Genomic Basis of Honey Bee Foraging Behavior

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Summary

Honey bee workers, like those of most other social insects, change their behavior with age. Nevertheless, there are differences between individuals in an age cohort. The observed differences could be partly genetic, but also influenced by social factors such as interactions with other nest mates. Chemical signals play an important role in the communication of insect societies. Our data show that in honey bee colonies, the queen plays an important role by using her mandibular pheromone (QMP) to influence gene activity in workers (**Chapter 1**). The ability to perceive chemical signals with the antennae, and then process them in the brain, is responsible for variation in foraging strategies, as some workers follow the social signals of others (e.g., waggle dance), while others do not (**Chapter 2**). Finally, rewards may induce foragers to maintain a foraging strategy by influencing their motivation to engage in a particular behavior (**Chapter 3**).

We have explored the genomic basis of foraging behavior in honey bees. While we know that many aspects of the colony environment influence worker behavior, the presence of the queen plays an important role in regulating the complex colony dynamics. Therefore, in **Chapter 1**, we investigated how exposure to the queen mandibular pheromone affects gene expression in different life stages of adult workers and how this affects later foraging. We found that there is a sensitive phase in early adulthood for responding to these queen signals that alters gene expression profiles but not foraging activity in foragers.

The external environment plays an important role in regulating the behavior of honey bee workers. They may decide to forage at foraging sites advertised by their nestmates or seek out those they have stored in their memory. Previous studies have examined the various environmental factors that influence a forager to use either social or private information. The focus of **Chapter 2** is to elucidate the molecular mechanisms underlying different information use strategies among foragers. We analyzed five tissues from the central and peripheral nervous systems: the antennae and in the brain: the antennal lobes, the mushroom bodies, the central brain, and the subesophageal ganglion. Surprisingly, we found no differences in gene expression in brain tissues between the two forager groups. However, we discovered that gene activity in the antennae was linked to foraging information use strategies. Our results suggest that honey bees do not rely on higher processing of cues in the central nervous system

during foraging, but rather on differences in sensory perception to decide which foraging strategy to use.

In **Chapter 3**, we wanted to further investigate whether the observed differences in gene expression patterns between foragers using different foraging strategies could be influenced by reward reinforcement. Differences in reward perception influence foraging and division of labor in honey bees. For example, regulation of pollen and nectar foraging in honey bees depends on reward perception. In turn, reward perception affects the use of memory in foraging by honey bees. We wanted to determine whether gene expression in the brain and antennae changes when foragers receive a reward for using a particular foraging strategy. We found that foragers that were consistently rewarded for using social or private information had a large number of differentially expressed genes, while the information use strategy itself had little effect on gene expression. Our results suggest that rewards elicit a strong gene expression signature in the brain, which increases the likelihood that a forager will use this foraging strategy again in the future.

In summary, this dissertation expands our understanding of the role that colony dynamics, information use strategies, and reward perception, and the molecular and neurogenomic signatures underlying these processes, play in influencing honey bee foraging behavior. We show for the first time, a sensitive phase in adult worker behavioral development in which queen signals have a long-lasting effect on gene expression. We also show that differences in antennal sensory perception in foragers affect their information use strategies. Food rewards enhance the use of specific foraging strategies and alter gene expression in the antennae and brain. Taken together, our results reveal a high degree of plasticity in foraging behavior and its transcriptional basis. By using the honey bee as a model organism, which exhibits the highest complexity of sociality, we can deepen and extend evolutionary insights into social behavior.

General Introduction

Anissa C. Kennedy

“Somewhere, something incredible is waiting to be known.”

— Carl Sagan

Social life evolved repeatedly and sociality exists across the animal kingdom in various complexities. Identifying ecological and social factors that drive and underlie social organization has been a central component of behavioral ecology. One particularly interesting observation is the wide range of group sizes, both within and among species. Group size affects many aspects of social life such as individual stress levels (Pride 2005; Snaith et al. 2008; Takeda et al. 2003), disease susceptibility (Nunn & Heymann 2005; Rifkin et al. 2012), reproductive and developmental rates (Borries et al. 2008), and individual and group behavior (Koenig 2002; Carpenter 2007; Nunn et al. 2009). Living in groups involves both conflicts and cooperations, which are mediated by the costs and benefits associated with living socially. When the benefits of living socially exceed the costs and risks of social life, scientists predict that cooperation will be favored. Social behavior consists of a set of interactions among individuals of the same species (McGlynn 2010). Many social behaviors of animals are adaptive, meaning that being social ultimately increases the inclusive fitness of the individual.

Social Organization

The most complex form of sociality, called eusociality, it is defined by three characteristics: 1) cooperative brood care, 2) overlapping generations, and 3) reproductive division of labor (Wilson 1971). Among these, the division of labor between individuals of different castes and ages is a central focus of current research as it relates to how individuals interact and respond to their environment both within and outside the colony (Smith et al. 2008; Beshers & Fewell 2001; Gordon 2016; Robinson 1992). The division of labor in social organization has been compared to multicellular organisms where there are reproductive germ cells and sterile somatic cells corresponding to the reproductive division of labor where there is a single, or few, reproductive individuals and the rest forgo reproduction to perform all other tasks (Bourke 2011; Smith & Szathmáry 1995). How colony dynamics are regulated and divided provides an opportunity to understand behavioral ecology and evolution on an individual level and how certain traits are selected for the benefit of the colony.

In social insects, division of labor means that individuals are specialized in particular tasks such as brood care, foraging, or reproduction (Robinson 1992). These tasks can be divided among individuals of different castes (females vs. males or queens vs. workers), called caste polyethism, and ages (within the same tasks: young vs. old), called age polyethism (Robinson 1992; Tripet & Nonacs 2004). Due to the specialization that exists within these societies, it is difficult to make conclusions regarding comparative gene expression analysis across the population. For example, comparisons focused on

understanding gene expression profiles between behaviroal tasks (*i.e.* reproductive vs. non-reproductive or nurse vs. forager) are confounded by differences in sex, size, age, and colony. Therefore, research focuses on reducing these factors by aiming comparative analysis within a specific behavioral task controlling for sex, age, size, and colony. By focusing comparisons within behavioral groups, researchers can help bridge gaps in knowledge of previous studies (*i.e.* previous comparisons between queens vs. workers can be improved by using reproductive vs. non-reproductive workers).

Honey bees as a sociobiological model

There are a number of research models used to study the links between genes and social behavior. This is due to the diversity of social behavior itself. There are many types of social behaviors, exhibited by species that differ dramatically in their level of sociality. There are solitary species that interact with conspecifics only when mating, and species that live in highly structured societies with complex social lives in which nearly all activities are influenced by interactions with conspecifics. Sociality, a term used to describe such all-encompassing social life, is highly derived and has evolved independently in many lineages of animals (Wilson 1975), providing diverse social systems for molecular analysis. Studying the most extreme manifestations of social behavior, exhibited by animals living in complex societies, provides experimental access to a process that is undoubtedly involved in all forms of social behavior, social regulation of gene expression. Studying diverse animal societies also allows for analysis, in molecular terms, of the roles of convergence and conservation in social evolution.

Honey bees (*Apis spp.)* serve as essential pollinators and drive the agricultural economy of many countries. Since 2006, honey bees have been on the decline in the U.S. (Johnson 2010) and there has been an increased interest in understanding their biology to promote better beekeeping practices. Although research using honey bees as model organism's dates back hundreds of years, recent studies aim to further explore honey bee behavior in order to gain evolutionary insights on both a fundamental and applied level. While there are ca. 8-10 species of honey bees and approximately 43 subspecies (Arias & Sheppard 2005; Raffiudin & Crozier 2007; Lo et al. 2010), this dissertation focuses on the western honey bee (*Apis mellifera*). Honey bees are the most studied social insect and colonies consist of a single reproductive queen, thousands of female workers, and few males (Winston, 1995). The females are separated into two castes: the queen and workers. The males are called drones, they contribute little to the colony aside from reproduction. Workers are functionally unable to mate and are facultatively sterile. Although workers typically do not activate their ovaries, if they do activate their

ovaries they can only produce haploid eggs that will develop into males. When the queen is present, workers will exhibit temporal polyethism, or division of labor, where they specialize in different tasks as they age (Seeley 1982; Robinson 1992). Younger workers perform the in-colony tasks while older workers perform the riskier outside tasks. Young workers (<7 days old) act as nurses, where they care for the developing brood by producing brood food from hypopharyngeal glands in their heads. Middle-aged workers (7-14 days old) perform other colony tasks, such as comb-building, food storing, or guarding. Older workers (>14 days old) act as foragers, where they leave the colony to collect nectar, pollen, water, or propolis (Seeley 1982; Robinson 1992).

Honey bee foragers are unique in their way of communication *via* the waggle dance, a striking example of social learning: learning that is influenced by other individuals, either by means of observation or interaction (for review, see Heyes, 1994). Returning foragers perform the dance to attract hive-mates and supply them with information about the precense, location, and odor of a food source (von Frisch 1967; Winston 1987; Seeley 1995). The dance follower's interest can be gauged by the number of waggle-runs followed (Fig. 1; Grüter & Ratnieks, 2011). Foragers can decide whether to follow and translate a dance and use the vector information (social information, recruitment) or to revisit a food source location that was memorized (private information, reactivation) during preceding foraging trips in the case of experienced foragers (Biesmeijer & Vries, 2001; Grüter & Ratnieks, 2011).

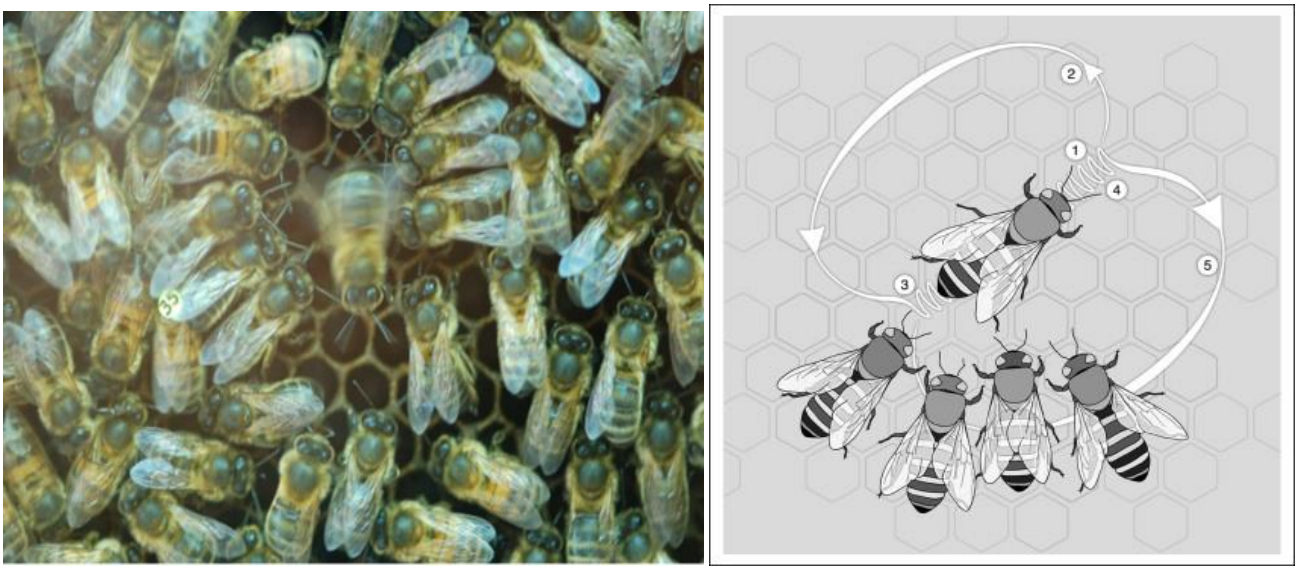

Figure 1: The waggle dance. *Apis mellifera* forager performing a waggle dance in the colony. The dancer strides forward while shaking her abdomen from side to side (waggle run, 1). Then she makes an abrupt turn to one side (2), circles back (3)

to restart the waggle run (4) and finally turns to the opposite direction (5). Bees following the waggle dance learn the distance to the food site from the duration of the waggle run. The direction relative to the sun is encoded in the angle of the waggle run (α) relative to gravity. The profitability of the food source is transmitted via the number of performed waggle runs. Additionally, followers receive chemical compounds like odor and hydrocarbons. *Figure and description modified from Grüter et al. (2009).*

Social versus. private information use

Social animals often exchange information about food, predators, or nest sites. Information can be obtained from direct communication but also from observing the behaviors of others, *i.e.* social cues (Seppänen et al. 2007; Schmidt et al. 2010). In this situation, an animal has to decide between the information gained from others through social interactions with the information it perceives directly from the environment. Animals usually only have partial, noisy information about the value of the options about mates, habitats, or food sources. Robust strategies for processing and integrating diverse sources of information are therefore central to decision-making, especially in uncertain environments. Individuals might benefit from a balance between acquiring new information through private exploration, using previously acquired private information, and using social information acquired by other individuals. Each strategy has its own costs and benefits. For example, existing private information incurs low costs and is usually reliable, but it can become outdated and it does not allow individuals to account for environmental changes. On the other hand, social information is cheaper to acquire than private information because of the energy costs and predation risks associated with exploration. However, social information is also less reliable, because animals make mistakes when interpreting social cues and may copy poorly informed individuals, leading to false informational cascades (Bicchieri & Dimant 2019; Rieucau & Giraldeau 2011; Grüter et al. 2011).

Insects and vertebrates use similar information use strategies to decide between ignoring social information and either explore the world individually or use private information obtained through prior experience. Individual honey bee foragers often need to decide between using private versus social information when choosing where to forage. Studies show that foragers usually prefer private information (Gruter et al. 2008), but increase the use of social information when private information becomes unrewarding (Grüter & Ratnieks 2011; Grüter et al. 2013; Wray et al. 2013) suggesting that honey bee foragers use a *copy-if-dissatisfied* social learning strategy (Gruter & Leadbeater 2014).

Types of foragers

Foragers are usually either pollen foragers or nectar foragers, and a minority collect both types of resources. Pollen is an important source of protein and lipids for many animals, including honey bees. Nectar is the main energy source that is a sugary fluid secreted by plants that workers modify into honey. It has been argued that having different individuals specialized for nectar or pollen collection is the most efficient strategy at the colony level (Ollerton & Waser 2006), due to the different spatio-temporal distributions of these major nutritional resources in the field and due to the need for specific behavioral skills to collect each of them. The division between these types of foragers is generally assumed to be true for honey bees where pollen and nectar foragers are considered as different behavioral castes (Free 1960; Robinson & Page 1989; Page et al. 2006; Page et al. 2012; Shapira et al. 2001). Pollen and nectar foragers differ in their brain neuropeptide profiles (Brockman et al. 2009), sucrose response threshold (Pankiw & Page 2000), ovary size (Amdam et al. 2006), levels of vitellogenin (a yolk precursor protein) (Nelson et al. 2007), and responses to social stimuli (pollen foragers rely more on dance communication) (Nürnberger et al. 2017). Together with evidence for genetic variation (Robinson & Page 1989; Page et al. 2012; Page et al. 1995), these observations suggest that pollen and nectar collection are evolved specializations within the foraging force of the colony (Page et al. 2012; Amdam et al. 2004; Siegel 2012).

Scouts are a separate class of foragers which are characterized by novelty seeking behaviors. They search for new food sources or nest locations. Food scouts, make up 5 to 25% of a colony's foraging force, search independently for new food sources and continue to do so even when plentiful sources have been found (von Frisch 1967; Seeley 1983). Nest scouts make up <5% of the population of a swarm, which is when a portion of the colony population leaves its natal nest to start a new colony. Nest scouts search independently for potential nesting cavities and collectively choose the best one (Seeley 2010).

Not all workers will transition to foraging at the same age. The onset of foraging depends on a variety of factors such as colony needs and worker physiology (Robinson 1992). If the colony is comprised of too many young workers, there will be an overrepresentation of nurses and younger workers will transition to foraging earlier (precocious foragers). On the other hand, if there are too few young workers to care for the developing brood, older foragers will revert back to nursing behavior (Robinson 1992). Although these two behavioral states have distinct physiological signatures, foragers

that have reverted back to nursing behavior can recover a similar physiological state as young nurses (Robinson 1998).

Regulation of foraging behavior: pheromones

Social insect colony conditions are controlled by an assortment of chemical signals called pheromones. The queen is an important source of pheromones and is responsible for releasing and maintaining the chemical balance contributing to the social order within the colony (Wyatt 2014; Naumann et al. 1991; Bortolotti et al. 2014; Provost et al. 2008). Pheromones are classified as either releaser or primer pheromones (Conte & Hefetz 2008; Billen & Morgen 2019; Abd El-Ghany 2020). Releaser pheromones cause a rapid but short-lived response. Primer pheromones are slower acting in influencing behavior via long-term physiologic effects. Primer effects are responsible for regulating colony organization, caste structure, and the division of labor (Slessor, Winston et al. 2005).

Behavioral transitions of workers are mediated by pheromones distributed throughout the colony. The queen's presence is vital to regulating the intricate colony dynamics among individual workers. In honey bees, the colony is alerted of the queen's presence and reproductive status through the dispersal of pheromones called queen mandibular pheromone (QMP). QMP is a blend of 5 compounds that influence worker attraction to the queen (retinue response), learning, reproduction, foraging ontogeny (Grozinger, 2015). Some effects of QMP are short-term (releaser) while others are long-lasting and take time to activate (primer). Exposure to QMP affects workers by changing brain expression profiles (Grozinger et al., 2003) and slows behavioral maturation from nursing to foraging, likely through its impacts on juvenile hormone (Pankiw et al. 1998). The effect of queen pheromones is particularly effective on young workers. QMP initiates a retinue response where young workers attend to the queen by feeding, licking, and grooming. Subsequently, QMP is spread throughout the colony by these workers who contacted the queen. This gives the colony a sense of being "queenright". The queen pheromones stimulate pollen foraging (Higo, Colley et al. 1992), elicit short-term inhibition of queen rearing (Winston et al., 1989, 1990, 1991; Pettis et al., 1995), calms queenless workers (Naumann et al., 1990), and represses ovary activation in workers while stimulating hypopharyngeal glands which nurses use to produce the protein rich food fed to developing larvae (Knecht and Kaatz 1990).

The life progression of workers from nurses to foragers is associated with physiologic changes induced by QMP, brood pheromones, and other worker pheromones (Grozinger et al. 2003; Pankiw et al. 1998; Traynor et al. 2014; Zayed & Robinson 2012). The transition from nurse to forager results in

alterations to their nutrient storage (Toth and Robinson 2005), hormonal expression (Guidugli, Nascimento et al. 2005), oxidative stress resistance (Seehuus, Norberg et al. 2006), mortality dynamics (Rueppell, Bachelier et al. 2007), and immune pathways (Amdam, Simões et al. 2004). Queenless colonies lack the influential effects of QMP and brood pheromones. Therefore, a queenless colony develops its own hive makeup independent of the queen. Identifying a queenless colony is straightforward based on the egg laying pattern, number of eggs in cells, position of egg(s) in cells, and drone brood in worker cells (Chapman, Oldroyd et al. 2009).

Colonies naturally experience periods of queenlessness. For example, during swarming when half of the colony leaves with the old queen to a new colony location. There is a period of time for the remainder of workers before the new queen emerges that they experience queenlessness. A lapse in QMP exposure of two days is enough to induce major gene expression changes in young workers and alter behavior (Grozinger et al. 2003). Still, little is known how short-term periods of queenlessness has consequences at the colony level.

Regulation of foraging behavior: reward perception

Foragers respond to changes in the quality of nectar resources. Increasing concentrations of sucrose in nectars result in increases in the numbers of foraging trips taken, the size of the load collected, and a decrease in the probability that a forager will abandon that resource (von Frisch 1967). Sugar concentration also affects the recruitment behavior of successful foragers. Higher concentrations of sucrose elicit higher probabilities that recruitment dances will be performed and dances are of longer durations (von Frisch 1978). Sweeter solutions also lead to faster and better associative learning (e.g. Scheiner et al. 1999). Therefore, foraging honey bees are capable of perceiving differences in sugar concentrations of nectar and adjust their foraging and recruitment behavior accordingly.

Aims of Thesis

The honey bee, *Apis mellifera,* is an ideal model organism to study the genomic basis of foraging behavior since we can train foragers to artificial food sources, a technique perfected by Karl von Frisch in the first half of the 20th century (von Frisch, 1967). The knowledge gained from the experiments in this thesis can aid in better understanding of the underlying molecular mechanisms that are associated with the various aspects of foraging behavior presented.

The colony environment plays a major role in regulating the transition between behaviors of

workers. Two main factors are brood and queen presence, both of which affect the transition between nursing and foraging behaviors. We decided to explore the long-term role of queen presence on gene expression and foraging activity during three crucial adult developmental stages: newly emerged, nurse age, and forager age. We aimed to understand how queen presence through queen pheromones regulates gene expression at different adult developmental stages and connect the associated gene expression profiles to changes in foraging activity measured as total foraging trips and average foraging duration. We present evidence for a sensitive period during which the adult worker is strongly affected by the exposure to queen pheromones. However, unexpectedly, the gene expression profile was not linked to changes in foraging activity. (**Chapter 1**).

Honey bee foragers have the choice between using socially provided information about food locations through the waggle dance or relying on previously acquired route memories (*i.e.* private information). Our goal was to establish whether a unique gene expression signature between these information use strategies exists by focusing on five brain tissues: antennae, antennal lobes, mushroom bodies, central brain, and subesophageal ganglion. While we did not find any gene expression differences in the brain tissues, we present a number of differentially expressed genes in the antennae (**Chapter 2**).

We know that experienced foragers often ignore the spatial dance information provided by the dancing bee and instead fly to food source locations they visited in the past. So, the waggle dance, in part, initiates foraging however, it is still unknown whether the decision to use or ignore waggle dance information depends on reward reinforcement. For example, if a forager locates a profitable food source utilizing a particular foraging strategy (social vs. private information) does this make the forager more likely to continue using the same strategy and does the reward reinforcement change gene expression in the brain? We further explored foraging strategies by examining how reward reinforcement (**Chapter 3**) influences genes expression patterns by comparing pre- and post-rewarded private and social information foragers. Some of the genes that are known to play a role in reward perception and novelty seeking in other animals have been shown to be upregulated in another class of foragers called scouts. Therefore, we compared scouts to pre- and post-rewarded SI and PI foragers to understand the role of the expression of genes that mediate reward perception as an important player in mediating the decision to use a particular type of foraging strategy.

CHAPTER 1

Early Life Exposure to Queen Mandibular Pheromone Mediates Persistent Transcriptional Changes in the Brain of Honey bee Foragers

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* Authors contributed equally to the study

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Abstract

How behavior in insect societies is regulated remains a fundamental question in sociobiology. In hymenopteran societies, the queen plays a crucial role in regulating group behavior by affecting individual behavior, physiology, and lifespan through worker gene expression. Honey bee (*Apis mellifera*) queens signal their presence via the queen mandibular pheromone (QMP). While QMP has been shown to influence the behavior of young workers, we know little about its long-term molecular impacts on workers and whether these pheromone effects depend on an early sensitive period in the life of a worker. Here we demonstrate that QMP treatment strongly impacts long-term forager gene expression in the mushroom bodies, antennal lobes, and antennae, but only if workers were treated early in life (1-2 days of age). Contrary to our expectation, foraging activity was not impacted by QMP treatment in the long-term, but genes important for division of labor, learning, chemosensory perception, and aging were differentially expressed in the antennae and brain tissues, suggesting that QMP influences diverse physiological and behavioral processes in workers. Overall, our study suggests a sensitive period early in the life of workers, where the queens' presence or absence has strong and potentially livelong effects on transcriptional activity in the central and peripheral nervous system.

Introduction

The ecological success of social insects depends, among other factors, on an effective division of labor and communication (Hölldobler & Wilson 2008; Duarte et al. 2011; Beshers & Fewell 2001; Gordon 2016). Ants, social bees, and social wasps show a reproductive division of labor between queens and workers, where queens are responsible for reproduction and all other colony tasks are performed by workers of different ages. In many species, young workers focus on tasks inside the colony, such as feeding the brood, whereas older workers perform tasks outside their nest, such as foraging (Robinson et al. 1992; Seeley 1982; Lindauer 1952; Shorter & Tibbets 2009; Grüter 2020; Siegel et al. 2013). The behavioral maturation from in-nest work to foraging is affected by genetic factors such as parent-specific gene expression from mothers (matrigenes) and fathers (patrigenes) (Robinson et al. 2008; Whitfeild et al. 2006; Ben-Shahar 2005; Robinson 2002) as well as sensory input from social and environmental stimuli (Grozinger et al. 2003; Ament et al. 2010; Leoncini 2004).

The queen influences worker behavior and colony dynamics through queen pheromones, with effects ranging from attracting workers to the queen (Slessor et al. 1988), suppressing reproduction in workers (Van Oystaeyen et al. 2014; Nunes et al. 2017) and other queens (Vargo 1992; Holman 2013), to altering the learning capacity of workers (Vergoz et al. 2007). Experiments with synthetic queen pheromones and mated versus non-mated queens have identified several bioactive components that promote altruistic behaviors and regulate caste differentiation (Van Oystaeyen et al. 2014; Holman et al. 2010; Smith et al. 2009; Matsuura et al. 2010; Oi et al. 2016). Queen pheromones of different social insect species are often chemically similar to cuticular hydrocarbons (CHCs), suggesting they evolved from conserved signals of a common ancestor (Van Oystaeyen et al. 2014). Probably as a result of this, cross-exposure between queen pheromones and workers of different species results in similar behavioral responses even in non-social insects (Croft 2017; Galang et al. 2019; Princen et al. 2020).

Honey bees represent an unusual case in that the primary queen signal is a 5-compound mixture that is secreted from the mandibular gland of queens, the so-called queen mandibular pheromone (QMP). In addition, CHCs might also act as queen pheromones in honey bees (Princen et al. 2019). QMP has both short-term and long-term effects. Short-term effects include the promotion of retinue behavior, swarm clustering, and drone attraction during mating (Grozinger et al. 2007), while suppressing aversive learning in young bees (Vergoz et al. 2007). Long-term effects of QMP comprise the inhibition

of queen rearing, worker reproductive development (Hoover et al. 2003; Traynor et al. 2014), lowering of the sucrose response threshold (Pankiw et al. 2003) and the modulation of worker activities such as the transition from nursing to foraging (Grozinger et al. 2007; Le Conte & Hefetz 2008). While there is ample experimental evidence to support the behavioral changes induced by QMP exposure, there is limited experimental evidence to show the upstream physiological changes that result in the subsequent behavioral changes induced by QMP. Understanding how QMP exposure influences worker neurophysiology, and how long these effects last, will provide better insights into the changes in areas related to learning and memory, the mushroom bodies (Maleszka et al. 2009; Hourcade et al. 2010; Molina et al. 2007) and olfaction, the antennal lobes (Jernigan et al. 2020), which are strongly associated with behavioral transitions.

The effects of QMP have been found to be most pronounced in young bees, which exhibit an increased sensitivity to QMP (Vergoz et al. 2009) and in which the expression of the biogenic amine dopamine is strongly altered shortly after QMP treatments (Beggs et al. 2007; Mustard et al. 2012). However, it remains unknown whether QMP influences transcriptional activity in the long-term, *i.e.* when workers reach forager age, and whether the effects of queen pheromone on gene expression depends on the life stage at which an individual worker experiences it.

It is common for honey bee colonies to experience temporal queen-absence due to swarming or queen-replacement, and long-term effects of the lapse in QMP exposure on the behavior and physiology of different age-cohorts are expected, possibly depending on an early sensitive period to QMP. Sensitive periods of exposure to social maternal factors have been studied in the social and biological sciences to understand time periods or life stages in which experience shapes a gene expression or behavior to a larger extend than if experienced in other time periods or life stages (Frankenhuis & Walasek 2020; Rosa-Salva et al. 2021). The responses are usually most pronounced during early life exposure and can carry over to influence phenotypes such as behavior, physiology, and morphology through the adult life. Current models examine how mechanisms of behavioral plasticity allow animals to optimally respond to experience across the lifespan (English et al. 2016; Frankenhuis et al. 2011; Panchanathan & Frankenhuis 2016). Nevertheless, we still require a better understanding of the neurophysiological and transcriptional basis of these social effects to account for the complexities of individual behavioral phenotypes.

In this experimental study, we investigated whether queen presence signaling (*i.e.*, QMP exposure) during different periods in the life of adult honey bees affects foraging behavior and gene expression at the age of foragers, which is the last phase in the life of a worker bee (Robinson 1992; Seeley 1982). We used synthetic QMP to elucidate the long-term consequences of QMP presence or absence by treating newly emerged workers, nurses, and foragers (1-19 days of age). When bees reached the foraging age at about three weeks of age, we analyzed gene expression in central and peripheral nervous system tissues thought to be critical for regulating worker behavior. We focused on the mushroom bodies, antennal lobes, and antennae to capture the entire pathway of odor perception, from pheromone binding in the antennae to processing in the antennal lobes to the mushroom bodies, where learning and memory and multimodal sensory integration occur (Ganeshina et al. 2001; Menzel 2012; Lin & Strausfeld 2012). We focused on the foragers for two reasons. First, foragers play a fundamental role for the nutritional health of a colony, but it remains unknown whether and how QMP affects the gene expression of forager-aged bees. Second, queen replacement leads to a gap in brood production, and requires bees of forager-age to rear the brood of the new queen. We might, thus, expect bees that experienced queenlessness at a young age to show strong changes in gene expression when reaching forager age. Finally, we tested whether the QMP treatment affected foraging behavior by observing our focal bees and quantifying foraging activity. We expected that the QMP treatment would alter transcriptional activities in foragers. Moreover, if QMP treatment has long-term consequences, we expected them to be most pronounced when treatment occurred early in life, as younger workers also responded more strongly to QMP than nurse/forager-aged bees (Beggs et al. 2007).

Materials and methods

Colony Set-up

Three Apis mellifera observation colonies were established from three regular sized colonies prior to the start of experiments from August to October 2019, each containing approximately 2000-3000 workers of mixed ages, from the Johannes Gutenberg University apiary in Mainz, Germany. Each observation colony was headed by a naturally mated unrelated queen and all observation colonies had three frames, brood, pollen, and honey reserves.

Sample Collection

One colony at a time was studied. Frames with ready-to-emerge workers were collected from the respective source colony to maintain a similar genetic composition in observation colonies. These frames were stored in a climate cabinet at 34℃ overnight. Newly emerged workers were carefully marked with randomly assigned cohort-specific colored number tags on their thorax and enamel paint on the abdomen. Marked bees were introduced to the respective observation colony and collected once the desired age for treatment was reached.

Brood frame collection was done in a staggered sequence to allow collecting of forager-aged bees (at 19-days), nurse-aged bees (at 7-days), and newly emerged workers (at 1-day of age) at the same time (Fig. S1). Mixed cohorts of ca. 150 bees consisting of forager-aged (n = 50) and nurse-aged (n = 50) workers, and newly emerged (n = 50) workers were evenly distributed and introduced to their designated cages (12 cm x 12 cm) with (QMP+; 1/10 amount of strip brand (the equivalent of 1 queen, Bee equipment, UK) or without artificial QMP (QMP-). They were kept in their respective cage for 2 days, with access to ad libitum queen candy. Queen candy was made by mixing powdered sugar and warm water until it was the consistency of putty. Afterwards, all cohorts were re-introduced to their observation colony (Fig. S1). We filmed the foraging activity of each cohort for 3 days once they reached 21 days of age, until 24 days of age, at which age they should typically be foragers. Foraging is the last task a worker bee performs before her death (Seeley 1982). After filming (see below), bees from the different cohorts were collected from observation colonies when they were 24 days old, using Eppendorf tubes and immediately preserved in liquid nitrogen. We also collected forager-age bees that remained in observation colonies for the duration of the experiments, *i.e.* workers that were never kept in a treatment cage but were returned to observation hives immediately after emergence and marking (henceforth called “control foragers”). All samples were stored in -80℃ freezer until further analysis.

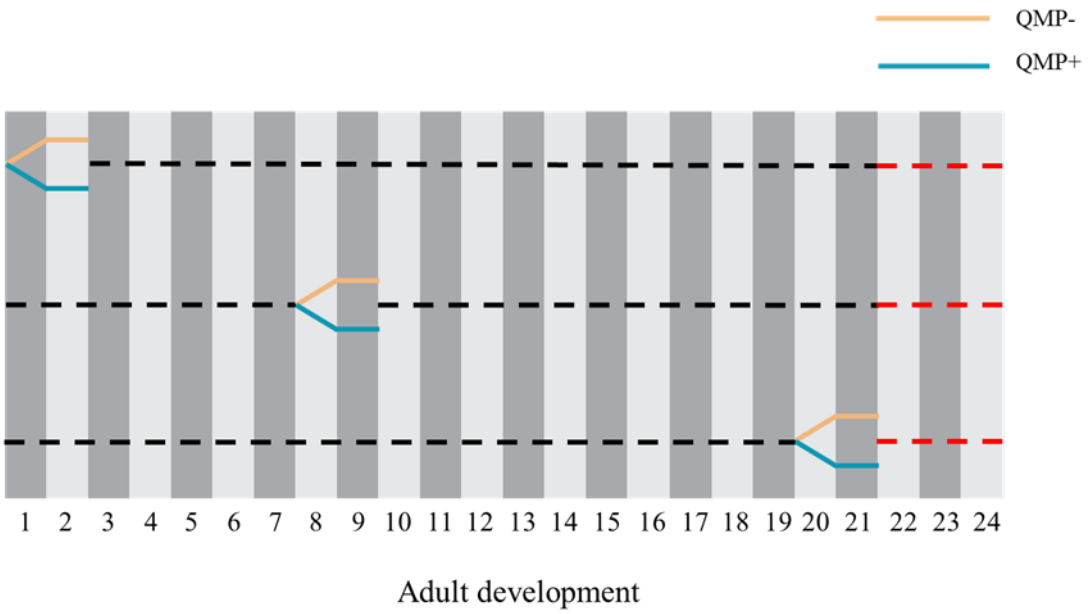

Figure S1: **Experimental design overview**. The black dotted line represents when workers were in the observation colony; the solid line represents when the workers were in the cage for treatment; the red dotted line represents when the workers were filmed in the observation colony.

Video Analysis

Each colony entrance was recorded for 3 days to quantify the foraging behavior of experimental bees at the age of 21 days. We filmed the entrance of the colonies for 8 hours/day, from 9 am to 5 pm and, subsequently, we analyzed foraging by noting the time of entry and departure of marked foragers. We logged behavioral observations using VLC Media Player and statistically analyzed the differences between colonies and treatment groups using generalized linear models (GLM) with the negative binomial distribution. We performed pairwise comparisons between age groups using the general linear hypothesis test (GLHT) with Bonferroni correction for multiple testing.

Brain dissection and RNA extraction

The heads from n = 42 workers were cut from the body and fixed on melted dental wax in a pre-chilled petri dish over ice. The antennae were cut off and stored in 100 µL of Trizol™ (Invitrogen, USA). The mushroom bodies and antennal lobes were removed by making incisions through the antennal base, eyes, compound eye, and ocellus (Kennedy et al. 2021). The cuticles, glands, retina and tissue around the brain were removed and the exposed tissues of the head were submerged with cooled bee saline (154 mM NaCl, 2 mM NaH_2PO_4, 5.5 mM Na_2HPO_4, pH 7.2). Each dissection, one brain dissected into

three tissues, was completed in less than five minutes to prevent degradation of RNA. Brain parts were stored in 100 µL of Trizol™ in -80 ℃ for later RNA extraction using RNAeasy Mini Extraction Kit™ (Qiagen, Germany) according to the manufacturers protocol. RNA was extracted from foragers treated immediately after emergence (newly emerged; n = 12), treated at nurse age (n = 12), treated at forager age (n = 12), and control foragers n = 6 (2 per colony) from a total of three colonies (4 samples from each colony consisting of 2 QMP+ and 2 QMP- in foragers that were treated immediately after emergence, at nurse age, at forager age and 2 samples from each colony consisting of 1 QMP+ and 1 QMP- in control foragers).

Transcriptome Analysis

Aliquots of RNA from each sample were sent to Beijing Genomics Institute (BGI) for sequencing using BGISeq to get 100 base pair (bp) paired-end reads, obtaining ~45 Mio clean paired reads per sample sequencing. Reverse transcription to cDNA was performed as part of the library preparation by BGI (Fig S2). Clean reads without adaptor sequences were provided by BGI and quality checked using FastQC v.0.11.8 (Andrews et al. 2015). Clean reads were aligned using HiSat2 v.2.1.0 (Kim et al. 2015) under default settings to the most recent honey bee genome HvA3.1 (Howe et al. 2020), with a mapping ratio of more than 95% per sample. To count how many aligned reads mapped to genes, we used HtSeq v.0.11.2 (Anders et al. 2015) to generate count tables under the default parameters. Count tables were generated separately for each sample and complied separately for each tissue (*i.e.* antenna, mushroom bodies, and antennal lobes), treatment (i.e. QMP+/-), and life-stage when experiencing the treatment (*i.e.* forager-, nurse-, and newly emerged-age).

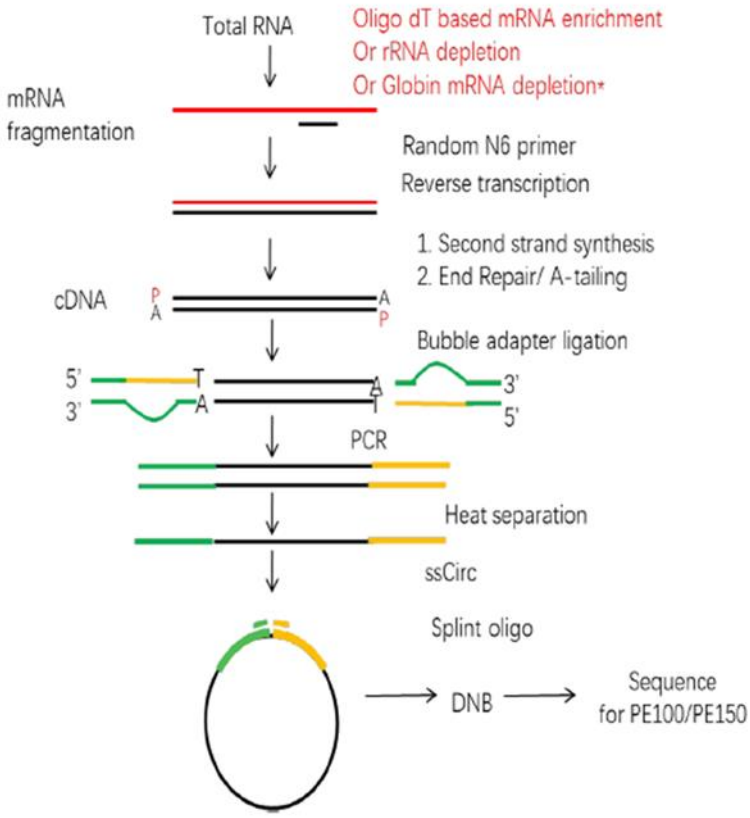

Figure S2: **RNA sequencing workflow provided by Beijing Genomic Institute (BGI).** Total RNA was extracted from 5 tissues (antennae; antennal lobes; mushroom bodies; central brain; and subesophageal ganglion) and sent to BGI for sequencing using BGISeq to get 100 base pair (bp) paired-end reads. We obtained ~45 Mio clean paired reads per sample sequencing.

Gene Expression Analysis

Gene expression differences were analyzed between treatments (QMP+ vs QMP-) for each tissue (mushroom bodies, antennal lobes, and antenna) and life-stage (newly emerged-forager, nurse-forager, forager-forager, and control-forager) using the R package DESeq2 v.1.24.0 (Love et al. 2014). Before the analysis, an additional filtering step was added to ensure that only genes with counts of at least 9 reads in n-1 of the smallest sample size were used in the gene expression analysis. We analyzed gene expression separately for each tissue and age. We tested the effect of treatment with QMP on gene expression by using the likelihood ratio test (LRT) approach whereby a full model with treatment (QMP+/-) and colony-ID as fixed factors is compared with a reduced model containing only colony-ID, taking into consideration colony effects. Genes were considered differentially expressed if the false

discovery rate (FDR) corrected p-value was < 0.05. All analysis was completed in R v.4.1.0 (R Core Team 2021). We performed permutations to test whether the overlap in differentially expressed genes between tissues differed from what could be expected to overlap by chance. We generated a gene universe that had the same total number of genes (n = 12, 320) which contained random gene IDs and zeros. We created a vector for number of permutations we wanted to generate (n = 1,000). We performed automatic iterations with the number of DEGs in each gene list to get overlap values with our randomly generated list. We plotted histograms of the distribution generated by the permutations against the overlap to see if what we found was within (expected by chance) or outside (not expected by chance) the normal distribution (Fig. S4).

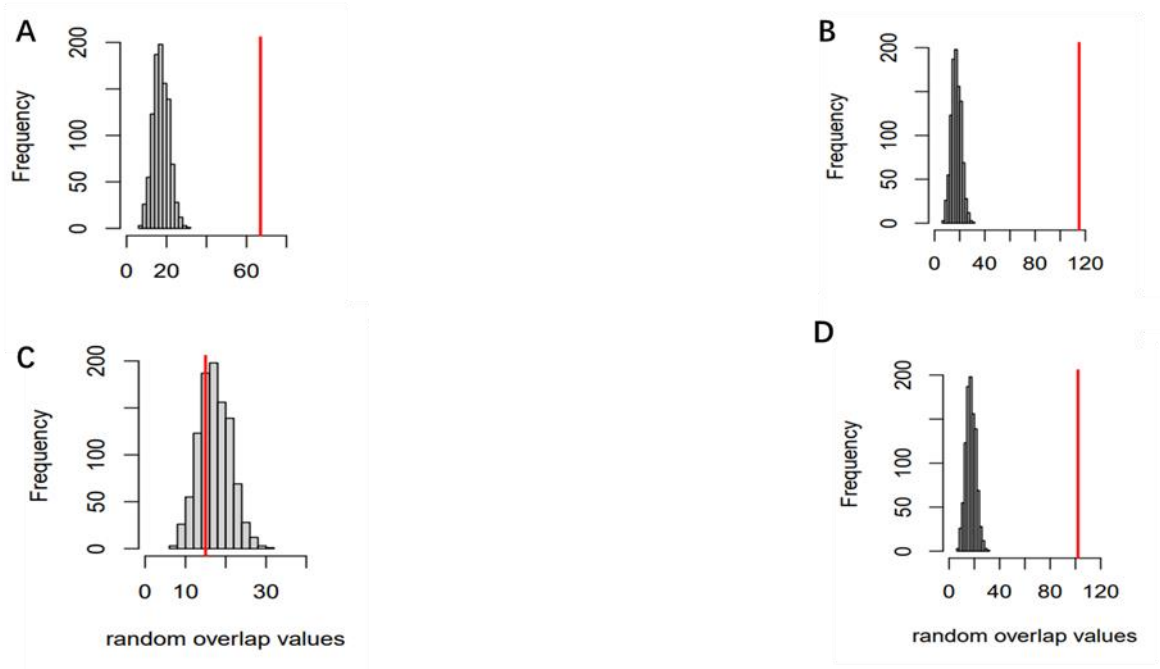

Figure S4: **Permutation results** for the gene expression overlap between tissues for samples treated with QMP as 1 day newly emerged bees. (**A**) mushroom bodies vs. antennal lobes (n = 67); (**B**) mushroom bodies vs. antennae (n = 115); (**C**) antennae vs. antennal lobes (n = 15); and (**D**) all tissue overlaps (n = 102). Red bar indicates the number of overlapped genes we found.

We performed both gene ontology (GO) and KEGG overrepresentation analysis on each differentially expressed gene list with the *clusterProfiler* package (Wu et al. 2021), using the honey bee annotation that can be retrieved with the R package *AnnotationHub* (Morgan & Shepherd 2022). To further investigate QMP treatment effects on specific genes, we focused on candidate genes involved in key individual and social behaviors and traits. We compiled lists of genes and molecular pathways

associated with foraging behavior and division of labor in honey bees (Kennedy et al. 2021; Forêt & Maleszka 2006; Schulz et al. 2003; Linn et al. 2020; Peng et al. 2021; Robinson 1987; Sullivan et al. 2000; Brito et al. 2021; Shpigler et al. 2021; Prato et al. 2021; Ihle et al. 2010; Nelson et al. 2007; Amdam et al. 2012; Johnson et al. 2016; Fussnecker & Grozinger 2008; Shpigler et al. 2010). Furthermore, we searched for genes that play roles in aging (Kennedy et al. 2021; Kuszewska et al. 2017; Haddad et al. 2007; Seehuus et al. 2006; Corona et al. 2005), immunity (Richard et al. 2008; Kennedy et al. 2021), and reproduction (Hoover et al. 2003; Traynor et al. 2014), *i.e.* processes known to be affected by queen signals. We cross referenced all of our DEG lists, including the overlapped DEGs, to the candidate gene list to find genes of interest from previous studies.

Results

Influence of QMP on gene expression

Transcriptome analysis across the three tissues showed that QMP treatment early in life produced the strongest response. Despite the longest time period between treatment and sampling, workers treated with QMP as newly emerged bees showed the most pronounced differences in gene expression: a total of 1,398 genes were differentially expressed in all tissues (Fig. 1). In contrast, nurse-aged or forager-aged bees exposed to our QMP treatments only altered the expression of 80 and 67 genes across tissues, respectively (Fig. 1). Tissue-specific analyses revealed strongest transcriptomic shifts in the mushroom bodies with 760 DEGs in foragers treated as newly emerged bees (Fig. 1C). The vast majority of differentially expressed genes were significantly upregulated in bees that experienced a lack of the queen signal during the first days of their life (Fig. 1, 68% in MBs, 85.7% in AL, 91.7% in AT.). Interestingly, the opposite pattern was found in bees treated at nurse-age (Fig. 1). Here, most differentially expressed genes were upregulated in QMP+ bees (Fig. 1) (94% in AL and 75.8% in AT). We found the most enriched GO terms of the DEGs in foragers treated with QMP as newly emerged bees. Many of these GO terms were associated with RNA processing and binding in the antennal lobes and in the mushroom bodies. We also found a number of behaviorally relevant candidate genes when comparing our DEGs with lists of genes found previously in the honey bee to be linked to foraging, division of labor, aging, reproduction and immunity genes.

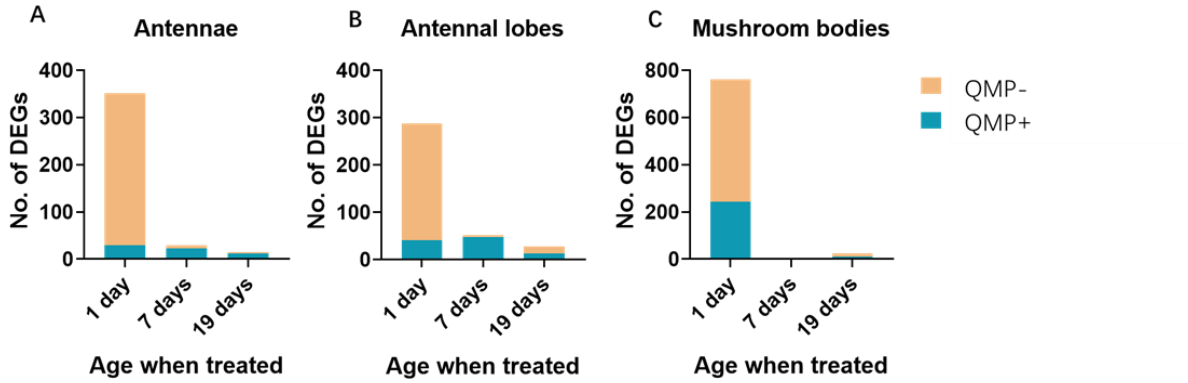

Figure 1: **Summary of the number of differentially expressed genes** (No. Of DEGs) for each tissue (**A**) antennae, (**B**) antennal lobes, and (**C**) mushroom bodies. On the y-axis, each bar indicates the total number of DEGs that are upregulated in each age group when QMP treatment occurred (1 day = newly emerged; 7 days = nurse; 19 = forager). Bars are separated by color to indicate 48h without QMP treatment (QMP-) or with QMP treatment (QMP+).

Mushroom bodies

Workers treated with QMP when newly emerged had differentially expressed genes associated with aging (DNA repair proteins, telomere associated proteins), division of labor (kruppel homolog 1), memory (metabotropic glutamate receptor), and foraging (serotonin receptor, D2-like dopamine receptor) (Fig. 3). Workers treated with QMP at forager age had differentially expressed genes associated with cellular maintenance and DNA repair.

Antennal lobes

Workers treated with QMP when newly emerged again had differentially expressed genes associated with aging (telomere associated proteins and DNA repair proteins) and learning (Allatostatin A receptor). Workers treated at nurse age had differentially expressed genes associated with foraging (e.g., dopamine receptor, D1) (Fig. 3). Workers treated at forager age had differentially expressed genes associated with learning (e.g., Allatostatin A receptor).

Antennae

Workers treated with QMP when newly emerged had differentially expressed genes associated with odor binding (odorant receptor 4-like, general odorant-binding protein 71) and aging (DNA repair proteins) (Fig. 3). Workers treated with QMP at nurse age had differentially expressed genes associated with odor binding (odorant receptor 53) (Fig.3). Workers treated with QMP as forager age had few (15) genes differentially expressed, many of which were uncharacterized.

Differentially expressed genes across tissues

Some genes were differentially expressed in more than one tissue. For the DEGs of the newly emerged bees, we found 15 genes that overlapped between the antennae and antennal lobes, 67 genes overlapped between the antennal lobes and mushroom bodies, 115 genes overlapped between the antennae and mushroom bodies, and 102 genes overlapped between all tissues (Fig. 2). Permutations showed that this gene expression overlap between all tissues, between mushroom bodies and antennae, and between mushroom bodies and antennal lobes were all outside of the range that could be expected by chance. However, the overlap between the antennae and antennal lobes was within the range of what could be expected by chance (Fig. S4). We found five genes that overlapped between the antennae and antennal lobes for samples treated with QMP at nurse-age. Note that there were no differentially expressed genes in the mushroom bodies. The gene expression overlap for nurse-age bees was within the range of what could be expected by chance. We found no overlap of DEGs between any tissues for samples treated at forager-age. All gene lists with a significant overlap were analyzed for matches against the candidate gene list as well as GO enrichment analysis. We did not find any GO enrichments or matches against the candidate gene list.

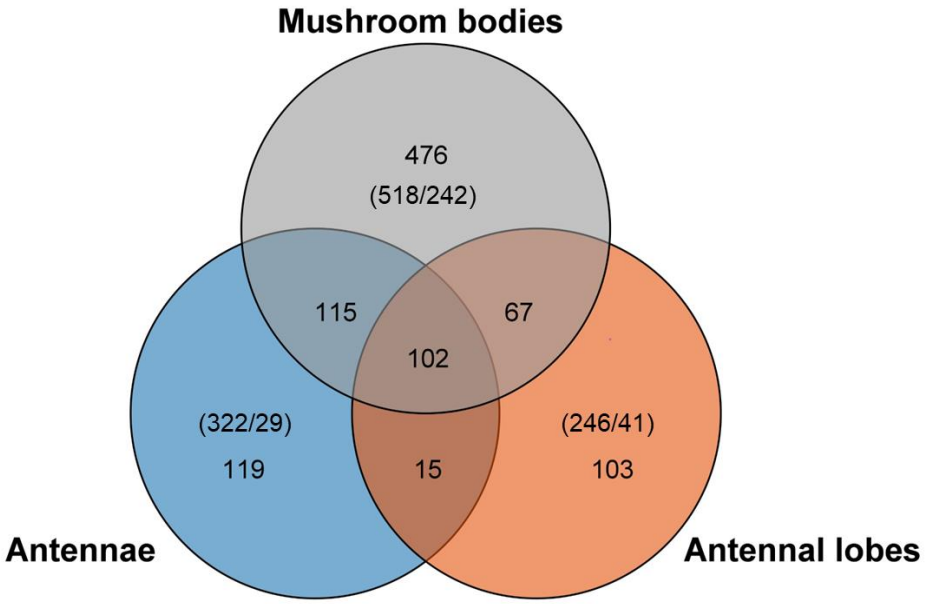

Figure 2: **Venn diagram representation of the overlap between DEGs** of tissues for foragers treated with QMP treatment as 1 day newly emerged bees. Each color circle represents a different tissue (blue = antennae; orange = antennal lobes;

grey = mushroom bodies). Numbers within each individual circle indicate the number of unique of DEGs for that specific tissue (*i.e.* 119 in the antennae; 103 antennal lobes; and 476 mushroom bodies). Numbers between circles indicate the number of shared DEGs between tissues (*i.e.* 15 between the antennae and antennal lobes; 67 between the antennal lobes and mushroom bodies; 115 between the antennae and mushroom bodies; 102 between all tissues). Numbers in Parenthesis indicate the number of upregulated genes (QMP-/QMP+) treated groups.

Foraging behavior

We found an overall effect of age when treated with QMP on the sum of foraging trips (GLM, negative binomial distribution, $X^2 = 17.91$, $p < 0.001$, Fig. 4) and average time spent foraging (LME, normal distribution, $X^2 = 12.25$, $p = 0.002$, Fig. 4), but no effect of the QMP treatment ($X^2 = 0.259$, $p = 0.6$; $X^2 = 0.705$, $p = 0.4$ respectively) and no interaction between QMP treatment and age when treated ($X^2 = 2.603$, $p = 0.272$; $X^2 = 4.18$, $p = 0.123$ respectively, Fig. 4).

We performed pairwise comparisons of the age when treated with QMP for both total number of foraging trips and average foraging duration using Bonferroni correction for multiple testing and found that bees treated as nurses had fewer foraging trips and spent on average less time foraging than those treated at foraging age (General Linear Hypothesis Test (GLHT): z-value = -3.214, p = 0.003 and GLHT: z-value = -3.202, p =0.004 respectively; Fig. 4) and newly emerged bees (GLHT: z-value = -2.813, p = 0.013; Fig. 4), while there was no difference between newly emerged and nurse-age (GLHT: z-value = -2.120, p = 0.068; Fig. 4) in total number of foraging trips. Forager-age and newly emerged bees treated with QMP did not differ in total number of foraging trips (GLHT: z-value = -1.616, p = 0.106; Fig. 4) or average foraging duration (GLHT: z-value = -0.960, p = 0.6; Fig. 4).

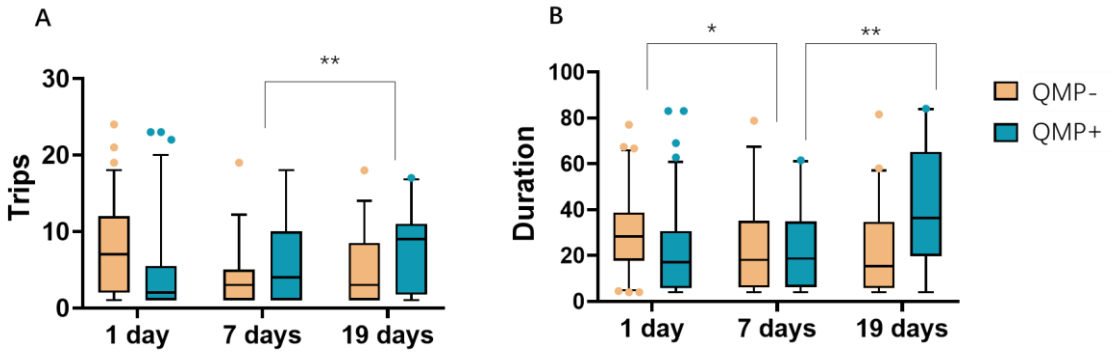

Figure 4: **Effect of QMP treatment on foraging activity** (**A**) total number of foraging trips and (**B**) average foraging duration. On the y-axis, each age group when treated with QMP is represented (1 day = newly emerged; 7 days = nurse; 19

days = forager). Box and wisker plots are color coded according to treatment (orange = 48h without QMP treatment (QMP-); blue = 48h with QMP treatment (QMP+)). There was no difference in foraging activity between QMP treatment groups. Pairwise tests between age groups were performed using General Linear Hypothesis Tests (GLHT) and differences were found in the total number of foraging trips (7 days nurse vs. 19 days foragers GLHT: z-score -3.214, p-value 0.00393.) and average foraging duration (1 day newly emerged vs. 7 day nurses GLHT: z-score -2.813, p-value 0.01340; and 7 days nurse vs. 19 days forager GLHT: z-score -3.202, p-value 0.00385).

Discussion

Our results demonstrate a sensitive period of exposure to QMP in newly-emerged bees, which results in long-term transcriptional changes in the central and peripheral nervous systems. These molecular consequences persist across behavioral transitions until bees reach foraging age (24 days in this study). QMP elicits multiple distinct behavioral and physiological responses in workers, as both a releaser and primer pheromone, and thus produces responses on vastly different time scales (Grozinger et al. 2007; Slessor et al. 2005). The long-lasting effects of QMP exposure on gene expression in bees treated after emergence (1,398 genes in all tissues combined; Fig. 1) was most pronounced in the mushroom bodies (highest number of DEGs: 785) and, interestingly, most DEGs were upregulated when young bees experienced a lack of QMP exposure (Fig. 1). This suggests that a brief period of queenlessness early in life leads to extensive and persistent upregulation of transcription. In contrast, there were only small to moderate effects in bees treated later in life (80 genes in workers treated at 7 days of age (nurse age) and 67 genes in workers treated at 19 days (forager age); Fig. 1), suggesting that the time window for large-scale QMP effects closes very early in the life of a worker bee.

Sensitive periods exist across the animal kingdom and are generally characterized by a limited duration for key developmental processes in response to external cues or stimuli (Frankenhuis & Walasek 2020; English & Barreaux 2020; Knudsen 2004). An individual's response to the cues or stimuli from the external environment is variable but the interaction can influence subsequent physiology and behavior. However, it is difficult to disentangle the role of the external environment in selecting for behavior in regards to learning plasticity versus the physiological traits of the individual in understanding how an organism responds to cues or stimuli. In humans, it is well-established that challenging conditions in utero, where an individual is buffered from the external environment, and early childhood shape adult coping strategies, behavior, and health (Almas et al. 2020; Duchesne et al.

2017; Schulz 2010).

This study provides evidence that the social signal, *i.e.* QMP exposure at a critical life stage (1-day-old, newly-emerged bees) is responsible for the previously observed worker phenotypes in response to pheromone signals. Young workers are more attracted to QMP, which results in the retinue response (Slessor et al. 1988; Slessor et al. 2005; Rangel et al. 2016), while older workers engaging in foraging are repelled by queen pheromones (Vergoz et al. 2009; Jarriault & Mercer 2012). This is consistent with the patterns in gene expression we found, where the transcriptional activity of 1-day newly emerged workers is drastically altered, while the transcriptional activity of 19-day foragers remains largely unchanged by QMP treatment (Fig. 1). Thus, forager-aged bees are largely resistant to QMP effects both in their behavior and upstream gene expression. Future studies could explore if long-term transcriptional effects observed during early life exposure to QMP are similar to pheromonal experiences during the larval and pupal stages.

QMP in high doses is reported to be repellent to bees (Moritz et al. 2001; Moritz et al. 2001) and can make workers aggressive (Vaitkevièienë & Budrienë 1999). QMP exposure during early adult life regulates the expression of D1-like, D2-like, and octopamine receptor genes (Vergoz et al. 2009; Beggs et al. 2007) which has been implicated in aversive learning in insects. We found that QMP exposure in early life led to long-lasting changes in the expression of D2-like dopamine receptor (*AmDop3*) and D1-like dopamine receptor (*AmDop1*) in the mushroom bodies and antennal lobes, respectively (Fig. 3). It has been suggested that QMP's effects on aversive learning may serve to prevent young workers that attend the queen from forming an association between the queen and any unpleasant effects of her pheromone (Vergoz et al. 2007), which is consistent with the observation that young workers are more attracted to QMP and why the formation of aversive olfactory memories is prevented (Vergoz et al. 2007, Vergoz et al. 2009).

Dopamine receptors have a crucial role in a broad range of behaviors, such as motor function, sensory processing, arousal, and reward signaling (McQuillan et al. 2012; Elsik et al. 2014; Mishra et al. 2018). *Amdop3* is widely expressed in the brain in both adults and pupae, with a unique pattern of expression compared to the other subtypes *Amdop1* and *Amdop2* (Suenami et al. 2016). Homovanillyl alcohol (HVA), a major component of QMP, has been shown to reduce the concentration of brain dopamine levels in the centers associated with learning and memory (*i.e.*, mushroom bodies). HVA selectively activates *Amdop3,* which blocks aversive learning in workers (Beggs & Mercer 2009),

possibly promoting the retinue response as seen in the upregulation of *Amdop3* in the mushroom bodies of bees treated after emergence. Since we also find an upregulation of *Amdop1* in the antennal lobes of bees experiencing the absence of QMP at nurse age, this could suggest an early trigger of non-nursing behaviors. We also show a serotonin receptor (*5-ht1*) to be upregulated in the mushroom bodies of bees treated with QMP as newly-emerged bees, suggesting its role in learning and memory. *5-ht1* injection prior to olfactory conditioning resulted in reduced memory storage and retrieval (Bicker & Menzel 1989; Menzel et al. 1999; Mercer & Menzel 1982), while high *5-ht1* levels in the central nervous system were associated with visual information processing in honey bee foragers.

Changes in QMP exposure also alter the morphology and gene expression in key brain tissues involved in olfaction, learning, and memory: the antennal lobes and mushroom bodies, under short-term QMP exposure conditions (Beggs et al. 2007; Mustard et al. 2012; Schulz & Robinson 1999; Velarde et al. 2009; Hameed 2009). The transcription factor *Krüppel-homolog 1* (*kr-h1*) is linked to hormone mediated social organization in honey bees, bumble bees, and ants (Shpigler et al. 2010; Grozinger et al. 2007; Gospocic et al. 2021). We found that QMP treatment induced an upregulation of *kr-h1* in the mushroom bodies of foragers that experienced no QMP exposure as newly-emerged bees. Experiments on young workers (<1 week) have shown that QMP exposure activates nursing genes and represses foraging genes, suggesting that QMP delays the transition between behavioral states by acting on *kr-h1* in the mushroom bodies (Grozinger et al. 2003). Although we do not find behavioral evidence to suggest that foraging activity was suppressed in 1 day newly emerged bees treated with QMP, future studies could use the evidence we provide to further investigate nursing behaviors after exposure to queenlessness.

QMP has varied effects on brain transcriptional activity depending on the behavioral state of a worker (Grozinger et al. 2003; Kocher et al. 2010) and our study shows that the pheromone information provided by QMP is processed differently at different adult stages. Accordingly, we find an upregulation in the expression of odor binding proteins (*odorant receptor 4-like* and *general odorant-binding protein 71*) and receptors (*odorant receptor 53*) in the antennae of workers treated with QMP as newly-emerged and nurse-aged bees (Fig. 3). A variety of odorant binding proteins (OBPs, ca. 21) and odorant receptors (ORs, ca. 180) have been characterized in the honey bee (Forêt & Maleszka 2006; Zhang et al. 2016) but their role in odor discrimination is still poorly understood. While we present further evidence for the role of some OBPs and ORs in the context of QMP signaling, more research is needed to understand

their functioning in the context of odor differentiation and subsequent behavioral regulation.

In summary, we show an early sensitive phase for QMP exposure and long-lasting effects on transcriptional activity of the brain and antennae of young honey bee workers. We build on current knowledge that queen pheromones not only attract young workers and affect gene expression, but that these changes can persist into late foraging age. We observed the strongest changes in the mushroom bodies in workers that experienced an absence of QMP in early life. The results suggest an impact on learning and memory, but surprisingly not on foraging activity. Future research should observe whether these long-lasting changes in gene expression triggered by early life treatment alter behaviors such as nursing or immune function as suggested by the observed gene expression patterns presented here and in previous studies.

CHAPTER 2

Use of waggle dance information in honey bees is linked to gene expression in the antennae, but not in the brain

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Abstract

Communication is essential for social animals, but deciding how to utilize information provided by conspecifics is a complex process that depends on environmental and intrinsic factors. Honey bees use a unique form of communication, the waggle dance, to inform nestmates about the location of food sources. However, as in many other animals, experienced individuals often ignore this social information and prefer to rely on prior experiences, *i.e.* private information. The neurosensory factors that drive the decision to use social information are not yet understood. Here we test whether the decision to use social dance information or private information is linked to gene expression differences in different parts of the nervous system. We trained bees to collect food from sugar water feeders and observed whether they utilize social or private information when exposed to dances for a new food source. We performed transcriptome analysis of four brain parts critical for cognition: the subesophageal ganglion, the central brain, the mushroom bodies, and the antennal lobes but, unexpectedly, detected no differences between social or private information users. In contrast, we found 413 differentially expressed genes in the antennae, suggesting that variation in sensory perception mediate the decision to use social information. Social information users were characterized by the upregulation of dopamine and serotonin genes while private information users upregulated several genes coding for odor perception. These results highlight that decision making in honey bees might also depend on peripheral processes of perception rather than higher-order brain centers of information integration.

Introduction

Exchanging information is essential in all animal societies. Communicating about resources, reproductive state, group membership, and threats are vital in ensuring the survival and success of the group. However, relying on social information is often not the only available option, *e.g.* to find a food source, but searching for a resource individually can often be the better choice (Laland, 2004; Kendal et al., 2009; Hoppitt & Laland, 2013; Dechaume-Moncharmont et al., 2005; I'Anson Price et al., 2019). Furthermore, an individual can rely on private information (*e.g.* spatial memory) about previously visited food source locations (Rendell et al., 2010; Grüter & Leadbeater, 2014). It is crucial for an organism to assess the different available options and their consequences to make the best decision in a given environment. Acquiring information through individual exploration, for instance, provides up-to-date information, but comes with the cost of trial-and-error learning. Social information avoids the costs of individual learning and exploration, but can involve the inefficient or erroneous transmission of information (Giraldeau et al., 2002; Dechaume-Moncharmont et al., 2005; Rieucau & Giraldeau, 2011; I'Anson Price et al., 2019). Thus, animals often employ flexible strategies for deciding between social or private information (Laland, 2004; Kendal et al., 2009; Hoppitt & Laland, 2013; Grüter & Leadbeater, 2014).

Social insects employ various methods to send signals to nestmates. Information exchange regarding resources is particularly well-studied and a wide range of communication behaviors are used, such as tandem running in ants (Alleman et al., 2019, Möglich et al., 1974; Glaser and Grüter, 2018) and trail pheromones in ants, and stingless bees (Jarau, 2009; Hölldobler & Wilson, 2009; Czaczkes et al., 2015). Honey bees (Apini) use a unique form of communication, the waggle dance that gives spatial information to nestmates about both distance and direction of a food source or a nest site in relation to the sun (von Frisch, 1967). In foraging, dances are performed by returning foragers as an advertisement for high quality food sources. Furthermore, waggle dancers emit floral odors and a blend of hydrocarbons that provide additional information and stimulate foraging in unemployed foragers (Gilley et al., 2018; Thom et al., 2007; Farina et al., 2012). Only a relatively small percentage of waggle dance followers use dance information to discover new food sources. The majority of waggle dances trigger experienced foragers to resume foraging at already familiar food sources, disregarding social dance information for private spatial information (Biesmeijer & Seeley, 2005; Grüter et al. 2008). While

various factors, like experience (Richter & Waddington, 1993; Biesmeijer & Seeley, 2005; Grüter & Ratnieks, 2011) and age (Tofilski, 2009; Woyciechowski & Moroń 2009) are likely to affect whether a bee uses social information, still little is known about the neuronal basis of dance communication and its use (Barron & Plath, 2017).

Numerous studies have shown that social insect behavior and responses to social information are linked to brain gene expression (Toth et al., 2010; Robinson et al., 2008; Zayed & Robinson, 2012; Ingram et al., 2011; Toth & Robinson, 2009). For example, foragers have a unique pattern of gene expression compared to nurses as they upregulate genes associated with synaptic plasticity and cognition (i.e spatial learning and memory), whereas nurses upregulate genes associated with intracellular signaling involved in the transition from nurse to forager (Whitfield et al., 2003). Even among foragers different gene expression patterns can be found. For example, pollen and nectar foragers differentially upregulate genes associated with regulating food intake (Brockmann et al., 2008). Behavioral variation within foragers seems to be strongly connected to the expression of genes that are important in biogenic amine signaling, such as dopamine, octopamine, tyramine, glutamate, and serotonin signaling (Liang et al., 2012; Scheiner et al., 2002; Schulz et al., 2003; Barron et al., 2002; Scheiner et al., 2017a). Indeed, manipulation of biogenic amine levels can alter foraging behavior (Liang et al., 2012; Peng et al., 2020; Linn et al., 2020) and perception of food rewards and odors (Mercer & Menzel, 1982; Barron et al., 2002; Scheiner et al., 2002). Most studies have focused on whole brains to reveal expression differences between behavioral groups (*e.g.* Whitfield et al., 2003; Liang et al., 2012; Alleman et al., 2019). However, different brain parts serve specific functions and are expected to differ in gene expression. For example, the antennal lobes receive input from the olfactory sensory neurons in the antennae (Paoli et al., 2016) and process olfactory information (Homberg et al., 1989; MaBouDi et al., 2017; Paoli et al., 2016). Insect mushroom bodies are key brain areas for multimodal sensory integration, learning and memory (Strausfeld et al., 2009; Collett & Collett 2018), whereas the central brain supports foraging behavior via motor control (Hanesch et al., 1989). Barron and Plath (2017) have suggested that the central brain might play a crucial role in the decoding of waggle dance information. Finally, the subesophageal ganglion mediates reward and taste perception (Kreissl et al., 1994; Dacks et al., 2005; Sinakevitch et al., 2005).

If and how these different brain parts are involved in dance communication and information-use is not well understood. Furthermore, we still know little about the role of the peripheral nervous

system for decision making and information processing (see *e.g.* Ozaki et al., 2005). The antennae, in particular, play important functions in social insect behavior, both within and outside the colony, such as mediating pheromone signaling (Nagari & Bloch, 2012; Vergoz et al., 2009; Grozinger et al., 2003; Pankiw, 2004), nestmate recognition (Ozaki et al., 2005; van Zweden & D'Ettorre, 2010) and odor learning (Robertson et al., 2006; Rogers & Vallortigara, 2008). An important role of the antennae in mediating different behaviors is also likely to explain why foragers and nurses show distinct antennal expression of chemical sensory and biogenic amine genes (Nie et al., 2018; McQuillan et al., 2012). Chemical stimuli differentiation and odor perception are not only important for task differentiation (Arenas & Farina, 2012; Balbuena & Farina, 2020), but could play a role in the decision to use social or private information (Thom et al., 2007).

Here we compared the gene expression of bees that used dance information (social information, SI) with those that preferred private information (PI) in different brain areas and the antennae in the honey bee *Apis mellifera.* We trained cohorts of workers to sucrose solution feeders and, subsequently, confronted them with conflicting social information about a new high-quality food source. As was shown for scouts, *i.e.* foragers that search for new food sources independently (Liang et al., 2012), we predicted that there are distinct neurogenomic signatures underlying the decision to use either social or private information. We compared different brain and peripheral chemosensory areas in both types of bees. We demonstrate that bees that decode and use waggle dance information differ in gene expression only in the antennae and provide evidence for roles of biogenic amine signaling and olfactory perception.

Materials and methods

Colony Set-up

A total of six observation hives of *Apis mellifera carnica* were studied from August through October 2016 (H1 – H3) and 2018 (C1 -C3), each containing approximately 2000-3000 workers of mixed ages. Colonies were established from the Johannes Gutenberg University apiary in Mainz, Germany, a few weeks prior to the start of experiments. Each of the observation colonies contained three frames, brood, food reserves and were headed by a naturally mated queen.

Training

Training was conducted one colony at a time. Workers were trained according to standard training procedures to collect sucrose solution at one of two artificial feeders (von Frisch, 1967; Linn et al.,

2020). First, a cohort of 50-60 workers was trained to the training feeder (T.F.). These workers were used as the samples that would later be designated as either social or private information users on test day. Then, a smaller cohort of ~20 foragers was trained to the dance feeder (D.F.). These workers would be designated as dancers on test day. Both feeders were 150m from the observation colonies with ca. 160 meters separating the training and dance feeder (see Linn et al., 2020, their Fig. 1). Workers were trained to their respective feeder with an unscented 0.8M sucrose solution and were individually marked with a number tag on the thorax. This spatial arrangement ensured that workers would visit only one feeder and no mixing of individuals between dance and training feeders occurred. The day after training, the sucrose solution was reduced to 0.3M at both feeders with the addition of an identical scent (5µL of essential oil /100mL sucrose solution). This concentration made sure that trained foragers would return to their respective feeder, but not recruit additional bees. Colonies were trained to a different odor: C1, H1 = sage, C2, H2 = jasmine, C3, H3 = peppermint. During 60 minutes, workers were allowed to visit their feeder repeatedly (2016: 5.24 ± 3.79 visits, N = 191; 2018: 8.09 ± 5.17 visits, N = 102). The 60-minute training with scented solution allowed workers to associate reward, scent, and location of the respective feeder.

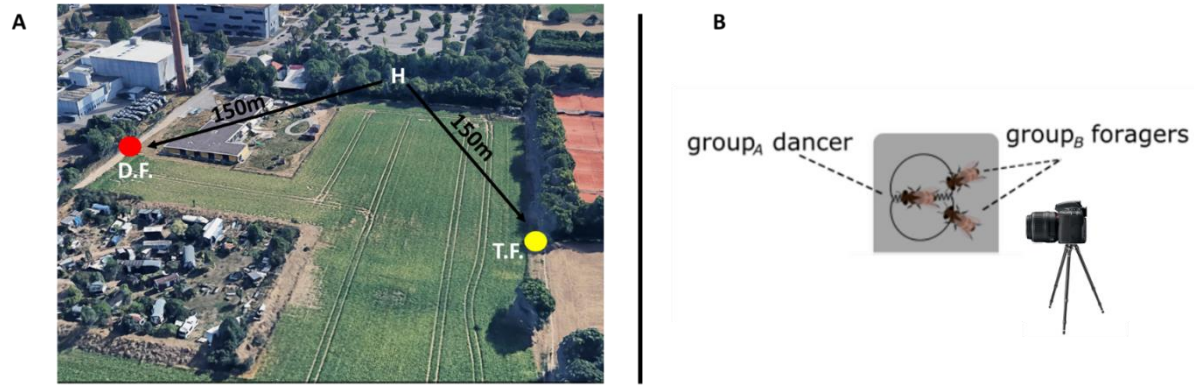

Figure S1: A) experimental set-up showing the location of the hives (H), dance feeder (D.F.), and training feeder (T.F.). Picture was taken from Google Earth (49° 59'13"N 8° 14' 08" E). Each feeder was 150m from the hives and 160m between each feeder. B) schematic design of filmed interactions inside the hive. On testing day, group$_a$ dancers (trained to D.F.) perform waggle dances inside the colony for feeder a location. Group$_b$ foragers (trained to T.F.) are faced with the decision to use social information (SI) and arrive at the D.F. or use private information (PI) and arrive at the T.F.

Sample Collection

On the test day, the day after the 60-minute odor training, 2M sucrose solution with the same scent as used during training was offered only at the dance feeder location, while the training feeder was empty. The sucrose concentration at the dance feeder was high to induce the collecting foragers to perform waggle dances. T.F. trained workers could then decide whether to use social information by following the waggle dances performed by the returning dancers (fly to the D.F.) or disregard the dance vector information and use private information (return to the T.F.). The arrival time and capture time of each individual bee was recorded. Dance and dance following behavior were recorded in the observation colony using a high-definition camera to quantify dance following behavior by T.F. foragers. Workers trained to the T.F. that arrived at the D.F location were collected in Eppendorf tubes and immediately preserved in liquid nitrogen; these workers were the social information users. Workers trained to the T.F. feeder that arrived at the T.F. feeder location were collected at a similar time; these workers were the private information users.

Video Analysis

Videos were analyzed using VLC Media Player. Dances and dance following behaviors were analyzed frame by frame. A worker was only counted as following a dance when she was within one antennal length of a marked dancer during the waggle run phase (Grüter et al., 2013; Linn et al., 2020), which is the component of the waggle dance that encodes the vector information (von Frisch, 1967). We compared the dance following behavior of private and social information users with linear mixed-effects models (LME and GLMM's). The nlme-package and linear mixed-effects models (LMEs) were used when the response variable was normally distributed (waggles per dance followed). The lme4-package and generalized linear mixed-effects models (GLMMs) were used when the response variable had a Poisson distribution (total number of dances followed) (Zuur et al., 2009). Colony-ID and year (2016 and 2018) were included as hierarchically nested random effects to account for their effects (Zuur et al., 2009).

Brain Dissection and RNA Extraction

In 2016, we dissected the calyxes of the mushroom bodies and antennal lobes from 14 workers (7 social information users and 7 private information users, 2-3 per colony and type). We confirmed that all social information users followed dances extensively. In 2018 we dissected central brains and subesophageal ganglions from 16 workers (8 social information users and 8 private information users, 2-3 per colony

and type), and the antennae from 11 different workers (1-4 per colony and type) (see Fig. 1 in Sen Sarma et al., 2009 for a schematic representation of the brain areas and cut-off lines). The additional handling of the samples after being flash frozen in liquid nitrogen caused the antennae of some samples to be brittle and easily break apart. Different workers were used to ensure that whole antennae could be used for equal extraction of RNA from all samples.

Heads from individual workers were cut from the body and fixed on melted dental wax in a pre-chilled petri dish over ice. The antennae were cut off and stored in 100 mL of TRIzol™ (Invitrogen, USA). Incisions were made at the antennal base, around the eyes, through the compound eye, and the ocellus. The cuticles, glands, retina and tissue around the brain were removed and the exposed tissues of the head were submerged with cooled bee saline (154 mM NaCl, 2 mM NaH_2PO_4, 5.5 mM Na_2HPO_4, pH 7.2). Subesophageal ganglion and central brain (which included the mushroom body peduncles, the bundled axons from the Kenyon Cells in the calyces), were removed by cutting off optic lobes, antennal lobes, and mushroom body calyces. All tissues called "mushroom body" refer to mushroom body calyces as it is extremely difficult to remove mushroom body peduncles. The calyces contain the intrinsic Kenyon cells, where a large part of mushroom body transcription takes place and, therefore, the calyces are often used to study mushroom body gene expression (Sarma et al., 2009; Reim & Scheiner, 2014; Humphries et al., 2003). Furthermore, the tissue called "central brain" refers to a brain region that also includes the mushroom body peduncles and putative differences in expression in this tissue should be interpreted carefully because of the different functions of these tissues. Each dissection was completed in less than 5 minutes to prevent RNA degradation. Brain parts were stored in 100 mL of Trizol™ (Invitrogen, USA) in -80 °C for later RNA extraction using RNAeasy Mini Extraction Kit™ (Qiagen, Germany) according to the manufacturers' protocol.

Transcriptome Analysis

For sequencing, aliquots of RNA from private and social information users were sent to Beijing Genomics Institute (BGI) for library construction and sequencing. In 2016, Hiseq 4000 was used to sequence 100 base pair (bp) paired-end reads, obtaining 40 Mio clean reads per sample. The total sample size was 28. In 2018, BGISeq was used to sequence 100 base pair (bp) paired-end reads, obtaining 70 Mio clean reads per sample. The sequencing failed for 1 sample and 1 sample was damaged during the travel (Eppendorf tube burst), decreasing our total sample size to 41. Raw reads were quality checked using *FastQC* v.0.11.8 (Andrews et al., 2010) followed by Illumina adapter removal using

Trimmomatic v.0.38. (Bolger et al., 2014). Clean reads were aligned using *HiSat2* v.2.1.0 (Kim et al., 2017) to the honey bee genome HvA3.1 as a reference (Wallberg et al., 2019). To count how many aligned reads mapped to genes, we used *HtSeq* v.0.11.2 (Anders et al., 2015) to generate count tables. Count tables for each part were analyzed separately for gene expression differences between social and private information users using the R package *DESeq2* v.1.24.0 (Love et al., 2014). Before the analysis, an additional filtering step was added to ensure that only genes with counts of at least 10 reads in at least 6 samples (n-1 of the smallest sample size) were used in the gene expression analysis. Information strategies were compared using the likelihood ratio test (LRT) approach whereby a full model with information type (SI or PI) and colony-ID as fixed factors is compared with a reduced model containing only colony-ID, taking into consideration colony effects. Genes were considered differentially expressed if the false discovery rate (FDR) corrected p-value was < 0.05. To ensure that the number of DEGs calculated by DESeq2 were not due to chance and to account for the uneven number of samples across bee types and colonies for the antennae, we additionally performed permutations by switching samples from opposite information user groups while maintaining colony structure (see methods in Libbrecht et al., 2016). For example, a sample from the same colony was switched for a different information user group and the number and distribution of DEGs was compared to those calculated from our model in DESeq2. We performed 28 permutations (14 times switching two samples for each group and 14 times switching three samples for each group) and recorded the number of DEGs in each permutation. We then compared this number to the numbers for all possible combinations of our samples to assess the number of DEGs that could be expected by chance.

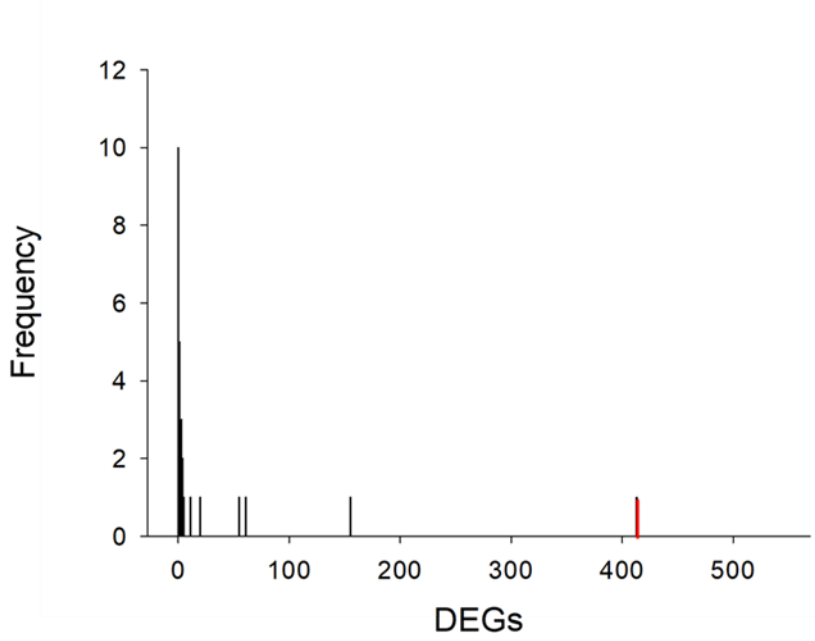

Figure S2: Distribution of differentially expressed genes (DEGs) expected by chance after permutation results (N = 28). The x-axis indicates the number of DEGs and the y-axis indicates the frequency of occurrences. Black bars indicate the results from the permutations, the red bar indicates our results.

We used the R package *DEGreport* v.1.20.0 (Pantano, 2019) to visualize any patterns for all genes going into the analyses and to identify clustering patterns across social and private information users by using the rlog function of DESeq2 to generate normalized count data and the default settings. PCAs (principal components analysis) based on all genes were performed for all tissues to visualize variation between samples. All analysis were performed in R v.3.5.0 (R Developmental Core Team, 2019).

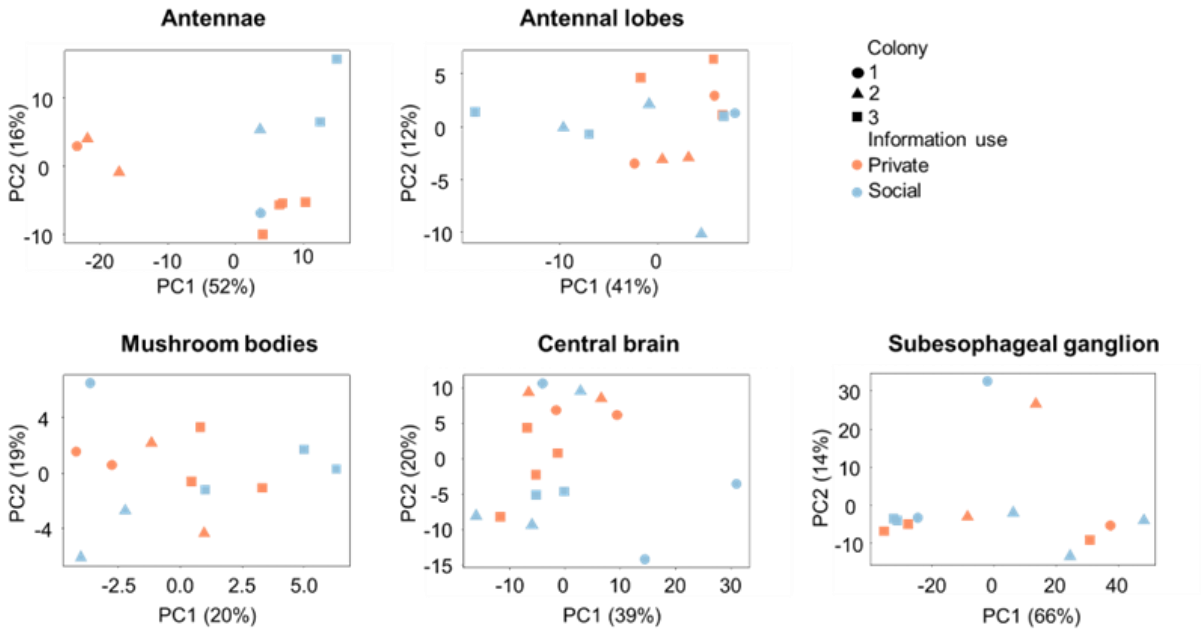

Figure 1: Principal Component Analysis (PCA) plots displaying variance between individual samples based on all genes for each tissue type. Samples are organized by color according to information use strategy (blue = social or red = private) and shapes by colony ID (circles = colony 1, triangles = colony 2, squares = colony 3).

2, squares = colony 3).

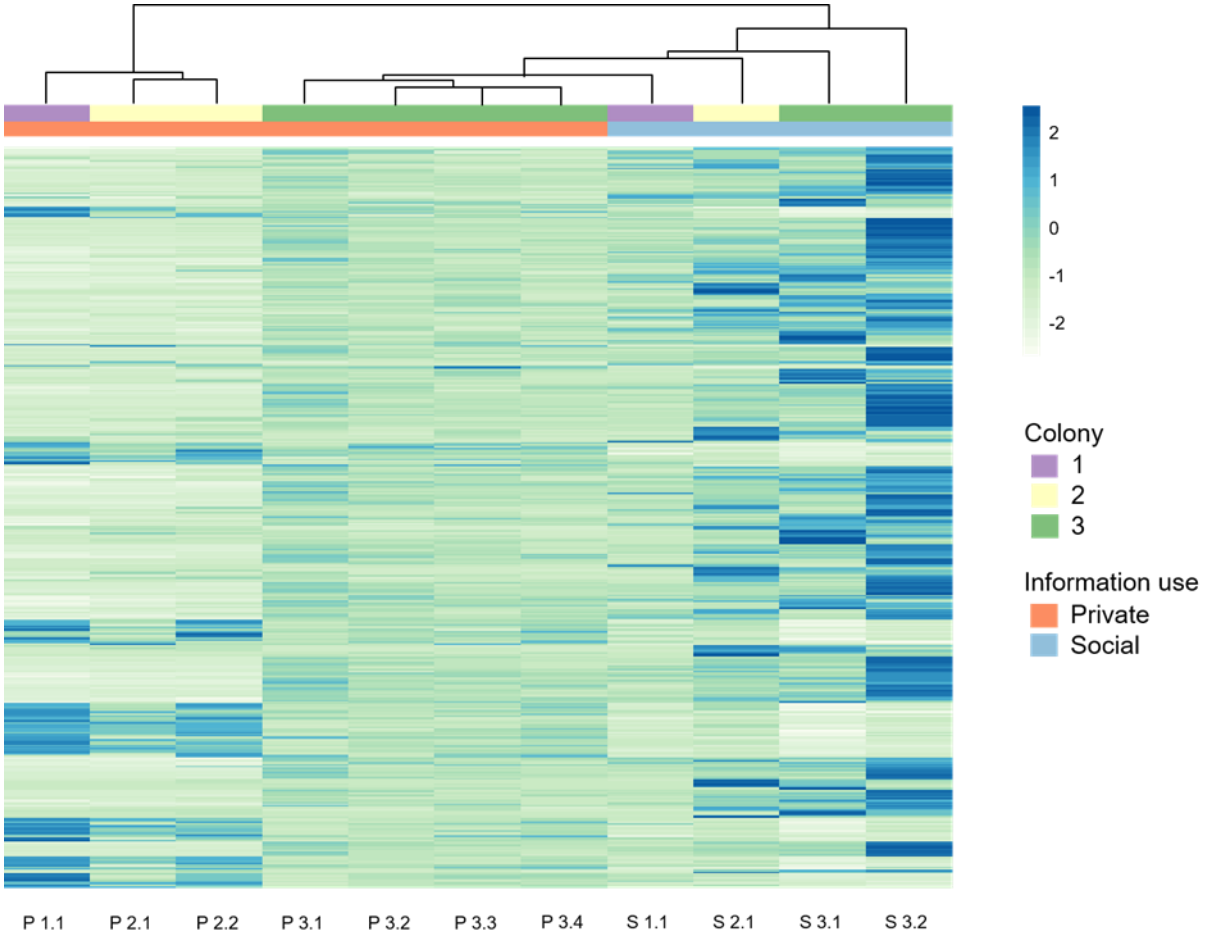

Figure S3: Heatmap of all differentially expressed genes (DEGs) in the antenna. Labels at the end of columns reference individual samples with information use strategies and colony IDs (i.e P 3.1 = third private information user from colony 1). Green indicates higher expression while blue indicates lower expression. All DEGs for the heatmap were not able to have colony ID included, colony ID is used to identify sample origins.

Gene Ontology Enrichment

DEGs were loaded in a BLAST search on the NCBI database against the honey bee genome HvA3.1 to find gene annotations. To further obtain information about Gene Ontology (GO) (Ashburner et al., 2000) and KEGG pathway (Ogata et al., 1999) enrichment we used *InterProScan* v.5.36-75.0 (Jones et al., 2014) on the protein sequences. The R package *topGo* v.2.36.0 (Alexa & Rahnenfuhrer, 2016) was used to perform an enrichment analysis of GO terms and a Fisher's exact test was performed on the list of biological processes.

Results

Dance following of social and private information users

Dance following behavior was analyzed by combining data collected from video analysis for both years. Using a linear mixed-effects model, we found that SI bees followed fewer dances than PI bees during the testing period (5 ± 0.7559 vs. 7.091 ± 1.546 dances) (LME: t-value = 2.218; p = 0.0396). However, SI bees followed dances for longer (more waggle runs per dance) than PI bees (27.214 ± 4.089 vs. 30.818 ± 6.5) (GLMM: z-value = -2.122; p = 0.0338).

Gene Expression Analysis

The likelihood ratio test (LRT) comparison of information use strategies revealed no differences in gene expression between the two information user groups in the central brain, antennal lobes, and subesophageal ganglion (Fig. 1). There was only one differentially expressed gene between social and private information users from our mushroom body calyxes' samples, which encodes for an uncharacterized protein (p = 0.026, gene ID: rna-XR_003305479.1). However, there were 413 differentially expressed genes in the antennae, 318 were higher expressed in social information users and 95 were higher expressed in private information users. To confirm these substantial differences in gene expression in the antennae, we used permutations of samples to assess how this affects the number of DEGs in the antennae. The permutations showed that only very few DEGs were found when 2-3 samples were swapped between the SI and PI groups within their respective colonies (colony ID as fixed factor: 11.89 ± 31.87, N = 28; colony ID not included: 3.25 ± 7.01, N = 28) (Fig. S2). This confirms that the substantial differences in gene expression in the antennae are linked to whether bees belonged to the SI or the PI group. PCA plots used transformed data of all genes to further explore whether there is a clustering of samples based on information use strategies and colony. While a clustering pattern based on information use and colony can be seen for the antennae (Fig. 1), the other tissues showed no clear clustering according to information use.

Exploring the list of DEGs in the antennae revealed that numerous odorant binding and chemosensory proteins differed in their expression in social and private information users. Specifically, we detected five genes for odorant or chemical perception among the upregulated genes in private information users (odorant binding protein 5,11, 19,7 and chemosensory protein 1) and two among the upregulated genes in social information users (odorant binding protein 7 and chemosensory protein 2)

(Fig. 2). Several genes involved in biogenic amine production or signaling were also differentially expressed. Social information users had a higher expression of tyrosine kinase Btk29A; dopamine N-acetyltransferase, tryptophan 5-hydroxylase 1, which are involved in the production of dopamine or serotonin (Vavricka et al., 2014; Coleman et al., 2005; Sasaki et al., 2012), while private information users had a higher expression of one gene tyramine receptor, transcript variant X1, which is associated with biogenic amine signaling (Mustard et al., 2005; Blenau et al., 2000) (Fig. 3). Social information users also had higher expression of the egg yolk precursor protein vitellogenin, a gene that is upregulated in nurses and downregulated in foragers fat bodies and brain (Amdam et al., 2002; Nunes et al., 2013) (Fig. 3).

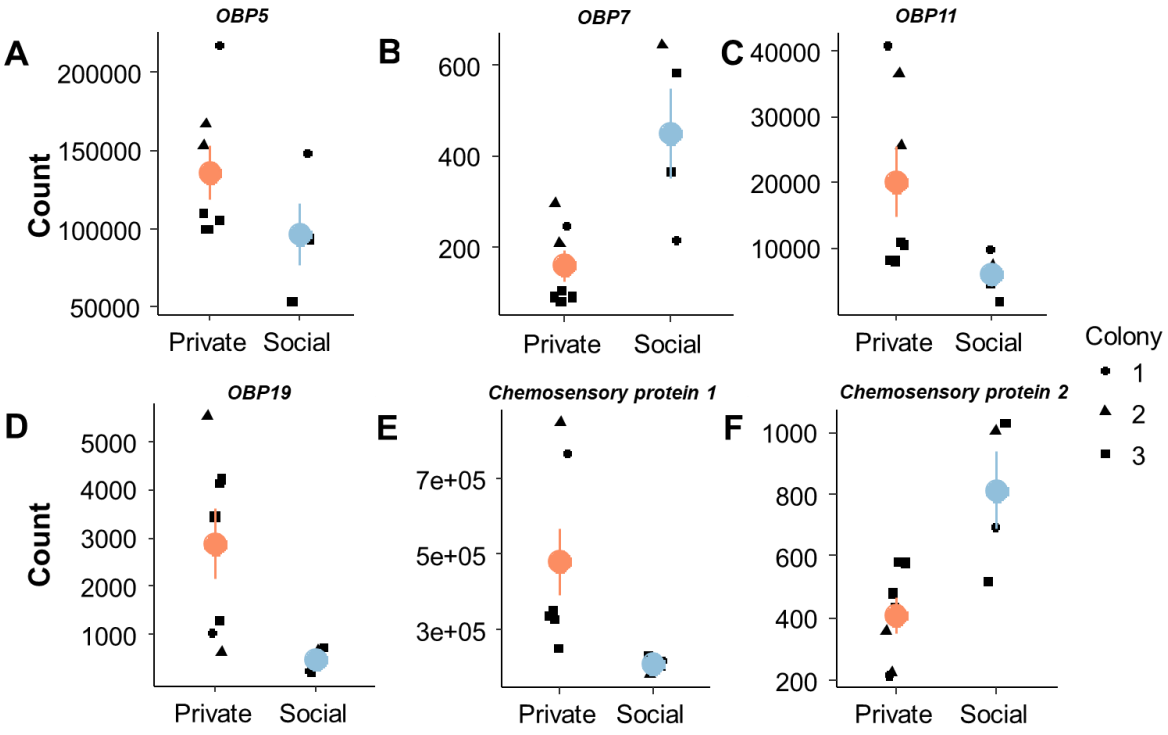

Figure 2: Plots of individual odorant binding protein and chemosensory protein genes. Black dots show counts for individual samples and shapes correspond to the colony ID (circle = colony1, triangle = colony 2, square = colony 3). Colored circles are representative of the average for the respective information strategy (red = private information user, blue = social information user) with confidence intervals. A) OBP11 ($p<0.001$), B) OBP19 ($p=0.001$), C) OBP5 ($p=0.03$), D) OBP7 ($p<0.001$), E) Chemosensory protein 1 ($p=0.009$) and F) Chemosensory protein 2 ($p=0.007$). P-values shown are after FDR correction.

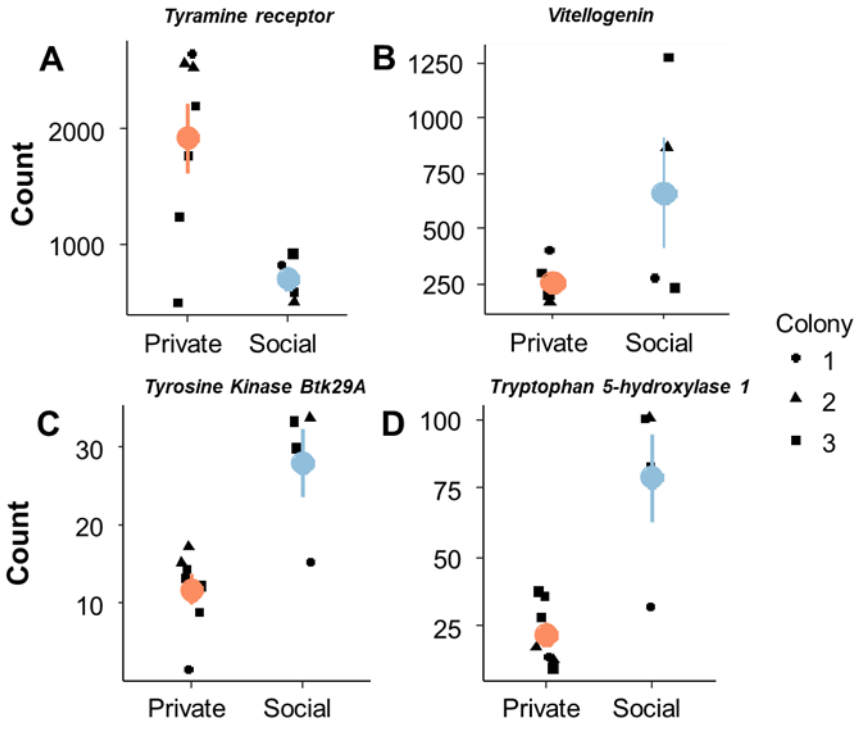

Figure 3: Plots of individual genes associated with biogenic amine production. A) Tyramine receptor (p=0.018), B) Vitellogenin (p = 0.045), C) Tyrosine Kinase Btk29A (p=0.006) and D) Tryptophan 5-hydroxylase 1 (p<0.001).

Gene function and enrichment analysis

Separate GO enrichment analyses of only upregulated genes for each information strategy showed a small number of enriched functions: 9 biological processes enriched in social information users connected mainly on carbohydrate (10 genes) and lipid (7) metabolic process and 18 enriched biological processes in private information users focused on oxidation-reduction process (7) and protein catabolic process (11).

Discussion

Information and its use in animals are an important topic in behavior, ecology, and evolution because information is a critical currency that allows animals to make adaptive decisions in a given situation (*e.g.* Danchin et al., 2004; Dall et al., 2005; Rieucau & Giraldau, 2011; Hoppitt & Laland, 2013). The decision of when to utilize social versus private information to best exploit potential opportunities while avoiding costs is crucial for success and has been studied within a variety of both social and non-social animals (*e.g.* Bonnie & Earley, 2007; Weimerskirch et al., 2010; Grüter & Ratnieks, 2011; Wray et al., 2011; Taborsky & Oliveira, 2012; Haak et al., 2020). However, it is still unclear if and how molecular and neurosensory factors determine an individual's preference for social or private information.

Here we explore whether gene expression differences between honey bee foragers are linked to the use of social or private information about food sources to uncover the potential molecular mechanisms that underlie the decision to decode and use waggle dances in honey bees. Contrary to our prediction, the transcriptomes of all four analyzed brain parts did not differ between bees using these two foraging strategies. Strikingly, however, we found substantial gene expression differences in the antennae. Over 400 genes were differentially expressed between social and private information users, suggesting that the sensory perception of these two forager types differs. This is further supported by expression differences related to odorant binding proteins, chemosensory proteins, and genes associated with biogenic amine production.

The lack of differences in the brain areas was unexpected given that Liang et al. (2012) found extensive differences in whole-brain gene expression between scouts and non-scout foragers (in their study, non-scout foragers could have included both private and social information users). We expected the mushroom bodies to show differences since it has previously been shown that they are involved in multisensory integration, learning, and place memory (*e.g.* Strausfeld et al., 2009; Collett & Collett, 2018). The antennal lobes are involved in odor recognition and memory through the interconnectivity of neurons with the mushroom body and were thus selected as another area of interest (Boeckh & Tolbert, 1993). The central brain has been suggested as an important area for dance communication (Barron & Plath, 2017), while the subesophageal ganglion plays important roles in reward perception and taste (Galizia et al., 2011). Together, these brain regions were thought to process reward and odor perception which could play an important role in the decision to use dance information. Our study

indicates that information use strategies may not primarily depend on integration of information in higher order centers, but that the antennae play a major role in decision-making when facing communication signals.

The 413 differentially expressed genes in the antennae present an array of gene families and functions. Of particular interest are genes coding for odorant binding proteins and those involved in biogenic amine production and signaling due to their potential roles in chemosensory perception. Thus, differences in the perception of chemosensory information cues and signals could result in divergent foraging strategies. While our study cannot disentangle whether gene expression is the cause or the consequence of the information use strategy, they suggest that chemosensory perception by the antennae could be involved in the decision to decode waggle dances and use social information. In many social insects, the antennae play an integral role in social recognition (Ozaki et al., 2005; Sharma et al., 2015; Balbuena & Farina, 2020). Studies in *Oecophylla smaragdina,* for instance, indicated that the density of antennal sensilla is important in regulating behavior, particularly in determining the aggression response behavior to non-nestmates (Gill et al., 2013; Cholé et al., 2019). Similar to other social insects, honey bee foragers first use their antennae to perceive and respond to a variety of chemical signals for navigation (Menzel & Greggers, 2013), efficient nectar/pollen collection (Arenas & Farina, 2012), and dance communication (Thom et al., 2007; Reinhard & Srinivasan, 2009; Gilley et al., 2012).

By transporting odorants*, e.g.* from antennal sensilla to odorant receptors, odorant binding proteins (OBPs) play important roles for olfactory sensitivity (Leal, 2013). They are hypothesized to be important in insect communication (Pelosi et al., 2005), including in the honey bee which use highly complex odors and pheromones to regulate their social activities (Farina et al., 2012; Baracchi et al., 2020). Of the 21 OBPs found in the honey bee, only 9 are exclusively expressed in the antennae. The remaining OBPs are active throughout the honey bee body or specific non-olfactory tissues (Forêt & Maleszka, 2006). Our analysis revealed that workers which rely on private information in the form of spatial memory show higher expression of four odorant binding proteins (*obp5*, *obp11*, *obp19*, and *obp7*), whereas workers that rely on socially acquired information upregulate one (*obp7*). Thus, ~25% of all OBPs found in honey bees were differentially expressed. Of the OBPs that were upregulated in private information users, *obp5* and *obp11* have been previously shown to be exclusively expressed in the antennae and suggest a chemosensory function (Forêt & Maleszka, 2006). Interestingly, *obp11* is mainly expressed in a rare type of antennal sensilla found only in female honey bees, the *sensilla*

basiconica, and is likely to facilitate the function of these sensilla (Kucharski et al., 2016). While the ligand of *obp11* remains unknown, there is evidence that the *sensilla basiconica* play important roles in the perception of cuticular hydrocarbons (CHCs) in ants (Sharma et al., 2015) and may play a similar role in honey bees (Kucharski et al., 2016). This is remarkable because CHCs emitted by dancing bees are known to trigger the use of private information in honey bees (Thom et al., 2007; Gilley et al., 2012). This raises the possibility that a higher expression of *obp11* increases the sensitivity of bees towards CHCs emitted by waggle dancers, thereby triggering private information use. The remaining differentially expressed OBPs (*obp19* and *obp7*) have been shown to be ubiquitously expressed, which suggests they may have additional molecular functions which we currently do not know. Overall, these results indicate a difference in perceptional sensitivity where workers which use private information perceive some chemosensory stimuli more or differently than social information users. This could have far reaching consequences for their behavior given the role that odors play in the decision-making and information use of a forager, e.g. in the identification and learning of floral resources or the perception of cuticular hydrocarbons (von Frisch, 1967; Johnson, 1967; Reinhard et al., 2004; Grüter et al., 2008; Gilley et al., 2012).

Chemosensory proteins serve a similar role as OBPs in transporting chemical stimuli through mechanisms that are not yet well understood. These proteins are heavily concentrated in antennal sensilla but are also expressed in non-olfactory tissues (Forêt et al., 2007; Calvello et al., 2005). Of the six chemosensory proteins found in honey bees (McKenzie et al., 2014), two were differentially expressed in social and private information users, chemosensory proteins 1 and 2. Both chemosensory proteins have been shown to be highly expressed in the antennae (Li et al., 2016), which further supports the view that the differences between the information strategies may be rooted in chemoreception.

Biogenic amines have been associated with regulating learning, foraging behavior, and the transition from in-hive tasks to foraging (Lehman et al. 2006). Biogenic amine signaling is known to change with age and tissue location in honey bees (*e.g.* McQuillan et al., 2012; Perry & Barron, 2013; Reim & Scheiner, 2014; Thamm et al., 2017). Specifically, dopamine, serotonin, octopamine, and tyramine titers in the brain were found to be linked to both task and age (Schulz & Robinson, 1999; Barron et al., 2002; Harris &Woodring, 1991; Kokay & Mercer, 1997). For example, tyramine levels have been linked to novelty seeking in scouting behavior (Cook et al., 2018; Liang et al. 2012), sucrose responsiveness (Scheiner et al., 2002; 2017a; 2017b), and division of labor between nectar and pollen foragers (Hunt

et al., 1995; Scheiner et al., 2001). Dopamine has been shown to modulate sucrose responsiveness (Scheiner et al., 2002), learning (Vergoz et al., 2007) and dance following (Linn et al., 2020), whereas serotonin influences foraging activity (Schulz et al., 2003) and regulates feeding in many animals (French et al., 2014; Blundell & Halford, 1998; Voigt & Fink, 2015). Our findings of an upregulation of genes associated with biogenic amine production, raise the possibility that social information users could differ in their sensory perception as well as sucrose response thresholds compared to private information users. It is noteworthy, however, that the differences we found in relation to biogenic amine signaling were not in the brain. Instead, higher expression of several genes associated with dopamine and serotonin production was found in the antennae of social information users. We did not control for foraging age or experience, which have already been shown to affect gene biogenic amine expression (Reim & Scheiner, 2014). However, the lack of differential expression in brain areas suggests that there was no systematic age bias in our samples.

Another interesting differentially expressed gene, *vitellogenin*, is best known as an egg yolk precursor protein for egg laying organisms. Under normal conditions in social insects, the queen is the main reproductive member and therefore produces the highest levels of *vitellogenin*. However, *vitellogenin* serves important roles for other behaviors and functions outside of reproduction (Nelson et al., 2007; Morandin et al., 2014). For example, nurses produce the next highest levels of *vitellogenin* in their hypherengeal glands to fortify brood food with protein (Amdam et al., 2003; Amdam et al., 2009; Wegener & Bienefeld, 2009). A characteristic feature of the transition from nurse to forager is the drop in vitellogenin levels (Amdam et al., 2003; Messan et al., 2018). Our finding is consistent with evidence that biogenic amine levels are linked to *vitellogenin* and foraging behavior (Linn et al., 2020; Koywiwattrakul et al., 2005), where social information have a similar physiological state to nurses.
Intrinsic factors such as genetic differences could also affect the decision to decode waggle dances. Honey bee queens can mate with more than 20 drones (Strassman, 2001), and the patriline composition of our samples is not known. It is well-known that different patrilines can differ in foraging behaviors, such as foraging age (Kolmes et al., 1989). Paternal effects can also impact gustatory responsiveness and learning abilities (Scheiner et al., 1999; 2001; 2005; Behrends et al., 2007; Scheiner & Arnold, 2009). It is unclear whether systematic patriline differences in the composition of PI and SI bees would lead to differential gene expression only in the antennae, but future studies should explore whether bees using private or social information differ in their patrilines.

Overall, our results suggest an important role of the antennae in mediating decision-making and information use. In particular, we suggest a link between chemosensory perception and the reliance on communication in honey bees. Further studies are needed to disentangle the potential effects of genetic differences (*i.e.* different patrilines), differences in foraging experience, and other factors on gene expression. In addition, we need studies to confirm our hypothesis that SI and PI bees differ in sensory perception such as sucrose response thresholds, odor learning, and electroantennograms.

CHAPTER 3

Sucrose rewards strongly influence transcriptional activity in the central and peripheral nervous system of private and social information using honey bee foragers

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Preparing:

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Abstract

Social animals often differ in their strategies of using information. In honey bees, some foragers rely on information gained from others by following waggle dances, whereas other foragers scout for new food sources or rely on private information gained during previous foraging trips. It is possible that these diverging behaviors are based on different responses to food rewards. In this study, we aimed to analyze whether honey bee foragers that use either private or social information differ in their transcriptional response to food rewards. In addition, we compared gene expression of private and social information users with scouts, *i.e.* foragers that search for new food sources independently. Our gene expression analyses of different parts of the brain and the antenna showed that the influence of reward is strong and similar in foragers utilizing different information strategies. Specifically, we find large-scale differences in gene expression of private and social information users that have been repeatedly rewarded compared to private and social information users that have not been rewarded, especially in the central brain and mushroom bodies, centers of higher information processing. We also find changes in gene expression in the antennae, an indication that reward perception changes following repeated sucrose rewards. Finally, we found that rewarded private information users were most similar to scouts in their gene expression patterns.

Introduction

Learning enables animals to store, integrate, and later use information from their external and social environment to make adaptive decisions. Social animals are especially adept at gaining information from their group members, so called social learning. Foraging behavior of social insects is well studied and numerous examples of social learning, *e.g.* about the identity and location of food sources, have been identified (Leadbeater & Dawson 2017; Dukas et al. 2010; Sumpter & Pratt 2003). The use of social information, e.g., in the form of communication among group members, improves the exploitation of temporary resources and has evolved in parallel in many social insects (Wilson 1971). For example, *Temnothorax* ants can teach their nestmates the route to a new nest site or a food source through tandem running (Franks & Richardson 2006; Franklin 2014). *Vespula germanica,* a social wasp, communicates through pilot flights and intranidal olfactory cues (Taylor et al. 2010; Schueller et al. 2010). In fact, many ants and some stingless bee species make extensive use of chemical communication (Grüter 2020; Morgan 2009; Gordon 2019; Czaczkes et al. 2015). The most complex form of communication is likely reached in the honey bees (*Apini*) through the unique waggle dance, which in combination with trophallaxis allows foragers to communicate the distance, direction, odor, and quality of nectar sources (von Frisch 1967).

The pursuit of a reward is an important motivator of behavior, and insects readily learn to modify their behavior to more easily obtain a reward or to respond to stimuli that are likely to elicit a reward (Perry & Barron 2013; Gruter & Czaczkes 2019). In honey bees, rewards, such as pollen and nectar, and positive social interactions often mediate learning in the context of communication. For example, followers of a waggle dance learn the location of a food source when they follow the dance and the smell of the plant species when they receive nectar samples during trophallaxis (Farina et al. 2005, 2012). Reinforcement and continued performance of behaviors, *e.g*., continued visits of a food source, is thus largely driven by how a reward is perceived by the learner. Individuals vary in their reward perception depending on their gene expression (Kennedy et al. 2021; Wang et al. 2013), age (Peng et al. 2021; Speth et al. 2015), experience (Finkelstein et al. 2019), and current physiological state. For example, hungry animals perceive a food reward often more positively than satiated ones (Pankiw et al. 2001; Crossley et al. 2018). In social insects, especially in Western honey bees (*Apis mellifera*), rewards were shown to modulate the motivation to perform foraging tasks (von Frisch 1967;

George & Brockmann 2019; Incorvaia et al. 2022; Detrain et al. 1999).

Social learning and the use of social information are not the only option a forager has during foraging. Experienced foragers also have the option to use private information gained from previous foraging trips. Private information can consist of either scouting the environment independently or from previously following social information. Numerous experiments show that experienced foragers often prefer private information while inexperienced foragers prefer social information (Grüter et al. 2013; Grüter & Leadbeater 2014; Detrain & Deneubourg 2008; Sumpter 2010). Foragers can associate floral scents with nectar or pollen rewards and reactivate foraging to known locations via the waggle dance (Grüter et al. 2008) or if the previous food source becomes unrewarding (*i.e.* the nectar falls below the individual threshold or is unyielding) a forager will switch to using social information (Grüter et al. 2013) depending on how profitable the forager determined the reward at the initial foraging site (Al Toufailia et al. 2013). Social insects are excellent model systems to study how reward and reward perception affect decision making and the use of either socially provided information from conspecifics as opposed to relying on past experience (private memory information).

The foraging behavior of social insects serves as an excellent behavioral context to explore the molecular basis of reward mediated behaviors. Honey bee foragers in particular, which are typically older female workers ranging from 2 to 6 weeks of age (Robinson 1992), and their foraging behavior have been extensively studied (e.g., von Frisch 1967; Seeley 1995; Menzel 1999; Giurfa 2007). Experiments demonstrate that food rewards influence both the speed of learning, the amount of information that can be learned, as well as how long information can be stored. Moreover, the physiological and molecular basis of reward mediated learning has also been well studied in insect brains, which show that octopamine and dopamine play a major role in signaling reward in the brain (Hammer & Menzel 1998; Burke et al. 2012; Kim et al. 2007; Krashes et al. 2009; Selcho et al. 2009; Linn et al. 2021).

Little is known about how reward perception and reward reinforcement mediate the decision to use social or private information foraging strategies. A previous study (Kennedy at al. 2021) showed that while there are few to no differences in brain gene expression between social information users that utilize the directional information gained from waggle dances and private information users, gene expression in the antennae differed considerably. These findings suggest that social information users perceive chemical cues within the colony differently than private information users. However, this

former study did not reward foragers after they used either foraging strategy. We hypothesize that if a forager is repeatedly rewarded for using either social or private information, this reward reinforcement will create different information-use phenotypes that show a corresponding gene expression signature in both the brain and antennae. This hypothesis is inspired by the self-reinforcement model of division of labor, which predicts that successfully performing a behavior increases the probability that an individual will perform this behavior in the future (Beshers & Fewell 2001; Ravary et al. 2007; Theraulaz et al. 1998).

We trained foragers to artificial sucrose feeders to study if repeated reward reinforcement leads to changes in gene expression via a proposed self-reinforcement process. We compared the gene expression of foragers just after using social or private information, but before being rewarded (pre-rewarded SI and PI foragers) to foragers that repeatedly experienced that the use of either social or private information leads to rewards (post-rewarded SI and PI foragers). We predicted that reward reinforcement would differentially alter brain gene expression in foragers using different information strategies and the strongest differences in gene expression would be expected in the subesophageal ganglion where reward perception is processed (Perry & Baron 2013; de Brito Sanchez 2011). Moreover, we were interested in transcriptomic patterns of scouts, which use neither social nor private information and search for new food sources (Liang et al. 2012). Liang et al. (2012) found vastly different whole brain gene expression in scouts and non-scout foragers. We explored this further by comparing scouts with pre- and post-rewarded foragers that used either social or private information and quantified gene expression in five tissues: antennae, antennal lobes, mushroom bodies, central brain, and subesophageal ganglion. We predicted that scouts differ most when compared to post-rewarded private information users since the latter are likely to show the least interest in new information.

Materials and methods

Colony Set-up

A total of three observation colonies were established prior to the start of experiments. Each colony was complete with three frames containing capped brood, food reserves, pollen, and some empty space. Each observation colony housed approximately 3,000-4,000 *Apis mellifera carnica* workers and a single naturally mated queen. When needed, additional food stores and emerging workers from larger independent source colonies from the apiaries at Johannes Gutenberg Universität in Mainz, Germany

were added. All experiments were performed from July through October 2019.

Training

We trained different cohorts of foragers to one of four feeders, three dance feeders (D.F.) and one training feeder (T.F., focal foragers) using standard training procedures (von Frisch 1967). Each feeder was ~150m from the hive and at least 160m form the nearest other feeder (Figure 1). This spatial arrangement ensured that each cohort only learned the location of one specific feeder location. The cohorts trained to the T.F. location was our focal cohort and foragers only had previous knowledge of one specific location. In order to keep track of individual foraging experiences, each forager was marked with an individual number tag.

Colonies were trained one at a time to reduce recruiting or re-discovery from previously trained colonies. Training was started with a T.F. and one D.F. location ($D.F._{Day1}$) using a 0.8M sucrose solution until the appropriate sample size was reached (T.F. n ~ 60 and D.F. n ~ 25). The following day, a new D.F. location ($D.F._{Day2}$) was introduced using a 0.8M sucrose solution and the previously used D.F. and T.F. locations were dropped to 0.3M sucrose solution to prevent further recruitment while maintaining a constant cohort size. The final day of training, the last D.F. location ($D.F._{Day3}$) was introduced using a 0.8M sucrose solution while all other feeder locations were reduced to 0.3M sucrose solution. Sucrose solutions were unscented to prevent feeders from being discovered by foragers from larger hives in the apiary.

Testing and Sample Collection

Trained foragers were tested for three days, one day per dance feeder. Each day of our 3-day experiment, only one of the three D.F. location was used while the T.F. location remained the same. This way the focal cohort of T.F. trained foragers could use either social information (arriving at the advertised D.F. location for that day) or private information (arrive at the T.F. location using memory). During the first day of testing, sugar solution (0.8 M) was offered at the $D.F._{Day\ 1}$ and the T.F. for 60 minutes. However, the T.F. feeder was empty for the first 30 minutes to ensure that the dance feeder had enough time for advertising to and recruiting potential social information users and at the same time to prevent foragers arriving at the T.F. location from being recruited via waggle dances. T.F. foragers could then use social information and be recruited to the $D.F._{Day1}$ feeder or use private information and return to the T.F. feeder. A small subset of foragers (2-4 foragers/colony per feeder) was collected as soon as they landed on either the $D.F._{Day1}$ or T.F. and killed in liquid nitrogen. These were designated as pre-rewarded social or

private information users, depending on whether they landed on the dance or the training feeder. The remaining focal foragers were recorded as either social or private information users, depending on the feeder they visited. Importantly, they were allowed to collect 0.8M sucrose solution for 60 minutes after using either social or private information to visit the $D.F._{Day1}$ or the T.F.. Their individual tag number and the number of visits to the respective feeder was recorded.

On the second day of testing, the procedure was repeated, but now we offered food at $D.F._{Day2}$ and delayed the sucrose reward at the T.F. location. We recorded the individual focal foragers that used either social (visited $D.F._{Day2}$) or private information (visited T.F.) again. They were again allowed to collect food from their respective feeder for 60 minutes. On the last day of testing, we offered sucrose reward at $D.F._{Day3}$ and T.F. for 60 minutes, with a 30 minute delay at the T.F. location, and collected only the individuals that had consistently used either social or private information for all testing days. Thus, these two groups of foragers consisted either of rewarded social information users that were recruited to all three dance feeders or rewarded private information users that visited the T.F. on days 1-3. All samples were collected in Eppendorf tubes and stored immediately in liquid nitrogen and stored in -80°C freezer for later analysis.

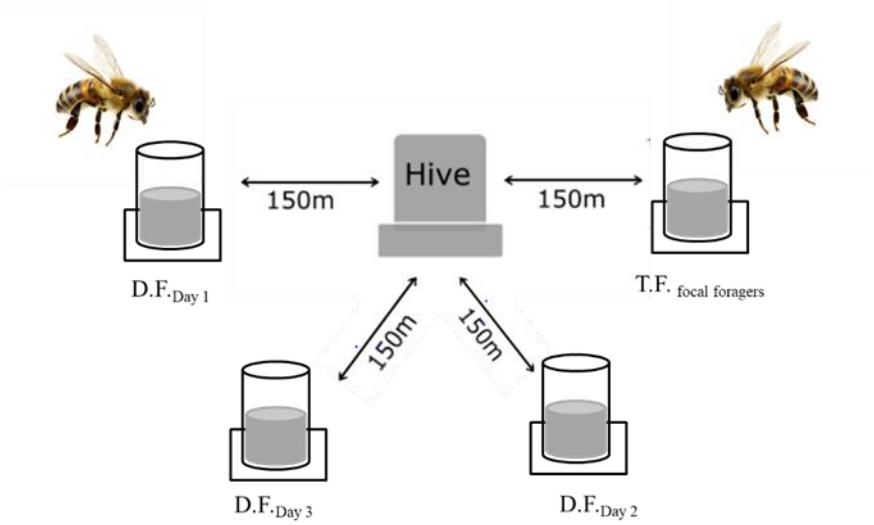

Figure 1: experimental design overview of the spatial arrangement of the behavioral experiment with four forager groups: foragers trained to $D.F._{Day\ 1}$, $D.F._{Day\ 2}$, or $D.F._{Day\ 3}$ were designated as dancers to advertise the location of the assigned feeder and recruit potential social information users (foragers trained to the $T.F._{focal\ foragers}$). During the training period, individual groups of foragers are trained to one of the four feeder locations. During the testing period, food was offered at one of the D.F. locations and delayed at the $T.F._{focal\ foragers}$ to allow for recruitment of social information users. Foragers trained to the T.F. location (our focal foragers) following these dances decide whether to use social information and fly to the D.F. feeder location or use personal information and return to their training feeder. Previous studies have shown that ca. 10-30% will use social information, whereas the rest will use private information (Grüter et al. 2008, 2013; Grüter & Ratnieks 2011; Wray et al. 2012;

Menzel et al. 2011).

Scouting assay

For two observation colonies, the scouting assay was performed after the previously outlined testing, for the last observation colony the scouting assay was performed before. Scouts were identified using a modified "feeder-discovery-assay" (Liang et al. 2012). For this, a new group of foragers were trained to a scented feeder offering a 0.8M sucrose solution a few meters from the colony and marked with colored number tags for individual identification. After 30min, a second feeder with a novel scent was placed near the first feeder. All foragers that discovered the second feeder were marked with acrylic paint on the abdomen. After an additional 30 min, a third feeder was placed close to the first feeder, with a new novel odor. We used sage, lavender, and peppermint as odors with a concentration of 5µl of the essential oil in 100ml sucrose solution. All foragers that discovered both new feeders were considered scouts (Liang et al. 2012). Scouts landing at the third feeder were killed immediately in liquid nitrogen and stored at -80°C until dissection.

Brain Dissection and RNA Extraction

Antennae, antennal lobes, mushroom bodies, central brains, and subesophageal ganglion were dissected from n = 12 pre-reward SI (day 1), n = 12 pre-reward PI (day 1), n = 12 post-reward SI (day 3), n = 12 post-reward PI (day 3), and n = 6 Scouts. Samples that were collected as social information users haven not yet been confirmed, however, previous research using similar methods has shown that the vast majority of foragers trained to a feeder will follow dances for a novel dance feeder. According to Grüter & Ratnieks (2011) study, 98% of foragers that landed on the dance feeder followed at least one waggle dance advertising the dance feeder location. These reports were based on video analysis of in-hive interactions. The head was cut from each sample and fixed to a sterilized petri dish using melted dental wax. The fixed head was placed over ice, using micro scissors cleaned with 70% ethanol, the antennae were cut at the base of the head and crushed in 100mL of TRIzol™ (Invitrogen, USA). A sterile blade was used to make incisions at the bottom of the antennal base, around the eyes, and the ocellus. The remaining tissues and exposed glands and fat were removed. The brain tissue was washed and submerged in chilled bee saline (154 mM NaCl, 2 mM NaH_2PO_4, 5.5 mM Na_2HPO_4, pH 7.2). The optic lobes were removed and discarded first, while the remaining brain tissues were cut in the following order: mushroom body calyces were cut at the base, the antennal lobes, the central brain (the mushroom body peduncles are included in what we refer to as the central brain), and the subesophageal ganglion.

All brain tissues were stored in 100mL of TRIzol™ in -80°C freezer until RNA extractions using the RNAeasy Mini Extraction Kit™ (Qiagen, Germany).

Transcriptome Analysis

A total of n = 150 samples (30 individuals,10 individuals per colony: 4 pre-reward (2 SI, 2 PI), 4 post reward (2 SI, 2 PI), and 2 scouts, dissected into 5 brain parts (antennae, antennal lobes, mushroom bodies, central brain, and subesophageal ganglion)) were sent to Beijing Genomic Institute (BGI) for BGISEQ-500 Transcriptome library preparation to get 100 base pair (bp) paired-end reads, obtaining ~45 Mio clean paired reads per sample sequencing. Raw reads were quality checked using *FastQC* v.0.11.8 (Andrews et al. 2015). Clean reads were aligned to the honey bee genome HvA3.1 using *Hisat2* v.2.1.0 (Langmead & Salzberg 2017). Aligned reads mapping to genes were counted using *Htseq* v.0.11.2 (Anders et al. 2015). Count tables were generated separately for each tissue. Before the gene expression analysis, an additional filtering step was added to ensure that only genes with counts of at least 10 reads in at least six samples ($n - 1$ of the smallest sample size) were used in *Deseq2* v.1.24.0 (Love, Anders, & Huber, 2014). We performed gene expression analysis according to tissue and behavioral group (*i.e.,* 5 tissues: x 5 behavioral groups: pre-reward (SI day 1 and PI day 1), post-reward: (SI day 3 and PI day 3), and scouts).

All analyses were performed in R version 4.1.2 (R Developmental Core Team, 2021). We tested for the interaction between the main effects of reward (*i.e*. day 1 vs day 3) and forager type (*i.e.* SI vs PI) using a likelihood ratio test (LRT) where a full model (containing the interaction of the main effects forager type: SI and PI foragers, and reward: day 1 and day 3) with colony ID as a fixed factor was compared to a reduced model that contained the main effects of forager type and reward without the interaction and colony ID as a fixed factor. Genes were only considered differentially expressed if the false discovery rate (FDR) adjusted p-value was less than 0.05. Subsequently, we performed pairwise comparisons between scouts and all pre- and post-rewarded samples using the same LRT approach where a full model was compared to a reduced model to test whether the term(s) removed in the reduced model explains a significant amount of variation in the data.

Differentially expressed genes (DEGs) were visualized by first normalizing the data using *rlog* function of *Deseq2* (Love et al. 2014) to generate normalize count data according to the default settings. PCA plots were created to visualize clustering patterns of DEGs between groups according to tissue using the R package *DEGreport* v.1.20.0 (Pantano, 2019) (Figure 2). We searched the list of DEGs for

the gene lists with the most DEGs: scouts vs pre-rewarded (day 1) PI foragers and scouts vs. pre-rewarded (day 1) SI foragers (Figure 4) by comparing these lists to our previous study (Kennedy et al. 2021) and our results for the effect of reward of central brain gene expression (Figure 3). We focused primarily on searching for genes that fell into the following classes: biogenic amine receptors, odorant binding proteins and receptors, and chemosensory proteins.

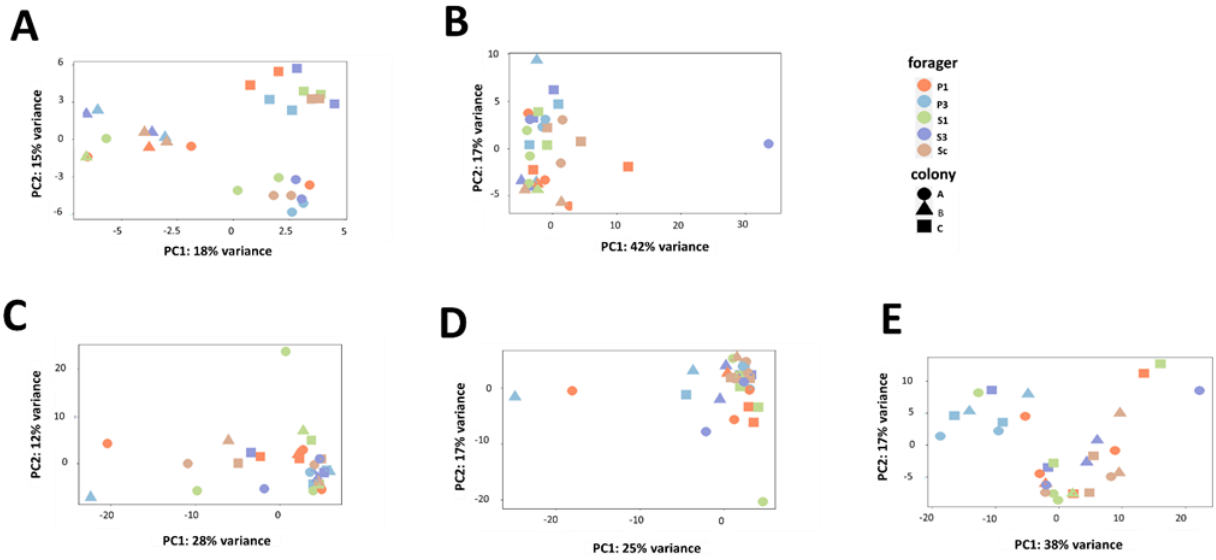

Figure 2: Principal component analysis (PCA) plots displaying variance between individual samples based on all genes for each tissue type (**A**) antennae, (**B**) antennal lobes, (**C**) mushroom bodies, (**D**) central brain, (**E**) subesophageal ganglion. Samples are organized by color according to information use strategy (red = pre-rewarded private, blue = post-rewarded private, green = pre-rewarded social, purple = post-rewarded social, and brown = scouts) and shapes by colony ID (circles = colony A, triangles = colony B, squares = colony C).

Gene Ontology Enrichment

We performed gene ontology (GO) enrichment analysis on each DEG list separately with the *clusterProfiler* (Wu et al. 2021) package using the honey bee genome annotation that can be retrieved with the R package *AnnotationHub* (Morgan et al. 2019) according to default settings and parameters. A Fisher's exact test was performed on the list of biological processes.

Results

Antennae

We found reward (day 1 vs. day 3) to have the largest effect on gene expression (n = 470 DEGs), while

information use (SI vs PI) had a negligible effect on gene expression (n = 2 DEGs). Only two genes interacted (*phospholipase B1, membrane-associated-like* (LOC551879) p = 0.01; *arylsulfatase B* (LOC552073) p = 0.01) between the main effects of reward or forager type (Figure 3). When we performed pairwise comparisons between scouts and the other four forager types (pre-rewarded; and post-rewarded SI and PI foragers), we found that scouts differed the most from pre-rewarded PI (day 1) foragers (n = 304 DEGs) and were most similar to post-rewarded PI (day 3) foragers (n = 3 DEGs). When we compared scouts to pre-rewarded (day 1) and post-rewarded (day 3) SI foragers we found a similar pattern with n = 113 and 78 DEGs respectively (Figure 4).

We only found GO enrichments for the effect of reward *(e.g.* day 1 or 3) on gene expression: chromatin (GO:0000785; **3**), nucleosome (GO:0000786; **3**), protein-DNA complex (GO:0032993; **3**), DNA packaging complex (GO:0044815; **3**), chromosome (GO:0005694; **3**), non-membrane-bounded organelle (GO:0043228; **3**), intracellular non-membrane-bounded organelle (GO:0043232; **3**), nucleus (GO:0005634; **3**); protein-containing complex (GO:0032991; **3**), membrane-bounded organelle (GO:0043227; **3**), intracellular membrane-bounded organelle (GO:0043231; **3**), protein heterodimerization activity (GO:0046982; **3**), protein binding (GO:0005515; **3**), protein dimerization activity (GO:0046983; **3**).

Antennal lobes

We found reward (day 1 vs. day 3) to have the largest effect on gene expression (n = 559 DEGs), while forager type did not have any effect on gene expression (n = 0 DEGs). We also did not find any interaction between the main effects of reward or forager type on gene expression (n = 0 DEGs) (Figure 3). When we performed pairwise comparisons between scouts and the other four forager types (pre-rewarded; and post-rewarded SI and PI foragers), we found that scouts differed the most from pre-rewarded SI (day 1) foragers (n = 346) and were most similar to post-rewarded PI (day 3) foragers (n = 3 DEGs). When we compared scouts to post-rewarded (day 3) SI and pre-rewarded (day 1) PI foragers we found n = 25 and 303 DEGs respectively (Figure 4).

We found no GO enrichment for DEGs of this tissue.

Central brain

We again found reward (day 1 vs. day 3) to have the largest effect on gene expression (n = 1372 DEGs). We did not find forager type to have any effect on gene expression (n = 0 DEGs). We did not find any effect of the interaction between the main effects of reward and forager type on gene expression (n = 0

DEGs) (Figure 3). When we performed pairwise comparisons between scouts and the other four forager types (pre-rewarded; and post-rewarded SI and PI foragers), we found that scouts differed the most from pre-rewarded PI (day 1) foragers (n = 1402 DEGs) and were most similar to post-rewarded PI (day 3) foragers (n = 1 DEG). When we compared scouts to pre- (day 1) and post-rewarded (day 3) SI foragers we found a similar patter with n = 781 and 130 DEGs respectively (Figure 4).

We only found GO enrichments for the pairwise comparison between scouts and pre-rewarded private information users (PI day 1): ion channel complex (GO:0034702; **4**), cation channel complex (GO:0034703; **4**), plasma membrane protein complex (GO:0098797; **4**), transmembrane transporter complex (GO:1902495, **4**), transporter complex (GO:1990351, **4**).

Mushroom bodies

We found reward (day 1 vs. day 3) to have the largest effect on gene expression (n = 602 DEGs), while no DEGs were found for the comparison between social and private information users. We did not find the interaction between the main effects of reward and forager type to have any effect on gene expression (n = 0 DEGs) (Figure 3). When we performed pairwise comparisons between scouts and the other four forager types (pre-rewarded; and post-rewarded SI and PI foragers), we found that scouts differed the most from pre-rewarded PI (day 1) foragers (n = 733 DEGs) and were most similar to post-rewarded PI (day 3) foragers (n = 4 DEGs). When we compared scouts to pre- (day 1) and post-rewarded (day 3) SI foragers we found a similar pattern with n = 476 and 93 respectively (Figure 4).

We only found GO enrichments for the pairwise comparison between scouts and pre-rewarded private information users (PI day 1): Nucleus (GO:0005634; **8**), membrane-bounded organelle (GO:0043227; **8**), intracellular membrane-bounded organelle (GO:0043231; **8**), chromosome (GO:0005694; **3**), intracellular organelle (GO:0043229; **8**), intracellular anatomical structure (GO:0005622; **9**), organelle (GO:0043226; **8**).

Subesophageal ganglion

We found reward (day 1 vs. day 3) to have the largest effect on gene expression (n = 253), while forager type had no effect on gene expression (n = 0 DEGs). We found two genes that showed a significant interaction between the main effects reward and forager type (Figure 3). When we performed pairwise comparisons between scouts and the other four forager types (pre-rewarded; and post-rewarded SI and PI foragers), we found that scouts differed the most from pre-rewarded PI (day 1) foragers (n = 172

DEGs) and were most similar to post-rewarded SI (day 3) foragers (n = 34 DEGs). When we compared scouts to post-rewarded PI (day 3) and pre-rewarded SI (day 1) foragers we found n = 36 and 170 DEGs respectively (Figure 4).

We only found GO enrichments for the pairwise comparison between scouts and pre-rewarded social information users (SI day 1): Chromatin (GO:0000785, **2**), nucleosome (GO:0000786; **2**), protein-DNA complex (GO:0032993; **2**), DNA packaging complex (GO:0044815; **2**), chromosome (GO:0005694; **2**).

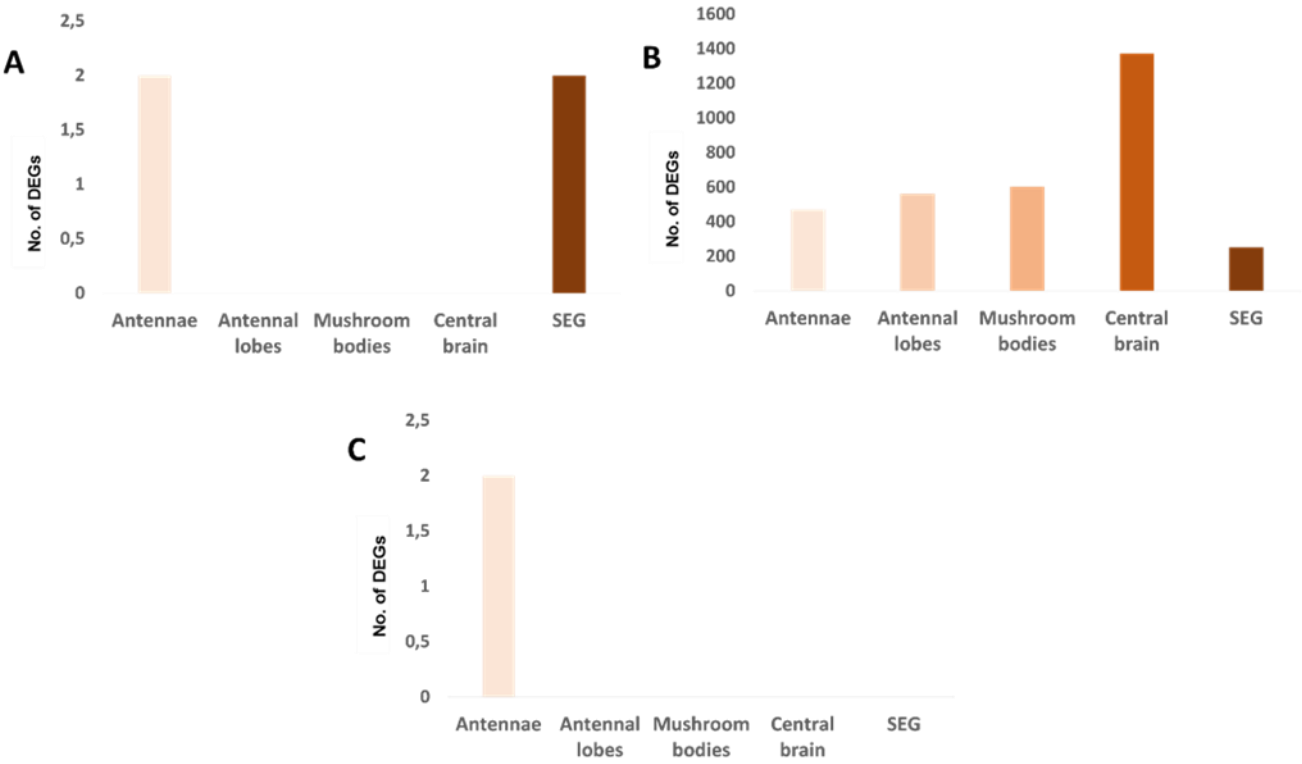

Figure 3: Summary of differentially expressed genes (DEGs) for each tissue analyzed: antennae, antennal lobes, mushroom bodies, central brain, and subesophageal ganglion (SEG). We used a likelihood ratio test (LRT) to analyzed **(A)** the interaction of the main effects reward (day 1 vs. day 3) and forager type (social information user vs. private information user) and main effects separately: **(B)** the effect of reward and **(C)** the effect of forager type.

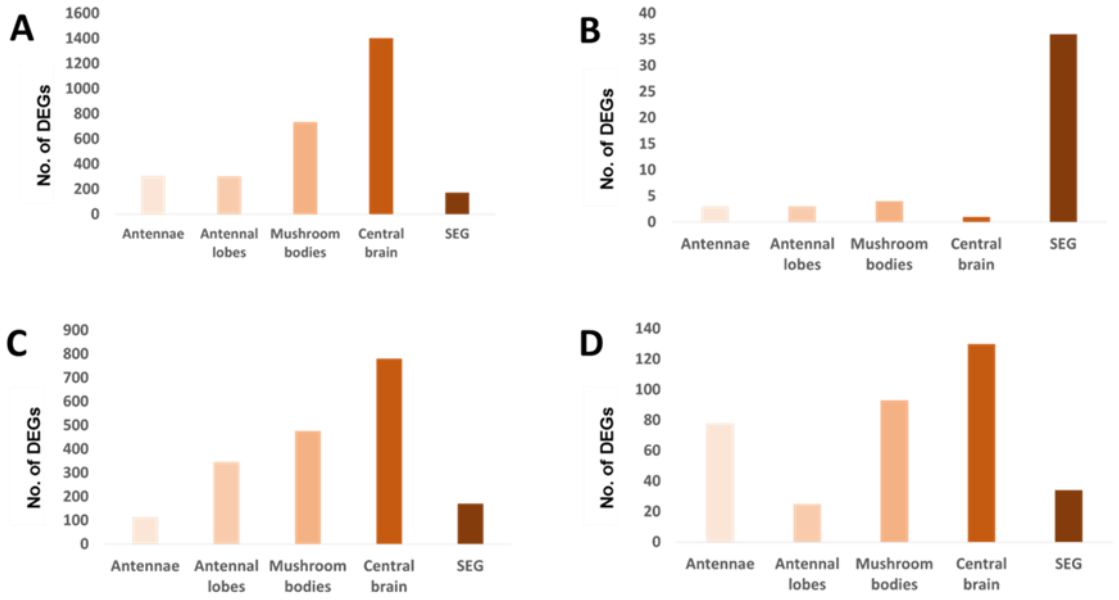

Figure 4*:* Summary of differentially expressed genes (DEGs) for each tissue: antennae, antennal lobes, mushroom bodies central brain, and subesophageal ganglion (SEG) analyzed separately. We performed pairwise comparisons between scouts and each forager type (**A**) scouts vs. pre-rewarded private information users (PI day 1); (**B**) scouts vs. post-rewarded private information users (PI day 3); (**C**) scouts vs. pre-rewarded social information users (SI day 1); and (**D**) scouts vs. post-rewarded social information users (SI day 3).

Candidate gene search

We compared the gene lists with most DEGs: scouts vs. pre-rewarded PI foragers and scouts vs. pre-rewarded SI foragers to our previous study (Kennedy et al. 2021) and our results for the effect of reward. We found that reward induced the upregulation of biogenic amine receptors: *tyramine receptor* (TyrR, p = 0.02), *octopamine receptor beta-3R* (LOC412994, p = 0.005), *D2-like dopamine receptor* (Dop3, p = 0.004), *dopamine receptor 2* (DopR2, p = 0.04); odorant binding proteins and receptors: *odorant binding protein 10* (Obp10, p = 0.003), odorant receptor 35 (Or35, p = 0.005); and chemosensory proteins: *chemosensory protein 6* (CSP6, p = 0.04) in the central brain (Table 1).

Table 1: results of candidate genes upregulated by reward in the central brain

biogenic amine		
Dop3	p = 0.004	post-reward
DopR2	p = 0.04	post-reward
LOC412994	p = 0.04	post-reward
TyrR	p = 0.02	post-reward
odorant binding		
Obp10	p = 0.003	pre-reward
Or35	p = 0.005	post-reward
chemosensory		
CSP6	p = 0.04	pre-reward

We only found a subset of these genes in the DEG list of scouts vs. pre-rewarded SI foragers: *D2-like dopamine receptor* (Dop3, p = 0.03), *chemosensory protein 6* (CSP6, p = 0.01), *odorant binding protein 10* (Obp10, p = 0.02) (Table 3). However, we found all of the genes associated with reward, except CSP6, in the DEG list of scouts vs. pre-rewarded PI foragers with the addition of: octopamine receptor (Oa1, p = 0.04), *chemosensory protein 2* (CSP2, p = 0.01), *odorant receptor 2* (Or2, p = 0.01), *odorant receptor 170* (Or170, p = 0.001) (Table 2).

Table 2: candidate genes differentially expressed between scout and pre-rewarded PI foragers

biogenic amine		
Dop3	p = 0.002	scouts
DopR2	p = 0.01	scouts
LOC412994	p = 0.005	scouts
TyrR	p = 0.04	scouts
Oa1	p = 0.04	scouts
odorant binding		
Or2	p = 0.01	scouts
Or35	p = 0.01	P1
Or170	p = 0.001	scouts
chemosensory		
CSP2	p = 0.01	scouts

We also compared the results of the effect of reward on the antennae to our previous study (Kennedy et al. 2021). We were only able to find one gene falling into the class of biogenic amine receptor: *serotonin receptor* (5-HT2alpha, p = 0.01).

Table 3: candidate genes differentially expressed between scouts and pre-rewarded SI foragers

biogenic amine		
Dop3	p = 0.03	scouts
odorant binding		
Obp10	p = 0.02	S1
chemosensory		
CSP6	p = 0.011	S1

Discussion

In this study, we investigated how food rewards and information use strategies in honey bee foragers affect gene expression by analyzing transcriptional activity in the peripheral (antennae) and central nervous system (different parts of the brain). Our data show that reward has a much stronger influence on gene expression, leading to large-scale transcriptional changes. In contrast, we did not find differences in most parts of the brain between foragers using private and social information, confirming our earlier findings (Kennedy et al. 2021). We also found no evidence for an interaction between information use strategy and reward reinforcement (Figure 3), suggesting that a food reward has a similar effect on brain activity of foragers relying on private information as those receiving information from their nestmates. However, an earlier study revealed not only two but several hundred genes to be differentially expressed between social and private information users in the antennae (Kennedy et al. 2021). We believe this is due to the differences in training foragers to the artificial sucrose feeders. One study used scented food sources (Kennedy et al. 2021), while in this study we used unscented food sources. If odor perception is important for some foragers, then our experiment without food odors might create a different pool of foragers that use social information.

The strongest transcriptional shifts with reward reinforcement were found in the central brain, important for locomotion and navigation, followed by the mushroom bodies, well-known for its role in learning and memory (Figure 3) (Pfeiffer & Homberg 2014; Collett & Collett 2018; Strausfeld et al. 2009). Our findings diverged from our initial predictions that we would find the most differences in transcriptional activity in the subesophageal ganglion. While this tissue has been shown to be responsive to taste (de Brito Sanchez 2011) and reward (Sinakevitch et al. 2005) we found little evidence that

rewards change gene expression in this tissue. We cannot discredit the role of the subesophageal ganglion in reward signaling. The role of the subesophageal ganglion could be to process the reward signal and therefore we find that rewards do not significantly change gene expression over the course of our three days of testing. If the main function of the subesophageal ganglion is to signal reward information, then we can expect for the gene expression to remain relatively constant over time as foragers are exposed and need to process rewards constantly in the environment and in the colony through trophallaxis.

Scouts, *i.e.*, foragers that independently search for new food sources, were most similar in their transcriptional activity to foragers that were rewarded repeatedly, especially those that use private information (Figure 4). Pre-rewarded foragers of either information use strategy showed vastly different gene expression compared to scouts, especially in the central brain (Figure 4). Scouts are characterized by their novelty seeking behavior (Liang et al. 2012; Seeley 1983), so we predicted that scouts would differ the most from post-rewarded PI (day 3) foragers because they were the least interested in new information (*i.e.* finding new foraging locations). However, our results are plausible because scouts repeatedly experienced rewards in our experiment, although they found them at different food sources. This is further support for the view that receiving food rewards rather than the information use strategy used by a forager has the biggest effects on brain gene expression and sheds new light on the findings of Liang et al. (2012). Their non-scout foragers probably consisted of a mix of private and social information users with varying amounts of reward experience. Their results might be explained by differences in reward experience, rather than the difference in foraging strategy.

While little is known about the molecular basis of differences in information use phenotypes among foragers (*i.e.* social and private information users), we know from previous studies that odor perception in the antennae (Kennedy et al. 2021) and reward perception and reinforcement in the brain have a potential role in regulating foraging strategies. Scouts have a unique gene expression pattern characterized by dopamine, octopamine, glutamate, and GABA signaling (Liang et al. 2012). The expression of these biogenic amines could result in scouts finding higher sucrose concentrations more rewarding than the average forager. Post-rewarded private information users could be similar in their resistance and reluctance to follow social information as they find the advertised sucrose reward, through trophallaxis, less desirable after reward reinforcement for using private information. In the future, we plan to compare the sucrose responsiveness of scouts, pre-reward PI and SI forager, and post-

rewarded PI and SI foragers.

In insects, most of what is known of reward processing is derived from studies of reward learning. This is localized to the mushroom bodies, subesophageal ganglion, and antennal lobes and organized by a network of hierarchically arranged modulatory circuits, especially those involving octopamine and dopamine (Perry & Barron 2013). Dopamine is regarded as the reward and motivation molecule in mammals (Dayane & Balleine 2002; Wise 2004). Octopamine has historically been considered to be the signal for reward in insects (Hammer 1993; Hammer & Menzel 1998; Schwaerzel et al. 2003). Dopamine and octopamine signaling is especially prominent in the mushroom bodies (Kenne & Waddell 2005; El-Kholy et al. 2015). Therefore, the presence of mushroom body peduncle tissue in the central brain could contribute to the robust results of gene expression in this tissue compared to the isolated mushroom body calyces.

We find an upregulation of dopamine (*Dop3* and *DopR2*) and octopamine (*LOC412994* and *Oa1*) receptors in the central brain. Dopamine and octopamine were both upregulated in scouts compared to pre-rewarded (day 1) SI and PI foragers (Table 2 and 3). According to Liang et al. (2012) neurochemical treatments with octopamine increased scouting probability while treatment with dopamine antagonist decreased the probability. When we compared the gene expression patterns to the profile of genes effected by reward, irrespective of foraging strategy, in the central brain we find a similar pattern between pre- and post-rewarded foragers (Table 1). Post-rewarded foragers upregulate dopamine and octopamine in a similar fashion as scouts. It has been shown that dopaminergic and octopaminergic neurons are found in the vertical lobes of the mushroom bodies (Schürmann et al. 1989; Sinakevitch et al. 2011; Sinakevitch et al. 2005) which further supports the presence of mushroom body tissue in the central brain contributing to such robust gene expression. The upregulation of the biogenic amine receptors dopamine and octopamine suggest that the neurotransmitter systems that appear to be involved in the regulation of scouting and novelty seeking behavior in honey bees are also involved in reward learning.

Alternatively, our results could be explained by differences in age or foraging experience as post-rewarded foragers were 2 days older and more experienced than pre-rewarded foragers. A number of genes that are important in reward processing are known to change their expression as a result of age and experience in honey bees (McQuillan et al. 2012; Reim & Scheiner 2014; Peng et al. 2021). While we deem it unlikely that the extensive changes we observe are due to age differences, more research is

needed to disentangle the effects of age, experience, and repeated rewards.

Overall, we provide evidence for the role of reward signaling through the successful use of a foraging strategy as a mechanism for creating a transcriptional footprint that increases the likelihood that a forager will continue to use the same strategy in future foraging decisions. We propose that there is positive feedback when an initial behavior causes experience that influences gene expression and the subsequent behavior. We also provide new insights on our understanding of scouting behavior through our comparisons of scouts to pre- and post-rewarded foragers. We show that the same genes involved in novelty seeking also can be attributed to reward learning and signaling We plan to disentangle gene expression differences that cause the use of a foraging strategy from gene expression differences that are caused by the successful use of a foraging strategy by linking the observed gene expression profiles to behavioral data. In the future, we will explore the dance following behavior of foragers that have found rewards using social or private information. We also intend to investigate whether the genes that respond to reward reinforcement play important roles in the putative self-reinforcement of social learning.

General Discussion

Anissa C. Kennedy

“The role of the infinitely small in nature is infinitely great.”

— Louis Pasteur

Main findings

In this thesis, we aimed to establish 1) the role of queen presence on both worker gene expression and subsequent foraging activity (**Chapter 1**), 2) if the decision to use a certain foraging strategy was driven by gene expression patterns in the central and peripheral nervous systems (**Chapter 2**), 3*a*) if rewarded foraging strategies are linked to the self-reinforcement model of division of labour and, 3*b*) how the gene expression signatures of foragers using different information use strategies compare to one another (**Chapter 3**). We found that queen pheromone plays a crucial role in gene expression patterns during a sensitive period in adult worker development, however the associated gene expression signature does not contribute to differences in foraging activity (**Chapter 1**). We decided to further explore intrinsic differences that influence foraging strategies focusing on gene expression patterns in different brain tissues. We explored a foragers decision to utilize either social or private information strategies. We did not find a gene expression signature difference in the central nervous system after analyzing four key brain regions: antennal lobes, mushroom bodies, central brain, and subesophageal ganglion. Surprisingly, we found a large-scale gene expression differences in the antennae of foragers implementing different information use strategies (**Chapter 2**). Lastly, we tested whether reward reinforcement for using a particular information use strategy creates different information-use phenotypes and corresponding gene expression signatures. We found that reward had a large influence on gene expression patterns between foragers that used different information use strategies. We then compare pre- and post-rewarded foragers to another category of forager observed in honey bees, scouts. We demonstrate that scout's gene expression signature is most comparable to post-rewarded private information users and the most divergent from pre-rewarded private information users (**Chapter 3**).

Queen presence in regulating worker gene expression and foraging activity

It is largely accepted that the evolution of social behavior acted on conserved mechanisms that control responses to stimuli in the environment (van Oystaeyen et al. 2014). Social cues, like other environmental cues, convey information critical for animal survival and reproduction. The basics of how individuals respond to their environment are similar across species and involve sensory structures, signal transduction cascades, and various forms of neural plasticity (Harris-Warrick 2000; Kandel et al. 2000). Genes involved in the perception and processing of sensory information and the responses that are then triggered are thus likely to be involved in social evolution. However, our understanding of social evolution is limited by the fact that the identification of genes involved in social behavior is still

in the early phases and no general principles have yet emerged.

The internal colony environment plays and integral role in regulating honey bee worker behavioral transitions from within the colony to foraging outside the colony. The majority of research focuses specifically on this transition from nurse to forager (Heylen et al. 2008; Jefferson et al. 2013; Amdam & Omholt 2003; Ament et al. 2008; Liu et al. 2019; Johnson et al. 2008). However, there is much plasticity within these individual behaviors. For example, foragers are typically older and perform the behavior during the last weeks of life. This process of behavioral development is sensitive to social feedback. If there is a decline in the number of foragers, in-hive workers accelerate their behavioral development and begin foraging precociously to compensate (Huang & Robinson 1996; Robinson et al. 1994). Similarly, if there are too many foragers and a lack of nurses, foragers can reverse their behavioral development and revert from foraging to nursing roles (Huang & Robinson 1996; Robinson et al. 1992).

Arguably the most important colony member responsible for regulating worker behavioral transition is the queen. Queen presence has been an understudied aspect of colony environment that has been attributed to regulate the ontogeny of worker behaviors (Whitfeld et al. 2006; Grozinger et al. 2003; Pankiw et al. 1998). However, there is little evidence to understand the underlying mechanisms that link how queen presence regulates workers behavioral transitions. Here, we use queen pheromone (QMP) to causally link gene expression patterns associated with QMP exposure to foraging activity by measuring total foraging trips and average foraging duration. We demonstrate for the first time that workers have a sensitive period during adult development to QMP exposure at around 1-2 days after emerging that lasts long-term throughout adult life (**Chapter 1, Fig. 1**). The majority of upregulated genes were found in treatment groups without QMP exposure and in the mushroom bodies (**Chapter 1, Fig. 1**). Interestingly after both GO and KEGG enrichment analysis, there were no particular processes that were enriched. We predicted, based on previous studies, that workers experiencing queenlessness would activate transcription factors that subsequently would activate regulatory pathways downstream selecting for nursing behaviors and repressing foraging behaviors. However, we were not able to link the gene expression signatures of either treatment group to foraging activity (**Chapter 1, Fig. 4**). Yet we show that queen presence influences upstream transcriptional activity indirectly through chemical signals. Although queen pheromones were long thought to be highly taxon-specific (Le Conte 2008), recent studies have shown that structurally related long-chain hydrocarbons act as conserved queen

signals across several independently evolved lineages of social insects (Holman et al. 2010; Holman et al. 2013; Van Oystaeyen et al. 2014; Holman 2014). These results imply that social insect queen pheromones are evolutionarily conserved and likely derived from an ancestral signaling system that was already present in their common solitary ancestors. The upstream gene expression changes that last throughout the life of a worker suggest that QMP alters transcription activity globally in the genome early in life, however we require future studies to further explore how this gene expression profile acts to influence worker behavior.

Intrinsic physiology's role in influencing the information use of foraging behavior

A major goal in the behavioral sciences is to identify genes that influence social behavior and understand how their protein products influence the structure and function of the nervous system. Studies conducted under ecologically relevant conditions make it easier to interpret molecular data within a broad integrative framework that includes both mechanistic and evolutionary perspectives (Wilson 1975; Robinson 1999; Robinson et al. 1999; Miklos & Maleszka 2000). The relations among genes, the brain, and social behavior are complex and span across several different time scales (Robinson 2004), ranging from organismal development and physiology all the way to evolutionary time. Genes do not directly translate to a specific behavior but rather encode molecular products that build and govern the functioning of the brain, through which behavior is expressed. Brain development, brain activity, and behavior all depend on both inherited and environmental influences. There is now evidence to support that social interactions can alter brain gene expression and behavior (Robinson et al. 2008; Lockett et al. 2016; Toth & Robinson 2007).

One of the fundamentals of social behavior is communication (Floreano 2007). Diverse social behaviors involve the production, reception, and interpretation of signals that influence individual behavior in a manner that depends on social context. Since the discovery of the unique dance language of the honey bee (von Frisch 1967) we have known that foragers have the choice to follow the information provided by the waggle dance. Dance following throughout the life of forager is not static. For example, an inexperienced forager can find her first food source either by scouting or being a recruit (Lindauer 1952; Seeley 1983). On the other hand, an older experienced forager can experience foraging that is interrupted either through weather, season, or food availability. This type of forager can be reactivated to foraging to previously known locations by observing waggle dances or by independently checking food sources (Gil & Farina 2002).

Here we classified foragers by the foraging strategies they used. The two types of information use strategies we focused on were social information users (SI), foragers that follow the information provided by the dance language and private information users (PI), foragers that rely on route memory to previously discovered locations from either a different waggle dance or scouting. While previous research has studied the various factors that drive the preference for one strategy over the other (Gruter & Ratnieks 2011; Gruter et al. 2013; Wray et al. 2012), empirical evidence suggests that at least some foragers readily switch between information use strategies.

This thesis highlights the missing link in genomic research regarding these two behavioral phenotypes among foragers. Traditional models of animal behavior present the brain as the control center; however, we show that the perception of signals is the major and potentially only distinguishing process necessary to make a decision on whether to use social or private information. We find over 400 DEGs in the antennae between foragers that use SI vs PI (**Chapter 2)**. Many of the genes fall into gene networks associated with odorant or chemical perception and biogenic amine production (**Chapter 2, Fig. 2 and 3**).

The role of reward in influencing information-use phenotypes of foragers

Behavioral scientists usually assume that animals use a motivational mechanism for decisions such as action selection and time allocation, allowing the maximization of their fitness. The choice of the best option can be defined in terms of satisfaction, welfare, and reinforcement or in fitness (Rangel et al. 2008). Animal decisions regarding foraging activities are studied in situations where the resource distribution is clumped in patches (Nonaka & Holme 2007; Hills et al. 2008; Wilke & Barrett 2009; Louâpre et al. 2010; Giraldeau & Dubois 2008). It is important for an individual to decide when to continue foraging on the current patch and when to leave the patch in order to maximize the yield. Behavioral ecologists have suggested that insects such as parasitoids and bumblebees use a motivational mechanism (Waage 1979; Driessen et al. 1995) to perform these tasks. According to the motivational hypothesis of making the decision to leave the patch, an animal enters a patch with an initial motivation that decreases as long as no rewarding item is found. Each time an item is discovered, the motivation increases with a positive reward or decreases with a negative reward. The animal leaves the patch when the motivation falls below a certain threshold or after satiation from finding many rewards.

Reward perception motivates a honey bee forager to return to the colony and perform the waggle

dance to recruit nestmates to further exploit the profitable resource (von Frisch 1967). We further investigated whether reward reinforcement in foragers that have decided on a particular foraging strategy (*i.e.* social vs. private information use) would induce a unique genomic signature and/or result in different information use phenotypes. We compared 5 tissues spanning across the central and peripheral nervous systems: antennae, antennal lobes, central brain, mushroom bodies, and subesophageal ganglion. We found that reward reinforcement did have a large impact on gene expression (*i.e.* SI vs. PI) in all tissues analysed (**Chapter 3, Fig. 3**). However, the central brain had the largest number of differentially expressed genes (DEGs) in response to reward reinforcement (**Chapter 3, Fig. 3**). However, contrary to our prediction, the information use strategy a forager implemented did not affect gene expression patterns (**Chapter 3, Fig. 3**).

Brain regions involved in reward processing are collectively known as the 'reward system', and are involved in orchestrating behavioral responses to various rewarding stimuli such as food and reproductive activity (McNeill et al. 2015). Honey bees and other social insects are well known for many intricately coordinated cooperative behaviors, but it is not yet well understood how reward systems are involved in regulating these behaviors. An animal's response to reward is complex, inducing changes in internal state that activate behavioral outputs. Several insect brain regions are known to respond strongly to sucrose ingestion. These include the antennal lobes, subesophageal ganglion, lateral protocerebrum, mushroom bodies, and antennal mechanosensory and motor centers (Giurfa & Sandoz 2012; Waddell 2010). Recent studies have suggested that rewarding and aversive sensory stimuli are integrated in the mushroom bodies, and mushroom body output neurons carry the internal state information required for correctly selecting a behavioral output (Aso et al. 2014; Giurfa 2013; Menzel 2014; Strube-Bloss et al. 2011).

For the purposes of this thesis, we divided foragers into five subgroups: pre-rewarded SI, pre-rewarded PI, post-rewarded SI, post-rewarded SI, and scouts. We find that scouts were overall more similar to post-rewarded PI foragers and differed the most overall from pre-rewarded PI foragers. A similar pattern was observed when scouts were compared with SI foragers (**Chapter 3, Fig. 4**). These results suggest that while post-rewarded foragers are least likely to switch foraging strategies or be interested in new foraging locations, these foragers are the most similar to scouts (**Chapter 3, Fig. 4**). Liang et al. (2012) has shown that scouts are different from other foragers in that they are consistently novelty seeking and have a unique gene expression profile. However, we uncover that food rewards as

demonstrated through post-rewarded foragers, also induce a similar gene expression profile as scouts (**Chapter 3, Table 1 – 3**).

Outlook

In this thesis we present relevant data on the molecular basis of honey bee foraging behavior. In **chapter 1** we have contributed new data that suggests that queen presence signalled through queen pheromones affects gene expression during a sensitive period during adult development. While the gene expression profiles are altered long-term, we could not find a correlation to foraging activity. Future research focusing on other foraging behaviors could elucidate how queen pheromones regulate the onset of foraging. In **chapter 2**, we show that foragers that use either a social or private information strategy have a unique gene expression profile in the antennae but not in brain tissues. We expected to see differences in gene expression in brain tissues as the central nervous system is the center for higher order processing. Our results suggest that social or private information foraging strategies differ in their sensory perception cues and future research can build upon these results by performing appetitive learning assays such PER to trained odors to link the gene expression differences in the antennae to potential differences in learning and memory. In **chapter 3**, we focused on reward reinforcement to determine if foragers would be more likely to re-use the same foraging strategy. We found that being rewarded over two consecutive days had a stronger impact on gene expression profiles than the foraging strategy used. We need further exploration to understand the differences that drive social information use and reward perception. Most of the appetitive learning assays make use of the proboscis extension response (PER), which occurs when the antennal tip of a worker is touched with sucrose solution. This response can be used to analyze responsiveness to sucrose It can also be used to compare chemoreception in groups of bees which differ in their foraging behavior, age or genetic background (Scheiner & Page 1999; Pankiw & Page 2000; Page et al. 1998). The large differences between post- and pre-rewarded foragers could also be explained by age effects, since post-rewarded foragers were 2 days older than pre-rewarded foragers. Previous studies have already demonstrated that forager age is linked to changes in gene expression (Peng et al. 2021). However, we deem it unlikely that age explains these large-scale differences because genes that have been shown to change with age were not among our differentially expressed genes. We plan to link our gene expression profiles to our behavioral data by comparing the dance following behavior of pre- and post-rewarded foragers

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Curriculum Vitae

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Education

2018-2022 University of Mainz, Mainz, Germany

PhD in Biology

Supervisors: PD Dr. Christoph Grüter & Prof. Dr. Susanne Foitzik

Title: **Genomic Basis of Honey Bee Foraging Behavior**

2016-2018 University of North Carolina at Greensboro, Greensboro, North Carolina USA,

MSc in Biology

Supervisor: Olav Rueppell

Title: **Increased Stress Resistance in Socially Manipulated Honey Bee (*Apis mellifera*) Workers.**

2012-2016 Winston-Salem State University, Winston-Salem, North Carolina USA

BSc in Biology

Research Summary

2018-2022

During my PhD, I studied the genomic basis of honey bee foraging behavior. I explored three influences on gene expression 1) social environment by manipulating exposure to queen pheromones, 2) intrinsic

gene expression differences of foragers that use private versus social information strategies, and 3) the role of reward perception and reinforcement in forager decision making. I found that queen pheromones, which signal the queen's presence, influence adult worker gene expression during a critical period of development that lasts long-term. However, I was not able to link the gene expression profiles to foraging activity. On the other hand, I was able to show that gene expression differences in the antennae contribute to a forager's preference for either social or private information. Lastly, I was able to demonstrate that reward overshadows foraging strategy effect on gene expression in the central and peripheral nervous system.

2016-2018

During my master program, my thesis focused on honey bee health. I explored how manipulating the social environment of workers by removing the queen would change their subsequent physiology. I was able to demonstrate a casual link between reproductive activation of workers in queenless colony with an increase resistance to oxidative stress to the herbicide paraquat and an increases immunity to the virus IAPV.

Publication List

[3] **Kennedy, A.,** Peng, T., Glaser, S. M., Linn, M., Foitzik, S., & Grüter, C. (2021). Use of waggle dance information in honey bees is linked to gene expression in the antennae, but not in the brain. Molecular Ecology, 30(11), 2676-2688.

[2] **Kennedy, A**., Herman, J., & Rueppell, O. (2021). Reproductive activation in honeybee (Apis mellifera) workers protects against abiotic and biotic stress. Philosophical Transactions of the Royal Society B, 376(1823), 20190737.

[1] Waiker, P., Baral, S., **Kennedy, A.,** Bhatia, S., Rueppell, A., Le, K., ... & Rueppell, O. (2019). Foraging and homing behavior of honey bees (Apis mellifera) during a total solar eclipse. The Science of Nature, 106(1), 1-10.

In Preparation and Submitted

[2] **Kennedy, A***., Peng, T*., Foitzik, S. and Grüter, C. (in preparation). Sucrose rewards strongly influence transcriptional activity in the central and peripheral nervous system of private and social

information using honey bee foragers (Co-first author).

[1] Peng, T*., **Kennedy, A*.,** Wu, Y., Foitzik, S. and Grüter, C. (submitted). Early Life Exposure to Queen Mandibular Pheromone Mediates Persistent Transcriptional Changes in the Brain of Honey bee Foragers (Co-first author).

Relevant skills

Animal handling: apiary management as a beekeeper, proficient in dissecting honey bee brain and ovaries, and training bees

Bioinformatics: ability to analyze RNAseq data using linux and R

Molecular method: RNA extraction, quantitative real-time PCR, microinjections,