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Original article

Olfactory associative behavioral differences in three honey bee *Apis mellifera* L. races under the arid zone ecosystem of central Saudi Arabia

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ABSTRACT

Apis mellifera jemenitica is the indigenous race of honey bees in the Arabian Peninsula and is tolerant to local drought conditions. Experiments were undertaken to determine the differences in associative learning and memory of honey bee workers living in the arid zone of Saudi Arabia, utilizing the proboscis extension response (PER). These experiments were conducted on the indigenous race (*A. m. jemenitica*) along with two introduced European races (*A. m. carnica* and *A. m. ligustica*). The data revealed that *A. m. jemenitica* is amenable to PER conditioning and may be used in conditioning experiments within the olfactory behavioral paradigm. The results also demonstrated that the three races learn and retain information with different capacities relative to each other during the experimental time periods. Native Arabian bees (*A. m. jemenitica*) exhibited significantly lower PER percentage during second and third conditioning trials when compared to exotic races. *Apis mellifera jemenitica* also exhibited reduced memory retention at 2 h and 24 h when compared to *A. m. carnica* and *A. m. ligustica.* Therefore, the native Arabian bees were relatively slow learners with reduced memory retention compared to the other two races that showed similar learning and memory retention. Three or five conditioning trials and monthly weather conditions (October and December) had no significant effects on learning and memory in *A. m. jemenitica.* These results emphasized a novel line of research to explore the mechanism and differences in associative learning as well as other forms of learning throughout the year among bee races in the harsh arid conditions of Saudi Arabia. This is the first study in Saudi Arabia to demonstrate inter-race differences regarding olfactory associative learning between native Arabian bees and two introduced European honey bee races.

1. Introduction

The Western honey bee (*Apis mellifera* L.) is a highly valuable insect owing to its use in managed agricultural ecosystems for pollination, including a broad array of economically critical crops, as well as for honey and several nutritional and therapeutic products (Southwick and Southwick, 1992; Free, 1993; Watanabe, 1994; Roubik, 2002). Honey bees have a range of sensory behavioral repertoires with sophisticated learning and memory capabilities, all of which are critical to colony success (Menzel and Giurfa, 2006; Giurfa, 2007). In fact, a honey bee colony's survival is dependent on foraging workers and their ability to learn and remember quickly, particularly in locating viable resources and recruit additional workers to such sources. Honey bees have the ability to associate different stimuli such as color, shape, and odour with nectar sources through associative learning (Menzel and Müller, 1996; Giurfa and Menzel, 1997; Erber et al., 1998; Faber and Menzel, 2001; Giurfa, 2004). The proboscis extension response (PER) is a well-established method for monitoring associative learning in conditioned honey bees under laboratory conditions (Sandoz et al., 1995; Menzel and Müller, 1996; Beekman, 2005; Farina et al., 2005; Giurfa, 2007; Smith and Burden, 2014).

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Apis mellifera has many geographical races over its wide distribution over Eurasia and Africa (Ruttner, 1976; Engel, 1999), with diverse behavioral and ecological adaptations. In Saudi Arabia, the most common bee races used for beekeeping are *Apis mellifera jemenitica* Ruttner (70–80%), *Apis mellifera carnica* Pollmann, and *Apis mellifera ligustica* Spinola. The first race is native to the Arabian Peninsula with 70–80% of the total bee colonies, whereas the last two are exotic European races with 30–70% of the total bee colonies in the country (Ruttner, 1976; Alqarni, 2006; Alqarni et al., 2011, 2014). These bee races may not exhibit response in similar way to thermal stress and their success corresponds to adapted response to ecological stresses (Abou-Shaara et al., 2012; Abou-Shaara et al., 2017).

Apis mellifera jemenitica has been observed to be the most successful of the races under the hot climatic conditions present in central Saudi Arabia, where summer temperature often exceeds 45 °C (Alqarni et al., 2011). Although high temperature has an impact on honey bee activity, many beekeepers reported tolerance and better performance of the native Arabian race in the hot-dry environment. Native Arabian bees produce a large brood and collect more pollen on average; however, once summer temperature approaches 40 °C, this leads to their rapid consumption of the stored honey (Alqarni, 1995). Native Arabian bees have the ability to quickly increase colony size with a large number of worker cells during periods of less rainfall and shorter flowering times (Chandler, 1975; Woyke, 1993).

In central Saudi Arabia, the peak of pollen-collection and brood-rearing seasons are between March–June (major period) and October–November (minor) (Alqarni, 1995). Native honey bees were successfully domesticated in modern bee hives by replacing the traditional log hives (Al-Ghamdi, 2005), and were more heat tolerant as compared to imported bees (Abou-Shaara, 2015). Nonetheless, native honey bees are too scant with relatively less output per hive to cope with the country's demand for honey and so there is a continued reliance on imported honey bee races (Al-Ghamdi et al., 2017).

The learning behavior of native Arabian honey bees remains to be addressed. Do native honey bees behave (learning and retain memory) differently than exotic races, particularly under the climatic conditions present in Saudi Arabia? Given differences in heat tolerance and body sizes between the races of honey bees in Saudi Arabia, the present study investigated the olfactory associative learning of the native honey bee race (*A. m. jemenitica*) in comparison with the two imported races from Europe (*A. m. carnica, A. m. ligustica*) by using the proboscis extension response (PER). We report for the first time experimental evidence for learning and memory retention in *A. m. jemenitica*. These preliminary findings demand further investigation regarding the impact of extreme arid conditions on the physiology and other behaviors of native Arabian honey bees. This study will also be helpful to manipulate the knowledge for improvement of native bee colonies in the field conditions.

2. Materials and methods

2.1. Experimental location

The experiments were performed in the Neurobiology Lab at the educational farm of King Saud University, Riyadh, Saudi Arabia during Oct–Dec 2016. The apiary of exotic (*A. m. carnica* and *A. m. ligustica*) and native (*A. m. jemenitica*) honey bee races was located in near vicinity of the laboratory at the educational farm which was free from any use of pesticides. The geographical coordinates of the site are 24.73°N, 46.61°E, and the height above sea level is 658 m. The colonies of native bees (*A. m. jemenitica)* were raised from queens of native origin, and of exotic races from queens imported from Egypt (*A. m. carnica)* and Jordan (*A. m. ligustica)* (Balhareth et al., 2012). A morphometric characterization showed the existence of these races in Saudi Arabia (Ruttner, 1976; Alattal et al., 2014).

2.2. Collecting and handling of bees

Adult foragers of both native and exotic races were captured in small glass vials from the entrance of hives. The bees were immobilized on ice in the laboratory and harnessed in small plastic tubes for subsequent behavioral tests as prescribed in previous studies (Iqbal and Mueller, 2007; Smith and Burden, 2014). All bees were fed 0.5 M sucrose solution till satiation and kept overnight in darkness in plastic containers until behavioral tests at artificially controlled room temperature (20–25 °C) in the lab.

2.3. Associative olfactory learning and memory tests

The overnight incubated forager bees were placed at the experimental site and left to acclimatize for at least 10 min. The initial motivation of individual bees for associative learning was done 10 min prior to actual learning trials by touching the antenna of the bee being tested with 0.5 M sucrose solution without feeding. The bees that did not show the reflex were discarded (Baracchi et al., 2018). 10 µl clove oil was applied to filter paper with a 20 ml plastic syringe for presenting the odour stimulus to bees (Smith and Burden, 2014). The bees were conditionally trained using classical Pavlovian conditioning of proboscis extension (Takeda, 1961; Müller, 2002). The conditioning trial consisted of pairing of an odour stimulus (conditioned stimulus, CS: clove oil for 5 s) with an appetitive reward stimulus (unconditioned stimulus, US: 1 M sucrose for 4 s). The bees were trained for three successive conditioning trials at 2 min Inter-trial intervals elicited all properties of long-term memory (Iqbal and Mueller, 2007; Merschbaecher et al., 2012; Müller, 2013). The bees not responding to the US (sucrose) during conditioning were excluded from the experiment. The memory tests were performed by stimulation with odour only at 2 h and 24 h after training. The percentage of bees exhibiting proboscis extension response (PER) to the conditioned odorant was used as a monitor to determine the level of learning and memory of the experimental bees. Multiple separate experiments were performed to detect any difference in learning and memory of the honey bee races being tested.

2.4. Data analysis

The datasets on associative learning and memory retention at different time periods were compared using a simple Chi-square test. Bonferroni correction was applied to control the risk of a false positive (Bland and Altman, 1995).

3. Results

3.1. Associative behavioral performance

The behavioral tests revealed a significant difference between learning and memory of native and exotic races. Fig. 1 shows that *A. m. jemenitica* (native race) exhibited a significantly lower PER percentage during 2nd ($\chi^2 = 14.450$; p = 0.0001) and 3rd ($\chi^2 = 17.215$; p = 0.0000) conditioning trials as compared to *A. m. ligustica* (exotic race). Moreover, the memory test at 2 h ($\chi^2 = 17.972$; p = 0.0000) and 24 h ($\chi^2 = 8.165$; p = 0.0043) after conditioning revealed reduced PER percentages in *A. m. jemenitica.*

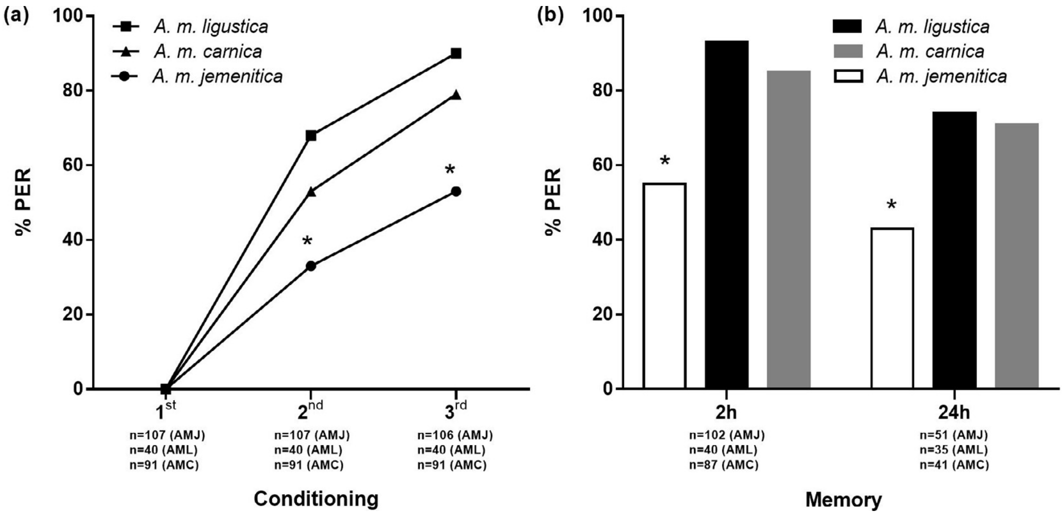

Fig. 1. Comparison of associative learning and memory retention among bee races (a) PER (%) explicit the significantly lower learning response during conditioning trials in *A. m. jemenitica* at 2nd (χ^2 = 14.450; p = 0.0001), (χ^2 = 8.109; p = 0.0044) and 3rd (χ^2 = 17.215; p = 0.0000), (χ^2 = 14.871; p = 0.0001) as compared to *A. m. ligustica* and *A. m. carnica*, respectively. The exotic bee races (*A. m. carnica* and *A. m. ligustica*) exhibited no differences in learning. (b) PER (%) showed the significant differences in memory retention of. *A. m. jemenitica* at 2 h (χ^2 = 17.972; p = 0.0000), (χ^2 = 19.884; p = 0.0000) and 24 h (χ^2 = 8.165; p = 0.0043), (χ^2 = 7.005; p = 0.0081) as compared to *A. m. ligustica* and *A. m. carnica*, respectively. The exotic bee races (*A. m. carnica* and *A. m. ligustica*) exhibited no differences in memory formation. The conditioning trial consisted of paring an odour stimulus with a subsequent sucrose reward to the antenna and proboscis. Odour alone was used during the memory retention tests. n = sample size below x-axis (AMJ = *A. m. jemenitica*, AML = *A. m. ligustica*, AMC = *A. m. carnica*). The asterisks indicate the significant difference between the groups (χ^2 test with Bonferroni correction; $^*p$ < 0.025).

Similarly, the learning and memory of the native race (*A. m. jemenitica*) was also significantly different from the other exotic race (*A. m. carnica*). A significantly lower PER percentage and memory retention values were exhibited by workers of *A. m. jemenitica* during 2nd (χ^2 = 8.109; p = 0.0044) and 3rd (χ^2 = 14.871; p = 0.0001) conditioning trials and at 2 h (χ^2 = 19.884; p = 0.0000) and 24 h (χ^2 = 7.005; p = 0.0081) when compared to workers of *A. m. carnica* (Fig. 1).

Worker honey bees of both exotic races showed identical trends regarding associative learning and memory retention tests. The PER of both races showed similar associative learning patterns during conditioning trials (Fig. 1). Likewise, memory retention at different time intervals after conditioning (2 h and 24 h) also showed no significant difference between the two European races. This indicates that workers of the native race (*A. m. jemenitica*) have olfactory behavioral differences with lower learning and memory retention relative to workers of exotic *A. m. carnica* and *A. m. ligustica.*

3.2. Monthly trend of associative learning in A. m. jemenitica

The experiments were performed during October and December 2016. We therefore attempted to look for any effect of monthly variations on associative learning in the native race (*A.m. jemenitica*). Accordingly, we compared the PER data for these two months (Fig. 2). No significant difference in PER percentages of the native race was found during the two months of learning trials and memory retention tests. Thus, any effect of monthly weather conditions on associative learning was ruled out.

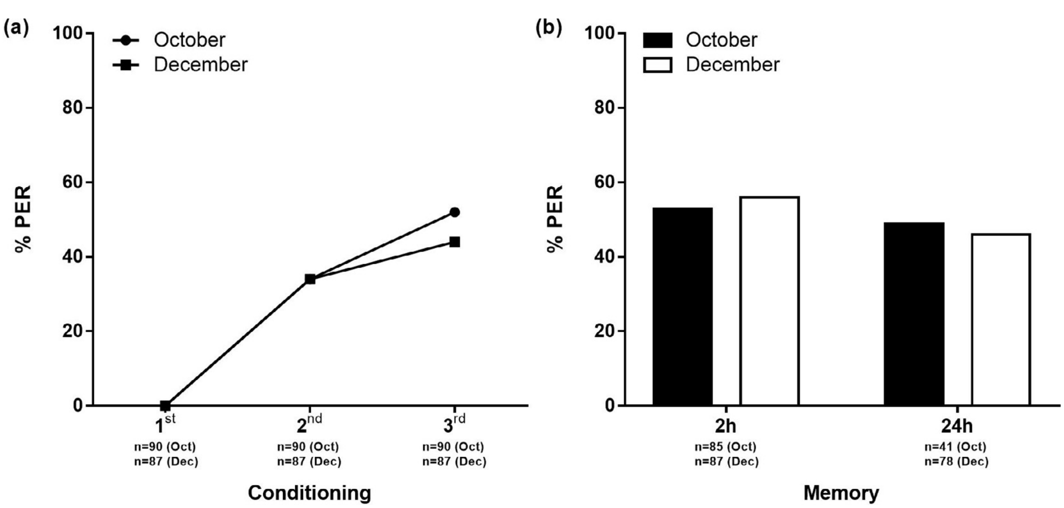

Fig. 2. Effect of monthly weather on learning and memory retention of *A. m. jemenitica*. PER (%) showed no differences in learning (a) and memory retention (b) during two different months (October & December). Thus, associative learning remained intact during October and December 2016. n = sample size below x-axis (Oct = October, Dec = December).

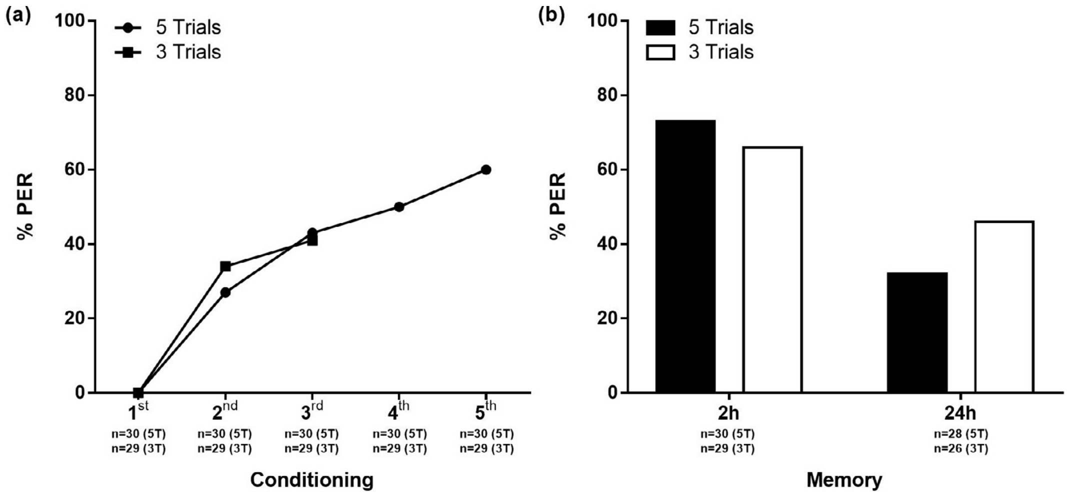

Fig. 3. Effect of number of conditioning trials on learning and memory of *A. m. jemenitica.* PER (%) showed no differences in learning (a) and memory (b) formation in *A. m. jemenitica* during 3 or 5 conditioning trials. Thus, associative learning remains intact irrespective of 3 or 5 conditioning trials**.** n = sample size below x-axis (5T = 5 Trials, 3T = 3 Trials).

3.3. Associative learning in A. m. Jemenitica after three and five conditioning trials

Since the native race (*A. m. jemenitica*) exhibited comparatively slow learning relative to the two exotic races, it was hypothesized that three conditioning trials might not be sufficient to elicit sufficiently robust learning and memory retention. Possibly more conditioning trials might lead to enhanced learning and memory in workers of the native race. Therefore, associative learning and memory was tested after three and five conditioning trails in workers of the native race. The data revealed no significant difference in learning and memory retention after three and five conditioning trials (Fig. 3). Consequently, any effect of the number of learning trials (three or five) on learning and memory was ruled out from our data.

4. Discussion

Olfactory foraging behavior in honey bees is crucial for the pollination of different crops (Free, 1993; Yang et al., 2012). This behavior is closely related to the impressive ability of honey bees to learn and memorize local features and routes, and to associate them with reliable nectar sources (Frisch and Chadwick, 1993; Menzel and Müller, 1996; Giurfa, 2004; Fry and Wehner, 2005; Leonard et al., 2011). The mechanism underlying learning and memory in honey bees is highly conserved (Kandel, 2001). However, a variation in the performance of the various races of honey bees cannot be ruled out due to many extrinsic as well as intrinsic factors such as genetic variation, the size of the bees, geographical area, local climatic conditions, evolutionary lability, and diversified foraging patterns (Riessberger and Crailsheim, 1997; Alqarni et al., 2014; Meixner et al., 2015). The cognitive abilities (learning and memory retention) of three different honey bee races were compared for the first time in the rather harsh climate of Saudi Arabia. It was observed that the honey bee races tested have different capacities in terms of learning and memory retention. Workers of *A. m. carnica* and *A. m. ligustica* showed no differences regarding learning and memory retention, a finding which is in accordance with previous studies (Hoefer and Lindauer, 1975). Similarly, no statistical difference was reported in the retention phase between *A. cerana* and *A. mellifera* (Wang and Tan, 2014).

Our data indicate that workers of *A. m. jemenitica* may also be taken as a potential honey bee race for neuroethological research. Native Arabian honey bees did not perform (learning and retaining memory) as well as the European races during the periods of testing. The inter-racial olfactory behavioral variations observed in the present study are strengthened by a previous report where African honey bees were also found to be slow learners in associative learning relative to their European counterparts (Couvillon et al., 2010). Honey bee races may have differential abilities to adopt the confined test conditions, reactions to harnessing, and to achieve satiation at different levels (Wang and Tan, 2014). Our preliminary results also reflect another possible reason that *A. m. jemenitica* bees might be more sensitive to the stress of whole harnessing situation than European bees that may lead to behavioral differences. Further investigations are proposed for aforementioned possible reasons behind the differential associative behavioral performance among bee races.

The small body size of native Arabian honey bees might be associated with their reduced learning and memory. An association of body size with learning differences was reported in bumble bees (Worden et al., 2005), and this is likely also the case among honey bees where there are notable size differences in morphometeric analysis among the various species and individual geographic races (Alattal et al., 2014)**.** An increase in foraging distance with larger body size has also been documented in honey bees (Greenleaf et al., 2007). The smaller body size of *A. m. jemenitica* aids its high thermal tolerance (Abou-Shaara, 2015), but begs the question as to whether such bees with greater heat tolerance truly learn slower and retain information for shorter duration, and why this should be the case? One possible explanation would be the difference in stress levels inside the body resulting from such temperatures and that might have a drastic effect on performance. Native Arabian honey bees may utilize a lot of energy to cope with heat stress, and for greater foraging activity when compared to exotic bees under the climatic conditions present in central Saudi Arabia around Riyadh (Alqarni, 2006; Abou-Shaara et al., 2012). High foraging activity causes high levels of oxidative stress in flight muscles that may be associated with a decline in cognitive performance (Williams et al., 2008; Behrends and Scheiner, 2010).

Moreover, native Arabian honey bees consume stored honey at a faster rate than exotic races, which is tied to their physical foraging activity as well as possibly increased metabolic rates (Alqarni et al., 2014). Exotic bees are commercially viable in Saudi Arabia but are facing quick depletion due to their poor survival under field conditions within the country (Al-Ghamdi et al., 2017). Indeed, the exotic races have the lowest value for foraging and tolerating summer temperatures (Alqarni, 2006; Abou-Shaara et al., 2012), even though their learning and memory retention is better than the native Arabian race, as demonstrated herein. Further experiments are necessary in order to compare the related behavioral performance of these races throughout the year and in different regions of the country where local climates can vary considerably relative to those of central Saudi Arabia. A relatively quick change in weather can have significant effects on survival of honey bee colonies (Riessberger and Crailsheim, 1997), with the highest rate of colony losses during October–December in Saudi Arabia (Al-Ghamdi et al., 2017). Given this, further experiments are needed to explore any differences in the learning capabilities of the native Arabian race between the hot summer months and winter season.

Apparently learning increases with an increase in brain size, and usually larger animals possess correspondingly bigger brains (Chittka and Niven, 2009). If this is universally the case, then the small brain size of native Arabian bees could be another reason for their decreased learning capacity relative to the two European races examined. However, it is debatable that brain size is a good predictor of behavior, with varied results linking this metric with the range of behavioural repertoires and cognitive capacity of bees (Couvillon et al., 2010; Chittka and Skorupski, 2011; Kotrschal et al., 2013).

Possible genetic based learning differences of native Arabian honey bees relative to exotic European honey bees could not be ruled out. Among European honey bees, the genetic factors are reported to contribute significantly to variability in associative learning among individuals (Brandes, 1991). Further investigation is needed in order to identify such genetic variations among individual honey bees among *A. m. jemenitica*, and to determine whether it can be related to behavioral performance. As of yet, no such variations have been identified among populations or individuals of *A. m. jemenitica*, or whether there are differences in learning capacity between such populations across the range of this honey bee race.

5. Conclusion

Our study has demonstrated inter-racial differences regarding learning and memory retention between three races of Western honey bees (*Apis mellifera*). The behavioral evidence concluded that classical PER conditioning can be applied to the native Arabian race of honey bees (*A. m. jemenitica*). Native Arabian bees were slower at learning and had reduced memory retention when compared to two exotic European honey bee races during October–December in central Saudi Arabia. No significant difference was observed between learning and memory performance of the two exotic European races. Further studies will be helpful to understand the mechanism behind the variation in associative learning and its link to other behavioral performances of the native Arabian honey bee race such as flight duration, flight distance, foraging frequency throughout the year and under the different climatic regions of the Arabian Peninsula.

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