Journal of Insect Physiology 57 (2011) 1385–1390

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Effects of cold immobilization and recovery period on honeybee learning, memory, and responsiveness to sucrose

Elisabeth H. Frost, Dave Shutler *, Neil Kirk Hillier

Department of Biology, Acadia University, 33 Westwood Avenue, Wolfville, Nova Scotia, Canada B4P 2R6

ARTICLE INFO

Article history:
Received 18 March 2011
Received in revised form 27 June 2011
Accepted 5 July 2011
Available online 13 July 2011

Keywords:
Honeybee
Cold immobilization
Recovery period
Proboscis conditioning
Sucrose response

ABSTRACT

In addition to human error and variation in laboratory conditions, there are numerous factors that can complicate comparisons among studies. Furthermore, differences in how experimental methods are executed can make it difficult to distinguish between effects of focal versus extraneous variables. Insect neural function is commonly evaluated using Pavlovian conditioning techniques; learning and memory in many species can be assessed using the proboscis extension reflex (PER). However, there are significant inconsistencies in methods used to immobilize insects prior to PER tests. We compared responses of honeybees immobilized in a refrigerator, on ice, and in a freezer, and evaluated influence of recovery interval before testing. Ice-chilling weakly decreased learning (response to an originally neutral odor) more so than refrigeration or freezing, but not 24-h recall of odor. We found no significant differences in responsiveness to sucrose relative to cooling method, but responsiveness was significantly lower among honeybees left to recover for only 0.75 h versus 1.5 or 3 h. Finally, we observed increased responsiveness to sucrose and geraniol between June and August. Our results suggest that inconsistencies in cold immobilization methods could confound interpretation and comparison of results from a large body of work on honeybee learning and memory.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Temperature plays an important role in insect development and can influence maturation rates (Porter, 1988; Pickup and Thompson, 1990), neural function (Groh et al., 2004; Peng et al., 2007), morphology (Crill et al., 1996; Frazier et al., 2008), and behavior (Han and Gatehouse, 1991; Becher et al., 2009). In honeybees (*Apis mellifera*), pupal rearing temperature affects brain development (Groh et al., 2004), memory acquisition and retention (Tautz et al., 2003; Jones et al., 2005), behavioral specialization (Becher et al., 2009), and susceptibility to various stressors including pesticides and parasites (Le Conte et al., 1990; Flores et al., 1996; Medrzycki et al., 2010). Among adult honeybees, temperature can alter frequency of flight departures (Burrill and Dietz, 1981), pollination activity (Benedek and Prenner, 1972; Cooper et al., 1985; Corbet et al., 1993), locomotory rhythms (Moore and Rankin, 1993), juvenile hormone titers (Lin et al., 2004), hoarding behavior (Free and Williams, 1972), and vulnerability to pesticides (Benedek, 1975). Here, we assess how variation in cold immobilization affects honeybee sensory responses.

Honeybee neural function can be evaluated using a Pavlovian conditioning method that focuses on the proboscis extension reflex (PER) (Bitterman et al., 1983). When their antennae contact sucrose, honeybees reflexively extend their proboscis to feed, but naïve animals will not respond to presentation of neutral odors. However, if an odor (conditioned stimulus) is presented immediately before a sucrose reward (unconditioned stimulus), honeybees can learn this association and may eventually extend their proboscis to the odor alone. Thus, associative learning can be assessed by measuring honeybees' ability to learn and retain associations between an originally neutral cue and a corresponding sucrose reward. In addition, responsiveness to sucrose provides an indication of honeybee health and propensity for learning (Scheiner et al., 1999, 2001, 2003), and can be quantified using assays that measure honeybees' responsiveness to increasing concentrations (Pankiw and Page, 2003; Kralj et al., 2007).

Although PER is widely used to assess learning and memory, there is substantial inconsistency in PER experimental design that remains largely unconsidered (Frost, 2011). These inconsistencies have potentially profound influences on how one interprets results of a large body of published work. A common inconsistency is the way in which honeybees are immobilized for handling; there has been little testing of whether variation in these methods affects honeybee cognition (Pankiw and Page, 2003).

Honeybees can be handled when active for marking and experimental manipulation (Mercer and Menzel, 1982; Gary and Lorenzen, 1988; Ichikawa and Sasaki, 2003; Pankiw and Page, 2003; Gil and De Marco, 2005), but the majority are immobilized

* Corresponding author. Tel.: +1 902 585 1354; fax: +1 902 585 1059.
E-mail addresses: 075309f@acadiau.ca (E.H. Frost), dave.shutler@acadiau.ca (D. Shutler), kirk.hillier@acadiau.ca (N.K. Hillier).

0022-1910/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jinsphys.2011.07.001

using low temperatures (e.g., Erber et al., 1980; Ray and Ferneyhough, 1997; Hori et al., 2006). Post-chilling effects on invertebrates include reduced foraging recruitment in *Bombus occidentalis* bumblebees up to several days after treatment (Wilson et al., 2006), prolonged copulation latency in *Drosophila melanogaster* fruit flies (Barron, 2000), disrupted kin recognition in *Aleochara bilineata* beetle larvae (Lizé et al., 2010), temporary amnesia in *D. melanogaster* (Xia et al., 1999; Knapek et al., 2010), and increased aggressive behavior in *Formica xerophila* ants (Tanner, 2009). Thus, consequences of how insects are immobilized, and length of recovery before behavioral experiments commence, should be carefully evaluated prior to selecting methods to assess learning (PER to odor during conditioning trials) and memory. (We note that although it is conventional to use PER to odor during extinction trials as evidence of memory, absence of PER does not necessarily mean that memory is impaired.) Honeybee memory formation is inhibited when individuals are cooled within 5 min after a conditioning trial (Erber et al., 1980). However, cold exposure prior to olfactory conditioning does not appear to affect learning or memory if honeybees are allowed to recover for at least 1 h (Pankiw and Page, 2003). Chilled honeybees actually exhibit a lower response threshold to sucrose after recovery, and Pankiw and Page (2003) concluded that, in part, immobilization prior to handling reduces associated stress.

Although exposure to cold temperatures is the most common method of immobilizing honeybees, within this general method techniques vary from refrigeration (Ray and Ferneyhough, 1997; Scheiner et al., 2003; Blažytė-Čereškienė and Skirkevičius, 2006; Hammer et al., 2009) to cooling on ice (Mamood and Waller, 1990; Masterman et al., 2001; Tautz et al., 2003; Hori et al., 2006; Kralj et al., 2007; Mattila and Smith, 2008; Carcaud et al., 2009) and temporary freezing (Erber et al., 1980; Deisig et al., 2006). Further, although individuals are usually cooled until immobile (thus duration of cooling varies), some researchers instead use a set interval (e.g., Erber et al., 1980; Ray and Ferneyhough, 1997; Carcaud et al., 2009; Hammer et al., 2009). In light of the variety of (often unspecified) methods employed, we studied effects of cooling method on honeybee learning, memory, and responsiveness to sucrose, and potential consequences for interpretation of PER results. Our results have significant implications for assessment of honeybee learning and memory.

2. Materials and methods

2.1. Honeybee collection

Conditioning experiments were conducted between 30 June and 15 July 2009; sucrose response experiments were conducted between 13 and 23 July 2010. To minimize variation in age of honeybees, we used only older individuals; these were foragers found on the outside of the hive (Higginson and Barnard, 2004). Honeybees were captured in small plastic scintillation vials (with air holes) near the hive entrance of colonies in Coldbrook, Nova Scotia, Canada. We did not discriminate between pollen and nectar foragers. Although forager role can affect responsiveness (Scheiner et al., 2003), individuals were randomly assigned to treatments. In any case, it is common for age of foragers (which typically dictates foraging role; i.e., pollen, nectar, or water) to be an uncontrolled variable in PER studies (e.g., Bitterman et al., 1983; Menzel, 1999; Scheiner et al., 2003). Honeybees were transported to the laboratory and cooled in their vials at temperatures we verified with a thermometer in a refrigerator (5 °C), a freezer (−18 °C), or on crushed ice (0 °C). Individuals were cooled for the minimum interval needed for immobilization (intervals to achieve this were recorded only for 2010 experiments). After cooling, honeybees were restrained inside modified 1000-μL plastic pipette tips (the tapered end was removed) with wax strips so only their antennae and mouthparts were free outside. Honeybees from each treatment group were assessed during the same trials in a random order, and PER responses were evaluated blind to treatment. All treatments were tested synchronously to reduce the influence of temporal variation on results.

2.2. Olfactory conditioning

Restrained honeybees were fed approximately 1 μL of 1.5 M sucrose solution using a wooden toothpick and left to recover for 3 h in the dark at room temperature, at which point they were taken to the laboratory for testing. A continuous flow of humidified air was provided at a flow rate of 0.8–1.0 L/min. Prior to training, honeybees were exposed to stimulus-free air for 30 s to acclimatize them to mechanosensory stimulation from a constant airflow. The conditioned stimulus was delivered by pipetting 3 μL of 'neat' geraniol (i.e. *trans*-3,7-Dimethyl-2,6-octadien-1-ol; Sigma–Aldrich; a monoterpenoid common in many plant oils and a component of the Nasonov pheromone, released by workers to attract nest mates) on filter paper, housed inside a syringe. A manual valve was used to switch between continuous air and the stimulus cartridge. Both continuous and stimulus flow lines were united at a mixing chamber (3 mm internal diameter; 10 mm in length) with the exodus held 10–15 mm from the anterior surface of an individual's head. Stimulus air was directed over an individual's antennae for a total of 6 s. The unconditioned stimulus, 1.5 M sucrose solution, was presented 3 s after the onset of the odor, for an overlap of 3 s. The right antenna was touched with a toothpick dipped in sucrose and then moved to the mouthparts for approximately 2 s of feeding. Vacuum flow was used subsequently to remove odors from the training arena.

Individuals were trained for 8 trials (a single trial consisting of accommodation, exposure to odor, and reward) with an inter-trial interval (ITI) of 9 min (published ITIs range from 30 s to more than 20 min; Erber, 1975; Gerber et al., 1998; Hellstern et al., 1998; Faber et al., 1999; Menzel, 1999; Menzel et al., 2001). Memory retention was monitored by assessing PER to the trained odor 24 h after conditioning trials (Hammer and Menzel, 1995). At this time, odor was presented in the absence of a reward (8 extinction trials; 9 min ITI).

A positive PER response was assigned when a honeybee extended its proboscis immediately following presentation of geraniol, before the sugar reward. For each trial, a score of 0 represented either no response or extension of the proboscis only after antennal stimulation with sucrose, and a score of 1 indicated proboscis extension to the odor alone. Although there were 8 trials, the maximum score for conditioning experiments was 7; positive response to geraniol on the first trial was spontaneous, suggesting prior exposure or an innate response (Gerber et al., 1996; Sandoz et al., 2000).

2.3. Sucrose responsiveness assay

To test sucrose responsiveness, bees were randomly assigned to one of the cooling methods. Following several authors (Masterman et al., 2001; Weick and Thorn, 2002; Decourtye et al., 2003; Jones et al., 2005; McCabe et al., 2007), honeybees prepared for sucrose response assays were fed approximately 1 μL of 1.5 M sucrose solution (by stimulating their proboscis directly, not their antennae) and left to recover for 0.75, 1.5, or 3 h. Honeybees then had their PER responses tested using a sucrose responsiveness technique that involves touching the antenna with water (0 M), followed by ascending sucrose concentrations (Pankiw and Page, 2000, 2003; Scheiner et al., 2001; Kralj et al., 2007) of 0.005, 0.01, 0.05, 0.1, 0.5, 1, 1.5 M to determine their responsiveness to

water and sucrose. The inter-stimulus interval was 3 min to prevent sensitization between successive sucrose stimulations. A positive PER response was assigned when a honeybee extended its proboscis following antennal stimulation. For each trial, a score of 0 represented no response, and a score of 1 indicated proboscis extension to the presented concentration, in this case with a maximum score of 8.

2.4. Statistical analyses

Statistical analyses were conducted in SPSS (Version 17.0, 2008, SPSS Inc., Chicago, IL, USA). Honeybee learning, memory, and responsiveness to sucrose were compared to test for differences in effects of cooling method and recovery period. Individuals had a total score for conditioning and extinction trials reflecting the number of positive responses to the conditioning odor (geraniol); gustatory scores represent sucrose responsiveness. Duration of cooling required for immobilization was compared among treatments using a general linear model; Tukey's tests were used for post-hoc pairwise comparisons. Because PER data were not normally distributed, scores among treatments were compared using Kruskal–Wallis Tests, and Mann Whitney U Tests were used for post-hoc testing. Because there were three pairwise comparisons, the Bonferroni adjusted cutoff p would be 0.05/3 = 0.017, although some authorities argue against too stringently applying this correction (Rothman, 1990; Nakagawa, 2004). Temporal change in responsiveness was assessed using a logistic regression. Means are reported ± 1 SD.

3. Results

3.1. Cooling duration

Likely because of a short-term fluctuation in temperature in the refrigerator, on one occasion three bees took >40 min to become immobilized; these bees were excluded from analysis because SPSS identified them as outliers. There was a significant effect of cooling method on duration of exposure required for immobilization ($F_{2,435} = 76.0$, $p < 0.0001$; Table 1). Cooling in the refrigerator was slower than both temporary freezing (Tukey tests, $p < 0.0001$) and cooling on ice ($p < 0.0001$). There was no difference between cooling duration for ice versus freezing ($p = 0.94$).

3.2. PER conditioning

Individuals responding spontaneously to either geraniol (6.6%, $n = 6$) or mechanical stimulation from airflow (4.4%, $n = 4$) were removed from statistical analyses. Average number of positive PER responses by honeybees during learning trials was 6.2 for refrigerator-cooled ($n = 30$), 5.7 for freezer-cooled ($n = 25$), and 5.0 for ice-cooled honeybees ($n = 24$), out of a possible 7 (Kruskal–Wallis $\chi^2_2 = 5.3$, $p = 0.07$). This low p suggested running pairwise comparisons of cooling methods although caution is warranted in interpreting these results; accordingly, ice-cooled honeybees had reduced learning compared to refrigerator-cooled conspecifics ($U = 238.5$, $p = 0.02$; not significant if Bonferonni correction is applied); remaining comparisons were not significant (refrigerator versus freezer: $U = 313.5$, $p = 0.25$; freezer versus ice: $U = 245.0$, $p = 0.25$). Average number of positive responses did not vary among treatments during extinction trials (4.7 for both refrigerator- and ice-cooled honeybees, $n = 28$ and 23, respectively, and 4.2 for freezer-cooled honeybees, $n = 25$; Fig. 1; Kruskal–Wallis $\chi^2_2 = 0.2$, $p = 0.91$).

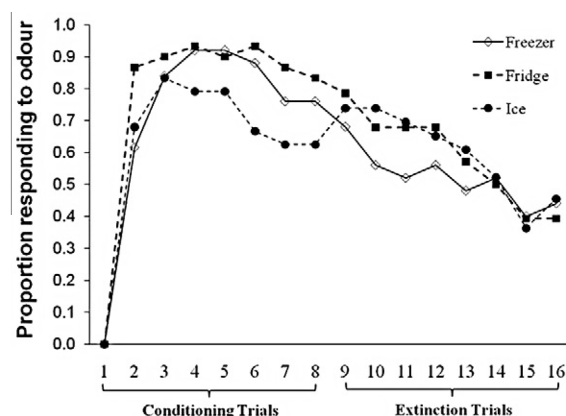

Fig. 1. Proportion of honeybees responding to geraniol prior to a presentation of 1.5 M sucrose solution. Extinction trials (memory) to assess proboscis extension to odor occurred 24 h after conditioning.

We estimated that less than 10% of approximately 60 honeybees had PER responses to sucrose in early June before we were satisfied that the assay was working. In contrast, approximately 95% ($n = 91$) of honeybees had PER responses to the first presentation of sucrose between 30 June and 15 July, with no significant trend with respect to day of the year within this interval (logistic regression, $Z_1 = 1.4$, $p = 0.24$).

3.3. Sucrose responsiveness

Honeybees that did not extend their proboscis, even to the highest concentration of sucrose (1.5 M), were excluded from analyses (4.1%, $n = 9$). There was no significant effect of cooling method on PER (Kruskal–Wallis $\chi^2_2 = 1.2$, $p = 0.56$). However, individuals left to recover for only 0.75 h had significantly lower response scores than honeybees recovering for either 1.5 h ($U = 2271$, $p = 0.002$) or 3.0 h ($U = 1470$, $p = 0.002$; Table 2 and Fig. 2; overall Kruskal–Wallis $\chi^2_2 = 14.2$, $p = 0.001$), whereas response to sucrose did not differ between individuals left to recover for 1.5 versus 3.0 h ($U = 1880$, $p = 0.87$).

4. Discussion

4.1. Effects of cooling method on interval to immobilization

In our study, there were significant differences in time to immobilization. Differences could arise from intra-colony genetic differences (Mattila and Seeley, 2007) or environmental conditions, each of which could contribute to variation in honeybee size, food reserves, and/or metabolic rate (Free and Spencer-Booth, 1960). In general, temperature thresholds needed to immobilize bees vary with caste (Robinson and Visscher, 1984), and are lower for workers than for drones or queens (Free and Spencer-Booth, 1960;

Table 1
Interval (min) required to immobilize honeybees for each cooling method.

Method	Mean	SD	Min	Max	n
Freezer	2.4	0.5	1.4	4.0	84
Refrigerator	9.0	4.4	3.5	25.3	76
Ice	2.5	0.8	1.0	5.5	85

Table 2
Number of positive responses to eight different sucrose concentrations (0–1.5 M; see Section 2) for honeybees permitted to recover from cold immobilization for different intervals.

Recovery Period (h)	Median gustatory response score	n
0.75	4	85
1.50	6	75
3.00	7	51

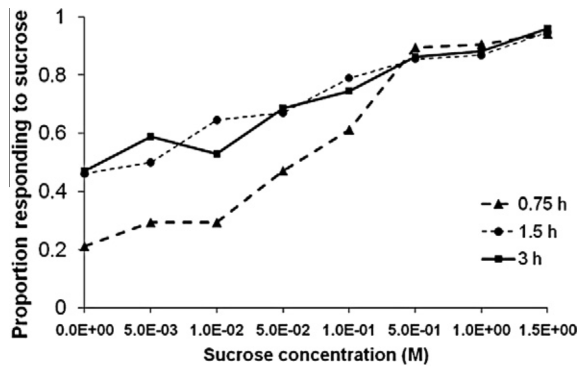

Fig. 2. Effect of recovery period from cold immobilization on honeybee responsiveness to different concentrations of sucrose.

Hrassnigg and Crailsheim, 2005). Among workers, foragers are most resistant to low temperatures, possibly because they spend more time in cooler parts of the hive and are exposed to cool temperatures outside the hive (Free and Spencer-Booth, 1960; Kovac et al., 2010). This highlights the need to consider potential intra-colony variation when evaluating whether it is appropriate to use a set interval of cold exposure to immobilize honeybees.

4.2. Effects of cooling method on learning and memory

Ice-chilled honeybees scored lower in PER assays than refrigerator-chilled ones during learning (conditioning trials); however, ice-chilling did not appear to affect their memory, nor was ice-chilling associated with lower response scores during extinction trials than was the case with other chilling methods. The lack of long-term differences may reflect benefits of a sufficient recovery period post-immobilization, which may be important for counteracting short–term consequences of chilling and handling stress. Pankiw and Page (2003) recommended that individuals be left to accommodate for at least 1 h prior to experiments, but our results suggest that honeybees in conditioning experiments may require a more lengthy recovery, especially after being chilled with ice. More generally, latent effects of cooling may skew selection of individuals that are retained for PER assays if honeybees show low responsiveness to sucrose or reduced propensity to learn an odor association (e.g., Chandra et al., 2000; Sandoz et al., 2000; Drezner-Levy et al., 2009). Results of these and similar studies may have been affected by these exclusion rules (Frost, 2011). Interestingly, a lower learning acquisition curve would appear to indicate a failure to learn an odor-reward association. Thus ice-chilled individuals appear to 'learn' the odor less readily than do freezer- and refrigerator-chilled honeybees. However, high odor recall among ice-chilled honeybees (comparable to individuals from other cooling techniques) during extinction trials suggests that an odor-reward association was learned despite a lack of behavioral evidence of learning (i.e., PER). This highlights the importance of testing both memory acquisition and retention (Abramson et al., 1999; Hussaini et al., 2009) when assessing honeybees' neural function, because learning and memory could be partially independent.

Our data also highlight that refrigerators may vary substantially in the temperatures they provide; temperatures will fluctuate depending on the sensitivity of temperature readers and the upper and lower tolerance settings. This will introduce additional inconsistency to comparisons among studies.

4.3. Effects of day of the year on learning and memory

In June 2009, we estimated <10% of honeybees responded to antennal stimulation with sucrose versus ⩾90% typically reported in other studies (e.g., Takeda, 1961; Hori et al., 2006; Deisig et al., 2007; Drezner-Levy et al., 2009). By the beginning of July, without changing our methods, honeybees began responding consistently to a sugar stimulus. This is likely a reflection of shifts in responsiveness to sucrose that is lowest among foragers in June and July (Scheiner et al., 2003). Temporal changes in responsiveness have been documented in studies using outdoor colonies (De Jong and Pham-Delègue, 1991; Ray and Ferneyhough, 1997; Blažytė-Čereškienė and Skirkevičius, 2006), with time of year significantly affecting memory acquisition, retention, and learning rate (Scheiner et al., 1999, 2001, 2003). Seasonal shifts may be affected by fluctuating levels of neuroendocrine secretions; Harris and Woodring (1992) found that levels of octopamine, dopamine, and serotonin peaked in honeybee colonies between June and September. These biogenic amines influence acquisition of appetitive memories; octopamine enhances sensitivity to olfactory stimuli, whereas dopamine and serotonin reduce responsiveness to a conditioned stimulus and inhibit memory retrieval (Mercer and Menzel, 1982; Harris and Woodring, 1992; Hammer and Menzel, 1998; Scheiner et al., 2002). Effects of food quality and availability may also modulate sucrose responsiveness and learning performance (Wahl and Ulm, 1983; Ray and Ferneyhough, 1997), although nutritional stress (poor quality pollen) alone does not affect olfactory learning, memory, or responsiveness to sucrose (Mattila and Smith, 2008), suggesting multidimensional effects of season.

In this study approximately 6.6% of foragers responded spontaneously to geraniol in July, similar to 6.2% in Smith (1991). However, in August/September 2009 and 2010, spontaneous responses increased to 28% and 17% respectively (Frost, 2011); this apparent temporal variation is likely affected by changes in forage plants that are available. Learned odor-reward associations can be transferred from natural settings to laboratory behavioral assays (Gerber et al., 1996; Arenas et al., 2007), and increase rates of spontaneous response to odors during PER conditioning (Gerber et al., 1996; Gil and De Marco, 2005). For this reason, most spontaneous responders are discarded from analyses (Erber et al., 1980; Mercer and Menzel, 1982; Bitterman et al., 1983; Dacher et al., 2005), as we did. However, we caution that this decision rule could also affect results and their interpretation.

4.4. Effects of cooling on honeybee responsiveness to sucrose

Published post-cooling recovery periods range from 0.5 to 24 h (Decourtye et al., 2003; Hammer et al., 2009; Mattila and Smith, 2008; Scheiner et al., 2003; Tautz et al., 2003), and are generally viewed as starvation windows. However, we also found a significant effect of recovery period on honeybee responsiveness to sucrose. Mean gustatory score, that measures overall responsiveness to sucrose, was significantly lower for honeybees left to recovery for only 0.75 h versus 1.5 or 3 h. This is consistent with Pankiw and Page (2003) who found a significant effect of cooling on sucrose responsiveness after 0.5 h, and recommended a recovery period of at least 1 h. This time-dependent responsiveness to sucrose likely represents a minimum period necessary to recover from the stress of immobilization. It is important to note that individuals recovering for >0.75 h had longer starvation windows; extending the interval between immobilization and the start of behavioral experiments could have increased responsiveness to sucrose due to hunger. However, all individuals were fed a small aliquot of sucrose following immobilization and because of the short duration of recovery periods (relative to 24-hr starvation windows often used in PER studies; e.g., Ray and Ferneyhough, 1997; Abramson et al., 2008), 'starvation' may not have been a major factor in responses. Furthermore, because of the small amount of sucrose delivered to the proboscis, satiation is also unlikely (Menzel et al., 2001).

5. Conclusions

Pavlovian conditioning experiments can provide valuable information on neural function, and can be used to investigate effects of numerous factors (e.g., environmental, genetic, chemical). PER is also favored for its procedural simplicity, broad range of applications, and low energy expenditure that make it easier to distinguish between physical and cognitive deficiencies. However, because of widespread variation in PER techniques (e.g., cooling method), the potential for experimental artefacts, and inherent environmental and genetic variation (e.g., age, caste, season, food availability), it is important to recognize and address inconsistency in techniques.

First, researchers must provide detailed accounts of PER protocols. Authors frequently fail to specify cooling method and duration of cold exposure, or discuss implications of immobilizing honeybees using low temperatures. However, this information may be vital when interpreting results, especially in light of differences among studies. For example, Free and Williams (1972) used 10 min at −5 °C and observed an increase in hoarding behavior, whereas Mardan and Rinderer (1980) used 3 min at −20 °C and observed a decrease; the latter authors attributed this disparity to cooling technique.

Second, it is important to consider age and physiology when selecting an immobilization protocol. Although individuals are typically cooled for the minimum time required for immobilization, some researchers instead use a set interval (e.g., Erber et al., 1980; Ray and Ferneyhough, 1997; Carcaud et al., 2009; Hammer et al., 2009). For example, Ebadi et al. (1980) suggested a 3-min exposure to −20 °C to immobilize foragers. However, this treatment duration was fatal to 85% of 1-day-old workers (Robinson and Visscher, 1984).

Finally, and more generally, the enormous potential for variation must be thoroughly addressed when interpreting results. In most instances, it will be impossible to fully elucidate which factor, or combination of factors, is affecting honeybee neural function. For example, Decourtye et al. (2003) found that pesticide toxicity (the causative factor being investigated) varied with season, and posited that this could reflect reduced nutritional quality in summer forage, particularly inadequate pollen supply. However, there are a number of additional factors that vary with season (e.g., temperature, barometric pressure, prevalence and intensity of diseases) that could influence honeybee responses.

Laboratory studies invariably introduce a host of variables that may not exist under natural conditions, and most are unavoidable. Although this does not negate the validity of laboratory results, or limit potential applications, more research is needed to examine potentially compounding and interacting effects of environmental, physiological, and experimental variation on honeybee learning and memory.

Acknowledgments

This research was supported by the National Sciences and Engineering Research Council of Canada, the Canadian Bee Research Fund, and Bee Maid Canada. Research assistance was provided by D. Deveau. K. Spicer provided bees, and members of the Hillier and Shutler labs provided various forms of assistance.

References

Abramson, C.I., Aquino, I.S., Ramalho, F.S., Price, J.M., 1999. The effect of insecticides on learning in the Africanized honeybee (*Apis mellifera* L.). Archives of Environmental Contamination and Toxicology 37, 529–535.

Abramson, C.I., Mixson, T.A., Çakmak, I., Place, A.J., Wells, H., 2008. Pavlovian conditioning of the proboscis extension reflex in harnessed foragers using paired vs. unpaired and discrimination learning paradigms: tests for differences among honeybee species in Turkey. Apidologie 39, 428–435.

Arenas, A., Fernández, V.M., Farina, W.M., 2007. Floral odor learning within the hive affects honeybees' foraging decisions. Naturwissenschaften 94, 218–222.

Barron, A.B., 2000. Anaesthetising *Drosophila* for behavioural studies. Journal of Insect Physiology 46, 439–442.

Becher, M.A., Scharpenberg, H., Moritz, R.F.A., 2009. Pupal developmental temperature and behavioral specialization of honeybee workers (*Apis mellifera* L.). Journal of Comparative Physiology A 195, 673–679.

Benedek, P., 1975. Effect of night temperature on the toxicity of field-weathered mevinphos residues to honeybees. Zeitschrift für Angewandte Entomologie 79, 328–331.

Benedek, P., Prenner, J., 1972. Effect of temperature on the behaviour and pollinating efficiency of honeybees on winter rape flowers. Zeitschrift für Angewandte Entomologie 71, 120–124.

Bitterman, M.E., Menzel, R., Fietz, A., Schäfer, S., 1983. Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). Journal of Comparative Psychology 97, 107–119.

Blažytė-Čereškienė, L., Skirkevičius, A., 2006. The effect of the season on the olfactory learning of worker honeybees (*Apis mellifera carnica* Pollm.) to queen bee pheromone. Acta Biologica Universitatis Daugavpiliensis 6, 45–50.

Burrill, R.M., Dietz, A., 1981. The response of honey bees to variations in solar radiation and temperature. Apidologie 12, 319–328.

Carcaud, J., Roussel, E., Giurfa, M., Sandoz, J.C., 2009. Odour aversion after olfactory conditioning of the sting extension reflex in honeybees. Journal of Experimental Biology 212, 620–626.

Chandra, S.B.C., Hosler, J.S., Smith, B.H., 2000. Heritable variation for latent inhibition and its correlation with reversal learning in honeybees (*Apis mellifera*). Journal of Comparative Psychology 114, 86–97.

Cooper, P.D., Schaffer, W.M., Buchmann, S.L., 1985. Temperature regulation of honey bees (*Apis mellifera*) foraging in the Sonoran Desert. Journal of Experimental Biology 114, 1–15.

Corbet, S.A., Fussell, M., Ake, R., Fraser, A., Gunson, C., Savage, A., Smith, K., 1993. Temperature and the pollinating activity of social bees. Ecological Entomology 18, 17–30.

Crill, W.D., Huey, R.B., Gilchrist, G.W., 1996. Within- and between-generation effects of temperature on the morphology and physiology of *Drosophila melanogaster*. Evolution 50, 1205–1218.

Dacher, M., Lagarrigue, A., Gauthier, M., 2005. Antennal tactile learning in the honeybee: effect of nicotinic antagonists on memory dynamics. Neuroscience 130, 37–50.

De Jong, R., Pham-Delègue, M.H., 1991. Electroantennogram responses related to olfactory conditioning in the honey bee (*Apis mellifera ligustica*). Journal of Insect Physiology 37, 319–324.

Decourtye, A., Lacassie, E., Pham-Delègue, M.H., 2003. Learning performances of honeybees (*Apis mellifera* L.) are differentially affected by imidacloprid according to the season. Pest Management Science 59, 269–278.

Deisig, N., Giurfa, M., Lachnit, H., Sandoz, J.C., 2006. Neural representation of olfactory mixtures in the honeybee antennal lobe. European Journal of Neuroscience 24, 1161–1174.

Deisig, N., Sandoz, J.C., Giurfa, M., Lachnit, H., 2007. The trial-spacing effect in olfactory patterning discriminations in honeybees. Behavioural Brain Research 176, 314–322.

Drezner-Levy, T., Smith, B.H., Shafir, S., 2009. The effect of foraging specialization on various learning tasks in the honey bee (*Apis mellifera*). Behavioral Ecology and Sociobiology 64, 135–148.

Ebadi, R., Gary, N.E., Lorenzen, K., 1980. Effects of carbon dioxide and low temperature narcosis on honey bees *Apis mellifera*. Environmental Entomology 9, 144–147.

Erber, J., 1975. The dynamics of learning in the honey bee (*Apis mellifica carnica*). I. The time dependence of the choice reaction. Journal of Comparative Physiology 99, 231–242.

Erber, J., Masuhr, T.H., Menzel, R., 1980. Localization of short-term memory in the brain of the bee, *Apis mellifera*. Physiological Entomology 5, 343–358.

Faber, T., Joerges, J., Menzel, R., 1999. Associative learning modifies neural representations of odors in the insect brain. Nature Neuroscience 2, 74–78.

Flores, J.M., Ruiz, J.A., Ruz, J.M., Puerta, F., Bustos, M., Padilla, F., Campano, F., 1996. Effect of temperature and humidity of sealed brood on chalkbrood development under controlled conditions. Apidologie 27, 185–192.

Frazier, M.R., Harrison, J.F., Kirkton, S.D., Roberts, S.P., 2008. Cold rearing improves cold-flight performance in *Drosophia* via changes in wing morphology. Journal of Experimental Biology 211, 2116–2122.

Free, J.B., Spencer-Booth, Y., 1960. Chill-coma and cold death temperatures of *Apis mellifera*. Entomologica Experimentalis et Applicata 3, 222–230.

Free, J.B., Williams, I.H., 1972. Hoarding by honeybees (*Apis mellifera* L.). Animal Behaviour 20, 327–334.

Frost, E., 2011. Using the proboscis extension reflex to evaluate effects of stressors on honeybee learning and memory. Master's Thesis, Acadia University, Wolfville, Nova Scotia, Canada.

Gary, N.E., Lorenzen, K., 1988. Substage illumination device for restraining flight of honey bees (Hymenoptera: Apidae) and positively phototactic insects. Annals of the Entomological Society of America 81, 700–703.

Gerber, B., Geberzahn, N., Hellstern, F., Klein, J., Kowalksy, O., Wüstenberg, D., Menzel, R., 1996. Honey bees transfer olfactory memories established during flower visits to a proboscis extension paradigm in the laboratory. Animal Behaviour 52, 1079–1085.

Gerber, B., Wüstenberg, D., Schütz, A., Menzel, R., 1998. Temporal determinants of olfactory long-term retention in honeybee classical conditioning: nonmonotonous effects of the training trial interval. Neurobiology of Learning and Memory 69, 71–78.
Gil, M., De Marco, R.J., 2005. Olfactory learning by means of trophallaxis in *Apis mellifera*. Journal of Experimental Biology 208, 671–680.
Groh, C., Tautz, J., Rössler, W., 2004. Synaptic organization in the adult honey bee brain is influenced by brood-temperature control during pupal development. Proceedings of the National Academy of Sciences USA 101, 4268–4273.
Hammer, M., Menzel, R., 1995. Learning and memory in the honeybee. The Journal of Neuroscience 15, 1617–1630.
Hammer, M., Menzel, R., 1998. Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. Learning and Memory 5, 146–156.
Hammer, T.J., Hata, C., Nieh, J.C., 2009. Thermal learning in the honeybee, *Apis mellifera*. Journal of Experimental Biology 212, 3928–3934.
Han, E.N., Gatehouse, A.G., 1991. Effect of temperature and photoperiod on the calling behaviour of a migratory insect, the oriental armyworm *Mythimna separate*. Physiological Entomology 16, 419–427.
Harris, J.W., Woodring, J., 1992. Effects of stress, age, season, and source colony on levels of octopamine, dopamine and serotonin in the honey bee (*Apis mellifera* L.) brain. Journal of Insect Physiology 38, 29–35.
Hellstern, F., Malaka, R., Hammer, M., 1998. Backward inhibitory learning in honeybees: a behavioral analysis of reinforcement processing. Learning and Memory 4, 429–444.
Higginson, A.D., Barnard, C.J., 2004. Accumulating wing damage affects foraging decisions in honeybees (*Apis mellifera* L.). Ecological Entomology 29, 52–59.
Hori, S., Takeuchi, H., Arikawa, K., Kinoshita, M., Ichikawa, N., Sasaki, M., Kubo, T., 2006. Associative visual learning, color discrimination, and chromatic adaptation in the harnessed honeybee *Apis mellifera* L.. Journal of Comparative Physiology A 192, 691–700.
Hussaini, S.A., Lisa Bogusch, L., Landgraf, T., Menzel, R., 2009. Sleep deprivation affects extinction but not acquisition memory in honeybees. Learning and Memory 16, 698–705.
Hrassnigg, N., Crailsheim, K., 2005. Differences in drone and worker physiology in honeybees (*Apis mellifera*). Apidologie 36, 255–277.
Ichikawa, N., Sasaki, M., 2003. Importance of social stimuli for the development of learning capability in honeybees. Applied Entomology and Zoology 38, 203–209.
Jones, J.C., Helliwell, P., Beekman, M., Maleszka, R., Oldroyd, B.P., 2005. The effects of rearing temperature on developmental stability and learning and memory in the honey bee, *Apis mellifera*. Journal of Comparative Physiology A 191, 1121–1129.
Knapek, S., Gerber, B., Tanimoto, H., 2010. Synapsin is selectively required for anesthesia-sensitive memory. Learning and Memory 17, 76–79.
Kovac, H., Stabentheiner, A., Schmaranzer, S., 2010. Thermoregulation of water-foraging honeybees – balancing of endothermic activity with radiative heat gain and functional requirements. Journal of Insect Physiology 56, 1834–1845.
Kralj, J., Brockmann, A., Fuchs, S., Tautz, J., 2007. The parasitic mite *Varroa destructor* affects non-associative learning in honey bee foragers, *Apis mellifera* L.. Journal of Comparative Physiology A 193, 363–370.
Le Conte, Y., Arnold, G., Desenfant, P.H., 1990. Influence of brood temperature and hygrometry variation on the development of the honey bee ectoparasite *Varroa jacobsoni* (Mesostigmata: Varroidae). Environmental Entomology 19, 1780–1785.
Lin, H., Dusset, C., Huang, Z.Y., 2004. Short-term changes in juvenile hormone titers in honey bee workers due to stress. Apidologie 35, 319–327.
Lizé, A., Clément, J., Cortesero, A.M., Poinsot, D., 2010. Kin recognition loss following anesthesia in beetle larvae (*Aleochara bilineata*, Coleoptera, Staphylinidae). Animal Cognition 13, 189–194.
Mamood, A.N., Waller, G.D., 1990. Recovery of learning responses by honeybees following a sublethal exposure to permethrin. Physiological Entomology 15, 55–60.
Mardan, M., Rinderer, T.E., 1980. Effects of carbon dioxide and cold anaesthesia on the hoarding behaviour of the honeybee. Journal of Apicultural Research 19, 149–153.
Masterman, R., Ross, R., Mesce, K., Spivak, M., 2001. Olfactory and behavioral response thresholds to odors of diseased brood differ between hygienic and non-hygienic honey bees (*Apis mellifera* L.). Journal of Comparative Physiology A 187, 441–452.
Mattila, H.R., Seeley, T.D., 2007. Genetic diversity in honey bee colonies enhances productivity and fitness. Science 317, 362–364.
Mattila, H.R., Smith, B.H., 2008. Learning and memory in workers reared by nutritionally stressed honey bee (*Apis mellifera* L.) colonies. Physiology and Behavior 95, 609–616.
McCabe, S.I., Hartfelder, K., Santana, W.C., Farina, W.M., 2007. Odor discrimination in classical conditioning of proboscis extension in two stingless bee species in comparison to Africanized honeybees. Journal of Comparative Physiology A 193, 1089–1099.
Medrzycki, P., Sgolastra, F., Bortolotti, L., Bogo, G., Tosi, S., Padovani, E., Porrini, C., Sabatini, A.G., 2010. Influence of brood rearing temperature on honey bee development and susceptibility to poisoning by pesticides. Journal of Apicultural Research 49, 52–59.
Menzel, R., 1999. Memory dynamics in the honeybee. Journal of Comparative Physiology A 185, 323–340.
Menzel, R., Manz, G., Menzel, R., Greggers, U., 2001. Massed and spaced learning in honeybees: the role of CS, US, the intertrial interval, and the test interval. Learning and Memory 8, 198–208.
Mercer, A.R., Menzel, R., 1982. The effects of biogenic amines on conditioned and unconditioned responses to olfactory stimuli in the honeybee *Apis mellifera*. Journal of Comparative Physiology A 145, 363–368.
Moore, D., Rankin, M.A., 1993. Light and temperature entrainment of a locomotor rhythm in honeybees. Physiological Entomology 18, 271–278.
Nakagawa, S., 2004. A farewell to Bonferroni: the problems of low statistical power and publication bias. Behavioral Ecology 15, 1044–1045.
Pankiw, T., Page Jr., R.E., 2000. Response thresholds to sucrose predict foraging division of labor in honeybees. Behavioral Ecology and Sociobiology 47, 265–267.
Pankiw, T., Page Jr., R.E., 2003. Effect of pheromones, hormones, and handling on sucrose response thresholds of honey bees (*Apis mellifera* L.). Journal of Comparative Physiology A 189, 675–684.
Peng, I.F., Berke, B.A., Zhu, Y., Lee, W.H., Chen, W., Wu, C.F., 2007. Temperature-dependent developmental plasticity of *Drosophila* neurons: cell-autonomous roles of membrane excitability, Ca^{2+} influx, and cAMP signalling. Journal of Neuroscience 27, 12611–12622.
Pickup, J., Thompson, D.J., 1990. The effects of temperature and prey density on the development rates and growth of damselfly larvae (Odonata: Zygoptera). Ecological Entomology 15, 187–200.
Porter, S.D., 1988. Impact of temperature on colony growth and developmental rates of the ant, *Solenopsis invicta*. Journal of Insect Physiology 34, 1127–1133.
Ray, S., Ferneyhough, B., 1997. Seasonal variation of proboscis extension reflex conditioning in the honey bee (*Apis mellifera*). Journal of Apicultural Research 36, 108–110.
Robinson, G.E., Visscher, P.K., 1984. Effect of low temperature narcosis on honey bee (Hymenoptera: Apidae) foraging behaviour. Florida Entomologist 67, 568–570.
Rothman, K.J., 1990. No adjustments are needed for multiple comparisons. Epidemiology 1, 43–46.
Sandoz, J.C., Laloi, D., Odoux, J.F., Pham-Delègue, M.H., 2000. Olfactory information transfer in the honeybee: compared efficiency of classical conditioning and early exposure. Animal Behaviour 59, 1025–1034.
Scheiner, R., Erber, J., Page Jr., R.E., 1999. Tactile learning and the individual evaluation of the reward in honey bees (*Apis mellifera* L.). Journal of Comparative Physiology A 185, 1–10.
Scheiner, R., Page Jr., R.E., Erber, J., 2001. Responsiveness to sucrose affects tactile and olfactory learning in preforaging honey bees of two genetic strains. Behavioural Brain Research 120, 67–73.
Scheiner, R., Plückhahn, S., Öney, B., Blenau, W., Erber, J., 2002. Behavioural pharmacology of octopamine, tyramine and dopamine in honey bees. Behavioural Brain Research 136, 545–553.
Scheiner, R., Barnert, M., Erber, J., 2003. Variation in water and sucrose responsiveness during the foraging season affects proboscis extension learning in honey bees. Apidologie 34, 67–72.
Smith, B.H., 1991. The olfactory memory of the honeybee *Apis mellifera*. I. Odorant modulation of short- and intermediate-term memory after single-trial conditioning. Journal of Experimental Biology 161, 367–382.
Takeda, K., 1961. Classical conditioned response in the honey bee. Journal of Insect Physiology 6, 168–179.
Tanner, C.J., 2009. Chill out: cooling promotes aggressive behavior in the ant *Formica xerophila*. Insectes Sociaux 56, 64–69.
Tautz, J., Maier, S., Groh, C., Rössler, W., Brockmann, A., 2003. Behavioural performance in adult honey bees is influenced by the temperature experienced during their pupal development. Proceedings of the National Academy of Sciences USA 100, 7343–7347.
Wahl, O., Ulm, K., 1983. Influence of pollen feeding and physiological condition on pesticide sensitivity of the honey bee *Apis mellifera carnica*. Oecologia 59, 106–128.
Weick, J., Thorn, R.S., 2002. Effects of acute sublethal exposure to coumaphos or diazinon on acquisition and discrimination of odor stimuli in the honey bee (Hymenoptera: Apidae). Journal of Economic Entomology 95, 227–236.
Wilson, E.E., Holway, D., Nieh, J.C., 2006. Cold anaesthesia decreases foraging recruitment in the New World bumblebee, *Bombus occidentalis*. Journal of Apicultural Research 45, 169–172.
Xia, S.-Z., Feng, C.-H., Guo, A.-K., 1999. Temporary amnesia induced by cold anesthesia and hypoxia in Drosophila. Physiology and Behavior 65, 617–623.