Abstract

The study of eusocial insects such as honeybees (*Apis mellifera*) has seen advancements in recent decades. This study offers a contribution to the understanding of choice behavior in honeybees, by conducting experiments on the *ligustica* and *carnica* races in a T-maze assay. Experiments were carried out using two different factors to motivate choice: In a social choice experiment newly emerged and adult *carnica* winter bees and newly emerged *ligustica* bees were presented with the choice between a familiar bee and an unfamiliar bee. In a feeding partner experiment adult *carnica* winter bees and two genetic strains of newly emerged *ligustica* bees, high pollen hoarders (highs) and low pollen hoarders (lows), were presented with the choice between two unfamiliar bees which had been fed high (50%) and low (10%) concentrations of sucrose.

Results showed that the T-maze can be a useful tool in studying the choice behavior in honeybees. Adult *carnica* winter bees showed a bias towards the unfamiliar bee, while newly emerged bees were consistent in that they did not share this preference, indicating a development in choice behavior with age. The low pollen hoarders were compared with the high pollen hoarders and showed a relative bias towards the high sucrose bees, possibly owing to their lower sensitivity towards sucrose. Results also indicate that the lows had a higher activity level than the high. However, inconsistency between the observed and calculated rates of movement suggests that the sampling frequency in this experiment was too low to accurately capture the activity level.

Sammendrag

Studien av eusosiale insekter som honningbia (*Apis mellifera*) har utviklet seg de siste tiår. Denne studien gir et bidrag til forståelsen av valgatferd hos honningbia, ved at eksperimenter med bier fra de to rasene *ligustica* og *carnica* har blitt utført i en T-labyrint. Experimentene utforsket to motivasjoner: I eksperimenter med nykrøpne- og voksne vinterbier av *carnica*-rasen,og nykrøpne bier av *ligustica*-rasen, ble sosiale valg utforsket ved biene fikk velge mellom en kjent og en ukjent bie. I fôringspartner-eksperimenter fikk voksnebier fra *carnica*-rasen og nykrøpne bier fra to genetiske stammer av *ligustica*-rasen, "high pollen hoarders" (highs) og "low pollen hoarders" (lows) , valget mellom to ukjente bier som hadde blitt fôret med høy (50%) og lav (10%) konsentrasjon av sukkervann.

Resultatene viste at T-labyrinten kan være et nyttig verktøy i studiet av valgatferd hos honningbia. Voksne *carnica*-vinterbier viste en tilbøyelighet til å velge ukjent bie, mens de nykrøpne var konsekvente i den forstand at de ikke delte denne preferansen, noe som indikerer at valgatferd utvikles med alderen. "Low pollen hoarders" ble sammenlignet med "high pollen hoarders", og utviste større preferanse for bia som hadde spist høy konsentrasjon av sukkervann, noe som kan forklares med at de er mindre sensitive overfor sukker. Resultatene indikerer også at lows hadde et høyere aktivitetsnivå enn highs, men beregnet aktivitetsnivå stemte imidlertid ikke overens med det som faktisk ble observert. Dette kan tyde på at frekvensen på prøvetakingen var for lav til at aktivitetsnivået ble nøyaktig målt.

Contents

1 Introduction

While the study of animal behavior is at least as old as livestock agriculture, its contemporary expression amongst other biological disciplines did not begin until the 20th Century. Ethology emerged from the desire to understand animal behavior, and has been defined as "the systematic study of the function and evolution of animal behavior" (Drickamer et al., 2002). Especially influential in the modernization of ethology has been the Nobel Prize-winning work of Konrad Lorenz and Niko Tinbergen. While Lorenz developed the concept of imprinting, that is, a phase-sensitive learning process amongst young animals (Brigandt, 2005; Drickamer et al., 2002), Tinbergen's work offers an analytical approach to questions of animal behavior that examines their causes, functions, development and evolution (Tinbergen, 1963). Such careful methodologies, along with technological innovations, have advanced the study of animal behavior in ways that allow contemporary researchers to consider their subjects with previously unattainable sensitivity and complexity.

Ethology studies a range of intraspecific relationships: from largely solitary animals (eagles and jaguars, which only get together for mating), to animals that live in monogamous pairs swans, animals which live in small groups or prides (lions), and those which sustain massive herds or complex family groups (caribou, foxes). The latter group also includes social insects such as honeybees, which like most bees, are "truly" social, or eusocial. Eusocial insects share several behavioral characteristics which separate them from others, mostly regarding their relationship to offspring, but also in other aspects of their society. These characteristics include, but are not limited to, the manner in which they cooperate in the care for the young: Responsibility is shared by several members of the population; due to overlapping generations, offspring have the task of raising their own siblings; and finally non-reproductive castes act as servants to the reproductive castes (Drickamer et al., 2002). For a more comprehensive description of eusocial insects, consult a textbook such as Drickamer et al. (2002)

Social insects represent a long-standing tradition in the field of ethology, and their study has seen advancements in recent decades, owing to the development of necessary conceptual and technical instruments for such research. Sociality is a complex strategy for survival that poses both advantages and disadvantages for the organisms that demonstrate such behaviors. For example, safety from predators and communal care of offspring are clearly examples of advantages, while on the other hand, disadvantages associated with sociality may include both significant intra-group competition (West-Eberhard, 1979) and an increased risk of parasitism and illnesses (Wilson et al., 2003).

Whether animals are solitary or live in groups, they must all make choices. While the adaptive choices are needed in order to survive and prosper, other choices may lead to a loss of resources, or even injury or death to the animal (Schultz et al., 1997). Animals must on some level weigh potential drawbacks against the expected reward and decide whether a choice is favorable or not. Factors influencing these choices include genetic predispositions, and learned behavior, which may include any changes in behavior that are acquired through lived experiences (Drickamer et al., 2002). Also, pertinent to the study of animal choices are such

transient considerations as social ranking (Bault et al., 2008) or even an animal's physiological and cognitive state, i.e. whether an animal is hungry, excited, relaxed, and so on. Each of these influences variably affects the decisions and behaviors animals may express at any given moment.

Many studies have been performed on rodents, calves and chickens to investigate animal choice behavior using apparatuses or chambers in laboratory conditions. Such instruments include elevated plus-mazes which are designed for detecting drug effects and anxiety in rats (Pellow et al., 1985), open field apparatuses which have been used to interpret fear and response to novelty objects in calves (Pajor et al., 1995), Y-mazes which have been used for exploratory and learning behavior in chickens (Krause et al., 2006), and T-mazes which are also widely used in preference- and memory experiments in rodents (Ras et al., 2002; Dudchenko, 2004). A variety of similar technical tools have also been applied to identify and understand contributing components of social behaviors, as they are manifested in honeybees, namely: bee learning and conditioning (Bitterman, 1996; Gil et al., 2008), responsiveness to stimuli (Roussel et al., 2009), and cognitive neuroethology (Giurfa, 2003).

Honeybee eusociality entails a whole suite of complex behaviors that are variably derived from both innate and acquired influences; among these include distinguishing colony-members from intruders, orchestrating successful thermoregulation for winterization, and communicating the location and quality of flower nectar (Tautz, 2009)[1].

Zhang et al. (1996) cites Weiss' (1953) landmark experiments on honeybees as the earliest example of the use of mazes in the study of insects. Since then, mazes have remained instruments to study bee behavior, learning and conditioning. For example, a series of maze-based experiments have been fundamental to recent investigations into such diverse topics as the honeybees' abilities to learn complex labyrinths through step-by-step training (Zhang et al., 1996); furthermore, the conditioning of bees using sucrose rewards and glass mazes has helped establish the capacity for bees to associate colors with rewards through experimental learning (Han et al., 2010). In another study involving a small Y-maze, Kevan and Lane have found that honeybees might use fine-scale tactile information to identify nectar sources (Kevan & Lane, 1985); and Wager and Breed have used T-mazes to examine behavioral responses in sting alarm pheromones (Wager & Breed, 2000).

My study uses maze-based techniques in the study of honeybee behavior (*Apis mellifera*). A T-maze provides a specific and constrained environment, and is suited to achieve the goal because of its reduced environmental variation. Through standardization of this test situation, which is facilitated by its specific and constrained design, it allows researchers to compare data from multiple groups of different bee phenotypes and genotypes. During my work, I performed series of choice experiments, where the bee was given two choices represented by one bee in each arm of the T-maze.

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[1] Von Frisch was a pioneer in the study of honeybees, investigating freely flying bees in their own natural habitat. He received the Nobel Prize in Physiology or Medicine in 1973 for his behavioral biology studies with bees alongside his aforementioned and similarly esteemed colleagues Lorenz and Tinbergen.

First, social choice experiments were conducted to detect preference towards a familiar and unfamiliar bee. This paradigm was performed with newly emerged bees of the *ligustica* and *carnica* race, and with adult winter bees, i.e. bees which were picked from an over-wintering colony, of the *carnica* race. Due to the kin recognition system in honeybees, the bees were expected to be able to discriminate between the familiar and unfamiliar bee, and prefer to stay with the familiar bee (Breed, 1981; Gadagkar, 1985).

Second, a feeding partner choice experiment was performed on adult winter bees of the *carnica* race and for genetic strains of high and low pollen hoarder honeybees. High and low pollen hoarder strains were created through selective breeding on descendant bees of *ligustica* race in California, USA. Hellmich et al. (1985) examined how a two-way selection for pollen hoarding affects colony organization and behavior of individuals within the colonies. This selection was further developed by Page and Fondrk (1995) using larger breeding populations over several generations, which established two genetic strains for use in research: High pollen hoarders, "highs", and low pollen hoarders, "lows". Highs and lows are distinguished by several traits: Highs are prone to forage for pollen, collect higher pollen loads, and the lows forage more for nectar (Page & Fondrk, 1995). The highs also start foraging earlier than the lows. However, of particular relevance for my experiment was that highs are more sensitive to sugar as well as more active walkers as measured when they emerge as new adults. These differences suggested to me that the two genetic strains would show diverging partner choices, with highs being more accepting of partner bees that presented food with a low sugar concentration; because they can sense this sugar while lows cannot. Moreover, highs were predicted to be more active in the T-maze.

With this study, I was able to reveal how honeybees respond to familiar and unfamiliar bees in a T-maze assay. I was also able to reveal different preferences towards feeding partner who have ingested specific sucrose solutions. My study advances maze-based techniques in the study of honeybee behavior.

2 Materials and Methods

2.1 Honeybee Sources

Different breeds of the honeybee, *Apis mellifera*, were used in assessing choice behavior using a T-maze. The honeybees used in this study may be divided into two major categories: newly emerged bees and winter (*diutinus*) bees. Populations of newly emerged bees used in this study may be subdivided according to the two different wild type races they represent at two locations, Ås, Norway and Tempe, Arizona. Distinctions between newly emerged bees from strains of high and low pollen hoarders account for yet a third dichotomy.

At the University of Life Sciences (UMB) in Norway, bees from the *carnica* race were used in the experiments. In September and October 2010, experiments using newly emerged bees were conducted. Experiments with winter bees from hibernating hives were used in experiments in January and February 2011. At Arizona State University (ASU) in Arizona, newly emerged bees of the Italian *ligustica* race and strains of high and low pollen hoarders were used in experiments, conducted in March and April 2011. The wild type bees at both locations were bred from open mated queens. The queens from the high and low pollen hoarders were instrumentally inseminated.

For the experiments using newly emerged bees, combs with capped brood were collected from two different hives and incubated at 34°C for one night. The bees were brushed off the combs into a box the next morning, and sampled for experiment preparations. For the experiments with winter bees, the bees were sampled from the hive on the day of the experimental preparations. Adult winter bees were collected from clusters of one overwintering colony in January.

2.2 The T-Maze

A T-maze was designed to conduct choice experiments with honeybees. The maze was manufactured at the Department of Mathematical Sciences and Technology at UMB, Ås, Norway (Bjørn Brenna), using materials from Ås Glass, Ås, Norway. The maze was shaped like the letter T, with two equal arms at the end of an open passage. It was made of Plexiglas, with each arm measuring 11 cm in length. The passage starting at the base of the T was called the *entry section*, and measured 7.5 cm. The Plexiglas was 0.5 cm thick, and the internal diameter was 3 cm (Figure 1). Consult Wager and Breed (2000) for reference on the design.
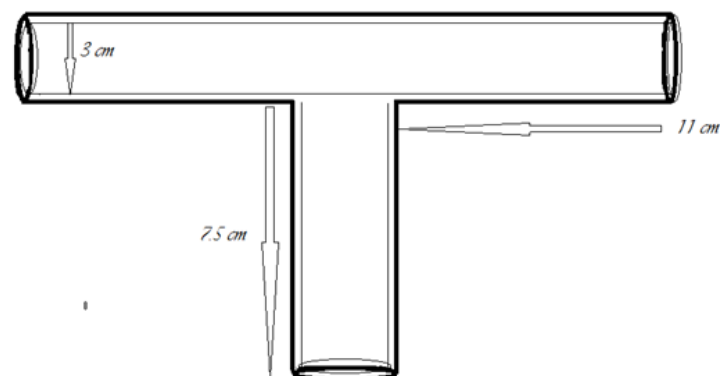

Figure 1: Diagram of the T-maze with measurements.

4

Each arm of the T-maze was fitted with a small cage, called *arm cage*, made from a short tube (Greiner centrifuge tube, Sigma-Aldrich, Norway), wire mesh, and a sponge (Figure 2). The wire mesh allowed a certain degree of interaction within the T-maze, while the sponge was used to close the cage in the other end of the tube. The arm cages contained a single bee each, called *arm bees*. The area in the T-maze, between the cages was called the *decision chamber*, and was divided into five equal parts, *time zones*, marked -2, -1, 0, 1, and 2, with 0 indicating the center (Figure 3). The number system was used in scoring and is discussed in Chapter 2.6.

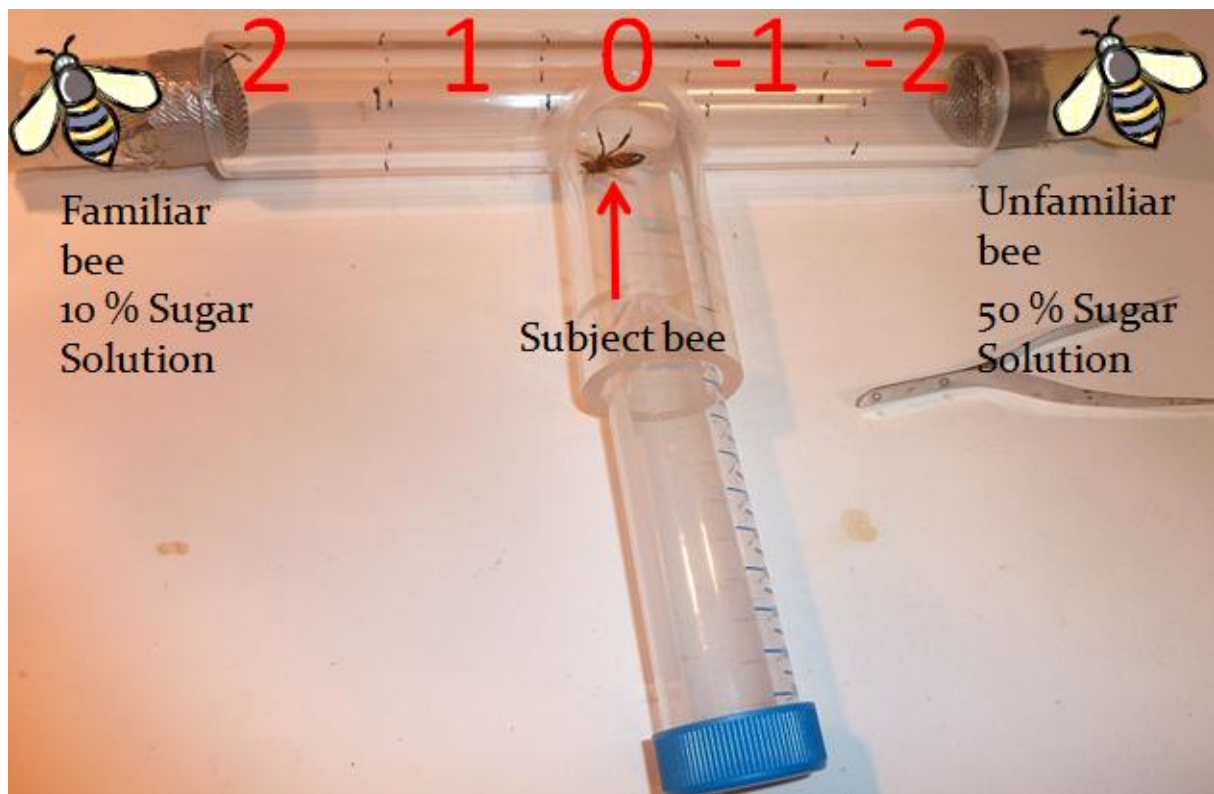*Figure 2:Arm cage. The wire mesh end was inserted into the T-maze to allow communication.*

Figure 3:The T-maze. The area between the cages was called the decision chamber, and was divided into five equal parts, time zones, marked -2, -1, 0, 1, and 2. This number system was used in scoring.

2.3   Choice Experiments: Preparations and Design

First, the bees in the sampling cages were fed ad libitum with 30 % sucrose solution to keep them from starving. Ten bees at a time were put on ice for three to five minutes, according to Roussel et al. (2009), until they stopped moving. The bees were put in individual plastic holders exposing only their heads, and were held still by two strips of tape, one between head and thorax, and one over the abdomen according to Bitterman et al. (1983). The bees were given five minutes to adjust to the new condition. Under the microscope, the eyes of the bees were covered with black, acrylic paint (Erber et al., 1998; Scheiner & Amdam, 2009), in order to eliminate visual factors (Figure 4). Thus, the bees were dependent on other senses for orientation in the T-maze. After ~10 minutes, when the paint had dried, the bees were removed from the holders and put in pairs in queen rearing cages (Nicot, Fay aux Loges, France).

Figure 4: The photo shows two bees harnessed in individual holders, held still by two strips of tape. The bees' eyes have been covered with black acrylic paint.

A queen rearing cage measured 8 cm long, 1 cm high, and 3.5 cm wide, and is shown in Figure 5. In the cage, the bees had a sponge soaked in sucrose solution. For the social choice experiments, all of the bees were fed 30 % sucrose according to Scheiner et al. (2003) and Page et al. (1998). In the feeding partner choice experiments, the bees were fed 10% and 50 % (see Chapter 2.5). The cages with bees were placed back into the incubator set to $28^{o}C$ overnight. Five extra cages were prepared for each group, and used in case bees died overnight or were discarded because they appeared ill or disabled.
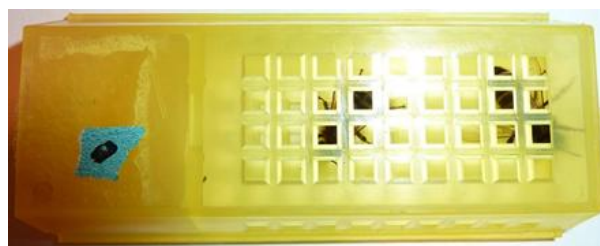

Figure 5: Queen rearing cage from Nicot, Fay aux Loges, France, was used to store the bees in the incubator.

An experimental round was started by placing one arm bee at each end of the arms of the T-maze. The subject bee was inserted into the entry section at the base of the T, and was followed by a plastic tube (Greiner centrifuge tube, Sigma-Aldrich, Norway), thus only allowing the bee to move towards the decision chamber (see Figure 3). Once the subject bee had entered the decision chamber, a timer was started and the location of the bee was noted on a check sheet every ten seconds over a time span of three minutes. This gave 18 observations for each bee, and an example of the corresponding check sheet is included in Appendix A. At the end of the session, the bee was given a score, which indicated bias towards one of the arm bees, and would be either a positive or negative value. Bees were discarded if they were damaged. They were also discarded if they did not move at all in the T-maze.

The T-maze was flipped horizontally between every other experiment. Also, the different bees were placed on alternating sides, switching between left and right side in each experiment. The T-maze was washed with dishwashing liquid (Zalo, Lilleborg, Norway and Ajax, Colgate-Palmolive Company, Arizona), and dried with a cloth between each of the 30 test trials, to remove pheromone footprints (Wager & Breed, 2000). All of this was done to reduce systematic errors, especially to eliminate the bees' possible attraction towards one or the other side of the T-maze.

2.4 Experiment 1: Social Partner Choice Experiment

The experiments on social partner choice were conducted with newly emerged bees and adult winter bees from the *carnica* race in Norway, and with newly emerged bees from the Italian *ligustica* race in the USA. This experiment examined a bees' degree of preference between a familiar bee from the same hive, which she had also shared a cage with over night, and an unfamiliar bee from another hive and cage.

For one experimental round, 60 bees were sampled from one hive and were called *familiar bees*, 30 bees were sampled from a different hive, and called *unfamiliar bees.* When the experiment began, one bee was placed at each arm of the maze. One was a familiar bee from the same cage as the subject bee, while the other was an unfamiliar bee. The procedure was carried out as described above.

There was conducted one replicate, so that 60 of both the newly emerged bees and winter bees were examined in Norway. Similarly, 60 newly emerged bees were examined in the USA.

2.5 Experiment 2: Feeding Partner Choice Experiment

The experiments on feeding partner choice were conducted on Norwegian adult winter bees of the *carnica* race, and strains of high and low pollen hoarders in Arizona. This experiment examined whether the subject bee's preference was affected when the bees located at the two arms of the T-maze, the *arm bees*, had been fed with different sugar concentrations.

The preparations of the Norwegian winter bees were similar to those for newly emerged bees, but they were grouped differently. After the bees had their eyes covered and put in cages, the cages were divided into three groups. Group three represents the test subjects, *subject bees*, while bees in group one and two were chosen as arm bees. All three bees in each of the 30 experimental rounds were selected from different groups, so that they were all unfamiliar to

one another. The bees from groups one and three were given 10 % sucrose solution, called *low sucrose bees*, while bees from group two was given 50 % sucrose solution, called *high sucrose bees*. All of the bees were then placed in the incubator set to 28°C overnight. Next day, the experiment was started by starving the subject bees from group three from two to four hours before beginning.

The feeding partner choice experiments using high and low pollen hoarders were somewhat different. Here, the experimental rounds was conducted using 15 bees of the high strain and 15 bees of the low strain in one round, and this procedure was carried out four times to directly compare the two different strains. 60 of the highs and 60 of the lows were examined in total.

2.6 Statistics

A total of 180 bees were used in each experiment, which was conducted with an initial round and a replicate round. All together, 6 experiments were conducted. In each experimental round, the choices of 30 bees were examined. Experiments using highs and lows were conducted through 4 rounds with 15 bees each. The chi-square test was done for each of the replicates and for the combined results, i.e. all the data from each experimental setup.

The chi-square tests convey which time zone has significantly high residuals. If zone 1 and 2 had significant residuals, then the bias is towards the familiar bee in the social choice experiments, or low sucrose solution bee in the feeding partner choice experiments. If zone -1 and -2 had significant residuals, then the bias is towards the unfamiliar bee or high sucrose solution bee. A significant residual in zone 0 indicated no bias at all.

Two methods were used to calculate the null distribution for the chi-square tests. For the social choice experiments and the feeding partner experiments using winter bees, the null distribution was calculated by simulating the behavior of 1.000.000 bees for 18 time steps, which is equal to one experimental trial, based on the activity level in the social choice experiment with newly emerged wild type bees from Arizona. The activity level of the bees was measured as the absolute value of the distance that a bee moved in one time step (10 seconds) an was calculated as Sum $(P_{t}-P_{t+1})/ n$, where P_{t} is the position of the bee at time t, given as -2, -1, 0, 1, or 2, and n is the numbers of observations for each bee, 18 in these experiments. For the experiments high and low pollen hoarding bees, the data from high pollen hoarding bees was used to calculate the null distribution for each round of 15 + 15 bees. Thus, the chi-square test was used to detect the difference between the two groups.

A student's t-test was performed to analyze the difference in activity level between the high and low pollen hoarders.

3 Results

This chapter includes raw data from each experiment and results from the statistical analysis. The p-values from all of the experiments are displayed in Appendix B. The results from each experiment are presented with average values for both the initial and the replicate rounds for each experiment in Appendix C.

3.1 General Honeybee Behavior in the T-Maze

When moving about within the T-maze, the bees did not seem to follow one typical pattern. While some bees walked straight to one end of the decision chamber and sat on the wire mesh for the entire three minutes the experiment lasted, others wandered back and forth from end to end and spent little time in one place. Some bees walked around the centre area, never reaching the end of the decision chamber, while others only move about in the same zone. These variations in movement pattern appear to have taken place on an individual level, and no correlation with either bee type, age, or any external factors was identified, although this was not examined in detail.

3.2 Social Choice Experiments

The experiments were conducted in two rounds and χ^2 tests were used to calculate bias using data from both rounds. In addition, consistency between the rounds within one experiment was evaluated by comparing the individual biases.

3.2.1 Newly Emerged *Carnica* Bees, Norway

No significant bias was found in this experiment. The experiment is inconsistent and one of the rounds showed a bias towards the unfamiliar bee ($p<0.001$). The data distribution, i.e. the sum of data points in each time zone for all the bees in this experiment, is shown in Figure 6.
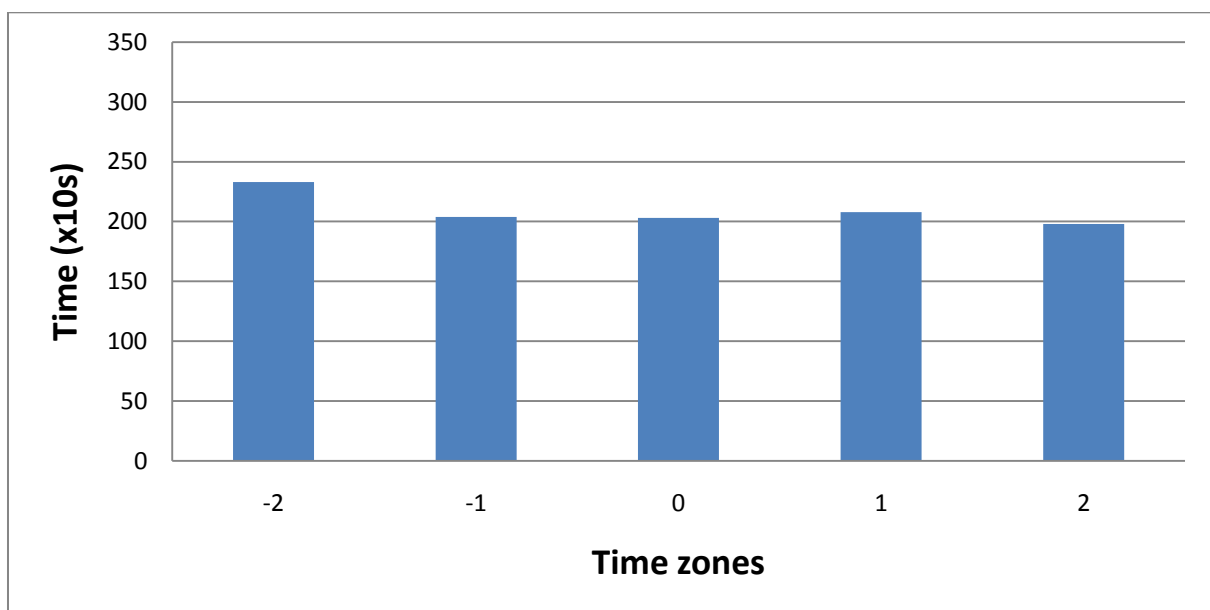

Figure 6: Histogram showing the total time spent in each of the five time zones of the T-maze, for the social choice experiment conducted on newly emerged carnica bees, Norway, N=60. The experiment did not show a significant bias. The experiment was inconsistent, and the initial round showed bias towards the unfamiliar arm bee (p<0.001).

3.2.2 Adult *Carnica* Winter Bees, Norway

The experiment conducted on winter bees in Norway showed a bias towards the unfamiliar bee ($p<0.001$). The data distribution from this experiment is shown in Figure 7.
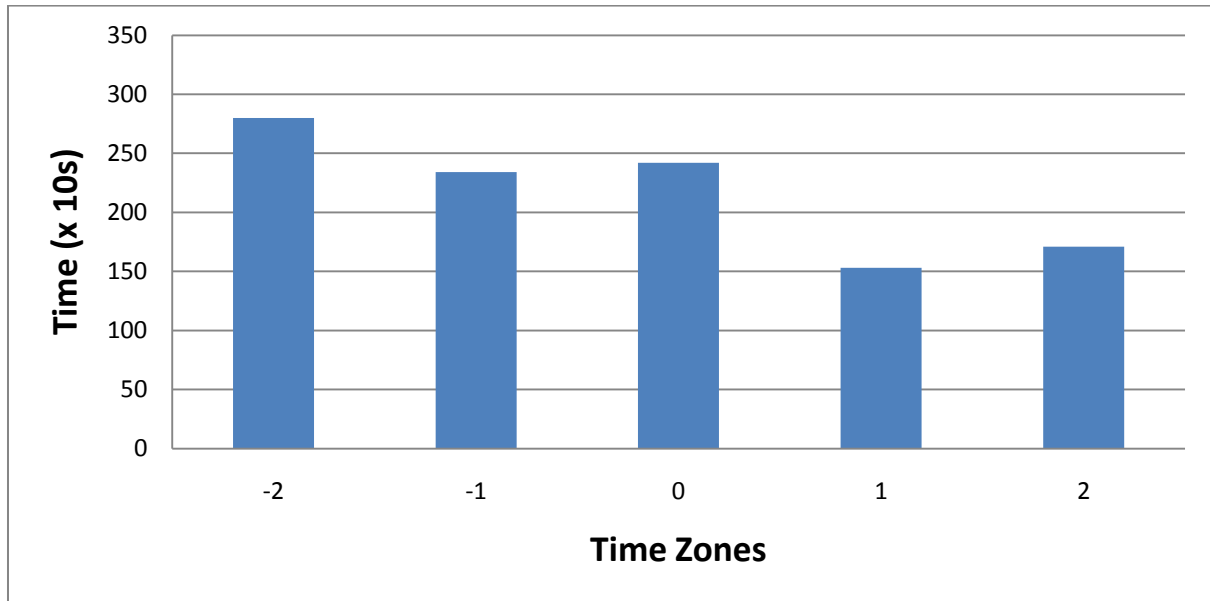

Figure 7: Histogram showing the total time spent in each of the five time zones of the T-maze, for the social choice experiment conducted on adult carnica winter bees, Norway, N=60. The experiment showed a significant bias towards the unfamiliar arm bee (p<0.001).

3.2.3 Newly Emerged *Ligustica* Bees, Arizona

Combined, the two rounds showed a significant bias towards the familiar bee ($p<0.001$), although the experiment conducted on American wild type bees was inconsistent. The initial round showed no bias, but the replicate showed a bias towards the familiar bee ($p<0.001$). The data distribution from this experiment is shown in Figure 8.
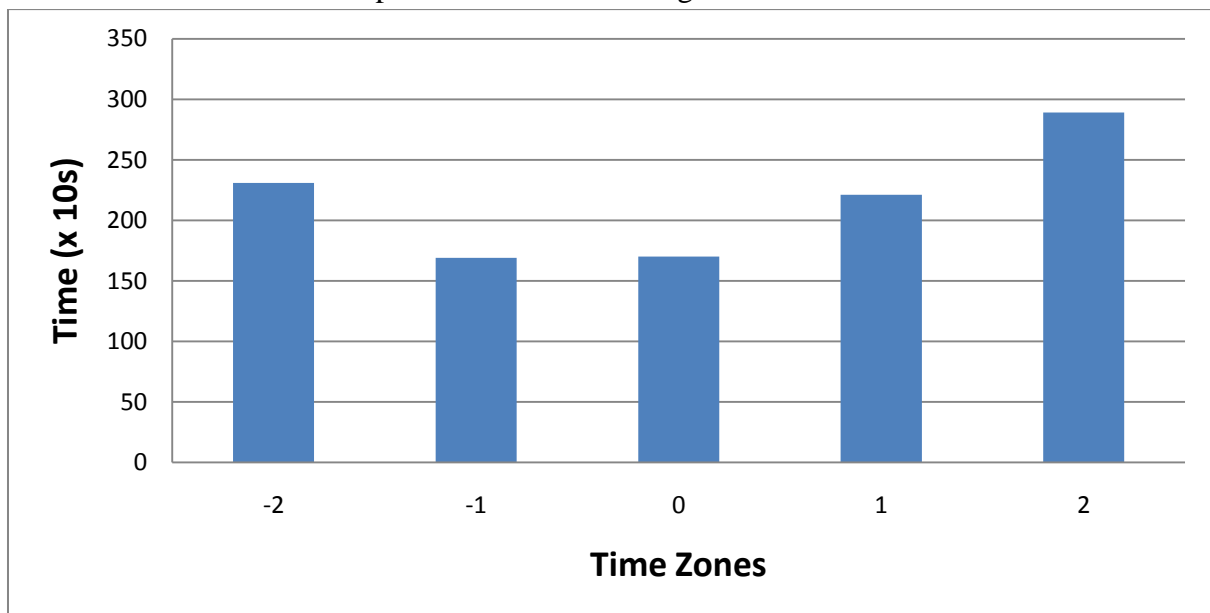

Figure 8: Histogram showing the total time spent in each of the five time zones of the T-maze, for the social choice experiment conducted on newly emerged ligustica bees, Arizona, N=60. The experiment showed significant bias towards the familiar arm bee (p<0.001).

10

3.3 Feeding Partner Choice Experiments

3.3.1 Adult *Carnica* Winter Bees, Norway

The experiments conducted on Norwegian winter bees show a bias towards the high sucrose bee ($p<0.001$). The data distribution from this experiment is shown in Figure 9.
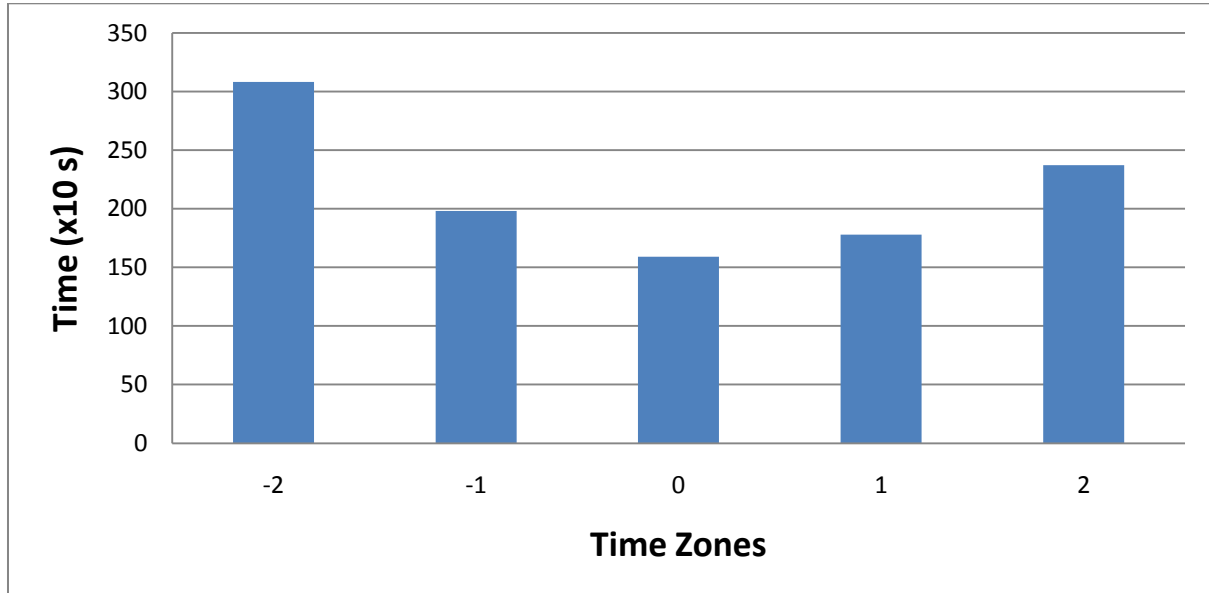

Figure 9: Histogram showing the total time spent in each of the five time zones of the T-maze, for the feeding partner choice experiment conducted on adult carnica winter bees, Norway, N=60. The experiment showed a significant bias towards the high sucrose arm be (p<0.001).

3.3.2 Newly Emerged High and Low Pollen Hoarders, Arizona

Data from high pollen hoarding bees was used to calculate the null distribution for all four experiments, and a chi-square test showed that, relative to the highs, lows prefer the high sucrose bee ($p<0.001$). The data distribution from this experiment is shown in Figure 10. Student's t-test was used to test the difference in activity level between the two genetic strains, and show that the activity was higher within the lows ($p=0.011$).
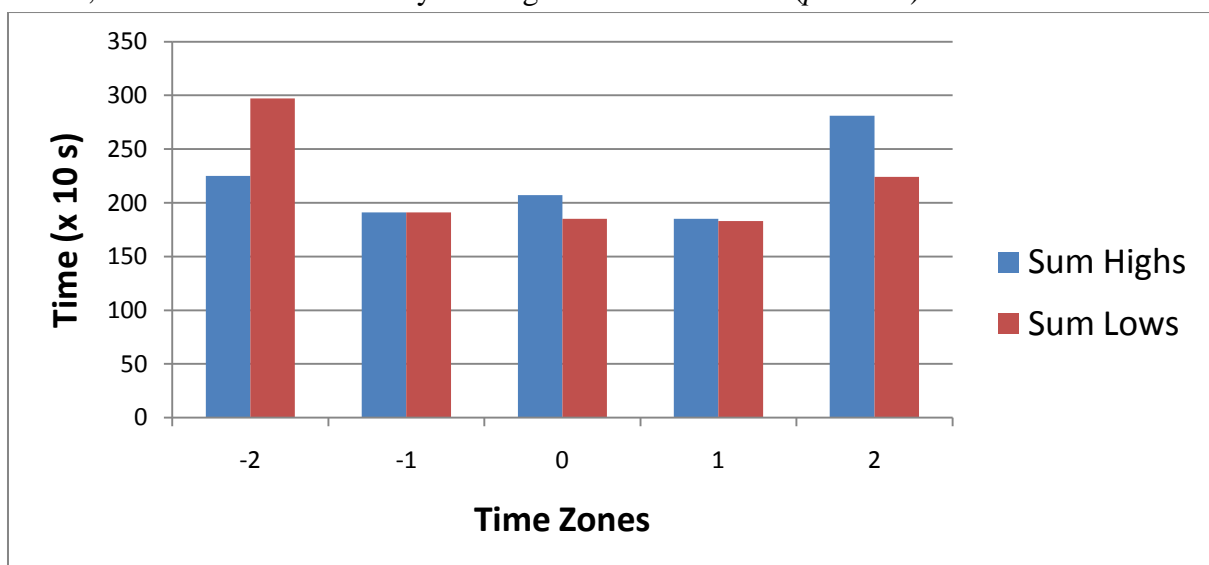

Figure 10: Histogram showing the total time spent in each of the five time zones of the T-maze, for the feeding partner choice experiments conducted on newly emerged high and low pollen hoarders, Arizona, N=120. Relative to the highs, lows showed a significant bias towards the high sucrose arm bee (p<0.001).

11

4 Discussion

4.1 General Handling and Feeding

Efforts were made to ensure that the conditions in the laboratory were the same during each experimental round: Weather conditions were as similar as possible during bee collections, laboratory preparations were done in the same place every time, using the same, clean equipment, and the same procedures were followed both during cooling and incubation. The bees were sampled from different hives for each experimental round to examine a larger sample of the honeybee population. This also eliminated any potential factors limited to one particular colony.

The number of bees used in one experimental round was adequate in means of work load for one day. However, increasing the sample size would strengthen the statistical analysis further, and testing more bees could eliminate, or at least reduce, inconsistencies. This is described in detail in Chapter 4.3.

We tested the bee cooling procedure prior to the experiments, to establish a system that worked and that could be repeated for every single bee. This included how long the bees were cooled before harnessing, and to make sure bees were never cooled twice. The bees that woke up before they were properly harnessed were difficult to handle, and they were discarded.

The bees in the social choice experiments were fed a solution of 30 % sucrose, which is a general concentration used in several conditioning and learning studies in honeybees (Bitterman et al., 1983; Page et al., 1998; Scheiner et al., 2003; Scheiner & Amdam, 2009). In addition to the safety of using a proven value, it may facilitate comparison with previously published results. Death rate inside the incubator never exceeded 4 %, indicating that the solution also was adequate for survival.

Hunger was used as a motivator in the feeding partner choice experiments. Preliminary studies revealed that bees would starve longer than four hours before showing signs of fatigue. Hence, the bees were never starved longer than this before being placed in the T-maze.

The different sucrose solutions that were given to the feeding partners were meant to represent two distinct alternatives. Prior to the feeding partner experiments, sucrose solutions of different concentrations were determined to represent high and low values. The 50% sucrose concentration used as the high value is large even when compared to the sucrose content even in flower nectar (Kreibich, 2011). To determine the low value, groups of 3x20 bees were fed 5, 8 and 10 % and put in the incubator. The mortality rate was noted over two days. Twice as many bees died in the 5% and 8 % group than in the 10% group, and 10 % was chosen as the low concentration in the feeding partner choice experiments.

4.1.1 Eye Paint

Bees use polarized light to orient themselves (Tautz, 2009). The bee's eyes were occluded with black acrylic paint in order to eliminate the influence of light on their decisions. Initial testing using an infrared light bulb in a dark room suggested that bees are able to see and are attracted to infrared light. Light was necessary for the observer to watch the bees in the T-maze, and thus the decision to paint the eyes and oscellies of the bees with acrylic paint was made. The procedure took some practice, but were more efficient and less time consuming along with the amount of practice. Some of the paint flaked off after a night in the incubator, probably due to the humid environment. This might have improved vision, causing the bees to spend more time at the end of the T-maze closer to the light source. Because the amount of flaking paint was the same in each experimental round, it should not have affected results between the rounds, but may have caused individual differences. Communication in honeybees is well developed in means of both pheromones and body language, which may concern information about food resources, swarming time and nest sites (Persson et al., 2008). Because bees are used to living in a dark environment inside the hive, blinding them should not be too much of a handicap. Care had to be taken not to paint the antennae which are used to perceive pheromone signals. Bees with painted antennae were not used in the experiments. The newly emerged bees used in the experiments were not yet able to fly at this age, while occluding the bee's eyes prohibited the adult winter bees from flying inside the T-maze.

4.2 The T-Maze

As mentioned previously, the design of the T-maze was inspired by and used the same measurements as that of Wager and Breed (2000). Preliminary experiments were conducted to test the design of the T-maze. We observed whether bees were able to move naturally in the apparatus and we determined how many minutes they should be observed. To decide how much time each bee should have in the T-maze, some bees were observed up to 30 minutes. Based on the movement we observed, the trial length for each bee was set to three minutes, because the bee did not seem to behave differently during subsequent time windows than during these first minutes of the test trial. Thus, the time span of three minutes seemed to capture the bees' behavior.

The bees were expected to make a choice in the T-maze by spending more time by one of the arm bees. The distance between the arm bees had to be short enough that the subject bee would know that there were two other bees in the maze, yet long enough that the subject bee did not experience that it had company by both arm bees and not make a choice.

The T-maze provides a spatial restraint which might stress the bees if they feel captured. In addition, the procedure where the bees were removed from their overnight-cages and put into the new environment in the T-maze might have been a stressful experience. Stress may cause the bees to act different than under normal circumstances, although we had no means of quantifying this. A more optimal design might consider giving the bees some time getting familiar with the environment in the T-maze, allowing their possible stress levels to decline before starting the timer. On the other hand, if the bee is already familiar with the two choices before the experiment actually starts, any initial motivation for contact might be eliminated.

Some of the bees were observed slipping on the smooth surface of the T-maze, particularly during the first 30 seconds of the experiment. This slipping did not occur at high frequency, but was most often observed in newly emerged bees of wild type and low pollen hoarders, and less with winter bees and high pollen hoarders. It is feasible that these differences could influence data recorded from the experiments, and introduce systematic error. A rougher surface inside the T-maze could have eliminated this possibility, but would have made washing and drying more time consuming and perhaps less efficient.

4.3 Data Collection

Time sampling was used to collect data for each bee. Time sampling is a simple, but powerful tool in observing animal behavior, especially in capturing a random sample of one particular behavioral state. While time sampling can provide more or less accurate data for large samples, its weakness is that it does not capture what happens between the sampling points, which were ten seconds apart in this study. Less information might be preserved using this method, and important behaviors might be lost between the sample points in each interval (Martin & Bateson, 2007). From the check sheet, a bee might appear to have never visited a particular time zone, even though the bee has actually been observed there. Some of the bees moved more rapidly than others, as was the case for the high pollen hoarders, increasing the difference between actual movement and the movement recorded in the check sheets. The sampling time, three minutes, was as mentioned previously determined through preliminary testing. In order to increase precision of the samples, one might consider increasing the sampling rate to 5 seconds, or perhaps even less. Increasing the number of trials, i.e. the number of bees in each experiment, would also increase precision.

4.4 Social Partner Choice Experiment

Based on knowledge of kin recognition, the bees were expected to prefer the familiar bee and the safety of their own.

4.4.1 Newly Emerged *Carnica* Bees, Norway

Data from this experiment revealed no bias. This lack of bias suggests that newly emerged bees do not have a distinct preference towards familiar and unfamiliar bees, and possible explanations will be discussed in the following paragraphs.

When bees are one day old, as in these experiments, they stay inside the hive cleaning the cells and combs, while after a few more days they start feeding larvae, and after yet a week or two some bees start guarding their hive from intruders, before the majority become foragers (Villumstad, 2004). This behavioral development indicates that the bee develops awareness about other bees over time, and that they are able to distinguish bees from their nest compared to other bees (potential intruders) after a while. Newly emerged bees are unaffected by adult experience, unable to fly and sting (Humphries et al., 2005). It is therefore possible that newly emerged bees do not have the experiential and physiological prerequisites to detect a difference between a familiar and unfamiliar bee, including pheromone or signal perception. Thereby, she also cannot discriminate between the two arm bees in my experiment. Further studies could be done twice on the same bees, first when they are newly emerged, and then again a few weeks later, to examine whether the bees make different choices as adults.

However, this would present challenges regarding the eye paint and storage in between experiments. In addition, changes in behavior caused by habituation to the T-maze might also be an issue.

4.4.2   Adult *Carnica* Winter Bees, Norway

As for newly emerged bees, the winter bees were expected to prefer the familiar bee over the unfamiliar one. However, the experiment was consistent and showed a significant bias towards the unfamiliar bee. This is a preference which serves to reflect the complexity of the choice they are making. Contrary to newly emerged bees, the winter bees are fully developed, adult bees with experience and perhaps more developed preferences than newly emerged bees. Their sensory apparatus also enable them to recognize and discriminate between bees, which probably contribute to their choices made.

The preference towards the unfamiliar bee can be explained by the bees having negative associations towards the familiar bee. A bee colony may easily contain 60 000 individuals, and different bees serve different tasks and fill social roles in the hive (Villumstad, 2004; Tautz, 2009). To spend many hours with one single bee in the cages during the night in the incubator might deprive the bees' need for social interactions, in addition to being isolated in pairs like this, which is not a normal situation for a bee to be in. Studies have shown that isolated bees are more aggressive than bees being reared in groups (Pearce et al., 2001). On the other hand, newly emerged bees at one day of age, are not known to be aggressive, and the bias towards the unfamiliar bee should not be due to a wish to attack the unfamiliar bee. The choice of the unfamiliar bee might therefore be explained by a need to socialize with new individuals, and an interest towards new bees, although this is highly speculative.

Honeybees guard their hives against intruders, and might perceive the unfamiliar arm bee as a threat in the social choice experiments (Breed et al., 2004). The presence of a stranger might trigger defensive behavior where the bee prioritizes protection of the hive over its own safety and comfort. The bees act for the benefit of the colony as a whole, and the worker bees must be able to discriminate between their own nest mates and intruders in this self-defense (Tautz, 2009). This is another factor which would explain why the winter bees spent more time in the arm of the T-maze containing an unfamiliar bee, but because the mesh cages limited interaction, one can only speculate as to the intentions of this preference. Since winter bees are adult bees which have gone through the life stages to develop as worker bees, they were believed to be more experienced than the newly emerged. The results demonstrate what could be interpreted as aggression towards the unfamiliar bee.

The winter bees behaved somewhat different in the T-maze than the newly emerged bees. According to the check sheets, the bees spent more time at one spot than the newly emerged bees, and they were occasionally observed standing still, vibrating their wings. This is termed *buzzing*, and is often performed by bees which have lost their queen, due to lack of queen pheromones, but is also serves as an alert to other bees that there is a danger present to the hive (Breed et al., 2004). The bees might not only feel threatened by the unfamiliar bee, but also by the experience of being in the T-maze. This would explain the buzzing, although my experiment was not designed to distinguish between these possibilities.

4.4.3 Newly Emerged *Ligustica* Bees, Arizona

Results from each round of this experiment were inconsistent: one experimental round showed a significant bias towards the familiar bee, and the other replicate showed no influence on behavior. The latter result is in line with my findings from newly emerged bees in Norway, with the initial result supporting the idea that young bees prefer the safety of their own. Although the results from the two locations, Norway and Arizona, are not identical, they are consistent in that none of them show a preference toward unfamiliar bees. I also believe that I can rule out effects from the experimental setup per se, since setups were the exact same for the social choice experiments at either location.

4.5 Feeding Partner Choice Experiment

In the feeding partner choice experiments, we wanted to examine if the subject bees could distinguish between the arm bees which had ingested different sugar solutions and whether they showed a preference towards high concentrations. As a suggestion to a different experimental setup, we could present the two sugar choices in a cup or on a sponge, and this would probably attract the bees more than when the arm bees have actually eaten the sugar, but then, we could not capture the social behavior aspect we were looking for.

The bees were able to perform trophallaxis and antenna contact across the wire mesh of the arm cages. Trophallaxis is a common phenomenon among honeybees which involves mouth-to-mouth feeding of nectar (Heilmann, 2009), and was to some extent expected in these experiments. The lack of trophallaxis indicates that other methods of communication are more important.

4.5.1 Adult *Carnica* Winter Bees, Norway

This experiment showed a bias towards the high sucrose bee, and is what was expected prior to the experiments. The result indicates that the bees are able to differentiate and choose between the two arm bees which have been eating food of different sugar value. Compared to the results for the winter bees in the social choice experiments, the bias towards the high sucrose bee indicates that the sucrose is a stronger motivation than the choice between bees that are familiar and unfamiliar. Social insects like honeybees are extremely altruistic, and act to serve the society (Krebs & Davies, 1993). The choices of the bees might not only be the result of a fine sensory apparatus which allows the subject bees to distinguish between high and low sucrose solutions (Kevan & Lane, 1985). As earlier mentioned, the bees perform a more vigorous recruitment dance after detecting a high quality flower, and they also give out samples of the flower nectar by trophallaxis in the recruitment process (Tautz, 2009). The arm bees with the sugar ingested might similarly communicate the sugar quality, although they did not dance, by using pheromones or vibratory signals, attracting the subject bee towards them.

4.5.2 Newly Emerged High and Low Pollen Hoarders, Arizona

By using high strain bees as a reference, we found that the low pollen hoarders showed a relative bias towards the high sucrose bee, which might be explained by their lower sucrose response. As mentioned in Chapter 1, the two pollen hoarding strains differ in their sucrose perception. The highs are more responsive to sucrose than the lows, and they might have perceived both arm bees as equally attractive feeding partners (Humphries et al., 1998;

Scheiner et al., 2003). This would explain why low strain bees, comparatively, showed a stronger bias toward the arm bee of higher sucrose value.

Based on observations during the experiments, there were distinct, behavioral differences between the highs and lows. A feature that was shared between the newly emerged wild type bees and the lows, was that they fell onto their backs and seemed to have trouble getting up. This is an indicator of them not being fully developed physiologically, and this behavior was not observed within the high strain genotype. Lows are known to have slower locomotor activity when they are young (Amdam & Page, 2010), and the lows were observed to be more sedate in their movements in the T-maze. The lows frequently spent more time in the entry section before reaching the decision chamber, and they often lingered at the 0 time zone in the beginning of a trial (although this behavior was not quantified directly).

The t-test showed higher activity level within the lows which is surprising, because it deviates from what was observed in the T-maze. Shorter sampling intervals would improve resolution and would capture the faster and more vigorous movement of the highs compared with the lows. The highs moved faster, and could benefit from a shorter sampling interval, for example five seconds instead of ten, in order to correctly capture the bees' movements in the T-maze (see Chapter 4.3). In other words, my sampling intervals were not sufficient to capture speed as rates of movements in the T-maze.

5 Concluding Remarks

The social choice experiments revealed that adult *carnica* winter bees showed a bias towards the unfamiliar bee, while newly emerged bees of both races were consistent in that they did not share this preference, indicating a development in choice behavior with age. In the feeding partner experiments, the adult *carnica* winter bees showed a preference towards the high sucrose bee, implying that bees were able to discriminate between the feeding partners. The high and low pollen hoarders differed both in physiological traits and in preference. The lows were compared with the highs and showed a relative bias towards the high sucrose bees, possibly owing to their lower sensitivity towards sucrose. Inconsistency between the observed and recorded activity levels show that a higher sampling frequency is needed to accurately capture the rates of movements in high and low pollen foragers.

The study offers a contribution to understanding the choices made by bees of different genotypes and phenotypes, regarding social preferences and sugar perception. The experiments revealed that the bees are able to make choices in the T-maze. Through standardization of the test situation the constrained environment of the T-maze can be a useful tool in studying the choice behavior in bees.

6 Acknowledgements

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8 Appendices

Appendix A – Check Sheet Used in Data Sampling

Date	28.okt								
Test #	2	L					R		
Animal #	1	Endmark					Endmark		
Age	1 day	x							
		Test bee					Test bees		
		-					+		

Time (s)	Ant C.	Trophal	-2	-1	0	1	2	Trophal	Ant C.
10			1						
20			1						
30					1				
40					1				
50							1		
60						1			
70				1					
80			1						
90			1						
100						1			
110							1		
120							1		
130							1		
140							1		
150							1		
160					1				
170					1				
180			1						

Figure A1: Check sheet used in data sampling. The position of the bee is noted every ten seconds over 3 minutes.

Appendix B – Table of Bias for the Experiments

Chi-square tests			
Experiments		**p-value**	**Bias**
Social choice experiments, newly emerged *carnica* bees, Norway	Initial round	3.76E06	Unfamiliar bee
	Replicate	0.25	None
	Combined	**0.1696**	None
Social choice experiments, *carnica* winter bees, Norway	Initial round	1.41E-05	Unfamiliar bee
	Replicate	6.62E-11	Unfamiliar bee
	Combined	**6.54E-10**	Unfamiliar bee
Social choice experiments, newly emerged *ligustica* bees, Arizona	Initial round	0.33	None
	Replicate	2.59E-08	Familiar
	Combined	**5.37E-05**	Familiar
Feeding partner choice experiments, *carnica* winter bees, Norway	Initial round	6.51E-05	High sucrose bee
	Replicate	1.7E-4	High sucrose bee
	Combined	**8.85E-07**	High sucrose bee
Feeding partner choice experiments, high and low pollen hoarders	Initial round	4.99E-05	High sucrose bee
	Replicate	3.34E-07	High sucrose bee
	Initial round	0.46	None
	Replicate	1.28E-07	High sucrose bee
	Combined	**1.64E-07**	High sucrose bee

Figure B1: Table of p-values for the choice experiments.

Appendix C – Histograms

The results from each experiment are presented with average values for both the initial and the replicate rounds for each experiment, presented with standard error.
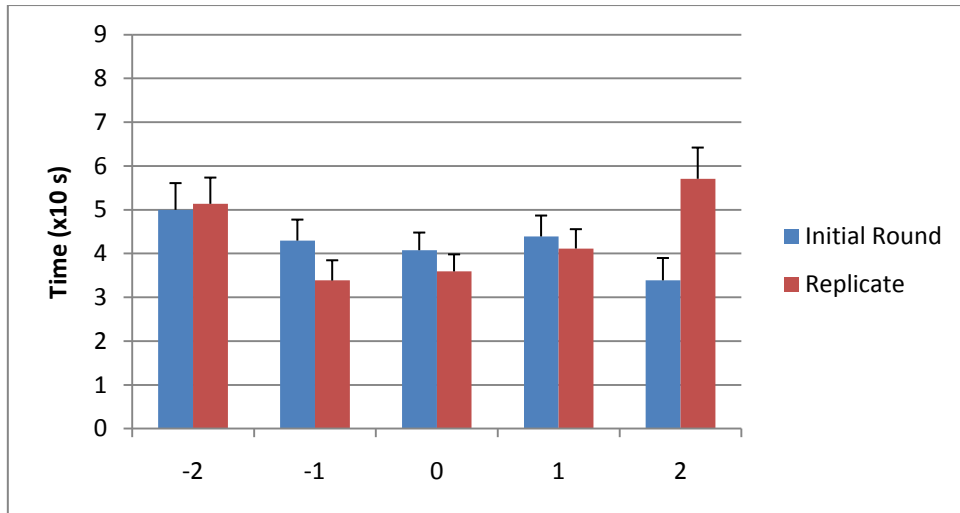

Figure C1: Average time spent in each time zone of the T-maze, social choice experiment, newly emerged carnica *bees, Norway.*
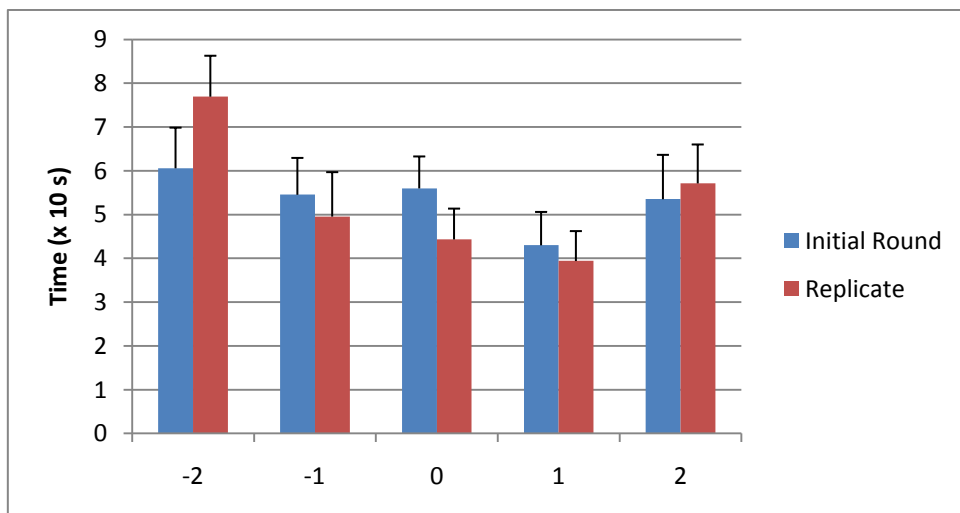

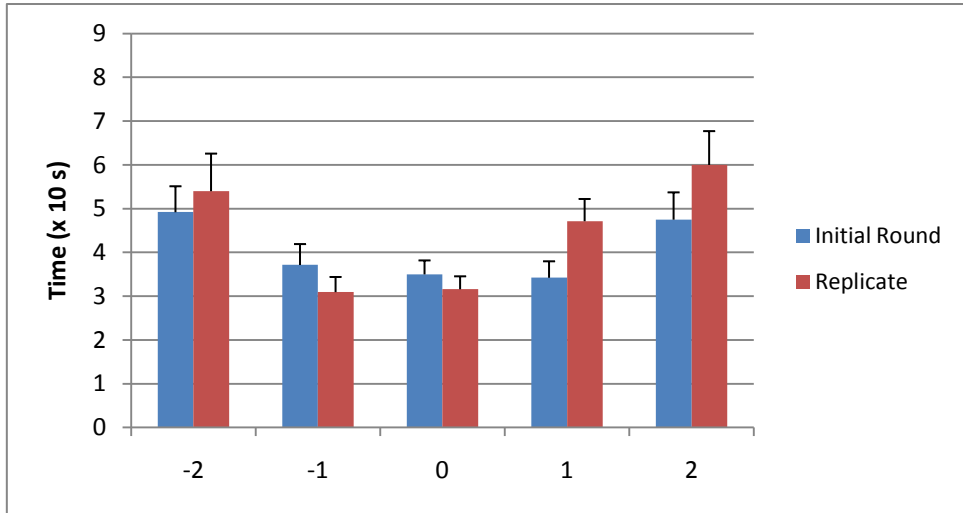*Figure C2: Average time spent in each zone of the T-maze, social choice experiment, adult* carnica *winter bees, Norway.*

Figure C3: Average time spent in each zone of the T-maze, social choice experiment, newly emerged ligustica *bees, Arizona.*
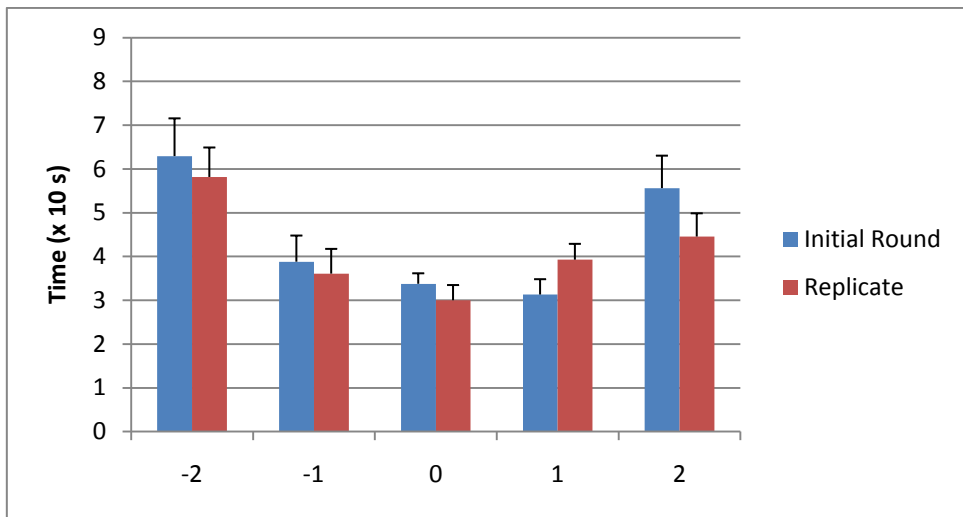

Figure C4: Average time spent in each zone of the T-maze, feeding partner choice experiment, adult carnica *winter bees, Norway.*
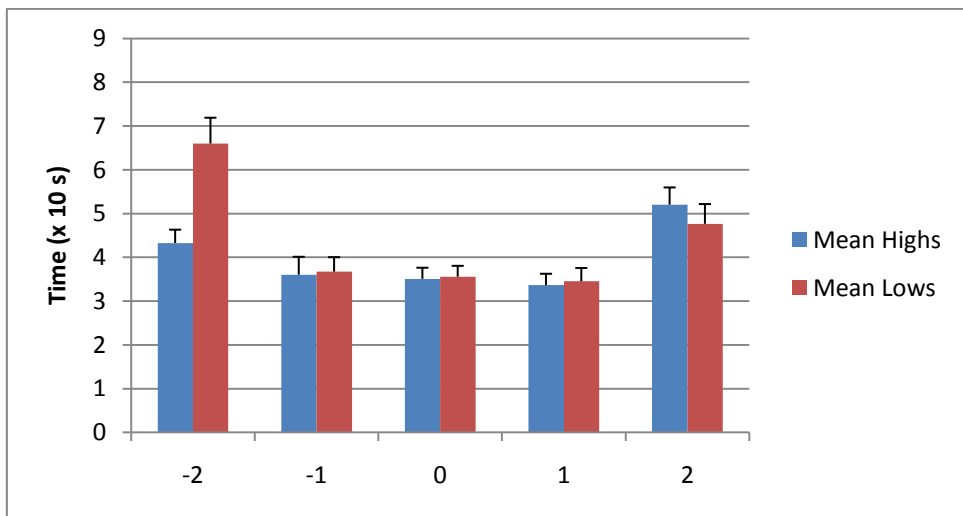

Figure C5: Average time spent in each time zones of the T-maze, feeding partner choice experiment, high and low pollen hoarders, Arizona.