University of Puerto Rico

Rio Piedras Campus

Biology Department

Master's Thesis

Title: Exploring mechanisms of behavioral flexibility and individual specialization through the comparison of members of multiple subspecies of honey bees

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Index

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Abstract

In this thesis, we explore the cognitive basis for the emergence of individual foraging strategies (IFS) across multiple honey bee subspecies using Reversal Learning assays and Free-Flying foraging problems. In Chapter 1 we studied the Reversal Learning ability of two different honey bee subspecies during a Proboscis Extension Reflex Assay. We found significant differences in how the subspecies learned. In Chapter 2 we studied the different foraging strategies honey bees could take when solving a Free-Flying foraging problem and found Subspecies is a strong factor in deciding individual's strategies.

General Background

Populations and species are cataloged as "generalists" or "specialists" according the variety of the resources they collect within their niche**.** Individual foraging specialization (**IFS**), on the other hand, is a phenomenon which is defined by "how many individuals in a group or the degree to which individuals use a subset of the overall resources available to the population" (Bolnick et al., 2003). For example: Banded Mongooses (*Mungos mungo*) are a "generalist" species that feeds on insects and occasionally small vertebrates. However, not all individuals follow the same diet; specialists seem to be foraging only a subset of the available food (Catherine E. Sheppard et al., 2018). Furthermore, individuals not only specialize in their diet, but they also specialize in how they forage. Whenever they must crack open a hard shelled prey, they will do this through one of two foraging strategies: biting or smashing open the item (Müller & Cant, 2010).

Individual foraging specialization (**IFS**) extends to more than 189 species across almost all taxa in the animal kingdom; probably due to the evolutionary advantages it provides to group living (Araújo, Bolnick, & Layman, 2011; Bolnick et al., 2003; Costa-pereira & Pruitt, 2019). Having specialized individuals within the group can: (1) increase group-stability by reducing competition for resources; (2) reduce the number of social interactions between group members, this could translate into disease protection since a disease would only affect one subgroup; (3) increased reproductive success by increasing the resources available to the community, in the case of eusocial insects it could decrease energy constraints on the queen (Araújo et al., 2011; Catherine Elizabeth Sheppard, 2016). At this point in time, there are four known factors which modulate IFS: Competition, Morphology, Predation, and Ecological Opportunity (Toscano, Gownaris, Heerhartz, & Monaco, 2016). In particular, the effects of predation and Ecological Opportunity have been extensively studied. Predation can reduce the viable foraging strategies of a population, thus it will promote homogeneity in the foraging strategies within a population (Mathot et al., 2011; Sih & Del Giudice, 2012; Toscano et al., 2016). On the other hand, an increase on Ecological Opportunity promotes the development and expression of IFS (Layman, Newsome, & Gancos Crawford, 2015).

However, even though IFS requires decision-making, learning, and memory, among other things, the relationship between cognitive processes and individual foraging specialization is still relatively explored (Araújo et al., 2011; Dingemanse & Wolf, 2013; Sih & Del Giudice, 2012). To address the cognitive basis of individual specialization some studies explored Behavioral flexibility, or the ability of an individual to modify their behavior with respect to the environment to adapt as optimally as possible (Beeler, 2012;

Alicia Izquierdo, Brigman, Radke, Rudebeck, & Holmes, 2016). These studies suggest that heterogeneous levels of behavioral flexibility promote IFS within a population and that the behavioral flexibility of a population can be modulated in different ways by long-term and short-term environmental factors (Barou Dagues, Hall, & Giraldeau, 2020; Dingemanse & Wolf, 2013; Mathot et al., 2011).

In the case of honey bees, behavioral flexibility has been shown to have a heritable impact on the learning ability and the foraging preferences of individuals (Ferguson, Cobey, & Smith, 2001; Latshaw & Smith, 2005). There is not, however, any research that explores the relationship between: (1) Behavioral Flexibility ability in laboratory conditions and the foraging strategy a forager will take when visiting flowers or (2) Behavioral Flexibility and IFS in Free-Flying honey bees.

Three *Apis mellifera* subspecies populations: *A. m. caucasica, A. m. syriaca*, and the gentle Africanized Honey bee (gAHb) would be ideal to explore this problem. In particular, the *A.m. caucasica* and *A.m. syriaca* subspecies have been studied for genetic and behavioral differences (Bodur, Kence, & Kence, 2007; Cakmak et al., 2010; Kence, Oskay, Giray, & Kence, 2013). Furthermore, they come from distinct ancestral habitats with unique selective pressures. *A.m. syriaca* inhabits southeast Anatolia, a generally dry habitat with longer seasonal foraging periods constrained by periodic blooms of one or few flowers (Kandemir, Kence, & Kence, 2000; Kandemir, Kence, Sheppard, & Kence, 2006). In this region there is a predatory wasp that can capture foraging honey bees, and bees of this region have adapted by reducing foraging activity (Butler, 1974; Çakmak, Wells, & Firatli, 1998; Ishay, Bytinski-Salz, & Shulov, 1967; Roubik, 1992; Ruttner, 1988). The bees from the subspecies *A.m. caucasica* inhabit temperate deciduous forests in the

northeast of Anatolia and the eastern Black Sea coast regions of Turkey. The gAHb inhabits Puerto Rico, a subtropical island the in the Caribbean with long foraging periods (Galindo-Cardona, Acevedo-Gonzalez, Rivera-Marchand, & Giray, 2013).

Here, we compare the Behavioral Flexibility of these two subspecies in different learning contexts and the distribution of their Individual Foraging Strategies.

Chapter 1:

As published in PeerJ in 2018

Doi: 10.7717/peerj.5918

Appetitive reversal learning differences of two honey bee subspecies with different foraging behaviors

Introduction

A honey bee colony shifts its foraging effort as the floral resources come and go in the environment (see Seeley, 1995). This dynamic allocation of foragers is thought to be adaptive since resources are harvested maximally. The basis of this constant response to changes in floral resources is the preference and foraging decisions of individual honey bees. Several mechanisms involving learning has been shown to be important in decisions of individual foragers (e.g. Ferguson, Cobey & Smith, 2001). We examined whether plasticity in appetitive learning will differentiate bees of *A.m. caucasica* subspecies that switch foraging preferences with ease, from bees of A.m. syriaca

147 subspecies that do not switch even when reward contingencies change (see Cakmak et
148 al. 2010).

149 Both the exploiting strategy of *A.m. syriaca*, and the exploring strategy of *A.m.*
150 *caucasica* could be adaptive in their respective environments. The hypothesis is that
151 specializing on a single flower type makes the bee faster both in finding the flower and in
152 handling the flower, and thus decreases the time spent outside, at risk, or exposure to
153 predators. Therefore, appetitive learning flexibility in the specialist subspecies, or *A.m.*
154 *syriaca* should be reduced to keep the bee focused on a single flower type. Alternately,
155 in low risk environment, a fully plastic foraging choice towards the most rewarding
156 resources is the best solution, and favors greater learning plasticity in the generalist
157 subspecies, or *A.m. caucasica*. This is then an example where phenotypic plasticity
158 comes with a cost of exposure, and reduced plasticity in learning is the compromise (see
159 DeWitt, Sih & Wilson, 1998; Murren et al., 2015).

160 Honey bees live in wide range of habitats, extending from tropical to subarctic,
161 either because of human intervention or because of evolutionary history of the
162 populations (Wallberg et al., 2014; Whitfield et al., 2006). These genetically distinct
163 populations are recognized as subspecies or races. Bringing members of different
164 subspecies together for experiments revealed many genetic differences in behavior and
165 its regulation (Alaux et al., 2009; Brillet, Robinson, Bues, & Conte, 2002; Büchler et al.,
166 2014; Çakmak et al., 2009; Cakmak et al., 2010; Giray et al., 2000; Kence et al., 2013).
167 Foraging choice differences across two subspecies from Turkey provides the ideal
168 situation to test the underlying learning plasticity differences across specialists and
169 generalists. Previously, *Apis mellifera syriaca* and *A.m. caucasica* bees have been

studied for genetic, colony and behavioral differences (genetics: Bodur, Kence & Kence, 2007; foraging behavior: Çakmak et al., 2009; colony traits: Cakmak et al., 2010; Kence et al., 2013).

The bees from the subspecies *A.m. syriaca* inhabit southeast Anatolia, a generally dry habitat with longer seasonal foraging periods constrained by periodic blooms of one or few flowers (Kandemir et al., 2000, 2006). For foraging *A.m. syriaca bees,* minimizing predation risk is important. In this region there is a predatory wasp that can capture foraging honey bees, and bees of this region are demonstrated to have specific behavioral adaptations against this Vespa species, such as reducing foraging activity (Ishay, Bytinski-Salz & Shulov, 1967; Butler, 1974; Ruttner, 1988; Roubik, 1992; Çakmak, Wells & Firatli, 1998). This response is absent in *A. m. mellifera* (Matsuura & Sakagami, 1973). In contrast, the bees from the subspecies *A.m. caucasica* inhabit temperate deciduous forests in the northeast of Anatolia and the eastern Black Sea coast regions of Turkey. Weather in these regions limits foraging to a short, three-month seasonal period, making it important to maximize collection rate.

One specific type of plasticity in learning, reversal learning, has been examined because of its potential relevance to tracking changing foraging resources (e.g Ferguson, Cobey & Smith, 2001). The bees learn to associate a stimulus (a floral odor) with a reward and learn to discriminate this from a second odor not associated with reward. Later bees are asked to switch the odor associations. Reversal learning measures behavioral flexibility, and either single or multiple reversions, and either two or more choices are utilized to examine extent of flexibility (rev. in Izquierdo et al. 2016). In comparison of bees of different ages (Ben-Shahar, Thompson, Hartz, Smith, & Robinson, 2000),

selected lines (Ferguson et al., 2001) and subspecies (Abramson et al. 2015), rate of reversal appears to differ, albeit the shape of reversal appears to remain similar (see Supplement Figure 1).

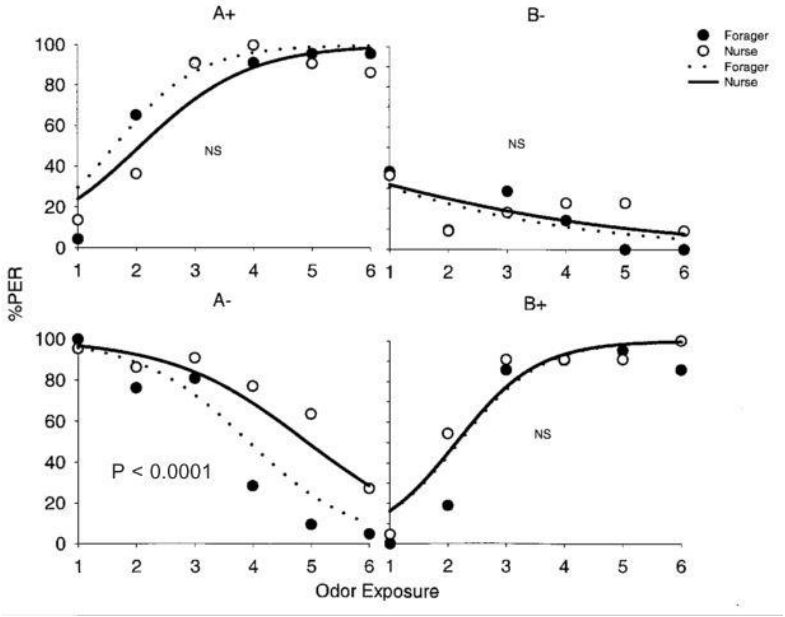
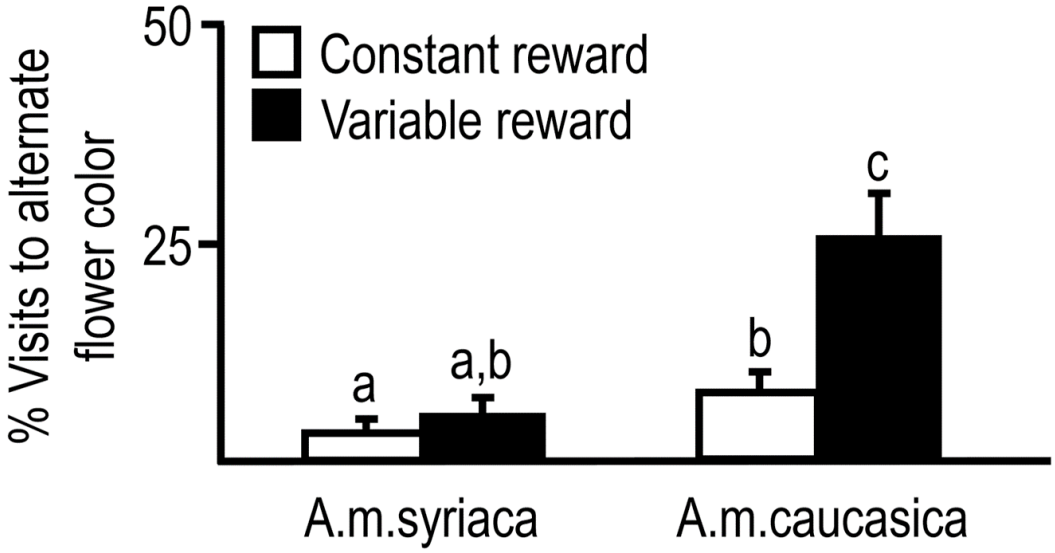


Figure S1. Reversal learning plot in Proboscis Extension Response Conditioning of bees from typical *Apis mellifera* colonies. (Ben-Shahar et al., 2000).

In the context of foraging behavior, reversal learning is similar to when a bee visits one flower providing nectar at that time, and later in the day switch to a different flower that is providing nectar then (e.g. Wagner et al., 2013). In addition, response of bees to variability in nectar availability is similar to response of other organisms such as vertebrates to variable reward or resources under experimental or natural conditions (rev. in Commons, Kacelnik & Shettleworth, 1987). For instance, if constant forage rate would provide energetic needs, organisms are likely to abandon variable reward for constant reward (see Caraco, 1981; Zalocusky et al., 2016). In previous work we have

demonstrated that bees from the temperate subspecies *A.m. caucasica* is more likely to switch to a different flower color morph. In contrast, bees from the subtropical



subspecies, *A.m. syriaca* are not sensitive to variability in reward, and continue to visit the same flower morph even when rate of reward is 1 in 3 visits (see Cakmak et al., 2010 and Figure 1.1).

Figure 1.1. Average percent visits to alternate flower color was significantly less for *A.m. syriaca* than *caucasica*. Bees first visited blue, white or yellow flowers. Later they visited alternates or initial preferred flowers with either constant reward (2µl 1M sucrose) or variable reward (only 1 of 3 flowers with 6 µl reward). Sample size: 6 colonies / subspecies, 30-35 bees /colony, 30-40 choices/bee. Error bars = SE. Factorial ANOVA indicated significant subspecies differences. Groups with different letters above bars are different at $P < 0.05$. (Cakmak et al., 2010).

We hypothesized that flower constancy even when faced with variable reward could be due to learning and memory differences of *A.m. syriaca* bees from other bees, including *A.m. caucasica*. We used Proboscis Extension Response (PER) conditioning (Charles I. Abramson, Craig, Varnon, & Wells, 2015) assay to examine differences in

appetitive learning behavior across bees from colonies of both subspecies maintained in a "common garden" apiary (see Kence et al., 2013).

Materials and Methods

Experimental Design:

Proboscis Extension Response Conditioning experiments were performed between June and July 2014 at the Middle East Technical University in Ankara, Turkey. In a preliminary work we also examined reversal in a non-appetitive aversive learning test, Electric Shock Avoidance conditioning (ESA, e.g. Agarwal et al., 2011; Dinges et al., 2013). To control for calendar variables associated with weather and field conditions, both PER and ESA (see supplement) conditioning assays were run simultaneously. In the PER series we investigated reversal learning of the proboscis extension response (PER) in bees harnessed in metal tubes and in the ESA series we investigated the reversal of spatial avoidance learning in honey bees confined to a shuttle box (ESA).

Foragers of two subspecies populations in Turkey were used. One subspecies was *Apis mellifera caucasica*, and the other subspecies was *Apis mellifera syriaca*. Both subspecies were maintained in a common garden under similar environmental conditions. Great care is taken to ensure that the subspecies lines are maintained and this is confirmed by use of genetic and morphological measurements, and acquiring new colonies or naturally mated queens from the geographically separated (>600 miles) locations (see Kence et al. 2013). We used three colonies from each honey bee subspecies to increase genetic variation within the samples for a total of 261 individuals

248 that were tested in learning and memory assays. One hundred thirty-seven bees (137),
249 divided in two equal groups (but for one bee), one for each subspecies, were recruited
250 for the PER assays where each experimental group consisted of 12 individuals, except in
251 occasion one or two bees were eliminated when not responsive. One hundred twenty-
252 four bees (124), divided in four equal groups, two for each subspecies, were recruited for
253 the supplemental ESA assays where each experimental group consisted of up to 34
254 individuals.

255 *Proboscis Extension Response (PER) Reversal Learning:*

256 In these experiments we trained the honey bees to discriminate between two
257 conditioned stimuli (CS) – one paired with a sucrose feeding (CS+) and the other not
258 (CS-). Following this phase, we reversed the CS+ and CS- roles such that the CS+ is now
259 the CS- and the CS- s now the CS+.

260 One CS consisted of lavender odor (Gilbertie's, Southampton, NY) and the other
261 cinnamon odor (Gilbertie's, Southampton, NY). The rationale behind the use of these
262 odors is that we have found them effective in our previous discrimination experiments in
263 Turkey (C I Abramson, Mixson, Çakmak, Place, & Wells, 2008; Charles I. Abramson et
264 al., 2015, 2010). The CS odor was applied to a 1 cm^2 piece of Whatman (#4) filter paper
265 using a wooden dowel and then secured to the plunger of a 20 cc plastic syringe with an
266 uncoated metal thumbtack. Our earlier work demonstrated this procedure produces
267 reliable results consistent with automated methods (Charles I Abramson & Boyd, 2001).

268 To remain consistent with our previous work: 1) a non-overlap procedure was
269 used in which the CS terminated before the US (C I Abramson, Aquino, Silva, & Price,

1997), 2) the CS duration was 3 seconds and the US duration was 2 seconds, and 3) the intertrial interval (ITI) between CS presentations was a fixed 5-minute interval. During the initial discrimination learning phase, each bee received 6 trials each with lavender and cinnamon for a total of 12 trials. During the reversal phase in which the role of the CSs were reversed, bees received 6 trials each with lavender and cinnamon for an additional 12 trials. The order of CS+ and CS- presentations were pseudorandom and identical for each bee. We used the order: Initial Discrimination training: CS+, CS-, CS-, CS+, CS-, CS+, CS+, CS-, CS+, CS-, CS-, CS+, Reversal Training: CS-, CS+, CS+, CS-, CS+, CS- , CS-, CS+, CS-, CS+, CS+, CS- for a total of 24 trials (12 CS+ and 12 CS-).

Honey bees from both subspecies were captured one day before the experiment. They were captured in glass vials and placed in ice. While sedated they were harnessed in metal tubes with a piece of duct tape placed between the head and thorax. Once awake they were fed 1.5 M sucrose solution in water until satiated and set aside in a fume hood. On the day of the experiment, the bees were removed from the fume hood and were placed in “squads” consisting of about 12 bees.

A conditioning trial was initiated by picking up a bee from its position in the squad and placing it in the fume hood. The purpose of the fume hood was to eliminate any lingering CS odors. After a few seconds, but never immediately upon placement, the CS was administered for 3 seconds and was immediately followed by the US. This procedure was necessary as bees can associate the “placement” with a feeding. The US was presented by touching the bee’s antennae with a filter paper strip containing 1.5 M sucrose and bees were allowed to lick the filter paper for 2 seconds after extending their proboscis. At the end of the 2-second feeding, the bee was removed from fume hood and

returned to its place in the squad at which time the next bee in the squad was placed in fume hood for its trial. This process continued until all the subjects in the squad received the required number of conditioning trials. During each trial, responses to the CS were recorded visually. If the bee extended its proboscis during the CS presentation, a positive response was recorded. If the bee did not extend its proboscis during the CS presentation, a “0” response was recorded. It should be noted that the experiment was run blind as the experimenter did not know what subspecies was being trained.

Each experiment consisted of two phases. The stage where memory of the paradigm was being acquired for the first time was termed *Acquisition* Phase. The step where we reverse the paradigm was termed *Reversal* Phase. During each trial we presented a CS+ and a CS-, each CS was a different odor. We used a model with two sets of experiments where each odor had the role of initial CS+ or initial CS- thus creating a counterbalance. The measured value was the PER response.

Supplemental Electric shock avoidance assay (ESA):

This experiment had two phases of 5 minutes each for a total of 10 minutes. During Acquisition phase, individuals were presented two colors, one as the punishment conditioned stimulus (CS+), this color was paired with electric shock (unconditioned stimuli), and the other as the no punishment conditioned stimulus (CS-), this color was not paired with electric shock. Here individuals learn to avoid punishment or one of the colors. That is to say, the bee learns to stay on one side of the box and not on the other. During the second or Reversal phase, the colors for the CS+ and CS- were switched.

Now the phase 1 CS+ is the phase 2 CS- and the phase 1 CS- is the phase 2 CS+. We do the switch by changing the side/color of the box that receives shock, and not by moving the colors, this way we avoid confounding position and color effects. Moreover, by moving the shock from one side of the box to another, the bee can only avoid the shock by making an active response; by moving from one side to the other.

To analyze the results from these experiments we first confirmed there is no color preference by bees from either subspecies when either blue or yellow was the CS- during Acquisition and Reversal Phases. Because we did not observe significant differences (results not shown) Color was not included as a variable in subsequent analyses. Instead, the first color associated with punishment is A+, and the second or Reversal phase this is A-, whereas the alternate color becomes B+.

We used a shuttle box apparatus as described before (Agarwal et al., 2011; Giannoni-Guzmán et al., 2014). The shuttle box measured 15 cm long by 2 cm wide and contained an electric shock grid with wires spaced .35 cm apart. The shock was presented to only one side of the apparatus identified by a specific color. Shock intensity was 6 V 50 mA DC from an analog power supply and was low enough not to produce a sting reflex. In one half of the shuttle box a color (CS) is paired with electric shock (US) to create a CS+, on the other half another color (CS) is not paired with the electric shock (US) to create a CS-. Time spent on the shock side was recorded by an observer, one observer for each individual. We used blue and yellow as we know from our previous experiments that bees can readily distinguish between them. We measured the mean amount of time spent on the shock side in sets of 60 seconds for a total of 5 sets or 300 seconds as was done previously (Agarwal et al., 2011).

Statistical Analysis:

Statistical analyses were performed using the GraphPad Prism 6 statistical software program. Analyses of the data from PER and the ESA assays were done with a two-way repeated measures ANOVA, we tested the data for significant phase (Acquisiton vs Reversal), subspecies, and interaction effects. A post-hoc Tukey-HSD test was used to examine trial to trial differences. We verified fit to a normal distribution using the Shapiro-Wilk's W test.

Results:

Proboscis extension response (PER) conditioning

We used the PER conditioning assay to determine if the honey bee subspecies *A.m. caucasica* and *A.m. syriaca* have olfactory learning differences. We first confirmed that the subspecies had no odor preference when either lavender or cinnamon was the CS+ or CS- during Acquisition phase. Two-way ANOVA comparison shows *A m. caucasica* has no significant odor preference between lavender and cinnamon for the Initial CS+ (P-value = 0.41, F(1,54) = 0.37) or the Initial CS- (P-value = 0.82, F(1,54) = 0.05). Likewise, *A m. syriaca* showed no significant odor preference between lavender and cinnamon for the Initial CS+ (P-value = 0.62, F(1,54) = 0.27) or the Initial CS- (P-value = 0.21, F(1,54) = 1.63). As a result, type of odor was excluded from further consideration, and the first CS+ odor is simply coded as A+, and the second CS+ as B+, the odors that are CS- are then B- in the Acquisition phase, and A- in the reversal phase.

We found that bees from both subspecies has a similar learning rate for the A+ in the Acquisition phase (see Figure 1.2. Panel A+). We also found that both subspecies showed discrimination and did not respond by proboscis extension to B- in the acquisition phase (see Figure 1.2. Panel B-). Surprisingly we found that during Reversal Phase *A m. syriaca*'s acquisition of B+ is impaired (Figure 1.2. Panel B+). This is unique to *A m. syriaca* as can be seen when our results are compared with those of similar experiments in the European honey bee from North America (a mix of the European *A.mellifera* subspecies, Ben-Shahar et al., 2000, Figure S1) or *A.m. anatoliaca* (Abramson et al. 2015). The Reversal Phase extinction of odor A (A-) was different, in that complete extinction did not occur, and extinction was slower for both *A.m. caucasica* and *A.m. syriaca* in comparison to bees from other subspecies (Figure S1, also see Figure 1.2. Panel A).

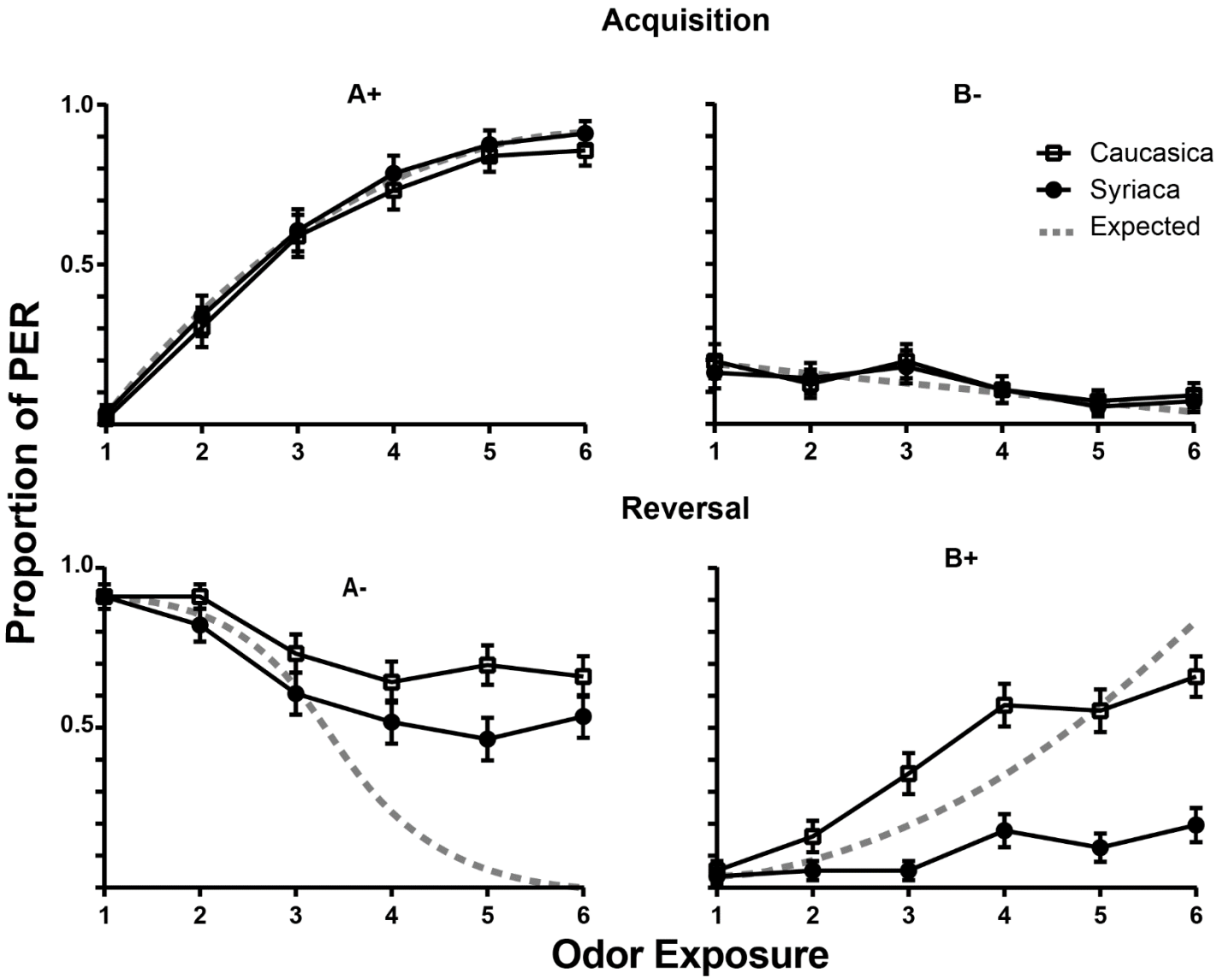


Figure 1.2. Comparison of responses to odors A and B between honey bee subspecies *A.m. caucasica* and *A.m. syriaca* during a proboscis extension response (PER) assay. Each data point shows the percentage (± standard error) of bees that showed PER during the assay. During the **Reversal for A-**, Sidak's multiple comparisons test confirms the observed differences in trial 5 (Alpha = 0.05, P-value < 0.05). During the **Reversal for B-**, Sidak's multiple comparisons test confirms the observed differences in trials 3 - 6 (Alpha = 0.05, P-value < 0.0001).

Supplemental Results:

Electric shock avoidance (ESA) conditioning

We used the ESA conditioning assay to determine if the honey bee subspecies *A.m. caucasica* and *A.m. syriaca* have spatial avoidance learning differences. Since each individual can be on one side of the apparatus at the same time, we only present the data for the CS+. Wilcoxon matched-pairs signed rank test shows there is no significant color preference for *A m. caucasica* between Blue and Yellow for the Initial CS+ (P-value = 0.31, W = -9.00). Likewise, *A m. syriaca* showed no significant color preference (P-value = 0.62, W = 0.13). We found that there are no differences between the learning rates for members of both subspecies during Acquisition (Phase I) or Reversal (Phase II) phases (Figure S2).

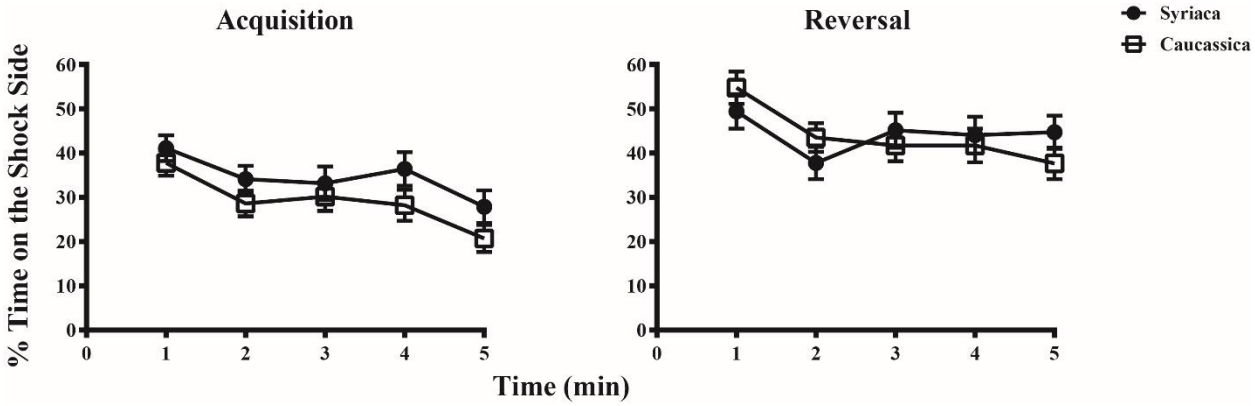


Figure S2. Comparison of spatial-avoidance learning rate between honey bee subspecies during an ESA assay. Each data point shows the percentage of time (± standard error) bees spent on the shock side during the trial. A two-way ANOVA test shows there are no differences between subspecies during Acquisition F (1, 109) = 2.315, P-value > 0.13 or during Reversal F (1, 109) = 0.0065, P-value > 0.93.

Discussion

The most significant finding of this study is that appetitive olfactory reversal learning differences across honey bee subspecies match differences in their foraging plasticity. These learning differences are specific to task since no differences across subspecies were observed for aversive conditioning. In appetitive olfactory reversal learning, bees from the subtropical subspecies *A.m. syriaca* do not show reversal, specifically they do not form association for the odor that is rewarded in the reversal phase. Unlike the typical reversal response of other organisms, such as other bee subspecies (see below), bees in this study continued to respond to the previously rewarded but now unrewarded odor in the reversal phase. Should these responses occur in the context of foraging, *A.m. syriaca* bees are expected to visit only flowers similar to a first learned flower. *A.m. caucasica* bees would be expected to visit an expanding repertoire of flowers with different features. These results suggest molecular substrates of learning and memory to be candidates for selection in adaptation to specific ecological conditions.

Specific learning differences across populations

This study is, to our knowledge, the first to demonstrate specific learning plasticity differences across genetically distinct populations of the same species. This could be due both to comparison of populations from contrasting environmental conditions and to use of a complex learning paradigm. In fact, the behavior of both of these subspecies, living at near extremes of honey bee distribution, differ from other subspecies such as *A.m.*

ligustica, *carnica*, and *anatoliaca* (Charles I. Abramson et al., 2015; Ben-Shahar et al., 2000; Hadar & Menzel, 2010). In these other subspecies similar paradigms result in complete switch from proper response to A+B- to proper response to A-B+, similar to other organisms (Izquierdo et al 2016).

The complexity of learning challenge

Using simple conditioning, differences can be observed across drug treatment and control groups (e.g. Abramson et al. 2010, Giannoni-Guzman et al. 2014), but this simple paradigm cannot differentiate age and job-related differences; for instance, across nurse and forager honey bees, or younger and older foraging bees (see Ben-Shahar et al. 2000). In these situations, reversal learning paradigms are used to better differentiate the learning abilities that change with age or disease. For example, only during the reversal phase of a reversal learning paradigm could it be shown that dogs and primates exhibit impaired spatial navigation as they age (Lai, Moss, Killiany, Rosene, & Herndon, 1995; Mongillo et al., 2013). In another recent study, reversal learning was necessary to show that an animal model of anorexia nervosa has impaired cognitive-flexibility, just like the human counterpart (Allen, Jimerson, Kanarek, & Kocsis, 2017; Tchanturia et al., 2011).

Reversal learning paradigms can probe deeper than its simple conditioning counterpart because it combines two related yet distinct conditioning phases: discrimination and reversal. Thus, we suggest the use of reversal learning paradigms could also be more appropriate when small differences in cognitive performance are expected in other organisms.

Neural substrates of reversal learning

In studies targeting mechanistic understanding of reversal learning, it is shown that in the first acquisition of rewarded vs non-rewarded stimuli, a type of discrimination learning, vs the second or reversal phase are shown to depend on different neural substrates (Izquierdo et al. 2016, in bees Devaud et al. 2007). The acquisition phase does not require mushroom body yet reversal phase requires the alpha-lobes of the mushroom bodies, as demonstrated by effects of anesthetics applied directly to this region only interferes with reversal phase but not with acquisition phase (Devaud, Blunk, Podufall, Giurfa, & Grünewald, 2007). Because neuropharmacological studies demonstrate the role of dopamine in reversal learning (Costa, Tran, Turchi, & Averbeck, 2015), it will be interesting to examine correlates of dopaminergic signaling in the mushroom bodies of *A.m. syriaca* and *A.m. cauc*asica bees.

A.m. caucasica versus A.m. syriaca

In this study, using the appetitive reversal learning paradigm we demonstrate that *A.m. caucasica* learns new associations, and keeps the previous associations. This is consistent with a highly plastic, generalist foraging behavior. *A.m. syriaca* shows very low plasticity in foraging choice (Cakmak et al. 2010, see Figure 1.1), and the lack of reversal learning in the appetitive reversal learning paradigm may underlie specialization to one or few resources. Specialization provides for speed of foraging and may reduce exposure to predators during foraging episodes. Foraging modeling (Becher et al., 2014) can help us further dissect the ecological importance of these observed differences.

Appetitive vs aversive learning

One interpretation of differences across *A.m. syriaca* and *A.m. caucasica* could have been greater learning ability in one vs the other subspecies. However, in that case learning effects would have been expected to be general, such as performance differences in all tasks across the two subspecies. This would be similar to comparing bees treated orally with ethanol and control group bees. For these two groups, both in appetitive and aversive learning tasks the 10% or higher ethanol treatment group performed poorly (Giannoni-Guzmán et al., 2014). However, in the current study different modes of learning, appetitive vs aversive, differed, and in aversive learning both *A.m. syriaca* and *A.m. caucasica* demonstrated complete reversal of punishment learning. This difference across learning modalities also supports ecological relevance of differences in appetitive reversal learning across subspecies.

Conclusion

In this study we demonstrated a match between ecology of foraging behavior and learning and memory differences of two honey bee subspecies. As a result we conclude molecular substrates of the foraging differences extend beyond modulation of the reward pathway as was demonstrated previously (e.g. Giray et al., 2015), and involves specific learning genes and their expression in different neural circuits. In future, it will be important to examine neurons involved in appetitive learning in the two subspecies, and examine expression of well-studied learning genes, and connections of aminergic cells and targets in relation to differences in acquisition and reversal phases. The molecular

485 targets that are linked with the obsessive-like behavior of *A.m. syriaca*, can also be
486 relevant for other normal or diseased learning contexts such as imprinting or addiction.

487 **Acknowledgements**

488 We like to thank members of Giray and Agosto laboratories for providing revisions and
489 critiques on earlier drafts of the work.

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Chapter 2:

Comparison of Individual Foraging Strategies Across Multiple Subspecies of Honey Bee

Introduction

Sucrose is a vital resource for honey bee foragers as they must constantly judge its quality and quantity to optimally forage a patch of flowers. However, each individual has different perceptions of what is the optimal foraging strategy which can be influenced by: (1) internal colony conditions like brood levels, population size, nectar and pollen stores; (2) heredity, which can influence the weight placed on resource quality vs the foraging effort; (3) and the environment which can influence ecological opportunity and predation risk, among other things (Anreiter & Sokolowski, 2019; Beeler & Mourra, 2018; Burke et al., 2012; Çakmak et al., 1998; Eckert, Winston, & Ydenberg, 1994; Huetteroth et al., 2015; Page, Waddington, Fondrk, & Hunt, 1995; Toscano et al., 2016). It is the existence of this diversity and constraints which can lead to the development of Individual Foraging Strategies (IFS) within a population.

In this study, we explore the influence of Reward-Effort valuation and Behavioral Flexibility in the expression of Individual Foraging Strategies (IFS) in three honey bee subspecies populations: *A.m. caucasica*, *A.m.* syriaca, and the gAHb. To this end, we used a Free-flying foraging assay where bees must balance energy budgets to judge between the effort exerted and the quality of the reward obtained in a changing artificial-environment (Çakmak et al., 2009). We also developed a new foraging strategy

classification method which considers the learning experience of every individual in the assay.

Given the research suggesting that environmental factors can select for specific IFS distributions, we hypothesized that the IFS distribution profiles of these honey bee subspecies would map to the one that is most beneficial in their ancestral habitats (Costa-pereira & Pruitt, 2019; Dingemanse & Wolf, 2013; Layman et al., 2015; Page et al., 1995; Parker & Hawkes, 2018; Pyke, 1984; Toscano et al., 2016).

Methods

I. Design and execution of the foraging assay

Artificial Flower Patch Design

We used the artificial flower and flower patch design of (Çakmak et al., 2009). Flowers had either short stamens (4mm) or long stamens (16mm). The flowers were painted blue (Testors ™ 1208) or white (Testors ™ 1245) on the underside. The flower patch was brown and had 18 blue and 18 white flowers spaced 75mm apart in a 6 row by 6 column Cartesian lattices. **Figure 2.1**

Foraging Assay

Training

Honey bee foragers were trained to visit the experimental site which was at least 50m from the colony. replicating the method used in Çakmak et al. (2009). To train the bees we would place a petri dish with pair of artificial flowers (blue and white) and sucrose at the colony entrance. As bees started visiting the petri dish, we would slowly move it until we arrived at the experimental site. The sucrose used for the training was 1M and had 1 µL of essential oil (cinnamon, lavender, or mint) to serve as an odorant.

Experiment

Each experiment uses a new set of uncaged, free-flying, naïve foragers that had no prior experience with the artificial flower patch. Each bee was be uniquely marked with paint following the methods of Seeley (1995).

The experiment consisted of three phases: (1) a control phase where both blue and white flowers had short stamens and each flower offered foragers 4µl of 1.25M

sucrose. The control phase ended when all the participant foragers reached 30 flower visits. (2) The control is followed by an acquisition phase where flowers offered foragers 4µl of 2.0M sucrose in long-stamen white flowers and 4µl of 0.5M sucrose in short-stamen blue flowers. The acquisition phase ended when all participating foragers reached 50 flower visits. (3) The last phase, the reversal phase, presented foragers with 4µl of 2.0M sucrose in long-stamen blue flowers and 4µl of 0.5M in short-stamen white flowers.

Once a bee finished their visits for each phase of the experiment, it would be placed in a cage (big enough to fly around) until the rest of the bees participating in the experiment would finish that phase. Once all participating bees finished the phase, we would switch out the flowers and let the bees fly out of the cage. Great care was taken to minimize the interactions with and stress to the bees during this process.

Team composition

The experiments were run by two teams. A team of experimenters refilled the flowers after every visit. This role was extremely important since bees should not visit empty flowers as this could add other variables to the results. The second team oversaw data acquisition. They followed each bee and annotated which flowers where visited.

Annotations

We annotated the visits of each phase in the following way: during the control phase, white flowers = 0 and blue flowers = 1; during the acquisition and reversal phases, easy/low-reward flowers = 0 and hard/high-reward flowers = 1.

II. Comparison and development of foraging strategy classification methods

Foraging Strategy Classification Methods

Method # 1: 50% Boundary Method

Prof. Harrington Wells from the University of Tulsa and his colleagues developed the foraging assay we used in our study and a classification method to describe the foraging strategies a bee could take in this assay (Çakmak et al., 2009; Giray et al., 2015). The foraging strategies he describes are: **Work Minimizing** (WM), **Color Constance** (CC), and **Energy Maximizing** (EM); each represents criteria used to solve the foraging assay.

To make the classifications, Prof. Well takes the percent of visits a bee makes to a flower type during the Acquisition and Reversal phases of the assay. If a honey bee goes more than 50% of the time to hard/high-reward flowers on both phases, it is an **EM**. Less than 50% of the time to hard/high-reward flowers on both phases, it is a **WM.** All other combinations are classified as **CC**.

Method #2: Chi-Square Statistic Method

A second classification method is proposed by Fanfan Noel, M.S in which he creates a new category. In his method, he uses Pearson's Chi-square test to discover when a bee has a significant preference of a specific flower type (Noel, 2019). Using this rationale, the boundary was drawn at 63% (31.5/50) flower visits. In practice, this results in the following way of classifying honey bees: If a bee goes more than 63% of the time to hard/high-reward flowers in both Acquisition and Reversal phases, it is an **EM.** If it goes less than 37% of the time to hard/high-reward flowers (which means 63% of the time to easy/low-reward flowers) in both phases, it is a **WM.** If the bee goes 63% and 37% or

37% and 63% in each phase; it is a **CC**. All other bees are pooled into a new category called **Generalists** (G). For a visual representation of this method look at **Figure 2**.

Method #3: Clustering Method

The previous methods of classification did not consider the Control phase of the foraging assay or the temporal dimension of the data when classifying the honey bees by strategy. Therefore, we developed our own classification method which considers all the data and used unsupervised machine learning to find inherent foraging strategies in our populations rather than prescribing labels for possible foraging strategies.

The specific machine learning algorithm we used was Consensus Clustering with K-Means and the Euclidean distance metric. This is a type of Ensemble machine learning which helps reduce output variability and improves the predictive power of our algorithms (Alpaydin, 2014c). It was implemented with the ConcensusClusterPlus library from the R programming language (Wilkerson & Hayes, 2010).

After running the algorithm, we used 2 different measurements to decide how many foraging strategies we would have: (1) The Consensus Matrix (heatmap) plot **Figure 3** and (2) the Delta Cumulative Distribution Function (CDF) plot **Figure S2**. The Consensus Matrix plots are an easy way to visualize the consensus values and boundaries of each foraging strategy; the “cleaner” and darker the squares in the Consensus Matrix, the better (Wilkerson & Hayes, 2010). The Delta CDF plot shows the point at which the foraging strategies reach their “maximum stability”; this maximum is usually at the inflection point of the plot (Alpaydin, 2014a).

III. Comparison of the foraging strategies distribution across multiple subspecies

Transportation of honey bee colonies

Preparation began one day before transportation: (1) Excess honey was removed to prevent colony deaths due to the honey melting. (2) Regular colony covers were substituted by ventilated screen tops and covers. (3) It was ensured that all the holes in the colonies were plugged. The entrance was sealed with screen material to allow ventilation. (4) Colonies were tied with ratchet straps.

Ten colonies were moved at a time and transportation occurred primarily at night.

Experimental animals

Foragers of three subspecies populations were used. Two subspecies from Turkey: (1) *Apis mellifera caucasica*, and (2) *Apis mellifera syriaca*. Both subspecies are maintained in a common garden under similar environmental conditions. Several measures were taken to ensure the subspecies lines are maintained. Mated queens or colonies for each subspecies were sourced and transported from the TEMA foundation for *Am caucasica* and from the Beekeepers Association of Hatay (HAB) for *Am syriaca*. Each year new queens are ordered from our sources. Once they arrive, they are tagged with paint to confirm their identity. Genetic and morphometric analysis would we used periodically to ensure the subspecies maintained their identity (Kence et al., 2013). The experiments with these subspecies were performed in the garden of the honey bee apiary at the Middle Eastern Technical University (METU), Ankara, Turkey.

One other bee population was from Puerto Rico: the gentle Africanized Honey Bee (gAHb) (Galindo-Cardona et al., 2013). This population is maintained at the Estación Experimental of the University of Puerto Rico in Gurabo, PR. Experiments with this population were carried out in the same place.

Testing for Weather influence on Foraging Strategy

Using the timestamps of our experiments we extracted weather data from the Weather Underground (wunderground.org) repository. We used available measurements like average temperature and dew point to test if these had any effect on the honey bees' foraging strategy.

IV. Statistical tests for analyzing the foraging strategy data

We used Pearson's chi-square test to compare the distribution of foraging strategies among the honey bee populations and find if they are different. Then we implemented the statistical software TETRAD to build a data-driven graphical hypothesis of the causal relationships between Weather, Subspecies, and Foraging Strategy (version 6.7, Center for Causal Discovery, 2019; Glymour, Scheiner, Spirtes, & Ramsey, 2015). We built our hypothesis using the Greedy Fast Causal Inference (GFCI) algorithm for mixed data (continuous and discrete data) (Glymour, Zhang, & Spirtes, 2019; Ogarrio, Spirtes, & Ramsey, 2016).

Results

I. Design and execution of the foraging assay

Here, we developed a new classification method for the foraging strategies which result from the assay in **Figure 2.1** and we used this new method to compare the distribution of foraging strategies across multiple honey bee subspecies.

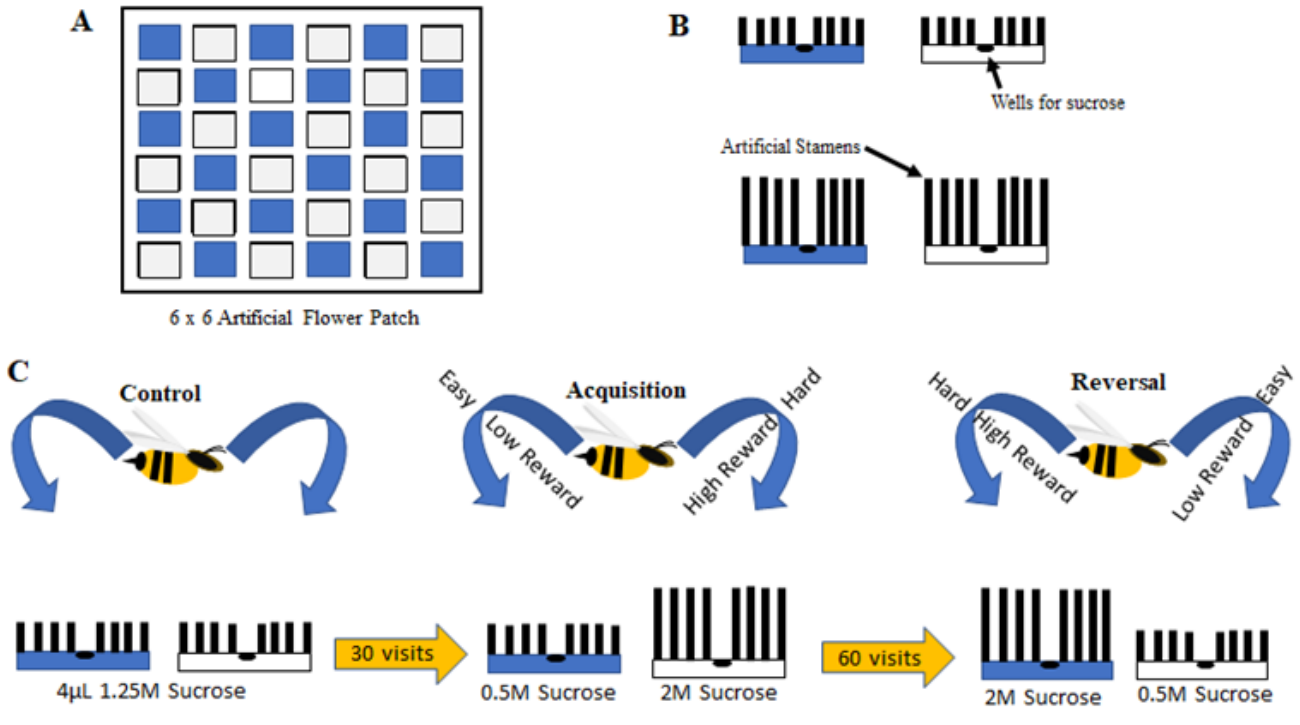

Figure 2.1: Foraging Assay
(A) The experiment is run in a 6 x 6 Artificial flower patch with flowers of different colors placed in a checkered manner. **(B)** There are 4 different types of artificial flowers: Long-Stamen White and Blue Flowers, Short-Stamen White and Blue Flowers. **(C)** There are 3 phases in this experiment. Each phase occurs without interruption. We follow bees that are uniquely marked. During Control we use Short-Stamen Blue and White flowers which contain 4µL of 1.25M Sucrose. After all bees do at least 30 visits, we switch out the Short-Stamen white flowers for Long-Stamen white flowers. During Acquisition we use Short-Stamen Blue flowers with 4µL of 0.5M Sucrose and Long-Stamen white flowers with 4µL of 2M Sucrose. After all bees do at least 50 visits, we switch out both flowers for Long-Stamen blue flowers and Short-Stamen white flowers. During Reversal, Long-Stamen blue flowers have 4µL of 2M Sucrose and Short-Stamen white flowers have 4µL of 0.5M Sucrose.

II. Comparison and development of foraging strategy classification methods

To analyze our results, it was necessary for us to classify the honey bees' foraging strategies into discrete groups. However, the existing classification methods had certain drawbacks. In this section we will compare both existing methods and justify the creation of a third option.

Method #1: 50% Boundary Method:

Prof. Harrington Wells from the University of Tulsa and his colleagues, developed the foraging assay we use in this study and the first classification method (Çakmak et al., 2009) **Figure 2.2A**. Their classification method is easy to implement, it draws a boundary at the 50% mark and from there it classifies the foraging strategy of a bee. This method has 3 problems: (1) It does not consider individuals who are very close to the 50% mark. A bee who went 51% of the time to hard/high-reward flowers is classified different than one who went 49% of the time. (2) This method assumes independence between the Acquisition and Reversal phases of the experiment and thus sets the boundary at the same level for both. However, we are learning that a honey bee's flower choice during the Reversal Phase is contingent upon its learning experience in the Acquisition phase. (3) This method also doesn't consider the temporal dimension of the data thus it further makes any learning processes invisible in the analysis. It could be that a bee has a learning bias for hard/high-reward flowers but, it learned slowly thought the course of the experiment in which case, the 50% boundary method would categorize this bee incorrectly as a Work Minimizer (**WM**) rather than as an Energy Maximizer (**EM**).

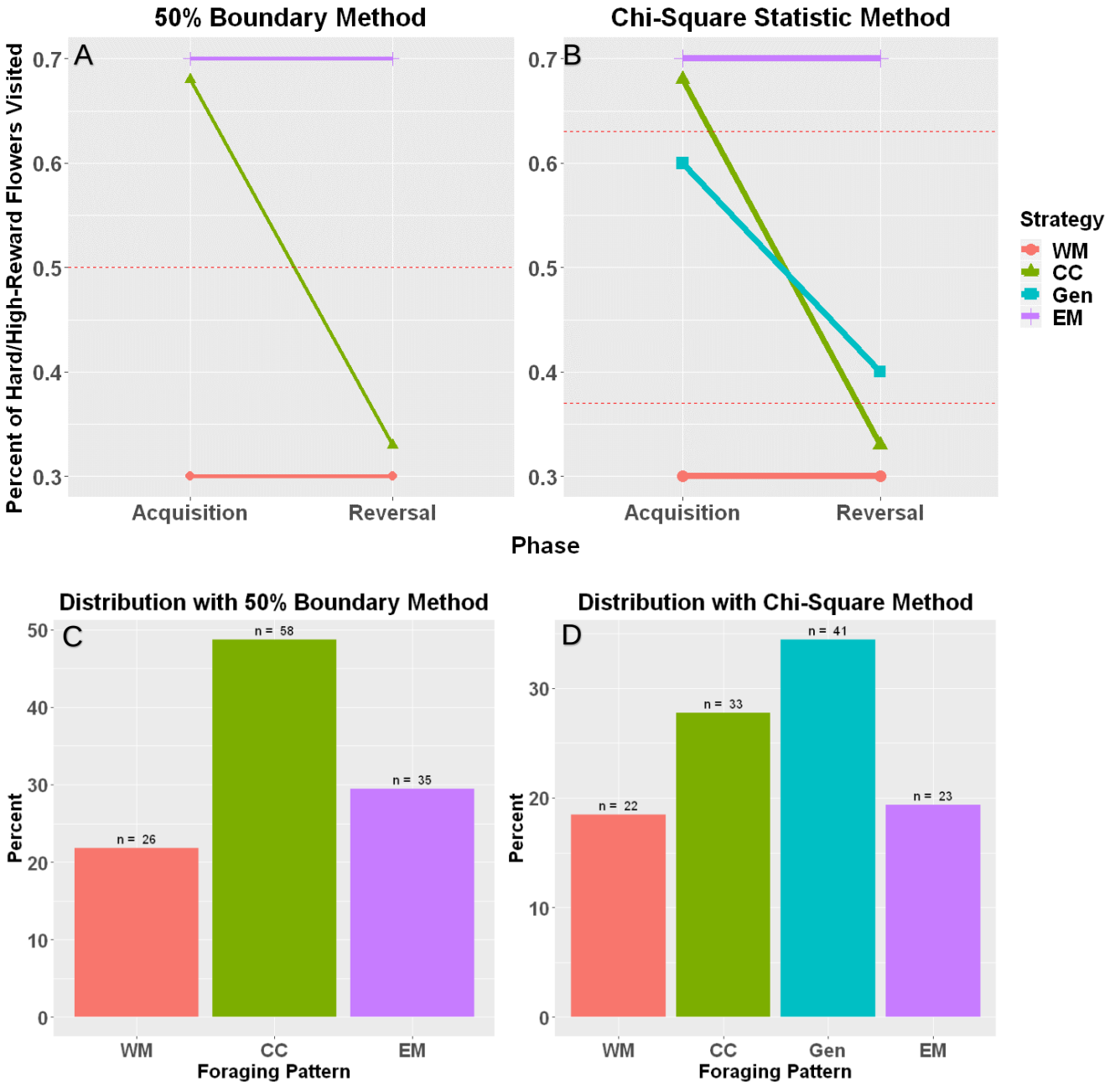

Figure 2.2: Current methods to classify individual foraging patterns in the foraging assay exhibit significant differences in the classifications.

(A) Example of 50% Boundary classification method: This method sets a boundary at 50% hard/high-reward flower visits. Bees change classification if they are above, below, or cris-crossing the boundary. The x-axis are the phases of the foraging assay. The y-axis is the Percent of Hard/High-reward flowers visited on each phase. **(B) Example of Chi-square classification method:** This method sets a boundary at 63% and 37% hard/high-flower visits. Bees change classification by being above the 63%, below 37%, cris-crossing 63% and 37%, or if they don't get to 37% or 63% in any phase. The x-axis are the phases of the foraging assay. The y-axis is the Percent of Hard/High-reward flowers visited on each phase. **(C) Distribution of foraging strategies with the 50% Boundary Method:** We applied this method to the data from our experiments. The y-axis is the percentage each foraging strategy represents in the distribution. The x-axis is each foraging strategy. **(D) Distribution of foraging strategies with the Chi-square Method:** We applied this method to the data from our experiments. The y-axis is the percentage each foraging strategy represents in the distribution. The x-axis is each foraging strategy. The new **Generalist** category works as a sink for individuals that didn't make the cut for the WM, CC, or EM strategies.

Method #2: Chi-square Statistic Method

To address the first problem with the 50% boundary method, Fanfan Noel (2019); proposes a new classification method in his master's thesis **Figure 2.2B**. In his method, Fanfan (2019) sets an upper and lower boundary at 63% and 37% visits to hard/high-reward flowers and creates a new category called **Generalists** (**Gen**) for bees that don't make the boundaries. However, this doesn't address the other 2 weaknesses of Prof. Well's method and has a drawback of its own. The new Generalist category works like sink which results in having bees under the same label which have very disparate foraging strategies **Figure 2.2D**. We could have a bee that had a score of 63% (Acquisition phase) and 38% (Reversal phase) in the same category as a bee with a score of 38% (Acquisition phase) and 63% (Reversal phase).

Method #3: Clustering Method

Therefore, we use the following justification to create our new classification method: **(1)** We assume that the results of the Reversal Phase are contingent upon the results of the Acquisition Phase. The foraging assay we employ is a learning assay, the result of each flower visit is contingent on all the flower visits before it. **(2)** Creating categories for the foraging strategies a priori brings the trouble of some of them working like a sink and not describing behavior accurately. For these reasons, our method considers the temporal dimension of the data and creates the categories based on the data itself.

With this in mind, we applied our data to the **Consensus Clustering Plus** algorithm which outputs measurements of how many foraging strategy clusters are likely to be in our data **Figure 2.3** and **Figure 2.4**. The measurement in **Figure 2.3** is a Consensus Matrix where, the "cleaner" and darker the squares the more stable the clusters of foraging strategies are. This method hinted at us having 4 foraging strategies in our data **Figure 2.3B**. Next, the Delta CDF Curve, has its inflexion point at 4 foraging strategies **Figure 2.4**. Therefore, we had enough reason to classify our bees into four strategies. **Figure 2.5** shows the typical behavior for bees of each strategy during the foraging assay.

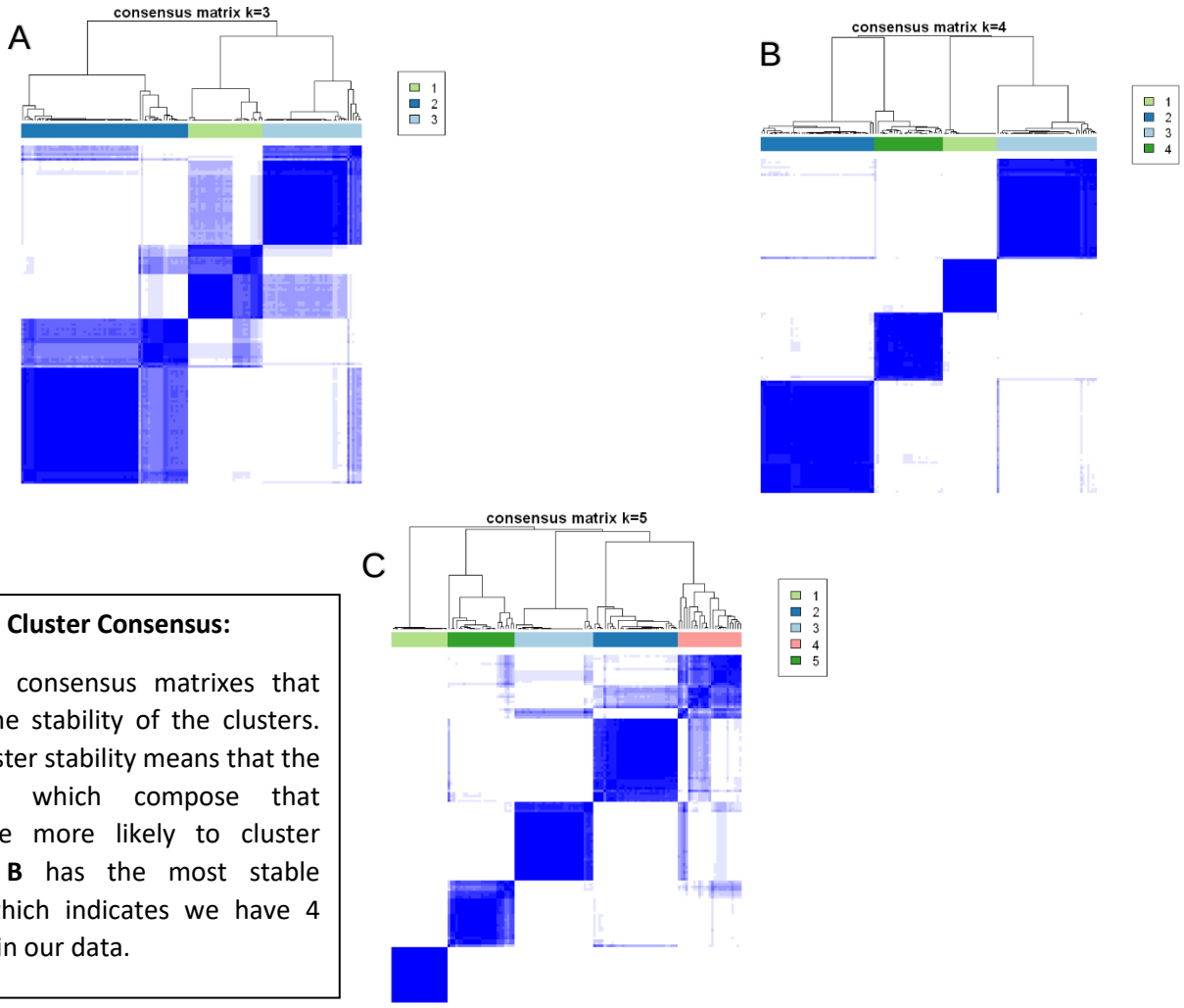

Figure 2.3: **Cluster Consensus:**

These are consensus matrixes that indicate the stability of the clusters. Higher cluster stability means that the individuals which compose that cluster are more likely to cluster together. **B** has the most stable clusters which indicates we have 4 strategies in our data.

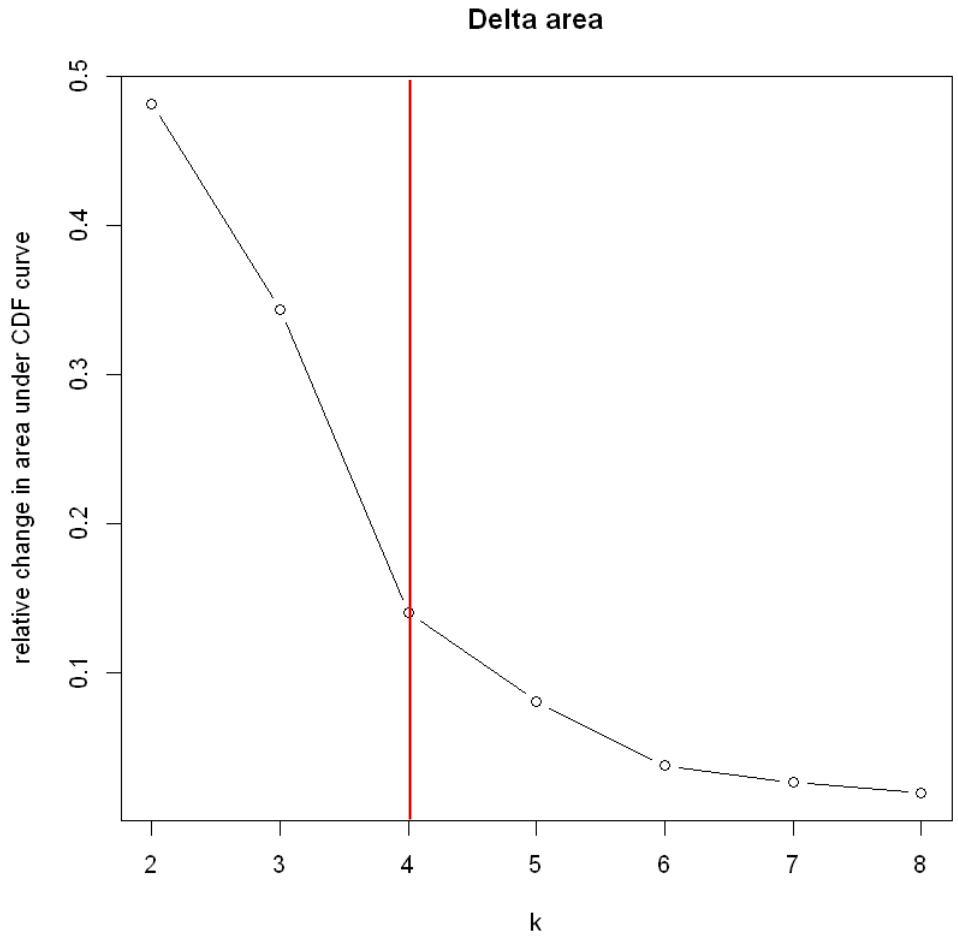

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Figure 2.4: Delta CDF Curve:

The x-axis represents the number of foraging strategy clusters. The y-axis is the change in area under the CDF curve. The red line indicates the inflection point of the curve at k = 4.

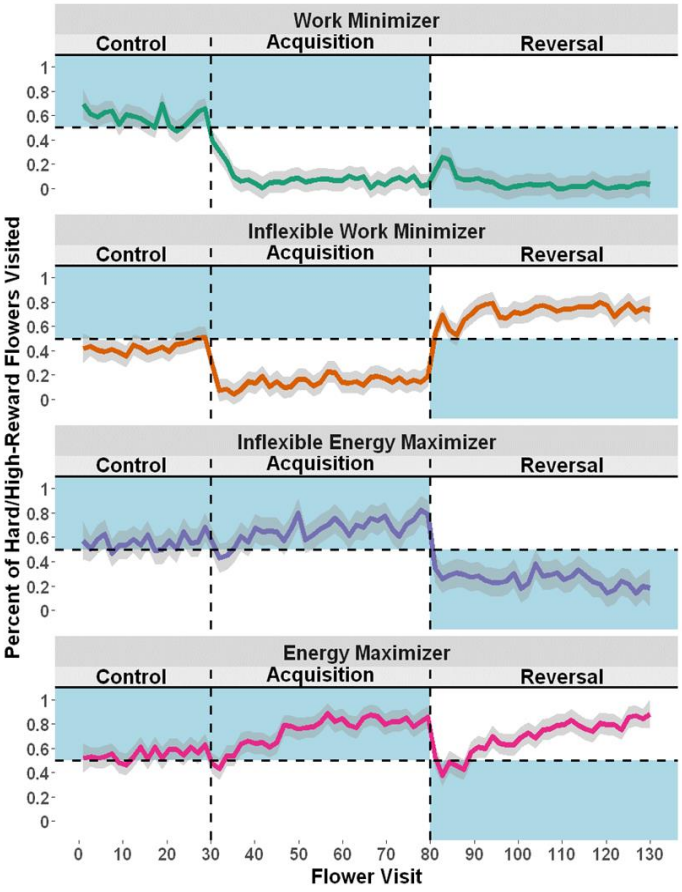

Figure 2.5: Foraging Strategy clusters by time:

Flower visits with different Difficulty & Reward by Foraging Pattern. The data was plotted using LOESS with a span of 0.1. The y-axis is the Percent of Hard/High-Reward Flowers Visited by honey bees from each strategy except during the Control Phase. During the Control Phase, the y-axis represents that flower color that became the Hard/High-Reward Flower during the Acquisition Phase. The x-axis is each Flower Visit of the honey bees.

An ANOVA test shows the foraging strategies are significantly different (Foraging Strategy **:** Phase **:** Trial, df = 6, F-value = 9.661, P-value < 0.0001) white the Control phase is not different (Foraging Strategy **:** Phase(Control), df = 3, F-value = 2.596, P-value = 0.0559).

Our naming for each of the foraging strategies in **Figure 2.5**, borrows from Prof. Well's original names and behavioral flexibility principles (Çakmak et al., 2009; DeWitt et al., 1998; Ferguson et al., 2001; A. Izquierdo, Brigman, Radke, Rudebeck, & Holmes, 2017; Xue et al., 2013). **(1) Work Minimizer (WM)** individuals follow easy/low-reward flowers through the Acquisition and Reversal phases of the experiment. **(2) Inflexible Work Minimizer (IWM)** individuals follow easy/low-reward flowers during the Acquisition phase and follow flowers of the same color during the Reversal phase. **(3) Inflexible Energy Maximizer (IEM)** individuals visit hard/high-reward flowers during Acquisition phase and follow flowers of the same color during the Reversal phase. **(4) Energy Maximizer (EM)** individuals follow hard/high-reward flowers during the Acquisition and Reversal phases.

Next, we applied these new foraging strategy labels and method to each of our honey bees to compare if they are evenly distributed across multiple honey bee subspecies.

III. Comparison of the foraging strategy distributions across multiple subspecies

Distribution of foraging strategies by subspecies

We found that each of the honey bee populations we surveyed has a preference for particular foraging strategies **Figure 2.6**: **(1)** *A.m. caucasica* individuals prefer the Energy Maximizing (IEM and EM) foraging strategies, specially where they visit hard/high-reward flowers during the Acquisition phase. These individuals either create a strong association with that color (IEM) or they can switch colors by following the reward (EM). **(2)** Most gAHb individuals follow a pattern of always going to easy/low-reward flowers (WM) or sticking to the color that was first associated with hard/high-reward flowers (IEM). **(3)** Overwhelmingly, most *A.m. syriaca* individuals follow foraging strategies where they visit hard/high-reward flowers during the Reversal phase (EM and IWM).

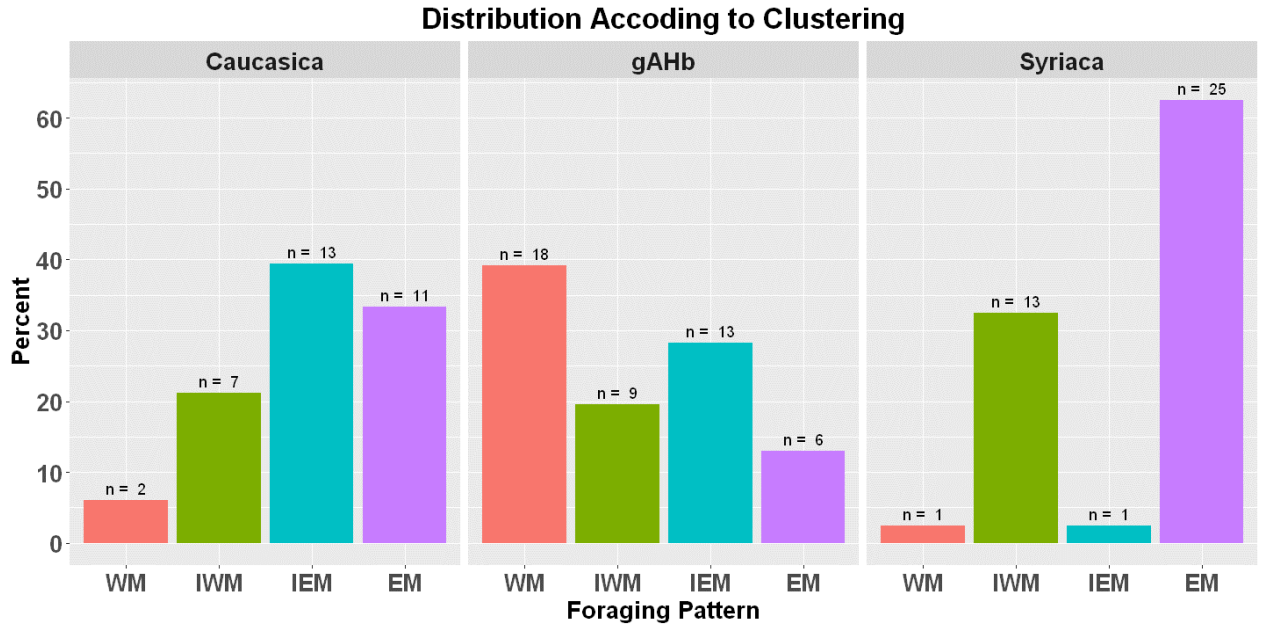

Figure 2.6: Distribution of Foraging Strategies across subspecies according to the clustering method:

Strategy is differentially distributed among subspecies. A chi-square test shows that the way individuals are distributed is significantly different among the subspecies (X-squared = 48.148, df = 6, P-value < 0.0001).

Causal hypothesis for the relationships between Temperature, Dew Point, Subspecies, and Foraging Strategy

We combined the data in **Figure 2.6** with weather data we extracted from the online repository "Weather Underground" to build a causal graph (Pearl, Glymour, & Jewell, 2016; Wheather Underground, 2020). Our goal was to discover a hypothesis for the causal relationships across: Foraging Strategy, Subspecies, Average Temperature, and Dew Point. For this, we used the causal inference software, TETRAD (version 6.7, Center for Causal Discovery, 2019). This program used our data to build a hypothesis on the causal relationships of our variables. The graph in **Figure 2.7** proposes that the Foraging Strategy of a honey bee is indirectly caused by Subspecies and that there are latent unmeasured variables in the chain of causation that are more proximate causes of Foraging Strategy. An example of what these unmeasured variables could be is the expression of genes associated with learning and reward valuation. Furthermore, the causal graph proposes there is not a direct relationship between a honey bee's foraging strategy and the weather conditions we have data on.

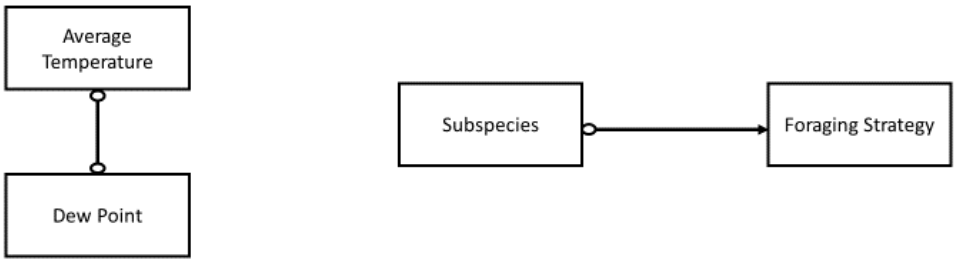

Figure 2.7 Causal hypothesis of Foraging Strategy:

We used the GFCI algorithm (Glymour et al., 2019; Ogarrio et al., 2016) for mixed (continuous and discrete) data. The "o->" arrow indicates Subspecies causes which Cluster an individual will belong to, but that there is a latent confounder in between both. "o-o" indicates that Average Temperature, Dew Point, Colony, and Subspecies have some relationship but the directionality of it is unknown and there may be latent unmeasured variables in between.

Discussion

In this study, we improved on previous foraging strategy classification methods by using Consensus Clustering with K-means to examine the temporal dimension of our foraging assay. With this method, we found four (4) distinct foraging strategies which honey bees used to solve our foraging assay: Work Minimizing (**WM**), Energy Maximizing (**EM**), Inflexible Work Minimizing (**IWM**), and Inflexible Energy Maximizing (**IEM**). The difference between each strategy stems from an interplay of each honey bee's approach to reward-effort valuation and their behavioral flexibility ability. WM and IWM individuals prefer to spend less effort (time and energy) to access resources even if they sacrifice reward quality. Opposite to these are the EM and IEM, individuals which prefer a to spend more effort if it means they'll get a higher quality reward. The IWM and IEM individuals are those which can't accurately adapt to the changing environment and thus can't fully express their reward-effort preference.

In the case of the honey subspecies populations we surveyed, each showed unique foraging strategy distribution profiles **Figure 2.6**. *A.m. caucasica* bees showed a preference for high-reward flowers, with most of its individuals being inflexible (IWM + IEM). In the case of *A.m. syriaca,* bees that showed a significant preference for Energy Maximization strategies had high behavioral flexibility while those that preferred Work Minimization showed low behavioral flexibility. Finally, while the gAHb population has a good balance between all strategies, its Work Minimizers are highly flexible and its Energy Maximizing individuals are mostly inflexible.

Consensus Clustering as a foraging strategy classification method

The 50% Boundary and Chi-square Statistic classification methods: (1) assumed independence between the phases of the foraging assay, (2) didn't take into account the temporal dimension of the data (learning), (3) and assumed a priori the strategies foragers would follow (**Figure 2.2**) (Çakmak et al., 2009; Giray et al., 2015; Noel, 2019). We chose to use Consensus Clustering as a simple computational method to address these 3 assumptions. It is important to discuss however, that our method does have drawbacks. It is sensitive to the size and complexity of the data set. As we collect more honey bee foraging data, the Consensus Clustering will become more accurate and we may even discover new foraging strategies, however, some of the individuals we've classified in this study may be labeled as belonging to another strategy that better fits their behavioral patterns (Wilkerson & Hayes, 2010). We could improve our method by: (1) Separating extreme individuals into their own categories by running "Outlier Detection" before the clustering (Alpaydin, 2014b). (2) Using fuzzy classification, a method where we get a score of how much an individual, pairs with each strategy.

The four strategies

With our classification method we found four major foraging strategies in the data. Although we could set the algorithm to find more than four strategies, the measurements used to asses cluster stability indicated that doing so would reduce the accuracy of our classification method **Figures 2.3 - 2.4**. Therefore, we left it at four and used a combination of the Energy Maximization models of Optimal Foraging Theory and Behavioral Flexibility principles to name our foraging strategies: Energy Maximizer, Inflexible Energy Maximizer, Work Minimizer, and Inflexible Work Minimizer (Çakmak et al., 2009; Parker & Hawkes, 2018; Pyke, 1984). Strategies where bees followed hard/high-reward flowers during the Acquisition Phase would be Energy Maximizers. Work Minimizing strategies would follow easy/low-reward flowers during the Acquisition Phase. Those strategies that failed to follow their acquired preference in the Reversal Phase were prefixed as “Inflexible” to denote these bees as having less behavioral flexibility than their counterparts.

Foraging strategy distributions across multiple subspecies

In this study, we controlled for the environment between the *A.m. caucasica* and *A.m.* syriaca subspecies and we still observe significant variation between their IFS distributions. This is consistent with the idea that pressures in the ancestral habitats of these bees could influence which IFS profiles are being selected for (Araújo et al., 2011; Page et al., 1995; Parker & Hawkes, 2018; Sih & Del Giudice, 2012). This idea is further reinforced by the causal graph we built which shows short-term environmental changes like Temperature and Dew Point have no direct causal connection to foraging strategy while Subspecies and what it entails, is a distal cause of Foraging strategy.

The causal hypothesis in **Figure 2.7** suggests there are hidden/unmeasured variables that are encompassed in Subspecies and affect the Foraging Strategy of each bee. These unmeasured variables could be the morphology of the bees or the expression of genes which modulate: learning & memory, reward valuation, lipid transport, and programmed cell death, among others (Naeger & Robinson, 2016). For example, genes for octopamine and dopamine receptors, which are related reward valuation, have different responses to training **Figure A1**. *A.m. caucasica* bees that have gone through the foraging assay show a significantly different expression of amDOP2 (dopamine receptor) and OA1 (octopamine receptor) while *A.m. syriaca* bees do not.

The role of octopamine and dopamine in modulating which foraging strategy a bee will follow has been further studied by Giray et al. (2015) and Fanfan Noel (2019) in his master's thesis. Giray and colleagues (2015) found that while octopamine antagonists or agonists do not affect foraging strategy, it will affect the fidelity a bee has to its strategy. On the other hand, Noel (2019) studied the effects of dopamine on the gAHb. This bee had the preference for the Work Minimizing strategy **Figure 2.6**. He found that a dopamine receptor antagonist shifts the bees to follow the Color Constant strategy as described in the Chi-square statistic method **Figure 2.2**. This Color Constant is a combination of the Inflexible Work Minimizer and Inflexible Energy Maximizer in our clustering classification method. This suggests the dopamine antagonist is reducing the bees' behavioral flexibility.

In the same study, a dopamine receptor agonist had the effect of shifting the bees into the Generalist strategy. This suggests the dopamine agonist, like the octopamine antagonist and agonist, make the bees have less fidelity to their strategy. The reasoning behind this is that the generalist strategy in the chi-square method is composed of all the bees that did fall into the Work Minimizing, Energy Maximizing, or Color Constant strategies. Therefore, bees that fall into the generalist strategy are those that have more variance in their foraging pattern.

Different selective pressures on a subspecies, could affect the expression of these genes which in turn influences which foraging strategy an individual will follow. This would support the idea that foraging strategy profiles are heritable in the populations. For example, *A.m. caucasica* and the gAHb come from area with high floral diversity which coincides with the thought that environments with high resource diversity promote the diversification of IFS in a population (Araújo et al., 2011; Layman et al., 2015; Toscano et al., 2016). *A.m. caucasica* which comes from deciduous forests with constrained blooming periods has most of its bees following the EM, IEM, and IWM foraging strategies (Adl, Gençer, Firatli, & Bahreini, 2007; Gençer & Firatli, 1999). The short blooming period could be a constraint for the development of behavioral flexibility while simultaneously promoting the preference for Energy Maximizing strategies (Komers, 1997; Mathot et al., 2011; Mathot, Wright, Kempenaers, & Dingemanse, 2012). *A.m. syriaca which* comes from a subtropical desertic rocky region that has few flowers blooming all year long and a predator which targets them when they are in flowers, mostly follow the EM and IWM strategies (Kandemir et al., 2000, 2006) The low floral diversity could be constraining the expression of IFS. On the other hand, the predation risk could be inflating the cost of each foraging trip and thus would push the individuals towards the EM strategy (Butler, 1974; Çakmak et al., 1998; Ishay et al., 1967; Mathot et al., 2012; Roubik, 1992; Ruttner, 1988). However, we can't explain the prevalence of the IWM strategy. It could be that these individuals forage closer to the colony and thus don't face as much predation risk as the bees that follow the EM strategy. Finally, the gAHb population which inhabits a subtropical island with an abundance of flowers blooming all year long and which has predator, has a distribution of IFS opposite to *A.m. caucasica.* Most of the gAHb honey bees follow the

WM, IWM, and IEM foraging strategies (Galindo-Cardona et al., 2013). The high floral diversity could be promoting a diverse IFS profile while the predator could skew the foraging preference towards WM, especially since these bees don't have a pressure to forage all they can before winter (Mathot et al., 2011; Mongillo et al., 2013; Toscano et al., 2016).

Limitations & Future Directions

The data set of our study was limited in how many individuals we could follow. This affected the predictions made the clustering algorithm, however, as we get more data the predictions will become more exact. In future experiments, we track the age of the bee and when it started foraging, since foraging experience could affect which strategy a bee will prefer. We should also do an extensive profiling of gene expression differences to dissect which genes are involved in modulating the behavior of the honey bees when going through our foraging assay.

General Discussion

In this thesis we explored the causes of individual specialization by studying the distributions of foraging strategy profiles across multiple subspecies of honey bee and the context-dependent behavioral flexibility in honey bees by comparing their performance in olfactory and foraging assays.

It seems like the foraging strategy of a honey bee has a heritable component. These foraging strategies must be modulated by reward-effort valuation processes and Behavioral Flexibility. Furthermore, we found that the behavioral flexibility ability in these honey bees is context dependent. In the Reversal Learning Proboscis Extension Reflex (PER) assay, *A.m. caucasica* reversed the associations faster than *A.m. syriaca* while the opposite was true in the Free-flying foraging assay. Most *A.m. syriaca* individuals followed flexible foraging strategies while the *A.m. caucasica* individuals followed inflexible strategies. To discover if the inverse relationship in behavioral flexibility ability holds across learning contexts, we could have each honey bee go through both the Foraging Assay and the PER so that we can compare their performance in both.

Appendix A:

Dopamine and Octopamine receptor expression of *A.m. caucasica* and *A.m. syriaca*

Methods

Experimental Animals

Two subspecies were used for these experiments: (1) *Apis mellifera caucasica*, and (2) *Apis mellifera syriaca*. Both subspecies are maintained in a common garden under similar environmental conditions. Several measures were taken to ensure the subspecies lines are maintained. Mated queens or colonies for each subspecies were sourced and transported from the TEMA foundation for *Am caucasica* and from the Beekeepers Association of Hatay (HAB) for *Am syriaca*. Each year new queens are ordered from our sources. Once they arrive, they are tagged with paint to confirm their identity. Genetic and morphometric analysis would be used periodically to ensure the subspecies maintained their identity (Kence et al., 2013). The experiments with these subspecies were performed in the garden of the honey bee apiary at the Middle Eastern Technical University (METU), Ankara, Turkey.

Naive honey bees were collected at the entrance of the colony as they return from foraging trips. Trained honey bees were collected at the artificial flower patch right after completing the reversal phase of the Free-Flying foraging problem **Figure 2.1**. After collection bees were placed in a -80℃ freezer until dissection.

Brain dissections

Brain dissections were done in a bed of dry ice. We removed the hypopharyngeal glands from each individual brain before placing it ‘intact’ in RNAlater®-ICE from

Ambion® by life Technologies™ for 24 hours at -20℃ to preserve the genetic material. Afterwards, we removed the optic lobes (OL) from each brain.

mRNA extractions and cDNA conversion

mRNA extractions were done using the RNeasy® Micro Kit from QIAGEN® following the standard protocol. The tissue disruptor in the RNeasy® Micro Kit protocol was replaced by a 1mL TB Syringe from BD, a new sterile syringe was used for each sample. For the RNA to cDNA conversion the ProtoScript® First Strand cDNA Synthesis Kit from New England BioLabs®Inc was used following the standard protocol. cDNA samples were kept at -20℃.

qPCR

All quantitative PCR reactions were carried out using the SYBR® Green qPCR supermix from Bio-Rad, the standard protocol was followed. Actin was used as the housekeeping gene. Primer design was as follows in **Table A**.

Gene	Forward Primer	Reverse Primer	Reference
amDop2	CCGAGGACCTCCAGGATCTC	TCTTCTCCTTGGCGAACTTGG	(Mustard, Pham, & Smith, 2013)
OA1	TAATACGACTCACTATAGGGAGACCACGAGACGAAGGCGGCGAAGACAC	TAATACGACTCACTATAGGGAGACCACCGTTTGCAGAAGCACTTGA CGATG	(Rein, Mustard, Strauch, Smith, & Galizia, 2013)
Actin	TGCCACACTGTCCTTTCTG	AGAATTGACCCACCAATCCA	(Scharlaken et al., 2008)

Table A. Primer selection for qPCR.

Results

We compared the gene expression of an Octopamine and Dopamine receptor in the brain of naïve honey bee foragers versus trained honey bee foragers of two different subspecies. We found that Training has a significant effect in the change of gene expression of both OA1 and amDOP2 on *A.m. caucasica* foragers (OA1: P-value = 0.047, t=2.621, df=5; amDOP2: P-value = 0.0011, t=5.891, df=6) **Figure A.1A**. For *A.m. syriaca*, training did not have a significant effect on the expression of OA1 (P-value = 0.1594, t=1.574, df=7) or amDOP2 (P-value = 0.2270, t=1.324, df=7) **Figure A.1B**.

There were no significant differences in expression between subspecies for: (1) the OA1 gene in naïve bees (**Figure A.2A,** P-value = 0.7350, t=0.3522, df=7); (2) the OA1 gene in trained bees (**Figure A. 2C,** P-value = 0.1433, t=1.735, df=5); (3) the amDOP2 gene in naïve bees (**Figure A.2B,** P-value = 0.6807, t=0.4291, df=7) or the amDOP2 gene in trained bees (**Figure A.2D,** P-value = 0.0915, t=2.008, df=6). However, there was a significant difference in the variance of gene expression of trained bees for gene amDOP2 (**Figure A.2D** P-value = 0.0013, F = 187.4, DFn = 3).

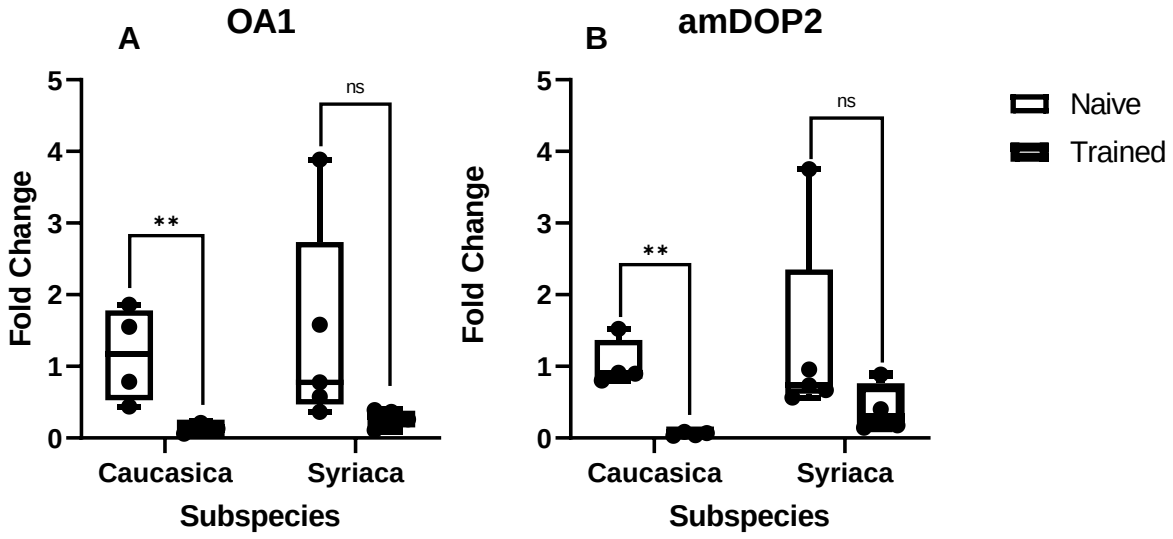

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Figure A.1 Effect of Training on Octopamine and Dopamine receptor gene expression

A t-test show the change in expression of the OA1 and amDOP2 genes is significant for *A.m. caucasica* after undergoing training (P-value < 0.05, df = 5), while not significant for *A.m. syriaca* (P-value > 0.05, df = 7).

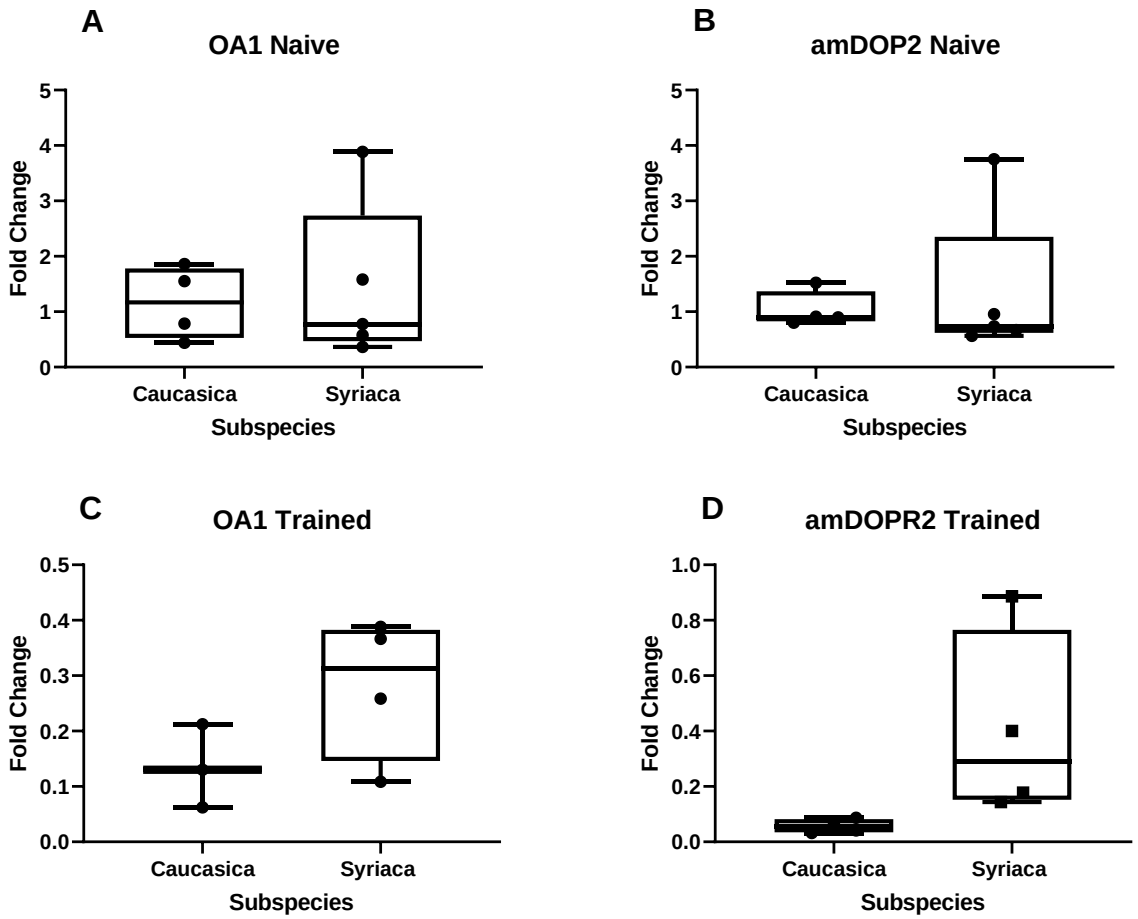

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Figure A.2 Subspecies differences in gene expression of Octopamine and Dopamine

A t-test shows that the expression of OA1 is not significantly different between the subspecies (P-value > 0.05). For amDOP2 while there wasn't a significant difference of expression for naïve bees (P-value > 0.05, df = 7) or for trained bees (P-value > 0.05, df = 5). However, an F-test shows a significant difference in variance of amDOPR2 expression between subspecies after training (P-value < 0.001, F = 187.4).

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Acknowledgements

I want to acknowledge the guidance from Prof. José Agosto and Prof. Tugrul Giray through my research career. Thanks to their support I have been able to develop into the scientist I am today. I want to acknowledge Prof. Patricia Ordoñez who guided my development as a data scientist and who introduced to the iBRIC program from the University of Pittsburgh where I will be continuing the next phase of my academic career. I want acknowledge Prof. Gregory Cooper, his student Bryan Andrews and Prof. Xinghua Lu for teaching me all I know about Causal Modeling and Clustering. Finally, I also want to acknowledge the members of my committee, the literature they've shared with me and their input, have in no small part helped me formulate the ideas written in this thesis.

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