Analysis of Spatial Learning in Honeybee Foragers Using the Food Search Box Assay

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Abstract

Given the under-representation of genetic analyses of spatial learning in honeybee literature, I designed an experiment to investigate gene expression profiles associated with spatial learning. The experimental design involved using the Food Search Box (FSB) assay to assess learning in Single Drone-Inseminated (SDI) foragers, then collecting the bees for RNA-seq analysis to generate and compare full-transcriptome gene expression profiles for five different stages of learning. From the first to the third learning trial, the SDI foragers did not decrease the number of mistakes they made in a significant way: they did not switch from chance to non-chance searching behaviour. In addition, during the memory recall stage, the bees made significantly more errors than chance, suggesting they did not remember their focal flower. Landmarks cues, inter-trial interval, motivation level for sucrose and genetics may each play a role in the ability of SDI foragers to learn vs. foragers from naturally mated queens.

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Chapter 1: Food Search Box Assay Using Honeybee Foragers

Introduction

Honeybees are eusocial insects. This characteristic means that these insects engage in highly organized social behaviours which rely on division of labour. These behaviours include brood rearing, distinct reproductive and non-reproductive castes, nest defense, food acquisition, cell cleaning, comb building, (Johnson 2003; Johnson 2010; Johnson and Frost 2012), etc. In order to adapt to changing conditions and colony needs, social insects must use chemical, auditory, visual and other cues to communicate with one another.

Honeybees are capable of communicating the location and quality of food resources using the waggle dance. This dance occurs when a foraging bee returns to the hive: on the dancefloor, the bee will move such that the angle of its dance indicates the food's direction relative to the sun while the duration indicates the distance from the hive (Tan et al. 2008). Additionally, returning forager bees will perform more cycles of the dance if the food resource is of high quality (accessible and abundant), which in turn, entices more dance attendees to visit the profitable location (Mattila et al. 2008; Okada et al. 2008). Honeybees can also encode information regarding risk associated with a given food resource. For example, Abbott and Dukas (2009) were able to show that bees will perform fewer waggle dances for a location if they perceive that predation risk is high. Another form of dance communication is involved in the nest-searching behaviour of colonies. When nest conditions change, the queen and several thousands of workers create a swarm in order to find a new home (Seeley and Morse 1977). This process involves the bees forming a cluster near the parent nest followed by scouts leaving the bee cluster to investigate the surroundings. These scouts will assess the quality of a candidate nest site and return to the swarm to communicate their findings using dance language which is very similar to

the waggle dance (Makinson and Beekman 2014). Given that many scouts engage in exploration of potential new homes, the cluster must make its final decision on where to establish its colony based on the combination of information provided by the individual scouts. Similar to the dance language associated with food resources, the liveliness and duration of the dance performed for a given location are proportional to the site's quality (Camazine et al. 1999; Passino et al. 2008; Seeley and Visscher 2008). The principles which link these two types of dance language are learning and memory. Specifically, the bees must not only use their foraging experience accumulated over their lifetime to assess the quality of a location, but they must also accurately recall vectoral features of these locations in order to translate their findings into dance language. Honeybee learning and memory are the focus of this project, specifically, the molecular biology of spatial learning.

Ever since the Proboscis Extension Response (PER) protocol was developed (Takeda 1961), research on learning in bees has been overwhelmingly skewed toward olfactory learning (Marfaing et al. 1989; Hammer and Menzel 1995; Scheiner and Arnold 2010; Frost et al. 2012; Wang et al. 2013; Claudianos et al. 2014; Bonnafé et al. 2015; Zhang et al. 2015; Guo et al. 2016). Other forms of learning include visual and spatial. Although these two learning faculties have overlapping features, they are different. Spatial learning relies on the ability to discern the positions of items relative to one another (VanderSal 2008), while visual learning pertains to the ability to discriminate items based on colour, shape, orientation, etc. (Bart et al. 2012). Given that spatiotemporal information is encoded in the honeybee waggle dance, spatial learning should be further investigated in order to better understand this dance behaviour. Currently, much of what is known about navigation and visual learning in bees addresses colour discrimination, shape discrimination, navigation strategies, dance communication and orientation

flights (Horridge 2005; Menzel et al. 2006; Menzel and Giurfa 2006; Giurfa 2007). However, a knowledge gap which needs to be filled with respect to spatial learning in honeybees is the role gene expression plays. My thesis aimed to help fill this gap.

We already know that gene expression profiles are not static. In bees, gene expression patterns change in response to navigation behaviours (Sen Sarma et al. 2009; Sen Sarma et al. 2010; Lutz and Robinson 2013); caste determination mechanisms and caste-specific behaviours (Shapira et al. 2001; Velarde et al. 2006; McQuillan et al. 2012; Singh et al. 2018; Ugajin et al. 2018); sensory information integration and memory formation (Schwärzel and Müller 2006; Claudianos et al. 2014; Zhang et al. 2014; Bonnafé et al. 2015; Guo et al. 2016; Li et al. 2018); and learning (Wang et al. 2013; Qin et al. 2014; Zhang et al. 2015; Naeger and Robinson 2016). In each of these cases, mRNA levels increase or decrease according to the needs of the insect during a given activity or the environment (Zayed and Robinson 2012). Currently, it is known that sensory information is integrated in the mushroom bodies of the bee brain, making this region an excellent target for investigating gene expression profiles pertaining to learning. (Meller and Davis 1996; Zars 2000; Shapira et al. 2001; Sen Sarma et al. 2009; Naeger and Robinson 2016). Also, Early Growth Response protein 1 (EGR1), a gene associated with learning in vertebrates, has been shown to be up-regulated in response to orientation flights in bees (Lutz and Robinson 2013). Other genes which are up-regulated in association with visual learning include cAMP response element binding protein (CREB), dopamine receptor 1 (dop1) and dopamine receptor 2 (dop2) (Zhang et al. 2014). During flight, gene expression patterns in the mushroom bodies and optical lobes change depending on a bee's perception of the distance it has flown (Sen Sarma et al. 2010). Given these findings on individual genes, not much is known about genome-wide patterns of expression.

Thus far, to my knowledge, no study has been undertaken to provide a genome-wide approach to determine how gene expression patterns change in response to learning under a spatial learning protocol. As of the development of this thesis project, I could identify only three learning- and memory-based studies which investigated genome-wide gene expression profiles in bees. However, two of these studies used visual, not spatial, learning protocols. The first study approached maze-based visual learning in bumblebees from the perspective of how miRNA expression modulates the expression of genes involved in learning and memory. Through the presence of co-expressed miRNA and coding mRNA, the researchers found that miRNA indeed played a role in regulating visual learning pathways (Qin et al. 2014). The second study investigated the molecular mechanisms underlying the formation of visual memory. This second group of researchers was able to provide evidence for the existence of two protein synthesis phases in the formation of long-term memory in honeybees (Li et al. 2018). The third study, which partially inspired the current thesis project, involved evaluating spatial learning by training honeybees to feeders at different times of day. The experimental design of this study was limited in the fact that the scientists could only make conclusions about how time of day and activity state (active vs. anticipating) affected gene expression, not the learning process itself (Naeger et al. 2011).

The current project aimed to answer the following question: how is spatial information encoded in the honeybee brain at the molecular level? As described above, there is a knowledge gap with respect to gene expression patterns as they relate to spatial learning in the honeybee. As evidenced by the studies and reviews cited above, honeybees can be trained relatively quickly to associate a reward with a desired behaviour. Using harnessed bees, drugs can be applied directly to the brain, without sacrificing the insect, to evaluate how these chemicals affect learning

(Avarguès-Weber and Mota 2016). The method I followed for this project was based on the Food Search Box (FBS) protocol developed by Tsvetkov et al (2018). This protocol involves training honeybees to identify an artificial flower, one of four options, using a sugar reward. After training trials, the ability of the bees to recall the spatial information is evaluated by releasing them into the training arena, without a sugar reward (Tsvetkov et al. 2018). By collecting these bees to perform a genome-wide analysis of brain tissue mRNA, I was hoping to identify gene expression profiles associated with different phases of spatial learning: no learning phase, training phase and memory phase. As honeybees naturally exhibit complex social behaviours which evolved independently of other invertebrate and vertebrate species, these expression profiles might have been able to elucidate the molecular mechanisms underlying their dance language and candidate genes in social learning and behaviours (Giurfa 2007; Dukas 2008).

In predicting the FSB learning assay results, I expected to obtain similar results to the Tvsetkov et al paper (Tsvetkov et al. 2018). In establishing the protocol, that group was able to show that the number of errors the bees made was not different from chance in the first training trial but decreased in a significant manner for trials 2 and 3 of training. They also found that the number of errors in the memory test was significantly different from chance. The Tsvetkov et al. paper defined errors as the number of incorrect visits to the arena flowers, without counting repeat visits, resulting in a maximum number of three errors. I decided to also take into account the absolute number of visits to incorrect flowers because this information provided insight into how the honeybees were behaving inside the arena. With respect to gene expression profiles of brain tissue, a previous study, which used a visual learning assay, was able to show distinct full transcriptome-level gene expression profile differences between naïve and trained bees (Li et al. 2018). Studies which investigated fewer genes demonstrated that such profile differences were

associated with transcriptional (Zayed and Robinson 2012; Lutz and Robinson 2013; Wang et al. 2013; Qin et al. 2014), translational (Meller and Davis 1996; Zhang et al. 2015) and epigenetic (Menzel 2012; Li et al. 2017) changes. In addition, the processes of consolidating and retrieving a memory have been shown to be associated with changes in both mRNA and protein synthesis (Schwärzel and Müller 2006). Altogether, the existing body of research on honeybee learning lead me to hypothesize that bees categorized into each learning phase would have different gene expression profiles such that the profiles of naïve bees would be most different from those of learning phase and memory phase profiles, while the learning and memory phase profiles would resemble each other the most. In addition, I suspected that there would be an overlap between differentially expressed genes (DEGs) associated with spatial learning and both visual and olfactory learning DEGs, such that some DEGs would be specific to spatial learning. The reason for this hypothesis is that honeybees use many cues to help them navigate a space, however each modality involves different senses and different types of interactions with the environment such that the brain receives unique bits of information from each type (Brown and Demas 1994; Menzel 2001; Horridge 2005; Menzel 2012). Unfortunately, the FSB learning assay results did not allow me to carry out the gene expression portion of this project. Nevertheless, I will discuss gene expression as it relates to various honeybee learning modalities later in this thesis.

Methods

Research Colonies

The honeybees (*Apis mellifera*) I used in this project were maintained at the Purdue University research apiary in West Lafayette, Indiana, U.S.A. In order to minimize genetic differences between individuals, Single Drone-Inseminated (SDI) queens were used to head the colonies. SDI queens produce offspring which are 75% related sisters compared to 50% related

sisters which come from naturally mated queens. (Oldroyd and Moran 1983; Mattila et al. 2008; Wang et al. 2013). Three SDI colonies were raised, however, only two were healthy enough to be used for experiments. In order to distinguish between the colony sources and determine age, the bees were paint-marked using non-toxic paint pens. One frame of brood was removed from the source colony and placed in an incubator overnight at 38°C and 65% relative humidity. The bees had one of two possible dates of emergence: June 28th or July 11th of 2019. The bees were paint-marked in two stages to ensure there were enough foragers for the duration of the experiment. Once the marking was completed, the bees were re-introduced into their source colonies. Bees emerged on June 28th were marked WHITE for SDI colony 1 or YELLOW for SDI colony 2. Bees emerged on July 11th were marked BLUE for SDI colony 1 or RED for SDI colony 2.

Food Search Box assay

When the time came to collect bees for the learning assay, only foragers were used because they are known to learn faster than nurses (Tsvetkov et al. 2018). Foragers were typically collected between 11h00 and 14h00 by placing an entrance reducer on the hive and using forceps to gently pick up paint-marked individuals and place them inside a plastic box with ventilation holes. This plastic box was identical to the FSB training arena (described later) in term of dimensions: 12.5cm x 12.5cm x 6.5cm and 908mL in volume. One plastic box was used per colony to collect between 20 and 30 foragers. As the bees were kept in an incubator overnight to acclimate (Naeger et al. 2011) at 35ºC and 65% relative humidity, the plastic box contained 30% sucrose (Sigma-Aldrich) *ad libitum* and two pollen patties measuring approx. 2cm x 3cm. The sucrose solution was placed in Eppendorf tubes with a cotton plug at the tube opening to give the bees access to the sucrose while preventing the solution from spilling inside

the plastic box. The following day, the plastic boxes containing the foragers were removed from the incubator and taken to a room at the apiary where the windows were covered with a thick sheet to prevent sunlight: this room is where the FSB learning assay was performed.

Before starting the FSB assay, the sucrose tubes were removed from the incubation boxes and the bees were allowed to acclimate to the room temperature training room for 10-20min. This acclimation period with no sugar water ensured the bees were hungry. The FSB learning assay occurred in two steps. The first step was the motivation test while the second step was the training phase. The motivation test consisted of placing a forager inside a Petri dish with ventilation holes containing a PCR tube cap filled with 1µL 30% sucrose. The bee was allowed to freely move around the Petri dish. When the bee extended her proboscis and drank the sugar solution, she was deemed a good candidate and moved on to the training phase. The training phase consisted of releasing a motivated bee into the FSB arena and closing the 1cm diameter entrance with tape. Before placing the bee inside the arena to begin her training trial, one of the four cotton swabs, i.e. artificial flowers, was randomly selected to be the focal flower and the cotton was soaked in 30% sucrose solution to serve as the sugar reward. The learning trial ended when she located the focal flower and extended her proboscis to drink. She was allowed to drink for approx. 5sec, then placed back inside her Petri dish to await her next trial with no food. Each forager was given a unique identifier and her visitation sequence between flowers, behaviour in the arena, time to complete the trial and any other pertinent notes were recorded in data sheets (tables 2 & 3).

Before the training phase could begin, the bee was randomly assigned a training group to represent each learning stage: motivation state, 1 learning trial, 2 learning trials, 3 learning trials and 3 learning trials with the memory test (table 1). During the training phase, the forager

completed her designated number of trials, with 20min inter-trial intervals, then was immediately flash-frozen using dry ice (-72ºC). She was later preserved at -80 ºC for long-term storage. Bees which did not complete their assigned number of trials within the allotted 30min per trial were discarded and not included in the analysis. Foragers which were assigned to the MS learning group were frozen without undergoing the training phase. Foragers which were assigned to the 3wMT learning group completed three learning trials, then a memory test where there was no sugar reward: none of the four cotton swabs were soaked in sugar water (Li et al. 2018; Tsvetkov et al. 2018). The memory test ended when she located the focal flower and extended her proboscis or stayed on the focal flower for longer than 5sec. This behaviour was noted as "REMEMBERED". Another memory test scenario was when the bee did not extend her proboscis or stay on the focal flower by the 30min mark. This second behaviour was noted as "DID NOT REMEMBER". Whether or not a forager remembered her focal flower, she was flash frozen for later comparison of gene expression profiles.

Food Search Box Variables

Described below are the variables I observed and measured for the learning assay portion of the project. For each of the variables, the "x" refers to the trial number, with "4" representing the memory test. A sample of data collected for trial 1 is given in table 2, while a sample of summary data obtained for a few bees is given in table 3.

- Bee ID = Code to uniquely identify each forager
- Sx = Visitation sequence for trial x. The order in which a forager landed on different flowers in the arena
- Vx = Total number of visits for trial x
- VECx = Total number of wrong visits before landing on the correct flower for trial x

- VERx = Total number of wrong visits before the sugar reward/end of trial for trial x
- ECx = Total number of errors before landing on the correct flower for trial x
- ERx = Total number of errors before the sugar reward/end of trial for trial x
- Tx = Time to complete trial x in seconds
- Colour = Paint mark to identify the bee's birth date and origin
- Origin = The source colony from which the foragers were raised. There were two SDI colonies which will henceforth be denoted as SD1 and SD2, respectively

It is to be noted that "wrong visits" are the absolute number of times a bee landed on any flower but the focal lower, while "errors" do not take into account repeated visits to the same flower. In essence, the maximum number of "wrong visits" cannot exceed the total number of visits, while the maximum number of errors is three. Also, "landing on the correct flower" means the bee landed on the correct flower but did not extend her proboscis while "reward" refers to scenarios where the bee landed on the correct flower and drank the sugar water, thus ending the trial. For the memory test, there was no sugar reward therefore, the trial ended when the bee landed on the focal flower and stayed there or extended her proboscis. With regard to the "origin" variable, I also used foragers which were raised from hives whose queens were naturally mated. This cohort of bees was not destined for gene expression analysis: their purpose was for me to practice the protocol and ensure I was doing the steps correctly. Throughout this thesis, these non-SDI bees will be referred to as "regular foragers".

Gene Expression

As mentioned earlier, I was not able to complete a gene expression analysis however, I will still provide a brief explanation of the protocol I planned to use to generate gene expression profiles for each learning stage. The mRNA extraction, assay and analysis phase was based on

the protocols used in the three large-scale transcriptome studies mentioned earlier (Naeger et al. 2011; Qin et al. 2014; Li et al. 2018). mRNA would have been extracted from the honeybee brains using a typical extraction kit such as RNeasy or Trizol reagent. Given that these kits both extract total RNA, I would have needed an affinity technique to purify the mRNA, which normally represents only 2% of total RNA in eukaryotic cells (Lowe et al. 2017). To obtain mRNA sequences spanning the entire genome, I would have employed high-throughput sequencing techniques. Although microarray has been used in the past to obtain genome-wide patterns of gene expression (Whitfield et al. 2003; Grozinger et al. 2007; Sen Sarma et al. 2009), RNA-seq is proving to be the method of choice for modern scientists due to its higher sensitivity, relative to microarray (McGettigan 2013). RNA-seq is a method which involves isolating RNA and converting it to cDNA using a reverse transcriptase. After amplification of cDNA using PCR, the cDNA strands are digested to facilitate the reading step within the analyzer instrument. Each cDNA fragment is read several times to ensure read accuracy, often reaching an order of magnitude of millions of times per fragment. Next, alignment ensures similar, but distinct genes, are read as different: in the case of this thesis project, the fragments would have been aligned to the honeybee reference genome found on GenBank (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/002/195/GCA_000002195.1_Amel_4.5). After alignment, a digital representation of gene expression levels across the genome would have been produced using software, likely various packages in R version 3.6.2 (The Honeybee Genome Sequencing Consortium 2006; Manfredini et al. 2015; McNeill et al. 2016; Lowe et al. 2017). In the end, I would have been able to demonstrate overall patterns of gene expression which would have been used to identify differentially expressed genes (DEGs) in trained vs. naïve honeybees. Using Gene Ontology (GO), the DEGs would have been categorized according to metabolic

function and respective pathway, then the change in DEGs would have been compared between learning trials and between memory test bees.

Statistics

With respect to statistical methods, I planned to use the Chi-square test (or its non-parametric counterpart, the Kruskal-Wallis test if the data were not normal) to analyze the visit and error data. In analyzing the error data, I compared the means to chance and determined which means were statistically different from each other using a Bonferroni correction to account for the number of comparisons. With respect to the gene expression analysis, I planned to use typical methods. These include creating and sequencing cDNA libraries, removing low-quality reads from the sequence data, checking the read alignment through comparison to a reference genome (Amel4.5), quantifying the number of reads to determine which genes are differentially expressed, using Gene Ontology terms to determine which metabolic pathways are associated with the DEGs then finally, generating gene expression profiles associated with each learning stage (Rajkumar et al. 2015; Naeger and Robinson 2016; Pertea et al. 2016; Corley et al. 2017).

Results

Number of Bees and Flower Position

For the regular foragers, I collected a total of 25 bees, each of which underwent 3 learning trials with the memory test (3wMT). Of the 25 regular foragers, 14 remembered their focal flower while the remainder did not remember. For the SD1 foragers, I collected a total of 33 bees which were split up between the learning categories. For the 3wMT category, 9 remembered their focal flower while 4 did not. With respect to the SD2 foragers, I collected a total of 37 bees which were split up between the learning categories. For the 3wMT group, 10 remembered their focal flower while 2 did not (table 4).

As part of basic exploratory data analysis, I first needed to determine if my data fit a normal distribution for the visit and error data for both regular and SDI foragers therefore, I used the Shapiro-Wilk test. With respect to the VEC data, the analysis showed that the data did not follow a normal distribution: all of the p-values were well below 0.05 for both regular and SDI foragers. With respect to the VER data, the data once again did not fit a normal distribution, with p-values well below 0.05 for both types of foragers. The same trends held for the EC and ER data: neither fit a normal distribution for the regular or SDI foragers (all p-values < 0.05). Given that both the visit and error data did not fit a normal distribution, I used non-parametric statistical tests for the remainder of my analyses.

Before analyzing the visit and error data more scrupulously, I wanted to ensure that the position of the flower had no effect on the number of attempts the bee made in locating the correct flower. I used the Kruskal-Wallis test to determine if any flower position showed more visits or errors. There was no statistically significant difference between flower positions for any of the forager groups: regular foragers' VEC (Kruskal-Wallis $\chi^2 = 1.11$, n = 14, df = 3, p-value = 0.774), regular foragers' VER (Kruskal-Wallis $\chi^2 = 2.31$, n = 14, df = 3, p-value = 0.512), regular foragers' EC (Kruskal-Wallis $\chi^2 = 1.257$, n = 14, df = 3, p-value = 0.739), regular foragers' ER (Kruskal-Wallis $\chi^2 = 2.203$, n = 14, df = 3, p-value = 0.531), SDI foragers' VEC (Kruskal-Wallis $\chi^2 = 3.97$, n = 19, df = 3, p-value = 0.265), SDI foragers' VER (Kruskal-Wallis $\chi^2 = 1.97$, n = 19, df = 3, p-value = 0.579), SDI foragers' EC (Kruskal-Wallis $\chi^2 = 4.378$, n = 19, df = 3, p-value = 0.223) and SDI forager' ER (Kruskal-Wallis $\chi^2 = 2.101$, n = 19, df = 3, p-value = 0.552). Altogether, these results indicate that flower position did not have an effect on number of visits or errors.

The remainder of my results will be presented in the order the analyses were performed. The discussion of the results will focus mainly on the visit data, the error data and differences in age. These sections will pay particular attention to the SDI foragers as these bees were the ones initially destined for transcriptomic analysis. A final exploration of gene expression in learning and memory in honeybees will be presented in chapter 2.

Visit Data

These data refer to the absolute number of times a bee landed on the incorrect flower. This variable gives a measure of how often a forager ignored the correct flower and explored her surroundings. I sub-divided this variable into two parts, the number of visits before the correct flower (VEC) and visits before the reward (VER). This division is relevant because it provides insight into the precise moment the bee drank the sugar reward.

Means and standard errors for the VEC and VER data were first calculated. For regular foragers, the mean VEC for trials 1 through 3 was 1.00, 0.77 and 0.14 with standard errors of 0.38, 0.28 and 0.097, respectively. The mean VEC for the memory test was 1.71 with a standard error of 0.57. The regular forager VEC means were statistically significantly different from each other (Kruskal-Wallis $\chi^2 = 10.27$, n = 14, df = 3, p-value = 0.016). Meanwhile, the mean VER for trials 1 through 3 was 1.14, 0.86 and 0.36 with standard errors of 0.40, 0.29 and 0.23, respectively. The mean VER for the memory test was 3.71 with a standard error of 0.96. The VER means for the non-SDI foragers were statistically significantly different from each other (Kruskal-Wallis $\chi^2 = 13.23$, n = 14, df = 3, p-value = 0.004) (see Figure 2). For all SDI foragers together, the mean VEC for trials 1 through 3 was 1.11, 0.79 and 0.79 with standard errors of 0.40, 0.34 and 0.34, respectively. The mean VEC for the memory test was 2.00 with a standard error of 0.39. The SDI forager VEC means were statistically significantly different from each

other (Kruskal-Wallis $\chi^2 = 9.33$, n = 19, df = 3, p-value = 0.025). Meanwhile, the mean VER for trials 1 through 3 was 1.11, 0.79 and 1.21 with standard errors of 0.40, 0.34 and 0.41, respectively. The mean VER for the memory test was 4.53 with a standard error of 0.69. The SDI forager VER means were statistically significantly different from each other (Kruskal-Wallis $\chi^2 = 24.57$, n = 19, df = 3, p-value = 1.88×10^{-5}) (see Figure 3). In order to determine which trials were different from each other in a statistically significant way, I used the Kruskal-Wallis pairwise comparison with Bonferroni correction, accounting for six comparisons. For the regular foragers, none of the pairs of VEC means were statistically significantly different from each other (p-value > 0.008 in all cases) however, VER3 with VER4 means showed statistical significance (p-value < 0.008, see Figure 3). For the SDI foragers, none of the pairs of VEC means were statistically significantly different from each other, although three pairs of VER means showed statistical significance (p-value < 0.008): trial 1 with the memory test, trial 2 with the memory and trial 3 with the memory test (see Figure 3).

Overall, the mean number of visits from trial 1 to 3 generally decreased, but the number for the memory test was higher than the trial 1 mean. Also, the data revealed that the increase in number of visits for the memory test, relative to the other trials, was statistically significant. From a biological perspective, we expected the bees to make fewer attempts, from trial 1 to trial 3, before reaching the focal flower, especially if she was motivated to find the sugar reward. The visit data indicated that the foragers were not decreasing their numbers of attempts over the course of the trials in a meaningful way. In comparing the means for VEC to VER, the data show that the foragers did not always drink from the focal flower the first time they landed on it because the means for VEC are slightly lower than the means for VER. With regard to the

memory test, the bees required more attempts than they did in trial 1 to land on the correct flower, suggesting they did not remember the correct flower.

Error Data and Simulation

Errors were defined as the number of mistakes a bee made before landing on the correct flower, without counting repeat visits to the same flower. This variable determines whether or not bees learned because we expect the number of mistakes to go down from trial 1 to 3. Hence, the maximum number of errors for a given trial is 3 and the number of errors which represent chance is 1.5. Like the visit data, this variable was divided into two parts: errors before the correct flower (EC) and errors before the reward (ER).

Means and standard errors for both the regular and SDI foragers' EC and ER variables were calculated. For the regular foragers, the EC means for trials 1 through 3 were 0.86, 0.57 and 0.14 with standard errors of 0.29, 0.17 and 0.097, respectively. The EC mean for the memory test was 1.21 with standard error of 0.28. The regular forager EC means were statistically significantly different from each other (Kruskal-Wallis $\chi^2 = 10.69$, n = 14, df = 3, p-value = 0.0135). Meanwhile, the ER means for trials 1 through 3 were 0.93, 0.64, 0.29 with standard errors of 0.31, 0.199 and 0.16, respectively. The memory test ER mean was 1.79 with a standard error of 0.32. The regular forager ER means were also statistically significantly different from each other (Kruskal-Wallis $\chi^2 = 13.156$, n = 14, df = 3, p-value = 0.00431) (see Figure 4). For the SDI foragers, the EC means for trials 1 through 3 were 0.74, 0.53 and 0.58 with standard errors of 0.21, 0.18 and 0.19, respectively. The EC for the memory test was 1.37 with a standard error of 0.22. The SDI forager EC means were statistically significantly different from each other (Kruskal-Wallis $\chi^2 = 10.16$, n = 19, df = 3, p-value = 0.0173). The ER means for trials 1 through 3 were 0.73, 0.53 and 0.84 with standard errors of 0.21, 0.78 and 0.23, respectively. The memory

test had a mean ER of 2.1 with a standard error of 0.22. The SDI forager ER means were also statistically significantly different from each other (Kruskal-Wallis $\chi^2 = 22.981$, n = 19, df = 3, p-value = 4.08×10^{-5}) (see Figure 5).

As the maximum number of errors was finite, I also wanted to know if the number of errors for each trial was different from 1.5. I used a Wilcoxon signed-rank test and found that the mean number of errors for each trial was significantly different from chance for all trials (p-value < 0.05), across both error variables and both bee origins, with the exception of means for regular foragers' trial 1 EC (lower than 1.5), trial 4 EC (lower than 1.5), trial 1 ER (lower than 1.5) and trial 4 ER (higher than 1.5), and SDI foragers' trial 4 EC (lower than 1.5) (table 5). Just like for the visit data, I used a Bonferroni-corrected Kruskal pairwise comparison to determine which pairs of EC and ER means were significantly different from each other for both bee origins: there were six comparisons for each bee origin-error variable combination. For the regular foragers, none of the pairs of EC means were statistically significantly different (all p-values > 0.008). With regard to the pairs of ER means, ER3 and ER4 values were significantly different from each other (p-value < 0.008, see Figure 4). For the SDI foragers, none of the pairs of EC means were significantly different from each other (all p-values > 0.008). However, three pairs of ER means were statistically significantly different from each other (p-value < 0.008): trial 1 with the memory test, trial 2 with the memory test and trial 3 with the memory test (see Figure 5).

Briefly, the error data tell a similar story to the visit data. From trials 1 to 3, the number of errors decreased but not in a significant way, while the number of errors made during the memory test was statistically significantly higher than any of other three trials. In addition, most of the mean numbers of errors were statistically different from chance. Biologically, these results indicate that although the bees found the sugar reward earlier than they would by chance, they

were still making just as many errors in trial 3 as they were in trial 1. They also required more attempts in the memory test than in the first trial to locate the focal flower. Ultimately, there was only weak evidence supporting the idea that the bees learned the location of their focal flower. The evidence supporting their memory at the recall stage of the experiment was even weaker.

Given that typical statistical testing did not demonstrate learning or memory in the foragers, the next step was to use a simulation written in R to represent the total number of errors the SDI foragers collectively made in each trial, especially trials 1 and 4, and then compare the simulated number to the real number encountered in the experiment. I used two simulations assuming different bee behaviours. The first simulation was based on a "Stepping-stone" behaviour and assumed that bees travelled from one flower to next only through adjacent flowers (see Figure 12). The second simulation was based on a "Random error" behaviour and assumed the bees randomly made one of four possible errors: 0, 1, 2 or 3 errors (see Figure 13). Simulations using similar principles have been used in other studies (Yoccoz et al. 1993; Motro and Shmida 1995; Kunin and Iwasa 1996). The parameters for both simulations were:

- Focal flower = A number randomly chosen between 1 and 4. Represented the focal flower
- Number of bees = The total number of real-world bees which were tested for a given trial (trials 1, 2, 3, or the memory test). This included bees from the 1TO, 2TO, 3TO and 3wMT learning groups
- Iterations = Number of times the simulation was run. We repeated each simulation 10 000 times
- Total errors = The simulated number of errors the bees collectively made for a given trial

Once the simulation was run, the script compared the number of errors made by the real-world foragers to the number of errors made by the simulation bees using a t-test. Because I expected the bees to behave randomly in trial 1 then non-randomly in subsequent trials, we expected the simulation to match the real-world foragers only in trial 1, i.e. the difference between the errors would not be significantly different from 0. If the simulation was able to show that the trial 1 foragers behaved in a way which resembled chance, this evidence may have demonstrated that the bees learned in the subsequent trials. Briefly, the simulation would have demonstrated that the foragers improved their performance by transitioning from random to focused behaviour and were able to find the focal flower in a non-chance event.

The results of the simulations revealed that the difference between the number of errors for real-world foragers vs. simulation bees was always statistically significantly different from 0 (p-value < 0.05), across all trials. As expected, the "Stepping-stone" simulation bees made many more errors than the real bees and the "Random error" simulation bees across all four trials. With respect to trial 1, the "Stepping-stone" bees made approx. 175 more errors than the SDI foragers while the "Random error" bees made approximately 30 more errors than the SDI foragers (table 6). Given that that both simulations' trial 1 differences were statistically different from chance, I cannot say the SDI foragers behaved in a way which resembled chance. Once again, the data did not indicate that the bees learned. Curiously, the SDI foragers made more errors than the "Random error" simulation for the memory test, suggesting they did not retain what they learned.

Age Differences

Given the lack of evidence supporting learning and memory in SDI foragers during the FSB assay, I decided to investigate the ways in which the foragers may have been different from each other. Previous studies have demonstrated that bees of different ages learn differently

(Gong et al. 2018). Part of the reason for this difference is that honeybee aging is associated with division of labour in the hive. This means that young workers, such as nurses, stay mainly inside the hive while older workers, like guards and foragers, perform their tasks outside of the hive. The cognitive demands of foragers and guards are arguably more extensive than nurses (Johnson 2003; Johnson 2010; Zayed and Robinson 2012).

As mentioned earlier, the SDI foragers had two possible dates of emergence: June 28th and July 11th, 2019. Thus, by the end of the experiment, the SDI bees' ages ranged from 17 to 42 days old. In order to determine how age affected learning performance, I focused on the SDI bees from the 3wMT learning category as these were the only foragers that completed all four stages of learning. The age distribution for this group of bees is given in Figure 8. I began by testing the correlation between bee age and the other variables (V, VEC, VER, EC, ER) for each trial. Using Spearman's rank correlation test, there were no statistically significant correlations between bee age and the number of visits or errors for a given trial (all p-values > 0.05). Generalized linear models (GLMs), based on Poisson distributions, were used to investigate the relationships further. Of the 20 variables tested, only a few yielded statistically significant slopes with bee age: V4, VEC4, VER4. The slopes for each of these variables were -0.023, -0.11 and -0.14, respectively (see Figures 7-11).

In sum, bee age did not seem to affect the number of visits or errors the SDI foragers made between learning trials. Because bee age did not seem to play a significant role, I did not use this factor as a possible explanation for why the SDI foragers bees did not seem to have learned. Biologically, these results indicate that the older bees in my experiment did not make more or fewer visits/errors than the younger bees. However, the only statistically significant slopes were all negative and represented the memory test, suggesting that older bees may have

better memory than younger bees, as older foragers made fewer incorrect visits to flowers relative to younger foragers. It is to be noted that this trend was not seen when considering the slopes for the error data and was therefore interpreted with caution.

Discussion

To recapitulate, the SDI foragers did not significantly decrease the number of visits or errors they made from trials 1 to 3. In addition, the mean number of errors the bees made during the memory test was relatively high. Together, these findings do not support the idea that the bees learned or remembered their focal flower.

Visits

I will discuss the visit data by reviewing foraging and navigation strategies from different species and comparing these to honeybees. When it comes to looking for food, animals can normally be divided into two categories: those which return to sites where they found food (win-stay) and those which do not return (win-shift). From the vertebrate literature, it is known that the predisposition to win-shift vs. win-stay depends on many factors including species (MacDonald et al. 1994; Heydarnejad and Purser 2016), diet (Sulikowski and Burke 2007), life experience (Wunderle and Martinez 1987), age (Olton and Schlosberg 1978; Reed 2016) and other contexts (Haig et al. 1983; Burke et al. 2002; Burke and Fulham 2003; Reed 2016). With respect to invertebrates, the preferences of different species have not been as thoroughly investigated. As far as I know, only one study has attempted to determine which strategy honeybees prefer. The researchers found that honeybees are pre-disposed to win-shift but can learn to win-stay (Demas and Brown 1995). Because the bees were able to learn both strategies, this finding suggests that the preference is likely to depend on other factors, not just species. In the current FSB experiment, both the VEC and VER means decreased from trial 1 to 3. This

observation can be interpreted as the bees returning to a rewarding location more often over the course of the experiment, suggesting that they learned to win-stay, like in the Demas and Brown (1995) study. By modifying the FSB training arena, the factors affecting a bee's propensity to win-shift vs. -stay could be investigated further. For example, the size of the arena could be increased, and the individual cotton swabs replaced with patches of cotton swabs. By designating multiple focal flowers and patches throughout the arena, various foraging behaviour experiments could be carried out which determine how often the bees return to the correct patches or correct artificial flowers.

Factors which may influence a bee's propensity to win-shift vs. win-stay include the quality of the reward and the rate of depletion of the reward. A previous study compared the behaviour of honeybees in visiting artificial flowers with three different nectar flow rates (1.02, 2.04 and 4.08μL/min). The researcher was able to demonstrate that the honeybees preferred artificial flowers with the highest flow rate. In addition, the bees were more likely to immediately re-visit the highest flow rate flowers relative to the other flowers (Giurfa 1996). Macpherson and Roberts (2010) found that dogs trained in a radial maze showed a similar preference for highly rewarding foods relative to less rewarding ones. In comparing Giurfa 1996's to my results, it is surprising that the differences between the VER and VEC means were not larger. Given that the bee's nectar options were 30% sucrose vs. 0%, I would have expected the bees to be immediately returning to the focal flower more often, resulting in lower VEC means overall. With respect to reward depletion rates, most of the experiments on this subject were done in vertebrates. In rats, researchers have shown that the animal is more likely to win-shift if it has depleted the food at a given site (Haig et al. 1983). In the FSB experiment, there was too much sugar water on the focal flower for it to be possible for the foragers to deplete the

nectar in one visit. The benefit of such an experimental design is that the bee is encouraged to return to the same place to obtain food, further influencing the bee to preferentially use a win-stay strategy, therefore resulting in fewer visits over the course of the experiment to drink from the focal flower.

Another factor which may affect a forager's likelihood to win-shift vs. win-stay is the delay in resource replenishment. Although the FSB learning assay did not involve resource depletion/replenishment, an understanding of how honeybees behave in these contexts can help to shed light on their behaviour in the FSB training arena. Honeybees are generalists so they feed on pollen and nectar from plants which bloom at different times of year (Baum et al. 2004; Mendes do Carmo et al. 2004; Decourtye et al. 2010; Deveci and Kuvanci 2012; Sponsler et al. 2020). As these floral resources can be depleted, nectar feeders must learn when it is appropriate to return to a previously depleted food site (Motro and Shmida 1995; Winter and Stich 2005; Sulikowski and Burke 2011). A study done in regent honeyeaters, a nectarivorous bird species, was able to demonstrate that the birds' propensity to win-shift vs. win-stay depended on the delay between training sessions. The birds were more likely to win-shift at 10min delays but win-stay at 3h delays (Burke and Fulham 2003). In the FSB learning assay, there was a 20min inter-trial interval. It may be possible that the inter-trial interval was not appropriate for encouraging the bees to learn a win-stay strategy such that the VEC or VER means were statistically different from each other. Alternatively, the number of training trials may not have been sufficient to show that the foragers' behaviour was changing in a significant way during the experiment.

As mentioned earlier, the differences between VEC and VER means were somewhat small. In fact, I noticed that the foragers would sometimes land on the correct flower but not

drink from it, then return to the focal flower later in the trial and extend their proboscis. The phenomenon where an individual visits previously unexplored sites is called spontaneous alternation (Montgomery 1951; Sutherland 1957; Izumi et al. 2013). Although this phenomenon has not been thoroughly studied in insects, it may explain why the VEC and VER means were not the same values. I suspect the bees were exploring their environment before deciding to feed on a particular flower, perhaps based on their perception of the value of the reward. For example, in both a bumblebee and a honeybee study, researchers found that the quality of the nectar reward found at the previous site influenced where the bee would go next. Over time, the bees tended to return to sites where the nectar reward was highest (Hodges 1985; Greggers and Menzel 1993). Such a behaviour results in flower constancy over the course of one foraging bout, especially when the bees must rely on visual cues to discriminate the more vs. less rewarding flowers (Hill et al. 2001; Gegear and Laverty 2004; Grüter et al. 2011). Goulson and Cory (1993) discussed the influences of flower constancy and infidelity on butterfly foraging. They suggested that is it advantageous for an animal to occasionally sample nearby food options in order to uncover new, possibly more rewarding foods (Goulson and Cory 1993). Given that nectar feeding insects can learn to associate flower traits (colour, odour, time of bloom, degree of damage, etc.) with varying levels of reward, some level of infidelity allows the insect to build their life experience over time and optimize the energetic costs of their foraging bouts (Motro and Shmida 1995; Thuijsman et al. 1995; Goulson et al. 2007). In essence, knowing how to identify and locate high quality food means the insect can exert less locomotive energy and gain more nutritive calories.

The foraging behaviour of the honeybee depends on its ability to navigate its environment. As mentioned earlier, spatial information is encoded in the waggle dance of the

honeybee, so this information must play an important role in foraging ability. In the context of the FSB learning assay, the foragers had to rely on mainly spatial cues to locate the focal flower during the training and memory phases, although the bee could have also used olfaction to a lesser degree during the training trials. Based on past research, honeybees are known to discriminate colours, shapes, sizes, distances/depths and orientations; rely on many cues at once to locate familiar places; be able to learn rules to obtain a reward; and engage in orientation flights to familiarize themselves with a new place (Horridge 2005; Menzel and Giurfa 2006; Avarguès-Weber et al. 2012; Collett et al. 2013). The results of the FSB assay showed that the foragers had trouble remembering the focal flower because they required more visits in the memory test relative to the first training trial. These results may indicate that the foragers were not able to optimize their spatial learning and memory to the point of easily locating the focal flower.

In terms of navigation, animals rely on one or more cues to decide where to go depending on their goals. These cues can be mainly divided into three categories: visual (colour, shape, orientation), olfactory (scent and taste) and spatial (the position of one thing relative to another). In experiments where honeybee foraging preferences were evaluated with respect to visual and olfactory cues, researchers found that high contrast blue vs. yellow cues induced colour-based (visual) preferences while low contrast blue vs. white cues induced reward quality-based (olfactory) preferences (Hill et al. 2001; Grüter et al. 2011). Based on these studies, it appears context may affect which cues take priority. In the FSB assay, the table was black, and the sheet used to cover the window was dark grey and speckled with small flecks of various colours, resulting in a low contrast background for the bees. Although the cotton swabs did not have colour, the amount of contrast within the training area, or in the area around the arena, may have

affected the perceived reward value of the focal flower, thereby affecting the hierarchy of cues the foragers used. If context affected the value of the spatial information the bees acquired during the training trials, the low contrast environment may explain why the VEC and VER means were relatively high during the memory test. More work would be required to determine how colour in the surroundings affects spatial discrimination during the FSB assay. Specifically, colours in the surroundings could serve as landmarks which help the foragers orient themselves. These future experiments could also use different sucrose concentrations on the artificial flowers to see how reward quality affects spatial discrimination.

Another aspect which is worth discussing with respect to cues is how they come together to create landmarks. A review by Collett et al. (2013) described the different guidance systems insects use to navigate and orient themselves in space. Path integration is the system where the animal finds its way by recalling where it travelled and for how long. This first system does not require *a priori* familiarity with the space. Alignment image matching is the system where the animal recalls a snapshot of what a location looks like and matches it perfectly to their current location. This second system requires some familiarity with the space in order to generate a snapshot of the area. The last guidance system the review describes is positional image matching. This third system involves matching some features of the snapshot with common features in the current location (Collett et al. 2013). As these guidance systems relate to the FSB assay, a bee which relied on both alignment image and positional image matching was likely to be more successful in locating the focal flower in fewer visits because it was using landmarks to situate itself within the training arena. Although they were not significantly different from each other, the VEC and VER means decreased from trial 1 to 3, especially in the regular foragers, suggesting that the foragers may have been using these two image matching guidance systems.

Confirmation of how bees use landmarks during the FSB protocol can be achieved by creating an experiment where the landmarks around the training arena are changed in terms of orientation. By comparing bees whose landmarks were consistent throughout the experiment to bees whose landmarks were changed, the impact of landmarks could be elucidated specifically in the context of the FSB assay.

Errors

Honeybees are capable of learning in a lab setting, as evidenced by PER and other well-established learning protocols. In the original FSB paper, Tsvetkov et al (2018) found that bees made fewer errors over the course of the experiment and that the mean number of errors was different from chance. One major difference between the current FSB assay and the original paper is that the current assay used SDI foragers while the original publication used bees from naturally mated queens. Colonies with greater genetic diversity tend to have greater colony fitness (Page et al. 1995; Palmer and Oldroyd 2000; Mattila and Seeley 2007). In addition, although small, a proportion of the variance in learning ability under PER can be explained by genetic differences between individuals (Latshaw and Smith 2005; Laloi and Pham-Delegue 2010). Together, these pieces of evidence suggest that the SDI foragers in the current FSB project may have been impaired in terms of learning due to low genetic diversity. More specifically, the use of SDI foragers may induce effects due to bee strain such that they learn differently than bees sourced from naturally mated queens. In fact, the regular foragers showed a gradual decrease in the number of errors while the SDI foragers did not, further supporting the idea that the SDI foragers may have been impaired. Even though controlling for genetic variation can make genetic analysis easier, there may be a trade-off between learning ability and degree of genetic variation when it comes to learning-based experiments. Because the FSB assay makes it

easy to control for different variables and compare learning ability in honeybees, it may be worthwhile using the assay to determine which amount of variation in learning may be attributed to different variables including bee age, foraging preference, sub-species, genetic variability, etc. Alternatively, a future full-transcriptomic study of learning in honeybees using the FSB protocol may benefit from simply using bees from colonies headed by naturally mated queens to increase the probability of detecting learning.

The method used to reinforce learning also affects the success of the honeybees when it comes to learning assays. For example, some studies use appetitive learning protocols (Bitterman et al. 1983; Guo et al. 2016; Søvik et al. 2016; Buatois et al. 2017) to reward desired behaviour while other use aversive learning to punish undesired behaviours (Vergoz et al. 2007; Carcaud et al. 2009; Mota et al. 2011). When it comes to bees, both types of consequences affect overall learning, however, context and genetics affect which type is favoured (Roussel et al. 2012; Junca et al. 2019), suggesting than one is not necessarily better than the other. Learning can also be reinforced by using different cues to stimulate a given sensory system appropriately. In an early PER-based paper, Bitterman et al. (1983) demonstrated that the association between the reward and the odour was learned better when both the antennae and the mouthparts were touched with sucrose. By touching one part after the other, the researchers were stimulating the olfactory system to a greater extent vs. touching only one part. In a different study investigating working memory in honeybees, Demas and Brown (1994) showed that removal of olfactory cues allowed the bees to perform better than expected in a spatial memory assay. They suspected the reason for the improvement in performance was that removal of the olfactory cue reduced the potential for conflicting signals, thereby reinforcing the spatial memory. To relate these previous studies to the current FSB experiment, a possible reason why the bees did not learn nor remember the

focal flower could be that a visual cue may have conflicted with the spatial cue. More specifically, the focal flower was a wet cotton swab while the non-focal flowers were dry. If the bees were not able to detect the difference between a wet or dry cotton swab, they would not have been able to use this visual cue to reinforce their spatial memory, resulting in the poor learning performance and poor memory observed during the FSB assay.

Here, I would like to delve deeper into how cues may affect learning in honeybees. From ant literature, the use of olfaction to return to a previously visited location or to recruit nestmates to profitable food patches is well documented. Ants use trail pheromones to accomplish these tasks (Wolf 2011; Steck 2012; Czaczkes et al. 2013). The same behaviours have also been documented in various bee species including bumblebees, honeybees and stingless bees (Granero et al. 2005; Wager and Breed 2006; Reichle et al. 2011; Schorkopf et al. 2011; Gilley 2014). Furthermore, a desert ant study showed that the insect's confidence in being able to find its nest depended on the cues it was trained to use. Ants trained with both cues located their nest entrance faster than ants trained with just an olfactory or just a visual cue (Steck et al. 2011). Given these findings from previous research, olfaction is likely a cue which enhances spatial learning. In the FSB assay, the error data suggested the bees did not learn. Because the honeybees in the FSB assay could have antennated the artificial flowers but may have also released trail pheromones, it is hard to know for sure if these possible olfactory cues indeed enhanced the bees' learning in any way during the assay. The possible relationship between different sensory cues during learning assays begs the question: can bees discriminate their own scent from their nestmates? If so, to what extent and how does this discrimination affect a bee's ability to locate a profitable food source? Research in stingless bees has shown that trail pheromone composition in different colonies from the same population are more distinct than

composition in colonies from different populations, possibly to minimize competition in the same place (John et al. 2012). As honeybees swarm to form new nests not too far away from the parent nest (Seeley and Morse 1977; Villa 2006; Laomettachit et al. 2015), these genetically overlapping but distinct colonies could serve as fertile ground to further investigate the correlation between spatial and olfactory cues with the use of the FSB assay.

A parameter which affects learning and memory in honeybees is inter-trial interval. This period designates the amount of time elapsed between trials during a learning assay. The reason this period is important is that memory consolidation occurs in phases and takes time to go from a fragile to a robust state (Menzel 2001; Giurfa 2007). During the current FSB assay, the inter-trial interval was 20min, resulting in a full assay lasting 80min, excluding the time taken to transfer the bees between the Petri dish and the training arena. Because different learning groups were subjected to different learning periods, the amount of memory retention may have differed between groups. From previous studies, we know that short-term memory is normally consolidated in the span of seconds and a few minutes, while intermediate-term memory is consolidated in the span of several minutes to hours (Giurfa and Sandoz 2012). Bitterman et al (1983) aimed to determine how different learning parameters affect PER performance. Results of that study showed that inter-trial intervals of 10min were optimal to achieve approximately 80% PER success. In addition, the researchers found that three learning trials were the minimum number required to generate robust memory which lasts longer, however, increasing the number of trials could also be beneficial (Bitterman et al. 1983). Menzel (1999) emphasized an interesting point related to learning parameters like inter-trial interval and number of trials. During each trial, the test subject must extract the appropriate information which will enable them to complete the task. If the appropriate information is not registered or retained by the test

subject, additional learning trials will not induce improved learning performance: the subject is likely to not learn at all or may stagnate in performance. In my study, the SDI foragers did not demonstrate learning or memory retention during the FSB assay. Because these types of foragers may be learning impaired due to genetics, a preliminary training study may be required to establish how various parameters like inter-trial interval and number of trials affect SDI bees' performance during the FSB assay. Such a preliminary study should involve varying the inter-trial intervals, the number of trials and sucrose concentration, then genotyping the bees to determine which genes/alleles may be associated with an improvement or reduction in performance (Bitterman et al. 1983; Giurfa et al. 2009; Laloi and Pham-Delegue 2010). Similarly, learning criteria could also be used instead of a discrete number of trials to assess performance. The benefit of learning criterion is that they ensure the learned behaviour is achieved, even though the number of trials may differ between individuals. The disadvantage of learning criterion-based studies is that they may require testing more subjects to achieve the appropriate threshold and they may be more labour intensive due to having to conduct more trials overall.

Further on the topic of inter-trial interval is if 20min is appropriate to keep the bee motivated to find the sugar reward. A review on spatial memory in insects briefly discusses how the motivational state of the animal affects the association between navigational memories and the travel goal. In short, the motivational state of the animal appears to affect which memories it uses to find food or its nest-site (Wehner et al. 2006; Collett et al. 2013). During the FSB assay, the motivation test helped to determine if the bee was responsive to 30% sucrose (Scheiner et al. 2001; Scheiner and Arnold 2010): bees which did not drink the sucrose were not included in the experiment. The question then becomes: were the foragers also motivated to find the sugar

reward in the training arena? An ant study demonstrated that unfed ants chose the food-ward path more often than the home-ward path relative to fed ants (Harris et al. 2005). Because the FSB foragers were not fed during the acclimation period in my study, I assumed they would have been in a food-motivated state. In addition, the appetitive learning assay studies referenced throughout this thesis typically used inter-trial intervals of anywhere between 3min to 2h, such that invertebrates were often deprived of food between trials for 5-10min. Based on my personal experience in carrying out the FSB assay, 20min may have been too long, resulting in bees which were too deficient in nutrients to fulfill the spatial learning task. Indeed, there were times when a forager completed a trial but appeared very lethargic. In these cases, I offered a very small amount of sucrose solution, allowing them to drink from a transfer pipette for 1-2sec. Bees which remained lethargic after this brief feeding often did not complete their next trial if they were in the 2TO, 3TO and 3wMT learning groups, and in some cases, died. Reducing the inter-trial interval to 10-15min minutes may increase the survivorship of individuals during the FSB assay.

The final factor I wish to highlight which may have negatively impacted learning in the FSB foragers is the location of the apiary. The Purdue University research apiary is located near many farms: a simple Google search shows that approximately eight farms are within a 10km radius of the apiary, which is within the foraging distance of honeybees (Dukas 2008). These eight farms do not include smaller operations or plots destined for residential use. The proximity to farmland, more often than not, means a proximity to pesticides. The pesticides of most concern when it comes to both wild and native bees are neonicotinoids. This category of pesticides interacts with the bees' nicotinic acetylcholine receptors, resulting in disruption of the central nervous system (Goulson et al. 2015; Klein et al. 2017; Siviter et al. 2018).

Neonicotinoids like imidacloprid, clothianidin and thiamethoxam have all been shown to negatively impact bees with respect to foraging behaviour, learning, memory, social behaviour and colony fitness (Kessler et al. 2015; Tsvetkov et al. 2017; Crall et al. 2018; Lämsä et al. 2018; Muth and Leonard 2019). Because the original study establishing the FSB protocol was conducted in Toronto, the risk of neonicotinoids to the bees may have been lower than in West Lafayette due to the research site being further away from farms. A recently published paper has also shown the negative impacts of commonly used acaricides on honeybee learning. That study demonstrated that of the five different chemicals tested at sub-lethal doses, formic acid produced the worst performance using the PER protocol (Gashout et al. 2020). Relating these findings to the current FSB project, the foragers may not have been able to learn the spatial task due to chronic exposure to pesticides in the surrounding fields. Moving forward, it would be wise to conduct the experiment further away from farms and to be mindful of the integrated pest management strategies used to manage the honeybee colonies to avoid impairing learning and memory.

Age

With respect to age and caste, both appear to play a role in learning. In PER-based studies, the researchers found that foragers with over 15 days of experience performed worse than bees with 6-13 days of experience. Nurse bees did not show this age-dependent difference in learning. A peculiarity of the bees aged 15 days or more was that their learning was very specific in the Behrends study: they did not respond to a new odour suggesting that their ability to generalize was impaired (Behrends et al. 2007; Baker et al. 2012). In the current FSB assay, the youngest bees in the 3wMT group were 17 days old. Based on the Behrends (2007) study, the reason for there not having been an age-related difference in visit or error numbers may be that

learning performance may decline until a plateau is reached. The SDI foragers may have already reached that plateau, resulting in no detectable decline in learning. Unfortunately, the effects of age on honeybee memory do not appear to be conclusive. Some studies say that there is a decline while others claim there isn't (Farris et al. 2001; Arenas et al. 2009; Scheiner and Amdam 2009; Münch et al. 2010). Given that during the FSB assay, older SDI foragers showed an improvement in memory relative to younger foragers for a few trials, more research would be required to determine if this improvement in memory is typical or unusual. Although these age-related effects on learning and memory are important to take into account for the sake of adequate experimental controls, these effects may not be biologically relevant at the colony-level. A foraging efficiency study, focused on pollen collection, found that bees were able to collect more pollen between foraging trips until a maximum of nine days after their transition to forager (Klein et al. 2019). This finding suggests that as long as an appropriate number of bees, with respect to number of larvae, are increasing their foraging performance during those nine days, the colony will be able to obtain enough provisions to sustain the developing larvae. In essence, it may not matter that memory retention changes with age, as long as there are younger bees sustaining the foraging effort in the colony.

Conclusion

Once again, the honeybee life cycle depends on foragers and scout bees being able to navigate their surroundings to find profitable resources like food patches or a new nest site. These insects use dance communication to tell their nestmates about these resources and recruit them to those sites. This form of communication requires the forager or scout to translate spatial and site quality information into a dance such that the dance angle relative to the hive indicates direction while the dance duration indicates distance. The bee may dance more or less vigorously

to indicate the perceived quality of the site. As bees learn about their surroundings through orientation flights and repeated foraging bouts, spatial learning appears to be an indispensable skill for honeybees. The purpose of this project was to use the FSB assay to investigate the molecular mechanisms associated with spatial learning, especially because it is an under-represented learning modality in honeybee literature.

In sum, the FSB learning assay was used to evaluate both the number of visits and number of errors SDI foragers made during the assay. As the initial goal had to been to compare gene expression profiles of different learning stages based on the number of learning trials and a memory test, the SDI bees were divided into five learning categories: MS, 1TO, 2TO, 3TO and 3wMT. In the end, after analyzing the data from the perspectives of visit behaviour, number of errors, simulated errors and age differences, the results repeatedly did not provide evidence for learning in the SDI foragers. Although the numbers of visits and errors decreased somewhat from trials 1 to 3, the number of errors were significantly different from chance in all three trials. If the SDI forager's EC1 and ER1 means had been statistically different from chance, I would have been able to demonstrate learning and would have moved on to the gene expression portion of the project. With respect to the memory tests, in all cases, the mean values were greater than any of the previous trial means, suggesting the bees did not remember their focal flower. Altogether, the results made it impossible to justify moving on the gene expression part of the project.

In trying to understand why my results were not what I expected, I learned many things. Firstly, both reward quality and the rate of depletion have been shown to affect an animal's propensity to return to a food site. These factors may explain why the VEC and VER means decreased somewhat from trial 1 to 3: the bees were not able to deplete the sugar reward,

encouraging them to return to the focal flower which had a high reward relative to the other artificial flowers. In addition, the phenomenon called spontaneous alternation may explain why the VEC and VER means were not the same values, although the differences were small. This phenomenon occurs when an individual is more likely to visit previously unvisited sites vs. previously visited ones. For a honeybee forager, this behaviour allows them to assess relative reward quality between food patches and possibly discover new food sources. This new information can be communicated to their nestmates using dance language, allowing the bees to optimize their exploitation of various food sources throughout the colony growth season. In flying between the nest and food resources, honeybees must rely on various navigation strategies to determine where to go. These navigation strategies involve creating images based on landmark cues, while also incorporating information about distance and direction travelled, to reach a goal efficiently. With respect to the FSB assay, bees which relied on position- and image-based guidance systems were more likely to perform well and require fewer visits to reach the focal flower relative to bees which relied only on path integration.

In dissecting the error data, I found that learning and memory are complicated processes. To achieve robust learning and memory, multiple learning trials are required. Although many studies use just three trials during honeybee learning assays, increasing the number of trials can induce better, longer-lasting memory. In addition, the inter-trial interval must be appropriately selected to ensure the different phases of memory formation are properly consolidated. Another challenge associated with selecting an inter-trial interval is balancing this duration with the insect's metabolism. Waiting too long between trials without feeding the forager can result in greater mortality rates of test subjects, as was seen during this project. In addition, previous studies have demonstrated that both visual and olfactory cues can be used to reinforce spatial

learning. However, if these cues are in conflict, the individual's spatial learning may be negatively impacted. This type of conflict between cues may explain why the SDI foragers did not learn: the visual cues within the training arena may have conflicted with the spatial cues. In the FSB assay, the bees may have been using olfactory cues from antennating the focal flower or following their own scent trails to reinforce spatial learning. The SDI foragers would have also benefited from using visual cues in the landmarks around the training arena to better orient themselves and make fewer errors before finding the focal flower.

Given the challenges I experienced with the FSB assay, I can suggest some modifications to the experimental design which may improve learning performance in the honeybees. In comparing the SDI foragers to the regular foragers, I noticed that the decrease in the mean number errors between trials was more evident in the regular vs. the SDI bees. This finding suggests that using bees from colonies headed my naturally mated queens is better than those from SDI colonies, even if the end goal of the experiment is to perform genetic comparisons. The use of regular foragers may increase the chance of detecting statistically significant learning trends. Alternatively, the use of learning criteria, as oppose to a discrete number of trials, allows the researcher to confirm that learning is occurring during the assay instead of making this confirmation later, such as during the statistical analysis. However, the use of learning criteria normally means that the experimental design is more labour intensive because the researcher must test more subjects in order to reach the pre-determined learning threshold. Additionally, the FSB training arena can be placed in a location where the surroundings have landmarks which are easily detected by honeybees. These landmarks may make it easier for the bee to locate the focal flower by providing visual cues which help the bee orient itself.

The design of the FSB assay makes it an easy protocol to use to study spatial learning. Various aspects of the assay can be modified to assess a given variable. For example, the learning proficiencies of different castes, bee species and source colonies can all be compared simply by subjecting the individuals to the same assay, then determining which ones are better or worse learners. As SDI bees are used less frequently in learning assays than regular bees, the different parameters of the FSB assay can be modified to determine which parameters optimize learning in a specific colony of SDI bees, such as inter-trial interval and number of trials. In addition, one could vary the surroundings of the training arena to determine if certain factors (like colour, degree of contrast, and shape) affect spatial learning. On another note, the FSB arena itself can be modified to better understand foraging behaviour in honeybees. As mentioned earlier, it appears only one study has attempted to determine the propensity of honeybees to win-shift vs. -stay. By increasing the size of the FSB arena and arranging the cotton swabs differently, the factors affecting win-shift vs. -stay behaviour in honeybees could be elucidated further. A different avenue for the FSB assay could be to use this protocol to investigate the nature of information communicated during the waggle dance. For example, if FSB trained honeybees are paint-marked and returned to their source colony, do the non-trained bees from that colony perform better relative to bees from colonies which have no FSB-trained bees? If the former set of bees perform better, such evidence may indicate that dance communication may relay more information between nestmates than initially thought. Finally, the gene expression portion of this project was never realized. In the future, the current FSB project could be repeated, using some of the modifications mentioned above, to investigate the genetic and epigenetic mechanisms associated with spatial learning.

Tables

Table 2 **: Possible learning categories.** These learning categories were randomly assigned to each forager before she began her set of trials.

Learning Assay Category	Abbreviation	Definition
Naïve Foragers	MS	Bees which demonstrated that they were motivated and drank the 30% sugar water from the cap within the Petri dish. These bees did not undergo a learning trial and were immediately frozen after demonstrating the motivation state
1 Learning Trial Foragers	1TO	After these bees demonstrated that they were motivated, they underwent one learning trial and were immediately frozen after completion
2 Learning Trial Foragers	2TO	After these bees demonstrated that they were motivated, they underwent two learning trials and were immediately frozen after completion
3 Learning Trial Foragers	3TO	After these bees demonstrated that they were motivated, they underwent three learning trials and were immediately frozen after completion
3 Learning Trial with Memory Test Foragers	3wMT	After these bees demonstrated that they were motivated, they underwent three learning trials with a memory test and were immediately frozen after completion

***Table 2*: Sample data collected for trial 1.** This table shows the type of data which were collected throughout the Food Search Box (FSB) project. Only the data for trial 1 are displayed however, the same variables were observed for each of the other trials as well. Columns V1 through ER1 represent the numbers of visits, mistakes and errors for trial 1. The time (T1) is given in seconds.

Trial Date	Bee Type	Bee ID	Experi-mental Group	Correct Flower	S1	V1	VEC1	VER1	EC1	ER1	T1
July 10, 2019	Purdue Apiary Regular Foragers	PRE5	3wMT	**4**	**2, 4**	2	1	1	1	1	470
July 10, 2019	Purdue Apiary Regular Foragers	PRE6	3wMT	**1**	**1, 2, 2, 3, 3, 3, 2, 2**	8	0	7	0	2	1800
July 10, 2019	Purdue Apiary Regular Foragers	PRE7	3wMT	**3**	**2**	1	1	1	1	1	494
July 12, 2019	Purdue Apiary Regular Foragers	PRF1	3wMT	**2**	**1**	1	1	1	1	1	1800
July 12, 2019	Purdue Apiary Regular Foragers	PRF10	3wMT	**3**	**3, 2, 4, 2, 1, 2, 1, 4, 1**	9	0	8	0	3	1319
July 12, 2019	Purdue Apiary Regular Foragers	PRF11	3wMT	**4**	**1**	1	1	1	1	1	1800
July 12, 2019	Purdue Apiary Regular Foragers	PRF12	3wMT	**2**		0	0	0	0	0	1800
July 12, 2019	Purdue Apiary Regular Foragers	PRF13	3wMT	**1**		0	0	0	0	0	1800

Table 3 : **Sample summary data collected for August 1st, 2019.** This table shows the type of data which were collected for each bee on August 1st, 2019. Similar information was summarized for each of the other bees which were included in the Food Search Box (FSB) project.

Trial Date	DOB	Colour	Bee ID	Origin	Group	Outcome
August 1 , 2019	N/A	N/A	PRP1	Purdue Apiary Regular Foragers	3 Trials with Memory Test	DID NOT REMEMBER
August 1 , 2019	N/A	N/A	PRP2	Purdue Apiary Regular Foragers	3 Trials with Memory Test	REMEMBERED
August 1 , 2019	N/A	N/A	PRP3	Purdue Apiary Regular Foragers	3 Trials with Memory Test	REMEMBERED
August 1 , 2019	N/A	N/A	PRP4	Purdue Apiary Regular Foragers	3 Trials with Memory Test	REMEMBERED
August 1 , 2019	July 11, 2019	BLUE	PS1H3	Purdue Apiary SDI Foragers I	Motivation State	COMPLETE
August 1, 2019	July 11, 2019	BLUE	PS1H1	Purdue Apiary SDI Foragers I	3 Trials Only	COMPLETE
August 1, 2019	July 11, 2019	BLUE	PS1H5	Purdue Apiary SDI Foragers I	3 Trials with Memory Test	DID NOT REMEMBER
August 1, 2019	July 11, 2019	BLUE	PS1H4	Purdue Apiary SDI Foragers I	3 Trials with Memory Test	REMEMBERED
August 1, 2019	June 28, 2019	YELLOW	PS2I5	Purdue Apiary SDI Foragers II	2 Trials Only	COMPLETE
August 1, 2019	June 28, 2019	YELLOW	PS2I3	Purdue Apiary SDI Foragers II	3 Trials with Memory Test	DID NOT REMEMBER
August 1, 2019	June 28, 2019	YELLOW	PS2I1	Purdue Apiary SDI Foragers II	Motivation State	COMPLETE

Table 4 **: Breakdown of number of foragers collected for each learning category.** The number of foragers which completed their randomly assigned number of trials varied between learning categories. SD1 and SD2 refer to two separate Single Drone-Inseminated (SDI) colonies.

Origin	Learning Category - Outcome	Number of foragers	Total Numbers	
Regular Foragers	3wMT – Remembered	14	25	95
	3wMT – Did not remember	11		
SD1 Foragers	Motivation Sate	7	33	
	1TO	5		
	2TO	4		
	3TO	4		
	3wMT – Remembered	9		
	3wMT – Did not remember	4		
SD2 Foragers	Motivation Sate	8	37	
	1TO	6		
	2TO	7		
	3TO	4		
	3wMT – Remembered	10		
	3wMT – Did not remember	2		

***Table 5* : Mean number of errors by trial for both regular and Single Drone-Inseminated (SDI) foragers.** This table compares which mean errors are statistically different from chance using a Wilcoxon ranked-sum test.

Origin	Trial	Number of Errors	Different from chance (p-value < 0.05)?
Regular Foragers	EC1	0.857	No
	EC2	0.571	Yes
	EC3	0.143	Yes
	EC4	1.21	No
	ER1	0.929	No
	ER2	0.643	Yes
	ER3	0.286	Yes
	ER4	1.79	No
SDI Foragers	EC1	0.737	Yes
	EC2	0.526	Yes
	EC3	0.579	Yes
	EC4	1.37	No
	ER1	0.737	Yes
	ER2	0.526	Yes
	ER3	0.842	Yes
	ER4	2.11	Yes

***Table 6* : Errors simulation results.** A simulation was carried out in R to approximate the collective number of errors for a given trial, based on two types of behaviour. The simulation compared the real-world number of errors to the simulated number of errors using a t-test.

Simulation	Trial	Number of Foragers Tested	Actual Errors	Approx. Difference in Errors (Simulation - Real-world)	Statistically Significant Difference (p-value < 0.05)?
Stepping-stone	1	49	46	175	Yes
	2	38	27	125	Yes
	3	27	21	100	Yes
	Memory Test	19	40	70	Yes
Random Error	1	49	46	30	Yes
	2	38	27	30	Yes
	3	27	21	20	Yes
	Memory Test	19	40	-10	Yes

Figures

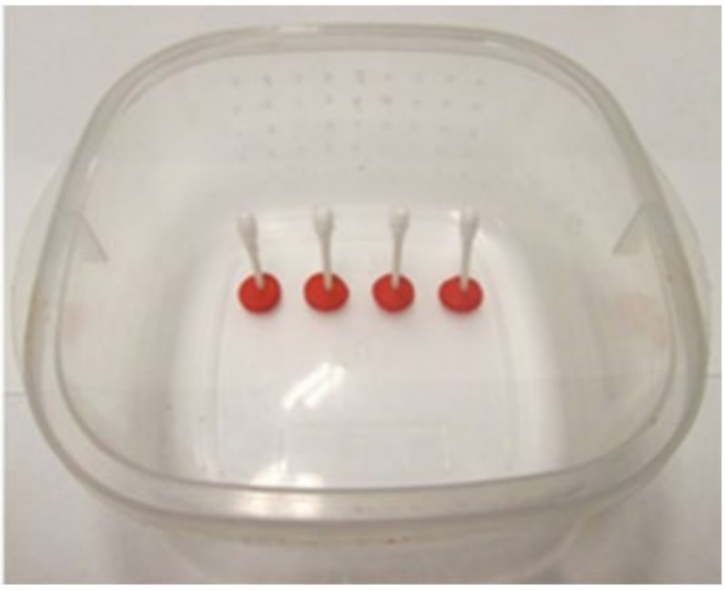

***Figure 2*: Food Search Box arena.** The honeybees were trained to identify an artificial flower in a plastic training arena measuring 12.5cm x 12.5cm x 6.5cm (908mL). The cotton swabs were maintained in place using plasticine and the arena was cleaned with 70% ethanol between trials to eliminate scent trails. (Tsvetkov et al. 2018). During training, the arena's lid was placed to prevent the bees from flying out. Figure reproduced with permission from Tsvetkov et al.

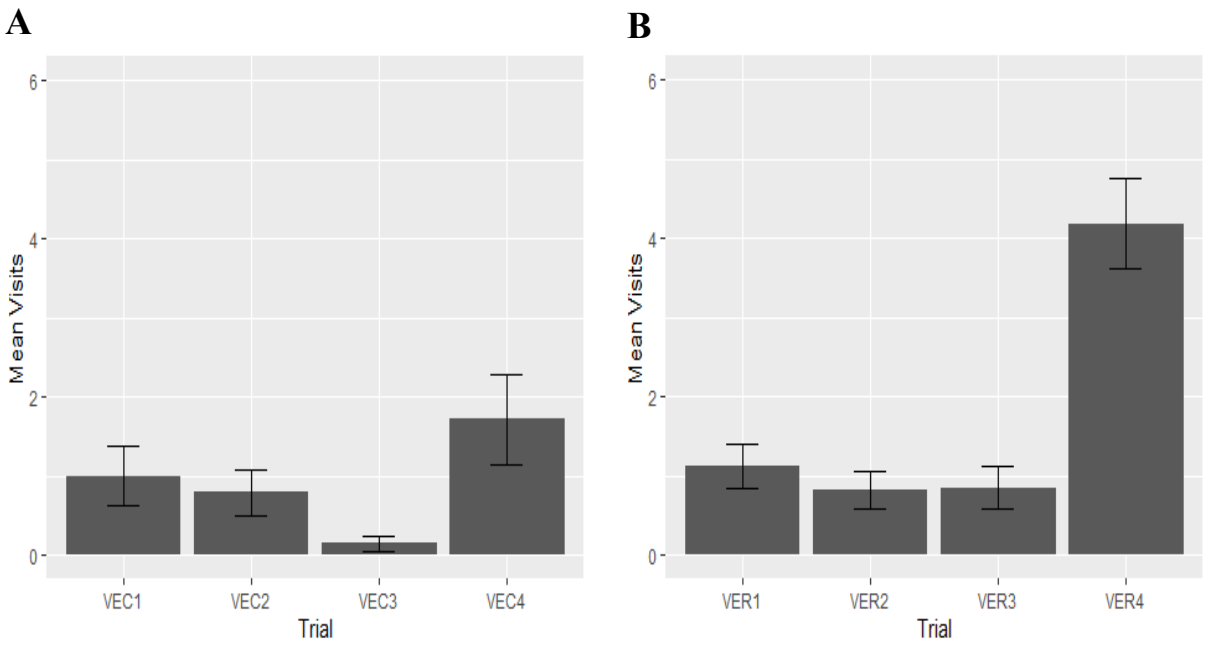

***Figure 2* : Number of visits for regular foragers. A)** This figure shows the mean number of visits before landing on the correct flower. **B)** This figure shows the mean number of visits before the reward. The confidence intervals represent the standard errors. Only the VER3-VER4 pair of variables were statistically different from each other (Kruskal pairwise comparison, p-value < 0.008, Bonferroni-corrected).

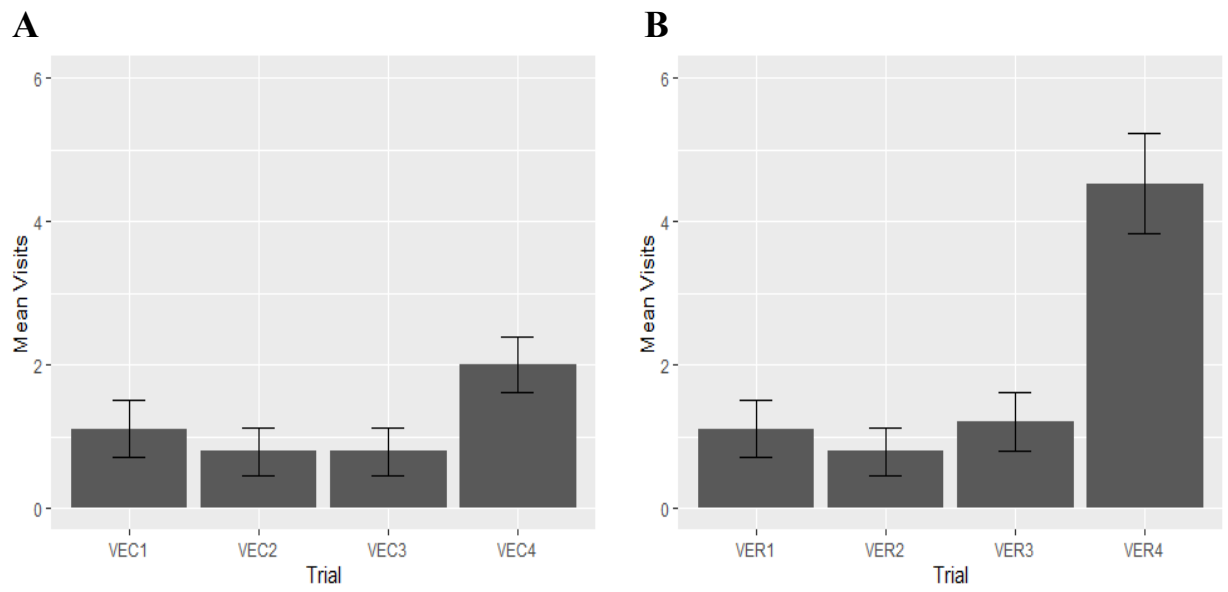

Figure 3 **: Number of visits for Single Drone-Inseminated (SDI) foragers. A)** This figure shows the mean number of visits before landing on the correct flower. **B)** This figure shows the mean number of visits before the reward. The confidence intervals represent the standard errors. Only the VER1-VER4, VER2-VER4 and VER3-VER4 pairs of variables were statistically different from each other (Kruskal pairwise comparison, p-value < 0.008, Bonferroni-corrected).

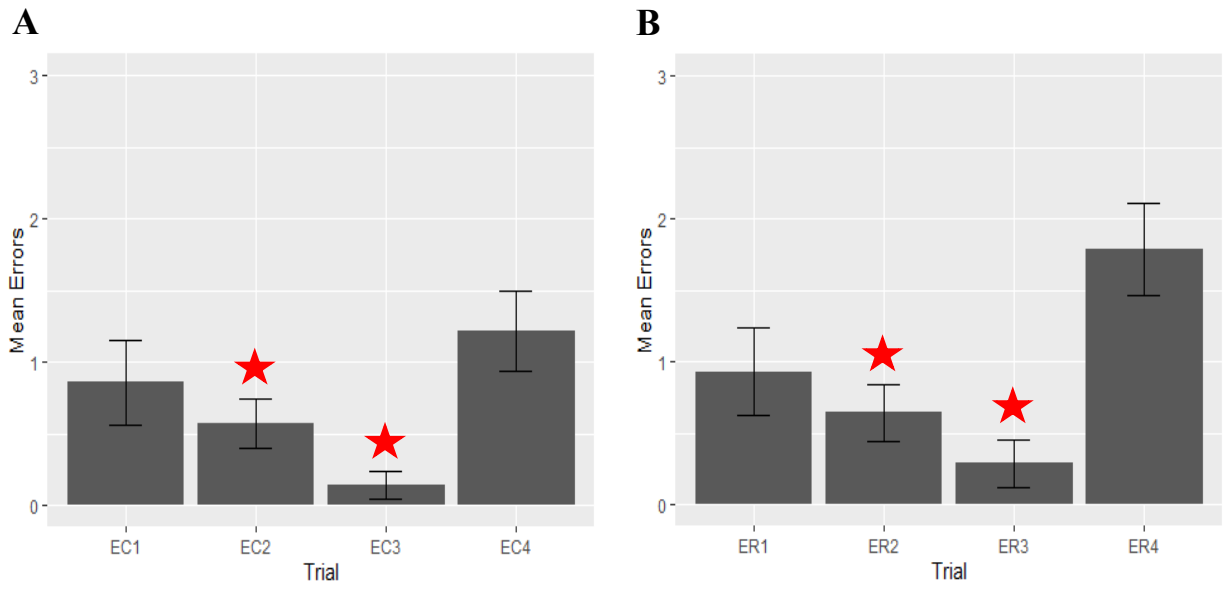

Figure 4 **: Number of errors for regular foragers. A)** This figure shows the mean number of errors before landing on the correct flower. **B)** This figure shows the mean number of errors before the reward. The confidence intervals represent the standard errors. Only the ER3-ER4 pair of variables were statistically different from each other (Kruskal pairwise comparison, p-value < 0.008, Bonferroni-corrected). Means which were significantly different from chance are indicated using a star (Wilcoxon signed-rank test, p-value < 0.05).

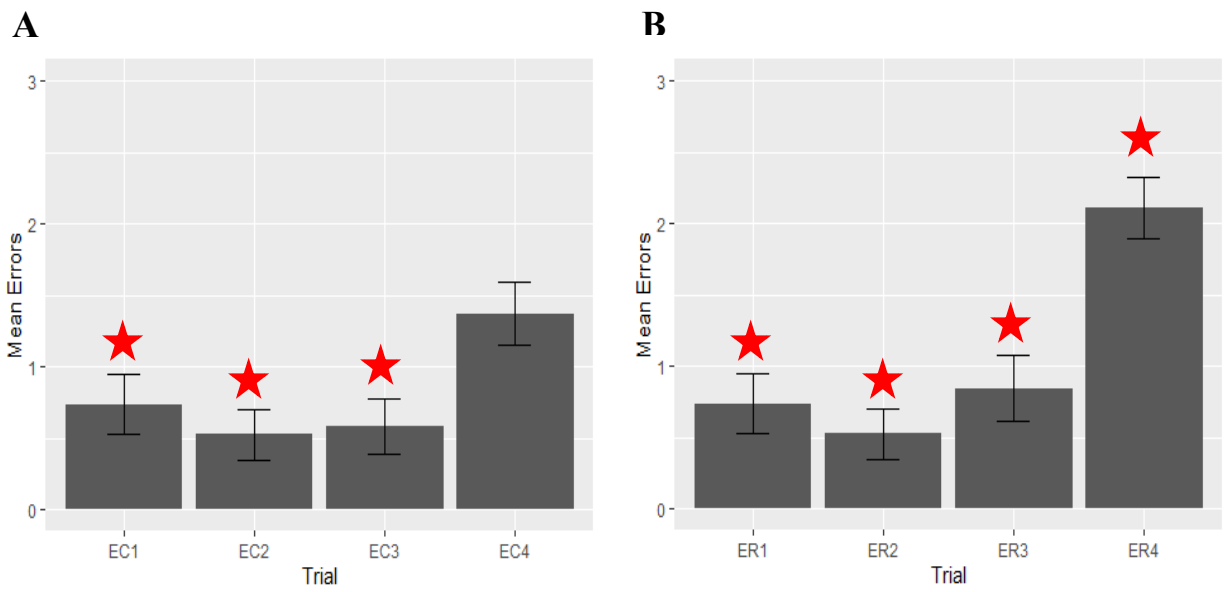

***Figure 5* : Number of errors for Single Drone-Inseminated (SDI) foragers. A)** This figure shows the mean number of errors before landing on the correct flower. **B)** This figure shows the mean number of errors before the reward. The confidence intervals represent the standard errors. Only the ER1-ER4, ER2-ER4 and ER3-ER4 pairs of variables were statistically different from each other (Kruskal pairwise comparison, p-value < 0.008, Bonferroni-corrected). Means which were significantly different from chance are indicated using a star (Wilcoxon signed-rank test, p-value < 0.05).

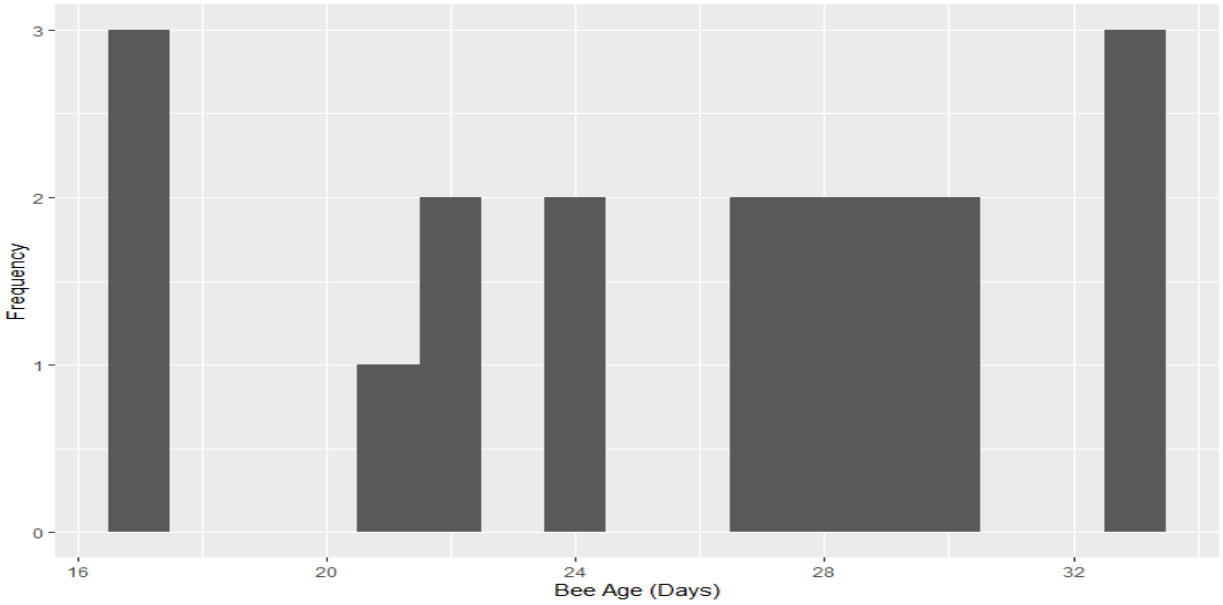

***Figure 6* : Histogram of Single Drone-Inseminated (SDI) forager ages.** This graph includes only bees which completed all three trials and the memory test (3wMT learning category).

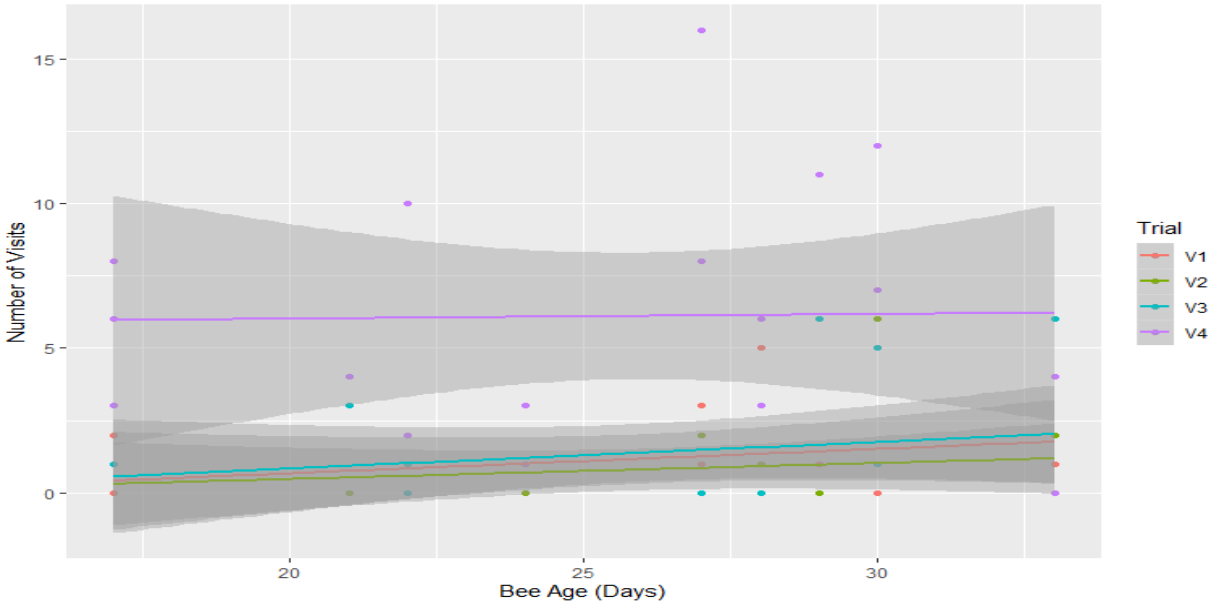

Figure 7 **: Relationship between bee age and total number of visits (V) for Single Drone-Inseminated (SDI) foragers.** This graph includes only bees which completed all three trials and the memory test (3wMT learning category). The shaded areas represent 95% confidence intervals. None of the correlations were statistically significant (all p-value > 0.05). Using a GLM based on the Poisson distribution, only the V4 slope was statistically significant (p-value < 0.05).

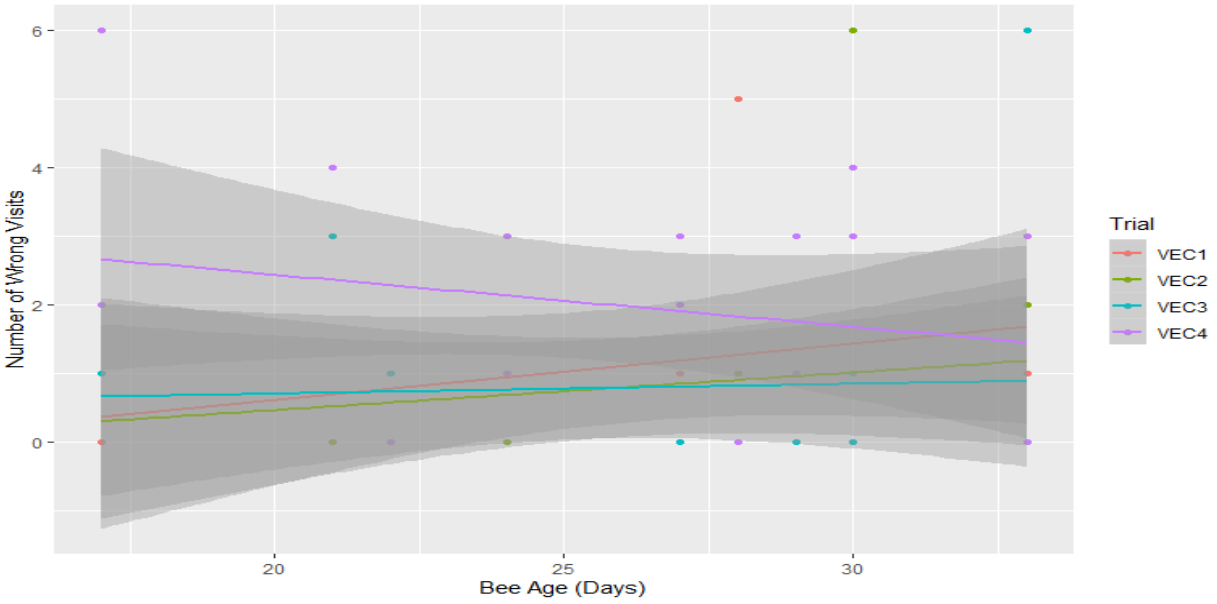

Figure 8 **: Relationship between bee age and number of visits before landing on the correct flower (VEC) for Single Drone-Inseminated (SDI) foragers.** This graph includes only bees which completed all three trials and the memory test (3wMT learning category). The shaded areas represent 95% confidence intervals. None of the correlations were statistically significant (all p-value > 0.05). Using a GLM based on the Poisson distribution, only the VEC4 slope was statistically significant (p-value < 0.05).

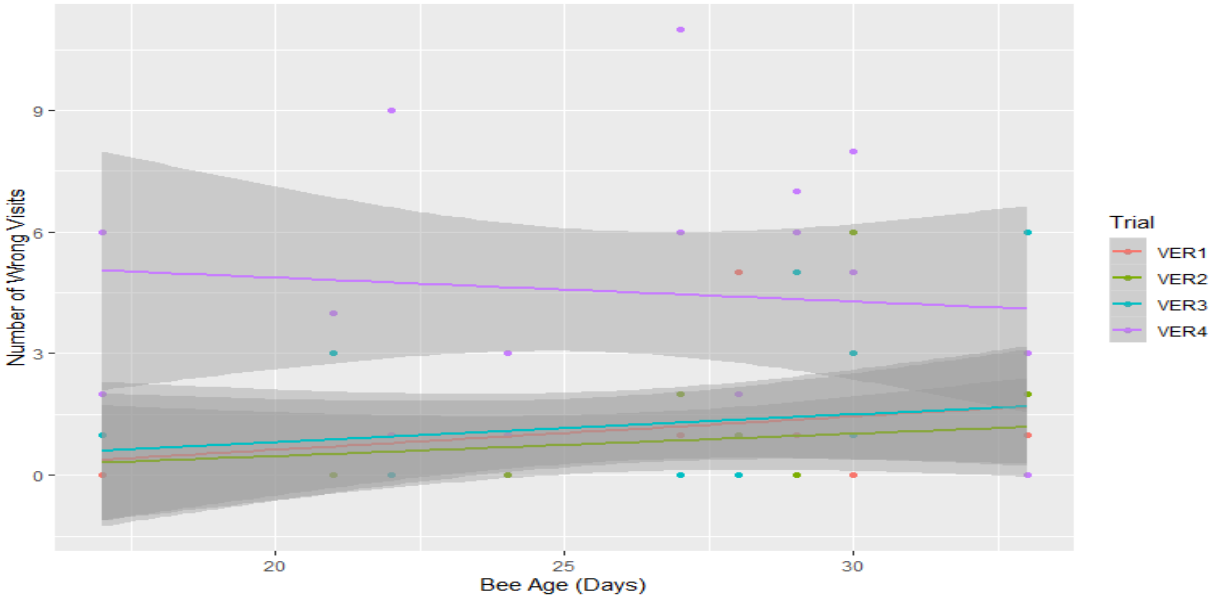

Figure 9 **: Relationship between bee age and number of visits before the reward (VER) for Single Drone-Inseminated (SDI) foragers.** This graph includes only bees which completed all three trials and the memory test (3wMT learning category). The shaded areas represent 95% confidence intervals. None of the correlations were statistically significant (all p-value > 0.05). Using a GLM based on the Poisson distribution, only the VER4 slope was statistically significant (p-value < 0.05).

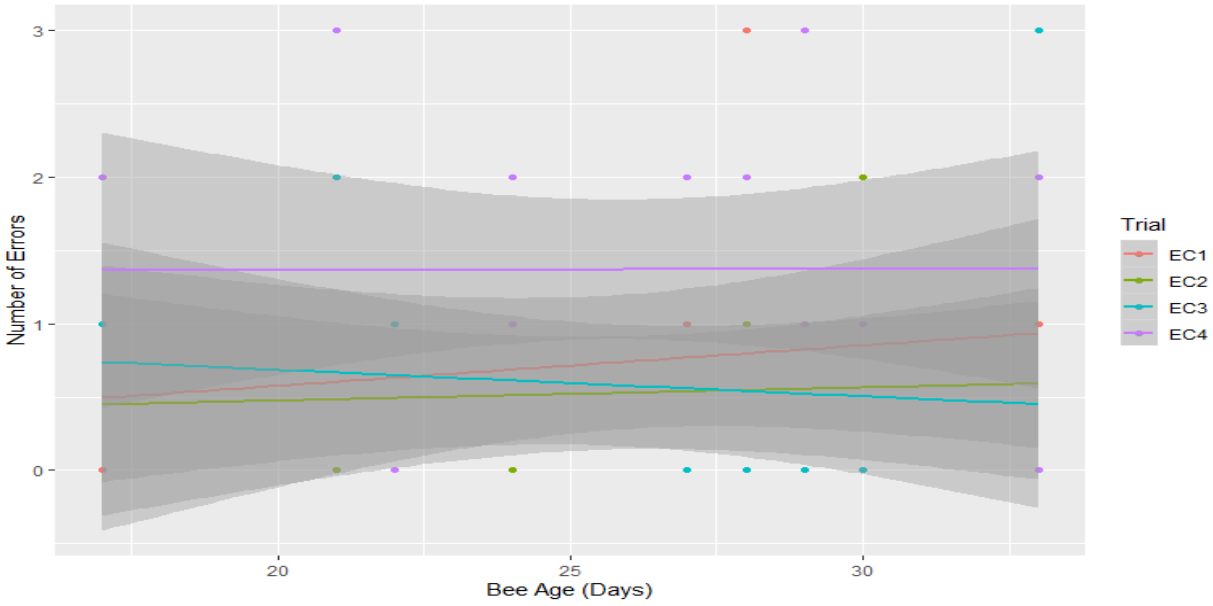

Figure 10 **: Relationship between bee age and number of errors before the correct flower (EC) for Single Drone-Inseminated (SDI) foragers.** This graph includes only bees which completed all three trials and the memory test (3wMT learning category). The shaded areas represent 95% confidence intervals. None of the correlations were statistically significant (all p-value > 0.05). Using a GLM based on the Poisson distribution, none of the slopes were statistically significant (all p-values > 0.05).

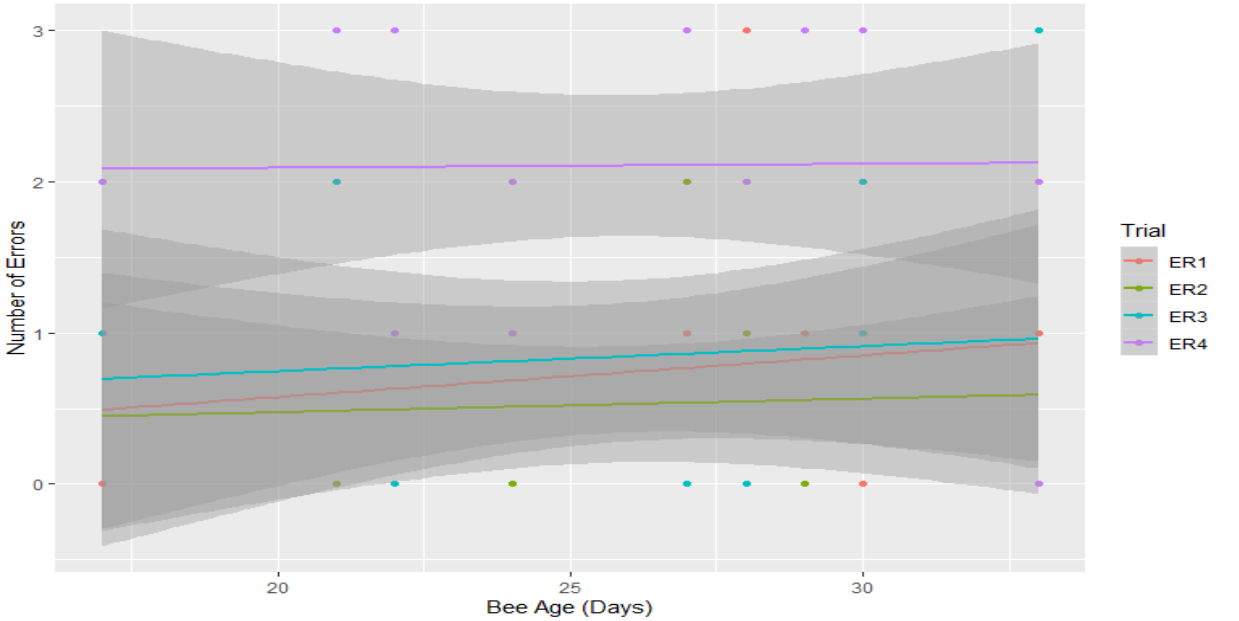

Figure 11 **: Relationship between bee age and number of errors before the reward (ER) for Single Drone-Inseminated (SDI) foragers.** This graph includes only bees which completed all three trials and the memory test (3wMT learning category). The shaded areas represent 95% confidence intervals. None of the correlations were statistically significant (all p-value > 0.05). Using a GLM based on the Poisson distribution, none of the slopes were statistically significant (all p-values > 0.05).

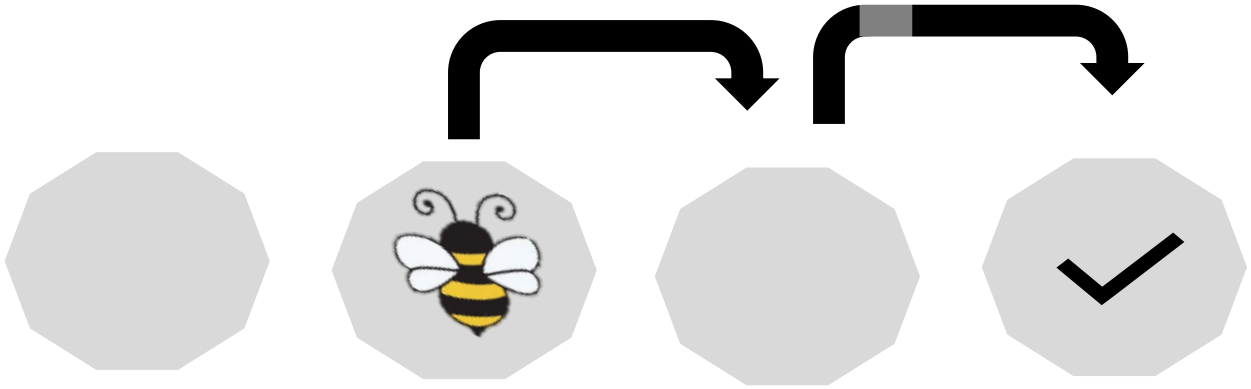

Figure 12 **: Graphical representation of the "Stepping-stone" simulation.** In this simulation, we assume the bee can only travel to the correct flower by first landing on an adjacent flower to her current position. It will take her more attempts to reach the correct flower.

Figure 13 **: Graphical representation of the "Random error" simulation.** In this simulation, we assume the bee can travel to the correct flower in whatever order she wants, so she can reach the correct flower in fewer attempts.

Chapter 2: Review of Gene Expression in the Context of Learning and Memory

Introduction

Although my FSB assay results did not provide strong evidence for learning or memory in the bees, I would still like to briefly review the existing body of literature on brain gene expression as it relates to learning and memory in honeybees. In this section, I will provide an overview of the molecular mechanisms underlying learning and memory then briefly discuss the associated genes and epigenetic factors, including predictions regarding genes which may be specifically associated with spatial learning in the context of the current FSB experimental design.

Molecular Processes

The molecular processes underlying learning and memory in honeybees take place in phases which occur in parallel and in sequence. These molecular processes allow learning to be encoded as memories which can be retrieved in a context-dependent way. As learning takes place, these memories are consolidated in different phases: early short-term (eSTM), late short-term lSTM, mid-term (MTM), early long-term (eLTM) and late long-term (lTLM) memory (Collett and Kelber 1988; Menzel 1999). Different phases of memory are consolidated in different brain regions. For example, the antennal lobes and mushroom bodies are associated with eSTM and lSTM, respectively. (Menzel 1999; Giurfa and Sandoz 2012). The mushroom bodies are known to receive many sensory inputs such as olfactory, visual and mechanosensory, so this brain region is suspected to be a site of sensory information integration (Menzel 2012; Collett et al. 2013). Using olfactory learning assays like PER, scientists determined that the VUMmx1 neuron is the major neuron which relays olfactory signals from the antennae and

proboscis to the brain. By replacing the sugar reward during PER assays with different neurotransmitters, they also found that this neuron is sensitive to octopamine such that it can replace the sugar reward entirely and still elicit PER (Hammer and Menzel 1995; Meller and Davis 1996; Menzel 1999; Menzel 2001; Schwärzel and Müller 2006; Giurfa and Sandoz 2012). In order to achieve robust early stages of memory, very few learning trials are required. Both STM and MTM do not require protein synthesis however, transcription may still occur depending on context (Schwärzel and Müller 2006; Giurfa 2015). The transition from consolidation of STM to MTM requires many learning trials, with appropriate timing. Too many trials too quickly lead to poor memory retention, which is why spaced trials are the preferred timing (Hammer and Menzel 1995; Menzel 1999; Giurfa 2015).

When it comes to cell signalling involved in learning and memory, some of the best characterized pathways are cAMP/PKA, calcium/PKC, CREB and acetylcholine. Through pharmacological studies, researchers found that low PKA activity specifically impairs LTM, but not the other phases, while blocking nicotinic acetylcholine receptors also induces LTM impairment (Meller and Davis 1996; Menzel 1999; Menzel 2001; Dupuis et al. 2012; Menzel 2012). In addition, the transition from MTM to LTM is associated with nitrous oxide activity, through protein kinase cell signalling. PKA activity begins to increase during MTM and remains high during LTM (Menzel 1999; Schwärzel and Müller 2006). Although LTM is characterized by both transcription and *de novo* protein synthesis, studies using various translation inhibitors revealed that different inhibitors affect LTM differently thereby suggesting that LTM is a two-stage process. Furthermore, LTM in honeybees is curious because memory up to three days after training trials appears to not require protein synthesis. This phenomenon reveals that much more work is required to elucidate the inner-workings of eLTM and lLTM (Meller and Davis 1996;

Menzel 1999; Eisenhardt 2014). Some factors which are known to modulate LTM formation are protein degradation mechanisms and changes in transcription which are due to alternative splicing of CREB (Menzel 1999; Eisenhardt 2014). Moreover, in addition to sensory information, honeybees must store vectoral and distance information in order to navigate. Together with sensory information, these navigation cues help to generate different types of memories which can be applied to everyday tasks such as foraging (Menzel et al. 2006).

Differentially Expressed Genes

The early stages of learning and memory are characterized by Immediate Early Genes (IEGs). These are a set of genes which are expressed differentially immediately after a new behavioural state is achieved. They are normally expressed rapidly and transiently (Sommerlandt et al. 2019). By comparing nurses vs. foragers and bees engaging vs. not engaging in orientation flights, Ugajin et al. identified possible candidate IEGs associated with learning. The researchers found that Kakusei, Early Growth Response protein 1 (EGR1), Hormone receptor 38, Src homology 3 domain-Binding Kinase 1 (SBK1) and Family with sequence similarity protein 46 (FAM46) were all differentially expressed between treatment groups (Ugajin et al. 2018). These findings are supported by similar studies which focused on foraging behaviour (Singh et al. 2018; Singh et al. 2020). Although the direct links to learning have not been established for these genes, their identification during behaviours which are known to rely on learning and memory provides good evidence to support their possible differential expression during learning assays like the FSB. Being IEGs, the change in expression for these genes would likely be detected in the MS and 1TO learning groups.

Multiple genes have been shown to be differentially expressed in response to learning and memory. Particularly, biogenic amines like octopamine, tyramine, dopamine and their receptors

have each been shown to have varying levels in response to a learning protocol or between honeybee castes. (Wagener-Hulme et al. 1999; Agarwal et al. 2011; Klappenbach et al. 2013; Sinakevitch et al. 2017; Mancini et al. 2018; Cook et al. 2019). Briefly, these neurotransmitters mainly interact with their own respective G protein-coupled receptors (GPCRs), and in some cases, the same receptors. When activated, these GPCRs go on to activate cAMP and calcium mediated pathways (Grohmann et al. 2003; Kurshan et al. 2003; McQuillan et al. 2012; Thamm et al. 2013; Raza and Su 2020). As mentioned earlier, both calcium and cAMP signalling play an important role in memory formation. The reason I believe these genes may be differentially expressed following the FSB assay is that their levels have already been shown to change in response to learning protocols. In addition, as the use of many cues at once can reinforce learning, the olfactory cues present during the FBS assay may lead to VUMmx1 neuron activation, a pathway which appears to overlap with octopamine signalling. As the other neurotransmitters are also part of the insect reward system (Perry and Barron 2013), their mRNA levels and the expression of their receptors (Bonnafé et al. 2015; Bonnafé et al. 2017) are likely to change during the FSB assay too. Through PER-based assays, another neurotransmitter called GABA has been shown to affect memory through the activity of glutamate-gated chloride channels (El Hassani et al. 2008; Démares et al. 2014). For the same reasons highlighted for the biogenic amines, I suspect that both GABA and chloride-gated channel mRNAs would be differentially expressed during the FSB assay, especially due to their role in neuron signalling at synapses (Ganeshina and Menzel 2001; Barbara et al. 2005).

Aside from neurotransmitters, other genes have also been associated with learning and memory. neuroligins and neurexins are proteins which bind to each other during sensory input and are preferentially expressed in mushroom bodies. Compared to untrained bees, their levels

show an increase in expression during PER assays in trained honeybees (Biswas et al. 2010; Cristino et al. 2014). As spatial learning involves integrating multiple visual cues like landmarks, I suspect a similar change in expression for both genes during the FSB assay. Because learning is a part of navigation, gene expression studies investigating behaviours like orientation flight, foraging and dance communication can help identify other candidate DEGs associated with spatial learning. Examples include the foraging gene (Ben-Shahar 2005), odour receptors (Claudianos et al. 2014) and circadian rhythm genes like slowpoke (Sen Sarma et al. 2009). The foraging gene is particularly interesting because it is associated with the onset of foraging in honeybees. It is highly expressed in mushroom bodies and its expression increases during the transition from nurse to forager. This transition is coupled with other physiological changes including an increase in size of the mushroom bodies, a change which is suspected of helping the bee handle the greater cognitive challenges of being a forager (Heylen et al. 2008; Antos et al. 2009; Matsumoto et al. 2014; Thamm and Scheiner 2014; Thamm et al. 2018). As the current FSB experimental design used foragers, I imagine that this gene would be highly expressed during the assay, possibly higher in high vs. low performance learners.

From full transcriptomic studies, a number of other candidate genes which may be differentially expressed during the FSB assay can also be identified. A study which focused on visual pattern discrimination is relevant because a portion of spatial learning involves visual cues: the bee must be able to use the surroundings to orient itself in space in order to locate the focal flower. In this visual pattern discrimination study, the most notable miRNAs detected were associated with nicotinic acetylcholine receptors, neuroactive ligand-receptor interactions, basic metabolic pathways and basic cell signalling pathways like MAPK (Qin et al. 2014). In two olfactory learning -based studies, the most important differentially expressed mRNAs or proteins

the researchers found were odorant binding proteins, a chemosensory protein (incidentally, the visual pattern study found miRNAs associated with the same chemosensory protein), neurotransmitter receptors (including GABA), acetylcholine receptors and synaptic proteins (Wang et al. 2013; Zhang et al. 2015). These olfaction-based studies are relevant to the FSB assay because during the training trials, the foragers possibly located the focal flower by smelling the sugar reward. From these three full transcriptome studies, it appears the following genes are consistently differentially expressed in response to any learning modality: acetylcholine receptors and neural function genes. In a future experiment using the FSB assay to determine which DEGs are associated with spatial learning, I would expect some degree of overlap with these three studies as many genes are required for both learning and memory consolidation. I would also expect for some patterns of gene expression to be specifically associated with spatial learning because it relies on proprioceptive processes which may not involve olfaction or visual perception.

Epigenetics

Gene expression is controlled by epigenetic factors. These include but are not limited to DNA methylation and histone modification. Most of what is currently known about honeybee epigenetics in learning and memory have to do with DNA methylation. For the first time in 2010, researchers found evidence to suggest DNA methylation inhibition affected memory retention in honeybees during the PER assay, depending on the timing of the inhibition treatment (Lockett et al. 2010). More work was done on this subject leading to the confirmation that DNA methylation plays a role in a bee's ability to discriminate a rewarded odour from a new unrewarded odour during PER (Biergans et al. 2012). In order to elucidate the molecular mechanisms underlying these methylation-related changes in learning and memory, researchers went on to perform DNA

methylation studies on specific genes or at a genome-wide level. In both studies, they found that following PER, learning- and memory-associated genes were differentially methylated like a GABA receptor, CREB, syntaxin, synaptojanin, neurexin and the foraging gene. The genes in this list are associated with cell signalling in neurons. In addition, they found that genes encoding methyltransferases were also differentially methylated, suggesting a feedback loop in epigenetic processes associated with learning and memory (Biergans et al. 2015; Li et al. 2017). Moreover, the FSB assay can be used to elucidate the epigenetic factors associated with spatial learning in a future study, as oppose to the many PER-based studies which focus on olfactory learning. Such an experiment should include the use of zebularine, a common methyltransferase inhibitor, to determine what impact DNA methylation may have. The study could also include a part which determines DNA methylation patterns in different genes, then compare these to known DEGs associated with memory. The benefit of this comparison would be to determine if the changes in gene expression are due to changes in methylation patterns.

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