Foraging in Eusocial Bees: Role of Biogenic Amine Signalling and Reward Perception

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Summary

People have been studying bees since thousands of years. These spritely creatures have attracted many people to explore their behaviours. In this dissertation, I mainly focus on the western honey bee (*Apis mellifera*) and a neotropical stingless bee (*Plebeia droryana*).

We know that bee foragers respond to reward stimuli, resulting in many different reward-seeking behaviours. Sugar is one of the most important rewards for bees. Therefore, In **Chapter 1**, we examined whether and how the concentration of sucrose solutions affects the foraging behaviour of *P. droryana.* We found that the concentration of sucrose strongly affected individual and collective foraging effort in *P. droryana*. Caffeine, on the other hand, which is a well-known stimulant for honey bees and bumblebees, had no effect. **Chapter 2** further investigates collective foraging and provides the first evidence that *P. droryana* foragers are able to communicate the location of high-quality food sources to nestmates.

The biogenic amine octopamine (OA) and dopamine (DA) are key neuromodulators of reward perception and they affect social interactions in other social insects. Reward perception, in turn, affects the reliance on memory during foraging in honey bees. However, little is known about how biogenic amines regulate foraging activities in the largest group of eusocial bees, the tropical stingless bees. In **chapter 3**, we found that OA treatment significantly increased the number of bees exploiting sucrose feeders and the individual foraging speed in *Plebeia droryana*. Our results highlight that OA might have similar effects on individual and social behaviours in honey bees and stingless bees, probably by lowering the sucrose response threshold.

Differences in reward perception affect foraging and division of labour in honey bees. For instance, the regulation of pollen and nectar foraging in honey bees depends on reward perception and, therefore, possibly on OA signalling. We thus investigated in **chapter 4** if OA treatment induces a transition from nectar to pollen foraging and whether OA circulating within the colony increases the ratio of bees collecting pollen. We indeed found that OA treatment caused more bees to collect pollen. Honey bees can be directed to profitable food sources by following waggle dances performed by other bees. Followers can often choose between using this social information or ignore it and rely on their own memory about food sources they have visited in the past, *i.e.* private information. In **chapter 5**, we experimentally tested whether OA and DA mediate this decision. This was expected because biogenic

amine signalling is likely to affect the food source evaluation and, therefore, the motivation to switch to alternative food sources. We show that OA and DA have contrasting effects on the interactions between dancers and dance followers, which confirm that OA and DA signalling in the bee brain affect the use of social information provided by dancers and, thus, information flow in the honey bee hive. In **chapter 6**, we discovered that the expression of OA and DA receptor genes in the mushroom bodies (importance centres of sensory integration, memory formation and the organisation of complex behaviours) varied with honey bee forager age, motivation and daytime but not with forager experience. Taken together, our results reveal complex links between forager state and biogenic amine signalling in the mushroom bodies. The data presented in **chapter 7** explored the molecular basis of the decision to use social or private information by analysing gene expression in different parts of the nervous system of honey bee foragers. Results show that genes were only differentially expressed in the antennae, suggesting that variation in sensory perception affects the decision to use social waggle dance information.

In conclusion, this dissertation expands our understanding of how biogenic amines signalling influences the behaviour of highly eusocial bee foragers. We also highlight the essential role of reward perception and its links with biogenic amine signalling for the regulation of foraging behaviours. Finally, we made a first step towards a better understanding of the molecular basis of honey bee information using strategies.

General Introduction

Tianfei Peng

“The most important reason for my success in science is my love of science and my persistence in long-term exploration.”

— Charles Darwin

Humans are not the only examples of social animals in nature. In fact, there are many examples of highly social lifestyles in animals, such as in social insects. Over the last decades, social insects have become model research organisms to study the biological basis and evolution of sociality. One extreme degree of sociality recognised by sociobiologists is eusociality, which evolved as a survival strategy in response to evolutionary pressures and includes features such as overlapping generations, reproductive division of labour and cooperative brood care (Wilson, 1971). These characteristics of social groups are thought to be beneficial for the development and success of groups and societies (Korb, 2009).

Importance of Highly Eusocial Bees

For decades, social bees have served as research models for a variety of areas such as ethology, toxicology and neurobiology (O' Toole and Raw, 1991; Galizia et al., 2011; Kessler et al., 2015). Many bees stimulate economies around the world by serving as essential pollinators of plants and some contribute valuable products such as honey and wax. Social bees have evolved complex social behaviours, including communication and division of labour. Social bees are divided into different groups: highly eusocial bees (Apini and Meliponini), primitively eusocial bees (Bombini), facultatively eusocial (Xylocopinae and some Halictinae) and less social arrangements that include communal bees, semisocial bees, quasisocial bees, parasocial bees and subsocial bees (Michener, 2000; Cane, 2008).

Social bees, like many other social insects, live in well-organised colonies. The number of individuals in social insect colonies is variable. Some have small colonies with only a few individuals, whereas the colonies of others contain thousands, or even millions of individuals (Queller and Strassmann, 1998). Social bees also vary in many other aspects. Colonies may be founded by a single individual, e.g. in bumblebees, or by a large cohort leaving the parent colony, e.g. in honey bees and stingless bees. In some species, colonies only exist for a short period; in others, they may last for many years (Queller and Strassmann, 1998).

To date, most research focused on the worker caste due to the high behavioural variability and the sheer number of individuals that can be studied. For example, the workers use their cognitive abilities to exchange information, learn, make decisions and navigate. Some species, like the honey bee *Apis mellifera*, are relatively easy to rear and they can be used as model species to answer both applied and fundamental research questions. The knowledge gained from such studies contributes to the greater understanding of biological processes.

Physiology and Behaviour of Highly Eusocial Bees

This thesis focuses on the biologically diverse highly eusocial bees. Two taxonomic groups of highly eusocial bees exist, both in the family Apidae: the honey bees (Apini) and the stingless bees (Meliponini). The species in these two groups are similar in both morphology – they both belong to the corbiculate bees – and social organisation. Although honey bees and stingless bees have many similar social characteristics and behaviours, the difference between them suggests an independent evolution of higher eusociality (Martins et al., 2014).

The genus *Apis* is comprised of approx. 10 species which are mainly spread across Asia. The western honey bee *Apis mellifera* has over 20 recognised subspecies with four evolutionary lineages in Europe, Africa, Asia, and the Middle East (Bradbear, 2009). It is also the only honey bee species that has undergone substantial domestication. Stingless bees include approximately 550 species and are distributed around the world in tropical and subtropical regions (Rasmussen and Cameron, 2010; Bradbear, 2009).

Colony Organization of Highly Eusocial Bees

Honey bee and stingless bee colonies demonstrate the hallmarks of eusocial societies: 1) The show a functional specialisation of group members resulting in a division of labour between members of different castes (reproductive queens and non-reproductive workers) and different ages (among workers, *i.e.* nurses and foragers), 2) overlapping generations and 3) cooperative brood care (O' Toole and Raw, 1991).

The colonies typically consist of three types of adults. The females are separated into morphologically distinct castes, a single queen and many workers. The males or drones do not work and their role is restricted to reproduction. The queen is usually the sole reproductive female, but in some stingless bee species, workers contribute to the production of males (Tóth et al., 2002; Grüter, 2018). The workers cooperate to maintain the colony by nest building, brood rearing, and foraging. Workers mostly divide tasks based on age, with younger workers performing in-nest tasks such as brood rearing and comb building while older workers perform outside tasks, such as foraging. In some stingless bees, morphology also affects division of labour (Hammel et al., 2016). The social structure of the colony is maintained largely by the presence of the queen and supported by the workers. Colony coordination depends on a complex system of communication that involves the distribution of pheromones among colony members (e.g. Van Oystaeyen et al., 2014).

Colony activity varies depending on the season and geographic location. For example, honey bees in the temperate zones adjust their egg laying, brood rearing, and resource collection (pollen and nectar) according to the time of year. Colonies with a large population of young workers and/or ample stores of honey and pollen start brood rearing and egg laying as early as in December – January to have strong worker populations when spring starts (Schmid-Hempel and Wolf, 1988).

During the early spring, the colony increases brood rearing and foragers collect water to liquefy any granulated honey stores for brood food. Later in spring, the population increases rapidly with many young workers present. However, in subtropical, tropical, and mild-winter climates, egg laying and brood rearing never stop (Terada et al., 1975). As the population increases, the number of foragers increases as well. Foragers will collect more pollen and nectar to maintain brood rearing and egg laying. During this time of rapid expansion, swarming is common. During the early summer months, the daylight is longest, resulting in extended daily foraging activity. After the swarming season, drones are forced out of the colony to preserve food stores. The colony also reaches its peak population of foragers during this time and shifts to the foraging for winter stores (Winston et al., 1983).

Foraging Behaviour of Highly Eusocial Bees

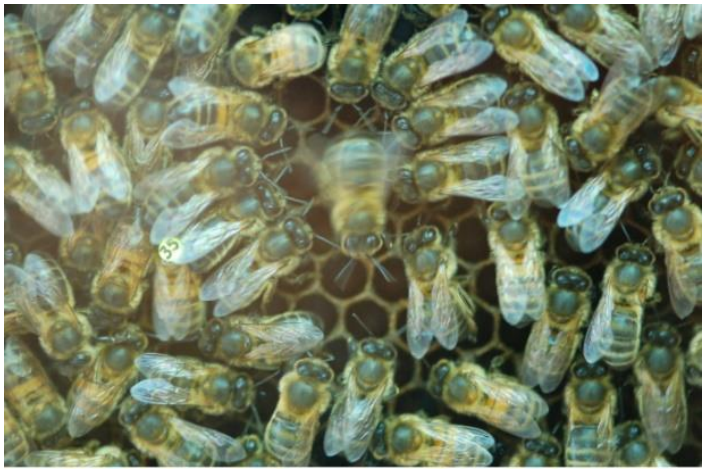
The foraging behaviour of highly eusocial bee colonies represents a remarkable example of cooperation in the insect world (von Frisch, 1967). The division of labour within these colonies is based on behavioural caste and age (Johnson, 2010; Huang and Robinson, 1996). Age-based division of labour is referred to as age-polyethism: bees generally progress through behaviours in five phases: callow, nurse bee, processor bee, guard and forager (Michener, 1974; Robinson, 1992; Bloch and Robinson, 2001; Sakagami, 1982; Cepeda, 2006). After the transition to foraging, workers perform this task until they die. The age of onset of foraging varies across species and depends on colony needs. Thus, to understand foraging behaviour of highly eusocial bee species, studies need to focus on both the behaviour of the individual worker and the colony-level needs (Seeley, 1995).

Information Flow and Organization of Honey bee Foraging

Foraging is cognitively demanding as foragers have to learn food locations, odours, floral shapes and colours (von Frisch, 1967; Biesmeijer and Slaa, 2004; Abou-Shaara, 2014). Foragers must also combine this information with the time of day a resource is most profitable because flowers bloom at specific times of day (Beling, 1929; Moore, 2001; Zhang et al., 2006). Consequently, finding a good food source

can be challenging for a forager and getting the information about how and where to find a good food source from a nestmate can significantly simplify this task.

The waggle dance of honey bees is a unique example of insect communication. It is often referred to as the "dance language", which was first decoded over 70 years ago by Karl von Frisch and has been studied extensively ever since (Figure intro 1) (von Frisch, 1967; Dyer, 2002; I'Anson Price et al., 2019; Gould, 1975; I'Anson Price and Christoph, 2015). Traditionally, researchers have distinguished between the round dance and the waggle dance. It was thought that only the waggle dance indicates the distance and direction of the advertised food source, whereas round dances imply only the presence of a high-quality resource somewhere nearby (<100 m from the hive). In the last few years, studies have reported that the honey bee round dance does seem to convey distance information, just not precise direction information (Gardner et al., 2008). Dancers also emit a unique blend of hydrocarbons to recruit and stimulate foraging (Thom et al., 2007; Gilley et al., 2012).



(Photo by Christoph Grüter)

Figure intro 1: *Apis mellifera* forager performing a waggle dance in the colony.

Although the location information encoded in the waggle dance can be deciphered by dance following foragers, 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 (Grüter et al., 2008, 2013; Grüter and Farina, 2009; Grüter and Ratnieks, 2011; Menzel et al., 2011; Biesmeijer and Seeley, 2005). Thus, experienced bees often prefer private information (navigational and spatial memories) to visit already familiar food sources over social information to visit new food sources. The third group of foragers shows a tendency to neither follow waggle dances nor use private information and instead searches for new food sources by individual exploration, so-called scouting (Seeley, 1995). The

proportion of scouts in the honey bee foraging force varies from about 5-25% (Seeley, 1995), the proportion of foragers that prioritise social information is around 10-30% and the proportion of foragers prioritising private information is estimated to be approx. 50-90% (Biesmeijer and Seeley, 2005; Grüter et al., 2008; Wray et al., 2012). Empirical and theoretical studies suggest that the benefits of scouting, social information and private information depend strongly on the spatiotemporal distribution of food sources. However, the colony's needs also likely influence individuals using different foraging strategies (Beekman and Lew, 2008; Dornhaus and Chittka, 2004; I'Anson Price et al., 2019; Schürch and Grüter, 2014).

Information Flow and Organization of Stingless Bee Foraging

Stingless bees (Apidae, Meliponini) display a great variety in lifestyle and ecology (Roubik, 1989). The food source communication mechanisms vary among different species, ranging from simply motivating nestmates to leave the nest and search for food to the precise communication of the food source location by depositing pheromone trails. Some stingless bee species are even more efficient at recruiting nestmates to food sources than honey bees (Lindauer and Kerr, 1960; Barth et al., 2008; Aguilar et al., 2005).

Recruitment mechanisms can be divided into intranidal and extranidal mechanisms. Numerous studies have described the intranidal recruitment communication in stingless bees. Generally, after stingless bee foragers encountered a high-quality food source, they perform "zigzag" or "jostling" runs inside the nest, which usually occur close to the nest entrance. The jostling runs are important for activating potential foragers (Lindauer and Kerr, 1958, 1960; Hrncir et al., 2000; Nieh, 1998; Hrncir, 2009). Buzzing sounds appear during the jostling runs and during trophallaxis, which is produced with thoracic muscles (Kerr et al., 1963; Esch et al., 1965; Nieh and Roubik, 1998; Aguilar and Briceño, 2002; Nieh et al., 2003a). Buzzing sounds may play a role in stimulating the vibrated receivers to initiate foraging (Hrncir et al., 2006; Hrncir et al., 2008; Hrncir and Barth, 2014; Krausa et al., 2017). Meanwhile, through trophallaxis, nestmates can learn about the quality and odour of a food source (Nieh et al., 2000; Aguilar et al., 2005; Jarau, 2009; Krausa et al., 2017).

Extranidal recruitment communication in stingless bees can be found in the form of foragers depositing a scent beacon close to the food source or laying a scent trail when returning to the nest from a food source. These chemicals guide other foragers to the food source (Nieh, 1998; Hrncir et al., 2004; Jarau et al., 2004). Moreover, it has been speculated that in some species, location-specific recruitment

can also be achieved by the visual tracking of guiding flights performed by foragers flying from the nest to the food source. Successful recruitment could also rely on a combination of these mechanisms (Nieh et al., 2003b; Nieh, 2004; Kerr and Lindauer, 1958, 1960; Aguilar et al., 2005; Jarau, 2009; Alavez-Rosas et al., 2017). Nevertheless, not all the stingless bees seem to use pheromone trails to recruit nestmates, which may depend on the colony size of species. For example, in small colonies, the number of workers might not be enough to maintain the volatile pheromone trails. Furthermore, for larger colonies it could be easier to dominate a food source, which makes the use of pheromone trails more beneficial (Nieh, 2004; Beekman and Dussutour, 2009; Aguilar et al., 2005). There also seem to be some species that are able to recruit to specific locations without the use of pheromone trails, but the mechanism of recruitment is still a mystery, e.g. in *Partamona orizabaensis* (Flaig et al., 2016). There are also species where successful foragers seem to communicate only the direction of food sources, without communicating precise distance information (Jarau et al., 2000; Nieh et al., 2000; Nieh, 2004; Aguilar et al., 2005). These examples show that there is great variation among species in how they communicate about food sources and many mechanisms are not well understood. One problem is that foraging communication has mostly been studied in three genera: *Melipona*, *Scaptotrigona* and *Trigona*, which means that our understanding is dominated by these groups. Hence, recruitment communication in most stingless bees has not been studied. Exploring other species could help us understand how complex recruitment communication systems have evolved in social bees and how it is related to ecological parameters.

During my PhD, I focused on *Plebeia droryana*, a small (~3 mm long) and common species in South America (Figure intro 2). It has been reported that thoracic vibrations (buzzing sound) are produced by successful foragers inside the nest, which could improve the foraging activity of the colony (Lindauer and Kerr, 1960). *Plebeia* is one of the largest and most common Neotropical genera (~40 species). However, we have very little insight into the foraging strategy of this group of important generalist pollinators. Studies on *Plebeia* would help us get a better understanding of how recruitment strategies are different in different genera.

(Photo by Christoph Grüter)

Figure intro 2: the left photo shows *Plebeia droryana* visiting the flowers of *Euphorbia milii*. The right photo shows the entrance of a *P. droryana* colony.

Molecular Mechanisms Underlying Foraging Behaviours in Highly Eusocial Bees

How genes modulate social behaviours has been the subject of extensive debate and controversy (Robinson et al., 2008). This controversy is not easily resolved because of the complexity of the relationship between genes, the brain, and social behaviour: genes have no direct influence on behaviour but they encode the molecular products that form and influence the working of the brain which in turn is responsible for the expression of behaviours.

The advent of genomics has revolutionised the study of behaviour and with hundreds or even thousands of animal genome sequences being available soon, behavioural biology is becoming more and more "genome powered". With the help of high-throughput sequencing technologies researchers have demonstrated that responses to social stimuli can be massive, involving hundreds or even thousands of genes at once, leading to different “neurogenomic signatures” of gene expression (Whitfield et al., 2003; Cummings et al., 2008; Robinson et al., 2008; Liang et al., 2012). This research has demonstrated that the genome, which was once thought to be a relatively passive blueprint guiding organismal development, is highly responsive to many different stimuli associated with social behaviour. In other words, social and environmental information lead to changes in brain gene expression and, subsequently, to changes in social behaviour (Robinson et al., 2008).

One of the key model species in the study of the genomic basis of social behaviour or “sociogenomics” is the honey bee (*Apis mellifera*) (Dolezal and Toth, 2014). In one of the first studies to compare genome-wide brain gene expression patterns of workers performing different behaviours,

microarrays were used to compare the expression patterns of workers performing nursing tasks with those of workers carrying out foraging tasks (Whitfield et al., 2003). This study found that more than 1000 genes were differentially expressed between the two task groups. Another early "sociogenomics" study found that the perception of alarm pheromone by honey bees alters the expression of hundreds of genes in the brain over a period of days to weeks (Grozinger et al., 2003). Liang et al. (2012) compared honey bee foragers using a scouting strategy with non-scouting foragers and found more than 1200 differentially expressed genes. Moreover, some of those genes are also known to be involved in the novelty seeking behaviour in mammals, including humans. While these studies focused on whole-brain gene expression, more recent work started to focus on particular brain regions to explore their role in affecting behaviour (Suenami et al., 2016, 2018). Taken together, these studies have demonstrated that genome responses to social information can be large (many genes are affected) and rapid (within minutes) (Dolezal and Toth, 2014).

Reward Perception Modulates Foraging Behaviour in the Highly Eusocial Bees

Individual honey bee and stingless bee foragers specialise in the collection of different types of resources: water, nectar, pollen or nesting material. In some extreme cases, foragers can also collect wax from scale insects and some stingless species have evolved carnivorous or robbing lifestyles (Dimou and Thrasyvoulou, 2007; Camargo and Roubik, 1991; Barth et al., 2008; Grüter et al., 2016). For most bee species pollen is the primary protein source and is fed to the larvae. Nectar is a carbohydrate source that ensures the survival of adult workers and is also an important "fuel" for the workers to heat the brood (Seeley, 1995; Kleinhenz et al., 2003). Even though many plants offer both resources at the same time, most foragers specialise in collecting one of the two resource types (Free, 1960). However, tasks specialisation is not fixed, and some foragers can switch from one resource type to the other in response to changing environmental or colony conditions (Biesmeijer and Tóth, 1998; Rotjan et al., 2002; Arenas and Kohlmaier, 2019).

In the honey bee, a critical factor for determining the type of resource that is collected is reward perception, which can be assessed by testing the sucrose response threshold. For example, water foragers have the lowest sucrose response thresholds, followed by the nesting material (Simone-Finstrom et al., 2010) and pollen foragers. Nectar foragers have the highest threshold. The threshold in foragers who collect both pollen and nectar is intermediate between the pollen and nectar foragers (Pankiw and Page, 2000). Honey bee foragers with very high sucrose response thresholds typically

return empty, probably because they attempt to locate very high-quality nectar sources.

The sucrose response threshold is a colony-level feature with a genetic component (Hunt et al., 1995), and is, among other things, affected by the genotype of bees (Pankiw et al., 2002). Page and Fondrk (1995) selected colonies for differences in pollen collection propensity, creating a high and a low pollen hoarding strain. The high pollen hoarding strain stored on average 6 times more pollen than the low pollen hoarding strain by the third generation. Consequently, high strain colonies have more pollen foragers than low strain colonies. As would be expected, pollen foragers from high strain colonies have lower sucrose response thresholds than low strain colony pollen foragers.

Much less is known about the links between reward perception and resource foraging behaviour in stingless bees, but a recent study demonstrates that reward perception indeed also plays a role in foraging behaviours in this group. Non-pollen foragers show higher sucrose response thresholds than pollen foragers and guards in *Tetragonisca angustula* (Balbuena and Farina, 2020). Moreover, *Trigona recursa*, a scent trail-laying stingless bee, has been found to recruit more foragers to the more profitable of two food sources (Schmidt et al., 2006). Also, in the African stingless bee *Plebeina hildebrandti*, individual foraging behaviours like the foraging cycle duration, food uptake, and intranidal behaviours like trophallaxis are influenced by food profitability (Krausa et al., 2017). Thus, food profitability seems to promote various aspects of the foraging process in stingless bees.

Biogenic Amine Signalling and Foraging Behaviour

In the central nervous system (CNS), biogenic amines play important roles in regulating neurophysiological responses and consequently many behaviours (Evans, 1980; David and Coulon, 1985; Roeder, 1994; Monastirioti, 1999). Octopamine (OA) and dopamine (DA) are biogenic amines present in the CNS as neurotransmitters, neurohormones and neuromodulators that have been widely studied in the honey bee (Hewlett et al., 2018; Roeder, 1994).

DA plays a crucial role in learning and memory, particularly in aversive learning (Schwaerzel et al., 2003; Riemensperger et al., 2005; Vergoz et al., 2007; Agarwal et al., 2011; Tedjakumala et al., 2014). Moreover, DA is also related to motor behaviour and activity level in honey bees as well as other insects (Mustard et al., 2010; Harano et al., 2008). Among foragers, DA titres in the brain have been found to differ significantly in pollen and nectar foragers (Taylor et al., 1992).

OA has an arousing effect and mediates the reward information in honey bees (Hammer and

Menzel, 1998). It is also known that OA levels are associated with the processing of visual and olfactory information (Grohmann et al., 2003) and it has a positive effect on the waggle dance behaviour of foragers (Barron et al., 2007). Previously, OA and DA signalling pathways were considered to be functionally separated, where OA is involved in reward signalling and DA in punishment signalling. However, more recent research in *Drosophila* suggests that DA and OA signalling pathways are tightly intertwined (Perry and Barron, 2013).

OA and DA signalling in the brain of bees is also involved in regulating the division of labour within colonies (Barron and Robinson, 2005; Wagener-Hulme et al., 1999). For example, OA and DA are connected to the transition from nursing to foraging, with foragers having higher brain levels of OA and DA (Wagener-Hulme et al., 1999, Schulz and Robinson, 1999; Taylor et al., 1992). Additionally, precocious foraging is observed in bees treated with OA (Scheiner et al., 2002; Schulz and Robinson, 2001). Higher OA levels were found in the optic lobes of pollen foragers, which displayed a significantly reduced phototaxis (Scheiner et al., 2014). In experiments investigating honey bee foraging behaviour, OA showed a trend to induce foragers to collect more dilute nectar or water (Giray et al., 2007), as a result of a lowered sucrose response threshold (Scheiner et al., 2002).

Biogenic amines act by binding to receptors in the brain (Roeder, 1994). DA and OA receptors have been well studied in many organisms and belong to the G-protein coupled receptor (GPCR) family. Several earlier studies show that the connection between stimulation of biogenic amine receptors and behaviour is mediated by the mushroom bodies (Durst et al., 1994; Farris et al., 2001; Ismail et al., 2006). In insects, the mushroom body is important for various cognitive functions, such as sensory integration, memory formation and the organisation of complex behaviours (Menzel et al., 2006; Giurfa, 2007; Heisenberg, 2003). The importance of the mushroom bodies for foraging behaviour is further corroborated by an increase in size and change in other anatomical features of the mushroom bodies in an age-dependent way and during the foraging period (Gronenberg et al., 1996; Fahrbach et al., 1998; Farris et al., 2001).

To date, three dopamine receptor genes and five octopamine receptor genes have been identified in the honey bee (Mustard et al., 2003; Humphries et al., 2003; Beggs et al., 2005; Grohmann et al., 2003; Balfanz et al., 2014). AmDOP1 and AmDOP2 belong to the subfamily of dopamine receptors called D1-like receptors (Kokay et al., 1999; Mustard et al., 2010). Similar to the function of D1-like receptors in other organisms, activation of AmDOP1 stimulates adenylyl cyclase, which leads to an

increase in the concentration of cAMP (Blenau and Baumann, 2001). *Amdop1* is expressed in the Kenyon cells of the mushroom body and has been assumed to participate in the processing of olfactory information (Blenau et al., 1999). *Amdop2* is evolutionarily related to *AmoctαR1* and, similar to AmOCTαR1 and AmDOP2, is coupled to both cAMP and Ca^{2+} signalling via phospholipase C activation (Beggs et al., 2011). AmDOP3 is a D2-like receptor that reduces intracellular cAMP when activated (Blenau et al., 1999; Humphries et al., 2003; Beggs et al., 2005). AmOCTαR1 belongs to a class of octopamine receptors called alpha-adrenergic-like receptors (OctαR), which mediates Ca^{2+} signalling. AmOCTβR1, βR2, βR3, βR4 belong to the class of beta-adrenergic-like receptors (OctβR), which are cAMP-coupled receptors, however, AmOCTβR3 and – βR4 receptors are two splice variants of the same gene (Balfanz et al., 2014).

Expression of biogenic amine receptor genes is elevated in the mushroom bodies of foragers' brain compared to the brain of bees working in the hive (Humphries et al., 2003; McQuillan et al., 2012). Thus, in accordance with studies on biogenic amine titers, the studies assessing receptor gene expression found that the mRNA level of biogenic amine receptor genes increases during the behavioural development of adult honey bees.

However, despite our understanding of the importance of biogenic amine in modulating honey bee behaviour, many basic questions remained unanswered, e.g. how do biogenic amines affect the behaviour after bees have transitioned to foraging? What kind of foraging behaviours are influenced by biogenic amines? What is the role of reward perception in biogenic amine signalling regulation of foraging behaviours? How does biogenic amine signalling affect foraging in stingless bees? The aim of this dissertation was to address these questions and identify new questions for further research.

Aims of My Dissertation

The honey bee *Apis mellifera* and the stingless bee *Plebeia droryana* are feasible model organisms to study foraging since we can train them to artificial food sources, a technique perfected by Karl von Frisch in the first half of the 20th century (von Frisch, 1967). Additionally, they play important roles in pollination (O' Toole and Raw, 1991). My research was split into two parts: part 1 focused on the stingless bee *Plebeia droryana* and part 2 on the honey bee *Apis mellifera*.

Part 1

Food quality influences the foraging behaviour of *Apis mellifera* foragers, e.g. by affecting the

persistence of foragers to feeding locations or their dance behaviour (Al Toufailia et al., 2013; Seeley et al., 2000). Much less is known about the role of reward perception for modulating individual and collective foraging behaviours in stingless bees. Therefore, we tested whether the concentration of sucrose in a solution affects the foraging behaviour of *Plebeia droryana*. Reward perception can also be affected by secondary plant compounds present in nectars. Caffeine, for example, is known to increase foraging, recruitment and learning in honey bees and foraging effort in bumblebees (Couvillon et al., 2015; Thomson et al., 2015). It has been suggested that plants might trick bees into collecting mediocre food sources by adding caffeine to nectar, thereby potentially harming bee colonies, while saving energy for themselves. However, the studied honey bee and bumblebee colonies were not exposed to plants that caffeinate their nectar. *P. droryana* is the most common native bee visitor of *Coffea* and *Citrus* in the State of São Paulo, Brazil, thus they have a long history of exposure to caffeinated nectar and pollen (Cortopassi-Laurino et al., 2009). Exposure to caffeine over evolutionary time periods might allow pollinators to adapt to such potential exploitation. For this reason, we also tested whether caffeine affects the foraging behaviour of *P. droryana* (**chapter 1**).

In their pioneering study, Lindauer and Kerr (1958, 1960) found no evidences for location specific communication in *Plebeia droryana*. Nevertheless, in **chapter 1**, we found evidence that *P. droryana* foragers might provide nestmates with specific location information. There are large gaps in our understanding of these mechanisms across stingless bees. To test this hypothesis, we studied wild colonies and tried to confirm whether or not foragers provide resource location information to their nestmates (**chapter 2**).

In honey bees, OA modulates both individual and social behaviours. OA enhances appetitive learning, mediates division of labour and increases recruitment communication, most likely because it enhances reward perception (Hammer and Menzel, 1998; Barron et al., 2007; Barron and Robinson, 2005). Almost nothing is known about the role of OA in mediating behaviour in stingless bees. If OA lowers the sucrose response threshold and, thus, increases the perceived value of the food source also in stingless bees, then we would expect that OA treatment increases individual and collective foraging in *P. droryana* (**chapter 3**).

Part 2

Studies discovered that individual experience and biogenic amines influence the reward perception in honey bee, which in turn affects foraging behaviours (Scheiner et al., 2002; Pankiw et al., 2002). We

explored two hypothesises. First, the regulation of pollen and nectar foraging is linked to differences in sucrose response thresholds. Pollen foragers have a lower sucrose response threshold than nectar foragers (Pankiw and Page, 2000). So, in **chapter 4**, we tested the hypothesis that OA signalling affects the division of labour between pollen and nectar foragers. We predicted that increased OA intake leads to an increase in pollen foraging at the individual and colony level.

Honey bees use the waggle dance to communicate about high-quality food sources. As mentioned before, 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. It is still not known whether this decision to use or ignore dance information depends on reward perception. Since oral and topical treatment of foragers with OA increases the motivation to perform waggle dances (Barron et al., 2007), we tested the hypothesis that OA and DA signalling would also affect interest in waggle dance communication and the use of dance information. Thus, in **chapter 5**, we used OA and DA to treat trained foragers and we then quantified their interest in dances for an alternative food source.

OA and DA do not work alone but in combination with receptors. Thus, we wanted to better understand the expression of OA and DA receptor genes of bees that have transitioned to foraging. In **chapter 6**, we quantified the expression of all OA and DA receptor genes, *Amdop1*, *Amdop2*, *Amdop3*, *AmoctαR1*, *AmoctβR1*, *AmoctβR2* and *AmoctβR3/4* in the mushroom bodies – important centres of sensory integration, memory formation and the organisation of complex behaviours – to test whether forager age, experience, motivation and the time of the day are linked to OA and DA amine signalling.

Foraging decisions are likely to be mediated by complex molecular networks in different brain areas. One way to gain initial insights into the molecular basis of behaviours is transcriptome sequencing. While **chapter 5** focused on OA and DA signalling as mediators of interest in dance information, **chapter 7** tested the prediction that bees that use social dance information and those using private information show differences in endogenous gene expression in the brain. In particular, we asked the question whether there are differences in the expression of genes involved in reward perception or biogenic amine signalling when comparing bees that use social information differently. We focused on gene-expression patterns in five important parts of the honey bee brain, the mushroom bodies, the antennal lobes, the central brain, the subesophageal ganglion and the antennae (**chapter 7**).

CHAPTER 1

Resource profitability, but not caffeine, affects individual and collective foraging in the stingless bee *Plebeia droryana*

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Abstract

Plants and pollinators form beneficial relationships with plants offering resources and, in return, they get pollinated. Some plants, however, add compounds to nectar to manipulate pollinators. Caffeine is a secondary plant metabolite found in some nectars that affects foraging in pollinators. In honey bees, caffeine increases foraging and recruitment to mediocre food sources, which might benefit the plant, but potentially harms the colonies. For the largest group of social bees, the stingless bees, the effect of caffeine on foraging behaviour has not been tested yet, despite their importance for tropical ecosystems. More generally, recruitment and foraging dynamics are not well understood in most species. We examined whether caffeine affects the foraging behaviour of the stingless bee *Plebeia droryana*, which frequently visits plants that produce caffeinated nectar and pollen. We trained bees to food sources containing field-realistic concentrations of sugar and caffeine. Caffeine did not cause *P. droryana* to increase foraging frequency and persistency. We observed *P. droryana* recruiting to food sources, however, this behaviour was also not affected by caffeine. Instead we found that higher sugar concentrations caused bees to increase foraging effort. Thus, unlike in other pollinators, foraging behaviour in this stingless bee is not affected by caffeine. As the Brazilian *P. droryana* population that we tested has been exposed to coffee over evolutionary time periods, our results raise the possibility that it may have evolved a tolerance towards this central nervous system stimulant. Alternatively, stingless bees may show physiological responses to caffeine that differ from other bee groups.

Introduction

Plants attract pollinators by providing resources, mainly nectar and pollen. In turn, they receive visits that facilitate plant reproduction through the transfer of pollen by the pollinators (Burkle et al., 2013; Mitchell et al., 2009). Since pollinators can use pollen and nectar either for themselves or to feed their offspring, this relationship between plants and pollinators usually benefits both parties. However, sometimes pollinators or plants cheat. For example, some plants attract pollinators by imitating floral signals or mating signals while not offering rewards (Schiestl, 2016; Bohman et al., 2016; Oelschlägel et al., 2015). Also, nectar-robbing bees make holes in flowers to extract nectar while providing little or no pollination service (Irwin et al., 2001; Inouye, 1980; Leadbeater and Chittka, 2008).

In nature, secondary metabolites are produced by plants as pharmacologically active toxins whose main function is to reduce leaf damage by herbivores (Bennett and Wallsgrove, 1994). Recent research has shown that secondary metabolites like caffeine (e.g. from the genera *Coffea*, *Citrus* and *Tilia*) or nicotine are added by some plants to the nectar they secrete (Kretschmar and Baumann, 1999; Wright et al., 2013; Thorburn et al., 2015; Heil, 2011). The effects of secondary metabolites on pollinators are complex and context dependent. For example, in bumblebees (*Bombus impatiens*), nicotine decreases parasite loads under varying temperature conditions, while at constant temperatures, it has the opposite effect (Thorburn et al., 2015). Several studies on the European honey bee (*Apis mellifera*) show that the presence of caffeine in nectar alters honey bee foraging behaviour: it increases the amount of nectar the bees drink, improves the learning performance and increases recruitment and persistency to the nectar sources (Couvillon et al., 2015; Singaravelan et al., 2005; Wright et al., 2013). Similar effects of caffeine on foraging have been found in bumblebees (Thomson et al., 2015). These studies suggest that field-realistic concentrations of caffeine enhance the reward perception of temperate honey bee and bumblebee foragers. In other words, the addition of caffeine to nectar seems to have a similar effect on bee foraging behaviour as an increase in sugar content.

It has been hypothesised that plants releasing caffeine into nectar might trick pollinators into increasing foraging rates and, therefore, pollination success without offering higher quality food (Couvillon et al., 2015). The presence of caffeine could even lead to detrimental effects on honey bee colonies as it might cause colonies to focus their foraging effort on caffeinated nectar sources containing relatively low quantities of sugar (Couvillon et al., 2015; Koch and Stevenson, 2017). In the worst case,

colonies could die because they are tricked into collecting low quality resources (Koch and Stevenson, 2017). Such effects might have ecological implications through changes in plant-pollinator interaction networks and, potentially, biodiversity.

With more than 500 described species, stingless bees (Meliponini) represent the largest group of highly eusocial bees and they play key roles as pollinators in tropical and subtropical habitats (Heard, 1999; Giannini et al., 2015). Despite their number and their importance, relatively little is known about the foraging behaviour of most species (Rasmussen and Cameron, 2010; Stangler et al., 2009; Aleixo et al., 2017; Hrncir et al., 2016). Stingless bees are known to naturally forage on flowers of species belonging to *Coffea* and *Citrus* (Heard, 1999; Ricketts, 2004; Ricketts et al., 2004). Coffee, for example, has been in Brazil for nearly 300 years and Brazil has been the largest producer of coffee in the word for the last 150 years, currently producing about a third of all coffee consumed (Fausto, 2014; Neilson and Pritchard, 2009). However, it remains unknown how stingless bees respond to caffeine and whether the collection of caffeinated nectar could have detrimental effects on tropical pollinators.

The foraging response of stingless bees and honey bees to compounds present in nectar may differ considerably. For example, the nectar of the avocado tree (*Persea americana*) contains minerals that have been shown to repel honey bees, which are not the natural pollinators of this plant, while pollinators from the native range of the plants, among which are two stingless bee species, were much less affected by these secondary nectar compounds (Afik et al., 2014). This points to taxon specific differences in the physiological and neural response to plant compounds. Furthermore, the long-term coexistence of plants and their pollinators may allow pollinators to adapt to their preferred plants, e.g. by evolving resistance to the effects of nectar compounds. To gain a better understanding of the potential effects of caffeinated nectar on plant-pollinator interactions in the tropics we studied the Brazilian stingless bee *Plebeia droryana*, which was found to be the most common native bee visitor of *Coffea* and *Citrus* in the state of São Paulo, Brazil (Nogueira-Neto et al., 1959; Imperatriz-Fonseca et al., 1989).

Unlike honey bees who use the waggle dance to recruit nestmates to profitable food sources (von Frisch, 1967), *P. droryana* is not known to recruit to particular foraging locations, but successful foragers are known to produce thoracic vibrations (buzzing sound) inside the nest, which could have the function of increasing the foraging activity of the colony (Lindauer and Kerr, 1960). After discovering a food source, some stingless bee species adjust their foraging frequency, their recruitment probability or their willingness to fight for a food source according to the quality of the resource

(Johnson and Hubbell, 1974; Biesmeijer et al., 1998; Jarau, 2009; Schorkopf et al., 2016). Likewise, the decisions on whether to return on the next day (persistency) depends on the combination of the bees' ability to memorise food locations and information about the food source quality (Biesmeijer and Slaa, 2004; Al Toufailia et al., 2013).

Here, we examined the effects of field-realistic concentrations of sugar and caffeine on the visitation rate and foraging persistency of *P. droryana*. Additionally, since the foraging method of *P. droryana* is not well studied, we explored if foragers might recruit nestmates to food locations offering high quality food.

Materials and methods

Study species and field site

We performed our experiments on the campus of the University of São Paulo in Ribeirão Preto, Brazil. This area has a high diversity of wild stingless bee species (Cortopassi-Laurino et al., 2009) and flowering plant species belonging to *Coffea* and *Citrus* (the campus is a former coffee farm). *Plebeia droryana* is a common species at our field site. When experimenting with foragers of one colony we could not exclude the possibility that individuals from other colonies, e.g. up in trees or hidden from view, would also arrive at our experimental set-up. However, by marking bees individually we could determine that the majority of foragers usually came from one focal colony. In total, we studied ten focal colonies at ten locations, while ensuring that locations were at least 800m apart from each other. Five of those locations were used for Part 1 and the other five for Part 2. Data were collected during four weeks in March 2018 on days with good foraging conditions.

Experimental setting

We used standard training procedures to train the foragers from the focal colony to artificial feeders: two artificial feeders containing a 50% sucrose solution were placed next to the nest entrance and on differentially coloured backgrounds (yellow vs blue) to stimulate foragers to start visiting the feeders. After a group of foragers was established on each feeder, the feeders were moved to the final destination while the foragers were drinking from the solution. Bees would then learn the new location of each respective feeder when returning to their nest (von Frisch, 1967; Nieh, 2004). We used this method to set up two feeders and, in order to help trained bees learn the location and prevent switching between feeders, we used different coloured backgrounds on a chair (height 0.5 m) (Couvillon et al., 2015). The

final destinations of the two feeders were 10 m away from the main colony and separated by 7 m from each other. We began training in the morning and after ~10 foragers were trained to each of the feeders, we marked all foragers individually with acrylic paints using a combination of colour dots. In all experiments we used unscented sucrose solutions.

Part 1: Do caffeine and sugar content affect foraging in stingless bees?

In a previous study, the sugar content of nectar of coffee in Sao Paulo state was found to be about 38% (range from 32.8% to 45%) (Nogueira Neto et al., 1959) and the caffeine concentration in the nectar of three Brazilian species of *Coffea* (*C. canephora, C. liberica and C. arabica*) increase from 0.003mM to 0.253mM as the sugar concentration decreases (Wright et al., 2013; Santos and Lima, 2009; Govaerts, 2009). To test whether caffeine affects foraging we used two sucrose solutions (30% and 40%) and two caffeine concentrations (25 ppm or ~0.14mM and 50 ppm or ~0.28mM). For experiment 1, colonies were tested with one feeder offering a 40% sucrose solution (control feeder) and a second feeder offering a 40% sucrose solution containing a medium dose of (25 ppm) caffeine (treatment feeder). In experiment 2, colonies were tested with one feeder offering a 30% sucrose solution (control feeder) and a second feeder offering a 30% sucrose solution containing a high dose of (50 ppm) caffeine (treatment feeder). The medium and high concentrations are found naturally in the nectar of coffee plants and we mimicked the negative correlation between sucrose and caffeine concentration (Wright et al., 2013; Couvillon et al., 2015).

During the training phase, we offered a 50% sucrose solution without caffeine to attract bees. After the training (but on the same day), the feeders were cleaned with water and filled with the solutions described in the previous paragraph for the treatment phase. During 120 minutes, we recorded how many bees visited each feeder every five minutes and those counted bees included the individually marked bees and unmarked bees. We continuously recorded how often the individually marked bees were at the feeders (foraging frequency) during the experimental period. For foraging frequency, we divided the total visit times of individually marked bees by the treatment time (120 minutes).

To not disturb the bees while they were getting familiar with the new solutions, the first count was done 10 minutes after the beginning of the treatment. Observers switched position every 20 minutes to exclude any biases caused by the attraction of the bees to one particular observer. Treatment-background (blue or yellow) combinations were randomised for each trial. In total, we marked 170 bees individually in our experiment. Among them, 27 bees had switched between the treatment and control

feeder during the treatment period. Of the bees that switched 48% had visited one feeder more than 90% of all visits. For the data analysis, we included the bees that never switched and the bees that had switched but visited one feeder for > 90% of all visits. We excluded the remaining bees that switched (8% of all marked bees) from the statistical analysis.

In order to know whether caffeine affects foraging persistency in *P. droryana*, i.e. the probability to return to the food source the day after treatment (Couvillon et al., 2015; Al Toufailia et al., 2013), we set up the same coloured backgrounds with empty, unscented feeders at the same location in the morning of the day after treatment and observed the feeders for 150 minutes. During these 150 minutes, we recorded the time when bees landed on each feeder. Then, both individually marked bees and unmarked bees were counted. We used these data to calculate the visitation rate to the now empty feeder every five minutes. Furthermore, we also calculated the probability of the individually marked bees to return to the empty feeder. We only counted the bees that landed on the feeder. The observations of persistency were done by only one observer who checked both feeders regularly for the presence of bees.

Part 2: Does P. droryana show recruitment to high quality resources?

The results of part 1 suggested that sucrose concentration affects the foraging motivation. To explore this further we performed a third experiment (experiment 3). To test whether *P. droryana* might recruit nestmates to feeders offering high quality food, we provided different concentrations of sucrose solution (30% and 40%) at the two feeders, while placing both on a yellow background. Concentration-location combinations were randomised in all trials. Every five minutes, we counted how many bees visited each feeder. The counting method was the same as described below the heading "Experimental setting".

Statistical analysis

For data analysis, we used generalised linear mixed-effect models (GLMM) and linear mixed-effects models (LME) in R version 3.4.4 (R Core Team, 2017), as implemented in the lme4 package and nlme package (Bates et al., 2015; Zuur et al., 2009). The focal colony was used as a random effect to account for the non-independence of observations from the same colony (Zuur et al., 2009). Depending on the error distribution of the response variable, we used normal (log and square root transformed), binomial or Poisson distribution. We used colour, caffeine treatment and time of measurement (10 to 120 min or 10 to 150 min, depending on the experiment) as fixed effects. In part 1, we were also interested in the two-way interaction between the caffeine treatment and time of measurement because the change of foraging behaviour over time might depend on the presence of caffeine. To test for the significance of

interactions, we used likelihood ratio tests (LRT). The interaction between these two fixed-effects was removed from the final model if it was not significant ($p > 0.05$). The final model always included all three fixed-effects. To test the significance of the main effects, we used Wald tests (Zuur et al., 2009).

Results

Part 1: Does caffeine affect foraging in stingless bees?

When the bees were offered 40% sucrose solution at one feeder with and one feeder without caffeine (Figure 1.1A), the number of bees at both feeders increased with time. However, there was no significant difference in the growth trend (i.e. the interaction between time and caffeine presence) between the two feeders (GLMM, time × treatment: LRT = 0.33, $p = 0.56$). We found no effect of the medium dose of caffeine or background colour on the number of bees at the feeders (treatment: z = 0.37, $p = 0.71$; time: z = 11.10, $p < 0.001$; colour: z = 1.45, $p = 0.15$). The number of foragers at the feeders did not increase over time when they were offered 30% (GLMM, time: z = -0.83, p = 0.41; time × treatment: LRT = 0.03, p = 0.87; Figure 1.1B). Also, the presence of a high dose of caffeine had no effect on the number of bees at the feeder (treatment: z = -0.21, p = 0.83). However, more bees visited feeders on the yellow background compared to blue background (colour: z = 10.30, $p < 0.001$).

The foraging frequency of the individually marked bees to feeders containing a 40% sucrose solution was not affected by caffeine (LME, treatment: $t = -0.39$, df = 44, $p = 0.70$; Figure 1.1C) and bees did not show a preference for a feeder based on the colour background (colour: $t = -0.45$, df = 44, $p = 0.66$). Similarly, the number of foraging visits of individually marked bees to 30% sucrose solution did not go up or down depending on the high caffeine dose or the colour of the background (LME, treatment: $t = -1.19$, df = 33, $p = 0.24$; colour: $t = -1.54$, df = 33, $p = 0.13$; Figure 1.1D).

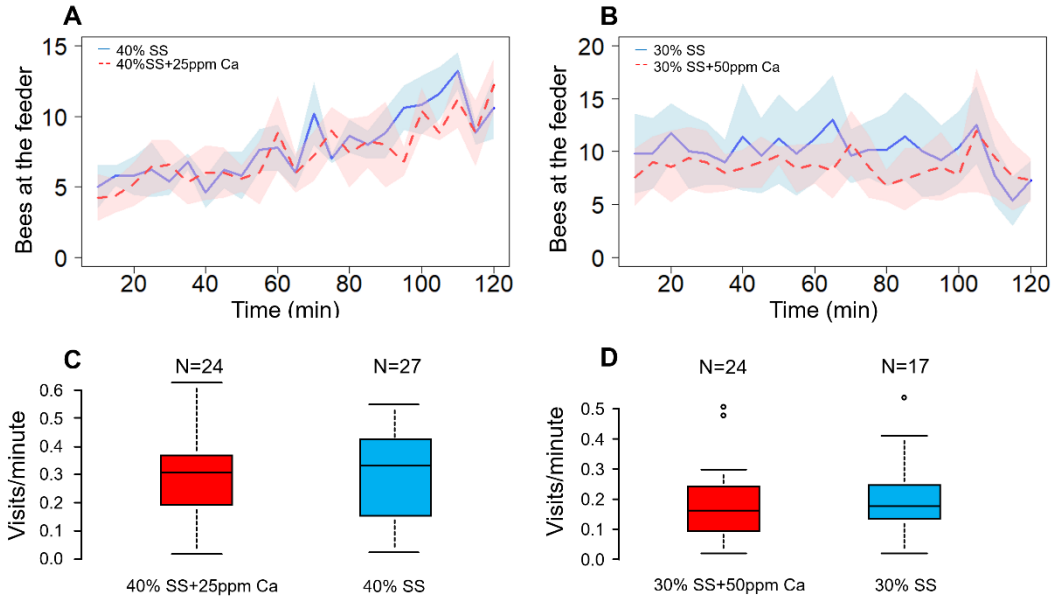

Figure 1.1: A) The mean number of bees visiting the 40% sucrose solution (SS) feeders, with and without a medium dose of caffeine (Ca), over 120 minutes. The shaded areas represent the SE of the mean. B) The mean number of bees visiting the 30% syrup feeders over time, with and without a high dose of caffeine. The shaded areas as in A. C) The visits per minute made by individually marked bees to the 40% sucrose solution for the caffeinated and non-caffeinated feeder. The horizontal bars of the boxplots indicate the medians and the boxes delimit the first and third quartile. N represents the number of individually marked bees. D) The visits per minute made by individually marked bees to the 30% sucrose solution for the caffeinated and non-caffeinated feeder. Boxplots as in C.

The bees that were treated with caffeine did not show higher persistency compared with control bees (which only received sucrose solution without caffeine) irrespective of the dose. More specifically, in the medium dose-40% sucrose solution treatment, the number of bees (both marked and unmarked) at a feeder was not affected by background colour during training, observation time, nor whether the feeder offered caffeinated or non-caffeinated solution during training (GLMM, treatment: $z = 1.68$, $p = 0.094$; time: $z = 1.49$, $p = 0.14$; colour: $z = 1.33$, $p = 0.18$; time × treatment: LRT = 0.87, df = 1, $p = 0.35$; Figure 1.2A). Likewise, in the high dose-30% sucrose solution treatment, the presence of caffeine in solution and observation time did not affect the number of bees (both marked and unmarked) at a feeder the next day (GLMM, treatment: $z = -0.59$, $p = 0.56$; time: $z = -0.28$, $p = 0.78$; time × treatment: LRT = 0.95, df = 1, $p = 0.33$; Figure 1.2B). More bees (both marked and unmarked), however, landed on the yellow feeder (which was also the yellow feeder on the previous day) than the blue feeder (colour:

z = 4.45, $p < 0.001$).

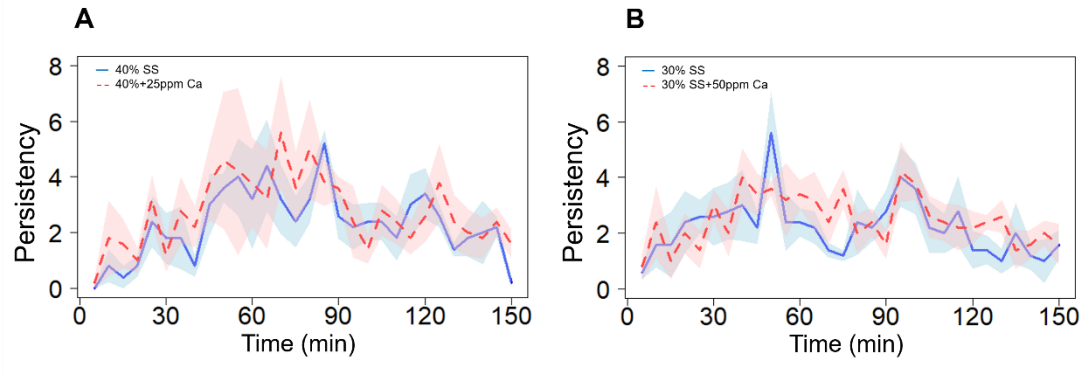

Figure 1.2: Persistency, i.e. bees return to the empty feeder the day after treatment. A) The day after treatment with 25ppm caffeine (Ca) dissolved in a 40% sucrose solution (SS), the lines show the mean number of bees returning to the empty feeders over 150 minutes. B) The day after treatment with 50ppm caffeine in 30% SS, the lines show the mean number of bees returning to the empty feeders over 150 minutes.

Additionally, we also examined the persistency of individually marked bees. Neither the medium dose of caffeine (Binomial GLMM, treatment: z = -0.84, $p = 0.4$; colour: z = 0.13, $p = 0.90$), nor the high dose of caffeine affected persistency (Binomial GLMM, treatment: z = -1.18, $p = 0.24$; colour: z = 0.79, $p = 0.43$). Because there was no effect of caffeine on the persistency of marked bees, we pooled the data of all bees to test if sucrose concentration had an effect on the persistency of marked bees. However, no significant difference was found between 30% and 40% sucrose solution treatment on persistency of marked bees (Binomial GLMM, treatment: z = 0.92, $p = 0.36$; colour: z = 0.14, $p = 0.89$).

Part 2: Does P. droryana show recruitment to high quality resources?

To examine whether the quality of the food source affects the number of bees at the feeders, we offered *P. droryana* colonies again two feeders, but one contained a 40% sucrose solution and the other one a 30% sucrose solution. The background colour was kept constant (yellow). The number of stingless bees foraging at the two feeders was significantly different between 30% sucrose solution and 40% sucrose solution (Poisson GLMM, treatment: z = -5.86, $p < 0.001$; Figure 1.3A). Additionally, there was a significant interaction between sucrose concentration and time (time × treatment: LRT = 35.40, df = 1, $p < 0.001$): More specifically, the number of foragers at the feeder with 40% sucrose solution increased over time (Poisson GLMM, time: z = 2.27, $p = 0.02$), whereas the number of foragers at the 30% feeder significantly decreased over time (Poisson GLMM, time: z-value = -5.42, $p < 0.001$). Focusing only on

individually marked foragers, we found a significantly higher foraging frequency at 40% sucrose solution feeder than 30% sucrose solution feeder, that is, 35.6% more visits per minute (LME, treatment: $t = 3.67$, df = 92, $p < 0.001$; Figure 1.3B).

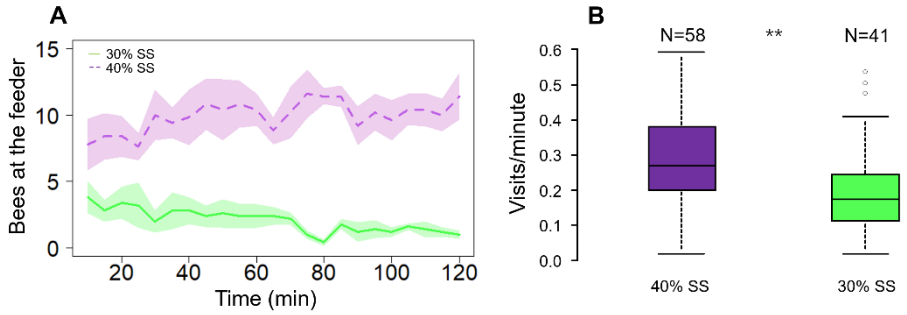

Figure 1.3: A) The lines show the mean number of bees visiting the 30% and 40% sucrose solution feeders over 120 minutes. B) The visits per minute made by individually marked bees to the 30% sucrose solution and 40% sucrose solution (SS) feeder. ** represents the p value <0.01.

Discussion

Our results suggest that caffeine does not affect individual and collective foraging effort in the stingless bee *P. droryana* in the range of tested concentrations in our study area. Compared to the control, a sucrose solution with caffeine did not alter the number of foragers at the food sources, the foraging frequency of individually marked bees and their persistency. We used two doses of caffeine, 25ppm (~0.14 mM) and 50ppm (~0.28 mM), which is comparable to what has been measured in the nectar of plants of the genus *Coffea* (0.003mM to 0.253mM) (Wright et al., 2013). It could be argued that *P. droryana* foragers do not pay attention to the perceived energy content of solutions but prioritise other factors, such as foraging distance or the flow rate of nectar. This could explain why caffeine did not affect foraging. However, this is unlikely to explain the lack of an effect of caffeine, because *P. droryana* foragers did increase their visitation rate when we offered solutions with higher sucrose concentrations. When the concentration of sucrose solution was 40%, the number of bees at the feeders increased over time, whereas the number of foragers remained constant or decreased at 30% feeders. Additionally, the visit per minute rate at the feeders was significantly higher at feeders offering 40% sucrose solution compared with the feeders offering 30%. This suggests that *P. droryana* adjusts its foraging behaviour to the perceived value of the food sources, but that caffeine does not modulate the perceived value.

Our finding that caffeine does not affect foraging effort in *P. droryana* contrasts findings from

European honey bees: caffeine intake caused changes in honey bee learning performance, foraging effort, recruitment behaviour and persistency (Wright et al., 2013; Couvillon et al., 2015). It has been suggested that plants might add caffeine to nectar to stimulate bee visitations resulting in pollination, while offering a smaller energetic reward than perceived by the bees, thus cheating in the plant-pollinator mutualism. In the tropics, stingless bees have long been recognised for their important role in pollination of *Coffea* (Heard, 1999; Ricketts, 2004; Nogueira et al., 1959). One explanation for the absence of an effect of caffeine on the foraging effort of *P. droryana* could be that this population has evolved a tolerance towards the effects of caffeine. *Plebeia droryana* has a long history of exposure to caffeinated nectar and pollen in the state of São Paulo and, thus, had many generations to adapt to caffeine in the study area.

An alternative explanation could be that the effects of caffeine and other secondary plant compounds vary among bee groups due to physiological and neural differences among different bee taxa (see e.g. Afik et al., 2014). In *A. mellifera*, caffeine functions as an adenosine receptor antagonist and affects the mushroom body neurons involved in olfactory learning and memory. The interaction of caffeine and adenosine receptors could lead to increased activation of Kenyon cells in projection neurons (Chittka and Peng, 2013; Wright et al., 2013). Ultimately, caffeine affects long-term memory by blocking adenosine receptors. However, in *P. droryana* caffeine might be broken down before it reaches the brain. Previous work has shown that caffeine was degraded in the gut of the coffee berry borer (*Hypothenemus hampei*) and that this activity was eliminated by experimental inactivation of the gut microbiota (Ceja-Navarro et al., 2015). Studies on *Drosophila* (Bhaskara et al., 2006; Willoughby et al., 2006) and honey bees (Kucharski and Maleszka, 2005) show that caffeine regulates the genes of the cytochrome P450 family (CYP proteins) which are involved in the detoxification metabolism by increasing their expression. However, information about the absorption, tissue distribution, and metabolites of caffeine in invertebrates is still sparse and more research is needed to understand the caffeine transport mechanism in stingless bees. Due to potential differences in physiology in different insect taxa, caffeine may have different effects. Similarly, octopamine and dopamine (which also affects reward signalling in bees) can affect different behaviours in different ant species (Kamhi and Traniello, 2013).

In honey bees, the profitability of food sources increased the persistence of foragers to an unrewarding feeding location (Toufailia et al., 2013). In our study, the persistence of *P. droryana*

foragers was not affected by the concentration of the sucrose solution and only 23% of the marked bees returned to the empty feeder the day after treatment with 40% sucrose solution. This suggests that *P. droryana* shows much lower day-to-day persistence than *Apis mellifera,* possibly because they forage on food sources that are more ephemeral.

Honey bees famously use the waggle dance to communicate the location of a profitable resource to their nestmates (von Frisch, 1967). Among the over 500 different stingless bee species (Rasmussen and Cameron, 2010), different methods are found to recruit nestmates to resources (reviewed in Nieh, 2004; Hrncir, 2009; Jarau, 2009). Stingless bees can be recruited to specific locations by other foragers; alternatively, they can be induced to search for food sources in the environment in a spatially unspecific way. For example, returned foragers of some species would vigorously run through the nest, therefore jostling their nestmates (Hrncir, 2009). Additionally, the foragers produce thoracic vibrations (sounds). Both behaviours were considered potential mechanism to improve the recruitment abilities of nestmates (Lindauer and Kerr, 1960). A different strategy is used by species that recruit to specific food source locations using chemical compounds (Jarau, 2009; Leonhardt, 2017; Nieh, 2004). Our finding that the number of foragers at a 30% sucrose feeder decreased over time, but increased at a nearby feeder that offered 40% solution could be explained by different processes. For instance, the different food qualities could lead to different rates of abandoning a food source. Also, discovery of a high quality food source often stimulates the foraging activity of a colony (Schorkopf et al., 2016), which, in combination with local enhancement (the visual attraction of a food source that is occupied by other foragers; Slaa et al., 2003) could explain why forager numbers decreased at the 30% feeder, but increased at the 40% feeder. Furthermore, the findings could also be explained by foragers switching from the 30% to the 40% feeder, but this was not noticed by the observers during the experiment. Alternatively, *P. droryana* foragers might be able to recruit nestmates to food sources. It could be, for example, that the bees deposit pheromones to advertise a high-quality food source. In *Trigona recursa*, for instance, a feeder baited with pheromones can attract nestmates (Jarau et al., 2004) and colonies recruit more bees to food sources of higher quality (Schmidt et al., 2006). However, we did not observe *P. droryana* foragers deposit odour trails near the food source. On the other hand, *P. droryana* is a very small bee (c. 3 mm long) and marking behaviour might be difficult to observe. Alternatively, bees might also be attracted by footprints left on and near the feeder by foragers (Hrncir et al., 2004; Jarau et al., 2004b; Jarau, 2009). Footprint chemicals are often not very volatile, which means that other bees would have to be relatively

close to perceive them (within 1 m in the much larger *M. seminigra*; Hrncir et al., 2004). Lindauer and Kerr (1960) found no evidence for site-specific recruitment in *P. droryana*, but their experiments should be repeated with a more representative sample size. Thus, a next step will be to further test if *P. droryana* recruits to particular food sources and, if this is indeed the case, elucidate the underlying mechanism

CHAPTER 2

Foragers of the stingless bee *Plebeia droryana* inform nestmates about the direction, but not the distance to food sources

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Peng, T., Pedrosa, J., Batista, J.E., Nascimento, F. & Grüter, C. (2020) Foragers of the stingless bee *Plebeia droryana* inform nestmates about the direction, but not the distance to food sources. *Ecological Entomology*.

Abstract

1. The tropical stingless bees have evolved intricate communication systems to recruit nestmates to food locations. Some species are able to accurately communicate the location of food, whereas others simply announce the presence of food in the environment.

2. *Plebeia droryana* is a tiny Neotropical stingless bee that, until recently, was thought to use a solitary foraging strategy, i.e. without the use of a recruitment communication system. However, recent research has indicated that *P. droryana* might be able to recruit nestmates to specific food source locations.

3. We tested this by studying whether foragers can guide nestmates in the direction and the distance of artificial feeders placed in the vicinity of the colony. We trained bees to a scented sucrose solution feeder at 10 m and placed different feeders either in different directions (experiment 1) or in different distances (experiment 2). We found that *P. droryana* directs newcomers in the right direction, but distance information does not seem to be communicated.

4. Moreover, we then tested whether newcomers use chemical and visual cues originating from nestmates foraging at the food source, but found no evidence for the use these social cues provided by conspecifics.

5. The potential mechanism that *P. droryana* may use to orient recruits toward the food source, however, remains unknown and requires further study.

Introduction

Social insects have evolved a remarkable diversity of communication mechanisms to guide nestmates to food locations (Wilson and Edward, 1971; Hölldobler and Wilson, 1990; Jarau and Hrncir, 2009). These communication mechanisms allow colonies to allocate workers to food sources that are too large to be exploited by an individual and, thereby, collect more food for the colony. Communication mechanisms can be divided into extranidal and intranidal mechanisms. The waggle dance in honey bees is a striking intranidal communication mechanism that has been studied extensively (von Frisch, 1967; Gould, 1975; Dyer, 2002; I'Anson Price et al., 2019). A dancing bee indicates the distance and direction of food sources to its followers (von Frisch, 1967; Dyer, 2002; Couvillon, 2012). At the same time, followers gain information about the odour of the food source, e.g. during trophallaxis (Gil and De Marco, 2005; Farina et al., 2005 Grüter and Farina, 2009). Extranidal communication mechanisms include the laying of pheromone trails (in many ants) or tandem running (Hölldobler and Wilson, 1990; Franklin, 2014; Czaczkes et al., 2015).

Stingless bees (Apidae, Meliponini) are a large group of eusocial hymenopterans that live in diverse tropical and subtropical habitats (Roubik, 1989). Several hundred species exist that show a great diversity in lifestyle and ecology. While most genera play a crucial role as pollinators, a few species have evolved carnivorous or robbing lifestyles (Camargo and Roubik, 1991; Barth et al., 2008; Grüter et al., 2016). Food source communication mechanisms in stingless bees are diverse and seem to be species-specific, ranging from simply motivating nestmates to leave the nest and search for food to the precise communication of the food source location by using pheromone trails. Some species are more efficient at recruiting nestmates to food sources than honey bees (Lindauer and Kerr, 1960; Aguilar et al., 2005; Barth et al., 2008).

Intranidal recruitment communication is well-known in stingless bees. Successful foragers of many species perform "zigzag" or "jostling" runs inside the nest (Lindauer and Kerr, 1958, 1960; Hrncir et al., 2000), which usually takes place close to the nest entrance (Nieh, 1998; Hrncir, 2009). The jostling runs appear to play an important role in activating potential foragers (Hrncir et al., 2000). For example, in *Melipona seminigra*, inactive foragers significantly increased their own jostling activity after they were jostled by a recruiting bee (Hrncir, 2009). During the jostling runs and during trophallaxis, buzzing sounds are generated with thoracic muscles (Kerr et al., 1963; Esch et al., 1965; Nieh and Roubik, 1998;

Aguilar and Briceño, 2002; Nieh et al., 2003b), which may stimulate the vibrated receivers to initiate foraging (Hrncir et al., 2006; 2008; Hrncir and Barth, 2014; Krausa et al., 2017). At the same time, the information of quality and odour of a food source is potentially shared inside the colony by performing trophallaxis (Nieh et al., 2000; Aguilar et al., 2005; Jarau, 2009; Krausa et al., 2017).

Extranidal recruitment communication can involve foragers laying a scent trail when leaving a food source, as found in *Cephalotrigona*, *Scaptotrigona* and *Trigona* (Lindauer and Kerr, 1958, 1960; Nieh et al., 2003a, 2004; Jarau, 2009) or the deposition of a scent beacon near the food source, which can attract other foragers (Nieh, 1998; Hrncir et al., 2004; Jarau et al., 2004a; Alavez-Rosas et al., 2017). Furthermore, for some species it has been suggested that visual tracking of guiding flights performed by recruiting foragers from nest to food source explain location specific recruitment (Kerr and Lindauer, 1960; Aguilar et al., 2005). Recruitment success could also be the result of a combination of these mechanisms (Barth et al., 2008). However, most stingless bees do not seem to use pheromone trails to recruit nestmates, which may be due to the relatively small colony size of many species (Nieh, 2004) as the number of workers in small colonies is not sufficient to sustain the volatile pheromone trails (Beekman and Dussutour, 2009). In addition, for larger colonies it could be easier to dominate a food source, which makes using pheromone trails potentially more beneficial (Aguilar et al., 2005). Thus, strong competition might favour recruitment communication in species with large colonies, but select against recruitment in species with small colony or body sizes (Johnson and Hubbell, 1974). Rapid and accurate communication could also be important for patchily distributed high-quality food sources, such as carrion (Noll, 1997).

Some species are able to recruit to specific locations without the use of pheromone trails. For example, *Partamona orizabaensis* foragers can communicate the location of food sources to their nestmates but the mechanism is still a mystery (Flaig et al., 2016). Furthermore, there are species where foragers seem to only use information about the direction of food sources, without using accurate distance information (Jarau et al., 2000; Nieh et al., 2000; Nieh, 2004; Aguilar et al., 2005). More generally, recruitment communication remains poorly understood in most stingless bees. This, in turn, has hampered our understanding of how complex recruitment communication systems have evolved in social bees and how foraging strategies are related to the lifestyle of different species.

In *Plebeia droryana*, a small (~3 mm long) species commonly found in South America, foragers

have been shown to produce buzzing sounds to alert nestmates about the presence of a food source, but Lindauer and Kerr (1958, 1960) found no evidences for specific location communication in this species. Peng et al. (2019), on the other hand, found that the number of *P. droryana* foragers steadily increased over time at a high-quality food source, suggesting that *P. droryana* foragers might provide nestmates with specific location information. The two studies differed in both the number of colonies observed and the foraging distance tested. While Lindauer and Kerr (1958, 1960) used only a single colony and a relatively large foraging distance for such a small bee, Peng et al. (2019) studied 5 colonies at a nearby food source (10 m). Since food source distance affects recruitment probability (Nieh et al., 2003a, 2004; Stangler et al., 2009), it is possible that foragers were not motivated to recruit in Lindauer and Kerr (1958, 1960). Here, we studied if *P. droryana* foragers can potentially provide direction and distance information to nestmates. Since foragers of some species are attracted by visual and chemical cues of conspecifics at food sources, so-called local enhancement (Slaa et al., 2003; Slaa and Hughes, 2009), we also explored if the presence of nestmates or their footprints at food sources affects *P. droryana* forager allocation.

Materials and methods

Study species and field site

We performed all experiments on the campus of the University of São Paulo in Ribeirão Preto, Brazil. This area has many different stingless bee species (Cortopassi-Laurino et al., 2009), and *Plebeia droryana* is among the most common ones. Wild colonies nest in tree cavities or in cavities in the walls of buildings. We used eight wild colonies for our experiments. Wild colonies were at least 100m from each other. To prevent bees from other colonies to visit our feeders, we closed all the visible colony entrances within a 10 m radius around the focal colonies. Data were collected in February and March 2019 on days with good foraging conditions.

Experimental procedures

Foragers from the tested colonies were trained to artificial feeders by standard training procedures (see von Frisch, 1967; Peng et al., 2019; 2020). First, we placed one artificial feeder with a 50% sucrose solution next to the nest entrance to attract foragers to start collecting the sucrose solution. After a group of foragers was established, the feeder was moved to the final location 10m from the tested colony while the foragers were drinking sucrose solution. Foragers would learn the new feeder location when

returning to their nest. The proximity of the feeder compared to Lindauer and Kerr (1958, 1960) increased the chance that foragers would recruit, which was important to test whether this species has the *potential* to recruit. In all experiments, the feeder was located on a chair (height 0.5 m) with a yellow background, and the sucrose solution contained a scent, like eucalyptus, lavender, orange or mint. Each colony was tested with only one odour (5 µl essential oil per 100 ml sucrose solution; Primavera Life GmbH, Oy-Mittelberg, Germany), which aids in the formation of spatial memories in bees (Menzel, 1999). We used the same scent in the training and testing phases in a given trial.

Experiment1: do P. droryana foragers provide direction information?

To test site-specific recruitment communication in *P. droryana*, we assessed direction (Experiment 1) and distance information (Experiment 2). For experiment 1, we began training in the morning and after ~10 foragers were trained to the feeder, we marked 10 foragers individually with acrylic paint on the thorax. The position of this training feeder was either on the left-hand side (4 trials) or right-hand side (4 trials) of the entrance of a tested colony (Figure 2.1A). During the following 20 minutes, we allowed only these marked bees to visit the feeder by catching all the unmarked bees using an aspirator and the marked foragers continued to visit the feeder for 20 minutes. According to Peng et al. (2019), foragers perform 5-6 foraging trips on average during 20 minutes to a high-quality food source at this distance. This allowed us to make sure that only foragers from the focal colony were collecting food from our feeder. Secondly, the foragers had sufficient time to deposit chemical compounds on the feeder (therefore called F_{Chem}) and chair if they did.

After this 20-minute phase, the training feeder F_{Chem} was replaced by a new, clean feeder at the training location (F_{TraLoc}). This new feeder was placed on an identical chair and yellow background. The original feeder F_{Chem}, the chair it was placed on, and the yellow background were moved to a different location that was also 10 m from the nest (Figure 2.1B). Additionally, we introduced a third feeder F_{New}, which was placed on an identical chair with yellow background 10 m from the nest, but in a different direction (Figure 2.1B). During the following testing phase, all three feeders offered 50% scented sucrose solution. In half of the trials, F_{Chem} was placed between F_{TraLoc} and F_{New}, whereas in the other half of the trials, F_{New} was placed between the other two feeders (Figure 2.1B). During the testing phase, the marked bees continued to collect food at the training location, now from feeder F_{TraLoc}.

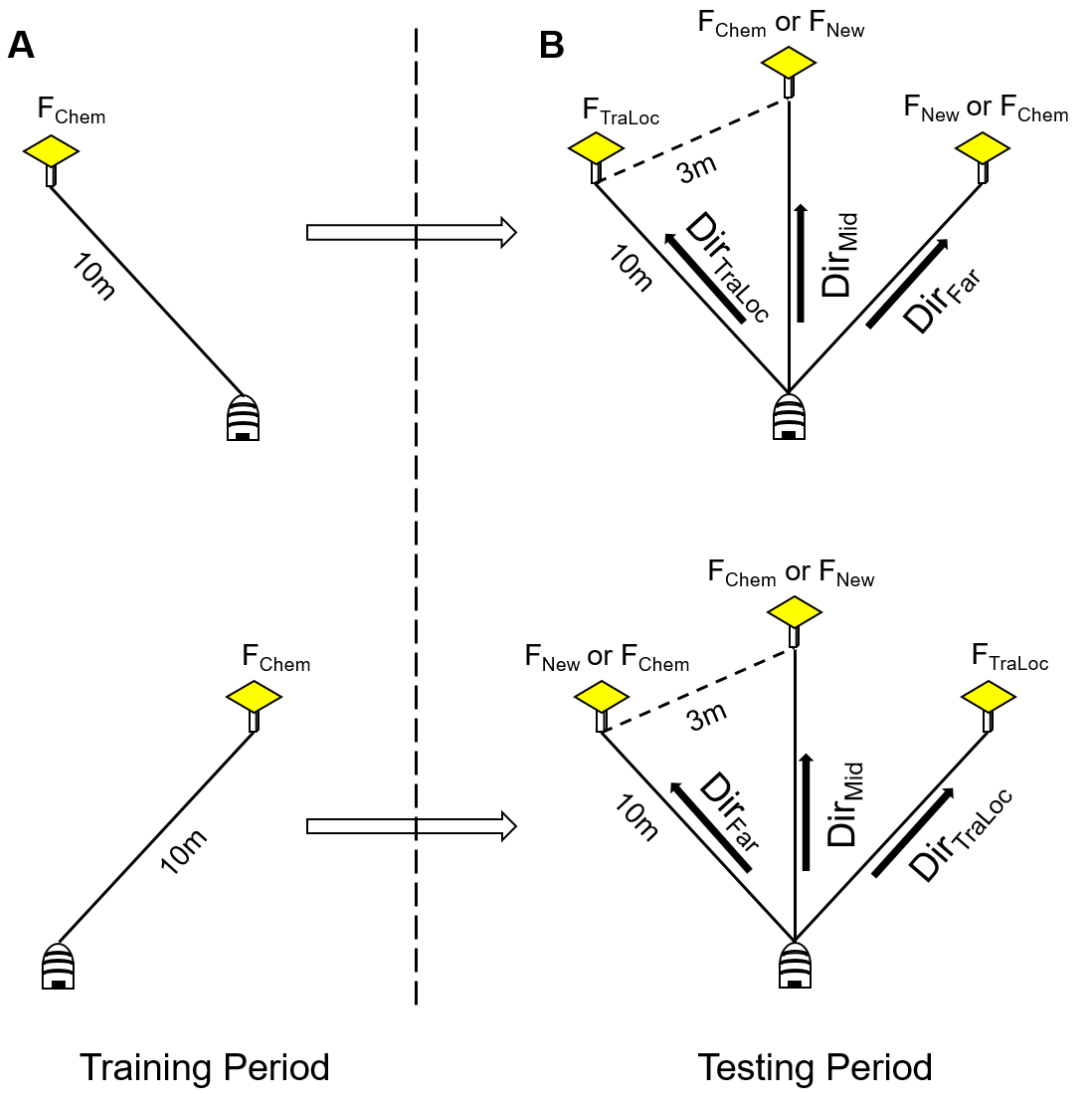

Figure 2.1: Direction experimental setup. A) The location of feeder F_{Chem} during the training period. B) The distribution of feeder F_{Chem}, F_{TraLoc}, F_{New} in the testing period.

During the testing period of 120 min, we caught all unmarked bees that landed on the three feeders, while allowing the marked foragers to collect food at F_{TraLoc}. If a marked bee landed on one of the other two feeders, we also caught them while on the empty feeder. However, this happened very rarely. Eight trials were carried out, one with each colony.

Experiment 2: do P. droryana foragers provide distance information and do newcomers use local enhancement?

In order to find out whether nestmates acquire distance information and whether local enhancement is used by newcomers, we performed a second experiment. We set up one feeder 10 m from the tested colony as described above (Figure 2.2A). Ten individually marked foragers were again allowed to visit

this feeder (called F_{Tra10m}) during 20 minutes, while unmarked bees that landed on the feeder were caught. Then, two new feeders on identical chairs and backgrounds were placed halfway between the training feeder and the nest (5 m from the nest). The two new feeders were separated by 1 m from each other. All three feeders were placed on a Petri dish (radius of 7 cm). One of the Petri dishes placed under one of the new feeders (henceforth called F_{Bee5m}) contained seven individually marked live foragers from the same colony to provide visual cues to approaching foragers (Figure 2.2B). Before the foragers were transferred to the Petri dish, we chilled them on ice for 2 min to immobilise them. After carefully moving them to the Petri dish, we tightly sealed the Petri dish with tape to prevent chemical compounds from leaving the Petri dish. The other two Petri dishes did not contain bees. During the following 120 min testing phase, we captured and counted all newcomers landing on each feeder. Marked bees were only allowed to visit feeder F_{Tra10m}, otherwise we caught them. The location of feeders F_{Bee5m} and F_{Emp5m} (the new feeder on an empty Petri dish) was randomized in each trial (left or right from the direction between the nest and the original training feeder F_{Tra10m}).

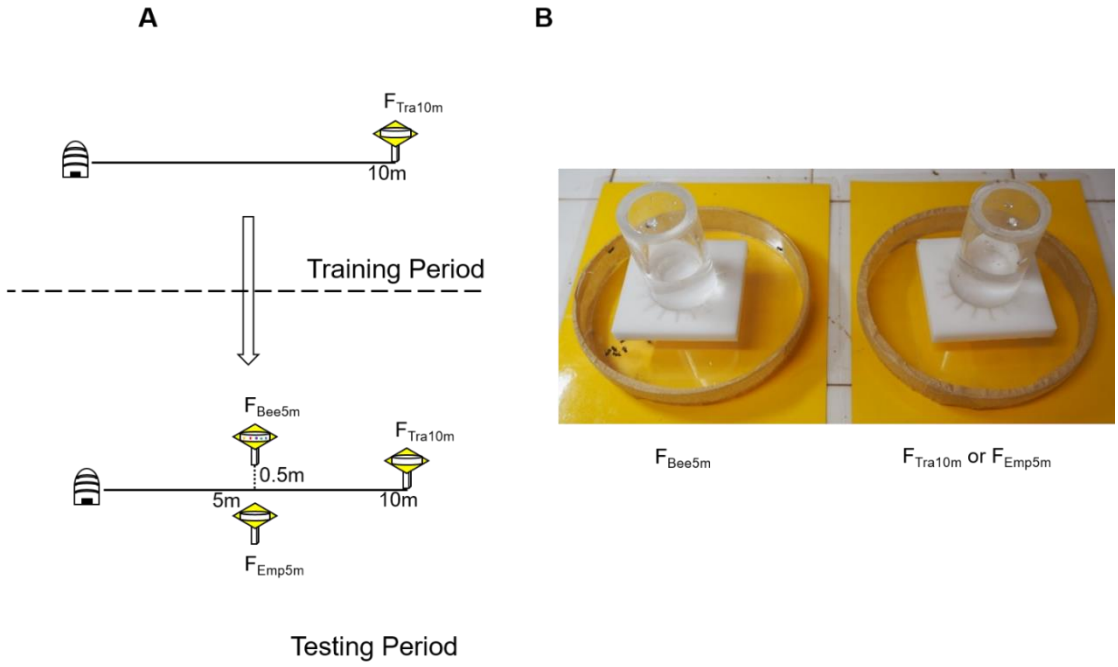

Figure 2.2: Distance and Local enhancement experimental setup. A) The location of feeder F_{Tra10m} in the training period and the distribution of feeder F_{Tra10m}, F_{Bee5m}, F_{Emp5m} in the testing period. Coloured dots at the feeder F_{Bee5m} represent imprisoned bees. B) The schematic diagram of F_{Tra10m} and F_{Bee5m} or F_{Emp5m}.

Statistical analysis

In experiment 1, we expected that if trained foragers provided direction information in the form of a

pheromone trail, visual cues or guidance flights, most newcomers should arrive at F_{TraLoc} (the direction of this feeder was called Dir_{TraLoc}). If foragers left chemical cues on the feeder or chair (a chemical "beacon"), then newcomers should land more frequently on feeder F_{Chem} than F_{New}. If foragers did not recruit nestmates to a food source location, then the number of newcomers should be equal on all three feeders. Thus, we first used the proportion of unmarked bees at each feeder (F_{Chem}, F_{TraLoc} and F_{New}) as response variable to test if the foragers show a preference for a feeder and whether they could use chemical marks to choose a feeder. We also compared the effect of the spatial distribution of feeders (Dir_{Mid}: feeder direction was closest to Dir_{TraLoc}, Dir_{Far}: feeder direction was most distant to Dir_{TraLoc} and Dir_{TraLoc}).

In experiment 2, we expected that if marked foragers provided distance information, then more newcomers would arrive at F_{Tra10m}. If newcomers use local enhancement, then they should prefer F_{Bee5m} and F_{Tra10m} over F_{Emp5m}. If bees used neither distance information nor visual cues, then they should arrive in equal numbers at all three feeders. We used the proportion of unmarked bees at each feeder (F_{Tra10m}, F_{Bee5m} and F_{Emp5m}) as the response variable.

All statistical tests were performed in R version 3.4.4 (http://www.R-project.org/). In experiment 1 and 2, we used paired sample *t*-test to test specific predictions about distance and direction communication. P-values were adjusted with the sequential Bonferroni correction if we used the same data for multiple comparisons (Sokal and Rohlf, 1995).

Results

Experiment 1: do P. droryana foragers provide direction information?

Most unmarked bees arrived at feeder F_{TraLoc}, which was exploited by the marked bees during the testing phase. On average 50.53% ± 4.62% of all recruits arrived at this feeder, while 19.19% ± 2.51% arrived at the training feeder F_{Chem} and 30.28% ± 4.38% at the feeder F_{New} (Figure 2.3A, Table 2.1). The proportion of newcomers at F_{Chem} was significantly lower than at F_{TraLoc} (paired *t*-test: t=-5.22, df=7, *P*-adjusted =0.0037), whereas there was no difference between the feeders with and without putative scent marks (F_{Chem} vs F_{New} *t*-test paired: t=2.04, df=7, *P*-adjusted =1.00; F_{TraLoc} vs F_{New} *t*-test paired: t=-2.34, df=7, *P*-adjusted=0.10).

Table 2.1: The number of foragers and average of proportion at each feeder or location in experiment 1 and 2. The value represents mean±SE.

Experiment 1			Experiment 2		
Feeders	*Average Number of Foragers*	*Average Proportion*	**Feeders**	*Average Number of Foragers*	*Average Proportion*
F_{Chem}	20.00±4.07	19.19% ± 2.51%	F_{Tra10m}	60.63±19.73	33.69% ± 4.34%
F_{TraLoc}	60.63±18.27	50.53% ± 4.62%	F_{Bee5m}	47.75±11.04	33.43% ± 2.47%
F_{New}	39.38±13.41	30.28% ± 4.38%	F_{Emp5m}	45.63±10.10	32.88% ± 3.31%
Location	*Average Number of Foragers*	*Average Proportion*			
Dir_{TraLoc}	60.63±18.27	50.53% ± 4.62%			
Dir_{Mid}	38.75±13.33	30.57% ± 3.56%			
Dir_{Far}	20.63±4.69	18.90% ± 3.44%			

As mentioned, most unmarked bees (50.53% ± 4.62%) arrived at the direction of F_{TraLoc} (direction Dir_{TraLoc} in Figure 2.3B), whereas 30.57% ± 3.56% landed on the feeder closest to F_{TraLoc} (direction Dir_{Mid} in Figure 2.3B). Only 18.90% ± 3.44% of newcomers landed on the feeder that was most distant to the feeder visited by the marked bees (direction Dir_{Far} in Figure 2.1B) (Figure 2.3B, Table 2.1). Thus, significantly more newcomers arrived at feeder visited by the marked bees compared to the more distant feeder (*t*-test paired: t=4.32, df=7, *P*-adjusted=0.01). The feeder placed between the other two feeders showed intermediate attractiveness (Figure 2.3B) (paired: *t*-test, Dir_{Mid} vs. Dir_{TraLoc}: t=-2.66, df=7, *P*-adjusted=0.065, Dir_{Mid} vs. Dir_{Far}: t=2.22, df=7, *P*-adjusted=0.065).

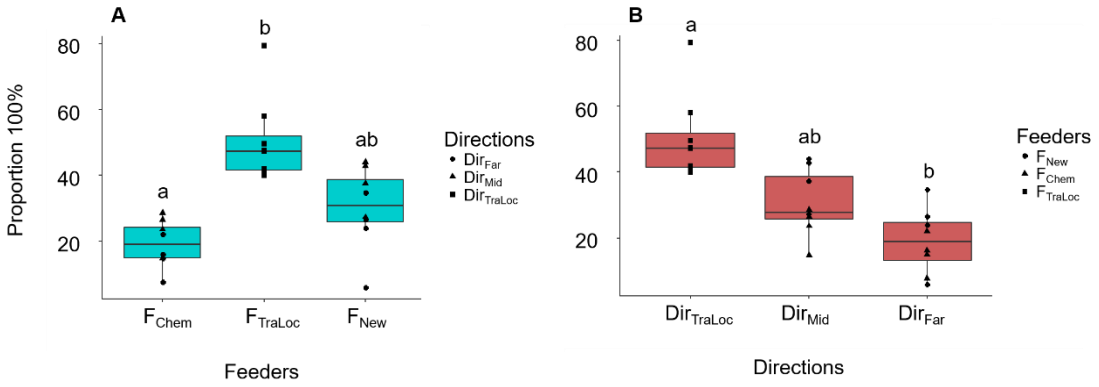

Figure 2.3: Newcomer arrival at different feeders (A) or directions (B). Different letters indicate significant differences ($P < 0.05$) between the percentages of newcomers at the feeders or directions. The boxplots indicate the medians, the 25%- and 75% quartiles. The different shape of dots at the box points individual datapoints according to their directions (A) or feeders (B).

Experiment 2: do P. droryana foragers provide distance information or do newcomers use local enhancement?

To test whether recruits use distance information to specific food locations or the visual presence of other bees at a food source, we set up one feeder on top of an empty Petri dish at 10 m (F_{Tra10m}), one

feeder on top of a Petri dish with live nestmates (F_{Bee5m}) at 5 m and another feeder on an empty Petri dish at 5m from the tested colony (F_{Emp5m}) (Figure 2.2A). Recruits arrived in similar proportions at the three feeders F_{Tra10m} (33.69% ± 4.34%), F_{Bee5m} (33.43% ± 2.47%) and F_{Emp5m} (32.88% ± 3.31%) (paired *t*-test: F_{Tra10m} vs F_{Bee5m}, t=-0.17, df=7, *P*-adjusted=1.00; F_{Tra10m} vs F_{Emp5m}, t=0.27, df=7, *P*-adjusted=1.00; F_{Bee5m} vs F_{Emp5m}, t=0.012, df=7, *P*-adjusted=1.00, Figure 2.4, Table 2.1).

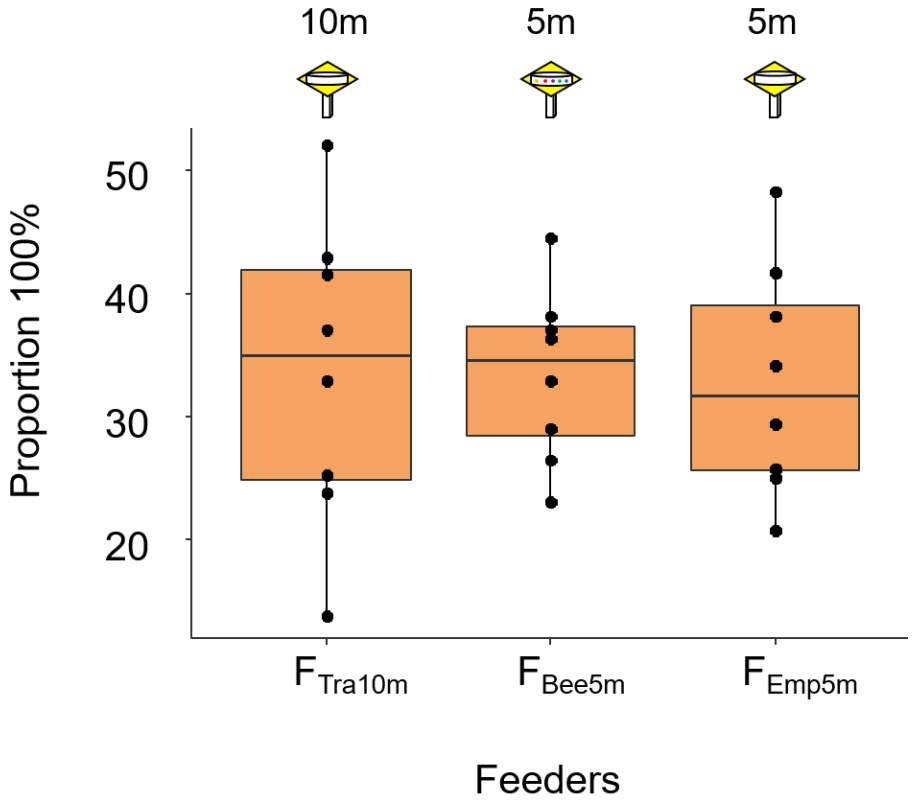

Figure 2.4: The percentages of *P. droryana* newcomers that arrived at different feeders. Black dots at the feeder F_{Bee5m} represent imprisoned bees. The boxplots indicate the medians, the 25%- and 75% quartiles. Coloured dots at the box represent the proportion of each colony. Black dots represent individual datapoints

Discussion

Our results suggest that *P. droryana* foragers transmit information about the direction of food sources to nestmates, but not about its distance. In addition, neither the visual presence of nestmates nor chemical marks *per se* affected the number of newcomers. The finding that foragers provide directional information to nestmates is consistent with the findings of a recent study (Peng et al., 2019), but contrasts with earlier observations that have suggested that this species does not recruit to food sources (Lindauer and Kerr, 1958, 1960). We used a shorter training distance compared to these earlier studies

(10 m vs 150 m), which is likely to affect both the efficiency and the likelihood of recruitment in *P. droryana*. The proximity of the food source in our study made it more probable that foragers would recruit and, thus, reveal whether this species has the potential to recruit nestmates.

Chemical cues left at the food source do not seem to explain the recruitment pattern we found because newcomers did not land more on the feeder that was visited by marked bees during the training phase (F_{Chem}) compared to a new feeder at the same distance from the colony (F_{New}) that was not visited by marked bees earlier. This is unexpected as one way to attract nestmates is to mark the food sources using chemical compounds (Jarau, 2009). For example, *Melipona panamica* foragers deposit scent marks that function as an olfactory beacon for themselves and for nestmates (Nieh, 1998). The same has been found in *Trigona corvina* and *Nannotrigona testaceicornis*, where newcomer bees are attracted to feeders that foragers previously visited compared to clean feeders (Schmidt et al., 2005; Boogert et al., 2006). One reason could be that the scent marks left on food sources have a short attractive range, which the *P. droryana* newcomers might not have entered. In *Melipona seminigra*, scent marks were perceived and attractive at a distance of about 1 m and their effect lasted about two hours (Hrncir et al., 2004). In *Scaptotrigona* aff. *depilis*, this attraction range was up to 20 m (Schmidt et al., 2003). Our results indicate that *P. droryana* foragers either did not leave significant amounts of scent marks at the feeding place or that this information was not used by recruits at the distances between food sources used in our experiment.

Alternatively, *P. droryana* foragers might use pheromone trails to lead recruits from the nest to a food resource, similar to what has been found in several species of stingless bees (Lindauer and Kerr, 1958, 1960; Nieh et al., 2003a, 2004; Jarau et al., 2010; Reichle et al., 2011). The feeder that was furthest away from the feeder visited by the marked bees attracted some, but fewer bees. This suggests that recruits are sent out in the approximate, rather than the precise direction. One possible explanation is that *P. droryana* foragers deposit pheromone trails in a winding pattern when returning to the nest, similar to *Scaptotrigona postica* (Lindauer and Kerr, 1958, 1960). Also, *P. droryana* foragers do not always fly in a direct line towards the nest, but frequently perform lateral movements (Collevatti et al., 2000). This would allow recruits to discover food sources in a certain corridor, rather than a specific point in space. Even in honey bees, waggle runs vary considerably in their angle (Towne and Gould, 1988; Al Toufailia et al., 2013; Beekman et al., 2015) and flight vectors of recruits show substantial

scatter (Riley et al., 2005). If the recruitment mechanism in *P. droryana* is of a similar stochastic nature, we would expect that the number of newcomers at other food sources decreases the more the route to these food sources deviates from the route between the nest and the advertised food source. Scent marking behaviour was never observed, which casts doubt over the use of pheromone trails by *P. droryana* foragers. On the other hand, *P. droryana* foragers are very small (ca. 3mm body length), which makes it difficult for human observers to follow their flight paths and observe them landing on vegetation to deposit pheromone when they return to the nest. An alternative hypothesis is the following of guiding flights by recruiting foragers. Lindauer and Kerr (1958) suggested that *Scaptotrigona postica* recruits might use visual guidance or follow an "aerial odour tunnel" created by the recruiting forager during flight as additional information to locate a food source. These guidance flights were also observed in *Trigona corvina* (Aguilar et al., 2005). It is important to note that there might not be one single recruitment mechanism, but that *P. droryana* foragers might combine several mechanisms to recruit nestmates.

Another argument against pheromone trails is our finding that newcomers did not predominantly arrive at the food source at the correct distance. Approximately equal numbers of newcomers arrived at the 3 feeders in experiment 2. This suggests that the recruitment method of *Plebeia droryana* is similar to *Plebeia tica* and *Tetragonula carbonaria*, which communicate the direction but not distance of food sources (Nieh et al., 2000; Aguilar et al., 2005). We cannot rule out that experienced foragers provide information about the distance to recruits, but that recruits do not use this information if they encounter a food source on the way to the advertised food source. Because we used identical odours for all three feeders, *P. droryana* newcomers might have searched for the food source using a combination of odour-based searching with other yet to be discovered recruitment mechanisms after leaving the nest (Aguilar et al., 2005).

Local enhancement has been shown to affect foraging decisions in honey bees, bumblebees, wasps and stingless bees (Goulson et al., 2001; Leadbeater and Chittka, 2005; Kawaguchi et al., 2007;). However, the effect of the visual presence of other individuals at food sources varies among species (Slaa and Hughes, 2009). In *Trigona amalthea*, for example, local enhancement depends on the foraging experience of bees (Slaa et al., 2003). In our study, we did not find that newcomers were more attracted to the feeder that had conspecifics in its vicinity (Figure 2.4), which suggests that local enhancement

does not explain why more newcomers landed on the feeder F_{TraLoc} in experiment 1. It is also possible that local enhancement exists in this species, but requires the demonstrator bee to be immobile and in immediate proximity of the food source.

Conclusions

We found that significantly more newcomers arrived in the direction of an exploited food source, whereas distance information does not seem to be transmitted during recruitment. The recruitment mechanism underlying this arrival pattern is still unclear. Future research should explore in more detail how foraging distance affects the efficiency and likelihood of recruitment. The recruitment method used by *P. droryana* seems to be less accurate than in some mass-recruiting species, but is probably still more efficient than a random search by alarmed nestmates. Recruitment communication is similar to *P. tica*, which raises the possibility that the communication mechanism is conserved in this genus. However, this requires further research since *Plebeia* represents a large and probably non-monophyletic genus (~40 species) (Rasmussen and Cameron, 2010). Studying communication strategies in a diverse range of species is needed to gain better insights into the behavioural evolution of Meliponini and how this depends on ecological factors. Future research should also assess nest-based behaviours to find clues as to the nature of the mechanism of recruitment in *P. droryana*.

CHAPTER 3

Octopamine increases individual and collective foraging in a neotropical stingless bee

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Abstract

The biogenic amine octopamine is a key modulator of individual and social behaviours in honey bees, but its role in the other group of highly eusocial bees, the stingless bees, remains largely unknown. In honey bees, octopamine mediates reward perception and affects a wide range of reward-seeking behaviours. Thus, we tested the hypothesis that octopamine increases individual foraging effort and collective food source exploitation in the neotropical stingless bee *Plebeia droryana*. Octopamine treatment caused a significant increase in the number of bees at artificial sucrose feeders and a 1.73-times higher individual foraging frequency. This effect can be explained by octopamine lowering the sucrose response threshold and, thus, increasing the perceived value of the food source. Our results demonstrate that similar to its effects on honey bees, octopamine increases both individual and collective food source exploitation in *P. droryana*. This suggests that, despite having evolved many complex behaviours independently, OA might have similar regulatory effects on foraging behaviours in the two groups of highly eusocial bees.

Introduction

Biogenic amines play crucial roles by regulating neurophysiological responses and, ultimately, many behaviours (David, 1985; Evans, 1980; Monastirioti, 1999; Roeder, 1994). For example, a large body of research has revealed that octopamine (OA) plays important roles in the central nervous system and peripheral sensory systems of invertebrates, including in arthropods, annelids, nematodes and molluscs (David, 1985; Farooqui, 2012; Monastirioti, 1999; Roeder, 1994; Roeder, 1999). Honey bees (*Apis mellifera*) have been particularly well studied regarding the role that OA plays in regulating individual and social behaviours. For example, OA reduces the responsiveness to light stimuli (Scheiner et al., 2014), enhances the appetitive learning ability (Behrends and Scheiner, 2012; Perry and Barron, 2013) and increases recruitment communication, most likely via its effects on reward perception (Barron et al., 2007; Pankiw and Page, 2003). These effects of OA are relatively short-term, i.e. treatment can affect behaviours within minutes. However, more profound changes in social behaviours have also been observed: OA modulates temporal polytheism by accelerating the transition from in-hive worker to outside forager (Barron and Robinson, 2005; Schulz and Robinson, 2001; Schulz et al., 2002).

Stingless bees are the only other group of highly eusocial bees and with more than 500 tropical and subtropical species they represent the largest group of social bees (Rasmussen and Cameron, 2010). They live in perennial colonies and are the most important group of pollinators in many tropical habitats (Roubik, 2006). However, it is largely unknown whether and how OA modulates behaviour and physiology in stingless bees. Honey bees (Apini) and stingless bees (Meliponini) have separated about 80 mya (Martins et al., 2014) and have evolved many complex social traits independently. The two groups vary considerably in their division of labour (Grüter et al., 2017), their recruitment communication (Nieh et al., 2004) and stingless bees differ from honey bees in how they respond to some neuroactive chemicals (Peng et al., 2019). This raises the question whether OA plays similar roles in stingless bees as in honey bees. We are aware of only one study that has explored the effects of OA in stingless bees: Mc Cabe et al. (Cabe et al., 2017) found that in the stingless bee *Melipona scutellaris,* workers treated with OA showed an increased sucrose responsiveness, similar to what has been found in *Apis mellifera* (Cabe et al., 2017; Scheiner et al., 2002). They found both time- and dose-depended effects of OA on sucrose responsiveness.

Here we tested for the first time, whether OA modulates the individual and collective foraging

behaviour in a meliponine bee. We tested the prediction that OA increases short-term foraging effort in the common Brazilian stingless bee *Plebeia droryana*. This would lead to an increased foraging tempo as well as potentially promoting the recruitment behaviour in *P. droryana* (Peng et al., 2019). We manipulated wild *P. droryana* colonies to assess OA effects in the natural environment of this species.

Materials and methods

We studied 20 wild nests of the stingless bee *Plebeia droryana*, located on the campus of the University São Paulo, Ribeirão Preto, Brazil. The 20 colonies were divided into ten pairs according to their estimated colony size, which we based on the traffic of returning foragers (Grüter et al., 2017). Traffic was counted three times on a day with normal foraging conditions (11:30, 14:30 and 17:30), two minutes per count. The average number of foragers in one minute is shown in Table 3.1. The paired colonies were tested on the same day to reduce variation. Control and treatment were allocated randomly for each pair. Due to bad weather and robber bee attacks, we had to exclude some trials (see Table 3.1).

Table 3.1: Ten pairs of colonies. Control and treatment colonies were categorized by colony size, which was estimated based on foraging traffic (average number of bees per minute entering a colony in two minutes, measured three times on a day with good foraging conditions). Colonies in bold were excluded from the experiment, either due to rain (colony 10 and 20) or robber bee attacks (colony 18).

Pair	Control	Foraging Traffic±SE	Octopamine-Treated	Foraging Traffic±SE
1	Colony 15	6.00±1.29	Colony 5	4.83±0.60
2	Colony 19	3.50±0.85	Colony 17	3.17±1.14
3	Colony 9	8.83±1.28	Colony 14	9.50±1.23
4	Colony 12	8.17±0.87	Colony 11	8.17±1.19
5	Colony 3	1.00±0.45	Colony 4	1.00±0.37
6	Colony 8	9.67±1.69	Colony 13	9.83±1.92
7	Colony 6	10.67±1.52	Colony 16	10.00±1.83
8	Colony 2	12.83±1.83	Colony 7	11.50±2.47
9	Colony 1	7.00±0.97	**Colony 20**	5.33±0.61
10	**Colony 10**	1.67±0.42	**Colony 18**	3.50±0.89

Each trial consisted of a training phase, a treatment phase and a testing phase. During the training phase, a 35% (w/w) unscented sucrose solution, offered in artificial feeders, was used (a typical concentration of nectars collected by stingless bees) (Nogueira-Neto et al., 1959). Training started in the morning and two colonies comprising a pair were trained to a feeder at ca. 10 m from the respective hive at the same time (see Peng et al., 2019 for more details on the training methods). On the same day and before the treatment period started, 10 trained bees per colony were marked individually while

drinking at the feeder. During the treatment phase (0-60min), cleaned feeders again offered 35% unscented sucrose solution. At the treatment feeder, we added 0.01M OA (Sigma Adrich), whereas the control feeder did not offer OA (Colonies that were offered an OA feeder during the treatment phase will be called OA-colonies, whereas colonies offered only sucrose solution will be called control-colonies). This OA concentration effectively lowered the sucrose response threshold in *Melipona scutellaris* (Cabe et al., 2017) and recruitment communication in honey bees (Barron et al., 2007). The treatment period lasted 60 min, during which all marked and unmarked bees at the feeders were counted at 5 min intervals. The first measurement was made at 5 min. Additionally, the number of visits of marked bees was recorded to calculate the foraging frequency (visits/min). After the treatment phase, control and treatment feeder were removed simultaneously for 20 min before the testing phase started. For the testing phase (minute 80-170 since start of treatment), we offered a 30% unscented sucrose solution without OA at either feeder. We used 30% sucrose solution because it was not very attractive for *P. droryana* foragers in a previous study (Peng et al., 2019). The first measurements were made at minute 85. During the testing phase, foragers at the feeders were counted as described for the training phase. For data collection, each feeder was observed by one observer. For each paired trial, only one (randomly chosen) observer knew which feeder was control or treatment and, during a trial, observers did not know how many bees were at the other feeder. Generally, the number of bees at a feeder was easy to count (mostly 10-20 bees per count).

All data were analysed with R 3.4.4 (http://www.R-project.org/). We used linear mixed-effects models (LME) and the nlme package to analyse the data (Zuur et al., 2009). We used *treatment* (OA vs control) and *time* (treatment phase 0-60 min, testing phase 80-170 min) as fixed effects. We also tested the interaction between the two fixed effects using a likelihood ratio test (LRT). We used focal colony ID and pair ID as random effects. To reduce the variation due to differences in colony size, we standardised the count data to form proportions of foragers relative to the average number of foragers counted during the treatment period. This was done for each colony separately. Wald tests were used to test the significance of the fixed effects (Zuur et al., 2009). We used LME's to compare the foraging frequency (visits/min) of control and treatment bees. Additionally, we used *experiment phase* (treatment vs testing) as a fixed effect to test if foraging activity differed between treatment and testing phase.

Results

Effects of octopamine on foraging

During the treatment phase, when bees were offered 35% sucrose solution, the absolute number of bees at both feeders increased over time irrespective of whether the solutions contained OA or not (LME, treatment×time: LRT=0.26, p=0.61; time: t=8.79, p<0.0001). We found no effect of the OA treatment on the absolute number of bees at the feeders during the treatment phase (treatment: t=-0.24, p=0.81). For the subsequent analyses, we used the proportion of bees at a feeder relative to the average number of bees drinking during the training phase. In the testing period, we found a significant interaction between treatment and time (LME, treatment×time: LRT=30.16, p<0.0001, Figure 3.1A). In order to better assess the changes in the number of bees visiting the feeders over the time during the testing period, we analysed control- and OA-colonies separately. In both the OA- and control-colonies we found an increase in the relative number of bees visiting the feeders over time (LME, OA-colonies: t=11.01, p<0.0001; control-colonies: t=5.32, p<0.0001, Figure 3.1A), but this increase was stronger in OA-colonies (Figure 3.1 A). As a result, we found a significant treatment effect at the last count of the testing the phase (OA-colonies had about 60% more foragers at feeders than control colonies) (LME, treatment: t=2.70, p=0.016, Figure 3.1A).

When comparing the foraging frequency of individually marked bees during the treatment phase, there was no difference between OA and control feeders containing 35% sucrose solution (LME, treatment: t=1.67, p=0.11, Figure 3.1B). However, during the testing phase when both feeders offered a 30% sucrose solution, bees visiting the feeder that offered OA-solution during the treatment phase had a significantly higher foraging frequency (1.73 times) than bees visiting the control feeder (LME, treatment: t=5.69, p=0.0001). When comparing the foraging frequency of the control group during the treatment and testing phase, we found that the foraging frequency was significantly lower during the testing phase (LME, phases: t=4.08, p=0.0001, Figure 3.1B). On the other hand, the OA-treated bees showed no difference in foraging frequency between phases (LME, phases: t=-0.94, p=0.35).

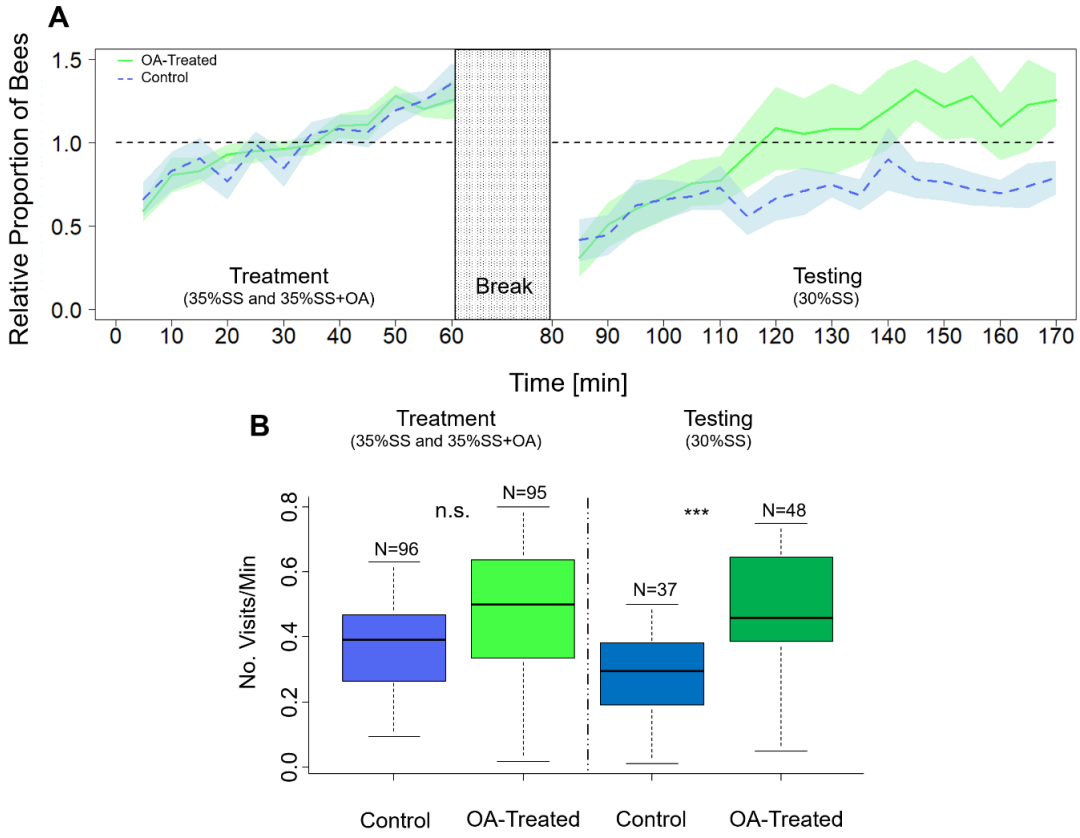

Figure 3.1: Effects of octopamine feeding on foraging behaviour in *P. droryana*. **A**, relative proportion of bees at the feeders during the treatment phase (35% sucrose solution, SS) and the testing phase (30% sucrose solution). The y-axis shows the proportion of bees at a feeder in relation to the average number of bees at that feeder during the treatment phase. The shaded areas represent the SE of the mean. **B**, the number of visits per minute by individually marked bees during the treatment and the testing phase. The boxplots indicate the medians, the 25%- and 75% quartiles. N represents the number of individually marked bees. *** represents the p-value <0.001, n.s. represents the p-value >0.05.

Discussion

We found that oral treatment of foragers with octopamine enhanced individual and collective foraging effort in *P. droryana*. During the treatment phase, OA-treated bees did not differ in their foraging effort compared to bees from control colonies, but when colonies were offered identical 30% sucrose solution during the testing phase, more foragers from OA-colonies visited the feeders. Moreover, during the testing phase, the number of bees from OA-colonies increased more strongly over time than at feeders visited by foragers from control-colonies (Figure 3.1A). The observation that the OA treatment effects only became apparent during the testing phase suggests that in *P. droryana* it may take around 30 minutes before significant changes in behaviour occur, which is slightly longer than was found in honey

bees, fruit flies and the stingless bee *Melipona scutellaris* where OA feeding affected behavioural responses within the range of minutes after uptake (Barron and Robinson, 2005; Barron et al., 2007; Cabe et al., 2017; Hoyer et al., 2008; Pankiw and Page, 2003; Schulz and Robinson, 2001). Furthermore, the foraging frequency was about 1.73 as high for bees treated with OA during the testing period. In honey bees, several studies have found that increasing endogenous OA levels increase the sensitivity to olfactory, visual or gustatory stimuli (Behrends and Scheiner, 2012; Erber et al., 1993; Pankiw and Page, 2003; Spivak et al., 2003). Thus, it is likely that the higher foraging frequency was mediated by an increase in reward sensitivity in *P. droryana* foragers. Foragers from control-colonies significantly decreased their foraging rate during the testing phase compared to the treatment phase, whereas the foraging rate did not change in OA-colonies between treatment and testing phases even though sucrose concentration was 5% lower during the latter phase (Figure 3.1B). This suggests that the OA treatment compensated for the drop in sucrose concentration.

In honey bees, oral OA treatment has a positive effect on waggle dancing, resulting in more recruitment to food sources (Barron et al., 2007). *P. droryana* is able to recruit nestmates to nearby food sources (Scheiner et al., 2002), thus, recruitment behaviour may also be influenced by the OA in *P. droryana*. This could explain the faster increase in the number of bees at the feeders of OA-colonies compared to control-colonies during the testing phase. Additionally, foragers that visited the feeder during the treatment period may have been more motivated to inspect the feeder after the break between the treatment and testing period. Honey bee foragers were more likely to return to food sources they perceived as more rewarding (Al Toufailia et al., 2013). Either process leads to an increased collective exploitation of the feeder by OA-colonies.

In summary, we found that OA increases foraging effort in wild colonies of a common Brazilian stingless bee. Additionally, oral OA treatment caused a substantial increase in the number of bees at a feeder. This increase in foraging motivation is likely to be linked to changes in reward sensitivity in *P. droryana* foragers (Cabe et al., 2017). Stingless bees and honey bees have been on separate evolutionary trajectories for about 80 million years and have independently evolved a highly eusocial lifestyle (Cardinal and Danforth, 2013; Martins et al., 2014). Stingless bees differ from honey bees in important aspects of their sociobiology, including many foraging behaviours and recruitment communication (Barth et al., 2008). Our results indicate that, despite this divergence, OA has overlapping effects on the individual and collective foraging behaviours in these two groups of eusocial bees. Still little is known

about the neurobiological basis of behaviour in stingless bees. A better understanding of the neurophysiological basis of stingless bee behaviour would help reveal whether there are general patterns in how neurotransmitters regulate complex behaviours in social bees. Further research could, for example, explore how biogenic amines such as OA or dopamine regulate behaviours like sleeping, learning, aggression and division of labour in stingless bee.

CHAPTER 4

Correlation between octopaminergic signalling and foraging task specialization in honey bees

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Abstract

The regulation of pollen and nectar foraging in honey bees is linked to differences in reward perception. Octopamine (OA) participates in the processing of reward-related information in the bee brain, being a good candidate to mediate and modulate the division of labour among pollen and nectar foragers. Here we tested the hypothesis that OA affects the resource preferences of foragers. We first investigated whether oral administration of OA is involved in the transition from nectar to pollen foraging. We quantified the percentage of OA-treated bees that switched from a sucrose solution to a pollen feeder when the sugar concentration was decreased experimentally. We also evaluated if OA circulating within the colony increases the ratio of bees collecting pollen. Finally, we quantified OA receptor gene expression of pollen and nectar foragers in different parts of the brain, as a putative mechanism that affects the decision-making process regarding to the resource type collected. OA administration modified the probability that foragers switch from nectar to pollen collection. The ratio of pollen/non-pollen foragers also increased after feeding colonies with OA-containing food. Furthermore, the expression level of the *AmoctαR1* was upregulated in foragers arriving at pollen sources compared with those arriving at nectar feeders. Using age-matched pollen and nectar foragers that returned to the hive, we detected an upregulated expression of a tyramine receptor gene (a precursor of octopamine) in the suboesophageal ganglia (SOG). These findings support our prediction that OA signalling affects the decision in honey bee foragers to collect pollen or nectar.

Introduction

Task specialization and division of labour are essential features for the ecological success of insect societies (Oster and Wilson, 1978; Wilson, 1985). Division of labour enables different activities to be performed simultaneously by groups of specialized individuals of the worker caste (Jeanne, 1986; Oster and Wilson, 1978). Workers performing different tasks, have different physiological states, distinct neurochemical and hormonal profiles and often, differ in how they perceived and respond to task-related stimuli (Robinson, 1987; 1992). In many social insects, these different internal states are linked to the age of the workers. In the honey bee *Apis mellifera*, for example, workers make a transition from in-hive tasks to the search and collection of resources outside at an age of 2-3 weeks (Winston, 1987).

The biogenic amine (BA) octopamine (OA) is an important driver of the regulation of honey bee division of labour. OA increases in the bee brain soon before the onset of foraging (Wagener-Hulme et al., 1999), but it does not change during periods of foraging inactivity or with different amounts of foraging experience (Schulz et al., 2003). Foragers present higher OA and tyramine (TYR) titres and have an upregulated gene expression of some OA (*AmoctαR1*) and TYR (*Amtyr2*) receptors compared to nursing bees (Schulz and Robinsion, 1999; McQuillan et al., 2012; Reim and Scheiner, 2014; Scheiner et al., 2017). By functioning as a neuromodulator (Bicker and Menzel, 1989; Roeder, 1994), OA enhances behavioural responsiveness to both gustatory (Scheiner et al., 2002) and olfactory stimuli (Mercer and Menzel, 1982; Hildebrandt and Müller, 1995; Barron et al., 2002; Spivak et al., 2003), a modulation that might enable bees to better asses foraging-related stimuli (Schulz and Robinson, 2001).

Because of the effects of OA on bees´ chemosensory responsiveness, it might affect division of labour among foragers. In honey bees, the collection of food sources, mainly protein and carbohydrates, is achieved by individuals specializing in pollen or nectar foraging, (but also on water, propolis, etc.). It is well known that the tendency to forage for pollen or nectar is predicted by the sensitivity to sucrose (Page et al., 1995). In behavioural bioassays, the offering of increasing concentrations of sugar solutions showed that pollen foragers exhibit significantly lower sucrose response thresholds (SRT) than nectar foragers indicating that both groups differ in how they perceive and evaluate the quality of nectar resources (Page et al., 1998; Waddington et al., 1998; Pankiw and Page, 2000; Arenas and Kohlmaier, 2019). Pharmacological activation of OA and TRY signalling has been shown to increase the gustatory responsiveness of nectar foragers to the level of pollen foragers (Scheiner et al., 2002; Scheiner et al.,

2017), which raises the question whether OA and TYR signalling affect the behavioural regulation between pollen and nectar foragers.

Behavioural experiments suggest that OA influences the type of material collected by foragers, since OA-treated foraging bees were more likely to collect water than non-treated foragers (Giray et al., 2007). TYR administration leads to an intermediate response. Schulz and co-workers (Schulz et al., 2004) explored the link between the amount of OA and the probability of bees to forage either pollen or nectar. They quantified OA levels in the brain of honey bees selected for high and low pollen-hoarding (Page and Fondrk, 1995) and found that despite an increasing level of OA with age, there were no differences between both strains. Similar results were obtained from the mushroom bodies (MB) of non-selected (wild type) pollen and nectar foragers (Scheiner et al., 2014). On the other hand, Scheiner et al. (Scheiner et al., 2014) found that pollen foragers displayed significantly higher mRNA expression of the *Amtyr1* in the brain compared with nectar foragers.

Tasks specialization for nectar and pollen is not fixed, as some foragers can switch from one resource type to the other in response to sudden changes in environmental or colony conditions (Rotjan et al., 2002; Arenas and Kohlmaier, 2019). Whilst neurochemical factors like OA and TYR seem to work more proximally to foraging initiation (Schulz et al., 2003), early endocrine factors (like higher levels of juvenile hormone (JH) and vitellogenin (Vg) protein at adult emergence), might be responsible for the control of forager development over a longer time scale (Schulz et al., 2004). There is evidence that the temporal dynamics of JH and Vg production, both related to reproductive maturation of insects, promotes pollen collection (Amdam et al., 2006), a resource fundamental for brood rearing (Seeley, 1995).

Here we explore the effect of OA on the specialization of food collection. We hypothesize that OA affects forager preferences for pollen and nectar by means of changing gustatory responsiveness. To elucidate to what extent OA signalling affects pollen and nectar foragers, we focused on three levels. At the individual response level, we investigated whether OA administration influences the probability to switch from sucrose to pollen collection. To this end we quantified the percentage of bees switching from sucrose solution to pollen feeders when the sugar concentration was decreased experimentally, while the quality/availability of the pollen source remained unaltered. We expected OA-treated foragers to show a higher probability of switching behaviour than control bees. At the colony level, we explored whether OA influences foraging activity patterns. We assessed changes in the ratio of incoming pollen

and non-pollen foragers before and after offering either sugar solution or sugar solution with OA inside hives. We reasoned that the feeding of OA would increase the proportion of pollen foragers. Finally, we assumed that pollen and nectar foragers perceive the resources differently, in part, due to naturally different levels of OA signalling. Even when there is no evidence for differences in BA titres between nectar and pollen foragers, regulation of foraging division of labour could be related to sensitivity to, rather than the amount of, circulating BAs. Therefore, we compared gene expression of five OA and one TYR receptors in the brain of foragers that have been collecting pollen or nectar while controlling either for their foraging motivation or the age. Receptor expression was quantified in the mushroom bodies (MBs), the antennal lobes (AL) and the suboesophageal gland (SOG), neuropils highly involved in the processing of odours and gustatory information (Erber et al., 1993; Perry and Barron, 2013; Galizia et al., 2011).

Materials and methods

Study species and field site

Behavioural experiments were carried out during the summer seasons of 2018/2019 in the Experimental Field of the School of Exact and Natural Sciences of the University of Buenos Aires (34°32′S, 58°26′W). For these experiments we used *Apis mellifera ligustica.* For the individual foraging response, we trained bees from two colonies to visit artificial feeders that offered 30% w/w sucrose solution. The feeders were located approximately 120m from the hives. To study the collective foraging response, we used fourteen 10-frame Langstroth hives (about 15000 worker bees), all containing a mated queen, 4–5 brood frames, and 1–2 frames with food reserves.

Receptor gene expression was quantified in *Apis mellifera carnica,* from colonies located on the campus of the Johannes-Gutenberg University in Mainz, Germany. In a first experimental series, we trained foragers from three different colonies to collect at feeding stations that simultaneously offered pollen and sugar solution. In a second experimental series we introduced marked newly emerged bees into a hive, which were then captured 18 days later when they returned to the colony with either nectar or pollen.

All experiments complied with the animal care guidelines of the National Institute of Health (1985) and the current laws of Argentina and Germany.

Experiment 1: OA effect on switching behaviour from sugar solution to pollen feeders

In this experiment, we studied how oral administration of OA influences the transition of bees between sucrose and pollen collection. To this end, we quantified the percentage of nectar foragers that switched to pollen gathering as the profitability of the sugar solution they were collecting, steadily deteriorated (Arenas and Kohlmaier, 2019). Switches were measured during four tests (T30%, T10%, T3%, and T1%) during which the concentration of sugar solution offered at the feeder decreased from 30% to 1% w/w.

Before the evaluations, honey bees previously trained to forage at the artificial feeder, were reactivated to a feeder that offered 30% w/w sucrose solution. According to the experimental design (see below), the sucrose solution did or did not contain OA (0.01M). To establish the focal group of bees that would be followed during the evaluations, reactivated foragers were marked with acrylic paint whilst they fed on the solution for 15 min. Afterwards, tests began. Each testing phase (T30%, T10%, T3% and T1%) lasted 40 min. Throughout these tests, an *ad libitum* pollen feeder (Petri dish of 9 cm in diameter) containing commercially available multiflora crushed bee-collected pollen (7g), was presented next to the sugar solution feeder. Colour-marked foragers were considered to have switched to the pollen feeder as soon as they gathered pollen and formed incipient pollen loads on their hind legs. Bees that switched were captured, killed in the freezer (-18 °C) and inspected for colour marks. Unmarked bees were discarded as we could not confirm that they belonged to the focal group. Once T30% finished, we removed the pollen feeder and replaced the content of the feeder with a 10% w/w sugar solution. By decreasing the profitability of the sucrose source, we expected a reduction in the number of bees that keep on foraging under the new rewarding condition, but also an increased likelihood to switch to pollen (Page et al., 1995). Before T10% started, we re-labelled the bees with a second colour for 10 minutes in order to count the number of bees that due to their higher sensitivity to the sucrose, continued foraging on the 10% sugar solution. Once all the remaining bees were counted, the pollen feeder was presented again for T10% to initiate. Test3% and T1% were done following the same procedure. Switching behaviours were obtained from 14 independent groups of bees, each group tested on different days. Seven groups were fed sucrose solution with OA and 7 groups sucrose solution without OA. Three independent groups fed with sucrose solution containing OA and 3 groups fed with sucrose solution alone came from the same hive, the other groups came from the second hive.

Experiment 2: OA effect on the rate of incoming foragers

Here we addressed whether the administration of OA diluted in the food of the colonies altered the incoming rate of pollen and non-pollen foragers (presumably nectar foragers). To this end, we evaluated the foraging activity patterns for colonies that were fed sucrose solution (30% w/w) with or without OA (0.01M). Sucrose solution (80ml) was offered by means of entrance feeders, a 5cm x 20cm x 1cm plastic container that was slid through the entrance of the hive to its interior. It took the colonies 60/100 min to empty the feeder. The number of incoming bees was obtained from videos taken with a digital camera (SONY) at the entrances of 14 hives. Ten-minute videos were recorded immediately before and 10min after the food either containing OA (7 hives) or not (7 hives) was finished. Incoming bees carrying pollen loads on their hind legs were identified as pollen foragers. Based on the rates of pollen and non-pollen foragers calculated before (T0) and after the treatment (T1), we calculated delta of incoming bees (Δ: T1-T0) for each group.

Experiment 3 and 4: OA receptor gene expression in pollen and nectar foragers

Experiment 3: Sampling pollen and nectar foragers at the beginning of the foraging visit

We trained bees of unknown age to forage for pollen and nectar at a feeding station 30m from the colony that simultaneously provided a sucrose solution (40% w/w) and a pollen feeder (as described above). As soon as the foragers showed their preference for either pollen or nectar, a few seconds after landing, they were captured and frozen in liquid nitrogen and shortly afterwards, stored in a -80 °C freezer until brains were dissected. Using this procedure, we aimed to control for the motivation of the bees that, when captured, were still largely empty and motivated to forage. At the same time, using this procedure we controlled for the potential effect of the location of the feeders and the resources quality (i.e. the feeder contained 40% sucrose solution)

Experiment 4: Sampling age-matched pollen and nectar foragers that returned to the hive

Because the age of foragers could be linked to BA signalling (Peng et al., submitted), we designed an experiment to control for the age of the foragers. About 4000 colour-marked newly emerged bees were introduced into a host hive. Newly emerged bees were obtained from 3 sealed brood frames taken from different colonies, placed in an incubator at 32 °C, 55% RH and darkness (Arenas and Farina, 2008). Every day about 1000, 0/1-day-old workers were collected from the frames and labelled with the traceable colour on the thorax to control for their age. This procedure was repeated for 4 consecutive days. Two weeks later, we started watching the entrance of the hive to find the labelled bees that became

foragers. Labelled bees that entered the hive were captured in plastic tubes. Pollen foragers were identified as they carried pollen loads on their hind legs. Nectar foragers were recognized as they exhibited a distended abdomen and regurgitated their gut content as soon as they were gently squeezed. Empty bees were discarded, and we kept only those bees that carried nectar of 15% w/w sugar or more (measured with a hand-held refractometer).

Brain dissections, RNA isolation, cDNA Synthesis and qPCR

Bee heads were cut off with dissection scissors and immediately fixed on a small piece of dental wax on an ice-cooled Petri-dish. We opened the head capsule with a scalpel to dissect the antennal lobe (AL), the suboesophageal gland (SOG), mushroom body (MB) calyces immersed in cooled PBS (phosphate-buffered saline) and over ice. Brains were dissected in about 5 min. Only the calyces of the mushroom bodies were used. The paired AL and MB and the SOG were transferred into different vials with TRIzol® (Invitrogen, USA) for RNA extraction. For each sample we pooled the brains of either three pollen or three nectar foragers in order to reduce the variability among different samples.

The RNA extraction was performed with RNeasy Mini Kit (Qiagen, Germany) according to the manual. The Quanti Tect Reverse Transcription Kit (Qiagen, Germany) was used to remove the genomic DNA from the previously isolated RNA. The Kit was also used to synthesize the cDNA of the genes we were interested in. All qPCR reactions were performed on the mic qPCR cycler (Bio Molecular Systems, Australia). The thermal cycling protocol compromises 40 cycles of denaturation at 95 °C for five seconds and annealing at 60 °C for 20 seconds. We focused on the receptor genes *AmoctαR1, AmoctβR1, AmoctβR2, AmoctβR3/4* and *Amtyr1* (Beggs et al., 2011; McQuillan et al., 2012), as well as the housekeeping genes *GAPDH* and *eiF3-S8*. Primer sequences are taken from the published literature (Grozinger et al., 2003; Reim et al., 2013). The $2^{-\Delta CT}$ method was used for calculating of the relative gene expression (Schmittgen and Livak, 2008).

Statistical analysis

All data were analysed using generalized linear mixed models (GLMM or generalized linear models (GLM) in the R environment (http://www.R-project.org/) (McCullagh and Nelder, 1989; Bolker et al., 2009). Differences in switching behaviour were assessed by means of GLMM with binomial distributions (Crawley, 2007). Here we explored the role of two fixed effects on switching behaviour, "treatment (i.e. bees that collected sugar solution with or without OA)" and "testing phase (T30%, T10%, T3% and T1%)". The day of the experiment was considered as a random effect, specified via

the model formula. We checked for overdispersion (Zuur et al., 2009). We used the glmer function of the lme4 package (Bates et al., 2011; R Development Core Team, 2011). The lme4 package (glmer function) uses Wald Z-tests to approximate p-values for GLMMs (Bolker et al., 2009).

Delta of incoming bees between colonies were analysed by means of GLMM with normal distribution. Here we analysed the effect of "treatment (offering of sugar solution or sugar solution with OA)" as fixed effect. Colonies were considered as a random factor. Homoscedasticity and normality assumptions were checked (Levene and Shapiro–Wilk tests, respectively). Dunnet's test was used for contrasts using the glmer package (Lenth, 2016).

To test the differences in biogenic amine receptor gene expression, we explored the impact of two fixed effects, "forager type (pollen or nectar)" and "brain part (AL, SOG and MB)". To compare the expression of biogenic amine receptor genes between treatments and brain parts, pairwise comparisons were performed, and a sequential Bonferroni correction was applied to adjust p-values for multiple testing (multcomp package in R).

Results

Experiment 1: OA increased switching behaviour from sugar to pollen feeders

The percentage of foragers that switched from the sugar feeder to the pollen feeder increased through the successive testing phases, which means that as the concentration of the sucrose solution offered at the feeder decreased, bees were more likely to switch from sucrose to pollen. More importantly, switching behaviour was influenced by the OA administration (Figure 4.1), as the percentage of bees that switch during single testing phases was higher if they previously collected sugar solution containing OA. Consistent with these results, the analysis showed a significant interaction between the tested factors (treatment×test: $F_{3,9} = 3.442$, $p = 0.006$). Main effects confirmed that switching behaviour of OA treated foragers was higher than controls in T30% ($Z = 3.904$, $p = 0.0001$), T10% ($Z = 2.480$, $p = 0.013$) and T3% ($Z = 1.986$, $p = 0.047$). In other words, OA-treated foragers were more likely to switch from nectar to pollen foraging than control bees, as it was found at least, for the first three testing phases.

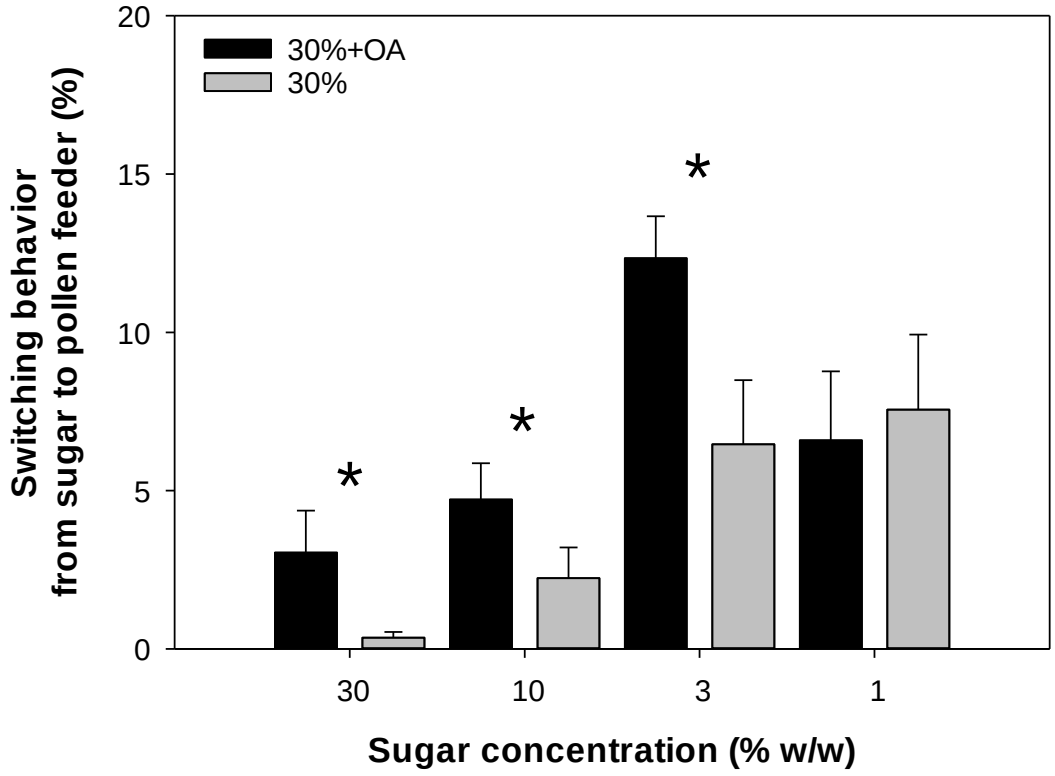

Figure 4.1: Switching behaviour from sucrose to pollen feeders. Percentage of labelled honey bees that changed their foraging preferences to pollen throughout four phases. Switching behaviour was quantified in foragers that had access either to a 30% w/w sugar solution or to a 30% w/w sugar solution with OA (0.01M). Bars show medians ± SE of 7 independent groups of bees for each treatment. Asterisks indicate statistically significant differences (*$p < 0.05$; Tukey's test).

Experiment 2: OA effect on the rate of incoming foragers

Additional evidence for the role of OA was found when looking at the colony response. Colonies fed with sugar solution containing OA exhibited positive Δ-values for pollen foragers, meaning that the rate of incoming pollen-loaded bees increased after the offering of food containing OA. On the other hand, Δ-values for pollen foragers of colonies fed sucrose solution alone were negative, indicating that the rate of incoming pollen foragers after the offering of food was lower than before the food offering. In line with the above, the analysis showed that Δ-values of pollen foragers in colonies fed sucrose solution with OA were significantly higher than Δ-values of the same group of foragers that belonged to colonies fed sucrose solution alone (treatment: $F_{1,10} = 6.3591$, $p = 0.03$; Figure 4.2A). Interestingly, Δ-values of non-pollen foragers did not differ between colonies treated with or without OA (treatment: $F_{1,10} = 0.2764$, $p = 0.61$; Figure 4.2B). These results indicate that OA affected the individual foraging preferences for pollen and modified the pollen foraging activity of the colony.

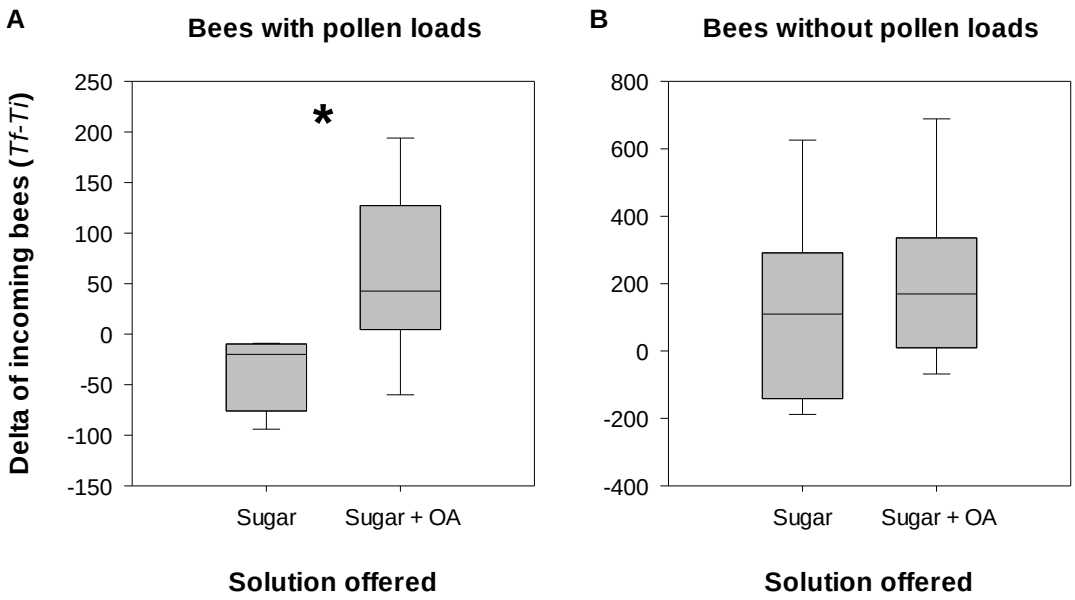

Figure 4.2: Delta (final rate minus initial rate) of incoming bees with **(A)** and without **(B)** pollen loads obtained from colonies fed either sugar solution or sugar solution with OA (0.01M). Box plots show medians, quartiles, and 5th and 95th percentiles from 12 hives. Asterisks indicate statistical differences (*$p < 0.05$).

Experiment 3 and 4: Variation in expression of OA and TYR receptors between pollen and nectar foragers

When the expression of receptor genes was studied in pollen and nectar foragers captured at the very beginning of a foraging visit, we detected significantly higher mRNA levels for *AmoctαR1* in the brain of pollen foragers compared to nectar foragers (forager type: $F_{1,66} = 5.336$, $p = 0.024$; Figure 4.3A), irrespective of the part of the brain. No differential expression linked to forager type was found for *AmoctβR1, AmoctβR2, AmoctβR3/4* or *Amtyr1* receptor genes (Figure 4.3B, C and D).

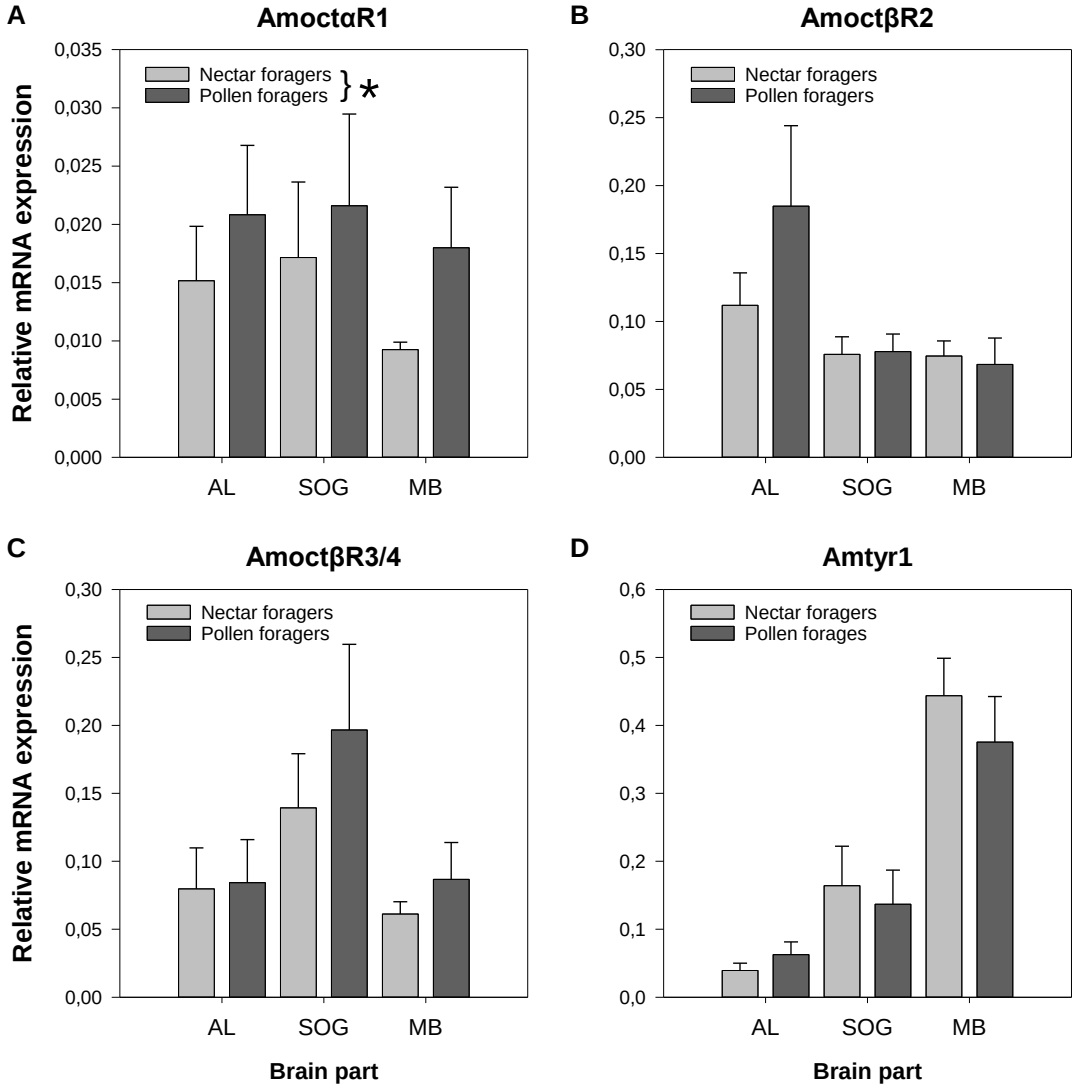

Figure 4.3: Biogenic amine receptor gene expression in different parts of the bee brain (AL: antennal lobe; SOG: suboesophageal ganglia and MB: mushroom bodies) of nectar and pollen foragers captured when arriving at artificial feeders. Bars show mean expression levels relative to the two reference genes (*GAPDH* and *eiF3-S8*) ± SE. Each bar shows the mean for 10-12 samples, each one made by pooling 3 different bees from the same hive and foraging sub-caste. Asterisk indicates an overall significant difference between nectar and pollen foragers (*$P < 0.05$).

When expression was analysed in age-matched pollen and nectar foragers captured at the entrance of the hive (end of the foraging visit), a significant difference was found for the receptor gene *Amtyr1*, with pollen foragers having an up-regulated expression compared to nectar foragers. Regarding the expression of this gene (*Amtyr1*), our analysis revealed a significant interaction between the factors (forager type×brain part: $F_{2,65} = 2.972$, $p = 0.05$; Figure 4.4E). The difference between foraging groups was explained by a higher receptor gene expression in the SOG of pollen foragers (Dunnett's test; pollen vs nectar foragers: $Z = -2.280$, $p = 0.0226$, Figure 4.4E). Expression of *AmoctαR1, AmoctßR1,*

AmoctβR2 and *AmoctβR3/4* was not affected by foraging type (Figure 4.4A, B, C and D).

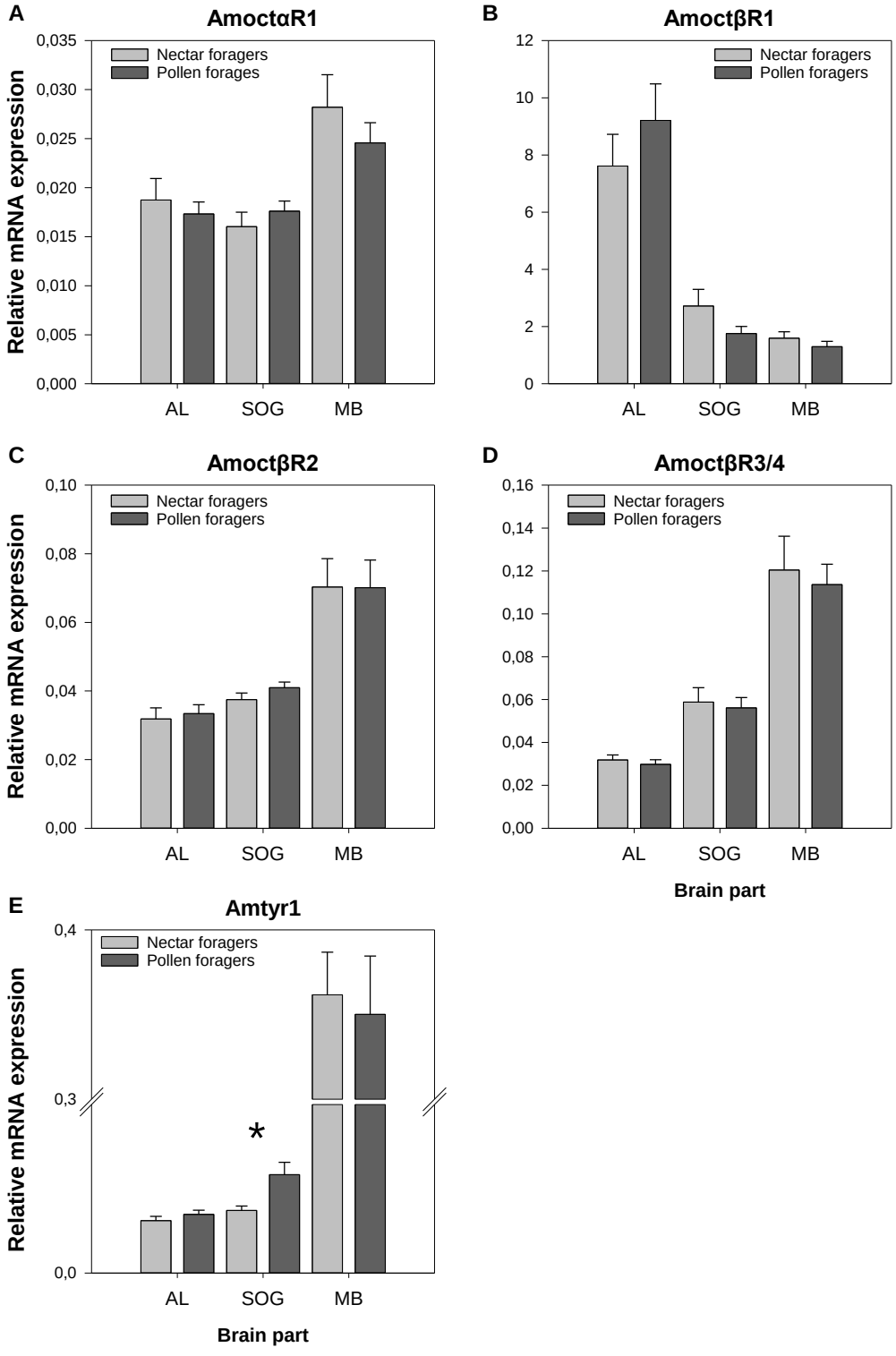

Figure 4.4: Biogenic amine receptor gene expression in different parts of the bee brain (AL: antennal lobe; SOG: suboesophageal ganglia and MB: mushroom bodies) of aged-matched nectar and pollen foragers, captured at the entrance of the hive. Bars show mean expression levels relative to the two reference genes (*GAPDH* and *eiF3-S8*)±SE. Each bar shows the mean for 11-12 bees of 18 days of age, that returned to the colony loaded either with pollen or nectar. Asterisk indicates an

overall significant difference between nectar and pollen foragers (*$P < 0.05$).

Discussion

The combined experimental approaches performed in this study suggest that OA and TYR signalling are involved in the short-term regulation of foraging division of labour in honey bees. At the individual level, foraging bees treated with OA were more likely to switch from nectar to pollen than bees of the control group. OA also impacted on the collective response, in which OA-treated colonies showed higher rates of incoming pollen foragers than control colonies. The behavioural choice regarding the preferred resource type correlated with an overall difference in the expression of receptor gene *AmoctαR1* in the brain of bees as they started to collect either pollen or sucrose solution and, consistent with Scheiner et al., with a change in the expression of receptor gene *Amtyr1* in the SOG of age-controlled bees that returned to the hive either carrying nectar or pollen (Scheiner et al., 2004).

Octopamine influences individual foraging preferences for pollen

Our results indicate that OA treatment is involved in the regulation of the transition of foragers between nectar and pollen collection. Arenas and Kohlmaier recently observed that switching between resource types can be an active decision of the bees in response to changes in sugar profitability of the feeding site (Arenas and Kohlmaier, 2019). In general terms, bees that persisted in visiting the feeding station when it offered low-quality sucrose solutions, presumably due to lower sugar response thresholds, were more likely to switch to pollen than those bees foraging only on highly concentrated solutions. Our results are consistent with previous findings (Arenas and Kohlmaier, 2019) and go further showing that OA-treated bees are more likely to switch than controls, probably through OA effects on the perception) of sugar and pollen reward-related stimuli (Scheiner et al., 2002). Because high OA levels in the brain modulate response thresholds for stimuli like odours (Mercer and Menzel, 1982) and sucrose (Scheiner et al., 2002), it is plausible that OA-treated bees were more sensitive and responsive to certain chemosensory cues of pollen, such as volatiles and tastes (Arenas and Farina, 2012), which are responsible for attracting the bees and eliciting pollen foraging behaviour (Pernal and Currie, 2002; Arenas and Farina, 2014). Furthermore, because bees showing lower SRTs are less demanding regarding the reward (Scheiner et al., 2004), they might also be attracted by non-nutritional compounds available in the pollen.

As expected, not all OA-treated bees became pollen foragers. This suggests that this behavioural

plasticity is not only controlled by OA, but probably depends on the interplay between endocrinal and neurochemical factors (Schulz et al., 2004). Regarding foraging specialization, it has been suggested that the temporal dynamics of different endocrinal factors, like higher levels of juvenile hormone (JH) and vitellogenin (Vg) protein at adult emergence, promote pollen collection (Amdam et al., 2006). Behavioural development under the control of early endocrine events and changes in OA signalling might ultimately drive foraging preference for either pollen or nectar.

Octopamine affects pollen-foraging activity of colonies

Consistent with changes in individual switching behaviour at the foraging site, we found that OA treatment also affects colony foraging activity towards nectar and pollen resources. Here, we observed that the ratio of bees carrying pollen increases after feeding the colonies sucrose solution with OA. This result is in line with a previous finding in which foragers treated with OA were more likely to collect water than non-treated foragers (Giray et al., 2007). Taken together, this evidence supports a role of OA in foraging regulation between nectar and resources that do not necessarily provide an immediate energy reward (e.g. pollen, water or resin).

It is known that circulation of gustatory information inside the hive modulates sugar response thresholds of workers (Pankiw et al., 2004; Ramírez et al., 2010) and is responsible for the re-allocation of foragers among nectar sources of different profitability (Seeley, 1995). Furthermore, it has recently been observed that the modulation of sugar thresholds also drives a re-allocation between nectar and pollen sources. Arenas & Kohlmaier observed that the ratio of pollen vs non-pollen foragers increased after feeding a colony low-quality sugar solution (3% w/w) and decreased after the feeding of a high-quality sugar solution (50% w/w) (Arenas and Kohlmaier, 2019). With increasing sugar responsiveness due to OA administration, we would expect more foragers to become responsive to low-quality nectars and also to pollen-related stimuli, a situation that would promote recruitment, activation of new foragers (Schulz and Robinson, 2001), and/or reactivation of experienced foragers to pollen sources. Adjustments in the amount pollen collected also include foragers carrying heavier pollen pellets and intensification of the frequency of their foraging bouts (Fewell and Winston, 1992; Fewell and Page, 1993). However, whether OA treatment also impacts on individual efforts remains to be tested.

In our experiments, behavioural responses were only tested with OA. However, whether other BAs like dopamine or serotonin (Taylor et al., 1992; Erber et al., 1993) could also affect resource selection, remains unknown. Our study (and Scheiner et al., 2004) suggests that TYR signalling is

involved in foraging specialization. Because OA in high concentrations can also bind, to some extent, to TYR receptors (Blenau et al., 2000; Verlinden et al., 2010), we cannot discard the possibility that OA administration affects the regulation of foraging behaviour via TYR receptors. TYR might also impact on motor activity (Scheiner et al., 2004), which is crucial for pollen collecting manoeuvres by which foragers brush pollen with their legs from body hairs to their hind legs, where they accumulate as pellets in the corbiculae.

OA and TYR receptor expression correlates with nectar or pollen collection

Octopamine achieves its effects on behaviour through binding to OA receptors, five of which have been described: one α receptor and four β receptors (Hauser et al., 2006; Balfanz et al., 2014). Receptor AmOCTαR1 leads to an increase in the Ca^{2+} signalling, whilst β receptors increase intracellular cyclic adenosine monophosphate (cAMP) levels when activated. For TYR there are at least two receptors (Hauser et al., 2006; Cazzamali et al., 2005). The AmTYR1 (the one we tested) decreases intracellular cAMP (Beggs et al., 2011; Blenau et al., 2000; Mustard et al., 2005), while AmTYR2 increases cAMP after activation (Reim et al., 2017). Recent findings support the hypothesis that mRNA expression of AmoctαR1 is more related to the social task than the age, whereas the expression of receptors from the β family is more likely to change with age (Reim and Scheiner, 2014) rather than with the social role. Indeed, lower expression of AmOCTβR1 was recently found in younger than older foragers (Peng et al., submitted).

Our results from Experiment 3, in which receptor gene expression was obtained from foragers captured immediately after landing at the feeder, showed that there was an overall higher expression for receptor gene *AmoctαR1* in the brain of foragers that landed at the pollen feeder. It is noteworthy that receptors of the β family did not show differences. However, we currently cannot exclude that the greater expression in pollen foragers was due to age effects as it has been reported that pollen foragers initiate foraging at slightly younger age than nectar foragers, a trait related to the accelerated behavioural development under the control of early endocrine processes (Amdam et al., 2006). However, because *AmoctαR1* seems to be linked to social task rather than the age and given that older bees have higher mRNA expression of OA receptors than younger bees (McQuillan et al., 2012; Reim and Scheiner, 2014), we deem it is unlikely that the upregulation of *AmoctαR1* in pollen foragers was linked to age differences.

Interestingly, pollen and nectar foragers of similar age (18-days) captured at the end of their

foraging bout (experiment 4) did not show differences in *AmoctαR1* expression, but for *Amtyr1* in the SOG. These findings match with a previous study that sampled pollen foragers at the entrance of the hive and found a higher expression of the *Amtyr1* in the SOG, but not in the AL or MB (Scheiner et al., 2004). Because the SOG is located in the ventral nerve cord, between the brain and the thoracic and abdominal ganglia, it could serve as a relay centre for information descending and ascending along the ventral nerve cord, which might be important for both the assessment of pollen with their tarsi for the control and coordination of leg movements during pollen gathering. In addition, ventral unpaired median neurons, all octopaminergic neurons (Kreissl et al., 1994), innervate different parts of the SOG and the brain, and might provide a neural substrate for the modulatory function of pollen. Together, our results suggest a role of TYR receptor AmTYR1 in the SOG for the division of labour among pollen and nectar foragers.

The different findings of Experiments 3 and 4 suggest a complex role of BAs in the regulation of resource selection. On the one hand, it is plausible that the expression of *AmoctαR1* receptor gene is higher at the beginning of the foraging trip but down regulated as the foragers become satiated and ready to leave the foraging site. Once inside the hive, and according to colony conditions, receptors might be up regulated again, driving the bees to resume pollen foraging. More investigations are necessary to examine whether changes in OA and TYR receptors expression relate to different phases of the pollen foraging bout.

We cannot rule out that the location of the foraging sites and the identity and/or quality of the resources were at least in part, responsible for the differences between Experiment 3 and 4. In Experiment 4, bees collected nectar and pollen from natural food sources. The sugar concentration in nectars sampled from the crops of returning foragers ranged from 15 to 20% w/w. Pollens were observed to belong to at least three different species (as revealed by the different colours of the pollen pellets). Bees from Experiment 3 foraged 30m from the hives on *ad libitum* feeders (10cm apart) either offering 40% w/w sucrose solution or crushed multifloral-bee collected pollen. These differences might have amplified differences in *AmoctαR1* receptor gene expression between foragers due to the more pronounced contrast between the profitability of both resource types. Nonetheless, our results suggest that pollen and nectar foragers differ in their assessment of gustatory and olfactory stimuli, and possibly in motor activity too, and that this is related to OA- and TYR signalling via the AmOCTαR1 and AmTYR1 receptors.

A combination of higher receptor expression in the brain of pollen foragers plus increased OA titres would lead to a higher sensitivity, and the capacity to elicit stronger responses with small changes in the amount of BAs. So far, there is no evidence for differences in OA levels neither in primary integration centres of odour (AL) and gustatory (SOG) information, nor in the higher processing centre of the bee brain (MB) between pollen and nectar foragers (Schulz et al., 2004; Scheiner et al., 2014). However, Schulz et al. discussed that a lack of differences between OA titres between high and low pollen-hoarding strains (Schulz et al., 2004; Page et al., 1995) could be because bees were tested before they became foragers. Furthermore, Scheiner et al. did not test OA titres in the AL or SOG, brain regions that are involved in the processing and evaluation of olfactory and gustatory stimuli (Scheiner et al., 2014). Thus, so far, no study analysed the amount of TYR in the brain of pollen and nectar foragers, which would allow us to link TYR levels in the brain with changes in the expression of *Amtyr1* (Scheiner et al., 2004 and this study). Likewise, BA titres at different stages of foraging bouts have not yet been compared.

Our study provides new insights into the underlying physiological processes and mechanisms involved in the control of resource collection. Quantitative but also qualitative differences in OA and TYR receptors among brain neuropils might reflect the complex patterns of gene expression that determine reward value representation in pollen and nectar foragers at different phases of the foraging bout. This, in turn, could affect the decision of foragers to collect either pollen or nectar.

CHAPTER 5

Octopamine and dopamine mediate waggle dance following and information-use in honey bees

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Submit as:

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Abstract

Honey bees can be directed to profitable food sources by following waggle dances performed by other bees. Followers can often choose between using this social information or relying on memories about food sources they have visited in the past, so-called private information. While the circumstances that favour the use of either social or private information have received considerable attention, still little is known about the neurophysiological basis of information-use. We hypothesised that octopamine and dopamine, two biogenic amines with important functions in reward signalling and learning, affect dance use in honey bees. We orally administered octopamine and dopamine when bees collected food at artificial feeders and tested if this affected interest in dance information about a new food source. We predicted that octopamine reduces interest in dances and strengthens private information use *via* an increase in the perceived value of the previously exploited resource. Since dopamine has been shown to lower reward perception, we expected it to act in the opposite direction. Octopamine treated foragers indeed followed 32% fewer dances than control bees and increased the use of private information. Dopamine treated bees, on the other hand, followed dances 15% longer than control bees, but surprisingly did not use social information more. Overall, our results suggest that biogenic amine signalling affects interactions among dancers and dance followers and, thus, information flow about high quality food sources.

Introduction

Social learning is learning that is influenced by other individuals or their products, either through observation or interaction (Heyes, 1994; Hoppitt and Laland, 2013). Honey bees, *Apis spp.*, use a unique form of social learning, the waggle dance communication (Couvillon, 2012; Dyer, 2002; l'Anson Price and Grüter, 2015; Seeley, 1995; von Frisch, 1967). During their waggle dances, dancers attract hivemates and provide them with information about the location and odour of a food source (Couvillon, 2012; Seeley, 1995; von Frisch, 1967; Grüter and Farina, 2009; Riley et al., 2005; Schürch et al., 2019). Experienced foragers can decide to follow dances and decode this vector information (social information) or to revisit food sources they remember from previous foraging trips (private information) (Biesmeijer and Seeley, 2005; Grüter et al., 2008; Grüter et al., 2013; Grüter and Ratnieks, 2011; Menzel et al., 2011; Wray et al., 2012). The dance follower's interest in social information can be gauged by the number of waggle runs followed, with bees that decode waggle dances following more waggle runs (Biesmeijer and Seeley, 2005; Grüter et al., 2013; Grüter and Ratnieks, 2011). A third strategy, called scouting, is to ignore both social and private information about foraging locations and search for a new food source independently (Seeley, 1995; von Frisch, 1967, Grüter and Leadbeater, 2014). Empirical and theoretical studies suggest that the benefits of independent exploration, social information and private information depend strongly on the spatiotemporal distribution of food sources (Beekman and Lew, 2008; Dornhaus et al., 2006; Dornhaus and Chittka, 2004; I'Anson Price et al., 2019; Schürch and Grüter, 2014).

While social information-use has been studied extensively from a behavioural ecological perspective (Hoppitt and Laland, 2013; Grüter and Leadbeater, 2014; Kendal et al., 2009; Laland, 2004; Rendell et al., 2010), less is known about the molecular and neurophysiological basis of the decision to use social vs private information. Previous research suggests that, in honey bee foragers, the perception of rewards is likely to play an important role in the use of social and private information. When foragers experience that their food source is no longer rewarding, they increase their dance following and social information use (Grüter et al., 2013; Grüter and Ratnieks, 2011), whereas foragers that experienced higher quality rewards in the past use private information more (Al Toufailia et al., 2013). Likewise, when foragers exploit more distant and, thus, less profitable food sources, they are more likely to use social dance information (Wray et al., 2012). This suggests that neurophysiological mechanisms of

reward perception play an important role in the decision to use waggle dance information *vs.* private information. Octopamine (OA) and dopamine (DA) are biogenic amines that function as neuromodulators in the central nervous system of invertebrates and they play important roles in reward signalling in honey bees (Barron et al., 2007; Hammer, 1997; Mercer and Menzel, 1982; Perry and Barron, 2013; Scheiner et al., 2002). They bind to specific membrane proteins mainly belonging to the family of G-protein-coupled receptors in different parts of the brain (Beggs et al., 2011; McQuillan et al., 2012; Mustard et al., 2012; Roeder et al., 2003), such as the mushroom bodies and the antennal lobes, *i.e.* brain areas with important functions in the processing and integration of information (Giurfa, 2007; McNeill et al., 2016; Zars, 2000). OA mediates the reward information during reward learning and, if administered to honey bees, increases the responsiveness of bees to sucrose (Mercer and Menzel, 1982; Scheiner et al., 2002; Giray et al., 2007; Pankiw and Page, 2003) and to olfactory stimuli (Perry and Barron, 2013; Hammer, 1997; Spivak et al., 2003). Additionally, oral or topical treatment of foragers with OA increases the motivation to perform waggle dances, most likely by increasing the perceived value of rewards (Barron et al., 2007). Interestingly, some instances of OA signalling in the *Drosophila* mushroom bodies require DA neurons (Burke et al., 2012; Søvik et al., 2015). In honey bees, DA has been found to reduce the response to sucrose rewards and conditioned olfactory stimuli (Mercer and Menzel, 1982; Perry and Barron, 2013; Scheiner et al., 2002). DA has various other effects, e.g. on avoidance learning, scouting and locomotion (Agarwal et al., 2011; Liang et al., 2012; Mustard et al., 2010), which could directly or indirectly affect waggle dance communication and the use of private information.

We hypothesized that OA would reduce the use of new social information and strengthen the use of private information by increasing the perceived value of a currently exploited food source. As a result, we expected a decrease in the interest in waggle dances by OA-treated foragers. DA effects are more difficult to predict since DA signalling seems to also complement OA signalling in *Drosophila* during reward learning (Burke et al., 2012; Søvik et al., 2015). But due to the contrasting effects of DA on sucrose responsiveness and extinction, we suspected that treatment with DA would reduce the use of private information about previous foraging sites and increase interest in waggle dances advertising new food sources. To test these predictions, we trained bees to collect sucrose solution with or without biogenic amines and then exposed these foragers to dances for an alternative, unknown food source. We quantified the interest of trained foragers in these alternative dances and recorded whether they used

private information or social information provided by the dance when deciding which feeder to visit.

Materials and methods

Study species and field site

Experiments took place from August to October 2016. We used three colonies (H1-H3) of *Apis mellifera carnica* housed in glass-walled observation hives in a hut situated on the campus of the University in Mainz, Germany. The colonies consisted of 2000-3000 workers, a queen, brood, pollen and honey reserves.

Experimental procedure

One hive at a time was studied and two trials per hive were performed (one with OA, one with DA; 6 to 14 days between the two trials). Each trial lasted 3-4 days and consisted of 1-2 days of training, followed by a treatment day and the test day. We used standard training procedures (von Frisch, 1967) to train two groups of 50-60 foragers to two feeders (unscented 0.8 M sucrose solution – a sugar concentration that induced bees to perform waggle dances) at a distance of 150 m from the hive and 7 m from each other (Figure 5.1). One group was trained to a feeder with a blue underlay (TFa) and the other group to a feeder with a yellow underlay (TFb). Colours were randomly assigned for each trial. The distance of 7 m between the two feeders and the two different colours made sure that trained foragers would visit just one of the two feeders. Afterwards, usually on the same day, we trained a third group of 10-20 foragers to a third feeder (DF, dance feeder) 160 m from the TFs and 150 m from the hive (Figure 5.1). All trained foragers were individually marked with numbered tags of different colours glued to the notum (Opalithplättchen). On the day after training, all feeders provided 0.3 M of identically scented sucrose solution (5µl essential oil per 100 ml sucrose solution; Primavera Life GmbH, Oy-Mittelberg, Germany). For each hive, we used a different odour: sage for H1, jasmine for H2 and peppermint for H3. On this treatment day, sucrose solution was provided for 60 min, from about 12.00 to 13.00 h. The sugar concentration was lower in order to prevent the recruitment of more bees, but make sure trained bees returned to their feeder. The duration of 60 min allowed foragers to learn the association between location, reward and scent and to form a long-term memory (Menzel, 1999). The number and time of each visit were noted for all marked bees during the 60-min treatment time.

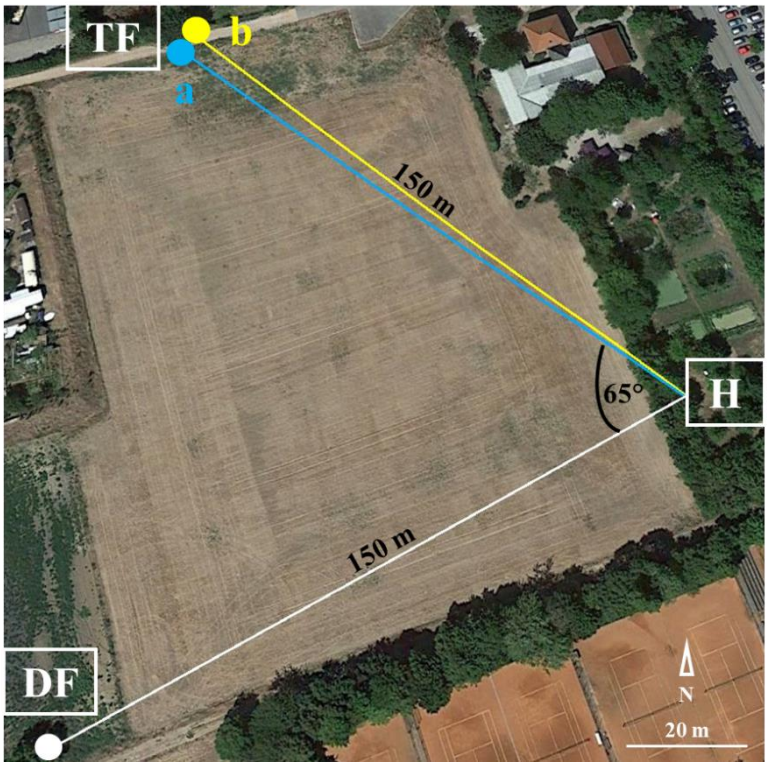

Figure 5.1: Experimental set-up. Location of the hive (H), dance feeder (DF) and training feeders (TF). The distance between DF and TF was 160 m. Picture taken from Google Earth (49°59'15.63" N, 8°14'07.20" E).

Additionally, at one TF (either a or b) we added 2 mg/ml of biogenic amine (octopamine or dopamine hydrochloride, Sigma Aldrich) during the treatment period. This concentration has induced behavioural changes in previous studies (Barron et al., 2007; Agarwal et al., 2011; Schulz and Robinson, 2001). The other TF served as a control (untreated bees). All solutions (training, treatment and test) also contained 1.75 mg/ml ascorbic acid (Sigma Adrich) to reduce oxidation of the biogenic amines (Scheiner et al., 2002). Orally administering biogenic amines has been shown to have similar effects on behaviour as other administration methods, such as topical application (Barron et al., 2007; Pankiw and Page, 2003; Schulz and Robinson, 2001; Barron et al., 2002).

On the test day, the day after the treatment, DF foragers were allowed to collect 1.8 M sucrose solution for 60 to 180 min (approx. 12.00-15.00 h) at the DF, whereas both TFs remained empty. This sucrose concentration made sure DF bees were likely to perform waggle dances advertising the DF location. The sucrose solution at the DF contained the same scent as during training. During this test period, 5-10 DF dancers made repeated foraging trips and performed waggle dances inside the hive. Meanwhile, TF foragers following these dances could decide whether to decode the dances advertising the DF, i.e. use social information, or use private information to fly to the TFs. Previous studies have

shown that experienced foragers are attracted to dancers carrying a familiar scent, which made it likely that a large proportion of TF foragers interacted with DF dancers (von Frisch, 1967; Grüter and Ratnieks, 2011). The arrival times of all bees at all feeders were noted. At the same time, we filmed the "dance floor" (Seeley, 1995) to record DF dances and the dance-following behaviour of TF foragers with high-definition video cameras.

A waggle dance usually consists of many waggle runs (range: 1 to >100) (Seeley, 1995; von Frisch, 1967). While waggle dances are frequently attended by both social and private information users, bees that attempt to decode dances follow more waggle runs (Biesmeijer and Seeley, 2005; Grüter et al., 2013; Grüter and Ratnieks, 2011). We defined dance following as directing the head towards a dancer and being within a distance of one antenna length during the waggle run phase (Al Toufailia et al., 2013; Tanner and Visscher, 2009). If a bee stopped dancing for at least 5 seconds we considered this dance to have ended (Al Toufailia et al., 2013; Tanner and Visscher, 2009). We analysed the time, the number of dances TF foragers followed as well as the number of waggle runs they followed.

Statistical analysis

Statistical analyses were performed in R 3.2.3 (https://www.r-project.org/). The data was analysed using generalized linear mixed-effects models (GLMM) for Poisson and binomial distribution. For normally distributed data we used linear mixed-effects models (LME). R fitted these models with the packages "lme4" and "nlme" (Bates et al., 2015; Pinheiro et al., 2019). In the case of zero-inflation or overdispersion (estimated with the "Dharma" package), we used GLMMs for zero-inflated data with the "glmmADMB" (Poisson distribution) and the "glmmTMB" (negative binomial distribution, nb) functions (Brooks et al., 2017). As random effects, we chose "hive" and "trial" to account for any hive or day effects. Occasionally, models failed to converge. In this case, we used only "trial" as a random effect because "trial" effects were stronger. We tested for differences in the number of dances followed, the number of waggle runs followed, the visited test feeder (DF or TF) and the recruitment probability between the two treatments (OA, DA) versus the control. Interactions between two fixed-effects were tested by comparing a model with and a model without the interaction using a likelihood ratio test (LRT) (Crawley, 2007). By means of a survival analysis for a constant hazard with exponential distribution ("survival" package), we compared the time of leaving the hive between the three treatment groups.

Results

During the six trials (two trials per hive), DF dancers performed 678 dances and a total of 10,789 waggle runs (Table 5.1). Overall, 259 bees were trained to the TF (5.24 ± 3.79 visits during the treatment time) and of those, 84% followed DF dances. Of this latter group, 40% were recruited to the DF by the end of the test period, whereas the remaining 60% exclusively visited the TF (Table 5.1).

Table 5.1: Dancing and dance following behaviour. Data shown are sample size or the mean ± StDev. DF = dance feeder; TF = training feeder. [1]Number of TF foragers that followed DF dances. [2]Average number of DF waggle runs followed per dance by TF foragers. [3] Number of DF dances followed per TF forager. [4]Number of TF foragers recruited to the DF. [5]Number of visits of the TF by TF foragers during testing.

Hive	Trial	Dances to DF	Waggle runs performed	Trained to TF	Bees followed[1]	Waggle runs followed	Waggle runs/dance[2]	Dances followed[3]	Recruited[4]	Visits to TF[5]
1	OA	60	1040	48	40	626	6.7±3.0	2.6±1.7	15	1.7±1.1
1	DA	79	1447	36	31	644	5.5±2.1	3.5±2.8	12	2.6±2.0
2	OA	182	2706	42	31	1673	4.8±1.3	10.9±8.3	13	3.0±1.8
2	DA	102	979	35	28	578	6.8±3.3	3.4±2.4	17	2.7±1.7
3	OA	114	2717	40	34	849	6.5±3.0	3.7±2.2	12	2.1±1.3
3	DA	141	1900	58	57	2440	5.4±1.6	7.9±4.4	15	4.1±2.5

(a) Dance-following behaviour

Overall, TF foragers followed 4.7 ± 5.1 dances with an average number of 5.9 ± 2.5 waggle runs per dance (Table 5.1). Bees that were recruited to the DF followed dances ~20% longer than bees visiting only the TF feeder (6.70 ± 2.72 vs. 5.6 ± 1.78) (LME: t = 2.25, p = 0.026), but there was no difference in the number of dances followed (nb GLMM: z = -1.73, p = 0.08) or the total number of waggle runs followed (LME: t = -1.25, p = 0.21).

OA-treated foragers followed 3.4 ± 5.7 dances and 27.5 ± 34.3 waggle runs in total, the control group followed 5.0 ± 5.2 dances and 30.2 ± 26.4 waggle runs. DA-treated foragers followed 5.3 ± 3.8 dances and 34.7 ± 24.4 waggle runs in total (Figure 5.2a, b). OA-treated foragers followed significantly fewer DF dances than control bees (Poisson GLMM: z = -3.1, p = 0.0017). Considering only the bees that followed at least one dance, OA-treated foragers also followed fewer waggle runs (Poisson GLMM: z = -2.4, p = 0.016) compared to the control group. We found no difference in the number of dances followed between DA-treated foragers and control bees (Figure 5.2a) (Poisson GLMM: z = 1.42, p =

0.14). However, DA-treated foragers that followed dances followed significantly more waggle runs in total (Figure 5.2b) (Poisson GLMM: $z = 5.6$, $p < 0.0001$). We found no differences between the treatment groups in the average number of waggle runs followed per dance (LME, OA vs. C: $t = 0.36$; $p = 0.72$; DA vs. C: $t = 1.37$; $p = 0.17$).

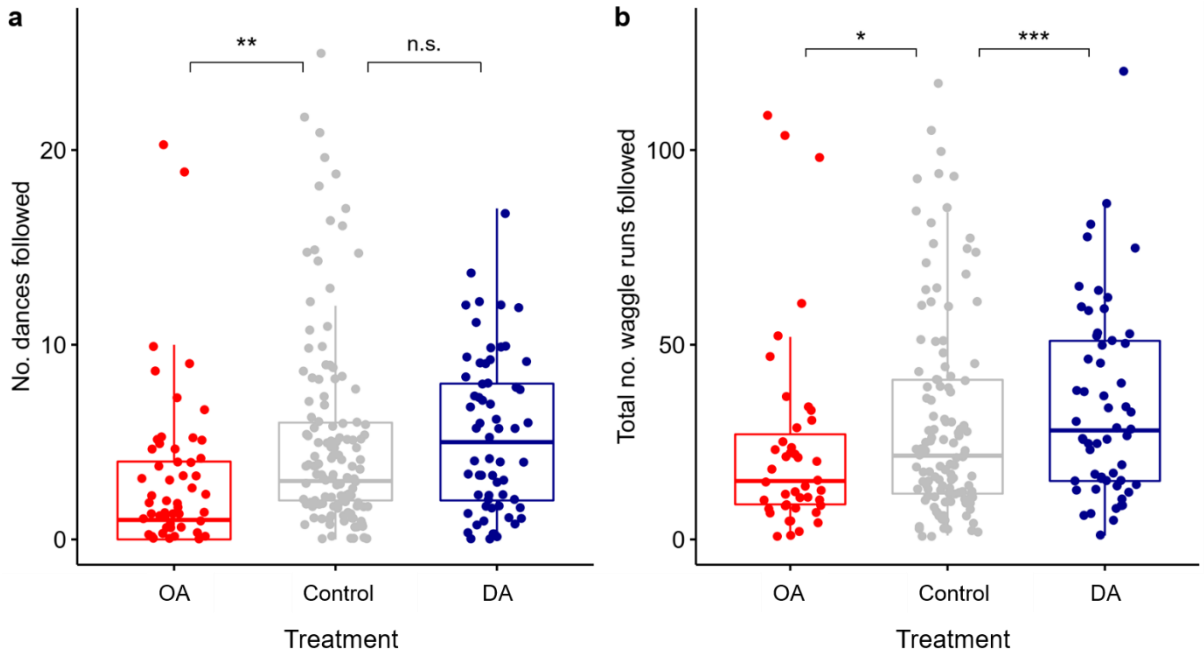

Figure 5.2: Effect of biogenic amine treatment on dance following behaviour. a) The number of waggle dances bees followed after oral treatment with octopamine (OA), control solution (C) and dopamine (DA). b) The effect of OA, C and DA on the total number of waggle runs followed by TF bees that followed at least one dance. Boxplots show medians, interquartile ranges (top line 75% quartile, bottom line 25% quartile) and whiskers show the 5% and 95% percentile). n.s. = $p > 0.05$, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.001$). Control bees from both trials are combined.

We also tested whether there was an interaction between treatment and the number of treatment visits. Indeed, these two factors significantly interacted in their effects on the number of dances followed (Poisson GLMM: LRT = 11.93; $p = 0.003$) and the total number of waggle runs followed (Poisson GLMM: LRT = 19.4; $p < 0.0001$). We, therefore, analysed the effect of feeder visits for each treatment group separately. The number of treatment visits had no effect on the number of dances followed in control- and DA-foragers (Poisson GLMM, control: $z = 0.04$, $p = 0.97$; DA: $z = -0.97$, $p = 0.33$), but we found a positive relationship between treatment visit number and the number of dances followed in OA-treated bees ($z = 1.98$, $p = 0.048$). Likewise, treatment visits did not affect the total number of waggle runs followed in control and DA-treated bees (Poisson GLMM, control: $z = 0.27$, $p = 0.79$; DA:

z = -1.24, p = 0.22), but we again found a positive effect of the number of treatment visits in OA-treated bees (nb GLMM: z = 3.1, p = 0.002).

(b) Feeder visitation probability

The DF was visited by 33% of OA-foragers, 54% of control foragers and 45% of DA-foragers (Figure 5.3). Of all bees visiting either feeder, OA-treated bees were significantly less likely to visit the DF than control bees (binomial GLMM: z = -2.6, p = 0.0085), but significantly more likely to visit only the TF (binomial GLMM: z = 2.5, p = 0.011). OA-foragers also visited the TF more often than control bees (Poisson GLMM: z = 2.7, p = 0.0080). Conversely, the probability to visit the DF or the TF did not differ between DA-foragers and control group foragers (binomial GLMM: DF: z = -0.6, p = 0.54; TF: z = 0.7, p = 0.47). Also the number of visits of the TF did not differ between these two groups (Poisson GLMM: z = -1.0, p = 0.30).

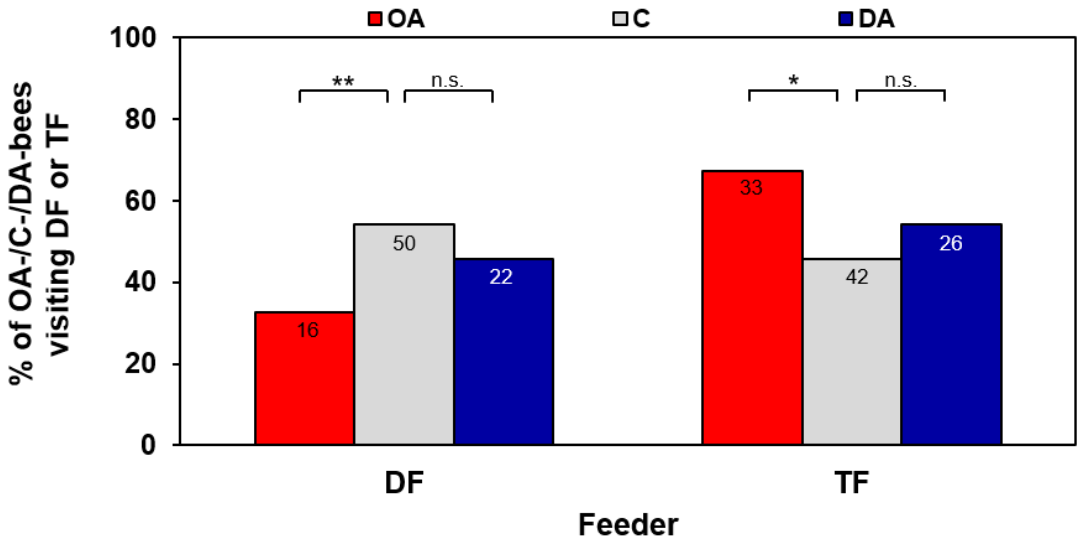

Figure 5.3: Effect of biogenic amine treatment on visitation probability. The percentage of bees that visited the dance feeder at least once, *i.e.* was recruited (DF, left) or exclusively visited the training feeder, *i.e.* only used private information (TF, right) after oral treatment with octopamine (OA), control solution (C) and dopamine (DA). Numbers in bars represent the number of bees.

With a survival analysis, we studied the temporal dynamics of the arrival times at the TF during testing. In this analysis, we included all bees that visited a feeder during the 60-minute treatment period (including those that did not visit a feeder during the testing). Again, more OA-treated visited the TF than control bees (Figure 5.4) (survival analysis for exponential response: z = -1.6, p < 0.001) and this effect seems especially clear at the beginning of the test period. A larger number of DA-bees visited the

TF than control bees (survival analysis for exponential response: $z = -0.8$, $p < 0.001$). This difference became apparent after approximately 20 minutes (Figure 5.4).

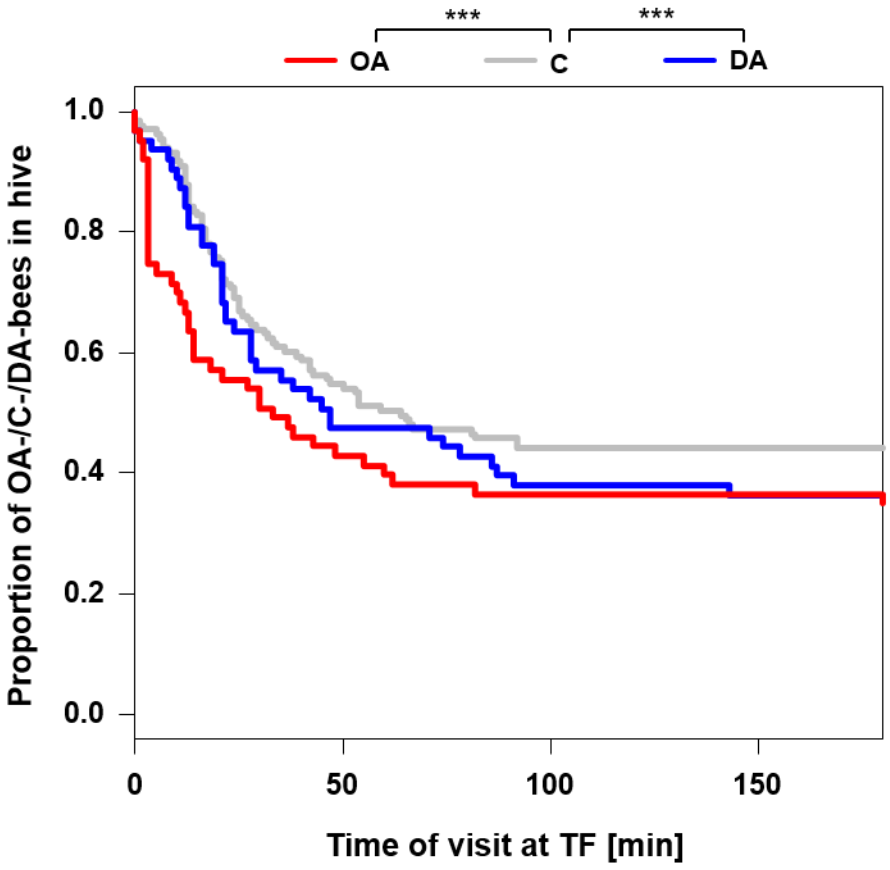

Figure 5.4: Proportion of bees not yet visiting the training feeder (TF) during the testing period. The first visit of a bee at the TF counted as the beginning (time = 0 min). A survival analysis suggests that there are differences in the temporal dynamics when comparing octopamine-treated foragers (OA, n = 62) vs bees that were fed with a control solution (C, n = 134) and when comparing dopamine-treated bees (DA, n = 63) with control bees.

Discussion

We found that oral treatment of honey bee foragers with octopamine and dopamine affected dance following behaviour and information-use. Foragers treated with OA followed fewer waggle dances and, if they followed dances, they followed fewer waggle runs compared to control bees. This is consistent with our prediction that OA-treated bees are less interested in new social information. Despite experiencing that the food source they exploited in the past (TF) was not presently rewarding, these bees mostly relied on their private information and inspected this feeder more often than control bees. Site-fidelity is well-known in honey bees, even if the visited foraging site does not currently offer

rewards (Grüter et al., 2013; Grüter and Ratnieks, 2011; Al Toufailia et al., 2013). A possible explanation for an increased use of private information by bees treated with octopamine is that OA increased the reward perception of bees collecting food at the TF during the treatment period. OA plays a crucial role in reward signalling and has been shown to increase responsiveness to sucrose, learning and retrieval of information in honey bees (Barron et al., 2007; Mercer and Menzel, 1982; Scheiner et al., 2002; Pankiw and Page, 2003).

Octopamine could also directly reduce the use of social information. Boulay et al. found that OA negatively affects social interactions in ants (Boulay et al., 2000). Low levels of OA brain titres, on the other hand, are associated with an increased motivation to engage in trophallaxis, which represents an important mechanism of social learning in ants and honey bees (Farina et al., 2005; Farina and Grüter, 2009; Provecho and Josens, 2009). Thus, OA-treatment might have reduced dance following by reducing the motivation of bees to interact with hivemates. This is consistent with the findings that OA-treatment increases scouting, *i.e*. the search for food without following dances (Liang et al., 2012), and that scouts have higher tyramine titres, a precursor of octopamine, than recruits (Cook et al., 2019). Thus, OA might not only strengthen the use of private information by increasing the perceived value of the reward offered at the TF, but also reduce social information-use by lowering the motivation to engage in social interactions, such as following waggle dances. The negative effects of OA on dance following are also consistent with the observation that older and more experienced foragers appear to rely more on private information and follow dances less (Gil and Farina, 2002; Biesmeijer and Seeley, 2005): OA titres change with age and are higher in older bees (Harris and Woodring, 1992; Schulz and Robinson, 1999; Wagener-Hulme et al., 1999). Surprisingly, OA-treated foragers showed more interest in dances if they visited the OA-feeder more often during the treatment period. It could, thus, be that the OA-treatment has a weaker inhibitory effect on foragers that are more motivated to forage, *i.e.* those that performed more visits during the treatment time. For instance, a larger dose of OA could induce molecular mechanisms that attenuate OA signalling in the brain, thereby reducing signalling when OA titres are very high (Böhm et al., 1997). More research is needed to better understand the relationship between experience, communication behaviour and biogenic amine signalling.

While DA-treated bees did not follow more dances overall, those bees that did follow dances followed significantly more waggle runs than control bees (Figure 5.2). Interestingly, despite their increased interest in dances, dopamine treated bees were not more likely to be recruited to the advertised

feeder, suggesting that an increased interest in waggle dances does not necessarily increase the decoding and use of social information. On the contrary, we found evidence that DA increased the use of private information. A survival analysis that included all treated bees found that DA-treated bees were significantly more likely than control bees to visit the training feeder (Figure 5.4). In other words, while DA caused bees to follow dances more thoroughly, it may also have increased their use of private information. These contradictory effects are puzzling but could be explained by the diverse and complex roles DA plays in the insect brain. Felsenberg et al., for example, demonstrated that there are different subsets of dopaminergic neurons in *Drosophila* mushroom bodies (Felsenberg et al., 2017; see also Tedjakumala et al., 2017). One subset neutralizes or extinguishes previously gained memory whereas the other subset reconsolidates the original memory. Furthermore, DA signalling is involved in both aversive and reward learning in fruit flies and is suspected to signal the nutritive value of a reward, while OA signals sweetness (Burke et al., 2012; Søvik et al., 2015). Much less is currently known about the role of DA in reward signalling in honey bees (Søvik et al., 2015). Distinct functions of DA together with the discrete compartmentalization of dopaminergic neurons in the mushroom bodies (McQuillan et al., 2012; Felsenberg et al., 2017; Krashes et al., 2009) might explain the complex effects on information use we found. Disentangling these effects would require a much more targeted way of treating honey bee foragers, e.g. by injecting dopamine into specific parts of the brain and the mushroom bodies.

It is possible that there are distinct types of information-users, *i.e.* private information users that consistently persist at familiar feeding sites (Biesmeijer and de Vries, 2001; Wagner et al., 2013; Grüter and Ratnieks, 2011) and social information users that have a high propensity to abandon their food source if it is below a certain threshold and follow dances to find better ones. Scouting bees, *i.e.* bees that have a high propensity to search for new food sources without following dances, differ substantially in their brain gene expression and learning performance compared to non-scouting bees (Liang et al., 2012; Cook et al., 2019). The probability to follow one of these three strategies seems to be influenced by biogenic amines in complex ways (see also Cook et al., 2019). We currently have a limited understanding of how biogenic amines affect the use of different types of information, but social insects are excellent model systems that can help us uncover the role of biogenic amines in individual decision-making and the coordination of foraging activities of colonies.

CHAPTER 6

Forager age and motivation, but not cumulative foraging experience, affect octopamine and dopamine receptor gene expression in the honey bee mushroom bodies

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Abstract

Foraging behaviour is crucial for the development of a honey bee colony. Biogenic amines are key mediators of learning and the transition from in-hive tasks to foraging. Bees that have become foragers vary considerably in their behaviour, but whether and how this behavioural diversity depends on biogenic amines is not yet well understood. For example, forager age, cumulative foraging experience or foraging motivation may all be linked to biogenic amine signalling. Furthermore, expression levels may fluctuate depending on day time. We tested if these intrinsic and extrinsic factors are linked to biogenic amine signalling by quantifying the expression of octopamine and dopamine receptor genes in the mushroom bodies. We found old foragers had a significantly higher expression of *Amdop1*, *Amdop2*, *AmoctαR1* and *AmoctβR1* compared to young foragers. Surprisingly, our measures of cumulative foraging experience were not related to the expression of the same receptor genes in the mushroom bodies. Furthermore, we trained foragers to collect sucrose solution at a specific time of day and tested if the motivational state of time-trained foragers affected receptor gene expression. Bees engaged in foraging had a higher expression of *Amdop1* and *AmoctβR3/4* than inactive foragers. Bees anticipating to forage were intermediate in the expression of this receptor, suggesting that bees upregulate some receptor genes in the mushroom bodies in preparation for foraging. Finally, the expression of *Amdop1*, *Amdop3* and *AmoctαR1* also varied with daytime. Our results show that receptor gene expression in forager mushroom bodies is complex and depends on both intrinsic and extrinsic factors.

Introduction

In the honey bee *Apis mellifera*, like in most other social insects, division of labour is partly age-dependent (Michener, 1974; Robinson, 1992). During the first 2-3 weeks after emergence, worker bees perform tasks in the hive like feeding the brood, building honey combs and processing nectar. After this time, they start to forage until they die at the age of approx. five to seven weeks (Seeley, 1982; Johnson, 2008; Johnson, 2010). However, task performance is not tightly linked to a specific age, but can change according to each colony's needs (Robinson et al., 1989; Huang and Robinson, 1996; Bloch and Robinson, 2001). The final task of foraging is cognitively demanding, as foragers have to learn food locations, odours, floral shapes and colours (von Frisch, 1967; Dukas and Visscher, 1994; Abou-Shaara, 2014). Because flowers often bloom at specific times of the day, foragers also have to learn at which time of the day particular nectar and pollen sources are available (Beling, 1929; Moore, 2001; Zhang et al., 2006).

Biogenic amines are important for both division of labour and learning (Taylor et al., 1992; Schulz and Robinson, 1999; Schulz and Robinson, 2001; Wagener-Hulme et al., 1999). They act as neurotransmitters, neurohormones and neuromodulators in the central nervous system (CNS) of animals. In insects, they mediate different physiological states and behaviours (Evans, 1980). Dopamine (DA), for example, plays a key role in learning and memory, particularly in aversive learning (Schwaerzel et al., 2003; Riemensperger et al., 2005; Vergoz et al., 2007; Agarwal et al., 2011; Tedjakumala et al., 2014). In addition, DA is linked to motor behaviour and activity level in insects in general and honey bees in particular (Harano et al., 2008; Mustard et al., 2010). Octopamine (OA) has an arousing effect and mediates the reward information during classical appetitive conditioning in honey bees (Hammer and Menzel, 1998). This function as a reward signal is likely to explain why OA stimulates waggle dancing in foragers (Barron et al., 2007). OA and DA levels in the brain of bees are also related to division of labour (Wagener-Hulme et al., 1999; Barron and Robinson, 2005; Schulz et al., 2002). Compared to young nurse bees, for example, foragers have higher brain levels of OA and DA (Taylor et al., 1992; Schulz and Robinsion, 1999; Wagener-Hulme et al., 1999) and the age of first foraging is reduced in OA treated bees (Schulz and Robinsion, 2001; Scheiner et al., 2002). Traditionally, octopaminergic and dopaminergic pathways were considered to be functionally separated, with OA being involved in reward signalling and DA in aversive signalling. However, more recent research in

Drosophila suggests that OA and DA signalling pathways are tightly intertwined (Perry and Barron, 2013).

Biogenic amine receptors belong to the G-protein coupled receptor (GPCR) family that are heavily expressed in different parts of the bee brain, particularly in the mushroom bodies (Durst et al., 1994; Roeder, 1994; Farris et al., 2001; Ismail et al., 2006). The mushroom bodies are an important centre for various cognitive functions, such as sensory integration, memory formation and the organization of complex behaviours (Heisenberg, 2003; Menzel et al., 2006; Giurfa, 2007). The observation that (*i*) mushroom bodies increase in size and change in anatomical features during the foraging period and when bees are older (Farris et al., 2001; Withers et al., 1995; Fahrbach et al., 1998) and that (*ii*) OA and DA receptor genes show higher expression in the mushroom bodies of foragers than in bees performing in-hive tasks (Humphries et al., 2003; Reim and Scheiner, 2014) further highlights the importance of the mushroom bodies for foraging.

While the role of OA and DA for the transition from in-hive to foraging tasks is relatively well studied, much less is known about the role of OA and DA after bees have transitioned to foraging. For example, OA and DA are likely to be important to understand the diversity of foraging-related behaviours and states, such as the tendency to be a scout or a non-scout or the likelihood to collect pollen instead of nectar (Pankiw and Page, 2000; Cook et al., 2019). Biogenic amine receptor gene expression has been shown to be a good indicator of behavioural states (Humphries et al., 2003; Reim and Scheiner, 2014; Liang et al., 2012; McQuillan et al., 2012). However, little is known about how forager age, cumulative foraging experience or the motivational state of foragers are linked to octopaminergic and dopaminergic signalling. Here, we explore these putative links between octopaminergic and dopaminergic signalling and forager age and cumulative foraging experience. Foraging experience is not only reflected in the cumulative amount of foraging (e.g. the number of foraging trips or the time spent foraging), but also in the immediate foraging state, which may vary during the course of a day (Tenczar et al., 2014). Naeger et al. (2011), for example, found that inactive foragers differ in their whole-brain gene expression from bees that are expecting to forage in the immediate future (anticipating bees) (Naeger et al., 2011). This study did not include bees that were actively engaged in the foraging process and it is, thus, unclear if active foraging is accompanied or preceded by changes in gene expression of OA and DA receptors. Additionally, it remains unclear whether gene expression differences linked to different foraging states can be found in the mushroom

bodies. Thus, we trained bees to collect food at particular times of the day and compared foragers that were inactive, anticipating to forage or engaged in foraging. Finally, gene expression might depend on the time of day as, for instance, cell adhesion genes that may be involved in learning and memory processes have been found to vary with time of day in honey bees (Naeger et al., 2011; Ingram et al., 2011). Furthermore, the *foraging* gene is more expressed during the daylight hours when foragers of the ant *Pogonomyrmex occidentalis* are foraging outside the nests (Welzl and Stork, 2003). Thus, we tested if OA and DA receptor genes expression fluctuates with the time of the day.

Materials and methods

Study species and field site

We used nine *Apis mellifera carnica* colonies (three for each of the three experiments; see below) housed in 3-frame observation hives on the campus of the Johannes-Gutenberg University in Mainz, Germany. Each observation hive had ~3000-4000 workers, brood, honey and a naturally mated queen. We kept the observation hives in a wooden shed for protection against the weather and sun exposure. The observation hives were made from a wooden casing comprising a translucent glass pane on each side and a transparent tube serving as an exit/entrance reaching the outside of the shed. The bees were allowed to adapt to the new environment for at least 1 week before further manipulations were performed.

Experimental procedure

The objective of this study was to explore whether forager age (Experiment 1), cumulative foraging experience (Experiment 2) and forager motivational state (Experiment 3) are linked to the expression levels of OA and DA receptor genes in the mushroom bodies of honey bee foragers. In experiment 1, the effect of the time of the day was also studied (Experiment1: daytime).

Experiment 1: Does the expression of octopamine and dopamine receptor genes depend on forager age?

The following procedure was performed for each observation hive, one at a time. Two frames of capped late-stage brood (shortly before eclosion) were removed from the original full-sized colony (from which the observation hive was built) and stored in a humidified climate chamber (35°C) overnight. The next day the newly emerged honey bees were removed from the brood frames and marked with coloured Opalith number plates to the thorax of bees. Then, the newly emerged, marked bees were introduced

into the observation hive. Each colony received 100 newly emerged bees of this age group, which constituted the "old forager" group. To obtain the "young foragers", the marking procedure was repeated exactly two weeks after the introduction of the first marked honey bees, using newly emerged bees from two different brood combs, but from the same original full-sized colony. This time Opalith number plates of a different colour were used to separate the two age groups.

Thirty-five days after the introduction of the first age cohort, we captured both young and old foragers. Thus, young foragers were 21 days old, whereas old foragers were 35 days old. Bees usually transition to foraging between the age of 2-3 weeks (Robinson, 1992; Seeley, 1982). However, they can also start to forage at a younger age if the weather is consistently good (Farris et al., 2001). In our study, video analysis (see details in Experiment 2) confirmed that all bees captured for Experiment 2 performed one or more field excursions when reaching the age of 21 days, indicating the commencement of foraging prior to this age. Also, bees were caught at four different times of the day: in the morning (~9:00), around noon (~12:00), in the afternoon (~16:00) and at night (~22:00). To catch the bees, the windows of the observation hives were carefully and slowly removed to access all the numbered bees inside the hive. Bees were collected individually with 5 ml Eppendorf tubes and immediately put into liquid nitrogen to maintain the state of gene expression in the mushroom bodies. Afterwards the samples were transferred to a -80°C freezer. For each time period, in total 12 bees were caught (60 bees in total, 20 from each of the 3 observation hives). The number of captured young foragers was the same as the number of old foragers. This experiment was performed between May and July 2017.

Experiment 2: Does cumulative foraging experience affect the expression of octopamine and dopamine receptor genes?

In Experiment 1, we studied if the age could affect receptor genes expression, however, the cumulative foraging experience of foragers was not controlled for. For example, a previous study has shown that long durations of foraging could cause a cognitive decline in honey bees (Behrends et al., 2007), which might also influence receptor gene expression. Therefore, we repeated the experiment the following year using video cameras to quantify the cumulative foraging experience of foragers that had the same age. We used 3 different observation hives and introduced 150 newly emerged, marked bees from the original full-sized colony (from which the observation hive was built) into the observation hive, as described for Experiment 1 (450 bees in total). When the marked bees were 10 days old and until the

age of 21 days, the entrance to the hives were filmed (JVC, model GZ-GX1BE) daily from 7 am to 7 pm in order to quantify the foraging activity of marked bees. To catch the bees on day 22, the same method was used as described above. The marked bees were caught between 12:30 and 13:30 using Eppendorf tubes and killed immediately with liquid nitrogen. For further storage, the bees were placed in a -80 °C freezer.

When reviewing the video material, the time of exit and return of the marked bees were noted. From these recordings, we calculated the following parameters: the total (i.e. cumulative) number of foraging trips performed over the entire observation period, the total cumulative foraging time, the total foraging days and the average duration of foraging trips. We excluded hive excursions of less than 4 min because they are unlikely to represent foraging trips. The total cumulative foraging time was the sum of the times of all trips one bee completed during the filming period. The total foraging days were calculated by counting the number of days a bee performed foraging trips. The average duration of foraging trips was determined by dividing the total cumulative foraging time of a bee by its number of cumulative foraging trips. These parameters indicated how much foraging experience bees had cumulative by the time of capture.

Among all the captured bees, 15 bees were selected from each observation hive for the qPCR analysis. The bees were selected so that they showed variation in their cumulative foraging experience (total number of cumulative foraging trips, total cumulative foraging time, total foraging days and average duration of foraging trips). This experiment was performed between May and July 2018.

Experiment 3: Does the expression of octopamine and dopamine receptor genes depend on the foraging-related motivational state?

We used three different observation hives (see above for description), one hive at a time. We trained two different groups of bees from an observation hive to collect food from two different feeders. Both feeders were 100 m from the hive, but in opposite directions. One group of bees was trained in the morning (MO bees), between 9:30 and 11:30. The other group was trained in the afternoon (AF bees) from 15:30 to 17:30. To establish a training group, we used standard procedures to train a group of 30-50 foragers to a feeder offering unscented 50% sucrose solution (von Frisch, 1967). By doing so, the bees would learn the location of their respective artificial feeder.

One day after the two groups of foragers were established, the bees trained to the feeders were numbered with Opalith number plates for individual identification. Training continued for 12 days to

maintain an adequate number (N≈30) of trained and marked bees that only appeared at a single training time at the respective feeder. During this training time, both feeders offered 30% unscented sucrose solution and bees could acquire a time-place memory for the respective feeder and training time. This reward represents an average food source (Seeley, 1986), which reduced the likelihood that bees would perform dances and recruit nestmates from the other training group. Meanwhile, the arrival of foragers was recorded for each foraging trip to make sure foragers only visited their own training feeder. Most bees exhibited allegiance to a single training time, the remaining bees (31.5%±1.9%) that visited both feeders were removed before the sample collection phase.

On collection day, the feeders were set up with 30% unscented sucrose solution during the respective training time. We collected three types of bees: anticipating bees whose training time was approaching were captured 15 minutes before offering food at the morning feeder location or afternoon feeder location (Table 6.1); foraging bees who foraged during the training time and inactive bees whose catching time was at least 4 hours earlier/later than the training time. Anticipating bees were caught close to the entrance of the hive as previous research has shown that time-trained bees often wait there shortly before the anticipated reappearance of the food source (Naeger et al., 2011; Körner, 1940; Moore et al., 1989; Van Nest et al., 2016). Inactive bees move to different hive areas, often further away from the hive entrance (von Frisch, 1967; Naeger et al., 2011; Kaiser and Steiner-Kaiser, 1983). Foraging bees were also immediately caught inside the observation hive near the entrance when they went back to the hive from the feeder. Using the number tags, we made sure that these bees were actively engaged in the foraging cycle, which includes hive-stays to unload food (Seeley, 1989). Bees engaged in foraging were caught less than a minute after they left the feeder. It is, therefore, unlikely that OA and DA receptor transcription levels changed significantly since the bees left the feeder (see e.g. Ugajin et al. 2013 for the temporal dynamics of expression changes of immediate early genes (IEGs) in honey bees).

Table 6.1: The design of Experiment 3 and the six behavioural groups analyzed in this study.

Time of Collection	States of Bees	
	Morning Trained (MO bees)	**Afternoon Trained (AF bees)**
09:15-09:30	Anticipating	Inactive
10:45-11:00	Foraging	Inactive
15:15-15:30	Inactive	Anticipating
16:45-17:00	Inactive	Foraging

Thus, all bees used for the qPCR analysis were collected while they were inside the hive and the bees were used for qPCR analysis only if they successfully trained to their corresponding feeder location for at least 7 days to make sure that they had learned the time of food availability (Lehmann et al., 2011). The method for catching bees was the same as described above. Thus, in total 6 different behavioural groups of bees were analyzed in experiment 3: anticipating, actively foraging and inactive bees, both for the MO and the AF group (Table 6.1). At least 12 bees from each of the 6 behavioural groups (four from each hive, a total of three hives) were used for molecular analysis. This experiment was done between July and September 2018.

Brain dissections, RNA isolation and cDNA Synthesis

Heads were removed from the body and fixed with dental wax on an ice-cooled Petri-dish to dissect the mushroom body calyces with cooled bee saline (154 mM NaCl, 2 mM NaH_2PO_4, 5.5 mM Na_2HPO_4, pH 7.2) over ice as quickly as possible (dissection lasted less than 3 minutes). We used sharp tweezers (FST, Canada) to remove the calyces of mushroom bodies along the bottom of the calyces (see Sarma et al., 2009, their Figure 1). While the paired mushroom bodies are made up of the pedunculus connected to the two cup-like calyces (a lateral and a medial calyx), we only used the calyces of the mushroom bodies because of the difficulty to completely remove the mushroom bodies from other brain parts. The calyces contain the intrinsic Kenyon cells, where a large part of mushroom body transcription takes place and the calyces are often used in mushroom body gene expression studies (Sarma et al., 2009; Lutz et al., 2012). The calyces were directly transferred into 100 µl TRIzol® (Invitrogen, USA) for RNA extraction. According to the manual, RNA was extracted from isolated calyces using RNeasy Mini Kit (Qiagen, Germany). Samples obtained with our method have high RNA integrity numbers (≥6.0) for analysis (McQuillan et al., 2012). The Quanti Tect Reverse Transcription Kit (Qiagen, Germany) was used to synthesize the cDNA. Before we synthesized the cDNA, the DNase digestion step took place according to the manufacturer instructions. For cDNA synthesis, we used 10 ng total RNA for each reaction.

Real-time quantitative PCR

Until now, three DA receptors and five OA receptors have been identified in the honey bee (Humphries et al., 2003; Grohmann et al., 2003; Mustard et al., 2003; Beggs et al., 2005; Balfanz et al., 2014), all of which were analysed in our study. AmOCTαR1 is a α-type OA receptor, which mediates Ca^{2+} signalling. Four AmOCTβ receptors belong to the OA β-receptors, which are cAMP-coupled receptors

(Evans et al., 2005; Sinakevitch et al., 2011). DA receptors have been categorized into two groups in the honey bee. AmDOP1 and AmDOP2 belong to D1-like receptors. Activation of D1-like receptors leads to an increase in intracellular cAMP levels (Blenau et al., 1999). AmDOP3 is a D2-like receptor that reduces intracellular cAMP when it is activated (Humphries et al., 2003; Beggs et al., 2005; Blenau et al., 1999). AmOCTβR3 and – βR4 receptors are two splicing variants of the same gene and we used AmOCTβR3/4 to represent the AmOCTβR3 and – βR4 receptors in our study (Balfanz et al., 2014; Reim and Scheiner, 2014).

PCR was performed on a mic qPCR cycler (Bio Molecular Systems, Australia) using the Blue S'Green qPCR mix Separate ROX (BioZyme, USA). Gene primers were based on published sequences and Primer Premier 3.0 (Table 6.2). All primers were synthesized by Biolegio (Netherlands). Each reaction volume of 20 µL contained 10 µL Blue S'Green qPCR mix, 0.25 µM of each primer, 2 µL cDNA and DNase/RNase free distilled water. The following cycling parameters were used: 95 °C for 2 min; 40 cycles of 95 °C for 5 s and 60 °C for 20 s. The fluorescence signal was measured at the end of each extension step at 60 °C. Quantification cycle (C_q) values were determined at the same fluorescent threshold for each gene by the micPCR Version2.6 software (Bio Molecular Systems, Australia). The transcript levels of the target genes were expressed as normalized transcript abundance using *GAPDH* and *eiF3-S8* as internal reference genes (Grozinger et al., 2003; Reim et al., 2013). Using the software package NormFinder version 0.953 (Andersen et al., 2004). we examined the stability of the reference genes and found that combining the two reference genes was more stable than using a single reference gene. The relative gene expression was calculated using the $2^{-\Delta CT}$ method using the following formula: Normalized = $2^{-(C_q\text{Targer}-C_q\ \text{Reference})}$ (Schmittgen and Livak, 2008). PCR efficiency (E) values were calculated by the software the micPCR Version2.6 software (Bio Molecular Systems, Australia) for each gene from the given slope after running standard curves and the following formulate $E=2^{-1/\text{slope}}-1$(Taylor et al., 2010).

Table 6.2: Primers used in real-time RT-PCR.

Primer name	Sequence (5′–3′)	Reference
Amdop1-F	ACA GAA TTC CGA GAA GCG TTC A	Liang et al., 2012
Amdop1-R	ATT CGC TAG TCG ACG GTT GAT TT	
Amdop2-F	ACA CGG AAT TGG TTC TCC ATC T	Liang et al., 2012
Amdop2-R	TCC CGT AAC CGG CTG TCA	
Amdop3-F	CGT TGC AAA CTG TCA CCA AT	Beggs et al., 2007
Amdop3-R	GAC GTC CAT TGC GAT GTA AA	
AmoctαR1-F	ACG AAG GCG GCG AAG AC	Liang et al., 2012
AmoctαR1-R	CGC GCA CCA AGT ACA TTG TG	
AmoctβR1-F	CAG CAC CGT CTC CAT ACT CC	Primer primer 3
AmoctβR1-R	GAG GTG TTT CTC GGT GGT GT	
AmoctβR2-F	AGC GTT GGC CGA CAT GTT	Liang et al., 2012
AmoctβR2-R	AGC CAT TTG CCG GTC AAT T	
AmoctβR3/4-F	CAC TTC GAT ACG ACA ACA AAC G	Primer primer 3
AmoctβR3/4-R	GGT TCA GGG CGC TGT TGA	
GAPDH-F	ACC TTC TGC AAA ATT ATG GCG A	Reim et al., 2013
GAPDH-R	CAC CTT TGC CAA GTC TAA CTG TTA AG	
eIF3-S8-F	TGA GTG TCT GCT ATG GAT TGC AA	Grozinger et al., 2003
eIF3-S8-R	TCG CGG CTC GTG GTA AA	

Statistical analysis

All data were analyzed using linear mixed-effects models (LME) with the nlme package 3.1-137 in the R environment version 3.4.4 (http://www.R-project.org/). The Shapiro-Wilk test was used to test for the normality of the residuals. If necessary, data were log- or square root transformed to achieve a Gaussian distribution of the model residuals. Colony ID was always included as random effect to account for the non-independence of observations from the same colony (Zuur et al., 2009).

Experiment 1: To test the significance of age/daytime-dependent biogenic amine receptor gene expression, we explored the role of two fixed effects, age and daytime. We removed the interaction between the fixed effects from the final model, because the interaction was never significant (all p-values > 0.05). To compare the expression of biogenic amine receptor genes between the four different daytimes, pairwise comparisons were performed and a sequential Bonferroni correction was applied to adjust p-values for multiple testing (multcomp package 1.4-8 in R).

Experiment 2: To test for relationships between forager experience and biogenic amine receptor gene expression, we again used LME's to explore the four measures of foraging experience (total number of foraging trips, total foraging time, total foraging days and average trip duration).

Experiment 3: To study how the motivational state of a forager was related to the biogenic

amine receptor expression, we used motivational state and training group (MO and AF group) as fixed effects. For post-hoc analyses of pairwise differences among the three motivational states, we again used the multcomp package 1.4-8 and the sequential Bonferroni correction to adjust p-values following multiple testing.

Results

Age- and daytime effects on biogenic amine receptor gene expression

The expression of receptor genes *Amdop1*, *Amdop2*, *AmoctαR1*, *AmoctβR1* showed a significant difference among age-groups, with the old foragers having an up-regulated expression (LME, *Amdop1*: *LRT*=11.26, *p*=0.0008; *Amdop2*: *LRT*=5.95, *p* = 0.015; *AmoctαR1*: *LRT*=5.96, *p*=0.015; *AmoctβR1*: *LRT*=7.50, *p*=0.0062, Figure 6.1A, B, D, E). *Amdop3*, *AmoctβR2*, *AmoctβR3/4* expression did not show a trend in the same direction (LME, *Amdop3*: *LRT*= 0.16, *p*=0.68; *AmoctβR2*: *LRT*=3.13, *p*=0.077; *AmoctβR3/4*: *LRT*=2.07, *p*=0.15 Figure 6.1C, F, G).

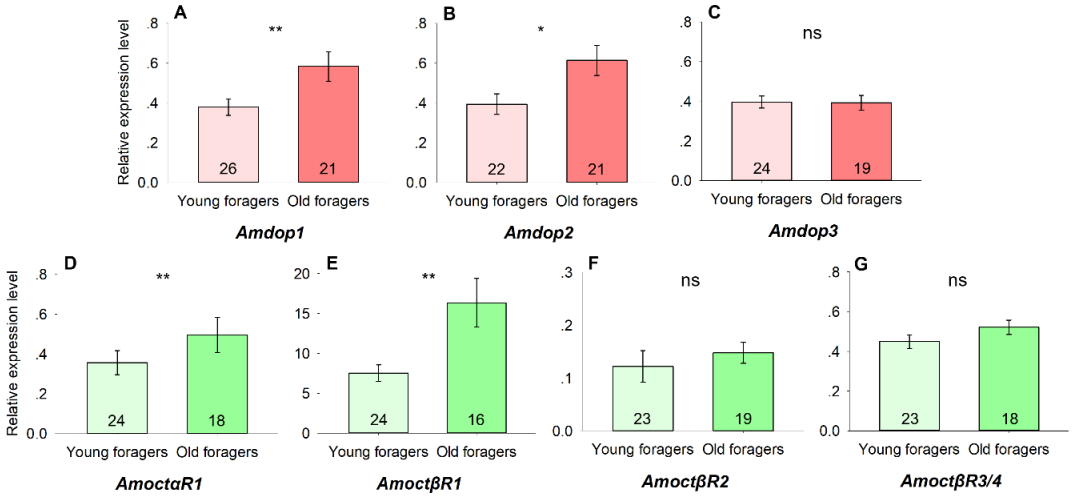

Figure 6.1: Biogenic amine receptor gene expression (A–E) in the mushroom bodies of young and old foragers. Bars show mean expression levels relative to the two reference genes (*GAPDH* and *eiF3-S8*)±SE. Numbers in bars indicate sample size. Asterisks indicate significant differences between young and old foragers (*$P \leq 0.05$, **$P \leq 0.01$).

We found that time of day affected gene expression in *Amdop1*, *Amdop3* and *AmoctαR1* (LME, *Amdop1*: *LRT*=30.75, *p*<0.0001; *Amdop3*: *LRT*=30.80, *p*<0.0001; *AmoctαR1*: *LRT*=36.46, *p*<0.0001). Pairwise comparison tests revealed that levels of *Amdop1* were significantly lower in the morning (~9:00) and at night (~22:00) compared to the other periods (Table 6.3, Figure 6.2A). The expression of *Amdop3* was significantly lower in the morning (~9:00) compared to other periods (Table 6.3, Figure 6.2C). *AmoctαR1* transcript levels, however, were significantly higher in the morning (~9:00) compared

to other periods (Table 6.3, Figure 6.2D). The expression of *Amdop2*, *AmoctβR1*, *AmoctβR2*, *AmoctβR3/4* did not change during the day (LME, *Amdop2*: LRT=2.62, *p*=0.45; *AmoctβR1*: LRT=2.67, *p*=0.45; *AmoctβR2*: LRT=0.85, *p*=0.84; *AmoctβR3/4*: LRT=6.16, *p*=0.10, Figure 6.2B, E-G).

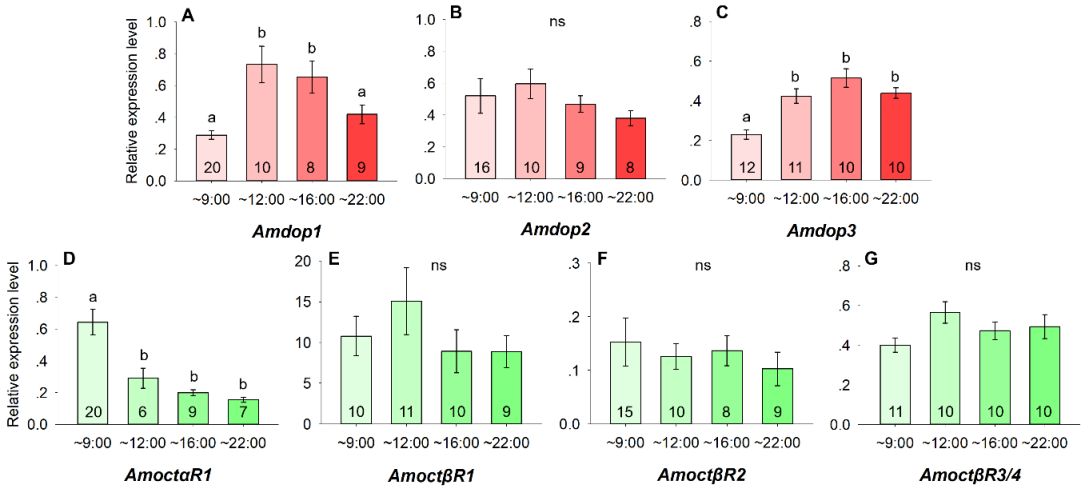

Figure 6.2: Biogenic amine receptor gene expression (A–E) in the mushroom bodies depends on time of day. Bars show mean expression levels relative to the two reference genes (*GAPDH* and *eiF3-S8*) ± SE. Numbers in bars indicate sample size. Letters indicate significant differences between groups. Bars not sharing a letter differ significantly (p <0.05). Bars without letters mean no significant.

Table 6.3: *P*- and *z*-values determined by linear mixed models for the pairwise comparison tests.

Pairwise comparison	***LME***	***Amdop1***	***Amdop3***	***AmoctαR1***
~9:00 vs ~12:00	*p*	<0.0001	0.0001	0.0094
	z	5.45	4.2	-3.04
~9:00 vs ~16:00	*p*	<0.0001	<0.0001	<0.0001
	z	4.43	5.99	-5.34
~9:00 vs ~22:00	*p*	0.22	<0.001	<0.0001
	z	1.59	4.4	-6.01
~12:00 vs ~16:00	*p*	0.59	0.19	0.34
	z	-0.53	1.87	-1.36
~12:00 vs ~22:00	*p*	0.0055	0.76	0.086
	z	-3.2	0.3	-2.19
~16:00 vs ~22:00	*p*	0.037	0.25	0.34
	z	-2.5	-1.53	-0.99

Cumulative foraging experience effects on biogenic amine receptor gene expression

From the recorded videos, we were able to determine the departure and return time of a tagged forager leaving the hive in 89.84% of all trips. This is considerably higher than in studies using RFID tags (Tenczar et al., 2014). In total, 1494 trips (trip ≥ 4 minutes duration) performed by 199 marked bees were observed in the three observation colonies. The distribution of the number of trips performed per forager was highly right-skewed (Figure 6.3). This means that most bees performed only a small number of trips by the age of 21 days, while a few did many trips. The foraging trip duration increased with the age of the bees (LME, t=8.53, p<0.001; Figure 6.4). While the mean trip duration of 10-day old bees was 13.9±14.5 minutes, it increased to 44.7±39.8 minutes for the 22-day old bees. However, we found no significant relationships between the gene expression of biogenic amine receptors and our measures of cumulative foraging experience (Table 6.4).

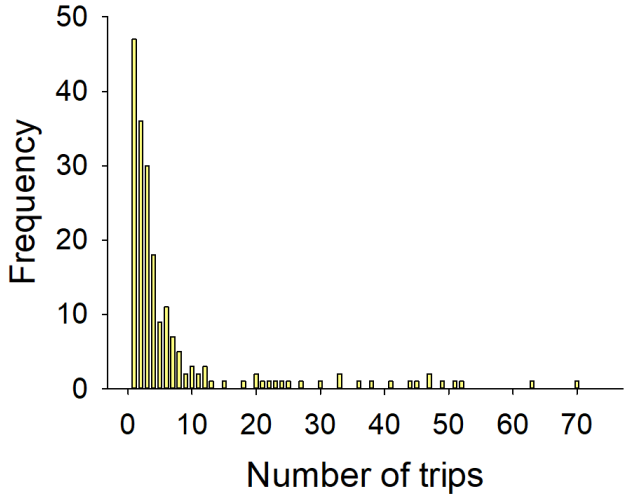

Figure 6.3: Frequency [%] of the number of performed trips.

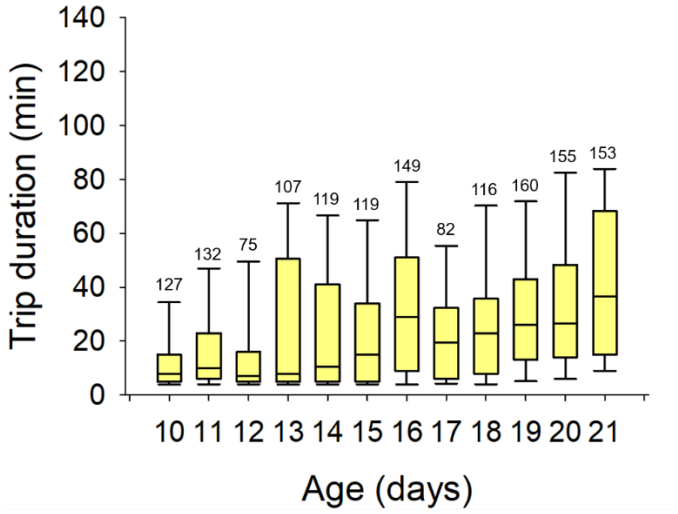

Figure 6.4: The effect of age in days on the trip duration in minutes. Boxplots show the medians, the 25%- and 75% quartiles. Numbers above bars indicate sample size.

Table 6.4: *P*- and *t*-values determined by linear mixed models for the relationships between the foraging parameters and the relative expression values of the biogenic amine receptor genes. N indicate the sample size.

Parameters	LME	*Amdop1*	*Amdop2*	*Amdop3*	*AmoctαR1*	*AmoctβR1*	*AmoctβR2*	*AmoctβR3/4*
Total number of foraging trips	*p*	0.57	0.2	0.19	0.71	0.82	0.17	0.099
	t	0.57	1.31	1.32	0.38	0.24	1.39	-1.69
	N	45	40	45	33	44	45	45
Total foraging time	*p*	0.42	0.51	0.29	0.57	0.84	0.22	0.14
	t	0.82	0.66	1.08	0.58	-0.2	1.23	-1.5
	N	45	40	45	33	44	45	45
Average trip duration	*p*	0.39	0.99	0.38	0.6	0.63	0.27	0.25
	t	0.87	-0.012	0.87	0.54	-0.48	1.11	-1.18
	N	45	40	45	33	44	45	45
Total foraging days	*p*	0.93	0.33	0.35	0.83	0.78	0.97	0.6
	t	0.09	0.99	0.94	0.21	-0.29	-0.041	-0.53
	N	45	40	45	33	44	45	45

Biogenic amine receptor gene expression and motivational state

We tested if biogenic amine receptor gene expression was linked to forager motivational state and training group in time-trained foragers. The expression of *Amdop1* was influenced by forager motivational state (LME, motivational state, LRT=25.25, p<0.0001; training group, LRT=1.31, p=0.25 Figure 6.5A). More specifically, *Amdop1* showed significantly higher mRNA levels during foraging than when bees were anticipating to forage or inactive (LME, foraging vs anticipation, z=2.69, p=0.014;

foraging vs inactive, z=-5.34, p<0.0001) and when anticipating compared to when bees were inactive (LME, anticipation vs inactive, z=-2.25, p=0.024).

For *AmoctβR3/4*, the gene expression differed among bees with different motivational state, whereas the training group showed no effect (LME, motivational state, *LRT*=9.17, p=0.010; training group, *LRT*=1.31, p=0.25 Figure 6.5G). The expression of *AmoctβR3/4* was higher in bees engaged in foraging compared to inactive bees (LME, foraging vs inactive, z=-2.96, p=0.0093). Bees in anticipation showed intermediate expression and did not differ significantly from either foraging or inactive foragers (LME, anticipation vs foraging, z=1.20, p=0.23; anticipation vs inactive, z=-1.59, p=0.22). The expression of *Amdop2*, *Amdop3*, *AmoctαR1*, *AmoctβR1* and *AmoctβR2* did not depend on either of the investigated factors (LME, *Amdop2*, motivational state, *LRT*=4.15, p=0.13; training group, *LRT*=1.03, p=0.31; *Amdop3*, motivational state, *LRT*=4.15, p=0.13; training group, *LRT*=0.22, p=0.64; *AmoctαR1*, motivational state, *LRT*=5.76, p=0.056; training group, *LRT*=0.40, p=0.53; *AmoctβR1*, motivational state, *LRT*=2.49, p=0.29; training group, *LRT*=0.041, p=0.84; *AmoctβR2*, motivational state, *LRT*=3.08, p=0.21; training group, *LRT*=1.05, p=0.31, Figure 6.5B-F)

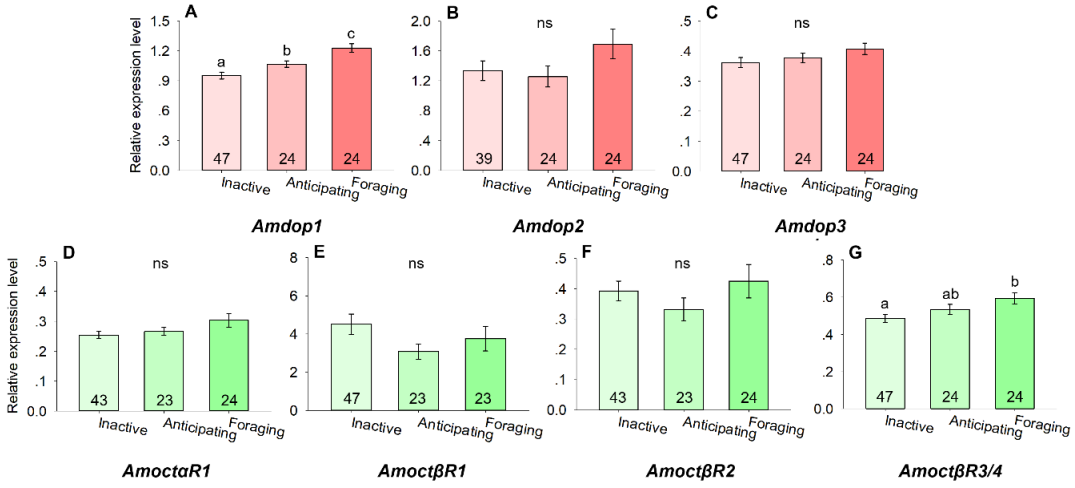

Figure 6.5: Biogenic amine receptor gene expression depends on foraging motivation (A–E) in the mushroom bodies. Bars show mean expression levels relative to the two reference genes (*GAPDH* and *eiF3-S8*) ± SE. Numbers in bars indicate sample size. Letters indicate significant differences between groups. Bars not sharing a letter differ significantly (p <0.05). Bars without letters mean no significant.

Discussion

Our findings suggest that forager age and motivational state are linked to octopaminergic and dopaminergic signalling in the mushroom bodies. In addition, the expression of some OA and DA

receptor genes depended on the time of day. Our measures of cumulative foraging experience, on the other hand, did not correlate with the expression of the receptors included in this study.

Forager age, but not cumulative foraging experience, predicts biogenic amine signalling

Foraging is a complex behaviour associated with physiological changes in the mushroom bodies that correlate with age and/or foraging experience (Farris et al., 2001; Withers et al., 1995; Maleszka et al., 2009). However, whether both of these factors affect gene expression after workers switched to foraging is not well known. We found that OA and DA receptor genes are more expressed in the mushroom bodies of old foragers. In line with our results, previous research has shown that expression levels of *Amdop2* in the Kenyon cells of the mushroom body calyces is higher in foragers than in newly-emerged cell cleaners (Humphries et al., 2003). Similar increases in the levels of expression of *Amdop1*, *Amdop2*, *Amdop3, AmoctαR1* were found when comparing the brain of pollen foragers to that of younger in-hive bees (<15 days old) (McQuillan et al., 2012). However, in the latter two studies, foragers were compared with non-foragers. Our results show that age continues to be associated with gene expression after workers have switched to foraging.

Since octopaminergic and dopaminergic signalling are important in aversive and appetitive learning in honey bees (Vergoz et al., 2007; Agarwal et al., 2011), forager age could correlate positively with learning performance. Ruepell et al. (2007), for example, found that older forager-aged bees tended to have a better learning performance than younger workers (Ray and Ferneyhough, 1997; Rueppell et al., 2007). In *Drosophila*, the orthologue of AmDOP1 and AmDOP2 have been shown to play a role in aversive learning (Kim et al., 2007; Selcho et al., 2009). This raises the possibility that *Amdop1*, *Amdop2*, *AmoctαR1* and *AmoctβR1* might mediate age-dependent learning performance in honey bees after the transition to foraging.

Bees usually start foraging after about two to three weeks after emergence (Seeley, 1982; Johnson, 2008), but earlier foraging can often be observed (Robinsion, 1992). In our study, hive excursions were observed in relatively young bees and they increased in duration with increasing age (Figure 6.3 and Figure 6.4). Some of the very early and short excursions may have been orientation flights (Becker, 1958; Degen et al., 2015). An increase in foraging trip duration could indicate that bees gained experience about where to find the most profitable food sources. It could also be that the foraging skills improved with age (Abou-Shaara, 2014) and an increase in foraging performance occurs as a result of learning (Schippers et al., 2006). On the other hand, extensive foraging experience (many days

of foraging or long foraging trips) has been found to correlate negatively with associative learning performance, regardless of age (Behrends et al., 2007; Tolfsen et al., 2011). Thus, we might have expected that the expression of receptor genes varied with cumulative foraging experience. Surprisingly, we found no relationship between biogenic amine receptor gene expression and different measures of cumulative foraging experience. It is possible that the foraging experience of our foragers (4 to 12 days of foraging) did not vary as much as in other studies that found effects of foraging experience, e.g. on learning performance (Scheiner and Amdam, 2009).

Taken together, our results suggest that the changes in biogenic amine receptor gene expression in the mushroom bodies of foragers are explained by age, rather than cumulative foraging experience. Future research should explore whether different measures of foraging experience are linked to receptor gene expression in other regions of the brain.

Foraging state influences biogenic amine receptor gene expression

We found that *Amdop1* expression was increased when bees were foraging compared to inactive time-trained bees. Gene expression levels were intermediate in anticipating bees. This is consistent with a study that used microarray analysis of whole-brain gene expression: *Amdop1* had a higher expression in anticipation bees than in inactive bees (Naeger et al., 2011). Additionally, in our study *AmoctβR3/4* expression was higher in foraging bees compared to inactive bees, but the function of this gene is still poorly understood. OA signalling might increase before foraging starts to upregulate the responsiveness to foraging-related stimuli (Cook et al., 2019; Barron et al., 2002). Possibly, *AmoctβR3/4* alters the motivational state in foragers. These findings indicate a potential association between biogenic amine receptor gene expression and behavioural plasticity in foragers.

In contrast, the expression of *Amdop2*, *Amdop3*, *AmoctαR1*, *AmoctβR1* and *AmoctβR2* were independent of foraging motivational state or time of training. Thus, these genes might be important for functions other than the regulation of foraging activity. For instance, knockdown of *Amdop2* mRNA expression has been found to affect the time honey bees spend grooming or walking (Mustard et al., 2010). It should be noted again that our study only used the mushroom bodies and the receptor genes could have different expression profiles in different neuroanatomical areas. Future studies could use RNA interference targeting specific brain areas to better understand the roles of biogenic amine receptor genes in regulating forager behaviour.

Daytime and biogenic amine signalling

Daytime is an important extrinsic factor in the life of a honey bee forager because it determines whether certain flower species produce pollen and nectar (Zhang et al., 2006; Lehmann et al., 2011). Honey bees keep track of the daytime by using a time-compensated sun compass, which allows them to visit food sources at the right time of day (von Frisch, 1967; Moore, 2001). So far, little is known about whether the time of day also affects biogenic amine receptor gene expression. In Experiment 1, we found that *Amdop1* showed significant down-regulation in the morning (~9:00) and at night (~22:00. Likewise, *Amdop3* showed significantly lower expression in the morning (~9:00), whereas *AmoctαR1* showed significant up-regulation in the morning. The expression of the other genes did not change during the day (Figure 6.2). Knockdown of *AmoctαR1* can cause impaired olfactory acquisition and recall (Farooqui et al., 2003; Rein et al., 2013). Thus, expression changes during the day could affect olfactory learning performance. Indeed, honey bees exhibit better olfactory learning performance in the morning compared to the afternoon (Lehmann et al., 2011). Also our finding that *Amdop3* is more expressed in the morning could indicate that it might improve the retrieval of appetitive memory in the morning, but this requires further testing. RNA interference (Farooqui et al., 2003; Rein et al., 2013; Farooqui et al., 2004) or null mutation of receptor genes (Kohno et al., 2016) could be used to explore if receptor gene expression in the mushroom bodies mediates olfactory learning performance.

Alternatively, daytime effects could be related to the sleep-wake cycles in foragers. Unlike nurses, foragers show rhythmicity by being active during the day and showing sleep-like behaviour at night (Kaiser and Steiner-Kaiser, 1983; Klein et al., 2008). In *Drosophila*, activating OA signalling has been suggested to cause a decrease in sleep (Crocker and Sehgal, 2008) and D1 dopamine receptor (DA1) mediates the arousal effect of dopamine in *Drosophila* (Andretic et al., 2005; Kume et al., 2005; Ueno et al., 2012). Therefore, our findings raise the possibility that the differential expression of *AmoctαR1* and *Amdop1* in the morning is related to the transition from sleep-like states to a more active state.

Queen mandibular pheromone (QMP) has been shown to affect the olfactory system of bees and influence brain gene expression (Slessor et al., 2005; Grozinger et al., 2003). For example, bees have lower *Amdop1* transcript levels and lower activity levels following QMP-treatment compared to controls (Beggs et al., 2007). The finding that *Amdop1* was down-regulated at night could be explained by foragers being more exposed to queen pheromone than in the morning and during the day. In

summary, our results reveal complex links between forager state and biogenic amine signalling in the mushroom bodies. They also highlight that more research is needed to understand if and how biogenic amine receptor expression is linked to extrinsic and intrinsic factors.

CHAPTER 7

Waggle dance decoding in honey bees is linked to gene expression patterns in the antennae rather than in the brain

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Preparing:

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Abstract

Communication is essential for social animals. Deciding how to utilize the information provided by conspecifics depends on environmental and intrinsic factors. Honey bees *Apis mellifera* use a unique form of communication, the waggle dance, to inform nestmates about both distance and direction of a food source in relation to the sun. However, as in many other animals, experienced individuals often ignore social information and prefer to rely on private information. Why individuals differ in their reliance on social information is not well understood. Here we test whether the decision to use social waggle dance information or private information is linked to gene expression differences in different parts of the nervous system. We trained workers to 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 that are critical for cognition, the subesophageal ganglion, the central brain, the mushroom bodies, and the antennal lobes, but detected no differences between social or private information users. Strikingly, we found 413 differentially expressed genes in the antennae, suggesting that variation in sensory perception affects the decision to decode waggle dance information. Social information users were characterized by the upregulation of dopamine and serotonin genes while private information users overexpressed odorant binding genes. These results highlight that decision making in social insects can also depend on peripheral processes of perception rather than higher-order brain centres of information integration.

Introduction

Exchanging information is essential in all animal societies. Communicating resources, reproductive state, group membership, and threats are vital in ensuring the survival and success of the group. However, relying on communication is often not the only available option, e.g. to find a food source, and searching for a resource individually can often be the better choice (Dechaume-Moncharmont et al., 2005; I'Anson Price et al., 2019). Furthermore, an individual can rely on private information (or spatial memory) about previously visited food source locations (Rendell et al., 2010; Grüter and 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 inefficient or erroneous transmission of information (Dechaume-Moncharmont et al., 2005). Thus, animals often employ flexible strategies for deciding between social or private information (Laland, 2004; Grüter and Leadbeater, 2014).

Social insects employ various methods to send signals to nestmates. Communication regarding resource exploitation is particularly well-studied and a wide range of communication behaviours 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 (Czaczkes et al., 2015). Honey bees (Apini) use a unique form of communication, the waggle dance that gives vector information to nestmates about both distance and direction of a food source or a nest site in relation to the sun (von Frisch, 1967). Dances are performed by returning foragers as advertisement for high quality food sources. Additionally, waggle dancers emit floral odours and a blend of hydrocarbons that provide additional information and stimulate foraging in unemployed foragers (Gilley et al., 2012; Thom et al., 2007; Farina et al., 2012). Only a small percentage of waggle dances are used to discover new food sources, while the majority of dances triggers experienced foragers to resume foraging at already familiar food sources, disregarding social dance information for private spatial information (Biesmeijer and Seeley, 2005; Grüter et al., 2008). While various factors, like experience (Richter and Waddington, 1993; Biesmeijer and Seeley, 2005; Grüter and Ratnieks, 2011) and age (Tofilski, 2009; Woyciechowski and 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 and Plath, 2017).

Social insect behaviour and responses to social information are linked to brain gene expression (Toth et al., 2010; Robinson et al., 2008; Zayed and Robinson, 2012; Ingram et al., 2011; Toth and Robinson, 2009). Behavioural variation within foragers seems to be strongly connected to the expression of genes that are important in biogenic amine signallings, such as dopamine, octopamine, tyramine, glutamate, and serotonin signalling (Liang et al., 2012; Scheiner et al., 2002; Schulz et al., 2003; Barron et al., 2002; Scheiner et al., 2017). Indeed, manipulation of biogenic amine levels can alter foraging behaviour (Liang et al., 2012) and perception of quality food sources (Barron et al., 2002; Scheiner et al., 2002). Most studies have focused on whole brains to discover expression differences between behavioural groups (e.g. Whitfield et al., 2003; Liang et al., 2012; Alleman et al., 2019). However, different brain parts serve specific functions. For example, the antennal lobes receive input from the olfactory sensory neurons in the antennae (Zacharuk, 1980) and process olfactory information (Homberg et al., 1989). The mushroom bodies in insects are a key brain tissue for learning and memory (Strausfeld et al., 2009). Foraging behaviour is also supported by the central brain primarily for motor control (Hanesch et al., 1989) and Barron and Plath (2017) have suggested that the central brain might play a crucial role in the decoding of waggle dance information. The subespohageal ganglion mediates reward and taste perception (Kreissl et al., 1994; Dacks et al., 2005; Sinakevitch et al., 2005).

If and how these different brain tissues are involved in dance communication and information-use is not well understood. Furthermore, the insect brain only controls a subset of functions and behaviours. Decision making and information processing also occur in the peripheral nervous system (e.g. Ozaki et al., 2005). The antennae, in particular, has important functions in social insect behaviour, both within and outside the colony, such as mediating pheromone signalling (Nagari and Bloch, 2012; Vergoz et al., 2009; Grozinger et al., 2003; Pankiw et al., 2004), nestmate recognition (Ozaki et al., 2005; van Zweden, 2010) and odour learning (Robertson et al., 2006; Rogers and Vallortigara, 2008). Also, 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 odour perception are not only important for task differentiation (Arenas and Farina, 2012), but could play a role in the decision between social and 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 feeders and then 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 olfactory tissues in both types of bees. We demonstrate that bees that decode waggle dance information differ in gene expression only in the antennae and provide evidence of a role of biogenic amine signalling and olfactory perception.

Materials and methods

Study species and field site

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). 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. Both feeders were 150m from the observation colonies with ca. 160 meters separating the training and dance feeder (Grüter et al., 2008). 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 odour: 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.

Sample Collection

On the test day, the day after the 60-minute odour training, 1.5M 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 was recorded. Dance and dance following behaviour was recorded in the observation colony using a high definition camera to quantify dance following behaviour 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 behaviours 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 (Al Toufailia et al., 2013), which is the component of the waggle dance that encodes the vector information (von Frisch, 1967).

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 subesophegeal 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). The additional handling of the samples caused some of the antennae to break apart and different workers were used to ensure equal RNA extraction.

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, including mushroom body peduncles, were removed by cutting off optic lobes, antennal lobes, and mushroom body calyx. All tissues called "mushroom body" refer to mushroom body calyces as it is extremely difficult to remove mushroom body peduncles and has been previously reported (Sarma et al., 2009; Reim and Scheiner, 2014; Humphries et al., 2003). Furthermore, all tissues called "central brain" refer to brain regions that also include 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 degradation of RNA. 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

In order to sequence and obtain reads for the approximate 15,000 genes present in the honey bee genome, aliquots of RNA from private and social information users were sent to Beijing Genomics Institute (BGI) for library construction. In 2016, Illumina 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, 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 (Crozier and Crozier, 1993). 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 tissue 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 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. This allows to account for colony effects. Genes were considered differentially expressed if the 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 in the antennae tissue, we additionally performed permutations by switching samples from opposite treatment groups while maintaining colony structure (Libbrecht et al., 2016). For example, a sample from the same colony was switched for a different treatment group and the number and distribution of DEGs was compared to those calculated from our model in *DESeq2*. We performed 28 permutations 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.

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 was performed in R v.3.5.0 (R Developmental Core Team, 2019).

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 and 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 sampled SI and PI bees

The SI bees captured in 2016 followed 5.0 ± 3.1 dances and 7.71 ± 1.7 waggle runs per dance before being captured, whereas PI bees followed 8.43 ± 6.1 dances and 4.43 ± 0.79 waggle runs per dance. The number of dances followed did not differ between both types of bees (Mann-Whitney *U* test: W = 32, p = 0.32), but SI bees followed more waggle runs per dance (W = 0, p = 0.002). In 2018, SI bees

captured followed 2.08 ± 1.78 dances and 11.0 ± 3.29 waggle runs per dance before being captured. PI bees followed 2.0 ± 1.26 dances and 8.5 ± 2.64 waggle runs per dance. The number of dances (Mann-Whitney *U* test: W = 17, p = 0.38) and waggle runs (Mann-Whitney *U* test: W = 14, p = 0.21) followed between each group did not differ between both types of bees.

Gene Expression Analysis

The likelihood ratio test (LRT) comparison of information use strategies revealed no differential gene expression in the tissues containing the central brain, antennal lobes, and subesophageal ganglion. There was only one differentially expressed gene present in the mushroom body calyces, 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 we swapped some of the PI and SI bee samples 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) when 2-3 samples were swapped between the SI and PI groups. 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 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 (Figure 7.1), the other tissues showed no clear clustering according to information use.

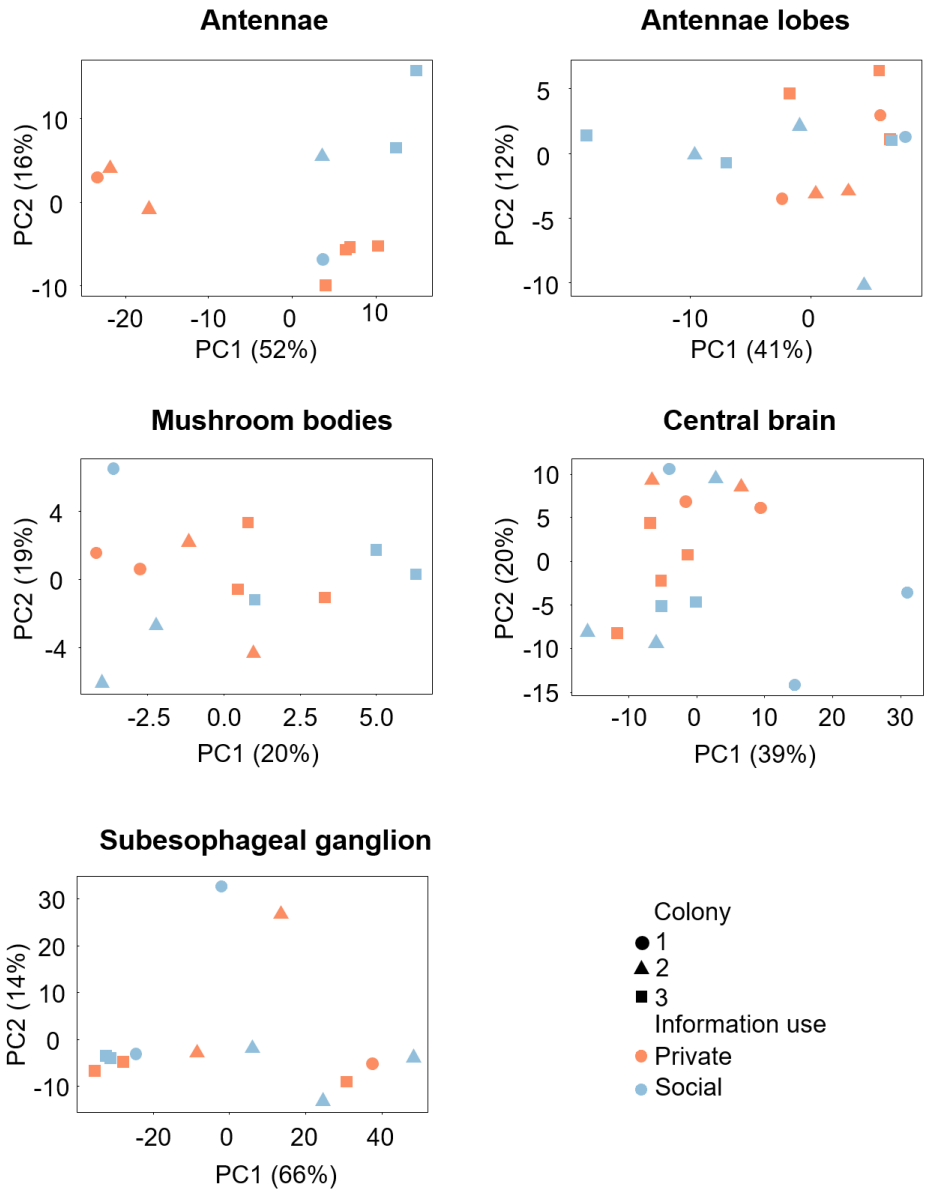

Figure 1.1: Principal Component Analysis (PCA) plots displaying variance between individual samples based on all genes for each tissue type. Samples are organized by color according to information use strategy (blue = social or red = private) and shapes by colony ID (circles = colony 1, triangles = colony 2, squares = colony 3). All genes displayed were unable to have colony ID factored into the variance, colony ID is used to identify sample origins.

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 5,11,19,71 and chemosensory protein 1*) and two among the upregulated genes in social information users (*odorant binding protein 7 and chemosensory protein 2*) (Figure 7.2). Several genes involved in biogenic amine production or signalling were also differentially

expressed. Social information users had a higher expression of *tyrosine-protein kinase Dnt; tyrosine hydroxylase, 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 only had a higher expression of one gene *tyramine receptor, transcript variant X1*, which is associated with biogenic amine signalling (Mustard et al., 2005; Blenau et al., 2000) (Figure 7.3). 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 (Amdam et al., 2003).

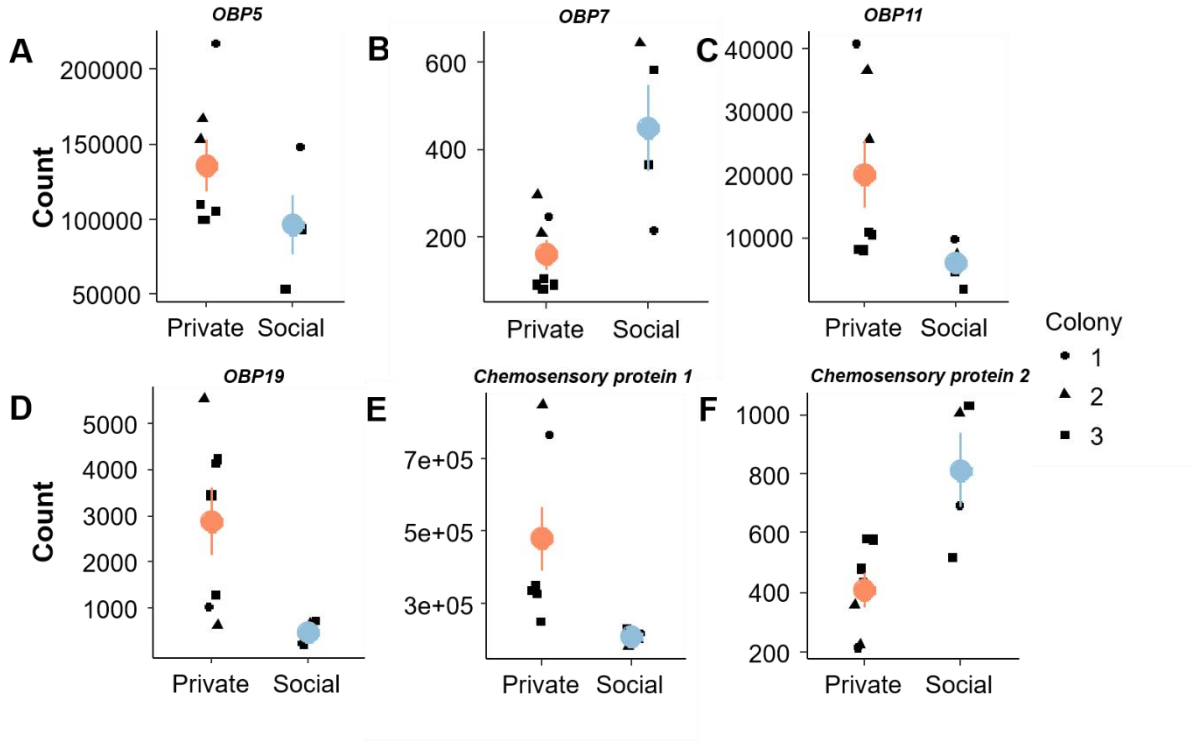

Figure 7.2: Plots of individual odorant binding protein and chemosensory protein genes. Black dots are representative of counts for individual samples and shapes correspond to the colony ID (circle = colony1, triangle = colony 2, square = colony 3). Colored circles are representative of the average for the respective information strategy (red = private information user, blue = social information user) with confidence intervals. A) OBP5 ($p=0.03$), B) OBP7 ($p<0.001$), C) OBP11 ($p<0.001$), D) OBP19 ($p=0.001$), E) Chemosensory protein 1 ($p=0.009$), F) Chemosensory protein 2 ($p=0.007$)

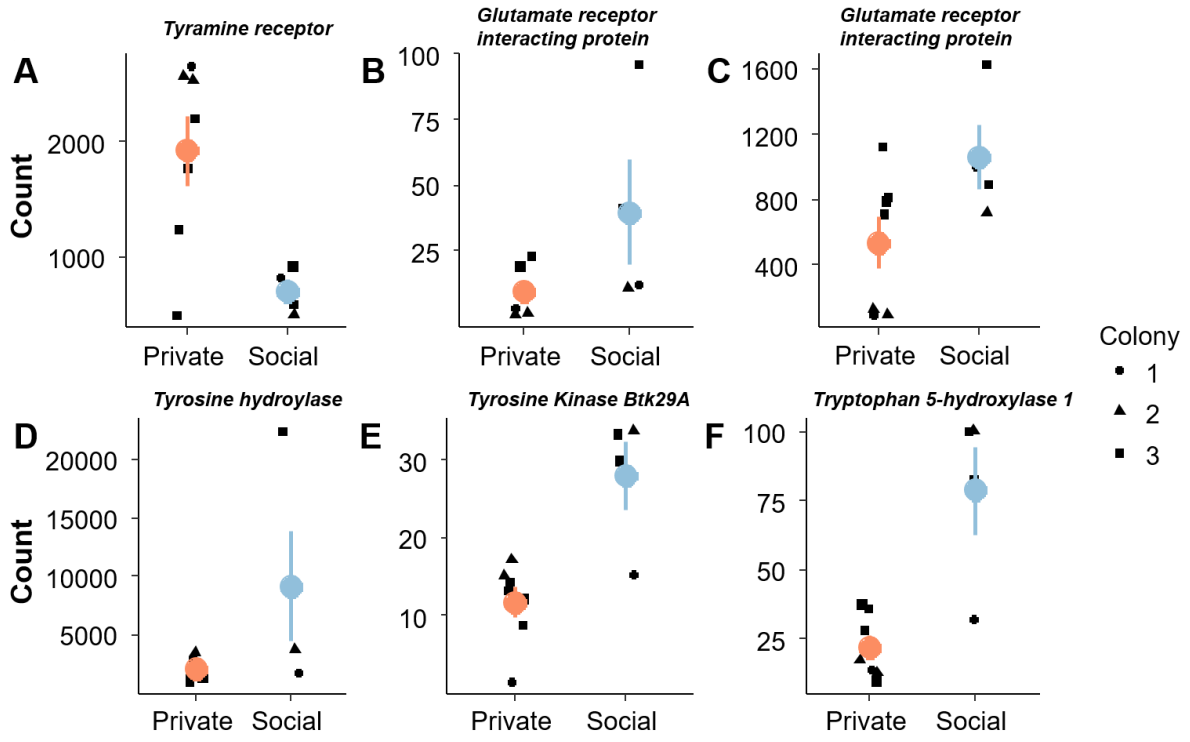

Figure 7.3: Plots of individual genes associated with biogenic amine production. Black dots are representative of counts for individual samples and shapes correspond to the colony ID (circle = colony1, triangle = colony 2, square = colony 3). Colored circles are representative of the average for the respective information strategy (red = private information user, blue = social information user) with confidence intervals. A) Tyramine receptor (p=0.018), B) Glutamate receptor interacting protein (p<0.001), C) Glutamate receptor interacting protein (p=0.006), D) Tyrosine hydroxylase (p=0.01), E) Tyrosine Kinase Btk29A (p=0.006), F) 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 focused mainly on *carbohydrate (10) 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

Previous studies have shown that honey bee foragers often rely on private information, but switch to social information when private information is unrewarding (Grüter and Ratnieks, 2011) or more costly (Wray et al., 2012). However, little is known about the neurobiological mechanisms that explain why some bees use dance information, while others ignore it. Here we explore gene expression differences between foragers that use social or private information to uncover the potential molecular mechanisms

that underlie the decision to decode waggle dances in honey bees. The transcriptomes of all four analyzed brain parts did not differ between bees using these two foraging strategies. Strikingly, however, we found substantial 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 brain gene expression between scouts and non-scout foragers. Furthermore, we expected the mushroom bodies to show differences since it has previously been shown that they are involved in odour and associative learning and place memory (Strausfeld et al., 2009). The antennal lobes are involved in odour recognition and memory through the interconnectivity of neurons with the mushroom body and were thus selected as another tissue of interest (Boeckh and Tolbert, 1993). The central brain has been suggested as an important tissue for dance communication (Barron and Plath, 2017), while the subesophageal ganglion has been shown to be involved in reward perception and taste (Galizia et al., 2011). Reward perception could play an important role as treatment with octopamine and dopamine, biogenic amines that signal rewards, affects dance following and information use (Linn et al., in press). Our study indicates that information use strategies may not depend strongly on integration of information in higher order centres, 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 signalling. Thus, differences in the perception of chemosensory information cues and signals could result in divergent foraging strategies. While our study cannot disentangle whether the gene expression is the cause of the information use strategy, they suggest that chemosensory perception bye the antennal 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 nestmate recognition (Ozaki et al., 2005; Sharma et al., 2015). Studies in *Oecophylla smaragdina* indicated that the density of antennal sensilla was important in regulating behaviour, particularly in determining the aggression response behaviour to non-nestmates (Gill et al., 2013; Cholé et al., 2019). Similarly, foraging behaviour requires integration and interpretation of chemical signals for navigation, efficient

nectar/pollen collection, and dance communication (Gilley et al., 2012; Thom et al., 2007).

Odorant binding proteins (OBPs) are essential for all insect communication, including in the honey bee due to the highly complex odours and pheromones that are exchanged among nestmates (Pelosi et al., 2005). Of the 21 OBPs found in the honey bee, only 9 are exclusively expressed in the antennae. The remaining OBPs are distributed throughout the honey body or specific non-olfactory tissues (Forêt and Maleszka, 2006). It was previously believed that honey bees lacked the chemosensory/olfactory discrimination capacities of other insects such as *Drosophila melanogaster, Anopheles gambiae,* and *Tribolium castaneum* that have at least twice the number of OBPs (Forêt and Maleszka, 2006). Our analysis revealed that workers which rely on spatial memory show higher expression of four odorant binding proteins (*obp5*, *obp11*, *obp19*, and *obp71*), whereas workers which rely on socially acquired information upregulate one (*obp7*). Thus, ~25% of all OBPs 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. The remaining differentially expressed OBPs have been shown to be expressed in other tissues as well, which suggests other functions for these proteins. 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 is consistent with the role that odours play in triggering the use of private information in honey bees (von Frisch, 1967; Johnson, 1967; Reinhard et al., 2004; Grüter et al., 2008; Gilley et al., 2012).

Chemosensory proteins serve a similar a role as OBPs in transporting chemical stimuli through poorly understood mechanisms. These proteins are heavily concentrated in antennal sensilla but also expressed in non-olfactory tissues (Forêt et al., 2007; Pelosi et al., 2005). Of the six chemosensory proteins found in honey bees (McKenzie et al., 2014), two were differentially expressed between 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) and support that the differences between the information strategies may be rooted in chemoreception.

Biogenic amines are the best-known neuromodulators for both vertebrates and invertebrates alike, and biogenic amine signalling is known to change with age and tissue location in honey bees (McQuillan et al., 2012). Their broad distribution throughout the brain and other tissues enables the modification of the effects of hormones and neurotransmitters resulting in various nervous system

responses to stimuli (Vergoz and Oldroyd, 2012). Specifically, dopamine, serotonin, and octopamine titers in the brain were found to be linked to both task and age (Schulz and Robinson, 1999; Barron et al., 2002; Harris and Woodring, 1992; Kokay and Mercer, 1997; Cook et al., 2018). The antennae of social information users showed a higher expression of several genes associated with dopamine and serotonin production. Dopamine has been shown to modulate sucrose responsiveness (Scheiner et al., 2002) and learning (Vergoz et al., 2007), whereas serotonin influences foraging behaviour (Schulz et al., 2003) and regulates feeding in honey bees (French et al., 2014). While we did not control for foraging age or experience, the upregulation of biogenic amine production in social information users suggests that foragers engaging in social information use as a strategy could be due to a different sucrose response threshold. The lack of differential expression in brain tissues suggests that there was no systematic age bias in this study since age is known to affect gene expression in the brain (Reim and Scheiner, 2014).

It is still not well understood why bees from the same colony vary so much in gene expression. Some external and environmental differences such as foraging experience or perceived predation risk could be a key factor affecting the use of social dance information (Grüter and Ratnieks, 2011; Grüter and Czaczkes, 2019; Nieh, 2010). Here we do not control for individual foraging experience before or after training times. Workers collected and trained to the T.F locations have been foraging for unknown time periods. It is possible that the cohorts trained to the T.F had an overrepresentation of experienced or unexperienced foragers. For example, workers are randomly collected and trained to a feeder location, if there are many experienced or unexperienced foragers present this could affect the SI and PI collected on testing day and consequently the observed gene expression patterns.

Colony dynamics during development and early life also affect gene expression. Changes in queen mandibular pheromone (QMP) exposure during periods of queen loss and requeening could result in workers that have different expression patterns that affect foraging preferences, learning, and possibly social information use (Grozinger et al., 2003; Pankiw et al., 1998; Grozinger and Robinson, 2007; Beggs et al., 2007; and Pankiw and Page, 2003). We created observation colonies from larger colonies, workers that were of foraging age during the training time could have experienced periods of lower levels of QMP when they were younger. Training occurred during the later summer months and early fall (August-early October). This time period is after the typical swarming season, i.e. the process when a colony splits into two distinct colonies, with the old queen leaving with a portion of the workers before

the new virgin queen emerges (Seeley, 2010). Swarming creates a vulnerable time for both the new and original colonies. For example, the workers in the swarm only have provisions of nectar and honey stored in their stomachs, if a new nest site is not quickly founded with a good nectar source the swarming colony will starve. The original colony is usually well provisioned with food, but experiences an interruption in the brood cycle. The new queen can be lost or eaten while on her mating flight or poor weather conditions could prevent the mating flight altogether. These short periods of changing colony dynamics could have long-term effects on the gene expression of individual workers from the same colony.

Intrinsic factors such as genetic differences could also affect the motivation to decode waggle dances. For example, it is well-known that different patrilines can impact foraging behaviours, such as foraging age (Kolmes et al., 1989). Paternal effects can also impact gustatory responsiveness and learning abilities (Scheiner and Arnold, 2009). Individuals of the same colony can differ strongly in their memory and learning performance, even individuals sharing the same patriline. The mechanisms involved in learning and memory are in part driven by genetics and these mechanisms partially underlie the sucrose responsiveness where bees that have a higher sucrose response learn better than those that have a lower sucrose response (Scheiner et al., 1999, 2001, 2005; Behrends et al., 2007).

Overall, our results suggest an important role of the antennae in mediating decision-making. In particular, we suggest a link between chemosensory perception and the reliance on communication in honey bees. Further studies are needed to disentangle if gene expression differences observed are due to genetic differences (i.e different patrilines), differences in foraging experience, or minor differences in age to understand gene expression differences between SI and PI users.

General Discussion

Tianfei Peng

“Diligent bees have no time to sorrow.”

— William Blake

The evolution of sociality has enabled ant, wasp, bee, and termite species to become dominant and abundant organisms in many terrestrial ecosystems. Eusociality has arisen about 8 times in hymenopteran insects alone and 5 times in bees. Highly eusocial bees are found in the family Apidae and are represented by two groups, the stingless bees and honey bees. Almost all of these species use pollen and nectar from flowering plants as their main food sources. One major factor affecting foraging behaviour and the dynamics of information sharing in bee colonies is the sugar content of carbohydrate sources, which in turn allows colonies to preferentially forage on plant species offering highly concentrated nectar. The majority of research in eusocial bees focuses on the honey bees (Apini), which contain approx. 10 recognized species and a single predominant recruitment mechanism during foraging, the waggle dance (Bradbear, 2009; von Frisch, 1967). Stingless bees represent a unique opportunity to study and understand the adaptive value of behaviour comparatively due to them having more than 500 species that rely on a wide range of communication mechanisms (Rasmussen and Cameron, 2010).

Current research on stingless bees can help broaden the knowledge and understanding of foraging behaviours across social bee species. This thesis contributes to this endeavour and addresses: 1) the relationships between caffeine and reward perception in stingless bee, 2) communication mechanisms and the role of biogenic amines in stingless bee foraging, 3) provide additional evidence to support the role of octopamine (OA) and dopamine (DA) in the honey bee in the context of waggle dance following, information use strategies, and foraging task specialization and, finally, 4) explores the molecular mechanisms underlying foraging decision making in the honey bee.

Reward Perception and Biogenic Amines Modulate Foraging Behaviours in a Stingless Bee

Excitatory effects of caffeine have been shown in some insects, i.e. the increase of foraging activity, recruitment and learning in honey bees and foraging effort in bumblebees (Couvillon et al., 2015; Thomson et al., 2015). It has been suggested that plants might "cheat" bees into collecting average food sources by adding caffeine to nectar, consequently lowering the sucrose response threshold in honey bees (Couvillon et al., 2015; Singaravelan et al., 2005; Wright et al., 2013). However, previous studies used honey bee and bumblebee colonies in temperate areas as models, which may not naturally encounter plants that caffeinate their nectar. Exposure to caffeine over evolutionary time periods might allow pollinators to adapt to such potential exploitation. The Brazilian stingless bee *Plebeia droryana*

presents an opportunity to understand the relationship between caffeine and foraging effort due to the long history of exposure to caffeinated nectar and pollen as one of the main visitors of coffee in Brazil.

We used field-realistic doses of caffeine and found that more foragers were willing to visit feeders with higher concentrations of sucrose, but that caffeine did not affect individual or collective foraging efforts in *P. droryana*. Additionally, our experiment provided the first evidence that *P. droryana* foragers are able to communicate the location of high-quality food sources to nestmates. The lack of an effect of caffeine is in contrast with evidence from honey bees and bumblebees and is consistent with the hypothesis that *P. droryana* possesses mechanisms of tolerance towards caffeine, which might be the result of a coevolutionary process (**chapter 1**).

Lindauer and Kerr (1958, 1960) found no evidence for location specific communication in *P. droryana*. The two studies (**chapter 1**) differ in that Lindauer and Kerr (1958, 1960) performed trials with only one colony and used a relatively large foraging distance (150 m) for such a small bee, whereas in chapter 1 we studied five colonies and used a nearby food source (10 m). Since recruitment probability is influenced by food source distance (Nieh et al., 2003a, 2004; Stangler et al., 2009), it is possible that foragers were not motivated to recruit in Lindauer and Kerr (1958, 1960). To study this further, we used 8 wild colonies to test potential communication strategies and found that foragers provide directional information, but not distance information, to their nestmates (**chapter 2**). Moreover, recruits did not use visual or chemical cues at food sources. Most likely, *P. droryana* foragers deposit pheromone trails in a winding pattern when returning to the nest as was found in *Scaptotrigona postica* (Lindauer and Kerr, 1958, 1960). An alternative explanation is that *P. droryana* foragers might combine several mechanisms like pheromone trails, visual guidance and an "aerial odour tunnel" created by the recruiting forager to recruit nestmates (Aguilar et al., 2005; Lindauer and Kerr, 1958, 1960). Even a less accurate recruitment strategy in *P. droryana* is probably still more efficient than a random search by alarmed nestmates and is similar to *Plebeia tica* (Aguilar et al., 2005), which raises the possibility that the communication mechanism is conserved in the *Plebeia* genus.

In honey bees, biogenic amines are important for regulating reward-seeking behaviours. In the stingless bee *Melipona scutellaris*, OA increases sucrose responsiveness in a dose dependent manner (Mc Cabe et al., 2017). Thus, octopaminergic signalling is a potential candidate for the organization of complex behaviours in highly eusocial bees. We tested the role of OA on individual and collective foraging in wild colonies of *P. droryana*. We found that OA treatment strongly increases individual

foraging rate and the total number of foragers at artificial food sources. We suggest that this is the result of OA lowering the sucrose response threshold in treated foragers. This mirrors the situation in honey bees where oral OA treatment has a positive effect on waggle dancing, which leads to increasing recruitment to food sources (Barron et al., 2007). Thus, our findings provide evidence that OA has a common function across in these two groups of eusocial bees, even though stingless bees and honey bees have evolved independently for ~80 million years and developed many different foraging strategies (Cardinal and Danforth, 2013; Martins et al., 2014) (**chapter 3**).

Reward Perception and Biogenic Amines Modulate Foraging Behaviours in Honey Bee

In honey bees, the regulation of the division of labour between pollen and nectar foragers is linked to differences in reward perception. Thus, the neurophysiological factors that underlie reward perception could explain why some bees collect nectar while others collect pollen. We investigated whether OA administration influences foraging preference, specifically the probability of switching from nectar to pollen collection. We found that OA administration modified the individual foraging preference by increasing the probability of foragers switching from nectar to pollen collection. Second, OA administration increased the ratio of incoming pollen and non-pollen foragers at the colony level (**chapter 4**).

In this study, we also compared the expression of OA receptor genes in the mushroom bodies between pollen and nectar foragers and discovered that the expression level of *AmoctαR1* was higher in foragers arriving at pollen feeders compared with those arriving at nectar feeders. When we controlled the foragers' age, then the different expression of the *AmoctαR1* disappeared. However, those two experiments were in the different contexts, also *AmoctαR1* seems to be linked to social task rather than the age and given that older bees have higher mRNA expression of OA receptors than younger bees (McQuillan et al., 2012; Reim and Scheiner, 2014), we deem it is unlikely that the upregulation of *AmoctαR1* in pollen foragers were linked to age differences (**chapter 4**).

Previous research suggests that, in honey bee foragers, the perception of rewards is likely to play an important role in the use of different strategies to find food. For example, foragers that experienced higher quality rewards in the past use private information more often (Al Toufailia et al., 2013). This suggests that neurophysiological mechanisms of reward perception play an important role

in the decision to use private information vs waggle dance information. As we mentioned before, OA affects reward perception and increases dancing in honey bee foragers. DA, on the other hand, reduces the response to sucrose rewards and conditioned olfactory stimuli (Mercer et al., 1982; Perry and Barron, 2013; Scheiner et al., 2002). We trained foragers to collect sucrose solution with or without OA and DA and observed if this affected interest in dance information about a new food source and the use of private information. OA treated foragers followed fewer dances and used private information more than control bees that only received sucrose solution. A possible explanation is that OA increased the reward perception of foragers collecting food at the feeder during the treatment period and, thereby, lowered their interest to switch to other food sources. Moreover, OA has been shown to affect the social interactions in ants (Boulay et al., 2000). Thus, high levels of OA brain titres may be associated with an increased motivation to engage in trophallaxis that is an important mechanism of social learning in ants and honey bees (Farina and Grüter, 2009; Provecho and Josens, 2009). Therefore, OA-treatment might also have reduced dance following by lowering the motivation of foragers to interact with hivemates. DA treated foragers followed dances longer than control bees, but did not use social information more than control bees. The effects of DA on honey bee information use is perplexing, but could be explained by the diverse and complex roles DA play in the insect brain. For example, in fruit flies, DA signalling is related to both aversive and reward learning and is suspected to signal the nutritive value of a reward, while OA signals sweetness (Burke et al., 2012; Søvik et al., 2015). Overall, our results confirm that OA and DA signalling in the bee brain affect the use of social information provided by dancers and, thus, information flow in the honey bee colony (**chapter 5**).

In **chapter 6**, we explored if honey bee forager age, experience, motivation and the time of day are correlated with biogenic amine signalling. As expected, the expression of the receptor genes *Amdop1*, *Amdop2*, *AmoctαR1* and *AmoctβR1* depended on forager age. Since octopaminergic and dopaminergic signalling are important in aversive and appetitive learning in honey bees (Vergoz et al., 2007; Agarwal et al., 2011), forager age could correlate positively with learning performance (Ray and Ferneyhough, 1997; Rueppell et al., 2007). This raises the possibility that *Amdop1, Amdop2*, *AmoctαR1* and *AmoctβR1* might mediate age-dependent learning performance in honey bees after the transition to foraging. Surprisingly, we did not find a relationship between biogenic amine receptor gene expression and cumulative foraging experience. It is possible that the foraging experience of our foragers (4 to 12 days of foraging) did not vary as much as in other studies that found effects of foraging experience, e.g. on

learning performance (Scheiner and Amdam, 2009). Moreover, short-term foraging experience did affect OA and DA receptor expression: *Amdop1* and *AmoctβR3/4* expression was significantly increased in foragers that were collecting food compared to those that were inactive. Bees that expected to forage soon (anticipating bees) already upregulated *Amdop1* and *AmoctβR3/4* before the onset of foraging, suggesting an increase in "readiness" to forage.

The expression of *Amdop1*, *Amdop3* and *AmoctαR1* also varied with the time of day, which points to a role of biogenic amine signalling plays a role in regulating circadian activities in foragers. For example, *AmoctαR1* has a lower expression in the morning, which could affect olfactory learning performance (Farooqui et al., 2003; Rein et al., 2013). Indeed, honey bees exhibit better olfactory learning performance in the morning compared to the afternoon (Lehmann et al., 2011). *Amdop3* is more expressed in the morning, which could indicate that it might improve the retrieval of appetitive memory in the morning, but this requires further testing. Furthermore, the differential expression of *AmoctαR1* and *Amdop1* in the morning could be related to the transition from sleep-like states to a more active state (Crocker and Sehgal, 2008; Andretic et al., 2005; Kume et al., 2005; Ueno et al., 2012). Finally, the lower *Amdop1* expression during the night could be explained by foragers being more exposed to queen mandibular pheromone (QMP) than in the morning and during the day. QMP is known to affect brain gene expression such as DA receptor genes (Beggs et al., 2007) (**chapter 6**).

As mentioned before, experienced foragers can choose to pursue private information about food source locations or follow instructions provided by dancers (Grüter and Ratnieks, 2011; Wray et al., 2012; Grüter et al., 2013; Grüter and Leadbeater, 2014). **Chapter 5** discovered that OA and DA signalling play important roles in mediation of the interest in dance information. In **chapter 7,** we broadened our focus to look into the gene expression patterns of social and private information using bees to identify genes that are associated with how honey bees use dance communication.

We found no differential gene expression in the central brain, the antennal lobes, or the subesophageal ganglia. There was one differentially expressed gene present in the mushroom bodies which was uncharacterized. Strikingly, there were 413 differentially expressed genes in the antennae, 318 were upregulated in social information users and 95 were upregulated in private information users. Among the differentially expressed genes were several genes related to odorant or chemical perception and biogenic amine signalling. This provides evidence that differences in information use strategies are not centred in the brain, but instead that they may depend on the chemosensory perception in the antenna.

Odorant binding proteins are essential for all insect communication, especially in the honey bee due to the highly complex odours and pheromones that are readily exchanged among the members of a colony (Pelosi et al., 2005). Thus, these results suggest a difference in perceptional sensitivity where workers which use private information are able to perceive some olfactory stimuli better than social information users. This would fit with the role that odours play in triggering the use of private information in honey bees (von Frisch, 1967; Johnson, 1967; Reinhard et al., 2004; Grüter et al., 2008; Gilley et al., 2012). In general, chemosensory proteins and odourant binding proteins share similar functions although they belong to different protein classes (Vieira and Rozas, 2011), however the proteins in our study (*CSP1, CSP2, OBP5, OBP11, OBP19 and OBP71*) have been shown to be mainly expressed in olfactory tissues (i.e the antenna and head) (Li et al., 2016). However, some are expressed to a lesser extent in non-olfactory tissues such as the thorax, leg and wing (Forêt et al., 2007). Thus, the differences between the information use strategies may be also rooted in early developmental differences. Moreover, the antenna of social information users showed a higher expression of several genes associated with dopamine production, which is consistent with the data of **chapter 5** that dopamine influences foragers information using strategies.

Outlook

We have contributed new data that help elucidate how biogenic signalling and reward perception are linked to the foraging behaviours of eusocial bees. However, based on our research, new questions have emerged that need further exploration. In **chapter 1**, we demonstrate a lack of a response to caffeine in a stingless bee. However, the location of our experiment was unusual and might not be representative for the distribution range of *P. droryana* as a species, since data were collected on a former coffee farm with many coffee plants still present. Thus, as a next step we could address the several questions: how do different stingless bee species or populations of the same species respond to caffeine (or nicotine, biogenic amines) in different areas and do different groups of bees show different physiological responses to secondary plant compounds? In **chapter 2**, we demonstrated that a higher concentration of sucrose solution indeed elicited recruitment behaviour in *P. droryana*. But we still do not know what the actual mechanism is that underlies *P. droryana* recruitment. Furthermore, do foragers perform nest-based recruitment behaviours which could explain the patterns that we found? In **chapter 3**, we followed the question how *P. droryana* foragers respond to biogenic amines. We only used the OA in

our experiments. Other biogenic amines, such as DA, should be studied as well.

In **chapter 4**, we found that OA signalling was involved in the decision to collect nectar or pollen. DA is an important biogenic amine that has been found to reduce the response to sucrose rewards and conditioned olfactory stimuli in honey bee (Mercer and Menzel, 1982; Perry and Barron, 2013; Scheiner et al., 2002). Thus, DA signalling could also influence division of labour between pollen and nectar foragers. This assumption should be tested in the future. **Chapter 5** explored how OA and DA affect honey bee information use strategy and **chapter 7** discovered the molecular correlates of social information use. We still have a limited understanding of how biogenic amines affect the use of different types of information. However, the results of these two chapters can offer insights into the molecular basis of how biogenic amines might influence social information use. Several studies have started to use RNA-sequencing to elucidate how the gene expression changing is associated with the pharmaceutical treatment (Wu et al., 2017; Kucharski and Maleszka, 2005). Using RNA-seq technology can help us understand how exogenous biogenic amines and individual experience affect information use via changes in gene expression. In **chapter 6**, we found that OA and DA receptor gene expression depended on the forager age, the motivational state of foragers and the time of day. Here, we only focused on the mRNA expression level, whereas biogenic amines directly act on receptors. Thus, future studies should analyse the protein level of the OA and DA receptors in the different conditions. Moreover, the knockdown of individual OA and DA receptor genes would be valuable to highlight and further dissect the roles of each receptor in modulating honey bee foraging behaviours.

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