Sugar perception and sugar receptor function in the honeybee (*Apis mellifera*)

Zuckerwahrnehmung und Zuckerrezeptorfunktion in der Honigbiene (*Apis mellifera*)

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Content

Summary

In the eusocial insect honeybee (*Apis mellifera*), many sterile worker bees live together with a reproductive queen in a colony. All tasks of the colony are performed by the workers, undergoing age-dependent division of labor. Beginning as hive bees, they take on tasks inside the hive such as cleaning or the producing of larval food, later developing into foragers. With that, the perception of sweetness plays a crucial role for all honeybees whether they are sitting on the honey stores in the hive or foraging for food. Their ability to sense sweetness is undoubtedly necessary to develop and evaluate food sources. Many of the behavioral decisions in honeybees are based on sugar perception, either on an individual level for ingestion, or for social behavior such as the impulse to collect or process nectar. In this context, honeybees show a complex spectrum of abilities to perceive sweetness on many levels. They are able to perceive at least seven types of sugars and decide to collect them for the colony. Further, they seem to distinguish between these sugars or at least show clear preferences when collecting them. Additionally, the perception of sugar is not rigid in honeybees. For instance, their responsiveness towards sugar changes during the transition from in-hive bees (e.g. nurses) to foraging and is linked to the division of labor. Other direct or immediate factors changing responsiveness to sugars are stress, starvation or underlying factors, such as genotype.

Interestingly, the complexity in their sugar perception is in stark contrast to the fact that honeybees seem to have only three predicted sugar receptors.

In this work, we were able to characterize the three known sugar receptors (AmGr1, AmGr2 and AmGr3) of the honeybee fully and comprehensively in oocytes (**Manuscript II, Chapter 3 and Manuscript III, Chapter 4**). We could show that AmGr1 is a broad sugar receptor reacting to sucrose, glucose, maltose, melezitose and trehalose (which is the honeybees' main blood sugar), but not fructose. AmGr2 acts as its co-receptor altering AmGr1's specificity, AmGr3 is a specific fructose receptor and we proved the

heterodimerization of all receptors. With my studies, I was able to reproduce and compare the ligand specificity of the sugar receptors *in vivo* by generating receptor mutants with CRISPR/Cas9. With this thesis, I was able to define AmGr1 and AmGr3 as the honeybees' basis receptors already capable to detect all sugars of its known taste spectrum.

In the expression analysis of my doctoral thesis (**Manuscript I, Chapter 2**) I demonstrated that both basis receptors are expressed in the antennae and the brain of nurse bees and foragers. This thesis assumes that AmGr3 (like the *Drosophila* homologue) functions as a sensor for fructose, which might be the satiety signal, while AmGr1 can sense trehalose as the main blood sugar in the brain. Both receptors show a reduced expression in the brain of foragers when compared with nurse bees. These results may reflect the higher concentrated diet of nurse bees in the hive. The higher number of receptors in the brain may allow nurse bees to perceive hunger earlier and to consume the food their sitting on. Forager bees have to be more persistent to hunger, when they are foraging, and food is not so accessible. The findings of reduced expression of the fructose receptor AmGr3 in the antennae of nurse bees are congruent with my other result that nurse bees are also less responsive to fructose at the antennae when compared to foragers (**Manuscript I, Chapter 2**). This is possible, since nurse bees sit more likely on ripe honey which contains not only higher levels of sugars but also monosaccharides (such as fructose), while foragers have to evaluate less-concentrated nectar.

My investigations of the expression of AmGr1 in the antennae of honeybees found no differences between nurse bees and foragers, although foragers are more responsive to the respective sugar sucrose (**Manuscript I, Chapter 2**). Considering my finding that AmGr2 is the co-receptor of AmGr1, it can be assumed that AmGr1 and the mediated sucrose taste might not be directly controlled by its expression, but indirectly by its co-receptor. My thesis therefore clearly shows that sugar perception is associated with division of labor in honeybees and appears to be directly or indirectly regulated via expression.

The comparison with a characterization study using other bee breeds and thus an alternative protein sequence of AmGr1 shows that co-expression of different AmGr1 versions with AmGr2 alters the sugar response differently. Therefore, this thesis provides first important indications that alternative splicing could also represent an important regulatory mechanism for sugar perception in honeybees.

Further, I found out that the bitter compound quinine lowers the reward quality in learning experiments for honeybees (**Manuscript IV, Chapter 5**). So far, no bitter receptor has been found in the genome of honeybees and this thesis strongly assumes that bitter substances such as quinine inhibit sugar receptors in honeybees. With this finding, my work includes other molecules as possible regulatory mechanism in the honeybee sugar perception as well. We showed that the inhibitory effect is lower for fructose compared to sucrose. Considering that sugar signals might be processed as differently attractive in honeybees, this thesis concludes that the sugar receptor inhibition via quinine in honeybees might depend on the receptor (or its co-receptor), is concentration-dependent and based on the salience or attractiveness and concentration of the sugar present.

With my thesis, I was able to expand the knowledge on honeybee's sugar perception and formulate a complex, comprehensive overview. Thereby, I demonstrated the multidimensional mechanism that regulates the sugar receptors and thus the sugar perception of honeybees. With this work, I defined AmGr1 and AmGr3 as the basis of sugar perception and enlarged these components to the co-receptor AmGr2 and the possible splice variants of AmGr1. I further demonstrated how those sugar receptor components function, interact and that they are clearly involved in the division of labor in honeybees. In summary, my thesis describes the mechanisms that enable honeybees to perceive sugar in a complex way, even though they inhere a limited number of sugar receptors. My data strongly suggest that honeybees overall might not only differentiate sugars and their diet by their general sweetness (as expected with only one main sugar receptor). The found sugar receptor mechanisms and their interplay further suggest that honeybees might be able to discriminate directly between

monosaccharides and disaccharides or sugar molecules and with that their diet (honey and nectar).

Zusammenfassung

Beim dem eusozialen Insekt Honigbiene (*Apis mellifera*) leben tausende sterile Arbeitsbienen zusammen mit einer fortpflanzungsfähigen Königin in einem Volk. Alle Aufgaben in der Kolonie werden von diesen Arbeiterinnen erledigt, während sie eine altersabhängige Arbeitsteilung durchlaufen. Als Stockbienen beginnend übernehmen sie Aufgaben im Stock wie die Reinigung oder die Produktion von Larvenfutter und entwickeln sich später zu Sammlerinnen. Das Wahrnehmung von Süße spielt für alle Honigbienen eine entscheidende Rolle, egal ob sie auf den Honigvorräten im Stock sitzen oder nach Nahrung suchen. Ihre Fähigkeit Süße zu wahrzunehmen ist zweifellos notwendig, um Nahrungsquellen zu identifizieren und zu bewerten. Viele der Verhaltensentscheidungen bei Honigbienen basieren auf ihrer Zuckerwahrnehmung, entweder auf individueller Ebene für die Nahrungsaufnahme oder für soziales Verhalten wie beispielsweise das Sammeln oder Verarbeiten von Nektar. Honigbienen zeigen auf vielen Ebenen ein komplexes Spektrum bei der Wahrnehmung von Süße. Sie können mindestens sieben Zuckerarten wahrnehmen und sammeln diese für ihren Stock. Darüber hinaus scheinen sie zwischen diesen Zuckern unterscheiden zu können oder zeigen zumindest klare Präferenzen beim Sammeln. Außerdem ist die Zuckerwahrnehmung bei Honigbienen nicht starr. Ihre Zuckerwahrnehmung ändert sich, wenn sie von einer Stockbiene (z. B. Ammen) zum Nahrungssammeln außerhalb des Stockes übergehen, und ist somit mit ihrer Arbeitsteilung verbunden. Andere direkte oder unmittelbare Faktoren, die die Reaktion auf Zucker verändern, sind Stress, Hunger oder zugrunde liegende Faktoren wie der Genotyp.

Interessanterweise steht die Komplexität der Zuckerwahrnehmung in starkem Kontrast zu der Tatsache, dass Honigbienen bisher anscheinend nur drei mögliche Zuckerrezeptoren haben.

In dieser Arbeit konnten wir die drei bekannten Honigbienenzuckerrezeptoren (AmGr1, AmGr2 und AmGr3) in *Xenopus*-Oozyten vollständig und umfassend charakterisieren (**Manuscript II, Chapter 3 und Manuscript III, Chapter 4**). Wir konnten zeigen, dass AmGr1 ein breitdetektierender Zuckerrezeptor ist, der auf Saccharose, Glukose, Maltose, Melezitose und Trehalose (der Hauptblutzucker bei Honigbienen), aber nicht auf Fruktose reagiert. AmGr2 fungiert als ein Co-Rezeptor, der die Spezifität von AmGr1 verändert. AmGr3 ist ein spezifischer Fruktoserezeptor und wir haben die Heterodimerisierung der Rezeptoren überprüft. Mit meinen Studien konnte ich die gefundene Ligandenspezifität der Zuckerrezeptoren *in vivo* reproduzieren und vergleichen, indem ich Rezeptormutanten mit CRISPR/Cas9 generierte. Dabei konnte ich AmGr1 und AmGr3 als die Basisrezeptoren von Honigbienen definieren, die bereits alle Zucker ihres bekannten Geschmacksspektrums detektieren können.

In der Expressionsanalyse meiner Doktorarbeit (**Manuscript I, Chapter 2**) konnte ich zeigen, dass beide Basisrezeptoren in den Antennen und im Gehirn von Ammenbienen und Sammlerinnen exprimiert werden. Diese Arbeit geht davon aus, dass AmGr3 (wie das Homologe in *Drosophila*) als Sensor für Fruktose fungiert, die das Sättigungssignal sein könnte, während AmGr1 Trehalose als Hauptblutzucker im Gehirn wahrnehmen kann. Beide Rezeptoren zeigen eine reduzierte Expression im Gehirn von Sammlerinnen im Vergleich zu Ammenbienen. Diese Ergebnisse könnten die höher konzentrierte Ernährung der Ammenbienen im Stock widerspiegeln. Die höhere Anzahl an Rezeptoren im Gehirn könnte es den Ammenbienen ermöglichen frühzeitiger Hunger wahrzunehmen und die Nahrung, auf der sie sitzen aufzunehmen. Sammelbienen dagegen müssen beim Sammeln und dem reduzierten Nahrungsangebot ausdauernder sein. Die gemessene reduzierte Expression des Fruktoserezeptors AmGr3 in den Antennen von Ammenbienen entsprechen meinen anderen Ergebnissen, wonach Ammenbienen im Vergleich zu Sammelbienen an den Antennen auch weniger empfindlich auf Fruktose reagieren (**Manuscript I, Chapter 2**). Dies ist möglich, da Ammenbienen eher auf reifem Honig sitzen, der nicht nur einen

höheren Zuckergehalt, sondern auch vermehrt Monosaccharide (wie Fructose) enthält, während Sammelbienen weniger konzentrierten Nektar bewerten müssen.

Meine Untersuchungen zur Expression von AmGr1 in den Antennen von Honigbienen ergaben keine Unterschiede zwischen Ammenbienen und Sammlerinnen, obwohl Sammlerinnen empfindlicher auf den entsprechenden Zucker Saccharose reagieren. Angesichts unserer Ergebnisse, dass AmGr2 der Co-Rezeptor von AmGr1 ist, kann die Hypothese aufgestellt werden, dass AmGr1 und der vermittelte Saccharose-Geschmack möglicherweise nicht direkt durch seine Expression, sondern indirekt durch seinen Co-Rezeptor reguliert werden. Meine Dissertation zeigt somit deutlich, dass die Zuckerwahrnehmung bei Honigbienen mit Arbeitsteilung verbunden ist und direkt oder indirekt über die Expression geregelt zu werden scheint.

Der Vergleich mit einer anderen Charakterisierungsstudie, durchgeführt an anderen Bienenrassen und damit einer alternativen Proteinsequenz von AmGr1, zeigt, dass die Co-Expression verschiedener AmGr1-Varianten mit AmGr2 die Zuckerantwort unterschiedlich verändert. Daher liefert diese Arbeit erste wichtige Hinweise darauf, dass alternatives Spleißen auch bei Honigbienen einen wichtigen Regulationsmechanismus für die Zuckerwahrnehmung darstellen könnte.

Des Weiteren habe ich herausgefunden, dass der Bitterstoff Chinin die Qualität der Belohnung in Lernexperimenten für Honigbienen senkt (**Manuscript IV, Chapter 5**). Bisher wurde kein Bitterrezeptor im Genom von Honigbienen gefunden und diese Arbeit deutet darauf hin, dass Bitterstoffe wie Chinin Zuckerrezeptoren in Honigbienen hemmen. Mit dieser Erkenntnis schließt meine Dissertation auch andere Moleküle als mögliche Regulationsmechanismen in die Zuckerwahrnehmung der Honigbiene ein. Wir haben gezeigt, dass die hemmende Wirkung bei Fruktose im Vergleich zu Saccharose geringer ist. Unter der Berücksichtigung, dass Zuckersignale bei Honigbienen möglicherweise unterschiedlich attraktiv verarbeitet werden, kommt meine Arbeit zu dem Schluss, dass die Hemmung der Zuckerrezeptoren durch Chinin

bei Honigbienen abhängig ist von der verwendeten Konzentration, der Bedeutung bzw. Attraktivität des Zuckers und seiner Konzentration.

Mit meiner Doktorarbeit konnte ich das Wissen über die Zuckerwahrnehmung der Honigbiene insgesamt erweitern und einen komplexen, umfassenden Überblick formulieren. Ich konnte den mehrdimensionalen Mechanismus aufzeigen, der die Zuckerrezeptoren und damit die Zuckerwahrnehmung von Honigbienen reguliert. Ich konnte AmGr1 und AmGr3 als Basis der Zuckerwahrnehmung definieren und diese Komponenten auf den Co-Rezeptor AmGr2 und die möglichen Spleißvarianten von AmGr1 erweitern. Ich habe außerdem gezeigt, wie diese Zuckerrezeptorkomponenten funktionieren, interagieren, und dass sie eindeutig an der Arbeitsteilung bei Honigbienen beteiligt sind. Zusammenfassend beschreibt meine Dissertation die Mechanismen, die es Honigbienen ermöglichen, Zucker auf komplexe Weise wahrzunehmen, selbst wenn sie eine begrenzte Anzahl von Zuckerrezeptoren besitzen. Meine Daten deuten stark darauf hin, dass Honigbienen Zucker und ihre Nahrung nicht nur aufgrund ihrer generellen Süße unterscheiden können (wie dies mit nur einem Hauptzuckerrezeptor zu erwarten wäre).

Die gefundenen Zuckerrezeptormechanismen und deren Zusammenspiel legen nahe, dass Honigbienen möglicherweise direkt zwischen Monosacchariden und Disacchariden bzw. Zuckermolekülen und damit zwischen ihrer Nahrung (Honig und Nektar) unterscheiden können.

1 General Introduction

The very name of the honeybee (*Apis mellifera*) indicates that sweetness, and the ability to perceive it, is an important part of the bees' environment and physiology. Due to the industrial production of sugar, the very sweet product honey has lost some importance to humans but not the fascinating, social insect itself (Allsop & Miller, 1996; Eggleston, 2019). For the honey production honeybees visit many flowers to collect nectar but they also collect pollen as a source for protein (Seeley, 1985; Winston, 1991). In doing so, these eusocial insects perform significant pollination, providing a massive, as well as economic value for modern agriculture and nature (Gill, 1990; Brittain et al., 2013). Foraging for food is therefore just one of many tasks in the colony performed by thousands of female worker bees (Seeley, 1985; Winston, 1991). These sterile individuals initially take on tasks within the hive and thereby undergo an age-dependent division of labor (Ben-Shahar, 2005). Those honeybee workers first perform tasks within the beehive and only later develop into foragers. During this transition their access to and the type of diet changes drastically as well as their task specific behavior towards it (Scheiner et al., 2004). Additionally, they are also able to communicate with each other and pass on information from the inner hive or food sources outside onto other individuals (Michelsen, 2003; George & Brockmann, 2019). This complex social organization, the eusociality, the division of labor, the learning ability, the cognition, and the behavior along with its already sequenced genome makes the honeybee an important model organism for various research questions.

For all these scientific areas it is undoubtedly interesting how the honeybee's most anticipated and expected stimulus sweetness is perceived, processed, regulated, and set into the context of division of labor.

1.1 Perception of sweetness is an important ability for honeybees to perform division of labor

Honeybees are eusocial insects. The only queen of the colony is responsible for its growth, and new queens and drones (in summertime) are responsible for its propagation (Butler & Fairey, 1963; displayed as colony development in Figure 1). All other tasks are performed by the sterile worker bees. Other than drones (emerging from unfertilized eggs), queens and workers emerge from fertilized eggs. Worker larvae are fed a less rich diet, pupate after about 13 days and hatch after 21 days (see individual development Figure 1). Young workers undergo an age-dependent division of labor starting with tasks within the hive such as cleaning (Seeley, 1985; Calderone et al., 1989; Ben-Shahar, 2005). Later they develop into nurse bees, begin to build wax combs, guard the nest entrance, and perceive food before they participate in their first orientation flights to become foragers (Seeley, 1985; worker development in Figure 1).

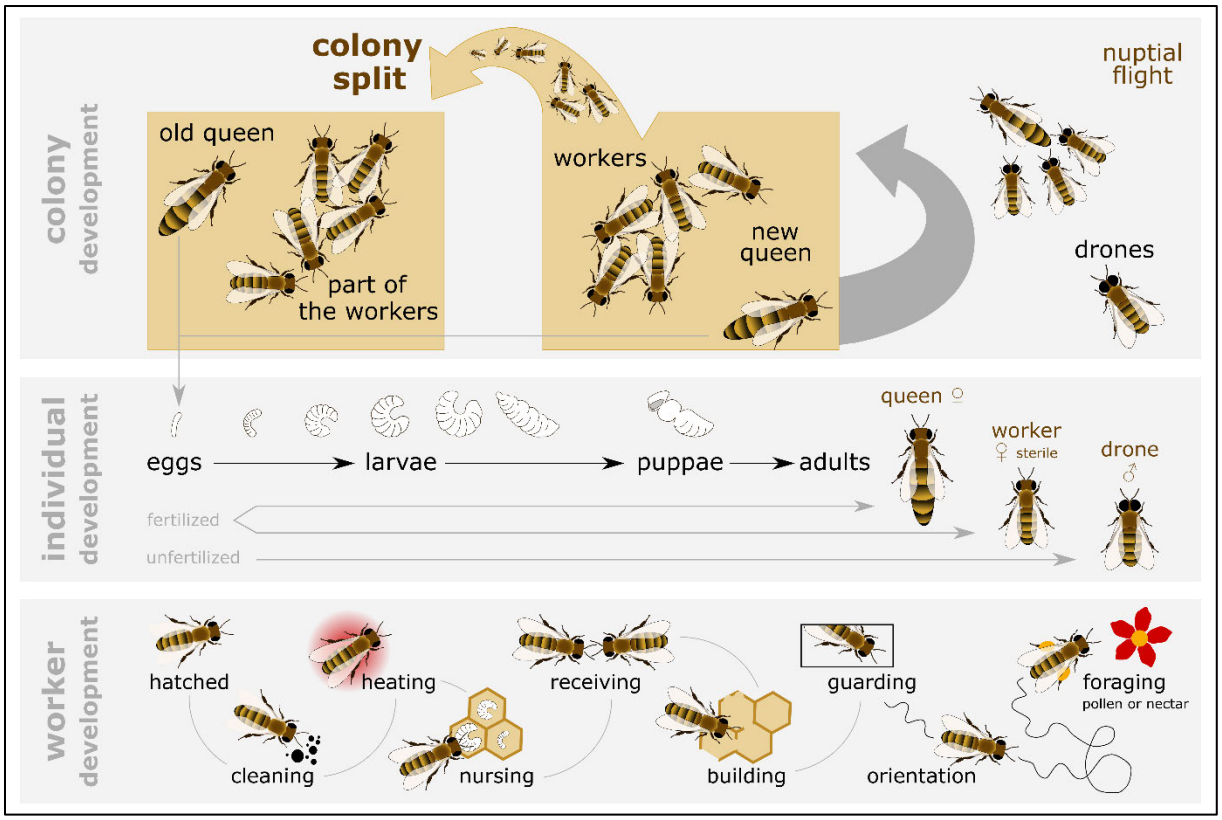

Figure 1: Overview of the biology of the eusocial insect honeybee adapted from Winston (1991). Honeybee colonies consist of one fertile queen and up to 30,000 worker bees. For colony propagation a portion of the worker bees and the old queen leave the colony just before a new queen hatches. When they have found another hive, the old queen begins to lay eggs and the new colony grows. Meanwhile the freshly hatched queen in the old colony is mated by several drones on the nuptial flight and begins to lay eggs to continue the old colony. From these eggs the honeybee larvae hatch after 3 days, develop into pupae before the adults emerge. Workers and new queens can develop from fertilized eggs. Male drones hatch from unfertilized eggs. The development time of the drones is the longest with 24 days, queens only need 16 days, workers 21 days. After hatching as imago, the honeybee workers undergo age-dependent division of labor which is flexible and can be adapted. They start to clean the brood cells, and care for the brood with heating. As nurse bees they feed the larvae with food from their hypopharyngeal glands. Later they receive the incoming nectar. Worker bees develop wax glands for building the wax combs. They protect the nest entrance before performing the first orientation flights. At the end of their lives (around 23 days in summer) they forage for pollen, nectar, water or plant resins (propolis).

In all these tasks taste is not only an important sense for honeybee workers to find and ingest their own diet but also necessary to fulfill taste-related social behaviors (Seeley, 1985; Winston, 1991). Stored pollen provides the protein source for honeybees and is consumed by freshly emerged bees. But besides that, nurse bees use pollen to produce the nutrition rich larval food with their hypopharyngeal glands which they develop from about the third day (Seeley, 1985; Winston, 1991). Additionally, it is well known that all honeybee workers feed on carbohydrates which they collect mainly from the nectar of flowering plants (Brodschneider & Crailsheim, 2010), and therefore particularly depend on the ability to sense sweetness. Hereby, the food supply in the

hive contains a wide spectrum from freshly harvested and low-concentration nectar to ripe honey. With that bees in the hive depend on their perception of sugar for choosing the right food for intake. Further, honeybees can differentiate between unripe nectar and ripe honey (Doner, 2003). Un-ripe nectar is taken up by in-hive bees, enriched with enzymes, and the water content is evaporated until they start to cap the ripe honey (Seeley, 1985). This clearly demonstrates that sugar perception and evaluation is also crucial for different behavioral responses of in-hive bees towards stored food. When bees later turn into receiver bees they can verifiably assess and evaluate the pollen and the nectar brought in by the foragers (Michelsen, 2003; George & Brockmann, 2019). It has been shown that in-hive bees give the foragers feedback on the quality of their forage but also on the hives stock level. Thus, the sugar perception of receiver bees and their subsequent behavioral feedback have a significant influence on foraging behavior of the whole colony (Schulz et al., 1998; Farina et al., 2007). As foragers honeybees must find and evaluate food sources considering quality, concentration, colony needs, and external supply based primarily on the pollen and nectar of flowering plants. Therefore, the gustatory ability of foragers, and in particular their perception of sweetness, is crucial for the inflow of food and nectar of the colony. Most of the foragers specialize on either pollen or nectar exclusively (Scheiner et al., 1999; Scheiner et al., 2001), while some collect both, water or propolis (a plant resin used as an antibacterial nest material; Hunt et al., 1995; Page, et al., 2000). Honeybee foragers also use their ability to perceive sweetness when they decide whether to communicate the direction and distance of the food source when back in the hive (von Frisch, 1967; Riley et al., 2005). All of this shows that the perception of sweetness plays an important role for worker bees during their lifespan and when carrying out different tasks.

Although division of labor depends on age, even colonies consisting of same-aged bees (i.e., single-cohort-colonies; Huang & Robinson, 1996; Schulz et al., 1998) perform sophisticated task partitioning. The generally accepted hypothesis, trying to explain division of labor, assumes that differences in sensory response thresholds lie at the basis of social organization in a honeybee colony. Assuming that division of labor is not

strictly age-dependent, can be reversed and can still occur in colonies of same-age bees, an additional mechanism must be involved. The most settled model here assumes that bees inhere an individual responsiveness towards task-related stimuli, for instance the food-related sweetness, that may differ (Huang & Robinson, 1992; Theraulaz et al., 1998). In this so-called response threshold model (Huang & Robinson, 1992; Theraulaz et al., 1998) the appropriate behavioral response is triggered when the task-related stimulus intensity reaches the bee's individual threshold for it. The examples show that the factors influencing the threshold does not have to be exclusively age. Genetic variations due to sisterhood and different drones as fathers, or epi-genetical changes, may also influence those thresholds (Page & Robinson, 1991; Pankiw & Page, 1999). Experiments examining the behavior of honeybees to a specific stimulus support the assumptions of the response threshold model. For example, it was shown that nurse bees have a lower responsiveness for light stimuli compared to foragers, matching their behavioral differences inside and outside the hive (Ben-Shahar, 2005; Erber et al., 2006; Scheiner et al., 2014). Additionally, a different responsiveness to sucrose was measured in honeybees performing different tasks. Nurse bees were less responsive compared to foragers (Scheiner et al., 2004). This may result in bees foraging for lower concentrated nectar outside the hive, while in-hive bees prefer to feed on high-concentrated honey. All of this shows that the honeybees' social organization is strongly associated with perception of sweetness as well as nutritional cues (reviewed by Ament et al., 2010).

Importantly, most experiments on sugar responsiveness in relation to division of labor focus on sucrose. This is astonishing since honeybees can detect a variety of sugars (as described before) and their diet comprises sucrose, fructose as main sugars (also glucose, as described in detail in the next section). Experiments that investigate the responsiveness of different bee cast (such as nurse bees and forager) towards other sugars, such as fructose, are missing.

1.2 Taste in honeybees is related to their natural context and their sweet diet

The total sugar concentration of nectar can vary from 5% to 80% (Graham, 1992). During the ripening process honeybees remove water for preservation. Furthermore, long sugar chains are broken down by enzymes (diastase, invertase and glucose oxidase). That is why honey contains a higher sugar percentage (up to 80% and more; Doner, 2003), in addition to minerals, enzymes, amino acids and vitamins. Honey contains a higher fraction of fructose and glucose but a lower fraction of sucrose (or other di- and oligosaccharides) than nectar (Harborne, 1994). Sucrose is often the main component of nectar as in the plant families *Laminacea* or *Ranuculacea,* and their representatives mint, buttercup and clematis (Chalcoff et al., 2006; Ball, 2007). Yet, there are nectars that naturally contain larger portions of fructose and glucose, and a lower portion of sucrose, such as oilseed rape (Bertazzini & Forlani, 2016). Already a very early study by Percival categorized these plant nectars based on the distribution of these three main sugars (see Table 1) into (a) nectars high in sucrose, (b) nectars with roughly evenly distributed sugars, and (c) nectars in which only glucose and fructose predominate (Percival, 1961).

Table 1: Exemplary plant families and their nectars classified according to the categories defined by Percival (1961). A distinction is made between nectars rich in sucrose (a), nectars with an even distribution of sugar (b; sucrose, glucose and fructose) and nectars without, or with only small amounts of sucrose (c; only glucose and fructose). A tendency towards only one specific nectar category (a, b or c) was only found in some plant families (shown here as an example). However, the species within a family often show a wide variety of the nectar categories, so that most plant families cannot be assigned a, b or c (like Geraniales[+]).

exemplary plant family	three nectar categories*			detailed description of the findings
	(a)	(b)	(c)	
Balsaminaceae	X			all tested species showed (a) nectars
Ranunculacea	X			all tested species showed (a) nectars
Ranales	X			all tested species showed rich (a) nectars
Berberidales	X			all tested species showed rich (a) nectars
Fumariaceae	X			all tested species showed rich (a) nectars
Geraniales[+]	X	X	X	no specific nectar type was found among the species
Boranginales		X		tested species rich in the intermediate products
Lamiales		X		tested species rich in the intermediate products
Compositae	(x)		X	most tested species showed (c) nectars, smaller section (a)
Umbelliferae			X	all tested species showed (c) nectars
Limnanthaceae			X	all tested species showed (c) nectars
Cruciales			X	all tested species showed (c) nectars, 1 exception
Oxalidacea			X	all tested species showed (c) nectars, 1 exception
Cruciferae			X	all tested species showed (c) nectars, 1 exception
Saxifragaceae			X	almost all tested species showed (c) nectars, few exceptions
Rosaceae	(x)	(x)	X	most tested species showed (c) nectars, few were (a) and (b)

*three nectar categories:		
(a)	high sucrose	(oligosaccharide)
(b)	evenly fructose, sucrose, and glucose	(oligosaccharide & intermediate breakdown products)
(c)	predominant glucose and fructose	(intermediate breakdown products)

Besides this, nectars also contain other sugars such as maltose (found particularly in clover according to Furgala et al., 1958) or small amounts of raffinose and melezitose (Bosi & Battaglini, 1978; de Brito Sanchez et al., 2007; Stanley et al., 2013). In addition to nectar, honeybees collect honeydew, a sugary metabolic by-product excreted by aphids that contains large amounts of melezitose. Very early on von Frisch gained fundamental insights into the bees' sense of taste in free-flight experiments in which he showed that bees only accept 7 of the 30 sugars tested (von Frisch, 1934). Not surprisingly, he found that honeybees collect sugars that occur in their natural diet (sucrose, glucose, fructose, melezitose, maltose; von Frisch, 1934). Additionally, bees

collected α-methyl glucoside and trehalose which was later found to be the main blood sugar of honeybees and most insects (Woodring et al., 1993; Blatt & Roces, 2001). Since organisms must be able to sense the internal blood sugar level, honeybees seem to be able to perceive their type of blood sugar (trehalose) externally as well. Although sugar plays a very prominent role, honeybees are also able to perceive amino acids, fatty acids and salt. For instance, they prefer pollen or even sugar solutions enriched with amino acids (Harborne, 1994; Kim & Smith, 2000; Cook et al., 2003; Bertazzini et al., 2010) and are able to discriminate pollen enriched with amino acids or fatty acids in a learning experiment (Ruedenauer et al., 2021). Further, foragers prefer a certain salt concentration to distilled (totally clean) water or higher concentrations (Butler & Fairey, 1963; Bonoan et al., 2017). Honeybees in general are attracted to those lower concentrations, whereas high salt contents are aversive as they are also to mammals (Oka et al., 2013). Similar to the effect on mammals, bitter substances also seem to have an aversive effect on honeybees. For example, quinine can be used as a punishment stimulus in learning experiments (Finke et al., 2021).

As described honeybees cover a wide range of substances when evaluating their food but tasting sugars accounts for the largest part. Their ability to sense sweetness includes a wide range of concentrations as well as different types of sugar. This shows that the taste of sweetness in honeybees covers a much wider spectrum than previously studied, and analyses of the responsiveness to sugars, others than sucrose, are still lacking. In addition, it has not yet been proven at the receptor level how all sugars detected or foraged by honeybees can also be detected by the sugar receptors, when characterized, and which sugar receptors are particularly involved.

1.3 Sugar perception in honeybees is performed with only a small set of gustatory receptors

The external organs responsible for taste perception in honeybees are the antennae, the tarsi and the mouthparts (de Brito Sanchez et al., 2007). With the sugar sucrose it was clearly shown that bees perceive sweetness in these organs and react accordingly with the extension of their tongue (PER, Proboscis Extension Response; Takeda, 1961; Bitterman et al., 1983). On the surface of the three sensory tissues there are specific structures, the so-called gustatory sensilla, also referred to as bristles. They can be visualized as hair-like structures by electron microscopy (Galić, 1971; Whitehead & Larsen, 1976). Sensilla bear a compound of several gustatory receptor neurons (GRN) presenting the chemoreceptors at their apex (Haupt, 2007; Simcock et al., 2017). Receptors that respond towards non-volatile substances via contact, such as sugar molecules, are referred as gustatory receptors or Grs (Mitchell et al., 1999). In electrophysiological studies electrodes were placed in the sensilla, which could identify gustatory neurons by their signaling when presented with a sweet stimulus. By that a response could be measured in labial palps, a subsection of the mouthparts, when stimulated with sucrose or fructose (Whitehead, 1978). Furthermore, a stimulation with sucrose could surge a signal in the last segment of the antennae (Haupt, 2007; de Brito Sanchez et al., 2015), the third and fourth tarsomeres of the pre-tarsi (de Brito Sanchez et al., 2014) or the galea as another subsection of the mouthparts (Miriyala et al., 2018). It is known that these signals are initially triggered when the tested sugar binds to the corresponding receptor (Cuatrecasas, 1974). In insects some of these gustatory receptors (Grs) appear to have seven transmembrane domains and have been suggested to function as ion-gated channels when performing structural analysis with the genomic sequences (Benton et al., 2006; Sato et al., 2008; Zhang et al., 2011; Hull et al., 2012; Hopf et al., 2015). Homology analyses with other insect receptors showed that the fruit fly (*Drosophila melanogaster*) has 68 Grs, the mosquito (*Anopheles gambiae*) has 76 Grs, but the honeybee (*Apis mellifera*) has only ten gustatory receptors (AmGr1-10; Robertson & Wanner, 2006; Simcock et al., 2017). This

is remarkable, since honeybees can perceive several different tastes, including at least seven types of sugar alone (as described above). So far, only three of the gustatory receptors (AmGr1-AmGr3) have been identified and partially characterized as sugar receptors (Robertson & Wanner, 2006; Jung et al., 2015; Takada et al., 2018).

Since bees can perceive a wide range of sweetness, a broad set of the respective sugars need to be included in a comprehensive characterization of these receptors. For example, melezitose is accepted by free-flying bees and is present in honeydew and nectar but has not yet been tested in such experiments. Although AmGr2 appears to function as a co-receptor for AmGr1, detecting a broad sugar spectrum, it is not yet clear to what extent all three known sugar receptors (AmGr1, AmGr2, and AmGr3) interact with each other in different combinations of co-expression. AmGr3 is suspected to be a specific fructose receptor. But its specificity needs be proven, also verified in the living animal, and its specific and unique role needs to be set and interpreted in a larger context with the overall sugar perception of honeybees.

1.4 Investigation of sugar receptor regulation to understand sugar perception in honeybees

For a comprehensive understanding of the receptor-based perception of sweetness in honeybees it is necessary to investigate gene expression. Cells of many species and organisms regulate the amount of protein produced by their gene expression, providing important clues for honeybees as well (Rockmann & Kruglyak, 2006). Scheiner et al. (2017) already showed that nurse bees and forager bees differ in their sucrose responsiveness, but the gene expression of the putative sucrose receptor (AmGr1) has not yet been investigated in the two casts (Scheiner et al., 2017). Yet, qPCR is already established for many honeybee genes and can be transferred to another gene whose sequence is known, like the gustatory receptors (Reim et al., 2013).

Both early PER experiments and electrophysiological measurement indicated that antennae, tarsi and mouthparts are capable for sucrose taste in honeybees (Whitehead, 1978; Scheiner et al., 2004; Haupt, 2007; Simcock et al., 2017). Besides those tissues, the expression of AmGr1 and AmGr2 was also detected in the brain (Simcock et al., 2017) and AmGr3 was found in the ganglia, glands, and midgut but not in the brain so far (Takada et al., 2018). This is not surprising since sweet taste receptors in other organisms are already found in many extra-gustatory tissues and are proposed to regulate metabolic processes (Laffitte et al., 2014).

As described, sugar responsiveness changes when worker bees undergo age-dependent division of labor. It is questionable whether differences in the expression of individual sugar receptors in the tissues are linked to the differences in behavior of the honeybees. Such studies can provide important insights and compare the individual sugar responsiveness (for instance of sucrose and fructose) with the expression of the respective receptors (AmGr1 and AmGr3) detecting them. Whether these changes in behavior are linked to a change in gene expression in the organs and tissues involved in taste perception, will be studied in **Manuscript I, Chapter 2**.

1.5 Genomic manipulation as a tool to study sugar receptor specificity and regulation in the honeybee

When studying the sugar receptor homologues in *Drosophila,* mutants with an inactive receptor were generated via transposons (Lim & Simmons, 1994). Such mutants showed a measurably different satiety behavior compared to those with functioning receptors in their brain. This technique of genetic manipulation, as well as the production of knock-out or knock-in mutants, is frequently performed in *Drosophila* but generally not available or successful in honeybees (reviewed by Lin et al., 2014). The advent of the CRISPR/Cas9 method has made it possible to perform gene editing in many organisms where it had not been possible previously. CRSIPR/Cas9 has been employed successfully in mammals as well as in numerous insects (respectively Bassett

& Liu, 2014; Singh et al., 2015; Kistler et al., 2015; Zeng et al., 2016). The enzyme Cas9 and artificial RNA fragments (sgRNAs) thereby introduce site-specific double-strand breaks into the genomic DNA which lead to nonsense mutations and non-functional proteins. For honeybees this means that adult worker bees, hatched from eggs injected and mutated with Cas9 and such specific sgRNA, will carry this mutation throughout their cells (Doudna & Charpentier, 2014). Thus, Roth et al. was able to produce fully mutated worker bees lacking a functional feminizer (fem) protein via CRISPR/Cas9. Mutants in *fem,* even under normal nutrition, developed queen-like reproductive organs proving the gene as a genetic switch for size-control for those animals (Roth et al., 2019). As another example, Kohno et al. produced mosaic mutated queens in the major royal jelly protein gene (*mrip1*) to obtain mutated drones from them and, after backcrossing, fully mutated worker bees in turn (Kohno et al., 2016). Since these first studies many genes in honeybees have been successfully edited with CRISPR/Cas9 and this method can now be considered as established (for example Hu et al., 2019; Nie et al., 2021; Wang et al., 2021). Accordingly, the CRISPR/Cas9 method is very useful to reveal sugar receptor function and regulation in live honeybee worker.

Although the sugar receptors have been characterized partly in the *in-vitro Xenopus* cell system, their ligand affinity has not been studied in live animals yet. To prove function and specificity of the sugar receptors in honeybees, it is important to study the behavior and responsiveness of their respective mutants towards the most common sugars. With CRISPR/Cas9 technique it would be possible, for the first time, to verify the role of sugar receptors in taste perception *in vivo* in honeybees. This is utmost important, since the fruit fly *Drosophila* (as described above) differs not only in the number of sugar receptors but also in the ability for taste perception and the associated behavior.

1.6 Bitter substances interfere with the sugar perception of honeybees

Sugar receptors and the taste they mediate are an important factor for honeybees to determine food quality while foraging and consuming carbohydrates. The nectar of bee-pollinated plants can contain many other compounds besides sugars (Adler, 2000). It is possible that these include substances that are harmful or difficult for honeybees to digest. Such substances can include alkaloids, amino acids, phenols and other secondary compounds (Liu et al., 2004; Singaravelan et al., 2005; Liu et al., 2007). Most organisms, such as humans, recognize inedible ingredients by their bitter taste and therefore avoid them (Wooding et al., 2021). This behavior is also found in many other insects. In the fruit fly *Drosophila,* for instance, bitter taste induces aversive reactions and inhibits feeding (French et al., 2015). Also, the mosquito (*Aedes aegypti*) perceives the most common insect repellent DEET as bitter via contact with the tarsi and avoids it. But evidence for the avoidance behavior of honeybees when exposed to bitter taste is rare. The most prominent indirect evidence is found in learning experiments, where bitter substances in combination with sugar were used as aversive stimulus. These experiments unequivocally show that bees can be punished with bitter substances, such as quinine, to enhance their learning (Chittka et al., 2003; de Brito Sanchez et al., 2014; de Brito Sanchez et al., 2015; Finke et al., 2021). For this experiment quinine, an alkaloid that does not occur naturally in nectar but is extracted from the bark of *Cinchona sp.,* was mixed with sucrose (Barreiro et al., 2012). Overall, those experiments clearly show that honeybees perceive bitter, however, not all studies are conclusive. Von Frisch postulated in 1967 that honeybees are not sensitive to bitter substances alone (von Frisch, 1967). It also can be shown that harnessed honeybees trained for odor, as another stimulus, drink bitter and toxic substances, even when those have malaise effects (Ayestaran et al., 2010).

Most organisms have bitter receptors that can detect such substances prior to ingestion (Behrens, & Meyerhof, 2006; Weiss et al., 2011; Sparks & Dickens, 2016). Nevertheless, among the reduced set of gustatory receptors of the honeybee (see

section above), no bitter receptor has yet been found (Robertson & Wanner, 2006). Homology analyses with the fruit fly gave no indication of bitter receptors in the genome of the bee, even if for *Drosophila* there are already five "core-bitter receptors" known among many other substance specific receptors (Moon et al., 2009; Lee et al., 2010; Weiss et al., 2011; French et al., 2015). Interestingly, in addition to the bitter receptors, there seems to be a second perception pathway in the fruit fly for bitter substances that mediates their avoidance. Flies with ablated bitter-sensitive cells were still able to avoid the bitter substance strychnine mixed with sucrose (French et al., 2015). Such experiments suggest that their perception of sugar is influenced by the bitter substances rather than tasting bitter. The mechanism behind this has not yet been studied in honeybees but might apply for their bitter perception as well. For instance, honeybees in PER (Proboscis Extension Reflex) experiments react less readily when exposed to a sucrose mixture with bitter substance, such as quinine at the antennae (de Brito Sanchez et al., 2005). Current studies about quinine as inhibitory bitter substance focus on the sugar sucrose which is detected by the sugar receptor AmGr1. However, fructose is specifically perceived by another receptor (AmGr3, described previously).

How bitter substances affect sugar receptors, and whether honeybees inhere bitter receptors at all, is still unknown. It is also unclear how bitter substances, mixed with various sugars, influence or interfere the behavior of honeybees (such as foraging). In the last Manuscript (**IV, Chapter 5**) I show results that might deliver important evidence that bitter substances, like quinine, are indirectly perceived by the sugar receptors.

1.7 Thesis Outline

Sugar perception is the most important trait for the honeybee to find, evaluate and ingest their carbohydrate food sources and to perform several tasks during division of labor. For this, honeybees supposedly only have three sugar receptors (AmGr1, AmG2 and AmGr3) which are the particular focus of my investigations.

The results of these research approaches are presented in the following **Manuscripts I-IV in Chapter 2-5**.

In **Manuscript I (Chapter 2)** I investigated the question whether social organization is related to differential sugar sensing. I studied the differences in sugar perception of nurse bees and pollen foragers with PER (Proboscis Extension Reflex) experiments not only for sucrose but also for fructose as another comparative sugar. Since the pronounced behavioral change occurs in the nurse forager transition, I further investigated the gene expression within these roles. I quantified the mRNA expression of the sugar receptors which are capable for sucrose detection (with AmGr1) and fructose detection (with AmGr3) in the antennae and brains of individual nurse or forager honeybees.

This enabled me to uncover differences in preference for these sugars (fructose and sucrose) of bees of different roles (nurse bees and foragers). Furthermore, it was possible to show that both roles (nurse bees and foragers) have different expression levels of the related sugar receptors (AmGr1 and AmGr3) in their tissues. I was able to correlate between sucrose and fructose responsiveness. Ultimately, based on my experiments, a connection between expression patterns of the sugar receptors in the tissues and sugar perception can be established.

In **Manuscript II (Chapter 3)** I focused on the function of the sugar receptor AmGr3. A detailed characterization of this receptor could be carried out with the support of Fábio Luiz Rogé Ferreira. It was cloned in *Xenopus* oocytes and analyzed via the Patch Clamp approach to reveal that it is a hyperpolarization-activated and a specific fructose

receptor, even when tested for a broader sugar spectrum. With that it was able to show that its activation mediates different cation currents and is therefore nonselective. I was able to test how the absence of the functional receptor AmGr3 affects the sugar responsiveness of honeybees. To do this, I generated *AmGr3* nonsense mutants via the CRISPR/Cas9 method and compared their fructose and sucrose responsiveness to wildtype bees. I showed for the first time in honeybees that a mutation in a taste receptor caused by CRISPR/Cas9 has a direct impact on behavior.

In **Manuscript III (Chapter 4)** I have extended my comparative study of the function of all three sugar receptors to gain the full picture of the molecular basis of honeybee sugar reception. Therefore, I generated CRISPR/Cas9 nonsense mutants for one of the respective receptors and measured their PER to different sugars. The co-expression of two receptors (but the absence of the third), performed by Fábio Luiz Rogé Ferreira, enabled me to compare his characterization experiments with the behavior of the mutants missing the third receptor. In these experiments I placed particular emphasis on expanding the sugar spectrum for both characterization and behavioral testing. This comparative study was particularly advantageous, because it not only reveals the broad perception or specificity of the receptors, but also their possible role as a co-receptor. Expression and co-expression were controlled using YFP-tags and BIFC.

In **Manuscript IV (Chapter 5)** I investigated how honeybees evaluate bitter substances in the context of behavioral PER and learning experiments. I placed a comparative focus on sucrose and fructose, since these two sugars are perceived by two different receptors and might react differently to an inhibition through bitter. I therefore compared the olfactory learning of honeybees, when rewarded with pure sucrose or fructose or those sugar solutions added with the bitter substance quinine. I focused in these learning experiments on two different quinine concentrations for both sugars and was specifically interested in whether the inhibition of the responsiveness with quinine is comparable to a lower sugar reward per se.

In the **General Discussion (Chapter 6)** I will discuss in detail how these different findings of my investigations **(Manuscript I-IV found in Chapter 2-5)** draw a comprehensive picture about the sugar perception of honeybees. Furthermore, I will classify how the perception of sweetness is regulated at the molecular level regarding the three known sugar receptors (AmGr1, AmGr2 and AmGr3). I will discuss how these findings relate to the existing models of the division of labor and the response threshold theory. Finally, I will discuss how sugar receptor perception can be modulated by other substances, such as bitter substances, and can reduce the value of sugar for honeybees. I will summarize these findings with a formulated hypothesis in an overview and describe it in detail.

2 Manuscript I: Responses to sugar and sugar receptor gene expression in different social roles of the honeybee (*Apis mellifera*)

This chapter is a peer-reviewed manuscript published in 2018 in the Journal of Insect Physiology (Vol. 106, p. 65-70).

Authors are Laura Değirmenci, Markus Thamm and Ricarda Scheiner.

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Responses to sugar and sugar receptor gene expression in different social roles of the honeybee (*Apis mellifera*)

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ABSTRACT

Honeybees (*Apis mellifera*) are well-known for their sophisticated division of labor with each bee performing sequentially a series of social tasks. Colony organization is largely based on age-dependent division of labor. While bees perform several tasks inside the hive such as caring for brood ("nursing"), cleaning or sealing brood cells or producing honey, older bees leave to colony to collect pollen (proteins) and nectar (carbohydrates) as foragers. The most pronounced behavioral transition occurs when nurse bees become foragers.

For both social roles, the detection and evaluation of sugars is decisive for optimal task performance. Nurse bees rely on their gustatory senses to prepare brood food, while foragers evaluate a nectar source before starting to collect food from it. To test whether social organization is related to differential sensing of sugars we compared the taste of nurse bees and foragers for different sugars. Searching for molecular correlates for differences in sugar perception, we further quantified expression of gustatory receptor genes in both behavioral groups.

Our results demonstrate that nurse bees and foragers perceive and evaluate different sugars differently. Both groups, however, prefer sucrose over fructose. At least part of the taste differences between social roles could be related to a differential expression of taste receptors in the antennae and brain. Our results suggest that differential expression of sugar receptor genes might be involved in regulating division of labor through nutrition-related signaling pathways.

1. Introduction

Sugar is the main carbohydrate source for honeybees. The sugar contents of flowers can vary hugely from 5% to 80% (Graham, 1992). In many nectars like that of the plant families *Lamiacea* (mints) or *Ranaculacea* (buttercups and clematis), sucrose is the main sugar (Chalcoff et al., 2006; Ball, 2007). Other plants, such as oilseed rape, which has become a frequent monoculture in Europe that is highly attractive to honeybees (Stanley et al., 2013), produce nectar containing very little sucrose but high concentrations of glucose and fructose (Bertazzini and Forlani, 2016).

Honeybees are social insects. When they collect nectar from flowers, they do not consume it but bring it back to the colony where it is processed into honey. The task of collecting nectar is performed by specialized workers, which are normally among the oldest bees in the colony. Other tasks, such as providing food for the larvae, are performed by much younger bees (Seeley, 1995). Although division of labor is generally dependent on age, this parameter *per se* does not regulate social organization, since even young bees can be foragers and old bees can revert to nursing tasks, if necessary (Rösch, 1930; Huang and Robinson, 1992).

In the last few years, evidence has accumulated that social organization in a honeybee colony might be linked to nutrition-related signaling (review by Ament et al., 2010). The available nutrients within the colony, for example, influence the age of the first foraging flights (Schulz et al., 1998). Honeybees of food-deprived colonies start foraging earlier than foragers of well-fed colonies. Further, while nurse bees store large amounts of lipids in their fat bodies, lipid storage is strongly reduced during the nurse-forager transition (Toth and Robinson, 2005). The loss of lipids is not caused by the flight activity of foragers, because the lipid loss could even be observed in bees which were inhibited from flying out. Inhibition of fatty acid synthesis in individuals could induce them to forage precociously (Toth et al., 2005), suggesting a tight connection between changes in metabolism and the nurse-forager transition. These examples imply that division of labor is influenced by the nutritional requirements of the colony and should thus affect the evaluation of nutrients by a worker bee.

Using the proboscis extension response assay (Scheiner et al., 2013)

Abbreviations: GRS, gustatory response score; PER, proboscis extension response

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it has been shown that bees performing different tasks, such as nurse bees and foragers, differ hugely in their evaluation of sucrose solutions (Thamm and Scheiner, 2014). Foragers extend their proboscis when their antennae are stimulated with a low concentrated sucrose solution, while nurse bees only respond at higher sucrose concentrations (Scheiner et al., 2004, 2017; Thamm and Scheiner, 2014). Importantly, all of these experiments were performed using the sugar sucrose. Very little is known about the perception and evaluation of other sugars such as fructose or glucose, although they are dominant in many nectars. In addition, little is known about the molecular mechanisms controlling responses for sugars.

Although the honeybee genome harbors a multitude of olfactory receptor genes that enable workers to perceive various floral odors, it contains less than a dozen gustatory receptor genes (Robertson and Wanner, 2006). Among these, at least three genes encode for putative sugar receptors (*AmGR1*, *AmGR2* and *AmGR3*), as suggested by homology analyses (Robertson and Wanner, 2006). Two of them, *AmGR1* and *AmGR2*, are homologous to eight candidate sugar receptor genes in *Drosophila*. These *Drosophila* genes likely do not encode stand-alone sugar receptors. It seems that the respective receptors contribute to a complex interaction of multimers and co-receptors, which enables the detection more than one sugar (Dahanukar et al., 2001; Chyb et al., 2003; Slone et al., 2007; Jiao et al., 2008; Wisotsky et al., 2011; Miyamoto et al., 2013; Fujii et al., 2015).

AmGR1 is most likely the honeybee receptor for sucrose, glucose, trehalose and maltose (Jung et al., 2015). AmGR2 alone does not respond to sugars when expressed in *Xenopus* oocytes, but can act as a co-receptor by modulating the AmGR1 sugar response *in vitro* (Jung et al., 2015). However, it has yet to be shown that the response characteristics of these receptors are also valid for other expression systems. Interestingly, the putative sugar receptor gene *AmGR3* is an orthologue to fructose receptor genes from diverse insect species (*HvCr4* in *Heliothis virescens*, *BmGr9* in *Bombyx mori*, *AgGr25* in *Aedes aegypti*, and *DmGr43a* in *Drosophila melanogaster*; Robertson and Wanner, 2006; Miyamoto et al., 2012). However, the *Drosophila* DmGr43 a receptor also responds to stimulation with sucrose (Miyamoto et al., 2012), which would be different to honeybees.

Overall, there is reason to assume that *AmGR3* likely has a conserved function in perceiving fructose (and possibly other sugars), and until now no duplication or loss in other insect species has been reported (Robertson and Wanner, 2006).

All three putative sugar receptors might be involved in regulating social organization through nutrition-related sensory response thresholds (Ament et al., 2010). We here correlate the responsiveness to fructose and sucrose in different behavioral groups of honeybees with the expression of the generic sugar receptor gene *AmGR1* and the specific fructose receptor gene *AmGR3* in the antennae and brain. The results of these experiments are essential for understanding the molecular level of the taste regulation in worker bees.

2. Material and methods

2.1. Preparation of bees

Bees were collected between July and August 2015 from a hive kept in the apiary of the University of Würzburg. The colony was headed by an artificially inseminated queen (inseminated with 12 related drones) to minimize genetic variations. Bees were collected in stable weather conditions during late morning. On each test day, at least 20 individuals with defined social role (nurse bee and pollen forager) were collected. Nurse bees were collected from a comb containing open brood and while feeding a larva. Only bees poking their head into an open brood cell for at least 10 s were considered as nurse bees. Returning foragers were identified by their pollen loads. Bees were immediately placed in small glass vials and immobilized on ice. When they showed first signs of immobility, they were mounted in brass tubes as described before (Scheiner et al., 2013). The bees were then individually fed with 30% sucrose solution until satiation. They rested one hour in a dark humidified chamber maintained at 22 °C before the onset of the behavioral experiments.

2.2. Measuring responsiveness to sucrose and fructose

All bees were tested for the proboscis extension response (PER) after the consecutive stimulation of both antennae with a sugar droplet as described in Scheiner et al. (2013). Contaminations of the antennae with sugar water were immediately removed and antennae were subsequently rinsed with water. To uncover possible influences of test order, experiments alternatingly started with fructose or sucrose. After a test with water, the following sugar concentrations were tested in ascending order: 0.1%, 0.3%, 1%, 3%, 10%, and 30% (w/v)), which corresponds to a logarithmic series of approximately −1, −0.5, 0, 0.5, 1, 1.5. The order of the concentrations does not affect sucrose responsiveness. For each individual, it was noted whether it showed a PER to a certain concentration or not. Inter-trial intervals were 2 min to prevent intrinsic sensitizations (Scheiner et al., 2013). The sum of the responses to one sugar and the water pretest amounts to the gustatory response score (GRS) of a bee for fructose or sucrose.

2.3. Analysis of sugar receptor mRNA levels in antennae and brain

Receptor mRNA levels were quantified using quantitative real-time PCR (qPCR). Therefore, bees were briefly anesthetized, pairs of antennae and brain tissues were micro-dissected individually and immediately flash frozen using liquid nitrogen. RNA extraction, cDNA synthesis and quantitative real-time PCR on a Rotor-Gene® Q (Qiagen) were performed from tissues of individual bees according to the protocol of Reim et al. (2013). Gene-specific TaqMan® probes and primers are shown in Table 1. Each sample was tested in triplicates. The qPCR runs were analyzed with the Rotor-Gene® Q-Pure detection software (version 2.3.1; Qiagen) using the ΔΔCT method. CT-values of our reference gene (*ef1α*) did not differ between groups ($P > 0.05$). Relative gene expression data were normalized to the mean expression of nurse bees, which was set at 1.0.

2.4. Statistics

The IBM SPSS® software (version 23.0.0.0, Chicago) was used for all analyses. Kolmogorov-Smirnov-Tests were applied to test for normal distribution of data. GRS of different groups were compared using two-tailed Mann-Whitney-*U*-tests, since data were not distributed normally (Kolmogorov-Smirnov-Test: $p \leq 0.05$ for all groups tested). The fructose gustatory response scores were compared with sucrose gustatory response scores using Wilcoxon-Signed-Ranks-Tests. Gustatory receptor gene expression was only compared between different groups if mRNA expression of *Ef1α* did not differ ($P > 0.05$; T-test for independent samples). To compare the relative receptor mRNA expression between the behavioral groups, *T*-Tests were employed, because data were distributed normally (Kolmogorov-Smirnov-Test: $P > 0.05$ for *AmGR2* and *AmGR3* in the antennae and brain). Correlation analyses were performed using Spearman's-Rank-Correlation-Tests (data not distributed normally) or Pearson correlation tests (data distributed normally).

3. Results

Both nurse bees and foragers clearly preferred sucrose over fructose and responded more frequently to the sucrose solutions. This is reflected by a significantly higher gustatory response score for sucrose compared to fructose of both behavioral groups (Fig. 1; nurse bees: $Z = 6.21$, $n = 118$, $P < 0.001$; foragers: $Z = 6.33$, $n = 104$, $P < 0.001$). In fact, nurse bees showed almost no response at all to

Table 1
Overview of the oligonucleotides used in the quantitative real-time PCR (qPCR). The table displays data of the gene, in which they are placed, the internal naming, and the sequence (5′→3′). Furthermore, the length of the oligonucleotides and the length of the expected PCR products are noted as well as the type of end modification (detectable fluorescence markers in TaqMan probes).

	Gene		Label	Sequence 5′→3′	Modification	Tracking (NCBI)	Region
Housekeeping	Ef1α	Fw	AmEF1alphaqF	GAACATTTCTGTGAAAGAGTTGAGGC		NM_001011628	939–964
	Ef1α	Rv	AmEF1alphaqR	TTTAAAGGTGACACTCTTAATGACGC		1386 bp	1307–1332
	Ef1α	Fw	AmEF1alphaTM	ACCGAGGAGAATCCGAAGAGCATCAA	6FAM – BBQ Green		1159–1184
G. of Interest	AmGR1	Fw	Amgr1F	GGTGATGACATTTGCATTAGTTGAG		XM_016912472.1	1137–1161
	AmGR1	Rv	Amgr1A	CCTTCCATAAACTGTACGAGGTTC		(X1)	1277–1300
	AmGR1	Fw	Amgr1 TM	TCGATAATCCACGGTTACTTCAAGGCGA	YAK – BBQ Yellow	2066 bp	1171–1198
G. of Interest	AmGR3	Fw	Amgr3 S	GAAAATGTCCAGAGACAGCTCATG		XM_001121326.3	728–751
	AmGR3	Rv	Amgr3 R	AGACTGCATCACAAAGCGAAGA		1992 bp	873–844
	AmGR3	Fw	Amgr3 TM	TATGGAACCTCAAACCAAAACGTCAAATTG	YAK – BBQ Yellow		770–799

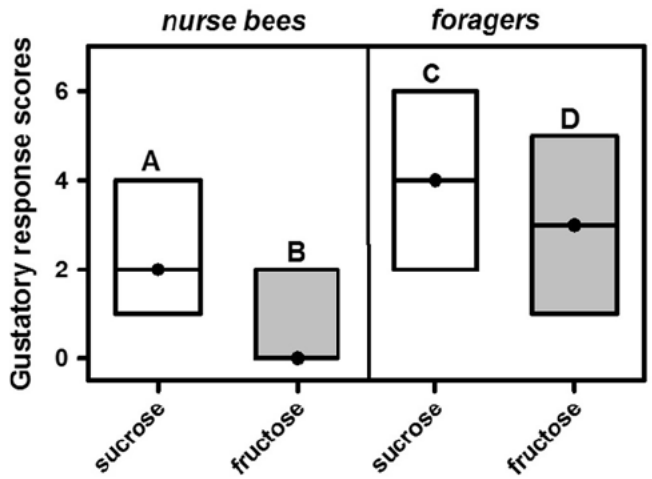

Fig. 1. Honeybees are more responsive to sucrose than to fructose and differ in their sugar responsiveness between social roles. Median gustatory response scores (dots) for sucrose and fructose for nurse bees and foragers. The 75 and 25 quartiles are represented by upper and lower lines, respectively. Gustatory response scores for sucrose are higher than those for fructose in both behavioral groups. Foragers were generally more responsive than nurse bees. Different letters indicate significant differences between groups (P at least < 0.001). For further details on statistics see Section 3. Numbers of bees tested: Nurse bees: 118, foragers: 104.

fructose. Their median fructose GRS was zero. Responsiveness to sucrose correlated highly significantly with responsiveness to fructose (Fig. 2A; nurse bees: rho = 0.57; P < 0.001; Fig. 2B: foragers: rho = 0.81, P < 0.001; Spearman rank correlation coefficient). Individuals with higher responsiveness for sucrose were also more responsive to fructose. This relationship was found both for nurse bees and foragers.

Foragers were significantly more responsive than nurse bees to fructose and to sucrose and accordingly displayed significantly higher gustatory response scores for both sugars (Fig. 1; sucrose: Z = 5.30, P < 0.001; fructose: Z = 6.04, P < 0.001; Mann Whitney *U* test). These data confirm a tight link between gustatory responsiveness and social organization.

Searching for molecular correlates of the differential gustatory responsiveness of nurse bees and foragers we quantified mRNA expression of two gustatory receptor genes which are assumed to mediate primarily perception to sucrose and fructose, respectively: *AmGR1* (Jung et al., 2015) and *AmGR3* (Sato et al., 2011; Miyamoto et al., 2012; Jung et al., 2015). Because floral nectars are primarily evaluated by the antennae of a bee, we quantified sugar receptor expression in the antennae of both behavioral groups. In addition, we measured sugar receptor expression in the brain, where gustatory information from the

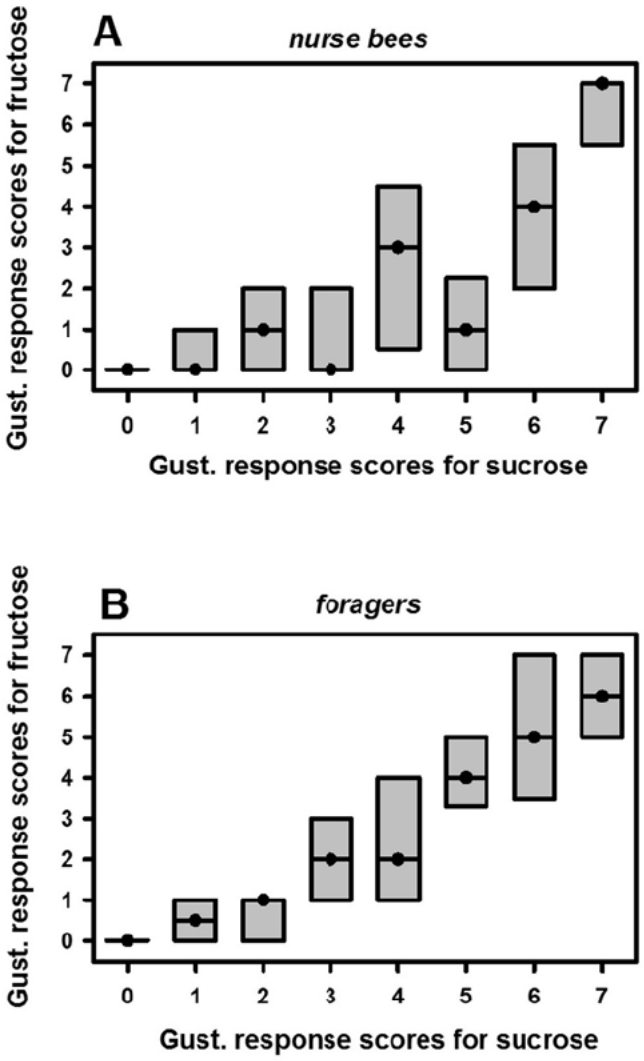

Fig. 2. Spearman's-Rank correlation between gustatory ("gust.") response scores for sucrose (x axis) and for fructose (y axis). **A.** Nurse bees. **B.** Foragers. Gustatory response scores for sucrose correlated highly significantly with gustatory response scores for fructose in nurse bees (rho = 0.57, P < 0.001), and foragers (rho = 0.81, P < 0.001). Medians (dots) 75 and 25 quartiles are shown (upper and lower lines, respectively). The number of bees tested in the group of nurse bees are for each sucrose response score class in ascending order: 29, 26, 27, 15, 13, 10, 13, 5 and for foragers: 5, 12, 19, 11, 15, 12, 17, 31.

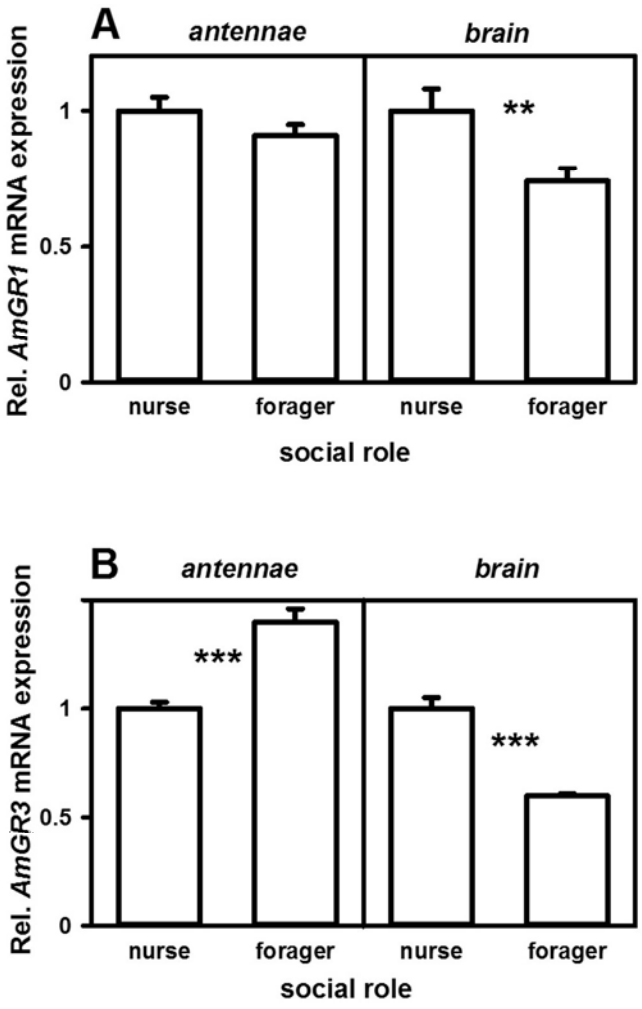

Fig. 3. Relative mRNA expression of the sucrose receptor gene *AmGR1* (and other sugars, Jung et al., 2015) and *AmGR3*, a putative fructose receptor gene of the honeybee. Expression was measured in the antennae and brains. CT-values of the housekeeping gene did not differ between groups (*ef1α*, T-test: $P > 0.05$ for all groups tested). Relative gene expression was normalized to the mean value of nurse bees, which was set to 1. Means and standard errors are displayed. **A.** *AmGR1*. **B.** *AmGR3*. Foragers displayed a significantly lower expression of both receptors in the brain and a significantly higher expression of *AmGR3* in the antennae. Significant differences between groups are indicated by asterisks. **: $P < 0.01$, ***: $P < 0.001$. *T* test. For details of statistics see Section 3. Numbers of individual tissues tested: *AmGR1*, pairs of antennae, nurse bees: 13, foragers: 14, brains, nurse bees: 11, foragers: 11; *AmGR3* pairs of antennae, nurse bees: 18, foragers: 20, brains, nurse bees: 9, foragers: 8.

antennae is processed and where gustatory receptors might act as nutrient sensors (Miyamoto et al., 2012; Simcock et al., 2017).

Expression of the putative general sugar receptor *AmGR1* in the antennae did not differ between both social roles (Fig. 3A; $T = 1.47$, $n_{nurse} = 13$, $n_{forager} = 14$; $P > 0.05$, *T*-Test). But nurse bees displayed a significantly higher expression of *AmGR1* in the brain compared to foragers (Fig. 3A; $T = 2.87$, $n_{nurse} = 11$, $n_{forager} = 11$; $P < 0.01$). In contrast to *AmGR1*, expression of the putative fructose receptor *AmGR3* in the antennae differed significantly between nurse bees and foragers (Fig. 3B; $T = 5.05$, $n_{nurse} = 18$, $n_{forager} = 20$; $P < 0.001$). Foragers displayed a significantly higher expression than nurse bees. *AmGR3* mRNA was expressed more strongly in the brains of nurse bees than in those of foragers (Fig. 3B; $T = 7.81$, $n_{nurse} = 9$, $n_{forager} = 8$; $P < 0.001$).

4. Discussion

Our results demonstrate that nurse bees and foragers were less responsive to fructose than to sucrose when tested sequentially at their antennae. Interestingly, Wykes (1952) similarly showed that under free-flying conditions and in laboratory experiments honeybees had a higher sucrose uptake compared to their fructose uptake. This supports the notion that a higher responsiveness to a certain sugar correlates with a higher uptake of this sugar. In how far the higher responsiveness to sucrose compared to fructose is linked to the use of foraging resources has not been addressed. Also, we do not know whether honeybees will prefer sucrose over fructose during the entire foraging season and in different climatic regions. Other factors which might affect sugar preferences are the yield of the foraging resource, the availability of food sources and temporal fluctuations. Donkersley at el. (2014) demonstrate that the composition of sugars in the beebread varies during the season and between different landscapes. Because individual gustatory responsiveness even of hive bees is influenced by the sugar concentration of a feeder (Pankiw et al., 2004), we assume that nectar intake has a large influence on sugar preferences of all bees in a colony.

It will be interesting to investigate the relationship between preferences for different sugars and the sugar composition of the nectars collected by the respective honeybees in future studies.

Naturally, honeybees perceive gustatory stimuli not only with their antennae but have taste sensilla at their mouth parts and at their forelegs too. Different gustatory receptors are expressed in the different taste organs, but most of the taste receptors are located in the antennae (Robertson and Wanner, 2006; de Brito Sanchez, 2011). Haupt (2004) showed with electrophysiological recordings that in addition to the high number of sensilla on the antennae, individual sensilla on the antennae displayed a much higher sensitivity for sucrose than those at the proboscis. The threshold for detecting sucrose at the antennae was below 0.1%, while the threshold for sucrose detection at the proboscis was about 0.34%, as had been shown earlier by Whitehead and Larsen (1976) and Whitehead (1978). The tarsae are even less responsive to sugars than the mouthparts. Marshall (1935) showed that only a 35% sucrose solution could elicit a PER when bees were stimulated at their tarsae, while a 2.85% sucrose solution was sufficient to elicit a PER when the bees were stimulated at their antennae. Similar results were obtained by de Brito Sanchez et al. (2008). The different perception of sugar at antennae, mouthparts and proboscis thus seems to be related both to a different number of gustatory receptors and to a differential sensitivity of individual receptors in the different taste organs. The high sensitivity of the antennae for sucrose and other sugars highlights the fundamental role of antennal gustatory receptors for behavioral decisions and were the main reason for us to focus on the antennae.

Our experiments further show that nurse bees and foragers differ in their responsiveness to sucrose and fructose, supporting earlier data on sucrose responsiveness (Thamm and Scheiner, 2014; Scheiner et al., 2017) and the hypothesis that social organization is controlled by different sensory response thresholds (Robinson, 1992; Ament et al., 2010;Theraulaz et al., 2002).

These differences might be linked to the varying requirements for the tasks that the bees perform and the sugar concentrations they are primarily confronted with. Nurse bees consume stored honey which contains large amounts of fructose and sucrose. Therefore, their responsiveness to these sugars should be lower than that of foragers. The latter need to collect nectar containing lower concentrations of both sugars and, particularly in late summer, it is sometimes better for the colony if foragers accept even nectar of low sugar concentrations than returning empty to the colony.

The fact that nurse bees hardly responded at all to fructose might be related to the higher abundance of fructose in processed nectar, i.e. honey, compared to freshly collected nectar, because fructose is converted from sucrose during the maturation of honey (Ball, 2007). For foragers it has been shown that a higher sucrose concentration of a feeder results in a lower responsiveness of the bees for this sugar (Pankiw et al., 2004). Therefore, it would not be surprising to find that

nurse bees are unresponsive to fructose, because they are mainly confronted with very high fructose concentrations. However, this issue clearly deserves further investigation, correlating the concentrations of the different sugars in the honey the nurse bees consume with their gustatory responsiveness to each sugar.

In contrast to nurse bees, pollen foragers do not only have contact to high-concentrated sugar solutions, because they only collect pollen and do not have to evaluate nectar sources. In fact, Pankiw and Page (2000) demonstrated that one-week-old bees which later in life become pollen foragers are almost as responsive to low-concentrated sucrose solutions as future water collectors. Nectar foragers should display a differential sucrose responsiveness, depending on the sugar concentrations available in the field. Indeed, we demonstrated earlier that sucrose responsiveness of nectar foragers varies throughout the season but is always higher than that of pollen foragers (Scheiner et al., 2003).

For bees of both social roles we could show a positive correlation between their sucrose and fructose responsiveness. The more responsive the bees were to sucrose, the more frequently they also responded to fructose. At first glance, one might speculate that both sugars are identified by the same receptor. According to our homology analysis, both receptors are capable of detecting fructose and sucrose (i.e. AmGR3 and AmGR1). However, Jung et al. (2015) showed that when expressed in an oocyte cell line, AmGR1 did not respond to fructose, although responding robustly to sucrose, glucose, maltose, and trehalose. Second, expression of *AmGR1* and *AmGR3* in the antennae did not correlate ($P > 0.05$, Pearson coefficient = 0.04, n = 32). We can therefore speculate that the perception of both sugars at the antennae is mediated by the different receptors.

Our experiments separately tested responsiveness for sucrose and fructose. Little is known how bees perceive and evaluate mixtures of both sugars or mixtures containing glucose, as are frequent in plant nectars. Whitehead and Larsen (1976) reported a synergism between glucose and fructose in the activation of taste neurons of the honeybee. It could be that a mixture of different sugars is more attractive for the honeybee than sugar solutions containing only one sugar, as found for other insect species such as mosquitoes (Ignell et al., 2010). This question should be addressed in future experiments.

Intriguingly, expression of both receptor genes was highly correlated in the brain ($P < 0.01$, Pearson coefficient = 0.70, n = 17). This suggests that both *AmGR1* and *AmGR3* might have a related function in nutrient sensing. For the *Drosophila* homologue of *AmGR3*, i.e. *DmGr43a*, a function in mediating satiety has indeed been demonstrated. The receptor is apparently able to promote feeding in hungry flies and to suppress feeding in satiated fruit flies (Miyamoto et al., 2012). In honeybees it was recently shown that *AmGR3* mRNA was increased in starved bees compared to bees provided with sucrose *ad libitum,* and feeding with different sugars resulted in differential expression of the candidate sugar receptor gene *AmGR1* in the brains of foragers (Simcock et al., 2017). However, more experiments which specifically manipulate gene expression of individual gustatory receptor genes are necessary to verify a role of these receptors in mediating satiety.

Nurse bees exhibited a significantly higher mRNA expression of both *AmGR3* and *AmGR1* in the brain compared to pollen forager bees, suggesting a differential role of these genes in both behavioral groups. Simcock et al. (2017) showed that nectar and water foragers can have a significantly higher mRNA expression of both genes than newly emerged bees. Although the two studies cannot be compared directly due to different criteria for selecting bees, the findings of both studies taken together suggest a complex pattern of mRNA expression of honeybee sugar receptors in the brain during their adult behavioral maturation, pointing towards a relationship between sugar receptor expression and behavioral differences. In how far expression of sugar receptors in the brain is linked to behavioral decisions has yet to be shown.

With our data it is possible to speculate that AmGR1 and AmGR3 might have functions in mediating starvation. Nurse bees generally have higher nutrient stores than foragers (Toth and Robinson, 2005). Therefore, they should be less likely to experience starvation. Foragers, in contrast, which are of a rather lean phenotype due to their flight-related metabolism, might be more exposed to starvation, at least in the short-term range. These differences in metabolism and availability of food could be reflected in a differential expression of starvation-mediating genes or genes involved in the perception of blood sugars. Because both *AmGR3* and *AmGR1* mRNAs are expressed more strongly in the brains of nurse bees compared to foragers, we assume an involvement of one or both sugar receptors in mediating starvation/nutrition in honeybees. For *AmGR3* a relationship between food deprivation and gene expression was already demonstrated in honeybee foragers (Simcock et al., 2017). Here, however, food-deprived bees displayed a significantly higher *AmGR1* mRNA expression in the brain compared to fed bees. In addition, feeding with different sugars resulted in a differential expression of the *AmGR3* mRNA in the brain.

In contrast to the brain, nurse bees displayed a significantly lower expression of the putative fructose receptor *AmGR3* in their antennae compared to foragers, while expression of *AmGR1* did not differ between the two groups. Simcock et al. (2017) showed that newly emerged bees had a significantly higher expression of both *AmGR1* and *AmGR3* in their antennae compared to those of foragers. This suggests again a differential role of the honeybee sugar receptors in adult behavioral transitions.

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3 Manuscript II: CRISPR/Cas 9-Mediated Mutations as a New Tool for Studying Taste in Honeybees

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Authors are Laura Değirmenci, Dietmar Geiger, Fábio Luiz Rogé Ferreira, Alexander Keller, Beate Krischke, Martin Beye, Ingolf Steffan-Dewenter and Ricarda Scheiner.

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Original Article

CRISPR/Cas 9-Mediated Mutations as a New Tool for Studying Taste in Honeybees

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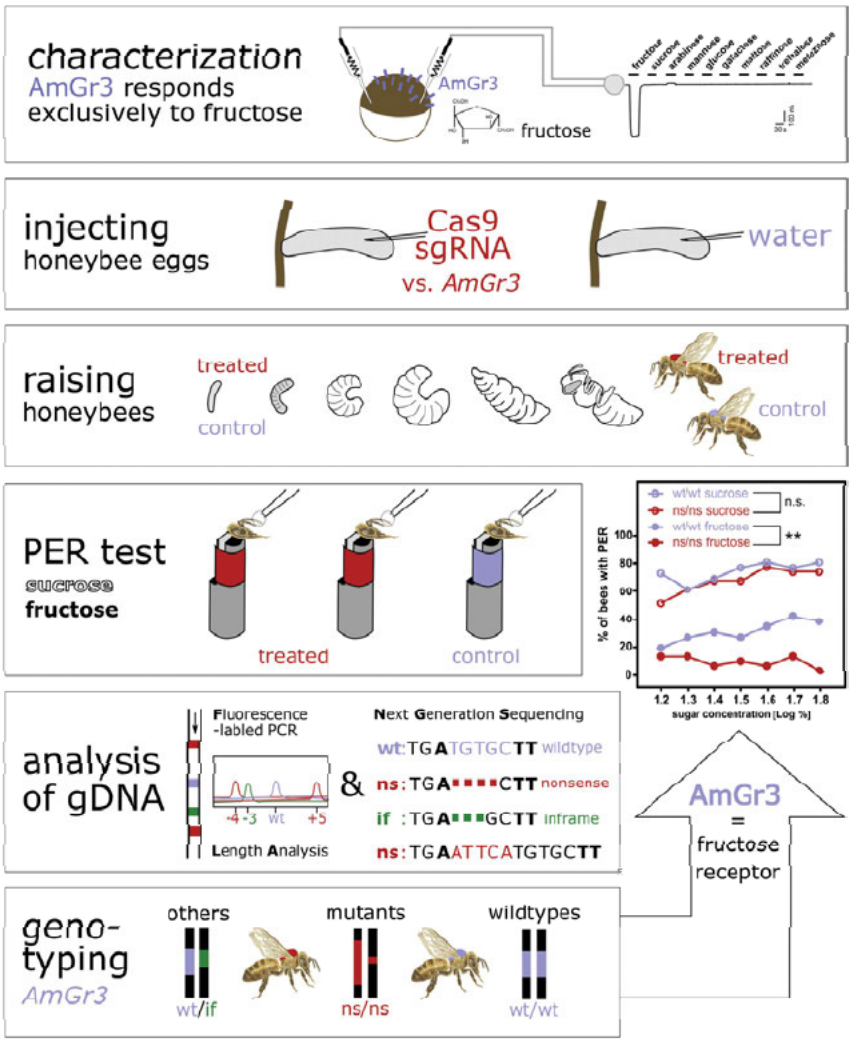
Abstract

Honeybees rely on nectar as their main source of carbohydrates. Sucrose, glucose, and fructose are the main components of plant nectars. Intriguingly, honeybees express only 3 putative sugar receptors (AmGr1, AmGr2, and AmGr3), which is in stark contrast to many other insects and vertebrates. The sugar receptors are only partially characterized. AmGr1 detects different sugars including sucrose and glucose. AmGr2 is assumed to act as a co-receptor only, while AmGr3 is assumedly a fructose receptor. We show that honeybee gustatory receptor AmGr3 is highly specialized for fructose perception when expressed in *Xenopus* oocytes. When we introduced nonsense mutations to the respective *AmGr3* gene using CRISPR/Cas9 in eggs of female workers, the resulting mutants displayed almost a complete loss of responsiveness to fructose. In contrast, responses to sucrose were normal. Nonsense mutations introduced by CRISPR/Cas9 in honeybees can thus induce a measurable behavioral change and serve to characterize the function of taste receptors *in vivo*. CRISPR/Cas9 is an excellent novel tool for characterizing honeybee taste receptors *in vivo*. Biophysical receptor characterization in *Xenopus* oocytes and nonsense mutation of *AmGr3* in honeybees unequivocally demonstrate that this receptor is highly specific for fructose.

Graphical Abstract



In "CRISPR/Cas 9-Mediated Mutations as a New Tool for Studying Taste in Honeybees" (2020) we show that honeybee mutants of the gustatory receptor AmGr3, which has also been characterized in *Xenopus oocytes*, show a reduced responsiveness to fructose. These results demonstrate that AmGr3 is a highly specific fructose receptor in the honeybee.

Keywords: *AmGr3* gene, *Xenopus* characterization, CRISPR/Cas9, fructose responsiveness, proboscis extension response, gustatory receptors

Introduction

Honeybees (*Apis mellifera*) are not only important pollinators worldwide. The highly social insects perform an intricate division of labor and are well known for their astonishing skills in learning and communication. When it comes to taste, however, honeybees display a rather poor set of receptors. Because plant-derived nectar is their sole source of carbohydrates, sugar perception is naturally of utmost importance for honeybees. The bees sense the sugar composition of a food source with only a few fine contact chemoreceptors on their antennal tip (Haupt 2007). In contrast to many other insects such as the fruit fly (*Drosophila melanogaster*) with 68 genes and mosquitoes (*Anopheles gambiae*) with 75 genes, the genome of the honeybee comprises only 10 genes coding for gustatory receptors (Grs) (Robertson and Wanner 2006). Among these only 3 code for sugar receptors: AmGr1, AmGr2, and AmGr3 (Robertson and Wanner 2006; Simcock et al. 2017). The taste receptors are expressed in the brain, the antennae, mouthparts, tarsi, and the gut of the honeybee. With this small set of receptors, honeybees evaluate a diverse set of sugars such as sucrose, fructose, maltose, and melicitose in nectar in varying composition and in amounts ranging from 5% to 80%. In flowers of mint plants (*Laminacea*), buttercups, and clematis (*Ranunculaceae*), for example, sucrose is the main sugar, whereas other flowers such as those of oilseed rape contain relatively more glucose and fructose (Stanley et al. 2013; Bertazzini and Forlani 2016). The sugar trehalose, in contrast, acts as blood sugar (Graham 1992; Chalcoff et al. 2006).

How honeybees recognize the different sugars in nectar and in their inner organs with this small set of receptors is unclear. While AmGr1 was shown to detect a variety of sugars (sucrose, fructose, glucose, and trehalose), AmGr2 seems to function as co-receptor only (Jung et al. 2015). AmGr3 appears to specifically perceive fructose (Takada et al. 2018), but not all of the relevant sugars have been tested so far. The AmGr3 receptor is an ortholog of the *Drosophila* fructose receptor DmGr43a (Robertson and Wanner 2006) and is similarly affine for fructose as the BmGr9 of the silkworm *Bombyx mori* (Sato et al. 2011). Because AmGr3 appears to selectively respond to one sugar, it is an interesting candidate for characterizing its function through a nonsense-mutation in the *AmGr3* gene. The function of insect and mouse taste receptors has been frequently characterized using electrophysiological techniques with heterologously expressed receptors in frog oocytes (*Xenopus* oocytes; Jung et al. 2015; Takada et al. 2018), human embryonic kidney cells (HEK cells; Sato et al. 2011) or plant cells (*Arabidopsis* mesophyll protoplasts; Yoo et al. 2007). However, experimental indications from heterologous expression systems need to be verified in the original organism using knock out or knock down mutants, such as has frequently been performed in fruit flies (for review, see Lin et al. 2014).

While techniques of genetic manipulation are generally not very successful in honeybees, the CRISPR/Cas9 system is a promising new genome-editing technique which has been employed successfully in numerous insects such as *D. melanogaster*, *Aedes aegypti*, and *B. mori* (Bassett et al. 2013; Basu et al. 2015 and Zeng et al. 2016, respectively). Applications in honeybees are still rare. Kohno et al. (2016) managed to produce mosaic queens and mutated drones lacking a major royal jelly protein (*mrjp1* gene) (Kohno et al. 2016). Roth et al. (2019) introduced a somatic mutagenesis approach using the CRISPR/Cas9 method and in vitro rearing to produce the first fully mutated worker bees. Worker bees were mutated on both alleles at high frequency in the absence of wild-type alleles and phenotypes, demonstrating that this somatic mutagenesis approach enables genetic studies directly in worker bees (Roth et al. 2019).

We characterized the function of the putative fructose receptor AmGr3 classically by heterologous expression in *Xenopus laevis* oocytes and elucidated its cation transport characteristics through 2-electrode voltage-clamp (TEVC) technique. In addition, we employed the somatic CRISPR/Cas9-mediated mutagenesis approach to induce specific mutations of this receptor in living honeybees and tested their responsiveness to different sugars as 1-week old adults.

Material and methods

To characterize the putative fructose receptor from *Apis mellifera*, we cloned the respective cDNA and expressed AmGr3 heterologously in *X. laevis* oocytes. To elucidate the sugar perception and cation transport characteristics of AmGr3, its functional analysis was performed using the TEVC technique. The electrical characteristics of AmGr3 were studied with respect to its sugar specificity, fructose affinity, cation selectivity, and voltage dependency.

Confirming its function as a fructose receptor in vivo, we used CRISPR/Cas9 in honeybee eggs (Kohno et al. 2016; Roth et al. 2019). Mutated honeybees were raised in the laboratory (Schmehl et al. 2016). At 1-week of age, these animals were tested for their response to fructose and sucrose (Scheiner et al. 2004, 2013). The success of the mutation was controlled by fluorescence length analysis (FLA; Ramlee et al. 2015) and next generation sequencing (NGS; Vogel et al. 2004; Shendure and Ji 2008).

Xenopus oocyte preparation

Investigations on AmGr3 were performed in oocytes of the African clawed frog *X. laevis*. Permission for keeping *Xenopus* exists at the Julius-von-Sachs Institute and is registered at the government of Lower Franconia (reference number 70/14 and 55.2-2532-2-1035). Mature female *X. laevis* frogs (healthy, non-immunized, and not involved in any previous procedures) were kept at 20 °C at a 12/12 h day/night cycle in dark grey 96 L tanks (5 frogs/tank). Frogs were fed twice a week with floating trout food (Fisch-FitMast 45/7 2 mm, Interquell GmbH). Tanks are equipped with 30 cm long PVC pipes with a diameter of around 10 cm. These pipes are used as hiding places for the frogs. The water is continuously circulated and filtered by a small aquarium pump. For oocyte isolation, mature female *X. laevis* frogs were anesthetized by immersion in water containing 0.1% 3-aminobenzoic acid ethylester. Following partial ovariectomy, oocytes were treated with collagenase I in Ca^{2+}-free ND96 buffer (10 mM HEPES, pH 7.4, 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$,) for 1 to 1.5 h. Subsequently, oocytes were washed with Ca^{2+}-free ND96 buffer and kept at 16 °C in ND96 solution (10 mM HEPES, pH 7.4, 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) containing 50 mg/l gentamycin. For electrophysiological experiments, 10 ng of AmGr3 cRNA was injected into each stage V or VI oocyte. Oocytes were incubated for 2 to 3 days at 16 °C in ND96 solution containing gentamycin.

RNA extraction and cDNA synthesis

For RNA extraction, frozen honeybee antennae, mouthparts, and tarsi were broken up in 750 µL TriFast (peqGOLD, VWR) in a 2-mL Eppendorf tube using Stainless Steel Beads (5 mm) and the TissueLyzer (Qiagen). After an incubation time of 5 min, 200 µL chloroform were added, mixed, centrifuged, and the aqueous phase was applied to a PerfectBind RNA Column of the Total RNA Kit (peqGOLD, VWR). Further extraction of total RNA was performed according to the kits protocol. RNA was precipitated with 3 M sodium acetate, washed with ethanol, dried and the pellet was resolved to adjust the concentration. Synthesis of cDNA was carried out with the AccuScript Hi-Fi cDNA Synthesis Kit (Agilent Technologies) using Oligo(dT) primer (18mers) according to the manufactures' instructions and required concentrations. RNA was digested enzymatically with RNAse H (NEB) following the protocol. According to the instructions, a large-scale Phusion PCR (NEB) was performed using a forward (5′-GAATTGTCTCGTTCGCAAATAC-3′) and a reverse primer (5′-CCGCTATTTACGAAAATTGG-3′) covering the predicted open reading frame (ORF) of the *AmGr3* gene (NCBI: XM_016913387.1). The PCR product was applied and run on a 1% (w/v) agarose gel. The appropriate band (1595 bp) was excised and purified as recommended by the Wizard SV Gel and PCR Clean-Up System (Promega). The blunt end PCR product was A-tailed with a 20-min incubation step at 72 °C by adding 0.2 mM dATP, taq polymerase, and its required buffer (NEB).

Cloning and cRNA synthesis

Via T/A ligation the fragment was inserted in the pGEM-T vector following the manufactures' recommendations (Promega). Competent *Escherichia coli* cells (*E. coli* JM109; Promega) were incubated with the ligation mixture on ice for 30 min and then transformed by a 45-s heat shock at 42 °C. After cooling on ice, the cells could regenerate on the shaker (300 rpm) at 37 °C for 45 min in 500 µL LB medium (Carl Roth). They were subsequently plated on agar plates (LB agar; Carl Roth) containing Carbenicillin (100 µg/mL; Carl Roth) and IPTG (1 M, 2.5 µL per plate; Carl Roth) and X-Gal (240 mM,

diluted in dimethylformamide, 37.5 µL per plate; Carl Roth) and could grow over night at 37 °C. Using blue-white selection, clones were picked, cultivated in a liquid overnight culture (LB and 100 µg/mL Carbenicillin; Carl Roth), pelleted and purified by the Plasmid Miniprep Kit I (peqGOLD). Inserts of the isolated plasmids were verified by sequencing. The cDNA of AmGr3 was then subcloned into oocyte expression vector pNBIu (based on pGEM vectors) by an advanced uracil-excision-based cloning technique using PfuX7 polymerase, as described by Nour-Eldin et al. (2006) and Nørholm (2010). All constructs were verified by sequencing. For functional analysis, cRNA was prepared with the AmpliCap-Max T7 High Yield Message Maker Kit (Cellscript according to the manufacturer's specifications.

Oocyte recordings

Solutions: In 2-electrode voltage-clamp studies, oocytes were perfused with Tris/Mes-based buffers. The standard solutions contained 30 mM NaCl, 10 mM Tris/Mes (pH 7.4), 1 mM $CaCl_2$, 1 mM $MgCl_2$, and either 160 mM d-sorbitol (control solution) or 160 mM fructose. Solutions for cation selectivity measurements based on the standard solutions where NaCl was replaced by 30 mM LiCl, KCl, RbCl, or CsCl. For sugar specificity measurements, d-sorbitol was exchanged by 160 mM of fructose, glucose, sucrose, mannose, galactose, maltose, arabinose, raffinose, trehalose, or melezitose. Osmolarity was adjusted to 220 mOsmol/L with d-sorbitol. For the determination of the fructose affinity of AmGr3, the fructose concentration in the standard solution varied between 0 and 500 mM. To balance the osmolarity, we compensated changes in the fructose concentration with d-sorbitol. Due to the high sugar concentration during the fructose dose-response measurements, the osmolarity was around 560 mOsmol/L, which was tolerated by the oocytes.

Electrical recordings and data analysis: For steady-state current (I_{SS}) recordings with AmGr3 expressing oocytes, the standard voltage protocol was as follows: Starting from a holding potential (V_H) of 0 mV, single 200 ms voltage pulses were applied from +40 to −150 mV in 20 mV decrements, unless otherwise stated in the figure legend. Fructose-induced currents were derived by subtracting the currents in the absence of fructose from the currents in its presence. For the calculation of the relative cation permeability of AmGr3, reversal potentials (V_{rev}) were determined with either 30 mM KCl, LiCl, NaCl, RbCL, or CsCl in the presence of 160 mM fructose. The relative permeability was calculated using the following equation (Becker et al. 1996):

$$\frac{P_X}{P_K} = \frac{([K^+]_o)}{([X^+]_o)} e \frac{(E_X - E_K)F}{RT}$$

where $[K^+]_o$ is the external potassium concentration and $[X^+]_o$ is the external concentration of the test cation. E_K is the reversal potential with potassium and E_X is the reversal potential for the external test cation. F and R are the Faraday and gas constants, respectively, and T is the absolute temperature. For the calculation of EC_{50} values, the fructose dose–response curves at different membrane potentials were fitted with a hill equation:

$$\theta = I_{base} + (I_{max} - I_{base})/\{1 + (EC_{50}/x)]^{n_H}\}$$

where I_{base} is the current in the absence of fructose, I_{max} is the current in the presence of saturating fructose concentrations, EC_{50} is the ligand concentration where the half maximal activity of AmGr3 is reached, x is the ligand concentration, and n_H is the Hill-coefficient.

Preparation of sgRNA

Appropriate sites for single guide RNAs (sgRNAs) were found in the first exons of the ORF of the putative fructose receptor *AmGr3* in the genome of *A. mellifera*. Using benchling (https://benchling.com), we defined the target-specific crRNA to be 20 bp long, next to an NGG pam site and to start with a guanine base (for position within the gene also see Figure 3). A sequence with a minimal on-target score of 50% and an off-target score of at least 97% were chosen. The secondary structure of the whole sgRNA was tested with the Vienna sgRNA fold program (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi, University of Wien, Austria) to assure that its stable part (tracrRNA) folds into the interaction structure for the Cas9 enzyme and the 20 bp of the variable part (crRNA) is still freely accessible and can thus bind the genomic target. Two primers with overlapping sequences were designed. The forward primer was containing a T7 promoter and the certain crRNA sequence (5′-GAAATTAATACGACTCACTATA-**GCAACTTGTAGTGATGTGCT**-*GTTTTAGAGCTAGAAATAGC*-3′), the reverse primer was containing the tracrRNA sequence (5′-AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTT-*GCTATTTCTAGCTCTAAAAC*-3′). Both were processed by an overlapping Phusion PCR (NEB) and purified with Monarch PCR & DNA Cleanup Kit (5 µg) (NEB), checked on an 1% (w/v) agarose gel and quantified (NanoDrop BioPhotometer plus; Eppendorf). The PCR product was the template for the sgRNA synthesis according to the protocol of the RiboMAX Large Scale RNA Production Systems with T7 RNA polymerase (Promega). After the kits DNAse digestion, sgRNA was purified with MEGAclear Transcrition Clean-Up Kit (Invitrogen), checked on an agarose gel and quantified (NanoDrop BioPhotometer plus; Eppendorf). The sgRNA was aliquoted and frozen in portions. Initially, we produced 3 different sgRNAs and tested them in different concentrations. In this preliminary experiment (data not shown), we defined the hatching and mutation rates for each sgRNA and their best ratio with Cas9 enzyme. During the experiment, a fresh aliquot with 46 ng/µL sgRNA and 3.13 µM commercial Cas9 enzyme (Cas9 Nuclease, *S. pyogenes*, 20 µM; NEB) was used for each day and stored on ice.

Honeybee egg harvest

Nine hives with related and naturally inseminated queens of *A. mellifera carnica* were kept at the bee station of the Julius-Maximilians-University of Würzburg in July and August 2018. Bees were allowed to forage freely. In order to stimulate the oviposition of the queen, the colonies were fed with ApiInvert or ApiFonda (Südzucker) during bad weather or insufficient floral nectar flow. For egg harvest, the queens were locked in the JENTER system (Karl-Heinz Jenter) the evening before. As a result, they were forced to lay their eggs through a comb-like cell grid and onto removable JENTER plug-in cells. All plug-in cells were placed on prefabricated plates so that they could be easily exchanged at once. The queens were left in the system for 3 days and the overnight eggs were discarded. Eggs were microinjected 0 to 1.5 h after deposition to gain fully mutated embryos with no detectable wild-type (wt) allele. Roth et al. (2019) demonstrated via somatic mutational screening that this approach is sufficient to mutate all nuclei in the embryo. They

showed that frequently both alleles were mutated (double mutants) and that the entire bee was affected (abscence of mosaicism). The entire mutational effect was demonstrated by the absence of wt alleles in FLA and NGS analysis and the absence of wt phenotypes (Roth et al. 2019). Yu and Omholt (1999) had shown earlier that the first division of the zygote is completed at 120 ± 6.9 min. For the transport of the eggs, we used an isolated transport box with preheated packs (35 °C, kept in the climate chamber).

Microinjection of eggs

According to the protocol of Roth et al. (2019), eggs were processed and injected in a climate chamber maintained at constant 35 °C with no humidity regulation. For this purpose, the egg-containing plug-in cells were removed from the plates and fixed vertically on petri dishes (VWR) with plastiline (Pelikan Schindellegi). Thus, the tops of the eggs were easily accessible on the outer ring, while the eggs were attached to the cell at their bottom. The injection area was surrounded by a box with a glass lid and a liquid reservoir to ensure humidity during the injection process. In this area, the rings could be rotated with one hand while the injection needle entered it through a small hole. The ICSI Glass Pipettes used (BioMedical Instruments) were controlled with the Singer Mk1 micromanipulator (SINGER Instruments) and inserted into the upper quarter of the eggs. For injection, the PLI-100A picolitre injector (Warner Instruments) with a footswitch was used and operated by the climate chamber's air system (max. 7 bar, with intermediate filter). Each egg was injected with 400 pL of either water or sgRNA6 with Cas9 (prepared as described above; injection time: 120 ms; P_{bal}: 5 kPa; P_{inje}: 60 kPa). Thereafter, the rings with injected eggs were placed in plastic boxes with a sulfurous atmosphere (1 mL of 16% sulfuric acid per liter of volume, separated from the rings by a grid) to keep the puncture site sterile. One day after the injection, burst or dried eggs were removed. The 24 h mortality rate reflects the failure of the egg to develop due to injection, because it is approximately equal for eggs injected with water or sgRNA and Cas9 enzymes. A few hours before hatching the sulfuric acid was washed off well and replaced with water. Immediately after hatching, the larvae were removed carefully with a modified Chinese grafting tool. The hatching rate displays the tolerance of the sgRNA with Cas9 enzyme, since it is over 95% when the eggs were injected with water only. In our experiment, we performed 2 replicates of injection weeks.

Artificial rearing of honeybees

The freshly hatched honeybee larvae were carefully detached from the plug-in cells with a modified Chinese grafting tool dipped in larvae food. They were placed laterally in prepared Nicot-wells (NICOTPLAST) containing larval food. Care was taken not to contaminate the lateral breathing holes of the upper side. The food and rearing procedures are described in detail by Schmehl et al. (2016) with some deviations from the protocol. The Nicot-wells in which the larvae were placed were already filled with "larval food A and B." They were placed in 48-well NUNC plates (ThermoFisher) in which they lay on cotton wool slices soaked with 0,4% MBC (methylbenzethoniumchlorid chloride, w/v) and glycerol (84.5% and 15.5%, v/v). The closed 48-well plates rested in a separate box in the incubator at 35 °C for the duration of larval development. As described by Schmehl et al. (2016), the box contained a K_2SO_4 buffer which adjusted the humidity to approximately 94%. After the larvae consumed the food of all conducted feedings (for feeding ingredients and times see also Schmehl et al. 2016), they were transferred to sterile filter paper in a fresh 48-well plate. During pupation, the animals were left to develop in approximately 75% humidity, which was achieved by NaCl buffer. After hatching within the 48-well plates, the adult bees were individually marked with colored number plates (opalith queenmarking plates) using super glue. After cutting of a wing for easier handling and safety reasons, they were placed in a cage with pollen and sugar water (20% sucrose, 10% fructose, and 10% glucose, w/w/w/v). All bees of one replicate shared one cage, including the labeled control animals, and were kept in an incubator maintained at 28 °C.

Testing responsiveness to sucrose and fructose

Bees were tested for their proboscis extension response (PER) to increasing concentrations of sucrose and fructose at 1 week old. For this test, each bee was immobilized on ice, carefully mounted in brass tubes and fixed with adhesive tape (Scheiner et al. 2013). At each test, both antennae were stimulated with a droplet of a certain sugar water concentration. Both sugars, alternatingly starting with fructose or sucrose, were tested. After a test with water, the test of a sugar solution was carried out with the following increasing concentrations 16%, 20%, 25%, 32%, 40%, 50%, and 63% (w/v) which corresponds to a logarithmic series of approximately 1.2; 1.3; 1.4; 1.5; 1.6; 1.7; 1.8. Contaminations occurring at the antennae were immediately removed and rinsed with water. It was already shown that sucrose responsiveness is not affected by the order of the concentrations tested (Scheiner et al. 2013). The positive PER for each concentration was recorded individually for each sugar (sucrose or fructose) and each bee. To prevent intrinsic sensitization, there was an intertrial interval of 2 min (Scheiner et al. 2013). The sum of the responses to water and the ascending concentrations of the certain sugar displays the gustatory response score (GRS) of a bee for fructose or sucrose.

Genotyping via FLA and NGS

Directly after the behavioral test, the bees were individually immersed in liquid nitrogen and stored at −20 °C. Their heads were dissected, placed in 2 mL Eppendorf tubes and disrupted with a pre-cooled Stainless-Steel Beads (5 mm) in the TissueLyzer (Qiagen). The genomic DNA (gDNA) of each bee was relieved by 200 µL CTAB lysis buffer (1% CTAB (w/v), 50 mM Tris (pH 8), 10 mM EDTA, 0.75 M NaCl) and 2 µL protein kinase K (NEB) during 2 h at 60 °C. It was isolated by phenol/chloroform/isoamyl alcohol (25:24:1, pH 7.5–8.0), washed with chloroform (250 µL) and precipitated with natrium acetate (3 M; 20 µL) and ice-cold ethanol (100%; 450 µL). After washing with 70% ethanol, final centrifuging and drying, the pellets were resolved in nuclease-free water (100 µL each). With the obtained gDNA samples, a PCR was performed with a hex-labeled forward primer (5′-HEX-TGCGTACTTGTATTACTACTTAG TGC-3′) and a reverse primer (5′-AACAAGTTGCAAATATTTCCAA CGG-3′), both framing the sgRNA site. In 96-well quality PCR plates (for FLA, Kisker Biotech), 1 µL of each PCR product was edited with Hi-Di Formamide (20 µL, ThermoFisher) and Gene Scan 500 ROX dye Size Standard (0.5 µL, ThermoFisher) and examined in a FLA via the HEX label. The obtained peaks accurately display length deviations of only 1 bp from the wt (evaluated with PeakScanner2; ThermoFisher). To ensure that these shifted peaks represent mutations in the genomic DNA, we performed NGS (performed with GENEWIZ) with all candidate samples. Samples were first indexed with 2 tags (5′-CTGTGATG-3′ and 5′-GCGCAATA-3′) for multiplexing and amplified with adapter overhangs (complete sequences,

forward: 5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTC TGTGATGtgcgtacttgtattactacttagtg-3′ and reverse: 5′- ACACTCTT TCCCTACACGACGCTCTTCCGATCTGCGCAATAtgcgtacttgtatta ctacttagtg-3′) for a second multiplexing process to be performed at GENEWIZ directly before sequencing on a Illumina HiSeq 2500 (2 × 250 bp, Rapid Run). We demultiplexed the samples by the barcoding using HMMer v3.2.1 (Eddy 2011). Forward and reverse reads were merged and subsequently quality filtered (maxEE = 1, minlen = 100) using USEARCH v11 (Edgar 2010). We then identified indel lengths and counted variants with an own perl script for each sample. Since each animal has 2 alleles, we classified each of them with "wt" for wildtype and "if" when in-frame indels were a multiple of 3 bps, leaving the open reading frame intact—not shown in the analysis). Nonsense alleles were labeled with "ns," including open reading frame shifts and leading to nonfunctional proteins (see Figure 3).

By sequencing the corresponding sgRNA target site in the *AmGR3* gene in each individual bee, we followed the genotyping approach of Roth et al. (2019) as described above. We only included animals that showed either only wt reads (in the following wt/wt) or only mutated reads. Furthermore, we only analyzed mutated animals showing indels (insertions or deletions) in their alleles which were not a multiple of 3. It can be assumed that the ORF is shifted and these animals only carry nonsense mutations in the *AmGR3* gene (in the following ns/ns). Since we genotyped the entire head tissue by deep sequencing of amplicons and excluded individuals with mosaicism, we ensure that the antennae of the bees studied for sugar responsiveness were mutated on both alleles with a ns/ns genotype. The somatic mutation approach has the advantage over germline mutations that it does not require raising and crossing of mutated queens and drones and keeping whole colonies under standards for genetically modified organisms.

Quantification and statistical analysis

All electrophysiological experiments were performed at least twice (independent experiments with oocytes from different batches). Sample size, *n*, and statistical details (mean ± standard error, SE or standard deviation, SD) are given in the figure legends for each experiment. For statistical analysis, the software Igor Pro 8 (waveMetrics, Inc.) and Excel (Microsoft Corp.,) was used.

For structural prediction of the AmGr3 protein (Figure 3), the sequence was modeled to the Cryo-EM structure of *Apocrypta bakeri* Orco (PDB 6c70A; Butterwick et al. 2018) using I-Tasser (University of Michigan; Yang and Zhang, 2015; Zhang et al. 2017) and compared with other predictions (PHYRE2, Imperial College London and TMHMM, DTU Bioinformatics Denmark).

The GraphPad Prism software (version 7.03; GraphPad Software) was used for analyzing survival and hatching. Fisher's exact tests were used to compare 24 h survival and hatching of eggs in both replicates either injected with sgRNA and Cas9 or with water. Chi-square tests were applied to compare these in total values additionally including not injected eggs. The fructose and sucrose gustatory response curves were analyzed with the IBM SPSS software (version 23.0.0.0; IBM) via logistic regression [factor genotype] and graphical displayed in Graph Pad Prism.

Results

AmGr3 represents a hyperpolarization-activated fructose receptor

Our results demonstrate that AmGr3 is clearly a fructose receptor. Upon addition of 160 mM fructose to the external solution, AmGr3-expressing oocytes elicited inward cation currents (negative currents) with amplitudes of several hundred nano amps at a holding potential of −80 mV (Figure 1A). Removing the fructose from the bath medium, the inward currents returned to the prefructose level. Control oocytes did not show any fructose-induced currents (Figure 1A, lower panel). To study the voltage dependence of AmGr3-mediated currents, 200 ms test voltage pulses were applied in the range from +10 to −150 mV in 20 mV decrements in the absence and presence of fructose (Figure 1B). Fructose-induced currents were derived by subtracting the currents in the absence of fructose from the currents in its presence (Figure 1B and C). The derived fructose-induced currents are characterized by time-dependent activation kinetics (Figure 1B) and hyperpolarization-dependent activation (Figure 1C).

Fructose-activated AmGr3 mediates nonselective cation currents

Gustatory receptors represent a group of (non-GPCR) 7-transmembrane receptors that detect tastants (nonvolatile compounds) via contact chemo sensation (Robertson 2019). Upon ligand binding, these receptors elicit cation currents finally leading to the firing of action potentials in gustatory neurons (Sato et al. 2011). To test the selectivity of the receptor for cations, oocytes expressing the gustatory receptor AmGr3 were perfused with external solutions containing 30 mM of different monovalent cations. The fructose-induced ionic currents were recorded at a membrane potential of −140 mV. In response to fructose perfusion, negative current deflections appeared in all cationic conditions tested (Figure 1D). To calculate the relative permeability of AmGr3 for cations, reversal potentials in the presence of different cations and fructose were monitored. Reversal potentials appeared similar between the cations tested. AmGr3 thus seems to be a rather nonselective cation channel with a relative permeability sequence for monovalent ions of $K^+ = 1 \pm 0 > Rb^+ = 0.97 \pm 0.05 > Cs^+ = 0.91 \pm 0.05 > Na^+ = 0.83 \pm 0.09 > Li^+ = 0.70 \pm 0.06$ (permeability of K^+ was set to 1, mean of $n = 5$ oocytes ± SE). These relative permeability values align with the Eisenman IV sequence for monovalent ions (Eisenman and Horn 1983).

Fructose is the only sugar inducing AmGr3-derived currents

In 2018, Takada et al. reported that the gustatory receptor AmGr3 responds only to fructose when transiently expressed in *Xenopus* oocytes (Takada et al. 2018). To confirm these results and to broaden the list of sugars tested (by additional use of arabinose, raffinose, and melicitose), we successively perfused AmGr3-expressing oocytes with different mono-, di-, and trisaccharides (160 mM each) at a membrane potential of −80 mV (Figure 2A). Among the 10 sugars tested, AmGr3 only responded to fructose, suggesting that AmGr3 is indeed a fructose specific receptor (Figure 2A and B; cf.; Takada et al. 2018).

Stepwise increases in fructose concentrations resulted in a gradual rise in AmGr3-mediated currents at a membrane potential of −80 mV (Figure 2C). When the steady-state currents, recorded in the presence of rising extracellular fructose concentrations (3 up to 500 mM), were plotted as a function of the fructose concentration, AmGr3 currents increased upon membrane hyperpolarization and started to saturate between 300 and 500 mM fructose (Figure 2D). A Hill function sufficiently described the individual fructose saturation curves at the given membrane potentials between −60 and −140 mV (Figure 2D). The apparent affinity constant EC_{50} of AmGr3 was 210 mM at −100 mV. Plotting the calculated EC_{50} values as

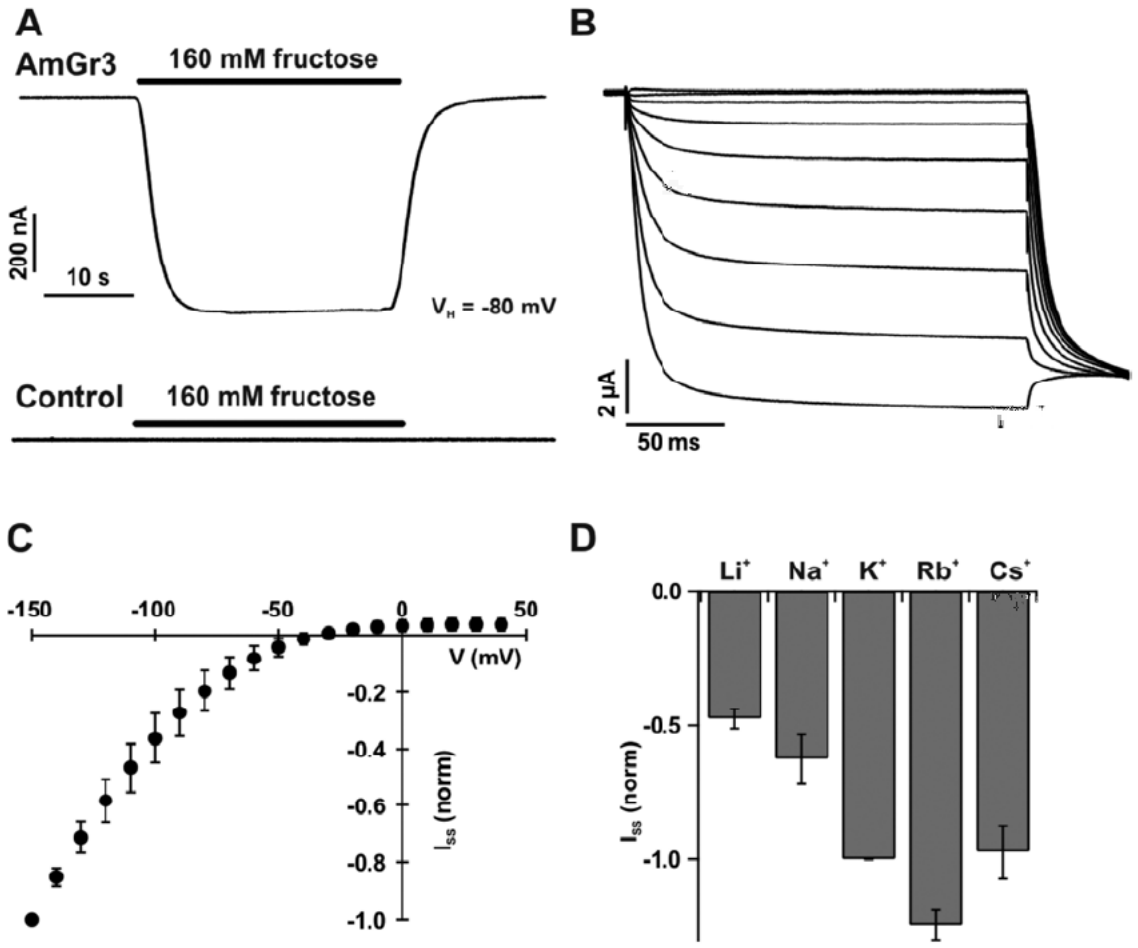


Figure 1: AmGr3 represents a hyperpolarization activated fructose receptor. **(A)** Representative whole oocyte currents recorded at –80 mV in response to perfusion with fructose in standard solution. Upper panel: AmGr3 expressing oocyte; Lower panel: non-injected control oocyte. **B)** Representative fructose-induced whole oocyte currents in response to a series of 200 ms test pulses ranging from +10 to –150 mV in 20 mV decrements**.** Each test pulse was followed by a constant voltage pulse to –140 mV. The holding potential was at 0 mV. Currents were recorded in standard solution containing 160 mM fructose. **(C)** Fructose-induced steady-state currents (I_{ss}) from AmGr3 expressing oocytes were plotted as a function of the applied membrane potential. Fructose-induced currents were derived by subtracting the currents recorded in standard solution containing 160 mM sorbitol from the currents in standard solution containing 160 mM fructose ($n = 3 \pm$ SD)**.** **(D)** Fructose-induced I_{ss} was recorded in the presence of 30 mM of different monovalent cations (as indicated) and 160 mM fructose at a membrane potential of –140 mV. AmGr3-derived currents were normalized to the currents in K^+-based media (mean of $n = 5$ oocytes ± SE).

a function of the membrane potential (Figure 2E), it becomes apparent that hyperpolarizing voltages increased the apparent affinity of AmGr3 from 325 ± 60.4 mM at –60 mV to 170 ± 7.8 mM at –140 mV.

Thus, our data show that AmGr3 is indeed a highly selective fructose receptor when expressed in *Xenopus* oocytes, leading to the question whether a nonsense mutation of this gene in live honeybees could affect their behavioral response to fructose.

CRISPR/Cas9 confirms AmGr3 as a specific fructose receptor in live honeybees

We used CRISPR/Cas9 to introduce indels (insertions or deletions) which are not a multiple of 3, leading to nonfunctional proteins of AmGr3 (Prykhozhij et al. 2017) (for sgRNA target site and the location of the introduced frame shift in relation to the entire ORF, exons and introns of *AmGr3*, see Figure 3). Two replicate experiments were performed, the second with a reduced sample size due to the extreme hot and dry summer 2018 (honeybee queens adapt their egg laying performance to the nectar flow and robustness during in vitro rearing decreases with low humidity). Around 80% of all eggs injected tolerated the injection. The treatment showed a 9.6% hatching rate (13.9% in replicate B). Between 53.3% and 69.9% of the control bees hatched into larvae (Table 1). The survival to adult emergence varied from day to day (59–86%), likely due to the manual transferring steps (after hatching on food, before pupation on filter paper, not shown in the table). All 1-week-old adult bees were tested for their responses to fructose and sucrose. Only double nonsense (ns/ns) mutants and wildtypes (wt/wt) were included in the evaluation.

Double nonsense mutations of the putative fructose receptor AmGr3 were not lethal during larval development and the first week of adult life. This indicates that *AmGr3* is not essential for life-preserving behaviors such as food intake. To prescreen the effectiveness of our treatment, we performed a FLA based on capillary gel electrophoresis with HEX-labeled PCR products of the bees. With FLA, we detected 36.0% double-nonsense mutants in the treatment group (49.1% in replicate B) and 91.7% wt (8.3% still with 1 wt and 1 ns allele) in the control group (100% in replicate B). We subsequently sequenced the respective amplicons of all primal genotyped

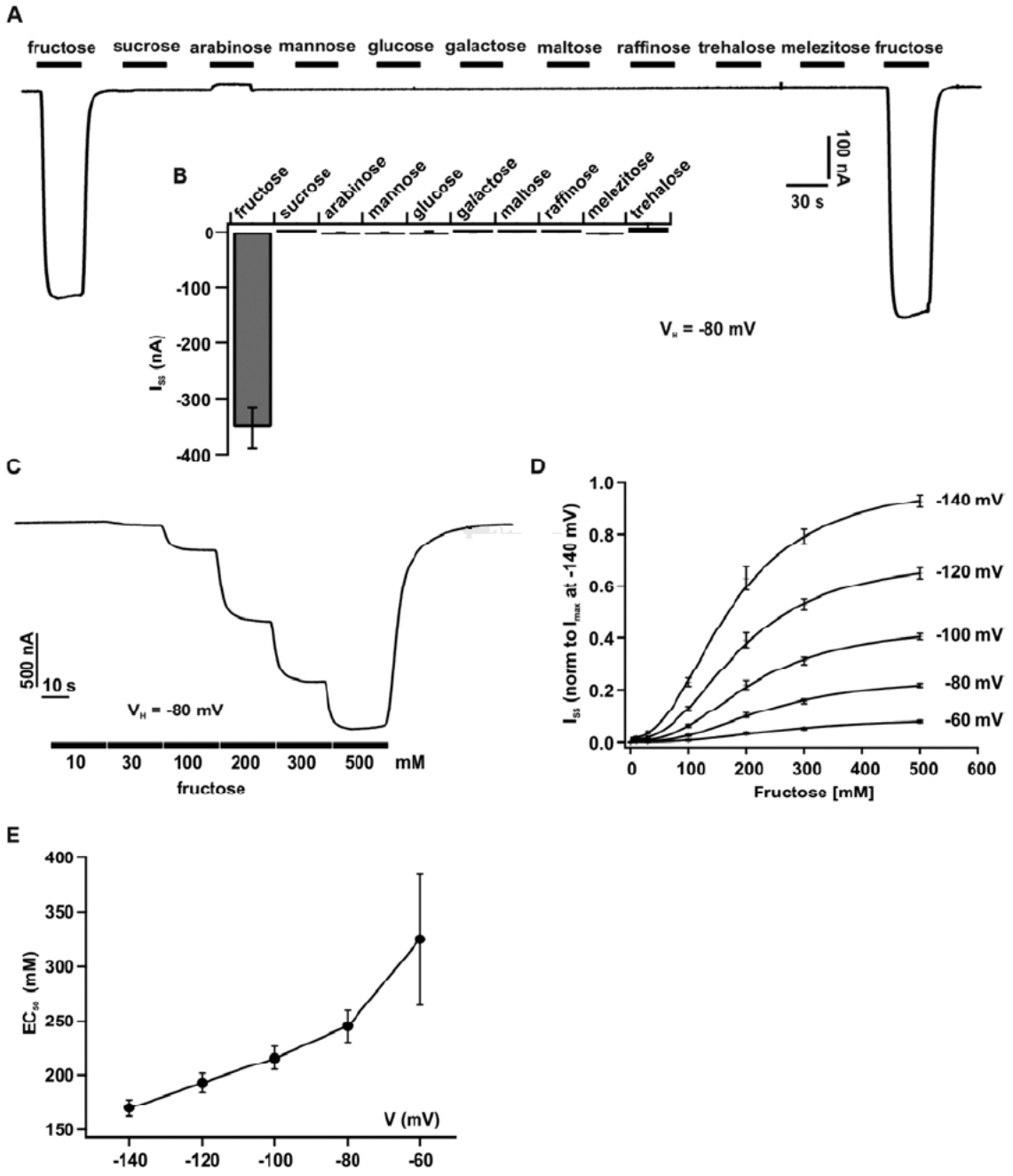


Figure 2: Fructose is the only sugar inducing AmGr3-derived currents. (**A**) Representative whole oocyte currents from oocytes expressing AmGr3 were recorded at –80 mV in response to perfusion with 160 mM of different mono-, di-, and trisaccharides in standard solution. Perfusion with test sugars are indicated by black bars. (**B**) Statistical analysis of the sugar selectivity of AmGr3 expressed in *Xenopus* oocytes. Steady-state currents in the presence of the indicated sugars were monitored at a membrane potential of –80 mV (mean of $n = 9$ oocytes ± SE). (**C**) Whole oocyte current recording from an AmGr3 expressing oocyte at a membrane potential of –80 mV. Successive elevation of the fructose concentration (black bars indicate the applied fructose concentration) in the standard solution gradually increased the AmGr3-mediated currents. (**D**) I_{SS} from AmGr3--injected oocytes were recorded in the presence of rising extracellular fructose concentrations and plotted as a function of the fructose concentration. A Hill function was fitted to the individual fructose saturation curves at the indicated membrane potentials (black solid line; mean of $n = 11$ oocytes ± SE). (**E**) The apparent affinity constants EC_{50} derived from fits such as shown in (D) were plotted as a function of the membrane potential (mean of $n = 11$ oocytes ± SE).

mutants (ns/ns) and wildtypes (wt/wt) using NGS. Our results include all individual with assured wildtype or mutant genotype via deep sequencing of the target amplicons (NGS proofed 85 samples (86.7%) of the FLA prescreened genotypes). All other genotypes (allele combinations of wt, ns and if (in-frame) were disregarded, since a clear statement about the presence and functionality of their AmGr3 proteins and the measured behavior is not possible.

In both replicates, double mutants (ns/ns in fructose receptor gene *AmGr3*) displayed a significantly reduced responsiveness to fructose, unlike wildtypes (wt/wt) (Figure 4, statistics also in Table 2) when

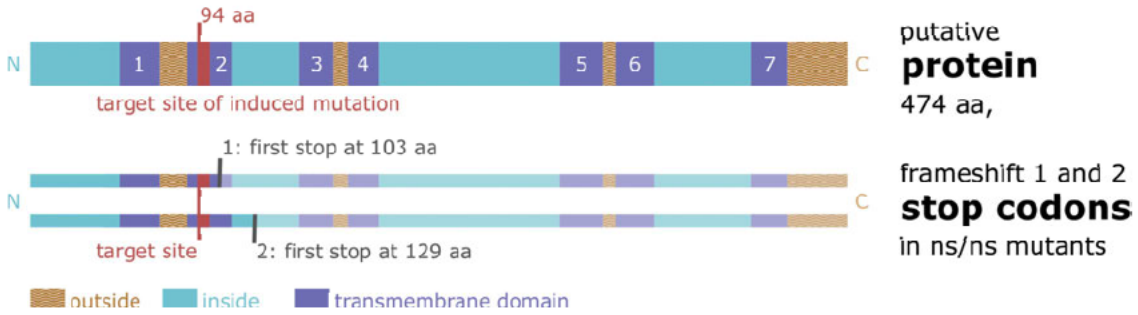


Figure 3: CRISPR/Cas9 induced nucleotide changes at the *AmGr3* gene target the second putative transmembrane domain and introduce double-nonsense mutations (ns/ns) at high frequency. Topology predictions (I-TASSER, PHYRE-Protein, and TMHMM) show that the mRNA target site for *AmGr3* (5-gcaacttgtagt gatgtgcttgg-3′) is placed within the putative second transmembrane domain (TMD). The 2 possible frameshift mutations (not a multiple of 3) driven from the sgRNA target-site introduce either a stop codon at position 103 aa or 129 aa (amino acids) of the deduced sequence and are followed by multiple stops. As a consequence, 5 TMDs of the AmGr3 proteins are lacking in double-nonsense (ns/ns) mutants and it is assumed that the receptor does not function as a fructose receptor at all.

Table 1: Survival and hatching numbers and rates of the sgRNA and Cas9 injected and control honeybee eggs or eggs with no injection under artificial rearing conditions

Treatment	Replicate A		Replicate B		Total	
	# 24 h	# hatched	# 24 h	# hatched	# 24 h	# hatched
sgRNA and Cas9 injected	1,436	200	1,116	107	2,552	307
Water injected	90	48	94	65	184	113
No injection	-	-	-	-	251	183
Treatment	% 24 h	% hatched	% 24h	% hatched	% 24 h	% hatched
sgRNA and Cas9 injected	82.0	13.9*	78.3	9.6*	80.3	12.0**
Water injected	83.3	53.3	85.5	69.1	84.4	61.4*
No injection	—	—	—	—	96.9***	72.9

The 24 h rate shows the percentage of eggs that tolerated the injection and were still intact after 24 h. The hatching rate shows the percentage of larvae that hatched from the surviving eggs. The frequencies of 24 h survival differ not when injected with sgRNA6 and Cas9 nuclease or water only (% 24 h survival; replicate A: n.s., $P = 0.7970$; replicate B: n.s., $P = 0.0895$; Fisher's exact test). The survival of microinjected eggs of 24 h is statistically different from eggs that were not injected (% 24 h survival; total: ***, $P < 0.0001$, Chi-square test). The hatching rate after 3 days decreases statistically when injected with water (% hatched; replicate A: ***, $P < 0.0001$; replicate B: *, $P = 0.0126$; Fisher's exact test) and even lower when injected with sgRNA and Cas9 (% hatched; total: ***, $P < 0.0001$; Chi-square test).

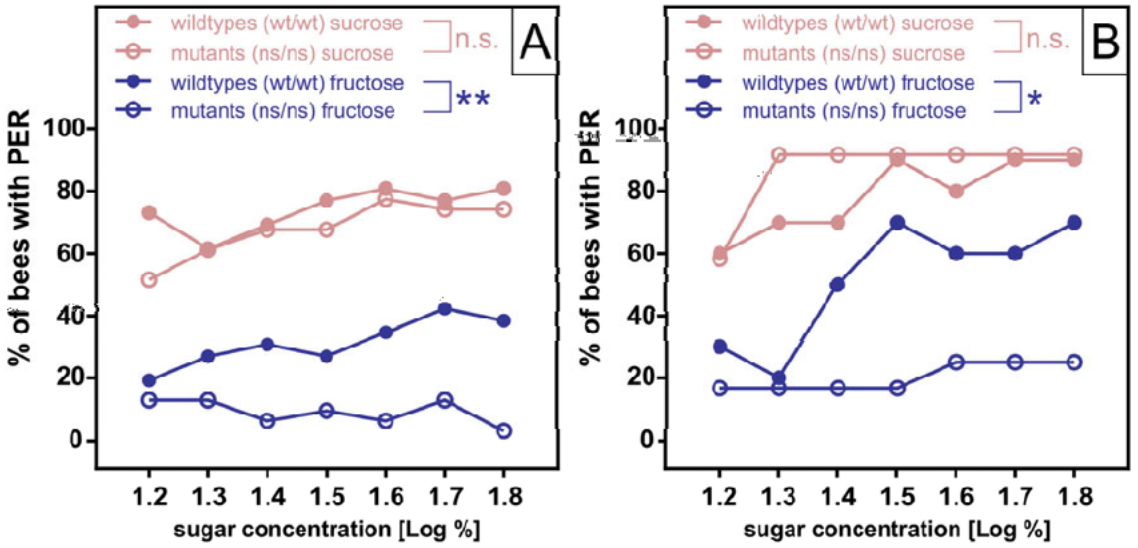


Figure 4: *AmGr3* mutants display a reduced responsiveness to fructose but not to sucrose. The figures (of replicate A and replicate B) show the percentage of bees responding to a defined sugar concentration of either fructose or sucrose (16%, 20%, 25%, 32%, 40%, 50%, and 63%, corresponding to a log of 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, and 1.8). Honeybee mutants of the fructose receptor *AmGr3* gene (ns/ns—double mutants, empty circles) are less sensitive to increasing fructose concentrations (darker lower lines) than wildtype bees (wt/wt, filled dots) (logistic regression [factor genotype] for fructose A: $^{**}P = 0.005$, $\chi^2_{1,399} = 8.026$, $N_{(wt/wt)} = 26$, $N_{(ns/ns)} = 31$ and fructose B: $^{*}P = 0.022$, $\chi^2_{1,154} = 5.265$, $N_{(wt/wt)} = 10$, $N_{(ns/ns)} = 12$). The same groups do not differ in their sucrose responsiveness (lighter upper lines) (logistic regression [factor genotype] for sucrose A: n.s. $P = 0.502$, $\chi^2_{1,399} = 0.451$, $N_{(wt/wt)} = 26$, $N_{(ns/ns)}=31$ and sucrose B: n.s. $P = 0.446$, $\chi^2_{1,154} = 0.504$, $N_{(wt/wt)} = 10$, $N_{(ns/ns)} = 12$). For statistics also see Table 2.

Table 2: Statistical values of logistic regression for Figure 4 displaying that wildtype (wt/wt) and AmGr3 mutant bees (ns/ns, double nonsense) differ statistically in their response to fructose but not to sucrose

Compare sugar response curves (wt/wt vs. ns/ns of fructose OR sucrose)—logarithmic regression										
See Figure 4	Test groups (*N*)		log. regression		Fructose responsiveness			sucrose responsiveness		
	wt/wt	ns/ns	df	N	Sign.	*P*	χ^2	Sign.	*P*	χ^2
Replicate A	26	31	1	399	**	0.005	8.026	n.s.	0.502	0.451
Replicate B	10	12	1	154	*	0.022	5.265	n.s.	0.446	0.504

Logistic regression was performed with the factor genotype for comparing the sugar curves (fructose OR sucrose) of both treatment groups (ns/ns vs. wt/wt). Mutant bees show a reduced fructose responsiveness but both groups do not differ in their sucrose response. For graphical display see Figure 4.

tested at their antennae with rising sugar concentration (Scheiner et al. 2013). Responses to sucrose, in contrast, were unaffected in both groups (Figure 4, statistics also in Table 2).

Discussion

Although honeybees rely on feeding nectar, their genome only encodes for a small set of gustatory receptors. The behavioral repertoire linked to a limited number of sugar resources (Scheiner et al. 2001, 2004; Wright et al. 2009; Scheiner et al. 2013; for review, see de Brito Sanchez et al. 2007) and the low number of taste receptors predestine the honeybee as an interesting organism to investigate the mechanisms of taste perception. Of the 10 putative honeybee taste receptors, AmGr1 (AmGr2 as its possible co-receptor) was characterized as a sugar receptor for various ligands (Jung et al. 2015) while AmGr3 is regarded as a conserved ortholog of the fructose receptor of flies and moths (Sato et al. 2011; Miyamoto et al. 2012; Takada et al. 2018).

Here we reconstituted the responses of AmGr3 to various sugars in the heterologous expression system of *Xenopus* oocytes. Two-electrode voltage-clamp studies revealed a fructose-specific nonselective cation current conductance in AmGr3-expressing oocytes (Takada et al. 2018). Although genetic studies using *D. melanogaster* suggest that co-expression of multiple Grs is necessary for sugar perception (Slone et al. 2007; Jiao et al. 2008), AmGr3 did not require the co-expression of other Gr subunits to respond to fructose in oocytes, just like BmGr9 and DmGr43 (Sato et al. 2011). The broad unspecific cation conductance we found is well in line with the studies of the AmGr3 ortholog from silkworm (*B. mori* Gr9, BmGr9; Sato et al. 2011). Sato et al. (2011) demonstrated that BmGr9 constitutes a ligand-gated nonselective cation channel. Just like BmGr9, AmGr3 conducted all monovalent cations tested with a relative permeability sequence for monovalent cations reminiscent of the Eisenman IV sequence (Eisenman and Horn 1983; Figure 1D). Besides conducting monovalent cations, both BmGr9 and DmGr43a are permeable for extracellular calcium ions, which was demonstrated through Fura-2-based Ca^{2+} imaging approaches in HEK293T and COS7 cells, respectively (Sato et al. 2011). Thus, it is tempting to speculate that AmGr3 is also permeable for calcium although experimental data is still lacking.

Very recently, a Cryo-EM-derived 3-dimensional structure of the olfactory receptor Orco (Odorant receptor co-receptor) from the parasitic fig wasp *A. bakeri* was resolved at 3.5 Å resolution (Butterwick et al. 2018). The 3D structure shows that the functional receptor consists of 4 monomers symmetrically arranged around a central ion-conducting pore. Since Ors and Grs share the same gene structure, a predicted topology with 7-transmembrane domains, an intracellular N-terminus and an extracellular C-terminus (Benton et al. 2006; Zhang et al. 2011) and a conserved motif in TM7 (Robertson 2019), it is tempting to speculate that functional Grs consist of 4 subunits, too. Whether AmGr3 assembles to heterotetrametric receptors with other members of the honeybee Gr-family and how this might influence the ligand specificity of the receptors remains to be shown. In future, the 3D structure of AbOrco will guide structure–function research not only of Ors but also of the related Gr family members.

Interestingly, fructose-induced cation currents across AmGr3 appeared activated by hyperpolarization and thus inward rectifying (Figure 1C; cf. Sato et al. 2011). Moreover, AmGr3 showed a time-dependent activation kinetics at hyperpolarized membrane potentials (Figure 1B) and voltage-dependent EC_{50} values for fructose (Figure 2D and E). These voltage-dependent electrical characteristics of AmGr3 require the presence of a voltage sensor domain/sidechains that sense the electrical field across the membrane.

However, the predicted topology of Grs does not contain a voltage sensor domain like the well-described Shaker-type voltage-gated potassium channels (Bezanilla 2005). In 2016, Barchad-Avitzur et al. showed that the agonist binding affinity of the GPCR M2 muscarinic acetylcholine receptor (M2R) is modulated by voltage, just like the EC_{50} values of AmGr3 (Figure 2E; Barchad-Avitzur et al. 2016). Using biophysical techniques in combination with site-directed mutagenesis, the authors identified a noncanonical tyrosine-based voltage sensor that appeared crucial for the voltage dependence of agonist binding to the M2R receptor. Whether the voltage dependence of AmGr3 is also based on tyrosine residues within the electrical field of the membrane and whether the voltage dependence of the fructose receptor plays a crucial physiological function for the perception of sugar concentrations in honeybees remains to be shown.

However, it is important to verify the heterologous expression in the original organism to define the function of a receptor. In the fruit fly *D. melanogaster*, this is often done via knock out or knock in mutants. In honeybees, there are no transposons available and RNAi works to a limited extend in nerve tissue, which makes the new CRIPR/Cas9 technique a very promising method for such scientific questions. Our study is the first to demonstrate that CRISPR/Cas9 is a successful method to investigate the function of taste receptors in adult honeybees on a behavioral level. Our results show that AmGr3 is a specialized fructose receptor in the honeybee, from both the biophysical characterization in oocytes and the behavioral perspective tested in honeybees. Healthy honeybees recognize both sucrose and fructose and respond more readily to increasing concentrations (Değirmenci et al. 2018). In our experiment, double nonsense mutations of the AmGr3 receptor led to a strong inhibition of responses to fructose, while responses to sucrose remained unaffected (Figure 4, statistics in Table 2). Intriguingly, some bees

with double nonsense mutations still responded to fructose. We cannot exclude the possibility that the sugar receptor AmGr1 and its co-receptor AmGr2 perceive fructose in a reduced manner when co-expressed in the same gustatory neuron (for sugar taste in *D. melanogaster* a co-expression of multiple Grs is assumed to be necessary (Slone et al. 2007; Jiao et al. 2008), although these receptors normally do not respond to fructose. We assume that the animals were in the same satiation and hydration state during the period when they could feed ad libitum in the cages prior to the PER experiment. Since we did not quantify the food volume consumed by each individual, satiation may have had a minor impact on the basal responses to fructose of the ns/ns workers. This is particularly conceivable if other receptors in the antennae may react to water, the tactile stimuli or the osmolarity of the testing solution and thus generate the baseline measured for fructose. Nevertheless, AmGr1 and AmGr2 did not show any reaction towards fructose when tested in *Xenopus* oocytes (Jung et al. 2015). Alternatively, one or several of the uncharacterized honeybee gustatory receptors might be able to perceive fructose, possibly through perceiving the molarity of liquids per se. In addition, fructose might be structurally similar to ligands of other gustatory receptors. Further characterization and investigation of the other taste receptors of the honeybee will bring clarity to these questions in the future.

Our experiments demonstrate that CRISPR/Cas9 is an efficient tool to characterize taste receptors and other behaviorally relevant proteins in the honeybee. With the advent of this genetic tool, the honeybee has now a high potential for genetic manipulation. Taken together with the rich behavioral repertoire of this insect and its unique behavioral characteristics such as division of labor, learning ability, and dance language (Seeley 2009), this makes the honeybee an ideal model organism for studying gene function in a live insect. Furthermore, our data demonstrate that the AmGr3 receptor is not essential for larval development and that it is a specific fructose receptor in honeybee workers.

Conflict of interest

None declared.

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Author contributions

D.G., L.D., M.B., A.K., and R.S. designed research and wrote the paper. I. S.-D. devised experiments and contributed to manuscript revision. D.G. and F.L.R.F. performed biophysical characterization experiments and analyzed the data. L.D. performed CRISPR/Cas9 experiments and data analysis. B.K. was involved in artificial rearing of honeybees. A.K. performed bioinformatics for genotyping.

Data availability

Correspondence and requests for materials should be addressed to L.D., R.S., and M.B. for CRISPR/Cas9, to L.D. for in vitro rearing of larvae, to D.G. and F.L.R.F. for characterization and to A.K. for bioinformatics.

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4 Manuscript III: Sugar perception in honeybees

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Sugar perception in honeybees

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Honeybees (*Apis mellifera*) need their fine sense of taste to evaluate nectar and pollen sources. Gustatory receptors (Grs) translate taste signals into electrical responses. *In vivo* experiments have demonstrated collective responses of the whole Gr-set. We here disentangle the contributions of all three honeybee sugar receptors (AmGr1-3), combining CRISPR/Cas9 mediated genetic knock-out, electrophysiology and behaviour. We show an expanded sugar spectrum of the AmGr1 receptor. Mutants lacking AmGr1 have a reduced response to sucrose and glucose but not to fructose. AmGr2 solely acts as co-receptor of AmGr1 but not of AmGr3, as we show by electrophysiology and using bimolecular fluorescence complementation. Our results show for the first time that AmGr2 is indeed a functional receptor on its own. Intriguingly, AmGr2 mutants still display a wildtype-like sugar taste. AmGr3 is a specific fructose receptor and is not modulated by a co-receptor. Eliminating AmGr3 while preserving AmGr1 and AmGr2 abolishes the perception of fructose but not of sucrose. Our comprehensive study on the functions of AmGr1, AmGr2 and AmGr3 in honeybees is the first to combine investigations on sugar perception at the receptor level and simultaneously *in vivo*. We show that honeybees rely on two gustatory receptors to sense all relevant sugars.

KEYWORDS

AmGr1, AmGr2, AmGr3, *Xenopus* oocytes, sugar responsiveness, proboscis extension response (PER), gustatory receptors (Grs), honeybee taste perception

Introduction

Honeybees depend on floral nectars and honeydew as carbohydrate sources. These comprise the sugars sucrose, glucose, fructose, melezitose and small amounts of other sugars (Ball, 2007; Pita-Calvo and Vázquez, 2017). Honeybees prefer these sugars as well as maltose and trehalose when foraging (Wykes, 1952; Graham, 1992; Roces and Blatt, 1999; Chalcoff et al., 2006; Stanley et al., 2013; Bertazzini and Forlani, 2016; Ryniewicz et al., 2020). Honeybees have only 10 Gr genes in their genome (Robertson and Wanner, 2006; Robertson and Wanner, 2006; Simcock et al., 2017). This number is very low compared to other insects such as the fruit fly (*Drosophila melanogaster*) with 68 genes and the mosquito (*Anopheles gambiae*) with 76 genes. With three Gr genes recognizing sugars, the honeybee has a comparatively reduced set of receptors for the yet broad sugar spectrum. So far, it has remained unknown how these receptors AmGr1, AmGr2, AmGr3 interact with each other. The sugar receptors are located on the antennal tips, the pre-tarsi and the mouthparts of the honeybees, but also internally in brain and gut (Haupt, 2007; Simcock et al., 2017). Honeybees possess one specific fructose receptor (AmGr3; Takada et al., 2018; Değirmenci et al., 2020) and one broadly tuned receptor (AmGr1) detecting sugars such as sucrose, glucose, trehalose and maltose (Jung et al., 2015). The third receptor, AmGr2, has been assumed to act as co-receptor

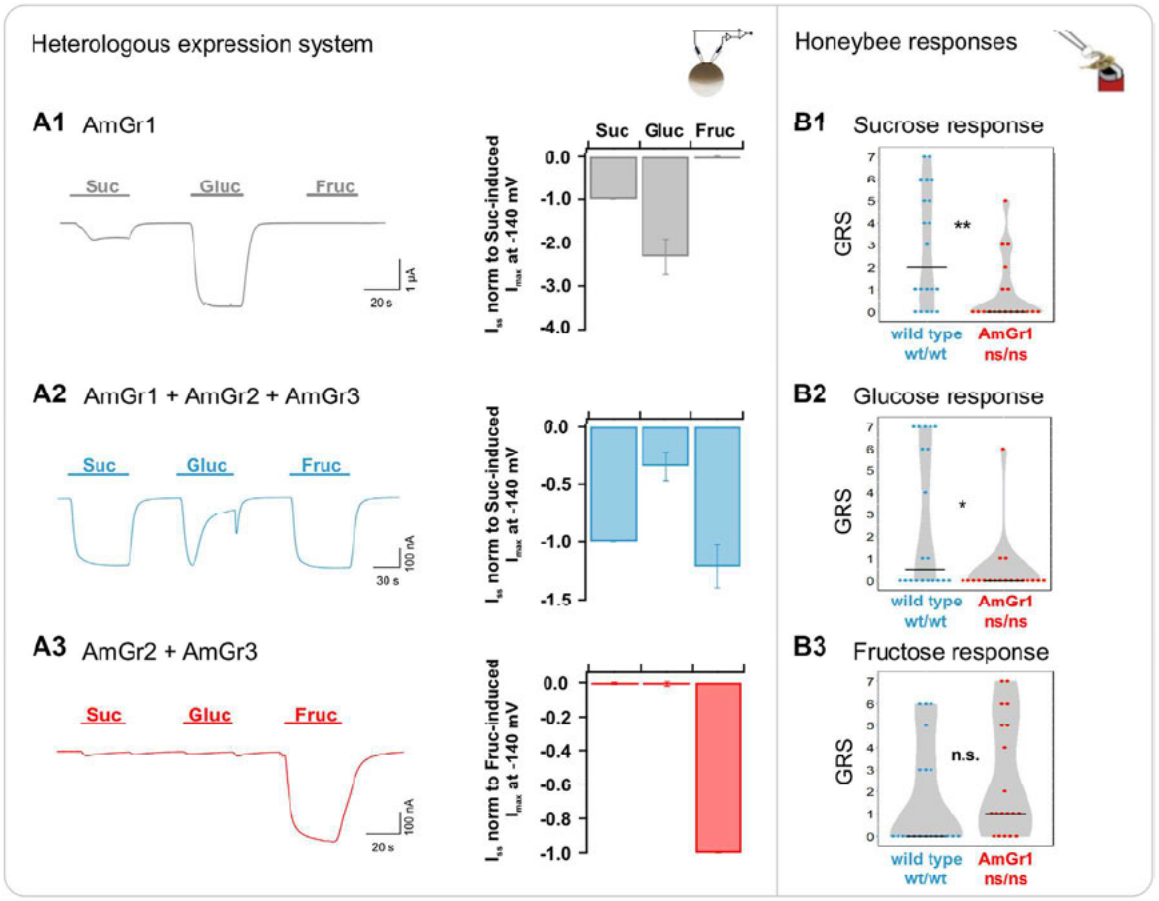

FIGURE 1
Functional analysis of *A. mellifera* gustatory receptor AmGr1 using a matched heterologous expression system and *in vivo* comparative approach. **(A1–A3)**: two-electrode voltage clamp measurements: Current traces were recorded at a holding potential of −80 mV in response to perfusion with sucrose (Suc), glucose (Gluc) and fructose (Fruc) in standard solution (left panel). Sugar-induced steady-state currents (I_{SS}) were recorded at a membrane potential of −140 mV (middle panel). **(A1)** Representative whole oocyte current trace of AmGr1-expressing oocyte (left panel); Quantification of sugar-induced I_{SS}. Currents were normalized to sucrose-evoked I_{SS} at −140 mV (mean of n = 16 oocytes ± SD; middle panel). **(A2)** Control: Inward whole oocyte currents from AmGr1/AmGr2/AmGr3-expressing oocyte (wild-type mimicry; left panel); Quantification of sugar-induced I_{SS} that were normalized to sucrose-evoked I_{SS} at −140 mV (mean of n = 10 oocytes ± SD, middle panel). These same values are displayed in all figures as consistent control. **(A3)** Whole oocyte currents from AmGr2/AmGr3 co-expressing oocyte (left panel); Quantification of sugar-induced I_{SS}. Currents are normalized to the fructose-evoked I_{SS} at −140 mV (mean of n = 9 oocytes ± SD; middle panel). **(B1–3)**: behavioural evaluation through proboscis extension response (PER, *in vivo*). Wild-type (wt/wt, N = 20) and AmGr1 mutant bees (ns/ns; N = 19) were presented a series of sugar concentrations (16%, 20%, 25%, 32%, 40%, 50% and 63% (*w/v*); representing .47 M, .58 M, .73 M, .93 M, 1.17 M, 1.46 M and 1.84 M) of all three sugars sucrose **(B1)**, glucose **(B2)** and fructose **(B3)**. The sum of the responses (PERs) towards the concentrations of one of the sugars was recorded as a sugar-specific GRS (gustatory response score). The distribution of all GRS values of all measured bees is shown as data points and the resulting medians as lines. AmGr1 mutants were less responsive to sucrose when compared with wild-type bees and had significantly lower GRS **(B1)**; Mann-Whitney-U, ns/ns vs. wt/wt, p = .0032, **). Glucose responsiveness in AmGr1 mutants was significantly lower than that in wild-types **(B2)**; Mann-Whitney-U, ns/ns vs. wt/wt, p = .0125, *). Both groups did not differ in fructose GRS **(B3)**; Mann-Whitney-U, ns/ns vs. wt/wt, p = .0779, n.s.).

of AmGr1 (Jung et al., 2015; Takada et al., 2018; Değirmenci et al., 2020). Based on the taste range of honeybees and the structural similarity of many sugar molecules, AmGr1 and AmGr2 (as co-receptor) might also respond to other sugars, but we are far from understanding the interaction of these receptors and require conclusive co-expression analyses. We assume that AmGr1 is capable of detecting many more sugars in the taste spectrum of honeybees than have been reported so far by interacting with AmGr2 as its co-receptor. With respect to the seven other putative Grs, only AmGr10 —assumedly a broad amino acid sensing receptor– has been characterized recently (Lim et al., 2019).

We here combine a refined electrophysiological analysis of honeybee gustatory receptors heterologously expressed with genetic knock-out of individual receptors and behavioural analysis of bees. Our data reveal new insight into the interaction of the three sugar receptors in honeybees and can explain the discrepancy between comparatively low number of sugar receptors and a broad taste spectrum in this insect.

Results

AmGr1 is essential for sucrose and glucose perception *in vivo* and when expressed heterologously

Our two-electrode voltage-clamp (TEVC) measurements confirm AmGr1 as a receptor for sucrose and glucose, but not fructose, when

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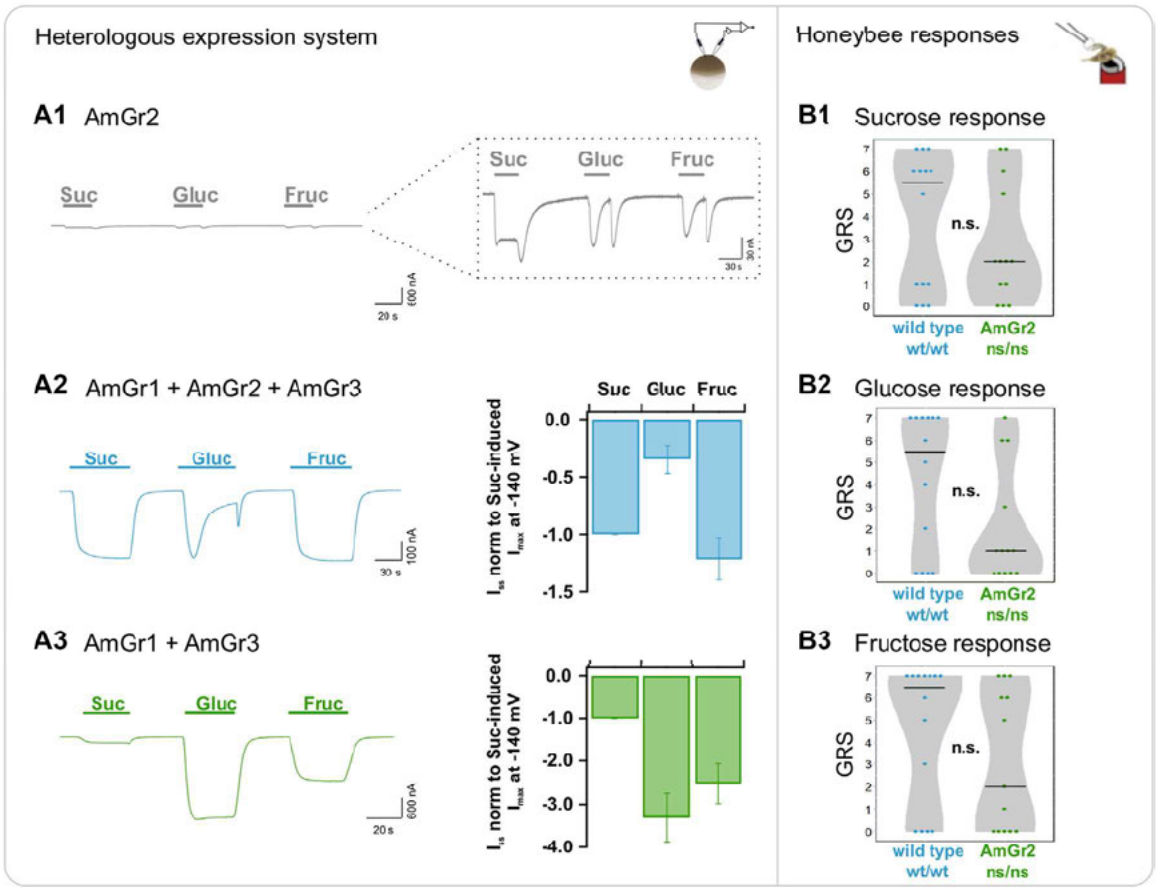

FIGURE 2
Functional analysis of *A. mellifera* gustatory receptor AmGr2 using a matched heterologous expression system and *in vivo* comparative approach. **(A1–A3)**: two-electrode voltage clamp measurements: Current traces were recorded at a holding potential of −80 mV in response to perfusion with sucrose (Suc), glucose (Gluc) and fructose (Fruc) in standard solution (left panel). Sugar-induced I_{SS} were recorded at a membrane potential of −140 mV and normalized to the currents in sucrose solution (middle panel). **(A1)** Representative whole oocyte current trace of AmGr2-expressing oocyte. Inset: Magnification of the current trace reveals microscopic sustained or transient inward currents upon sugar application. **(A2)** Control (for clarification displayed again): The inward whole oocyte currents from AmGr1/AmGr2/AmGr3-expressing oocyte (wild type mimicry, left panel); Quantification of sugar-induced I_{SS} that were normalized to sucrose-evoked I_{SS} at −140 mV (mean of n = 10 oocytes ± SD; middle panel). These same values are displayed in all figures as consistent control. **(A3)** Whole oocyte currents from AmGr1/AmGr3 co-expressing oocyte (left panel); Currents are normalized to the sucrose-evoked I_{SS} at −140 mV (mean of n = 13 oocytes ± SD; middle panel). **(B1–B3)**: behavioural evaluation through proboscis extension response (PER, *in vivo*). Wild-type (wt/wt, N = 14) and AmGr2 mutant bees (ns/ns; N = 13) were presented a series of sugar concentrations (16%, 20%, 25%, 32%, 40%, 50% and 63% (*w/v*); representing .47 M, .58 M, .73 M, .93 M, 1.17 M, 1.46 M and 1.84 M) of all three sugars sucrose **(B1)**, glucose **(B2)** and fructose **(B3)**. The sum of the responses (PERs) towards the concentrations of one of the sugars was recorded as a sugar-specific GRS (gustatory response score) of each respective bee. The distribution of all GRS values of all measured bees is shown as data points and the resulting medians as lines. AmGr2 mutants (ns/ns) did not show any significant differences in their responsiveness towards all three sugars when compared to wild-type (wt/wt) bees, neither to sucrose **(B1)**; Mann-Whitney-U, ns/ns vs. wt/wt, p = .5351, n.s.), to glucose **(B2)**; Mann-Whitney-U, ns/ns vs. wt/wt, p = .0909, n.s.) or to fructose **(B3)**; Mann-Whitney-U, ns/ns vs. wt/wt, p = .2536, n.s.).

transiently expressed in *Xenopus* oocytes (Figure 1A1). Oocytes co-expressing all three sugar receptors (AmGr1-3, representing a wildtype-like set of sugar receptors) elicited sustained inward currents of several nano amps when flushed with sucrose and fructose, whereas currents elicited during glucose application displayed a differed trace shape (Figure 1A2). Although glucose was present for 60 s, the glucose-induced currents appeared only transiently for about 20 s (see below). To verify the impact of AmGr1 on overall sugar responses, co-expression of only AmGr2 and AmGr3 in oocytes was tested, simulating honeybee mutants lacking AmGr1. Under this scenario, only fructose-induced macroscopic currents occurred (Figure 1A3), indicating that AmGr2 does not modulate the fructose-specific receptor AmGr3 in *Xenopus* oocytes. Our behavioural assay revealed that mutant bees lacking a functional AmGr1 were significantly less responsive to sucrose and glucose than wildtype bees (Figure 1B1, B2). However, their responses to fructose did not differ from those of wildtypes (Figure 1B3).

AmGr2 is a functional receptor and operates as a co-receptor for sucrose and glucose perception

Expression of AmGr2 in oocytes was confirmed by an N-terminal fused YFP as a genetically encoded reporter protein (Supplementary Figure S3A). Intriguingly, AmGr2-expressing oocytes did not reveal any macroscopic sugar-induced currents in TEVC (Figure 2A1).

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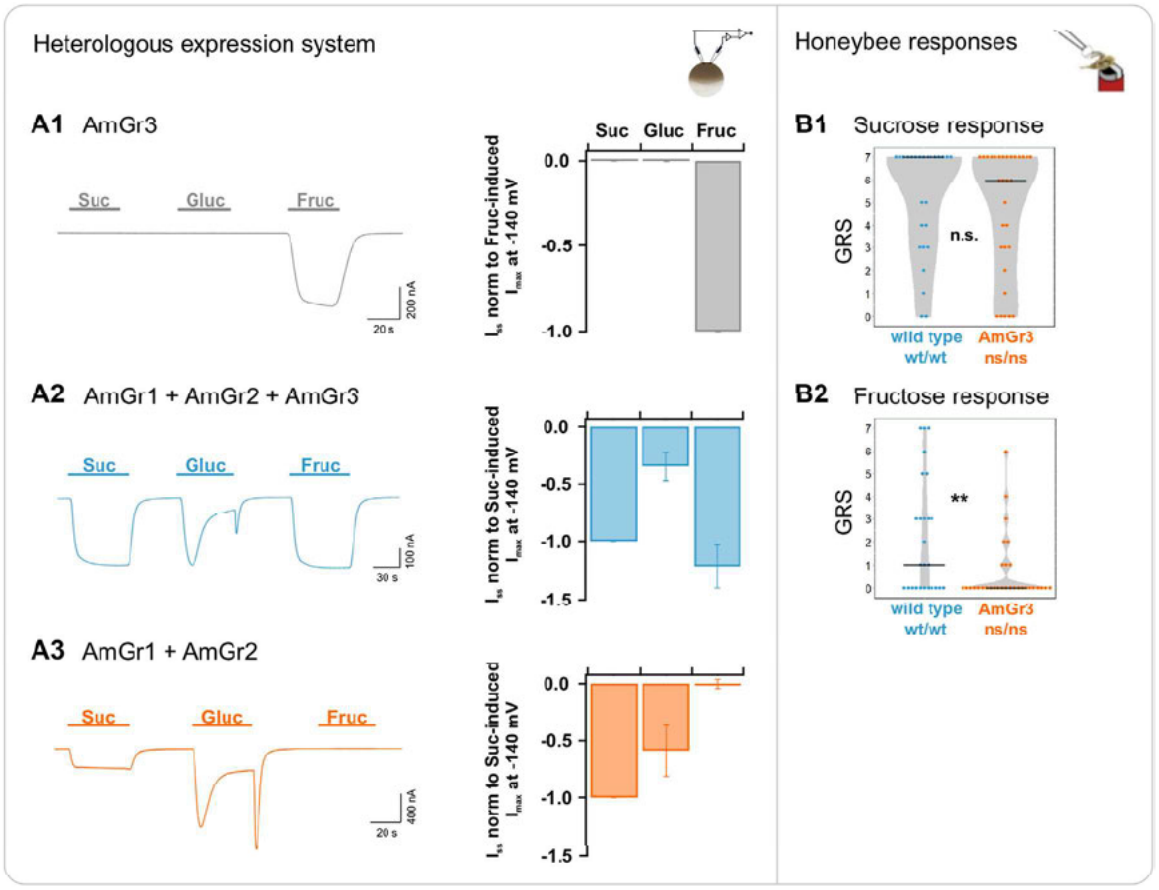

FIGURE 3
Functional analysis of *A. mellifera* gustatory receptor AmGr3 using a matched heterologous expression system and *in vivo* comparative approach. **(A1–A3)**: two-electrode voltage clamp measurements: Current traces were recorded at a holding potential of −80 mV in response to perfusion with sucrose (Suc), glucose (Gluc) and fructose (Fruc) in standard solution (left panels). Sugar-induced I_{SS} were recorded at a membrane potential of −140 mV (middle panels). **(A1)** Representative whole oocyte current trace of AmGr3-expressing oocyte (left panel); Currents are normalized to the fructose-evoked I_{SS} at −140 mV (mean of n = 8 oocytes ± SD; middle panel). **(A2)** Control (for clarification displayed again): Inward whole oocyte currents from AmGr1/AmGr2/AmGr3-expressing oocyte (wild-type mimicry, left panel); currents were normalized to sucrose-induced I_{SS} at −140 mV (mean of n = 10 oocytes ± SD; middle panel). These same values are displayed in all figures as consistent control. **(A3)** Whole oocyte currents from AmGr1/AmGr2 co-expressing oocyte (, left panel); currents were normalized to sucrose-induced I_{SS} at −140 mV (mean of n = 13 oocytes ± SD; middle panel). **(B1–B2)** (as published previously in Degirmenci et al., 2020): behavioural evaluation through proboscis extension response (PER, *in vivo*). Wild-type (wt/wt, N = 26) and AmGr3 mutant bees (ns/ns; N = 31) were presented a series of sugar concentrations (16%, 20%, 25%, 32%, 40%, 50% and 63% (*w/v*); representing .47 M, .58 M, .73 M, .93 M, 1.17 M, 1.46 M and 1.84 M) of the sugars sucrose **(B1)** and fructose **(B2)**. The sum of the responses (PERs) towards one of the sugars was recorded as a sugar-specific GRS (gustatory response score). The distribution of all GRS values of all measured bees is shown as data points and the resulting medians as lines. AmGr3 mutants did not show any difference in GRS when compared with wild-type bees **(B1)**; Mann-Whitney-U, ns/ns vs. wt/wt, p = .4279, n.s.). Fructose GRS of AmGr3 mutants were significantly lower than those of wild-types **(B2)**; Mann-Whitney-U, ns/ns vs. wt/wt, p = .0062, **). Because of experimental limitations (such as short survival of the AmGr3 mutants), glucose measurements were not implemented and cannot be pursued retrospectively.

However, reproducible microscopic inward current deflections in response to sugar application –albeit very low– were recorded and reached current amplitudes in the range of tens of nano amps overall (Figure 2A1, inset). Sustained inward currents could only be generated by application of sucrose but not with other sugars. Analogous to the glucose-induced current responses in the wildtype simulation in oocytes (Figure 2A2), AmGr2-expression revealed transient inward currents during glucose application followed by a rapid remission to the baseline. The peak amplitude of the currents lay in the same range as those recorded for sucrose. A similar transient current deflection was observed upon washout with reference solution. Perfusion with fructose evoked a comparable current response pattern to that of AmGr3-expressing oocytes either alone or in combination with AmGr1 and/or AmGr2. Bimolecular fluorescence complementation (BiFC) confirmed physical interaction of AmGr2 subunits, indicating the assembly of homomeric AmGr2 receptors of low electric activity (Supplementary Figure S3B). Taken together, the sugar-induced inward currents, along with the physical interaction between AmGr2 subunits proven with BiFC suggest that AmGr2 assembles to a functional homomeric channel building up an ion pore, thus being able to perform ligand-gated channel activity with low conductance in oocytes by itself. Sucrose and glucose stimulation of oocytes co-expressing AmGr1 and AmGr3 led to a similar current pattern as that seen for the sole expression of AmGr1, with additional fructose-induced currents upon fructose application (Figure 2A3). Honeybee AmGr2-mutants did not differ from wildtype bees in their responses to sucrose, glucose or fructose (Figures 2B1–B3).

AmGr3 is a specific fructose receptor

The sole expression of AmGr3 in oocytes revealed only fructose-induced current responses (Figure 3A1). No other sugar acted as ligand of AmGr3. Oocytes co-expressing AmGr1 and AmGr2, simulating the honeybee AmGr3 knock-out mutant, did not elicit any fructose-induced currents in the cells (Figure 3A3). Honeybee AmGr3 homozygous mutants displayed a significantly reduced response to fructose compared to wild-type bees. This behavioural difference was not observed when tested with sucrose. These findings show that AmGr3 is unequivocally a fructose-specific receptor in the honeybee.

Modulation of sugar-induced signals by receptor co-expression

Xenopus oocytes expressing all three receptors showed robust current deflections in TEVC when exposed to sucrose, glucose or fructose. Interestingly, glucose-induced inward currents were of transient nature, showing a decay over the course of application (displayed throughout all figures: Figure 1A2; Figure 2A2; Figure 3A2). Following the decay, glucose-induced I_{SS} reached similar levels of maltose, trehalose and melezitose (Supplementary Figure S1F, bar diagram). This behaviour is also apparent when AmGr3 is absent (Figure 3A3; Supplementary Figure S1C). However, when expressed alone, AmGr1-elicited glucose currents were stronger and did not decay over time (Figure 1A1). AmGr2 itself did not show macroscopic sugar-induced currents (Figure 2A1) which is well in line with honeybee AmGr2 mutants that did not show significant differences in responses to sucrose, glucose or fructose compared to wild-type bees (Figures 2B1–B3). Moreover, our BiFC experiments indicate a direct physical interaction between AmGr1 and AmGr2 and strongly suggest that heteromerization occurs (Supplementary Figure S3B). Thus, AmGr2 potentially acts exclusively as co-receptor for the sucrose signal of AmGr1, modulating strength and time-dependent characteristics of glucose-induced signals.

For fructose-induced currents by AmGr3 activation we did not see any modulation when co-expressing with the other receptors (Figure 1A3 or Figure 2A3). Nevertheless, a physical interaction with AmGr2 on the protein level seems to be possible, even if it did not modulate the fructose specificity of AmGr3 (Supplementary Figure S3). We did not detect any heteromeric formation with AmGr1 in our BiFC experiments (Supplementary Figure S3). Mutant bees expressing AmGr1 and AmGr3 but lacking AmGr2, as well as those expressing AmGr2 and AmGr3 but lacking AmGr1 did not show any significant differences in their fructose response compared to wild-type bees (Figure 1B3; Figure 2B3). Thus, AmGr3 is irreplaceable for fructose perception in honeybees and its electrophysiological properties cannot be modulated by neither AmGr1 nor AmGr2.

Discussion

Sugar taste plays a critical role when evaluating profitable food sources in terms of concentration and type of sugar in honeybees (de Brito Sanchez et al., 2007), since they rely on nectar as their main source of carbohydrates. Honeybees only have 10 Gr genes and hardly anything is known about their gustatory perception (Robertson andWanner, 2006; Simcock et al., 2017). Given that honeybees respond to a large variety of sugars, it is interesting that they achieve this with only three candidate sugar receptors AmGr1-3 (Wykes, 1952; Robertson & Wanner, 2006; Simcock et al., 2017). We hypothesized that honeybees rely on a complex interaction of these receptors to identify the different sugars and investigated for the first time Gr interaction using electrophysiology and behavioural assays.

AmGr1 elicited sugar-induced responses to sucrose, glucose, maltose and trehalose (consistent with Jung et al., 2015) when heterologously expressed. Further, our study reveals that AmGr1 is also capable of perceiving melezitose (Supplementary Figure S1). Melezitose is collected from honeydew, making up to 70% of its sugar fraction (Seeburger et al., 2020), rendering it as an alternative food source (Meiners et al., 2017). However, excessive melezitose intake can lead to health problems in bees, including reduced foraging activities, hair loss and necrotic appearances in the midgut (Horn, 1985; Imdorf et al., 1985; Seeburger et al., 2020). The fact that AmGr1-expressing oocytes recognize melezitose similarly to sucrose and glucose suggests that melezitose evokes a positive response by bees. We here propose that honeybees are unable to discriminate between melezitose from beneficial sugars, exposing a health risk under unfavourable foraging conditions (e.g. over-breeding of aphids; Seeburger et al., 2022). This could be a major reason for the occurrence of honeydew flow disease reported by beekeepers (Alfonsus, 1935).

Furthermore, we have shown for the first time that AmGr1 is directly involved in the evaluation of sucrose, glucose (Figure 1) and maltose at the behavioural level but not in the perception of fructose or arabinose (Supplementary Figure S4). Maltose is found in both honeydew and nectars of many plants, whereas arabinose seems to be present only in traces (Wykes, 1952; Manzanares et al., 2011; Akšić et al., 2020). The substrate specificity of AmGr1 for sucrose, glucose, maltose, trehalose and melezitose (rather than arabinose) might reflect the natural occurrence of these sugars in honeybee resources or in its haemolymph sugar. With this promiscuous ligand specificity, AmGr1 is important for taste perception of honeybees, thereby counterbalancing a comparatively small set of Grs. This shows that AmGr1 inheres a ligand cross-reactivity based on sugar ligands with at least one accessible D-glucose unit. Ligand cross-reactivity is also known in other organisms of this receptor family (see review in *Drosophila*: Slone et al., 2007; Freeman et al., 2014). In contrast, we could not detect any responses to the less relevant sugars such as arabinose, mannose or galactose without glucose unit. Additionally, no signal was generated by raffinose since we assume that its critical glucose unit is embedded and difficult to access. Future experiments combining structure-related functional analysis with glucose analogues might provide new insights regarding its sugar stereospecificity.

Our study provides first evidence that AmGr2 forms a functional receptor, though it does not provide sufficient ion channel performance on a comparable scale to AmGr1 or AmGr3. Furthermore, co-expression of AmGr2 and AmGr3 tagged with complementary YFP-halves revealed fluorescence signals (Supplementary Figure S3B), indicating that AmGr2 can potentially form a heteromer with AmGr3. BiFC results must be carefully interpretated, as cases of false-positives have been reported in the

literature. Nevertheless, TEVC experiments provided neither a gain nor a loss of function in oocytes co-expressing both receptors, suggesting that AmGr3 is not modulated by AmGr2. Here, co-expressing AmGr2 with AmGr1 displays a clear co-receptor function, that is in contrast to Jung et al. (2015). It tunes the broad sugar perception of AmGr1 into a specific sucrose receptor by drastically affecting glucose-induced signals. This was observed over the course of long sugar applications (current decrease occurs after 10–15 s; Figure 3A3). We assume that a current remission of transient nature might have been overlooked in the study of Jung et al. (2015), because therein sugar applications lasted 10 s overall and no steady-state currents were used for analysis. The inactivation property of AmGr2 only occurs in co-expression with AmGr1, suggesting that the heteromer adopts an altered, yet fine molecular gating mechanism restricting the ion passage when interacting with ligands other than sucrose (substrate-induced inhibition). When we stimulated oocytes longer than 10 s, inactivation occurred at glucose concentrations higher than 50 mM, with larger doses leading to stronger inactivation (Supplementary Figure S2). Thus, a broad spectrum of sugar taste in honeybees can be fine-tuned to a small set of sugars and AmGr1-2 heteromerization broadens their functional diversity (Xicluna et al., 2007; Geiger et al., 2009). Some of the contrasting results of our study and that by Jung et al. (2015) might be related to differences in the protein sequence used for AmGr1 (here: GenBank accession OP546539). While Jung et al. (2015) used hybrids of *Apis mellifera carnica*, *ligustica* and *caucasica* (H. Kwon, personal communication) derived from Korea, our bees were *Apis mellifera carnica* from a German source (Supplementary Table S1).

AmGr2 mutants did not differ in their responses to sucrose, glucose or fructose compared to wild-type bees. Although our behavioural paradigm works excellently for sugar evaluation in honeybees (Değirmenci et al., 2020), it might be rather unspecific for characterizing a co-receptor like AmGr2 in behaviour. In taste tissues of insects there is a variety of different sensory receptors expressed which might produce overlapping stimuli, so that fine-tuning signals from a co-receptor may be blurred in behaviour (Thorne et al., 2004; Amrein, 2016; Miriyala et al., 2018). In contrast, AmGr1 and AmGr3 mutants, which are directly and exclusively responsible for sugar perception, produce clear phenotypes but not a total loss of sugar responsiveness. This indicates that testing behaviour *in vivo* cannot exclude the influence of other interfering stimuli (Geiger et al., 2009). For instance, it was shown that fixation influences behavioural responses to sugar by inducing stress (Pankiw and Page, 2003). Our electrophysiological results suggest that AmGr2 appears to only act as a co-receptor by modulating sugar signals.

Earlier experiments showed that freely moving or caged bees prefer sucrose over other sugars (von Frisch, 1934; Wykes, 1952; Bachmann and Waller, 1977) comparable to fixed bees in more recent behavioural experiments (Ayestaran et al., 2010; Simcock et al., 2018). In all TEVC experiments, however, the sucrose signals measured were mostly weaker or similar to those of glucose. The yet uncovered co-receptor function of AmGr2 or differences in general receptor expression might thus be factors modulating the receptor signal and the actual behaviour, but these points need further investigation.

Our results prove AmGr3 to be a specific fructose receptor. Cells without this receptor led to an absence of inward currents after applying fructose. Honeybee AmGr3 mutants were significantly less responsive to fructose than wildtypes. For the first time, we can thus prove that AmGr3 is not influenced by any other sugar receptor. Intriguingly, a single receptor seems to be responsible for fructose perception, while AmGr1 detects multiple sugars. We hypothesize that AmGr3 may not only function as a sugar receptor in the peripheral taste perception but may further function as an internal sensor, which is supported by the presence of AmGr3-mRNA in the antennae and brain (Değirmenci et al., 2018). Furthermore, AmGr3 was suggested to detect the nutritional level of haemolymph sugar (Simcock et al., 2017). Levels of haemolymph fructose as well as AmGr3 expression together might orchestrate an intricate mechanism to drive starvation sensation and metabolic responses. Similarly, the receptor homolog of AmGr3 in *Drosophila* (DmGr43a) functions both as receptor and nutrient sensor (Slone et al., 2007; Miyamoto et al., 2012; Freeman et al., 2014; Fujii et al., 2015). Further studies are necessary to precisely unravel the internal role of AmGr3 in the honeybee.

Overall, our matched *in vivo* and functional analyses provide a powerful tool to characterize taste perception at different levels of the system. Thus, the repertoire of sugar taste in honeybees could be expanded and mimicked to full extent of all possible combinations of sugar receptor ensembles in *Xenopus* oocytes. Furthermore, we were able to assign a direct physiological role to these Grs *in vivo*. This approach can be adapted to further uncover taste perception in honeybees, which has been largely ignored due to lack of suitable approaches. It is convincible that honeybees inhere a reduced set of receptors due to co-evolution with plants resulting in a narrow food ecology. Nevertheless, their complex interaction provides an enhanced perception capacity.

Similar to colour vision, which can be achieved by just three photoreceptors (trichromatic vision; Dominy and Lucas, 2001), the broad sugar taste in honeybees can be covered by three sugar receptors. Surprisingly, two receptors (AmGr1 and AmGr3) are sufficient for the basic perception of sugars in honeybees, regardless of the fine-tuning by the co-receptor AmGr2.

Materials and methods

RNA extraction and cDNA synthesis

RNA (taste tissue) was extracted according to Değirmenci et al. (2020). RNA was then purified, precipitated, washed, dried and resolved (as described; Değirmenci et al., 2020). The cDNA synthesis was followed by an RNA H digestion. Large scale Phusion PCRs were performed (Supplementary Table S1). PCR products were proven on gel, purified and A-tailed with taq-polymerase as described before (Değirmenci et al., 2020).

Cloning and cRNA synthesis

PCR fragments were cloned into pGEM-T vector *via* T/A cloning following our previous protocol (Değirmenci et al., 2020). *E. coli* were transfected, selected and cultivated overnight (described in Değirmenci et al., 2020). The plasmid was then isolated and verified through sequencing. Each cDNA was sub-cloned into pNBIu, YFP-fusion and BiFC (Bimolecular Fluorescence Complementation) vectors and respective cRNAs were accomplished using the techniques described in Değirmenci et al. (2020). YFP and complementary YFP-halves were cloned upstream of

the respective cDNA (Supplementary Figure S3) and verified by sequencing.

Xenopus oocyte recordings

Oocytes were injected with either 25 ng AmGr1, 50 ng AmGr2 or 50 ng AmGr3 cRNA (sole or co-expression combinations) and incubated for 2–5 days at 16°C in ND96 solution (10 mM HEPES pH 7.4, 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) containing 50 mg/l gentamycin. Electrophysiological experiments were performed using the two-electrode voltage-clamp (TEVC) technique. Standard voltage protocols: holding potential of 0 mV followed by 200 ms voltage pulses (+20 to −140 in 20 mV decrements); single-pulse at −80 mV holding potential. TEVC solutions: 30 mM NaCl, 10 mM Tris/Mes (pH 7.5), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM KCl and either 160 mM D-sorbitol (reference solution) or 160 mM of the tested sugars (sucrose, glucose, fructose, maltose, arabinose, mannose, galactose, raffinose, trehalose or melezitose). Sugar-induced steady-state currents (ISS) were derived by subtracting the currents in the absence of sugar from the currents in the presence of sugar and normalized either to sucrose- or fructose-induced I_{SS} at −140 mV (depending on the AmGr-ensemble).

Bimolecular fluorescence complementation (BIFC) assay

YFP- or BiFC-derived fluorescence in oocytes were excited with an argon laser line (514 nm) and YFP fluorescence emission was monitored (500–580 nm). Pictures were taken with a confocal laser scanning microscope (Leica TCS SP5; Leica Microsystems GmbH) equipped with a L25×/0.95W objective. Oocytes were injected with 50 ng cRNA of each BiFC construct.

Preparation of sgRNA

Target-sites for the sgRNAs (single guide RNAs) were found in the first exons of the open reading frames (ORFs) of the respective genes following strict criteria (Supplementary Table S2; Supplementary Figure S6; Değirmenci et al., 2020). The PCR template for sgRNA production was generated with specific primers (for each sgRNA, Supplementary Table S2, Supplementary Figure S6) in an overlapping phusion PCR and purified as described in Değirmenci et al. (2020). According to that work, we produced receptor specific sgRNA for which best hatching and mutation rates were pre-tested. During the experiment, a fresh aliquot of sgRNA and Cas9 enzyme was used per day and stored on ice (concentrations in Supplementary Table S2; protocol of Değirmenci et al., 2020).

Honeybee egg harvest

The beehives had related and naturally inseminated queens of *Apis mellifera carnica*, which were maintained outdoors at Würzburg University and fed if necessary. Time-monitored eggs were harvested with the JENTER system as described in Değirmenci et al. (2020). Eggs were microinjected 0–1.5 h after deposition (with either sgRNA for AmG1, AmGr2 or AmGr3 and water controls; two replicates; results of replicates in Supplementary Figure S5), assuring mutational events during single-cellular state, leading to fully mutated embryos without mosaic patterns (honeybee zygote division: Yu and Omholt, 1999). This method was proven in our previous work (Değirmenci et al., 2020) and by Roth et al. (2019). Mutations were controlled *via* NGS (next generation sequencing).

Microinjection of eggs and artificial rearing of honeybees

Following the protocol of Roth et al. (2019), eggs were processed and injected with 400 pl volume (water or sgRNA/Cas9; Supplementary Table S2), using the same conditions, set-up, procedure and material described in our prior work (Schmehl et al., 2016; Değirmenci et al., 2020). Eggs were treated until hatching and the larvae were artificially reared as we described previously (Değirmenci et al., 2020) and based on the protocol of Schmehl et al. (2016). As described, adult bees were individually marked and one wing was removed. All marked bees of one replicate (raised in the same batch, the treatment group with one respective sgRNA/Cas9 and the water control group) were kept in a cage under same conditions described in Değirmenci et al. (2020).

Testing responsiveness to sugars

All animals tested were raised, kept and tested for sugar responsiveness in the same set-up randomized and with covered marking. Bees were mounted and tested for their proboscis extension response (PER) to increasing concentrations of each of the sugars sucrose, glucose and fructose (alternatingly starting with one of them: 16, 20, 25, 32, 40, 50% and 63% (w/v); representing .47, .58, .73, .93, 1.17, 1.46 and 1.84 M) (Scheiner et al., 2013; Değirmenci et al., 2020). Because of experimental limitations (short survival of the mutants) glucose measurements were not implemented for AmGr3-mutants. Sugar responsiveness is not influenced by the order of concentrations (Scheiner et al., 2013). For each sugar and each bee, the positive PERs towards the concentrations were recorded. The sum of responses to all seven concentrations of a sugar constitutes the individual gustatory response score (GRS, for each sugar) of a bee (Scheiner et al., 2003a; Scheiner et al., 2003b; Scheiner et al., 2004; Scheiner et al., 2013; Değirmenci et al., 2020).

Genotyping *via* next generation sequencing (NGS)

Honeybee gDNA was isolated as described before (Değirmenci et al., 2020). The gDNA samples of putative mutants and the control group were pre-selected *via* a hex-labelled PCR and fluorescence length analysis (Supplementary Table S2). Subsequently, we performed NGS in multiplex approach with indexed samples as described previously (with GENEWIZ, Leipzig, Germany, Supplementary Table S2; Değirmenci et al., 2020). Sequencing, bioinformatic analysis, demultiplexing, merging of all reads (forward and reverse) and filtering was performed according to

Değirmenci et al., in 2020 (Edgar, 2010; Eddy, 2011). Using software and scripts from our previous work, we identified and counted variants of each sample, dereplicated, aligned them with the reference and counted indel positions (Değirmenci et al., 2020).

For AmGr1, the alignment was split into segments to cover only the relevant site to account for splice variants at other positions (Edgar, 2010) before counting indels. Alleles were classified: wild-type as "wt"; in-frame (with indels multiple of 3, intact ORF) as "if"; nonsense (frame shift 1 or 2 leading to a non-functional protein, Supplementary Figure S6) as "ns". We thus followed the proved genotyping approach of Roth et al. (2019) and only included animals with a proven homozygous mutant (ns/ns) or homozygous wildtype (wt/wt) genotype according to our previous work (Değirmenci et al., 2020).

Quantification and statistical analysis

At least two independent TEVC experiments (oocytes from different batches) were performed. Sample size *n* and statistical details (mean ± standard deviation, SD) are given in the figure legends. For the behavioural analysis the GRS of mutant and wild-type bees of each sugar were compared using the Mann-Whitney-U test, since data was not normally distributed.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://datadryad.org/stash, https://doi.org/10.5061/dryad.prr4xgxpt.

Author contributions

LD, FF, DG, and RS designed research and drafted the manuscript. DG and FF performed biophysical characterization experiments and analysed the data. LD and AV performed CRISPR/Cas9 experiments and data analysis. CH was involved in experimental support and artificial rearing of honeybees. AK performed bioinformatics for genotyping. All authors contributed to the final manuscript and approved it.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.1089669/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Representative TEVC recordings of sugar-induced currents derived from *Xenopus* oocytes expressing different Gr-ensembles.

SUPPLEMENTARY FIGURE S2
Glucose dose response of AmGr1 and AmGr1+AmGr2 expressing oocytes.

SUPPLEMENTARY FIGURE S3
Fluorescence-based studies of Xenopus oocytes expressing different YFP-fused AmGr1-3 constructs.

SUPPLEMENTARY FIGURE S4
Behavioural PER test with AmGr1 mutants (ns/ns), half-mutated and half in-frame-mutated bees of AmGr1 (ns/if) and wildtype bees (wt/wt) with a 30% solution of several other sugars (glucose, sucrose, fructose, maltose, arabinose) and water.

SUPPLEMENTARY FIGURE S5
Replicates of behavioural evaluation through proboscis extension response (PER, in vivo) according to the section B of each figure (Figures 1–3).

SUPPLEMENTARY FIGURE S6
Overview of the receptors (AmGr1, AmGr2 and AmGr3) gene sequences and the early stop codons introduced by mutational frameshifts.

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5 Manuscript IV: Role of the bitter alkaloid quinine on sugar perception in honeybees (*Apis mellifera*)

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Authors are Elena Reiriz Martínez, Laura Değirmenci and Ricarda Scheiner.

20 pages (56-75)

Role of the bitter alkaloid quinine on sugar perception in honeybees (*Apis mellifera*)

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Keywords

Quinine, bitter taste, sugar responsiveness, proboscis extension response (PER), olfactory conditioning, gustatory receptors (Grs), sucrose, fructose.

Abstract

The nectar of honeybee (*Apis mellifera*)-pollinated flowers contains many compounds besides sugars, including bitter-tasting alkaloids. Protocols investigating honeybee cognitive behavior often use bitter compounds as an aversive taste stimulus. They frequently employ the alkaloid quinine, although it is not encountered by bees in nature. Intriguingly, no specific receptors for bitter substances have been found yet among the ten *A. mellifera* gustatory receptors (AmGr) known. We studied the influence of the bitter substance quinine on the responsiveness of honeybee nectar foragers to two of the most common nectar sugars, fructose and sucrose. When we stimulated the antennae of the bees with a sugar-quinine mixture, their responsiveness (measured through proboscis extension response) was lower compared to a pure sugar solution. Learning performance in olfactory conditioning was also lower when the sugar reward contained quinine, most likely because quinine lowers the reward value of the solution. A high quinine concentration of 60 mM showed a stronger effect than a low concentration of 1 mM. Generally, the effect of quinine on the behavior of the animals also depended on the sugar type and sugar concentration. Our findings support the hypothesis that bitter substances like quinine are sensed by honeybees indirectly, through inhibition of their sugar perception.

Introduction

The sense of taste is fundamental for Western honeybee (*Apis mellifera* L.) workers to find high-quality forage for their colony, mainly pollen and nectar (Winston, 1991). The main sugars found in flower nectar are sucrose, glucose, and fructose (Adler, 2000). Out of these, bees show a natural preference for sucrose over fructose, which could be linked to a differential expression of sugar receptors (Değirmenci et al., 2018). With a total of ten gustatory receptors (*Grs*), bees have a relatively small set compared to other insects such as *Drosophila melanogaster* (Honey Bee Genome Sequencing Consortium, 2006; Smith et al., 2011). So far, only three of the gustatory receptors in the honeybee have been identified as sugar receptors (Robertson & Wanner, 2006; Değirmenci et al., 2020). The honeybee receptor *AmGr1* and its co-receptor *AmGr2* react to various sugars, including sucrose, glucose, maltose, and trehalose, while *AmGr3* is a specific fructose receptor (Jung et al., 2015; Takada et al., 2018; Değirmenci et al., 2018). Taste receptors in *A. mellifera* are located in gustatory neurons within sensilla on the antennae, the mouthparts, and the tarsi (de Brito Sanchez et al., 2005). The antennae, in particular, play a fundamental role in evaluating potential food sources (Winston, 1991). Stimulation of the antennal sensilla with sucrose elicits the so-called proboscis extension response (PER; Takeda, 1961; Bitterman et al., 1983). PER is often used in behavioral preference or learning experiments to reward bees' responses to different stimuli (Scheiner et al. 1999, 2001, 2003a, b; Wright et al., 2010; Scheiner et al., 2013, 2014; Buckemüller et al., 2017).

The nectar of bee-pollinated plants contains a myriad of compounds besides sugar (Adler, 2000). Among these are alkaloids, i.e., nitrogen-containing compounds with a bitter taste (Palmer-Young et al., 2019). Alkaloids are mainly produced by plants as a defence against herbivores and are typically found in leaves, fruits, or bark (Grinkevich & Safronich, 1983), but they are also present in the pollen and nectar of some plants (Palmer-Young et al., 2019). The biological effects of nectar alkaloids on flower visitors are concentration-dependent (Stevenson et al., 2017) and range from being deterrent or even toxic (Baker, 1977) to making the nectar more attractive to pollinators. The latter is the case of caffeine, present both in the leaves and, in lower concentrations (in the range of 0.003-0.253 mM), in the nectar of *Coffea* spp. and *Citrus* spp. (Wright et al., 2015). Bitter alkaloids have been used in many studies as a punishment to enhance learning and memory formation through aversive conditioning (de Brito Sanchez et al., 2015). A well-established unconditioned stimulus in aversive conditioning in honeybees and bumblebees is quinine. It is typically applied in concentrations ranging between 0.1 and 100 mrM (de Brito Sanchez et al., 2014; Finke et al., 2021). Quinine is an alkaloid not naturally present in flower nectar but is isolated from the bark of *Cinchona sp.* (Barreiro et al., 2012).

Taken together, this clearly shows bitter substances have an impact on honeybee behavior. But not much is known about the perception of bitter taste of honeybees. In fact, their capacity to detect bitter substances at all was already questioned by von Frisch and is nowadays still under discussion (von Frisch, 1934; de Brito Sanchez et al., 2014). It seems reasonable to expect bees to possess specific receptors for bitter substances, as do other insects (French et al., 2015; Xu et al., 2016; Kanost et al.,

2016). However, neither evidence in favour of specific bitter receptor cells on the sensilla nor a clear homolog of a bitter taste receptor gene in the honeybee genome have been found (de Brito Sanchez et al., 2005; Robertson & Wanner, 2006; de Brito Sanchez et al., 2014). This may indicate that honeybees perceive bitter substances only indirectly, through inhibition of their sugar receptors (one of the main mechanisms by which plant secondary compounds deter herbivores; Chapman, 2003; Schoonhoven et al., 2005).

We evaluated the influence of quinine in honeybee responsiveness to two sugars in nectar, i.e. fructose and sucrose, which are detected by distinct gustatory receptors. We investigated the effect of different quinine concentrations mixed into the sugar reward on olfactory conditioning, and whether there are differences in the quinine effects depending on the sugar used. We assumed that adding quinine lowers the reward value of the sugar solution. We tested this hypothesis at the behavioral level with a modified PER assay, quantifying responsiveness to different sugar solutions with and without quinine. Our experiments reveal for the first time the interplay of sugar salience and quinine concentration in the modulation of taste aversiveness of a sugar-quinine solution. Furthermore, our results support the hypothesis that bitter tastants like quinine are perceived through inhibition of sugar perception. This has important implications for understanding honeybee behavioral output to bitter tastants, given the presence of these secondary compounds in the nectar of bee-pollinated flowers.

Materials and methods

Honeybee sampling and preparation

Honeybees were collected from colonies maintained at the departmental apiary at the university of Wuerzburg during the late summer of 2019 and 2020. Returning honeybee nectar foragers, differentiated by swollen abdomens and empty pollen baskets (Scheiner et al., 2013), were captured in the morning of each experimental day. Bees were caught in individual glass vials and cooled on ice for immobilization. They were then transferred into brass tubes and secured with fabric tape while their antennae and probosces could move freely (Scheiner et al., 2013). For the learning experiments, bees were fed with 5 µL of sucrose solution (30 % w/v) to be sufficiently motivated for the learning assay (Scheiner et al., 2003, 2005) and kept in a dark, humid chamber at room temperature to acclimatise for three hours. For the sugar responsiveness experiments, they were fed *ad libitum* with the same solution, which usually amounted to up to 40 µL, depending on the bees' starvation state. After feeding, they were left to acclimatise for one hour.

Olfactory conditioning

To study the effect of quinine on the learning ability of the bees, we used an absolute olfactory conditioning paradigm. We conditioned the bees to extend their probosces in response to a specific odour, as described by Bitterman et al. (1983) and Scheiner et al. (2013, 2021). Before the start of each

experiment, the sucrose responsiveness of each bee was tested using the PER assay (Bitterman et al., 1983; Scheiner et al., 2013). The antennae of each bee were stimulated by gently tapping them with a toothpick dipped in a sugar solution of increasing concentration. The sugar concentrations tested were 0.1 %, 0.3 %, 1 %, 3 %, 10 % and 30 %, corresponding to a logarithmic series of -1, -0.5, 0, 0.5, 1 and 1.5. Inter-trial intervals of at least 2 minutes were kept throughout all experiments to prevent intrinsic sensitization (Scheiner et al., 2003). The response was scored as a binary variable, '1' for proboscis extension or '0' for no response. The sum of responses to the different sucrose concentrations represents the gustatory response score (GRS) of a bee, ranging from 0 to 6 (Scheiner et al., 2013). Since sucrose responsiveness usually correlates with learning performance (Scheiner et al., 2004; Scheiner et al., 2021), only bees with a GRS of 3 or higher were chosen to be conditioned. For each experiment, two groups were formed with the same number of bees and an equal GRS median.

For each learning experiment, the restrained bees were placed in a constant air stream. The odorant was delivered manually for 3 seconds, using a 20 mL syringe containing a 1 cm^2 filter paper strip soaked with 5 µl of pure odour solution. The conditioned stimulus (CS) was 1-nonanol (Sigma-Aldrich; St. Louis, MO, USA). Bees showing spontaneous responses to this odour were discarded before the first experimental trial Scheiner et al., 2013). The unconditioned stimulus (US) was a sugar solution, delivered manually to the antennae (to elicit proboscis extension) and the proboscis using a toothpick dipped into the solution. It was presented for a total of three seconds, first overlapping with the CS for two seconds. Depending on the experiment, each of the two bee groups to be compared received a different US (in the following 'reward'), consisting of a sugar solution of either sucrose or fructose. For one group, this solution was pure; for the other one, it contained quinine, at a concentration of either 1 mM (a low concentration which already induces 'malaise-like' behaviors in honeybees; Hurst et al., 2014) or 60 mM (a high concentration typically used in behavioral experiments with honeybees; e.g.: Finke et al., 2021). It must be noted that the 60 mM quinine solution was made from quinine hydrochlorate dihydrate rather than pure quinine because of the low solubility of quinine in water. In a further test, the effects of a reward with a 30 % sugar solution on learning performance were compared to that of a lower sugar concentration of 5.6 % (logarithmic difference of approximately 0.75). This amounted to a total of six different experiments, each of which used a distinct set of 52-74 bees (see **Table 1** for experimental design). After the presentation of CS and US, each bee was left in front of the clean airflow for a few additional seconds, to remove all traces of the scent (Scheiner et al., 2013).

In each experiment, the presentation of the stimuli (CS and US) was performed for a total of six times. Inter-trial intervals of five minutes were used, representing a 'spaced' conditioning task, known to promote memory formation (Menzel et al., 2001). The response of each bee during the conditioning trials was recorded as a binary variable, with '1' meaning a conditioned response (proboscis extension to CS before presenting US) and '0' implying no conditioned response (proboscis extension only after US presentation). The sum of conditioned responses constitutes the learning score (LS) of a bee. After

the conditioning phase, bees were exposed to two odours with no reward: first, to a new, unconditioned scent, eugenol (clove leaf oil; Sigma-Aldrich), and then again to the CS, 1-nonanol.

Sugar responsiveness

We asked if adding quinine would mask the sweetness of sugar or activate aversive bitter receptors by mixing it in the sugar solution and quantifying the responses of the bees to antennal stimulation using a modified PER assay (Scheiner et al., 2013). Instead of presenting increasing concentrations of a single sugar solution in sequence, we here compared responses to one sugar concentration with those to the same sugar concentration enriched with quinine. Each bee was first stimulated with the pure sugar solution and then with a solution of the same concentration enriched with quinine. Water tests were introduced between each trial as described in Page et al. (1998) to act as a control of the effects of a repeated stimulation with sugar, which can potentially cause an increased sensitization of the response (Page et al., 1998).

We performed a total of eight PER assays. Each of these used a different group of 71-142 bees. Each assay employed a different combination of three factors: sugar type, sugar concentration series and quinine concentration. The sugar type was either fructose or sucrose. The concentration steps of this sugar followed either a “low” concentration series (0.1 %, 0.3 %, 1 %, 3 %, 10 % and 30 %, corresponding to a logarithmic series of approximately -1, -0.5, 0, 0.5, 1 and 1.5; Scheiner et al., 2021; Schilcher et al., 2021) or a “high” concentration series (with 8 %, 11 %, 16 %, 25 %, 32 %, 45 % and 63 %, corresponding to a logarithmic series of approximately 0.90, 1.05, 1.20, 1.35, 1.50, 1.65 and 1.80; Değirmenci et al., 2020). The sugar solution was enriched with a quinine concentration of either 1 mM or 60 mM.

Statistics

In both experiments, absolute non-responders (bees not responding during any trial or the pre-test) and non-discriminators (bees which responded to every single stimulus) were excluded from statistical analysis (Scheiner et al., 1999).

In the pre-tests for olfactory learning experiment, median GRS were compared between different groups with a Mann-Whitney U test using GraphPad Prism 6.00 for Windows (GraphPad Software; La Jolla, CA, USA; www.graphpad.com), because data were not distributed normally. The percentage of bees reacting positively to the odorant was plotted as a function of conditioning trial number, and the resulting curves were analysed using generalized estimating equations (GEE) for repeated-measures logistic regression, with the type of reward (as a factor), the trial number (as a covariable) and their interaction as explanatory variables (Scheiner et al., 2021). Additionally, the percentage of bees responding to each odour during the learning test was compared using Fisher’s exact test (GraphPad Prism).

Learning tests were analysed similarly, plotting response curves from the percentage of bees displaying a PER in each trial. The binary responses of the bees were analysed using GEE, with treatment (pure

sugar or sugar with quinine; factor), the sugar concentration (covariable) and their interaction as explanatory variables. These analyses were performed separately for fructose and sucrose according to the experimental design. For each experiment, each of the two water response curves (one for the water trials immediately before a pure sugar trial and the other one for the water trials immediately before a sugar and quinine trial) were tested independently for sensitization effects, with sugar concentration following water stimulation as a covariable. All generalised linear models were analysed in IBM SPSS Statistics version 23.0 for Windows (IBM Corp, Armonk, NY, USA). Learning and response curves were displayed graphically using GraphPad Prism.

Results

A high quinine concentration reduced learning performance

Bees rewarded with sucrose enriched with 60 mM quinine showed a significantly poorer learning performance than those being rewarded with a pure sucrose solution, with the difference growing larger from trial to trial (**Figure 1A**, GEE, interaction: $P < 0.05$; see **Table 2** for an overview of the statistics). A similar trend was observable with the fructose reward being enriched with 60 mM quinine, even though the difference was not significant here (**Figure 1B**, GEE, reward and interaction: $P > 0.05$). In the learning test following training, bees conditioned with sucrose and 60 mM quinine showed a significantly reduced memory formation compared to bees trained with pure sucrose (**Figure 1C**, **Table S1**, Fisher's exact test: $P < 0.01$). For fructose, there was a similar, marginally significant trend when bees trained with fructose and 60 mM quinine were compared to bees trained with fructose only (**Figure 1D**, Fisher's exact test: $P = 0.06$).

Sugar water rewards enriched with the lower quinine concentration of 1 mM did not lead to significantly poorer learning performances compared to pure sugar rewards, neither for sucrose (**Figure 2A**, GEE, reward and interaction: $P > 0.05$) nor fructose (**Figure 2B**, GEE, reward and interaction: $P > 0.05$). In the learning test following training, there were also no significant differences between groups trained with sugar enriched with 1 mM quinine and those trained with sugar alone, irrespective of the sugar used (**Figure 2C-D**, Fisher's exact test: $P > 0.05$).

We hypothesized that the reduced learning performance of bees rewarded with sugar and the high quinine concentration of 60 mM was related to a lower incentive value of the sugar solution and trained bees with a low (5.6 %) pure sugar solution (sucrose or fructose) vs. a high sugar solution of 30 % (sucrose or fructose), expecting a similar difference in learning performance as observed in bees trained with sugar vs. those trained with sugar and 60mM quinine. Rewarding bees with the low sucrose concentration resulted in a significantly lower learning performance during acquisition (**Figure 3A**, GEE, reward: $P = 0.05$). The lower fructose concentration resulted in a weaker learning performance during conditioning compared to reward with the attractive 30 % fructose, but this trend was not significant

(**Figure 3B**, GEE, reward and interaction: $P > 0.05$). Intriguingly, the probability of showing conditioned PER during the test was slightly but not significantly higher in the group receiving the low-concentration reward (sucrose: **Figure 3C**, fructose: **Figure 3D**; both: Fisher's exact test, $P > 0.05$). No significant differences were found in any of the learning tests for the non-conditioned odour eugenol (Fisher's exact test: $P > 0.05$), indicating that bees were conditioned to the specific odour used during the experiment, 1-nonanol, and not to the presence of any odour in general.

Quinine inhibits responsiveness to sugar

Responsiveness to both series of increasing sucrose concentrations was significantly higher compared to the same solutions enriched with a 60 mM quinine solution, indicating an inhibitory effect of quinine on sugar evaluation. In the low concentration series, this difference is apparent for sucrose concentrations of 1 % (log unit: 0) and higher (**Figure 4A,** $P < 0.001$, GEE). In the high concentration series, the difference is clear for every sugar concentration (**Figure 4B**, $P < 0.05$). The low quinine concentration did not affect responsiveness to the sugars (**Figures 4C-D**, $P > 0.05$; for an overview of the sucrose statistics, see **Table 3**).

Bees were apparently unable to differentiate between a low-concentrated fructose solution and the same solution mixed with 60 mM quinine (**Figure 4E**, $P > 0.05$, GEE). Responses to antennal stimulation with fructose solutions up to 10 % (log 1.0) did not differ from those to fructose solutions enriched with quinine. But when the bees were stimulated with 30 % (log 1.5) fructose solutions, responsiveness to the pure sugar solution was clearly higher than to the sugar solution mixed with 60 mM quinine. This difference in responsiveness becomes even more pronounced with higher fructose concentrations (**Figure 4F**, $P < 0.01$). Correlating with the results from the learning experiment, the low (1 mM) quinine concentration appears not to be recognized by the bees, since responses to the different fructose concentrations did not differ from those to fructose solution mixed with quinine (low concentration series: **Figure 4G**; high concentration series: **Figure 4H**; for both: $P > 0.05$). (For an overview of the fructose statistics, see **Table 4.**)

Before touching the antennae of each with a fructose solution or with a fructose-quinine solution, bees were presented with water to test for sensitization effects (curves shown in grey in **Figure 4**). Responses to these water stimulations increased significantly throughout some of the experiments, especially in those testing high-sugar concentration series (see **Table 3** and **Table 4** for details).

Discussion

Our experiments investigated how honeybees evaluate sugar solutions with or without the addition of the bitter tastant quinine. We hypothesized that both in tests analysing responsiveness to sugar and in appetitive learning tests, a high quinine concentration (60 mM) should reduce performance, whereas a low quinine concentration (1 mM) might not be noticed by the bees. Two frequent nectar sugars, i.e., sucrose and fructose were used in each experiment.

Honeybees show a poorer learning performance with high quinine concentrations in their sugar reward

The olfactory conditioning experiments revealed that a reward with a sugar solution containing quinine generally resulted in a poorer learning performance than a reward with a pure sugar solution, leading to a lower percentage of bees showing the conditioned extension of the proboscis during training and testing. However, the strength of the "quinine effect" depended on the sugar used and on the quinine concentration added.

In our experiments, honeybees rewarded with a strongly quinine-enriched sucrose solution showed a significantly poorer learning ability than those rewarded with sucrose only (**Figure 1A**). We saw a similar difference in performance with an equally bitter fructose reward (**Figure 1B**). When the quinine concentration was too low, however, there were no differences in learning with either type of sugar reward (**Figure 2**).

An experiment by Guiraud et al. (2018) recently suggested that honeybees are unable to distinguish between bitter stimuli and water using an adapted conditioning paradigm. They proposed that the rejection of sugar-bitter mixtures may sometimes not reflect avoidance of a distasteful stimulus but simply of a non-sugary substance (Guiraud et al., 2018). Conditioning assays with *D. melanogaster* similarly showed that their ability to discriminate between different rewards was based on the intensity or palatability of the solution rather than its chemical identity, whether the rewards used were in the same taste modality (sweet or bitter) or a mixture of two (sweet-bitter; Masek & Scott, 2010). Our findings suggest that quinine is not only perceived as "unsweet" in honeybees but that it may also place a lower value on a sugar solution it is mixed with.

Learning performance in honeybees is lowered by the bitter substance quinine similar to a reward with a lower sugar concentration

The reduced learning performance with added quinine appears directly comparable to that observed with poor sugar water rewards (**Figure 3**). A reduced sugar concentration led to a lower learning performance compared to a higher sugar concentration. For both sugars, performance in the memory test was slightly better for the bees rewarded with the lower sugar concentration. We suggest that the lower sugar concentration of the reward plays a role in how many conditioning trials are necessary for memory acquisition, but not necessarily for the strength of the memory. Memory retention tests at

different points in time after each experiment are a way of clarifying this point in the future. Nevertheless, both a lowered sugar concentration and a quinine-enriched solution resulted in a lower learning ability when compared to a pure sucrose solution of 30%. We, therefore, assume that the bitter substance quinine is not directly perceived by bees, but rather inhibits the perception of sugar. This is likely, since the inhibition of sugar-sensing by bitter molecules such as quinine is one of the main mechanisms by which plant secondary compounds deter herbivores (Chapman, 2003; Schoonhoven et al., 2005) and has long been observed in insects (Morita & Yamashita, 1959). However, no single mechanism has been found to explain this phenomenon so far. De Brito Sanchez et al. (2011) propose that quinine modifies the membrane properties of taste neurons unspecifically. This conclusion is derived from their previous findings that sensilla normally responding to sucrose are inhibited by a sucrose-quinine mixture, and sensilla responding to NaCl are similarly inhibited by a NaCl-quinine mixture (de Brito Sanchez et al., 2005). Further studies may help understand in greater detail the mechanisms responsible for this inhibition.

Bitter perception in honeybees might be indirect through inhibition of sugar receptors

For a more precise understanding of taste perception, it is necessary to consider recent findings at the receptor level in honeybees. Intriguingly, these insects do not appear to perceive bitter substances through their direct activation of specific receptors, as no specific bitter receptors have been found so far (Robertson & Wanner, 2006). There seems to be a trend in insect genomics that the more polyphagous an insect is, and the more noxious substances it can potentially encounter, the more expanded its gustatory receptor family, and especially the candidate receptors for bitter taste (see examples in Li et al., 2019). In comparison, honeybees have a relatively limited range of food sources, and thus a limited risk of being exposed to harmful molecules, which could explain their lack of bitter receptors. Nevertheless, it is still possible that honeybees possess bitter receptors. Out of the gustatory receptors identified in *A. mellifera* so far, there are two whose specificity is still unknown, but which appear to cluster with the *DmGr28a/b* complex (Robertson & Wanner, 2006), which in *D. melanogaster* has been identified in bitter taste neurons in the legs and taste sensilla in the proboscis (French et al., 2015). Future research should characterize these receptors using both electrophysiology techniques *in vitro* and CRISPR/Cas 9-mediated mutations *in vivo,* (Değirmenci et al. 2020). The fact that the sugars sucrose and fructose are perceived by two different receptors (AmGr1 and AmGr3, respectively; Jung et al., 2015; Takada et al., 2018; Değirmenci et al., 2020) is reflected in all our experiments. Sucrose produces a greater percentage of proboscis extension responses or a better learning performance than fructose (see **Figure 1A and 1B** or **Figure 4A and 4E**). The different effects of quinine in combination with these sugars could be due to a different inhibitory effect of quinine on their specific receptors, which characterization could also help identify.

Responsiveness of honeybees towards a solution is determined by its salience or attractiveness (including concentration of the bitter substance or the sugar and type of sugar)

In all experiments, the responsiveness of the bees to each stimulus was determined by the salience or attractiveness of the solution, which depends on the interplay of sugar type, sugar concentration and quinine concentration. On the one hand, the learning experiments showed that a high quinine concentration reduces learning performance more than a low quinine concentration. This dose-dependency was also visible in the percentage of bees reacting in the PER assays. There, a high quinine concentration was able to reduce honeybees' responsiveness towards sucrose, both for low and high sugar concentrations (**Figure 4A and 4B**). The same was true for the high fructose concentration series (**Figure 4F**), but not for the low fructose concentration series (**Figure 4E**). A low quinine concentration did not affect bees' responsiveness towards any of the sugars, neither at high nor at low concentrations (**Figure 4C, 4D, 4G, 4H**).

On the other hand, the attractiveness of the sugar solution plays a fundamental role in the aversive effect of quinine in combination with each sugar. Sucrose, as a disaccharide, probably has a higher nutritional value than the monosaccharide fructose, and bees show a natural preference (manifested through higher responsiveness) to sucrose over fructose (Değirmenci et al., 2018). This preference strongly suggests that the salience of sucrose masks the aversive power of a low concentration of quinine. This is visible in our olfactory conditioning experiment since 1 mM quinine is not sufficient to reduce learning ability compared to a pure sucrose solution (**Figure 2A**). In our study, we use a fructose-quinine mixture for the first time as a reward in olfactory conditioning. Since a fructose reward already results in a poorer learning performance than a sucrose reward, it is generally not used as a conditioning reward (Simcock et al., 2018). Moreover, Wright et al. (2007) showed that only bees rewarded with sucrose form memories that last longer than 10 minutes (Wright et al., 2007). This might explain why learning performance with fructose in our olfactory conditioning assays was generally too weak to see significant differences due to the addition of quinine (**Figure 1B** and **Figure 2B**). Learning performance was stronger in the groups rewarded with sucrose, and it is here that we see significant differences with the addition of a high quinine concentration. In the PER assays, the final factor affecting the salience of a solution was the sugar concentration. Higher sugar concentrations are more attractive and can mask the effect of quinine more easily. For example, when using fructose and 60 mM quinine, the differences only appeared at fructose concentrations beyond 25 %. With sucrose and 60 mM quinine, the differences between treatments only appeared at sucrose concentrations higher than 11%, whereas in the high concentration series, both curves show an almost parallel trajectory. All in all, quinine concentration, sugar type and sugar concentration all clearly determine the effect of a sugar-quinine solution on honeybee behavior. An explanation offered by Masek & Scott (2010) for *D. melanogaster* seems to also apply to *A. mellifera*. In these insects, taste discrimination is based on the intensity or palatability of the solution (Masek & Scott, 2010). These factors need to be taken into consideration when incorporating quinine into experimental protocols.

Biological relevance for indirect bitter perception via sugar receptor inhibition

In the past, several studies have shown that honeybees and the closely related bumblebees can detect bitter compounds in sucrose rewards and learn to avoid the floral traits associated with their presence. However, the concentrations of toxins used in these studies were typically several degrees of magnitude higher than their actual concentrations in flower nectar. For example, Wright et al. (2015) found that bees were more likely to reject sucrose solutions that contained caffeine concentrations greater than 1 mM (Wright et al., 2015). In contrast, caffeine appears in the nectar of *Coffea* sp. and *Citrus* sp. at concentrations in the range of 0.003-0.253 mM (Wright et al., 2015). Tiedeken et al. (2014) set out to clarify this: they performed a paired-choice experiment on bumblebees using different concentrations of several toxins present in the nectar of bee-pollinated flowers (Tiedeken et al., 2014). They found that bumblebees were not deterred by ecologically relevant concentrations of any of them, that is, the concentrations that are naturally present in nectar. The toxin that showed the lowest deterrence threshold of all, despite not being encountered by bees in nature, was in fact quinine, avoided in concentrations as low as 0.01 mM. Wright et al. (2010) similarly showed that honeybees were more sensitive to quinine than to amygdalin, although both are equally toxic for them (Wright et al., 2010). On the other hand, Barlow et al. (2017) found that lycaconitine and aconitine, the nectar alkaloids of *Aconitum* sp., are deterrent to the nectar-robbing bumblebee *Bombus terrestris* at concentrations as low as 20 ppm (≈ 0.03 mM), while it takes concentrations of 10-20 times higher to deter the real pollinator species, *B. hortorum* (Barlow et al., 2017). In other words, true pollinators are not deterred by the low toxin concentrations that deter nectar robbers. Taken together, this suggests that honeybees, as true pollinators, are not deterred by the bitter compounds they encounter in flower nectar. In fact, the presence of some bitter compounds such as caffeine and nicotine may actually have the opposite effect, encouraging pollinators. The higher sensitivity of bees to quinine in comparison to nectar toxins could be due precisely to the fact that quinine does not appear in their natural environment and plays no role in pollination.

Conclusions

The present study shows the role of the bitter alkaloid quinine on the behavioral responses of honeybee foragers to sugar solutions. The aversive effect of quinine is manifested as lower responsiveness to a sugar-quinine solution compared to a pure sugar solution, and as lower learning performance in olfactory conditioning when the sugar reward contains quinine. In both cases, quinine is perceived by the antennae, before ingestion, and it appears to reduce the value of the sugar solution that contains it. This effect was already visible with quinine concentrations as low as 1 mM, but it was especially strong with a high 60 mM concentration. The effect of quinine is modulated by the attractiveness of the solution it is mixed with, as its aversiveness can be overshadowed by the high appeal of, for example, a high sucrose concentration. But overall, quinine affects responses to fructose and sucrose in a similar way. This implies that these effects could be generalised to other sugar-quinine combinations, including glucose, the third sugar in nectar.

This evidence supports the hypothesis that bitter tastants, like quinine, are perceived through their inhibition of sugar perception. Considering that, so far, no bitter gustatory receptors have been identified in *Apis mellifera*, this is an important insight into honeybee gustatory perception. Furthermore, understanding the honeybee's behavioural output to bitter tastants is important given the presence of these secondary compounds in the nectar of bee-pollinated flowers.

Figures

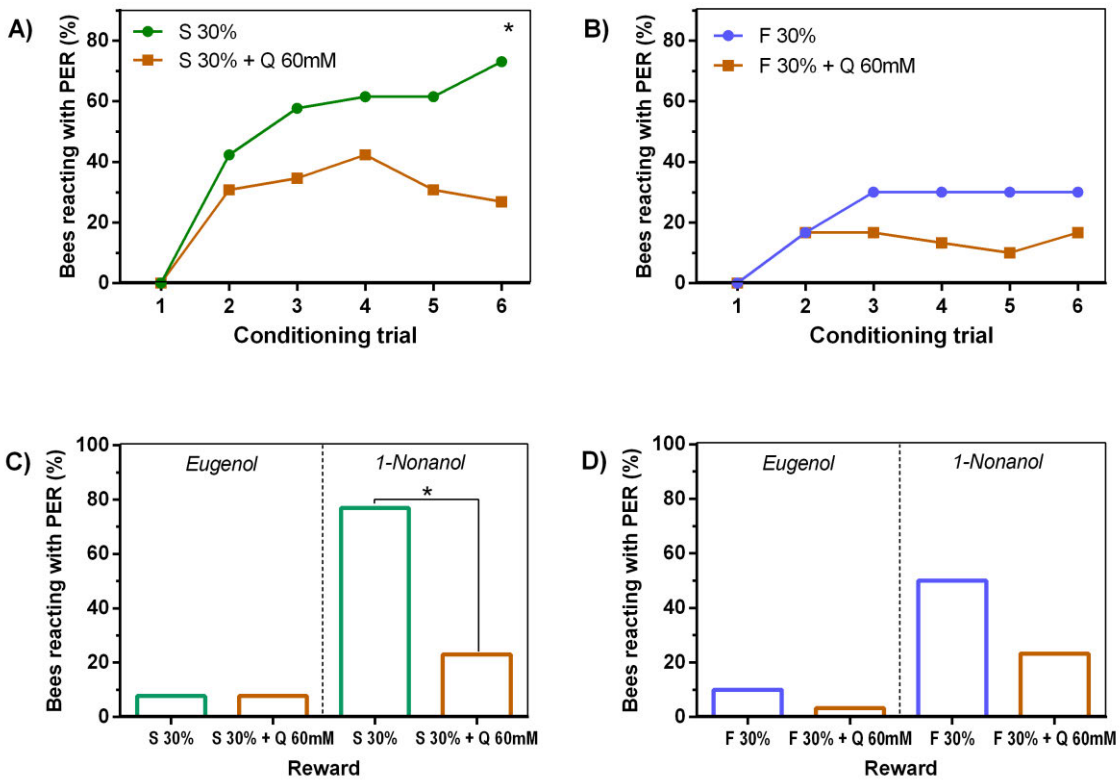
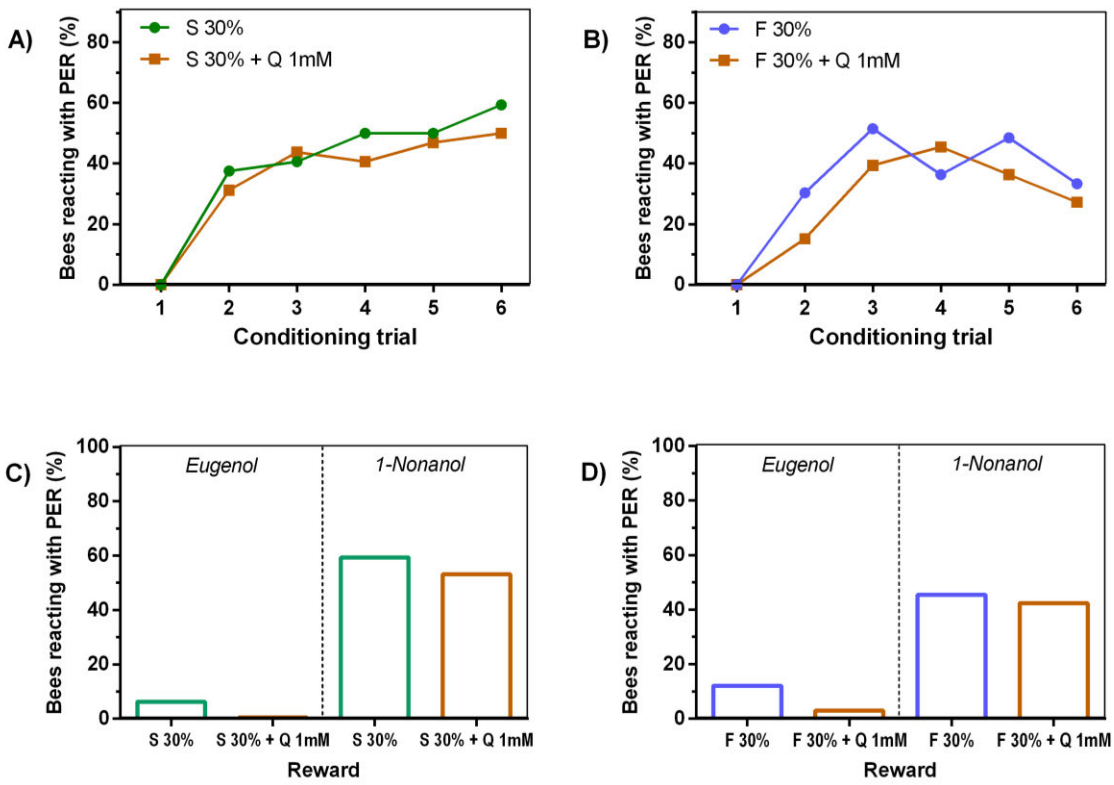


Figure 1: Appetitive learning with sugar and high quinine concentration (60 mM). Olfactory learning performance of honeybee foragers rewarded with 30 % sugar solution (sucrose or fructose) and those rewarded with sugar + quinine (60 mM). **A**: Frequency of conditioned proboscis extension responses (PER). A reward with sucrose and a high quinine concentration (S 30% + Q 60 mM) generates a significantly lower learning performance compared to that of a pure sucrose reward (S 30%) ($P < 0.05$, GEE, *). **B:** Learning performance with a reward of fructose and a high quinine concentration (F 30 % + Q 60 mM) does not differ significantly from that with a pure fructose reward (F 30 %) ($P > 0.05$). However, the learning rate was lower with fructose rewards compared to that with a sucrose reward. **C**: Post-conditioning test of the alternative odour (eugenol) and conditioned odour (1-nonanol). Percentage of bees displaying PER to the conditioned odour is significantly lower when the reward contained a high quinine concentration (S 30% + Q 60 mM) compared to when the reward was a pure sucrose solution (S 30%) ($P < 0.05$, Fisher's exact test, *). There are no significant differences in the alternative odour test ($P > 0.05$). **D:** Post-conditioning test of the alternative odour (eugenol) and conditioned odour (1-nonanol). There are no significant differences between the pure fructose reward (F 30 %) and the reward with quinine (F 30% + Q 60mM), neither for 1-nonanol nor eugenol ($P > 0.05$). $N_{A,C} = 52$, $N_{B,D} = 60$. See **Table 2** for detailed statistics of the learning curves and **Table S1** for the post-conditioning test.



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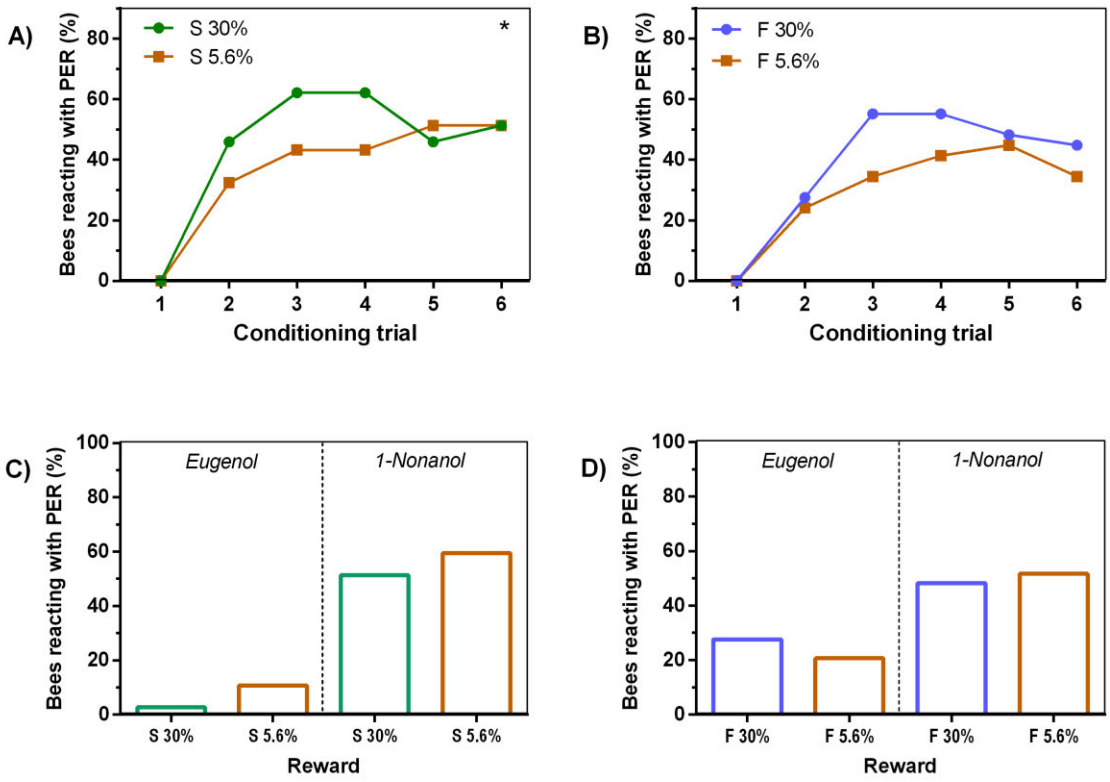
407 **Figure 2: Appetitive learning with sugar and low quinine concentration (1 mM).** Olfactory conditioning of honeybee nectar
408 foragers rewarded with a 30 % sugar solution (either sucrose or fructose) compared to those rewarded with a low quinine
409 admixture (1 mM). **A**: Frequency of conditioned proboscis extension responses (PER). Learning performance with a reward of
410 fructose and a low quinine concentration (F 30% + Q 1mM) does not differ significantly from performance with a pure fructose
411 reward (F 30%) ($P > 0.05$, GEE). **B*:*** Frequency of conditioned proboscis extension responses (PER). Learning performance with
412 a reward of fructose and a high quinine concentration (F 30% + Q 60mM) does not differ significantly from performance with a
413 pure fructose reward (F 30%) ($P > 0.05$). **C**: Post-conditioning test of the alternative odour (eugenol) and conditioned odour (1-
414 nonanol). There are no significant differences between the pure sucrose reward (S 30%) and the reward with quinine (S 30% +
415 Q 1mM), neither for 1-nonanol for eugenol ($P > 0.05$, Fisher's exact test). **D:** Post-conditioning test of the alternative odour
416 (eugenol) and conditioned odour (1-nonanol). There are no significant differences between the pure fructose reward (F 30%) and
417 the reward with quinine (F 30% + Q 1mM), neither for 1-nonanol nor eugenol ($P > 0.05$). $N_{A,C} = 64$, $N_{B,D} = 66$. See **Table 2** for
418 detailed statistics of the learning curves and **Table S1** for the post-conditioning test.



419

420 **Figure 3: Appetitive learning with low sugar concentration (5.6%).** Olfactory conditioning of honeybee nectar foragers
421 rewarded with a high 30 % sugar solution (either sucrose or fructose) compared to those rewarded with a low 5.6 % solution of
422 the same sugar. **A**: Frequency of conditioned proboscis extension responses (PER). Reward with a high sucrose concentration
423 (S 30%) generates a significantly higher learning performance than a low sucrose concentration (S 5.6%) ($P < 0.05$, GEE, *). **B:**
424 Frequency of conditioned proboscis extension responses (PER). Performance does not differ between bees being rewarded with
425 a high fructose concentration (F 30%) and those being rewarded with a low fructose concentration (F 5.6%) ($P > 0.05$, GEE). **C**:
426 Post-conditioning test of the alternative odour (eugenol) and conditioned odour (1-nonanol). There are no significant differences
427 between the high sucrose concentration (S 30%) and the low sucrose concentration (S 5.6%), neither for 1-nonanol nor for eugenol
428 ($P > 0.05$, Fisher's exact test). **D*:*** Post-conditioning test of the alternative odour (eugenol) and conditioned odour (1-nonanol).
429 There are no significant differences between the high fructose concentration (F 30%) and the low fructose concentration (F 5.6%),
430 neither for 1-nonanol nor eugenol ($P > 0.05$). $N_{A,C} = 74$, $N_{B,D} = 58$. See **Table 2** for detailed statistics of the learning curves and
431 **Table S1** for the post-conditioning test.

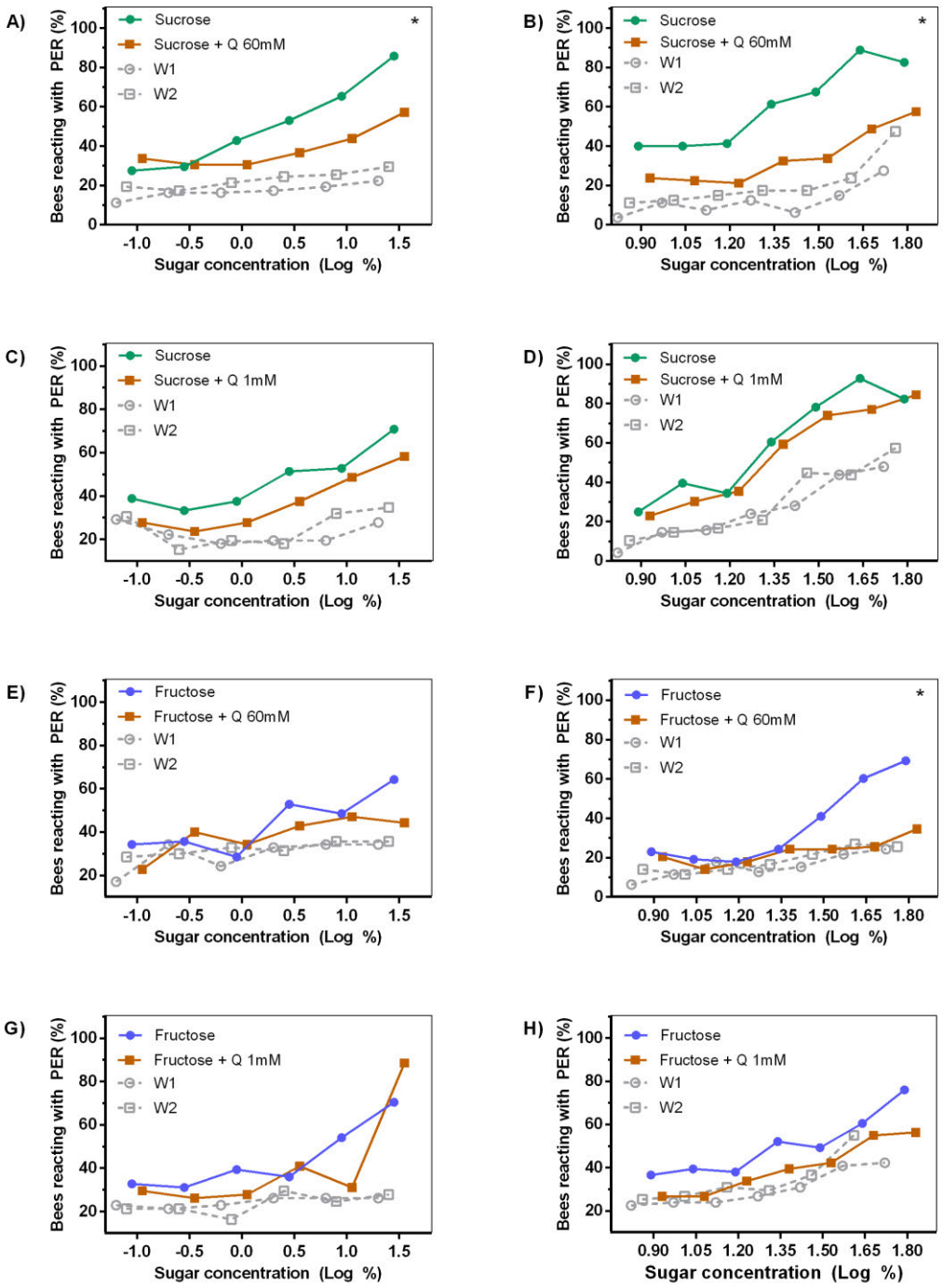


Figure 4: Antennal responsiveness to sugar and sugar with quinine. Percentage of honeybee nectar foragers reacting with proboscis extension response (PER) to water and increasing sugar concentrations (sucrose, **A-D**, or fructose, **E-H**), alternately pure or mixed with quinine in a low concentration (1 mM) or a high concentration (60 mM), following a low sugar concentration series (log % = -1.0 – 1.5) or a high sugar concentration series (log % = 0.90 – 1.80). Bees are significantly less responsive to sucrose solutions mixed with a high quinine concentration (60 mM) in the low sugar concentration series (**A**, $P < 0.05$, GEE, *) and in the high sugar concentration series (**B**, $P < 0.05$, *). Similarly, responses to pure fructose solutions in the high concentration series were significantly more frequent than to fructose solutions mixed with the high quinine concentration of 60 mM in the high concentration series (**F**, $P < 0.05$, *). In all other cases (**C-E**, **G-H**) no significant differences ($P > 0.05$) were detected in the responses to the pure sugar solution and the responses to sugar solutions mixed with quinine. Grey lines show responses to stimulation with water before each pure sugar trial (W1) or before each sugar with quinine trial (W2). $N_A = 72$, $N_B = 96$, $N_C = 98$, $N_D = 80$, $N_E = 61$, $N_F = 71$, $N_G = 70$, $N_H = 78$. See **Table 3** and **Table 4** for detailed statistics.

Tables

Table 1: Experimental design of the olfactory conditioning: solutions offered as reward in each group.

Experiment	CS+	CS-
1	Sucrose 30 %	Sucrose 30 % + Quinine 60 mM
2	Fructose 30 %	Fructose 30 % + Quinine 60 mM
3	Sucrose 30 %	Sucrose 30 % + Quinine 1 mM
4	Fructose 30 %	Fructose 30 % + Quinine 1 mM
5	Sucrose 30 %	Sucrose 5.6 %
6	Fructose 30 %	Fructose 5.6 %

Table 2: Overview of statistical results of binomial models (GEE) of the olfactory conditioning experiments.

	Fructose 30 %			**Sucrose 30 %**		
	vs. F 30 % + Q 60mM	*vs. F 30 % + Q 1mM*	*vs. F 5.6 %*	*vs. S 30 % + Q 60mM*	*vs. S 30 % + Q 1mM*	*vs. S 5.6 %*
N (per group)	60	66	58	52	64	74
Trial number	$\chi^2_{1,360}$ = 12.530	$\chi^2_{1,396}$ = 30.554	$\chi^2_{1,348}$ = 50.425	$\chi^2_{1,312}$ = 43.488	$\chi^2_{1,384}$ = 87.979	$\chi^2_{1,444}$ = 53.833
	***P* < 0.001**	***P* < 0.001**	***P* < 0.001**	***P* < 0.001**	***P* < 0.001**	***P* < 0.001**
Reward	$\chi^2_{1,360}$ = 0.040	$\chi^2_{1,396}$ = 1.021	$\chi^2_{1,348}$ = 0.682	$\chi^2_{1,312}$ = 0.242	$\chi^2_{1,384}$ = 0.000	$\chi^2_{1,444}$ = 3.858
	P = 0.842	*P* = 0.312	*P* = 0.409	*P* = 0.623	*P* = 0.991	***P* = 0.050**
Interaction	$\chi^2_{1,360}$ = 1.390	$\chi^2_{1,396}$ = 0.117	$\chi^2_{1,348}$ = 0.033	$\chi^2_{1,312}$ = 8.786	$\chi^2_{1,384}$ = 0.362	$\chi^2_{1,444}$ = 1.109
(Trial × Reward)	*P* = 0.238	*P* = 0.732	*P* = 0.857	***P* = 0.003**	*P* = 0.547	*P* = 0.292

Table 3: Overview of statistical results of binomial models (GEE) of the antennal responsiveness experiments with sucrose.

Sucrose				
	Low sugar concentration series		*High sugar concentration series*	
	vs. Quinine 1mM	***vs. Quinine 60mM***	***vs. Quinine 1mM***	***vs. Quinine 60mM***
N	72	98	96	80
Sugar concentration	$\chi^2_{1,864} = 48.110$	$\chi^2_{1,1176} = 105.379$	$\chi^2_{1,1204} = 219.500$	$\chi^2_{1,1099} = 95.587$
	***P* < 0.001**	***P* < 0.001**	*P* < 0.001	***P* < 0.001**
Treatment	$\chi^2_{1,864} = 1.735$	$\chi^2_{1,1176} = 2.832$	$\chi^2_{1,1204} = 0.192$	$\chi^2_{1,1099} = 4.050$
	P = 0.188	*P* = 0.092	*P* = 0.662	***P* = 0.044**
Interaction	$\chi^2_{1,864} = 0.109$	$\chi^2_{1,1176} = 20.626$	$\chi^2_{1,1204} = 0.065$	$\chi^2_{1,1099} = 2.474$
(Sugar conc. × Treatment)	*P* = 0.741	***P* < 0.001**	*P* = 0.798	*P* = 0.116
Water control 1	$\chi^2_{1,432} = 0.096$	$\chi^2_{1,588} = 5.289$	$\chi^2_{1,602} = 60.248$	$\chi^2_{1,560} = 13.249$
	P = 0.757	***P* = 0.021**	***P* < 0.001**	***P* < 0.001**
Water control 2	$\chi^2_{1,864} = 1.735$	$\chi^2_{1,1176} = 2.832$	$\chi^2_{1,1204} = 0.192$	$\chi^2_{1,1099} = 4.050$
	P = 0.188	*P* = 0.092	*P* = 0.662	***P* = 0.044**

Table 4: Overview of statistical results of binomial models (GEE) of the antennal responsiveness experiments with fructose.

Fructose				
	Low sugar concentration series		*High sugar concentration series*	
	vs. Quinine 1mM	***vs. Quinine 60mM***	***vs. Quinine 1mM***	***vs. Quinine 60mM***
N	61	70	71	78
Sugar concentration	$\chi^2_{1,732} = 48.463$	$\chi^2_{1,840} = 19.933$	$\chi^2_{1,994} = 35.842$	$\chi^2_{1,1092} = 41.307$
	***P* < 0.001**	***P* < 0.001**	***P* < 0.001**	***P* < 0.001**
Treatment	$\chi^2_{1,732} = 1.027$	$\chi^2_{1,840} = 0.034$	$\chi^2_{1,994} = 0.778$	$\chi^2_{1,1092} = 1.000$
	P = 0.311	*P* = 0.854	*P* = 0.378	*P* = 0.317
Interaction	$\chi^2_{1,732} = 1.000$	$\chi^2_{1,840} = 0.834$	$\chi^2_{1,994} = 0.053$	$\chi^2_{1,1092} = 9.360$
(Sugar conc. × Treatment)	*P* = 0.317	*P* = 0.361	*P* = 0.818	***P* = 0.002**
Water control 1	$\chi^2_{1,366} = 0.51$	$\chi^2_{1,420} = 3.319$	$\chi^2_{1,497} = 10.167$	$\chi^2_{1,546} = 8.172$
	P = 0.475	*P* = 0.068	***P* = 0.001**	***P* = 0.004**
Water control 2	$\chi^2_{1,366} = 1.358$	$\chi^2_{1,420} = 1.041$	$\chi^2_{1,497} = 12.261$	$\chi^2_{1,546} = 8.374$
	P = 0.244	*P* = 0.308	***P* < 0.001**	***P* = 0.004**

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Supplements

Table S1: Statistical values of the post-conditioning learning tests with the conditioned odour (1-nonanol) and the alternative odour (eugenol). Displayed are the number of tested bees (N) and the values of Fisher's exact test comparing reward groups.

	Fructose 30 %			**Sucrose 30 %**		
	vs. F 30 % + Q 60mM	*vs. F 30 % + Q 1mM*	*vs. F 5.6 %*	*vs. S 30 % + Q 60mM*	*vs. S 30 % + Q 1mM*	*vs. S 5.6 %*
N (per group)	60	66	58	52	64	74
Conditioned odour	*P* = 0.060	*P* = 1.000	*P* = 0.314	***P* = 0.002**	*P* = 0.801	*P* = 0.640
Alternative odour	*P* = 0.612	*P* = 0.355	*P* = 0.760	*P* = 1.000	*P* = 0.492	*P* = 0.358

6 General Discussion

6.1 The complex spectrum of sugar perception in honeybees is contrary to the reduced set of sugar receptors

For honeybees, the perception of sweetness plays a crucial role to detect and evaluate their food sources such as carbohydrates in nectar and honey (Seeley, 1985). Many of their behavioral decisions are therefore based on sugar perception, either individually for ingestion or for social behaviors, like collecting or processing nectar (Winston, 1991). Sugar perception is by no means rigid in honeybees and changes during the transition from in-hive bees (nurses) to foragers (Scheiner et al., 2001; Scheiner et al., 2017). Honeybees seem to have a broad spectrum of different detectable sugar molecules when perceiving sweetness on many levels. They can detect at least seven types of sugars and decide to forage for them when offered (Vogel, 1931; von Frisch, 1934). They thereby seem to show a clear preference for certain sugars such as sucrose (Wykes, 1952). Astonishingly, the broad spectrum of molecules of the sugar perception in honeybees is contrary to the fact that they seem to have only three putative sugar receptors (Robertson & Wanner, 2006).

In the following chapters I expand how the presented results of the thesis draw a more comprehensive picture of sugar perception in honeybees. I discuss the underlaying mechanisms that allow only three sugar receptors (AmGr1, AmGr2 and AmGr3) to provide the well-known (and with the results shown, also expanded) complexity and flexibility of sugar perception in honeybees. Further, I speculate to what extend the role of the sugar receptors and receptor components can enable honeybees to discriminate sugars. Additionally, I will discuss how the receptors might be involved in the detection of other substances, such as bitter ones, or be modulated by them. This allows me to put the ability to sense sweetness in relation to the overall ability of taste perception in honeybees and to draw an outlook for future research to a certain extent.

6.2 AmGr3 is the only specific fructose receptor

The gustatory receptor AmGr3 seems to be exclusively reserved for the perception of fructose when characterized in the *Xenopus* cell-system. It is a non-specific cation channel (**Manuscript II, Chapter 3**; also see Takada et al., 2018). No signals from AmGr3 were detected in the presence of other sugars (such as arabinose, mannose, raffinose and galactose). However, AmGr3 did also not react towards sugars that honeybees can clearly perceive (such as sucrose, glucose, maltose, melezitose and trehalose; for which we later showed that AmGr1 is capable; **Manuscript III, Chapter 4;** for honeybees' sugar preference see: Vogel, 1931; von Frisch, 1934; Wykes, 1952). My experiments proved that AmGr3 does not depend on the co-receptor AmGr2, in contrast to AmGr1.

Further, the results displayed AmGr3 as a specific fructose receptor *in vivo,* since mutants lacking it as a functional protein were less responsive to fructose, but not as a sucrose receptor (**Manuscript II, Chapter 3**). The presented results indicate that fructose seems to inhere an outstanding specific role for the honeybee at the receptor level, for instance, their sensilla reacts less readily to fructose than to sucrose (Whitehead & Larsen, 1976). According to this, the PER experiments of this work demonstrated that honeybees are also less responsive to fructose than to sucrose, whether they are nurses or foragers (**Manuscript I, Chapter 2**). Additionally, this work proved that the learning performance for odors is lower when bees get rewarded with fructose instead of sucrose (**Manuscript IV, Chapter 5**). This confirms early choice experiments in which caged bees or foragers prefer sucrose and additionally glucose over fructose (Vogel, 1931; Wykes, 1952). With AmGr3 a separate and specific receptor for fructose was found, although this sugar seems to be less relevant in taste perception at first glance.

Interestingly, this study discovered AmGr3 not only to be expressed in the antennae, but also in the brain (**Manuscript I, Chapter 2**). Therefore, an additional internal role for this specific receptor and fructose might be likely, even if the sugar is not preferred externally. A possible function of such an internal sensor can be found in the homology

analysis with the gustatory receptor DmGr43a in the fruit fly *Drosophila melanogaster* (Miyamoto et al., 2012). The AmGr3 homolog DmGr43a was shown to mediate feeding in hungry flies and to suppress food intake when flies were satiated. In addition, it has been demonstrated that the hemolymph fructose level, even if it is only a small component of total blood sugar of the flies, increases dramatically up to 4- or 10-fold immediately after feeding (Miyamoto et al., 2012). Therefore, fructose could also play a similar role in the honeybee as a signal for recent food intake but not for the actual energy level in the blood. This might explain the specificity of AmGr3 as a possible internal sensor for satiation.

When comparing the expression levels of AmGr3 in the brains of foragers and nurse bees, we found a significantly higher expression in nurses (**Manuscript I, Chapter 2**). For nurse bees, sitting directly on the food stores, a quick response to hunger (promoted by AmGr3) can be beneficial for consuming energy for production of larval food (Seeley, 1985; Crailsheim, 1990; Crailsheim et al., 1992). In contrast, foragers must be more resilient to starvation when foraging. When they have reached and validated the nectar source they are supposed to take up as much as possible and not to stop the intake due to early satiation (Fewell & Winston, 1996). Studies that directly compare feeding to satiety in nurses and foragers are still missing, while satiety affects the learning performance of nurse bees and foragers differently (Ben-Shahar & Robinson, 2001). When measuring AmGr3 expression levels via qPCR in the antennae of nurse bees, they show a significant lower expression of the specific fructose receptor AmGr3 when compared with the antennae of foragers (**Manuscript I, Chapter 2**). Honey contains much higher portions of monosaccharides, such as fructose, than polysaccharides, such as sucrose, compared to nectar (Doner, 2003). As in-hive bees, nurse bees rather sit on honey stores than on nectar, and it seems relevant to have fewer fructose receptors in the antennae and therefore be less sensitive to this highly concentrated diet (Seeley, 1985). In line with this, there was an overall higher responsiveness of the foragers to fructose (also sucrose) when compared with nurses (**Manuscript I, Chapter 2**). Most nectars, whether they mainly contain sucrose or

fructose (and glucose), are less concentrated than honey (Percival, 1961; Doner, 2003). The found expression differences of AmGr3 in nurse bees and foragers can be set directly in context with the known behavior of both bee casts. As an internal fructose sensor, its higher expression in the brain of nurse bees might promote a quicker response to satiety and hunger. A high antennal expression of AmGr3, as external receptor, enables foragers to accurately assess and validate nectar as a food source for their foraging.

Nevertheless, the individual sucrose responsiveness of honeybees, whether nurse bees or foragers, correlates with their fructose responsiveness (**Manuscript I, Chapter 2**). This alone could falsely indicate that an overall sugar concentration would also be sufficient for food validation, which could have been carried out by just one external receptor for sugars. For example, humans cannot discriminate between different sugars and sweet taste perception of all sugars seems to be carried out mainly by one heteromeric G-protein-coupled receptor complex in the taste buds (T1R2 and T1R3; Zhao et al., 2003). In contradiction to this, the antennal responsiveness (for sucrose and fructose) in honeybees is based on two different receptors (AmGr1 and AmGr3; **Manuscript III, Chapter4**), while one of them inheres an outstanding and specific role as fructose receptor (**Manuscript II, Chapter 3**). Taking this into account, I hypothesize that the two taste receptors enable honeybees to differentiate between nectar and ripe honey, independently from their individual overall sugar responsiveness (Eyer et al., 2016). With that, two distinct receptors enable the honeybee to detect a ratio (of the monosaccharide fructose and other polysaccharides, such as sucrose), which delivers more information about the ripeness of the diet than sugar concentration alone.

With the results shown, we demonstrated that only two taste receptors (AmGr1 and AmGr3) build the base for the perception of the sugar spectrum of honeybees. The specific fructose receptor AmGr3 might be used to detect satiation (internally) and to discriminate the ripeness of the diet (externally). In this context, AmGr3 shows a direct expressional regulation in the brain and antennae according to the bees' age and task,

and is therefore linked with division of labor. However, we found that sugar perception in honeybees is even more complex and relies on further regulatory mechanisms when we focused on the other sugar receptors (AmGr1 and AmGr2).

6.3 The gustatory receptor AmGr1 is a broad sugar receptor that is regulated by its co-receptor AmGr2 and expressional differences

The presented studies demonstrate that AmGr1 acts as a broad sugar receptor detecting many different sugar molecules, when expressed in *Xenopus* oocytes (**Manuscript III, Chapter 4**). AmGr1 reacts to sucrose, glucose, maltose, trehalose and melezitose, but not fructose or other sugars that honeybees do not prefer (such as arabinose, mannose, raffinose and galactose; for honeybees sugar preference see Vogel, 1931; von Frisch, 1934; Wykes, 1952). Furthermore, these findings for AmGr1 were supported by *in vivo* experiments, since its mutants generated with CRISPR/Cas9 showed a reduced responsiveness towards sucrose and glucose but not towards fructose (**Manuscript III, Chapter 4**). The co-expression of AmGr1 and AmGr2 in our experiments alters the strength and time-dependent characteristics of sucrose-, glucose- and maltose-induced signals (**Manuscript III, Chapter 4**), while CRISPR/Cas9 mutants and wildtypes of the co-receptor AmGr2 do not differ in their sugar responsiveness (**Manuscript III, Chapter 4**). A total loss of the responsiveness towards the respective sugars was never measurable in all our receptor mutants, even if it was clearly significant (for AmGr1 and AmGr3). Live animals in experiments are exposed to many different stimuli, and the measured behavior (such as in PER experiments) is influenced by many regulatory mechanisms upstream from receptor signaling (Thorne et al., 2004; Amrein, 2016; Miriyala et al., 2018; Scheiner et al., 2013). With that the modulating effect of AmGr2 in its mutants seems to be weaker and harder to measure in living animals than in the oocyte cell system. Nevertheless, our overall finding about AmGr1 and the co-expression with AmGr2 is well in line with the experiments by Jung

et al. (2015). Both studies show that AmGr1 is a broad sugar receptor whose specificity for certain sugars can be modulated and is thus regulated by the co-receptor AmGr2.

However, in our experiments the co-expression of AmGr1 and AmGr2 shows a different modulation pattern compared to Jung et al. (2015). Both studies only used the cDNA sequences driven from mRNA found in the taste organs of different honeybee hybrids *lingustica and caucasia* (**Manuscript III, Chapter 4** ; Jung et al., 2015). The discrepancy between the two studies is an important indication that different protein sequences of AmGr1 already influence the receptor and co-receptor interaction. Focusing on the annotated mRNA-Sequences of AmGr1 in the hybrid of our study (*Apis mellifera carnica),* different splice variants can be found (see LOC727431; Consortium Honeybee Genome Sequencing, 2006). Splicing is a known process in many genes of higher eukaryotic cells that also results in different protein sequences and is often used as a regulatory mechanism for the respective genes (Cooper, 2005). It is very likely that splicing is a similar additional regulation mechanism for AmGr1's specificity, since there are different splice variants and the comparison of two studies shows that a different protein structure alters the interaction with its co-receptor (AmGr2).

However, structural analyses investigating the detailed relationships between the protein sequences and their functional regions, and the specificity and interaction of the sugar receptors in honeybees, are still lacking. Compared to Jung et al. (2015) our study already draws a more comprehensive picture when proving the heteromerization and interaction of AmGr1 and its co-receptor AmGr2 by bimolecular fluorescence complementation (BiFC; **Manuscript III, Chapter 4**). Additionally, it was found that AmGr1 also detects the sugar melezitose excreted by aphids and rarely found in nectar (**Manuscript III, Chapter 4**; Bosi & Battaglini, 1978; de Brito Sanchez et al., 2007; Stanley et al., 2013). Excessive foraging and consumption of the so-called honeydew and possibly resulting negative effects are reported by beekeepers as the honeydew disease in bee colonies (Horn, 1985; Imdorf et al., 1985). Findings that one receptor (AmGr1) detects the healthy sugars, as well as the un-healthy melezitose, suggest that honeybees are unable to discriminate them (Seeburger, 2021). This might

be the explanation of the occurrence of the honeydew disease (when aphids over-breed or honeydew is the only alternative to un-favorable nectar flow; Meiners et al., 2017).

The earlier study of this thesis found that honeybee foragers are more responsive to sucrose when compared to nurse bees, while there are no significant differences in the expression of the respective sucrose receptor (AmGr1) in their antennae (**Manuscript I, Chapter 2**). The behavioral data alone is well in line with previous findings of Scheiner et al. (2017). The regulation of antennal sucrose responsiveness of AmGr1 might be more influenced by its co-receptor AmGr2 (or other regulatory mechanisms) than its own overall expression level. A recent example for a co-receptor inhering an important regulatory function is CD28 in the human immune system (in b-cells). A reduced expression or lack of this regulatory co-receptor leads to autoimmune diseases (Okazaki et al., 2002). Nevertheless, my study revealed a significantly higher expression level of AmGr1 in the brain of nurse bees compared to foragers (**Manuscript I, Chapter 2**; similar to AmGr3). Thus, AmGr1 might also be an internal sugar sensor which is related to division of labor. The broad spectrum of sugars that AmGr1 can perceive suggests that it is rather a sensor for the main blood sugars, which are the main energy carrier (such as trehalose, see Božič & Woodring, 1997). The higher expression level of AmGr1 in the brain may indicate that nurse bees can react more readily to changes in the overall energy level of their hemolymph. Foragers might be more endurable to a dropping energy level during foraging when they have less expression of the internal receptor sensing it (Blatt & Roces, 2001). With this, the receptor AmGr1 functions as both, an internal energy sensor, as well as an external taste receptor. It seems to be regulated by direct expression (in the brain) or indirectly (via its co-receptor, splice variants or unknown mechanisms).

In my studies, I was able to proof the interaction of the broad sugar receptor (AmGr1) and its co-receptor (AmGr2) in the *Xenopus* cell system (**Manuscript III, Chapter 4**) and to measure the expression of AmGr1 in the antennae of honeybees in different bee casts (**Manuscript I, Chapter 2**). Comparable expression analyses of its co-receptor

AmGr2 are lacking. Nevertheless, my findings suggest a differentiated expression of both receptors in different taste neurons of honeybees (de Brito Sanchez et al., 2007). For *Drosophila,* so-called sweet neurons were found to show different distinct expression patterns of sugar receptors. They can even be categorized into anatomically and functionally distinct groups, valuing sweetness differently to promote or reject sucrose diet (Chen et al., 2022). Accordingly, it is convincing that the sugar receptors (AmGr1, AmGr2 but also AmGr3) are also not expressed in the same levels and combinations in all taste neurons of the honeybee. Subsequently the modulating effect of AmGr2 on AmGr1 might be different in separate neurons (or neuronal groups). For odors, activating and inhibitory neurons were already found in the sensory organs of honeybees, when studying olfactory perception and neurophysiology of the honeybee (Denker et al., 2010; Galizia et al., 2011). I assume that the broad sugar receptor AmGr1 and its co-receptor (but also AmGr3) differ in their expression patterns in different honeybee neurons and enable an even more complex evaluation of diet. Whether there are special sweet neurons, or even activating or inhibitory sweet neurons in the honeybee, has to be investigated in further studies.

Summarizing these results, the knowledge of the sugar perception of the honeybee is expanded to a more complex picture. The receptor AmGr1 alone appears to be a broad sugar receptor that can perceive the sugars sucrose, glucose, maltose, trehalose and melezitose. The expression level of AmGr1 is directly regulated in the brain according to the bees' age and task and is therefore linked with division of labor. It can be assumed that it inheres a role in the internal sensing of the overall sugar and energy level in the hemolymph. Initially, honeybees seem not to be able to differentiate between all sugars externally detected by AmGr1, as it is the case of melezitose and the resulting honeydew disease. Contrastingly, the results of this thesis show that AmGr2 is the co-receptor of AmGr1, altering and fine-tuning its sucrose and maltose signals. The comparison with other studies suggests that splice variants and a varying protein structure also influence the specificity of the AmGr1 receptor. It is justifiable to hypothesize that both, the existence of the co-receptor and possible splice variants,

enable honeybees to an even more differentiated perception or even discrimination of the sugars detected. This might be possible when different neurons (or tissues) inhere different sets and combinations of AmGr1, its splice variants or its co-receptor, and honeybees are able to compute such incoherent signals.

6.4 The complexity of sugar perception in honeybees includes non-sugar molecules

The results of this work show that the multidimensionally regulated and complex picture of sugar perception might be expanded to the presence of other molecules. It was possible to clearly show that the bitter substance quinine (in high concentration), added to a sucrose solution, weakens the learning performance of honeybees (**Manuscript IV, Chapter 5**). Similar effects were observed in learning when rewarding with high-concentrated sucrose solution, compared to a lower concentrated sucrose reward. Up to now, there is no bitter receptor found in honeybees (Robertson & Wanner, 2006). Together with experiments investigating learning differences with water and bitter stimuli, my results suggest that honeybees are not able to distinguish and therefore cannot sense bitter substances in general (Guiraud et al., 2018).

In the fruit fly *Drosophila,* there are clearly bitter receptors located in bitter-sensitive cells. Nevertheless, it was shown that the flies' bitter avoidance is also based on the inhibition of sugar perception (French et al., 2015). According to that study, my results show that honeybees rewarded with fructose or sucrose and quinine display bitter-related differences in their learning ability even it was not significant (**Manuscript IV, Chapter 5**). A high-concentrated fructose reward compared to a low-concentrated fructose reward, both without the bitter substance, did not show a significant difference. As discussed previously, fructose seems to be less attractive to honeybees even if it is perceived by a single specific receptor (Vogel, 1931; von Frisch, 1934; Wykes, 1952; **Manuscript I, Chapter 2**). I assume, similar to the found mechanism in fruit flies (French et al., 2015), bitter perception in honeybees might be indirect

through inhibition of the sugar receptors. The findings, that both sugars (sucrose and fructose) are perceived by different receptors (AmGr1 and AmGr3; **Manuscript II, Chapter 3**, **Manuscript III, Chapter 4** and **Manuscript I, Chapter 2**), indicate that bitter substances (such as quinine) inhibit the sugar receptors differently, and this can therefore represent a further regulatory mechanism.

It remains to be clarified to what extent inhibitory learning effects of quinine are only limited to AmGr1 or can also affect AmGr3 on the receptor level but are not visible to its less attractive ligand fructose (**Manuscript I, Chapter 2**). Since my results clearly show that sucrose perception involves a co-receptor (AmGr2, co-receptor for AmGr1; **Manuscript III, Chapter 4)** which is not the case for fructose perception (AmGr3 only), it could be that that bitter compounds have a greater influence on this co-receptor (AmGr2) than on the defined basic sugar receptors (AmGr1 and AmGr3). This must be proven in further characterization experiments in the presence of bitter substances (**Manuscript III, Chapter 4**; Jung et al., 2015; Takada et al., 2018). Subsequent PER experiments with high-concentrated fructose, high-concentrated sucrose, or low-concentrated sucrose solution clearly confirmed the inhibitory effect, when adding high-concentrated quinine (**Manuscript IV, Chapter 5**). There was no inhibition in the PER reaction in low-concentrated fructose when quinine was added. This underlines again, that bitter substances might have a lowered inhibitory effect on the fructose perception of AmGr3.

When adding a lower quinine concentration to the sugar series for PER experiments, there was no effect whether sucrose, fructose or a lower or higher concentration series was used (**Manuscript IV, Chapter 5**). In conclusion, it can be hypothesized that the inhibitory effect of bitter substance on the sugar perception is concentration-dependent, based on the salience or attractiveness and concentration of the present sugar (Scheiner et al., 2004).

My results indicate that the different inhibitory effects of bitter substances are due to the interplay of the found receptors (AmGr1, AmGr2 and AmGr3; see **Manuscript II,**

Chapter 3 and **Manuscript III, Chapter 4**) and their susceptibility to inhibition. Studies examining these effects in *Xenopus* oocytes are still lacking. Since my studies were performed with fixed bees, free-choice or foraging choice experiments are essential to fully uncover the picture of bitterness perception in honeybees and their associated behavior. It would therefore be interesting to investigate how other bitter substances (apart from quinine) affect the sugar perception of the receptors of the honeybee. Furthermore, understanding bitter substances, their perception and avoidance behavior in honeybees might have an advantage for agriculture and the protection of the insect. Enriching pesticides with substances that deter the pollinator might prevent its contamination or poisoning.

6.5 Summarizing in an overview how sugar perception is regulated and can be accomplished by a reduced set of sugar receptors

Summing up the discussion of the results of this work, an expanded picture of sugar perception in honeybees can be drawn. Following this picture displayed in **Figure 2**, first detectable sugars and their occurrence in the environment of honeybees can be determined. Further, the defined basis of sugar perception (the receptors AmGr1 and AmGr3 are already capable of perceiving all sugars) can be extended to include the co-receptor AmGr2 and the unknown, but highly suspected, splice variants (alternative protein structures) of AmGr1. With this, all receptor components for sugar perception in honeybees are enumerated. Furthermore, it is possible to clarify more detailed the designation of the receptor components: AmGr1 as the broad sugar receptor, AmGr1s splice variants as possible broad sugar receptors with altered ligand specificity or heterodimerization pattern, AmGr2 as a co-receptor for it and AmGr3 a specific-fructose receptor. In **Figure 2** all findings based on a hypothesis from the previous discussion are marked with an asterisk (*) to distinguish them from the clear results of this work or other studies (without asterisk).

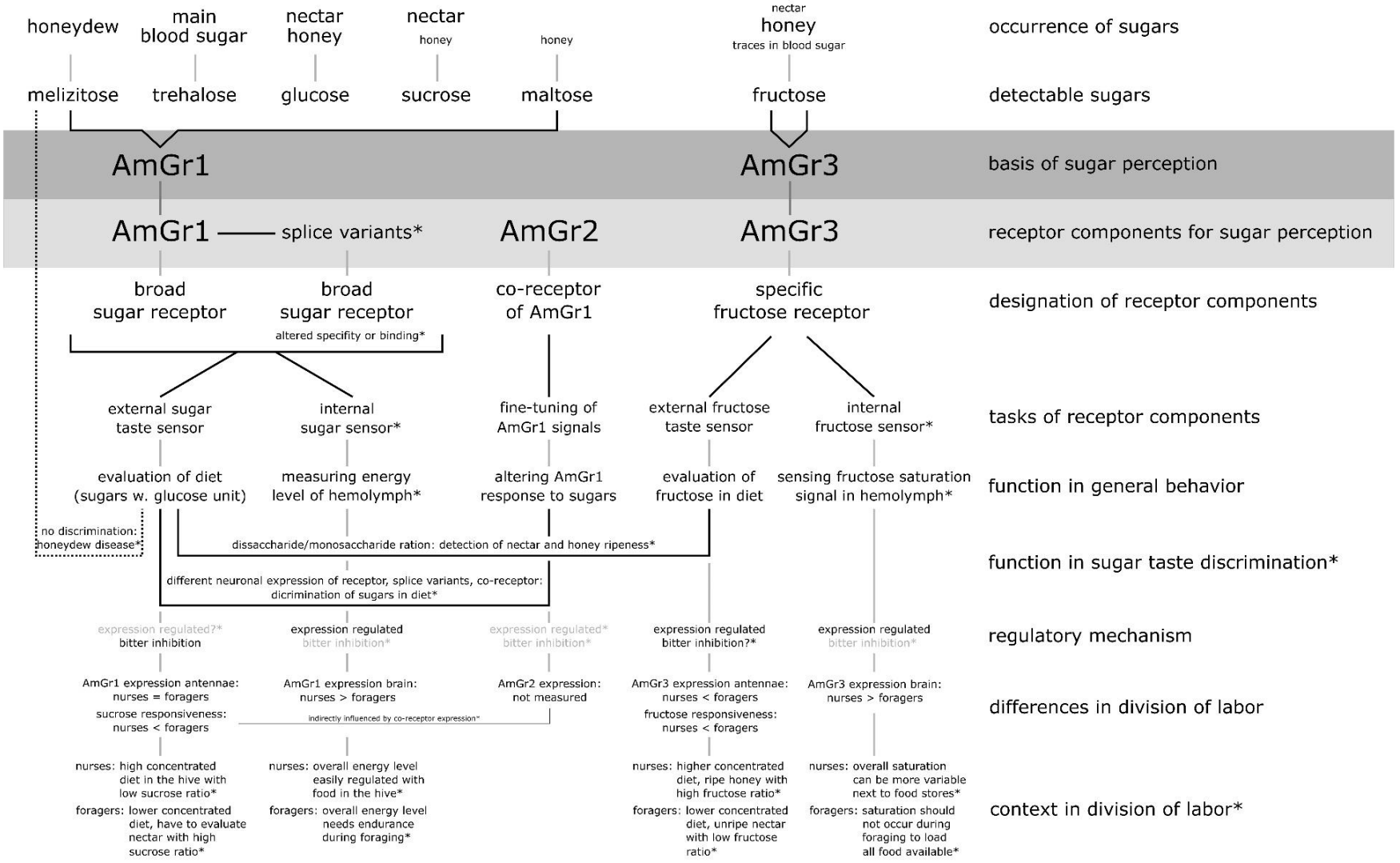

Figure 2: Overview of the known facts, results of this thesis and derived hypotheses about the sugar receptors, their components, their regulation, and the sugar perception in the context of division of labor of honeybees. The basis receptors AmGr1 and AmGr2, already capable of detecting occurring and detectable sugars, were extended by further receptor components for sugar perception (dark gray and light gray). The designation of the receptor components, their tasks and function in the general behavior of honeybees could be defined. It is possible that the found receptor components inhere an important function in sugar taste discrimination in honeybees. Hypotheses like the interaction of receptor and co-receptor (including splice variants), may enable sugar discrimination, or a specific fructose receptor enables to discriminate ripe or unripe honey, are marked with asterisks(*). Further regulatory mechanisms via bitter substances or expression and already found differences in division of labor are displayed. Such results, as well as differences in the responsiveness of different bee casts towards different sugars, are set in the context of division of labor of the honeybee (also marked as hypotheses with an asterisk(*)).

For the receptor components the presented investigations provided first indications about the comprehensive tasks of the sugar receptors in the physiology of honeybees.

A possible function in general behavior of AmGr1 and AmGr3 is an external and internal taste sensor. In combination with splice variants and the co-receptor function of AmGr2 for AmGr1, they provide important roles in in sugar taste discrimination. The combination of AmGr1 and AmGr3 signals may enable honeybees to discriminate ripe honey from nectar via the ratio of mono- and disaccharides. The shown investigations found receptor expression as one of the possible regulatory mechanisms of the sugar perception which clearly shows differences in division of labor. Sugar receptors seem to be inhibited by bitter substances and, up to now, no bitter receptor was found in the bee genome. AmGr1 was found to be differentially expressed internally in the brain but not externally in the antennae of nurse bees and foragers. A direct regulation of AmGr1 in other tissues has not been investigated but is still likely. The expression of the splice variants and the co-receptor (AmGr2) was not measured, but a different expression of those, or a regulation via splicing or modulation of the heterodimerization, can be speculated. Such assumptions, which contradict the measurements of our studies or were judged to be less improbable, were marked as hypotheses (*), with a question mark (?) and are also marked in gray in **Figure 2**.

Further, as regulatory mechanisms for AmGr3, a different expression levels internally (in the brain), as well as externally (in the antennae), was shown. These findings and the different responsiveness of the honeybee casts (nurse bees and forager) towards their ligands (sucrose and fructose) suggest that the regulatory mechanisms of sugar receptors are linked and have to be discussed in the context of division of labor. In this context, nurse bees are exposed to higher concentrated diet and are therefore less responsive to sugars (fructose and sucrose) than foragers. This is also reflected in their expression of the fructose receptor, but not the sucrose receptor. Nurse bees may react more readily to starvation or a dropping internal energy level since both sugar

receptors (AmGr1 and AmGr3) are higher expressed in their brain tissue. Foragers might have to be more responsive to sugars to ensure a more differentiate evaluation of food. A lower internal expression of both sugar receptors (AmGr1 and AmGr3) could make them less sensitive to hunger or low blood sugar and more persistent in foraging.

6.6 Conclusion and Outlook

The most important regulatory mechanisms of the sugar receptors and honeybee sugar perception this thesis found, are the receptor and co-receptor interaction and its alteration of the sugar specificity (**Manuscript III, Chapter 4**), as well as the possibility of splice-variants and alternative protein structures (**Manuscript III, Chapter 4**). Further, this thesis showed a clear different responsiveness of bee cast towards sugars and a possible linked expressional regulation (direct or indirect, based on tissue or bee cast; **Manuscript I, Chapter 2**). Additionally, it revealed the outstanding separate role of the specific fructose receptor AmGr3 (**Manuscript II, Chapter 3**), or even non-sugary ligands that can influence and inhibit the receptors activity (**Manuscript IV, Chapter 5**).

The results presented, and the hypotheses derived, may provide incentives for further examinations. Structural analysis and further characterizations of all receptor components for sugar perception (**see Figure 2**) are necessary to fully reveal their interaction, regulatory function, ligand specificity and their inhibition by bitter substances. Behavioral experiments can strengthen those findings, as well as the presented very probable hypothesis, that sugar receptors act as internal sensors. The presented experiment discovering differences in sugar receptor expression and sugar responsiveness can be extended to further tissues and organs, all shown receptor components (the co-receptor and the splice variants) or other bee casts (such as other task, or age-marked bees) to extend the understanding of receptor regulation and division of labor in a greater detail.

Additionally, it would be very exciting to confirm the overarching hypotheses of this work, that honeybees are able to discriminate sugars and diet, through future research. Complex behavioral assays in the beehive would be conceivable, in which the behavior of CRISPR/Cas9 mutants of the sugar receptors can be compared with wildtype bees. In this way, one could not only gain further insights into sugar perception and division of labor, but also test whether bees are capable to discriminate sugars, honey and nectar (see **Figure 2**, function in sugar taste discrimination* and context of division of labor*).

Investigations of upstream integration and neuronal computing of the sugar perception in the neurons and the brain of the honeybee were not part of the research question of this work. Nevertheless, such future research questions have to be addressed, since this thesis might provide an important basis for those significant further studies.

Considering all known gustatory receptors of honeybees, the here investigated sugar receptors (AmGr1, AmGr2 and AmGr3) already represent a large proportion with three out of ten (Robertson & Wanner, 2006). This is not surprising, since the perception of sweetness is an important part of their environment and physiology of the honeybee (Seeley, 1985). Due to the co-evolution and the resulting mutualistic relationship with flowering plants, honeybees are more dependent on other stimuli (such as odors or visual stimuli, for finding) than on the sense of taste (Winston, 1991; Robertson & Wanner, 2006).

In this greater context, the presented studies suggest that not only this co-evolution contributed a reduced gustatory receptor set in honeybees. Also, the uncovered mechanisms of this thesis enable the insect to a complex perception and evaluation of sweetness, despite having fewer sugar receptors than other insects (Robertson & Wanner, 2006). Future research, and the comprehensive and complete characterization of all gustatory receptors, will reveal the full understanding of sugar perception. In addition, the comprehensive picture found about multidimensional

regulatory mechanisms of the sugar receptors of this work (**Figure 2**; described in detail in the previous chapter) can then be compared to the overall ability of honeybees to perceive taste. Even if the found complexity of sugar perception presented in this work seems to be already sufficient, the future characterization of all gustatory receptors will reveal whether additional sugar receptors, additional bitter receptors or receptor components can be found and have to be added to the overall results of this thesis (**Figure 2**).

To summarize, the studies of this doctoral thesis expanded the knowledge of sugar perception in honeybees and draws a comprehensive picture about multidimensional regulation mechanisms of the sugar receptors (**Figure 2**; described in detail in the previous **Chapter 6.5**). The known basis of receptors for the perception of sugar could not only be extended by further components, but this thesis also clarifies how they function, interact, and are involved in the division of labor in honeybees and presents considerable hypothesis for its findings. The most important finding of this work is that, despite the seemingly limited number of receptors, various mechanisms enable honeybees to perceive sugar in a complex way, a differentiation of sugars might even be possible, and their sugar taste is tightly linked with division of labor.

7 Literature

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Abbreviations

Aedes aegypti	species name of the mosquito
AmGr1	*Apis mellifera* Gustatory Receptor 1
AmGr1-10	*Apis mellifera* Gustatory Receptor 1-10
AmGr2	*Apis mellifera* Gustatory Receptor 2
AmGr3	*Apis mellifera* Gustatory Receptor 3
Apis mellifera carnica	species name of the carniolan honeybee
Apis mellifera caucasia	species name of the caucasian honeybee
Apis mellifera lingustica	species name of the italien honeybee
BiFC	bimolecular fluorescence complementation, to validate protein interactions
Cas9	CRISPR associated protein 9; used for genome engineering
CD28	co-receptor inhering an important regulatory function is CD28 in the human immune system in b-cells
Cinchona sp.	species of plants known as fever trees; their bark contains quinine
co-receptor	a receptor binding the primary receptor to facilitate ligand recognition
CRISPR/Cas9	genetic engineering method to modify genome by introducing double-strand breaks
DNA	deoxyribonucleic acid
DEET	N,N-Diethyl-meta-toluamide or diethyltoluamide, an active ingredient in insect repellents
DmGr43a	*Drosophila melano*gaster gustatory receptor 43a, a homolog of the honeybees' receptor AmGr3
Drosophila melanogaster	species name of the fruit fly
fem	feminizer protein that switches "ON" the machinery that is required for sensing the worker nutrition and for implementing the size polyphenism in honeybees
gDNA	genomic deoxyribonucleic acid
GRN	gustatory receptor neurons
Grs	gustatory receptors
in-vitro	studies are performed with microorganisms, cells, or biological molecules outside their normal biological context
in-vivo	studies that are performed in living organisms or cells (Latin for "within the living")
Laminacea	plant species of flowering plants commonly known as the mint, deadnettle, or sage family
mrip1	major royal jelly protein gene
mRNA	messenger ribonucleic acid
PER	Proboscis Extension Response
qPCR	real-time quantitative polymerase chain reaction (real-time PCR, or qPCR)
Ranuculacea	plant species of flowering plants commonly known as buttercup or crowfoot family
sgRNA	singe-stranded guide ribonucleic acid
T1R2 and T1R3	heteromeric G-protein-coupled receptor complex in the taste buds in humans
Xenopus	species name of the African clawed frog
YFP-tags	yellow fluorescent protein tags

Appendix

Affidavit

I hereby confirm that my thesis entitled "Sugar perception and sugar receptor function in the honeybee (*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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Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation "Zuckerwahrnehmung und Zuckerrezeptorfunktion in der Honigbiene (*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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Ort, Datum Unterschrift

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Study Design Methods Development	M.T.	L.D.	R.S.		
Data Collection	L.D.				
Data Analysis and Interpretation	L.D.	R.S.	M.T.		
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	L.D.	R.S.	M.T.		

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Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	L.D. (M.B.) & F. L. R. F.	R.S. & D.G.	B.K.		
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Explanations (if applicable): The paper includes results of two methods of two working groups each with one author and one supervisor (L.D. & R.S. and F. L. R. F. & D.G.). The CRIPSR/Cas9 method was learned in the lab of M.B.

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Study Design Methods Development	L.D. & F. L. R. F.	R.S. & D.G.			
Data Collection	L.D. & F. L. R. F.	R.S. & D.G.	A.V.	C.H.	
Data Analysis and Interpretation	L.D. & F. L. R. F.	R.S. & D.G.	A.K.		
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Ricarda Scheiner	16.02.2023	Würzburg	
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