

The proboscis extension reflex to evaluate learning and memory in honeybees (*Apis mellifera*): some caveats

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Received: 29 March 2012 / Revised: 18 July 2012 / Accepted: 20 July 2012 / Published online: 7 August 2012
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Abstract The proboscis extension reflex (PER) is widely used in a classical conditioning (Pavlovian) context to evaluate learning and memory of a variety of insect species. The literature is particularly prodigious for honeybees (*Apis mellifera*) with more than a thousand publications. Imagination appears to be the only limit to the types of challenges to which researchers subject honeybees, including all the sensory modalities and a broad diversity of environmental treatments. Accordingly, some remarkable insights have been achieved using PER. However, there are several challenges to evaluating the PER literature that warrant a careful and thorough review. We assess here variation in methods that makes interpretation of studies, even those researching the same question, tenuous. We suggest that the numerous variables that might influence experimental outcomes from PER be thoroughly detailed by researchers. Moreover, the influence of individual variables on results needs to be carefully evaluated, as well as among two or more variables. Our intent is to encourage investigation of the influence of numerous variables on PER results.

Keywords *Apis mellifera* · Classical conditioning · Honeybee · Learning · Proboscis extension reflex

Introduction

Social insects have sophisticated learning and memory capabilities, providing valuable models of cognition and behaviour (Bonabeau et al. 1996; Giurfa and Menzel 1997; Leadbeater and Chittka 2007; Rogers and Vallortigara 2008;

Haase et al. 2011) that frequently parallel vertebrate systems (Menzel 1983; Laska et al. 1999; Giurfa 2003). The honeybee, *Apis mellifera*, is characterized by acute sensory acuity, a repertoire of complex behaviours and a simple central nervous system (Menzel and Müller 1996; Faber et al. 1999; Giurfa et al. 2001; Menzel and Giurfa 2001; Giurfa 2003, 2007). Honeybees' ability to integrate, distinguish and respond to a diverse range of environmental stimuli is well documented. Under experimental conditions, they can be trained to differentiate between similar odours (Getz and Smith 1987; Laska et al. 1999; Fröhlich et al. 2000), colours and other visual patterns (Giurfa et al. 1999; Zhang et al. 1999; Menzel and Shmida 1993; Chen et al. 2003; Dyer et al. 2008; Niggebrügge et al. 2009), and tactile cues (Erber et al. 1998; Bernadou et al. 2009). Moreover, they can identify stimuli based on recognition of abstract concepts of 'similarity' and 'difference' (Giurfa et al. 2001; Avarguès-Weber et al. 2011). Additionally, a honeybee's central nervous system is easily accessible for physiological recording, so underpinnings of learning phenomena can be investigated at cellular levels alongside behavioural observations (Faber and Menzel 2001; Galizia and Menzel 2001; Deisig et al. 2006; Peele et al. 2006). Despite all the benefits of using honeybees as model organisms, variation in methods used to assess neural function prompts questions about interpretability of the existing literature. This review delves into some potentially important issues in the current implementation of a traditional learning/memory paradigm and makes several recommendations for improved reporting of methods; ultimately, we hope that a standardized set of methods will be developed that nonetheless retains flexibility for addressing particular questions.

The proboscis extension reflex

When honeybees' antennae contact sucrose (Bitterman et al. 1983) or pollen (Grüter et al. 2008), they reflexively extend

Communicated by: Sven Thatje

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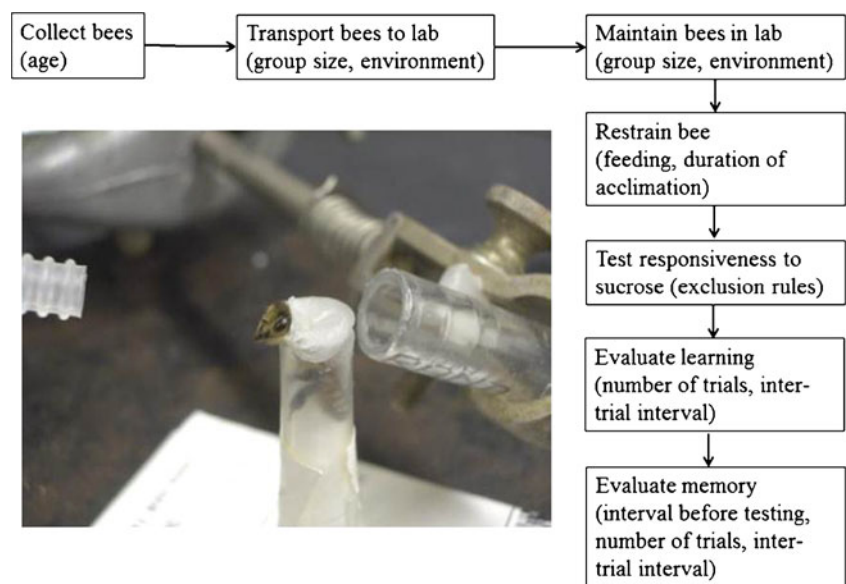
their probosces to feed. Researchers have capitalized on this proboscis extension reflex (PER) by using a Pavlovian conditioning technique to evaluate learning and memory (Takeda 1961; reviewed in Giurfa and Sandoz 2012). Honeybees can be trained to respond to an originally neutral stimulus (conditioned stimulus [CS]) by pairing it with a sugar reward (unconditioned stimulus [US]) that evokes the unconditioned response of proboscis extension. If associative learning is unimpeded, presentation of the CS elicits PER, suggesting that the insect has learned that the CS predicts a reward (Faber et al. 1999; Faber and Menzel 2001). For simplicity, in this review, we use the term “learning” to mean evidence of associating the CS with a reward and “memory” as evidence of storing and retrieving that association after some time interval.

PER methods are generally executed with a broadly similar set of steps (Fig. 1). Honeybees are usually collected from a field site and returned to a lab that maintains humidity and temperature that approximate hive conditions. Honeybees may be allowed to acclimate to lab conditions, alone or in groups, before PER experiments begin. Prior to training, honeybees are immobilized and restrained so that only their antennae and mouthparts are free (Bitterman et al. 1983; Getz and Smith 1987; Hammer and Menzel 1995). Honeybees are fed a sucrose solution and left to acclimate to restraint (Masterman et al. 2001; Weick and Thorn 2002; Decourtye et al. 2003; Jones et al. 2005; Kralj et al. 2007; McCabe et al. 2007). After acclimation, responsiveness to sucrose (US) is evaluated before conditioning begins, and individuals failing to extend their proboscis are usually removed from further testing (Bitterman et al. 1983; Latshaw and Smith 2005). Pulsing odours into a continuous air stream is the most common method of delivering a CS.

Individuals are often exposed to unscented air before training to eliminate confounding effects of mechanosensory stimulation by airflow (Decourtye et al. 2003; Tautz et al. 2003). In traditional forward pairing, a CS is presented for several seconds alone and then followed by, or presented concurrently with, an US (Getz and Smith 1987; Deisig et al. 2007). Honeybees are trained for a fixed number of trials (Gerber et al. 1998; Morgan et al. 1998; Ray and Ferneyhough 1999; Abramson et al. 2008). Memory is tested using extinction trials, wherein a CS is presented without an US (Fig. 1; Hammer and Menzel 1995; Sandoz et al. 1995; Gerber et al. 1998; Morgan et al. 1998; Tautz et al. 2003; Decourtye et al. 2005; Mattila and Smith 2008). PER to odour during extinction is assumed to reflect memory of odour–reward associations, although the absence of PER need not indicate loss of memory.

Research supports the ecological relevance of PER (Sandoz et al. 1995; Beekman 2005; Farina et al. 2005). Odour discrimination is usually comparable between honeybees trained under restrained and free-flying conditions (Mauelshagen and Greggers 1993; Pham-Delègue et al. 1993; Laloi et al. 2000) and olfactory memories appear resistant to changes of context (Gerber et al. 1996; Sandoz et al. 2000; Chaffiol et al. 2005; Carcaud et al. 2009), including shifts from natural to artificial environments (Gerber et al. 1996; Farina et al. 2005). Information gathered from PER is also used in developing hive-level (Masterman et al. 2000; Pham-Delègue et al. 2002; Decourtye et al. 2005) and human (Abramson et al. 2006; Rains et al. 2008) applications. For example, Masterman et al. (2000) used differential conditioning to evaluate honeybee detection of chalkbrood caused by the fungal pathogen *Ascosphaera apis*. Odour of live, healthy pupae was paired with a sucrose reward and odour of chalkbrood-infected pupae was

Fig. 1 Steps involved in using the proboscis extension reflex to evaluate memory and learning. Variables that differ among studies are in parentheses. A restrained bee in a test setup is shown



paired with an aversive NaCl solution. Hygienic-bred honeybees were better able to discriminate between healthy and diseased brood than were individuals from a non-hygienic genetic line (Masterman et al. 2000, 2001).

PER of restrained honeybees has been used to evaluate responses to olfactory (Hammer and Menzel 1995; Guerrieri et al. 2005; Mattila and Smith 2008), mechanosensory (Scheiner et al. 2003; Giurfa and Malun 2004; Dacher et al. 2005), thermal (Hammer et al. 2009) and visual (Hori et al. 2006; Letzkus et al. 2008; Mota et al. 2011) stimuli (Table 1). Studies have assessed the influence of environment (Scheiner et al. 2003; Tautz et al. 2003), genotype (McCabe et al. 2007; Couvillon et al. 2010), stimulants and depressants (Si et al. 2005; Abramson et al. 2007; Mustard et al. 2008; Barron et al. 2009), pesticides (Mamood and Waller 1990; Weick and Thorn 2002; Decourtye et al. 2005; El Hassani et al. 2008) and a variety of other factors on honeybee learning (Giurfa and Sandoz 2012; Table 1).

In sum, there is an abundant literature that has taken advantage of the honeybee PER paradigm not only to address a remarkably broad suite of questions but also, in many cases, to address the same questions. Giurfa and Sandoz (2012) review

the history of achievements obtained with PER, but they also wrote that “subtle modification of experimental parameters ... may lead to radically different conclusions, some of which may be misleading.” Moreover, variation in methods makes it difficult for new researchers to decide on how to begin using PER despite the potential power of the method.

Variation in application of PER methods

Complete standardization of behavioural assays is rare for various reasons, including logistical constraints, variation in researcher experience and variation in what techniques are successful for a given researcher (e.g. Lemly and Smith 1986; Feng et al. 2004; see Table 2 for examples of variation in PER techniques). In addition to human error and variation in laboratory conditions (e.g. pressure, humidity, temperature), there are numerous environmental and physiological variables that can affect honeybee learning and memory (Gerber et al. 1996; Ray and Ferneyhough 1997; Sandoz et al. 2000; Menzel et al. 2001; Scheiner et al. 2003; Behrends and Scheiner 2009; Hussaini et al. 2009). Unfortunately, it can

Table 1 Conditioning cues used to investigate a range of factors and their effect on honeybee learning and memory using the proboscis extension reflex

Conditioning cue(s)	Parameters evaluated	Source
Olfactory	Age and queenlessness	Morgan et al. (1998)
Olfactory	Age, caste, and genotype	Bhagavan et al. (1994)
Visual	Antennae removal, caste, etc.	Hori et al. (2006)
Tactile	Antenna-specific learning	Bernadou et al. (2009)
Olfactory	Biogenic amines	Mercer and Menzel (1982)
Visual	Brain lateralization	Letzkus et al. (2008)
Olfactory	Conditioning procedures	Sandoz et al. (1995)
Olfactory	Conditioning procedures	Gerber et al. (1998)
Olfactory	Conditioning procedures	Menzel et al. (2001)
Olfactory	Environment (season)	Blažytė-Čereškienė and Skirkevičius (2006)
Tactile/olfactory	Environment (season)	Scheiner et al. (2003)
Olfactory	Environment (temperature)	Tautz et al. (2003)
Olfactory	Chemical	Mamood and Waller (1990)
Olfactory	Chemical	Decourtye et al. (2003)
Tactile	Foraging specialization	Scheiner et al. (1999)
Olfactory	Genetic	Getz et al. (1986)
Olfactory	Genetic	Latshaw and Smith (2005)
Olfactory	Genetic	McCabe et al. (2007)
Olfactory	Genetic	Couvillon et al. (2010)
Tactile	Nicotinic antagonists	Dacher et al. (2005)
Olfactory/visual	Stimulant (caffeine)	Si et al. (2005)
Olfactory	Stress (nutritional)	Mattila and Smith (2008)
Olfactory	Stress (sleep deprivation)	Hussaini et al. (2009)
Tactile	Tactile learning	Erber et al. (1998)
Thermal	Thermal learning	Hammer et al. (2009)
Olfactory	<i>Varroa destructor</i>	Kralj et al. (2007)

Table 2 Selected examples of variation in methods used for absolute (single odour/positive reward) conditioning, including conditioned stimuli (CS), concentration of the sucrose unconditioned stimulus (US), inter-trial interval (ITI), number of conditioning (CT) and

extinction (ET) trials, recovery period (RP) and duration of airflow before presentation of the CS. Blank cells indicate that information was not reported

CS	US	CS duration (s)	US duration (s)	ITI (min)	CT	ET	Time between CT and ET (min)	RP (h)	Airflow duration (s)	Source
Thyme	0.9 M	6	3	8	8	0		3		Taylor et al. (1987)
Orange, lavender	0.9 M	2	3		1	1	15	0.5		Morgan et al. (1998)
Geraniol	1.5 M	8	3	20	3	1	1,440	2		Ray and Ferneyhough (1997)
Citronella	0.9 M	2			1	1	10	24	45	Tautz et al. (2003)
Neroli	1.2 M	2	3		1	1	1 or 12	3	45	Kralj et al. (2007)
Cinnamon	1.8 M	3	2	10	12	12	1,440	24		Abramson et al. (2008)
Linalool	Pollen, 1.8 M	6	3	15	3	5	15	1	20	Grüter et al. (2008)

be difficult to distinguish relative effects of the causative factor(s) being studied from extraneous variables, the latter being frequently excluded from statistical analyses and interpretation of results. The necessity of designing original research experiments for publication further complicates the issue. Although multiple authors may investigate effects of the same variable [e.g. Lambin et al. (2001), Decourtye et al. (2003), Guez et al. (2003), Decourtye et al. (2004), and Decourtye et al. (2005)—all investigated the chloronicotinyl insecticide imidacloprid], there are invariably differences in methods (e.g. pesticide concentration, honeybee age, season, colony health, geographic location, PER protocols). Moreover, focal and extraneous variables may interact, potentially obscuring identification of which factor(s) is (are) affecting learning and memory, thereby threatening the reliability and reproducibility of PER results. We review here a few of these variables and show how they may influence results among studies.

Effects of season

Season is associated with changes in learning (De Jong and Pham-Delègue 1991) and response to stressors (Dacher and Smith 2008). Menzel et al. (2001) list a number of factors affected by season, including weather and food availability, that likely influence olfactory learning and physiology. Similarly, intra-colonial factors (e.g. queen health and age, honey stores, virus/mite loads; Scheiner et al. 2003) may contribute additional temporal variation. This may be especially pertinent in temperate regions where honeybees often overwinter inside hives for several months, restricting most behavioural research to late spring and summer.

Temporal changes in both honeybee learning ability and rate have been documented in studies using outdoor colonies (De Jong and Pham-Delègue 1991; Ray and

Ferneyhough 1997; Blažytė-Čereškienė and Skirkevičius 2006). Honeybees learned more successfully in early autumn (August–November) and less successfully in spring and early summer (March–July; Blažytė-Čereškienė and Skirkevičius 2006). However, no temporal changes were observed for indoor colonies conditioned using PER, possibly because of more consistent food quality and availability (Ray and Ferneyhough 1997).

Because honeybees failing to respond to a sugar stimulus are usually removed from further analysis, seasonal variation may affect the stringency of exclusion criteria for determining which bees are retained for PER testing. This variability in olfactory learning performance may be strongly correlated with seasonal shifts in honeybee responsiveness to sucrose and water (Scheiner et al. 2003). Nectar foragers are least responsive to low concentrations of sucrose in June and July, which can affect memory acquisition and retention (Scheiner et al. 1999; 2001; 2003) and may be influenced 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, corresponding with periods of high foraging activity that may also be associated with changes in hive population size, nutrition or brood-rearing. The aforementioned biogenic amines influence the 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). Interestingly, dopamine mediates aversive [stinger extension reflex (SER)] learning and may convey information about negative stimuli in honeybees and other insects (Schwaerzel et al. 2003; Vergoz et al. 2007), although there is no apparent correlation between an individual's responsiveness to sucrose and their responsiveness to shock (Roussel et al. 2009).

Season can also alter honeybees' susceptibility to sublethal effects of pesticides. Honeybees exposed to imidacloprid experienced learning impairment at lower concentrations in summer (outdoors) than winter (reared indoors; Decourtye et al. 2003). This may reflect lower nutritional quality in the diet of outdoor colonies (Ray and Ferneyhough 1997; Decourtye et al. 2003); pesticide toxicity increases under conditions of inadequate pollen supply, which is likely a result of protein deficiency (Wahl and Ulm 1983). However, Mattila and Smith (2008) found that nutritional stress from poor-quality pollen alone did not affect olfactory learning, memory or responsiveness to sucrose, indicating that the effect of season is multidimensional.

Effects of prior learning

Honeybee PER elicited by neutral olfactory stimuli prior to the first presentation of the US are classified as spontaneous responses and occur at variable rates [~ 0.7 % in Ray and Ferneyhough (1999); ~ 4 % in Mercer and Menzel (1982); ~ 21 %, but up to 40 %, in Skirkevičius and Bląpytė-Èereðkienė (2005)], which can be affected by season (Erber et al. 1980; also see earlier discussion). Although some continue to train spontaneous responders (De Jong and Pham-Delègue 1991; Sandoz et al. 1995), these honeybees are usually discarded from analysis because a spontaneous PER suggests a previously established odour–reward association (Erber et al. 1980; Mercer and Menzel 1982; Bitterman et al. 1983; Getz et al. 1986; Pelz et al. 1997; Ray and Ferneyhough 1997; Morgan et al. 1998; Dacher et al. 2005). Excluding spontaneous responders could potentially have significant influences on results. Spontaneous responses are enhanced by octopamine (seasonal fluctuations described earlier; Harris and Woodring 1992); 10–27 % of bees injected with octopamine displayed spontaneous PER, whereas the rate was ≤ 3 % for individuals injected with Ringer's solution, dopamine or serotonin (Mercer and Menzel 1982).

Gerber et al. (1996) investigated the transfer of olfactory information learned in the wild to PER conditioning in the laboratory. Foraging encounters with basswood (*Tilia* sp.) significantly increased spontaneous PER (~ 60 %) to the corresponding odour. This suggests that context plays a relatively weak role in retrieving olfactory memories established during foraging (Gerber et al. 1996; Sandoz et al. 2000; Arenas et al. 2007) or earlier in development (Sandoz et al. 2000; Grüter et al. 2006; Arenas et al. 2009a, b). In free-flying bees, Reinhard et al. (2004) found that when odours that were learned previously at a sugar feeder are blown into a hive they induce flight and navigation to feeders. Similarly, honeybees' successful orientation to conditioned odours in a wind tunnel (Chaffiol et al. 2005) and a four-sourced airflow arena (Sandoz et al. 2000) was significantly enhanced by prior olfactory exposure. The opposite

outcome may occur as latent inhibition. Here, if a bee is exposed to an odour but receives no reward, that same bee may be inhibited in learning to associate that odour with a paired reward later on (Chandra et al. 2010).

Associative learning also occurs within hives (Arenas et al. 2007; Grüter et al. 2009) and odours experienced by honeybees in the first week following emergence are prioritized when these individuals become foragers (Arenas et al. 2009a). A primary mode of information transfer involves odour/reward pairings via trophallaxis (Farina et al. 2005; Grüter et al. 2006), when nectar brought back by foragers is moved among colony members. Recipient honeybees detect odours clinging to a forager's body and within nectar (Grüter et al. 2006; Díaz et al. 2007). In this environment, nectar acts as the US and accompanying odours function as the CS. Associations learned via trophallaxis can be retrieved in the context of PER (Farina et al. 2005, 2007). In addition to heightening responsiveness to odour stimuli, prior exposure significantly increases resistance to memory extinction (Gerber et al. 1996). Fortunately, honeybees apparently do not remember into adulthood stimuli from substances that they were fed as larvae (Sandoz et al. 2000) so that researchers need not be overly concerned about variation in responses being introduced from early (pre-eclosion) experience.

Certain odorants elicit more appetitive responses than others after the same conditioning procedure (Smith and Menzel 1989; Smith and Cobey 1994; Bonod et al. 2003), suggesting a bias for strong associations between particular odours and a reward (Smith and Menzel 1989; Smith 1991). For this reason, some researchers use novel odours for PER [cinnamon by Stone et al. (1997); cinnamon, fennel and citronella by Abramson et al. (2006); cinnamon and wintergreen by Abramson et al. (2008)] that still result in high memory acquisition. However, these novel odours are complex blends that may still contain single compounds that are found in natural odours. Furthermore, the use of potentially biologically irrelevant odours may pose issues in terms of the basic ability of the honeybee to detect and process such compounds. This suggests that more experimental control is obtained using single-molecule odorants, but there are clearly differences of opinion in the literature.

Effects of inter-trial interval and trial number

The influence of conditioning techniques on memory acquisition and resistance to extinction can be difficult to determine. A common variant in PER protocols is the inter-trial interval (ITI). In massed conditioning, ITIs are usually 30–60 s (Sandoz et al. 1995), whereas in spaced trials ITIs are 10 to 30 min (Mamood and Waller 1990; Stone et al. 1997; Weick and Thorn 2002; Decourtye et al. 2005). Similarly, the number of trials used to test simple odour–stimulus pairings can range

from 1 to >10 (Frings 1944; Taylor et al. 1987; Mamood and Waller 1990; Smith 1991; Menzel and Müller 1996; Stone et al. 1997; Gerber et al. 1998; Morgan et al. 1998; Ray and Ferneyhough 1999; Weick and Thorn 2002; Decourtye et al. 2005; Abramson et al. 2008). Massed trials usually have more trials, but this is not always the case. Further, conditioning paradigms differ from simple paired US–CS presentations to differential conditioning, aversive conditioning and complex stimuli discrimination that require additional trials (Giurfa 2003, 2004).

Although Sandoz et al. (1995) found equal performance with massed (1-min ITI) and spaced (10-min ITI) conditioning in honeybees, spaced trials typically elicit better long-term memory based on PER (Gerber et al. 1998; Menzel et al. 2001; Deisig et al. 2007; Hammer et al. 2009) and SER paradigms (Giurfa et al. 2009). Effects of temporal spacing between successive conditioning trials on memory consolidation and recall are called trial spacing effects (Barela 1999; Menzel et al. 2001; Deisig et al. 2007) and have been documented in bumblebees (*Bombus impatiens*; Toda et al. 2009), rats (*Rattus norvegicus*; Barela 1999; Sunsay et al. 2004; Sunsay and Bouton 2008) and humans (*Homo sapiens*; Ambridge et al. 2006; Litman and Davachi 2008).

Erber (1975) suggested that massed trials are less effective for honeybees than spaced trials are because of weaker consolidation of stored information; Menzel et al. (2001) found that spaced conditioning improved learning and led to better memory, particularly at long intervals, but with no difference in learning for ITIs of 10, 20 or 30 min. Massed trials may also affect responsiveness (a consequence of feeding after short intervals), although satiation is unlikely because of the small amount of sucrose delivered to the proboscis (Menzel et al. 2001). Massed conditioning may also lead to interference between memory consolidation processes and new learning events (Gerber et al. 1998; Hellstern et al. 1998; Menzel et al. 2001). Very short ITIs (especially under 30 s) can induce backward conditioning, leading to an inhibitory effect between the US of trial n and the CS of trial $n+1$ (Hellstern et al. 1998). ITIs of 30 s and 3 min reduced memory, but there was no significant difference in memory between ITIs of 1 and 20 min (Gerber et al. 1998); Faber et al. (1999) found that an ITI of 1 min facilitated successful discrimination between two similar odours. However, Menzel (1999) found that ITIs of 30 s or 1 min resulted in slower acquisition and reduced memory compared to trials spaced at 5 and 10 min.

Multiple (≥ 3) conditioning trials promote long-term memory, increasing consolidation of learned associations and resistance to extinction (Sandoz et al. 1995; Menzel and Müller 1996) because of the sequential organization of olfactory memories (Menzel et al. 1993). Learning acquisition curves show that a single conditioning trial can lead to consolidation of the CS–US association (≥ 60 %) and a

consistent response (≥ 80 %) can be reached after three trials (Bitterman et al. 1983; Taylor et al. 1987; Sandoz et al. 1995; Menzel and Müller 1996). Sandoz et al. (1995) assessed several common learning paradigms: (1) a single conditioning trial, (2) three trials with a 10-min ITI and (3) three trials with a 1-min ITI. For each conditioning procedure, they assessed short- (30 s to 3 h post-conditioning), middle- (1–4 days post-conditioning), and long-term (1–2 weeks post-conditioning) memory. They did not find significant differences between spaced and massed presentations of three conditioning trials (consistent with Bitterman et al. 1983) on learning or memory consolidation. However, although there is evidence that odour memories can last for the lifetime of a honeybee after a single association, individuals from one-trial conditioning were more susceptible to memory extinction when tested repeatedly (Sandoz et al. 1995).

Other influences

Rather than belabour additional documented variation in PER that may arise from various experimental design factors, we instead list some important ones here: genotype and phenotype (e.g. age, caste) of bees that are tested (Bhagavan et al. 1994; Pankiw and Page 2000; Laloï et al. 2001; Maleszka and Helliwell 2001; Scheiner et al. 2001; Ichikawa and Sasaki 2003; Arenas and Farina 2008; Behrends and Scheiner 2009; Drezner-Levy et al. 2009; Couvillon et al. 2010), social and physical environmental conditions in the lab (group size, temperature, humidity, air flow, cage size, cage colour, cage materials, etc.; Tautz et al. 2003; Jones et al. 2005; Hussaini et al. 2009; Amdam et al. 2010), acclimation interval and stimulus intensity (Pelz et al. 1997).

Interpreting PER studies

Clearly, there is widespread variability in PER methods; understandably, much of this variability (e.g. study climate, human error) and constraints (financial, temporal, logistic) will be unavoidable and in some cases necessitated by the questions being posed. Unfortunately, the relative influence (and potentially synergistic interaction) of a multitude of variables on PER results is often overlooked and has scarcely been evaluated (Frost et al. 2011). For example, the inclusion/exclusion of extinction trials in PER paradigms is an important difference among experimental designs. Although pesticides and other stressors do not always affect learning (Taylor et al. 1987; Decourtye et al. 2005; Hussaini et al. 2009), they may significantly influence memory (Abramson et al. 1999; Hussaini et al. 2009), thereby altering perceived risks to honeybee health. However, memory is

not always assessed in PER studies (e.g. Taylor et al. 1987; Decourtye et al. 2005; Blažytė-Čereškienė and Skirkevičius 2006). Generally, learning an association is only advantageous if it can be recalled accurately and consistently. Even when memory is assessed, there is variability in when it is assessed (Table 2). Some of this variability is guided by the hypotheses being tested (e.g. timing may differ for physiological versus ecological questions).

Because of variation in PER methods, at best only cautious inferences can be made from evaluating inter- versus intra-laboratory findings (e.g. Crabbe et al. 1999; Pankiw and Page 2003; Wahlsten et al. 2003) or comparing laboratory results to data collected under more natural conditions (Downs and Ratnieks 1999; Mujagic and Erber 2009; Niggebrügge et al. 2009). The absence of scholarly discussion of variation in PER may stem from the multitude of paradigms, each with a unique set of variants. However, there must be increased awareness about this issue, and we suggest that authors provide clearer documentation of conditions under which PER is evaluated. It is imperative that researchers provide more thorough descriptions of their experimental designs and highlight/discuss the potential consequences of variation on the reliability and reproducibility of PER findings. Appropriate PER protocols need to be designed for common conditioning methods (e.g. absolute, differential, negative and positive patterning), including standardization of trial number, frequency, acclimation periods, etc. In cases where researchers deviate from these standards, alterations should be justified and analysed as a potential influence on PER performance. Finally, researchers need to specifically examine interacting effects of environmental, physiological and experimental variation on honeybee learning and memory. This research will hopefully point to future design of PER protocols.

Acknowledgments Four reviewers made excellent suggestions that significantly improved this manuscript. We thank Daniel Deveau and Cate Little for help in collecting data, Kevin Spicer for supplying beehives, and Teams Shutler and Hillier for input. Funding was provided by NSERC via a PGS A scholarship to Frost and a Discovery Grant to Hillier, the Canadian Bee Research Fund, and Bee Maid.

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