# Modulation of Sensing and Sharing Food-Related Information in the Honey Bee

by

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ABSTRACT

Food is an essential driver of animal behavior. For social organisms, the acquisition of food guides interactions with the environment and with group-mates. Studies have focused on how social individuals find and choose food sources, and share both food and information with group-mates. However, it is often not clear how experiences throughout an individual's life influence such interactions. The core question of this thesis is how individuals' experience contributes to within-caste behavioral variation in a social group. I investigate the effects of individual history, including physical injury and food-related experience, on individuals' social food sharing behavior, responses to food-related stimuli, and the associated neural biogenic amine signaling pathways. I use the eusocial honey bee (*Apis mellifera*) system, one in which individuals exhibit a high degree of plasticity in responses to environmental stimuli and there is a richness of communicatory pathways for food-related information. Foraging exposes honey bees to aversive experiences such as predation, con-specific competition, and environmental toxins. I show that foraging experience changes individuals' response thresholds to sucrose, a main component of adults' diets, depending on whether foraging conditions are benign or aversive. Bodily injury is demonstrated to reduce individuals' appetitive responses to new, potentially food-predictive odors. Aversive conditions also impact an individual's social food sharing behavior; mouth-to-mouse trophallaxis with particular groupmates is modulated by aversive foraging conditions both for foragers who directly experienced these conditions and non-foragers who were influenced via social contact with foragers. Although the mechanisms underlying these behavioral changes have yet to be resolved, my results implicate biogenic amine signaling pathways as a

potential component. Serotonin and octopamine concentrations are shown to undergo long-term change due to distinct foraging experiences. My work serves to highlight the malleability of a social individual's food-related behavior, suggesting that environmental conditions shape how individuals respond to food and share information with group-mates. This thesis contributes to a deeper understanding of inter-individual variation in animal behavior.

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My dissertation work led me to new friends, mentors, and colleagues who not only made this dissertation possible, but continue to inspire my academic and personal trajectories.

Choosing Gro Amdam's lab for my PhD is one of the best decisions I've ever made. Under Gro's guidance, I was able to pursue my own questions without any intellectual limitations and yet with the utmost support. I still remember our first in-person meeting; after I babbled on for a while about my interests, Gro nodded and summed it all up in one clear statement that suddenly illuminated my path forward. I knew after this meeting that the Amdam lab was the right place for my graduate career, and this was confirmed after every subsequent meeting, not matter how daunting the science sometimes seemed.

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# CHAPTER 1

# INTRODUCTION

This thesis focuses on how external and internal factors can modulate the sensing and social sharing of food-related information. My collaborators and I show that external foraging conditions impact social food sharing behavior between honey bees (*Apis mellifera)* and give rise to lasting modulation of individual biogenic amine signaling and food responsiveness. Internal conditions such as physical health are demonstrated to alter appetitive reactivity to food-related olfactory stimuli. I discuss potential neural mechanisms of the modulation of individuals' sensing food-related stimuli as well as channels for the social sharing of food-related information. My work contributes to the field of animal behavior, providing new insight into how inter-individual differences arise as a function of individual history.

## BACKGROUND

Social animal behavior is driven by the acquisition and transmission of food-related in-formation. From the forms taken by schools of fish (Hoare and Krause 2003) to the collective foraging decisions made by honey bee colonies (Seeley et al. 1991) to the acoustic calls of dolphins (King and Janik 2015), prairie dogs (Kiriazis et al. 2006), and cliff swallows (Brown et al. 1991), social systems give rise to unique approaches to focus individuals' foraging efforts on optimal food sources while avoiding the various dangers to which they are meanwhile exposed. The food-related decisions made by a highly social organism arise from a rich interplay of the individual's current state, genotype, history, and socially transmitted information.

### Inter-individual Variability in Food Preferences

Food is one of the most fundamental rewards an organism encounters. Yet, sensitivity and preference for particular nutrients can be highly variable both between individuals and within a single individual's life. Early life experience as well as genetics can lead to sustained individual differences in responsiveness to nutrients. In humans, amniotic fluid and breast milk contain tastants that influence infants' feeding behaviors and preferences (Ventura and Worobey 2013). The concentration of sucrose fed to young honey bees influences their sensitivity to sucrose; the higher the concentration they have experienced, the higher their threshold for responding appetitively (Martinez and Farina. 2008). In humans, there is a correlation between chemosensory alleles and preference for vegetables and alcohol (Dineheart et al. 2006). Sucrose preference can also change with age; as human infants mature, they grow to prefer sucrose less, and rats similarly exhibit a reduced preference for sucrose at the onset of puberty (Mennella and Bobowski 2015; Ventura and Mennella 2011; Wurtman and Wurtman 1986). In the short term, food preferences can change due to recently eaten food quality or due to state of hunger. Feeding behavior can thus aid in the maintenance of physiological homeostasis. The intake of carbohydrates has been shown in mice to cause the liver to release a growth factor that acts on the paraventricular nucleus of the hypothalamus to suppress sugar intake (Holstein-Rathlou et al. 2016). Similarly, fat metabolism regulates satiety behavior in the nematode *Caenorhabditis elegans* (Hyun et al. 2016). Satiety as well as the type of recently eaten sucrose correlates with gustatory receptor expression in the honey bee brain and peripheral taste organs (Simcock et al. 2017). Beyond the transient effects of recent food-related experience, some organisms experience changes in food preference due to chronic depression or stress. Anhedonia, or reduced sucrose preference, is correlated with depressive symptoms in horses (Fureix et al. 2015), humans (Pizzagalli 2014), rats (Matheus et al. 2016), and non-human primates (Felger et al. 2013). Sickness can also change food preferences. Studies in vertebrate model

systems suggest that infection induces synthesis of cytokines in the brain, which leads to a decrease in food intake and lowers the rejection threshold for aversive tastes (Aubert and Dantzer 2004). In honey bees, viral infection increases sucrose responsiveness (Li et al. 2013).

**Social Modulation of Food-Related Behavior**

For many social animals, food-related behavior is modulated not only by their own experience, but also by that of conspecifics within their group. Social modulation can occur via chemical or behavioral mechanisms. Alarm pheromones in social vertebrate (Brechbuhl et al. 2013) as well as social insect (Norman et al. 2017) systems are used to alert individuals of danger sensed by a group member, causing receivers to respond aggressively or defensively rather than focusing on food collection. Some species of ants use chemical trail pheromones to recruit nestmates to food sources (Attygalle and Morgan 1985). In vertebrate as well as invertebrate species, social transmission of food preference causes individuals to prefer food that has been previously consumed by group-mates; this has the putative adaptive value of biasing individuals towards food more likely to be safe to eat. In rats, social transmission of food preference requires concurrent detection of odors from the food source with detection of social odors present on the demonstrator's breath (Galef 1983; Posadas-Andrews and Roper 1983; Galef and Kennett 1987). Rabbits transmit food preference across generations via mothers' fecal pellets in the nest, prenatal experience in utero, and during nursing (Bilko et al. 1994). In addition to chemical signals, social insects also use behavioral mechanisms of signaling experience with food sources. Honey bees use a referential dance language to signal the location and value of food sources and a vibrational stop signal to prevent recruitment to food

sources where danger was previously experienced (Schurch et al 2016; Nieh 2010). Some species of ants use physical contact as a method of recruitment, for example tandem leading to food sources (Franks and Richardson 2006). Mouth to mouth food exchange, which in some species is referred to as "trophallaxis," results in preference for the socially exchanged food in meerkats (Thornton 2008), ants (Provecho 2009), and honey bees (Arenas et al. 2008).

**The Honey Bee**

The honey bee system is ideal for furthering our knowledge of how food-related information is sensed and shared, due to the manifold of possible social interactions as well as plasticity in individual food-related behavior. A honey bee colony consists of a reproductively active queen, up to 50,000 sterile female workers, and several hundred males depending on colony size and season (Seeley 1995). Task specialization in a honey bee colony changes throughout workers' lives, rather than being morphologically predetermined (Menzel et al. 2006). Young workers stay inside the hive, moving from cleaning duties to nursing brood and eventually to receiving and storing food. After approximately two weeks - though this timeline can change due to weather and demands inside the colony - older workers initiate foraging for pollen, nectar, water, and propolis (Seeley 1995). Food resource information is transmitted throughout these different worker castes via several different forms of communication. Foragers recruit one another to food sources through the referential dance language previously mentioned (Seeley 1995), and limit recruitment to dangerous sources by using a vibrational stop signal to halt dances (Nieh 2010). Potential dangers encountered while foraging include predation (Monceay and Thiery

2016), con-specific competition (Rogers et al. 2013), and plant toxins (Stevenson et al. 2016). Workers inside the nest receive collected food from foragers and distribute it to one another through mouth-to-mouth trophallaxis, a process that transmits information about food quality and associated olfactory cues (Gruter et al. 2006). Socially transferred food can confer preference to food smelling of particular scents and can change sucrose response threshold (Pankiw et al. 2004). There is a high level of inter-individual variability in sucrose response threshold and variation throughout the season (Scheiner et al. 2003), likely arising from a combination of experience and innate sensitivity. Well established laboratory protocols can quantify individual responsiveness to sucrose as well as appetitive learning ability and memory retrieval (Matsumoto et al. 2012). Potential neurochemical modulation of both food sensing and information sharing has been revealed by pharmacological studies. Manipulation of the biogenic amine octopamine through feeding, topical application, and injection changes how bees respond to food reward as well as how foragers report resource quality during recruitment dances (Scheiner et al. 2002; Barron et al. 2007). This richness of social information transfer, tractability of quantifying individual responsiveness, and known neurochemical modulation of both social and individual food-related behavior provide an exciting system in which to explore the modulation of food sensing and sharing.

## SIGNIFICANCE

This thesis contributes new basic insight into animal behavior; historically, the effects of experience have been studied from the perspective of changed responses to conditioned cues that predict food or danger. Our work investigates the potential for

lasting impact of experience on responsiveness to food reward itself. The results demonstrate new ways for external environment and internal health to influences how social organisms respond to food-related stimuli. For the field of social insect communication, our experiments work to uncover a potential new channel for transmission of information about food quality, as well as a way for aversive information concerning food sources to change social information dynamics within a colony. These latter findings contribute to the understudied area of how aversive, rather than appetitive, experience can be socially transmitted. And finally, some of what we have found can be useful for the agricultural management of honey bees, in terms of understanding the influence of aversive conditions such as pesticide use and crowded conspecific competition on social feeding dynamics inside a colony.

## PURPOSE

The purpose of this work is to show how internal and external conditions affect honey bee food-related behaviors. These studies clarify the influence of foraging experience and physical health on how individuals respond to food-related stimuli and how they share this information with groupmates. I demonstrate the impact of olfactory and aversive stimuli experienced during foraging on sucrose responsiveness, neural biogenic amine signaling, and mouth-to-mouth food sharing. I also assess the effect of physical injury on appetitive responsiveness to novel scent.

## APPROACH

This thesis employed several new behavioral paradigms in conjunction with established honey bee laboratory assays. For two of the units, I designed a cage that

allows manipulation of foraging conditions and observation of freely moving bees (see Chapters 2 and 5). Aversive conditions were created by rigging feeders with mild electric shock, an aversive stimulus commonly used in honey bee laboratory assays (Vergoz et al. 2007). To identify changes in social dynamics, bees were individually labeled with tags on their thoraxes, and trophallaxis events were recorded during spot-checks (see Chapter 2). To explore volatile chemical signals that could provide a substrate for communicating the quality of bees' crop contents, I worked with an undergraduate team to design a paradigm for probe capture of volatile emission from live honey bees for Gas Chromatography/Mass Spectrometry analysis (see Chapter 3). Experience-induced change in individual sucrose responsiveness was quantified in restrained bees using the classical Proboscis Extension Response (PER) protocol (Smith and Burden 2014). Underlying neurochemical changes in different brain regions were investigated with quantitative real-time PCR analysis of biogenic amine receptor expression and High Powered Liquid Chromatography analysis of biogenic amine concentrations (see Chapter 4). To better understand how internal factors, such as physical health, influence individuals' sensing of food related information, a method to simulate extensive surgery on honey bees was employed (see Chapter 5).

CHAPTER 2

# PUNISHING FORAGING CONDITIONS ALTER DOWNSTREAM SOCIAL FOOD SHARING IN HONEY BEES

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Contribution: I designed, performed, and analyzed all experiments, and wrote the manuscript, under the guidance of Dr. Gro Amdam. Aspects of experimental setup, such as collecting bees and labeling with tags, were facilitated by a stellar team of undergraduate students (see acknowledgments).

## Introduction

Collection of food is the driving force behind much of the activity within a eusocial insect colony. Sterile or functionally sterile workers are responsible for foraging, distribution, and processing of nutrients. In some species, such as leafcutter ants, behavioral specialization is morphologically fixed during development, while in other cases behavioral specialization can be modulated by social and environmental factors (Mertl and Traniello 2009; Page and Amdam 2007). Whether roles change throughout an individual's life or remain fixed, an essential facet of the resulting division of labor is that in any given moment a proportion of members do not forage and can experience external conditions only through a cascade of social interactions.

Honey bees, *Apis mellifera*, perform a wide array of behaviors inside and outside the nest. Individuals progress through various kinds of work throughout their lives in a progression that is partially determined by genetics and age but is also influenced by queen pheromone, brood pheromones, food availability, and season (Seeley 1982; Pankiw et al. 1998; Barron and Robinson 2005). Some worker traits

correlate specifically with behavioral role, such as circadian rhythm (Moore 2001), metabolism (Ament et al. 2008), blood levels of vitellogenin and juvenile hormone (Guidugli et al. 2005), and expression of octopamine receptor *OA1* in the subesophageal ganglion and antennal lobes (Reim and Scheiner 2014). Other worker traits are better correlated with age rather than role, such as sucrose responsiveness (Behrends et al. 2007) and expression of octopamine receptor in the mushroom body (Reim and Scheiner 2014). Without a disruption of the age distribution in a colony, approximately 0-5 day old workers specialize in cleaning cells, ~3-12 day old bees act as nurses for developing brood, ~12-14 day old bees receive and store food, and older bees forage outside the hive (Seeley and Kolmes 1991).

Trophallaxis, the mouth-to-mouth exchange of liquid, is a primary mechanism of food and information transfer inside a honey bee colony. The receiving bee places her proboscis between the mandibles of the donor and the two rapidly antennate as liquid is passed from donor to receiver (Korst and Velthuis 1982). Foraging conditions and the olfactory cues associated with incoming food influence the dynamics of ensuing trophallaxis. Nectar flow rate determines the rate at which foragers unload to a receiver, and subsequently the rate at which the first receiver unloads to the following receiver (Goyret and Farina 2005). The presence of odor in nectar increases frequency of trophallaxis events in a hive (Arenas 2012).Novel odorants in the crops of donating foragers have been shown to reduce the occurrence of trophallaxis if a receiving forager has had prior appetitive olfactory experience (Gil and Farina 2003).

The process of collecting food involves a rich slew of sensory experiences, and can generate aversive experience as well as appetitive reward. Foragers are

exposed to con-specific competition and predation from other arthropods including praying mantids (Greco 1995), wasps (Morse 1986), and spiders (Greco and Kevan 1995; Evans and ONeill 1988) or birds (Fry 1983), and noxious secondary compounds excreted by various plants (Ibanez et al. 2012). Aversive encounters lead to memories that can be transmitted through social interactions within the colony. Returning foragers find others recruiting with nectar samples smelling of an odor they recall as being aversively associated. They make contact with the dancer and vibrate, halting the dance, thus minimizing recruitment to a potentially aversive food source. This is called the "stop signal" (Nieh 2010) and can also be triggered by long wait times or competition due to conspecifics at feeders (Lau and Nieh 2010). To date, this is the only example of a honey bee behavior performed due to aversive experience in the field.

In the present study, we were interested in how foragers' punishing experiences influence food sharing dynamics among bees further down the network of food transfer. To test this we allowed one group of bees access to feeders which provided different foraging experiences, while another group was separated from feeders by mesh and fed only through social trophallaxis. Negative foraging conditions were simulated using feeders rigged with mild electric shock. Electric shock is the form of punishment used most commonly in the lab to explore bees' aversive learning abilities as well as unconditioned behavioral responses (Tedjakumula and Giurfa 2013). Our results confirm the hypothesis that punishing conditions influence downstream food sharing. We discuss likely behavioral mechanisms by which foragers' aversive experiences modulate non-foragers'

trophallaxis frequency, and the implications for how aversive external conditions can influence individuals that have not yet left the hive.

## Materials and Methods

*Bee Collection and Experimental Setup:* Pre-foraging aged *Apis mellifera ligustica* bees were collected on frames containing honey and no brood, first touched lightly with forceps to cause foragers to fly off. For each replicate, bees were collected from three different hives and mixed to prevent hive effects. Bees were immobilized in glass vials on ice, labeled with a queen bee number tag (The Bee Works Queen Marking Kit, Ontario, Canada) on the thorax, and placed inside an experimental cage in which they regained motility. Each cage was divided by mesh to separate bees into two groups: 17 bees with feeder access in the bottom compartment and 13 bees with no feeder access in the top compartment (Fig. 1). Empty honey comb lined the back wall in both compartments. The cages were kept in constant light conditions at room temperature (20-26 C). Cage locations within the room were alternated.

Six replicates were run at Arizona State University in Tempe, Arizona from April - June 2015. Each replicate consisted of four cages, differing only in what was offered at the feeders during the first two days of treatment. In two of the boxes, the first two days of treatment consisted of 2 Kimwipes (Kimberly-Clark Professional, Roswell, Georgia, USA) soaked in 1.5M sucrose in a feeder that delivered 4.2 V of electric shock to feeding bees (Fig. 1). In one of these boxes, the sucrose was scented with linalool or phenylacetaldehyde (Sigma Aldrich, St. Louis, MO, USA) (50ul odorant per liter of 1.5M solution). The other two boxes received identical treatment

but without the electric shock. Two feeder slots at the bottom of each cage allowed food location to be alternated every 6-8 hours to reduce spatial associative pairing.

After two days of treatment, the feeder slots at the bottom of all boxes were cleaned with a Kimwipe dampened with ethanol to remove traces of sucrose, odor, or secretions from honey bees. All feeder slots were filled with bottle caps containing two Kimwipes soaked in 0.5M sucrose. These were replaced to maintain constant saturation. After three days, a randomly selected majority of bees were chilled on ice to be harnessed for sucrose responsiveness assays, while a smaller portion of bees was flash frozen in liquid nitrogen for another study.

*Trophallaxis and Foraging:* Each box was observed for one hour, 30 min per day. The time of day and observer (either A. B. Finkelstein or one of two undergraduate students) were alternated between treatments. During the hour of observation, we recorded instances of foraging from feeders, trophallaxis between forager bees, trophallaxis between bees in the bottom and top compartments, and trophallaxis between bees in the top compartment. Bees were identified by the colored number tag on their thoraxes.

*Sucrose Responsiveness:* Bees were collected from boxes into glass vials, chilled on ice until immobile, and harnessed as described in Smith and Burden 2014. Labeled tape on each harness encoded treatment and bee identity. After resting for about 30 min, bees from the top compartments were fed with 2 ul of 0.5M sucrose, under the assumption that they may have been less sated than bees in the bottom compartment

with access to *ad libitum* sucrose. Three hours later, all bees were tested with retention tests in which the treatment scent and a novel scent were presented in a pseudo-random order and proboscis extension was observed for each odor. Afterward, all bees were allowed to feed on 0.5M sucrose until sated. Bees were considered sated when touching the antennae with a droplet of sucrose did not cause proboscis extension. The following day, 96 hours after the end of treatment, sucrose responsiveness was determined by presenting randomly ordered bees with 0.1%, 0.3%, 1%, 3%, 10%, 30% sucrose interspersed with water between each trial to prevent sensitization. Both antennae were touched with a droplet of sucrose or water on a pipette tip, and proboscis extension response was recorded to produce a Gustatory Responsiveness Score (GRS) of 0-6.

*Octopamine Receptor Gene Expression Analysis:* To analyze gene expression, bees were flash frozen in liquid nitrogen after behavioral assays for dissection of mushroom bodies, antennal lobes, and subesophageal ganglion. We were interested in octopamine *OA1* receptor differences between foraging and non-foraging bees in benign conditions, to exclude any additional effect of stressful aversive experience. RNA was extracted from each neuropile using a trizol/protocol (ThermoFisher Scientific, Waltham, MA, USA). cDNA libraries were created with the Taqman Reverse Transcription Reagents Kit (ThermoFisher Scientific, Waltham, MA, USA. Using established *OA1* primers (Reim et al. 2014) we performed quantitative real-time Polymerase Chain Reaction (qPCR) using the ABI PRISM® 7000 Sequence Detection System ((ThermoFisher Scientific, Waltham, MA, USA to compare expression levels between bees performing foraging and non-foraging roles in cages.

For normalization of the receptor transcripts we used *elongation factor 1 alpha* as the reference gene, as in Reim et al. 2014, because in full colonies it is stably expressed in nurse bees and foragers.

*Statistics:* All statistical analyses were performed in Statistica v12.7. Frequency counts for trophallaxis occurrence for each individual bee ranged from 0 to 5 and did not adhere to assumptions of homogeneous variance and normality requisite for parametric tests. We thus used the Mann-Whitney U Test to determine the effect of scent and electric shock on frequencies of each behavior, and Spearman Rank Order Correlations to identify correlations between, with missing data deleted pairwise. Gustatory responsiveness scores did not follow a normal distribution, therefore we used the non-parametric Mann Whitney U Test to compare groups. Octopamine receptor expression followed a normal distribution and displayed equal variances, so the parametric Student's two-tailed *t*-test was used to compare expression levels between groups.

Fig. 2.1 Experimental Paradigm

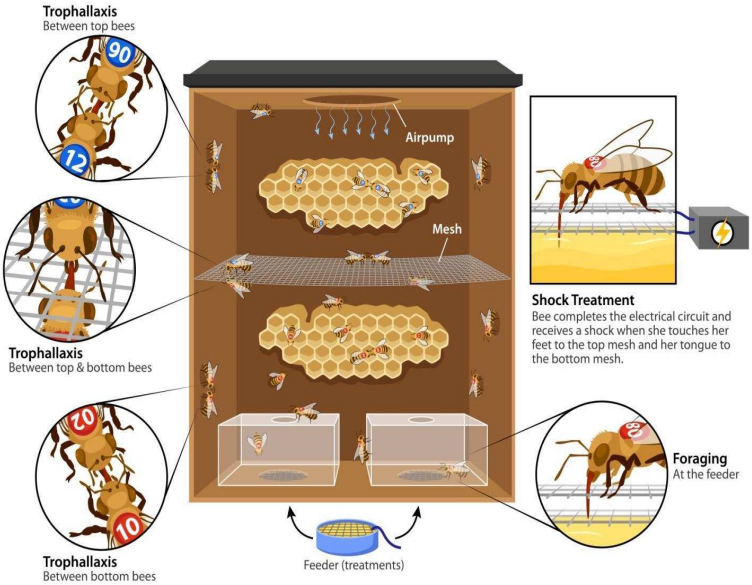


**Fig. 2.1**

**Experimental Paradigm:** In a novel cage design, bees are separated into two compartments, of which only the bottom provides the opportunity for foraging at feeders. For the first 48 hours, cages offered four different treatments:

1. feeders offering sucrose
2. feeders offering scented sucrose
3. feeders offering sucrose paired with electric shock
4. feeders offering scented sucrose pared with electric shock

## Results

*Self-specialization of feeding behavior in cages:*

Bees observed to enter feeders and collect sucrose at least once are considered "foragers" and bees never observed inside feeders were considered "non-foragers." Foragers were much less likely to be observed engaging in trophallaxis with either other bees that had feeder access (Mann Whitney U Test, U=3851.5, N= 166 (foragers), 80 (non-foragers), Z-adjusted=-6.167, $p<0.0000001$) or bees behind mesh that did not have feeder access (Mann Whitney U Test, U=4643, N= 166 (foragers), 80 (non-foragers), Z-adjusted=-5.501, $p<0.000001$) (Fig. 1a and 1b).

*Correlations between sucrose responsiveness and food sharing behavior*

For non-foragers, sucrose responsiveness was inversely related to trophallaxis with other bees in their compartment (Spearman Rank Order Correlations, R=-0.402, N=25, $p<0.05$). Considering both foragers and non-foragers, sucrose responsiveness was positively related to trophallaxis with bees that lacked feeder access (Spearman Rank Order Correlations, R=0.241, N=74, $P<0.04$).

*Octopamine receptor expression and foraging behavior:*

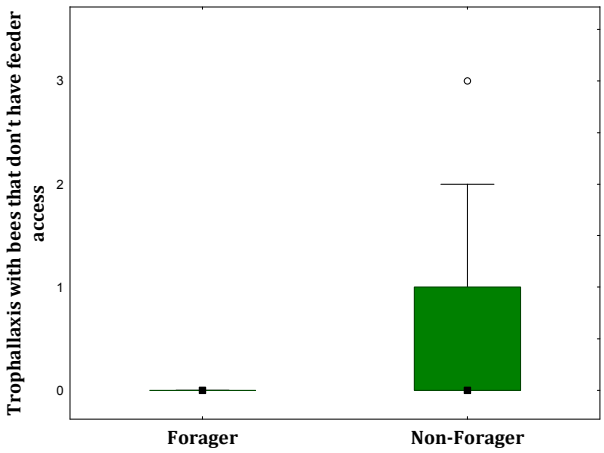
Octopamine receptor *OA1* expression in the subesophageal ganglion was not different between foragers and non-foragers (Student's t-test, t-value=-0.164, df=12, N=9 (foragers), 5 (non-foragers), $p<0.9$) (Fig. 2).

*Effect of foraging conditions on social food sharing:*

Considering foraging for both scented and unscented food, aversive foraging conditions caused foragers to trophallax more with bees behind the mesh (Mann Whitney U Test, U=3119, Z-adjusted=-1.97, N=86 (benign conditions), 80 (aversive conditions), $p<0.05$). When food was scented, this effect was eliminated in foragers (Mann Whitney U Test, U=876, Z-adjusted=-1.44, N=45 (benign conditions), 42 (aversive conditions) but the social food sharing behavior of non-foragers was changed in the same way to trophallax more with bees behind the mesh (Mann Whitney U Test, U=85, Z-adjusted=-2.60, N=17 (benign conditions), 18 (aversive conditions), $p<0.01$). When food was unscented, the effect of aversive conditions on non-foragers' food sharing with bees behind mesh was eliminated (Mann Whitney U Test, U=201, Z-adjusted=1.103, N=27 (benign conditions), 18 (aversive conditions), $p<0.3$). (Fig.3 A-C).

**Fig. 2.2 Self specialization of social food transfer behavior**

A. Trophallaxis with bees that did not have feeder access




B. Trophallaxis with bees that had feeder access

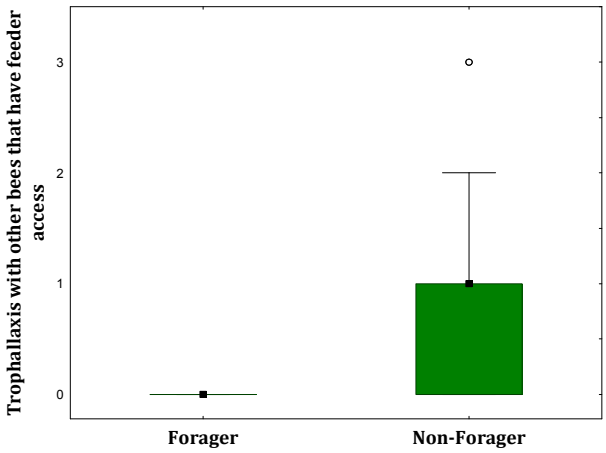



**Fig. 2.2** *Boxes represent 25-75 percentiles, bars show the non-outlier range, points show outliers, squares represent medians.Forager bees exhibited extremely low rates of trophallaxis compared to non-forager bees, who were much more like to participate in distributing food throughout the cage.* ***A.*** *Foragers trophallax less with bees behind mesh than non-foragers (Mann Whitney U Test, U=4643, N= 166 (foragers), 80 (non-foragers), Z-adjusted=-5.501, p<0.000001).* ***B.*** *Foragers trophallax less with bees with feeder access than non-foragers (Mann Whitney U Test, U=3851.5, N= 166 (foragers), 80 (non-foragers), Z-adjusted=-6.167, p<0.0000001)*

**Fig. 2.3 Octopamine expression in subsesophageal ganglion of foragers vs. non-foragers**

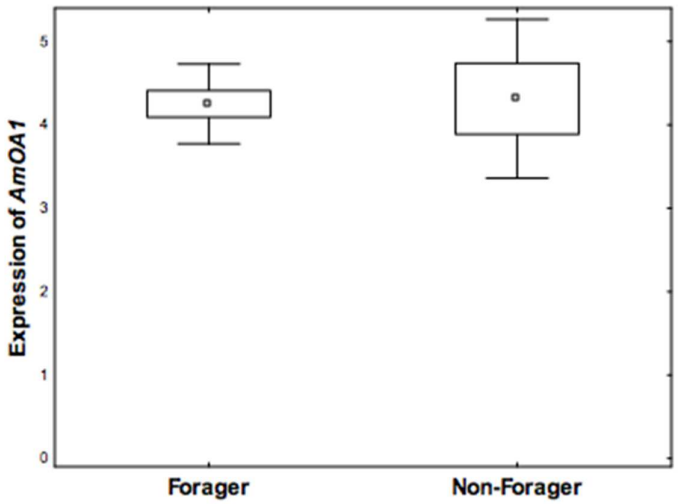
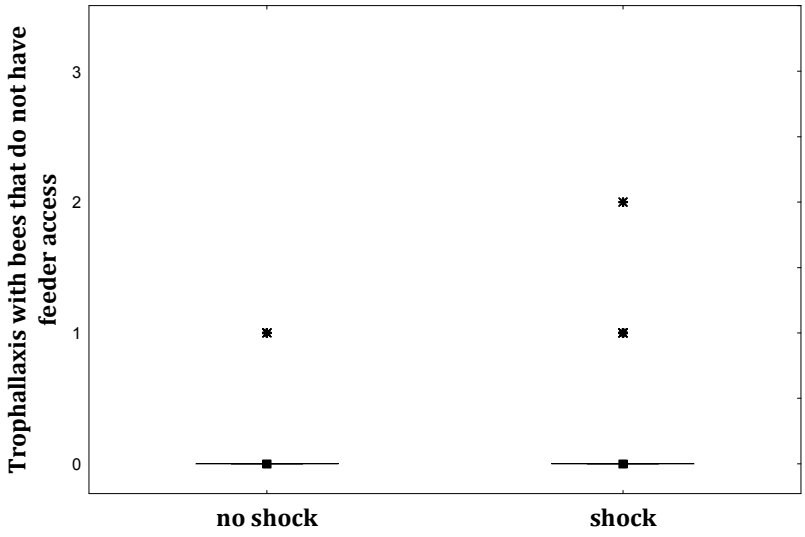
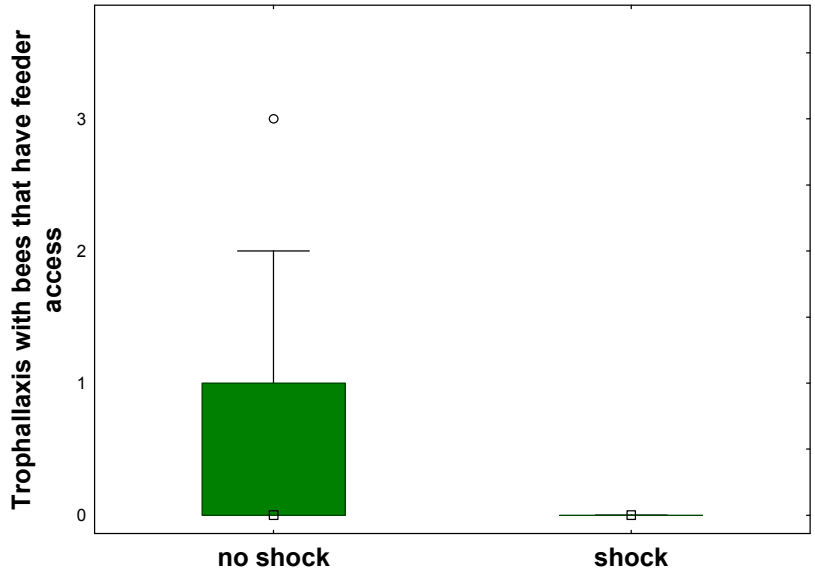



**Fig. 2.3** *Boxplots show relative expression levels of octopamine receptor OA1 in foragers vs. non-foragers. Boxes show mean+/-standard error, whiskers show mean +/-standard deviation, squared represent means. There is no difference in expression levels (Student's t-test, t-value=-0.164, df=12, N=9 (foragers), 5 (non-foragers), p<0.9).*

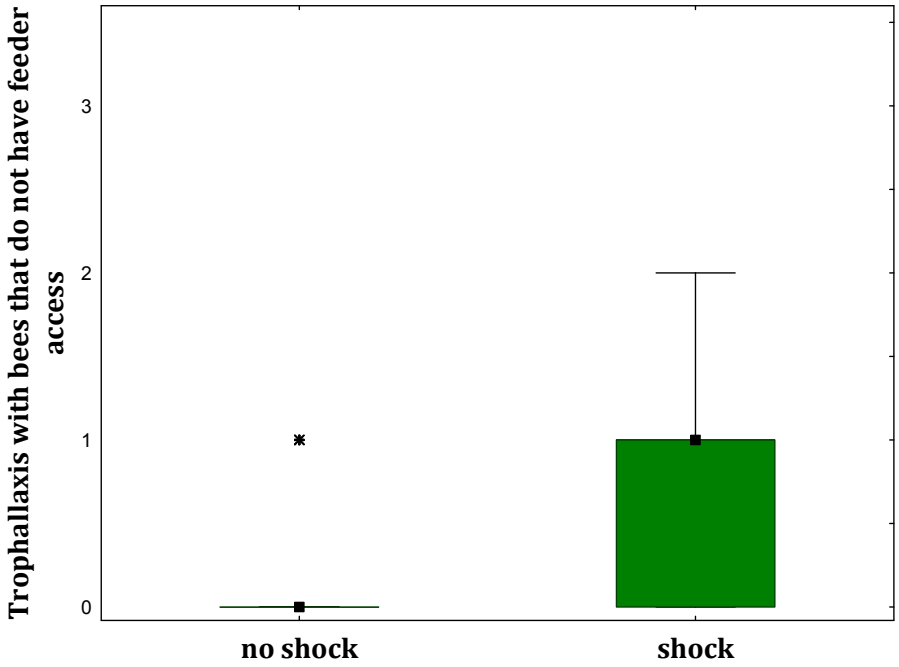
**Fig. 2.4: Effects of foraging conditions on food sharing dynamics**

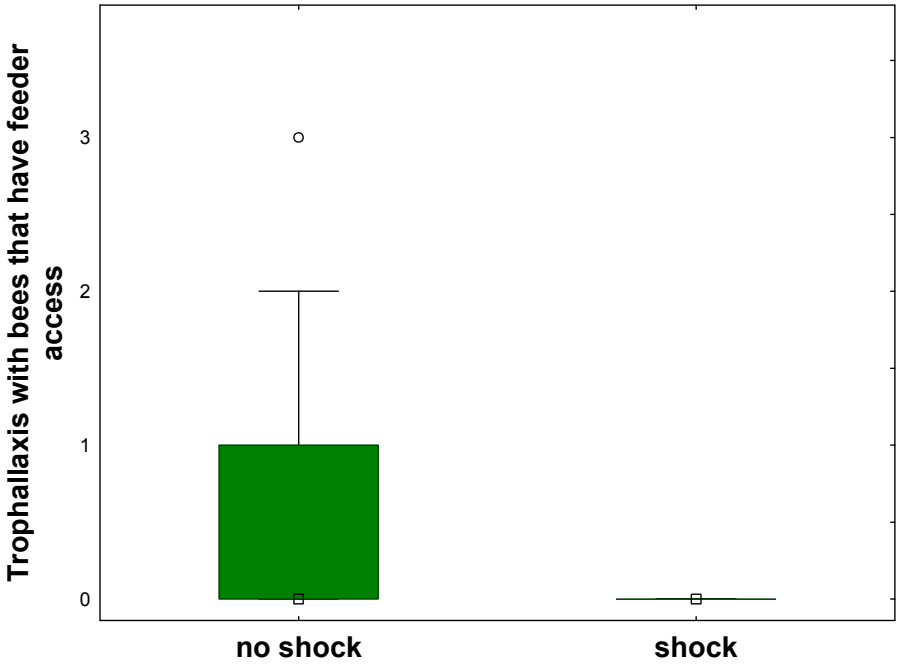
**A. Foragers: trophallaxis with bees that have and do not have feeder access**






**B. Non-foragers, when food is scented**






**C. Non-foragers, when food is unscented**

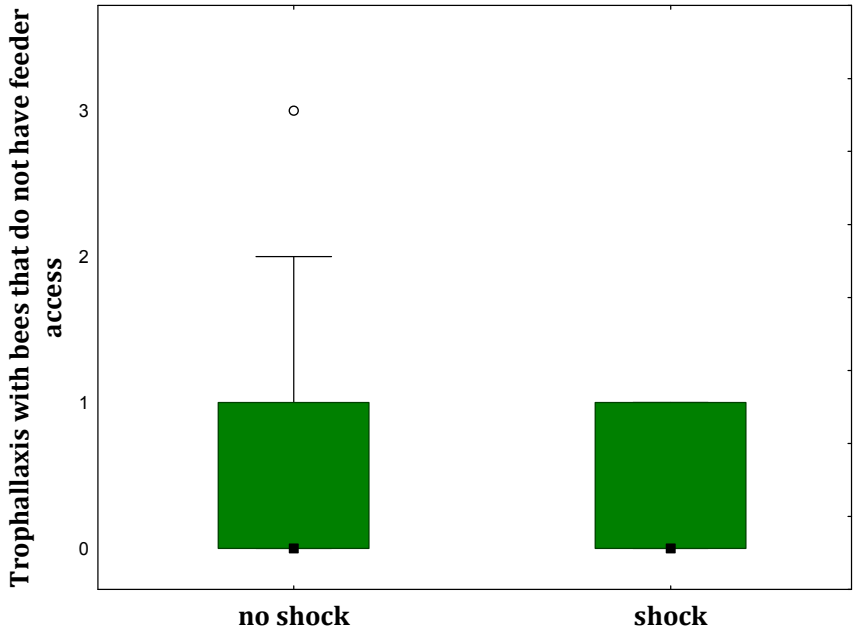



***Fig 2.4*** *Box and Whiskers plots showing trophallaxis in different foraging conditions. Boxes represent 25-75 percentiles, bars show the non-outlier range, points show outliers, squares represent medians.* ***A.*** *Foragers trophallax significantly more with bees that do not have feeder access in aversive conditions but not with bees that have feeder access.* ***B.*** *When food is scented, aversive conditions increase the trophallaxis*

*of non-foragers with bees that do not have feeder access but not with bees that have feeder access.* ***C.*** *Without scent, this effect is eliminated.*

**Discussion**

In natural honey bee colonies, forager bees returning to the nest unload their crop to a single non-foraging receiving bee, only sharing with additional receivers if the crop load is more than can be imbibed by just one (Huang and Seeley 2003). The receiving bee trophallaxes the nectar to other hive mates or deposits it in honey comb (Seeley 1995). Our cage paradigm gives rise to a similar self-specialized distribution of behavior. The bees observed to forage at feeders once or more were foraging for 78% of all their observed behaviors, sharing food with other bees in the same compartment with feeder access 16%, and sharing food with bees in the upper compartment without feeder access 5%. In contrast, bees never observed to forage were sharing food with other bees in the same compartment 61% and with bees in the upper compartment 39% of all their observed behaviors. We will call the first group "foragers" and the second group "non-foragers."

To better understand the behavioral roles that emerged inside cages, we quantified sucrose responsiveness as well as expression levels of octopamine receptor *OA1,* a gene known to be differentially expressed in the antennal lobes and subesophageal ganglion of same-aged foragers and non-foragers in a single cohort outdoor colony (Reim and Scheiner 2014). Our results do not conclusively show differences in *OA1* expression between foragers and non-foragers, suggesting that this is not necessary for the determination of foraging role at least in an artificial laboratory setting. We find that the higher a non-foragers' sucrose responsiveness, the lower her frequency of trophallaxing with another bee in the bottom compartment. When considering all individuals with feeder access, including both foragers and non-

foragers, there is a positive relationship between sucrose responsiveness and trophallaxis with bees behind mesh, who do not have feeder access. Our observations did not specify directionality of food transfer, so it is unclear whether the relationship is specific to donating food or to receiving it. Future studies will be necessary to resolve the causality of the relationship between food sharing and sucrose responsiveness.

Our study finds that aversive foraging conditions change the food sharing behavior of not only foragers, but also of non-foragers that have not directly experienced the aversive conditions. The latter effect is eliminated when food is unscented. Foragers that had experienced punishment were more likely to trophallaxis with bees behind the mesh, without changing trophallaxis with other bees that had feeder access. When food was scented, aversive conditions caused non-foraging bees to likewise increase their frequency of trophallaxis with bees behind the mesh without altering trophallaxis with bees that had feeder access.

Mechanistically, these results suggest that punishing foraging experience induces foragers to produce a local signal during the unloading of scented food to receiving non-foragers, which acts to change the latter's food sharing behavior. While at first glance one might postulate that foragers' stress responses following aversive stimulation can alone cause a change in others' behavior, this would not explain why the effect is present only when food is scented. Moreover, any wide-reaching effects of alarm pheromone (Boch et al. 1971) or some other form of stressful contagion would be expected to influence food sharing indiscriminately rather than specifically with the bees behind mesh. We show that there is no effect of aversive conditions on how often non-foragers trophallax with other bees that have feeder access or on how

often bees without feeder access trophallax with one another. We speculate that foragers transmit some form of signal or cue as they unload their crops to receiving non-forager bees that go on to distribute the food.

Researchers have long known that trophallaxis likely serves communication purposes beyond the sharing of food; when trophallactic interactions in small groups of bees were studied, less than 5% of the interactions actually resulted in food transfer (Korst and Velthuis, 1982). Social mammals such as rats (Galef and Stein 1985), mice (Valsecchi and Galef 1989), Mongolian gerbils, (Valsecchi et al. 1996; Galef et al. 1998) and spiny mice (McFadyen-Ketchum and Porter 1989) confer long-lasting food preference in conspecifics using semiochemicals found on the breath and in urine and feces. These preferences are sustained even for toxic or unpalatable food (Kelliher and Munger 2015). Negative feedback transmitting food source aversion is less common; *Lasius niger* ants deposit less trail pheromone when a trail is crowded, a reduction in positive signal which serves as a self-organized mechanism of negative feedback (Czaczkes et al. 2013). In honey bees, several behaviors are known to be modulated by aversive experience. Foragers that have had an aversive experience at a food source identity other foragers dancing to recruit to a similarly scented location, and stop the dance with a vibrational "stop signal" (Nieh et al. 2010). This reduces recruitment to aversively associated sites. In a laboratory setting, honey bees produce a hissing sound when presented with an odor they have learned to associate with electric shock (Wehmann et al. 2015). It is not yet clear whether this hiss is similarly produced in the natural context of a hive or whether it acts as a social signal. Both the vibrational stop signal and the hiss are activated by aversively associated scents, providing a potential explanation for why aversive conditions modulate non-foragers'

behavior only when food is scented. As foragers unload scented sucrose to non-foragers, it is possible that foragers sense the aversively associated scent while liquid is transferred during trophallaxis, and are thus triggered to engage in a behavior such as hissing or vibrating. Food receivers in colonies of the stingless bee *Melipona seminigra* food are vibrated in this manner on their thorax by foragers during unloading, though the effect on the receiver is not known (Hrncir et al. 2006). We propose that such a behavior could underlie the increase in non-foragers' food sharing behavior. We occasionally observed bees running at higher speeds than usual inside cages and vibrating in place or while touching other bees, but it was not possible to identify the bees' tag numbers during such rapid movement. It was also not possible to hear individual hisses through the plastic window covering cages, due to interference from the air pump as well as bees' buzzing. Although we have not identified the mechanism by with foragers' aversive experience leads to increased trophallaxis of non-foragers with downstream bees, our results are consistent with the existence of such as signal.

From the ultimate standpoint, it is at first glance surprising that punishing foraging conditions would increase, rather than decrease, the frequency at which receiver bees feed those downstream. In a natural colony, young bees can be fed by older receiver bees as well as by eating stored food (DeGrandi-Hoffman and Hagler, 2000). The substances that these young bees are fed influence their future foraging decisions. Bees as young as a few days after emergence are primed by the odors foragers bring into the nest, forming associative memories that can be retrieved once they reach foraging age (Arenas and Farina 2008). Bees prefer flowers smelling of nectars they have consumed in the nest (Farina et al. 2007). Likewise, *Camponotus*

*mus* ants that have received scented solution in a single trophallaxis event prefer this scent when teste in a Y-maze (Provecho and Josens 2009). Based on this, it would be expected that increasing the sharing of scented food when it has been collected in punishing conditions would have the maladaptive consequence of priming bees to choose previously punishing food sources. We postulate that receiver bees not only increase their rate of trophallaxis with nest bees, but also pass on a signal or cue of the punishing association. Since the original scent is still dissolved in the food that is being transferred, this would adaptively serve to teach as many bees as possible that the scent is associated with a dangerous food source.

The findings of this study suggest that aversive conditions experienced during foraging can change the social food sharing behavior of not only foragers, but also of non-foragers that have not yet experienced external conditions firsthand. We hope to spur future studies exploring how aversive conditions such as predation and con-specific competition influence food sharing between bees of different behavioral castes, and to test the hypothesis that receiving aversively associated food through trophallaxis influence pre-foragers' decisions once they initiate foraging.

**Acknowledgments**

I would like to thank Zamzam Hashi, Achal Patel, and Austin Huang for helping with spot-check observations and experimental setup, Dan Punch for helping design the cages, and Sabine Deviche for her graphic design illustrating the experimental setup.

CHAPTER 3

# ADAPTIVE DISCRIMINANT BEHAVIOR OF HONEY BEES (*APIS MELLIFERA*) IN A T-MAZE ASSAY

**Contributing authors:** Mari Høiland[1], Abby Finkelstein[2], Austin Huang[2], Katie Ennis[2], Kevin Flores[2], Claus Kreibich[1], Gro V. Amdam[1,2,†]

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Contribution: I led a team of undergraduate students in developing a method to compare the volatile compounds emitted by bees. My work adds a proximate mechanistic element to this project, demonstrating a potential way for bees to sense the information they need in order to exhibit the behavioral decision-making described by my collaborators. I also revised the manuscript and contributed to the introduction and discussion sections.

## Introduction

Honey bees (*Apis mellifera*) are highly social animals. They show a reproductive division of labor between females in which queens are responsible for egg-laying, while functionally sterile helpers, called workers, take care of brood-rearing, colony defense and foraging (Winston, 1987). Worker bees express discriminant behaviors that contribute to colony fitness. For example, workers can discriminate between healthy and infected brood and discard the infected from the colony (Spivak, 1996). They can distinguish between nestmates and unfamiliar bees and evict or kill intruders (Robinson & Page, 1988). Workers also discriminate between nectars of different concentrations and can promote the most profitable foraging sites through dance language, a communication that includes regurgitation of food samples (Frisch, 1967; Seeley et al., 1991; Seeley & Tovey, 1994). Collected nectars are shared between the members of the colony, while surplus is stored as honey (Winston, 1987).

Worker discriminant behavior is influenced by several factors including age and genotype (Robinson & Page, 1988; Hunt et al., 1995; Arathi & Spivak, 2001; Johnson et al., 2002; Lapidge et al., 2002; Rueppell et al., 2004). Immature (<24-48h old) bees can discriminate between sugar concentrations (Pankiw & Page, 2000), but workers usually do not express hygienic, guarding or foraging behavior before 10-15 days of age (Seeley, 1995; Goode et al., 2006). It is likely that the worker brain must complete maturational processes before certain behaviors are performed (Whitfield et al., 2006; Adams et al., 2008). Breeding experiments demonstrate that honey bee colonies and workers also show selectable variation for traits like nest hygiene, defensive behavior and sugar sensitivity (Lapidge et al., 2002; Rueppell et al., 2006; Hunt et al., 2007). These results have made important contributions to the general understanding of insect sociality, animal behavior and behavioral genetics by elucidating the genetics of insect social behavior (Robinson et al., 2008).

Discriminant worker behavior can release reflex responses such as stinging and proboscis (tongue) extension. These responses can be easily monitored and used in conditioning paradigms to understand brain function (see (Srinivasan, 2011) for a recent review). During conditioning, a stimulus that is to be learned (conditioned stimulus, CS) is paired with an unconditioned stimulus (US), e.g. with an electric shock punishment that elicits stinging or with a sugar reward that elicits proboscis extension. Such experiments are used to ask how well workers learn a punished CS(-) or a rewarded CS(+), and how well individuals discriminate a CS from an alternative neutral stimulus. Classical conditioning is a powerful research tool, but a complicating factor is that individuals differ in their subjective perception of the US. In the laboratory this subjectivity varies with age and genotype in worker bees, which

parallels their expression of discriminant behavior in the colony (Scheiner et al., 2001; Scheiner et al., 2005; Behrends & Scheiner, 2009).

Several areas of research, therefore, can gain from new approaches and insights into the innate discriminant behaviors of honey bees, including animal behavioral ecology, genetics and neuroscience. The T-maze is a relevant behavioral tool that is used for learning and memory tests but also for exploring variation in innate behavioral responses in a wide variety of animals. For example, the T-maze was used to examine how worker bees respond to biochemical compounds of alarm pheromone that can release defensive behavior (Wager & Breed, 2000).

Here we used a T-maze to study the behavior of worker bees that were asked to discriminate between conspecifics. The conspecific bees were manipulated to vary for ecologically relevant parameters that can elicit discriminant behavior. In the first set of experiments, we explored behavior toward familiar and unfamiliar individuals. These tests contrasted mature and immature wild-type (unselected) workers, with the expectation that mature workers could discriminate between bees that were familiar versus unfamiliar to them. Immature workers would not normally express defensive behavior, but it was unknown whether they could convey discriminant abilities in the T-maze. In the next set of tests, we studied behavior toward individuals that had fed on solutions with different concentrations of sugar (10% versus 50%). Immature wild type bees are only weakly sensitive to sugar (Behrends & Scheiner, 2009), and only mature bees will forage for sugar-containing nectars. Sugar sensitivity and foraging behavior, however, have significant and overlapping genetic components (Scheiner et al., 2001; Rueppell et al., 2006). We therefore included two honey bee strains that were bidirectionally selected for foraging behavior (Page & Fondrk, 1995), which

results in differences in the sucrose responsiveness of immature bees. One strain has reduced sugar sensitivity, and mature foragers collect nectars with high sugar concentration. The other strain has heightened sucrose sensitivity, and mature foragers accept nectars with low sugar concentration (Page et al., 2006). We predicted that mature wild-type bees[AB1] and the (selected) immature bees with reduced sugar sensitivity would discriminate against individuals that had food loads of only 10% sugar.

We found that immature wild type bees did not express consistent biases toward workers that were familiar or unfamiliar to them, while the remaining results followed the predicted outcomes of the experiments. These data demonstrate that mature worker honey bees can express quantifiable and adaptive discriminant behavior toward conspecifics in a T-maze. Furthermore, the behavior of the experimental bees in the maze suggests that both mature and immature workers can use volatile cues to assess the quality of nectars carried by other individuals. We establish a new protocol to allow comparison of volatiles emitted by live honey bees using Gas Chromatography/Mass Spectrometry, identifying several potential chemical targets that could be used to communicate food value. Our findings clarify the effects of age and genotype on honey bee social food-orienting and aggressive behaviors, and exemplify the potential of the T-maze to reveal discriminant behaviors essential to highly social organisms.

Methods

**Honey bees**. Wild-type workers came from freely mated European honey bee queens. The selected genotypes were the high and low pollen-hoarding strains, which provide model systems for worker behavior (Amdam & Page, 2010). The pollen-hoarding

strains were bidirectionally bred from European wild type based on a single colony trait: the amount pollen stored in the nest (Page & Fondrk, 1995). This selection affected a suite of correlated traits, including worker sugar sensitivity <24h after adult eclosion (emergence) and the sugar concentration of nectars that mature workers collect. Similar trait correlations are found in wild type, which also vary for the amount of pollen stored in the next (Amdam & Page, 2010). Pollen-hording strains are out-bred to phenotypically similar wild type colonies every 3-4$^{th}$ generation to maintain within-strain genetic variation.

Immature worker bees were obtained from wax combs with mature pupae from wild type, high and low pollen-hoarding strain colonies. At least two combs from separate colonies of each genotype were incubated in the laboratory overnight at 34$^{o}$C and 55-70% relative humidity. The next day, <24h old workers could be collected from the incubator. Mature workers were >20 day-old nest bees (i.e., non-foragers) that were collected directly from the colonies. Mature foragers were not used because they show a heightened activity level and a strong propensity for flight in the T-maze (M. Høiland, C. Kreibich, pers. obs.). Such diverging behavior could influence and potentially confound data interpretation.

After collection the bees were placed on ice for three to five minutes until they stopped moving. Thereafter, each bee was immobilized in an individual plastic holder before her eyes were covered with black acrylic paint in order to eliminate visual factors (Scheiner & Amdam, 2009). Similar procedures are typically used in tests of honey bee tactile learning and memory (Scheiner et al., 1999; Scheiner et al., 2005; Scheiner & Amdam, 2009). We adopted the procedure after pilot experiments in infrared light failed to produce consistent walking behavior in the T-maze: Under

infrared light, walking was interrupted by bursts of flight that made reliable data recording difficult. When the paint had dried (about 10 min) the bees were removed from the holders and put in pairs into small cages (queen cages by Nicot, Fay aux Loges, France). The cages were incubated at 28ºC overnight with *ad libitum* access to a sugar solution of 10, 30 or 50% sucrose, depending on the experiment (details below). Mortality never exceeded 4%.

Efforts were made to ensure that environmental conditions were the same for each experiment and replicate. Bees were collected under similar weather conditions, laboratory preparations were done in the same manner every time, with the same operator (M. Høiland) using the same, clean equipment. Bees were sampled from different sets of colonies for each replicate to ensure that patterns in the behavioral data were not due to traits that were specific to a single set of honey bee hives.

**T-maze.** The maze was manufactured in 0.5 cm thick Plexiglas at the Department of Mathematical Sciences and Technology at the Norwegian University of Life Science in Ås, Norway. The internal diameter was 3 cm. The passage starting at the base of the T (entry section) was 7.5 cm long. Each arm measured 11 cm in length, and ended in a fitted, 3 cm deep cylindrical cage (arm cage) made of wire mesh.

Each test began with the placement of one worker bee in each arm cage before the experimental bee was placed in the entry section. A timer was started as soon as the bee reached the branching point of the two arms, and subsequently, the location of the bee was recorded every 10 sec for three min. Both arms were divided into two recording zones, one proximal and one distal to the arm cage. We also recorded the central zone by the entry section, while the entry section tube was blocked off as soon

as the bee entered the arms for the first time. For every experimental bee, these methods resulted in 18 data points collected over the three min; each point corresponding to one of five locations in the maze: central zone; distal or proximal to the left arm cage; distal or proximal to the right arm cage (see **Figure 1** for an example).

Between each test run of one experimental bee, the maze was washed with dishwashing liquid and dried with cloth to remove potential pheromone footprints. The specific placement (left versus right arm cage) of the unfamiliar versus familiar bee, or the 10% versus 50% sugar-fed bee, was swapped between each experimental worker that entered the maze. Between every second run, moreover, the maze was flipped horizontally so the left arm became the right. These iterations were done to ensure that unknown sources of error (e.g., such as a hypothetical propensity to always walk to left) would not create spurious patterns in the data.

**Discrimination between familiar versus unfamiliar bees.** Each experimental bee was caged overnight with a same-aged companion worker obtained from the same colony. This companion worker became the familiar bee in the subsequent T-maze test. The unfamiliar worker was same-aged, but she was collected from a different colony and stayed in a separate cage overnight together with a same-aged bee from her own colony. For each replicate of the experiment, each of 30 experimental workers was tested toward her familiar bee (i.e., we used 30 familiar bees in total) and an unfamiliar bee (30 in total) that were placed in the separate arm cages of the T-maze. For mature bees, the experiment was replicated twice at the University of Life Sciences ($N = 60$). For immature bees, the experiment was replicated twice at

University of Life Sciences, Aas, Norway (i.e., $N = 60$), and twice at Arizona State University, Tempe, USA ($N = 60$).

**Discrimination between bees with 10% versus 50% sugar loads.** Experimental bees were caged together in pairs and received a diet of 30% sugar overnight. The workers that the experimental bees were tested against were caged in pairs and received 10% or 50% sugar overnight. For each test run, an experimental bee was studied for her behavior toward one bee fed 10% sugar and another bee feed 50% sugar. Both bees in the arm cages were thus unfamiliar to the experimental worker. The experimental bee, furthermore, was starved for 2h before she entered the maze, while the other workers had access to their diets of 10% or 50% sugar until three min before the test runs began. For mature bees, the experiment was replicated twice at the Norwegian University of Life Sciences ($N$=60). For immature bees of the high and low pollen hoarding strains, the experiment was replicated four times at Arizona State University. Each replicate contained 15 high and 15 low strain workers, so that each was tested toward a 10% versus 50% sugar-feed bee of their own genotype. This setup gave a total sample size of 60 bees per strain genotype.

**Chemical volatile emissions of bees that have consumed 10% versus 50% sucrose** For each replicate, four pre-foraging honey bees were collected as described above at the Arizona State University campus in Tempe, AZ, USA. The bees were chilled on ice until immobilized, then restrained in harnesses so that eyes could be painted with black acrylic paint as described above. Once the paint dried, approximately two minutes after application, bees were removed from harnesses with soft forceps and placed in pairs inside queen cages. Cotton balls soaked in either 10% or 30% sucrose

were added to each cage, and cages were stored overnight in an incubator at 28 C. A bucket of water at the bottom of the incubator as well as a wet paper towel directly below cages increased humidity.

In the morning, cages were removed from the incubator and transported in dark boxes to another facility. An entire cage was kept on ice until bees were immobilized, then one bee was removed from the cage with soft forceps and placed inside a volatile collection apparatus. This apparatus consists of a small glass cylinder (need manufacturer) into which L-shaped wire mesh is inserted. Aluminum foil was wrapped around the top of the apparatus to minimize volatile dispersion. A Solid Phase Microextraction (SPME) 100 um polydimethylsiloxane probe (Sigma Aldrich, St. Louis, MO, USA) was inserted through the foil, and the fiber extended to collect volatiles in the space above the wire mesh preventing the bee at the bottom of the apparatus to touch the fiber. The fiber collected volatiles for 1.5 hours. Concurrently, a blank run was performed to clear contaminants from the Gas Chromatography/Mass Spectrometry (GC-MS) system. After 1.5 hours, the fiber was inserted into the GC-MS system and a run begun. The fiber was retracted from the machine three minutes later.

**GC-MS Protocol:**

Oven:

Initial temp: 60 C
Initial time: 2 min
Max temp: 350 C
Equilibration time: 0.50 min
Ramps:

| # | Rate | Final Temp | Final time |
|---|---|---|---|
| 1 | 10 | 250 | 5 |

Run time: 26 min

Front Inlet (SPLIT/SPLITLESS) Back Inlet (CIS3)

mode: spitless
initial temp: 260
pressure: 12.32 psi
purge flow: 50 mL/min
purge time: 2 min
Total flow: 53/8 mL/min
Gas saver: on
Saver flow: 20 mL/min
Save time: 2 min
Gas: helium

mode: split
initial temp: off
pressure: off
Total flow: 45 mL/min
Gas saver: off
Helium

**Statistics.** To simplify statistical processing, the raw data were re-organized before analysis. In the actual experiment, the specific (arm) placement of the unfamiliar versus familiar bee, and the 10% versus 50% sugar-fed bee was quasi-randomized. To simplify analysis, data were re-organized so that the unfamiliar bee was always at the 'left end' of the maze while the familiar bee was at the right end. Similarly, the 50% sugar-load bee was always left and the 10% sugar-load bee was right.

Chi-square tests were used for each experimental replicate to determine significant biases toward the left or right ends of the maze, corresponding to the different treatment sets of bees. Data were cumulatively binned within the 5 maze zones, which were assigned integer values from -2 to 2 (from the left to the right end of the maze: left distal: -2, left proximal: -1, central zone: 0, right proximal: 1, right distal: 2). Replicates were also aggregated and chi-square tested to determine the total bias toward the left or right ends of the maze within each experiment. A bias toward the left end of the maze was inferred if the left distal or left proximal zones had significant chi-square residuals (> 2), and similarly for the bias toward the right end of the maze.

We calculated a theoretical null (TN) distribution for use in every chi-square test. The TN distribution was generated by simulating the behavior of 1,000,000 bees

for 18 time steps with 10 sec intervals, i.e. similar to the data recoded in the experiments. In these simulations, bees were initially placed in the central zone, and at each time step they randomly chose to either stay in the same zone or move to an adjacent zone. The probability of movement, $P_M$, to an adjacent zone (i.e., a measure of relative speed) was the average of the experimentally measured activity level of 30 immature wild type bees in the T-maze. Thus, the probability to move from zone -2 to -1 or from zone 2 to 1 was equal to $P_M$. When the simulated bee was in zones -1, 0, or 1, the probabilities to move left or right in one time step were both equal to half of $P_M$. The experimentally measured activity level of a wild type bee was calculated as the average of |P(t)-P(t+1)|, where t is a multiple of 10 seconds, t = 0, … , 17, and P(t) is the position of the bee at time t which takes on the values -2, -1, 0, 1, or 2, corresponding to the locations left distal, left proximal, central zone, right proximal, or right distal, respectively.

**Results**

**Discrimination between familiar versus unfamiliar bees.** We tested whether mature bees discriminated between unfamiliar and familiar bees in the T-maze by comparing the experimental data against the TN distribution. The mature bees showed a significant bias toward the unfamiliar bee in each of the two ($N = 30$) replicates ($\chi^2$= 28.21, $df = 4$, $P = 1.13e\text{-}5$, and $\chi^2 = 54.01$, $df = 4$, $P = 5.23e\text{-}11$, respectively), as well as among the aggregated (total) data from the experiment ($\chi^2 = 49.65$, $df = 4$, $P = 4.27e\text{-}10$, $N = 60$), **Figure 1**).

In contrast, the immature bees showed a significant bias toward the unfamiliar bee in the first experimental replicate ($\chi^2$= 30.49, $df = 4$, $P = 3.9e\text{-}6$), while no bias

was found for the second experimental replicate ($\chi^2$ = 5.29, *df* = 4, *P* = 0.2592) or in the total data (*N* = 60) from the two replicates conducted in Norway ($\chi^2$= 6.09, *df* = 4, *P* = 0.1925, **Figure 1**). Also, no bias was inferred from the third experimental replicate ($\chi^2$= 4.51, *df* = 4, *P* = 0.341) while a significant bias toward the familiar bee was present in the forth ($\chi^2$= 40.56, *df* = 4, *P* = 3.31e-8) and among the total data (*N* = 60) from the two replicates conducted in the USA ($\chi^2$= 24.34, *df* = 4, *P* = 6.83e-5, **Figure 1**). When these four replicates where analyzed together (*N* =120), the immature bees showed no significant bias toward either the familiar or unfamiliar bee ($\chi^2$= 7.46, *df* = 4, *P* = 0.113).

**Discrimination between bees with 10% versus 50% sugar loads.** Next, we tested whether mature bees discriminated between bees with 10% versus 50% sugar loads by comparing experimental data against the TN distribution. Mature bees showed a significant bias toward the 50% sugar-fed bee in both experimental replicates ($\chi^2$ = 24.59, *df* = 4, *P* = 6.09e-5, and $\chi^2$= 22.21, *df* = 4, *P* = 1.82e-4, respectively), as well as among the aggregated data ($\chi^2$ = 33.65, *df* = 4, *P* = 8.76e-7, *N* = 60), **Figure 2**).

Finally, we tested how immature bees from the bidirectionally selected high and low pollen-hoarding strains discriminated between 10% versus 50% sugar-fed bees. Between the four replicates with high pollen-hoarding strain bees (*N* = 15), only the third replicate showed a significant bias ($\chi^2$= 24.09, *df* = 4, *P* = 7.63e-5), which was toward the 10% sugar-fed. The total data (*N* = 60) also showed a significant bias toward the bee fed 10% sugar ($\chi^2$= 9.94, *df* = 4, *P* = 0.041, **Figure 2**). In contrast, three of the four replicates with low pollen-hoarding strain bees (*N* = 15) showed a significant bias toward the 50% sugar-fed bee, while one replicate indicated a bias

toward the bee fed 10% sugar). The total data, though, demonstrated that low pollen-hoarding strain bees displayed a significant bias toward the bee fed 50% sugar ($\chi^2$ = 19.42, *df* = 4, *P* = 6.51e-4, **Figure 2**).

**Fig. 3.1 Discrimination of familiarity in mature and immature bees**

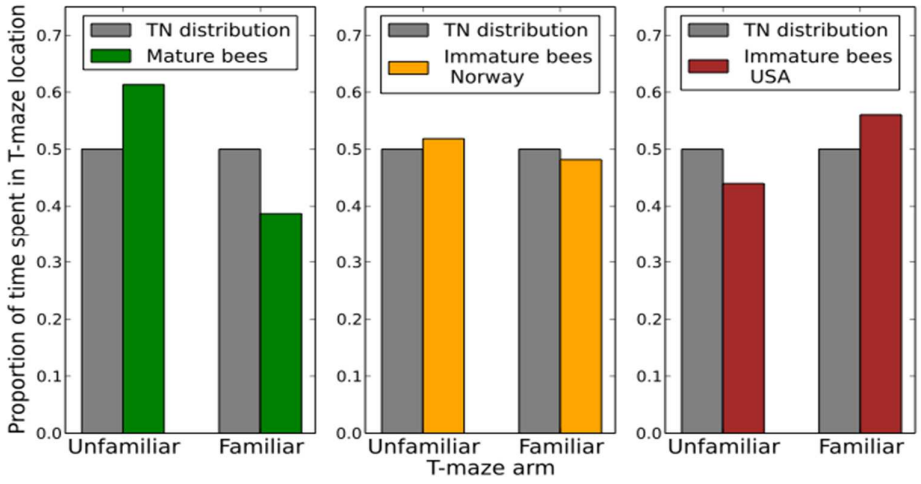



**Fig. 3.1** Mature bees spend significantly more time in the arm at the end of which was an unfamiliar bee rather than a familiar bee. Immature bees do not spend more time in an arm depending on familiarity.

**Fig. 3.2 Discrimination of crop contents by mature and immature bees**

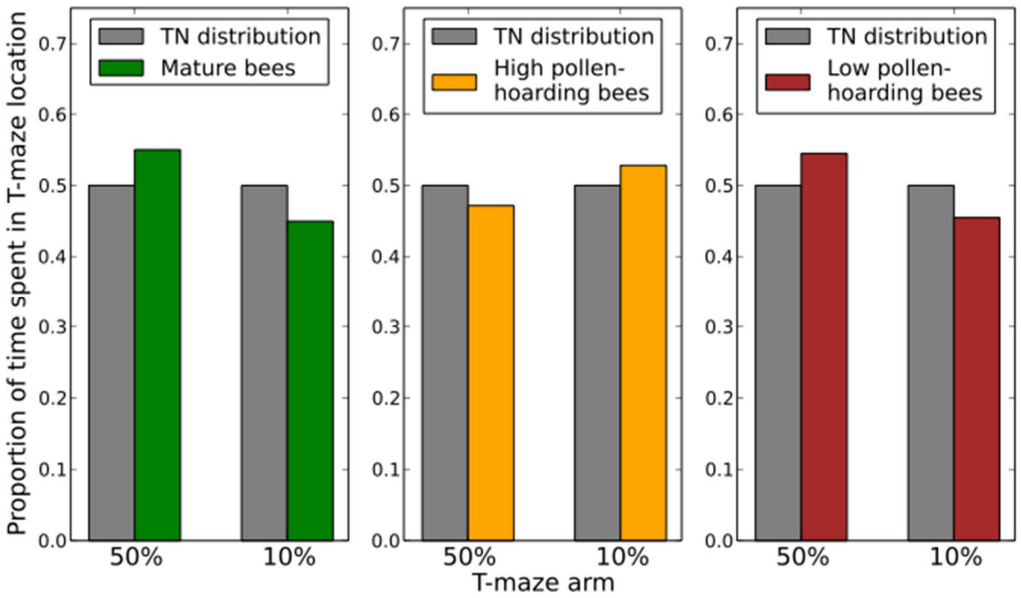
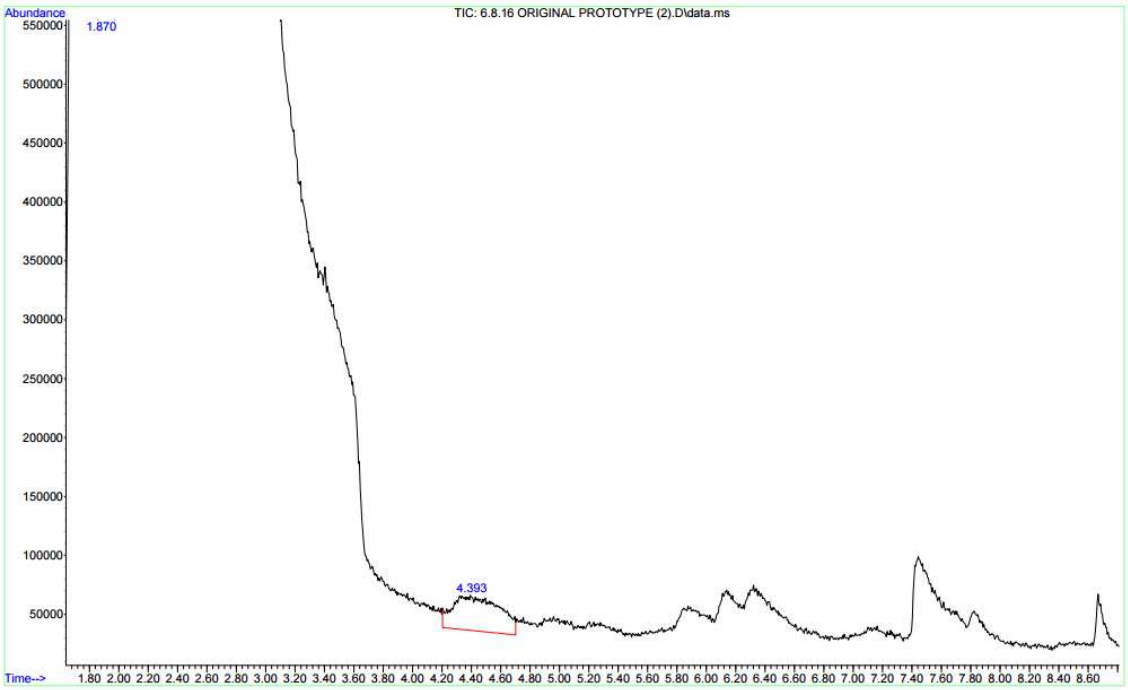



**Fig. 2** Mature bees spend significantly more time in the arm at the end of which was a bee that had fed on 50% sucrose rather than 10% sucrose. For immature bees, high pollen-hoarders spent more time in the arm at the end of which was a bee that had fed on the lower concentration, while the opposite was true of low pollen-hoarders.

**Fig. 3.3 Characterization of volatile profiles emitted by honey bees**



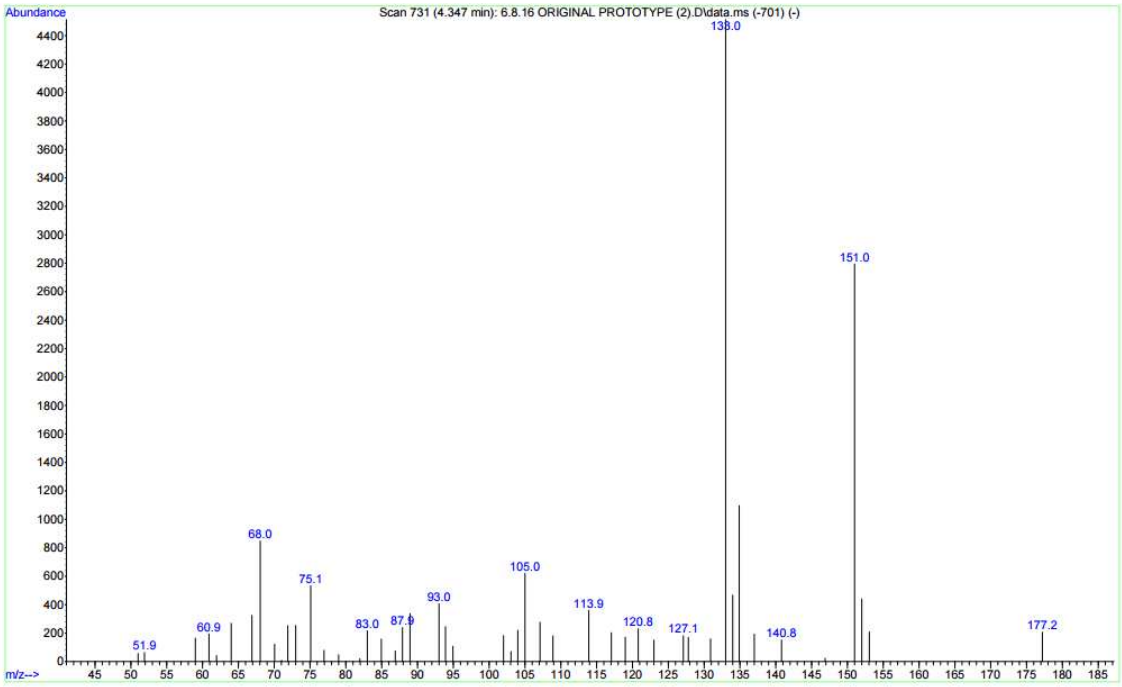



**Fig. 3.3** Identified volatile compound: Oxime-, methoxy-phenyl has also been found in honey extracted from honey bee hives (Wolski et al. 2006), suggesting as a feasible candidate for a volatile that can transmit information concerning consumed sucrose concentration.

**Discussion**

Our experiments suggest that immature honey bee workers do not consistently differentiate between bees that are familiar or unfamiliar to them, while mature bees do. The data further indicate that both mature and immature workers can differentiate between bees that carry 10% versus 50% sugar loads. We tested mature wild type workers, which consistently preferred the bee with the 50% sugar load. We also tested immature workers with documented differences in sucrose sensitivity. Those with low sucrose sensitivity (low strain) showed a more consistent bias toward the 50% sugar-fed bee compared to the workers that have high sucrose responsiveness (high strain).

We show that bees' volatile emissions can be characterized using Gas Chromatography, and propose an experiment to compare volatile emissions between bees that have consumed high vs low concentrations of sucrose.

**T-maze assay and implications:** Painting over bees' eyes causes bees' discriminant behaviors, as observed in the maze, to rely on sensory modalities other than vision. This behavioral context would be typical for the pre-foraging bees we used in our experiments. Prior to foraging, worker bees perform tasks inside the dark nest-area of the colony, where tactile, olfactory and vibratory information is ostensibly more important than visual cues.

Data recording was done by a time sampling method, i.e. by recoding the bees' location in the maze every 10 sec for 3 min. Time sampling is a simple but powerful tool to study behavior (Powell et al., 1977). Alternative methods, such as continuous data capture by video, have many benefits but also tend to produce complex outputs that are time-consuming to analyze. We cannot exclude that such alternative methods would have produced richer datasets. Yet, the time sampling technique is a valid experimental approach (Gershuny, 2004) that produced reasonable and significant results in this study.

Our data were recoded in two locations, one in Norway and one in the USA. The test of whether immature bees would discriminate between familiar and unfamiliar bees was performed in replicate at both locations, leading to the same conclusion. The other experiments were conducted in replicate in either Norway or the USA. For these data, we cannot guarantee that the observed outcomes and conclusions are valid for both European and US bee stocks. This limitation, however,

equally applies to any experiment that is performed on only one continent, which would account for the majority of honey bee research today.

**Discrimination between familiar versus unfamiliar bees.** Our experiments showed that immature bees (<48 h old at the time of the T-maze assay) do not consistently discriminate between same-aged bees that are familiar or unfamiliar to them, while mature bees do. The mature bees showed a consistent bias toward the unfamiliar worker that might be interpreted as an adaptive defensive response. Aggressive behavior toward unfamiliar conspecifics is an important social mechanism in honey bees that defends the society's honey stores against robber bees from other colonies (Winston, 1987). Visual information was not available to our test animals, but odors provide important nestmate recognition cues in social insects and honey bees may use additional contextual cues, such as the presence or absence of threats, to adjust their permissiveness in discrimination between nestmates and non-nestmates (Ratnieks et al., 2011). Our results indicate that recognition and possibly contextual cues were available in the T-maze assay to aid discrimination by mature bees.

Immature bees have a soft cuticle and stinger (when < 24h old) and do not usually express aggressive behavior. They can also be transferred to an unfamiliar colony without evoking aggression from mature worker bees. Immature workers, in other words, are unable to afflict damage to intruders and they are not the subjects of aggressive behavior, including from mature bees that are unfamiliar to them. The immature bees in our experiment, furthermore, were only in brief contact with the wax combs of their native nest (<24h). Wax is an important source of odor recognition cues in honey bees (Breed et al., 1989). Thus, it may not be expected that

these immature bees express significant discriminant behavior toward other immature workers. We did not ask, however, if immature bees can differentiate between mature workers that are familiar or unfamiliar to them, or vice versa. Such contrasts can be tested in future experiments. Until then, our data might suggest that immature bees do not show a negative bias toward unfamiliar individuals, and support the hypothesis this discriminant ability develops with age in bees.

**Discrimination between bees with 10% versus 50% sugar loads.** We found that mature wild-type worker bees as well as the immature bees of the high and low pollen-hoarding strains behaved differently toward bees that had fed on 10% or 50% sugar solutions. Trophallaxis (food exchange by mouth), provides a direct method for a bee to assess the sugar concentration of nectar in another worker's stomach (Frisch, 1967; Farina & Núñez, 1993). This behavior, however, was not regularly observed during our experiments (only 4 occurrences recorded, Høiland, M. pers. obs.). Worker honey bees also have sugar receptors on the antennae, so an alternative explanation is that the experimental individuals detected sugar loads tactilely, e.g. by touching the mouth-parts of the bees. Yet, antennal contact was also uncommon in our assay (only 5 occurrences recorded, Høiland, M. pers. obs.), suggesting that bees can communicate sugar loads without direct sampling. A possible route for this communication is volatile cues. Sucrose, the sugar used in our experiments, is not volatile but the volatility of other compounds can be influenced by sucrose in solution (Covarrubias-Cervantes et al., 2004; Hort & Hollowood, 2004; Pfeiffer et al., 2006). Such compounds include esters (Covarrubias-Cervantes et al., 2004) which are important components of honey bee odors such as pheromones found in the gut (Le

Conte et al., 1994; Le Conte et al., 2001; Leoncini et al., 2004). Thus, we speculate that worker odors might change with crop content. We develop a Gas Chromatography/Mass Spectrometry method to characterize and compare the volatile emissions of bees that have consumed different concentrations of sucrose.

The mature workers consistently preferred the 50% sugar-fed bee. Under natural conditions wild-type honey bee colonies can discriminate between food sources (flower patches) because the behavior of returning foragers is conditional on the quality of the food source: Foragers can promote a highly rewarding patch by dancing more vigorously, or for longer periods of time (Seeley, 1989). Dancing communicates the direction, distance and quality of the food, and can include regurgitation of nectar samples (Frisch, 1967). The likelihood that an individual begins dancing is affected by the time it takes to unload her nectar (Seeley & Tovey, 1994). The longer a forager waits, the lower the likelihood of dancing. It is unclear whether food storer bees, which the forager unloads to, preferentially accept food loads of high quality. If they do, then the colony has an additional mechanism that promotes highly rewarding flower patches at the expense of those that are less rewarding. Our finding that mature (nest) bees consistently discriminate between workers that carry food loads of different profitability provides some indirect support for this hypothesis.

Between the pollen-hoarding strains, the genotype that is less sensitive to sugar (low strain), showed the stronger behavioral bias toward bees with 50% sugar loads in the T-maze. Under natural conditions, low strain bees forage for nectars of higher sugar concentration and store more nectar relative to pollen in their colonies than high strain bees do (Page et al., 1998). In laboratory assays, high strain bees

respond reflexively with proboscis extension to lower sugar (sucrose) concentrations on average than low strain bees. These concentrations can be as low as 0.1% sucrose (Scheiner et al., 2001; Scheiner et al., 2005). We suggest that, because of these documented genotype-differences in gustatory perception, the 10% sugar-load bees were accepted by high strain workers but discriminated against by low strain workers. An acceptance of the 10% sugar-load bee by high strain bees, however, does not fully explain why this genotype showed an overall bias toward bees with 10% sugar load. This finding is intriguing because only a small fraction of the tested workers engaged in trophallaxis or antennal contact, suggesting that workers can use currently unidentified cues that communicate the food load of other bees. We identify a volatile compound that could serve as a way for bees to transmit information about the quality of food in their crops, paving the road for future experiments to expand our understanding of honey bee communication.

**Acknowledgement**

We thank Daniel Alcantar and Nicholas Baker for technical assistance with behavioral assays and honey bee colonies, and Nicholas Baker, Daniel Münch and Nicola Plowes for helpful comments on the manuscript.

CHAPTER 4

# FORAGING EXPERIENCES INDUCE ENDURING CHANGE TO HONEY BEES' SUCROSE RESPONSIVENESS AND ANTENNAL LOBE BIOGENIC AMINE LEVELS

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Contribution: I designed and performed all experiments and analyses, with the exception of preparing tissue post-dissection for high-performance liquid chromatography and calculating biogenic amine concentrations from the output data. I wrote this manuscript, with the guidance and feedback of Dr. Colin Brent and Dr. Gro Amdam.

## Introduction

Foraging for food is central to the lives of many organisms. However, exploration for resources exposes foragers to predation and conspecific competition. A large body of literature focuses on how organisms learn to approach or avoid locations, smells, visual cues, and other stimuli that they have experienced in association with food or danger (Pearce 2008; Honey et al. 2014). In some species individuals diverge not only in their responses to food-associated stimuli, but in their sensitivity to food quality. The mechanisms by which they acquire and retain these food preferences are not well understood. One area that has received attention is the sucrose response threshold, which is known to vary between individual rats (Tõnissaar et al 2006), humans (Dias et al. 2015), and social insects (Scheiner et al. 2013; Muller 2011).Research has focused on the genetic correlates of such variability

(Dias et al. 2013), or otherwise on the short-term effects wrought by hormonal changes (Curtis et al. 2005; Jyotaki et al. 2010), hunger (Hanci et al. 2016), or stress (Ileri-Gurel et al. 2013). However, there has been little exploration of the long-term effects of specific food-related experiences on the sucrose response threshold. This study proposes that the aversive and appetitive experiences acquired during foraging contribute to lasting inter-individual differences in sucrose responsiveness.

The sucrose responsiveness of honey bee (*Apis mellifera*) foragers varies between individuals and causally affects appetitive learning ability (Scheiner et al. 2005). Honey bees' response threshold can be quantified using the Proboscis Extension Response (PER) protocol, in which honey bees restrained in harnesses raise their proboscis when the concentration of a droplet of sucrose touched to their antennae is high enough to elicit a feeding reaction (Pankiw and Page, 1999, 2000, 2003). Response thresholds in honey bees are influenced by genotype (Page et al. 1997) and the expression of cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) (Thamm and Scheiner 2014). Additionally, response thresholds can be modulated in the short-term by recent experience: the consumption of scented sucrose acutely increases sucrose responsiveness (Ramirez et al. 2010) while satiation (Friedrich 2004), stress (Pankiw and Page 1999), and the taste of highly concentrated sucrose (Pankiw et al. 2001) have the opposite suppressive effect. Nectar foragers are less responsive to sucrose than pollen foragers (Pankiw and Page 2000), but the overall responsiveness of each group changes throughout the foraging season, particularly dramatically in nectar foragers (Scheiner et al. 2013).

Pharmacological manipulations of biogenic amine circuits have revealed a role in the regulation of reward and punishment responsiveness. Honey bees' sucrose

responsiveness is increased by injection or ingestion of octopamine or its precursor tyramine (Scheiner 2002). The opposite effect can be induced by injection of dopamine into the thorax, or by either injection or ingestion of the dopamine receptor agonist 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN). Drugs that antagonize dopamine *D1* and *D2* receptors or serotonin *5-Ht2a* and *5-Ht2b* receptors elevate electric shock responsiveness (Tedjakumula 2014). It has not been demonstrated that naturally occurring variation in individuals' responsiveness to sucrose or electric shock correlates with differences in biogenic amine pathways, but adult honey bees typically progress through a series of roles as they mature and age, and some behavioral changes correlate with, and can be induced by, alterations in biogenic amine signaling (Schulz et al. 2002; Reim 2014). Octopamine is one of the major neurotransmitters implicated in the transition from working inside the nest to foraging. Higher levels of octopamine in the antennal lobes is associated with foraging behavior and injection can induce an early transition to foraging (Schulz et al. 2002). Likewise, octopamine *OA1* receptors are up-regulated in foragers' antennal lobes and subesophageal ganglion compared to same-aged honey bees working inside the nest (Reim 2014). The process of foraging provides individuals with a variety of potential experiences such as encounters with plants' olfactory bouquets and nectars, aversive con-specific competition (Rogers et al. 2013), predation from arthropods and birds (Bromley 1984; Suttle 2003), as well as noxious secondary compounds secreted by plants (Stevenson et al. 2016). We propose that such diverse foraging experiences induce sustained inter-individual differences in sucrose responsiveness through biogenic amine pathways, continuing to shape individuals' behavior after acute effects have subsided.

To elucidate the long-term effects of foraging experience, we simulated diverse foraging conditions using cages in which free-roaming bees could collect food from feeders offering sucrose paired with floral scents and/or electric shock. Electric shock is the most commonly used form of aversive stimulation in the laboratory for honey bees as well as other model organisms (Tedjakumula and Giurfa 2013). Bees can form long term memories associating electric shock with different contexts or scents (Tedjakumula 2014), and individual response threshold for electric shock correlates with nest guarding and foraging behavior in the field (Roussel et al. 2009). We predicted that bees that had experienced different feeder conditions would exhibit divergent sucrose responsiveness, antennal lobe biogenic amine titers, and receptor expression in the subesophageal ganglion and antennal lobes, the same regions implicated in a worker's transition from nurse to forager behaviors.

## Methods

### *Bee Collection*

Honey bee (*Apis mellifera ligustica*) foragers were collected from hives at the Arizona State University Campus in Tempe, AZ from April-June 2015 in the morning. For each test date, bees were taken from three different hives and mixed together to minimize hive-specific effects. Mature but pre-foraging nest bees were chosen to avoid variation due to aging (Behrends et al. 2007). We chose bees by lifting the outer honey comb frames, where younger nest-bees are unlikely to be found (Seeley and Kolmes 1991), out of the hive box, and collecting only those bees that did not fly away when gently touched with soft forceps. Bees were placed in glass vials, and cooled on ice until they stopped moving.

*Cage Paradigm*

Once immobilized inside the glass vials on ice, each individual was labeled with queen bee number tags (The Bee Works Queen Marking Kit, Ontario, Canada) on the thorax, and placed inside an experimental cage in which they regained motility and became acclimatized for three hours before experiment initiation. Each cage was divided by mesh to separate bees into two groups: 17 bees with feeder access in the bottom compartment and 13 bees with no feeder access in the top compartment (Fig. 1). Empty honey comb lined the back wall in both compartments. Six replicates were run, each consisting of four cages, differing only in the feeder conditions for the first two days. The cages were exposed to constant fluorescent lighting and were kept at room temperature (21-26 C). The 4 locations for cages within a room in our laboratory were kept constant, and the location of each experimental condition was alternated between replicates. In the two boxes simulating aversive foraging conditions, the first two days of treatment consisted of 2 Kimwipes (Kimberly-Clark Professional, Roswell, Georgia, USA) soaked in 1.5M sucrose in a feeder that delivered 4.2 V of electric shock to feeding bees (Fig. 1). In one of these boxes, the sucrose was scented with linalool or phenylacetaldehyde (Sigma Aldrich, St. Louis, MO, USA) (50ul odorant per liter of 1.5M solution, as previously used in Ramirez et al. 2013). The other two boxes received identical treatment but without the electric shock, simulating benign foraging conditions. Two feeder slots at the bottom of each cage allowed food location to be alternated every 6-8 hours to reduce spatial associative pairing. After two days of treatment, the feeder slots at the bottom of all boxes were cleaned with a Kimwipe dampened with ethanol to remove traces of

sucrose, odor, or secretions from honey bees. All feeder slots were filled with bottle caps containing two Kimwipes soaked in 0.5M sucrose. These were replaced to maintain constant saturation.

After three days of *ad libitum* feeding, cages were laid horizontally so that bees could be collected for subsequent assays. We alternated between collecting bees in glass tubes to be chilled on ice for behavioral assays, and collecting bees in plastic Eppendorf tubes to be flash frozen in liquid nitrogen for biogenic amine analysis. Biogenic amine concentrations can change rapidly in bees (Harris et al. 1992), especially due to stress (Chen et al. 2008), so we chose to analyze the biogenic amine profiles immediately following removal from cages rather than after behavioral assays.

*Biogenic Amine Analysis*

Flash frozen bees were stored at -80 °C. Antennal lobes were later dissected from frozen samples and pooled in groups of five pairs per sample. High-performance liquid chromatography (HPLC) was used for analysis of biogenic amine content. A detection limit of 25 pg precluded the analysis of individual heads. Dissected brains were placed in a 1.5-ml centrifuge tube and homogenized with a pestle in 20µl of chilled perchloric acid (0.2 M) containing dihydroxybenzylamine (DHBA, 87pg/µl; Sigma-Aldrich, St. Louis, MO, USA) and synephrine (50pg/µl; Sigma-Aldrich) as internal standards. Samples were then sonicated for 5 min in a covered ultrasonic bath (Branson 2510, Branson Ultrasonics Corp., Danbury, CT, USA) filled with an ice water slurry. After sonication the samples were allowed to sit in the water bath for an additional 20 min to maximize amine extraction. Samples were spun at 12,000 RCF

for 10 min in a refrigerated (4°C) centrifuge, then kept on ice in a covered container until analysis.

Only six samples were prepared at a time to minimize the delay between removal from the freezer and amine quantification. The biogenic amine content of 10 µl of supernatant was determined on an HPLC system (ESA, Chelmsford, MA, USA) consisting of a Coularray model 5600A with a 4 channel electrochemical detector (Ch1 650 mV, Ch2 = 425 mV, Ch3 = 175 mV, Ch4 = -125mV), a model 582 pump, and a reverse-phase catecholamine HR-80 column. Samples were manually injected (Rheodyne 9125, Rohnert Park, CA, USA) into a 20-µl loop. Mobile phase (flow rate = 0.5 ml min-1) consisted of polished water, 15% methanol, 15% acetonitrile, 1.5 mmol $l^{-1}$ sodium dodecyl sulfate, 85 mmol $l^{-1}$ sodium phosphate monobasic, and 5 mmol $l^{-1}$ sodium citrate. Phosphoric acid was used to adjust the buffer pH to 5.6. Results are expressed on a per head basis. The size of resultant peaks were compared to a serial set of external standards (hydrochloride forms of DA, OA, 5-HT, TA; Sigma-Aldrich) run before and after each set of 6 samples to determine the equivalent quantity in picograms.

*Behavioral Assays: Retention, Sucrose Responsiveness, and Differential Conditioning*

Bees in glass vials were chilled on ice until immobilized, and then restrained individually in metal harnesses with strips of duct tape (Duck Brand, Avon, OH, USA) as described in Smith and Burden 2014. After a 20 minute rest period, bees from the compartments without feeder access were fed with 2 ul of 0.5M sucrose from a pipette tip by first touching the antennae to elicit proboscis extension. We did not feed the bees that had feeder access to unlimited sucrose, as these bees were

already presumed to have fed and our goal was to bring all bees to a similar state of satiation.

Three hours later, all bees were tested for retention ability by presenting the treatment scent and a novel scent in a random order and observing proboscis extension. Afterward, all bees were allowed to feed on 0.5M sucrose until sated. Bees were considered sated when touching the antennae with a droplet of sucrose did not elicit proboscis extension.

The following morning, 96 hours after the end of treatment, sucrose responsiveness was determined by presenting randomly ordered bees with a progressive sequence of 0.1%, 0.3%, 1%, 3%, 10%, 30% sucrose interspersed with water between each trial to prevent sensitization. Both antennae were touched with a droplet of sucrose or water on a pipette tip, and proboscis extension response was recorded to produce a Gustatory Responsiveness Score (GRS) of 0-6.

Four hours after sucrose responsiveness assays, bees were tested on an olfactory discrimination assay in which one odor (CS+) predicted reward of 1.5M sucrose while the other (CS-) did not predict reward. The odor used as CS+ was alternated between replicates. Each trial consisted of a few seconds of acclimatizing to the testing context, followed by a 4 second presentation of the odor stimulus by pushing air through an odor cartridge. Three seconds after odor onset, the CS+ odor was forward-paired with 0.6 µl of 1.5M sucrose while the CS- odor was not paired with sucrose. Bees' responses were recorded as binary "yes" or "no" with "yes" indicating that bees exhibited a proboscis extension reflex (PER) – extension beyond an imaginary line between the opened mandibles – during presentation of scent and before presentation of sucrose (Smith and Burden 2014). Afterward, the bee was left

in position for several seconds. Each bee had an inter-trial interval of at least 10 minutes. Approximately 30 minutes following conditioning, we tested short-term retrieval by exposing each bee to a single unreinforced test trial with each odor, alternating the order of odors presented. The presence or absence of PER was once again recorded as a "yes" or "no."

During discrimination assays, which occurred 7 days after bees were first placed into cages, bees were visibly stressed as exhibited by drooping heads, slightly extended proboscises, reduced locomotion inside harnesses, and a lack of proboscis extension response when touched on the antennae with 1.5M sucrose during the conditioning trials. All but a few bees failed to learn during the conditioning. We therefore did not include discrimination learning and retention performance in our analyses, as bees were not healthy enough by this time to provide an accurate representation of differences in learning ability.

*Octopamine Receptor Gene Expression Analysis*

To analyze gene expression, bees were flash frozen in liquid nitrogen after behavioral assays for dissection of mushroom bodies, antennal lobes, and subesophageal ganglion. We were interested in octopamine *OA1* receptor differences between bees that had exhibited differences in performance on behavioral assays due to foraging conditions. As shown in the results, only bees with feeder access in benign conditions exhibited behavioral differences; bees foraging for scented sucrose exhibited different sucrose responsiveness from bees foraging for unscented sucrose. Therefore, only bees with feeder access in benign conditions were used for analysis of octopamine receptor expression. RNA was extracted from each neuropile using a

trizol/chloroform protocol (ThermoFisher Scientific, Waltham, MA, USA). cDNA libraries were created with the Taqman Reverse Transcription Reagents Kit (ThermoFisher Scientific, Waltham, MA, USA. Using established *OA1* primers (Reim et al. 2014) we performed quantitative real-time Polymerase Chain Reaction (qPCR) using the ABI PRISM® 7000 Sequence Detection System ((ThermoFisher Scientific, Waltham, MA, USA) to compare expression levels between bees that had foraged for scented vs unscented sucrose. For normalization of the receptor transcripts we used *elongation factor 1 alpha* as the reference gene, as in Reim et al. 2014, because it is stably expressed in nurse bees and foragers, castes that are known to exhibit different sucrose responsiveness.

*Statistics*

All statistical analyses were performed in Statistica v12.7 (Dell Inc, Round Rock, TX, USA) Antennal lobe biogenic amine levels and sucrose responsiveness did not follow normal distributions; therefore we used the non-parametric Mann Whitney U Test to compare groups. All comparisons were planned (i.e. prior to collection of the data), and accordingly, corrections for multiple comparisons were not required (Keppel & Wickens, 2004)

The proportion of bees responding to the long-term retrieval tests for previously experienced scents were plotted as percentage of bees exhibiting PER in each trial (% PER). We used the Pearson's Chi-square test to identify differences between groups in the total bees displaying PER when presented with the novel and treatment scents during retrieval assays.

Octopamine receptor expression followed a normal distribution and displayed equal variances, so the parametric Student's two-tailed *t*-test was used to compare expression levels between groups. All comparisons were planned (i.e. prior to collection of the data), and accordingly, corrections for multiple comparisons were not required (Keppel & Wickens, 2004).

**Fig. 4.1 Experimental Paradigm**

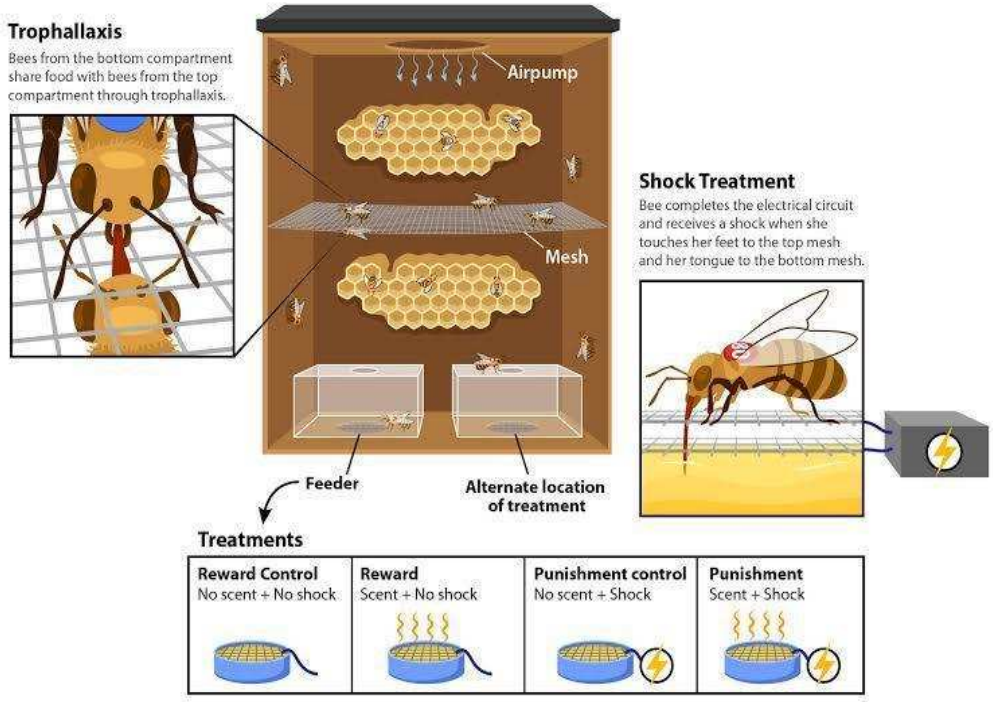



**Fig. 4.1**
A new experimental paradigm provides free ranging honey bees with feeders paired with electric shock and/ or odor. Each replicate of the study included four separate cages, each offering different foraging conditions: scented sucrose, unscented sucrose, scented sucrose with electric shock, unscented sucrose with electric shock. Each cage contained an internal control of bees exposed to the same cage environment, but fed only through trophallaxis.

## Results

### *Sucrose responsiveness*

Scented sucrose reduced foragers' gustatory response scores (GRS) four days after the conclusion of foraging conditions relative to unscented sucrose (Mann Whitney U test: n=43 (no scent) and n= 44(scent), U= 576.5, z-adjusted=3.133, p= 0.0012) (Fig. 1a). Aversive conditions eliminated the effect of floral scent dissolved in sucrose on subsequent sucrose responsiveness (Mann Whitney U test: n=17 (no scent), n=13 (scent), U = 94, z-adjusted=-0.347, p-value = 0.728) (Fig. 1b). In contrast, control bees that did not have feeder access and were fed scented or non-scented sucrose only through trophallaxis through wire mesh did not exhibit differences in sucrose responsiveness (Fig 2) (Mann Whitney U test: n=26 (no scent), n=20 (scent, U = 817, z-adjusted = 0.129, p-value = 0.889).

### *Biogenic amine titers*

In aversive foraging conditions, the presence of floral scent dissolved in mesh elevates serotonin concentration in antennal lobes (Mann Whitney U Test, n=7,7, Z Adjusted = -2.044, p=0.0409) (Fig. 3a). No other biogenic amine levels were changed (Fig. 3b-d): Antennal lobe octopamine was not influenced by scent in aversive conditions (Mann Whitney U Test, N=7, 7, U=15, z-adjusted=-1.150, p-value=0.250). Antennal lobe dopamine was not influenced by scent in aversive conditions (Mann Whitney U Test, N=7, 7, U=19, z-adjusted=0.523, p-value=-0.639). Antennal lobe tyramine was not influenced by scent in aversive conditions (Mann Whitney U Test, N=7,7, U=60, z-adjusted=-0.894, p-value=-0.894)

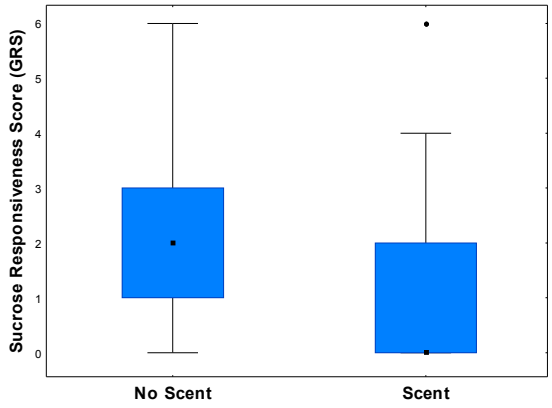
When food was unscented, aversive conditions relative to benign reduced concentrations of octopamine and serotonin in antennal lobes relative to benign conditions (octopamine: Mann Whitney U Test, Z adjusted=2.172180, p=0.029843, U=8, N=7,7 and serotonin: Mann Whitney U Test, Z adjusted=2.04441, p=0.040914, U=7, N=7,7) (Fig 4a and b). Dopamine and tyramine concentrations were independent of foraging conditions (dopamine: Mann Whitney U Test, z-adjusted = 1.79, U=10, p=0.074, N=7,7 and tyramine: Mann Whitney U Test, z-adjusted=1.53, U=12, p=0.125, N=7,7) (Fig 4c and d).

***Octopamine receptor expression***

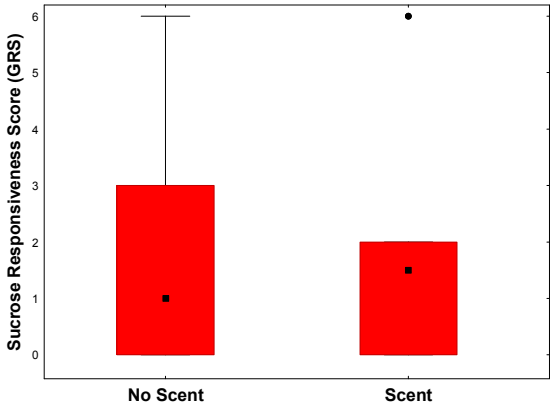
In contrast to sucrose responsiveness, octopamine receptor expression in specific brain regions did not between bees that had feeder access to scented vs. unscented sucrose in benign conditions. Antennal lobes: Student's T Test, t-value=-0.424, df=14, $N_{no\ scent}$=7, $N_{scent}$=9, p=0.678. Subesophageal ganglion: Student's T Test, t-value=-1.248, df=14, $N_{no\ scent}$=6, $N_{scent}$=10, p=0.233. Mushroom bodies: Student's T Test, t-value=-0.312, df=13, $N_{no\ scent}$=5, $N_{scent}$=10, p=0.76.

**Fig. 4.2 Effect of scented sucrose on sucrose responsiveness under benign and aversive foraging conditions**

**A. Benign Conditions**




**B. Aversive Conditions**




*Box-and-whiskers plots showing sucrose responsiveness of bees with feeder access, measured 96 hours after scented food was removed. Boxes represent 25-75 percentiles, bars show the non-outlier range (1.5*height of the box), points show outliers, and squares represent medians.* ***A.*** *In comparison to unscented control, scented food reduced sucrose responsiveness three days after removal of scents (Mann Whitney U test: n=43 (no scent) and n= 44(scent,) U=576.5, z-adjusted=3.236, p= 0.0012)* ***B.*** *With the addition of electric shock at the feeders, sucrose responsiveness was equivalent between bees fed with scented and unscented food (Mann Whitney U test: n=17 (no scent), n=13 (scent), U = 94, z-adjusted=-0.347, p-value = 0.73).*

**Fig. 4.3 Effect of scented food on sucrose responsiveness of non-forager bees in benign conditions**

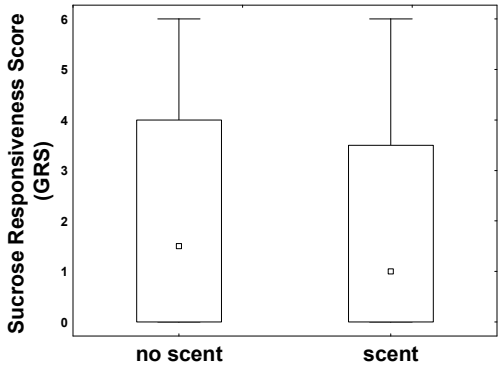
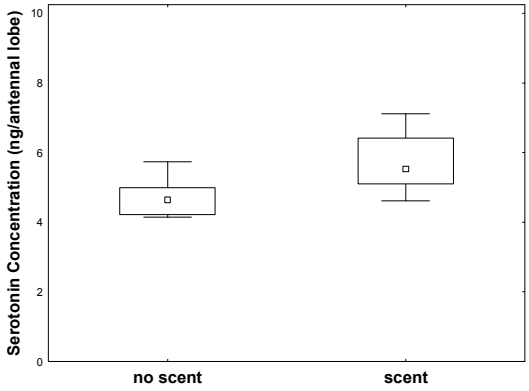



**Fig. 4.3**

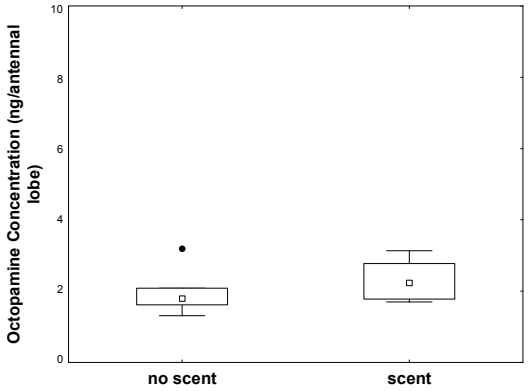
*Box-and-whiskers plots showing sucrose responsiveness of bees without feeder access, measured 96 hours after scented food was removed. Boxes represent 25-75 percentiles, bars show the non-outlier range (1.5*height of the box), points show outliers, and the square represents the median. In contrast to bees with feeder access, the sucrose responsiveness of bees fed through trophallaxis is not altered by the presence of scent in trophallaxed food. Mann Whitney U test: n=26 (no scent), n=20 (scent, U = 817, z-adjusted = 0.129, p-value = 0.889).*

**Fig. 4.4 Effect of foraging for scented vs unscented sucrose in aversive conditions on antennal lobe biogenic amine levels**

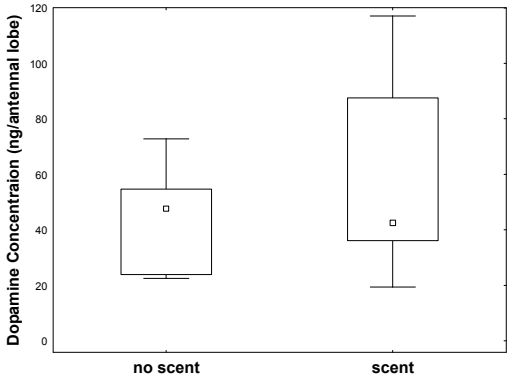
**A. Antennal lobe serotonin**



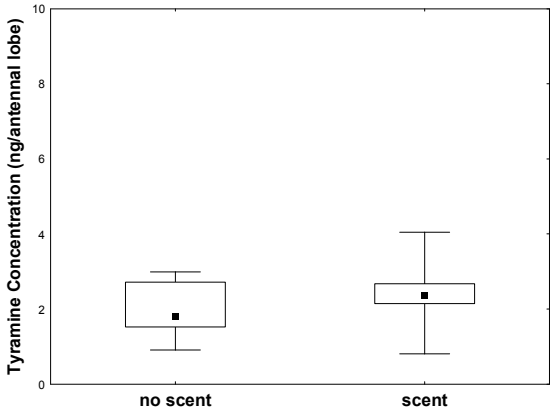
**B. Antennal lobe octopamine**




**C. Antennal lobe dopamine**




**D. Antennal lobe tyramine**

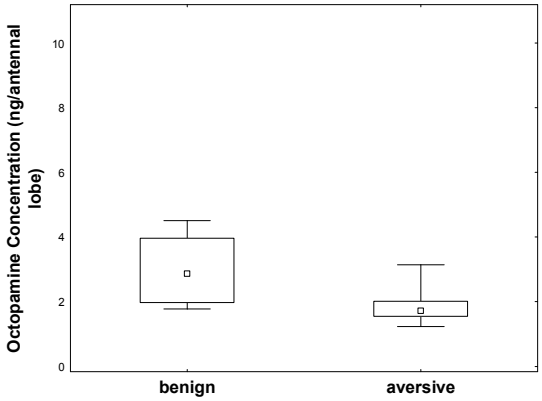



*Box-and-whiskers plots showing antennal lobe biogenic amine concentrations in aversive foraging conditions with and without scent dissolved in food. Boxes represent 25-75 percentiles, bars show the non-outlier range (1.5*height of the box), points show outliers, and squares represent medians.* ***A.*** *Antennal lobe serotonin was significantly higher when food was scented in aversive conditions (Mann Whitney U Test, N=7, 7, U=8, z-adjusted = -2.044, p-value = 0.04090).* ***B.****Antennal lobe octopamine was not influenced by scent in aversive conditions (Mann Whitney U Test, N=7, 7, U=15, z-adjusted=-1.150, p-value=0.250).* ***C.*** *Antennal lobe dopamine was not influenced by scent in aversive conditions (Mann Whitney U Test, N=7, 7, U=19, z-adjusted=0.523, p-value=-0.639).* ***D*** *Antennal lobe tyramine was not influenced by*

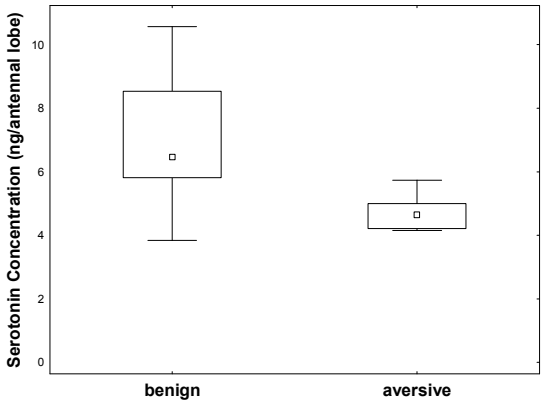
*scent in aversive conditions (Mann Whitney U Test, N=7,7, U=60, z-adjusted=-0.894, p-value=-0.894)*

**Fig. 4.5 Effect of foraging for non-scented sucrose in benign vs aversive conditions on antennal lobe biogenic amine levels**

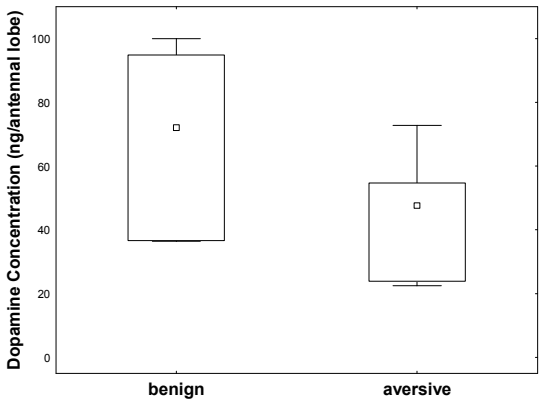
**A. Octopamine**



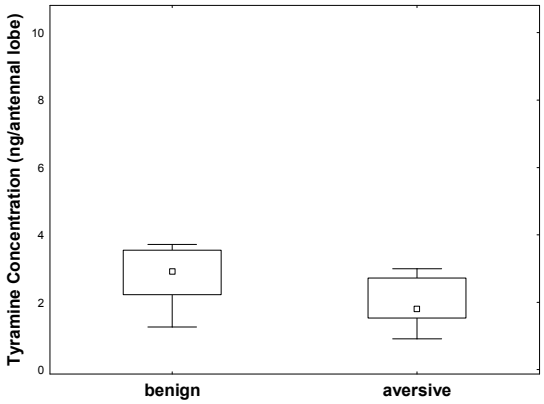

**B. Serotonin**




**C. Dopamine**



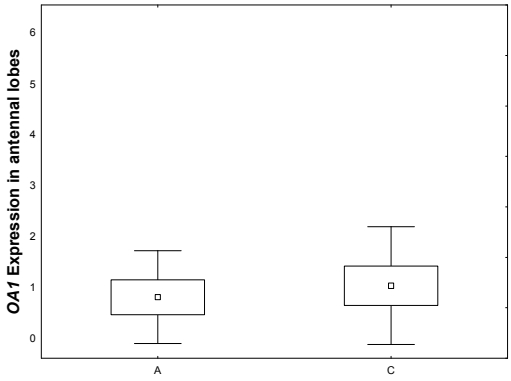

**D. Tyramine**



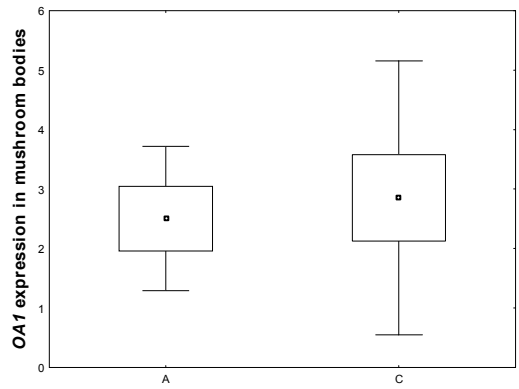

*Box-and-whiskers plots showing antennal lobe biogenic amine concentrations in aversive foraging conditions with and without scent dissolved in food. Boxes represent 25-75 percentiles, bars show the non-outlier range, points show outliers, squares represent medians. When food was unscented, aversive foraging conditions influenced antennal lobe biogenic amine titers as follows:* ***A.*** *octopamine (Mann Whitney U Test, Z adjusted=2.172180, p=0.029843, U=8, N=7,7).* ***B.*** *serotonin (Mann Whitney U Test, Z adjusted=2.04441, p=0.040914, U=7, N=7,7).* ***C.*** *dopamine (Mann Whitney U Test, z-adjusted = 1.79, U=10, p=0.074, N=7,7).* ***D****. tyramine (Mann Whitney U Test, z-adjusted=1.53, U=12, p=0.125, N=7,7)*

**Fig. 4.6 Effect of scented food on octopamine receptor *AmOA1* expression in benign foraging conditions in 3 neuropiles**

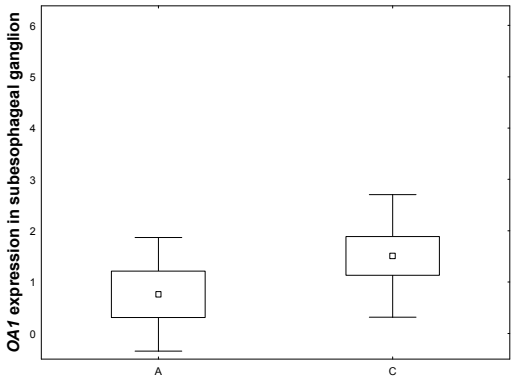
**A.**




**B.**




**C.**




*Box whiskers plot showing subesophageal ganglion expression levels of octopamine receptor AmOA1. The rectangles show mean+/-standard error, whiskers show mean+/-standard deviation, the square indicates the mean. AmOA1 expression level does not differ depending on whether sucrose is scented (group c is scented, group a is unscented).*

## Discussion

### *Overview of findings*

This study found lasting effects of different foraging experiences on honey bee' sucrose responsiveness and biogenic amine titers in antennal lobes, confirming our prediction. Foraging for highly concentrated sucrose paired with floral scent reduced sucrose responsiveness relative to foraging for unscented sucrose under benign foraging conditions but not in aversive foraging conditions. Foraging for sucrose paired with floral scent vs unscented sucrose in aversive foraging conditions elevated serotonin in antennal lobes. Aversive foraging conditions lead to reduced octopamine and serotonin concentrations in antennal lobe relative to benign conditions, but only when food is unscented. Our results suggest that dissimilar foraging experiences lead to inter-individual differences in sucrose responsiveness and biogenic amine signaling.

### *Experience-induced changes in biogenic amine levels, and the potential behavioral consequences*

Association with an olfactory cue changes the effect of aversive experience. We find that when food is scented under aversive foraging conditions, antennal lobe serotonin is elevated. Antagonists of serotonin elevate electric shock responsiveness in honey bees, suggesting that elevated serotonin could lead to reduced responsiveness to aversive stimuli (Tedjakumula et al. 2013). A depression in aversive responsiveness following the pairing of electric shock with a cue is consistent with the Giurfa group's work showing that successful aversive learning leads to a reduction in shock responsiveness to intermediate voltages (Tedjakumula et

al., unpublished). The ecological equivalent of scented sucrose paired with electric shock is a hazard at a recognizable food source. Examples of such situations include predators that specializein hunting at specific types of plants (Bromley 1984; Suttle 2003), food patches located close to predator nests (Monceau and Thiery 2016), or types of plants producing bitter toxins (Stevenson et al. 2016). In such cases there is an adaptive benefit to avoiding the known, most risky food sites while becoming more accepting of less aversive stimuli at other food sites. Elevated serotonin levels perhaps provide a mechanism of reducing responsiveness to intermediate aversive stimuli.

Aversive conditions are not always predicted by a recognizable scent, as they are not always specific to a patch of flowers or type of flower. Predation can provide generalized, unpredictable danger; in some environments, overall predation pressure can vary between years (Monceau and Thiery 2016). Conspecific competition at plant sites is also aversive to honey bees (Rogers et al. 2013); it seems likely that crowding, such as in commercial beekeeping operations, could result in high overall con-specific competition at food sites. Within our paradigm, electric shock at feeders with no associated odor may give rise to a similar effect as unpredictable ecological danger. In these unscented and thus unpredictable conditions, we observe reduced antennal lobe serotonin and octopamine concentrations in bees that experienced aversive foraging conditions relative to bees that experienced benign conditions. Pharmacological studies have demonstrated several potential behavioral consequences to changes in octopamine and serotonin levels in honey bees. Foragers recruit one another to foraging sites by communicating location and value through a symbolic dance language (Seeley 1995). The reporting of foraging site value has been shown to

selectively increase with oral and topical treatment with octopamine (Barron et al. 2006). Reporting of reduced resource value after experiencing aversive foraging conditions would adaptively result in greater colony-level recruitment to benign sites. Reduced antennal lobe octopamine could contribute to changed dance behavior following aversive experience. Future studies should examine octopamine levels and dance behavior of foragers that have visited aversive vs. benign feeders. Octopamine has also been implicated in modulation of responses to aversive situations. Ingestion of octopamine causes bees to be less likely to learn to avoid an area where they receive mild electric, spending more time in the aversive area (Agarwal et al. 2011). It follows that reduced octopamine concentration in bees with aversive foraging experience could have the adaptive effect of accelerated learning to avoid aversive sites. Reduced serotonin concentrations after experiencing aversive condition could also change bees' subsequent behavior, by elevating aversive responsiveness (Tedjakumula et al. 2013).

The above described pharmacological studies manipulated octopamine and serotonin concentrations systemically, impacting levels not only in antennal lobes but in other brain regions as well. It is unclear whether altered levels specifically in antennal lobes would have the same effect. Moreover, in our study only antennal lobe concentrations were measured; it is possible that biogenic amine levels in other brain regions were affected in similar or opposite ways. Therefore, our biogenic amine data can only provide hypotheses for the effect of foraging experience on behavior. Our results are consistent with the hypotheses that unpredictable punishment leads to increased avoidance and responsiveness to aversive stimuli, or more "careful" bees, while predictable scent-paired punishment reduces aversive responsiveness to stimuli

of intermediate valence. We hope for future studies to test whether experiencing aversive foraging conditions can change bees' reporting of food site value through dance, avoidance of subsequently encountered aversive sites, and sting extension responsiveness to aversive stimuli.

*The role of associative learning in mediating the effects of foraging experience*

The effects of scent on the interpretation of aversive stimuli may be a by-product of the process of associative learning. Honey bees can form long-term appetitive and aversive memories (Bos et al. 2014), and although our experimental design did not result in successful memory retrieval when tested under restrained laboratory conditions, this does not necessary indicate that the bees did not learn or form memories. The foraging paradigm was in a free-moving and group-housed context, while retrieval tests were performed in a socially isolated, physically restrained, and more sensory sterile laboratory environment. It is possible that the discrepancy between the learning and retrieval contexts prevented demonstration of memory retrieval. Honey bees conditioned to an olfactory stimulus in a free-walking Y-maze assay do not respond to the odor when restrained in the typical laboratory assay 2 hours later, although they are able to transfer the memory from restrained conditioning to the Y-maze after 23 hours (Sanchez et al. 2015). Previous work has shown that bees tested in restrained laboratory testing conditions can typically retrieve memories formed during outdoor foraging (Gerber et al. 1996) as well as while group-housed in a cage (Farina 2005) within 24 hours of learning, but later retrieval ability has not been explored. Our laboratory assays were performed more than 72 hours after scent was removed; the longer time-frame between acquisition and

retrieval may cause greater sensitivity to changes in context. Another possibility is that the five days spent in a highly unnatural cage environment affected bees in such a way that they were not able to perform well after the additional stress of being cold anesthetized and transferred into a restrained laboratory protocol. We therefore cannot rule against the possibility that the process in which sucrose responsiveness is reduced is related to memory formation. However, bees fed with scented sucrose via trophallaxis can also learn the association and retrieve memories 24 hours afterwards (Farina et al. 2005). Therefore, learning alone does not explain why the internal control bees in our paradigm, fed through trophallaxis, did not exhibit a similar reduction in sucrose responsiveness as foraging bees.

*Non-global impact of foraging conditions on sucrose responsiveness*

The specificity of the effect of scented sucrose on the sucrose responsiveness of bees with access to feeders has ecological as well as mechanistic significance. It seems that either the behavioral context of foraging, or the physiological predisposition of a worker bee that has transitioned to foraging behavior, facilitates experience-modulated sucrose responsiveness. This suggests that collecting scented nectar can have long-term effects on the sucrose responsiveness of foragers that collect it but not on that of the pre-foraging bees inside the colony that receive it. Long-term reductions to the sucrose responsiveness of foragers with experience collecting high quality scented sucrose in non-aversive conditions could adaptively maintain selectivity for food sources, promoting returns to the same source so long as quality persists rather than settling for sources of lesser quality. In contrast, the foraging landscape is likely to have changed by the time the pre-foragers begin to

make their own foraging decisions so they have no selective advantage in long-term retention of modulated sucrose responsiveness. However, pre-foragers are still primed to experience at least short-term modulation, as they increase their sucrose responsiveness when assayed 24 hrs after consuming scented sucrose in both cages and outdoor colonies (Ramirez et al. 2010). We did not observe a sustained increase in responsiveness in our tests at 72 hours, but the diverging effects of scented sucrose on foraging vs non-foraging bees provide an intriguing direction for future studies.

*Mechanisms of reduced sucrose responsiveness*

We speculate that there are two, potentially interacting, neural mechanisms that could give rise to foragers' long-term change in sucrose responsiveness. The change in responsiveness could occur at the level of peripheral receptors. It has been shown in the cabbage moth *Mamestra brassicae* that biogenic amines modulate sensitivity of antennal olfactory neurons to components of sex pheromones. Octopamine increased likelihood of firing while serotonin inhibits firing (Grosmaitre et al. 2001). To determine whether scented sucrose induces peripheral sensory changes, future studies could quantify biogenic amines in antennae and perform electroantennograms on taste sensilla to compare firing rates. On a more cognitive level, it is possible that experiencing the addition of scent to sucrose directs attention away from incoming sensory information about sucrose quality to focus on incoming olfactory information about scents potentially predicting sucrose reward. Attention excites neurons responsive to attended features and inhibits neurons responsive to unattended features (Fritz et al., 2003, 2007, 2008; Jääskeläinen et al., 2007), and attention and prediction are dependent of each other (Hsu et al. 2014). In the honey

bee, such attentional modulation could occur in integrative structures such as the mushroom bodies (Heisenberg 2003). Electrophysiological work could clarify whether neuronal activity in mushroom bodies in response to sucrose is modulated by previous association with scent.

*Concluding remarks*

In summary, this study demonstrates that foraging experience can have relatively long term influence on honey bees. The pairing of a floral scent with sucrose induces a reduction in sucrose responsiveness of bees with feeder access, suggesting that foragers adaptively become more selective for quality food once they have experienced it in non-aversive conditions. In aversive conditions, serotonin concentrations in antennal lobes are elevated when food is scented, which could adaptively contribute to greater acceptance of sites of intermediate aversive salience after experiencing danger at predictable food sites. When food is not scented, octopamine and serotonin concentrations in antennal lobes are reduced, potentially reducing the reported value of food sites and increasing overall aversive responsiveness when environmental threats are not localized to a specific predictable food source. We hope our findings spur future studies exploring the behavioral consequences of experience-induced changes in biogenic amine levels.

# CHAPTER 5

# SURGERY IN OLD HONEY BEES REDUCES APPETITIVE RESPONSIVENESS TO UNKNOWN ODORS WITHOUT IMPAIRING OLFACTORY DISCRIMINATION

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Contribution: I wrote the introduction, methods, and discussion of the following manuscript, incorporating valuable feedback from Dr. Daniel Munch and Dr. Gro Amdam. Writing these sections required integrating and interpreting the data acquired by my collaborators in Norway, and reviewing the literature to place the findings in context.

## Introduction

Non-brain surgery can lead to impairments in neural functioning, with age as a main risk factor (Krenk et al., 2010, Monk et al., 2008). Clinical studies in humans have shown that postoperative cognitive dysfunction (POCD) can manifest in deficits in attention, working memory, long term memory, and cognitive flexibility (Hovens et al., 2010). Most patients recover fully within a month, but some continue to exhibit cognitive deficits (Rasmussen, 2006). Non-human studies with rats and mice show that they also exhibit impairments in learning and memory due to surgery (Hovens 2015, Fan et al. 2016) and aged rodent models of POCD have revealed potentially translateable therapies that can be used during or after surgery to prevent effects on cognition (Feng et al. 2017, Guo and Hu 2017). While other animal models for learning and memory have been established as efficient tools in biorgerontology, it is

not clear if they can be used to model POCD. However, alternative models may allow comparative approaches to explore generic physiological principles that govern POCD-like syndromes and to reveal effects of surgery that are less discernable in vertebrate behavioral paradigms.

Resilience to aging is correlated with cognition; several potentially interacting mechanisms have been proposed to explain this connection. A mechanism coined "cognitive reserve" is based on the idea that the process of learning can re-route synapse efficacy to compensate for areas with dysfunctions (Stern 2002). This could explain why exposing the brain to challenging mental activities may be protective against aging as well as against POCD (Kotekar et al. 2014). Cognitive reserve may contribute to the observation that patients with a level of education higher than high school have a lower incidence of POCD relative to those with lower educational levels (Kotekar et al. 2014, Tsai et al. 2010). Training in cognitive tasks during adulthood has also been shown to enhance cognitive task performance in aged male and female rats (Talboom et al. 2014). Another, more controversial, hypothesis is that innate intelligence is a predictor of resilience to aging and trauma. In humans, higher scores on intelligence tests early in childhood is associated with a reduced risk of mortality in adulthood, an effect not explained by early life socioeconomic status or within-family factors (Iveson et al. 2017). Similarly, honey bees' (*Apis mellifera*) performance in a pavlovian conditioning task is positively correlated with survival duration in hyperoxia, a measure of metabolic stress resistance classically related to overall lifespan (Amdam et al. 2010).

The honey bee system provides correlates of the cognitive senescence observed in vertebrates as well as ways to experimentally separate the easily

confounded variables of changed social context and chronological age. Honey bees senesce both cognitively and physiologically, but the process is not a linear function of chronological age (Ruepell et al. 2009). Unlike in humans (Burke and Barnes 2006) and flies (Grotewiel et al. 2005), in honey bees both functional and cellular senescence correlate with foraging role and time spent foraging (Seehuus et al. 2006, Munch et al. 2013, Behrends et al. 2007). Workers shift behavioral roles throughout their lives, beginning with tasks inside the nest and later moving to forage for pollen and nectar (Menzel et al. 2006). Functional cognitive senescence in honey bees is defined by reduced olfactory acquisition, a task in which bees learn to respond to odors paired with sucrose via the proboscis extension response (Behrends et al. 2006). Cellular senescence in the brain has been confirmed by lipofuscin accumulation and protein oxidation damage (Seehuus et al. 2006, Munch et al. 2013). In the honey bee aging model, senescence can be reversed or accelerated by manipulating the social structure of a colony, causing some individuals to change social roles and aging dynamics (reviewed by Munch et al. 2013). To explore the connection between learning ability and resilience to surgical trauma in aged individuals, this study capitalizes on the extensive information provided by the honey bee discrimination learning paradigm (Giurfa 2007). In this assay, bees are trained to associate one odor with a rewarding sucrose solution and another odor with an aversive salt solution. Bees' performance reveals their initial, unconditioned response to olfactory stimuli, their speed of acquisition, and their ability to retrieve long term memory when presented with the associated cue (Smith and Burden 2014). We predicted that learning performance would be correlated with resilience, and would therefore be inversely correlated with post-surgery mortality and cognitive impairment. We

collected bees that had foraged for more than 14 days, since that is the time point after which foragers exhibit slowed olfactory learning (Behrends et al. 2010). Bees were tested in the discrimination learning paradigm before undergoing a surgery in which the largest muscles in the thorax were extensively damaged. After surgery, we recorded mortality, retrieval of the previously learned memory, and performance in a new discrimination learning assay.

**Materials and methods**

*Honey Bee Collection*

The experiments were conducted during the summer 2011 at the Norwegian University of Life Sciences (UMB) in Ås, Norway. The honeybees used in this study were at the facilities of the UMB. To control for hive specific effects, individuals from two different colonies were mixed together.

Aged individuals that had foraged for at least 14 days were acquired by catching foragers of random age at the entrance of the hive, marking each with a felt-tip on the dorsal thorax, and releasing to be collected after another 14 days of foraging.

The day prior to the first learning test, marked honeybees were collected at the entrance of the hives. They were placed overnight in wooden boxes in high humidity and 30°C, with unlimited access to water and 30 % sucrose solution.

*Experimental Design*

The experiment began 1 day after collection (see figure 1 for overview). To avoid added variability in learning performance due to satiation state, individuals were food deprived for 4 hours. Afterwards, they were immobilized on ice and individually strapped into small plastic tubes in which they were only able to move their mouthpart and antennas. To reduce mortality due to hunger, the bees were force-fed with 1μL of 30% sucrose solution. The antennae were not exposed to sucrose to prevent habituation effects.

The bees were then trained in a differential learning paradigm. Before initiating the training procedure, the gustatory response score (GRS) was measured by monitoring the proboscis extension response to 20% sucrose solution. This was done by gently touching the antenna with 2μL of 20% sucrose solution, not followed by feeding. GRS measures the bee's subjective value of the sucrose solution. Since only bees that respond to sucrose can be rewarded by it, none responders were not used in this initial assay.

*Differential Olfactory Conditioning*

In the differential learning paradigm, bees were trained to associate odor A (CS+) with the reward sucrose solution and odor B (CS-) with the punishment 3M NaCl solution. The differential conditioning used two different odors, 2-Octanone and Hexanal. It was important that the bees were able to differentiate between the odors and that the odors had no similarities with floral species currently foraged. The four odors were selected based on Guerrieri et. al. 2005 and a pilot study determining the levels of spontaneous proboscis extension to each odor. The two odors were counter

balanced over days to prevent any odor-specific effect (See table 1 for overview). Each individual underwent 12 trials, six each with the rewarded and the unrewarded odors (A and B). The odor sequence was pseudo-randomized and equivalent for each animal in the test (ABBABAABABBA). A conditioning trial began with placing the bee in front of an exhaust fan (10 cm diameter) for 10 seconds. The bee was then acclimatized to the airflow before being exposed to CS and US. The odor was manually delivered with a 10 mL syringe containing 2 μL of pure odorant on a paper. CS was presented for 5 seconds, with US applied in addition after 3 seconds (see figure 2). The US was given by touching the antenna and mouthparts with 30% sucrose solution or the 3M NaCl solution. Bees that showed proboscis extension was allowed to feed (approximately 1μL). There was at least a 10 minute interval between conditioning trials (24). After differential conditioning, bees were placed in separate cages with unlimited access to 30 % sucrose solution and kept in an incubator overnight.

*Surgical Protocol*

On day two, bees were assigned to be damaged with surgical insult or to act as un-surgerized controls. The bees were divided according to the learning score of the rewarded odor in learning test one. Individuals with a similar learning score were divided between the two groups, ensuring an equal distribution of good and poor learners. Surgery consisted of immobilization on ice and piercing the dorsal thorax followed by an injection of 3μL of Millie-Q water into the flight muscle, without disturbing vital internal organs.

*Post-Surgery Assays*

On the third day, all individuals were again food deprived, for 4 hours, immobilized on ice, strapped in individual plastic tubes and checked for GRS. The memory retrieval test of odor A+ and B- consisted of presenting the odor without the US. Animals that responded, i.e. extended proboscis, got a score 1 and non-responders a score of 0. Retrieval tests were followed by a new differential learning test with two new odors, 2-Nonanol and 1-Hexanol.

**Table 5.1: Overview of odors used for each group in learning test 1 and 2**

| **Group** | **Learning test 1** | | **Learning test 2** | |
|---|---|---|---|---|
| | ***Odor A (CS+)*** | ***Odor B (CS-)*** | ***Odor C (CS+)*** | ***Odor D (CS-)*** |
| **1** | 2-Octanone | Hexanal | 2-Nonanol | 1-Hexanol |
| **2** | Hexanal | 2-Octanone | 1-Hexanol | 2-Nonanol |
| **3** | 2-Octanone | Hexanal | 2-Nonanol | 1-Hexanol |
| **4** | Hexanal | 2-Octanone | 1-Hexanol | 2-Nonanol |
| **5** | 2-Octanone | Hexanal | 2-Nonanol | 1-Hexanol |
| **6** | Hexanal | 2-Octanone | 1-Hexanol | 2-Nonanol |
| **7** | 2-Octanone | Hexanal | 2-Nonanol | 1-Hexanol |

**Table 5.1.** The odors used for discriminative learning were chosen to maximize bees' ability to differentiate them, and minimize similarity to floral odors that bees might have previously experienced

**Figure 5.1**: Overview of the experimental set-up in the laboratory.

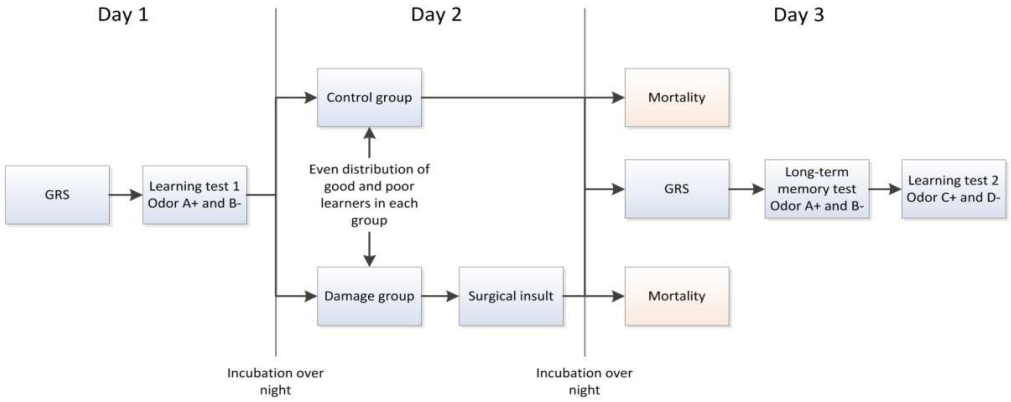
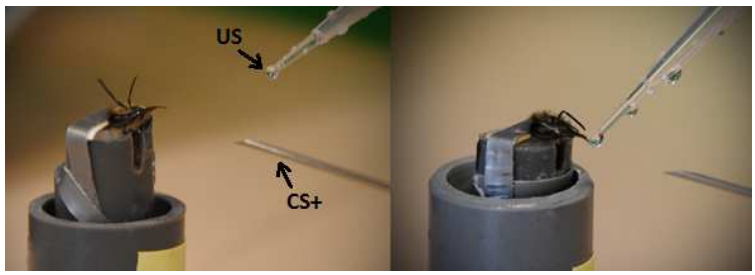



**Figure 5.2.** Conditioning trial: a honeybee that has learned to associate the odor with sucrose reward.




*Statistics*

We used the Pearson Chi-square test to compare groups, where individuals received dichotomous scores; these were between group comparisons of mortality, odor discrimination, memory retrieval and spontaneous responses. To analyze if test groups differed in their learning performance (learning scores) we applied the non-parametric Mann-Whitney U test (MWU) since the distribution of the learning score data was highly skewed. Correlations between two interval variables (learning scores, discrimination index) were analyzed by calculating the Pearson product-moment

correlation statistics. To test for correlations between interval variables (learning scores) and dichotomous variables (mortality, spontaneous response, memory retrieval) we used the point-biserial test. Data were processed and graphed in Excel, statistical analyses were performed with xx (point-biserial correlation) and Statistica v12.7 (Dell Inc, Round Rock, TX, USA) (all remaining statistical tests).

**Results**

*Mortality effects of the surgical protocol*

To establish a suitable protocol, we devised a surgical procedure that inflicts extensive damage to the honey bee's largest muscles in the thorax. While the brain was not harmed directly, the protocol should induce functional detriment that is can be detected as increased die–off in surgically treated, old individuals. In accord, Fig. 1 shows that surgery was associated with a moderate, but significant increase in mortality ($\chi^2_{160/143}$=4.71, *df*=1, $p$<0.05; Pearson Chi-square) – as compared to control individuals in which the thorax was left intact. Except for surgery, control and test group were both exposed to potential stressful treatments. These included restraining within holders, forced feeding and repeated testing. The detrimental effects of such stressors are likely reflected by a reduced survival also in the control group, which is in agreement with previous reports that tested bees in similarly long-lasting learning and memory assays (e.g., (Menzel, Manz et al. 2001, Münch, Baker et al. 2010)).

**Fig. 5.3.** Effect of surgery on mortality

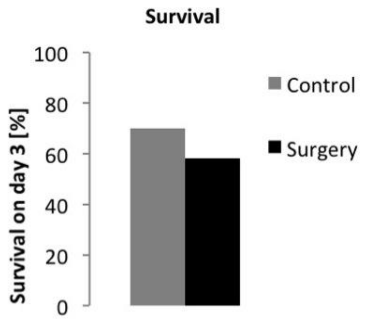



**Fig. 5.3. Survival was reduced by the surgical protocol.** Extensive injury inflicted to the thorax led to increased mortality in the surgically treated test group. Substantial mortality in the control group suggests that other stressors like restraining, force-feeding and repeated testing contribute to overall mortality in both groups.

*Effects of surgery on behavioral performances*

To assess pre- and post-surgery states of learning capacity, we tested individuals with a more complex, differential learning assay, where one of the two odors is rewarded and the other odor is punished. Fig. 2A shows the results of the differential learning assays prior prior to surgery (day 1). As expected, bees acquired a response that was specific for the rewarded odor A+, in that response to A+ increased and the response to the punished odor B- decreased (Fig. 2A). Our pre-surgery tests confirm that the two groups designated to either surgery or control displayed similar learning scores (LS) for the rewarded odor A+ as well as for the punished odor B- ($Z_{144/143}$=0.24, *df*=1, *p*= 0,8139 for A+; $Z_{144/143}$=0.67, *df*=1, *p*= 0,5006 for B-; MWU; Fig. 2B). Likewise, learning performance did not differ between the two colony replicates ($Z_{187/100}$=-1.03, *df*=1, *p*=0,3044; MWU). In contrast, testing for possible differences between actual odors used as A+ we found a significant difference in the

LS for the two odors, i.e. 2-Octanone and Hexanal ($Z_{153/134}$=-2.27, *df*=1, *p*<0,05; MWU). However, median learning scores for the two odors used as A+ were both high and differed only by 1 LS point with LS=5 and 4, respectively (data not shown). In addition both odors were balanced, in that 153 individuals were tested with the first and 134 individuals with the second odor.

On day 3, post-surgery, we first tested for effects of surgery on memory retrieval of the rewarded odor A+. However, we found no effect of surgery on the response to the previously learned odor A+ ($\chi^2_{85/73}$=0.49, *df*=1, *p*=0.4821; Chi-square; Fig. 2C). Similarly, we did not detect surgery related effects on the response to the previously punished order B- (Fig. 2B; $\chi^2_{85/73}$=1.56, *df*=1, *p*=0.2120; MWU; Fig. 2C).

To further assess post-surgery effects on learning and memory, bees were tested again for differential learning with two two novel odors used as rewarded (C+), respectively punished CS (D-).In contrast to memory retrieval (Fig. 2B), the differential learning tests showed that learning scores for the rewarded C+ and the punished D- were significantly lower in the surgery group than in the control ($Z_{85/73}$=3.18, *df*=1, *p*<0.005 for C+; $Z_{85/73}$=2.07, *df*=1, *p*<0.05 for D-; MWU; Fig. 2C, D). However, while both groups displayed virtually similar responses in the last C+/reward pairing (6$^{th}$ trial in Fig. 2D), we detected significantly fewer individuals with a spontaneous response to the initially unknown C+ in the surgery group ($\chi^2_{85/73}$=5.52, *df*=1, *p*<0.05; Chi-square; compare 1$^{st}$ trial in Fig. 2D). This indicates a dominant effect of surgery on CS+ responsiveness (‘odor response readiness’), rather than a reduced capacity to form new memory. This is further corroborated by the fact that removing all individuals with a spontaneous response to C+ (compare 1$^{st}$ trial in Fig. 2D) from the learning score analyses, also eliminates detectable surgery effects

on learning scores that are shown in Fig. 2D,E ($Z_{52/56}$=-1.17, *df*=1, *p*=0.2415 for C). The effect on CS (odor responsiveness is in contrast to the responsiveness for the US (the sugar reward), which all tested bees had shown a response to, even at a concentration lower than used in the reward.

**Figure 5.4. Learning and memory tests to study potential effects of surgical treatment**

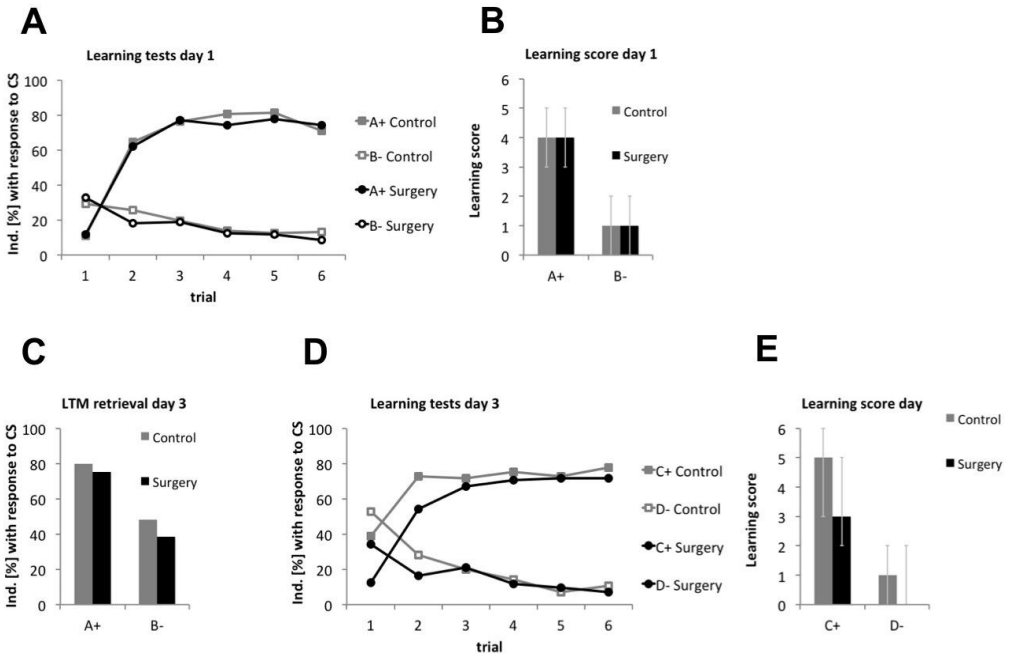



**Figure 5.4. Reduced olfactory learning scores after surgery are likely explained by a lower responsiveness rather than reflecting a reduced learning capacity.** Control and surgically treated test group were tested for learning capacity in differential learning assays, as well as for long term memory. **A** Learning curves for the rewarded odor A+ and the punished odor B- confirm similar learning performance for test and control before treatment. **B** Surgical treatment had no detectable effect on

memory retrieval as compared to the control on day 3. **C** Compared to the control, surgical treatment resulted in reduced learning scores for the rewarded odor (CS+; day 3 after surgery). However, controls showed a higher spontaneous response to the unknown CS+ in trial 1. This suggests that reduced learning scores after surgery maybe due to a lowered CS (odor) responsiveness, in contrast to a sensitive US (sugar) responsiveness displayed by all tested individuals (see Method section). The detailed statistics are given in the Results section.

*Testing links between performance before surgery and post-surgery outcome*

Next we separated groups based on their learning performance in the tests with the rewarded A+ on day 1, i.e. prior to surgery (compare Fig. 2A, B). This allowed testing if learning performance before surgery can be linked to survival and two performance measures after surgery. Briefly, individuals with a learning score from 4-6 to A+ were classed as good learners, those with learning scores from 0-3 were classed as poor performers. A learning score of 3 – i.e. the 25% percentile value for learning scores on day 1 (Fig. 2B) – was set as the separating cutoff value, to have only a deviant minority representing the poor performing group. We first tested if poor learning performance on day 1 is linked to poor survival outcome in the two treatment groups on day 3. While reduced survival was observed for poor learners of both treatment groups (Fig. 2A), those differences were not significant ($\chi^2_{62/98\ poor/good}$=2.43, *df*=1, *p*=0.1192 for control; $\chi^2_{47/96\ poor/good}$ =3.63, *df*=1, *p*=0.0568 for surgery treatment; Chi-square).

**Fig. 5.5 Effects of pre-surgery learning performance on different performance values after surgery.**

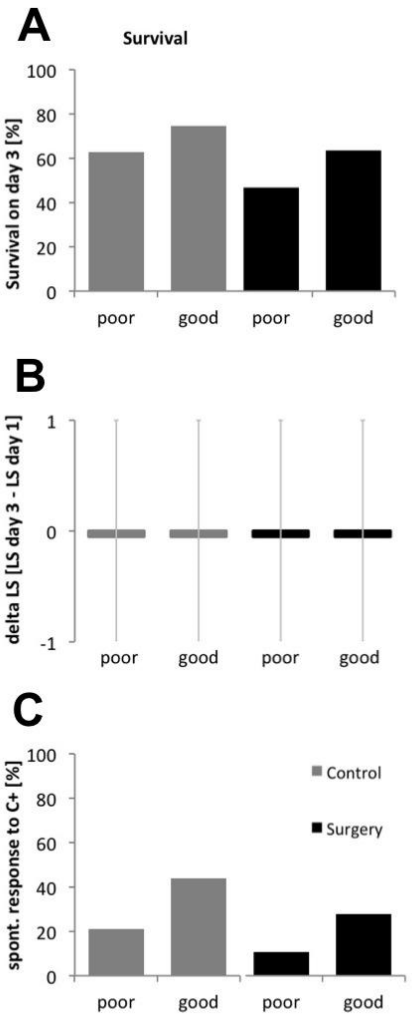



**Fig. 5.5. Lower learning capacity before surgery can be linked to a reduction in survival and behavioral measures after surgery.** The panels show how learning of the rewarded odor before treatment (learning scores for A+ on day 1) correlates with survival and behavioral measures after surgery (day 3). **A** Learning scores for the rewarded A+

before treatment correlate with survival after surgery. This association between learning performance and survival outcome was not detectable in the control. **B** A weak correlation between learning scores on day 1 (A+) and on day 3 (C+) was detected for the control but not for the surgically treated group. **C-D** show bar graphs with individual learning performances on day 1 depicted as poor (0-3) versus high (4-6) learning scores for A+. **C** In both, the control and surgically treated group a higher learning score for A+ on day 1 was associated with a higher spontaneous response to C+ on day 3 (compare trial 1 for C+ in Fig. 2 C). **D** A higher learning score for A+ on day 1 was associated with increased long-term memory retrieval for A+ after surgery. However this association between learning score and memory retrieval was not detectable in the control. **E** A higher learning score on day 1 was associated with increased discrimination between the rewarded A+ and the punished B-, but only in the surgically treated group. Detailed statistics are given in the Results section.

We then tested if pre-surgery reward learning performance can be used to predict changes in learning performance changes – for example a reduced learning capacity – after treatment (compare also Fig. 2A, B with Fig. 2D, E). To this end, each individual was assigned one of three categories: -1 for reduced, 0 for similar, and 1 for improved learning performance in tests with C+ and A+ on day 3 and 1, respectively. However, no difference in pre- to post surgery learning performance was found among the good and

poor performing individuals of both treatment groups (H=3,635730, *df*=3, *p*=0.3036; Kruskal-Wallis ; Fig. 3B).

Lastly we asked if pre-surgery learning performance correlates with post-treatment spontaneous response to the unknown C+ in learning trial 1 on day 3 (compare Fig. 2D). Again, we observed that good and poor learners were different, in that less individuals in the poor performer group showed spontaneous responses. Yet, those differences were not significant, neither in the control, nor the surgery group ($\chi^2_{poor/good}$=3.25, *df*=1, *p*=0.0713 for control; $\chi^2_{poor/good}$=2.34, *df*=1, *p*=0.1260 for surgery treatment; Chi-square).

**Discussion**

This study demonstrates that in old honey bees sensory responsiveness is reduced by surgical trauma, while performance on the tested cognitive tasks remains intact. Additionally, cognitive performance prior to surgery is not correlated with resilience to surgical trauma. We found that acquisition of discriminative olfactory learning was not affected by surgery and did not predict either survival or performance post-surgery. Retrieval of long term memories was also not impacted by surgery. Unconditioned appetitive responses to novel odors were reduced post-surgery.

The observed reduction in responsiveness to novel odors could arise post-surgery for several reasons. The proboscis extension response results from the integration of stimulus information that generates an appetitive response – a visible motor signal. Surgery in old individuals could alter one, or a combination, of these components. In humans as well as in model vertebrate species, age leads to diminished olfactory sensitivity (Mobley et al. 2014). Reduced olfactory sensitivity is associated with greater likelihood of mortality in humans, suggesting that loss of olfactory sensing might be a marker of deteriorating health (Ekstrom et al. 2017). If this is also the case in bees, it follows that olfactory sensitivity may be a more sensitive marker of ill health than discriminative learning performance. Changed olfactory sensitivity could mechanistically be due to the composition or excitability of antennal chemoreceptors, or a change in glomeruli in the antennal lobes receiving input from the olfactory receptor neurons. To test for increased olfactory sensitivity threshold, future studies could quantify behavioral

responses to different odor concentrations. To test whether surgery impacts antennal chemoreceptors, electroantennograms could be used to identify receptor signals in response to different compounds.

Response readiness, or the 'motivation to respond' to a potential reward could also contribute to the observed reduction in unconditioned olfactory responsiveness. In humans and vertebrate model systems, depression, anxiety, and reduced appetitive responsiveness have been implicated as consequences of some types of surgery (Gnoheim and O'Hara 2014; Popp et al. 2014). Bees that have undergone stressful shaking are more likely to predict punishment from an ambiguous stimulus (Bateson et al. 2011), suggesting that the stress of surgery could also cause a negative bias toward new odors and thus fewer appetitive responses that in control animals. Honey bees' responsiveness to unconditioned olfactory stimuli is enhanced by the injection of octopamine directly into the region of the thick ocellar neurons (Mercer and Menzel 1982), suggesting a possible neurochemical pathway by which surgery results in reduced octopamine concentration and thus alters one of the above described components of responsiveness. Studying octopaminergic signaling in the context of POCD, thus, may allow testing how central reward processing may contribute to the observed response decline after surgery.

Lastly, motor system dysfunction, i.e. impaired efferent motor circuits involved in the proboscis extension response, may have contributed to the observed effects. To address this, electromyograms could test motor neuron and muscle activity. One caveat is

that a honey bee's unconditioned response to novel odors may not be independent of cognition. Bees are able to generalize memories; a response to a novel stimulus could be due to generalizing it with one previously learned (Stach et al, 2004; Mota and Giurfa 2010). Therefore, the reduced response to unconditioned odors in surgerized bees could be a result of reduced generalization rather than reduced olfactory responsiveness. Although we did not find retrieval of previously learned memories to be impaired in surgerized bees, the process of generalization could require additional cognitive functions and be more sensitive to surgery.

Discrimination learning is a form of elemental learning, in which unique events are linked (Giurfa 2007). Although we did not observe an effect of surgery on this task, we do not know whether performance on other, especially non-elemental, cognitive tasks would be impacted. Moreover, we did not test the effect of surgery on ability to form mid-term or long-term memory; only retrieval of associations formed prior to surgery was tested. Studies in aged rodents suggest that postoperative cognitive impairment is limited to specific cognitive domains, especially spatial memory (Hovens et al. 2015). Future honey bee studies should explore the effect of trauma on spatial navigation abilities as well as performance on non-elemental learning tasks, such as negative patterning, categorization, or contextual learning (Giurfa 2003). In addition, it is possible that survival or performance after surgery is correlated with prior performance on a complex cognitive task, although it is not correlated with discriminative learning. Our

results do not conclusively argue against an effect of surgery on cognition, or against a connection between cognitive ability and resilience to surgery.

Our findings encourage studies on POCD which address not only cognitive dysfunctions but also assess sensory responsiveness. We show here that surgerized honey bees exhibit a reduction in unconditioned olfactory responsiveness relative to non-surgerized bees. Altered responsiveness, whether only to a specific modality such as olfaction or as a broader function of altered motivational drive, could influence both behavior and well-being, and is as important to understand as cognitive performance. For honey bee researchers in particular, our work indicates the importance of including similarly-surgerized control animals in any surgical manipulation, and demonstrates that the first trial in an olfactory acquisition paradigm provides distinct information about olfactory responsiveness, which can be modulated separately from acquisition ability. The unique system of aging in the honey bee, coupled with honey bees' spatial navigation (Cheeseman et al. 2014) and non-elemental learning abilities (Guirfa 2003), will make possible a deeper understanding of the vulnerability of the aging brain. We hope our work spurs future studies to investigate the relationships between surgical trauma, sensory responsiveness, and cognition in a variety of model systems for aging.

# CHAPTER 6

# DISCUSSION

## MAJOR FINDINGS

Chapters 2 and 3 reveal novel routes for the social sharing of food-related information. Chapter 2 shows that trophallaxis, or the mouth-to-mouth sharing of food and information, is affected by aversive foraging conditions and not only food quality as previously known. Aversive foraging conditions elevate trophallaxis between foragers and bees that do not have feeder access. When food is scented, aversive foraging conditions upregulate the trophallaxis of non-foragers with the bees that do not have feeder access. Chapter 3 shows that even before trophallaxis occurs, bees can decide which conspecific to approach based on familiarity and crop contents. We suggest that the quality of sucrose concentration in a bee's crop can be discriminated through volatile chemical emissions, and demonstrate a Gas Chromatography/ Mass Spectrometry protocol that makes it possible to compare volatile chemical profiles of live bees.

Chapters 3 and 4 focus on modulation at the individual level rather than the social. In Chapter 3, bees provided similar concentrations of sucrose but under aversive vs non-aversive conditions exhibited long-term differences in antennal lobe octopamine and serotonin levels. In non-aversive conditions, sucrose responsiveness is down-regulated following foraging for scented sucrose. Chapter 4 shows that individuals' bodily health also plays a role in appetitive responsiveness to stimuli. A surgical

procedure performed on old bees leads to intact discriminative learning and retrieval ability, but reduced appetitive response to new odors.

## MAIN LIMITATIONS

The study of social animals can necessitate the displacement of subjects of interest from their natural social context, thus disrupting the feedback mechanisms normally regulating their behavior and physiology. Honey bees' social environment, including number of brood in the colony, health of the queen, and number and activity of nestmates of various castes, regulates many aspects of individuals' behavior (Pankiw et al. 1998; Alaux et al. 2009; Amdam and Omholt 2002). The artificial group dynamic created in the cages housing experimental bees in Chapters 2 and 4 is lacking in several of these regulatory social components, most markedly a queen. Queens' pheromonal bouquets have many regulatory roles; most pertinent for the questions of interest in Chapters 2 and 4, queen pheromones influence biogenic amine circuitry and reduce aversive learning ability in young bees (Vergoz et al. 2007). In addition, the impact of foraging conditions on bees that forage by walking inside cages may be different from the impact of these foraging conditions on bees that fly long distances to food sources, an energy intensive process that requires complex spatial navigation (Neukirch 1982; Menzel et al. 2005). It is possible that the reported effects of foraging conditions are either amplified or dampened by the proximity of food sources in our studies. Due to these limitations, our findings concerning the effects of foraging conditions are not

conclusive for honey bees living in their natural environment, but rather offer novel, intriguing hypotheses for future field studies to verify.

## IMPLICATIONS AND FUTURE DIRECTIONS

Aversive conditions are shown to change the food sharing behavior of foragers, and of non-foragers when food is scented. Chapter 2 discusses the possibility of a signal or cue triggered in foragers by aversively associated scents as they unload food collected in aversive conditions to receiving non-foragers. Future studies could isolate and observe the trophallaxis of individuals that have foraged in benign vs aversive conditions, to identify the mechanism by which aversive conditions change change the food sharing behavior of receiving non-foragers. More broadly, the results suggests that social food sharing behavior is modulated by negative feedback due to aversive conditions and not only by food quality. See Chapter 2 for discussion of the potential adaptive value of changed food sharing patterns in aversive foraging conditions.

Honey bees are shown to be capable of differentiating between individuals that have consumed high and low qualities of sucrose. This may be mediated by the chemical volatiles emitted. Future work is necessary to compare the volatile profiles of bees that have crops full of different concentrations of sucrose, and to demonstrate causality by using hexane to extract the volatiles and determine their effect on preference to approach. See Chapter 3 for more extensive discussion of future experiments to resolve this

potential communicatory pathway for orienting toward specific individuals during social food sharing events.

Experiencing the same concentration of sucrose in aversive vs non-aversive conditions leads to long-term differences in honey bees' antennal lobe octopamine and serotonin levels. In non-aversive conditions, sucrose responsiveness is down-regulated following foraging for scented sucrose, suggesting that experience tunes honey bees' sucrose responsiveness to minimize settling for low sucrose nectar once they have experienced floral stimuli associated with high quality and non-aversive alternatives. It would be interesting to explore this phenomena in other species, including vertebrates and invertebrates that must forage for food in a variety of conditions, to ask whether there is a general phenomenon in which aversive foraging change how food response thresholds are modulated by food quality. See Chapter 4 for discussion of how and why aversive conditions mediate honey bees' neurochemistry and sucrose responsiveness.

Physical health and prior experience are demonstrated to alter appetitive responsiveness to stimuli including sucrose and novel scents. This raises the question of whether modulation occurs at the level of peripheral sensitivity, central integration of sensory information, or a combination of the two. Future studies can use electroantennograms to test the response of taste hairs and olfactory receptor neurons to unconditioned stimuli (Haupt 2004; Wright and Smith 2004), and whole cell in vivo recordings to determine changes in representation in the integrative central structure of the mushroom body (Turner et al 2008). The reduction in honey bees' appetitive

responsiveness to novel olfactory stimuli after surgical injury suggests that studies on Post-Operative Cognitive Dysfunction in humans and vertebrate model organisms should incorporate assays of sensory responsiveness as well as cognitive tests. See Chapters 4 and 5 for a more detailed discussion of suggestions for future work to resolve the neural mechanisms underlying the modulation of appetitive responsiveness.

## CONTRIBUTION TO THE FIELD OF ANIMAL BEHAVIOR

The perspective on inter-individual differences between individual conspecifics has historically revolved around genetics, early-life conditions, and learning. This thesis demonstrates ways in which the unconditioned behaviors of adult individuals are lastingly modified by experience. My work highlights the plasticity of a social organism's interactions with the environment and with group-mates, and suggests that such interactions are adaptively shaped by environmental and internal conditions. I hope that my results encourage future studies to explore the mechanisms and consequences of sustained, experience-induced changes in behavioral responses to unconditioned stimuli such as food.

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