Regulation of the nurse-forager transition in honeybees (*Apis mellifera*)

Regulation des Ammen–Sammlerinnen-Übergangs
in Honigbienen (*Apis mellifera*)

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Summary

Honeybees are among the few animals that rely on eusociality to survive. While the task of queen and drones is only reproduction, all other tasks are accomplished by sterile female worker bees. Different tasks are mostly divided by worker bees of different ages (temporal polyethism). Young honeybees perform tasks inside the hive like cleaning and nursing. Older honeybees work at the periphery of the nest and fulfill tasks like guarding the hive entrance. The oldest honeybees eventually leave the hive to forage for resources until they die. However, uncontrollable circumstances might force the colony to adapt or perish. For example, the introduced *Varroa destructor* mite or the deformed wing virus might erase a lot of in-hive bees. On the other hand, environmental events might kill a lot of foragers, leaving the colony with no new food intake. Therefore, adaptability of task allocation must be a priority for a honeybee colony.

In my dissertation, I employed a wide range of behavioral, molecular biological and analytical techniques to unravel the underlying molecular and physiological mechanisms of the honeybee division of labor, especially in conjunction with honeybee malnourishment. The genes *AmOARα1*, *AmTAR1*, *Amfor* and *vitellogenin* have long been implied to be important for the transition from in-hive tasks to foraging. I have studied in detail expression of all of these genes during the transition from nursing to foraging to understand how their expression patterns change during this important phase of life. My focus lay on gene expression in the honeybee brain and fat body. I found an increase in the *AmOARα1* and the *Amforα* mRNA expression with the transition from in-hive tasks to foraging and a decrease in expression of the other genes in both tissues. Interestingly, I found the opposite pattern of the *AmOARα1* and *AmTAR1* mRNA expression in the honeybee fat body during orientation flights. Furthermore, I closely observed juvenile hormone titers and triglyceride levels during this crucial time. Juvenile hormone titers increased with the transition from in-hive tasks to foraging and triglyceride levels decreased.

Furthermore, in-hive bees and foragers also differ on a behavioral and physiological level. For example, foragers are more responsive towards light and sucrose. I proposed that modulation via biogenic amines, especially via octopamine and tyramine, can increase or decrease the responsiveness of honeybees. For that purpose, in-hive bees and foragers were injected with both biogenic amines and the receptor response was quantified

using electroretinography. In addition, I studied the behavioral response of the bees to light using a phototaxis assay. Injecting octopamine increased the receptor response and tyramine decreased it. Also, both groups of honeybees showed an increased phototactic response when injected with octopamine and a decreased response when injected with tyramine, independent of locomotion.

Additionally, nutrition has long been implied to be a driver for division of labor. Undernourished honeybees are known to speed up their transition to foragers, possibly to cope with the missing resources. Furthermore, larval undernourishment has also been implied to speed up the transition from in-hive bees to foragers, due to increasing levels of juvenile hormone titers in adult honeybees after larval starvation. Therefore, I reared honeybees *in-vitro* to compare the hatched adult bees of starved and overfed larvae to bees reared under the standard *in-vitro* rearing diet. However, first I had to investigate whether the *in-vitro* rearing method affects adult honeybees.

I showed effects of *in-vitro* rearing on behavior, with *in-vitro* reared honeybees foraging earlier and for a shorter time than hive reared honeybees. Yet, nursing behavior was unaffected.

Afterwards, I investigated the effects of different larval diets on adult honeybee workers. I found no effects of malnourishment on behavioral or physiological factors besides a difference in weight. Honeybee weight increased with increasing amounts of larval food, but the effect seemed to vanish after a week.

These results show the complexity and adaptability of the honeybee division of labor. They show the importance of the biogenic amines octopamine and tyramine and of the corresponding receptors AmOARα1 and AmTAR1 in modulating the transition from in-hive bees to foragers. Furthermore, they show that *in-vitro* rearing has no effects on nursing behavior, but that it speeds up the transition from nursing to foraging, showing strong similarities to effects of larval pollen undernourishment. However, larval malnourishment showed almost no effects on honeybee task allocation or physiology. It seems that larval malnourishment can be easily compensated during the early lifetime of adult honeybees.

Zusammenfassung

Honigbienen gehören zu den wenigen Spezies, die in eusozialen Gemeinschaften leben. Die eierlegende Königin und die männlichen Drohnen dienen nur der Fortpflanzung. Alle anderen Arbeiten von den sterilen Arbeiterinnen ausgeführt werden. Die Arbeitsteilung wird meistens anhand des Alters der Bienen organisiert. Junge Arbeiterinnen bleiben im Inneren der Kolonie und führen beispielsweise Putzarbeiten und Ammentätigkeiten aus. Mit zunehmendem Alter verlagern sich ihre Tätigkeiten immer mehr in Richtung des Nestausgangs wo sie, unteranderem als Wächterbienen, den Stockeingang bewachen. Die ältesten Honigbienen verlassen das Nest, um Honig, Pollen, Wasser oder Propolis zu sammeln, bis sie am Ende sterben.

Allerdings können unvorhersehbare Ereignisse dazu führen, dass sich die Kolonie anpassen muss, um nicht unterzugehen. Krankheiten wie der Flügeldeformationsvirus oder die, durch den Menschen eingeführte, *Varroa destructor* Milbe können auf einen Schlag eine große Zahl an Bienen auslöschen. Des Weiteren können beispielsweise starke Unwetter dafür sorgen, dass etliche Sammlerinnen auf ihrem Sammelflug sterben und die Kolonie ohne neuen Nektar oder Pollen zurückgelassen wird. Es liegt auf der Hand, dass eine starre Arbeitsverteilung nicht ausreicht, um solchen Umständen entgegenzuwirken und, dass eine gewisse Flexibilität notwendig ist.

In meiner Dissertation habe ich eine weitreichende Anzahl an verhaltensbiologischen und molekularbiologischen Techniken verwendet, um die molekularen und physiologischen Mechanismen der Arbeitsteilung bei Honigbienen aufzuklären, vor allem im Bezug auf den Übergang von Ammenbienen zu Sammlerinnen.

Es ist seit langer Zeit bekannt, dass die Gene *AmOARα1*, *AmTAR1*, *Amfor* und *Vitellogenin* beim Übergang von Ammenbienen zu Sammlerinnen von zentraler Bedeutung sind. Deshalb habe ich die Expression dieser Gene, sowohl im Gehirn als auch im Fettkörper, in genau diesem Zusammenhang betrachtet und die unterschiedlichen Veränderungen der Expressionsmuster während dieser wichtigen Phase im Leben einer Honigbiene analysiert.

Ich konnte zeigen, dass sowohl die mRNA Expression des *AmOARα1* und des *Amforα* beim Übergang von Ammenbienen zu Sammlerinnen anstieg, während die Expression der anderen Kandidatengene im gleichen Zeitraum sowohl im Gehirn als auch im Fettkörper abfiel. Interessanterweise zeigten die Expressionsmuster des *AmOARα1* und des *Am-*

TAR1, während der Orientierungsflüge, genau in die entgegengesetzte Richtung. Zusätzlich habe ich mir bei denselben Bienen auch den Juvenilhormongehalt in der Hämolymphe und die Menge an Triglyceriden im Fettkörper angeschaut. Der Juvenilhormongehalt nahm schlagartig zu, als die Bienen mit dem Sammeln begannen. Die Menge an Triglyceriden nahm allerdings von Ammenbienen, über Bienen während des Orientierungsfluges zu Sammlerinnen konstant ab.

Des Weiteren war bereits bekannt, dass sich Ammenbienen und Sammlerinnen nicht nur auf genetischer, sondern auch auf verhaltensbiologischer und physiologischer Ebene voneinander unterscheiden. Zum Beispiel sind Sammlerinnen empfindlicher für Licht und Saccharose. Ich stellte die Hypothese auf, dass die Empfindlichkeit von Honigbienen für solche Schwellen durch biogene Amine, insbesondere Oktopamin und Tyramin, moduliert werden kann. Oktopamin sollte die Empfindlichkeit von Bienen erhöhen, wohingegen Tyramin diese verringern sollte.

Hierfür injizierte ich Stockbienen und Sammlerinnen beide biogenen Amine und analysierte die Rezeptorantwort mit einem Elektroretinogramm (ERG) und die Lichtempfindlichkeit in einer Phototaxisarena.

Oktopamininjektion führte dazu, dass die Rezeptorantwort im ERG erhöht wurde und dass beide Gruppen eine erhöhte Lichtempfindlichkeit aufwiesen. Tyramin hatte in beiden Experimenten genau den gegenteiligen Effekt.

Allerdings kann der Ammen-Sammlerinnen-Übergang nicht nur durch biogene Amine moduliert werden, auch die Ernährung hat einen großen Einfluss. Zum Beispiel fangen unterernährte Honigbienen eher an zu sammeln als satte Honigbienen. Des Weiteren sollte auch die larvale Unterernährung bereits einen Einfluss auf die spätere Arbeitsteilung haben, da man bei Arbeiterinnen, die im Larvenstadium bereits unterernährt waren, eine erhöhte Menge an Juvenilhormon festgestellt hatte. Dies sieht man auch beim Übergang von Ammenbienen zu Sammlerinnen.

Deshalb nutzte ich eine Methode zur artifiziellen Aufzucht von Honigbienen, um die Standarddiät, die diese normalerweise erhalten, zu variieren. Allerdings musste ich zuerst den Effekt der *in-vitro* Aufzucht auf im Stock aufgezogene Honigbienen untersuchen.

Ich konnte zeigen, dass die artifizielle Aufzucht das Sammelverhalten erwachsener Honigbienen signifikant beeinflusste, während das Ammenverhalten der *in-vitro* aufgezogenen Bienen nicht beeinflusst wurde. Artifiziell aufgezogene Honigbienen begannen, im Vergleich zu normalen Bienen, früher zu sammeln und sammelten für eine kürzere Zeit.

Danach zog ich unterernährte, normal ernährte und überfütterte Honigbienen *in-vitro*

auf. Ich fand Unterschiede im Gewicht zwischen den Behandlungsgruppen. Unterernährte Bienen waren die leichtesten und überfütterte Bienen wogen am meisten. Dieser Unterschied verschwand aber über die Zeit. Des Weiteren konnte ich keinen Einfluss der Ernährung auf das Ammenverhalten oder das Sammelverhalten zeigen.

Dieser Ergebnisse zeigen sowohl die Komplexität als auch das Anpassungsvermögen der Arbeitsteilung von Honigbienen. Sie zeigen, dass sowohl die beiden biogenen Amine Oktopamin und Tyramin, als auch die dazugehörigen Rezeptoren AmOARα1 und AmTAR1 bei der Modulation des Ammen-Sammlerinnen-Übergangs eine große Rolle spielen. Des Weiteren zeigen die Ergebnisse des Vergleichs von artifiziell und im Stock aufgezogenen Bienen, starke Gemeinsamkeiten zu einer larvalen Unterernährung mit Pollen. Jedoch scheint eine allgemeine larvale Unterernährung kaum einen Effekt auf den Ammen-Sammlerinnen-Übergang zu haben. Diese scheint während der ersten Lebenstage von Honigbienen relativ leicht kompensiert werden zu können.

1

General Introduction

1.1   Division of labor in honeybees

Honeybees (*Apis mellifera*) are eusocial insects that live inside highly complex colonies (Seeley, 1995). Eusociality is defined by division of labor into reproductive and non-reproductive groups and individuals living inside a single colony with overlapping generations of adults (Crespi and Yanega, 1995). Honeybee colonies consist of combs hanging vertically with two layers of horizontal cells (Seeley, 1995). Theses hexagonal cells are built in two sizes using wax. The larger hexagonal cells are used to rear drones while the smaller cells are used to rear worker bees. Queens are reared in specially constructed cup-like large queen cells. Both worker brood cells and drone brood cells are used to store resources like water, nectar, and pollen. Resources are generally stored schematically. The inner most layers of a comb are filled with brood, while honey or nectar is stored in the outer layers. Pollen is usually stored in a small ring between brood and honey (Seeley, 1995). Division of labor in honeybee colonies is twofold. Reproduction is taken care of by the queen and drones, while all of the other tasks are accomplished by sterile female workers (Winston, 1991). In spring, the queen lays thousands of diploid eggs from which female worker bees hatch. Male drones only hatch between May and July. They usually hatch about a month before virgin queens hatch and their only function

is to mate with a queen (Winston, 1991). Young drones are fed by nurse bees, while older drones tend to feed themselves from honey reserves. When drones reach sexual maturity, they undertake a few orientation flights until they leave the hive and aggregate at specific locations where they may mate with a virgin queen. After mating, the drones die. The queen hatches about a month after the drones hatched. About six days later, the virgin queen leaves the hive and flies to the congregation areas. After mating with up to 17 drones (Tarpy and Page, 2000), the queen returns to the hive. However, if her spermathecae is not filled completely, she leaves for up to four more mating flights until she starts laying eggs (Woyke, 1964).

Worker bees hatch from diploid eggs where the embryo consumes mostly egg yolk. After about three days, the larvae hatch and are fed by nurse bees with a mixture of honey, pollen and royal jelly produced in their hypopharyngeal glands (Seeley, 1995). Larval feeding stops after approximately five days when worker bees construct a wax cap that seals the larva in its cell. Afterwards, the larva transforms into a prepupa and then into a pupa until it eventually becomes an adult honeybee. The newly formed honeybee then gnaws through the wax and emerges into the hive as an adult worker bee.

A honeybee fulfills multiple tasks during her lifetime, mostly regulated by age (temporal polyethism). While physical appearance is fixed after emergence, glandular systems develop and degrade in correlation with the task currently executed (Seeley, 1995), e.g. hypopharyngeal glands are highly enlarged in young honeybees that usually perform nursing tasks, while they are degraded in old honeybees that already foraged (Crailsheim and Stolberg, 1989). Interestingly, Huang and Robinson (1996) showed that hypopharyngeal glands increase again if foragers revert to nurse bees, uncoupling hypopharyngeal glands´ development from age.

During the first few days, a newly emerged honeybee functions as a cleaning bee, cleaning brood cells recently vacated (Seeley, 1995). After approximately eight days, it functions as a nurse bee performing multiple tasks in conjunction with the brood nest like taking care of the larvae or the queen (Winston, 1991). After about twelve days, it will become a storage bee, processing incoming nectar in addition to pollen and storing them in the appropriate cells. Usually after 23 days, it will leave the hive to forage for resources like nectar and pollen until it eventually dies (see Figure 1 for details).

While the general sequence of tasks is relatively fixed, individual effort expended in any tasks varies quite vastly. One worker might never perform a certain task while others specialize in a single task for several days. For example, some honeybees might ventilate
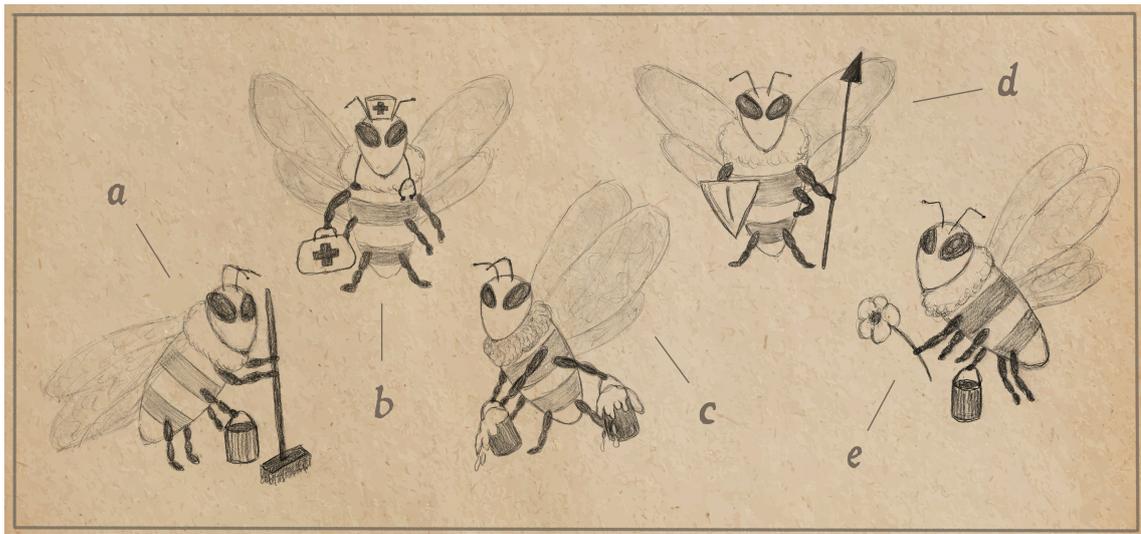
Figure 1: **Temporal polyethism of honeybee task performance** adapted from Winston (1991). a) During the first few days after emergence, a honeybee functions as a cleaning bee, cleaning recently vacated brood cells. b) After about eight days of age, it functions as a nurse bee taking care of the larvae and the queen, capping brood cells and feeding nest mates. c) At around twelve days of age, a honeybee functions as a storage bee, producing honey, packing pollen, grooming nest mates, and constructing cells. d) After approximately 20 days of age, a honeybee functions as a guard bee, protecting and ventilating the hive. e) At the end of its life, at around 23 days of age, it leaves the hive to forage for nectar, pollen, propolis or water.

the hive for several days but never guard the hive entrance while others might do the opposite (Seeley, 1995). This phenomenon can be explained by the widely accepted response threshold model (Robinson, 1992; Beshers et al., 1999; Barron and Robinson, 2008; Robinson and Page, 2019). This model sates that individual responses to a task-related stimulus differ between bees due to genetic variation. To stay within the same example as above, if some honeybees prefer cooler temperatures than others, they will start fanning earlier than their sisters. These different response thresholds are thought to be the results of genetic variability due to different fathers (Page and Robinson, 1991). Additionally, other factors are supposed to influence these response thresholds to adapt to changing conditions, like a loss of foragers or different temperatures (Seeley, 1995). Interestingly, studies could support the response threshold model experimentally (Scheiner et al., 2014, 2017b; Thamm and Scheiner, 2014). For example, nurse bees are known to have a lower sucrose responsiveness (Scheiner et al., 2017b) and a lower responsiveness to light (Thamm and Scheiner, 2014) than foragers. Furthermore, pollen
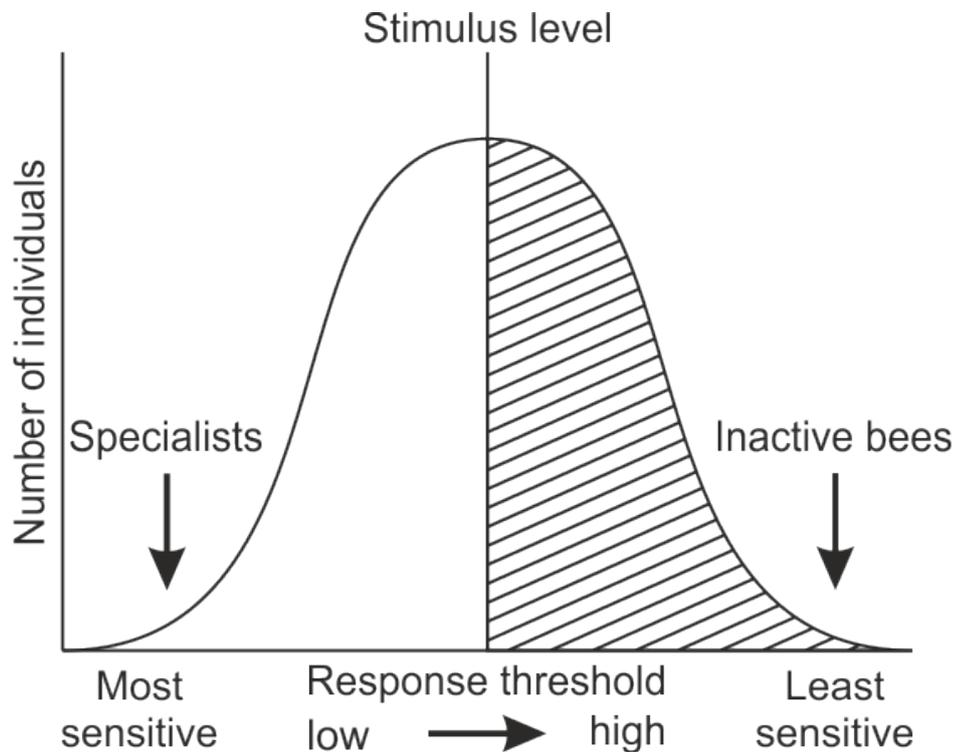
Figure 2: **Response threshold model** adapted from Barron and Robinson (2008). This graph shows a hypothetical distribution of the variation in response thresholds to task-related stimuli. Individuals with a response threshold higher than the stimulus level (shaded area) will not engage in the current associated task. Individuals with an extremely high response threshold ("Inactive bees") will rarely perform the task. Individuals with an extremely low threshold ("Specialists") will most likely perform the task.

foragers have been shown to have a higher responsiveness to light than nectar foragers (Scheiner et al., 2014).

1.2   Mechanisms of the division of labor

1.2.1   The role of juvenile hormone and vitellogenin (in the division of labor)

Juvenile hormone (JH) has long been considered one of the most important factors involved in division of labor (Robinson, 1987; Elekonich et al., 2001; Bloch et al., 2002). Initially, this hypothesis was based on the observation that JH levels increase with age in

the honeybee, similar to the age-dependent change in tasks (Rutz et al., 1976; Hagenguth and Rembold, 1978; Fluri et al., 1982; Robinson et al., 1987). Interestingly, of the seven naturally occurring JH isoforms (Riddiford, 1994), JH III is the only isoform occurring in honeybees (Hagenguth and Rembold, 1978) and even other Hymenoptera (Nijhout, 1975). Initially, Robinson (1985) showed that treatment of honeybees with the JH analog methoprene leads to a shift of worker honeybees from brood nest behaviors to peripheral behaviors, connecting JH and division of labor. Furthermore, Sasagawa (1986) showed afterwards that treatment with methoprene induces workers to forage prior to their usual foraging age, so-called precocious foragers. Additionally, they showed that the *corpora allata*, the site of JH synthesis, increases in bees when they transition from in-hive tasks to foraging, while JH also increases with age (Huang et al., 1994). Furthermore, treating honeybees topically with methoprene speeds up the major tasks involved in division of labor (Robinson, 1987), while the treatment also degrades the hypopharyngeal glands (Brouwers, 1983).

All these findings led to the hypothesis that juvenile hormone might be *the* trigger or one of the triggers involved in the transition from nursing to foraging. However, JH would need to increase prior to the onset of foraging to support this hypothesis. Elekonich et al. (2001) conducted a study to investigate whether JH levels increase with the nurse-forager transition. While they found that nurse bees show low levels of JH, they could not find differences in JH levels between bees on their first or later foraging flights, indicating that JH should increase prior to foraging. However, concrete evidence has not been produced so far.

It has rather been speculated that JH might rather be involved in the timing of division of labor than the direct initiation (Sullivan et al., 2000). They showed that even when they removed the *corpora allata*, honeybees still began to forage, albeit later. This effect was reversible via methoprene treatment.

As ambiguous as is the concrete function of JH so is its mode of action. A study by Amdam and Omholt (2003) proposed the double repressor hypothesis to explain the function of JH in the nurse-forager transition. They state that JH production is repressed by an external repressor and by vitellogenin (vg) which, in turn, is repressed by secretion of JH. Vitellogenin is a common yolk precursor protein of oviparous animals (Brandt et al., 2005). However, it seems to have evolved pleiotropic functions in the eusocial honeybee like protection against oxidative stress (Seehuus et al., 2006) or possible involvement in division of labor (Amdam et al., 2003; Nelson et al., 2007). Vitellogenin generally

decreases with honeybee age (Fluri et al., 1982). Knocking down *vg* through RNA interference (RNAi) can lead to precocious foraging (Nelson et al., 2007; Marco Antonio et al., 2008), thus dissociating age and task.

While both JH and vg seem to play important roles in the honeybee division of labor, multiple other factors have been implied to be involved. Some of them will be discussed below.

1.2.2 The role of nutrition (in the division of labor)

Unlike bumblebees, honeybees collect resources not only to reproduce and feed the colony for one season but store nectar to survive the winter and to start into the next season without founding a new colony (Doeke et al., 2015). Therefore, a colony should be able to regulate food intake in times of dearth. As has been shown by Schulz et al. (1998), honeybee colonies starved via honey restriction increased the number of foragers compared to colonies fed well, which led to the hypothesis that division of labor can be modulated by changing the nutritional state of a colony. The first step in proving this hypothesis was to observe differences in lipid levels between nurse bees and foragers. It has been shown that abdominal lipid stores differ between nurse bees and foragers (Toth and Robinson, 2005). Nurse bees have significantly more triglycerides (TGs) than foragers. This effect was also observed in precocious foragers and foragers that reverted to nursing tasks, so-called reverted nurse bees, which shows that adult maturation can be dissociated from age in honeybees.

Later, Toth et al. (2005) showed that feeding honeybees with TOFA (5-tetradecyloxy-2-furanocarboxylic acid) while at the same time restricting pollen consumption (as a way of synthesizing TGs), led to a decrease in abdominal lipid stores and an increase in foraging activity. This demonstrates a possible link between food reduction and increased foraging activity. Furthermore, researchers showed that newly emerged honeybees only feeding on sugar syrup displayed an up-regulated expression of two genes involved in the synthesis of juvenile hormone compared to honeybees feeding on beebread (Bomtorin et al., 2014). Interestingly, multiple studies (Frias et al., 2016; Wegener et al., 2018) found that pollen consumption leads to higher vg titers, linking high pollen consumption and high vg titers. According to the double repressor hypothesis discussed above (Section

1.2.1), JH synthesis is repressed via the internal repressor vg. Therefore, it seems possible that nutrition might influence the internal repressor and thus influence the division of labor.

The foraging gene (Amfor) has long been associated with the honeybee division of labor (Ben-Shahar, 2005) and nutrition (Thamm and Scheiner, 2014). It has been shown that foragers have a significantly higher *Amfor* expression than nurse bees (Ben-Shahar, 2005) and Thamm and Scheiner (2014) showed that activation of the putative cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG), the protein *Amfor* encodes for, increases sucrose responsiveness. However, in the desert locust *Schistocerca gregaria* Tobback et al. (2013) showed that starved locusts show higher expression levels of the *for* gene compared to normally fed individuals.

Recent studies went even further and connected larval starvation to precocious foraging. Scofield and Mattila (2015) showed that honeybees that were deprived of pollen as larvae became poorer foragers. Fewer honeybees became foragers and the ones that did forage started foraging sooner and for a shorter amount of time. Furthermore, starving honeybees during the fifth larval instar increased hemolymph JH titers in the hatched adult worker bees (Wang et al., 2016).

1.3 Modulation of the division of labor via biogenic amines

Biogenic amines have long been linked to division of labor (Wagener-Hulme et al., 1999). They can function as neurohormones, neurotransmitters and neuromodulators (Evans, 1980; Erber et al., 1993; Roeder, 1994, 1999; Blenau and Baumann, 2001; Scheiner et al., 2006). The biogenic amines serotonin, histamine and dopamine fulfill similar functions in both vertebrates and invertebrates (Scheiner et al., 2006). However, in invertebrates the biogenic amines octopamine (OA) and tyramine (TA) are speculated to act analogous to epinephrine and norepinephrine in vertebrates (Roeder, 2005). Both amines have been implied in the modulation of the honeybee division of labor (Barron et al., 2002; Hunt et al., 2007).

Octopamine is assumed to regulate the onset of foraging in conjunction with the juvenile hormone (Kaatz et al., 1994; Schulz et al., 2002a). Treating honeybees with methoprene increases OA and JH titers and induces precocious foraging (Schulz et al., 2002a).

Furthermore, removing the *corpora allata* (allectomized bees) and treating honeybees with OA increases the number of foragers compared to allectomized honeybees without OA treatment. However, the highest number of foragers was seen in colonies when allectomized honeybees were treated with methoprene and OA. These experiments suggest that OA acts more proximal than JH. A conclusion that has also been drawn from experiments by Kaatz et al. (1994) who showed that OA increases JH release from the *corpora allata*. A later study showed that the relative gene expression of the *Apis mellifera octopamine receptor α 1* (*AmOARα1*) is significantly higher in foragers compared to nurse bees (Reim and Scheiner, 2014), further emphasizing the importance of OA in division of labor.

Much less is known about tyramine. One of the earliest studies showed that while OA induces precocious foraging, TA induces the opposite effect (Schulz and Robinson, 2001). The scarcity of studies about TA is mainly due to its disregard as a precursor of OA with no function on its own (Roeder et al., 2003). However, this has changed in recent years (Lange, 2009). Hunt et al. (2007) investigated into quantitative trait loci in the honeybee. These loci are gene sections that correlate with quantitative phenotypic traits. They showed that the *Apis mellifera* tyramine receptor 1 (AmTAR1) is located on a quantitative trait locus linked to foraging behavior (Hunt et al., 2007). Furthermore, an interesting study showed that foragers have significantly higher TA brain titers and that TA increases the sucrose responsiveness of honeybees, which also increases as honeybees transition from nurse bees to foragers (Scheiner et al., 2017b).

1.3.1 Opposing actions of octopamine and tyramine

It has long been hypothesized that OA and TA act oppositely and thus behave similar to their vertebrate counterparts epinephrine and norepinephrine (Roeder et al., 2003; Roeder, 2005, 2020). Studies showed the opposing functions of both amines on a behavioral level (Saraswati et al., 2004; Fussnecker et al., 2006; Ma et al., 2015). For example, Fussnecker et al. (2006) showed that honeybees treated with OA showed an increase in flying behavior while honeybees treated with TA showed a decrease in flying behavior. Saraswati et al. (2004) showed in *Drosophila melanogaster* larvae that elevated TA and reduced OA levels lead to increased pause periods, while feeding OA

rescued the effect. However, both amines can also induce similar effects on behavior (Behrends and Scheiner, 2012; Scheiner et al., 2017b). Behrends and Scheiner (2012) showed that treating honeybees with OA increases sucrose responsiveness. In a later study, they showed that the same effect can be achieved when honeybees are treated with TA (Scheiner et al., 2017b).

It is hypothesized that these different modes of actions might be due to different receptors activated by OA and TA (Scheiner et al., 2017b). The biogenic amines OA and TA both activate G protein-coupled receptors in the honeybee. For OA, five receptor coding genes have been identified (Hauser et al., 2006), while only two are known for TA (Cazzamali et al., 2005). The AmOARα1 releases Ca^{2+} from intracellular stores (Grohmann et al., 2003). The *Apis mellifera* octopamine receptors β 1-4 activate the adenylyl cyclase and increase intracellular cAMP (Balfanz et al., 2014). Two tyramine receptors have been identified so far. The AmTAR1 inhibits the adenylyl cyclase and therefore decreases intracellular cAMP (Blenau et al., 2000). The function of the second *Apis mellifera* tyramine receptor (AmTAR2) has recently been described showing an increase in intracellular cAMP after AmTAR2 activation (Reim et al., 2017). Therefore, activating different sets of receptors could either lead to same or opposing effects depending on the tissue. However, it remains inconclusive, how OA and TA interact with one another on a behavioral and molecular level.

1.4 Link between nutrition and biogenic amines (in the division of labor)

As mentioned before (Section 1.2.2), nutrition seems to be a key regulator in division of labor. Starvation has been linked to an increase in octopamine titers in honeybees (Harris and Woodring, 1992). In *Drosophila melanogaster*, OA has shown to be crucial for starvation-induced foraging activity (Yang et al., 2015).

A good indicator for starvation in honeybees is the sucrose responsiveness, as starved honeybees show an increased responsiveness towards sucrose compared to satiated honeybees (Scheiner et al., 2003). Other studies showed that feeding an octopamine receptor antagonist reduces the sucrose responsiveness of honeybees (Buckemüller et al., 2017), while feeding OA increases sucrose responsiveness (Behrends and Scheiner, 2012). An increase in sucrose responsiveness also becomes apparent when nurse bees become for-

agers (Scheiner et al., 2017b).

Even less is known for tyramine. Scheiner et al. (2017a) showed an increase in sucrose responsiveness after treatment with TA in foragers and in another study in nurse bees (Scheiner et al., 2017b). Furthermore, a study by Wang et al. (2016) found that honeybee adults starved during the fifth larval instar show lower expression levels of the *AmTAR1*.

1.5   Thesis outline

The superorganism honeybee is a uniquely qualified model organism to study division of labor and the underlying molecular and physiological mechanisms. I investigated the transition from nurse bees to foragers in great detail, especially the involvement of the candidate genes connected to division of labor and to nutritional changes. During this doctoral thesis I combined behavioral field experiments, larval *in-vitro* rearing and molecular biological and analytical methods to conduct my experiments. In the following, I will outline the chapters of my thesis, which was aimed at revealing the mechanisms underlying the division of labor in honeybees and how nutrition might have an influence on it.

In **Chapter 2**, the main focus lay on the general molecular mechanisms underlying division of labor. Numerous factors were thought to be involved in the transition (Kaatz et al., 1994; Elekonich et al., 2001; Schulz et al., 2002b; Ben-Shahar, 2005; Toth and Robinson, 2005; Behrends and Scheiner, 2012; Scheiner et al., 2017a). To investigate these factors, I removed marked honeybees at different time points from the hive and studied the candidate genes (*AmOARα1*, *AmTAR1*, *vitellogenin*, *Amfor*), juvenile hormone and triglycerides during the transition from in-hive tasks to foraging, focusing on honeybees during orientation flights. I hypothesized that factors increasing or decreasing during the orientation flight period might be one of the main triggers for the division of labor in honeybees. Especially the gradual increase in *AmOARα1* expression from nurse bees to foragers led me to believe that it fulfills a pivotal role in pacing the nurse-forager transition in honeybees.

Afterwards I conducted larval *in-vitro* rearing experiments (**Chapters 3, 4**). I wanted to investigate, whether the increase in JH titers after larval starvation (Wang et al., 2016) affects the timing of division of labor.

Initially (**Chapter 3**), I studied the effects of *in-vitro* rearing on adult honeybee work-

ers. Contrary to my hypothesis, *in-vitro* rearing significantly affected honeybee workers. *In-vitro* reared honeybees started foraging earlier and foraged for a shorter time span compared to honeybees reared in a normal colony.

Afterwards (**Chapter 4**), I analyzed whether larval undernourishment and overfeeding influences the division of labor of *in-vitro* reared honeybee workers. I found no effect of malnourishment on adult workers besides difference in weight during early adulthood. I hypothesized that *in-vitro* rearing affects the nurse-forager transition similarly to pollen undernourishment during larval development, leading to stronger effects than the experiments I conducted in **Chapter 4**.

In **Chapter 5** I further investigated the effects of the two most promising biogenic amines involved in division of labor. I found opposing actions of octopamine and tyramine on the sensory input level and the behavioral output level of honeybees. This led me to believe that both biogenic amines modulate division of labor oppositely.

In **Chapter 6** (General Discussion) I will discuss in detail how the different factors studied in this thesis contributed to the regulation and modulation of division of labor in honeybees. Further, I will suggest important future studies to test new hypothesis derived from my findings.

2

Cause or effect? - Novel insight into the mechanisms of the nurse-forager transition in honeybees

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Abstract

Temporal polyethism in honeybees has long been a major focus in eusociality research. While external factors involved in division of labor have long been identified, the molecular mechanisms involved have remained a mystery. Here we investigated for the first time the complex relationship between candidate genes, juvenile hormone (JH) and triglycerides (TGs) at different time points during the nurse-forager transition. Marked newly emerged bees were inserted into a honeybee colony. At appropriate time points, we collected (1) nurse bees, (2) bees performing their first orientation flights, (3) young foragers and (4) old foragers from these cohorts. Juvenile hormone titers and TG levels were quantified. Messenger RNA expression was measured of our candidate genes *Apis mellifera octopamine receptor 1* (*AmOARα1*), *Apis mellifera tyramine receptor 1* (*AmTAR1*), the egg yolk precursor *vitellogenin* (*vg*), and the two splice variants of the *Apis mellifera foraging* gene (*Amforα* and *Amforβ*) in the honeybee brain and fat body. In addition, we treated honeybees topically with JH at two time points and quantified the same factors. We found an increase in JH and *AmOARα1* mRNA expression in the brain and fat body with the transition from in-hive bees to foragers. Triglycerides and *AmTAR1* mRNA expression showed an inverse pattern to that of JH and *AmOARα1* expression. *Amforα* mRNA expression generally increased, while *Amforβ* expression generally decreased with the transition from nurse bees to foragers in both tissues. Juvenile hormone treatment significantly increased JH levels in the hemolymph and decreased *AmTAR1* and *vg* expression in the fat body linking these factors directly to JH. We thus show for the first time that JH does not affect the *AmOARα1* mRNA expression in the fat body but actually suppresses the *AmTAR1* mRNA expression, providing further evidence that octopamine acts more proximal than JH. Additionally, we found clear expression patterns of our candidate genes during the transition from in-hive bees to foragers and possibly varying actions of JH in the brain and fat body. Furthermore, the results insinuate opposing actions of the *AmOARα1* and the *AmTAR1* receptor genes in the honeybee division of labor.

2.1 Introduction

Polyethism in honeybees has long been a focus of eusociality research (Robinson, 1987; Fahrbach and Robinson, 1996; Scheiner et al., 2004; Schmickl and Crailsheim, 2004; Ben-Shahar, 2005; Bloch, 2010; Johnson, 2010). While honeybee task allocation is known to be regulated by age, with young honeybees performing in-hive tasks and older honeybees working at the periphery and later outside the hive, multiple factors have been identified in the regulation of this temporal polyethism. Most of the research focuses on the transition from nurse bees to foragers, the most drastic change in the life of a honeybee. Juvenile hormone has been identified as an important factor in this transition (Robinson et al., 1987). Experiments revealed that removing the *corpora allata*, the organ of JH synthesis (Gade et al., 1997) leads to a delayed onset of foraging. This effect was reversed with methoprene, a JH analog (Sullivan et al., 2000). Interestingly, the age of the first orientation flight was not affected. Additionally, Huang et al. (1991) showed that JH titers in the hemolymph of honeybees increase with age, especially once honeybees have become foragers. Supposedly, JH represses the egg yolk protein vitellogenin (Amdam and Omholt, 2003) and *vg* gene expression is upregulated in nurse bees compared to foragers (Peso et al., 2016). Knockdown of *vg* expression via RNAi resulted in an earlier onset of foraging (Nelson et al., 2007; Marco Antonio et al., 2008) and an increase in JH hemolymph titers (Guidugli et al., 2005). However, JH alone cannot explain how the nurse-forager transition is regulated, especially because bees lacking the *corpora allata* still became foragers, albeit delayed (Schulz et al., 2002b). Another factor intertwined in this mechanism is octopamine (OA). Similar to other biogenic amines, OA can act as a neurohormone, neurotransmitter or neuromodulator (Scheiner et al., 2006). The relative expression of the *AmOARα1* is higher in foragers compared to nurse bees (Reim and Scheiner, 2014), even when investigating reverted worker bees, which are bees that already foraged and changed their tasks back to nursing. Furthermore, feeding honeybees with OA leads to a relatively higher proportion of foragers compared to controls (Schulz and Robinson, 2001). Octopamine most likely acts more proximally than JH but both lead to a higher percentage of foragers (Schulz et al., 2002b). However, these interactions show an incomplete picture. The Amfor coding for a cGMP dependent protein kinase (PKG) is also involved in the behavioral transition (Thamm and Scheiner, 2014; Thamm et al., 2018). *Amfor* mRNA expression increases from nurse bees to foragers (Ben-Shahar, 2005; Thamm and Scheiner, 2014), even when these are

forced to forage precociously (Ben-Shahar et al., 2002). These findings indicate a role of *Amfor* with task performance rather than an ageing effect. Treatment with cGMP, which activates PKG, can increase the proportion of foragers (Ben-Shahar et al., 2002) similar to OA. In addition, it increases responsiveness to light (Ben-Shahar et al., 2003) and responsiveness to sucrose (Thamm and Scheiner, 2014) just like octopamine (Behrends and Scheiner, 2012; Schilcher et al., 2021b). Furthermore, foragers are normally more responsive to both stimuli than nurse bees (Scheiner et al., 2017a,b; Schilcher et al., 2021b).

In a previous study, we showed that injection of OA increases the phototactic response of honeybees, while tyramine (TA) acts oppositely (Schilcher et al., 2021b). Octopamine also increased the relative proportion of foragers while TA decreased it (Schulz and Robinson, 2001), possibly by binding to the AmTAR1. The AmTAR1 is located on a quantitative trait locus related to foraging (Hunt et al., 2007). These experiments support the general hypothesis that OA and TA fulfill similar roles in invertebrates as adrenaline and noradrenalin do in vertebrates (Roeder, 2020). However, other experiments showed that TA can also act in the same direction as OA, e.g. it can increase responsiveness towards gustatory stimuli (Behrends and Scheiner, 2012; Scheiner et al., 2017a). An increase in gustatory responsiveness is often used as an indicator of the nurse-forager transition because nurse bees have a lower gustatory responsiveness than foragers (Scheiner et al., 2017b). Tyramine also improves the appetitive learning performance of honeybees, similar to octopamine (Behrends and Scheiner, 2012; Scheiner et al., 2017a) and foragers typically display a better learning performance than nurse bees (Scheiner et al., 2017b).

A reduction in triglycerides is often used as a further indicator of the transition from nursing to foraging, because lipid levels typically decrease from nurse bees to foragers, independent of age (Toth and Robinson, 2005). An artificial reduction in TG levels can further increase the number of foragers (Toth et al., 2005).

In this study, we investigated a number of highly important molecular and hormonal factors (JH and TGs , relative gene expression of the *AmOARα1*, *AmTAR1*, *Amfor* and *vg*) during the nurse-forager transition and treated a second group of bees with JH to resolve their temporal and causal relationships and to unravel their function in one of the most fascinating behavioral transitions of social insects.

2.2 Material and Methods

2.2.1 Animals

Honeybees (*Apis mellifera carnica*) for the orientation flight experiments were reared at the departmental apiary of the University of Würzburg, Germany. Honeybee brood combs with larvae about to emerge were placed in an incubator maintained at 35 °C. The next day all honeybees that had emerged over night were color-marked and placed into a nuke colony inside a mesh net tent on the grounds of the University. A pollen source of daily ground pollen and a sugar water source (50 % sucrose) were placed inside the tent. The colony was left undisturbed for three days, so that the newly inserted honeybees had enough time to be accepted by the hive bees.

We collected honeybees from four different time points during adult maturation. We collected nurse bees, honeybees during their orientation flights, young pollen foragers and old pollen foragers. Nurse bees were collected by opening the hive and removing honeybees with their heads in brood cells using spring steel tweezers and by placing them in snap lid jars. These bees were only collected in the afternoon after orientation flight observations. To collect bees performing their orientation flights, honeybees were observed every day until they left the hive for the first time. Bees returning from their orientation flights were collected using snap lid jars (Capaldi and Dyer, 1999). Foraging honeybees, which were roughly three weeks of age, were identified once they had landed on the pollen source and started to collect pollen. We collected young pollen foragers as soon as the marked bees started foraging on pollen and older pollen foragers about one week later. After sampling, the honeybees were transferred into the lab and we measured levels of juvenile hormone and triglycerides and relative expression of our candidate genes in the brain and fat body as described below.

For the topical JH experiments, honeybee brood combs with larvae about to emerge were placed in an incubator maintained at 35 °C. The next day all honeybees that had emerged over night were trapped in a plastic tub. The sides of the tub were rubbed with petroleum jelly to prevent the honeybees from climbing out.

2.2.2   Juvenile hormone analysis

Honeybees were immobilized on ice and fixed with needles onto a Styrofoam plate. Then, the cuticle was pierced in between the fourth and fifth abdominal segments using glass micro capillaries (servoprax®, A1 0115; servoprax GmbH; Germany). We extracted 5 µL of hemolymph, flash froze it in liquid nitrogen and stored it at -80 °C until analysis. Juvenile hormone levels in the hemolymph were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Waters Acuity ultrahigh-performance liquid chromatography system coupled to a Waters Micromass Quattro Premier triple quadrupole mass spectrometer (Milford, MA) as described before (Scholl et al., 2014; Schilcher et al., 2021a).

Topical JH treatment

Newly emerged honeybees were treated topically with JH (Sigma Aldrich, CAS 24198-95-6, Germany). Juvenile hormone was solved in dimethylformamid (DMF) to a concentration of 20 µg/µl. Honeybees were taken out of the plastic tub (Section 2.2.1) and separated using snap-on jars. The control group was treated with DMF by immobilizing them on ice and fixing them with needles onto Styrofoam plates, carefully fixing the wings to the side. Each bee received 3.5 µl of DMF on their abdomen. After the solvent dried, the honeybees were transferred to a cage and provided with water and 30 % sugar water *ad libitum*. The treatment group was treated in the same manner and received 3.5 µl of the JH - DMF solution to a concentration of 70 µg JH per honeybee as done before (Amsalem et al., 2014). Honeybees were sampled one day and eight days after the treatment for analysis.

2.2.3   Triglycerides

After JH collection, we froze the honeybees in liquid nitrogen. Half of their fat bodies were crushed using a cooled mixer mill (MM 400; Retsch) and zirconia beads. We added chloroform (1 ml), methanol (0.5 ml) and two triacylglycerol (TAG) standards (2.5 µg each, 10:0 TAG and 17:0 TAG) to extract the lipids. After centrifugation the supernatant was collected. This step was repeated and 0.88 % aqueous KCl (0.75 ml) was added, discarding the upper phase. 0.25 ml methanol and 0.25 ml H2O were

added to the lower phase and placed into a rotational vacuum concentrator (RVC 2-25 CDplus; CHRIST) at 50 °C until completely dry. The dried residue was dissolved in 100 µl isopropanol and frozen at -20 °C until analysis with an ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC–qTOF-MS, Synapt G2 HDMS , Waters, Milford, MA) as described in Mueller et al. (2015). The data was analyzed using MassLynx™software from Waters®. Only the ten most frequently appearing TGs were selected for statistical analysis as they represent more than 80 % of all TGs as we did before (**Chapter 4**, Supplementary Figure 1).

2.2.4   Gene expression analysis

Table 2.1: **Primers used for the gene expression analysis.** The respective gene, NCBI Gene ID, the sequence of the corresponding forward and reverse primers, TaqMan probes, and the expected length of the product are shown. *Vitellogenin* primers and probe were ordered as published before by Peso et al. (2016).

Gene	Gene ID	Primer	Sequence	Length [bp]
Vitellogenin	406088	forward	5´-AGTTCCGACCGACGACG-3´	63
		reverse	5´-TTCCCTCCCACGGAGTCC-3´	
		Probe	YAK-CCGTACGCCTCGTTCAAAGCCA-BBQ	
AmOARα1	406068	forward	5´-GCAGGAGGAACAGCTGCGAG-3´	154
		reverse	5´-GCCGCCTTCGTCTCCATTCG-3´	
		Probe	6FAM-TCCCCATCTTCATCACCCTTGGCTTCTCC-BBQ	
AmTAR1	406110	forward	5´-AGCCGACCGAGGTCACGATAG-3´	169
		reverse	5´-CCCATTATCACGCCCAATGTCC-3´	
		Probe	Cy5-AACGAGATCCTCTGCCTCTCCTCGATGAA-BBQ	
Amforα	406092	forward	5´-CTTGACACCGACGAAACCC-3´	131
		reverse	5´-CTGCTTTGATCAGTTCACGAGATC-3´	
		Probe	6FAM-TTGTCAGCGTGGCAAGCTCTTGA-BBQ	
Amforβ	406092	forward	5´-GTCGAGGGAGGACGAATACA-3´	150
		reverse	5´-CTCGATTCGCTGTCACTGGTC-3´	
		Probe	Cy5-TGTTCTTGGGGATGGCCGC-BBQ	
AmEF1α	408385	forward	5´-CGATTGTCACACCGCTCATATC-3´	249
		reverse	5´-TAAAGGTGACACTCTTAATGACG-3´	
		Probe	6FAM-ACCGAGGAGAATCCGAAGAGCATCAA-BBQ	
AmRpL32	406099	forward	5´-AgTAAATTAAAgAgAAACTggCgTAA-3´	182
		reverse	5´-TAAAACTTCCAgTTCCTTgACATTAT-3´	
		Probe	Cy5-TGGCAACATATGACGAGTTTTTTTGTT-BBQ	

The other half of the fat body (Section 2.2.3) and the corresponding brains were used for gene expression analysis. Additionally, we also analyzed fat body tissue of the honeybees

treated topically with JH. The tissue was dissected under liquid nitrogen. We used GenUP Total RNA Kit (biotechrabbit, Henningsdorf, Germany) to extract total RNA following the standard protocol provided by the manufacturer. In addition, we included an extra DNase I digestion step, after binding of the RNA to the Mini Filter RNA. We then added 50 µl of a DNase mix containing 30 U RNase-free DNase I (Lucigen Corporation, Middleton, USA) together with the corresponding buffer and incubated the samples for 15 min at room temperature. For the following polymerase chain reaction (PCR) experiment, we extracted total RNA from the individual tissues. We used 100 ng of total RNA of each tissue for cDNA synthesis using the Biozym cDNA Synthesis Kit (Biozym, Hessisch Oldendorf, Germany). We used 20 µl reactions (4 µl 5x cDNA Synthesis Mix, 1 µl 20x Rtase, 4 µl RNA template, 11 µl H_2O) with the following protocol: 42 °C for 30 min and 85 °C for 10 min. The cDNAs were analyzed afterwards in either 20 µl triplex PCR reactions (5 µl cDNA, 1.4 µl H_2O, 10 µl 2x qPCR Blue Probe Mix (Biozym, Hessisch Oldendorf, Germany), 1.2 µl of each primer (0.2 µM, Table 2.1)) or 20 µl duplex PCR reactions (5 µl cDNA, 2.6 µl H_2O, 10 µl 2x qPCR Blue Probe Mix (Biozym, Hessisch Oldendorf, Germany), 1.2 µl of each primer (0.2 µM, Table 2.1)). We analysed PCR triplicates of each cDNA (5 µl) using the following real-time quantitative PCR (qPCR) protocol: 95 °C for 2 min and 40 cycles at 95 °C for 5 s and 60 °C for 30 s on a Rotor368 Gene Q (Qiagen, Hilden, Germany). Gene expression was quantified relative to the reference genes *AmEF1α* and *AmRP49* using the R package 'EasypcR - V. 1.1.3' as published by Hellemans et al. (2007); Le Pape (2012).

2.2.5 Statistics

Statistical analyses were conducted using R (V. 4.1.1 including 'stats', R Core Team, 2021). and the R package 'rstatix - V. 0.7.0' (Kassambara, 2021). Data was analyzed for normal distribution using a Shapiro-Wilk test. For the orientation flight experiments, normally distributed data was analyzed using an ANOVA and data that was not distributed normally was analyzed using a Kruskal-Wallis test. Multiple comparison analyses were conducted using either a Tukey post hoc test (following an ANOVA) or a Wilcoxon signed rank test (following a Kruskal-Wallis test). Significant differences of the post hoc analyses were adjusted using the false discovery rate (fdr) adjustment. For the results from topical JH application, data was analyzed using a generalized linear mixed model (GLMM) with a Gaussian distribution using the R package 'glmmTMB - V. 1.1.2.3'

(Brooks et al., 2017). Post hoc analyses were conducted using the R package 'lsmeans - V. 2.30-0' (Lenth, 2021) with a Zukey adjustment. The models were fitted using the R package 'DHARMa - V. 0.4.4' (Hartig, 2021). Graphs were constructed using the packages 'ggplot2 - V. 3.3.5' (Wickham, 2016) and 'ggpubr - V. 0.4.0' (Kassambara, 2020).

2.3 Results

2.3.1 Juvenile hormone and Triglyceride levels change as honeybees become foragers

Closely observing the behavior of the bees during the nurse-forager transition, we can show that juvenile hormone levels were low in nurse bees and bees performing their first orientation flights but increased significantly in regularly foraging honeybees (Figure 3A; Table 2.2), implying that JH titers change in response to transition to foraging and not preceding it. After the initiation of foraging, they stayed at a high level.

Similar to JH levels, triglyceride levels differed strongly between regularly foraging honeybees and nurse bees as well as honeybees that performed their first orientation flights (Figure 3B; Table 2.2). Here, the change seems to be more gradual, but bees that performed orientation flights did not differ significantly from nurse bees. Interestingly, foragers tended to lose more TGs with experience, although this trend was not reflected by significant differences between groups.

2.3.2 Brain gene expression suggests a causal role for an octopamine receptor in the nurse-forager transition

Our results reveal a very interesting pattern of changes in candidate gene expression during the transition from in-hive tasks to foraging. The mRNA expression of the octopamine receptor gene *AmOARα1* appeared to increase rather gradually from nursing over orientation flights to foraging performance and increased even further with foraging experience (Figure 4A; Table 2.3), suggesting a causal role for this receptor in the nurse-forager transition. Messenger RNA expression of the tyramine receptor gene *AmTAR1* reveals a very different picture. Gene expression appeared to decrease during the nurse-forager transition in a stepwise manner. While bees performing their orientation flights had a comparatively high mRNA expression which did not differ from that of nurse bees, mRNA expression of *AmTAR1* sharply dropped in young foragers (Figure 4B; Table 2.3). Experienced foragers did not decrease further in their *AmTAR1* mRNA levels.
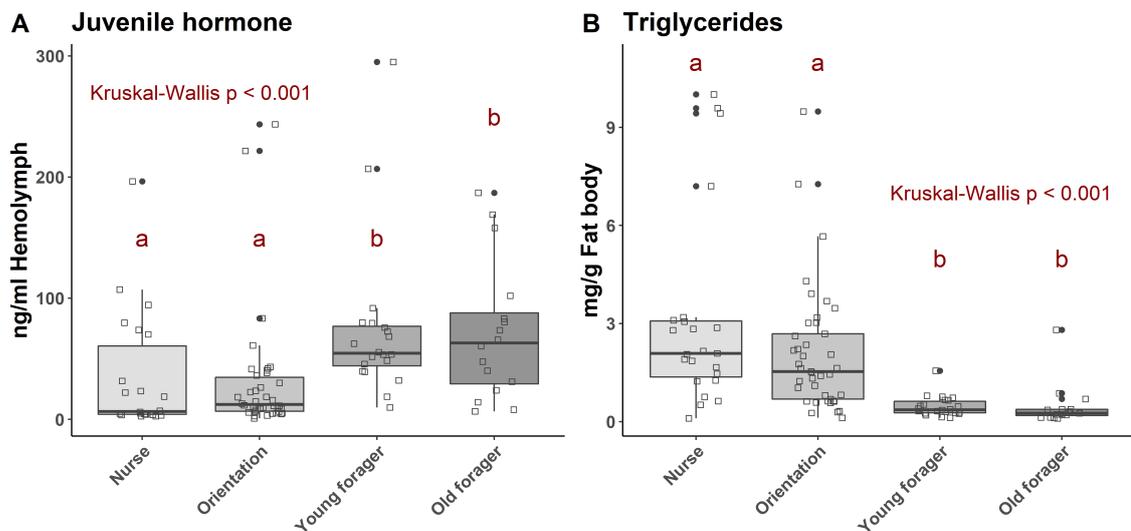
Figure 3: **Juvenile hormone titers and triglyceride levels during the nurse-forager transition.** (A) Task significantly influenced JH levels. Nurse bees and bees performing their first orientation flights did not differ in their JH levels. However, as soon as the honeybees began to forage, JH levels increased significantly. Young and old foragers did not differ in their hemolymph JH titers. (B) Nurse bees and bees performing their first orientation flights did not differ in their abdominal lipid levels. Yet, foragers showed significantly decreased lipid levels but young and old foragers did not differ significantly. For test statistics and sample size, see Table 2.2. Significant differences between the groups are indicated by lowercase letters.

One of the most interesting candidate genes is *vitellogenin*, which has long been linked to the nurse-forager transition. In our experiments, *vg* mRNA expression did not differ significantly between the groups (Figure 4C; Table 2.3). However, our close-up of the nurse-forager transition revealed a gradual decrease in *vg* mRNA expression with increasing age and while changing tasks from nursing over performing orientation flights to foraging, supporting an age-related change. This might be negatively coupled to the octopamine receptor gene *AmOARα1*. Neither of the *Amfor* splice variants *Amforα* or *Amforβ* differed significantly between social groups in the honeybee brain (Figure 4D, E; Table 2.3), but *Amforα* tended to increase in young foragers.

2.3.3 Opposite relative mRNA expression pattern of the *AmOARα1* and the *AmTAR1* in the fat body of honeybees

The honeybee nurse-forager transition is thought to be tightly linked to vitellogenin (Amdam and Omholt, 2003), which is not only expressed in the brain of the honeybee but also in the fat body (Engels et al., 1990). For that reason, we also looked at the expression of our candidate genes in the fat body.

There were no significant differences between individual behavioral groups in the relative *AmOARα1* mRNA expression (Figure 5A; Table 2.4) or *AmTAR1* mRNA expression in the fat body (Figure 5B; Table 2.4). Interestingly, the expression of the octopamine receptor gene *AmOARα1* seemed to decrease from nurse bees to bees performing their first orientation flights while expression of the tyramine receptor gene *AmTAR1* appeared

Table 2.2: **Test statistics for the analysis conducted in Figure 1.** Significant differences between the groups are indicated by asterisks (ns p > 0.05, ** p < 0.01, *** p < 0.001). Results are shown for nurse bees, bees during orientation flights (Orient), young foragers (Young for), old foragers (Old for) and the respective sample sizes (n).

| Analysis	Figure	Treatment	n	Test	Statistic	p
Juvenile hormone	3A	Nurse	23	Kruskal-Wallis	$\mathcal{X} = 24.347$	$2.114e^{-05}$
		Orientation flight	38			
		Young forager	20			
		Old forager	16			
		Nurse vs Orient			ns	0.48
		Nurse vs Young for			**	0.0064
		Nurse vs Old for		Wilcoxon	**	0.0096
		Orient vs Young for		fdr correction	***	0.000015
		Orient vs Old for			**	0.0022
		Young for vs Old for			ns	0.99
Triglycerides	3B	Nurse	23	Kruskal-Wallis	$\mathcal{X} = 44.52$	$1.17e^{-09}$
		Orientation flight	41			
		Young forager	22			
		Old forager	16			
		Nurse vs Orient			ns	0.15
		Nurse vs Young for			***	0.00000066
		Nurse vs Old for		Wilcoxon	***	0.0000049
		Orient vs Young for		fdr correction	***	0.00000066
		Orient vs Old for			***	0.0000033
		Young for vs Old for			ns	0.15

Relative gene expression - Brain
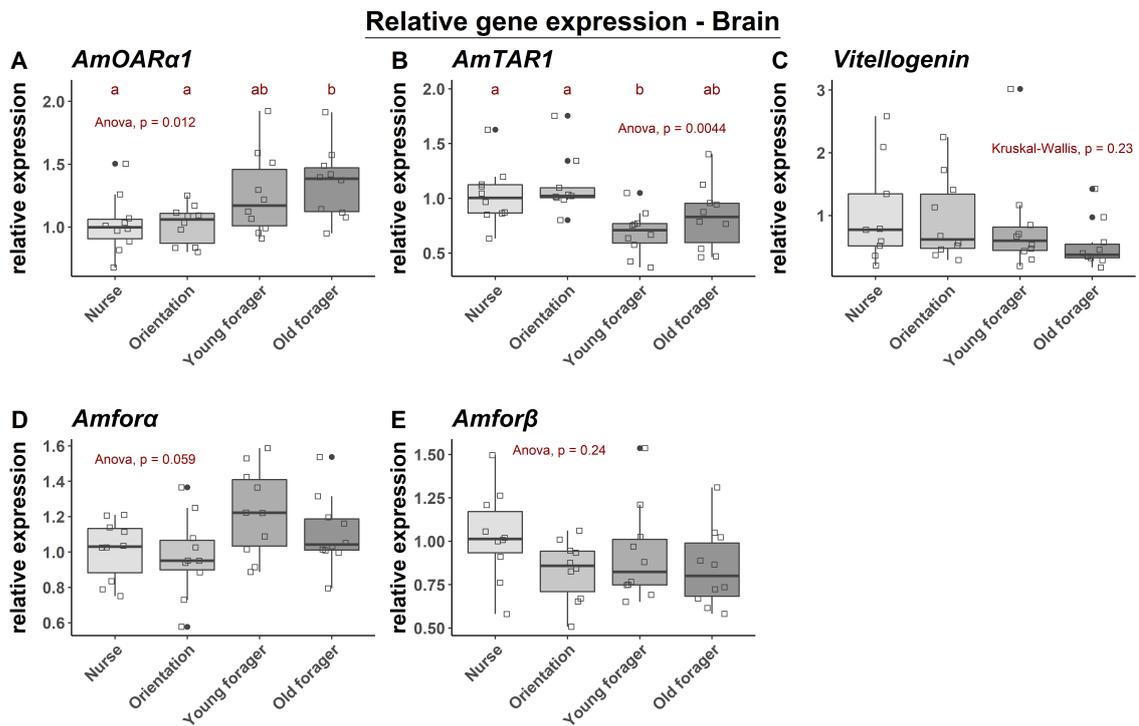
Figure 4: **Gene expression of *AmOARα1*, *AmTAR1*, *vitellogenin* and the *foraging* gene during the transition from nurse bees to foragers in the honeybee brain.** (A) *AmOARα1* gene expression differed significantly between the groups. Nurse bees and bees performing their first orientation flights (Orientation) showed significantly lower expression levels compared to old foragers. (B) Gene expression of the *AmTAR1* changed significantly with the transition from nurse bees to foragers. Nurse bees and bees performing their first orientation flights show significantly higher expression levels compared to young foragers. No significant differences were found for the expression levels of *vg* (C), *Amforα* (D) nor *Amforβ* (E). For test statistics and sample size, see Table 2.3. Significant differences between the groups are indicated by lowercase letters.

to increase, exactly opposite to our observations in the honeybee brain (Section 2.3.2). Yet, at the onset of foraging, *AmOARα1* increased and *AmTAR1* decreased.

Vitellogenin mRNA expression tended to decrease from nurse bees to foragers (Figure 5C; Table 2.4; p = 0.074). We observed the same pattern as in the honeybee brain (Section 2.3.2), further supporting an age-related change in *vg* mRNA expression.

We did not observe any significant difference in the *Amforα* mRNA expression in the fat body between the four behavioral groups (Figure 5D; Table 2.4). However, the *Amforα* expression seemed to increase in young foragers. *Amforβ* decreased significantly with

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the transition from nurse bees to foragers (Figure 5E; Table 2.4). Nurse bees showed the highest expression while old foragers displayed the lowest expression levels. This expression pattern resembles that observed in the brain for the same genes (Section 2.3.2).

Table 2.3: **Test statistics for the graphical analyses conducted in Figure 4.** Significant differences between the groups are indicated by asterisks (ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Results are shown for nurse bees, bees during orientation flights (Orient), young foragers (Young for), old foragers (Old for) and the respective sample sizes (n).

| Analysis	Figure	Tissue	Treatment	n	Test	Statistic	p
AmOARα1	4A	Brain	Nurse	10	ANOVA	F = 4.175	0.0123
			Orientation flight	10			
			Young forager	10			
			Old forager	10			
			Nurse vs Orient			ns	0.98
			Nurse vs Young for			**	0.12
			Nurse vs Old for		t-test	**	0.037
			Orient vs Young for		fdr correction	***	0.12
			Orient vs Old for			**	0.037
			Young for vs Old for			ns	0.64
AmTAR1	4B	Brain	Nurse	10	ANOVA	F = 5.22	0.0043
			Orientation flight	9			
			Young forager	10			
			Old forager	10			
			Nurse vs Orient			ns	0.49
			Nurse vs Young for			*	0.015
			Nurse vs Old for		t-test	ns	0.21
			Orient vs Young for		fdr correction	*	0.01
			Orient vs Old for			ns	0.092
			Young for vs Old for			ns	0.26
Vitellogenin	4C	Brain	Nurse	9	Kruskal-Wallis	$\mathscr{X} = 4.289$	0.2318
			Orientation flight	10			
			Young forager	10			
			Old forager	10			
Amforα	4D	Brain	Nurse	10	ANOVA	F = 2.718	0.0589
			Orientation flight	10			
			Young forager	10			
			Old forager	10			
Amforβ	4E	Brain	Nurse	10	ANOVA	F = 1.464	0.241
			Orientation flight	10			
			Young forager	10			
			Old forager	10			

Relative gene expression - Fat body
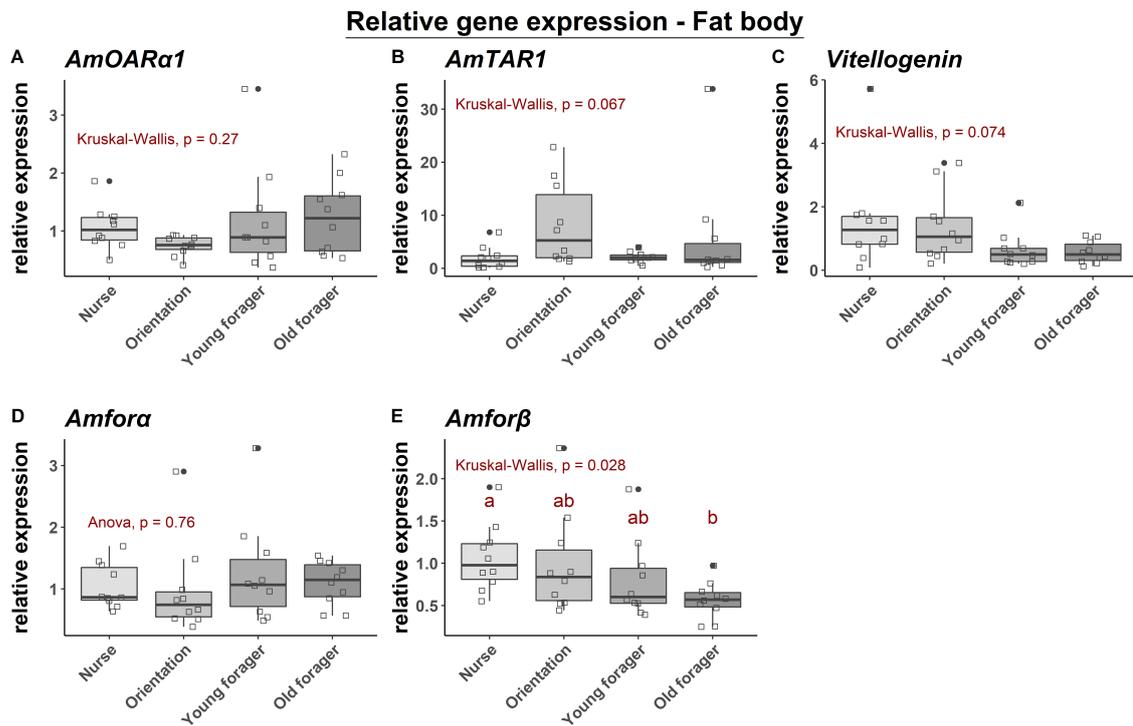
Figure 5: **Gene expression of *AmOARα1*, *AmTAR1*, *vitellogenin* and the *foraging* gene during the transition from nurse bees to foragers in the honeybee fat body.** The nurse-forager transition had no significant effect on the relative expression of *AmOARα1* (A), *AmTAR1* (B), *vg* (C) and *Amforα* (D). (E) *Amforβ* decreased significantly with the transition from nurse bees to foragers. Nurse bees showed the highest expression of *Amforβ* and differed significantly from honeybees performing their first orientation flights (Orientation). Both groups did not differ significantly from young foragers but from old foragers. For test statistics and sample size, see Table 2.4. Significant differences between the groups are indicated by lowercase letters.

2.3.4 Juvenile hormone levels increase after topical treatment but lipids are unaffected

Treating honeybees with juvenile hormone strongly increased JH levels. In addition, JH naturally increased with age (Figure 6A; Table 2.5). Triglycerides, in contrast, were unaffected by JH treatment and age (Figure 6B; Table 2.5).

2.3.5 Relative *AmOARα1* expression is unaffected by JH treatment while *AmTAR1* expression increases

AmOARα1 mRNA expression was unaffected by JH treatment but decreased between day 1 and day 8 in the honeybee fat body (Figure 7A; Table 2.6). The mRNA expression of *AmTAR1*, in contrast, decreased after JH treatment but was unaffected by honeybee age (Figure 7B; Table 2.6), even though a slight increase can be observed between day 1 and day 8.

Unlike in the brain, expression of *AmOARα1* and *AmTAR1* the octopamine receptor

Table 2.4: **Test statistics for the graphical analyses conducted in Figure 5.** Significant differences between the groups are indicated by asterisks (ns p > 0.05, * p < 0.05). Results are shown for nurse bees, bees during orientation flights (Orient), young foragers (Young for), old foragers (Old for) and the respective sample sizes (n).

| Analysis	Figure	Tissue	Treatment	n	Test	Statistic	p
AmOARα1	5A	Fat body	Nurse	10	Kruskal-Wallis	$\mathscr{X} = 3.881$	0.2746
			Orientation flight	10			
			Young forager	10			
			Old forager	10			
AmTAR1	5B	Fat body	Nurse	10	Kruskal-Wallis	$\mathscr{X} = 7.1693$	0.06669
			Orientation flight	10			
			Young forager	10			
			Old forager	10			
Vitellogenin	5C	Fat body	Nurse	10	Kruskal-Wallis	$\mathscr{X} = 6.9234$	0.07438
			Orientation flight	10			
			Young forager	10			
			Old forager	10			
Amforα	5D	Fat body	Nurse	10	Kruskal-Wallis	$\mathscr{X} = 2.9078$	0.4061
			Orientation flight	10			
			Young forager	10			
			Old forager	10			
Amforβ	5E	Fat body	Nurse	10	Kruskal-Wallis	$\mathscr{X} = 9.0834$	0.0282
			Orientation flight	10			
			Young forager	10			
			Old forager	10			
			Nurse vs Orient			ns	0.44
			Nurse vs Young for			ns	0.15
			Nurse vs Old for		Wilcoxon	*	0.013
			Orient vs Young for		fdr correction	ns	0.44
			Orient vs Old for			ns	0.15
			Young for vs Old for			ns	0.44
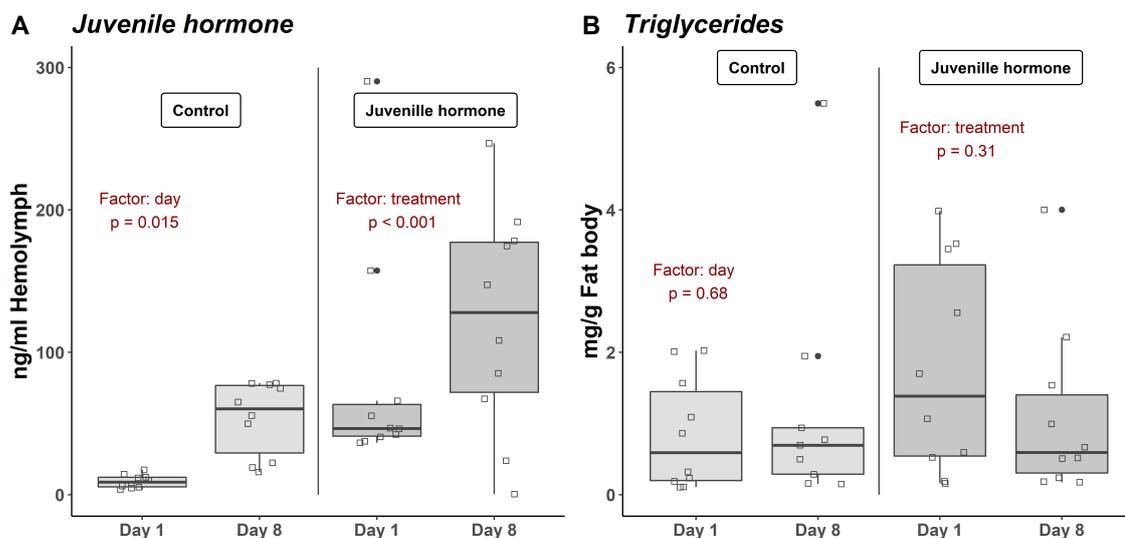
Figure 6: **Influence of topical juvenile hormone treatment on JH and triglyceride levels.**
(A) Juvenile hormone levels increased significantly in the control from day 1 to day 8 showing and effect of age on hemolymph JH levels. Additionally, the topical treatment also significantly affected hemolymph JH levels. (B) Triglyceride levels were unaffected by age and treatment. However, a median decrease in TG levels can be observed in the group treated topically with juvenile hormone. For test statistics and sample size, see Table 2.5

Table 2.5: **Test statistics for the graphical analyses conducted in Figure 6.** Honeybees were either treated with juvenile hormone (JH) or with the solvent DMF. Significant differences between the groups are indicated by asterisks (* $p < 0.05$, *** $p < 0.001$).

Analysis	Figure	Treatment	n	Test	Statistic	p
Juvenile hormone	6A	Control Day 1	10	GLMM		
		Control Day 8	8	Factor day	$\mathscr{X} = 5.8982$	0.01516
		JH Day 1	10	Factor treatment	$\mathscr{X} = 16.33735$	$5.201e^{-05}$
		JH Day 8	8			
		Day 1 vs Day 8		Tukey Factor day	*	0.0203
		Control vs JH		Tukey Factor treatment	***	0.0003
Triglycerides	6B	Control Day 1	10	GLMM		
		Control Day 8	8	Factor day	$\mathscr{X} = 0.1658$	0.6838
		JH Day 1	10	Factor treatment	$\mathscr{X} = 1.0419$	0.3074
		JH Day 8	8			

mRNA expression decreased and the tyramine receptor increased from day 1 to day 8 in the fat body. Thus, both receptors show the same expression pattern as we observed during the orientation flights (Section 2.3.3).

Vitellogenin mRNA expression shows a very interesting picture (Figure 7C; Table 2.6). It was comparatively high on day 1 in control bees and decreased strongly with age. Bees treated with JH already displayed very low *vg* mRNA expression on day 1 which did not change with age. We did not observe any effect of age or treatment on the *foraging* gene mRNA expression (Figure 7D, E; Table 2.6), which leads to the conclusion that the *foraging* gene is unaffected by increasing JH levels and that the decrease we observe in section 2.3.3 seem to be independent of age but seem to be rather an effect of task.
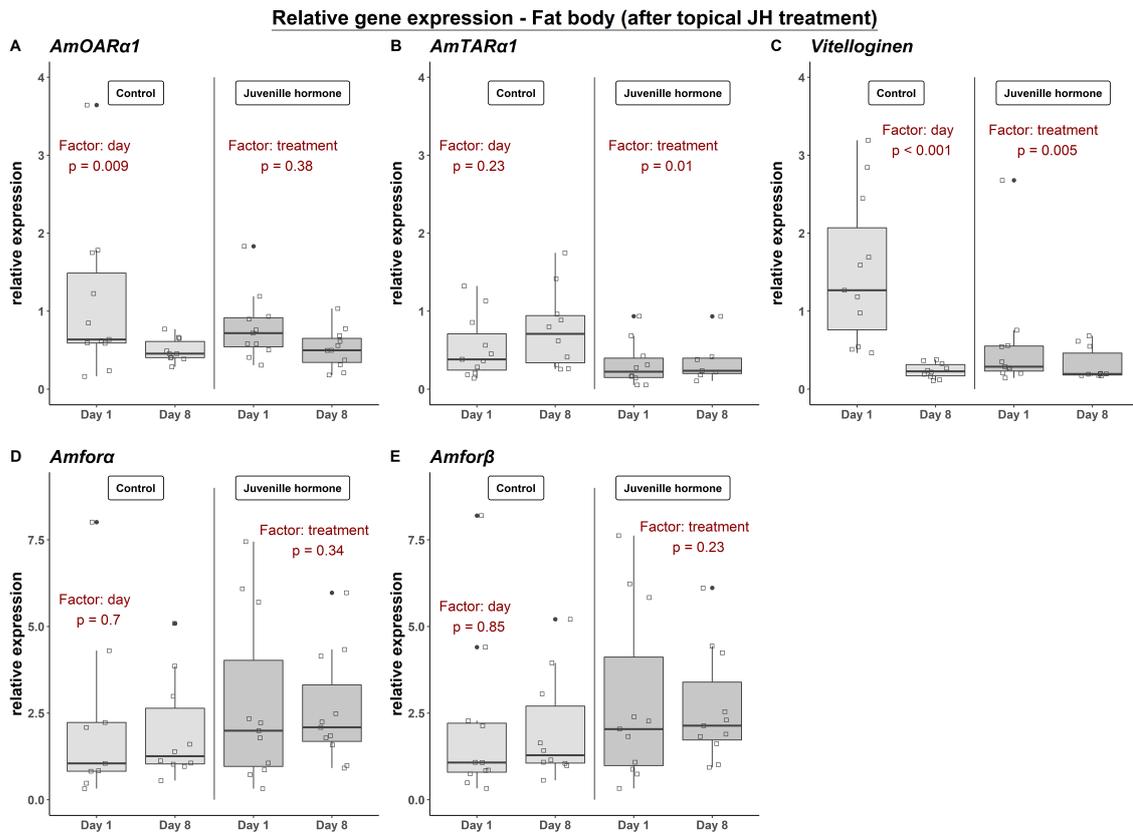
Figure 7: **Influence of topical juvenile hormone treatment on relative gene expression of *AmOARα1*, *AmTAR1*, *vitellogenin* and the *Amfor* in the fat body.** (A) Juvenile hormone treatment had no significant effect on the relative expression of *AmOARα1*. However, *AmOARα1* significantly decreased from day 1 to day 8. (B) Juvenile hormone treatment significantly increased the relative expression of *AmTAR1* in the honeybee fat body. No changes could be observed between day 1 and day 8. (C) *Vitellogenin* expression levels decreased with topical treatment of JH and between day 1 and day 8. Interestingly, the interaction between the treatment and day also showed a significant effect. Juvenile hormone levels significantly decreased from day 1 to day 8 in the control honeybees. However, no difference can be seen between topically treated animals on day 1 and day 8. Neither day nor treatment affected the relative expression of either *Amforα* (D) or *Amforβ* (E). For test statistics and sample size, see Table 2.6.

2.4   Discussion

Our results suggest that the honeybee octopamine receptor *AmOARα1* has a decisive integrative function in regulating social organization through pleiotropic effects on the

nurse-forager transition. Its brain expression increases gradually from nurse bees to foragers, while the expression in the fat body decreases during orientation flights and increases with foraging experience. Juvenile hormone titers in the hemolymph show a similarly gradual increase from nursing behavior over orientation flights to foraging behavior, while levels of triglycerides decrease in inverse manner to JH. Our data thus support a tight and causal link between OA signaling and JH (Schulz et al., 2002b). Based on the study by Corby-Harris et al. (2020) we speculate that the octopamine receptor *AmOARα1* is directly involved in the degradation of hypopharyngeal glands

Table 2.6: **Test statistics for the graphical analyses conducted in Figure 7.** The treatment group was treated with juvenile hormone (JH). The Control group (C) was treated with the solvent DMF. Significant differences between the groups are indicated by asterisks (ns p > 0.05, * p < 0.05, *** p < 0.001).

Analysis	Fig.	Tissue	Treatment	n	Test	Statistic	p
AmOARα1	7A	Fat body	Control Day 1	11	GLMM		
			Control Day 8	10	Factor day	$\mathscr{X} = 6.6389$	0.009977
			JH Day 1	11	Factor treatment	$\mathscr{X} = 0.7492$	0.386736
			JH Day 8	11			
			Day 1 vs Day 8		Tukey Factor day	*	0.0139
AmTAR1	7B	Fat body	Control Day 1	11	GLMM		
			Control Day 8	10	Factor day	$\mathscr{X} = 1.4103$	0.23500
			JH Day 1	10	Factor treatment	$\mathscr{X} = 6.2374$	0.01251
			JH Day 8	7			
			Control vs JH		Tukey Factor treatment	*	0.0175
Vitellogenin	7C	Fat body	Control Day 1	11	GLMM		
			Control Day 8	10	Factor day	$\mathscr{X} = 17.622$	$2.695e^{-05}$
			JH Day 1	11	Factor treatment	$\mathscr{X} = 6.3688$	0.011614
			JH Day 8	10	Factor interaction	$\mathscr{X} = 7.8840$	0.004987
			Day 1 vs Day 8		Tukey Factor day	***	0.0002
			Control vs JH		Tukey Factor treatment	*	0.0222
			C. Day 1 vs C. Day 8		Tukey	***	0.0001
			JH Day 1 vs JH Day 8		Factor interaction	ns	0.7600
Amforα	7D	Fat body	Control Day 1	9	GLMM		
			Control Day 8	10	Factor day	$\mathscr{X} = 0.1448$	0.7036
			JH Day 1	11	Factor treatment	$\mathscr{X} = 0.9026$	0.3421
			JH Day 8	11			
Amforβ	7E	Fat body	Control Day 1	11	GLMM		
			Control Day 8	10	Factor day	$\mathscr{X} = 0.0380$	0.8454
			JH Day 1	11	Factor treatment	$\mathscr{X} = 1.4384$	0.2304
			JH Day 8	11			

and the change from lipid metabolism to carbohydrate metabolism, which is needed to fuel the cost-intensive foraging flights. We can directly show that *AmOARα1* expression is unaffected by JH levels but increases with age. It has long been postulated that OA acts more proximal than JH and rather paces JH synthesis (Rachinsky, 1994; Schulz et al., 2002b). This hypothesis is based on the increase in JH synthesis via OA (Rachinsky, 1994) and a delayed foraging of allatectomized honeybees once treatment with OA was discontinued (Schulz et al., 2002b). Our results provide direct evidence for this hypothesis, further emphasizing the pacemaker function of OA on JH. However, further experiments should be conducted investigating the effects of OA on the factors investigated above.

While *AmOARα1* mRNA expression was unaffected by topical JH treatment, we can show a decrease in the *AmTAR1* mRNA expression following JH treatment (Figure 7A, B). Furthermore, the *AmTAR1* shows an inverse expression pattern to that of the *AmOARα1* receptor gene in all tissues (Figure 4A, B; Figure 5A, B). In the honeybee brain, the *AmOARα1* mRNA increases from nurse bees over bees performing orientation flights to foragers, while the *AmTAR1* mRNA expression decreases (Figure 4A, B). However, in the fat body of honeybees, the *AmOARα1* mRNA expression decreases during orientation flights (Figure 5A; Figure 7A) but increases afterwards, while the *AmTAR1* expression increases during orientation flights (Figure 5B; Figure 7B) and decreases afterwards, most likely due to increasing JH levels (Figure 7B).

Multiple studies have shown opposite actions of both OA and TA. While OA increases the phototactic response of honeybees, TA decreases it (Schilcher et al., 2021b). Honeybees treated with OA spent an increased time flying, while honeybees treated with TA spent a decreased time flying (Fussnecker et al., 2006). In general, it is hypothesized that both substances fulfill the roles of mammal epinephrine and norepinephrine in honeybees, respectively (Roeder, 2005). This can also be seen at the receptor level. The octopamine receptor AmOARα1 releases Ca^{2+} from intracellular stores upon activation (Grohmann et al., 2003). Yet shortly afterwards scientists came to believe that it also increases intracellular cAMP by activating adenylyl cyclase (Farooqui, 2007). The tyramine receptor AmTAR1 inhibits the adenylyl cyclase after activation by TA and therefore reduces intracellular cAMP (Blenau et al., 2000). However, inverse expression patterns of both receptors in the brain and fat body indicate different functions depending on the tissue, possibly regulated by JH. Interestingly, while JH treatment had no effect on the *AmOARα1* mRNA expression, expression significantly decreased from day 1 to day

8 in the fat body (Figure 7A). However, it increased continually with age in the brain (Figure 4A). Other studies have shown that octopamine brain levels not only increase with age but also with the transition to foraging (Wagener-Hulme et al., 1999). Young hive bees generally show lower brain levels of OA compared to older honeybees, i.e. foragers. Interestingly, foragers reverting back to nursing tasks also displayed lower OA levels while precocious foragers had increased OA levels. This suggests that there must be an internal repressor that can inhibit the levels of OA signaling.

We here propose that the AmTAR1 might be that repressor. The *AmTAR1* mRNA expression is known to differ between nurse bees and foragers (Scheiner et al., 2014) and its brain expression pattern has been thoroughly categorized (Thamm et al., 2017). Tyramine binding to the AmTAR1 protein leads to a decrease in internal cAMP (Blenau et al., 2000). While little is known in honeybees, it has been shown that changes in internal cAMP can lead to an increase or decrease in transcription factors (TF) like the cAMP response element-binding protein (CREB) (Lonze and Ginty, 2002), which is known to mediate gene expression (Kitagawa, 2007). Therefore, a decrease in TFs like CREB might delay or halt the transcription of other receptor genes like *AmOARα1*. While multiple TFs could be involved in such a cascade, CREB seems to be a likely candidate. It has been shown that honeybees responding to a conditioned stimulus after associative learning training show higher levels of phosphorylated honeybee CREB homolog (pAmCREB) than honeybees not responding to the stimulus (Gehring et al., 2016a). Furthermore, pAmCREB increases in the inner compact cells between day 8 and day 15 in the mushroom body basal ring in honeybees, an area that shows increased activity in foragers compared to nurse bees (Khamis et al., 2015; Gehring et al., 2016b). Additionally, it has been shown that CREB regulates the tyramine beta hydroxylase, the enzyme converting TA into OA (Burkewitz et al., 2015), and activating CREB led to an increase in the mRNA expression of *tyramine-β-hydroxylase-1*.

High expression of *AmTAR1* might therefore lead to an increase in AmTAR1 protein and thus a reduced expression of the *AmOARα1* possibly via TFs and a low conversion of TA into OA. An increase in JH synthesis could then decrease the expression of the *AmTAR1* mRNA, as we showed in this study (Figure 7B). This would allow for an increase in *AmOARα1* expression which could further increase JH synthesis, accelerating the transition from nurse bees to foragers. However, further experiments need to be conducted especially investigating biogenic amine content during the transition. For example, one would need to look at the TA levels after honeybee JH treatment and the

resulting OA levels over multiple days. Additionally, one could use tools to inhibit, knock down or knock out the *AmTAR1*. Inhibition would be achieved via specific receptor inhibitors like yohimbine (Reim et al., 2017). However, yohimbine does target AmTAR1, AmTAR2 as well as AmOARα1. So far, no inhibitors are available that only target the *AmTAR1*. Yet, RNAi, which knocks down the *AmTAR1* mRNA, has been shown to work in honeybees for the *AmTAR1* (Sinakevitch et al., 2017). CRISPR/Cas 9 would be a valid alternative, knocking out the *AmTAR1*. While CRISPR/Cas 9 has not been used for the tyramine receptor specifically, it has been shown to work for other genes in the honeybee (Değirmenci et al., 2020).

Additionally, JH also influences *vg* mRNA expression. Topical treatment with JH leads to a suppression of *vg* mRNA (Figure 7C). However, as we also see in the orientation flight experiments, *vg* mRNA decreases naturally over time in the brain (Figure 4C) and in the fat body (Figure 5C; Figure 7C). As shown before, JH levels increase from nursing to foraging (Elekonich et al., 2001), however we were mainly interested in the transition time point around the orientation flights and the onset of foraging. Interestingly, hemolymph JH levels did not increase significantly before foraging but rather increased drastically after honeybees became foragers (Figure 3A). This provides further evidence that JH is, in fact, not a direct trigger of nurse-forager transition but rather paces other factors under normal conditions. Juvenile hormone seems to be the *zeitgeber* for the decrease in *vg* mRNA expression and *AmTAR1* mRNA expression and indirectly the increase in mRNA expression of *AmOARα1*. These results experimentally support the double repressor hypothesis set up by (Amdam and Omholt, 2003) which states that JH synthesis is under negative control of an external and internal repressor.

The external repressor is rather unspecified, but it could be an external signal coming from other honeybees. This could be the brood ester pheromone, a pheromone that induces worker bees to take care of the brood rather than allocate energy to foraging activities (Le Conte et al., 2001). The internal repressor is proposed to be the vg protein. The *foraging* gene seems to be unaffected by JH (Figure 7D, E). While we can observe a decrease in the *Amforβ* mRNA expression in the brain with the transition from nurse bees to foragers (Figure 4E) and the fat body (Figure 5E), JH does not seem to be involved in that decrease. Interestingly, while *Amforα* mRNA expression is not affected by topical JH treatment either, it shows an interesting expression pattern in the brain (Figure 4D) and fat body (Figure 5D). In the brain, *Amforα* mRNA expression seems to increase in young foragers, as shown previously (Thamm and Scheiner, 2014) but

expression drops once the foragers mature. However, in the fat body gene expression seems to decrease during orientation flights and then gradually increases with foraging experience, further emphasizing the possible differences in function of the same genes between brain and fat body.

While topical JH treatment did not affect the *foraging* gene expression nor *AmOARα1* mRNA expression, we observed the expected increase in hemolymph JH levels (Figure 2.5A). However, TG levels were unaffected by JH treatment and age (Figure 6B), contrary to our predictions.

Most likely, the lipid free diet of honeybees held in cages during the topical experiments led to lipid accumulation only due to *de novo* synthesis from dietary carbohydrates (Toth et al., 2005). This synthesis pathway seems to be insufficient to increase the total TG amount during eight days. Yet, during the orientation flight experiments we observed a significant decrease in TGs once honeybees became foragers. However, contrary to common believe, lipids were still high during orientation flights indicating that lipids decrease due to foraging experience and not beforehand (Toth and Robinson, 2005). However, it remains unclear whether JH directly affects TG levels, re-conducting the tropical treatment experiments, with *ad libitum* access to pollen should reveal this information.

3

In Vitro Rearing Changes Social Task Performance and Physiology in Honeybees

Article

In Vitro Rearing Changes Social Task Performance and Physiology in Honeybees

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Simple Summary: The rearing of honeybee larvae in the laboratory is an important tool for studying the effects of plant protection products or pathogens on developing and adult bees, yet how rearing under artificial conditions affects the later social behavior and physiology of the honeybees is mostly unknown. We, here, show that honeybees reared in the laboratory generally had a lower probability for performing nursing or foraging tasks compared to bees reared under natural conditions in bee colonies. Nursing behavior itself appeared normal in in vitro honeybees. In contrast, bees reared in the laboratory foraged for a shorter period in life and performed fewer trips compared to bees reared in colonies. In addition, in vitro honeybees did not display the typical increase in juvenile hormone titer, which goes hand-in-hand with the initiation of foraging in colony-reared bees.

Abstract: In vitro rearing of honeybee larvae is an established method that enables exact control and monitoring of developmental factors and allows controlled application of pesticides or pathogens. However, only a few studies have investigated how the rearing method itself affects the behavior of the resulting adult honeybees. We raised honeybees in vitro according to a standardized protocol: marking the emerging honeybees individually and inserting them into established colonies. Subsequently, we investigated the behavioral performance of nurse bees and foragers and quantified the physiological factors underlying the social organization. Adult honeybees raised in vitro differed from naturally reared honeybees in their probability of performing social tasks. Further, in vitro-reared bees foraged for a shorter duration in their life and performed fewer foraging trips. Nursing behavior appeared to be unaffected by rearing condition. Weight was also unaffected by rearing condition. Interestingly, juvenile hormone titers, which normally increase strongly around the time when a honeybee becomes a forager, were significantly lower in three- and four-week-old in vitro bees. The effects of the rearing environment on individual sucrose responsiveness and lipid levels were rather minor. These data suggest that larval rearing conditions can affect the task performance and physiology of adult bees despite equal weight, pointing to an important role of the colony environment for these factors. Our observations of behavior and metabolic pathways offer important novel insight into how the rearing environment affects adult honeybees.

Keywords: honeybee; artificial rearing; behavior; in vitro; juvenile hormone; triglycerides; PER; foraging; nursing

1. Introduction

Eusociality and division of labour are key factors in a functioning honeybee colony. The colony strongly depends on the proper execution and appropriate timing of various tasks by its members [1,2]. Reproduction is in the hands of the queen and drones, while the other tasks are conducted by sterile, female honeybee workers. Young honeybees perform in-hive tasks such as brood care [2]. At about 20 days of age, honeybees leave the colony to collect resources for the colony, e.g., nectar and pollen [1,2]. However, this age-dependent behavioral shift is very plastic. Removing foragers from a colony can induce nurse bees to start foraging precociously [3]. Another factor influencing the transition from nursing to foraging is nutritional restriction. When honeybees are deprived of lipids, the number of precocious foragers increases [4]. Environmental stress, which can induce an increase in juvenile hormone (JH III) or the neurohormone and transmitter octopamine [5], is generally linked to earlier foraging behavior in adult honeybees. It has been shown that immune-stressed honeybees have significantly lower expression levels of the juvenile hormone esterase and the egg yolk precursor vitellogenin, which is used to produce brood food [6]. Another study showed that nurse bees infested with the varroa mite are less attracted to brood pheromone compared to uninfested honeybees [7]. How these environmental stressors can influence the colony on a developmental level is poorly understood.

In vitro rearing of honeybees is a common method for studying diverse questions related to environmental risk assessment [8], development [9], pathogens [10,11], pesticides [12] and behavior [13]. While the method itself has frequently been improved by making minor changes to the rearing protocol [14–18], very few studies have evaluated the "quality" of honeybees obtained through this protocol and their behavior as adults.

There are, naturally, huge differences in the social environment of honeybees developing in the colony and those reared in vitro. While bees reared in the colony were reported to be contacted by nurse bees up to 2785 times [19], bees reared in the laboratory are deprived of social contact and lack the chemical interactions and the regular tending of nurse bees [14]. Instead, they are provided with artificial food in a constant amount calculated for the average honeybee larva per day [15]. One recent study shows that honeybees reared in vitro on a diet equivalent to normal feeding by nurse bees were not only smaller but had smaller lateral mushroom body calyces than their sisters reared in the colony [9], which is probably a result of social deprivation. A reduced mushroom body size has even been reported for fruit flies following social deprivation [20]. This indicates that even the standard feeding protocol during in vitro rearing might act as a nutritional stressor during larval development. Nutritional stress during the fifth larval instar can increase starvation resistance compared to honeybee larvae fed on a normal diet [21]. However, the same treatment leads to higher JH III hormone titers in 24-h-old honeybees and in seven-day-old honeybees, indicating a faster maturation due to stress. At the same time, larval starvation decreased responsiveness to sugar in seven-day-old honeybees, which normally corresponds to low JH III titers. Pollen deprivation during larval development delays the onset of foraging and decreases mean foraging span and the lifespan of adult honeybee workers [22]. Nutritional deprivation during larval development can also influence adult ovary development. Thus, reduced pollen intake during the larval stages was shown to reduce ovary development in adult honeybees [23].

An interesting study addressing flight behavior of in vitro-reared honeybees demonstrated a lower metabolic rate in in vitro-reared bees compared to honeybees reared in the colony. The former could not fly as fast under tethered conditions [24]. Studies on the performance of in vitro-reared honeybees in a natural environment, however, are rare. It has been shown that in vitro-reared honeybees visited queen cells less frequently compared to their colony-reared sisters [13].

Our study aims to evaluate the behavioral performance of in vitro-reared honeybees in comparison to honeybees reared in the colony in a field study. We investigated how larval rearing can influence nursing performance and foraging flights—two of the most important tasks performed in the hive. Suboptimal food provisioning by humans during

in vitro rearing compared to optimal feeding by nurse bees in the hive, as well as the utilization of yeast as a pollen substitute in artificial rearing, might lead to developmental stress of the larvae. This stress may later lead to a reduced nursing activity in adult bees, possibly reducing colony fitness in the long run [7]. A reduced foraging performance might lead to malnourishment of the entire colony, strongly reducing winter survival. We further investigated whether in vitro rearing affects physiological traits that are important for task performance. In addition to testing the effects of artificial rearing on weight, we measured titers of JH III. This developmental hormone increases during the transition from nursing to foraging, with foragers normally having the highest titers of JH III [25]. Changes in JH III titers may point towards a different tempo in adult maturation [26]. We also measured abdominal triglyceride levels, which are related to the metabolic states of honeybees performing different tasks and considered to be negatively affected by JH III titers [27]. Thus, young honeybees typically have high triglyceride levels stored in the fat body while foragers are lean. JH III is also negatively correlated with the egg yolk precursor vitellogenin, an important factor in nursing behavior [28]. According to the double repressor hypothesis, JH and vitellogenin inhibit each other [28], and both have an opposite effect on sucrose responsiveness [29]. Sucrose responsiveness normally increases with age so that nurse bees have a lower sucrose responsiveness than foragers [30,31]. Our experiments not only allow us to link the rearing condition of larvae to later task performance, but aim to unravel the impact of larval rearing condition on important hormonal and metabolic aspects of adult maturation.

2. Materials and Methods

2.1. In Vitro Rearing

One group of honeybees could develop naturally in the colony (control), while another group of honeybees (treatment) was reared in the laboratory under the standard feeding diet as established previously [15] (160 µL diet; Table 1). We controlled for age by caging each queen onto an empty comb three days prior to the day of larval emergence. Cages were freely passable for workers. Upon emergence, the larvae were transported into the lab, individually grafted and placed into small plastic cups (Weisel cups, Heinrich Holtermann KG; Brockel, Germany). These cups, placed in a 48-well plate, contained 20 µL of diet "A". Well plates and cups were pre-heated to 35 °C and kept on 35 °C warm thermal mats (ThermoLux, Witte + Sutor GmbH, Murrhardt, Germany) while grafting. Afterwards, the well plates were transferred to an incubator maintained at 35 °C and 95% relative humidity (RH). Larvae were fed over six consecutive days according to Table 1. Once larvae transformed into pupae, they were transferred into fresh 48-well plates, transferred into a new incubator at 35 °C and 75% RH and left untouched, apart from sparse mortality check-ups, until emergence. For the group of bees reared in the colony, we removed frames with emerging brood and placed them in an incubator maintained at 35 °C and 75% humidity.

Table 1. Standard larval diet according to Aupinel et al. (2005). Feeding takes place over six days. On day one, larvae receive 20 μL of diet "A". On day three, larvae receive 30 μL of diet "B". On days four to six, larvae receive 30 μL, 40 μL, 50 μL, respectively.

Diet A	Royal Jelly	Fructose	Glucose	Yeast	Water
[%]	50	6	6	1	37
[g]	20	2.4	2.4	0.4	14.8
Diet B	Royal Jelly	Fructose	Glucose	Yeast	Water
[%]	50	7.5	7.5	1.5	33.5
[g]	20	3	3	0.6	13.4
Diet C	Royal Jelly	Fructose	Glucose	Yeast	Water
[%]	50	9	9	2	30
[g]	20	3.6	3.6	0.8	12

2.2. Comparison of Nursing Behavior

After emergence, in vitro-reared honeybees and colony-reared, newly emerged honeybees ("colony-reared controls") were marked using a colored number plate (Opalith Classic Garnitur; Heinrich Holtermann KG; Brockel, Germany) and superglue (UHU® Sekundenkleber blitzschnell Pipette; UHU GmbH & Co. KG; Bühl, Germany). After tagging, honeybees were transferred into cages (internal dimensions: 8 cm × 5 cm × 5 cm; three impenetrable walls and one wire-framed wall) containing 50% sugar water, normal tab water and a pollen source. These cages were left in an incubator overnight at 35 °C and 50% RH for the superglue to fully dry. Afterwards, the honeybees were integrated directly into a four-framed observation hive. Observations started the next day by removing the walls on the side of the observation hive and observing the honeybees through see-through Plexiglas walls.

Observations were conducted for four consecutive weeks. They started at 10:30 a.m. and finished at 2:30 p.m. every day. All four frames were scanned systematically in a pseudo-randomized order. Every visible honeybee bearing a number plate and putting its head into a brood cell was recorded. Every week, the brood area on the frames was marked to ensure that only nurse bees were recorded during the observations.

2.3. Comparison of Foraging Behavior

Newly emerged, in vitro-reared honeybees and colony-reared control honeybees were color-marked on the abdomen and tagged with a unique RFID (radio-frequency identification) chip (mic3-TAG 64-bit read only, carrier frequency: 13.56 MHz, Microsensys GmbH, Erfurt, Germany) using superglue. After tagging, honeybees were transferred into cages (same as above) containing 50% sugar water, normal tab water and a pollen source. These cages were left in an incubator overnight at 35 °C and 70% RH for the superglue to fully dry.

After the drying period, honeybees of all treatment groups were placed within the cages into a six-frame queen right mini-plus colony outfitted with two specifically designed scanners (MAJA Bundle Bee Identification System: iID 2000 ISO 15693 optimized, Microsensys GmbH). These scanners were positioned in front of the hive entrance so honeybees leaving or returning to the colony had to pass both scanners in a defined order. Data were acquired as previously established [32]. Only technical outliers were removed from the data sets. Data were excluded when the time interval between the first and second scans was larger than 300 s. Thus, we excluded foraging events when one of the scanners did not work. Additionally, only complete foraging trips were analyzed, i.e., when both events (leaving and returning to the hive) fulfilled our criteria. After one day, the cages were opened, and the tagged honeybees were able to freely move around the colony. This adaptation period was used to increase acceptance of the young honeybees once they had been released into the colony. Afterwards, the recordings began.

2.4. Weight, Juvenile Hormone, Triglycerides and Sucrose Responsiveness

Honeybees were treated in the same way as in Section 2.2. However, they were placed in cages into a single six-frame mini-plus colony as in Section 2.3. Every week, 15 honeybees of both groups were removed from the colony to perform various experiments.

Honeybees were immobilized on ice, mounted in metal holders and fed until saturation. After one hour of adjustment to the holders, the proboscis extension response (PER) experiment was conducted. The antennae of the honeybees were first touched with a droplet of water and, afterwards, sequentially with increasing sugar concentrations, as established previously, to determine the individual responsiveness to sucrose, with an intertrial interval of two minutes [31,33]. The occurrence of the proboscis extension was recorded for each stimulation of the antennae with water and the following sucrose concentrations with equal logarithmic distances: 0.1% sucrose, 0.3% sucrose, 1% sucrose, 3% sucrose, 10% sucrose and 30% sucrose. Afterwards, bees were individually weighed. Honeybees were then immobilized on ice for a second time and fixed with needles onto a Styrofoam plate. A 5 µL amount of hemolymph was extracted by piercing the cuticle in between the fourth and fifth abdominal segments using glass micro capillaries (servoprax®, A1 0115; servoprax GmbH, Wesel, Germany). The hemolymph was sampled for analyzing JH III titers. The 5 µL of hemolymph was blown out onto a parafilm surface using a thin hose and transferred into an Eppendorf Tube® (1.5 mL) using a pipette. The tubes were then stored in a container filled with liquid nitrogen. JH III in hemolymph was analyzed by LC-MS/MS using a Waters Acuity ultra-high performance liquid chromatography system coupled to a Waters Micromass Quattro Premier triple quadrupole mass spectrometer (Milford, MA, USA), as described before [34].

To analyze the triglycerides (TGs) of the fat bodies, one half of a honeybee's frozen fat body was crushed in a cooled mixer mill (MM 400, RETSCH GmbH, Haan, Germany) using zirconia beads. After a short centrifugation (Centrifuge 5424; Eppendorf, Hamburg, Germany), the triglycerides were extracted twice using chloroform (1 mL), methanol (0.5 mL) and two TAG standards (2.5 µg each, 10:0 TAG and 17:0 TAG). After mixing and centrifugation, the supernatant was collected and 0.88% aqueous KCl (0.75 mL) was added. After phase separation, the upper phase was discarded, and 0.25 mL methanol and 0.25 mL H_2O were added to the lower phase containing the lipid extract. Afterwards, the lower phase was dried under reduced pressure using a rotational vacuum concentrator (RVC 2-25 CDplus, Martin Christ Gefriertrocknungsanlagen GmbH; Osterode am Harz, Germany) at 50 °C. The dried residue was dissolved in 100 µL isopropanol and frozen at −20 °C until analysis with a UPLC–qTOF-MS (Waters Corporation; Milford, MA, USA), as described before [35]. The data were analyzed using MassLynx™ software from Waters®. Only the ten most frequently appearing TGs were used for statistical analysis.

2.5. Data Analysis

Statistical analyses and graph construction were conducted using GraphPad Prism (GraphPad Software Inc., V8, San Diego, CA, USA) and R (4.1.2) and the R packages "glmm TMB" v. 1.1.2.3 [36], "lme4" v. 1.1–27.1 [37], "DHARMa" v. 0.4.4 [38] and "lsmeans" v. 2.30-0 [39]. Proportional data were analyzed with a Chi-square test using GraphPad Prism. A Shapiro–Wilk test was used to test the nursing data for normal distribution. Since data were not distributed normally, a Mann–Whitney U test was used to analyze the nursing data using GraphPad Prism. Data for the foraging performance were not distributed normally (Shapiro–Wilk test). Effects of rearing condition on foraging performance was investigated with a generalized linear mixed model (GLMM) with treatment as fixed factor and the four different colonies as random factor and nbinom2 family. Physiological data were not distributed normally. Effects of rearing condition on physiology was investigated with a GLMM with treatment and weeks as fixed factors and nbinom2 family. Post hoc analysis was conducted using Tukey multiple comparison tests.

3. Results

3.1. Comparison of Nursing Behavior

In vitro rearing strongly affected the proportion of the bees performing nursing tasks (Figure 1A). Colony-reared honeybees had a significantly higher proportion of bees performing nursing behavior compared to in vitro-reared honeybees (Figure 1A and Table 2). However, the individuals actually performing nursing tasks did not differ in their task performance between rearing conditions, i.e., onset of nursing behavior (Figure 1B and Table 2), termination of nursing behavior (Figure 1C and Table 2) and nursing span (Figure 1D and Table 2).
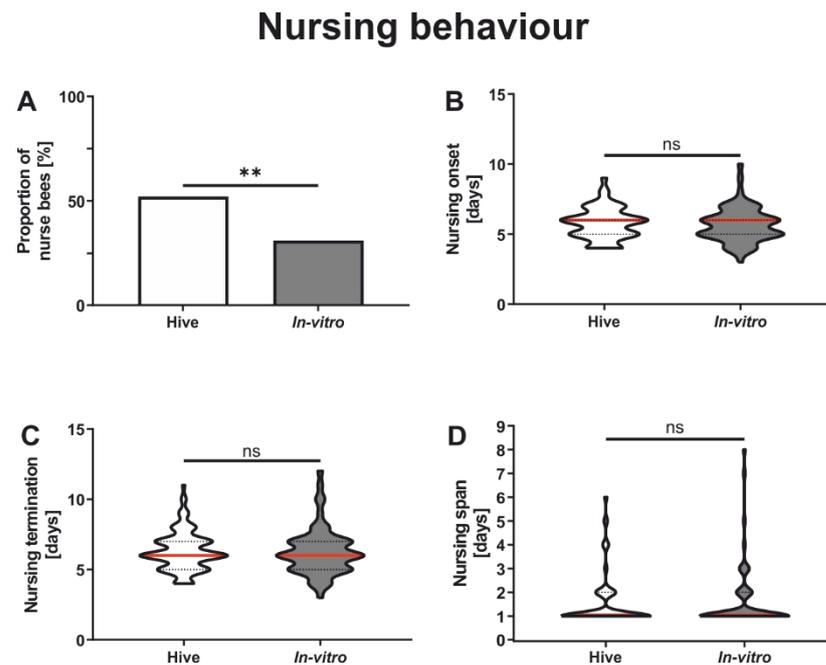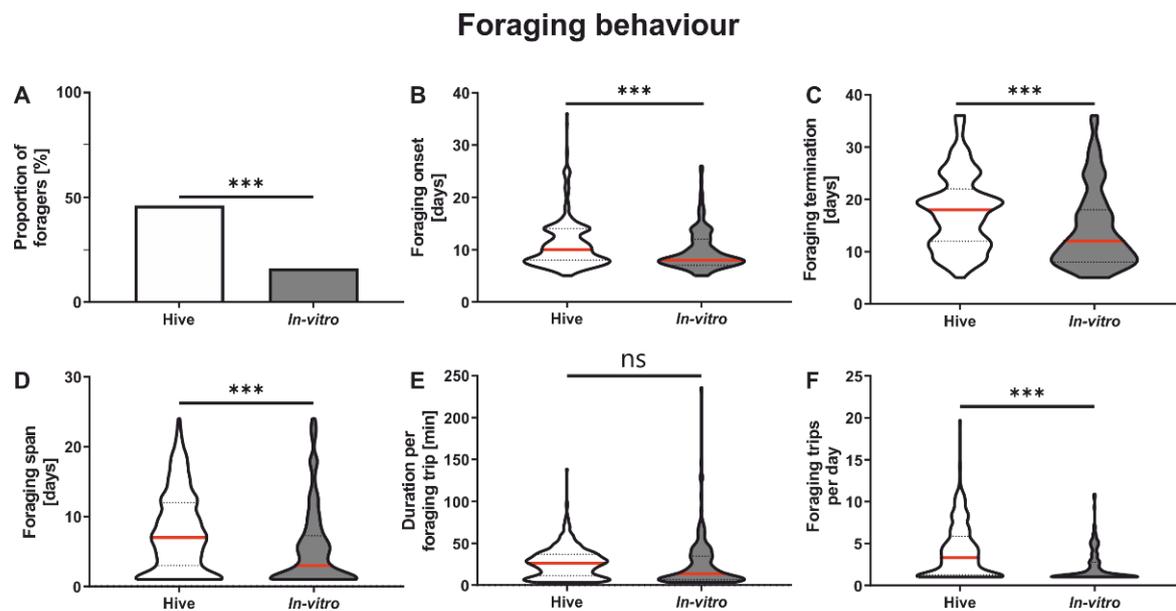

Figure 1. Influence of in vitro rearing on nursing behavior. (**A**): In vitro rearing significantly influenced the proportion of nurse bees. Significantly fewer in vitro-reared honeybees became nurses compared to colony-reared honeybees. (**B**): In vitro rearing did not affect the onset of nursing behavior. (**C**): In vitro rearing did not influence the termination of nursing behavior. (**D**): In vitro rearing did not affect nursing span. Significant differences between groups are indicated by asterisks (ns: $p > 0.05$, **: $p < 0.01$). For test statistics and sample size, see Table 2. Data in (**B–D**) display medians (red line) and 25% and 75% quartiles (lower and upper dotted lines, respectively).

Table 2. Test statistics for the analysis made in Figures 1 and 2.

Analysis	Figure	Treatment	Sample Size	Test	Test-Value	*p*
Nursing proportion	1A	Colony In vitro	199 192	Chi-square	χ = 8.16	<0.01
Onset of nursing	1B	Colony In vitro	104 62	Mann-Whitney	U = 3055	0.56
Termination of nursing	1C	Colony In vitro	104 62	Mann-Whitney	U = 3098	0.67
Nursing span	1D	Colony In vitro	104 62	Mann-Whitney	U = 3223	0.99
Foraging proportion	2A	Colony In vitro	1016 1005	Chi-square	χ = 21.04	<0.001
Onset of foraging Factor treatment	2B	Colony In vitro	472 164	GLMM	χ = 15.58	<0.001
Termination of foraging Factor treatment	2C	Colony In vitro	472 164	GLMM	χ = 34.14	<0.001
Foraging span Factor treatment	2D	Colony In vitro	472 164	GLMM	χ = 18.99	<0.001
Duration per foraging trip Factor treatment	2E	Colony In vitro	472 164	GLMM	χ = 0.03	0.8834
Foraging trips per day Factor treatment	2F	Colony In vitro	472 164	GLMM	χ = 54.31	<0.001

3.2. Foraging Behavior

　　In vitro rearing had a significant influence on the proportion of foragers (Figure 2A). A significantly higher proportion of colony-reared honeybees became foragers compared to in vitro-reared honeybees (Figure 2A and Table 2). We next focused on those bees of both groups which actually performed foraging flights.

Foraging behaviour

Figure 2. Influence of in vitro rearing on foraging behavior. (**A**): In vitro rearing significantly influenced the proportion of foragers. Significantly fewer in vitro-reared honeybees became foragers compared to colony-reared honeybees. (**B**): In vitro rearing significantly decreased the onset of foraging. (**C**): In vitro-reared honeybees terminated their foraging trips significantly earlier then colony-reared honeybees. (**D**): In vitro rearing significantly decreased the foraging span. (**E**): The duration per foraging trip was not influenced by the rearing environment. (**F**): In vitro-reared honeybees flew significantly fewer foraging trips per day then colony-reared honeybees. Significant differences between groups are indicated by asterisks (ns: $p > 0.05$, ***: $p < 0.001$). For test statistics and sample size, see Table 2. Data in (**B**–**F**) display medians (red line) and 25% and 75% quartiles (lower and upper dotted lines, respectively).

Foraging onset was significantly affected by rearing condition (Figure 2B and Table 2). In vitro-reared bees started foraging significantly earlier compared to colony-reared bees. The end of foraging was similarly affected by rearing environment (Figure 2C and Table 2) with in vitro-reared bees finishing foraging earlier than colony-reared bees. Since the onset and termination of foraging were earlier in the in vitro-reared group (Figure 2B,C), it is not surprising that foraging span was also significantly shorter in this group (Figure 2D and Table 2). While the rearing environment did not affect the duration of the foraging trips (Figure 2E and Table 2), it significantly affected the number of trips a forager performed per day (Figure 2F and Table 2).

3.3. Honeybee Morphology, Physiology and Sucrose Responsiveness

Honeybee weight decreased significantly with age across groups (Figure 3A and Table 3). Rearing condition did not affect honeybee weight (Figure 3A and Table 3). Titers of JH III increased significantly in both rearing groups during the first four weeks of adult life (Figure 3B and Table 3). However, treatment had a significant effect on JH III titers (Figure 3B and Table 3). In vitro-reared bees displayed a significantly lower JH III titer than colony-reared honeybees in week 3 and week 4 (Table 3), i.e., around the time when colony bees transition to foraging. Total lipids also changed significantly with the age of the bees after placement in the colony (Figure 3C and Table 3). In both rearing groups, lipids decreased over the four experimental weeks. Rearing condition affected lipid levels (Figure 3C and Table 3). In vitro-reared honeybees had significantly lower lipid levels in week 4 (Table 3). Individual sucrose responsiveness, measured as GRS, increased significantly with age in both rearing groups (Figure 3D and Table 3) and was unaffected by rearing environment (Figure 3D and Table 3).
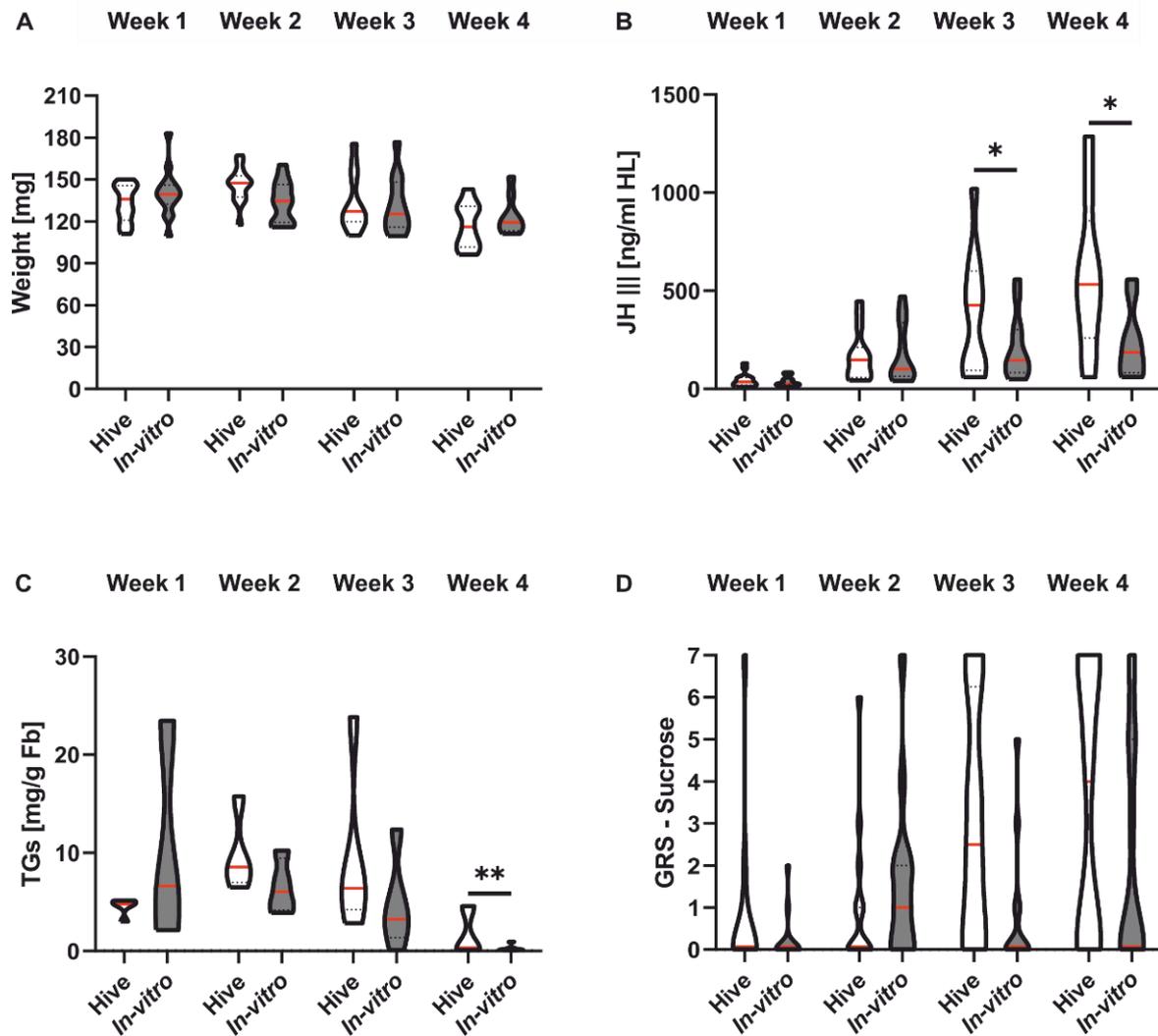
Figure 3. Body weight, JH III titers, TG levels and sucrose responsiveness of in vitro- and colony-reared honeybees in their first four weeks of life. (**A**): Honeybee body weight significantly decreased during the first four weeks of adult life. In vitro rearing did not influence honeybee weight. (**B**): Honeybee age significantly affected juvenile hormone tires (JH III), with older bees displaying higher JHIII titers. In addition, colony-reared bees had significantly higher JH III titers than in vitro-reared bees in week 3 and week 4. (**C**): Fat body triglyceride (TG) levels were significantly influenced by honeybee age, indicating a decrease in TG levels with increasing honeybee age. In vitro rearing significantly influenced TG titers in week 4. (**D**): Honeybee age significantly influenced the gustatory response scores (GRS). GRS generally increased with age. Rearing environment had no effect on GRS. For statistics, see Table 3. Significant differences between the two rearing groups are indicated by asterisks (*: $p < 0.05$; **: $p < 0.01$). The red line displays the median, and 25% and 75% quartiles are displayed by the lower and upper dotted lines, respectively.

Table 3. Test statistics for the analysis made in Figure 3.

Analysis	Figure	Treatment	Sample Size	Test	Test Value	p
Weight Factor Treatment	3A		Colony In vitro	63 66	GLMM	F = 0.06	0.804
Weight Factor Week	3A		Colony In vitro	63 66	GLMM	F = 43.27	<0.001
Weight Interaction Treatment and Week	3A		Colony In vitro	63 66	GLMM	F = 6.86	0.076
JH III Factor Treatment	3B		Colony In vitro	50 50	GLMM	χ = 5.36	<0.05
JH III Factor Week	3B		Colony In vitro	50 50	GLMM	χ = 146.70	<0.001
JH III Interaction Treatment and Week	3B		Colony In vitro	50 50	GLMM	χ = 6.04	0.11
JH III Pairwise Tukey Test	3B	Week 1	Colony In vitro	15 15	Tukey	t = 0.76	0.45
	3B	Week 2	Colony In vitro	15 14	Tukey	t = −0.30	0.77
	3B	Week 3	Colony In vitro	11 12	Tukey	t = 1.99	<0.05
	3B	Week 4	Colony In vitro	9 9	Tukey	t = 2.60	<0.05
TGs Factor Treatment	3C		Colony In vitro	17 21	GLMM	χ = 0.04	<0.05
TGs Factor Week	3C		Colony In vitro	17 21	GLMM	χ = 81.12	<0.001
TGs Interaction Treatment and Week	3C		Colony In vitro	17 21	GLMM	χ = 10.92	<0.05
TGs Pairwise Tukey Test	3C	Week 1	Colony In vitro	5 3	Tukey	t = −1.43	0.16
	3C	Week 2	Colony In vitro	4 4	Tukey	t = 0.70	0.49
	3C	Week 3	Colony In vitro	5 5	Tukey	t = 1.28	0.21
	3C	Week 4	Colony In vitro	3 9	Tukey	t = 3.31	<0.01
GRS Factor Treatment	3D		Colony In vitro	17 21	GLMM	χ = 3.69	0.055
GRS Factor Week	3D		Colony In vitro	17 21	GLMM	χ = 18.43	<0.001
GRS Interaction Treatment and Week	3D		Colony In vitro	17 21	GLMM	χ = 5.68	0.13

4. Discussion

This study investigated the influence of rearing environment on adult honeybee workers. Larvae were either raised in vitro in an established laboratory assay or in the colony under natural conditions. One main effect we observed concerned the likelihood that a worker bee performed nursing or foraging tasks. The likelihood for performing these tasks was significantly reduced in bees reared in vitro compared to naturally reared honeybees. Why fewer in vitro reared honeybees performed the tasks is unclear. One possible explanation is that naturally reared honeybees are more vital than in vitro-reared bees due to artificial rearing acting as a nutritional stressor. It has been shown that nutritional deficits during in vitro rearing can lead to under-developed honeybee workers [21], which, in turn,

might lead to the adult honeybees not nursing or foraging. In general, there are two main differences between the two rearing conditions. First, naturally reared larvae are fed by nurse bees according to their direct needs which are communicated by a brood pheromone signal [40]. Feeding larvae in the laboratory is based on an estimate of how much food a larva needs on average during development. Individual larvae might differ in their need for food, resulting in a larger span of possibly over-fed or under-fed larvae. The resulting honeybee workers might be less vital compared to their colony-reared sisters, leading to worker bees that are less capable to perform specific tasks. Another explanation might be the absence of fresh pollen in the diet of in vitro-reared larvae. During the in vitro rearing protocol, larvae are fed with a food mixture combining royal jelly with various concentrations of sugars and yeast [14,15,41]. However, while yeast is normally used as a pollen substitute, real pollen is not used during in vitro rearing to avoid moulding. This could negatively influence honeybee vitality because pollen quality and diversity are important for honeybees health and survival [42,43]. Additionally, it has been shown that bumblebees and probably also honeybees depend on fatty acids from pollen sources [44]. Increasing or decreasing fatty acid concentrations due to a yeast substitute could detrimentally affect honeybee health or behavior.

However, nurse bees which actually performed nursing tasks did not differ between rearing conditions. Honeybees reared in vitro started and terminated nursing tasks at a similar age as their naturally reared sisters. Another study investigating nursing behavior of in vitro-reared honeybees showed that these visited queen cells less frequently compared to colony-reared honeybees [13]. Uncovering the effects of in vitro rearing on the quality of worker and queen brood care could be an interesting question for future studies.

Intriguingly, in vitro rearing significantly influenced foraging behavior. In vitro-reared honeybees started foraging significantly earlier than bees reared in the colony but stopped foraging earlier than colony-reared bees, thus experiencing a significantly shorter foraging duration in total. Additionally, in vitro-reared honeybees also flew significantly fewer trips per day compared to naturally reared honeybees, indicating that in vitro-reared honeybees are not as strong as naturally reared honeybees and may not work as effectively as the latter. The duration of foraging trips was not significantly affected by in vitro rearing. However, Figure 2E suggests that the majority of in vitro-reared honeybees seem to have rather shorter foraging trips, lasting around 10 min, while the majority of hive-reared bees had flight durations of around 25 min, such as has been reported in other studies on untreated honeybees [45–47]. This question certainly deserves a more detailed investigation, since individual variation in flight duration can be large, as is also suggested by Figure 2E. Another study investigating the flight performance of in vitro-reared honeybees [24] did not find any differences in the distance flown between the two rearing conditions, which correlates with our study not finding any differences in the foraging duration. However, they showed differences in the maximum speed during the second flight with colony-reared honeybees reaching higher velocities than in vitro-reared honeybees. These results also indicate that naturally reared honeybees seem to be stronger and more vital compared to in vitro-reared honeybees.

To further investigate whether the difference in vitality also results in a different morphology or physiology, we looked at weight, JH III, TGs and GRS. We did not find any differences in weight between in vitro-reared and naturally reared honeybees. These results support earlier studies [48]. However, other studies also found in vitro-reared honeybees to be smaller [9,49]. These different results might be an effect of individually different metabolic needs, as discussed above. JH III increased with age, as frequently observed in honeybees [50–52]. However, during the third and fourth weeks of adult maturation, in vitro-reared honeybees had significantly lower JH III titers compared to colony-reared honeybees. These results seem contradictory, especially because in vitro-reared honeybees started foraging earlier. However, in vitro-reared honeybees stopped foraging around 14 days of age on average. Therefore, it is possible that in vitro-reared honeybees simply stopped foraging and returned to the colony to perform other tasks. This would correlate

with their lower JH III titers. It has been shown that reverted nurse bees (foragers that go back to nursing) also show lower JH III levels compared to same-aged foragers [53]. However, it could also mean that the sampled honeybees in week 4 never foraged and, therefore, had low JH III titers. Future studies should analyze both factors simultaneously to uncover why in vitro-reared honeybees show lower JH III titers.

Triglycerides generally increased until week two and decreased from week two until week four. This pattern is likely linked to the transition from nursing to foraging [54]. However, we also found a significant treatment effect in week four, with significantly lower TG levels of in vitro-reared honeybees compared to colony-reared honeybees. These results appear contradictory. Usually, foragers have high JH III titers and low TG levels and nurse bees show the opposite pattern [53,54]. However, in vitro-reared honeybees had a significantly lower probability of performing social tasks, so that the in vitro-reared bees whose hemolymph was sampled in week four may never have performed nursing and foraging tasks, thus displaying rather unusual TG titers, a conclusion further emphasized by the general activity during our observation experiments (Figure A1 in Appendix A). In addition, there seems to be no direct link between JH III titers and TG titers in in vitro-reared bees in week four.

Rearing conditions did not significantly influence GRS. However, a tendency can be observed that in vitro honeybees show a lower GRS than colony-reared honeybees (factor treatment $p = 0.055$, interaction treatment*weeks $p = 0.13$; Table 3). Similar effects have been found earlier. Three-week-old in vitro-reared honeybees were significantly less responsive towards sucrose than naturally reared honeybees [13]. These findings further support the hypothesis that our in vitro-reared foragers either reversed back to nursing behavior, or the honeybees analyzed during week three and four never started to forage because, in general, it was shown that nurse bees are less responsive to sucrose than foragers [31]. Future studies should investigate further the "fate" of in vitro-raised honeybees in the hive. Are they generally less likely to perform any task and rather serve as a "reserve" in the hive, or do they simply perform tasks which were not in the focus of our investigation?

5. Conclusions

Overall, we found that the probability for performing social tasks was significantly reduced by in vitro rearing, likely because these bees were slightly weaker, although their weight did not differ from colony-reared bees. The typical increase in JH III titers observed during the transition from hive tasks to foraging was much less pronounced in lab-reared bees, correlating with their lower likelihood of becoming a forager, indicating hidden physiological modifications due to in vitro rearing. Importantly, honeybees reared in vitro were still able to perform all tasks. Their foraging performance was slightly reduced compared to that of colony-reared bees and there was no difference in the performance of nursing tasks between both treatment groups. Our data thus show that the method of in vitro rearing is nonetheless suitable for investigating honeybee behavior and physiology, provided that comparisons between treatment groups are all based on in vitro-reared worker bees.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

We calculated the mean activity index (mAI) for honeybees used in the observation hive experiments (see Section 2.2) as shown by the formula below. Active honeybees are bees that performed any observed behavior while inactive honeybees performed no observable behavior at all. Thus, the higher the mAI, the more observable behaviors were performed by the honeybees. Colony-reared honeybees showed a significantly higher mAI compared to in vitro-reared honeybees (Figure 1A; *t*-test: *t* = 4.61, df = 56, *p* < 0.001).

$$mean\ activity\ index = \frac{(active\ honeybees - inactive\ honeybees)}{(active\ honeybees + inactive\ honeybees)}$$
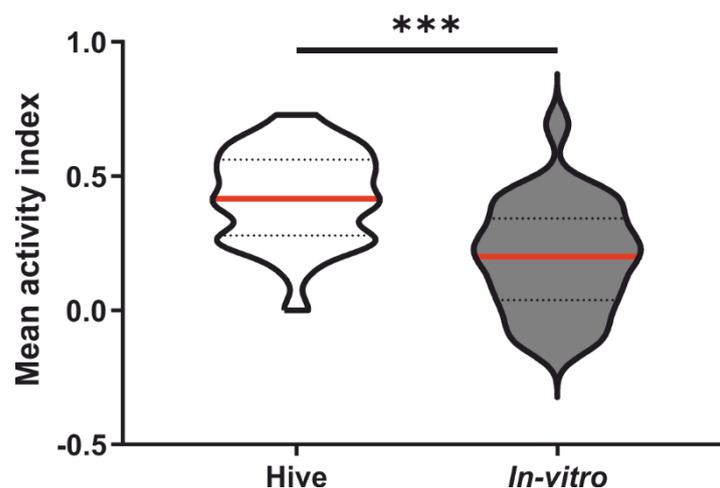
Figure A1. Mean activity index (mAI) of honeybees during the observation hive experiments. Colony-reared honeybees showed a higher mAI compared to in vitro-reared honeybees. Significant differences between the two rearing groups are indicated by asterisks (***: *p* < 0.001). The red line displays the median, and 25% and 75% quartiles are displayed by the lower and upper dotted lines, respectively. For details, see text.

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4

Honeybees are buffered against undernourishment during larval stages

1   # Honeybees are buffered against undernourishment during larval stages

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20   **Abstract**

21   The negative impact of juvenile undernourishment on adult behavior has been well reported for
22   vertebrates, but relatively little is known about invertebrates. In honeybees, nutrition has long been
23   known to affect task performance and timing of behavioral transitions. Whether and how a dietary
24   restriction during larval development affects the task performance of adult honeybees is largely
25   unknown.

26   We raised honeybees *in-vitro*, varying the amount of a standardized diet (150 µl, 160 µl, 180 µl in
27   total). Emerging adults were marked and inserted into established colonies. Behavioral performance
28   of nurse bees and foragers was investigated and physiological factors known to be involved in the
29   regulation of social organization were quantified.

30   Surprisingly, adult honeybees raised under different feeding regimes did not differ in any of the
31   behaviors observed. No differences were observed in physiological parameters apart from weight.
32   Honeybees were lighter when undernourished (150 µl), while they were heavier under the overfed
33   treatment (180 µl) compared to the control group raised under a normal diet (160 µl). These data
34   suggest that dietary restrictions during larval development do not affect task performance or
35   physiology in this social insect despite producing clear effects on adult weight. We speculate that

36 possible effects of larval undernourishment might be compensated during the early period of adult
37 life.

1   Introduction

39 Malnourishment has long been a topic of research when it comes to human development, especially
40 when observing malnourished children in third world countries. It has been shown that early
41 malnourishment can have sever cognitive, developmental and behavioral effects (Grantham-
42 McGregor, 1995; Berkman et al., 2002). However, little is known about long-term effects of
43 malnourishment in social insects such as honeybees. Especially in times of dearth, when beekeepers
44 add additional food to their honeybee colonies, inappropriate feeding might have severe side effects.

45 Honeybee colonies are highly complex superorganisms that depend on the proper execution and
46 timing of tasks by their members (Winston, 1987; Seeley, 1996). Apart from reproduction, all of the
47 colony tasks are executed by sterile female workers. Young honeybee workers perform in-hive tasks,
48 like nursing and cleaning, while older honeybees work at the periphery of the hive until they
49 eventually leave the hive to forage for resources. However, this temporal polyethism can be
50 accelerated, halted or even reversed (reviewed by Robinson, 1992). Depleting a honeybee colony of
51 foragers can lead to an increase in precocious foragers (Huang and Robinson 1996). However,
52 removing young bees from a colony leads to enlarged hypopharyngeal glands in foragers reverting to
53 nursing tasks. These glands are essential for producing brood food and normally deteriorate during
54 the transition from nursing to foraging. Schulz et al. (1998) showed that starving a colony by
55 removing most honey reserves can lead to an earlier onset of foraging most likely regulated by the
56 nutritional state of the bees. Toth et al. (2005) demonstrated that inhibiting honeybees from
57 synthesizing lipids similarly leads to an increase in precocious foraging.

58 However, multiple factors are involved in the transition from nursing to foraging, i.e. juvenile
59 hormone (JH), vitellogenin (VG) and responsiveness to sensory stimuli, to name but a few of them.
60 Treating honeybees with the JH analog methoprene started and stopped nursing tasks earlier
61 compared to the control (Robinson, 1987) and led to an earlier initiation of foraging. Nurse bees
62 generally have lower titers of JH than foragers but higher titers of VG (Fluri et al., 1982; Hartfelder
63 and Engels, 1998; Elekonich et al., 2001). This egg yolk precursor protein is generally assumed to be
64 the suppressor of JH, with JH possibly also suppressing VG by hitherto unknown mechanisms
65 (Amdam and Omholt, 2003). Once the suppressor VG is used up by nurse bees for producing brood
66 food, JH titers can increase and induce the nurse-forager transition. This transition coincides with an
67 increase in sucrose responsiveness which can serve as a behavioral indicator (Scheiner et al., 2004,
68 2013, 2017b; Scheiner and Erber, 2009). Additionally, downregulating *vitellogenin* gene expression
69 increases sucrose responsiveness of honeybees (Amdam et al., 2006). Other factors involved in the
70 transition are most likely biogenic amines, which are believed to modulate sensory responsiveness.
71 The involvement of octopamine in the transition from nursing to foraging has been shown by Schulz
72 et al. (2002). Treatment of honeybees with octopamine led to an increase in foraging. However, the
73 number of foragers increased even further when honeybees were treated with octopamine in
74 conjunction with methoprene. They concluded that octopamine acts more proximal than JH, possibly
75 modulating JH synthesis. Another study showed that octopamine can also increase sucrose
76 responsiveness of honeybees (Scheiner et al., 2002), linking division of labor and nutrition. The
77 metabolic precursor tyramine (Roeder, 2020) is also assumedly involved in the transition from
78 nursing to foraging (Hunt et al., 2007; Scheiner et al., 2017b). Scheiner et al. (2017a) showed that
79 injecting the neuromodulator tyramine into the fat body of honeybees can increase sucrose
80 responsiveness similar to octopamine. Another gene which is assumedly involved in the nurse-

81   forager transition is the *foraging* gene *Amfor* (Thamm and Scheiner, 2014; Thamm et al., 2018).
82   Foragers have higher levels of *Amfor* gene expression than nurse bees and activating protein kinase
83   G, which is encoded by *Amfor*, leads to an increased sucrose responsiveness, similar to that observed
84   when nurse bees transition to foragers.

85   Other studies have shown that some of these factors can be influenced by *Varroa destructor*, a
86   parasite feeding primarily on the fat body of honeybees (Ramsey et al., 2019), It has been shown that
87   infested honeybees show reduced nursing behavior, reduced hypopharyngeal gland development but
88   significantly higher JH titers (Zanni et al., 2018) and a premature initiation of foraging (Zanni et al.,
89   2017). Therefore, honeybee starvation, which directly affects the fat body (Corby-Harris et al., 2019),
90   might directly or indirectly affect the factors mentioned above, thereby decelerating or accelerating
91   the transition from nursing to foraging. Kaatz et al. (1994) showed that starving honeybees increases
92   JH production in foragers, but the increase was significantly stronger in nurse bees. However,
93   starvation does not only affect adult honeybees but also their larvae. Wang et al. (2016) showed that
94   starving honeybee larvae *in-vitro* can lead to increased juvenile hormone titers in newly emerged
95   workers and in seven-day-old worker bees, thus linking JH titers to starvation. We here test the
96   hypothesis that starvation during larval development induces a precautious increase in JH titers in
97   young adult worker bees, resulting in an earlier onset of foraging.

98   2   Material and Methods

99   2.1   *In-vitro* rearing

100   Honeybees were reared in the laboratory according to standardized *in-vitro* rearing protocol (Aupinel
101   et al., 2005, 2007, 2009; Crailsheim et al., 2015; Schmehl et al., 2016; Steijven et al., 2016;
102   Değirmenci et al., 2020; Schilcher et al., 2021). Three groups of honeybees were reared under
103   different diets (Table 1): 150 µl ("undernourished"), 160 µl ("normal diet") and 180 µl ("overfed").
104   Food was provided on six consecutive days according to Table 1 (Steijven et al., 2017). Honeybees
105   were reared according to Schilcher et al. (2021a). In short, age-controlled larvae were individually
106   grafted and placed into small plastic cups (Weisel cups, Heinrich Holtermann KG, Brockel,
107   Germany). These cups were transferred into 48-well plates and maintained in an incubator at 35 °C
108   and 95 % relative humidity (RH) over six days. Larval food contained royal jelly, fructose, glucose,
109   yeast and water according to Table 2. After pupation, the pupae were placed into fresh 48- well
110   plates, transferred into a new incubator and maintained at 35 °C and 75 % RH and left untouched
111   until emergence, apart from sparse mortality checkups.

112   **Table 1 Different feeding regimes for the three treatment groups.** Changes in food quantity accrued only on the 5th
113   and 6th days of feeding according to Steijven et al. (2017).

Treatment	Day 1 Diet A	Day 2	Day 3 Diet B	Day 4 Diet C	Day 5 Diet C	Day 6 Diet C
150 µl (undernourished)	20 µl	X	20 µl	30 µl	40 µl	40 µl
160 µl (normal diet)	20 µl	X	20 µl	30 µl	40 µl	50 µl
180 µl (overfed)	20 µl	X	20 µl	30 µl	50 µl	60 µl

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115

116 **Table 2 Standard larval diet according to Aupinel et al. (2005).** All treatment groups received the same diets
117 with variations in total food volume.

Diet A	Royal Jelly	Fructose	Glucose	Yeast	Water
[%]	50	6	6	1	37
[g]	20	2.4	2.4	0.4	14.8
Diet B	Royal Jelly	Fructose	Glucose	Yeast	Water
[%]	50	7.5	7.5	1.5	33.5
[g]	20	3	3	0.6	13.4
Diet C	Royal Jelly	Fructose	Glucose	Yeast	Water
[%]	50	9	9	2	30
[g]	20	3.6	3.6	0.8	12

118

119 **2.2 Behavioral experiments**

120 For the nursing behavior observations, emerging honeybees were marked using colored number
121 plates (Opalith Classic Garnitur; Heinrich Holtermann KG; Germany) and superglue (UHU®
122 Sekundenkleber blitzschnell Pipette; UHU GmbH & Co. KG; Germany). Afterwards, they were
123 transferred into cages (internal dimensions: 8 cm x 5 cm x 5 cm; three impenetrable- & one wire
124 framed wall) and were fed *ad libitum* with pollen, tab water and 50 % sugar water. After a night in an
125 incubator maintained at 35 °C and 50 % RH for the superglue to fully dry, the honeybees were
126 integrated into a four-frame observation hive. One day after the integration, the observations began
127 by removing one outside wall of the observation hive. Thus, the experimenter could observe the
128 honeybees through a see-through Plexiglas wall. Observations were conducted each day from 10:30
129 a.m. until 2:30 p.m. for four consecutive weeks. All four frames were scanned systematically in a
130 pseudo-randomized order, recording every visible honeybee with its head in a brood cell.

131 For the foraging behavior observations, honeybees were treated identically as before. However,
132 instead of colored number plates, radio-frequency identification (RFID) tags (mic3-TAG 64bit read
133 only, carrier frequency: 13.56 MHz, microsensys GmbH, Erfurt, Germany) were used to mark the
134 emerging honeybees (Hesselbach et al., 2020; Schilcher et al., 2021). After the drying period in the
135 incubator, the cages were placed into four six-frame queen-right mini plus colonies outfitted with two
136 specifically designed scanners (MAJA Bundle Bee Identification System: iID 2000 ISO 15693
137 optimized, Micro-Sensys GmbH). Both scanners were placed in front of the hive entrance and were
138 distinguishable by a unique number. Honeybees leaving from or returning to the colony had to pass
139 both scanners in a defined order. Data was acquired as established previously (Hesselbach et al.,
140 2020). Cages were opened after one day and the marked honeybees were able to move about freely in

141 the colony, while the recordings began. This adaptation period of one day was used to increase
142 acceptance of the young honeybees once they had been released into the hive.

143 **2.3   Weight, juvenile hormone, triglycerides and sucrose responsiveness**

144 Once in a week, five honeybees were removed from each colony and treatment to perform further
145 analyses. First, honeybees were immobilized on ice and weighted (Schilcher et al., 2021). Then they
146 were fixed in metal tubes and fed until satiation using 30 % sugar water (Scheiner et al., 2003a,
147 2003b). After one hour of adjustment, sucrose responsiveness was quantified using the proboscis
148 extension response (PER) assay (Scheiner et al., 2013, 2017b). First, the antennae of each bee were
149 touched with water. Afterwards, they were sequentially touched with increasing sucrose
150 concentrations of equal logarithmic distance (0.1 % sucrose, 0.3 % sucrose, 1 % sucrose, 3 %
151 sucrose, 10 % sucrose and 30 % sucrose) with an intertrial interval of 2 min to avoid intrinsic
152 sensitization (Scheiner et al., 2003a, 2003b). The occurrence of proboscis extension was recorded for
153 each stimulation of the antennae. The sum of the seven PER responses including water of an
154 individual honeybee represents the gustatory response score (GRS) as established previously
155 (Scheiner et al., 2004, 2013).

156 After quantifying individual sucrose responsiveness, honeybees of the different feeding regimes were
157 immobilized on ice for a second time and fixed with needles onto a Styrofoam plate. We extracted
158 5 µl of hemolymph by piercing the cuticle in between the fourth and fifth abdominal segments using
159 glass micro capillaries (servoprax®, A1 0115; servoprax GmbH; Germany). Hemolymph was stored
160 at -80 °C until analyzation. Levels of hemolymph JH were analyzed by LC-MS/MS using a Waters
161 Acuity ultrahigh-performance liquid chromatography system coupled to a Waters Micromass *Quattro*
162 *Premier* triple quadrupole mass spectrometer (Milford, MA) as described before (Scholl et al., 2014).
163 After the hemolymph extraction, the honeybees were frozen in liquid nitrogen and half of their fat
164 bodies was crushed in a cooled mixer mill (MM 400; Retsch) using zirconia beads. Afterwards, the
165 triglycerides were extracted twice using chloroform (1 ml), methanol (0.5 ml) and two triacylglycerol
166 (TAG) standards (2.5 µg each, 10:0 TAG & 17:0 TAG). After mixing and centrifugation, the
167 supernatant was collected and 0.88 % aqueous KCl (0.75 ml) was added. The upper phase was
168 discarded and 0.25 ml methanol and 0.25 ml H_2O were added to the lower phase containing the lipid
169 extract. Afterwards, the lower phase was dried under reduced pressure using a rotational vacuum
170 concentrator (RVC 2-25 CDplus; CHRIST) at 50 °C. The dried residue was dissolved in 100 µl
171 isopropanol and frozen at -20 °C until analysis with a UPLC–qTOF-MS (Synapt G2 HDMS; Waters)
172 as described in Mueller et al. (2015). The data was analyzed using MassLynx™ software from
173 Waters®. Only the ten most frequently appearing triglycerides (TGs) were selected for statistical
174 analysis as they represent more than 80 % of all TGs (Supplementary Figure 1).

175 **2.4   Statistics**

176 Statistical analyses were conducted using R (4.1.2). and the R packages "glmm TMB" V. 1.1.2.3
177 (Brooks et al., 2017), "lme4" V. 1.1-2s7.1 (Bates et al., 2015), "DHARMa" V. 0.4.4 (Hartig, 2021),
178 "rstatix" V 0.7.0 (Kassambara, 2021) and "lsmeans" V. 2.30-0 (Lenth, 2016). Proportional data was
179 analyzed with a χ^2 test. A Shapiro-Wilk test was used to test the data for normal distribution. Since
180 data was not distributed normally most of the time, effects of larval nutrition on task performance
181 were investigated with a generalized linear mixed model (GLMM). For the experiment studying
182 nursing behavior, larval nutrition was used as a fixed factor. For the experiment on foraging
183 behavior, larval nutrition was used as a fixed factor and the four different colonies were inserted into
184 the model as a random factor. The family was chosen according to the best fit in a DHARMa residual

185   analysis (Hartig, 2021). Physiological data was handled in the same way as the foraging data. Post-
186   hoc analyses were conducted using Tukey multiple comparison tests. Graphs were constructed using
187   R (4.1.2). and the R packages "ggplot2" V 3.35 (Wickham, 2016) and "cowplot" V 1.1.1 (Wilke,
188   2020)

189   **3   Results**

190   **3.1   Behavioral experiments**

191   Larval nutrition did not show strong effects on nursing behavior (Figure 1; Table 3). However, the
192   proportion of bees performing nursing tasks tended to decrease with increasing amounts of food (p =
193   0.075, χ^2 test, Figure 1A, Table 3). No effects were observed for the onset of nursing (Figure 1B;
194   Table 3), the termination of nursing (Figure 1C; Table 3) or the nursing span (Figure 1D; Table 3).
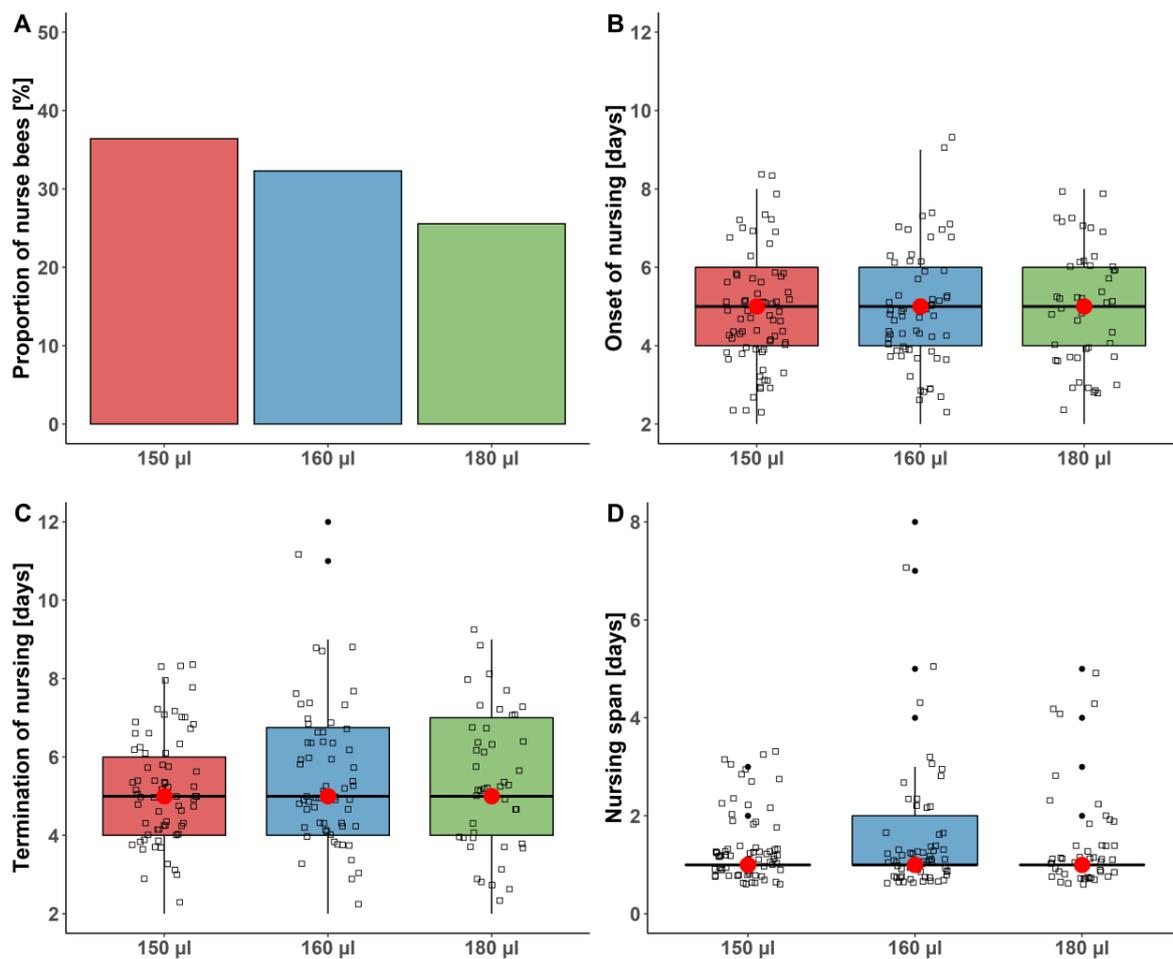
195

196   **Figure 1: Influence of larval nutrition on nursing behavior.** The red color indicates a diet of 150 µl
197   ("undernourished"), the blue color that of 160 µl ("normal diet"), and the green color indicates 180 µl of food supply
198   ("overfed"). (**A**) Effects of larval nutrition on the probability of performing nursing tasks. Overfed honeybees were less
199   likely to perform nursing tasks than undernourished honeybees and honeybees receiving the normal diet. *(B)* Larval
200   nutrition did not influence the onset of nursing. (**C**) Larval nutrition did not influence the termination of nursing. (**D**)
201   Larval nutrition did not influence the nursing span. For test statistics and sample size, see Table 3. Data in (A) is depicted

202 as bar graphs. Data in (B, C, D) is depicted as boxplots with upper quartiles (75 %) and lower (25 %) quartiles. The red
203 dots indicate the medians, black dots indicate possible outliers and square boxes indicate jittered individual data points.

204

205

206 **Table 3: Test statistics for the analysis conducted in Figures 1 and 2.**

Analysis	Figure	Treatment	Sample Size	Test	Test-Value	p
Nursing proportion	1A	150 µl 160 µl 180 µl	195 192 180	Chi^2	χ = 5.175	0.075
Onset of nursing	1B	150 µl 160 µl 180 µl	70 62 46	GLMM	χ = 0.681	0.712
Termination of nursing	1C	150 µl 160 µl 180 µl	70 62 46	GLMM	χ = 2.026	0.363
Nursing span	1D	150 µl 160 µl 180 µl	70 62 46	GLMM	χ = 2.518	0.284
Foraging proportion	2A	150 µl 160 µl 180 µl	1012 1005 1025	Chi^2	χ = 4.112	0.128
Onset of foraging Factor treatment	2B	150 µl 160 µl 180 µl	137 164 168	GLMM	χ = 0.211	0.899
Termination of foraging Factor treatment	2C	150 µl 160 µl 180 µl	137 164 168	GLMM	χ = 0.012	0.994
Foraging span Factor treatment	2D	150 µl 160 µl 180 µl	137 164 168	GLMM	χ = 0.283	0.868
Duration per foraging trip Factor treatment	2E	150 µl 160 µl 180 µl	137 164 168	GLMM	χ = 5.374	0.068
Foraging trips per day Factor treatment	2F	150 µl 160 µl 180 µl	137 164 168	GLMM	χ = 3.084	0.214

207

208 Larval nutrition did not affect foraging behavior (Figure 2; Table 3). Different feeding regimes had
209 no impact on the proportion of bees performing foraging tasks (Figure 2A; Table 3), the onset of
210 foraging (Figure 2B; Table 3), the termination of foraging (Figure 2C; Table 3), the foraging span
211 (Figure 2D; Table 3), the duration of a foraging trip (Figure 2E; Table 3) or the total number of
212 foraging trips per day (Figure 2F; Table 3). However, the duration of foraging trips tended to
213 decrease with increasing amounts of larval nutrition (p = 0.068, GLMM).

214
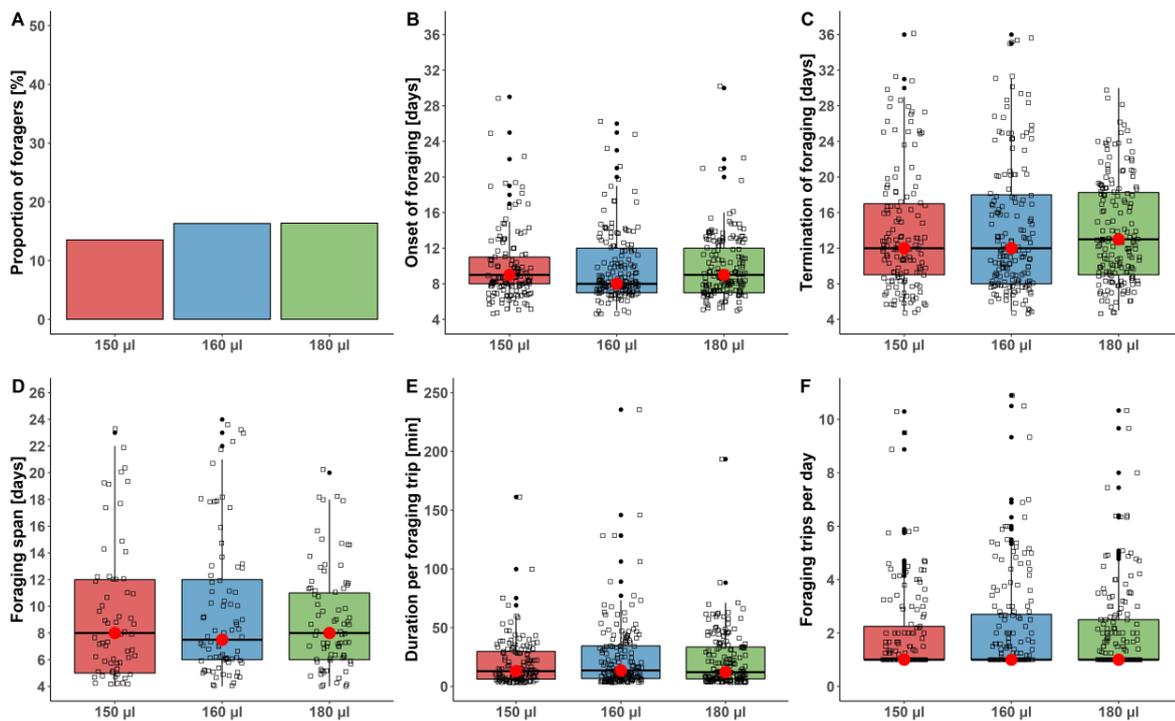
Figure 2: Influence of larval nutrition on foraging behavior. The red color indicates a diet of 150 µl
("undernourished"), the blue color that of 160 µl ("normal diet"), and the green color indicates 180 µl of food supply
("overfed"). **(A)** Larval nutrition did not influence the proportion of bees performing foraging tasks. **(B)** Larval nutrition
did not influence the onset of foraging. **(C)** Larval nutrition did not influence the termination of foraging. **(D)** Larval
nutrition did not influence the foraging span. (E) Larval nutrition did not influence the duration per foraging trip. (F)
Larval nutrition did not influence the foraging trips per day. For test statistics and sample size, see Table 3. Data in (A) is
depicted as bar graphs. Data in (B, C, D, E, F) is depicted as boxplots with upper quartile (75 %) and lower (25 %)
quartile. The red dots indicate the medians, black dots indicate possible outliers and square boxes indicate jittered
individual data points.

224

3.2 Weight, juvenile hormone titers, triglyceride levels and sucrose responsiveness

Larval nutrition had a significant effect on the weight of adult honeybees (Figure 3A; Table 4). As
we assumed, undernourished honeybees (150 µl) were lighter than bees receiving the normal diet
(160 µl) or overfed honeybees (180 µl). Interestingly, honeybee weight increased significantly with
age (Figure 3A; Table 4), especially in the undernourished group, indicating a way for honeybees to
compensate early food deprivation. However, the interaction between age and diet did not
significantly influence honeybee weight (Figure 3A; Table 4).

As expected, JH levels were significantly influenced by age, with older honeybees showing higher
JH levels then younger honeybees (Figure 3B; Table 4). However, neither diet nor the interaction
between age and diet significantly influenced JH levels (Figure 3B; Table 4).

Triglycerides were also affected by age. TG levels significantly increased in week 2 and decreased
from then onwards (Figure 3C; Table 4). However, neither diet nor the interaction between age and
diet significantly influenced TG levels (Figure 3C; Table 4). Sucrose responsiveness measured as
GRS was not influenced by neither diet nor time (Figure 3D; Table 4).

239
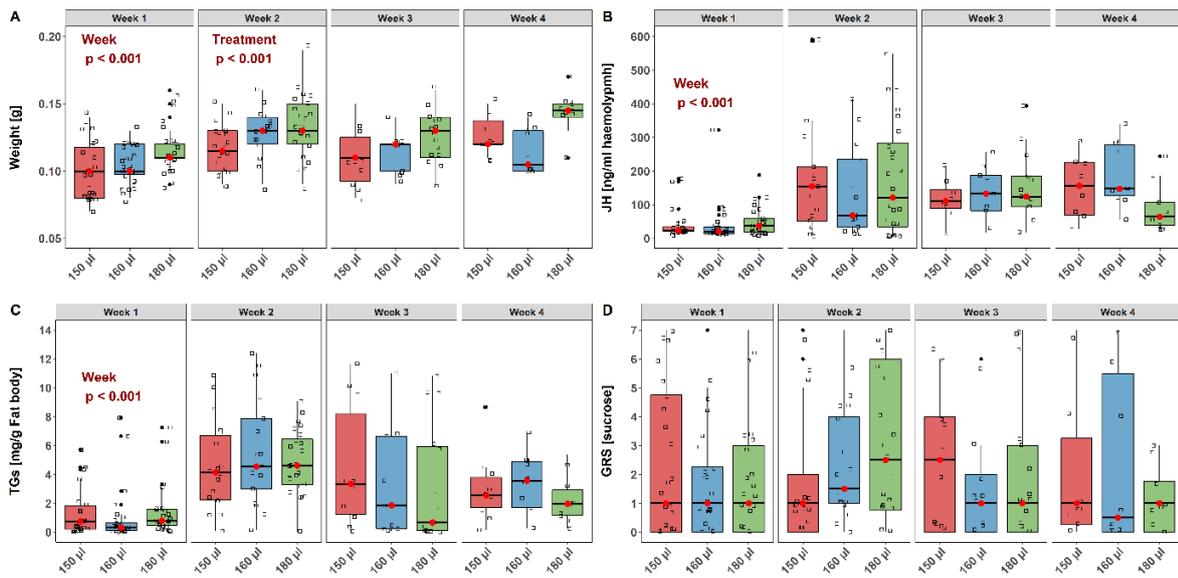
**Figure 3: Influence of larval nutrition on the body weight, JH III titers, TG levels and sucrose responsiveness of
adult honeybees in their first four weeks of life.** The red color indicates a diet of 150 µl ("undernourished"), the blue
color that of 160 µl ("normal diet"), and the green color indicates 180 µl of food supply ("overfed"). **(A)** Larval nutrition
significantly influenced adult honeybee weight, with honeybees receiving less food being significantly lighter. However,
weight increase with age. No significant interaction effect was found between age and diet. **(B)** JH levels were neither
affected by diet nor by the interaction between age and diet. However, age significantly increased JH levels. **(C)** TG
levels were neither affected by diet nor by the interaction between age and diet. However, TG levels increased in week
two and decreased afterwards. **(D)** Neither age nor diet affected the sucrose responsiveness measured as gustatory
response scores (GRS). For test statistics and sample sizes, see Table 4. Data is depicted as box plots with upper quartiles
(75 %) and lower (25 %) quartiles. The red dots indicate the medians, black dots indicate possible outliers and square
boxes indicate jittered individual data points.

251

Table 4: Test statistics for the analysis conducted in Figure 3.

Analysis	Figure	Treatment	Sample Size	Test	Test-Value	p
Weight Factor Week	3A	150 µl 160 µl 180 µl	66 65 74	GLMM	χ = 52.20	< 0.001
	Tukey Posthoc	*Weeks* *1-2* *1-3* *1-4* *2-3* *2-4* *3-4*	*p* *< 0.001* *0.08* *< 0.001* *0.048* *0.97* *0.047*			
Weight Factor Treatment	3A	150 µl 160 µl 180 µl	66 65 74	GLMM	χ = 33.74	< 0.001
	Tukey Posthoc	*Treatment* *150 µl – 160 µl* *150 µl – 180 µl* *160 µl – 180 µl*	*p* *0.424* *< 0.001* *< 0.001*			
JH Factor Week	3B	150 µl 160 µl 180 µl	61 65 72	GLMM	χ = 64.46	< 0.001

Honeybees are buffered against
undernourishment during larval stages

		Tukey Posthoc	*Treatment*	*p*			
			1-2	*< 0.001*			
			1-3	*< 0.001*			
			1-4	*< 0.001*			
			2-3	*0.587*			
			2-4	*0.058*			
			3-4	*0.583*			
JH Factor Treatment	3B	150 µl	61		GLMM	χ = 1.87	0.393
		160 µl	65				
		180 µl	72				
TG Factor Week	3C	150 µl	65		GLMM	χ = 80.79	< 0.001
		160 µl	64				
		180 µl	74				
		Tukey Posthoc	*Treatment*	*p*			
			1-2	*< 0.001*			
			1-3	*0.047*			
			1-4	*0.072*			
			2-3	*< 0.001*			
			2-4	*< 0.001*			
			3-4	*0.999*			
TG Factor Treatment	3C	150 µl	65		GLMM	χ = 0.58	0.748
		160 µl	64				
		180 µl	74				
GRS Factor Week	3D	150 µl	66		GLMM	χ = 2.36	0.534
		160 µl	65				
		180 µl	74				
GRS Factor Treatment	3D	150 µl	66		GLMM	χ = 0.06	0.958
		160 µl	65				
		180 µl	74				

253

4   Discussion

In this study we reared honeybees *in-vitro* under different larval diets with larvae receiving 150 µl of food ("undernourished"), 160 µl of food ("normal diet"), or 180 µl of food ("overfed"). We expected severe effects of undernourishment on adult honeybees as it has been shown multiple times that poor nutrition can severely affect honeybee colonies, especially during autumn when the flowers stop blooming (Brodschneider and Crailsheim, 2010; Döke et al., 2015; Ricigliano et al., 2018). As a reference point for undernourishment we used the standard artificial rearing diet which has been shown to be adequate for honeybee rearing (Aupinel et al., 2005, 2007, 2009; Schmehl et al., 2016; Schilcher et al., 2021). Earlier experiments showed effects of larval undernourishment on adult morphology, with undernourished larvae having slightly smaller thoraces and heads than honeybees reared under normal diet (Steijven et al., 2017). Similar to that earlier study we found clear effects of larval diet on morphology. We show that diet significantly affected the weight of adult honeybees. Undernourished honeybees were the lightest and overfed honeybees weighed the most (Figure 3A; Table 4) as shown before (Aupinel et al., 2005; Wang et al., 2016; Steijven et al., 2017). However, the clear differences seem to disappear over the weeks when weight generally increased (Table 4). Interestingly, undernourished honeybees tended to weigh even more then honeybees fed with the normal diet during the fourth experimental week. It seems that early starvation, which correlates with a reduced growth, can be compensated for during adult development.

Surprisingly, we found almost no effects of undernourishment on honeybee task performance and physiology, suggesting that physiology and behavior are not tightly linked to body weight and size.

Juvenile hormone titers increased from the first week until the last week. These results indicate a normal transition from in-hive tasks to foraging, as JH titers are known to increase with age and tasks

276   in normal honeybees (Robinson, 1987; Sullivan et al., 2000). Contrary to our expectation, we did not
277   observe an effect of diet on JH titers independent of age (Figure 3B; Table 4). An earlier study
278   showed that honeybees starved during larval development had increased JH hemolymph titers as
279   adults compared to controls (Wang et al., 2016). However, starvation treatment was conducted very
280   differently compared to our study. Wang et al. (2016) reared honeybee larvae inside a normal colony
281   and used pushing cages during the fifth larval instar to block nurse bees from feeding the larvae.
282   Older larvae consume an increasing amount of food (Aupinel et al., 2005, 2007, 2009; Schmehl et al.,
283   2016), leading to the conclusion that starvation during the fifth instar is more drastic than the
284   undernourishment we applied during our *in-vitro* rearing. Furthermore, honeybees without access to
285   food during the fifth larval instar lack pollen, royal jelly and more, while our undernourishment
286   protocol mainly restricts carbohydrates. Yet, pollen malnourishment has been shown to severely
287   effect bees (Scofield and Mattila, 2015; Kämper et al., 2016; Branchiccela et al., 2019; Grund-
288   Mueller et al., 2020). It seems likely that the honeybees can compensate well for larval
289   undernourishment during the first few days of adult emergence unless starvation becomes too drastic.

290   Triglyceride levels were not affected by the different diets but decreased significantly with age
291   (Figure 3C; Table 4) as has been shown before (Toth and Robinson, 2005), leading to the same
292   conclusion as posed for JH, i.e. slight undernourishment can be compensated during adult honeybee
293   development and does not affect lipid storage.

294   Furthermore, diet did not affect the proportion of honeybees performing either nursing or foraging
295   tasks (Figure 1A; Figure 2A; Table 3). However, we could observe a trend that honeybees receiving
296   less food also had a higher chance of performing nursing tasks (p = 0.075). Yet, this effect was lost
297   when observing foraging proportion (p = 0.128). This higher chance to become a nurse bee might
298   occur due to increased nutrient intake to compensate for undernourishment. Increased nutrient intake
299   has been shown to increase *vitellogenin* levels (Frias et al., 2016), which are also increased in nurse
300   bees (Peso et al., 2016) This further emphasizes the possible compensation of honeybees during adult
301   maturation.

302   These results seem to indicate that starvation resistance of honeybees might be two-fold. Pollen
303   availability during larval development and young adulthood seems to be crucial for honeybees. We
304   showed in a recent study that the *in-vitro* rearing protocol has a strong effect on adult honeybee task
305   performance and physiology (Schilcher et al., 2021). Honeybees reared *in-vitro*, which coincides
306   with a lack of pollen during development, performed significantly more poorly during foraging and
307   significantly fewer honeybees became foragers compared to hive reared controls. Scofield and
308   Mattila (2015) showed similar effects. Only 62 % of honeybees became foragers when larvae were
309   deprived of pollen, while about 82 % of honeybees became foragers when they were raised with an
310   abundance of pollen. They also showed that honeybees reared under pollen restricted conditions had
311   an earlier foraging onset and terminated their foraging trips sooner than honeybees reared with an
312   abundance of pollen (Scofield and Mattila, 2015). The same has been shown in our earlier study.
313   Honeybees reared *in-vitro* started and terminated their foraging flights earlier compared to controls
314   (Schilcher et al., 2021), while carbohydrate restriction did not have any effects (current study).
315   Additionally, Scofield and Mattila (2015) postulated an increase in mortality in pollen-restricted
316   colonies - an effect we also observed earlier (Schilcher et al., 2021). This effect cannot be seen under
317   carbohydrate restriction (current study), as diet had no effect on foraging span (Figure 2D; Table 3).

318   These results indicate carbohydrate restriction during larval development can be easily compensated
319   for during adult maturation but restriction of pollen cannot, possibly due to the lack of essential
320   amino acids, essential lipids or essential sterols during development (Feldlaufer et al., 1997; Roulston

321 and Cane, 2000; Nicolson et al., 2007; Sommano et al., 2020; Ruedenauer et al., 2021). Interestingly,
322 Scofield and Mattila (2015) showed significant effects of larval pollen deprivation on the weight of
323 adult honeybee workers. Pollen deprived honeybees weighed significantly less compared to
324 honeybees with an abundance of pollen, while honeybees reared under the standard *in-vitro* rearing
325 protocol weighed as much as honeybees reared in the hive (Schilcher et al., 2021). This indicates that
326 yeast supplement during *in-vitro* rearing supplies enough substance for growth but further
327 emphasizes the possibility of missing essential amino acids, essential lipids or essential sterols during
328 development.

329 In conclusion, the reduced amount of food supply during larval development appears not to lead to
330 gross behavioral deficits in contrast to a lack of pollen, suggesting that honeybees are well buffered
331 against this kind of nutritional stress, as long as essential amino acids, essential lipids or essential
332 sterols are present in normal proportions. Contrary to undernourishment in young humans
333 (Grantham-McGregor, 1995; Berkman et al., 2002), honeybees seem to be able to compensate for
334 short periods of larval undernourishment as long as they receive ample amounts of food as newly
335 emerged adults.

336

5   Conflict of Interest

338 The authors declare that the research was conducted in the absence of any commercial or financial
339 relationships that could be construed as a potential conflict of interest.

6   Author Contributions

341 Conceptualization, R.S. and I.S.-D.; methodology, F.S., L.H. and M.K..; data analysis, F.S., M.K.,
342 L.H. and M.A.; writing—original draft preparation, F.S.; writing—review and editing, F.S., M.K.,
343 M.A., L.H., R.S. and I.S.-D.; visualization, F.S.; supervision, R.S., I.S.-D. and M.J.M.; software, F.S.
344 and M.A.; project administration, I.S.-D., M.J.M. and R.S.; funding acquisition, I.S.-D. and R.S. All
345 authors have read and agreed to the published version of the manuscript.

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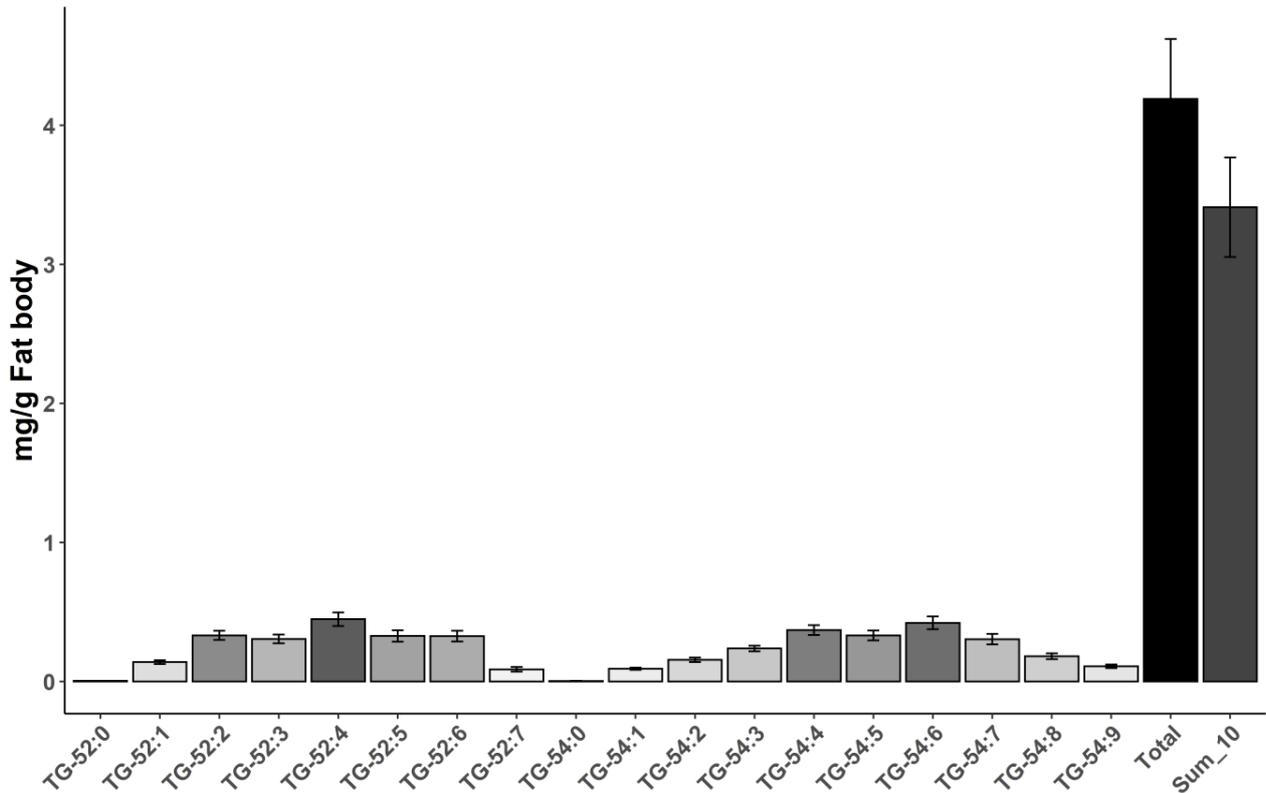
357

9   Data Availability Statement

359   Correspondence and requests for materials should be addressed to F.S. and R.S.

360

361   **10   Supplementary Material**

362

363   **Supplementary Figure 1.** Total amount of Triglycerides, sum of the ten most frequently appearing TGs and all TGs
364   measurable in the honeybee fat body of 130 honeybees. The sum of the ten most frequently appearing TGs is equal to
365   approx. 80% of the sum of all measured TGs in the honeybee fat body. The darker the color, the more mg TGs / g fat
366   body were present. Error bars show the standard error.

367

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Opposing Actions of Octopamine and Tyramine on Honeybee Vision

Article

Opposing Actions of Octopamine and Tyramine on Honeybee Vision

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Abstract: The biogenic amines octopamine and tyramine are important neurotransmitters in insects and other protostomes. They play a pivotal role in the sensory responses, learning and memory and social organisation of honeybees. Generally, octopamine and tyramine are believed to fulfil similar roles as their deuterostome counterparts epinephrine and norepinephrine. In some cases opposing functions of both amines have been observed. In this study, we examined the functions of tyramine and octopamine in honeybee responses to light. As a first step, electroretinography was used to analyse the effect of both amines on sensory sensitivity at the photoreceptor level. Here, the maximum receptor response was increased by octopamine and decreased by tyramine. As a second step, phototaxis experiments were performed to quantify the behavioural responses to light following treatment with either amine. Octopamine increased the walking speed towards different light sources while tyramine decreased it. This was independent of locomotor activity. Our results indicate that tyramine and octopamine act as functional opposites in processing responses to light.

Keywords: biogenic amines; neurotransmitter; phototaxis; *Apis mellifera*; ERG; behaviour; modulation; visual system; octopamine; tyramine

1. Introduction

The biogenic amines octopamine (OA) and tyramine (TA) play decisive roles in the modulation of honeybee behaviour. They can act as neurohormones, neurotransmitters and neuromodulators [1–7]. Octopamine is present in the honeybee brain in a concentration of up to 900 pg/brain [8,9]. Tyramine is present in the honeybee brain in much smaller quantities, ranging between 50 and 65 pg/brain [10]. Five different OA receptor genes and two TA receptor genes have been characterized in the honeybee. The activation of the respective receptor proteins can turn on very different intracellular second messenger cascades, which differentially affect physiology and behaviour. *AmOctαR1* leads to a change in intracellular calcium ($[Ca^{2+}]_i$) when activated [11]. The remaining receptors are coupled with the adenylyl cyclase and lead to changes in intracellular cAMP ($[cAMP]_i$) when activated. AmOctαR2 and *AmTAR1* decrease $[cAMP]_i$ [12,13], while AmOctβR1/2/3-4 and AmTAR2 increase $[cAMP]_i$ [14,15].

Based on the structural similarities of the receptor subtypes and intracellular signalling it is generally assumed that the insect OA/TA system is comparable to the deuterostome epinephrine/norepinephrine system [4,5,7,16,17]. Furthermore, some studies show that OA and TA can have opposite effects, indicating that both amines may act functionally antagonistically [18,19]. Octopamine has frequently been shown to increase responsiveness to different stimulus modalities in honeybees and other insects [1,4–7,20]. *Drosophila melanogaster* mutants lacking a functional tyramine β hydroxylase, which mediates the final step in OA synthesis, showed a significantly reduced responsiveness to sucrose due to the lack of OA

or an increase in TA titres [21]. Additionally, OA plays further important roles in associative learning and memory [1,4,22] by mediating and modulating the reward in appetitive learning [7,23,24]. In contrast to OA, the role of TA has been studied less intensively. The *Drosophila* mutant *honoka* displays a reduced expression of the TAR1 receptor [25]. These mutant flies are slightly hyperactive, have defects in olfactory perception and display a reduced TA-induced muscle contraction. Furthermore, TA was shown to rescue cocaine sensitization defects in *Drosophila* iav/TRPV channel mutants [26].

In the honeybee, OA and TA have been shown to have functionally similar effects in different taste-related behaviours. Both amines accelerate the rate of habituation of the proboscis extension response (PER) [27] and increase responsiveness to sucrose in honeybees [7,28,29]. They can both improve appetitive learning performance in the PER assay, most likely by increasing responsiveness to the reward [10,30]. Further, both OA and TA are involved in the social organisation of honeybees. Octopamine speeds up the adult behavioural maturation [31] and is associated with the transition from hive work to foraging [32]. Additionally, expression of the OA receptor gene *AmOctαR1* is higher in foragers compared to nurse bees [33] and the levels of both biogenic amines are higher in foragers compared to nurse bees as well [9,10]. The TA receptor gene *AmTAR1* is located on a quantitative trait locus linked to foraging behaviour [34]. However, how OA and TA affect division of labour in honeybees is unclear. They assumedly modulate sensory responses to sensory stimuli, i.e., gustatory, visual and olfactory cues, as has been shown for the gustatory system.

While some behavioural evidence suggests that OA plays in honeybee vision, little is known about the role of TA in the visual system. Octopamine was shown to enhance the direction-specific antennal responses during presentation of moving stripe patterns [35]. Whether TA affects this behaviour is unknown. Interestingly, in honeybee nectar foragers we showed earlier that TA enhances phototaxis without affecting locomotor behaviour. Octopamine had the opposite effect, i.e., reducing the walking speed towards a light source [20].

To differentiate effects of both amines on the periphery and on the decision-making processes in the central nervous system, we performed comparative studies using electroretinography (ERG) and phototaxis. To investigate whether the substances have a similar effect on honeybees of different ages or behavioural groups, we used young nurse-aged bees and forager bees.

2. Materials and Methods

2.1. Honeybees

Honeybees (*Apis mellifera carnica*) were reared at the departmental apiary and collected individually in uncoated bottles with snap-on caps. After collection, the honeybees were placed into cages and maintained in an incubator (30 °C—constant darkness) overnight with access to 50% sugar solution *ad libitum*. Bees were tested for behaviour and/or sensory responses on the following day. Different honeybee groups were chosen for the two experiments. For the ERG experiments, returning non-pollen foragers were caught directly at the hive entrance. For the phototaxis experiments, two groups of honeybees were collected. Foragers were sampled during winter and were therefore collected inside a glasshouse at a feeder containing a 50% sucrose solution. Young honeybees were sampled in spring. Here, we marked newly emerged honeybees and inserted them into an existing colony. They were collected 6 to 14 days later to be used for the experiments, thus representing hive bees. Due to the experimental design and a timespan of 6 months during the experiments, different bee groups had to be used for the different experiments. However, during the same experiment, the two treatment groups were identical.

2.2. Electroretinography

For the ERG, a honeybee was removed from the cage, immobilized on ice and mounted on a small acrylic glass block. After mounting, the honeybee's mandibles and neck were

fixed with low melting dental wax. A tiny window was cut into the head capsule between the antenna base and the ocelli. The window could be folded backwards to apply the substances. The glands and trachea dorsal to the honeybee´s brain were removed. A reference electrode (silver wire, diameter: 25 µm; Nilaco, Tokyo, Japan) was placed into one eye and connected to a common ground, whilst the recording electrode, located inside a glass capillary (1B100F-3, WPI, Sarasota, FL, USA) was pulled with a DMZ-Universal Puller as done before [36] and filled with 0.1 M KCl; it was then inserted into the contralateral eye. The signal was amplified 50x (Neuroprobe Amplifier 1600, A-M Systems, Inc, Sequim, WA, USA) and high-pass filtered by an acquisition board (Labtrax 4/16, WPI, Sarasota, FL, USA) and recorded using LabScribe 2 (iWorx Systems Inc., Dover, NH, USA).

A xenon light source (Perkin Elmer optoelectronics, XL2000 Fiber Optic Illumination System) emitting a daylight spectrum between 350 nm and 800 nm was used in combination with three greyscale intensity filters (36%–, 59%– and 100% light intensity) to stimulate the compound eye with a maximum light intensity of 4.95×10^{16} photons/cm^2. To open the light beam we used a manual shutter with a shutter time of 85 ± 6 ms. After placing a honeybee into the ERG setup, it was allowed to rest in constant darkness for 15 min before starting the stimulation with the three intensities in ascending order. Each intensity was applied four times with an inter-trial interval and an inter-stimulus interval of 1 min (Pre-test). Next, the head window was opened and 1 µL of Ringer (270 mM NaCl, 3.2 mM KCl, 1.2 mM CaCl$_2$, 10 mM MgCl$_2$, 10 mM 3-(*N*-morpholino) propanesulfonic acid, pH 7.4) or 1 µL of either OA or TA in one of three concentrations (10^{-3} mol/L, 10^{-4} mol/L or 10^{-5} mol/L—diluted in Ringer) was applied directly onto the honeybee brain. After another adaptation period of 15 min, the intensities were applied in the same way as in the Pre-test (Post-test). All concentrations for one substance as well as one Ringer control were tested each day using a new honeybee for each substance. The testing order of the substances was distributed randomly. Individual raw ERG data are shown for OA and TA respectively (Figure S1).

2.3. Phototaxis Assay

For the phototaxis experiment, a honeybee was removed from the cage, immobilized on ice and mounted in a small plastic tube. Thereafter, the median ocellus was punctured using a small micro dissecting needle. Afterwards, it was injected with 300 nL of Ringer (see above), tyramine (10^{-2} mol/L—diluted in Ringer) or octopamine (10^{-2} mol/L—diluted in Ringer) using a micro manipulator and glass capillaries (1B100F-4, WPI, Sarasota, FL, USA), pulled as described above. Each honeybee was allowed to rest in a Petri dish (diameter = 85 mm) for 15 min in constant darkness. Then it was placed inside the phototaxis arena to measure light responsiveness and walking parameters. The phototaxis assay was analysed as described before [20,36,37]. In short, dark-adapted honeybees were individually placed inside the phototaxis arena and their mean velocity in darkness was recorded for 2 min to measure their locomotor activity. Afterwards, the arena was illuminated with different green light-emitting diodes (LEDs, wavelength = 527 nm) of different light intensities (3%, 6%, 12%, 25%, 50% and 100% light intensity) with a maximum light intensity of 2.61×10^{14} photons/cm^2. For technical reasons, we used non-modifiable LEDs in the phototaxis assay and not the same white light source as in the ERG setup. Two LEDs with the same light intensity were placed opposite each other. Once a honeybee reached a light source, the LED was switched off, and the opposite LED with the same light intensity was switched on. This was repeated four times for each light intensity. Each experiment was started by switching on the LED with the lowest light intensity. The other LEDs were turned on in ascending order. The honeybee´s walking time towards each light source was recorded using a computer stopwatch (Comfort Software Group) [37]. Honeybees for one treatment and the corresponding Ringer controls were tested each day in a pseudo random order from 9 am until 4 pm. Honeybees were kept in the cage with ad libitum access to 50% sucrose solution under constant darkness until injection with the treatment or Ringer.

2.4. Data Analysis

For the ERG experiment, data were recorded using LabScribe 2 (iWorx Systems, Inc.). We extracted the maximal response amplitude of the ERG response to compare them before (pre) and after (post) treatment using a one-way ANOVA. Post hoc analyses were conducted using Dunnett's multiple comparison test. Differences in the ERG responses elicited due to the three light intensity filters were analysed with a repeated measures (RM) one-way ANOVA followed by a Bonferroni´s multiple comparison test. For the phototaxis experiment, the mean velocity during the dark-runs was analysed using a Student's *t*-test, since data were distributed normally. The means of one honeybee of four trials for each light intensity were calculated, compared and analysed with a RM 2-way ANOVA using GraphPad PRISM (GraphPad Software Inc., V7, San Diego, CA, USA). Post hoc analyses were conducted using Bonferroni´s multiple comparison test.

3. Results

3.1. The Effect of Octopamine and Tyramine on the ERG Response

Prior to treatment with either amine or the control solution, we investigated whether the three light intensities tested elicited differential receptor responses. In general, the more transparent the filter, the higher the receptor responses independent of the treatment (RM one way ANOVA, $p < 0.001$; for details see Table 1).

Table 1. Analysis of the ERG response of three light intensity filters before and after the application of octopamine/tyramine (treatment) or Ringer (control).

OA—Pre-Response	Friedmann Test		$\chi^2_{(2)}$	*p* Value	
Ringer—Control			14.63	<0.001	***
	Dunn´s test	*n* (Ringer)	*n* (Treatment)		
	36% vs. 59%	16	16	0.024	*
	36% vs. 100%	16	16	<0.001	***
	59% vs. 100%	16	16	0.867	n.s.
OA—post response Ringer—Control	Friedmann test		$\chi^2_{(2)}$ 16.63	*p* value <0.001	***
	Dunn´s test	*n* (Ringer)	*n* (Treatment)		
	36% vs. 59%	16	16	0.008	**
	36% vs. 100%	16	16	<0.001	***
	59% vs. 100%	16	16	0.99	n.s.
OA—pre-response OA—Treatment	Friedmann test		$\chi^2_{(2)}$ 26.79	*p* value <0.001	***
	Dunn´s test	*n* (Ringer)	*n* (Treatment)		
	36% vs. 59%	48	48	0.001	**
	36% vs. 100%	48	48	<0.001	***
	59% vs. 100%	48	48	0.99	n.s.
OA—post response OA—Treatment	Friedmann test		$\chi^2_{(2)}$ 38.17	*p* value <0.001	***
	Dunn´s test	*n* (Ringer)	*n* (Treatment)		
	36% vs. 59%	48	48	<0.001	***
	36% vs. 100%	48	48	<0.001	***
	59% vs. 100%	48	48	0.459	n.s.
TA—pre-response Ringer—Control	Friedmann test		$\chi^2_{(2)}$ 24	*p* value <0.001	***
	Dunn´s test	*n* (Ringer)	*n* (Treatment)		
	36% vs. 59%	12	12	0.043	*
	36% vs. 100%	12	12	<0.001	***
	59% vs. 100%	12	12	0.043	*

Table 1. *Cont.*

OA—Pre-Response	Friedmann Test		$\chi^2_{(2)}$	*p* Value	
TA—post response Ringer—Control	Friedmann test		$\chi^2_{(2)}$	*p* value	
			24	<0.001	***
	Dunn´s test	*n* (Ringer)	*n* (Treatment)		
	36% vs. 59%	12	12	0.043	*
	36% vs. 100%	12	12	<0.001	***
	59% vs. 100%	12	12	0.043	*
TA—pre-response TA—Treatment	Friedmann test		$\chi^2_{(2)}$	*p* value	
			70.06	<0.001	***
	Dunn´s test	*n* (Ringer)	*n* (Treatment)		
	36% vs. 59%	36	36	<0.001	***
	36% vs. 100%	36	36	<0.001	***
	59% vs. 100%	36	36	<0.001	***
TA—post response TA—Treatment	Friedmann test		$\chi^2_{(2)}$	*p* value	
			62.39	<0.001	***
	Dunn´s test	*n* (Ringer)	*n* (Treatment)		
	36% vs. 59%	36	36	<0.001	***
	36% vs. 100%	36	36	<0.001	***
	59% vs. 100%	36	36	<0.001	***

Significant differences are indicated by asterisks (n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Afterwards, we measured the ERG responses of honeybees following treatment with OA, TA or Ringer. Octopamine had a significant overall effect on the ERG response compared to the Ringer control at all three light intensities (Figure 1A–C; Table 2). It significantly increased the ERG response at all three light intensities for a concentration of 10^{-3} mol/L (Table 2). Tyramine had a significant overall effect on the pre-post response compared to the control at two out of three light intensities (Figure 1E–F; Table 2). It significantly decreased the ERG response at 59% light intensity and 100% light intensity for a concentration of 10^{-4} mol/L (Table 2).

Table 2. Statistical comparison of the octopamine/tyramine (treatment) or Ringer (control) pre-post receptor response in the honeybee retina.

OA—Pre-Post Response	1way ANOVA			F (3, 54)		*p* Value	
- 36% light intensity				4091		0.011	*
	Dunnett´s test	*n* (Ringer)	*n* (Treatment)	q	DF		
	R vs. OA 10-3	13	14	3457	54	0.003	**
	R vs. OA 10-4	13	15	1667	54	0.237	n.s.
	R vs. OA 10-5	13	16	1.37	54	0.386	n.s.
OA—pre-post response - 59% light intensity	1way ANOVA			F (3, 55)		*p* value	
				3176		0.031	*
	Dunnett´s test	*n* (Ringer)	*n* (Treatment)	q	DF		
	R vs. OA 10-3	13	14	3078	55	0.009	**
	R vs. OA 10-4	13	16	1768	55	0.197	n.s.
	R vs. OA 10-5	13	16	1544	55	0.293	n.s.
OA—pre-post response - 100% light intensity	1way ANOVA			F (3, 57)		*p* value	
				2821		0.0469	*
	Dunnett´s test	*n* (Ringer)	*n* (Treatment)	q	DF		
	R vs. OA 10-3	13	16	2744	57	0.022	*
	R vs. OA 10-4	13	16	1232	57	0.471	n.s.
	R vs. OA 10-5	13	16	2118	57	0.098	n.s.

Table 2. *Cont.*

| OA—Pre-Post Response	1way ANOVA			F (3, 54)		*p* Value	
TA—pre-post response - 36% light intensity	1way ANOVA			F (3, 39) 1.16		*p* value 0.337	n.s.
	Dunnett´s test	*n* (Ringer)	*n* (Treatment)	statistics	DF		
	R vs. TA 10-3	11	10	1404	39	0.37	n.s.
	R vs. TA 10-4	11	11	0.892	39	0.703	n.s.
	R vs. TA 10-5	11	11	0.232	39	0.991	n.s.
TA—pre-post response - 59% light intensity	1way ANOVA			F (3, 39) 3304		*p* value 0.03	*
	Dunnett´s test	*n* (Ringer)	*n* (Treatment)	q	DF		
	R vs. TA 10-3	11	11	2	39	0.211	n.s.
	R vs. TA 10-4	11	10	2677	39	0.029	*
	R vs. TA 10-5	11	11	0.162	39	0.997	n.s.
TA—pre-post response - 100% light intensity	1way ANOVA			F (3, 39) 3492		*p* value 0.025	*
	Dunnett´s test	*n* (Ringer)	*n* (Treatment)	q	DF		
	R vs. TA 10-3	11	10	2373	39	0.059	n.s.
	R vs. TA 10-4	11	10	2702	39	0.027	*
	R vs. TA 10-5	11	12	0.6678	39	0.844	n.s.

Significant differences are indicated by asterisks (n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$).
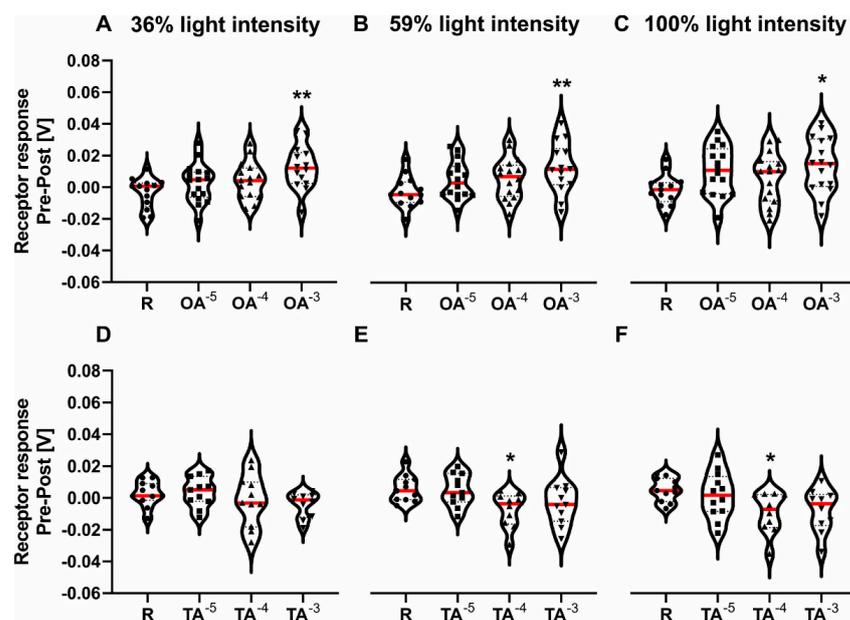
Figure 1. ERG−amplitude receptor response pre-post for three concentrations of octopamine (x-axis in **A**–**C**) and three concentrations of tyramine (x-axis in **D**–**F**) and the respective Ringer control (R). The different light intensities are shown at the top. The median is marked in red. Dots, squares, upper triangles and lower triangles represent individual data points for Ringer, OA/TA 10^{-5} mol/L, OA/TA 10^{-4} mol/L, OA/TA 10^{-3} mol/L, respectively. (**A**–**C**): Pre-post response after OA and Ringer were applied using 36%, 59% and 100% light intensity filters for A, B, and C respectively. A significant overall effect was found for all three light intensities. OA 10^{-3} mol/L significantly increased the amplitude (for statistics, see Table 2). (**D**–**F**): Pre-post responses after TA or Ringer were applied using 36%, 59% and 100% light intensity filters for D, E, and F respectively. No significant differences were found for the 36% light intensity filter. A significant overall effect was found for 59% and 100% light intensity. TA 10^{-4} mol/L significantly decreased the amplitude (for statistics, see Table 2). Significant differences between Ringer and either treatment are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$).

3.2. Tyramine and Octopamine Have Opposite Effects on the Phototaxis of In-Hive Bees and Foragers

Neither OA (Figure 2A, Table 3) nor TA (Figure 2B, Table 3) affected locomotor behaviour of in-hive bees in the dark arena. Light intensity significantly influenced phototaxis of in-hive bees in both experiments (Figure 2C,D; Table 3). Octopamine significantly decreased the time in-hive bees took to walk towards the switched-on light source compared to the control group (Figure 2C, Table 3), while TA significantly increased the time in-hive bees needed to reach the different switched-on light sources (Figure 2D, Table 3).
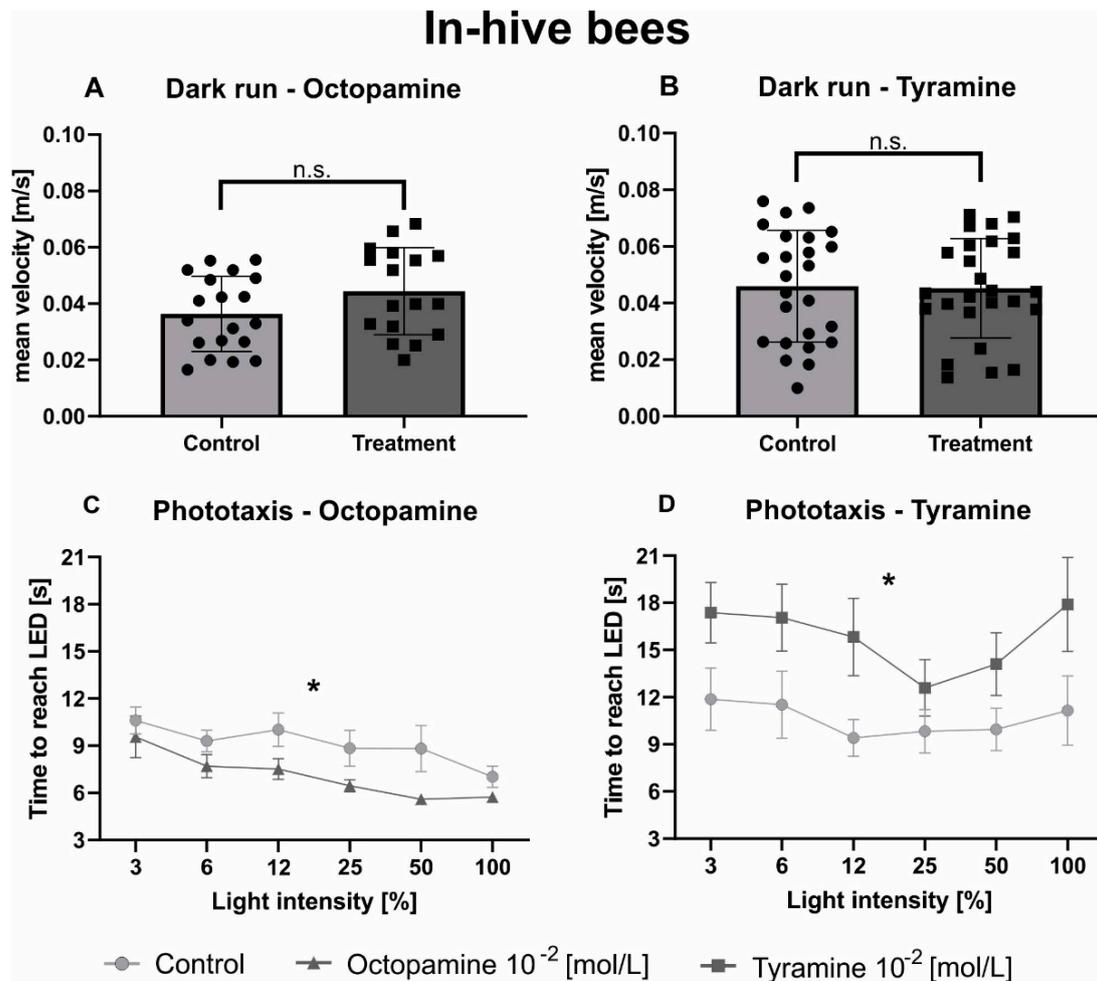
Figure 2. Octopamine and tyramine have opposite effects on the phototaxis of in-hive bees. Control bees are shown in light grey. Those injected with OA (10^{-2} mol/L) or TA (10^{-2} mol/L) are shown in dark grey. (**A**,**B**): Average velocity (mean + standard deviation) of honeybees during one minute of constant movement in the dark arena. Neither OA nor TA differed significantly from the Ringer control in their mean velocity in the dark (Table 3). (**C**,**D**): Average walking time (mean + standard error) towards the different switched-on light sources. The factor light intensity significantly influenced phototactic behaviour (Table 3). Octopamine decreased the time honeybees needed to reach the switched-on LEDs significantly compared to the control solution, while TA increased it (Table 3). Significant differences between Ringer and OA/TA are indicated by asterisks (n.s. $p > 0.05$, * $p < 0.05$).

Similar to hive bees, no effect of OA (Figure 3A; Table 3) nor TA (Figure 3B; Table 3) could be observed on the mean velocity of foragers in the dark arena. As expected, the factor light intensity significantly influenced phototactic behaviour of foragers in both experiments (Figure 3C,D; Table 3). Octopamine significantly decreased the walking time of foragers towards the switched-on light sources compared to the control group (Figure 3C, Table 3), while TA significantly increased the time foragers needed to reach the different switched-on light sources (Figure 3D, Table 3).

Table 3. Statistical comparison of the dark runs and the phototaxis of honeybees (either in-hive bees or foragers) treated with octopamine/tyramine (treatment) or Ringer (control).

Octopamine (In-Hive)	Unpaired *t* Test	Statistic	DF	*n* (Ringer)	*n* (Treatment)	*p* Value	
Dark run		$t = 1.679$	34	19	17	0.102	n.s.
Phototaxis	2way ANOVA						
	Intensity	$F_{(5, 170)} = 6.131$				<0.001	***
	Treatment	$F_{(1, 34)} = 5.750$				0.022	*
	Interaction	$F_{(5, 170)} = 0.625$				0.681	n.s.
	Bonferroni test						
	3%	0.824	204	19	17	0.999	n.s.
	6%	1258	204	19	17	0.999	n.s.
	12%	1960	204	19	17	0.308	n.s.
	25%	1874	204	19	17	0.374	n.s.
	50%	2531	204	19	17	0.073	n.s.
	100%	1017	204	19	17	0.999	n.s.
Tyramine (in-hive)	unpaired *t* test	statistic	DF	*n* (Ringer)	*n* (Treatment)	*p* value	
Dark run		$t = 0.139$	49	25	26	0.890	n.s.
Phototaxis	2way ANOVA						
	Intensity	$F_{(5, 245)} = 2.564$				0.028	*
	Treatment	$F_{(1, 49)} = 4.919$				0.031	*
	Interaction	$F_{(5, 245)} = 0.669$				0.647	n.s.
	Bonferroni test						
	3%	1916	294	25	26	0.338	n.s.
	6%	1931	294	25	26	0.327	n.s.
	12%	2236	294	25	26	0.157	n.s.
	25%	0.964	294	25	26	0.999	n.s.
	50%	1452	294	25	26	0.886	n.s.
	100%	2352	294	25	26	0.116	n.s.
Octopamine (forager)	unpaired *t* test	statistic	DF	*n* (Ringer)	*n* (Treatment)	*p* value	
Dark run		$t = 1.595$	38	20	20	0.119	n.s.
Phototaxis	2way ANOVA						
	Intensity	$F_{(5, 190)} = 4.342$				< 0.001	***
	Treatment	$F_{(1, 38)} = 5.223$				0.028	*
	Interaction	$F_{(5, 190)} = 1.514$				0.187	n.s.
	Bonferroni test						
	3%	2695	228	20	20	0.045	*
	6%	1630	228	20	20	0.627	n.s.
	12%	1700	228	20	20	0.543	n.s.
	25%	2492	228	20	20	0.081	n.s.
	50%	1610	228	20	20	0.653	n.s.
	100%	2545	228	20	20	0.070	n.s.
Tyramine (forager)	unpaired *t* test	statistic	DF	*n* (Ringer)	*n* (Treatment)	*p* value	
Dark run		$t = 0.123$	48	25	25	0.903	n.s.
Phototaxis	2way ANOVA						
	Intensity	$F_{(5, 245)} = 5.986$				< 0.001	***
	Treatment	$F_{(1, 49)} = 11.29$				0.002	**
	Interaction	$F_{(5, 245)} = 0.072$				0.996	n.s.
	Bonferroni test						
	3%	2412	294	26	25	0.099	n.s.
	6%	2838	294	26	25	0.029	*
	12%	2435	294	26	25	0.093	n.s.
	25%	2268	294	26	25	0.144	n.s.
	50%	2377	294	26	25	0.109	n.s.
	100%	2334	294	26	25	0.122	n.s.

Significant differences are indicated by asterisks (n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).
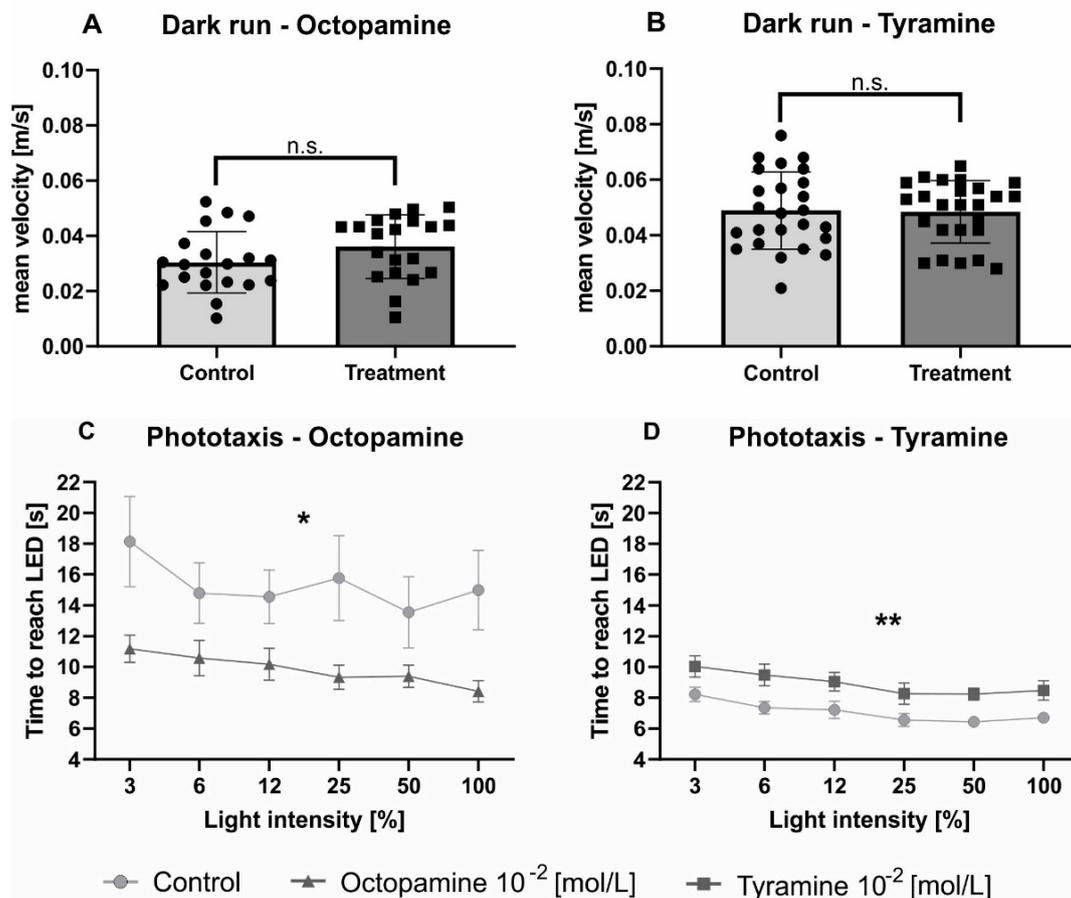
Figure 3. Octopamine and tyramine have opposite effects on the phototaxis of foragers. Control bees are shown in light grey. Those injected with OA (10^{-2} mol/L) or TA (10^{-2} mol/L) are shown in dark grey. (**A**,**B**): Average velocity (mean + standard deviation) of honeybees during one minute of constant movement in the dark arena. Neither OA nor TA did differ significantly from Ringer in their mean velocity in the dark (Table 3). (**C**,**D**): Average walking time (mean + standard error) towards the different switched-on light sources. The light intensity factor significantly influenced phototacic behavior (Table 3). Octopamine decreased the time honeybees needed to reach the switched-on LEDs significantly compared to the control solution, while TA increased it (Table 3). Significant differences between Ringer and OA/TA are indicated by asterisks (n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$).

4. Discussion

In this study we investigated the influence of octopamine and tyramine on honeybee responses to light. One major goal was to separate the effects of both amines at the sensory input level (ERG) and the behavioural output level (phototaxis). To understand whether the stronger attraction to light induced by OA was based on a higher perception of light at the sensory periphery we quantified ERG responses. In the ERG, OA (10^{-3} mol/L) elicited stronger receptor responses compared to controls. Tyramine (10^{-4} mol/L) had the opposite effect. The same pattern could be observed on the behavioural level. While OA elicited faster walking behaviour to light, indicating a stronger incentive value of the light, TA had the opposite effect. Neither amine affected the velocity during the dark runs and presumably also during the phototaxis assay. Our findings are in line with earlier studies showing that OA mainly has arousing functions in insects [5,7,32] and that it increases the perceived value of a food source of honeybee foragers [38]. Whether TA has similar or opposing effects is little-known so far. Only a few behavioural experiments were conducted including both biogenic amines. It has been shown that OA can decrease the walking

speed towards different light sources when applied chronically, while TA can increase the walking speed in foragers [20]. However, TA also influenced the general locomotor activity in those experiments, so it is impossible to state whether the change in walking times due to TA treatment was a locomotor effect, an effect of increased light perception or a mixture of both in those experiments. The present study differs mainly in the drug application. While we injected both monoamines locally, the authors in the earlier study [20] fed them over three consecutive days before conducting the experiments, possibly resulting in opposite effects. Other studies could also show an arousing effect of OA on the phototaxis. Feeding formamidines, toxins reported to inhibit the OA-stimulated adenylate cyclase, to *Drosophila* reduced phototactic behaviour, indicating a positive effect of OA on the phototaxis [39]. Different application methods could possibly lead to different target receptors. Octopamine and tyramine are known to elicit different effects depending on their targeted receptors [7]. However, due to the lack of suitable antibodies, the location of most OA/TA receptors in the adult honeybee is still unknown. Only the spatial distribution of the octopamine receptor *AmOctαR1* and of the tyramine receptor *AmTAR1* have been described in the honeybee brain. While strong labelling of *AmOctαR1* can be observed for the optic lobes [40], *AmTAR1* is not present here [41]. This indicates that *AmOctαR1* is a strong candidate for the observed effects in this study. In *Drosophila*, Kholy et al. [42] showed expression of *Oamb* and the *TyrRIII* in the optic lobes of *Drosophila melanogaster*. Similar to the *AmOctαR1*, the *Oamb* increases [cAMP]$_i$ [43]. However, a *TyrRIII* honeybee homologue, which decreases [cAMP]$_i$ [44] is currently unknown. Similar to flies, different OA/TA receptors should be present in the optic lobes of honeybees which might explain our results. Yet, our current experiments do not allow us to specify which receptors are activated by OA or TA. Here we suggest targeted knockout of individual receptor genes using CRISPR/Cas9 [45] or RNA interference [46] in future studies.

Both neurotransmitters are known to modulate not only the central nervous system (CNS) but also peripheral organs expressing respective receptors [47]. It has been shown that OA can target receptors in the CNS, as well as in the periphery, independently of the application method [30,48,49]. Therefore, application duration might be more important than the application itself. Application over three consecutive days might target the CNS and the periphery, while a local injection might preferably target receptors in the honeybee brain. Furthermore, TA is the metabolic precursor of octopamine [4]. Over time, TA might be converted into OA by the enzyme tyramine β hydroxylase. Thus, feeding TA over three consecutive days could lead to an OA effect rather than the expected TA effect. Additionally, a constant treatment of honeybees with either amine might lead to an internalization of the respective receptors which in turn could also lead to the opposite effect [49]. Here, it would be important in future investigations to quantify OA and TA brain titres directly after the phototaxis experiment. In addition to applying the CRISPR/Cas9 technique for a targeted knockout, RNA interference (RNAi) might be an interesting option to reduce receptor gene expression [50,51]. One could argue that an ocellus injection might have a strong negative impact on bee's behaviour, which might lead to different results. However, we did show that the ocellus injection, does not negatively affect honeybee behaviour during the assay (Figure S2).

To find out whether the opposing effects of OA and TA on walking speed towards light were related to perception, we performed ERG experiments. Octopamine increased the photoreceptor response, while TA decreased it. These results show the same pattern as the phototaxis experiments, leading to the conclusion that sensory input and behavioural output might be directly linked. Little is known about the influence of biogenic amines on the ERG response of honeybees. Lim and Wasserman [52] showed that washing OA-containing seawater over the eye of *Limulus polyphemus* increases the receptor potential in ERG experiments, while Battelle et al. [53] showed that OA increased the ERG amplitude of a *Limulus polyphemus* eye. Erber et al. [54] demonstrated that OA could increase the visual antennal response. However, Chyb et al. [55] found OA to be decreasing the ERG response in *Drosophila melanogaster*. As seen in the previously mentioned behavioural

studies, results of biogenic amine experiments can be contradicting. Another explanation might be that OA- and TA receptors are very similar. Tyramine does not only bind to TA receptors but also to OA receptors when applied in high concentrations, although TA has a much higher affinity towards TA receptors [14,15]. If TA bound to all TA receptors present, it might also have activated OA receptors. OA receptors could then elicit different or even opposite effects.

It seems likely that the results obtained in our study reflect the short-term modulation of the visual system in honeybees by OA and TA. Studies that obtained different results also differed in their application methods, indicating a difference between short- and long-term modulation of perception by OA and TA. This is supported by a study from Scheiner et al. [29]. They showed that injecting TA into the abdomen of honeybees leads to an increase in OA and TA in the honeybee brain. This effect is most likely a result of the tyramine β hydroxylase converting TA into OA. While the exact time point of the OA and TA titre quantification is not stated, PER experiments were conducted prior to the amine quantification of the same honeybees. This indicates that at least 1.5 hours passed between the injection and the quantification of both biogenic amines. This time seems sufficient for metabolizing TA into OA.

As stated before, the phototaxis and the ERG results both show the same opposing pattern for octopamine and tyramine. This coincides with the proposed hypothesis by Roeder et al. [4–6] stating that the OA/TA system in insects can be compared to the epinephrine/norepinephrine system in deuterostomes. This is also supported by other studies. Saraswati et al. [18] showed the opposing functions of OA and TA in the locomotion of *Drosophila melanogaster* larvae. Furthermore, Fussnecker et al. [56] showed that honeybees spent a significantly increased time flying when treated with OA, whereas those treated with TA spent a significantly decreased time flying compared to controls. Yet, when feeding or injecting OA and TA into the thorax, both increased the PER of honeybees [28]. This indicates that both substances fulfil complementary roles in some sensory systems but opposing functions in other systems. This study clearly shows the opposing functions of OA and TA on the visual system of honeybees. However, further experiments need to be conducted into differentiating long-term and short-term modulation as well as turning off or blocking single receptors to fully understand the modulating effects of both important biogenic amines.

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6
General Discussion

6.1 Models of division of labor

Over the years, multiple models have arisen that try to explain the division of labor in honeybees (Beshers and Fewell, 2001). One of the most prominent models is the response threshold model (Figure 2). This model was created around the hypothesis that different workers have individual response thresholds for different task-related stimuli (Robinson, 1992; Robinson and Page, 2019). The default state for a worker is assumed to be inactive and it will only conduct a task when a stimulus exceeds its individual response threshold. Division of labor is achieved, because some individuals are more responsive to a certain task-related stimulus than other bees. Variation in tasks performance can be manifold. One example is the genetic variability of honeybee workers due to multiple fathers (Page and Robinson, 1991; Robinson and Page, 2019).

A second model, the double repressor hypothesis (Amdam and Omholt, 2003), took a different approach to the division of labor phenomenon (Figure 8). In this model, juvenile hormone is under positive control by the allatoregulatory central nervous system, which, in turn, is under negative control of an external and internal repressor. Amdam and Omholt (2003) propose that vitellogenin might be the internal repressor, while a signal originating from other honeybees is supposed to be the external repressor. Loss
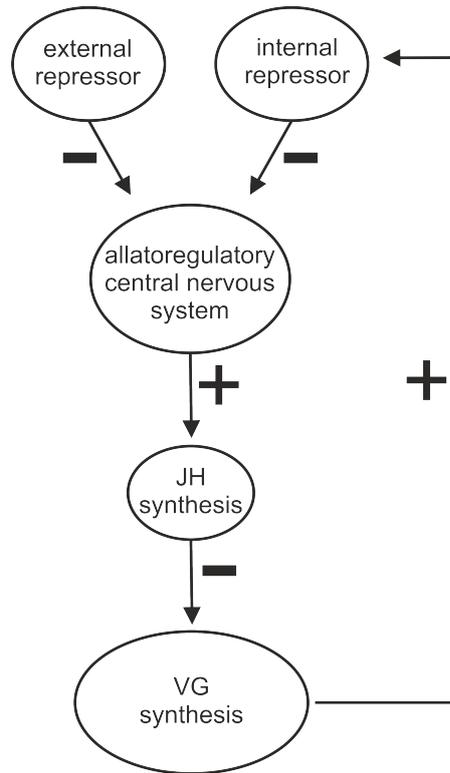
Figure 8: **Model of the double repressor hypothesis** adapted from Amdam and Omholt (2003). This model explains the honeybee division of labor via an internal repressor (vitellogenin) and an external repressor (pheromone signal). Both repressors negatively influence the allatoregulatory central nervous system (ACNS). Juvenile hormone synthesis is under positive control of the ACNS. Increasing titers of JH suppress the internal repressor which in turn increases JH even further. Loss of internal repressor or external repressor leads to an increase in JH which drives the transition to foragers by reducing vg synthesis.

of the external repressor activates the allatoregulatory central nervous system pathway, which stimulates the synthesis of JH, which suppresses vg synthesis and leads to the transition from in-hive bees to foragers (Amdam and Omholt, 2003). While they do not explicitly state which signal could fulfill such a function, brood ester pheromone seems likely (Le Conte et al., 2001). Brood ester pheromone is emitted from honeybee larvae. Increasing levels of brood ester pheromone in a honeybee colony induces honeybees to perform nursing tasks (Le Conte et al., 2001).

Other factors, e.g. starvation, are proposed to lead to a premature depletion of vg (Amdam and Omholt, 2003). Loss of the internal repressor than induces the same cascade leading to a faster transition from in-hive tasks to foraging.

In this discussion, I provide evidence supporting the double repressor hypothesis and provide insight into which factors might be involved in modulating the internal repressor or might be modulated by the external repressor. Additionally, I speculate about the differences of pollen undernourishment and general undernourishment and their role in the honeybee division of labor.

6.1.1 Evidence for the double repressor hypothesis

A key assumption for the double repressor hypothesis (Amdam and Omholt, 2003) is the importance of the internal repressor vitellogenin. Instead of a signal that triggers the nurse forager transition, honeybees will stay inside the hive until either vg titers or the external repressor decreases. Under normal conditions vg decreases with honeybee age (Engels and Fahrenhorst, 1974; Fluri et al., 1982). While we did not find any difference in *vg* mRNA expression in the brain and fat body between nurse bees, orientation-flight bees, young foragers and old foragers, we observed a gradual decrease in relative *vg* expression in both tissues (**Chapter 2**). It seems likely that honeybees stay inside the hive for as long as *vg* expression or vg protein is at a sufficient level. *Vitellogenin* mRNA expression and thus vg protein decreases with age under normal conditions, leading to a reduction of egg yolk proteins. This, in turn, enables honeybees to become foragers (natural loss of internal repressor). Depending on vg hemolymph titers, honeybees would be able to fulfill different tasks in the colony. For example, very high levels of vg would prime a honeybee for nursing tasks. Loss of vg would correlate with a transition from nursing tasks to for example food-processing tasks until they finally become foragers at very low levels of vg hemolymph titers. This is based on experiments showing that increased *vg* expression inhibits the onset of foraging (Nelson et al., 2007) but vg protein declines with age in workers (Fluri et al., 1982) and our results that show the gradual decrease in *vg* mRNA expression from nurse bees to foragers. Vitellogenin could thereby serve as a pacemaker for age polyethism and determine lifespan, as originally hypothesized by Amdam and Omholt (2002, 2003).

Furthermore, Amdam and Omholt (2003) states that vg synthesis is supposed to be repressed via juvenile hormone (Figure 8). Evidence for this has been shown before (Pinto et al., 2000; Corona et al., 2007). However, both of these studies used the JH analogue methoprene instead of JH. In our experiments, we treated honeybees topically with JH and observed effects on *vg* mRNA expression (**Chapter 2**). We clearly showed that

honeybees treated with JH show decreased levels of *vg* expression. Therefore, increasing JH titers inhibit the mRNA expression of *vg* and thus synthesis of the internal repressor should be inhibited as well, as shown before with methoprene (Pinto et al., 2000). According to the double repressor hypothesis, this should speed up the transition from in-hive bees to foragers.

It seems likely that juvenile hormone is the most important *zeitgeber* for vitellogenin degradation. To investigate this hypothesis, we observed JH hemolymph titers during the transition from in-hive bees to foragers (**Chapter 2**). We found that JH levels were low in nurse bees and orientation-flight bees and increased once the bees became foragers. Juvenile hormone levels did not increase further with foraging experience, which has been shown before (Robinson et al., 1987; Huang et al., 1991). Interestingly, other studies have shown an increase in JH in anticipation of foraging (Jassim et al., 2000; Elekonich et al., 2001). It seems likely that both findings are valid. Under normal conditions JH increases during the transition to foraging, but loss of the internal repressor *vg* might lead to increasing JH levels prior to foraging (Amdam and Omholt, 2003). Furthermore, we also treated honeybees topically with JH. As expected, JH levels increased significantly due to the treatment. However, JH titers also increased naturally with age, independent of our treatment. This shows the opposite picture to *vg*, which decreases naturally with age (Engels and Fahrenhorst, 1974; Fluri et al., 1982). It seems plausible that the natural decrease in the internal repressor (*vg*) allows for an increase in JH titers. In addition, we also studied the effects of juvenile hormone on triglycerides (**Chapter 2**) as it is known that vitellogenin synthesis also depends on lipid reserves (Chapman and Chapman, 1998). Normally, TGs degenerate with the transition from in-hive bees to foragers (Toth and Robinson, 2005), which we could also show (**Chapter 2**). Triglyceride levels were high in nurse bees and orientation-flight bees, but almost completely zero in young foragers and old foragers, showing a steep decrease in a relatively short period. Yet, treatment with JH did not affect TG levels. These results indicate that the effects of JH on *vg* gene expression are independent of lipid reserves.

We think that the double repressor hypothesis seems to be the closest model to reality. It completely removes the need for a trigger of the nurse-forager transition. Especially, when taking the response threshold model (Figure 2) as the basis for the transition, one needs to find a trigger mechanism that is responsible for the transition. Yet, until this day, a concrete trigger for the transition remains elusive. In the following section, we propose an addition to the existing hypothesis.

6.1.2 The extended double repressor hypothesis

This section should be seen as an addition to the existing double repressor hypothesis (Figure 8). Future experiments to provide further evidence for this hypothesis will be discussed at the end of this thesis (Section 6.3). We propose the following hypothesis:

"The *AmTAR1* mRNA expression is repressed via an increase in juvenile hormone or directly via loss of the external repressor. The following reduction of the AmTAR1 protein allows for increased *AmOARα1* mRNA expression and therefore an increase in the AmOARα1 protein. Activation of the AmOARα1 via octopamine in turn increases JH synthesis in the *corpora allata* and speeds up the transition from nurse bees to foragers via a decrease in vitellogenin mRNA expression and therefore a decrease in vg protein."

This hypothesis (Figure 9) originated due to the experiments we conducted in **Chapter 2**, investigating the transition from nurse bees to foragers. Initially we hypothesized that the *foraging* gene plays an important role in the honeybee division of labor. *Amfor* expression did not differ in the honeybee brain between nurse bees, orientation-flight bees, young foragers and old foragers. Yet, we found a gradual decrease in the *Amforβ* gene expression in the honeybee fat body from nurse bees to old foragers. Furthermore, we observed a tendency of *Amforα* expression to increase in foragers compared to nurse bees and bees during their orientation flights as shown before (Thamm and Scheiner, 2014). Neither treatment with juvenile hormone, nor age affected *Amfor* gene expression. The independence of *Amfor* expression on JH titers indicates that the foraging gene likely plays rather a subordinate role in the nurse-forager transition.
  Furthermore, we showed that treatment with JH did not affect the *AmOARα1* mRNA expression but that expression decreased with age. This further emphasizes that JH does not affect *AmOARα1* gene expression and likely neither the octopamine signaling. Yet, it seems that the synergistic effects of OA and JH (Schulz et al., 2002b) occur indeed due to OA influencing JH synthesis (Thompson et al., 1990; Kaatz et al., 1994). However, treatment with JH significantly decreased the *AmTAR1* mRNA expression, while honeybee age did not affect the *AmTAR1* expression, even though we observed a slight increase between day one and day eight. Thus, increased JH synthesis not only decreases the internal repressor (vg) but also the mRNA expression of the *AmTAR1*. A reduced *AmTAR1* mRNA expression likely leads to a decrease in the AmTAR1 protein.
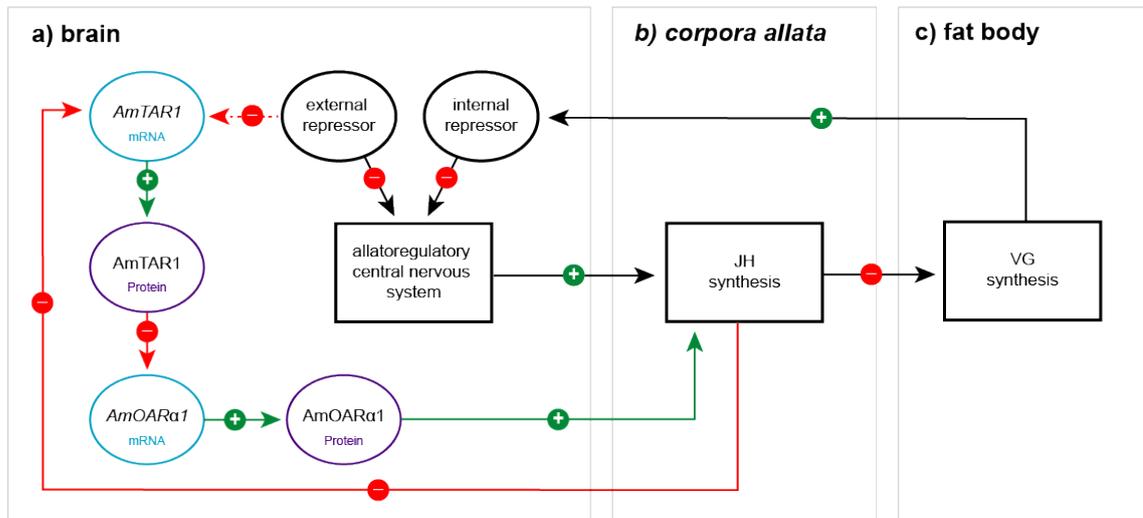
Figure 9: **Scheme of the extended double repressor hypothesis in the honeybee brain.**
In black, the standard double repressor hypothesis is depicted (Figure 8), as proposed by Amdam and Omholt (2003). The addition is shown in colors. The *AmOARα1* mRNA expression is under negative control of AmTAR1 protein in the brain (a). External factors like the loss of foragers might not only affect the allatoregulatory central nervous system but also *AmTAR1* mRNA expression (dotted red arrow). Loss in external repressor leads to loss of *AmTAR1* expression and thus, AmTAR1 protein, which allows for increased *AmOARα1* mRNA expression in the brain (a). The AmOARα1, via octopamine activation, increases juvenile hormone synthesis in the *corpora allata* (b). This leads to a suppression of vitellogenin synthesis (c) as well as *AmTAR1* expression in the brain (a).

Next, we showed opposite expression patterns of the *AmOARα1* mRNA expression and the *AmTAR1* mRNA expression in the brain and fat body (**Chapter 2**). We found an increase in the *AmOARα1* relative gene expression in the honeybee brain from nursing to foraging as shown previously. Reim and Scheiner (2014) showed an increase in *AmOARα1* mRNA expression in forager brains compared to nurse bee brains. *AmOARα1* expression also tended to increase from nurse bees to foragers in the honeybee fat body. However, in the brain the *AmOARα1* mRNA expression increased gradually unlike the expression in the fat body. Here, *AmOARα1* mRNA expression decreased from nurse bees to orientation-flight bees and only increased once honeybees started to forage. This leads us to believe that the AmOARα1 seems to be of gross importance for the transition from in-hive bees to foraging.

The *AmTAR1* mRNA expression showed the opposite picture in both tissues. In the brain, the relative *AmTAR1* gene expression was high in nurse bees and orientation-

flight bees and low in foragers. In the fat body the relative *AmTAR1* mRNA expression tended to decrease with the transition from in-hive tasks to foraging. Interestingly, during orientations flights, we saw an increase in *AmTAR1* mRNA expression in the fat body and a decrease once the honeybees began to forage.

While no one observed the opposite expression pattern of *AmOARα1* mRNA expression and *AmTAR1* mRNA expression before, opposite functions of the activating biogenic amines have been implied for a long time. Octopamine and tyramine have been implied to act oppositely like their vertebrate counterparts epinephrine and norepinephrine (Roeder et al., 2003; Roeder, 2005, 2020). We found concrete evidence for that hypothesis on honeybee vision (**Chapter 5**). Sensory sensitivity of the photoreceptors was increased via OA and decreased via TA. Furthermore, in in-hive bees and foragers, OA increased the phototactic response while TA decreased it, independent of honeybee locomotion.

Opposite expression patterns of *AmOARα1* and *AmTAR1* (**Chapter 2**) as well as opposite actions of OA and TA (**Chapter 5**) in addition to juvenile hormone decreasing the gene expression *AmTAR1* while not affecting the *AmOARα1* expression (**Chapter 2**) and OA increasing JH synthesis (Kaatz et al., 1994) led to the second assumption of the extended double repressor hypothesis (Figure 9): Expression of the *AmOARα1* is repressed by activation of the AmTAR1 protein via TA. It has been shown that activation of the AmTAR1 protein via TA leads to decrease in internal cAMP (Blenau et al., 2000). As discussed in **Chapter 2**, this change in internal cAMP can increase or decrease transcription factors like CREB (Lonze and Ginty, 2002). CREB can mediate the expression of other genes (Kitagawa, 2007), e.g. the *AmOARα1* expression. Furthermore, increased CREB activity can be linked to a faster nurse-forager transition. Increased pAmCREB is known to increase inner compact cells in the mushroom body, an area that increases its activity in foragers compared to nurse bees (Khamis et al., 2015; Gehring et al., 2016b).

Modulation of the division of labor independent of juvenile hormone

In the original double repressor hypothesis (Amdam and Omholt, 2003), if juvenile hormone is removed from the model, the tempo of the honeybee nurse-forager transition should be fixed (Figure 8). If JH is missing, there is no more substance that can set the pace of the nurse forager transition. In that case the only *zeitgeber* that remains

is the natural degradation of *vitellogenin* expression shown by us (**Chapter 2**) and vg protein as shown by others (Engels and Fahrenhorst, 1974; Fluri et al., 1982). However, studies have shown that even when the *corpora allata* is removed, the nurse forager transition can still be modulated with octopamine (Schulz et al., 2002b,a). Allectomized honeybees treated with OA sped up the transition to foragers compared to allectomized honeybees that were not treated with OA (Schulz et al., 2002b), showing modulation of division of labor even without the *corpora allata*. Our extended double repressor hypoth-
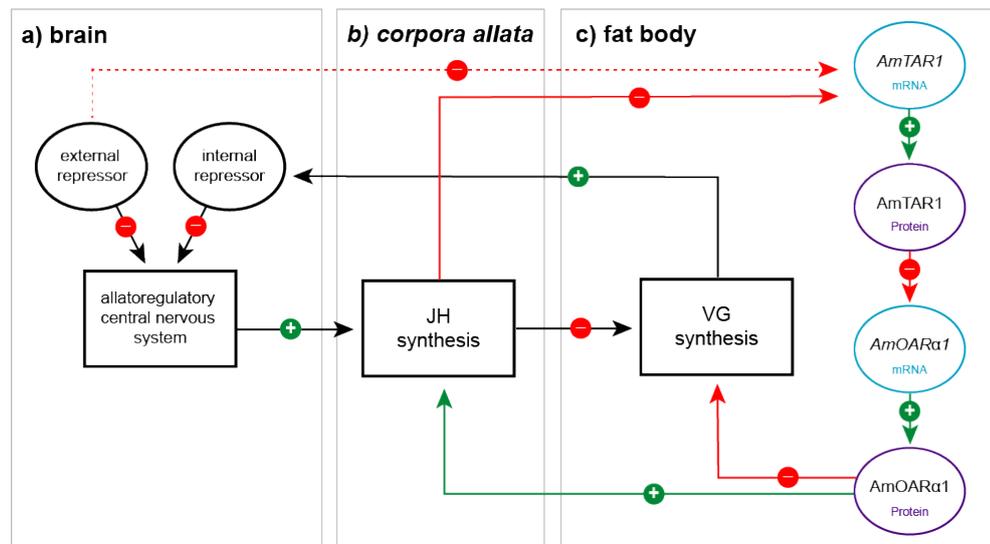
Figure 10: **Scheme of the extended double repressor hypothesis in the honeybee fat body.** In black, the standard double repressor hypothesis is depicted (Figure 8), as proposed by Amdam and Omholt (2003). The addition is shown in colors. As all ready shown in the brain (Figure 9), the *AmOARα1* mRNA expression is under negative control of AmTAR1 protein in the fat body (c). External factors like the loss of foragers affects the *AmTAR1* mRNA expression (dotted red arrow). Loss in external repressor (a) leads to loss of *AmTAR1* expression and thus, AmTAR1 protein which allows for increased *AmOARα1* mRNA expression in the fat body (c). The AmOARα1, via octopamine activation, increases juvenile hormone synthesis in the *corpora allata* (b) and thus suppresses the *AmTAR1* expression in the fat body (c). Furthermore, activation of the AmOARα1, via octopamine also leads to a decrease in vitellogenin synthesis.

esis addresses this flaw (Figure 10). As we showed in **Chapter 2**, expression patterns in the brain and fat body generally show a similar pattern (*AmOARα1* expression increases from nurse to forager; *AmTAR1* expression decreases) except during orientation flights in the fat body. Here, *AmOARα1* expression decreased while *AmTAR1* expres-

sion increased. It seems likely that the biogenic amines octopamine and tyramine might elicit different effects in modulating division of labor, depending on the tissue where the receptors are expressed.

We propose that the activation of the AmOARα1 via OA in the fat body represses the vg synthesis, allowing for modulation of the honeybee division of labor even in absence of juvenile hormone (Schulz et al., 2002b,a). Loss of the external repressor, leads to a decrease in *AmTAR1* mRNA expression (Figure 10). While actions of OA on vitellogenin have not been directly shown in honeybees, Tinikul et al. (2015) showed that OA decreases hemolymph vg levels in *Litopenaeus vannamei*.

In conclusion, JH synthesis is under the control of the allatoregulatory central nervous system as well as octopamine signaling. Increase in JH synthesis not only represses vitellogenin synthesis, but also tyramine signaling. This allows for an increase in octopamine signaling. Furthermore, the octopamine and tyramine signaling pathway can change vitellogenin synthesis, independent of juvenile hormone.

6.2 *In-vitro* rearing and larval malnourishment (in the division of labor)

Nowadays, it seems common knowledge that nutrition or lack thereof can increase the number of foragers in a honeybee colony (Schulz et al., 1998; Toth et al., 2005). For example, Toth et al. (2005) showed that lipid deprivation increases the number of foragers.

In our studies (**Chapters 3, 4**), we wanted to evaluate whether larval malnourishment can influence the number of foragers and the general foraging behavior of honeybees. For that, we first tried to evaluate whether the commonly used method of *in-vitro* rearing (Aupinel et al., 2005, 2007, 2010; Crailsheim et al., 2013; Schmehl et al., 2016; Steijven et al., 2017) has an effect on the behaviors observed in **Chapter 3**. Nursing behavior was unaffected by *in-vitro* rearing. Yet, significantly fewer *in-vitro* reared honeybees became nurse bees. The ones that did perform nursing tasks performed them equally well as their hive reared sisters. Contrary to our hypothesis, we found significant effects of *in-vitro* rearing on honeybee foraging behavior and physiology. Significantly fewer *in-vitro* reared honeybees became foragers. Furthermore, *in-vitro* reared honeybees started and terminated foraging significantly earlier and foraged for a shorter time in general,

indicating an earlier demise. However, we could not find a difference in foraging trip duration.

During the classical *in-vitro* rearing protocol, honeybees receive a standard diet. Within this diet, the normal pollen the larvae receive when fed by nurse bees is substituted by yeast (Aupinel et al., 2005, 2007, 2010; Crailsheim et al., 2013; Schmehl et al., 2016; Steijven et al., 2017) to prevent the larval food from pollution by mold. However, it has been shown that pollen consumption during early adulthood is crucial for honeybees. Scofield and Mattila (2015) deprived honeybee larvae of pollen and found almost the same results as we did. They also showed a decreased number of hive bees that actually became foragers, and the pollen-limited group also started their foraging trips earlier and had a shorter foraging span (Scofield and Mattila, 2015). Therefore, we propose that the observed differences between *in-vitro* reared and hive reared honeybees arise due to missing pollen during *in-vitro* rearing protocol. Furthermore, due to the experimental set-up, newly emerged honeybees had to be maintained in cages for approximately 72 hours to increase acceptance of the weaker *in-vitro* reared bees into the host colonies and for the superglue, used to mark the bees, to dry. However, honeybees consume large quantities of pollen during the first 42 hours to 52 hours after emergence (Seeley, 1995). Therefore, not only received the larvae less pollen during the *in-vitro* rearing but also during early maturation.

Pollen seems to be vital during larval development and early maturation. It has been demonstrated that pollen contains essential amino acids, lipids and sterols (Cane et al., 2000). Essential amino acids are known to promote honeybee gland and muscle development (Hendriksma et al., 2019). It seems clear that *in-vitro* rearing is a form of pollen undernourishment during larval development. Interestingly, this form of undernourishment leads to a premature development, resulting in an earlier onset of foraging but not affecting nursing behavior. It seems to affect honeybees similar to other stressors. For example, Hesselbach et al. (2020) showed that honeybees treated with the pesticide flupyradifurone initiated foraging significantly earlier than control bees. It is possibly, that nutritional stress during larval development via pollen affects the internal repressor vitellogenin (Figure 9, 10). In honeybees, it has been shown that increased pollen consumption can lead to higher vg hemolymph levels (Frias et al., 2016; Wegener et al., 2018). In the grasshopper *Romalea microptera* Fei et al. (2005) demonstrated that starvation can reduce vg levels. Therefore, it seems likely that *in-vitro* rearing affects honeybees similar to pollen undernourishment (Scofield and Mattila, 2015) and

other stressors (Hesselbach et al., 2020) in initiating an earlier transition to foraging by modulating the internal repressor (vg).

6.2.1   Larval undernourishment can be easily compensated by adult honeybees

The results discussed in Section 6.2 led us to believe that *in-vitro* rearing affects adult worker bees similarly to larval pollen undernourishment. Our experiment comparing *in-vitro* reared honeybees to hive reared honeybees clearly showed that artificial rearing affected foraging behavior without affecting nursing behavior. We hypothesized that the earlier initiation of foraging in *in-vitro* reared bees was due to a lack of pollen, which was unavoidable in our rearing protocol. However, it might have simply been malnutrition that led to the observed differences in behavior, because *in-vitro* reared bees are not fed as regularly as hive reared bees. Larvae raised in a normal colony transmit a pheromone that induces worker bees to feed the brood rather than allocating energy to foraging activities (Le Conte et al., 2001; Maisonnasse et al., 2010). To dissociate effects of a lack of pollen from those of malnutrition, we performed a second experiment with *in-vitro* reared bees (**Chapter 4**). This time, we varied the amount of food, while all groups of bees received yeast instead of pollen. Honeybees either were undernourished, received the standard diet or were overfed.

First, we hypothesized that overfed honeybees might stay inside the hive longer, possibly even nurse for a longer period of time as increased nutrition is directly linked to higher vitellogenin titers (Frias et al., 2016; Wegener et al., 2018). However, we found no evidence to support this, as our feeding regimes did not affect nursing or foraging behavior. This was surprising as we also expected undernourished bees to initiate foraging earlier, as shown before (Schulz et al., 1998). It seems that malnutrition does not affect task allocation but that lack of pollen during larval development does lead to an earlier initiation of foraging (**Chapter 3**). Furthermore, as increased pollen nutrition is linked to higher vg titers (Frias et al., 2016; Wegener et al., 2018), increasing amounts of pollen during larval development should lead to a slower transition from in-hive tasks to foraging.

We found differences in weight between the treatments. Undernourished honeybees were the lightest, overfed honeybees were the heaviest and normally fed honeybees were

in between. Interestingly, honeybees increased their weight overtime with undernourished honeybees increasing their weight the most (**Chapter 4**). This showed us, that honeybees seem to be able to compensate for larval undernourishment during adult maturation. It has been shown that starved honeybee colonies increase the number of foragers (Schulz et al., 1998). However, adding additional food to those colonies halts the increase in foragers (Schulz et al., 1998), showing the capability of honeybee colonies to compensate for changes in their nutritional state.

Compensation of nutritional deficits during early adulthood seems to be very important. For example, periodic food shortage during larval development should not lead to precocious foraging. It has been shown that starving honeybee larvae emit a pheromone (He et al., 2016), which can increase the number of foragers in a colony (Maisonnasse et al., 2010). Yet, if food stocks have been replenished by the time the starved larvae become adult worker bees, there is no need for them to forage precociously.

In conclusion, larval undernourishment can be compensated during early maturation (**Chapter 4**), while pollen deficits or major food deficits seem to be so severe that they cannot be compensated for, resulting in an accelerated maturation (**Chapter 3**). Additionally, pollen diversity seems to be of gross importance as it is known be important for stress resistance (Castle et al., 2022) as well as for growth and reproduction (Requier et al., 2020). This should be accounted for in future studies especially when honeybees are reared artificially to observe foraging behavior.

6.3   Conclusions and Outlook

This doctoral thesis gave further inside into the division of labor in honeybees, focusing on the transition from nurse bees to foragers. In **Chapter 2** I showed how the candidate genes (*AmOARα1*, *AmTAR1*, *Amfor* and *vitellogenin*) are expressed during the transition from nurse bees to orientation-flight bees to young foragers and old foragers. Furthermore, I found evidence on how octopamine and tyramine, to important biogenic amines in task allocation, affect in-hive bees and foragers (**Chapter 5**). In **Chapters 3, 4** I focused on larval nutrition and how it affects the transition from nurse bees to foragers.

However, while some questions have been answered by this dissertation, multiple questions remain unanswered. I showed that juvenile hormone significantly decreases *AmTAR1* mRNA expression (Section 6.1.2), which, among other results, led to the extended

double repressor hypothesis (Figure 9, 10). However, a key assumption of this hypothesis is that the AmTAR1 activation via tyramine inhibits *AmOARα1* expression, possibly via transcription factors like pAmCREB. To test this, it is seems necessary to reduce AmTAR protein activation. Protein activation could be reduced via pharmacological inhibitors like yohimbine (Reim et al., 2017). However, these receptor inhibitors are rather unspecific. For example, yohimbine not only binds to the AmTAR1 but also to the AmTAR2 and the AmOARα1. Therefore, one should reduce *AmTAR1* expression via knock down through RNAi (Scholl et al., 2015; Sinakevitch et al., 2017) or genetic knock out via CRISPR/Cas (Değirmenci et al., 2020). These experiments should lead to an increase in *AmOARα1* expression and AmOARα1 protein. Furthermore, it seems plausible that octopamine signaling and tyramine signaling can modulate division of labor independent of the juvenile hormone pathway (Section 6.1.2). To test this, allectomized honeybees should be treated with octopamine and tyramine. I would expect a decrease in vitellogenin hemolymph titers when allectomized honeybees are treated with octopamine. Furthermore, tyramine treatment should lead to either unchanged vitellogenin titers or an age related vitellogenin decrease that decreases slower compared to octopamine treated bees.

This extended double repressor hypothesis attributes a new causal function to tyramine signaling in the honeybee division of labor. Furthermore, the experiments conducted in **Chapter 2** attribute a rather subordinate role to the *Amfor* in division of labor. Expression of *Amfor* seems to increase rather due to foraging experience than due to its involvement in regulating the honeybee division of labor.

Another key element is the possible role of vitellogenin. I hypothesized that high levels of vitellogenin protein or *vitellogenin* mRNA expression prevent honeybees from becoming foragers (Section 6.1.1). However, the vitellogenin synthesis pathway remains elusive. Until today, we only know the structural prediction of vitellogenin (Leipart et al., 2022). A first step would be to use *Chlorella sorokiniana* algae, which are known to increase *vitellogenin* mRNA levels (Jehlik et al., 2019). This artificial increase in *vitellogenin* mRNA expression should delay the transition from nurse bees to foragers. Next, one should observe overwintering honeybees. The overwintering state is achieved, among others, by increasing levels of vitellogenin (Knoll et al., 2020), while little is known how honeybees return to the summer state. However, it seems likely that task allocation happens once more after honeybees return to the summer state. Therefore, honeybees leaving the hive after the winter should show lower vitellogenin titers compared to same

age honeybees that remained in the hive.

Furthermore, I showed that the lack of pollen during *in-vitro* rearing leads to an earlier initiation of foraging and a shorter life span (**Chapter 3**). It seems that pollen stress during larval development affects honeybees similarly to stress via pesticides (Hesselbach et al., 2020) or parasites (Zanni et al., 2018). Due to these results I hypothesized that pollen malnourishment should decrease the internal repressor (Figure 9, 10). This reduction in vitellogenin should lead to an earlier maturation. To test this, honeybee colonies should be deprived of pollen, which should lead to decreased vitellogenin protein and *vitellogenin* mRNA levels as well as to precocious foraging. Furthermore, in these experiments, juvenile hormone levels should increase prior to the onset of foraging, unlike in colonies that were not pollen stressed. Here juvenile hormone levels should increase once honeybees become foragers.

However, unlike pollen stress during *in-vitro* rearing, lack of nutrition during larval development was easily compensated by the emerged honeybee workers (**Chapter 4**), as I did not observe any effects on nursing or foraging behavior.

In total, these results greatly increase our knowledge in our understanding of how honeybees are able to coordinate thousands of individuals living together in massive colonies without central organization. Additionally, they show how the decentralized organization can be affected by malnourishment.

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7

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A

Appendix

Affidavit

I hereby confirm that my thesis entitled **"Regulation of the nurse-forager transition in honeybees (*Apis mellifera*)"** is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation **"Regulation des Ammen–Sammlerinnen-Übergangs in Honigbienen (*Apis mellifera*)"** eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

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Danksagung

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"Freunde sind diese rar gesähten Leute, die uns fragen, wie es uns geht und dann unsere Antwort abwarten."
Ed Cunningham

Statement of individual author contributions and of legal second publication rights to manuscripts included in the dissertation

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Participated in	Author Initials, Responsibility decreasing from left to right					
Study Design	RS	FS	LH			
Methods Development	FS	RS	LH	MT	MK	
Data Collection	FS	LH	MK			
Data Analysis and Interpretation	FS	LH	MK			
Manuscript Writing	FS	RS	LH	MK	MT	MJM
Writing of Introduction	FS	RS	LH	MK	MT	MJM
Writing of Materials & Methods	FS	RS	LH	MK	MT	MJM
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Explanations (if applicable):

*: Equal contribution

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In Vitro Rearing Changes Social Task Performance and Physiology in Honeybees *Insects* 13, 4.

Participated in	Author Initials, Responsibility decreasing from left to right										
Study Design	RS	ISD									
Methods Development	FS	LD	BK	MA	LH	LR	MK				
Data Collection	FS	LH	BK	LR	LD						
Data Analysis and Interpretation	FS	RS	MA	MK	BR						
Manuscript Writing	FS	RS	ISD	MK	MA	LH	LR	BK	BR	MJM	LD
Writing of Introduction	FS	RS	ISD	MK	MA	LH	LR	BK	BR	MJM	LD
Writing of Materials & Methods	FS	RS	ISD	MK	MA	LH	LR	BK	BR	MJM	LD
Writing of Discussion	FS	RS	ISD	MK	MA	LH	LR	BK	BR	MJM	LD
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Honeybees are buffered against undernourishment during larval stages

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Study Design Methods Development	R.S. FS	ISD MA	LH	MK			
Data Collection	FS	LH	MK				
Data Analysis and Interpretation	FS	RS	LH	MA	MK		
Manuscript Writing 　Writing of Introduction 　Writing of Materials & Methods 　Writing of Discussion 　Writing of First Draft	FS FS FS FS FS	RS RS RS RS	ISD ISD ISD ISD	MA MA MA MA	MK MK MK MK	LH LH LH LH	MJM MJM MJM MJM

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I also confirm my primary supervisor's acceptance.

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