et al.* (2009) [45] looked for possible asymmetric use of the antennae in the thermal learning by comparing two groups of honeybees. In the left group they applied the thermal stimulus only to the left antenna, whereas in the right group they applied the thermal stimulus only to the right antenna. Bees were trained to associate the thermal stimulus with a food reward over 24 trials: 12 CS+ and 12 CS- (unrewarded conditioned stimulus) trials. All the studies presented here deal with the asymmetrical use of the antennae of bees in processes such as olfactory learning and recall of the olfactory short- and long-term memory. Letzkus *et al.* (2006) [54] compared the number of the main olfactory receptor sensory organs in honeybees, *Sensilla placodea*, on the two antennae. Images of ten right antennae and ten left antennae (seven of these left-right pairs from the same individuals) were obtained using scanning electron microscopy (SEM) and the mean numbers of *Sensilla placodea* per flagellum on the two antennae were compared. The number was significantly higher on the right than on the left antenna (mean difference of 10%).

We have shown that *A. mellifera. meda* drones are amenable to conditioning of the proboscis extension response. *A. mellifera* consistently performs very well in PER conditioning and can therefore be used as a standard for comparison with other species. Our findings suggest that proboscis extension reflex (PER) conditioning may be an appropriate tool for comparative studies between all honey bee species.

6. Acknowledgement

The authors are grateful to the beekeepers for having kindly provided bee samplestwo exotic species Western hybrid honeybee (*A. mellifera. carnica*) and Eastern honeybee, (*A.ceranea. indica*) in Iran and we would like to thank Department of Honeybees, Animal Sciences Research Institute Karaj of Iran (ASRI) and Department of Entomology and Animal Science Research Varamin- Pishva University for their cooperation and excellent facilitation during study period. We are also grateful to three anonymous reviewers for their insightful comments and helpful suggestions for improvement of the manuscript.

7. Conflicts of interest

The authors declare that they have no conflicts of interest.

8. List of symbols and Abbreviations

(ANOVA): analysis of variance (LH): lateral horn
(CS): conditioned stimulus (CR): conditioned response
(US): unconditioned stimulus (STM): short-term memory
(PER): proboscis extension reflex (LTM): long-term memory
(AL): antennal lobe (MTM): mid-term memory
(MB): mushroom bodies (EAG): electroantennogram

9. References

1. Anfora G, Rigosi E, Frasnelli E, Ruga V, Trona F, Vallortigara G. Lateralization in the invertebrate brain: left-right asymmetry of olfaction in bumble bee, *Bombus terrestris*. PLos One. 2011; 6:4-9.
2. Anfora G, Frasnelli E, Maccagnani B, Rogers LJ, Vallortigara G. Behavioral and electrophysiological lateralization in a social (*Apis mellifera*) but not in a non-social (*Osmia cornuta*) species of bee. Behavioural Brain Research. 2010; 206:236-239.
3. Ache BW, Young JM. Olfaction: diverse species, conserved principles. Neuron. 2005; 48:417-430.
4. Admassu A. Botanical inventory and phenology in relation to the foraging behavior of the Cape honeybees (*Apis Mellifera* Capensis) at a site in the Eastern Cape, South Africa. Unpublished MSc Thesis, Rhodes University, South Africa. 2003, 67-73.
5. Abel R, Rybak J, Menzel R. Structure and response pattern s of olfactory inter- neurons in the honeybee, *Apis. mellifera*. Journal of Comparative Neurology. 2001; 437:363-383.
6. Arnold G, Masson C, Budharugsa S. Comparative study of the antennal lobes and their efferent pathways in the worker bee and the drone (*Apis mellifera*). Cell and Tissue Research. 1985; 242:593-05.
7. Becker M, Bruckner D, Crewe R. Behavioral response of drone honeybees, *Apis. mellifera carnica* and *Apis mellifera scutellata* to worker produced pheromone components. Journal of Apicultural Research. 2000; 39:149-52.
8. Bitterman ME. Comparative analysis of learning in honeybees. Animal Behavior. 1996; 24:123-141.
9. Bitterman ME. Vertebrate-invertebrate comparisons. NATO ASI Ser Intelligence Evolutionary Biology. 1988; 17:251-275.
10. Bitterman ME, Menzel R, Fietz A, Schafer S. Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). Journal of Comparative Psychology. 1983; 97:107-119.
11. Breed MD, Guzman Novoa E, Hunt GJ. Defensive behavior of honeybees: organization, genetics and comparisons with other bees. Annual Review of Entomology 2004; 49:271-98.
12. Brockmann A, Bruckner D. Structural differences in the drone olfactory system of two phylogenetically distant *Apis* species, *Apis florea* and *Apis mellifera.* Naturwissenschaften. 2001; 88:78-81.
13. Brockmann A, Bruckner D, Crewe R. The EAG response spectra of workers anddrones to queen honeybee mandibular gland components: the evolution of a social signal. Naturwissenschaften. 1998; 85:283-85.
14. Brockmann A, Dietz D, Spaethe J, Tautz J. Beyond 9-ODA: sex pheromone communication in the European honeybee, *Apis mellifera* L. Journal of Chemical Ecology. 2006; 32:657-67.
15. Cheng K, Wignall AE. Honeybees (*Apis mellifera*) holding on to memories: Response competition causes retroactive interference effects. Animal Cognition. 2006; 9:141-50.
16. Chaffiol A, Laloi D, Delegue MHP. Prior classical olfactory conditioning improves odor cued flight orientation of honeybees in a wind tunnel. Journal of Experimental Biology. 2005; 208:3731-737.
17. Davis RL. Olfactory memory formation in Drosophila: from molecular to systems neuroscience. Annual Review of Neuroscience. 2005; 28:275-302.
18. Eisthen HL. Why are olfactory systems of different animals so similar? Brain Behavior and Evolution. 2002; 59: 273-293.
19. Erber J, Masuhr T, Menzel R. Localization of short-term memory in the brain of the bee, *Apis mellifera*.

Physiological Entomology. 1980; 5:343-358.
20. Esslen J, Kaissling KE. Zahl und verteilung antenna ler sensillen bei der honigbiene (*Apis mellifera* L.) Zoomorphology. 1976; 83:227-51.
21. Felsenberg J, Gehring KB, Antemann V, Eisenhardt D. Behavioural pharmacology in classical conditioning of the proboscis extension response in honeybees (*Apis mellifera*). Journal of Visual Experiment. 2011; 47:e2282.
22. Frost EH, Shutler D, Hillier NK. Effects of cold immobilization and recovery period on honeybee learning, memory, and responsiveness to sucrose. Journal of Insect Physiology. 2011; 57:1385-90.
23. Frasnelli E, Vallortigara G, Rogers LJ. Right-left antennal asymmetry of odor memory recall in three species of Australian stingless bees. Behavioural Brain Research. 2011; 224:121-127.
24. Frasnelli E, Vallortigara G, Rogers LJ. Response competition associated with right-left antennal asymmetries of new and old olfactory memory traces in honeybees. Behavioural Brain Research. 2010; 209:36-41.
25. Frisch K. The dance language and orientation of bees. Harvard University Press; Cambridge, MA, USA; 1967.
26. Frisch K. Über den Geruchssinn der Bienen und seine blütenbiologische Bedeutung. Zool. Jb., Abt. Allg. Zoological Physiology. 1919; 37:1-238.
27. Frings H, Frings M. The loci of contact chemoreceptors in insects. A review with new evidence. American Midland Naturalist. 1949; 41:602-58.
28. Frings H. The loci of olfactory end-organs in the honey-bee, *Apis mellifera* Linn. Journal of Experimental Zoology. 1944; 88:65-93.
29. Goulson D, Park KJ, Tinsley MC, Bussière LF, Vallejo-Marin M. Social learning drives handedness in nectar robbing bumblebees. Behavioral Ecology Sociobiology. 2013; 67:1141-1150.
30. Giurfa M, Sandoz JC. Invertebrate learning and memory: fifty years of olfactory conditioning of the proboscis extension response in honeybees. Learning and Memory. 2012; 19:54-66.
31. Gerber B, Stocker RF, Tanimura T, Thum AS. Smelling, tasting, learning: *Drosophila* as a study case. Results Problem Cell Different. 2009; 47:139-185.
32. Galizia CG. Insect olfaction, in The Senses, *A Comprehensive Reference*, (Eds) Smith DV, Firestein S, Beauchamp GK. (London: Elsevier), 2008, 725-769.
33. Galizia CG, Szyszka P. Olfactory coding in the insect brain: molecular receptive ranges, spatial and temporal coding. Entomologia Experimentalis ET Applicata. 2008; 128:81-92.
34. Galizia CG. Neuroscience: brainwashing, honeybee style. Science. 2007; 317:326-27.
35. Giurfa M. Behavioral and neural analysis of associative learning in the honeybee: a task from the magic well. Journal of Comparative Psychology A-Neuroethology Sensory Neural and Behavior. 2007; 193:801-824.
36. Guerrieri F, Schubert M, Sandoz JC, Giurfa M. Perceptual and neural olfactory similarity in honeybees. Plos Biology. 2005; 3:e60.
37. Ghirlanda S, Enquist M. A century of generalization. Animal Behavior. 2003; 66:15-36.
38. Giurfa M. Cognitive neuroethology: dissecting non-elemental learning in a honeybee brain. Current Opinion Neurobiology. 2003; 13:726-35.
39. Giurfa M, Lehrer M. Honeybee vision and floral displays: from detection to close-up recognition. In Chittka L Thompson R. (Eds) Cognitive ecology of pollination. Cambridge University Press; Cambridge, UK: 2001, 61-82.
40. Galizia CG, Menzel R. The role of glomeruli in the neural representation of odours: results from optical recording studies. Journal of Insect Physiology. 2001; 47:115-129.
41. Gould JL, Marler P. Ethology and the natural history of learning. In The Biology of Learning (Eds. Marler P, Terrace HS), New York: Springer-Verlag. 1984, 47-74.
42. Hildebrand JG, Shepherd GM. Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. Annual Review of Neuroscience. 1997; 20:595-631.
43. Hammer M. The neural basis of associative reward learning in honeybees. Trends in Neurosciences. 1997; 20:245-252.
44. Hammer M, Menzel R. Learning and memory in the honeybee. Journal of Neuroscience. 1995; 15:1617-1630.
45. Hammer TJ, Hata C, Nieh JC. Thermal learning in the honeybee, *Apis mellifera*. Journal of Experimental Biology 2009; 212:3928-3934.
46. Kaspi R, Shafir S. Associative olfactory learning of the red dwarf honeybee *Apis florea*. Apidologie. 2013; 44:100-109.
47. Kells AR, Goulson D. Evidence for handedness in bumblebees. Journal of Insect Behavior. 2001; 14:47-55.
48. Kirschner S, Kleineidam CJ, Zube C, Rybak J, Grunewald B, Rössler W. Dual olfactory pathway in the honeybee, *Apis mellifera*. Journal of Comparative Neurology. 2006; 499:933-952.
49. Koeniger N, Koeniger G. Reproductive isolation among species of the genus *Apis*. Apidologie. 2000; 31:313-39.
50. Kuwabara M. Bildung des bedingten reflexes von Pavlovs Typus bei der honigbiene, *Apis mellifera*. Journal of Forensic Science. 1957; 13: 458-464.
51. Kenyon FC. The brain of the bee–a preliminary contribution to the morphology of the nervous system of the Arthropoda. Journal of Comparative Neurology. 1896; 6:134-210.
52. Leonard AS, Dornhaus A, Papaj DR. Flowers help bees cope with uncertainty: signal detection and the function of floral complexity. Journal of Experimental Biology. 2011; 214:113-121. doi:10.1242/jeb.047407.
53. Leon M, Johnson BA. Is there a space-time continuum in olfaction? Cellular and Molecular Life Sciences. 2009; 66:2135-2150.
54. Letzkus P, Ribi WA, Wood JT, Zhu H, Zhang SW, Srinivasan MV. Lateralization of olfaction in the honeybee *Apis mellifera*. Current Biology 2006; 16:1471-1476.
55. Letzkus P, Boeddeker N, Wood JT, Zhang SW, Srinivasan MV. Lateralization of visual learning in the honeybee. Biology Letters. 2008; 4:16-18.
56. Lledo PM, Gheusi G, Vincent JD. Information processing in the mammalian olfactory system. Physiological Reviews. 2005; 85:281-317.
57. Laloi D, Pham-Delegue MH. Bumble bees show asymmetrical discrimination between two odors in a classical conditioning procedure. Journal of Insect Behavior. 2004; 17:385-396.
58. Laloi D, Sandoz JC Picard-Nizou AL, Marchesi A, Pouvreau A, Taser JN, Poppy G, Pham-Delegue MH.

Olfactory conditioning of the proboscis extension in bumble bees. Entomologia Experimentalis ET Applicata. 1999; 90:123-129.
59. Laurent G. Olfactory network dynamics and the coding of multi- dimensional signals. Nature Reviews Neuroscience. 2002; 3:884-895.
60. Laska M, Galizia CG, Giurfa M, Menzel R. Olfactory discrimination ability and odor structure-activity relationships in honeybees. Chemical Senses. 1999; 24:429-438.
61. Matsumoto Y, Menzel R, Sandoz, JC, Giurfa, M. Revisiting olfactory classical conditioning of the proboscis extension response in honey bees: a step towards standardized procedures. Journal of Neuroscience Methods. 2012; 211:159-167.
62. Mc Cabe SI, Farina WM. Olfactory learning in the stingless bee *Tetragonisca angustula* (Hymenoptera, Apidae, Meliponini). Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavior. 2010; 196:481-490.
63. Mandairon N, Linster C. Odor perception and olfactory bulb plasticity in adult mammals. Journal of Neurophysiology. 2009; 101:2204-2209.
64. Masse NY, Turner GC, Jefferis GS. Olfactory information processing in *Drosophila*. Current Biology. 2009; 19:700-713.
65. Mc Cabe S, Hartfelder K, Santana W, Farina W. Odor discrimination in classical conditioning of proboscis extension in two stingless bee species in comparison to Africanized honeybees. Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavior. 2007; 193:1089-1099.
66. Mori K, Takahashi YK, Igarashi KM, Yamaguchi M. Maps of odorant molecular features in the mammalian olfactory bulb. Physiological Reviews. 2006; 86:409-433.
67. Mizunami M, Yokohari F, Takahata M. Further exploration into the adaptive design of the arthropod microbrain: I. Sensory and memory-processing systems. Zoological Science. 2004; 21:1141-51.
68. Menzel R, Giurfa M. Cognitive architecture of a mini-brain: the honeybee. Trends in Cognitive Science. 2001; 5:62-71.
69. Menzel R, Manz G, Menzel RM, Greggers U. Massed and spaced learning in honeybees: The role of CS, US, the inter-trial interval, and the test interval. Learning and Memory. 2001; 8:198-208.
70. Menzel R. Searching for the memory trace in a mini-brain, the honeybee. Learning and Memory. 2001; 8:53-62.
71. Müller D. Die olfaktorische Kodierung: Plastizität und Stabilität-Eine elektrophysiologische Analyse der Ausgangsneurone des Antennallobus der Honigbiene. PhD Thesis, FU Berlin. 2000.
72. Mori K, Nagao H, Yoshihara Y. The olfactory bulb: coding and processing of odor molecule information. Science. 1999; 286:711-715.
73. Menzel R. Memory dynamics in the honeybee. Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavior. 1999; 185:323-340.
74. Menzel R, Müller U. Learning and memory in honeybees: From behavior to neural substrates. Annual Review of Neuroscience. 1996; 19:379-404.
75. Menzel R, Gaio UC, Gerberding M, Nemrava EA, Wittstock S. Formation of longterm olfactory memory in honeybees does not require protein synthesis. Naturwissenschaften. 1993; 80:380-82.
76. Menzel R, Backhaus W. Color vision in insects. In Gouras, P (ed.) Vision and Visual dysfunction: the perception of color. MacMillan Press, London. UK: 1991, 262-288.
77. Masson C, Mustaparta H. Chemical information processing in the olfactory system of insects. Physiological Reviews. 1990; 70:199-45.
78. Menzel R. Learning, memory, and "cognition" in honeybees. In: Neurobiology of comparative cognition (Kesner RP Olten DS, Eds), Hillsdale, NJ: Erlbaum. 1990, 237-292.
79. Menzel R. Learning in honeybees in an ecological and behavioral context. In: Experimental behavioral ecology (Hölldobler B, Lindauer M, Eds), Stuttgart: Fischer Verlag), 1985, 55-74.
80. Menzel R. Neurobiology of learning and memory: the honeybee as a model system. Naturwissenschaften. 1983; 70:504-511.
81. Mobbs PG. The brain of the honeybee *Apis mellifera* I. The connections and spatial organization of the mushroom bodies. Philosophical Transactions of the Royal Society B-Biological Sciences. 1982; 298:309-354.
82. Menzel R. Behavioral access to short-term memory on bees. Nature. 1979; 281:368-369.
83. Menzel R, Erber J, Masuhr T. Learning and memory in the honeybee. In Experimental analysis of insect behavior (ed. L.B. Browne), Springer, Berlin: 1974, 195-217.
84. Nagaraja N, Brukner D. Olfactory learning and memory recall in drones of hive honeybee species. Journal of Entomological research. 2013; 37:29-32.
85. Pelz C, Gerber B, Menzel R. Odorant intensity as a determinant for olfactory conditioning in honeybees: roles in descrimination, overshadowing and memory consolidation. Journal of Experimental Biology. 1997; 200:837-47.
86. Peng YS, Fang Y, Xu S, Ge L. The resistance mechanism of the Asian honey bee, *Apis cerana* Fabr, to an ectoparasitic mite, *Varroa jacobsoni* Oudemans. Journal of Invertebrate Pathology. 1987; 49:54-60.
87. Pareto A. Die zentrale Verteilung der Fühlerafferenzbei Arbeiterinnen der Honigbiene, *Apis. mellifera* L. Z. Zellforsch. 1972; 131:109-140.
88. Pavlov IP. Conditioned reflexes. Oxford University Press, Oxford. 1927.
89. Qin QH, He XJ, Tian LQ, Zhang SW, Zeng Z.J. Comparison of learning and memory of *Apis cerana* and *Apis mellifera*. Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavior. 2012; 198:777-8.
90. Rogers LJ, Rigosi E, Frasnelli E, Vallortigara G. A right antenna for social behavior in honeybees. Scientific Reports. 2013; 3:2045.
91. Rigosi E, Frasnelli E, Vinegoni C, Antolini R, Anfora G, Vallortigara G, Haase A. Searching for anatomical correlates of olfactory lateralization in the honeybee antennal lobes: A morphological and behavioral study. Behavioural Brain Research. 2011; 221:290-294.
92. Riveros AJ, Gronenberg W. Olfactory learning and memory in the bumblebee *Bombus occidentalis*. Naturwiss. 2009; 96:851-856.
93. Rogers LJ, Vallortigara G. From antenna to antenna: Lateral shift of olfactory memory in honeybees. PLos One. 2008, 3:e2340.
94. Riddell CE, Mallon EB. Insect psychoneuroimmunology:

immune response reduces learning in protein starved bumblebees (*Bombus terrestris*). Brain Behavior Immunity. 2006; 20:135-138.
95. Rescorla RA. Behavioral studies of Pavlovian conditioning. Annual Review of Neuroscience. 1988; 1:329-352.
96. Shafir S, Kaspi R. Associative olfactory learning of the red dwarf honeybee *Apis florea*. Apidologie. 2013; 44:100-109.
97. Sandoz JC. Behavioral and neurophysiological study of olfactory perception and learning in honeybees. Frontiers in System Neuroscience. 2011; 5:98. http://www.frontiersin.org/Systems_Neuroscience/editorialboard.
98. Sandoz JC. Odour-evoked responses to queen pheromone components and to plant odours using optical imaging in the antennal lobe of the honeybee drone, Apis mellifera L. Journal of Experimental Biology. 2006; 209:3587-598.
99. Sandoz JC, Menzel R. Side-specificity of olfactory learning in the honeybee: Generalization between odors and sides. Learning & Memory. 2001; 8:286-294.
100. Schwaerzel M, Muller U. Dynamic memory networks: dissecting molecular mechanisms underlying associative memory in the temporal domain. Cellular and Molecular Life Science. 2006; 63:989-98.
101. Shafir S, Menda G, Smith BH. Caste-specific differences in risk sensitivity in honeybees, *Apis mellifera*. Animal Behavior. 2005; 69:859-868.
102. Strausfeld NJ. Organization of the honeybee mushroom body: representation of the calyx within the vertical and gamma lobes. Journal of Comparative Neurology. 2002; 450:4-33.
103. Shafir S, Wiegmann DD, Smith BH Real LA. Risk-sensitive foraging: choice behavior of honeybees in response to variability in volume of reward. Animal Behavior. 1999; 57:1055-1061.
104. Scheiner R, Page RE, Erber J. Responsiveness to sucrose affects tactile and olfactory learning in preforaging honey bees of two genetic strains. Behavioural Brain Research. 2001; 120:67-73.
105. Scheiner R, Erber J, Page RE Jr. Tactile learning and the individual evaluation of the reward in honeybee (*Apis mellifera* L.). Journal of Comparative Physiology. 1999; 185:1-10.
106. Srinivasan MV, Poteser M, Kral K. Motion detection in insect orientation and navigation. Vision Research. 1999; 39:2749-2766.
107. Seeley TD. The Wisdom of the Hive–The Social Physiology of Honey Bee Colonies. London: Harvard University Press. 1995.
108. Srinivasan MV. Pattern recognition in the honeybee: recent progress. Journal of Insect Physiology. 1994; 40:183-194.
109. Spear NE, Miller JS, Jagielo JA. Animal memory and learning. Annual Review of Psychology. 1990; 41:169-211.
110. Smith BH, Menzel R. The use of electromyogram recordings to quantify odor discrimination in the honeybee. Journal of Insect Physiology. 1989; 35:369-375.
111. Suzuki H. Convergence of olfactory inputs from both antennae in the brain of the honeybee. Journal of Experimental Biology. 1975; 62:11-26.
112. Tan K, Wang Z, Li H, Yang S, Hu Z, Kastberger G, Oldroyd BP. An 'I see you' prey-predator signal between the Asian honeybee, *Apis cerana*, and the hornet, *Vespa velutina*. Animal Behaviour. 2012a; 83:879-882.
113. Tan K, Yang S, Wang ZW, Radloff SE, Oldroyd BP. Differences in foraging and broodnest temperature in the honey bees *Apis cerana* and *A. mellifera*. Apidologie. 2012b; 43:618-623.
114. Thom C, Gilley DC, Hooper J, Esch HE. The scent of the waggle dance. PloS Biology. 2007; 5:e228.
115. Takeda K. Classical conditioned response in the honey bee. Journal of Insect Physiology. 1961; 6:168-79.
116. Vorel CA, Pitts-Singer TL. The proboscis extension reflex not elicited in megachilid bees. Journal of the Kansas Entomological Society. 2010; 83:80-83.
117. Vareschi E. Duftunterscheidung bei der Honigbiene-Einzelzell-Ableitungen und Verhaltensreaktionen Z. vergl. Physiologie. 1971; 75:143-73.
118. Vetter RS, Visscher PK. Influence of age on antennal responses of male honeybees, Apis mellifera to queen mandibular pheromone and alarm pheromone component. Journal of Chemical Ecology. 1997; 23:1867-880.
119. Wang ZW, Tan K. Comparative analysis of olfactory learning of *Apis cerana* and *Apis mellifera*. Apidologie. 2014; 45:45-52.
120. Wright GA, Mustard J, Kottcamp SM, Smith BH. Olfactory memory formation and the influence of reward pathway during appetitive learning by honey bees. The Journal of Experimental Biology. 2007; 210:4024-4033.
121. Wehner R. Spatial vision in arthropods. In Autrum HJ. (Ed.) Handbook of sensory physiology. Springer-Verlag; Berlin: Germany, 1981, 287-616.
122. Winston ML. The Biology of the Honey Bee. Cambridge, MA: Harvard University Press. 1987.
123. Yang GH. Harm of introducing the western honeybee *Apis mellifera* L. to the Chinese honeybee *Apis cerana* F. and its ecological impact. Acta Entomologica Sinica. 2005; 48:401-406.