The effects of acute stress on learning and memory in bumblebees

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ABSTRACT

Stress has complex effects on learning and memory, depending on both the type of stress and when the animal experiences it. Honeybees and bumblebees are agriculturally important pollinators for whom the effects of stress are extremely relevant. These pollinators are often transported long distances during which colonies experience severe physical disturbance, causing stress to individuals prior to their release for pollination. Under natural foraging conditions, bees are excellent at learning about the flowers they forage from, including associations between floral stimuli and rewards. However, it is not clear how stress might affect bees' abilities to learn and remember floral features. Here we address the effects of acute stress on learning and memory in the bumble bee, *Bombus impatiens*. Using the Proboscis Extension Response (PER) protocol, we look at stress effects on learning and memory in three experiments. After being trained to a conditioned stimulus, we addressed: (1) the effect of 24 h and (2) 30 min of stress on the recall of this learned association and (3) the effect of stress on subsequently learning an association. We found that 24 h of stress after learning appeared to improve memory recall, and there was a trend in the same direction for 30 min of stress. However, bees that were stressed prior to learning an association did not differ from unstressed bees in their ability to learn or remember an association. Our finding that stress has effects on memory in the bumblebee could have implications for their use in behavioral experiments and for promoting the success of both managed and wild populations.

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Stress affects all animals, but can do so in many different ways. Stress encompasses a broad range of experiences, including nutritional stress, physical stress (pain), social stress and psychological stress. Stressors elicit a variety of physiological responses, but can also be seen through behavioral responses. How stress affects an individual's ability to learn and remember is diverse, there being no simple relationship between stress and learning (Schwabe, Joëls, Roozendaal, Wolf, & Oitzl, 2012). The effects of stress on learning and memory depend on both the type of stress (chronic e.g., Pravosudov, 2003; or acute e.g., Shors, 2001), when the stress occurs (before or after learning, and how long before or after (Schwabe, Wolf, & Oitzl, 2010), and individual factors such as sex and age (Shors, 2006). In the current study, we address the effect of stress on learning and memory in a case not before investigated, that of the bumble bee.

As generalist foragers, honey bees and bumblebees are extremely adept at learning associations between floral features and nectar rewards, both in order to learn the most highly rewarding flowers to forage on (Chittka, Thomson, & Waser, 1999) and to learn how to handle flowers effectively (Chittka & Thomson, 1997; Heinrich, 1979; Laverty, 1980, 1994). Bees have

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emerged as a model system for the study of learning and memory (Giurfa, 2007), using both free-flying behavioral assays (Papaj & Lewis, 1993), and the Proboscis Extension Response (PER) paradigm (Bitterman, Menzel, Fietz, & Schäfer, 1983; Riveros & Gronenberg, 2009; Takeda, 1961). In the PER protocol, an individually harnessed bee is presented with an initially neutral stimulus (the conditioned stimulus; CS, e.g., an odor), paired with a sucrose reward (the unconditioned stimulus; US). After the animal learns this association, it will extend its proboscis (in order to drink the sugar reward) in response to the conditioned stimulus alone (the conditioned response; CR). PER has been used extensively in research on learning and memory in honey bees (reviewed in Giurfa & Sandoz, 2012) and, more recently, in bumble bees (Laloi et al., 1999; Riveros & Gronenberg, 2009). However, despite extensive study of learning and memory processes in bees (Menzel & Giurfa, 2001), and a parallel literature on their physiological stress responses (reviewed in Even, Devaud, & Barron, 2012), we know little about what effects stress might have on learning and memory in this system.

Honeybees and bumblebees are perhaps the most important insects for human agriculture, due to the pollination service they provide around the world (Gallai, Salles, Settele, & Vaissière, 2009). These commercial pollinators face a number of stressors, many of which have been linked to their recent decline, including but not limited to habitat loss and fragmentation, use of agrochemicals, pathogens, parasitic mites and climate change (Potts et al., 2010). Aside from these diverse stressors, one form of stress for commercial pollinators comes from their long-distance transportation (Ahn, Xie, Riddle, Pettis, & Huang, 2012). For example, honey bees are transported from Florida to California in the spring for almond pollination, and from Michigan to Florida over winter before being brought back for apple and cherry pollination. Bumble bees pollinate a wide variety of economically important crops, including kiwifruit, tomatoes, strawberries, eggplant, sweet peppers, cranberries and blueberries, and are shipped from the east to the west of America (e.g., Whittington & Winston, 2004). While being packed for this transportation, during transportation artificial and in unpacking afterwards, colonies are likely to experience physical disturbance (i.e., vibration). Bees within a colony are sensitive to such physical disturbance, and respond with behavior such as hissing (Kirchner & Röschard, 1999) and production of alarm pheromone (Jandt, Robins, Moore, & Dornhaus, 2012). Such disturbance (i.e., by shaking) also causes pessimistic cognitive biases in bees (Bateson, Desire, Gartside, & Wright, 2011). Once a colony reaches its destination and bees begin foraging, they will learn associations between features of the flowers they visit and the reward the flowers offer, as well as learning how to handle these flowers effectively. These same bees may then be transported again to the same or a different crop, thus experiencing more stress. Even among wild populations of bees, there is some evidence that queens prefer relatively "undisturbed" nest sites (e.g., those away from intensively managed fields; Barron, Wratten, & Donovan, 2000). However, we do not know what effects stress by physical disturbance has on bee learning and memory.

Stress is known to have both positive and negative effects on learning in animals, depending on a number of factors (reviewed in Joëls, Pu, Wiegert, Oitzl, & Krugers, 2006; Sandi & Pinelo-Nava, 2007). One factor that might be of particular relevance to commercial bees that are transported to the crops they pollinate is that of timing. At least in rats, which have largely been used as the model system for understanding stress effects on learning, when stress is encountered immediately after learning, it often has positive effects on memory consolidation, as opposed to when it is experienced prior to recalling a previously learned stimulus, where it is more likely to have detrimental effects (Roozendaal, 2002, 2003; Schwabe et al., 2012). To our knowledge, the only other study looking at stress effects on learning and memory in a bee addressed the effects of chronic nutritional stress on a honey bee colony (Mattila & Smith, 2008), a very different type of stress from the acute stress likely to also be experienced by commercial pollinators.

Here we tested the effects of stress on learning and memory on the Common Eastern bumble bee, *Bombus impatiens*, a species encountered across Eastern North America and sold commercially across the USA and Canada. Specifically we looked at the effect of the timing and duration of one stressor (physical disturbance by shaking), on a learning and memory task. We made the assumption that physical disturbance by shaking would be acutely stressful to bumble bees, as bees that encounter physical disturbance show behavior that is likely to indicate stress, such as hissing (Kirchner & Röschard, 1999) and production of alarm pheromone (Jandt et al., 2012). Furthermore, shaking in honeybees has been shown to have physiological effects, as it changes the bees' levels of biogenic monoamines, reducing constitutive levels of octopamine, dopamine, and serotonin in the bees' haemolymph (Bateson et al., 2011; Chen, Hung, & Yang, 2008). We used the PER paradigm as this method allows precise control of conditioning. We carried out three experiments to ask the following questions: (1) what effect does 24 h of stress have on an individual's ability to recall a previously learned association?; (2) what effect does 30 min of stress have on an individual's ability to recall a previously learned association?; and (3) how does 30 min of stress preceding conditioning affect bees' ability to learn and remember an association?

Methods

General methods

Subjects and maintenance

We used colonies of *Bombus impatiens* (Koppert Biological Systems, MI, USA) which were connected via plastic tubes to a central arena sized 98 cm × 96 cm × 91 cm (length × width × height) containing unrewarding artificial flowers (for enrichment). We provided bees with *ad libitum* pollen placed directly into their colonies, and allowed them to free-forage in the central arena on a white cotton-wicked artificial feeder containing 15% (w/w) sucrose solution. While the communal
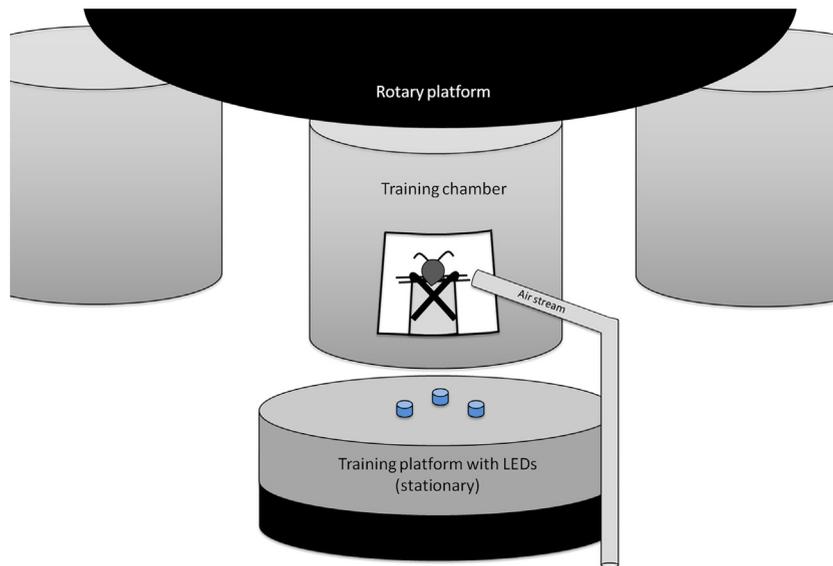
Fig. 1. Diagram of the Proboscis Extension Response apparatus.

foraging arena prevents us from assigning colony identity to individuals, four colonies were connected at any one time to the arena, ensuring multiple colonies were represented in each experiment.

Harnessing for PER

In all experiments, we removed 14–18 bees from the sucrose feeder in the central arena using a hand-held vacuum aspirator designed for use in insect research. We immediately placed these bees into individual test tubes and placed these tubes into a bucket of ice for 20 min for cold immobilization. After this, we mounted bees in plastic tubes such that their bodies were held inside the tube and only their head was outside. Bees' heads were held in place by two metal pins forming a "yoke" fitted between their head and thorax (following protocol designed by Riveros & Gronenberg, 2009). We then added tape to secure the bees' heads in a fixed position. We left the bees to habituate to their harnesses for at least 2 h. After this time, we gave each bee 50% (w/w) sucrose solution (first held between antennae and then to its proboscis) for 3 s. If a bee did not exhibit PER at this stage, it was excluded from the experiment.

PER training and testing

Our protocol is based upon that of Riveros and Gronenberg (2009, 2012), who successfully conditioned bumble bees to both color and scent. We mounted bees in a rotary apparatus (Fig. 1) made of a circular piece of foam board, 40 cm in diameter and raised 28 cm off the desk held in a clamp. We attached 12 equally-spaced training chambers (plastic tubes sized 5 cm in diameter and 7 cm in height) to the foam board. Chambers were painted black on the outside and covered in aluminum foil on the inside, except for an open window (sized 3.5 cm in width and 2 cm in height), which allowed access to an airflow controlled by an air pump. The back of the chamber was connected to a vacuum pump, to contain the spread of odorants used during training. The floor of the chamber was open to a platform with an array of blue LEDs, controllable by a remote power source. We placed the harnessed bees into the apparatus (one per chamber) such that their heads were oriented upwards and their probosces towards the experimenter (i.e., away from the apparatus). Once placed in the apparatus, we commenced the first training trial by exposing a bee to 4–5 s of charcoal-filtered air. After this, we exposed the bee to a multimodal compound stimulus (color + odor, hereafter referred to as 'bimodal') for 7 s (blue LED and the odor 1-hexanol) before immediately giving the bee a 50% sucrose reward for 3 s via a hand-held syringe. We used a bimodal stimulus as multimodal flower cues are more salient to bumble bees and facilitate learning (Katzenberger, Lunau, & Junker, 2013; Leonard, Dornhaus, & Papaj, 2011). To stimulate the bee to extend its proboscis, the sucrose was held between the bees' antennae for less than 1 s and then given to the bee when it extended its proboscis. We then rotated the PER apparatus such that the next bee was in the same position that the first bee had been in, and this bee was then given its first training trial. The rotational scheme resulted in an inter-trial-interval of approximately 10 min for each individual. During the first training trial, we were prepared to note whether any bees showed an innate PER to the color and odor used in the experiment, but this was never the case. After all bees had gone through the first round of training, we immediately continued to carry out seven additional training trials, all identical to the first training trial. If the bees expressed PER two or more times within the 7 s of CS (but before their antennae were stimulated with sucrose) over the eight training trials, they were considered to have learned to make the CR to the bimodal stimulus. If bees did not exhibit PER even when having their antennae stimulated by the sucrose more than once, they were considered non-responders and removed from the experiment.

During the training trials, we recorded whether the bee exhibited PER in response to the bimodal stimulus before being given the sucrose reward. In memory tests we presented bees with the bimodal stimulus and recorded the bee's response in the same way as during training, but without the sucrose reward.

Experiment 1: Bees trained, given 24 h of stress and then tested

We trained 147 harnessed bees following the procedure in Section "General methods" and then divided the bees that had learned the association ($n = 60$) into two treatment groups: (1) stressed ($n = 30$) and (2) control ($n = 30$). Approximately 5 min after the end of training we fed all bees to satiation and placed the stressed individuals on a customized shaker apparatus, which delivered a 1-s long vibrational stimulus every 5 min for 24 h. During this time, we left the control bees in the same room (at a distance from the shaker), but they were not shaken. All bees were left in their PER-harnesses during this time. We placed harnessed bees in a room undisturbed for 30 min before we returned them to the PER apparatus for a memory test using the bimodal stimulus.

Although the PER protocol is well-established in studies of bee learning, as a follow-up treatment to confirm that our training resulted in associative learning, we included 21 'stressed unpaired' bees. These bees were trained in the same way as the other two treatments, except that instead of being exposed to the conditioned stimulus and then the reward, they received the reward for 3 s and then the conditioned stimulus. This was to ensure that bees were not more likely to exhibit PER for a reason other than learning that the CS predicted the sucrose reward. After undergoing eight unpaired training trials, these bees were placed in the shaking apparatus for 24 h before being tested for memory recall in the same way as the other treatment groups.

Experiment 2: Bees trained, stressed for 30 min and then tested

We trained 188 harnessed bees following the procedure in Section "General methods" and then we divided the bees that had learned the association ($n = 77$) into two treatment groups: (1) stressed ($n = 39$) and (2) control ($n = 38$). We followed the same procedure as in Experiment 1 (Section "Experiment 1: Bees trained, given 24 h of stress and then tested"), with the exception that stressed bees were shaken for 30 min rather than 24 h (and control bees were left undisturbed for 30 min) before being tested 30 min later.

Experiment 3: Bees stressed for 30 min, then trained and tested

A total of 141 bees were harnessed for PER. Each experimental day, we placed half of the harnessed bees (the 'stressed' treatment) into the shaking apparatus that shook them for 1 s every 5 min for 30 min. Half of the bees (control treatment) were left undisturbed in the same room as the shaken bees for the same amount of time. Thirty min after the end of this period, we checked that bees would exhibit PER when stimulated with 50% sucrose, and those that did not were discarded. The remaining bees were placed into the PER apparatus for training ($n = 46$ bees responded to training). A memory test was conducted 30 min after the end of training, and then again 24 h later.

Data analyses

To compare learning curves, in all cases general linear models were fitted with the response being the proportion of PER, and the explanatory variables being the continuous variable 'trial number' (1–8) and the fixed factor 'treatment' (experimental and control). Maximal models were run initially and then if the interaction was non-significant, the model was re-run with the interaction removed. In all experiments, we measured bee head width posthumously, to account for the role of brain or body size in any differences observed between treatment groups (Worden, Skemp, & Papaj, 2005). Likewise, in the memory experiments (1 & 2), we retroactively compared the learning rates of bees assigned to different treatment groups, to confirm that any observed differences in performance did not precede the stress manipulation of interest. All analyses were carried out using R v.3.1.0 (R Development Core Team 2010).

Results

Experiment 1: Bees trained, stressed for 24 h and then tested

After the 24 h elapsed, 12 bees died (eight control and four stressed). Of the $n = 22$ control and $n = 26$ stressed bees remaining, those that were shaken for 24 h remembered the learned association better than bees that had not been shaken, as more shaken bees exhibited PER in response to the trained conditioned stimulus in the test (with no sucrose reward) than control bees (χ^2 test: $\chi^2_1 = 6.69$, $p = 0.010$; Fig. 2).

As expected, prior to being split into the experimental and control groups, bees in these two groups both learned the association across the eight trials, and there was no difference between experimental and control bees (GLM: trial: $F_{1,13} = 86.557$, $p < 0.001$; treatment: $F_{1,13} = 0.374$, $p = 0.347$; Fig. 2). Two of 21 bees (a proportion of 0.09) in the 'unpaired stressed' treatment
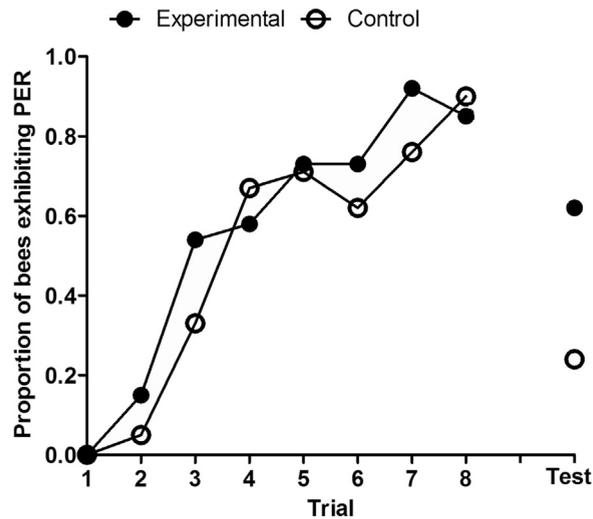
Fig. 2. Experiment 1: the proportion of bees exhibiting Proboscis Extension Response in response to the bimodal stimulus before being given sucrose reward across the eight training trials and test trial (no reward given). Between training and testing bees were either shaken (experimental treatment) or not shaken (control treatment) for 24 h.
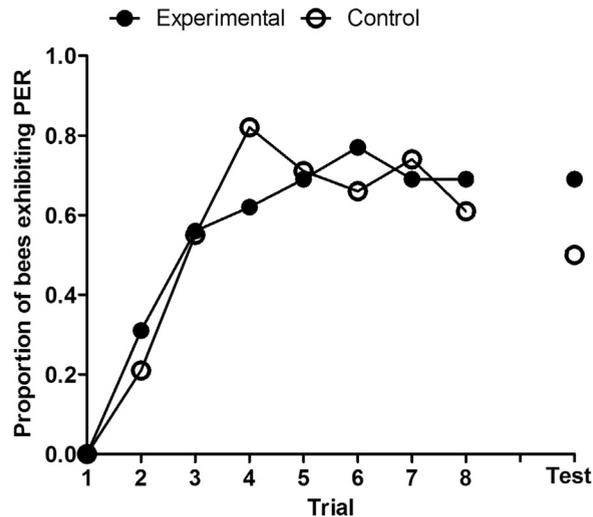

Fig. 3. Experiment 2: the proportion of bees exhibiting Proboscis Extension Response in response to the bimodal stimulus before being given sucrose reward across the eight training trials and test trial (no reward given). Between training and testing bees were either shaken (experimental treatment) or not shaken (control treatment) for 30 min.

group exhibited PER in the memory test. This was significantly lower than stressed bees that had received paired training (χ^2 test: $\chi^2_1 = 5.86$, $p = 0.016$).

Bees in the experimental and control treatment groups did not differ from each other in size (unpaired t-test: $t_{45} = 1.09$, $p = 0.282$). Bees that learned (responded with PER in test) were not any larger than bees that did not learn (control bees: $t_{19} = 0.247$, $p = 0.808$; experimental bees: $t_{24} = 0.245$, $p = 0.808$).

Experiment 2: Bees trained, stressed for 30 min and then tested

There was no statistical difference in ability to remember the learned association between bees that were shaken for 30 min after training and control bees, as groups exhibited similar levels of PER in the test trial (χ^2 test: $\chi^2_1 = 2.96$, $p = 0.085$; Fig. 3). However, the trend was in the direction of the experimental (shaken) bees exhibiting PER in the test more often than the control bees.

As expected, prior to being split into the experimental and control groups, bees in these two groups both learned the association across the eight trials, and there was no difference between experimental and control bees (GLM: trial: $F_{1,13} = 20.179$, $p < 0.001$; treatment: $F_{1,13} = 0.0018$, $p = 0.967$; Fig. 3). Likewise, bees in the experimental and control treatment groups did
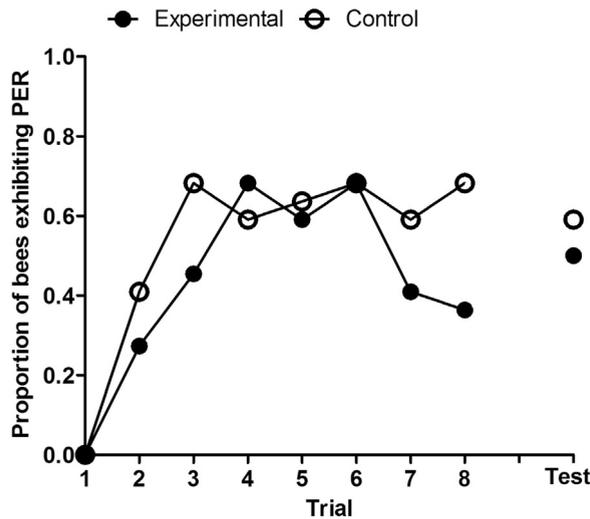
Fig. 4. Experiment 3: the proportion of bees exhibiting Proboscis Extension Response in response to the bimodal stimulus before being given sucrose reward across the eight training trials and test trial conducted 30 min after training (no reward given). Bees were either shaken (experimental treatment) or not shaken (control treatment) for 30 min prior to training and testing.

not differ from each other in size (unpaired t-test: $t_{45} = 0.641$, $p = 0.525$). Bees that learned (responded with PER in test) were not any larger than bees that did not learn (control bees: $t_{21} = 0.969$, $p = 0.344$; experimental bees: $t_{25} = 1.237$, $p = 0.228$).

Experiment 3: Bees stressed for 30 min, then trained and tested

Of the 70 experimental and 71 control bees that were trained after having been either shaken or not shaken, 24 and 22 bees responded to training, respectively. Thus the shaking treatment had no effect on bees' tendency to exhibit PER in response to the conditioned stimuli across training (χ^2 test: $\chi^2_1 = 0.175$, $p = 0.676$).

Of the 24 (stressed) and 24 (control) bees that responded to PER training, there was an increase in the number of bees that exhibited PER in response to the CS across trials, but this did not differ between treatment groups (GLM: trial: $F_{1,13} = 7.347$, $p = 0.018$; treatment: $F_{1,13} = 1.133$, $p = 0.306$; Fig. 4). There was also no difference in test performance across the two groups, either 30 min after (χ^2 test: $\chi^2_1 = 0.367$, $p = 0.545$) or 24 h after training (χ^2 test: $\chi^2_1 = 0.128$, $p = 0.721$).

Discussion

Bumble bees that were stressed by being shaken after learning an association between a conditioned stimulus (blue light and the 1-hexanol odor) and a sucrose reward were better at remembering that learned association than bees that had not been shaken. However, this effect was only found when bees were shaken for 24 h; after only 30 min of shaking there was only a trend in this direction. Bees that were shaken before learning an association between the bimodal stimulus and sucrose reward did not differ in their ability to learn and remember this association compared to undisturbed individuals. The physiological effects of stress have been well documented in the honey bee using a broad range of stressors (Even et al., 2012). However, we believe this is the first study to suggest an effect of acute stress on memory in a bee.

Bumble bees that were shaken for 24 h were better at remembering the association learned prior to the shaking stress than bees that had not been stressed in this way. At least in honeybees, consolidation of memories into 'early' long-term memory occurs within 24 h after learning (Menzel, 2001). Therefore, it seems plausible that in our experiment the experimental stress affected memory consolidation, as individuals encountered the 24 h stress (shaking) around 5 min after learning. As our shaken bees sat undisturbed for 30 min prior to being tested, it seems less likely that the stress interfered with memory recall, where stress, at least in rats, usually needs to occur immediately before or during retrieval (Roozendaal, 2002, 2003). Our results, therefore, appear to agree with previous studies in vertebrates that have generally found that stress immediately after an event improves memory consolidation, thus improving the recall of a previously learned event and that stress immediately before recalling an event generally impairs memory retrieval (reviewed in Roozendaal, 2002, 2003; Sandi & Pinelo-Nava, 2007). However, in order to determine whether our results do indicate this conclusively, we would need to manipulate the duration of time bees experienced after training before being stressed and after being stressed before their memory was tested. The current experiment at least suggests that 30 min of being undisturbed is enough time for bees to 'calm down' enough for stress not to have any negative effects on retrieval. If stress after learning an association enhances memory consolidation, it seems that 30 min of stress is not sufficient to have this effect, as bees that were stressed for this amount of time only showed a trend in improved memory performance. Instead, a longer period of time may be needed for

this effect on memory consolidation. Further experiments would reveal where this duration of stress falls between 30 min and 24 h.

A previous study using honeybees in a PER protocol (Hussaini, Bogusch, Landgraf, & Menzel, 2009) found that bees that were sleep-deprived through shaking for 15 h did not have impaired memory for a previously learned association. However, shaking affected the extinction of this memory: bees were given two extinction trials (CS presented alone), and those that had been shaken showed a greater PER response to the previously rewarded CS than bees that had not been shaken. Therefore, in this case, shaking seems to have interfered with the formation of the memory that the CS does not predict a reward. As this study differed from ours in a number of ways, it is difficult to identify which factor might be responsible for the difference in results, but possibilities include: a different species used, a difference in the conditioned stimulus used (olfactory only vs. bimodal), the timing of the shaking (both the total time and the duration of each shaking episode), the number of training trials prior to shaking and the duration after the end of shaking that the bees were tested for memory retention. Another study using honey bees found that after being trained, a stressful experience impaired memory after 24 h (Beckmann, 1974). Again, this study differed from ours in a number of features and it is difficult to conclude which one might be responsible for a potential difference in results.

An alternative explanation for our results is that a physiological process associated with the shaking procedure increased bees' tendencies to exert their prosci, and this was unrelated to the presentation of the bimodal stimulus we had conditioned them to. However, bees that we stressed in the same way (shaking for 24 h) but had previously had unpaired training (reward followed by CS) did not generally exert their prosci in response to the CS. Despite this control, it is still possible that being shaken for 24 h caused bees to be more likely to exert their prosci in the test for a reason that was not due to memory. In line with previous learning experiments that used the PER protocol (Bateson et al., 2011; Hussaini et al., 2009), we did not test for responsiveness to sucrose after shaking, and it is possible that 24 h of shaking stress may have affected hunger or motivation, which could influence a bee's tendency to exert its proboscis. However, both groups of bees were likely to be hungry: bees typically only survive for around 20–30 h without food (Moret & Schmid-Hempel, 2000). Furthermore, at least under certain conditions, shaken bumble bees seem to be equally able and motivated to exhibit PER to the same extent as unshaken bees (Bateson et al., 2011).

While stress improved bees' ability to remember a previously learned association, it had no effect on bees' ability to learn when they were stressed prior to learning. As with memory recall, a number of factors influence the effects of stress on learning (Shors, 2006), one of which is the timing of the stressful event. As we allowed our bees to sit for 30 min after experiencing the stressful event, it is plausible that any effects of stress disappeared over this time. Stressing bees either immediately prior to training or during training would be more likely to have effects on their ability to learn. Previous studies have found both facilitating and impairing effects of stress on animals' ability to learn. For example, rats stressed by being placed near a predator (a cat) were worse at subsequently learning a spatial task than unstressed rats (Park, Zoladz, Conrad, Fleshner, & Diamond, 2008). Similar results have been found using other protocols and in other species (Overmier & Seligman, 1967; Schwabe & Wolf, 2010; Shors, 1998; Shors, Weiss, & Thompson, 1992). On the other hand, studies with male rats that experience stress prior to eyeblink conditioning have consistently shown enhanced learning (Servatius & Shors, 1994; Shors, 2001; Shors et al., 1992). For example, rats that were stressed with electric shocks to the tail or being forced to swim without an escape route responded more to a conditioned stimulus than rats that had not experienced these stressors (Shors, 2001). However, this study also showed that the type of stressor can be key in determining this effect: other stressors (inescapable noise or shocks to the eyelid) did not enhance learning despite being correlated with comparable levels of the stress hormone corticosterone. In the current study, we used shaking as our stressor as it has previously been shown to have effects on bees' cognition (Bateson et al., 2011) as well as being representative of the physical disturbance pollinators might experience. However, it is likely that other types of stressors would have different or possibly interactive effects on learning and memory. For example, given evidence that commercial and wild bees may face chronic nutritional stress, whether vibrational disturbance would have a similar effect as on our laboratory-reared bees (fed nearly *ad libitum* on sucrose and pollen) is an obvious open question.

In our third experiment, both experimental and control treatments of bees appeared to show a higher response to the conditioned stimulus by trial 2 but then showed a plateau faster, with a smaller proportion of bees learning by the end of the eight training trials. It is difficult to compare the control groups between our three experiments, as they were conducted at different times with different colonies (well-documented to vary in learning ability; Raine, Ings, Ramos-Rodriguez, & Chittka, 2006). The timing of when the bees had last been fed also differed between experiments (in Experiment 3 bees were likely to be hungrier than bees in Experiments 1 and 2, as they had not been fed prior to being placed on the shaker for 30 min and then sitting for 30 min). Thus motivational and physiological differences in this group of bees might explain their difference in learning performance in this third experiment. Furthermore, in trial 8 bees in the different treatments appeared to be exhibiting different levels of PER, yet it is not clear why this might be the case, especially since there were no statistical differences in the bees' test response 30 min later.

One potential limitation to the current study is that all learning and memory assays were conducted using PER, which may have added additional stress to bees despite the advantages of using this protocol. However, similarly-harnessed honey bees do not have higher levels of HSP70 (a heat shock protein associated with the stress response) compared to unharnessed bees, even after having been harnessed for 24 h (Hranitz, Abramson, & Carter, 2010).

As there are a multitude of factors involved in mediating the effects of stress on learning and memory, more extensive research is needed to determine the effects stress such as physical disturbance have on pollinators. For example, factors that

have been identified as being key in determining whether stress has beneficial or deleterious effects on an animal's ability to learn and remember include the intensity, the duration, and the timing of the stressor, how predictable it is, the gender of the individual and whether the stressor occurs at the same time or in the same context as the stimulus being learned (reviewed in Joëls et al., 2006; Sandi & Pinelo-Nava, 2007). Some of these factors are likely to have influenced how stress affected bees in the current experiment. For example, it is possible that the shaking stress was predictable to bees after a certain period of time, whereas in natural or agricultural settings, physical disturbance may be sudden and unpredictable.

Many key aspects of pollinator decline have now been identified including the stressors involved and the physiological effects they have on bees. However, one key topic that has largely been neglected is how stress might affect an extremely well-studied aspect of behavior in honey bees and bumble bees: learning and memory. In the current study we highlight a protocol that can be used to easily investigate this topic. Using such a protocol, future research could investigate further how stress affects learning and memory, which could have implications for an ecologically- and agriculturally-important pollinator, as well as more broadly offering a system for better understanding the effects of stress on memory.

Acknowledgments

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