Original Article

No sex differences in learning in wild bumblebees

Felicity Muth,[a,●] Amber D. Tripodi,[b] Rene Bonilla,[c] James P. Strange,[b] and Anne S. Leonard[c]

[a]Department of Integrative Biology, University of Texas at Austin, 2415 Speedway, Austin, TX 78712, USA, [b]USDA ARS Bee Lab, Utah State University, Logan, UT 843225310, USA, and [c]Department of Biology, University of Nevada, Reno, NV, 89557, USA

Females and males often face different sources of selection, resulting in dimorphism in morphological, physiological, and even cognitive traits. Sex differences are often studied in respect to spatial cognition, yet the different ecological roles of males and females might shape cognition in multiple ways. For example, in dietary generalist bumblebees (*Bombus*), the ability to learn associations is critical to female workers, who face informationally rich foraging scenarios as they collect nectar and pollen from thousands of flowers over a period of weeks to months to feed the colony. While male bumblebees likely need to learn associations as well, they only forage for themselves while searching for potential mates. It is thus less clear whether foraging males would benefit from the same associative learning performance as foraging females. In this system, as in others, cognitive performance is typically studied in lab-reared animals under captive conditions, which may not be representative of patterns in the wild. In the first test of sex and species differences in cognition using wild bumblebees, we compared the performance of *Bombus vancouverensis nearcticus* (formerly *bifarius)* and *Bombus vosnesenskii* of both sexes on an associative learning task at Sierra Nevada (CA) field sites. Across both species, we found that males and females did not differ in their ability to learn, although males were slower to respond to the sucrose reward. These results offer the first evidence from natural populations that male bumblebees may be equally as able to learn associations as females, supporting findings from captive colonies of commercial bees. The observed interspecific variation in learning ability opens the door to using the *Bombus* system to test hypotheses about comparative cognition.

Key words: **Bombus bifarius, Bombus vancouverensis nearcticus, Bombus vosnesenskii, cognition**

INTRODUCTION

Cognitive abilities, like physical traits, are expected to evolve in relation to selection pressures from an animal's environment (Shettleworth 2010). Even within a species, individuals may vary in their cognitive abilities in a manner reflecting their lifestyle. For example, mountain chickadees *Poecile gambeli* vary in measures of cognition in accordance with ecological requirements (i.e., low vs. high elevation; Freas et al. 2012; urban vs. rural; Kozlovsky et al. 2017). Intraspecific comparisons carry the advantage that some of the confounding variables that exist when addressing differences between species (such as phylogeny and some ecological variables) are controlled for (Pravosudov and Clayton 2002). Intraspecific differences in cognition are perhaps most apparent in comparisons of males and females, which are often subject to different selection pressures. A classic demonstration of this comes from the prairie vole (*Microtus ochorogaster*) and meadow vole (*Microtus pennsylvanicus*; Gaulin and FitzGerald 1986). In monogamous prairie voles, males

and females have similar home ranges and similar requirements in terms of spatial cognition and navigation abilities; this is reflected in their similar performance in spatial learning tasks in the lab (Gaulin and FitzGerald 1986; Gaulin et al. 1990). On the other hand, polygamous meadow voles hold larger home ranges and likewise outperform females of the same species on spatial tasks (Gaulin and FitzGerald 1986; Gaulin et al. 1990).

While sex differences in cognition are most often studied in relation to spatial abilities (Caplan et al. 1985; Jacobs et al. 1990; Sherry et al. 1992; Grön et al. 2000; Jones et al. 2003; Jones and Healy 2006; Tello-Ramos et al. 2014), other cognitive processes may also differ between the sexes. For example, male and female chimpanzees learn termite-fishing skills in different ways (Lonsdorf et al. 2004), zebra finches differ in their discrimination of vocal signals (Vicario et al. 2001), and female rats outperform males in some classical and most operant conditioning paradigms, while males outperform females at other specific tasks (Dalla and Shors 2009).

Sex differences are most often investigated in lab-reared animals in artificial settings, where it may be difficult to disentangle

Address correspondence to F. Muth. E-mail: felicity.muth@austin.utexas.edu.

differences in cognitive performance from the influence of other confounding factors. For example, male rats *Rattus norvegicus* typically outperform females when tested in a spatial water maze task; however, females typically show more of a stress-related behavior (thigmotaxis: swimming close to the wall of the maze), which may explain the apparent difference in "cognition" between the sexes in this commonly used protocol (Harris et al. 2008). Although lab conditions could reduce the behavioral "noise" associated with different developmental environments, it is also plausible that sex-specific cognitive abilities may not develop in lab-reared animals as they would in wild-foraging animals. For example, a lack of environmental enrichment can lead to learning deficiencies (cuttlefish: Dickel et al. 2000; rats: van Praag et al. 2000) that could, in principle, exacerbate or mask sex differences. Thus, combining information about cognitive performance among both lab-reared and free-living individuals can yield a stronger holistic understanding of the extent to which sexes show cognitive differences.

Bees are a model system for learning and memory (Chittka and Thomson 2001), yet nearly all studies have involved female workers. In particular, social bees like honeybees and bumblebees are a common model for the study of how animals learn stimuli–reward associations in a foraging context. As generalists, bumblebee foragers can visit hundreds of flowers of various types each day (Goulson 2003) and, thus, it is advantageous for individuals to rapidly learn associations between multiple floral stimuli and rewards, such as nectar (Chittka and Thomson 2001; Dyer et al. 2006; Clarke et al. 2013) and pollen (Muth et al. 2015, 2016). This ability to learn associations between floral cues and rewards is correlated with higher foraging success in some cases, which may, in turn, lead to higher fitness (Raine and Chittka 2008; but see Evans and Raine 2014; Evans et al. 2017). However, despite bumblebees' wide use in the study of many aspects of learning (Sherry and Strang 2015), nearly all of these studies have been with female foragers, and the majority have involved work on captive, commercial species (*Bombus terrestris* for work based in the United Kingdom and Europe and *Bombus impatiens* for work based in the United States and Canada).

Why might flower-visiting male and female bumblebees exhibit cognitive differences? For primitively eusocial bumblebee colonies (the majority of the 250 *Bombus* species), all workers are female. Since workers do not mate, foragers spend most of their lifetime (~2–6 weeks, depending on species; Goulson 2003) collecting nectar and pollen from flowers, before returning with food to the colony to feed the developing brood. Individual foragers can visit a wide variety of flowers, making hundreds to thousands of visits in a day (Goulson 2003; Heinrich 2004). They learn associations between a wide range of floral stimuli (Dyer et al. 2006; Clarke et al. 2013; Foster et al. 2014) and rewards (Muth et al. 2015, 2016) and can display a wide range of "complex" abilities (Perry et al. 2017). As such, they have emerged as a model system for the study of cognition.

Surprisingly, given their prominence in behavioral research, only a handful of studies have addressed foraging in male bumblebees (Ranta and Lundberg 1981; Bertsch 1984; Ostevik et al. 2010; Wolf and Moritz 2014; Ogilvie and Thomson 2015; Roswell et al. 2019). Male bumblebees are produced later in the colony life cycle, typically around late summer or autumn (Ostevik et al. 2010). In contrast to foragers, males do not forage for their colony but, instead, only collect food for themselves to meet their own energetic needs, spending most of their time searching for mates (Goulson 2003) and, rarely, if ever, return to the natal nest. Sex differences in foraging behavior have previously been described in

commercial *B. impatiens*: in one study, males visited half as many flowers per minute as workers, were more likely to switch patches, and spent significantly longer handling each flower (Ostevik et al. 2010). Like workers, males prefer nectar of higher sucrose concentrations (Brown and Brown 2020) but, unlike workers, do not collect pollen. Therefore, while learning is no doubt important for both sexes, we might expect foraging females to have enhanced abilities to learn and remember floral associations, especially if those abilities come with a cost (Dukas 1999; Mery and Kawecki 2004; Jaumann et al. 2013).

The question of sex-based learning differences in bees has been addressed in three cases to our knowledge and has always been undertaken with commercially reared bumblebees maintained in lab conditions (Church et al. 2001; Lichtenstein et al. 2015; Wolf and Chittka 2016). These efforts have generally not found differences between the sexes, but the results are somewhat mixed. For example, male and female commercially reared *B. terrestris audax* did not differ in their ability to learn a series of associations between artificial flowers and sucrose rewards (Wolf and Chittka 2016). In another study, males and females of *B. impatiens* did not differ in their ability to learn a color association, but females performed better at learning a pattern association (Church et al. 2001). However, in this study, males were taken from one colony, while females were taken from another, thus making it difficult to disentangle sex-based from colony-based differences. In a third study, *B. terrestris* males and females did not differ in their ability to learn an association between a light cue and sucrose reward using the Proboscis Extension Response (PER) paradigm (where a bee is harnessed such that only their antennae and proboscis can move), but when they were removed from their harnesses and transferred to a new paradigm (a Y-maze), the female workers performed better than the males (Lichtenstein et al. 2015). This study compared males and females taken directly from a colony rather than foragers, which risks misjudging female performance, since bees with different roles may differ in learning performance.

Assessing cognitive performance with commercial colonies under lab conditions has its advantages. For example by taking individuals directly from a colony (as in Lichtenstein et al. 2015), the effects of individual experience are limited and, therefore, differences in innate learning ability may be more readily seen. On the other hand, sex-specific cognitive abilities may not develop as they would in wild-foraging bees; early experience in bumblebees shapes brain development, including the development of the mushroom bodies that support learning and memory (Jones et al. 2013). Lab-reared colonies are often maintained on a simple sucrose feeder and pollen is placed directly into a colony: this comparatively impoverished foraging scenario, lacking the wide array of colors, scents, and rewards offered by real flowers, could lead to altered cognitive development. Furthermore, it is not clear whether species that are from commercially reared stock might have experienced relaxed selection for cognitive abilities (which may be costly and trade-off with other traits that may be more important for commercial species; Mery and Kawecki 2003). This is a possibility since bees from commercial colonies would be expected to chiefly forage on one or a few readily available crop species (or be maintained on within-colony feeders), whereas wild foragers need to seek out rewarding flowers and likely visit a greater number of species with a greater diversity of stimuli and rewards. Therefore, although usually more difficult, testing cognition in wild populations is a necessary and, arguably, a more informative step to describe the potential for ecologically meaningful sex-based differences in

cognition. More generally, expanding the focus of behavioral research beyond a few model species is recognized as a critical effort for the development of the field of comparative cognition (Perry et al. 2013), as well as more broadly in the study of animal behavior (Rosenthal et al. 2017).

To this end, we recently developed a technique that allows us to compare cognitive traits in wild bumblebees using a protocol in which freely moving bees are tasked with learning a visual association ("Free-Moving Proboscis Extension Response" [FMPER]; Muth et al. 2017). Using this protocol, we compared the associative learning ability of wild-foraging male and female bumblebees of two common species in the Sierra Nevada mountains (CA; *Bombus vosnesenskii* and *Bombus vancouverensis nearcticus*, formerly *bifarius*; Ghisbain et al. 2020). If there has been stronger selection for female workers to learn floral associations compared to males, we expected that females would perform better on a color-learning task, making fewer errors during acquisition and/or a subsequent test phase.

METHODS

Between July and August 2016, we collected male and female *B. vosnesenskii and B. vancouverensis nearctius* bumblebees from flowers at two sites in Tahoe National Forest north of Truckee, CA (GPS coordinates 39°25'56.3"N, 120°15'22.7"W and 39°26'06.4"N, 120°16'29.5"W). Both sites covered an area of land of ~200 000 m², near Sagehen Creek. Flowers that we collected bumblebees from included *Erythranthe guttata*, *Potentilla gracilis*, *Sidalcea orgeana*, *Penstemon rydbergii*, and *Lupinus argenteus*. Between 8:00 and 9:00 AM on each day, we caught ~30 bees, with roughly equal representation of both species and sexes. We held bees in individual 12-dram plastic vials during this time, placed in a cooler (~15 °C) to prevent them from overheating.

Species identification and criteria for inclusion

We visually identified male and female *B. vosnesenskii* and *Bombus bifarius* (now *vancouverensis*) initially in the field prior to testing, but then clipped the terminal portion of the tarsus of the right mid-leg for later microsatellite analysis after testing (Holehouse et al. 2003). The area of study contained a number of bumblebee species that are morphologically similar enough to *B. vosnesenskii* (e.g., *Bombus vandykeii*, *Bombus californicus*, and *Bombus insularis*) or *B. vancouverensis* (e.g. *Bombus melanopygus*) workers or males in this area, and so we wanted to ensure that field IDs were correct. The final analysis only included females and males whose species had been thus confirmed with genetic analysis. Note that taxonomists have recently reassigned populations of *B. bifarius* from our sampling region to *B. vancouverensis nearcticus* (Ghisbain et al. 2020).

Training protocol

We trained and tested bees using FMPER, a protocol we developed to address cognitive traits in wild bumblebees (Muth et al. 2017). This protocol allows us to compare the visual learning performance of individual bees who can freely move while confined to individual chambers, and in which we can observe their tendency to exhibit PER to conditioned stimuli (colored strips of paper) over time (Figure 1). To accomplish this, each bee was transferred to an individual transparent plastic cylindrical tube (TAP Plastics) with holes for ventilation (L × D: 13 × 2.5 cm, wall thickness: 1.6 mm; Figure 1). The tube was sealed at one end by a rubber cap, and at the
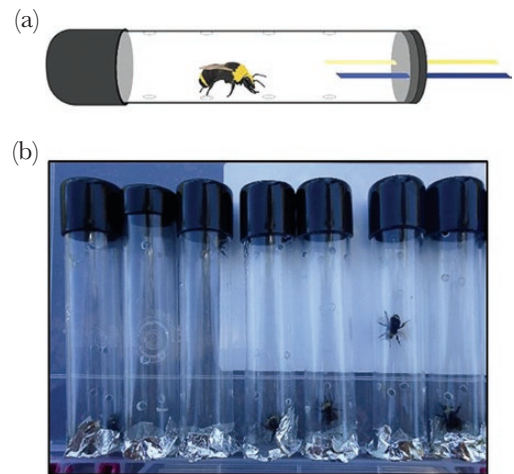

Figure 1
(a) Diagram of testing apparatus. Bees were contained in plastic tubes and presented with both colored stimuli for all "choice" trials and the final "test" trial. (b) Photograph of bees being tested within a block.

other by a transparent acrylic disc, affixed in place by metal tape, with two 3-mm diameter holes. This disc served as the location where the bee was trained and tested. We allowed bees to acclimatize to the apparatus for 2 h before we tested the first of two blocks, with each block consisting of up to seven bees being exposed to nine presentations of reward, followed by a test (details below). The second block of bees was tested immediately after the first (i.e., 3.5 h from initial capture).

We trained bees to a color discrimination task, where each bee was presented with a color cue (a strip of colored card, W × L: 1.0 × 20 mm) either dipped in 50% (w/w) sucrose (positively reinforced conditioned stimulus, CS+) or water (negatively or neutrally reinforced conditioned stimulus, CS−). Color stimuli used were either human blue or yellow. We counter-balanced our design such that, for both species and sexes tested, half of the bees were trained with blue as the CS+/yellow as the CS−, and half were trained to the opposite relationship. We successfully tested a total of 114 bees (*B. vancouverensis nearcticus* females $n = 45$; males $n = 17$; *B. vosnesenskii* females $n = 33$; males $n = 19$). In seven cases, bees died prior to or during testing.

The training protocol was as follows: for two "pretraining" trials, bees were presented with the CS+, followed immediately by the CS−. For each of these presentations, the strip was inserted 1 cm into the tube, oriented such that the bee could see it (to the front and side of its head) and placed on its antennae. The bee was allowed to drink from the strip for 3 s before it was removed. These two pretraining trials served to familiarize the bees to the protocol. For seven subsequent "choice trials," each bee was presented with a simultaneous choice between the CS+ and CS−: we dipped each strip in either the US+ (positive unconditioned stimulus, 50% (w/w) sucrose solution) or US− (negative or neutral unconditioned stimulus, water) and inserted them 1 cm into each of the holes at the end of the tube (spaced ~2cm apart from each other). We always did this when the bee was at the far end of the tube so that it had the opportunity to see both strips before making a choice. The bee's choice was recorded as the strip it approached and extended its proboscis toward; bees would typically extend their proboscis even to the unrewarding strip; however, if they only antennated the unrewarding strip

and did not extend their proboscis, this was still scored as an "incorrect" choice. The bee was allowed to drink from its chosen strip for 3 s. After this, we removed the chosen strip and offered the alternative (unchosen) strip for 3 s. Thus, all bees received both the CS+ and the CS− on each choice trial, but the order of presentation was determined by the bee rather than by the experimenter. After the bee had received seven choice trials, we presented it with a final trial, the "test', which was conducted in the same way as the other choice trials, the only difference being that both strips offered water only. As with the choice trials, we recorded the color that the bee first extended its proboscis to. The position of the CS+ was alternated both across individuals and between the two disc holes during training and testing. In previous work, we established that the FMPER protocol returns results in keeping with the general predictions of learning theory when various properties of the CS and US are manipulated (Muth et al. 2017). While we did not video record our behavioral trials, examples of this behavior can be seen at: https://methodsblog.com/2017/09/21/bee-cognition/.

All pretraining trials and choice trials were spaced ~10 min apart for each bee (i.e., "bee 1" would be given a choice trial, followed by "bee 2," "bee 3" etc., circling back to "bee 1" at approximately 10 min since its previous trial; the time that all trials were conducted was recorded. Occasionally, bees would stop moving mid-way through testing (B. vancouverensis nearcticus females $n = 7$; B. vancouverensis nearcticus males $n = 1$; B. vosnesenskii females $n = 4$; B. vosnesenskii males $n = 2$). In these cases, we presented the CS+ to the bee's antennae again (as in the pretraining trial) and allowed it to drink for 3 s before presenting it with the CS− for 3 s. This "refresher" trial was carried out in place of a regular "choice" trial and, as such, no data from such trials were included in the final analysis. If a bee required a refresher trial more than twice, we removed it from the experiment. Twenty-one bees required "refresher" trials: B. vancouverensis nearcticus females $n = 10$; males $n = 3$; B. vosnesenskii females $n = 6$; males $n = 2$).

After noticing that males seemed to be slower to respond to the rewarding stimulus, we then measured this to confirm our observations. Specifically, we measured (using a single stopwatch) the time it took for males to extend their proboscis to the rewarding strip of paper after it was first presented to their antennae. These data were collected for a subset of individuals included in the learning analysis, as well as another set of bees tested exclusively for their time to respond (final sample sizes: B. vancouverensis nearcticus female, $n = 17$; B. vancouverensis nearcticus male, $n = 36$; B. vosnesenskii female, $n = 13$; B. vosnesenskii male, $n = 22$). Of these, 47 bees were measured for their learning ability, and 41 were not. Since we carried this out while observing multiple bees (approx. 7) at once, it was difficult to be precise about the response time for each bee; instead, we measured response time as "1 s or less"; "1–10 s"; "10 s–1 min," "1–10 min"). A difference in the time it takes bees to respond may be an indication of their motivation to forage (see Discussion).

Immediately after the bees were tested, we placed them in a cooler (~5 °C) to immobilize them. We also clipped the terminal portion of the tarsus of the right mid-leg and stored it in 90% ethanol for later specific identification by genetic analysis. We then released the subjects; we avoided reusing any bees that had this portion of their tarsus missing.

Genetic analysis

DNA was extracted from each sample using a 5% Chelex-100 (Bio-Rad, Hercules, California) protocol (Strange et al. 2009).

Each specimen was characterized with 16 microsatellite loci (Supplementary Table S1) as described in Strange et al. (2009; Supplementary Material), including 17 reference specimens that were caught at the site and identified to species (with the assistance of Dr. Robbin Thorp, UC Davis Bohart Museum of Entomology; B. bifarius [since reclassified as vancouverensis nearcticus] = 9; B. vosnesenskii = 4; B. insularis = 3; Bombus flavifrons = 1) using morphological characters. Microsatellite data were used to assign each specimen to a species group using STRUCTURE v.2.3.4 (Pritchard et al. 2000), and the optimal number of groups was selected using the Evanno method (Evanno et al. 2005). A total of 242 bee specimens were genotyped at 16 loci, but 4 loci were removed because they did not consistently amplify across all specimens (Supplementary Material). The remaining 12 loci were used to assign each specimen to a population group. The plot of all delta-K over K showed a peak at $K = 3$ (Supplementary Figure S2). The three groups were classified based on the voucher samples they contained (B. vosnesenskii $n = 88$, B. bifarius/ vancouverensis nearcticus $n = 126$, and other, which included the voucher samples identified as B. insularis and B. flavifrons, $n = 28$; Supplementary Figure S3). We limited our analysis of behavior to samples that were assigned to either B. vancouverensis nearcticus or B. vosnesenskii since we only had both females and males of these species.

Data analysis

All analyses were carried out in R version 4.0.3. To determine whether learning differed between male and female bees for the two species, we carried out binomial generalized linear mixed models (GLMMs) with the response variable "correct" (1) or "incorrect" (0; for each bee's choice on each trial), the explanatory factors: trial (continuous); sex (male or female); species (B. vosnesenskii or B. vancouverensis nearcticus), color trained to (blue or yellow), and the random factor "individual bee," to account for the multiple measures taken per bee across trials. We conducted two models: one including the "choice" trials (1–7) only, and the second including the final "test" trial as the eighth trial. We included this eighth (test) trial in a second model since it improved the power of the model and because this trial was the same as the other trials with the exception of the rewards present (which the bees did not sample until after making a choice); we refer to these models as "Model 1" and "Model 2," respectively, in the results. Maximal models were run initially, and nonsignificant interaction terms removed. Using the final model, we then checked whether any other variables (not directly of interest) explained learning performance, including block number (1 or 2) and site collected from (1 or 2) (none did; see Results). For GLMMs, we used the glmer() function in the nlme package (Pinheiro et al. 2016) specifying a binomial distribution. We carried out a power analysis using the package simr (Green and MacLeod 2016).

To determine whether bees in each treatment had learned in the test phase, we carried out χ^2 tests comparing the number of bees making the correct choice in the test phase to the number of bees making the incorrect choice. To determine whether the four different treatments differed to each other in their test phase performance, we carried out a binomial GLM with the response variable "correct" (1) or "incorrect" (0) and the explanatory factors sex and species.

To determine whether sexes and treatments differed in how long the bees took to respond to sucrose on their initial exposure, we carried out a Cox proportional hazards model (survival analysis) using the R package survival (Therneau and Grambsch 2000), comparing

the time it took bees to respond across four times (by 1, 10, 60. and 600 s), including "sex" and "species" and explanatory factors in the model. Bees that never responded were excluded from this analysis.

RESULTS

Learning performance did not differ between male and female *Bombus*

Overall, bees learned the association between color and reward, choosing the rewarding color more often across trials (trial: Model 1: $z = 4.128$, $P < 0.0001$; Model 2: $z = 5.688$, $P < 0.0001$). Across both species, males and females did not differ in their ability to learn associations (Model 1: sex × trial: $z = 0.772$, $P = 0.439$; sex: $z = −0.405$; $P = 0.686$; Model 2: sex × trial: $z = 0.786$, $P = 0.432$; Figure 2). *B. vancouverensis nearcticus* had a steeper learning curve than *B. vosnesenskii*, with this effect being a trend when addressing 1–7 only but significant when the final test trial was included in the model (Model 1: species × trial: $z = −1.808$, $P = 0.071$; species: $z = 1.525$, $P = 0.127$; Model 2: species × trial: $z = −2.006$, $P = 0.045$). Addressing the test phase results alone, it is clear that bees learned: each group of bees was more likely to select the correct color than the incorrect color (χ^2 tests: *B. vancouverensis nearcticus* females: $\chi^2_1 = 20.455$; $p < 0.0001$; *B. vancouverensis nearcticus* males: $\chi^2_1 = 13.235$; $P < 0.001$; *B. vosnesenskii* females: $\chi^2_1 = 13.364$; $P < 0.001$; *B. vosnesenskii* males: $\chi^2_1 = 5.556$; $P < 0.05$). Test phase performance did not differ depending on species or sex (binomial GLM: species: $z = −0.958$; $P = 0.338$; sex: $z = −0.421$; $P = 0.673$). Learning performance was not affected by whether the bee was trained with yellow or blue as the CS+ (Model 1: $z = 1.566$, $P = 0.117$; Model 2: $z = 1.350$, $P = 0.177$), but this term was kept in the model since it was deemed to be an important part of the experimental design (c.f. Colegrave and Ruxton 2017). Learning performance was also not affected by whether the bee tested was in the first block or the second ($z = 0.050$, $P = 0.958$) or whether the bee was collected from the first or second site ($z = 0.249$, $P = 0.804$). A power analysis showed that the power to reject the null hypothesis of no effect of the factor "sex" was 79% for an effect size of 0.7 at an alpha level 0.05 for Model 1 (choice trials alone). When

the final test trial was included in the model, the power to reject the null hypothesis of no effect of "sex" increased to 85.3% for the same effect size and alpha level.

Time taken to respond to sucrose

When we compared groups in how long they took to respond (extend their proboscis) to the sucrose once it was presented to their antennae, there was no difference between the two species ($n = 88$, $z = 0.858$; $P = 0.391$); however, males took longer than females ($n = 88$, $z = −2.686$; $P < 0.01$; Figure 3).

DISCUSSION

Bees' interaction with flowers has been a long-standing model for understanding many aspects of behavior, yet few studies have investigated the cognitive abilities of male bees or compared their cognition to that of females. Those that have tackled this question in the past focused exclusively on lab-reared commercial colonies, which could be expected to differ from wild bees in several ways. Here, we carried out the first investigation into associative learning differences between the sexes in wild-foraging bumblebees of two species. Despite foraging likely playing a role as a determinant of fitness (Raine and Chittka 2008), we found that wild-caught male and female bumblebees did not differ in their ability to learn a color association. However, males typically took longer to respond to the sucrose reward, indicating sensory or motivational differences between the sexes.

Our finding that wild-foraging male and female bumblebees did not differ in their ability to learn supports similar findings from previous lab-based work with captive *B. terrestris* (Church et al. 2001; Lichtenstein et al. 2015; Wolf and Chittka 2016). Our results complement these studies by demonstrating that these findings hold not only among naïve bees from commercial sources that only experience artificial flowers but also among individuals from natural populations. This implies that the lack of an effect found previously was likely not due to a lab artifact, such as sensory or cognitive abilities not developing normally due to a lack of foraging experience.
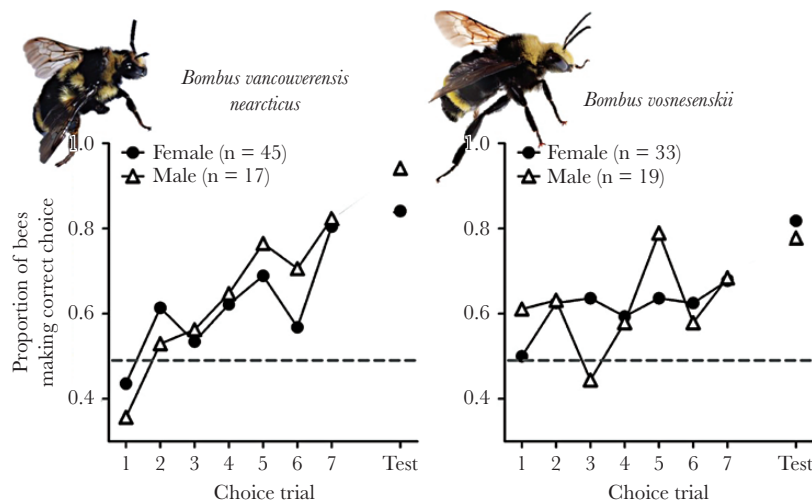

Figure 2
After two pretraining trials where bees were presented with the CS+ followed by the CS−, data are the proportion of correct choices made by bumblebees for one of two colored stimuli for (a) *B. vancouverensis nearcticus* and (b) *B. vosnesenskii* across seven learning trials and a single test (unrewarded) trial. Dotted gray line indicates chance performance.
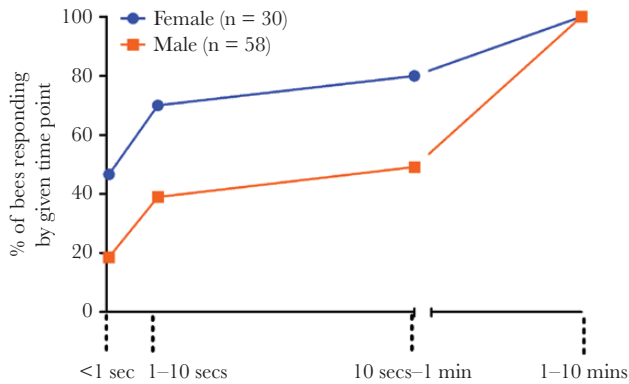
Figure 3
Latency of initial proboscis extension to sucrose, shown as the percentage of bees responding by a given time point (data shown cumulatively). *B. vancouverensis nearcticus* and *B. vosnesenskii* are pooled because there was no difference between species.

As interest grows in comparative cognition in invertebrates generally (Perry *et al.* 2013) and in *Bombus* specifically (Ings *et al.* 2009), it is an open question the extent to which findings from one or two species of commercially bred colonies align with those from wild bees of different species. In this specific case, we found that findings of previous lab-based work on commercial colonies, conducted in a variety of different ways, generally matched up with our findings among two species of wild bees. Despite being slower, foraging males may be as capable as females at learning simple associations.

"Negative" results such as these are not only critical to report for the field of comparative cognition more generally (Perry et al. 2013) but can also help guide future research to focus on other behaviors that may differ among species, sexes, or populations (i.e., commercial vs. wild). While we did not find differences in learning acquisition, males took longer to extend their probosces to the sucrose reward when it was presented to their antennae. This result could either be due to differences in detection ability, for example, males having different sensory thresholds to sucrose than females, or due to motivational differences. The former explanation seems unlikely since we used sucrose of a concentration (50% [w/w]) that is at the upper extreme of what is typically found in natural flowers (Pamminger et al. 2019) and is of a concentration that is highly preferred by both workers and males (Willmer 2011; Bailes et al. 2018; Brown and Brown 2020); thus, we would expect all bees to be highly responsive to it. Also, we would expect that if males did differ to females in their gustatory responsiveness to this concentration of sucrose, then this would translate into a difference in learning performance. At least in honeybees, workers that differ in their sucrose responsiveness (Pankiw and Page Jr. 1999; Scheiner et al. 2004) also vary in learning performance (Scheiner et al. 1999; Scheiner et al. 2001): individuals that have lower response thresholds (i.e., exhibiting PER to lower concentrations of sucrose) learn associations better since they perceive a given concentration of sucrose as more highly rewarding. Instead of an explanation based on sensory differences, we propose that motivational factors explain the difference in the time males took to exhibit PER to the sucrose: we noted that females would often run toward the colored strip, while the males tended to approach the strip more slowly and in a less direct fashion. These motivational differences may be due to differences in the roles of males and females: female foragers collect food not only for themselves but also for their colony and, as such,

are consistently motivated to forage. In contrast, male bumblebees forage only for themselves and thus may be less consistently motivated for sucrose, depending on when they last ate. Motivational differences have also been noted in previous studies, for example, in one study male bumblebees took longer than female workers between visiting flowers (Church et al. 2001). Similarly, commercial *B. impatiens* males were slower to visit flowers and spent more time on each flower (Ostevik et al. 2010). While the motivational differences did not lead to differences in learning performance here, they may play out in other scenarios or testing conditions. For example, when males performed worse when transferring learned information from a harnessed PER protocol to a Y-maze (Lichtenstein et al. 2015), these results could be explained by differences in motivation to forage after being restrained.

It is possible that we did not detect differences between the sexes because of the FMPER protocol we used, which gives a single data point per bee per trial (a binary response). This is equivalent to how learning is measured in a classic PER protocol (involving harnessed bees: Takeda 1961; Bitterman et al. 1983; Laloi et al. 1999) and allows for larger sample sizes at the cost of less data per bee (i.e., in contrast to free-flying behavioral studies that typically measure more choices per learning trial, e.g., Cnaani et al. 2006; Muth et al. 2016; Wolf and Chittka 2016). Nonetheless, measuring a binary response per trial limits the ability to explore the variation between the sexes, and it is possible that males and females differed in their variability in learning ability (i.e., see Branch et al. 2020). It is unlikely that we did not detect a difference between the sexes due to a lack of statistical power (see Results). It is also possible that the learning protocol was "too easy" and thus masked true differences. This latter explanation seems unlikely, given that the final learning performance in Trial 7 was only around 70% correct for *B. vosnesenskii* and 80% correct for *B. vancouverensis nearcticus*. We have used this protocol in the past to detect differences in learning, with results in the direction one would expect: learning performance is better when a more aversive punishment is used and when colors are easier to discriminate (Muth *et al.* 2017).

Aside from associative color learning, what other arenas might be worth exploring in terms of sex differences in wild *Bombus* populations? Given that females forage for both nectar and pollen, while males forage for nectar only, individuals likely visit different flower types (Smith et al. 2019). Thus, we might expect that females might be better at learning about multiple stimuli or reward types concurrently compared to males. We also expect that the sexes might differ in their preference for particular colors or scents depending on the flowers they had been foraging on. Male and female bumblebees might also be expected to vary in aspects of spatial cognition: *B. terrestris* females perform "learning flights" when leaving both the nest and flowers in order to learn their spatial location, whereas males only perform these flights when leaving flowers (Robert *et al.* 2016), indicating that the sexes may use location cues differently. Another promising research line is suggested by our finding of differences in learning performance between the two species we tested (a trend in one model, and a significant effect when the final test trial was also included), with *B. vancouverensis nearcticus* learning with a steeper incline than *B. vosnesenskii*: *B. vosnesenskii* learned better initially (performing above 50% after their two initial pretraining trials), but then did not improve as much after this point. Whether these are true species differences in learning or instead due to a factor such as hunger would need to be confirmed under more controlled conditions. Aside from body size,

our two species also differ in that *B. vosnesenskii* has larger colonies than *B. vancouverensis nearcticus* (Strange JP, unpublished) and larger foraging ranges (Rao and Strange 2012; Geib et al. 2015). Whether these or other traits might explain differences in learning would need to be tested over a larger number of species.

We believe that the protocol we used here opens the door to a wide range of species- and population-level questions about how environments might shape cognition across both developmental and evolutionary timescales. Having the ability to catch wild queens of multiple species, rear colonies in a lab environment, place colonies in different environments (i.e., translocation experiments), and test individuals in the wild makes bumblebees a useful system to disentangle developmental versus functional explanations of behavior, as well as whether particular cognitive traits are adaptive. While interest in sex differences in cognition has primarily been focused on mammals and birds, invertebrates may offer more tractable systems to investigate such questions (Perry et al. 2017). More broadly, the question of whether comparative cognition experiments conducted on lab- versus wild-living animals return similar answers transcends taxonomic divides (Morand-Ferron et al. 2016; Pritchard et al. 2016). If there is reason to expect that aspects of the developmental environment, for example, shape the degree to which the sexes differ in cognitive performance, then working on both lab and field populations will be essential to understand cognitive differences in context. In this system (*Bombus*), a number of studies, conducted in different ways on different species and on both lab and wild animals, point towards no robust sex differences in associative learning performance. This concordance may be one data point useful in assembling the bigger picture of how comparative cognition results translate from lab to field.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

FUNDING

Handling editor: Emilie Snell-Rood

REFERENCES

Bailes EJ, Pattrick JG, Glover BJ. 2018. An analysis of the energetic reward offered by field bean (*Vicia faba*) flowers: nectar, pollen, and operative force. Ecol Evol. 8:3161–3171.

Bertsch A. 1984. Foraging in male bumblebees (*Bombus lucorum* L.): maximizing energy or minimizing water load? Oecologia. 62:325–336.

Bitterman ME, Menzel R, Fietz A, Schäfer S. 1983. Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). J Comp Psychol. 97:107–119.

Branch CL, Sonnenberg BR, Pitera AM, Benedict LM, Kozlovsky DY, Bridge ES, Pravosudov VV. 2020. Testing the greater male variability phenomenon: male mountain chickadees exhibit larger variation in reversal learning performance compared with females. Proc R Soc B Biol Sci. 287:20200895.

Brown M, Brown MJF. 2020. Nectar preferences in male bumblebees. Insectes Soc. 67:221–228.

Caplan PJ, MacPherson GM, Tobin P. 1985. Do sex-related differences in spatial abilities exist? A multilevel critique with new data. Am Psychol. 40:786–799.

Chittka L, Thomson JD. 2001. Cognitive ecology of pollination. Cambridge (MA): Cambridge University Press.

Church D, Plowright C, Loyer D. 2001. Discriminations of color and pattern on artificial flowers by male and female bumble bees, *Bombus impatiens* (Hymenoptera: Apidae). Great Lakes Entomol. 34:85–95.

Clarke D, Whitney H, Sutton G, Robert D. 2013. Detection and learning of floral electric fields by bumblebees. Science. 340:66–69.

Cnaani J, Thomson JD, Papaj DR. 2006. Flower choice and learning in foraging bumblebees: effects of variation in nectar volume and concentration. Ethology. 112:278–285.

Colegrave N, Ruxton GD. 2017. Statistical model specification and power: recommendations on the use of test-qualified pooling in analysis of experimental data. Proc R Soc B Biol Sci. 284:20161850.

Dalla C, Shors TJ. 2009. Sex differences in learning processes of classical and operant conditioning. Physiol Behav. 97:229–238.

Dickel L, Boal JG, Budelmann BU. 2000. The effect of early experience on learning and memory in cuttlefish. Dev Psychobiol. 36:101–110.

Dukas R. 1999. Costs of memory: ideas and predictions. J Theor Biol. 197:41–50.

Dyer AG, Whitney HM, Arnold SEJ, Glover BJ, Chittka L. 2006. Bees associate warmth with floral colour. Nature. 442:525–525.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software structure: a simulation study. Mol Ecol. 14:2611–2620.

Evans LJ, Raine NE. 2014. Foraging errors play a role in resource exploration by bumble bees (*Bombus terrestris*). J Comp Physiol. 200:475–484.

Evans LJ, Smith KE, Raine NE. 2017. Fast learning in free-foraging bumble bees is negatively correlated with lifetime resource collection. Sci Rep. 7:496.

Foster JJ, Sharkey CR, Gaworska AVA, Roberts NW, Whitney HM, Partridge JC. 2014. Bumblebees learn polarization patterns. Curr Biol. 24:1415–1420.

Freas CA, LaDage LD, Roth TC, Pravosudov VV. 2012. Elevation-related differences in memory and the hippocampus in mountain chickadees, *Poecile gambeli*. Anim Behav. 84:121–127.

Gaulin S, FitzGerald RW. 1986. Sex differences in spatial ability: an evolutionary hypothesis and test. Am Nat. 127:74–88.

Gaulin SJ, FitzGerald RW, Wartell MS. 1990. Sex differences in spatial ability and activity in two vole species (*Microtus ochrogaster* and *M. pennsylvanicus*). J Comp Psychol. 104:88–93.

Geib JC, Strange JP, Galen C. 2015. Bumble bee nest abundance, foraging distance, and host-plant reproduction: implications for management and conservation. Ecol Appl. 25:768–778.

Ghisbain G, Lozier JD, Rahman SR, Ezray BD, Tian L, Ulmer JM, Heraghty SD, Strange JP, Rasmont P, Hines HM. 2020. Substantial genetic divergence and lack of recent gene flow support cryptic speciation in a colour polymorphic bumble bee (*Bombus bifarius*) species complex. Syst Entomol. 45:635–652.

Goulson D. 2003. Bumblebees: their behaviour and ecology. Oxford: Oxford University Press.

Green P, MacLeod CJ. 2016. SIMR: an R package for power analysis of generalized linear mixed models by simulation. Methods Ecol Evol. 7:493–498.

Grön G, Wunderlich AP, Spitzer M, Tomczak R, Riepe MW. 2000. Brain activation during human navigation: gender-different neural networks as substrate of performance. Nat Neurosci. 3:404–408.

Harris AP, D'eath RB, Healy SD. 2008. Sex differences, or not, in spatial cognition in albino rats: acute stress is the key. Anim Behav. 76:1579–1589.

Heinrich B. 2004. Bumblebee economics. Cambridge, MA: Harvard University Press.

Holehouse KA, Hammond RL, Bourke AFG. 2003. Non-lethal sampling of DNA from bumble bees for conservation genetics. Insectes Soc. 50:277–285.

Ings TC, Raine NE, Chittka L. 2009. A population comparison of the strength and persistence of innate colour preference and learning speed in the bumblebee *Bombus terrestris*. Behav Ecol Sociobiol. 63:1207–1218.

Jacobs LF, Gaulin SJ, Sherry DF, Hoffman GE. 1990. Evolution of spatial cognition: sex-specific patterns of spatial behavior predict hippocampal size. Proc Natl Acad Sci USA. 87:6349–6352.

Jaumann S, Scudelari R, Naug D. 2013. Energetic cost of learning and memory can cause cognitive impairment in honeybees. Biol Lett. 9:20130149.

Jones BM, Leonard AS, Papaj DR, Gronenberg W. 2013. Plasticity of the worker bumblebee brain in relation to age and rearing environment. Brain Behav Evol. 82:250–261.

Jones CM, Braithwaite VA, Healy SD. 2003. The evolution of sex differences in spatial ability. Behav Neurosci. 117:403–411.

Jones CM, Healy SD. 2006. Differences in cue use and spatial memory in men and women. Proc R Soc B Biol Sci. 273:2241–2247.

Kozlovsky DY, Weissgerber EA, Pravosudov VV. 2017. What makes specialized food-caching mountain chickadees successful city slickers? Proc Biol Sci. 284:20162613.

Laloi D, Sandoz J, Picard-Nizou AL, Marchesi A, Pouvreau A, Tasei JN, Poppy G, Pham-delègue MH. 1999. Olfactory conditioning of the proboscis extension in bumble bees. Entomol. Exp Appl. 90:123–129.

Lichtenstein L, Sommerlandt FM, Spaethe J. 2015. Dumb and lazy? A comparison of color learning and memory retrieval in drones and workers of the buff-tailed bumblebee, *Bombus terrestris*, by means of PER conditioning. PLoS One. 10:e0134248.

Lonsdorf EV, Eberly LE, Pusey AE. 2004. Sex differences in learning in chimpanzees. Nature. 428:715–716.

Mery F, Kawecki TJ. 2003. A fitness cost of learning ability in *Drosophila melanogaster*. Proc R Soc B Biol Sci. 270:2465–2469.

Mery F, Kawecki TJ. 2004. An operating cost of learning in *Drosophila melanogaster*. Anim Behav. 68:589–598.

Morand-Ferron J, Cole EF, Quinn JL. 2016. Studying the evolutionary ecology of cognition in the wild: a review of practical and conceptual challenges. Biol Rev. 91:367–389.

Muth F, Cooper TR, Bonilla RF, Leonard AS. 2017. A novel protocol for studying bee cognition in the wild. Methods Ecol Evol. 9:78–87.

Muth F, Papaj DR, Leonard AS. 2015. Colour learning when foraging for nectar and pollen: bees learn two colours at once. Biol Lett. 11:20150628.

Muth F, Papaj DR, Leonard AS. 2016. Bees remember flowers for more than one reason: pollen mediates associative learning. Anim Behav. 111:93–100.

Muth F, Tripodi AD, Bonilla R, Strange JP, Leonard AS. 2021. Data From: no sex differences in learning in wild bees. Behav Ecol. doi:10.5061/dryad.8pk0p2nmm.

Ogilvie JE, Thomson JD. 2015. Male bumble bees are important pollinators of a late-blooming plant. Arthropod-Plant Interact. 9:205–213.

Ostevik KL, Manson JS, Thomson JD. 2010. Pollination potential of male bumble bees (*Bombus impatiens*): movement patterns and pollen-transfer efficiency. J Pollinat Ecol. 2:21–26.

Pamminger T, Becker R, Himmelreich S, Schneider CW, Bergtold M. 2019. The nectar report: quantitative review of nectar sugar concentrations offered by bee visited flowers in agricultural and non-agricultural landscapes. PeerJ. 7:e6329.

Pankiw T, Page RE Jr. 1999. The effect of genotype, age, sex, and caste on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L.). J Comp Physiol A. 185:207–213.

Perry CJ, Barron AB, Cheng K. 2013. Invertebrate learning and cognition: relating phenomena to neural substrate. Wiley Interdiscip Rev Cogn Sci. 4:561–582.

Perry CJ, Barron AB, Chittka L. 2017. The frontiers of insect cognition. Curr Opin Behav Sci. 16:111–118.

Pinheiro J, Bates D, DebRoy S, Sarkar D. 2016. nlme: linear and nonlinear mixed effects models_. R package version 3.1–128. Available from: http://CRAN.R-project.org/package=nlme.

van Praag H, Kempermann G, Gage FH. 2000. Neural consequences of environmental enrichment. Nat Rev Neurosci. 1:191–198.

Pravosudov VV, Clayton NS. 2002. A test of the adaptive specialization hypothesis: population differences in caching, memory, and the hippocampus in black-capped chickadees (*Poecile atricapilla*). Behav Neurosci. 116:515–522.

Pritchard DJ, Hurly TA, Tello-Ramos MC, Healy SD. 2016. Why study cognition in the wild (and how to test it)? J Exp Anal Behav. 105:41–55.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. Genetics. 155:945–959.

Raine NE, Chittka L. 2008. The correlation of learning speed and natural foraging success in bumble-bees. Proc R Soc B Biol Sci. 275:803–808.

Ranta E, Lundberg H. 1981. Resource utilization by bumblebee queens, workers and males in a subarctic area. Ecography. 4:145–154.

Rao S, Strange JP. 2012. Bumble bee (Hymenoptera: Apidae) foraging distance and colony density associated with a late-season mass flowering crop. Environ Entomol. 41:905–915.

Robert T, Frasnelli E, Collett TS, Hempel de Ibarra N. 2016. Male bumblebees perform learning flights on leaving a flower but not when leaving their nest. J Exp Biol. 220:151126.

Rosenthal MF, Gertler M, Hamilton AD, Prasad S, Andrade MCB. 2017. Taxonomic bias in animal behaviour publications. Anim Behav. 127:83–89.

Roswell M, Dushoff J, Winfree R. 2019. Male and female bees show large differences in floral preference. PLoS One. 14:e0214909.

Scheiner R, Erber J, Page RE Jr. 1999. Tactile learning and the individual evaluation of the reward in honey bees (*Apis mellifera* L.). J Comp Physiol A. 185:1–10.

Scheiner R, Page RE, Erber J. 2001. Responsiveness to sucrose affects tactile and olfactory learning in preforaging honey bees of two genetic strains. Behav Brain Res. 120:67–73.

Scheiner R, Page RE, Erber J. 2004. Sucrose responsiveness and behavioral plasticity in honey bees (*Apis mellifera*). Apidologie. 35:133–142.

Sherry D, Jacobs L, Gaulin S. 1992. Spatial memory and adaptive specialization of the hippocampus. Trends Neurosci. 15:298–303.

Sherry DF, Strang CG. 2015. Contrasting styles in cognition and behaviour in bumblebees and honeybees. Behav Processes. 117:59–69.

Shettleworth S. 2010. Cognition, evolution, and behavior. 2nd ed. Oxford: Oxford University Press.

Smith GP, Bronstein JL, Papaj DR. 2019. Sex differences in pollinator behavior: patterns across species and consequences for the mutualism. J Anim Ecol. 88:971–985.

Strange JP, Knoblett J, Griswold T. 2009. DNA amplification from pin-mounted bumble bees (*Bombus*) in a museum collection: effects of fragment size and specimen age on successful PCR. Apidologie. 40:134–139.

Takeda K. 1961. Classical conditioned response in the honey bee. J Insect Physiol. 6:168–179.

Tello-Ramos MC, Hurly TA, Healy SD. 2014. Female hummingbirds do not relocate rewards using colour cues. Anim Behav. 93:129–133.

Therneau TM, Grambsch PM. 2000. Modeling survival data: extending the cox model. New York: Springer.

Vicario DS, Naqvi NH, Raksin JN. 2001. Sex differences in discrimination of vocal communication signals in a songbird. Anim Behav. 61:805–817.

Willmer P. 2011. Pollination and floral ecology. Princeton, NJ: Princeton University Press.

Wolf S, Chittka L. 2016. Male bumblebees, *Bombus terrestris*, perform equally well as workers in a serial colour-learning task. Anim Behav. 111:147–155.

Wolf S, Moritz RFA. 2014. The pollination potential of free-foraging bumblebee (*Bombus spp.*) males (Hymenoptera: Apidae). Apidologie. 45:440–450.