**Title:** Reproductive environment affects learning performance in bumble bees

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## Author Contributions

EL and LJE conceived the project. LJE, EL and NER designed the research experiments. LJE carried-out the experiments. LJE and EL performed the statistical analyses. LJE, EL and NER wrote the manuscript.

## Abstract

Despite a presumed fitness advantage for individuals with well-developed cognitive abilities, learning performance is usually found to be highly variable within a population. Although little is currently known about the mechanisms responsible for maintaining such variation there is correlative evidence to suggest that learning performance may be linked to reproductive physiology in the social insects. Bumble bee colonies naturally undergo an initial co-operative phase, when only the queen reproduces, and a subsequent competition phase when all colony members compete to produce male offspring. We experimentally induced these distinct phases by manipulating the presence/absence of the queen, and assessed changes in sucrose responsiveness and learning performance. We found that nest-based workers upregulated their reproductive potential in queenless colonies, and correspondingly, these bees were more responsive to sucrose than their queenright counterparts, performing better in an olfactory learning task as a result. These findings suggest that differences in ovarian development are responsible for at least some of the remarkable variation in learning performance that can be observed among very closely-related members of social insect colonies.

**Keywords: bumblebee *Bombus terrestris*, foraging behaviour, odour learning, reproductive environment, reproductive groundplan hypothesis, sucrose responsiveness**

### Significance Statement

Cognitive abilities are often assumed to be inherently adaptive, so the question of why individuals vary in their learning ability has received relatively little attention. Here, we focus on reproductive status as a proximate cause of variation in learning ability in the social insects. We show that a significant proportion of the surprising variation that exists between genetically similar colony members can be explained by worker ovarian development; reproductively active workers are more sensitive to food rewards and thus learn more quickly. Learning ability may be one of a suite of correlated traits that are linked to reproductive physiology in social insects, and therefore play an important role in the evolution of division of labour.

## Introduction

Animal cognitive abilities have generally been assumed to be associated with enhanced fitness, yet we see striking variation in cognitive traits within species, even in societies of closely related individuals. For example, within a single eusocial insect colony there are often individuals that consistently perform exceedingly well in particular learning tasks while others perform poorly (e.g. Scheiner et al. 1999, 2003; Raine et al. 2006a; Raine and Chittka 2012; Perez et al. 2013; Evans and Raine 2014a; Jandt et al. 2014). Although such variation is well-documented, both its ultimate function and proximate causes are still poorly understood (Jeanson and Weidenmüller 2014). Here we focus on a hypothesized proximate cause of variation in learning ability in the social insects using the bumble bee (*Bombus terrestris*) as our experimental model organism. We investigate whether individual learning abilities could be one of a suite of correlated traits that are closely linked to reproductive physiology.

Like many pollinators, foraging bumble bees are faced with a complex environment in which floral rewards can change quickly over short time periods (Raine et al. 2006b). The ability to learn about floral characteristics that predict high quality food rewards should enhance foraging efficiency, and correspondingly, learning performance should correlate with foraging success (Raine and Chittka 2008). Nonetheless, individual foragers vary markedly in their performance in sucrose-rewarded learning tasks (Raine et al. 2006a; Muller and Chittka 2012; Raine and Chittka 2012; Evans and Raine 2014a; Smith and Raine 2014). Studies from other social insects provide reason to hypothesize that this variability could derive from variation in reproductive physiology among nestmates, because reproductive physiology correlates with a suite of behavioural traits in social bees.

Perhaps of greatest interest here is a large body of evidence linking both learning and reproductive status to pollen/nectar foraging preferences within the worker caste of the bumblebee’s close relative, the honey bee *Apis mellifera*. For example, high ovariole numbers in young honey bee workers are associated with a subsequent foraging preference for pollen (Amdam et al. 2006; Nelson et al. 2007; Page and Amdam 2007; Linksvayer et al. 2009; Wang et al. 2010). Likewise, high vitellogenin (a yolk-precursor protein) and Juvenile Hormone (JH) titres in workers are associated with pollen-hoarding (Amdam et al. 2004; Schultz et al. 2004), and knockdown of the gene responsible for vitellogenin production results in a subsequent preference for nectar collection (Nelson et al. 2007). We hypothesize that variation in worker reproductive physiology might have impacts upon learning because honey bee preferences for pollen or nectar collection are also associated with their responsiveness to sucrose (Pankiw and Page 1999; Scheiner et al. 1999; Scheiner 2004; Latshaw and Smith 2005; Drezner-Levy et al. 2009), and correspondingly with their performance in sucrose-rewarded learning tasks (Scheiner et al. 2001a, 2001b). Nectar-specialist honey bee workers have higher sucrose responsiveness (SR), and consequently collect more concentrated nectar (because they are less likely to detect or respond to more dilute concentrations).

Although reproductive traits are linked to nectar/pollen preferences, and nectar/pollen preferences are in turn linked to sucrose-rewarded learning, the evidence for a direct link between reproductive traits and sucrose-rewarded learning remains correlative and indirect. In this study, we exploit a natural feature of the bumble bee life cycle to manipulate worker reproductive development and directly assay the consequences for sucrose responsiveness and odour learning performance. Towards the end of the annual bumble bee colony cycle, previously co-operative workers enter an all-out competition to produce male offspring (Van Honk and Hogeweg 1981; Bourke and Ratnieks 2001), which continues during colony decline and eventual death. In this study we artificially induced this reproductive development by splitting colonies to create matched queenright and queenless groups. We verified that our manipulation led to reproductive activation in queenless workers, and assayed differences between the two sub-colony types in terms of sucrose responsiveness and learning performance using the Proboscis Extension Reflex (PER) conditioning paradigm.

## Methods

*Colony setup*

Five young *B. terrestris* colonies, containing an average of 22 workers, were obtained from Biobest (Westerlo, Belgium). On arrival, each colony was transferred into a colony box (L x W x H = 280 x 155 x 110 mm). Colony boxes were connected, via rubber tubing, to individual foraging arenas (235 x 140 x 110 mm) containing a dish of defrosted, honey bee-collected pollen (Koppert Ltd UK; *ca*. 3g replaced every second day) and *ad libitum* 40% (v/v) sucrose solution in a feeder. All workers that emerged prior to splitting colonies (described below) were individually marked with numbered tags (Opalith tags; Christian Graze KG, Germany) on the day of emergence, so that their subsequent age when tested was known. Colonies were split when they contained 75-100 workers and were still in their ‘pre-competition phase’, when all egg laying is exclusive to the queen. Each colony was split to create one queenright (Q+) group, and one queenless (Q-) group: both the bees and brood were divided evenly and half was transferred to a new colony box. In small groups of queenless workers ovary development takes just five days (Rӧseler 1974), after which time workers of seven (or more) days old can be ready to lay eggs (Amsalem et al. 2014). Seven to ten days after splitting the colonies, we assessed the sucrose responsiveness and olfactory learning performance of workers from both queenless and queenright sides.

### *Behavioural observations of workers*

We expected any effect of the queenless state might vary with worker task specialization (nest workers have more developed ovaries in some bee species: West-Eberhard 1996) and/or worker dominance (dominant workers may have more developed ovaries: Amsalem and Hefetz 2010; Amsalem et al. 2013). To control for these variables in our statistical analysis we monitored individual worker behaviour both before and after colony splits. Activity in the foraging box was observed for 1-1.5 hours per day (2-3 x 30-minute intervals spread over the course of the day), five days per week. Foragers were defined as individuals that were observed foraging at one of the feeders on at least five occasions prior to splitting (as colonies were initially small they needed less pollen and sucrose solution) or eight times (range = 8-30) after splitting. Nest workers were defined as individuals observed on feeders on no more than four (range = 0 – 4) occasions. These simple definitions of “nest worker” and “forager” were used to increase the power of our analysis by accounting for task specialization (role) - a known correlate of variation in reproductive development among workers.

To identify individuals displaying dominant behaviour we performed focal observations of each queenless nest (no dominant behaviours were observed in queenright colonies) under red light twice daily for 15 minutes. Workers were considered to be dominant if they displayed any of the following behaviours: overt aggression, threatening behaviour, oviposition and/or egg eating (Van Doorn and Heringa 1986; Duchateau and Velthuis 1988; Bloch et al. 2000; Geva et al. 2005). When possible, the behaviours of aggressed individuals (workers receiving the aggressive behaviour) were also recorded, because competing workers are reported to focus their aggression on those workers signalling fertility (Van der Blom and Verkade 1991; Visscher and Dukas 1995; Amsalem et al. 2009). When selecting foragers and nest workers from the queenless treatment groups for learning and sucrose responsiveness assays, we selected bees that performed or received aggressive behaviours. In other words, we chose to test those individuals most likely to have responded to the queenless state.

### *Sucrose responsiveness*

We assessed the sucrose responsiveness (SR) of workers 7-10 days after the colonies were split using a proboscis extension reflex (PER) assay. Between 2-3 foragers and 2-3 nest bees were caught from each treatment group per day and chilled on ice until they became quiescent (*ca*. 12-15 min). They were then mounted in modified plastic syringe tubes (2ml volume, 8.2mm diameter cut down to 3cm long), with a notch cut out of the top of the tube to allow the bees to freely extend their proboscis. Subjects’ heads were held in place between two entomological pins (size 0) that were glued at one end and subsequently fastened at the other end. This pin yoke was secured with tape. A small ball of moistened cotton wool was inserted into the mounting tube behind the bee to prevent desiccation (Riveros and Gronenberg 2009; Smith and Raine 2014). The harnesses were individually numbered to ensure that testing was conducted blind with respect to treatment group.

Each subject’s sucrose responsiveness was assessed 2-3 hours after being harnessed, using an ascending concentration series of 0 (water; negative control), 1, 3, 5, 8, 10, 15, 25, 35 and 50% (v/v) sucrose solution. Stimulus presentation involved touching the subject’s antennae with one of the sucrose solutions, presented on a toothpick. Stimulation with detectable levels of sucrose evokes probsocis extension (Bitterman et al. 1983), with water included as a negative control (no subjects responded to the water solution). The time between stimulus presentations was 4-5 minutes, depending on the number of bees (n = 21-30) being tested. An individual’s SR score was the number of concentrations that evoked a full extension of the proboscis (0-10), with a high SR score indicating greater responsiveness to sucrose.

### *Olfactory learning performance*

After assaying sucrose responsiveness, bees were left overnight to ensure that they were sufficiently motivated for assessment of olfactory learning performance by PER the following morning. This period (~18h) is necessary to prevent bumble bees from ceasing to respond during PER testing. We used an absolute PER conditioning paradigm (Riveros and Gronenberg 2009; Smith and Raine 2014). Prior to testing, each bee’s antennae were stimulated with a 50% (v/v) sucrose solution, and if the bee extended its proboscis it was given a small droplet of sucrose. If a bee failed to respond after four attempts it was excluded from the test (n = 18 of 172 bees, including 10 bees that died overnight).

Bees were assessed on their ability to learn to associate a floral odour (lavender essential oil, Calmer solutions) with a 50% sucrose solution reward. Each harnessed bee was placed individually into an odour extraction hood during a training event. The bee was positioned in a marked location, 3cm away from an odour tube that delivered an air stream (containing the odour stimulus at the appropriate times). The odour tube contained a strip of filter paper soaked with 2µl of the essential oil. This concentration is similar to those used in other studies of olfactory learning in bumble bees (e.g. Riveros & Gronenberg 2009; Smith & Raine 2014) and is most likely high enough to overwhelm any individual differences in odour sensitivity between bees, which could otherwise affect learning (Spaethe et al. 2007). Filter paper strips were replaced after 20-30 uses for consistency of odour strength. A Programmable Logic Controller computer controlled the volume of the air, flow rate, and duration of the odour presentation. Bees were exposed to 5 seconds of clean air, followed by 10s of odour-containing air for each trial. Using a Gilmont syringe, each bee was provided with 0.8µl of sucrose solution after approximately 6s of exposure to the odour containing air.

A positive response was recorded in trials when a bee extended its proboscis during exposure to the odour stimulus prior to antennal stimulation with sucrose solution. On such occasions, the sucrose reward was provided immediately. Each bee was given 15 trials, with a 12-minute inter-trial interval. After each trial we recorded whether the bee responded before antennal sucrose stimulation (demonstrating an association between odour and reward), after stimulation (demonstrating a response to antennal stimulation with sucrose), or no response. Learning performance was quantified as the sum of trials in which an individual bee extended its proboscis prior to antennal stimulation. Bees that did not respond to the odour, or the subsequent antennal sucrose stimulation, for three consecutive trials were excluded from the learning experiment (n = 9 bees), as were unresponsive bees during the SR test (n = 10 bees).

Bees were immediately frozen at -20°C after testing. Thorax width measurements were taken for each tested individual and recorded as a measure of worker body size, which has the potential to affect learning performance (Worden et al. 2005; Sommerlandt et al. 2014; but see: Raine et al. 2006a; Raine and Chittka 2008; Evans and Raine 2014b; Smith and Raine 2014). Workers were then dissected to assess the presence of measurable oocytes in each of the eight (four per ovary) ovarioles (egg laying filaments). When no clearly defined oocytes were present in the ovarioles, it was noted that the ovarioles were present but undeveloped. Note that “ovarian development” was measured as a general indicator of reproductive activation, but it is just one component of the large network of physiological and endocrine reproductive traits that are likely to change following queen removal. Our analyses focus upon the effects of the queenless/queenright state, rather than specifically on the effects of ovarian development.

### *Statistical analyses*

All analyses were conducted in R v 3.0.2 (R Core Development Team 2014). We used a series of generalised linear mixed models, GLMM’s, (using the glmer function in package lme4: Bates et al. 2014, assuming a binomial error distribution) to examine whether either SR (Table 1a) and/or olfactory learning performance (Table 1b) were predicted by treatment (queenright/queenless). In both cases, we used an information theoretic approach to compare a series of candidate models containing all combinations of treatment (queenright/queenless), worker size, worker age and role (task specialization: nest worker/forager) as predictors (R package MuMIn: Bartoń 2016). We also included an interaction term (role:treatment) because initial data exploration indicated that the effects of treatment varied between the forager and nest worker groups. Each candidate model included “colony” as a random factor, including our null model (“basic model”), which otherwise contained only the intercept as a predictor. Parameter estimates are based on model averaging of the 95% confidence set.

**Results**

Overall, we found that both SR and olfactory learning performance varied significantly between the queenright and queenless groups, and that this effect was limited to the nest worker bees (Fig. 1; Fig. S1). For SR, model-averaged parameter estimates based on the 99% confidence set of models (Table 1a) indicated that treatment (model-averaged estimate: 1.46, 95% c.i. = 0.69 to 2.23), role (0.69; 0.34 to 1.04), and an interaction between treatment and role (-1.47; -1.99 to -0.95) significantly predicted SR. Worker age and worker size did not receive strong support (estimates and 95% c.i. = 0.02; -0.004 to 0.05 and -0.05; -0.42 to 0.31, respectively). Likewise, treatment (estimate: 2.38, 1.66 to 3.10), role (estimate: 0.51, 0.22 to 0.80) and their interaction (estimate: -1.93, -2.44 to -1.42) emerged as significant predictors of olfactory learning performance, and in this case both worker size (estimate:1.55, 1.18 to 1.92) and worker age (estimate: -0.03, -0.06 to -0.01] also received support (Table 1b). In summary, nest workers in queenless colonies were more responsive to sucrose and thus performed better in the olfactory learning task than their counterparts in the queenright colonies, but no difference was found among foragers.

Post-experimental dissections revealed that this marked difference between young nest workers and older foragers most likely reflects the differential effects of queen removal on the two worker types. In the presence of the queen, nest workers typically had undeveloped ovaries, but queen removal led to ovarian development. In contrast, forager ovaries were well developed in both queenless and queenright colonies (Fig. 2). However, replacing “treatment” with “ovarian development” in our full models did not provide a better fit to the SR data (AICc[treatment model]=1059.3, AICc[ovarian development model]=1111.3); nor to our learning performance data (AICc[treatment model]=948.0, AICc[ovarian development model]=961.2). This most likely reflects the fact that variation in ovarian development, at least when categorised using our methods, is unlikely to capture all the reproductive changes that occur in workers when the queen is removed from a colony.

## Discussion

In this study, we found that queenless bumble bee workers were more responsive to sucrose than queenright (control) bees. Correspondingly, they performed better in an olfactory learning task. Interestingly, this effect is limited to those bees that specialized upon nest-based tasks, rather than those bees leaving the colony to forage. Our ovarian dissections suggest an explanation for this: in a queenright colony, nest workers show less ovarian development than foragers, but this increases to forager-like levels following removal of the queen. This indicates that effects of queen removal may be greater for nest-based workers on the network of endocrine and physiological traits that underlie reproduction. In other words, for those individuals that switched to a reproductive phenotype upon removal of the queen, we saw a corresponding change in SR and learning ability.

Ultimately why should workers benefit from increased responsiveness to sucrose in the absence of a queen? Reproductive workers could be under selection to participate in foraging, to provide for their brood, but there is no obvious direct link between increased responsiveness to sucrose and foraging performance. Greater responsiveness is not likely to lead to collection of higher-quality nectar; in contrast, bees that are very responsive to sucrose should detect and accept less concentrated (and thus poorer quality) floral nectar rewards (Pankiw et al. 2001). Alternatively, it may be that the increase in sucrose responsiveness that we observed is a byproduct of selection for responsiveness to other stimuli in reproductive workers, because there is evidence to suggest that heightened sucrose responsiveness reflects a general increase in responsiveness to many stimuli (e.g. light; Erber et al. 2006; Tsuruda and Page 2009). For example, honey bee larvae produce a “brood pheromone” to stimulate pollen foraging (Pankiw et al. 1998), and it may be that reproductive bumble bee workers respond to a similar substance.

A third alternative is that increased sucrose responsiveness in reproductive workers has no adaptive function. The Reproductive Ground Plan hypothesis postulates that reproductive behaviour, foraging preferences and neurosensory responses are linked by a pleiotropic gene network that simply occurred at the same life-history stage in a solitary ancestor, and have been co-selected to produce division of foraging labour in extant social insects (Amdam et al. 2004, 2006; Page and Amdam 2007). Under this framework, the increase in sucrose responsiveness and corresponding improvement in learning ability that we observed could be simply a byproduct of reproductive up regulation.

Our findings reveal novel parallels with other Hymenoptera in terms of social biology. We found that only nest-based workers responded to the queenless state with reproductive activation. In honey bees (*A. mellifera*), it is the younger, nest-based workers are also more likely (than older foragers) to undergo oogenesis in the absence of the queen (Lin et al. 1999), just as we observed for bumble bees. Likewise, in the tropical wasps *Metapolybia aztecoides* and *Synoeca surinama* (both of which exhibit cyclical oligyny) a dead or missing queen is typically replaced by recently emerged females that were already present in the nest (i.e. they have not yet begun to forage). In the absence of a queen, these nest dwelling individuals will mate and develop mature ovaries (West-Eberhard 1996). However, there are also clear differences between the reproductive systems of these species and *B. terrestris*. In queenright *M. aztecoides*, *S. surinama* and *A. mellifera*, foraging individuals have regressed ovaries (West-Eberhard 1996; Pinto et al. 2000), while we found that queenright *B. terrestris* foragers exhibited high levels of ovarian development. This may reflect the fact that ovarian development in bumble bee colonies is actively supressed through the presence of the queen, through queen mandibular pheromone (QMP) and brood pheromone (Trynor et al. 2014). Since foragers spend much less of their time in the nest, they are likely to receive lower exposure to both pheromones, allowing for initiation of Juvenile Hormone (JH) secretion by the *corpora allata* (Rӧseler 1977). However, it is not clear why the same effect is not observed in other eusocial insects, since the mechanisms by which worker reproduction is suppressed are similar. One possible explanation stems from the fact that worker bumble bees are almost unique among social insects for their lack of age polyethism (Cameron 1989; Cameron and Robinson 1990). Throughout the social insects it is typically the youngest workers that develop ovaries in the absence of the queen, but only in bumble bees is it likely that some of these young workers will regularly leave the nest to forage. Alternatively, it is possible that in our experimental setup (foragers collecting food in a flight arena) foragers still had sufficient energy for ovarian development, which would perhaps not occur under natural conditions when foraging from flowers in the field (Foster et al. 2004; Williams et al. 2008; Jandt and Dornhaus 2011).

In summary, our results provide evidence that at least some of the appreciable intracolony variation seen in learning performance among bumble bees (Raine et al. 2006a; Muller and Chittka 2012; Evans and Raine 2014; Smith and Raine 2014; Sommerlandt et al. 2014) is likely to be attributable to individual variation in reproductive development. In queenright colonies, we see variation in ovarian development between nest workers and foragers, and correlated variation in sucrose responsiveness. Our results also lead to the testable prediction that in bumble bee colonies that have reached the “competition phase”, whereby the queen loses reproductive dominance, we may see similarly high levels of reproductive development between nest workers and foragers, and thus less variation in responsiveness to sucrose and learning behaviour than in early-stage colonies. On a proximate level, our study provides strong evidence for a link between reproductive physiology and cognitive traits in bees, inviting further investigation into the potential fitness benefits of this relationship.

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**Table/Figure legends**

**Table 1**

Candidate models to predict the **a)** sucrose responsiveness (n= 157 bees) and **b)** olfactory learning performance (n = 144 bees) for *B. terrestris* workers. Each model contained the intercept and “colony” as a random factor, together with fixed factors indicated by the colony names. The 95% confidence set, shown in bold, is the set of models in which we can at least be 95% sure that the best model lies.

**Fig. 1a** Sucrose responsiveness (number of sucrose concentrations that evoked full proboscis extension; a high SR score indicates high responsiveness to sucrose) in queenright (Q+: *darker coloured bars*) compared to queenless treatment groups (Q-: *lighter coloured bars*) and in foragers (F: *red bars*) compared to nest workers (N: *purple bars*). **1b** Learning performance in queenright (*darker coloured bars*) compared to queenless treatment groups (*lighter coloured bars*), and in foragers (*red bars*) compared to nest workers (*purple bars*). Column heights indicate the mean (± SE) values for SR (a) and learning performance (b) of bees from each group. Column heights indicate the mean (± SE) SRT A) and learning performance B) of bees from each group. Different letters indicate significant differences among groups (Tukey’s HSD, p ≤0.05).

**Fig. 2** Ovarian development in queenright (Q+: *darker coloured bars*) compared to queenless treatment groups (Q-: *lighter coloured bars*) and in foragers (F: *red bars*) compared to nest workers (N: *purple bars*).

**Figures**

**Figure 1**

FQ+ FQ- NQ+ NQ-

a

a

a

b

a

a

a

b

 FQ+ FQ- NQ+ NQ-

**Figure 2**

 FQ+ FQ- NQ+ NQ-