The production of melezitose in honeydew and its impact on honey bees (*Apis mellifera* L.)

Dissertation for Obtaining the Doctoral Degree of Natural Sciences (Dr. rer. nat.)

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And when the dew that lay was gone up, behold, upon the face of the wilderness there lay a small round thing, as small as the hoar frost on the ground, when the children of Israel saw it, they said one to another, it is manna, for they wist not what it was (Exodus 16, 14 -15).

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Authors contributionship

Parts of this dissertation have already been published with permission of the supervisor. The scientifical work of this dissertation has partly been absolved in cooperation with the University of Hohenheim and the University of Wuppertal. From the University of Wuppertal two additional publications with my co-authorship arose that are not direct part of my thesis.

I conceptualised the sampling of honeydew and environmental data together with Dr. Annette Schroeder and Prof. Dr. Martin Hasselmann. Sampling of the data and honeydew in the field were conducted by myself with the help of Tomas Danhel and Simay Yaycioglu. The sugar spectra of the honeydew samples were analysed by Basel Shaaban and validated by Prof. Dr. Gertrud Lohaus. I analysed all data with statistical advice by Dr. Karsten Schweikert.

For the publication “The trisaccharide melezitose impacts honey bees and their intestinal microbiota.” (Seeburger, 2020), I conceptualised the experimental design with Dr. Annette Schroeder and Prof. Dr. Martin Hasselmann. All feeding experiments including sample collection and processing were conducted by myself. I performed the microbial analyses that were validated by Dr. Paul D’Alvise. The sugar spectra analyses have been done by Basel Shaaban and validated by Prof. Dr. Gertrud Lohaus. I analysed all data with statistical advice by Dr. Karsten Schweikert. I carried out the writing of the original draft of the manuscript. Prof. Dr. Martin Hasselmann, Dr. Paul D’Alvise and Dr. Karsten Schweikert gave useful advises while reviewing the manuscript.

Stuttgart, 07.12.2020 

Place and Date Signature of the Supervisor

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1 Introduction

Honey is a high-value food product and a positive value-adding ingredient in the food market (Fairchild et al. 2003). Honeydew honey belongs to the main honey types produced in Europe (Persano Oddo and Piro 2004). This honey type is popular and achieves high market values due to its strong malty-aromatic taste (Castro-Vázquez et al. 2006; Vasić et al. 2019). In order to harvest honeydew honey, a complex two-step process involving different insect organisms have to be passed (Sanz et al. 2005). Under normal circumstances, honey bees feed on the nectar of different plant species and even selectively distinguish between the different nectar sources (Seeley et al. 1991). Honey bees process the nectar within their hives to blossom honey (Sanz et al. 2005). To achieve honeydew honey, honeydew flow seasons have to be present (Crane and Walker 1985). Beekeepers have to observe trees in temperate-zone forests, especially in parts of Europe, New Zealand and North America (Crane and Walker 1985) for a successful harvest of honeydew honey. This depends on aphid and scale insect species of the order *Hemiptera* that are producing the honeydew, main component of honeydew honey (Sanz et al. 2005). A honeydew flow season can only be established when specific honeydew producing species can grow up their population size. This is dependent on numerous environmental factors such as the number of natural predators, parasites and meteorological effects (Liebig et al. 1982).

1.1 Honeydew producing insect species

Insects of the order *Hemiptera* feed the phloem sap of coniferous or deciduous trees, absorb its included amino acids and excrete the sugars in so-called honeydew droplets (Douglas 2006). To maintain a honeydew flow season, the hemipteran species have to produce sufficient honeydew (Crane and Walker 1985). The most relevant species producing sufficient honeydew are species of the family Lachnidae (aphids) and of the family Coccidae (scale insects) (Liebig 1999). In comparison to scale insect species, aphid species are movable their whole lifetime, they have several (up to 15) generations per year and are overwintering in eggs. Scale insect species are only moving within the first and second larval stage, have one generation per year and are overwintering in the second larval stage (Kloft et al. 1985). Honeydew is produced in high volumes especially when the hemipteran species need the amino acids from the host plant species to produce

their offspring. Since the offspring of different hemipteran species is produced at different times of the year, the time of honeydew producing varies during spring and summer season. The species living on spruces (*Picea abies*), *Physokermes hemicryphus* (2.5-6 mm; Dalman, 1826) and *Physokermes piceae* (6.4-10.8 mm; Schrank, 1801) are producing honeydew mostly between April and June. In June, mainly *Cinara pilicornis* (2.8-4.5 mm; Hartig, 1841) is producing honeydew, but also *Cinara piceae* (4.5-6.7 mm; Panzer, 1800) until July. Living on firs (*Abies* sp.), *Cinara confinis* (5.4-7.8 mm; Koch, 1856), *Cinara pectinatae* (3.0-5.0 mm; Noerdlinger, 1880) and *Cinara curvipes* (4.0-5.3 mm; Patch, 1912) are producing honeydew between June and possibly until September (Binazzi and Scheurer 2009). The honeydew producer species are shown in Fig 1.

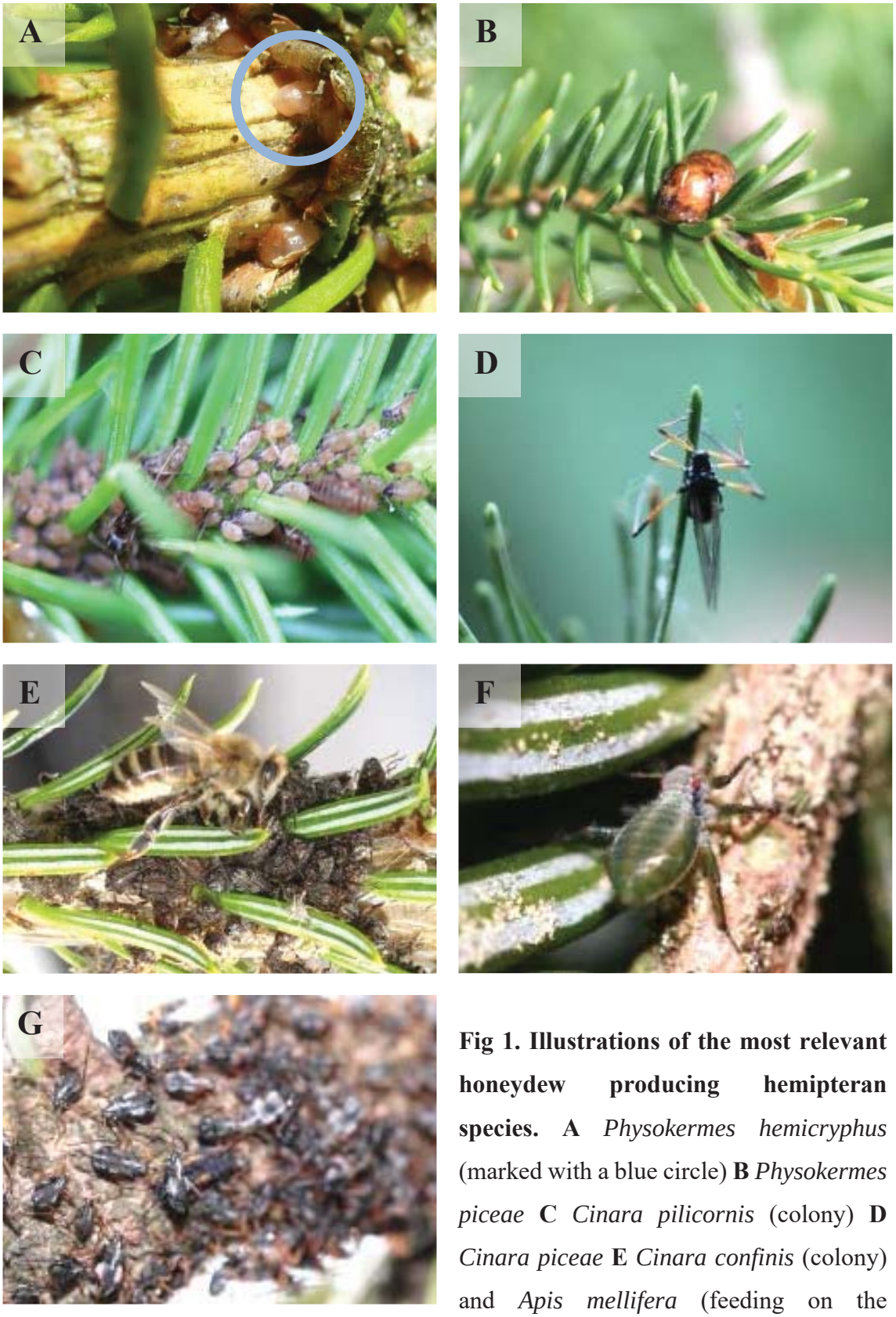

Fig 1. Illustrations of the most relevant honeydew producing hemipteran species. A *Physokermes hemicryphus* (marked with a blue circle) **B** *Physokermes piceae* **C** *Cinara pilicornis* (colony) **D** *Cinara piceae* **E** *Cinara confinis* (colony) and *Apis mellifera* (feeding on the honeydew) **F** *Cinara pectinatae* **G** *Cinara curvipes* (colony)

Observing different forest regions, beekeepers have to decide when and where they move their bee hives to collect honeydew honey (Liebig 1999). The amount of honeydew honey produced by the bees located near forest locations can be predicted by counting and estimating the individuals of the honeydew producer species. Nevertheless, the natural predators such as ladybugs, lacewings and hoverflies and parasites such as the ichneumon fly larva have to be considered (Liebig 1999). Subsequently, honey bees take up the honeydew droplets and process the substance into honeydew honey in their hives (Sanz et al. 2005). Fig 2 demonstrates the two-step process of honeydew honey.

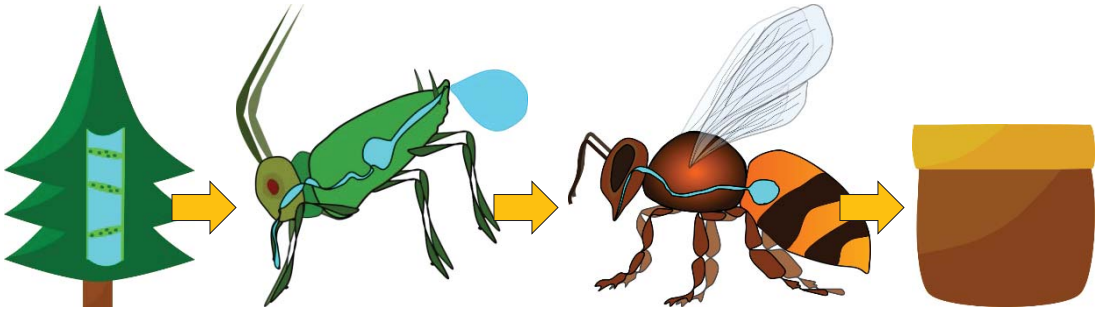

Fig 2. Phloem sap (light blue) of different plant species on the left side are absorbed by different hemipteran species. Hemipteran species are excreting the sugars of the phloem sap as so-called honeydew. Afterwards, honey bees collect the honeydew in the illustrated crop and process it to honeydew honey within their hives (drawing not to scale).

1.2 Honeydew production of hemipteran species

In comparison to nectar, which is mainly composed of the two monosaccharides glucose and fructose, and the disaccharide sucrose (Baker and Baker 1983), honeydew contains additionally the disaccharides maltose and melibiose and the trisaccharides erlose and melezitose (Shaaban et al. 2020). The hemipteran species that produce honeydew feed on the amino acids of the phloem sap of their host trees (Douglas 2006). The phloem sap consists of amino acids (Lohaus and Moellers 2000; Lohaus and Schwerdtfeger 2014), but primarily of sucrose (Fink et al. 2018). Since the honeydew consists of a variety of sugars (Shaaban et al. 2020) and it represents a waste product for the hemipteran species (Douglas 2006), the arising question is, why the insects produce these sugars.

It has to be considered that hemipteran species face the challenge of osmotic pressure while digesting the phloem sap (Douglas 2006). The ion concentration of the phloem sap is up to five times higher than within the haemolymph of the insect and leads to a high osmotic pressure within the hemipteran species (Douglas 2006). To counteract a loss of water within their haemolymph, hemipteran species reduce the ion-concentration by producing higher molecular sugars from contained sucrose (Fisher and Wright, J.P., Mittler, T.E. 1984). Whereas the fructose moiety is assumed to be assimilated efficiently, the glucose moiety is produced to the oligosaccharides erlose and melezitose with sucrose (Ashford et al. 2000). The effect of the phloem sap composition on the sugar production of hemipteran species have not been analysed in detail yet, however the osmoregulation of the hemipteran species leads to the suggestion that a higher molality of the phloem sap enhances the production of the oligosaccharides erlose and melezitose.

1.3 Melezitose-rich honeydew and honey production

Within the produced honeydew of hemipteran species, melezitose is the primarily occurring trisaccharide (Shaaban et al. 2020). Melezitose is produced within the gut of hemipteran species via α-glucosidase/transglucosidase of two molecules glucose and one molecule fructose (Price et al. 2007). The skeletal formula of the molecule is shown in Fig 3.

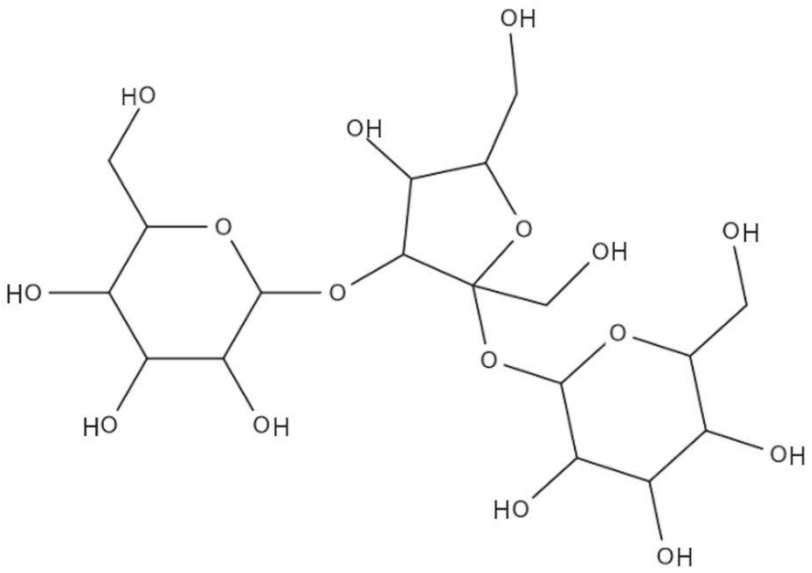

Fig 3. Skeletal formula of the molecule melezitose. Melezitose is a trisaccharide consisting of one fructose and two glucose molecules.

It is known that different hemipteran species produce different proportions of melezitose within the honeydew (Liebig 1979), especially the species living on spruces such as *Cinara piceae* and *Cinara pilicornis* producing melezitose proportions up to 70% (Shaaban et al. 2020). This can be explained by different environmental factors influencing hemipteran species, such as the attendance of ants (Woodring 2004) in *Cinara spp*. (Fischer and Shingleton 2001) or *Aphis fabae* (Vantaux et al. 2011). It is known that ant species (for example *Lasius niger*) live in a mutualistic symbiosis with honeydew producing insects (Völkl et al. 1999). Whereas ants are attracted by melezitose, they protect hemipteran species against natural predators (Banks 1962; Vantaux et al. 2015) and even locate themselves as close as possible to this food source (Samways 1983). Another effect on different proportions of sugars in the honeydew of the hemipteran species *Cinara pectinatae* (formerly named *Buchneria pectinatae*) was investigated in a previous study without ant attendance. This study focused on photoperiodic factors, revealing that *Cinara pectinatae* produces more melezitose in the daytime than at night (Liebig 1979). Honeydew producing scale insects and aphids mainly occur on the spruce species *Picea abies* and the fir species *Abies alba* in Germany or Middle Europe. Especially hemipteran species living on spruces are producing high proportions of melezitose (Shaaban et al. 2020). Spruces exist in regions with elevated air humidity, suffering from higher air temperatures and especially from heat and drought stress. In association with their shallow root system, spruces have no access to water sources in deeper soil layers (Nadezhdina et al. 2014). A higher molar concentration of phloem sap can be caused by the limited access of host trees to water (Woodruff 2014). On the contrary, firs are less vulnerable to unfavourable meteorological and soil nutrient conditions than spruces due to their deeper reaching root systems (Urban et al. 2013). It is therefore assumed that water availability plays an essential role in the molar concentration of phloem sap, especially in spruces. Furthermore, it has been shown that the osmolality of the phloem sap varies with altitude. The highest volumes of phloem sap were produced in trees were located above 500 meters and the highest brix content was found in trees between 0 and 100 meters and during the dry season (Lantemona et al. 2013).

Honey containing at least 20% of this sugar crystallises quickly and even before the honey is ripe for harvesting. The honey cannot be harvested by centrifugation and obstructs the

combs (Hudson and Sherwood 1919) leading to high economic losses for beekeepers. Furthermore, honey bees cannot take up the crystallised honey as a food source (Hudson and Sherwood 1919). This so-called cement honey presents an underestimated but substantial risk for beekeepers. Beekeepers in countries with high honeydew production are very well aware of this periodical occurring problem. Unfortunately, this phenomenon has so far hardly been recognised in research projects and in the scientific literature. Since the 1970s, there has been no scientific publication on this subject. However, articles in beekeepers' journals demonstrate that cement honey can occur wherever honey bees collect melezitose-rich honeydew and process it into honeydew honey.

1.4 Impacts of melezitose on the health of honey bees

Foraging is one of the most important tasks for each worker honey bee (Brodschneider and Crailsheim 2010). The first sign of health a beekeeper controls within a honey bee hive is the sufficiency of food. Usually, foraging honey bees are sampling nectar within their crops. In comparison to the complex sugar composition of honeydew excreted by hemipteran species, nectar is the phloem sap of flowering plant species, filtrated by their nectaries and contains mostly sucrose (Baker and Baker 1983). In other word, the differences of the sugar composition between nectar and honeydew are immense. Negative effects on the lifespan of parasitoid wasps fed on honeydew instead of nectar are previously described (Lee et al. 2004). The honey made of honeydew also has a mineral content that is up to five times higher than of blossom honey (Castro-Vázquez et al. 2006; Lachman et al. 2007) and contains more oligosaccharides than blossom honey (Sanz et al. 2005). In the absence of nectar in certain times of the year or when beekeepers locate their hives in forest regions, worker honey bees have to forage on honeydew. In periods with hemipteran species producing high proportions of melezitose, honey bees have to handle with the trisaccharide. The link between the fructose and glucose molecule of melezitose can be broken down by invertase (Detrain et al. 2010). The concentration of invertase in honey bees occurs constantly (Simpson et al. 1968) and it can be assumed, that bees are capable of breaking down melezitose. However, the so-called honeydew flow disease is often reported and the precise reason is still unknown (Bailey 1965; Hudson and Sherwood 1919). Several clinical symptoms, especially on winter bees feeding on honeydew honey have been recorded: changes of their behavioural patterns, necrotic appearances and collapsing colonies within short time (Horn 1985). Despite the

arising concerns about the symptoms, the exact reasons for the occurring disease remain unknown. Several studies have been conducted but could not show an effect of the different mineral contents between honeydew and nectar (Horn 1985), assuming that the reason for the disease must be anatomical effects on the bee gut, microbial changes or restricted assimilation of the nutrient leading to the higher mortality rate of the bees (Pohorecka, K. and Skubida, P. 2004). The oligosaccharides in honeydew have a lower nutritional value than nectar sugars (Lee et al. 2004). The sugar melezitose constitutes the main difference between honeydew and nectar (Shaaban et al. 2020) and it can be assumed that this difference causes the honeydew flow disease in honey bees.

1.5 Impacts of melezitose on the gut microbiota of honey bees

In general, the gut microbiome has become a high awareness over the last years as important part, not to say "organ", that contributes to organismic health. Most prominent research has been performed in humans (Claesson et al. 2017; Grice and Segre 2012; Hughes 2019) but also in many insect species (Almeida et al. 2017; Engel and Moran 2013b; Pernice et al. 2014). In honey bees, the research on gut microbiota also increased and gave deeper insight in dietary effects and health (D'Alvise et al. 2018; Engel et al. 2015; Kešnerová et al. 2020; Kwong and Moran 2016). The honey bee gut microbiota consists of only few species (Crotti et al. 2013; Engel et al. 2012) and sugar uptake systems are enriched in the species (Engel and Moran 2013a). Especially genes for sugar transport and carbohydrate breakdown are enriched in the gut microbial species *Gilliamella apicola*, *Bifidobacterium* spp. and the species clusters *Lactobacillus* Firm-4 and Firm-5 (Engel and Moran 2013a; Moran 2015) and are essential for honey bees in using the dietary sugars as energy sources (Engel et al. 2012). With digesting the carbohydrates and producing fermentation products, the gut microbial species enable energy metabolism in honey bees (Lee and Hase 2014). The gut microbiota could be essential for degrading oligosaccharides which otherwise may have toxic effects (Engel et al. 2012). Especially the oligosaccharides in honeydew, erlose and melezitose, may lead to a change of the gut microbial community which can give an insight in the impact of a diet on honeydew for honey bees.

1.6 Aims of the thesis

Honey with high amounts of the trisaccharide melezitose is harmful for bees and beekeepers; it crystallises quickly and obstructs the combs. Beekeepers cannot harvest the honey by centrifugation which means an economical loss and honey bees cannot take up the honey as food source (Hudson and Sherwood 1919). However, it is known that different melezitose proportions are produced from the same hemipteran species (Liebig 1979; Shaaban et al. 2020). Consequently, one main goal of my dissertation was to understand under which certain environmental circumstances melezitose production is enhanced in hemipteran species. Since it is known that hemipteran species produce oligosaccharides for osmoregulation (Douglas 2006), the hypothesis of the first aim of the thesis is that a higher molality in the phloem sap due to less access to water by the host trees indirectly leads to higher melezitose production of the hemipteran species. For a precise understanding of factors enhancing melezitose production, the sugar compositions of honeydew samples from the field were analysed, considering their zoological (hemipteran species), botanical (host tree species) and geographical (geo-coordinates) source as well as the meteorological data while the hemipteran species produced the honeydew. Furthermore, possible effects of the altitude of the sampling site, the sampling year, month and time were analysed.

The honeydew flow season is often associated with the honeydew flow disease (Horn 1985). Since it is still unknown, which factors exactly lead to this disease, the second part of this work discusses the diet of honey bees on melezitose. The hypothesis of the second part of my thesis is, that a melezitose-rich diet is one triggering factor for the honeydew flow disease in honey bees. To understand the impact of the trisaccharide on honey bees precisely, feeding experiments have been performed in three consecutive years. Honey bees of the experiments were fed with a melezitose rich sugar solution and the control groups were fed with sugars similar to a normal nectar sugar composition of sucrose, fructose and glucose. The gut microbial community can give a hint on honey bee health (Kwong and Moran 2016) and thus can help understand the role of the bacteria for the metabolism of melezitose. Regarding the honeydew flow disease as a possible consequence of the different sugar compositions in honeydew and nectar, the hypothesis is that the dietary effects of different sugar compositions on bees can be understand with analysing their gut bacterial community. A comparison of the microbiota between bees

fed with melezitose and fed with control sugar solution has been performed to analyse the melezitose metabolism efficacy of honey bees. The publication of this study is shown in the appendix.

2 Material and methods

2.1 Honeydew sample collection and analyses

2.1.1 Honeydew sampling and data collection

Honeydew samples of seven honeydew producing hemipteran species feeding on two different host tree species were collected between 2016 and 2019 during a period from April to August. All forest regions in Baden-Wuerttemberg (Germany) were monitored with digital scales (Wolfwaagen, Waidhaus, Germany) below the bee hives. The scales were connected to an online software, which transferred total weight data per hive three times a day. A high weight increase was indicative for the collection of honeydew by the honey bees due to no other rich food source in forest regions. This has been taken as signal that honeydew is available and the sampling was initiated. In total, 620 honeydew samples were taken directly from the hemipteran species using micropipettes. The samples were taken while the hemipteran species excreted the honeydew. To obtain enough sample material, one sample from different specimen of the same hemipteran colony was collected at the same end of the branch of the host tree. The individuals were attributed to one colony when they were positioned at the same end of the branch of the host tree and within an area of 5 cm. Each sample contained at least 1 µl and was stored frozen in small plastic tubes until analysis.

The hemipteran species observed belong to the families Coccidae (scale insect species *Physokermes piceae* and *Physokermes hemicryphus*) and Lachnidae (aphid species *Cinara pilicornis* and *Cinara piceae*) and were found during sampling by feeding on their specific host tree species *Picea abies*, as well as hemipteran species belonging to the family Lachnidae (aphid species *Cinara confinis*, *Cinara curvipes* and *Cinara pectinatae*) which were found feeding on their specific host tree species *Abies alba*. All samples were collected within Baden-Wuerttemberg, South-Germany between latitude 47 and 49 and between longitude 008 and 009. Beekeepers participated in this study voluntarily on their personal ground. Due to privacy protection of the beekeepers the exact coordinates of the sampling sites are not shown. The sampling sites within Baden-Wuerttemberg are located in five different natural areas. The natural areas are classified based on their geomorphological and hydro-geographic specification: “Neckar- und Tauber-

Gaeuplatten" (NTG) consisting of sandstone, "Schwaebische Alb" (SA) with soil based on limestone and karst soil, "Schwaebisches Keuper-Lias Land" (SKLL) built from limestone and red sandstone, "Schwarzwald" (S) consisting of clayey red sandstone and "Voralpines Huegel- und Moorland" (VHM) with soil based on swamp, sand and grave soil (Meynen et al. 1953-1962). The different natural areas in Southern Germany are prime candidates to study the effect of different geomorphological and hydro-geographic parameters on the composition of honeydew. Precipitation before honeydew season in the sampling region Baden-Wuerttemberg is usually highest in January, but the rainfall differed substantially between the sampling years: the annual precipitation for 2016 was 123.1 l/m², for 2017 43.5 l/m², for 2018 157.8 l/m² and for 2019 95.6 l/m² (Deutscher Wetterdienst 2019). An overview of the honeydew sampling sites and sample information are described in Fig 4 and Table 1.

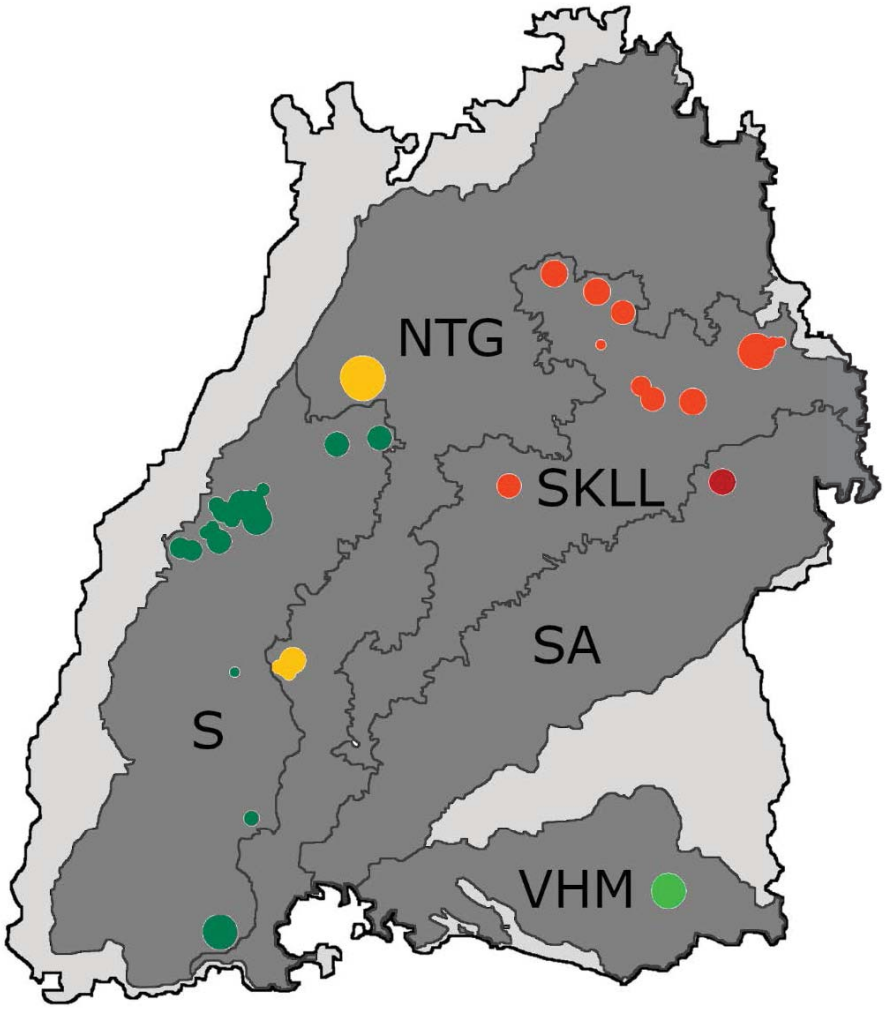

Fig 4. Map of the sampling sites within the State of Baden-Wuerttemberg in Southern Germany. A total of 620 samples were taken, the size of the dots illustrates the number of samplings taken, the position of the dots shows their geo-coordinates. The dots are highlighted with different colours according to the natural areas (dark grey) (LUBW 2010) where the samples are taken: NTG (Neckar- und Tauber-Gaeuplatten, yellow), SA (Schwaebische Alb, red), SKLL (Schwaebisches Keuper-Lias-Land, orange), S (Schwarzwald, dark green), VHM (Voralpines Huegel- u. Moorland, light green). The colours (from dark green via light green, via yellow, via orange to red) symbolise areas with expected good to limited water reservoir capacities (Meynen et al. 1953-1962). Reprinted from Karte Naturraeume 3. und 4. Ordnung in Baden-Wuerttemberg nach Meyen and Schmithuesen et al. under a CC BY license, with permission from LUBW (2010): Naturraeume Baden-Wuerttembergs. https://pd.lubw.de/96935, original copyright 2020.

Table 1. Sample numbers of the collected honeydew droplets from different hemipteran species living on different host tree species and the different natural areas "Neckar- und Tauber-Gaeuplatten" (NTG), "Schwaebische Alb" (SA), "Schwaebisches Keuper-Lias Land" (SKLL), "Schwarzwald" (S) and "Voralpines Huegel- und Moorland" (VHM) and years between 2016 and 2019 in which the hemipteran species were found.

Hemipteran species	Host tree species	Natural areas	Sampling years 2016-2019	Samples
Physokermes piceae	*Picea abies*	NTG, SA, SKLL, VHM	17	119
Physokermes hemicryphus	*Picea abies*	NTG, SKLL, S	16, 17	30
Cinara pilicornis	*Picea abies*	NTG, SA, SKLL, S	16, 17, 19	140
Cinara piceae	*Picea abies*	S, VHM	17	35
Cinara confinis	*Abies alba*	NTG, SKLL, S	16, 17, 18, 19	48
Cinara curvipes	*Abies alba*	NTG, SKLL, S	17	22
Cinara pectinatae	*Abies alba*	NTG, SKLL, S	16, 18, 19	226

In accordance with the guidelines of the authors' institutions' and the applicable regulations, no ethics approval was required or obtained for the present study. This study was carried out in Baden-Wuerttemberg (Germany). Scale insect and aphid species of the

order *Hemiptera* are no subjects of the German Animal Protection law and neither endangered nor protected species were involved in this study. For all locations of honeydew sampling specific permission was not required.

The date, time, coordinates, altitude and meteorological data were recorded simultaneously at each sampling site. Hemipteran and host tree species were morphologically identified. Coordinates and barometric altitude were recorded with the GPS-tracker Montana 610 (Garmin Deutschland GmbH; Garching bei Muenchen, Germany). Air temperature and relative humidity were recorded with a psychrometer PCE-320 (PCE Deutschland GmbH; Meschede, Germany).

2.1.2 Sugar analyses of the honeydew

The 1 µl of honeydew was added to 1000 µl ultrapure water and stored at -80°C until analysis. The sugars in honeydew were analysed according to Lohaus and Schwerdtfeger (2014). Melezitose and erlose proportions in honeydew were analysed via high performance anion exchange chromatography coupled with pulsed amperometric detection. Standards of melezitose and erlose were measured in parallel. The identification of melezitose and erlose was based on the comparison of their chromatographic peak areas with the standard calibration curves using an integration program (Chromeleon 7.2, Dionex Corp, Sunnyvale, CA, USA). Furthermore, the results obtained were regularly checked using the standard addition method (Shaaban et al. 2020).

2.1.3 Statistical analyses of the honeydew sampling

Statistical analyses were conducted using R software (version 3.6.2). Honeydew samples were categorised in the different natural areas of Baden-Wuertemberg using the geo-coordinate data and grouped in different time categories (morning 07:01-13:00, afternoon 13:01-16:00, evening 16:01-22:00) using the time data. Following the hypothesis that various environmental factors may contribute differently to the production of melezitose, a two-part model was estimated. The dataset contains a substantial number of samples (25.81%) with zero melezitose content (see Fig 5 and Table 2) and a single index model for the proportion of melezitose in honeydew would not be desirable from a statistical perspective. Consequently, the choice of models was guided by the idea that the proportion of melezitose is a mixed discrete-continuous random variable. First, a logit

model was considered to explain the binary outcome of occurrence/ non-occurrence of melezitose in honeydew conditional on the explanatory variables: air temperature, relative humidity, altitude, hemipteran species, natural area, sampling year, month and time. In the second part of the model, it has been tested whether those environmental factors have an effect on the relative proportion of melezitose in honeydew (the variable is bounded between 0 and 1). A fractional logit analysis has been run (Ramalho et al. 2011) using all samples with non-zero melezitose proportion and the described explanatory variables. Additionally, linear regression analyses or ANOVA with post-hoc Tukey tests have been separately conducted for each explanatory variable. The logit and fractional logit two-part models have also been estimated for the occurrence/ non-occurrence and proportion of erlose within honeydew. Significance level was set at $\alpha = 0.05$. The plots were produced with ggplot2.

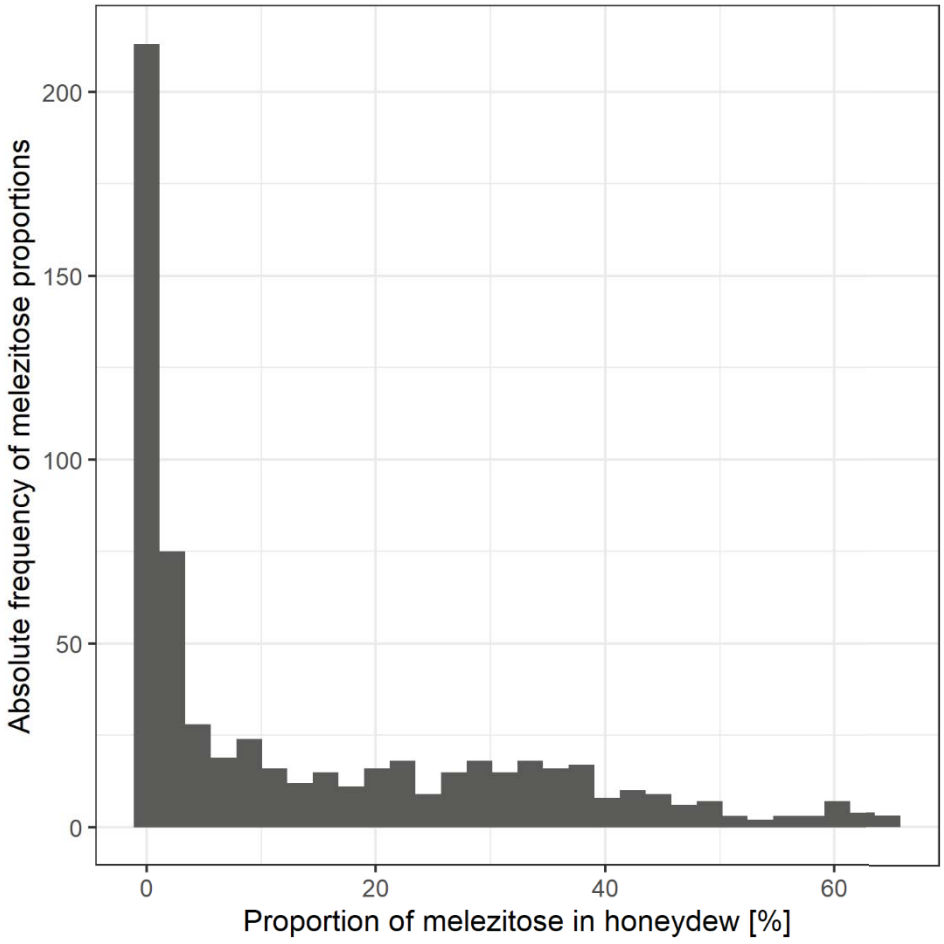

Fig 5. Histogram of the frequency of the different melezitose proportions in all collected honeydew samples in percentage.

Table 2. Crosstable with sample numbers of the collected honeydew droplets from the seven different hemipteran species (first column) and the occurrence of melezitose in the honeydew.

	No melezitose occurs	Melezitose occurs
Cinara confinis	13	35
Cinara curvipes	10	12
Cinara pectinatae	72	154
Cinara piceae	2	33
Cinara pilicornis	8	132
Physokermes hemicryphus	12	18
Physokermes piceae	43	76

2.2 Feeding experiment and microbiota analyses

2.2.1 Performance of the feeding experiments

Four feeding experiments were performed during summer of the years 2017, 2018 and 2019. European honey bees (*Apis mellifera*) were collected from the hives of the Apicultural State Institute (University of Hohenheim, Germany). For every experiment, six brood combs without adult bees were removed from three different donor colonies, caged and incubated at 33°C for 24 h. Newly emerged bees (day 1) were collected and pooled. Out of the pooled bees, 50 bees were randomly collected and placed in each one of twelve experimental cages. Bees were held in cages, as previously described (Ziegelmann et al. 2018). The cages were placed in a darkened climate chamber in randomised block design at the typical brood nest temperature of 35°C. Melezitose does

not crystallise at this temperature. The bees were fed *ad libitum* with control feed (39% (w/v) fructose, 31% glucose, 30% sucrose) or melezitose feed (50% melezitose, 19.5% fructose, 15.5% glucose), which mimicked the sugar spectrum of honeydew honey with high melezitose content.

Six cages were supplied with control feed and six cages with melezitose feed. Both sugar solutions were treated in the same way, dissolved in an ultrasonic bath that heats up in 30 min from 23°C to 70°C. 2 ml of the respective sugar solution was freshly prepared daily to ensure same viscosity and no effect on the degree of crystallisation. Solutions were administered simultaneously with vials in each cage until all bees had died.

In the first cage experiment, all bees were frozen at day 21. This was necessary for crop content analysis in order to prove the ability of all bees to collect and process the food solutions with different sugar compositions. The crop content, if present, was collected for sugar analysis (34 crops of control-fed bees and 69 crops of melezitose-fed bees). The sugars were analysed according to Lohaus and Schwerdtfeger (2014).

In the second cage experiment, bees of all six melezitose-fed cages collected the sugar solution in small honey combs that they built from the provided wax foundation rectangle. The sugar proportions of the collected, processed feed were analysed (Lohaus and Schwerdtfeger 2014) for each cage (six processed sugar solutions).

In the third cage experiment, a preliminary gut microbiome analysis has been performed. At day 10, one live bee was collected from one control-fed cage, and two live bees from two different melezitose-fed cages. At day 15 and 20 this procedure was repeated. The collected bees (N=9) were frozen immediately at -80°C. Further description of the preliminary gut microbiota analysis is provided below. In the third cage experiment, distilled water was supplied *ad libitum* in centrifuge tubes; in addition to the sugar feed solution. Again, the bees in two of the melezitose-fed cages collected the sugar solution in honey combs, and the sugar proportions were analysed (Lohaus and Schwerdtfeger 2014) for each cage (two processed sugar solutions).

In the fourth cage experiment, gut microbiota analysis was implemented. The results of the preliminary microbiota analysis in cage experiment three indicated that for acquisition of a complete gut microbiota, the caged bees needed contact to nurse bees (Martinson et

al. 2012; Powell et al. 2014). To ensure this, the experimental design was adjusted. 1'832 newly emerged bees from the donor colonies described above (day 1) were marked with liquid water-proof marker in a colour representing their colony (1, 2 and 3). Later, on the same day, the marked bees were placed back into their donor colonies. Four days later (day 5), 20 marked bees from each donor colony were placed in each of the nine experimental cages. Six cages were fed with the control solution and three cages with the melezitose solution. Moreover, from day 10 on, three of the six control-fed cages were changed to melezitose diet ("changed diet").

For the gut microbiota analysis, bees were collected at different time points. On day 5, six bees from each donor colony (3 donor colonies x 6 bees = 18 bees) were collected. On day 10, six bees were collected from each of the three control and three melezitose-fed cages. Constantly two of the collected bees per cage originated from donor colony 1, two from donor colony 2 and two from donor colony 3, as identified by their colour marks (2 treatment groups x 3 cages x 3 donor colonies x 2 bees = 36 bees). On day 15, six bees were collected from each cage (3 treatment groups x 3 cages x 3 donor colonies x 2 bees =54 bees). The collected bees (N=108) were frozen immediately at -80°C. Further procedures in the gut microbiota analysis are described below.

Additionally, at five sampling sites in the black forest (Southern Germany), 100 bees from three bee colonies per sampling site were collected during honeydew season in 2017 and 2018. The crop contents from each hive were pooled for sugar analysis (5 sampling sites x 3 colonies x 2 years = 30 analyses).

2.2.2 Analyses of the aspects of honey bee health and gut microbiota

In order to measure the aspects of bee health (see Table 3) in feeding experiments 2-4, food uptake per cage was recorded daily by weighing of the food [mg]. The food uptake was then calculated difference to the food weight given the day before. Mortality was recorded by counting the dead bees exactly every 24 hours. The whole body weights without crop and the weights of the dissected guts of the first ten dead bees in each cage were recorded.

Table 3. Overview of the analyses as well as the number of bees and cages per treatment of the feeding experiments performed between 2017 and 2019.

Feeding experiment	Year	Sugar analysis	Microbiota analysis	Aspects of bee health	Treatment groups	Cages per treatment	Bees per cage
1	2017	Cf, Cc, Fw	/	/	Co, Me	6	50
2	2017	Cc, Fw	/	FU, GW, ST	Co, Me	6	50
3	2018	/	9	FU, GW, ST	Co, Me	6	50+3
4	2019	/	108	FU, GW, ST	Co, Me, M10	3	60+12

The sugar content was analysed in crops of bees in the field (Cf), crops of bees from the feeding experiments (Cc) and from feed the bees transported into the cells of the beeswax foundation rectangle in the cages (Fw). Aspects of bee health monitored by daily food uptake per cage (FU), gut-body weight ratio of dead bees (GW) and survival of all bees per cage (ST). The treatment groups were fed a control solution of sucrose, glucose and fructose (Co) or with a 1:1 solution of control and melezitose (Me), in 2019 control-fed bees were fed with melezitose from day 10 (M10). The extra bees for microbiota analysis were marked and put in the cages additionally (noted with +).

To profile the gut microbiota, DNA of nine bees from the feeding experiment in 2018, as well as from 108 bees from the feeding experiment in 2019 was extracted using a TRIzol protocol. Whole single bees were extracted using the described standard protocol because DNA and RNA can be extracted simultaneously and be used for further experiments. The bees were placed in a 2 ml lysis tube with five 0.8 mm steel beads, roughly 50 µl 0.1 mm glass/zirconia beads and 0.5 ml TRIzol (Invitrogen). The bees were homogenised on a FastPrep24 (MP Bio) at 5.5 m/s for 50 s. After 5 min of incubation at RT, 100 µl

chloroform was added and the contents were mixed by vigorous shaking, followed by 5 min of incubation at RT. The two phases were separated by 15 min centrifugation at 12.000 *g* and 4°C. The aqueous phase was transferred to another tube for RNA extraction. 250 µl back extraction buffer (4 M guanidine thio-cyanate, 50 mM sodium citrate, 1 M TRIS base) was added to the rest of the homogenate and mixed by vigorous shaking. After 10 min of incubation at RT and centrifugation for 15 min at 12.000 *g* and 4°C, the aqueous phase was transferred to a new tube with 200 µl isopropanol and mixed by repeated inverting. After 5 min of incubation at RT and 15 min of centrifugation (12.000 *g*, 4°C), the supernatant was removed, the pellet was washed with 80% ethanol, dried for 10 min at RT and centrifuged again (12.000 *g*, 4°C) for 5 min. The supernatant was removed, the pellet dried for 5 min at RT and redissolved in 50 µl 8mM NAOH. After another centrifugation for 10 min (12.000 *g*, RT) to remove the membrane lipids, the supernatant was transferred into 4.25 µl 0.1 M HEPES and 0.5 µl RNAse A (Amresco 10 mg/ml), mixed carefully and incubated for 1 h at 37°C. DNA concentrations were determined using Qubit fluorometer (Thermo Fisher Scientific). The resulting DNA concentrations ranged between 10.1–94.6 ng/µl. Amplicons from the V3-V4 region of the 16S-rRNA-gene were generated and Illumina-sequenced using 20 ng template DNA (Eurofins Genomics, Ebersberg, Germany). The PCR conditions, library preparation, sequencing and initial data preparation were described previously (D'Alvise et al. 2019). After demultiplexing by demultiplexor_v3.pl (Perl 5.30) and initial quality filtering, OTU binning (97% identity) was done by USEARCH 8.0 (Edgar 2010), as well as quality filtering and Chimera filtering by UCHIME (Edgar 2013) (with RDP set 15 as a reference database). The sequencing data were analysed on the Integrated Microbial NGS platform (Lagkouvardos et al. 2016), using a 0.1% total abundance threshold. This is a UPARSE based analysis pipeline reporting OTU sequences with ≤1% incorrect bases in artificial microbial community tests (Edgar 2013). Primary taxonomic classification was done by RDP classifier version 2.11 training set 15 (Wang et al. 2007) and sequence alignment was done by MUSCLE (Edgar 2004). The taxonomic classification was controlled and refined by BLAST-searching the representative OTU sequences in the NCBI database (https://blast.ncbi.nlm.nih.gov). Normalisation, taxonomic binning, and statistical analyses were carried out using the RHEA scripts (Lagkouvardos et al. 2017) on R studio version 1.1.456.

In accordance with the guidelines of the authors' institutions' and the applicable regulations, no ethics approval was required or obtained for the present study. This study was carried out in Baden-Wuerttemberg, Germany. Honey bees are no subjects of the German Animal Protection law. Additionally, neither endangered nor protected species were involved in this study.

2.2.3 Statistical analyses of the feeding experiments

The daily food uptake per bee was calculated in consideration of the number of bees alive on the respective day. The gut-body weight ratio was calculated from the weight of the recorded bee bodies (without crop) and their removed guts. In order to visualise the results for both measures, box plots were created for each group in the respective year. A linear regression was used to estimate the group differences in daily food uptake controlling for the number of bees alive, daily and annual effects. Since the gut-body weight ratio range between 0 and 1, a fractional logit regression model was employed to estimate group differences controlling for year effects and the age of bees. Survival of bees was analysed in a Cox proportional hazards model. Differences between bacterial species in the treatment groups were analysed with the Rhea R pipeline (Lagkouvardos et al. 2017). All statistical tests were conducted and graphs were drawn using R version 3.5.2.

3 Results

3.1 Analysis of environmental effects on melezitose production

To gain insights into the impact of potential environmental effects on melezitose production, all possible influence variables on the occurrence (multiple McFadden R^2 of 0.183 (Cox&Snell 0.188; Nagelkerke 0.277): $F(18,618)= 7.186$; $p < 0.001$, N=620) and proportion (multiple R^2 of 0.540, N=460) of melezitose were analysed simultaneously (Table 4). Overall influence factors were the air temperature and the relative humidity. Whereas an air temperature increase of one degree Celsius increased the proportion of melezitose by an average of 0.6 percentage points ($p = 0.004$), an increase of relative humidity by one percentage point reduced the melezitose proportion by 0.2 percentage points ($p = 0.008$). Furthermore, the relative humidity also had a highly significant negative effect on the occurrence of melezitose in honeydew ($p < 0.001$). The occurrence of melezitose was observed significantly more frequently at sampling sites with slightly higher altitudes ($p = 0.008$). Negative influence factors of the overall analysis on the occurrence of melezitose were the scale insect species *Physokermes piceae* ($p = 0.002$), *Physokermes hemicryphus* ($p = 0.012$) and the aphid species living on *Picea abies*: *Cinara curvipes* ($p = 0.004$) and *Cinara pectinatae* ($p = 0.008$). Site-specific factors also had an influence on melezitose production. The trisaccharide was more likely to occur in samples collected from the natural areas “Schwaebische Alb” ($p = 0.002$) and “Voralpines Huegel und Moorland” ($p = 0.036$). The highest effect on an increasing melezitose proportion in honeydew were observed within samples taken from the natural area “Schwaebisches Keuper-Lias-Land“ ($p = 0.005$), followed by the “Schwaebische Alb” ($p = 0.017$). Furthermore, the year, month and time of the sampling had an effect on melezitose production. The highest average melezitose proportion was observed in the sampling year 2019 ($p < 0.001$), followed by 2017 ($p = 0.002$). The average melezitose proportion in the samples increased significantly from the baseline category April to May ($p = 0.006$) to June ($p = 0.006$), to July ($p = 0.001$) and to August ($p < 0.001$). The lowest likelihood for the occurrence of melezitose was observed for honeydew sampled of the afternoon ($p = 0.001$).

Table 4. Results of the logit and fractional logit analysis: Estimate and p-value of the constant, hemipteran species, air temperature, relative humidity, altitude, natural area, sampling year, month and time data (*p < 0.05, ** p < 0.01, *** p < 0.001). The multiple McFadden R^2 of the logit analysis is 0.183 (Cox&Snell 0.188; Nagelkerke 0.277): $\chi 2(18,619)= 129.34$; p < 0.001, N=620; the multiple R^2 of the fractional logit analysis is 0.540, N =460.

Coefficients	**Occurrence of melezitose in honeydew**	**Proportion of melezitose in honeydew**
[a] Constant	3.205** (p = 0.008)	-
Air temperature	0.045 (p = 0.104)	0.006** (p = 0.004)
Relative humidity	-0.047*** (p < 0.001)	-0.002** (p = 0.008)
Altitude	0.002** (p = 0.008)	0.000 (p = 0.678)
Hemipteran species living on *Picea abies*		
Physokermes piceae	-2.079** (p = 0.002)	-0.121 (p = 0.083)
Physokermes hemicryphus	-1.673* (p = 0.012)	-0.101 (p = 0.258)
Cinara pilicornis	-0.669 (p = 0.361)	-0.082 (p = 0.186)
Cinara piceae	-0.578 (p = 0.565)	0.045 (p = 0.488)
Hemipteran species living on *Abies alba*		
Cinara curvipes	-2.072** (p = 0.004)	0.076 (p = 0.113)
Cinara pectinatae	-1.370** (p = 0.008)	-0.003 (p = 0.954)
Natural area		
Schwaebische Alb	2.562** (p = 0.002)	0.133* (p = 0.017)

Schwaebisches Keuper-Lias-Land	0.753 (p = 0.056)	0.148** (p = 0.005)
Schwarzwald	0.728 (p = 0.064)	0.117 (p = 0.053)
Voralpines Huegel- u. Moorland	1.290* (p = 0.036)	0.046 (p = 0.436)
Sampling year		
2017	0.279 (p = 0.576)	0.162** (p = 0.002)
2018	-0.656 (p = 0.070)	-0.069 (p = 0.054)
2019	0.390 (p = 0.618)	0.254*** (p < 0.001)
Sampling month		
May	-	0.224** (p = 0.006)
June	-	0.234** (p = 0.006)
July	-	0.256** (p = 0.001)
August	-	0.306*** (p < 0.001)
Sampling time		
Afternoon	-0.928** (p = 0.001)	-0.031 (p = 0.097)
Evening	-0.560 (p = 0.102)	0.027 (p = 0.114)

The base categories for the logit model are the hemipteran species *Cinara confinis*, the natural area Neckar-Tauber-Gaeuplatten, the sampling year 2016, month April and time Morning (expected mean value of melezitose proportion when all explanatory variables are 0).

[a] The constant absorbs the baseline categories that are needed to interpret the coefficients as partial effects.

3.1.1 Regression analyses of the air temperature, relative humidity and altitude effects on the production of melezitose in honeydew

In Fig 6, the measured air temperature of all samples can be seen (between 11 and 31°C). Overall, the aphid species living on *Picea abies* produced the highest melezitose proportions. All aphid species produced more melezitose at higher temperatures (*Cinara*/*P. abies*; p = 0.008 and *Cinara*/*A. Alba*; p = 0.002). The air temperature did not have a significant effect on the melezitose production by scale insect species (p = 0.113).

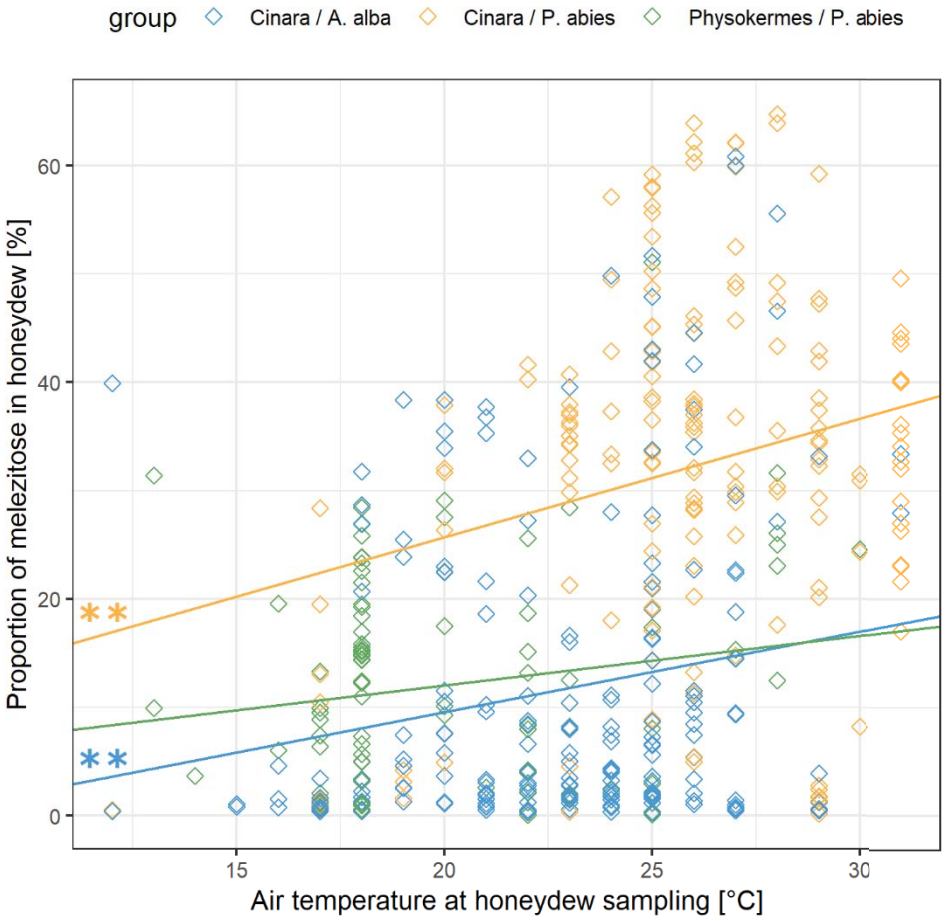

Fig 6. Scatterplot of melezitose proportion of 620 honeydew samples produced at different air temperatures from aphid species (*Cinara* sp.) living on *Abies alba* (blue; slope= 0.742; p = 0.008) and living on *Picea abies* (yellow; slope= 1.094; p = 0.002) and from scale insect species (*Physokermes* sp.) living on *Picea abies* (green; slope= 0.459; p = 0.113). Significance levels are highlighted by the asterisks on the respective regression line (*p < 0.05, ** p < 0.01, *** p < 0.001).

In Fig 7, all measured relative humidity levels can be seen (between 32-90%). The aphid species living on *Abies alba* ($p = 0.025$) and the scale insect species ($p < 0.001$) produced less melezitose at lower relative humidity levels. The aphid species living on *Picea abies* did not produce less melezitose with a relative humidity increase ($p = 0.060$).

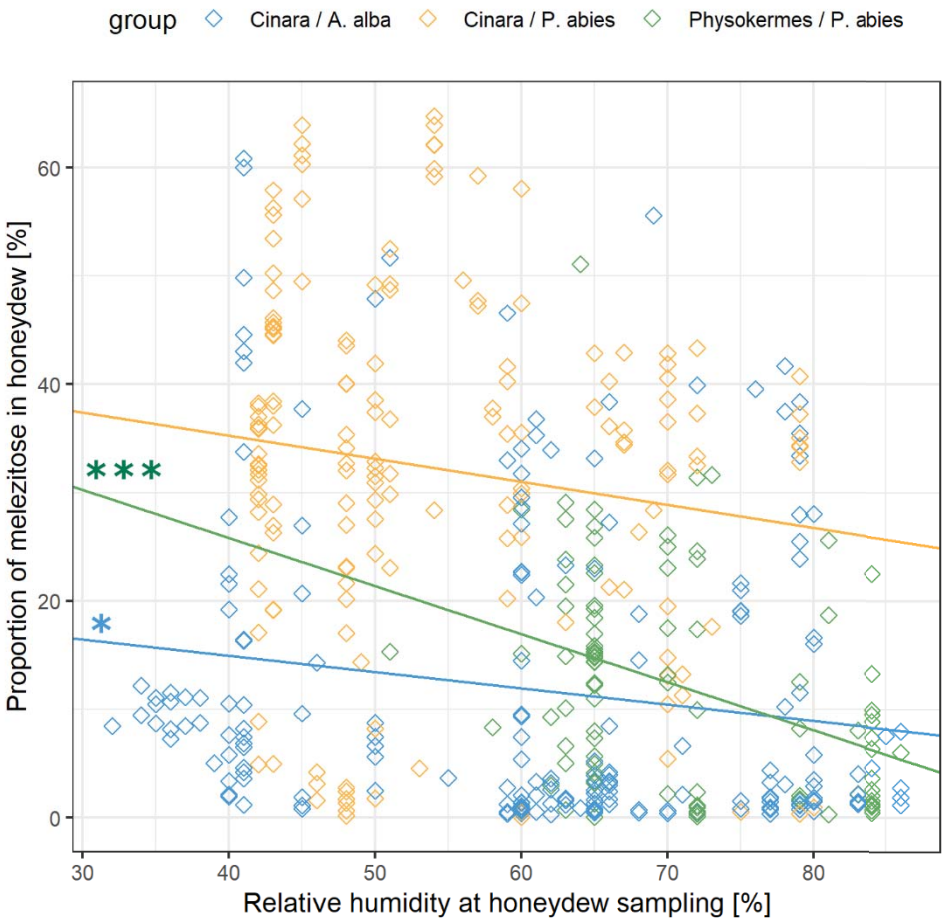

Fig 7. Scatterplot of melezitose proportion of 620 honeydew samples produced at different relative humidity levels from aphid species (*Cinara* sp.) living on *Abies alba* (blue; slope= -0.150; p = 0.025) and living on *Picea abies* (yellow; slope= -0.213; p = 0.060) and from scale insect species (*Physokermes* sp.) living on *Picea abies* (green; slope= -0.444; p < 0.001). Significance levels are highlighted by the asterisks on the respective regression line (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

All honeydew samples were collected between 252 and 982 meters. The minimum and maximum altitude of the natural areas were: NTG 352 -702 meters; SA 385-673 meters; SKLL 252-536 meters; S 282-982 meters; VHM 283-780 meters. In Fig 8, the melezitose proportion of all honeydew sampled at different altitudes is given. The scale insect species produced more melezitose with increasing altitude ($p = 0.030$). In contrast, the aphid species living on *Picea abies* produced more melezitose with increasing altitude ($p = 0.021$). The altitude did not significantly affect the melezitose production of the aphid species living on *Abies alba* ($p = 0.670$).

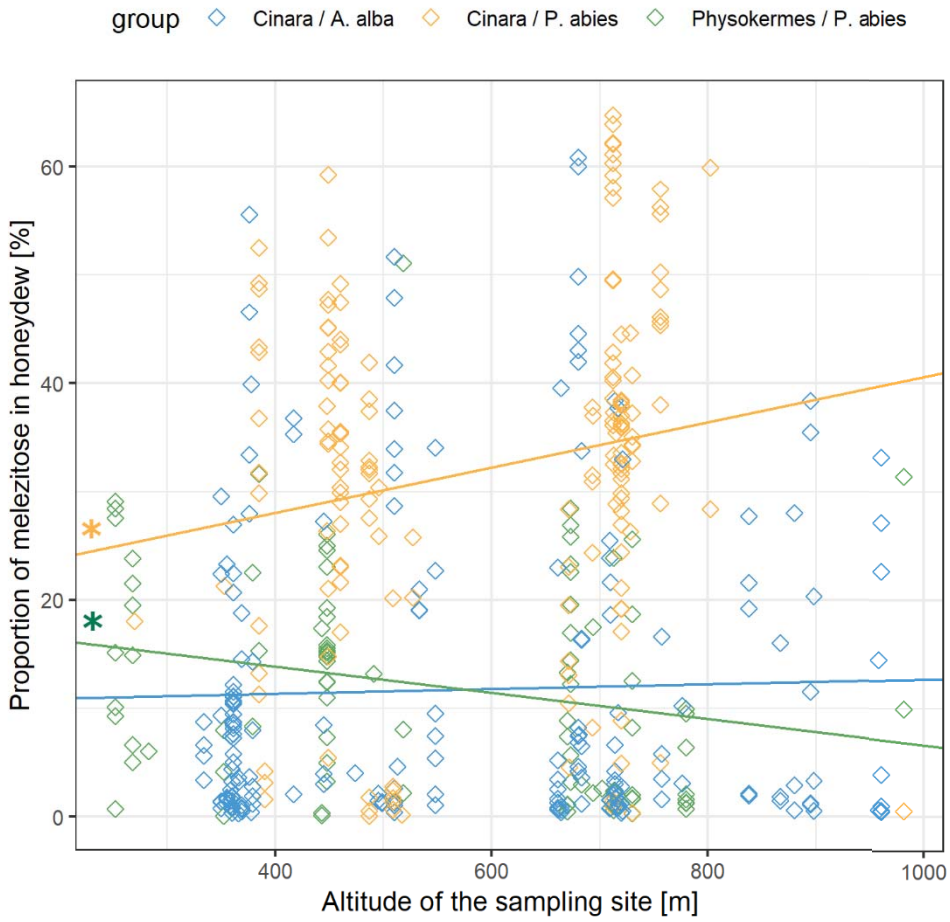

Fig 8. Scatterplot of melezitose proportion of 620 honeydew samples produced at different sampling site altitudes from aphid species (*Cinara* sp.) living on *Abies alba* (blue; slope= 0.002; p= 0.670) and living on *Picea abies* (yellow; slope= 0.021; p= 0.021) and from scale insect species (*Physokermes* sp.) living on *Picea abies* (green; slope= -0.012; p= 0.030). Significance levels are highlighted by the asterisks on the respective regression line (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.1.2 Analyses of variances of the hemipteran species, natural area, year, month and time on the production of melezitose in honeydew

The aphid species *Cinara piceae* produced significantly the highest proportions of melezitose, followed by *Cinara pilicornis*, both species living on *Picea abies* (Fig 9). The scale insect species and the aphid species living on *Abies alba* produced significantly less melezitose. The results are based on an one-way ANOVA ($F_{(6,453)}$: 42.780, $p < 0.001$) and post-hoc Tukey tests. Consequently, both aphid species living on *Picea abies* had the highest sample numbers with occurring melezitose (94.29% and 94.29%).

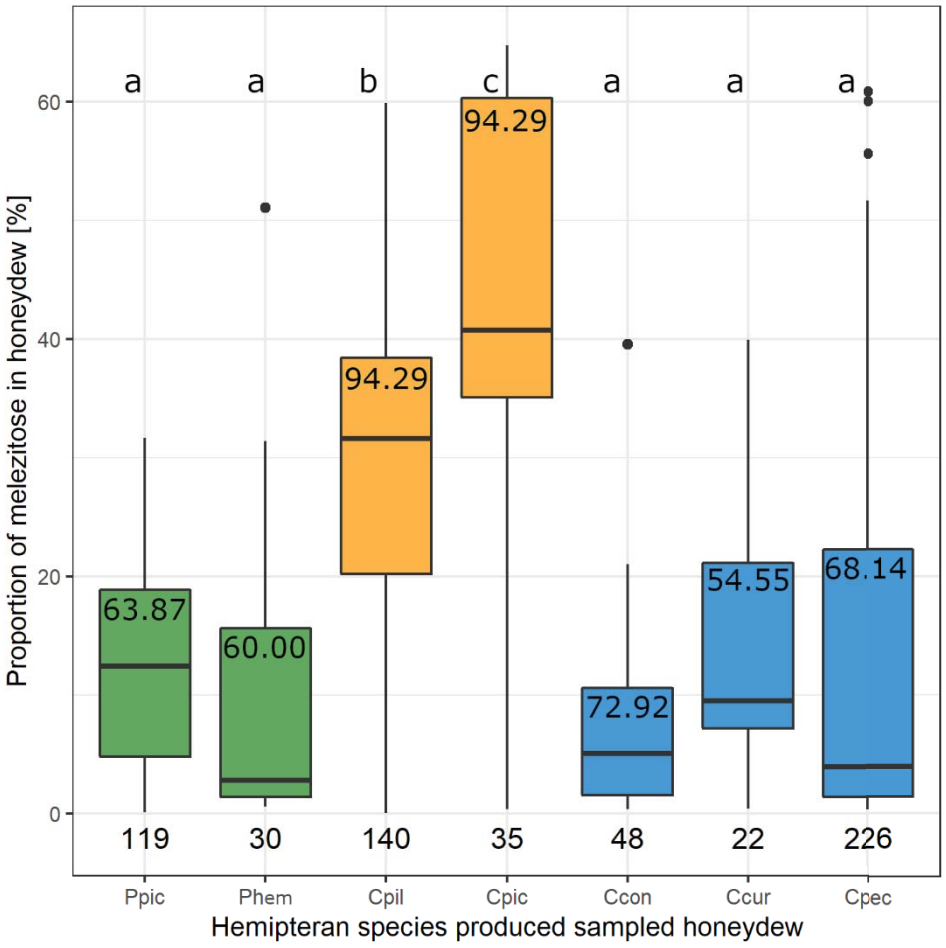

Fig 9. Boxplot of melezitose proportion of 620 honeydew samples from seven different hemipteran species. Coccidae: *Physokermes piceae* (Ppic; mean= 12.4 ± SE 9.0) and *Physokermes hemicryphus* (Phem; mean= 10.3 ± SE 13.9) living on *Picea abies* (green), Lachnidae*: Cinara pilicornis* (Cpil; mean= 29.4 ± SE 14.9) and *Cinara piceae* (Cpic; mean 43.4 ± SE 17.9) living on *Picea abies* (yellow), *Cinara confinis* (Ccon; mean= 7.6 ± SE 7.9), *Cinara curvipes* (Ccur; mean= 13.8 ± SE 11.5) and *Cinara*

pectinatae (Cpec; mean= 12.5 ± SE 15.3) living on *Abies alba* (blue). Significantly different groups are highlighted by the letters a, b and c based on an one-way ANOVA ($F(6,453): 42.780$, $p < 0.001$) and post-hoc Tukey tests. Numbers in the boxes show the percentage of samples with occurring melezitose. Numbers below the boxes show the sample numbers.

Fig 10 reports the melezitose proportion of all honeydew samples across natural areas. The natural areas “Schwaebisches Keuper-Lias-Land“ and “Schwarzwald” had the highest proportions of melezitose. The results are based on an one-way ANOVA ($F(4,455): 13.360$, $p < 0.001$) and post-hoc Tukey tests. Nevertheless, the “Schwaebische Alb” had the highest number of samples with occurring melezitose (95.00%).

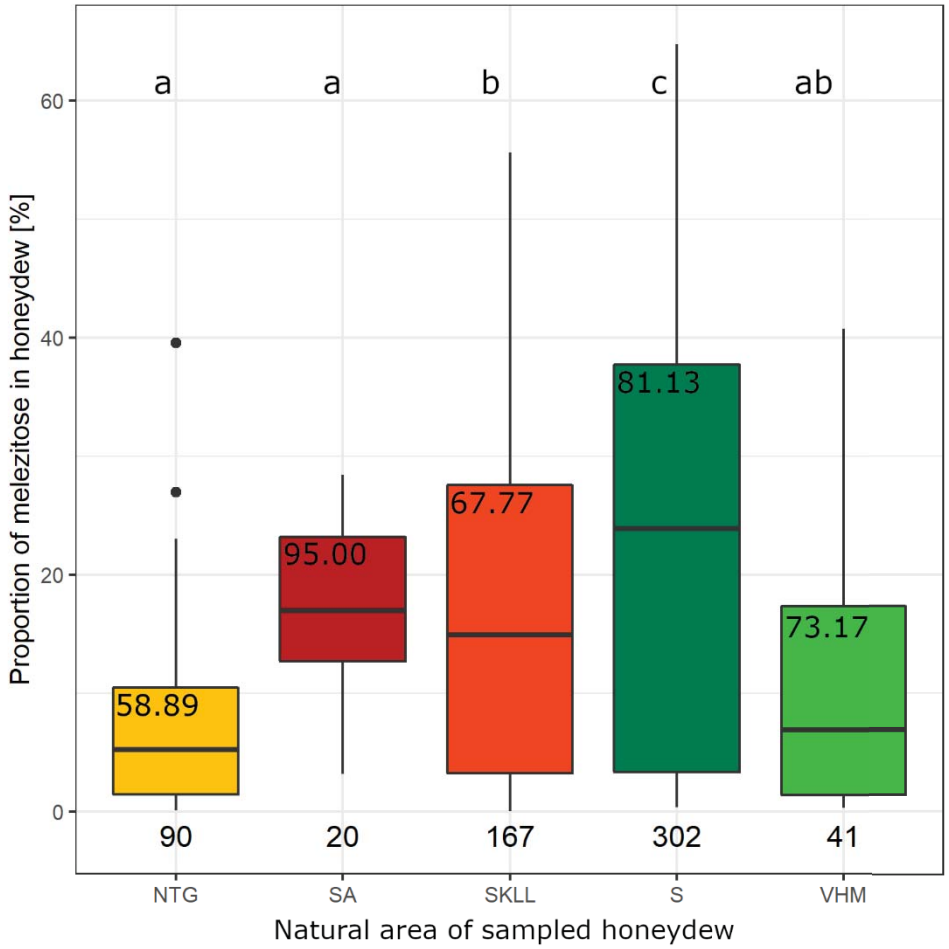

Fig 10. Boxplot of melezitose proportion of 620 honeydew samples from five different natural areas. The boxplots are highlighted with different colours accordingly to the natural areas shown in Fig 4 (LUBW 2010) where the samples are taken: S (Schwarzwald, dark green, mean= 23.7±SE 19.2), NTG (Neckar- und Tauber-Gaeuplatten, yellow, mean= 7.4±SE 7.9), SKLL (Schwaebisches Keuper-Lias-Land, orange, mean= 17.0±SE 14.6), SA (Schwaebische Alb, red, mean=17.3±SE 7.9), VHM (Voralpines Huegel- u. Moorland, light green, mean= 11.9±SE 13.5). The colours (from dark green via light green, via yellow, via orange to red) symbolise areas with expected good to limited water reservoir capacities, respectively (Meynen et al. 1953-1962). Significantly different groups are highlighted by the letters a, b and c based on an one-way ANOVA ($F(4,455): 13.360$, $p < 0.001$) and post-hoc Tukey tests. Numbers in the boxes show the percentage of samples with occurring melezitose. Numbers below the boxes show the sample numbers.

The data show significantly less melezitose in the sampling years 2016 and 2018 than in 2017 and 2019. In Fig 11, the melezitose proportion of all honeydew samples between the sampling years can be seen. The results are based on an one-way ANOVA (F (3,456): 27.780, $p < 0.001$) and post-hoc Tukey tests. Consistently, the highest sample numbers with occurring melezitose were collected in 2017 (77.82%) and 2019 (92.60%).

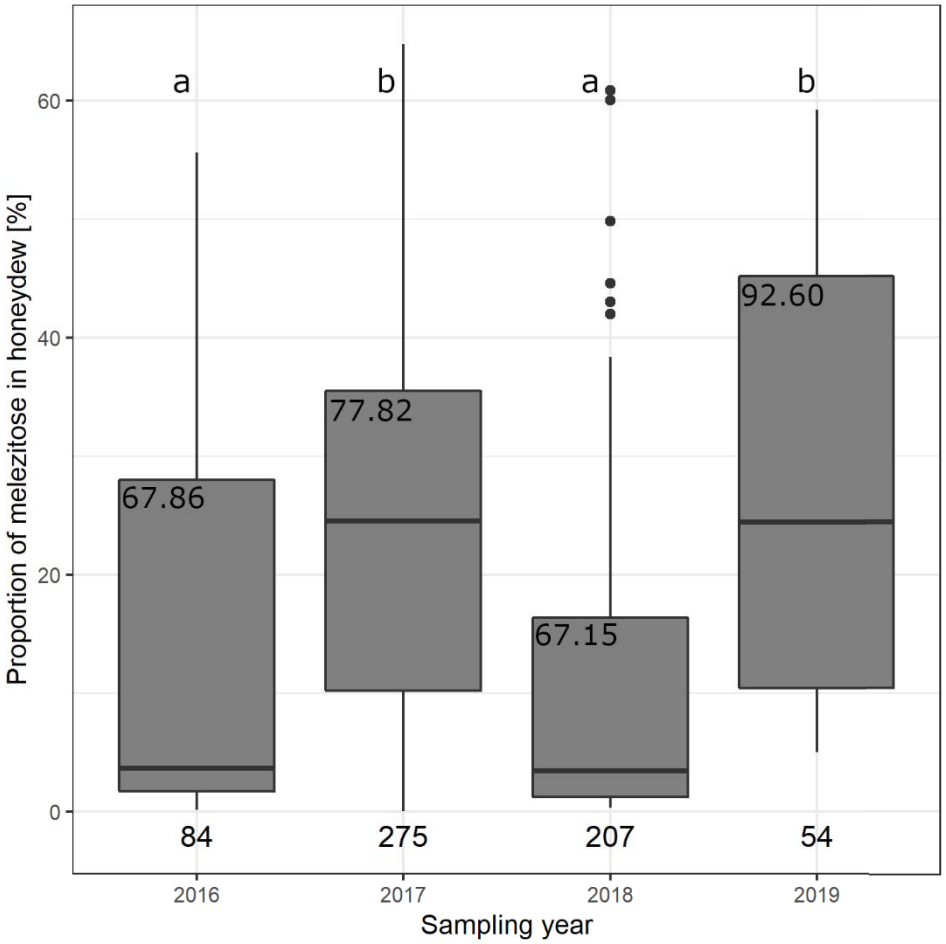

Fig 11. Boxplot of melezitose proportion of 620 honeydew samples from four different sampling years. 2016 (mean= 13.7 ±SE 17.0), 2017 (mean= 24.3 ±SE 16.6), 2018 (mean= 10.4 ±SE 13.5), 2019 (mean= 27.3 ±SE 18.3). Significantly different groups are highlighted by the letters a and b based on an one-way ANOVA (F (3,456): 27.780, $p < 0.001$) and post-hoc Tukey tests. Numbers in the boxes show the percentage of samples with occurring melezitose. Numbers below the boxes show the sample numbers.

In Fig 12, the melezitose proportion of all honeydew samples between the sampling months can be seen. In June and July, the significantly highest melezitose proportions were sampled. The results are based on an one-way ANOVA (F (4,455): 9.433, $p < 0.001$) and post-hoc Tukey tests. All samples collected in April contained melezitose (100.00%), whereas the sample numbers with occurring melezitose between May (60.71%), June (82.38%), July (72.79%) and August (77.23%) differed.

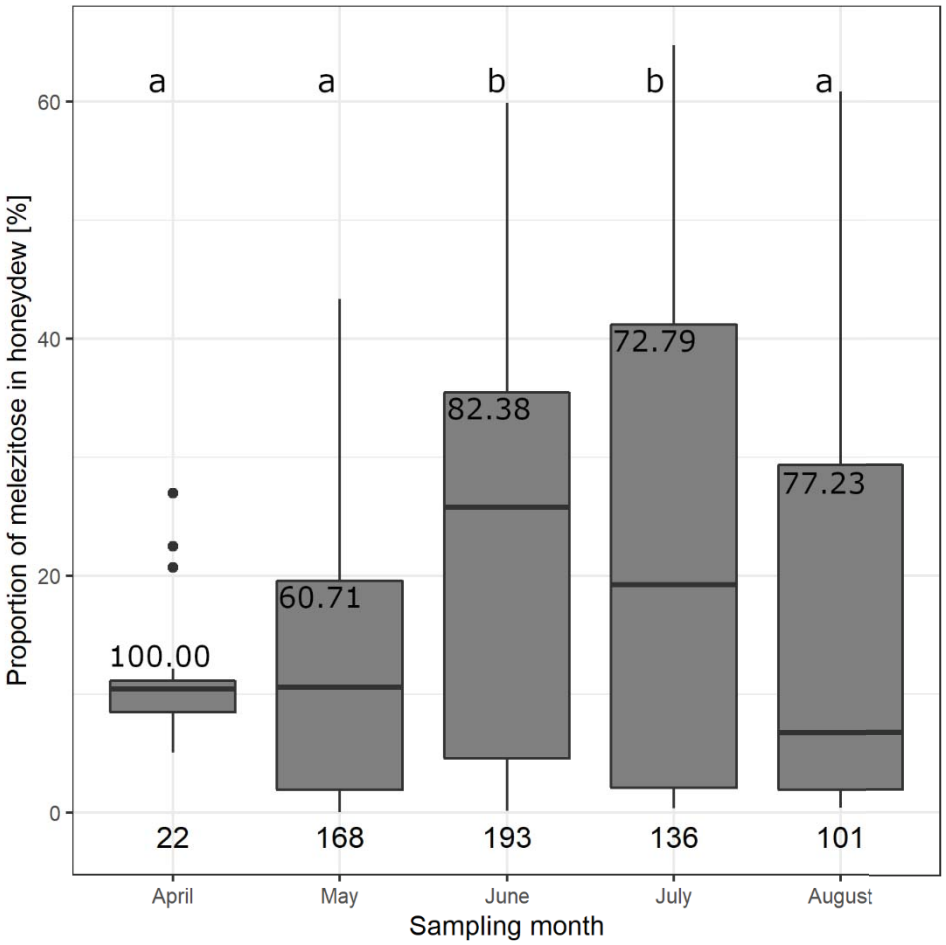

Fig 12. Boxplot of melezitose proportion of 620 honeydew samples from five different sampling months. April (mean= 11.2 ±SE 5.4), May (mean= 12.8 ±SE 11.4), June (mean= 23.0 ±SE 16.1), July (mean= 23.7 ±SE 22.2) and August (mean= 16.11 ±SE 17.7). Significantly different groups are highlighted by the letters a and b based on an one-way ANOVA (F (4,455): 9.433, $p < 0.001$) and post-hoc Tukey tests. Numbers in the boxes show the percentage of samples with occurring melezitose. Numbers below the boxes show the sample numbers.

In Fig 13, the melezitose proportion of all honeydew samples between the sampling time categories can be seen. The significantly highest melezitose proportions were sampled in the evening, between 16:01 and 22:00. The results are based on an one-way ANOVA (F (2,457): 7.206, p < 0.001) and post-hoc Tukey tests. The highest sample number containing melezitose was also collected in the evening (80.18%).

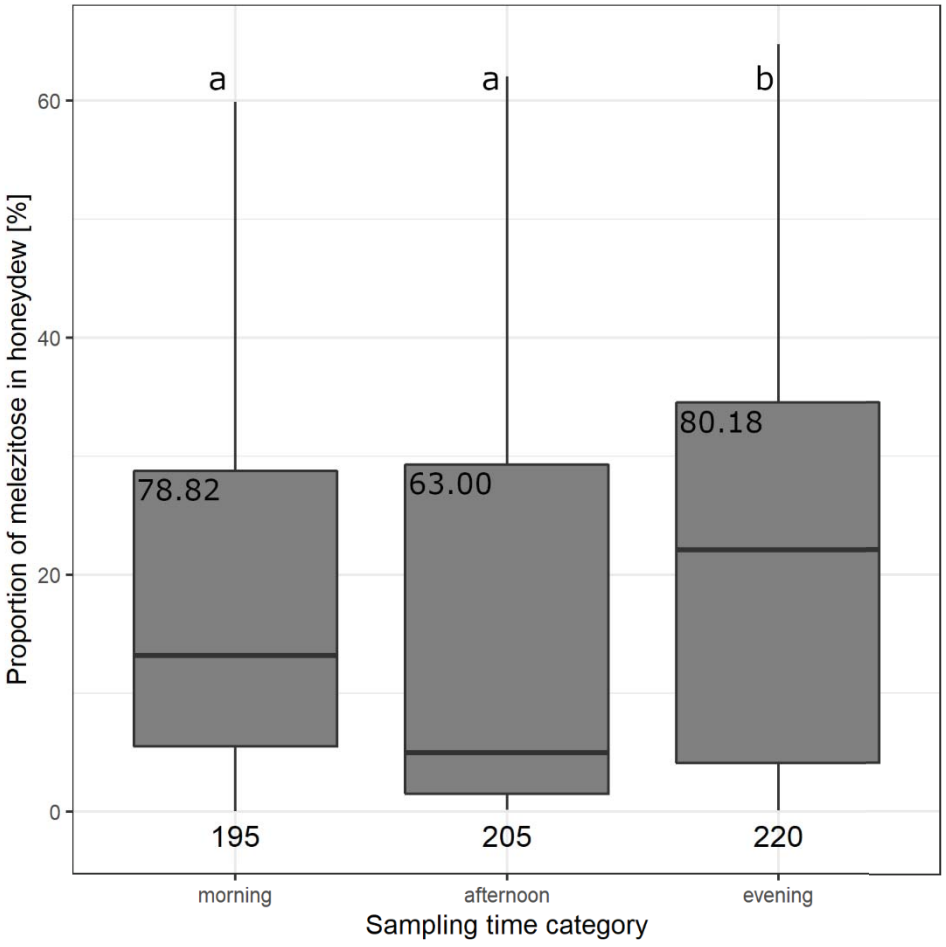

Fig 13. Boxplot of melezitose proportion of 620 honeydew samples from three different time categories. Morning (mean= 18.2 ±SE 15.5), afternoon (mean= 15.4 ±SE 16.9) and evening (mean= 22.7 ±SE 18.6). Significantly different groups are highlighted by the letters a and b based on an one-way ANOVA (F (2,457): 7.206, p < 0.001) and post-hoc Tukey tests. Numbers in the boxes show the percentage of samples with occurring melezitose. Numbers below the boxes show the sample numbers.

3.2 Analyses of melezitose effects on honey bees

The bees fed with melezitose in the experiments showed disease symptoms related to their physiological condition and generic behaviour. Bees fed with the control diet stayed predominantly on the beeswax foundation rectangle (Fig 14A) and bees fed with melezitose were observed to crawl mostly on the bottom of the cage (Fig 14B). In fact, towards the end of the experiment, this behaviour was exhibited more frequently. Additionally, melezitose-fed bees also moved more often and faster than the bees in the control-fed cages. Swollen abdomens, abdomen tipping, impaired movement, twitching and terminal paralysis were observed during all feeding experiments in the melezitose-fed bees (Fig 14 C-D).

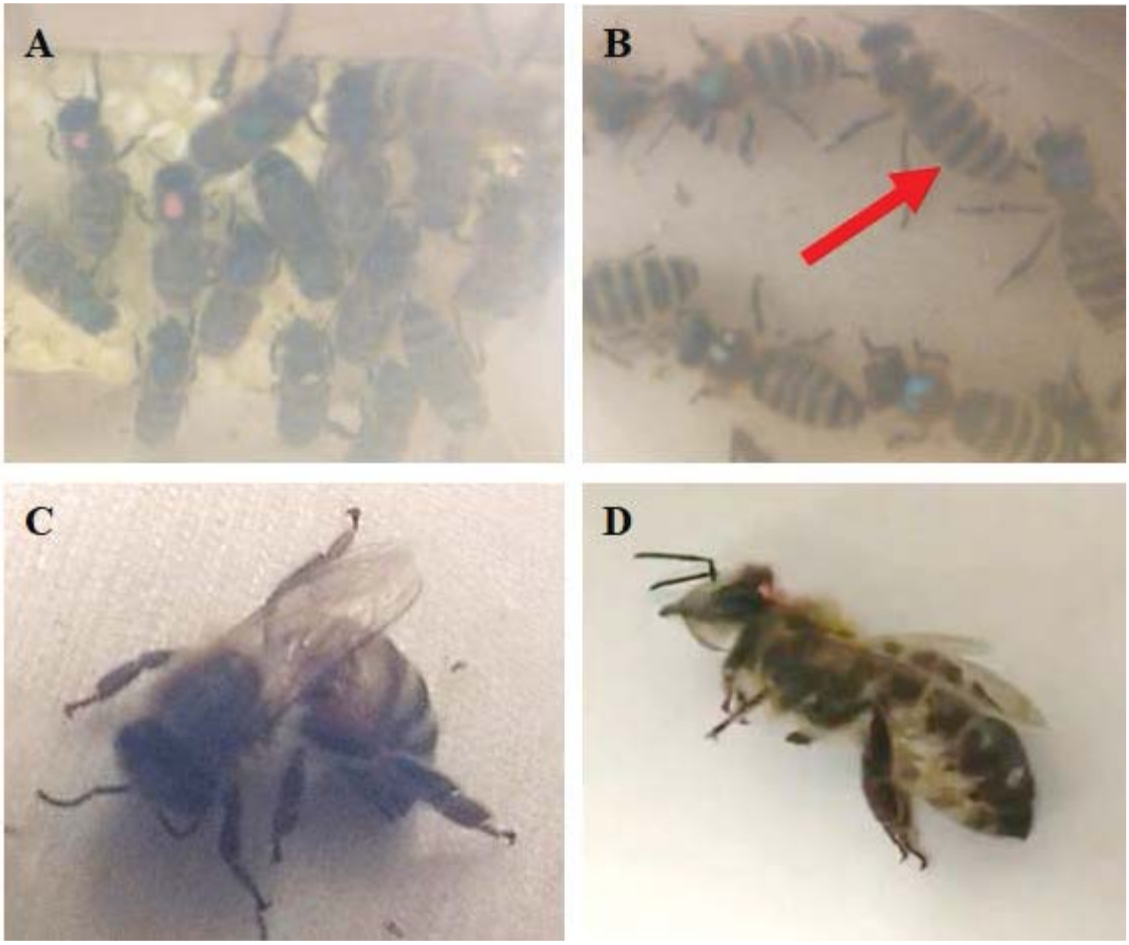

Fig 14. Images of honey bees fed with different diets. A Honey bees fed with low-molecular sugars located at the wax rectangle. **B** Red arrow shows a honey bee fed with melezitose being on the bottom of the cage and moving atypical. **C** The abdomen of the shown melezitose-fed honey bee is swollen and the bee has problems with its movement. **D** Symptoms on melezitose-fed honey bees can become worse until no movement is possible.

Following the hypothesis that melezitose affects the health of honey bees, several multivariate regression analyses were conducted investigating multiple aspects of honey bee health. Using daily food uptake per bee, gut-body weight ratio and survival time as dependent variables, melezitose had a highly significant negative effect on honey bee health. The results of the individual regression models are presented in Table 5.

Table 5. Results of the linear regression model (daily food uptake), fractional logit regression model (gut-body weight ratio) and Cox proportional hazard model (survival).

Coefficients	Estimate	St. Error	Pr(>|t|)
Panel I: Daily food uptake			
Intercept	0.1162	0.0096	<0.001
Melezitose	0.0027	0.0029	0.947
Melezitose day +10	0.0184	0.0061	0.003
2018	0.0109	0.0030	<0.001
2019	0.0151	0.0040	<0.001
Days	-0.0018	0.0003	<0.001
Bees_alive	-0.0017	0.0001	<0.001
Panel II: Gut-body weight ratio			
Intercept	-0.0520	0.1099	0.636
Melezitose	0.0866	0.0154	<0.001
Melezitose day +10	0.0550	0.0260	0.034
2018	0.0157	0.0136	0.248
2019	0.0028	0.0142	0.842
Days	0.0012	0.0016	0.440
Panel III: Survival			
Melezitose	0.9319	0.0555	<0.001
Melezitose day +10	2.2514	0.1073	<0.001
2018	-0.1144	0.0584	0.050
2019	-0.8595	0.0771	<0.001

The multivariate R^2 for the linear regression in Panel I is 0.253 and the F-test for overall significance yields $F(6.893)= 50.4$ ($p < 0.001$). For the fractional logit regression in Panel II, average partial effects are reported. The multivariate R^2 is 0.174 based on 330 observations. The log-rank test (4.1740) yields 628.1 ($p < 0.001$).

3.2.1 Sugar analyses of crop contents and of processed feed

The results of the sugar spectrum analysis in the crops of the caged bees point out that the trisaccharide melezitose was taken up and degraded into the small molecule sugars

trehalose, sucrose, glucose and fructose. While the melezitose proportion in the food was 50%, the mean value in the crops from 69 bees was 18.88%.

The sugar analyses of the contents from the field and laboratory experiments showed that the bees ingested melezitose. Presence of liquid in the crop indicated active feeding. The crops of the field-collected bees contained up to 10.8% melezitose. Besides melezitose, these crops contained (in ranking order) mainly glucose and fructose, less than 10% sucrose, trehalose, turanose, maltose and erlose. Furthermore, less than 1% consisted of melibiose, raffinose and stachyose.

The mean melezitose content in the processed feed in the combs formed from the beeswax foundation rectangles in the cages was 28.92% (8 processed sugar solutions of melezitose-fed cages) (Table 6).

Table 6. Fructose, glucose, sucrose and melezitose proportion of the feed solution, crop content of honey bees collected from the first feeding experiment, field experiment and the processed melezitose feed.

Analysed solution	Fructose [%]	Glucose [%]	Sucrose [%]	Melezitose [%]
Food solution	19.50	15.50	15.00	50.00
Crop content of feeding experiment (N=69)	38.82	36.04	6.10	18.88
Crop content of field experiment (N=15)	45.30	35.50	4.60	3.70
Processed sugar solution (N=8)	24.71	37.59	8.79	28.92

3.2.2 Effects of melezitose feeding on water and food uptake

Bees did not take up more water when fed with melezitose feed, as compared to control solution and also did not show a significantly higher food uptake than control-fed bees (Fig 15). In contrast, the food uptake of bees fed with melezitose starting from day 10 was 20 mg higher ($p < 0.01$) than that of bees fed with the control solution and higher than that of bees fed with melezitose from the first day onwards ($p < 0.01$). To illustrate the relative increase in food uptake, the average uptake per bee was determined, which was 20 mg for control-fed bees. Calculating from this value, a change from control to melezitose diet caused the bees to approximately double their food intake (Fig 15).

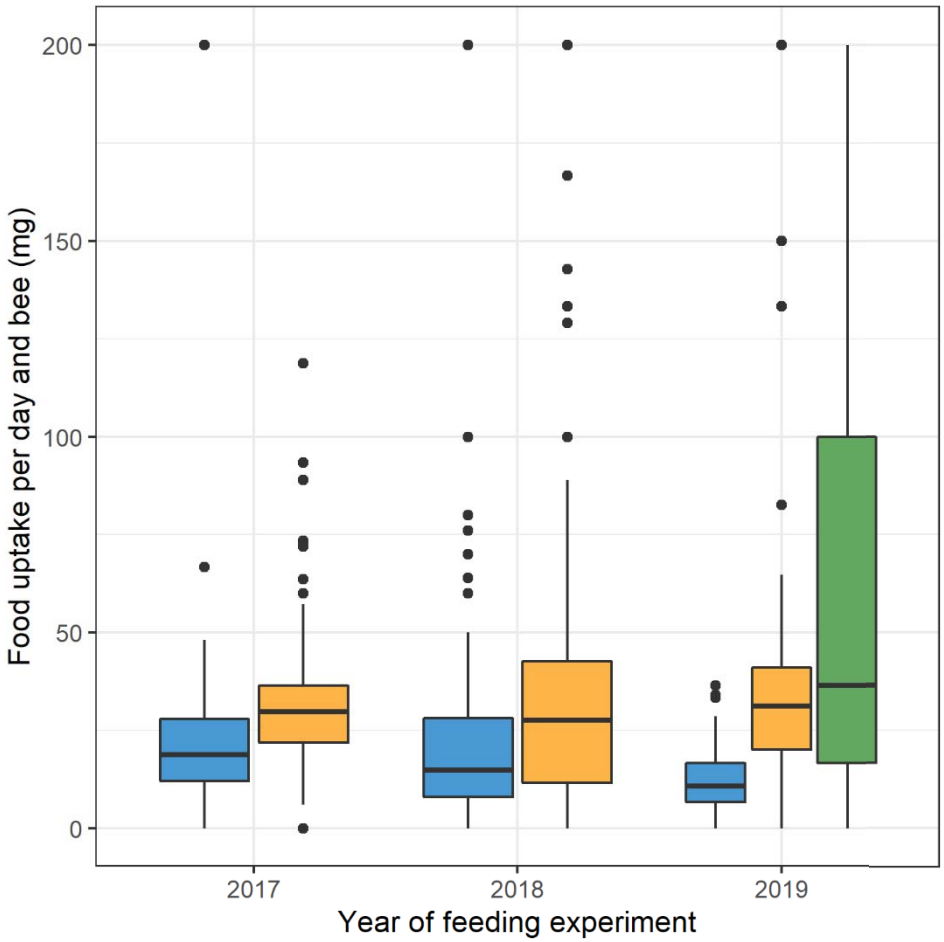

Fig 15. Boxplot of food uptake per day and bee in milligram (mg) in all cage experiments. The three treatment groups are highlighted with different colours. The mean value of daily food uptake per bee fed with control feed (blue) was 20 mg per day (n=445) and 37 mg per day (n=388) for bees fed with melezitose feed (yellow) and 70 mg per day (n=67) for bees fed with melezitose feed from day 10 (green) of all years. The vertical boxplots depict the interquartile range (lower bound/ upper bound of the box correspond to the 25/75% quantile), the median (horizontal line in the box) and outlying observations (points outside the box).

3.2.3 Effects of melezitose feeding on viscosity and weight of guts

The proportion of gut weight of the respective bee bodies was 52% in control-fed bees, 60% in melezitose-fed bees and 56% in bees fed with melezitose from day 10 (Fig 16). A fractional logit regression was conducted ($R^2(5,330)=0.174$) and found that both groups fed with melezitose had significantly higher gut-body weight ratios than the control group ($p < 0.001$ for bees fed with melezitose from day 1 and $p < 0.05$ for bees fed with

melezitose from day 10). The effect of the higher gut-body weight ratio was numerically slightly weaker (six percentage-points increase of gut-body-weight ratio instead of nine percentage points increase) for bees fed with melezitose starting from day 10, which implies that a longer exposure to melezitose seems to enlarge the gut of bees.

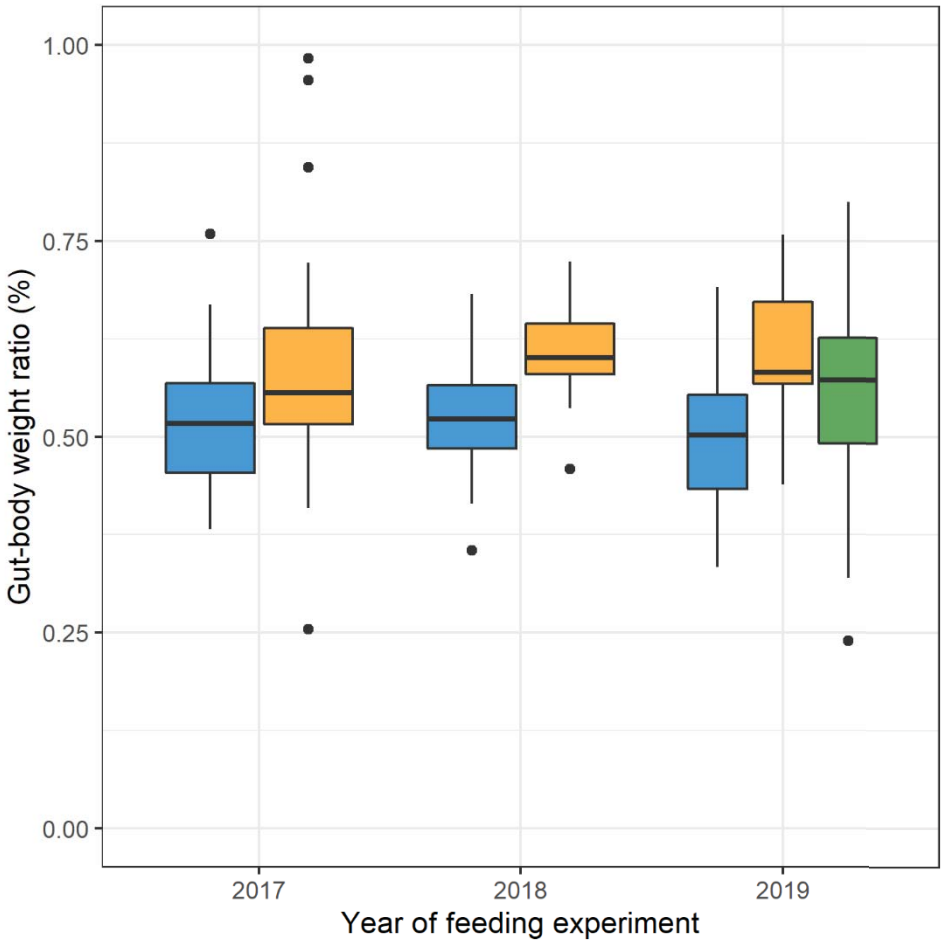

Fig 16. Boxplot of gut-body weight ratio of honey bees in all cage experiments. The three treatment groups are highlighted with different colours. The mean value of gut-body weight ratio per bee fed with control feed (blue) was 52 % (n=150) and 60% (n=150) for bees fed with melezitose feed (yellow) and 56% (n=30) for bees fed with melezitose feed from day 10 (green). The vertical boxplots depict the interquartile range (lower bound/ upper bound of the box correspond to the 25/75% quantile), the median (horizontal line in the box) and outlying observations (points outside the box).

3.2.4 Effects of melezitose on survival

Estimated survival times for all feeding experiments are depicted in Fig 17. In general, the survival time of bees fed with melezitose was lower compared to the survival time of bees fed with the control solution in each year ($p < 0.001$). No significant differences between the feeding experiments in 2017 and 2018 could be found. However, the median survival rates in 2019 were significantly higher than those in 2017 and 2018 ($p < 0.001$). In the control group of the feeding experiment 2019, 50% of the bees had died after 29 days, while 50% of the melezitose-fed bees had died already after 25 days. Interestingly, the bees fed with melezitose starting from day 10 showed a more rapid inset of mortality than the bees fed with melezitose from the start of the experiment ($p < 0.001$). Their median survival time was only 17 days.

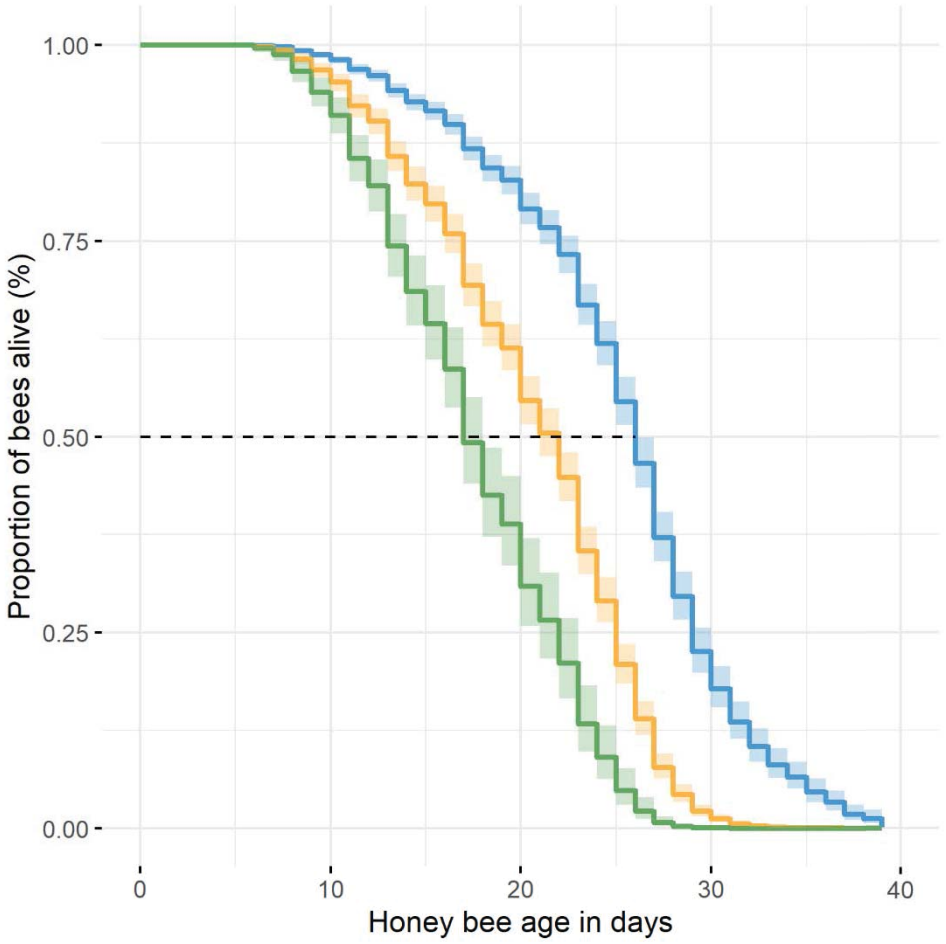

Fig 17. Overall survival probability plots in all cage experiments. The survival rate of the honey bees in the control (blue), in the melezitose group (yellow) and in the bees fed with melezitose from day 10 (green) (Cox regression, Log-rank (4,1740): 628.1, $p < 0.001$).

3.2.5 Effects of melezitose on the gut microbiota

A preliminary, small scale (N=9) microbiota analysis was conducted in the second experiment, to determine if there were any effects of the treatments on the microbiota. Surprisingly, the core microbiota members *Gilliamella apicola* and *Snodgrassella alvi* were absent from all bees analysed (Fig 18).

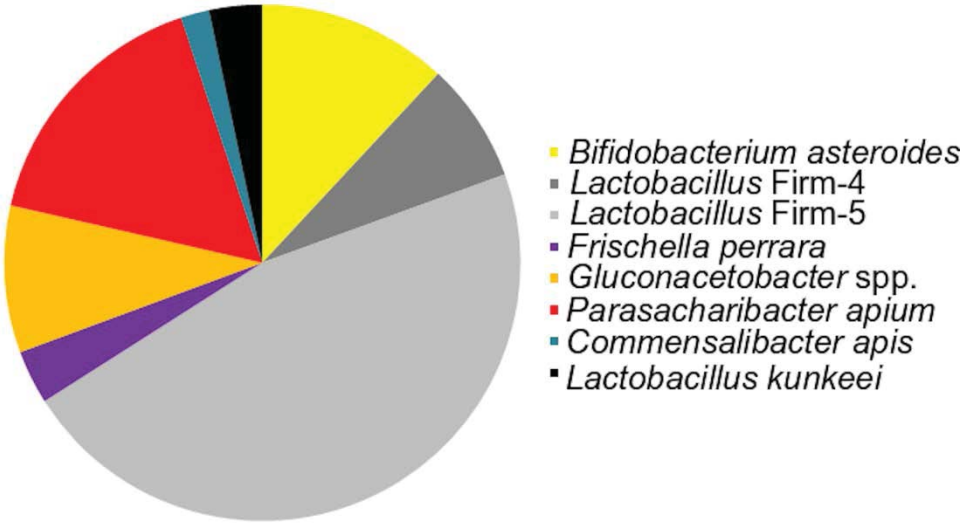

Fig 18. Overall gut microbiota of sequenced DNA of honey bees from the third feeding experiment (mean value of N=9 analysed gut microbial communities). Honey bees were removed from their colonies directly after emerging and had no contact to nurse bees. Bacterial species are highlighted by colour and shown in the legend. Core-members of the honey bee gut microbiota are written in bold.

This is remarkable, as they are usually, together with the *Lactobacillus* species, the most common and persistent bacteria in free-living adult bees. This absence of two core-microbiota bacteria was attributed to the circumstance that the bees used in the first three experiments had emerged from their cells and were put into the cages without contact to adult nest members. Therefore, it was decided to modify the experimental setup for the third experiment in a way that the age-standardised bees come into contact with their adult nest members for five days to allow natural development of the intestinal microbiota (Fig 19).

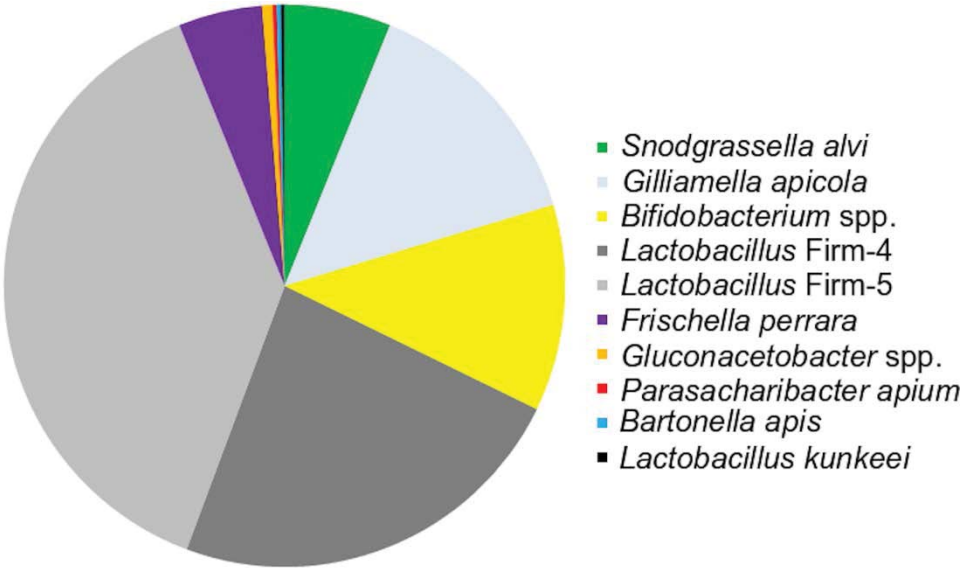

Fig 19. Overall gut microbiota of sequenced DNA of honey bees from the fourth feeding experiment (mean value of analysed gut microbial communities of N=108 bees). Honey bees lived in bee hive colonies with contact to nurse bees until day 5. Bacterial species are highlighted by colour and shown in the legend. Core-members of the honey bee gut microbiota are written in bold.

Diversity analyses of the 16S-amplicon sequence data revealed no significant differences in the community composition between the treatment groups of the feeding experiment (Fig 20).

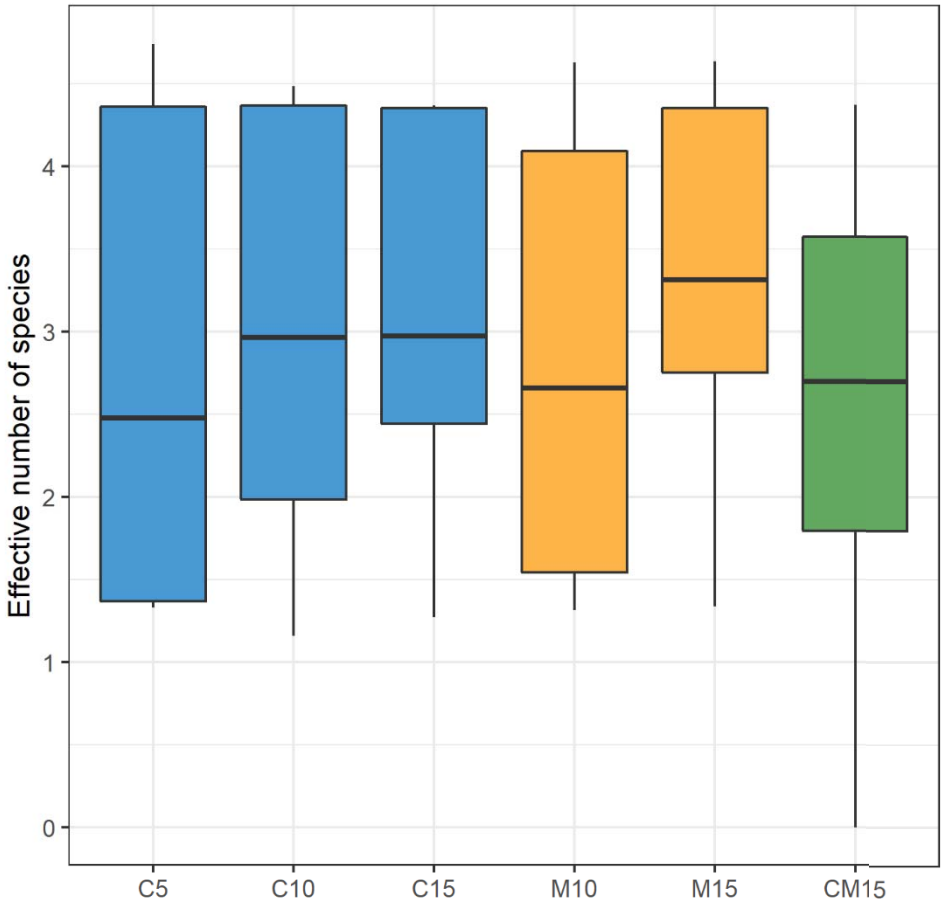

Fig 20. Differences in α-diversity, i.e. Shannon effective number of species, in the gut microbiota of control (blue) and melezitose (yellow) fed bees and bees with a changed diet (green) based on 16S RNA gene amplicon sequencing. No significant differences between the groups were detected (Kruskal-Wallis chi-squared = 1.8162, df = 5, p-value = 0.8739). For each treatment group and honey bee age, 18 honey bee individuals were used for analysis. C = control-fed bees (blue), M = melezitose-fed bees (yellow), CM = bees first fed with control and from day 10 with melezitose (green); 5, 10 and 15 shows the honey bee age in days. The vertical boxplots depict the interquartile range (lower bound/ upper bound of the box correspond to the 25%/ 75% quantile) and the median (horizontal line in the box).

In the bees from the fourth experiment (N = 108) a total of ten gut bacteria species could be detected. The five core members are *Snodgrassella alvi*, *Gilliamella apicola*, *Bifidobacterium* spp., *Lactobacillus* Firm-4 and *Lactobacillus* Firm-5, as well as bacteria from the species *Frischella perrara*, *Gluconacetobacter* spp., *Parasaccharibacter apium, Bartonella apis* and *Lactobacillus kunkeei* (Fig 21).

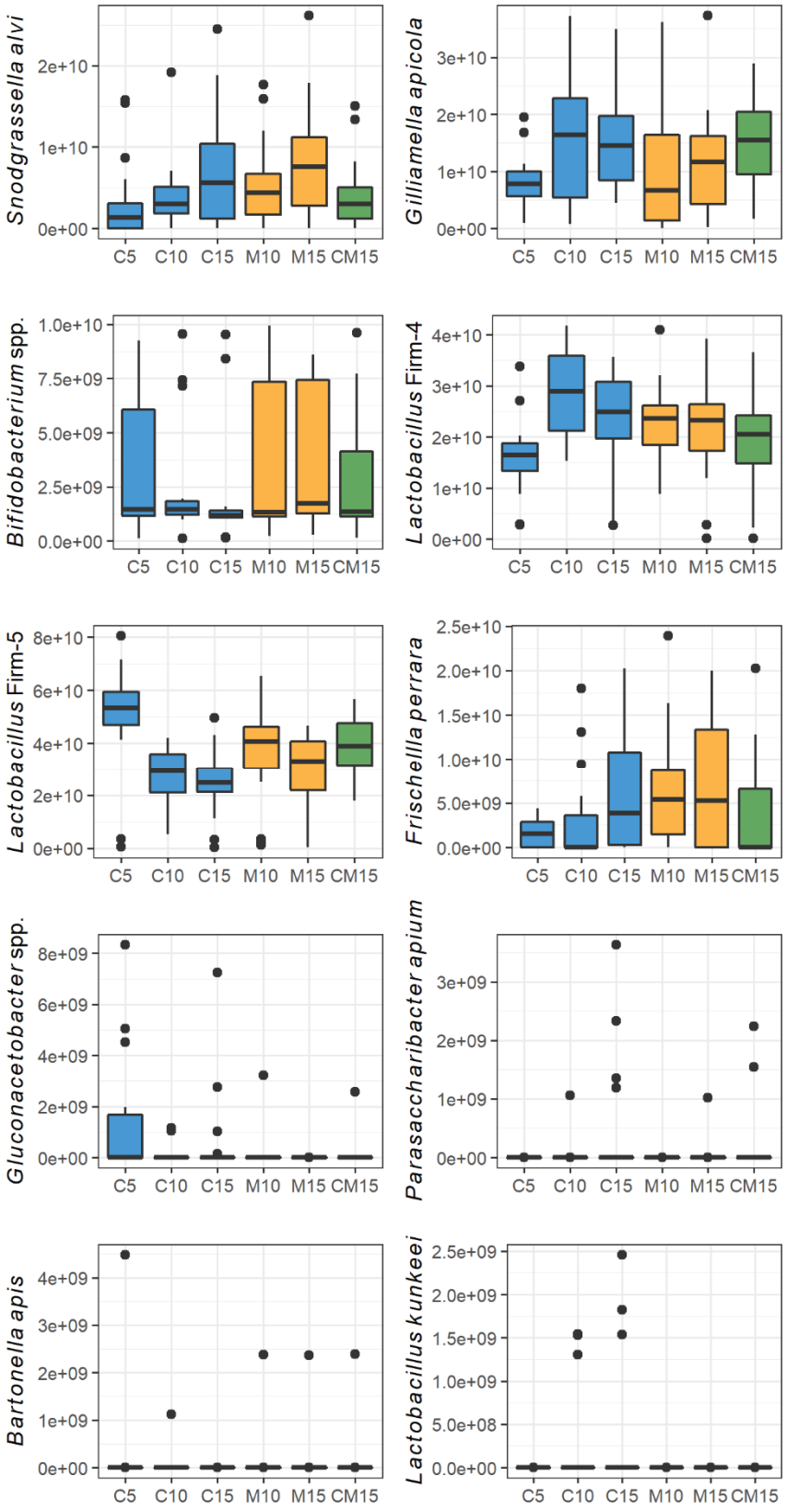
Snodgrassella alvi
0e+00
1e+10
2e+10
Gilliamella apicola
0e+00
1e+10
2e+10
3e+10
Bifidobacterium spp.
0.0e+00
2.5e+09
5.0e+09
7.5e+09
1.0e+10
Lactobacillus Firm-4
0e+00
1e+10
2e+10
3e+10
4e+10
Lactobacillus Firm-5
0e+00
2e+10
4e+10
6e+10
8e+10
Frischellla perrara
0.0e+00
5.0e+09
1.0e+10
1.5e+10
2.0e+10
2.5e+10
Gluconacetobacter spp.
0e+00
2e+09
4e+09
6e+09
8e+09
Parasaccharibacter apium
0e+00
1e+09
2e+09
3e+09
Bartonella apis
0e+00
1e+09
2e+09
3e+09
4e+09
Lactobacillus kunkeei
0.0e+00
5.0e+08
1.0e+09
1.5e+09
2.0e+09
2.5e+09
C5 C10 C15 M10 M15 CM15

Fig 21. Absolute abundances of ten microbiota members monitored in the feeding experiment 2019. Absolute abundance of the ten monitored phylotypes: *Snodgrassella alvi*, *Gilliamella apicola*, *Bifidobacterium* spp., *Lactobacillus* Firm-4, *Lactobacillus* Firm-5, *Frischella perrara, Gluconacetobacter* spp., *Parasaccharibacter apium, Bartonella apis* and *Lactobacillus kunkeei*. The ten plots show the cumulative abundances for each bee. For each treatment group, 18 honey bee individuals were used for analysis. C = control-fed bees (blue), M = melezitose-fed bees (yellow), CM = bees first fed with control and from day 10 with melezitose (green); 5, 10 and 15 shows the honey bee age in days. Significant differences between the treatment groups could be shown for all *Lactobacillus* species and are demonstrated in Fig. 22-24. The vertical boxplots depict the interquartile range (lower bound/ upper bound of the box correspond to the 25%/ 75% quantile), the median (horizontal line in the box) and outlying observations (points outside the box).

At first glance, the microbial community appeared to be unaffected by the melezitose treatment. However, on closer examination of the OTU composition, the proportions of the lactic acid bacteria differed between the treatment groups. *Lactobacillus kunkeei* increased over time in bees fed with the control diet, but was not present at all in bees fed with melezitose, and it was very low in bees fed with melezitose from day 10 (Fig 22; F-statistic: 2.66 on 5 and 102 DF, $p = 0.03$).

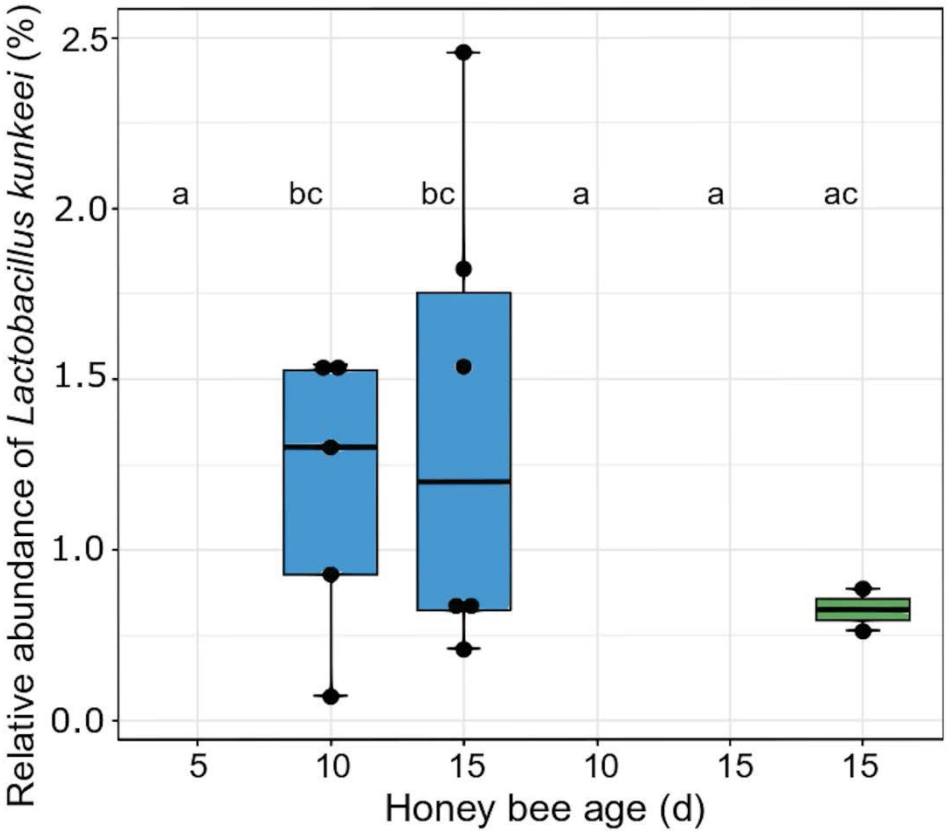

Fig 22. Relative abundance of *Lactobacillus kunkeei* in the gut microbiota. The three treatment groups are highlighted with different colours: control (blue), melezitose (not detected at all time points) and melezitose from day 10 (green). Significantly different groups are highlighted by the letters a, b and c (F-statistic: 2.66 on 5 and 102 DF, p = 0.027). For each treatment group and each bee age, 18 individuals were used for analysis. The vertical boxplots depict the interquartile range (lower bound/ upper bound of the box correspond to the 25%/ 75% quantile), the median (horizontal line in the box) and outlying observations (points outside the box).

Also, the relative abundance of *Lactobacillus* Firm-4 increased in control-fed bees and decreased in bees fed with melezitose from day 10 (Fig 23; F-statistic: 4.245 on 5 and 102 DF, p = 0.002).

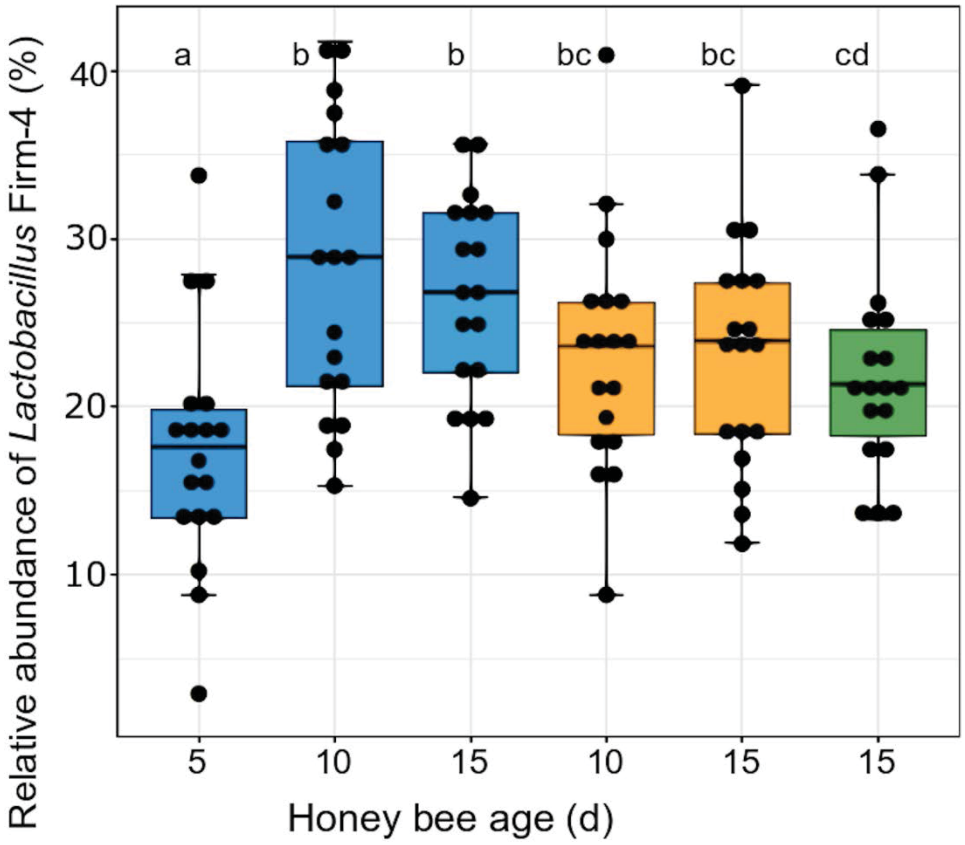

Fig 23. Relative abundance of *Lactobacillus* Firm-4 in the gut microbiota. The three treatment groups are highlighted with different colours: control (blue), melezitose (yellow) and melezitose from day 10 (green). Significantly different groups are highlighted by the letters a, b, c and d (F-statistic: 4.245 on 5 and 102 DF, p = 0.002). For each treatment group and each bee age, 18 individuals were used for analysis. The vertical boxplots depict the interquartile range (lower bound/ upper bound of the box correspond to the 25%/ 75% quantile), the median (horizontal line in the box) and outlying observations (points outside the box).

In contrast, the relative abundance of *Lactobacillus* Firm-5 decreased in bees fed with the control diet, but increased significantly in bees fed with melezitose and bees fed with melezitose from day 10 (Fig 24; F-statistic: 7.048 on 5 and 102 DF, $p < 0.001$).

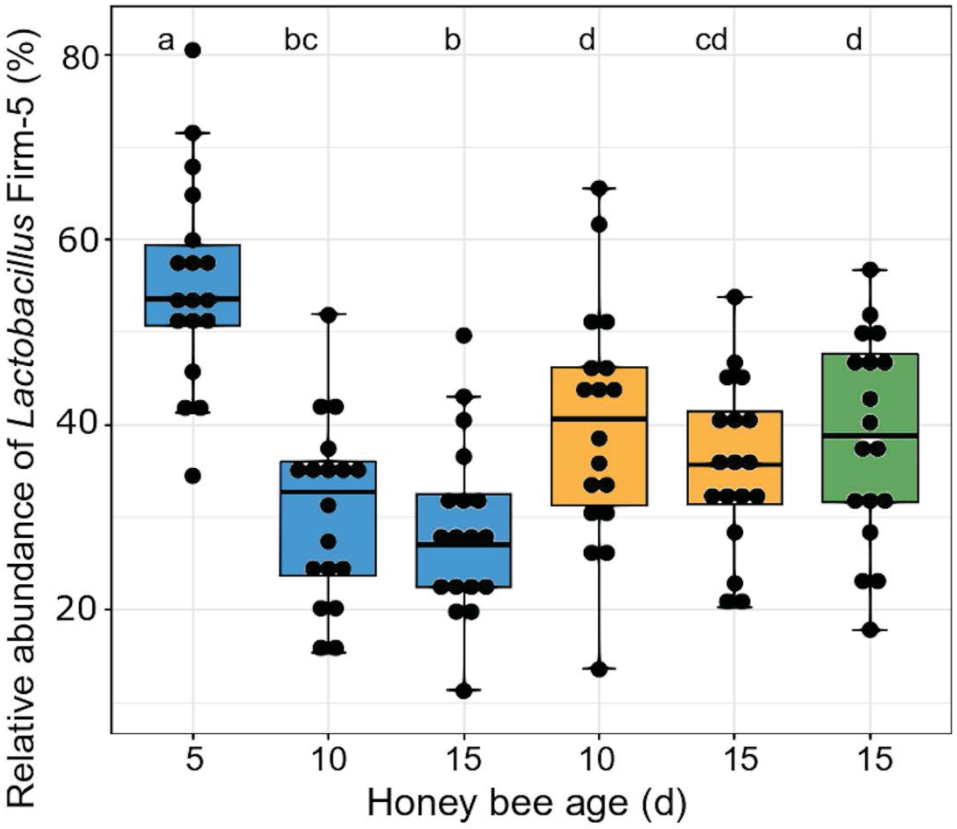

Fig 24. Relative abundance of *Lactobacillus* Firm-5 in the gut microbiota. The three treatment groups are highlighted with different colours: control (blue), melezitose (yellow) and melezitose from day 10 (green). Significantly different groups are highlighted by the letters a, b, c and d (F-statistic: 7.048 on 5 and 102 DF, $p < 0.001$). For each treatment group and bee age, 18 honey bee individuals were used for analysis. The vertical boxplots depict the interquartile range (lower bound/ upper bound of the box correspond to the 25%/ 75% quantile), the median (horizontal line in the box) and outlying observations (points outside the box).

4 Discussion

The here presented work provides new information about the process between phloem sap of coniferous trees and the final product honeydew honey as well as the impact of a honeydew diet on the health and intestinal microbiota of honey bees. It could be shown that the hemipteran species relevant for the production of honeydew honey increased the proportions of the trisaccharide melezitose within honeydew under environmental factors similar to drought stress. High air temperatures, low relative humidity levels as well as the hemipteran species living on shallow rooted spruces and in natural areas with limited water reservoir capacity enhanced the production of melezitose within honeydew. All results of the performed feeding experiments lead to the conclusion that melezitose affects the health of honey bees. Bees fed with melezitose showed intestinal anomalies, increased food uptake, gut weight and mortality as well as a shift in the lactic acid bacteria of their gut microbiota. These multiple effects indicate that the trisaccharide may accumulate in the gut over time and consequently lead to the described honeydew flow disease.

4.1 Discussion of the methods

For this thesis, a complex variety of different methodological approaches have been conducted. In the field study, I sampled honeydew from living hemipteran species and analysed the sugar production biochemically considering a multitude of environmental factors to understand the physiological interactions within the hemipteran species and their host trees while processing the phloem sap to honeydew. Feeding experiments with honey bees in a laboratory study and the microbiological comparison of the gut microbiota from honey bees fed on different sugar compositions provided new evidence in the comprehension of the honeydew flow disease.

In the first part of this thesis, I collected a variety of honeydew samples in an area of almost 36 km². This field work could be enabled by coworking with over 30 “honeydew flow beekeepers” that voluntarily took part in the study. The beekeepers as well as own project bee hives with scales indicated honeydew flow in several parts of the field. The beekeepers were observing their own bee hives and the surrounded forest regions for the possible occurrence of hemipteran species producing high volumes of honeydew. The scales below the project bee hives of the study indicated honeydew flow season as well

with gaining weight. A higher weight of the bee hives usually means that the bees fill their combs with honey and when they are located in forests without the possibility to collect nectar, the honeydew flow season could be assumed. With these signs, it was possible to collect the necessary amount of honeydew samples for analysing the effect of different environmental factors on the production of melezitose by hemipteran species. Furthermore, I recommend the usage of a glass micropipette to collect honeydew as a nice and easy method to get exactly what is wanted (Fig 25). With capillary force, the honeydew was absorbed directly from the hemipteran species and the micropipette could be quick-frozen immediately to avoid possible changes of the sugar composition after the excretion of the hemipteran species. Since the hemipteran species produced the honeydew in the moment when I collected it and measured the meteorological factors with a hygrometer on site, it was possible to precisely analyse the effect of the environmental factors on the production of melezitose.

Fig 25. Honeydew sampling with a micropipette on *Physokermes piceae* living on *Picea abies*.

The statistical analyses of the factors influencing the melezitose production in honeydew was challenging, since I collected data from a variety of explanatory variables on the

melezitose production. I compared the explanatory variables air temperature, relative humidity, altitude, hemipteran species, natural area, sampling year, month and time with the proportion of melezitose in honeydew. Since approximately a quarter of the samples did not include melezitose, I first analysed the effect of the explanatory variables on the occurrence and absence on melezitose. Afterwards, I analysed the effect of the explanatory variables on the proportion of melezitose within the samples with occurring melezitose. This two-part statistical analysis could illustrate the power of the single explanatory variables under consideration of all variables simultaneously and provided a clear overview how the environmental factors affect melezitose production. For more detailed clarification, I conducted multiple regression analyses for the continuous variables with grouping the hemipteran species in aphid species living on firs (*Abies alba*), aphid species living on spruces (*Picea abies*) and scale insect species living on spruces and ANOVA for the nominal variables. I also conducted interaction analyses to understand the interaction effects between the meteorological effects on the hemipteran species. This comprehensively statistical analyses helped to understand the environmental factors on the production of melezitose. However, as I sampled all present honeydew during the study, it resulted in asymmetrical sample numbers between the hemipteran species and a more detailed analyses of especially the interaction effects could be conducted with higher sample numbers for some hemipteran species. In order to avoid this problem, laboratory experiments are advisable. In follow-up experiments, the hemipteran species could be fed on different sucrose concentrations to clarify the direct effect a high molality in the phloem sap on the osmoregulation of hemipteran species and with that the production of melezitose. This excludes additional environmental and meteorological effects and outlines the effect of drought stress on the production of melezitose by aphids. Nevertheless, the data of this field study are essential for the evidence that the different factors are effectively influencing the hemipteran species and for the comprehension of the complexity of this phenomenon. It has to be mentioned that the highly significant effect of the air temperature on the increase in melezitose proportion in honeydew could also be explained by the increased activity of most enzymes with increasing temperatures. However, this comprehensive study showed that there is more than one factor influencing the melezitose production and that the results give a first tool to understand the hemipteran biology in producing melezitose.

For the second part of this study, I conducted feeding experiments in cages allowing a standardised quantification of the effect of different sugar diets on honey bees. I suggest the usage of the simple setup with approved plastic cages with syringes (Fig 26 A) for the cage experiments. Despite previous literature showed that the contact to nurse bees is ubiquitous for the development of an intact gut microbiota (Martinson et al. 2012; Powell et al. 2014), I was surprised by the fact that the honey bees of the first three cage experiments of this study did not show any presence of the two core member gut bacterial species *Snodgrassella alvi* and *Gilliamella apicola*. These results once more demonstrate the major importance of the naturally given contact between newly emerged bees and nurse bees. This is the reason why I highly recommend the methods used for the fourth cage experiment of this study. I marked newly emerged honey bees and put them back in their bee hives to give the opportunity to achieve a usual microbiota in the first five days of the lifetime. Afterwards I could find the honey bees by their markings and put them in the cages of the feeding experiments. This is also shown in Fig 26 B-C.

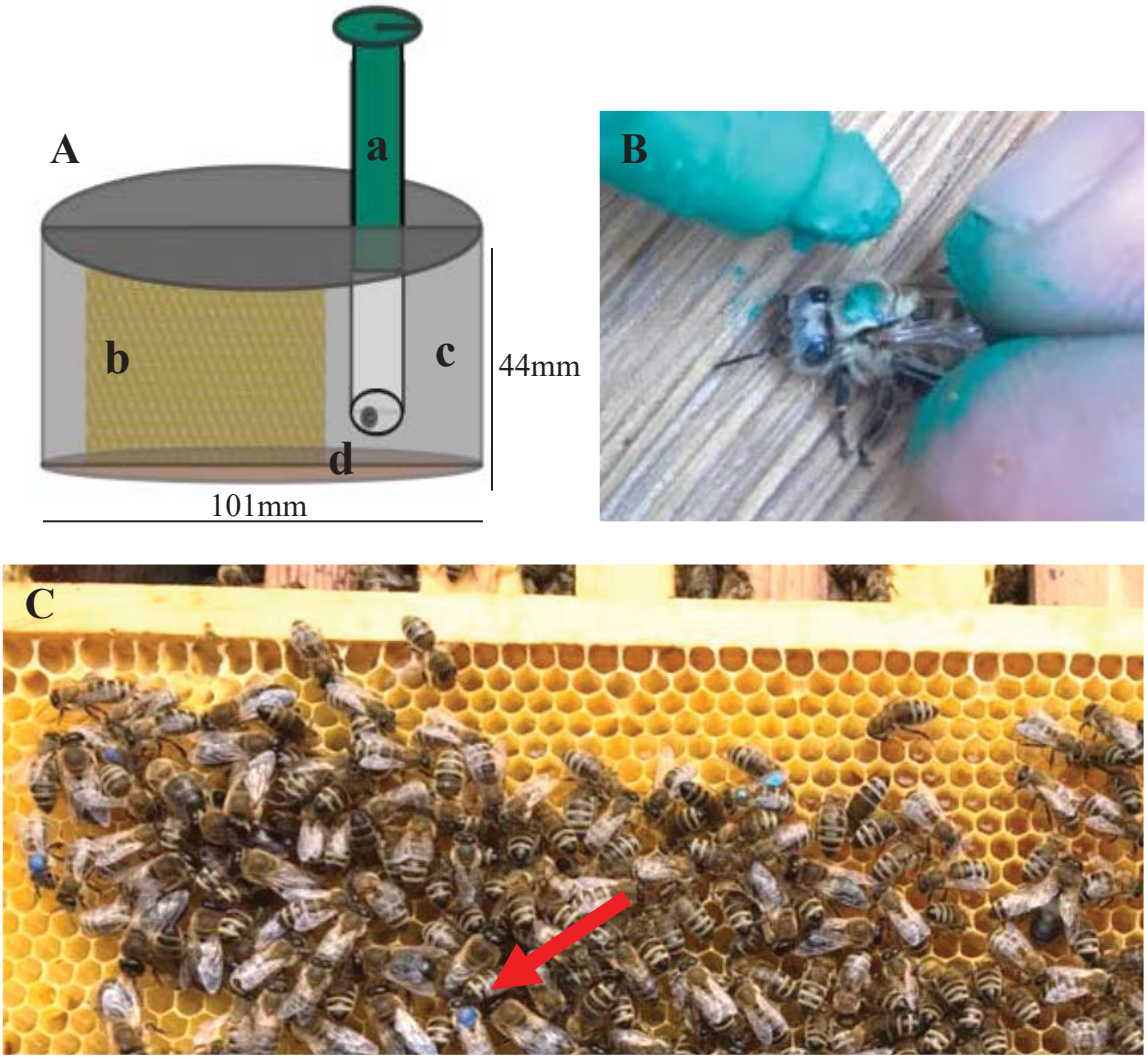

Fig 26. Drawing and images of the feeding experiments. A Drawing of one cage of the feeding experiments. a = 10 ml syringe for feeding sugar solution; b = rectangle of bee wax with honeycomb structure fixed on the top (100x30 mm); c = cage of approved plastic (101x44 mm), d = net of 10 DEN. In each cage, 50 honey bees were feed, daily food uptake, gut weight and mortality was recorded and gut microbiota was analysed. **B** Newly emerged bee with its typical bright-whitish hair. Bee is marked with a colour specific to its hive. **C** Red arrow shows that bees can be found in the hives after five days and put in the cages of the feeding experiment.

The feeding experiments gave an important tool to understand the effect of melezitose on honey bees, which can be one factor triggering the honeydew flow disease. It is advisable to repeat the experiments in different years for the possibility to statistically exclude year effects such as a year with high virus loads in the bee hives. Furthermore, it can be

assumed that several other factors can lead to the shown intestinal problems. A further step to understand the occurrence of the disease would be additional field experiments with comparing honey bee hives situated in flowering plant regions and forest regions during honeydew flow season. For evaluating the magnitude of the negative effect of melezitose, further cage feeding experiments have to be realised with other characteristic honeydew sugars such as the trisaccharide erlose. It should be considered, why honey bees collect honeydew with high contents of melezitose in the first place. It is not known yet, whether the bees can recognise melezitose within the droplets and with that, whether they have the possibility to avoid the sugar. Supplementary cage experiments with the simultaneously feeding of artificial nectar and artificial honeydew have already been conducted and showed a clear preference of the honey bees on artificial nectar. These experiments can be continued in future research, as well as a comparison of a melezitose and an erlose diet and could be eye-opening to answer this question.

Moreover, it is necessary to understand the impact of melezitose at the molecular level and also the role of the microbial species in the digestion of melezitose. In detail, it is important to find out the influence of the gut microbiota in honey bees on the digestion of melezitose. This can be realised by understanding the functional genes of the lactic acid bacteria clusters. Functions of *Lactobacillus* genes that are responsible for digesting higher molecular sugars can give a hint to a possible adaptation of the microbiota to the diet of honey bees. Furthermore, there are more than different microbiota clusters, but also multiple different bacterial strains within the clusters. A microbiota strain comparison of the melezitose-fed bees and the bees fed with the characteristic nectar-sugars would be an important tool for a deeper understanding. Even within the not-differentiating bacteria clusters of this experiment, such as *Snodgrassella alvi*, there could be significant differences between the strain compositions of this clusters between the experimental groups. The 16S rRNA sequences of this study gave a first overview to possible adaptations on different diets. With more insights into the roles of the gut microbiota on the different diets of honey bees, the administration of special bacterial inoculations could help metabolising the poorly digestible trisaccharide melezitose. Feeding experiments with different bacterial inoculation compositions can be realised to see the direct effect on the honey bees depending to different diets. The potential of the

conducted cage experiments of this study can be used for alternative analyses of the effect of different diets on honey bees.

4.2 Discussion of the results

This thesis provides new scientific results in the biology of honey bees and hemipteran species. The trisaccharide melezitose occurs in different proportions in honeydew of hemipteran species dependent on environmental factors. It has been shown that the sugar is not only harmful for beekeepers but also for honey bees showing various disease symptoms. The comprehension of the circumstances that enhance melezitose production and the precise effect of the sugar on the health of honey bees and their microbiota is a first step to understand the complexity of honeydew production and the associated honeydew flow disease. All results lead to the assumption that less water access of the host trees increases the osmolality in the phloem sap and with that the osmoregulation by melezitose production in the hemipteran species. Especially meteorological conditions as high air temperatures and low relative humidity levels enhanced the melezitose production of hemipteran species indirectly.

Furthermore, it could be shown that melezitose trigger intestinal symptoms, an increased food uptake, gut weight and mortality of honey bees. Furthermore, 16S-amplicon sequencing pointed out a shift in the species composition of the lactic acid bacteria community within the gut microbiota of honey bees fed with melezitose. The lactic acid bacteria species *Lactobacillus kunkeei* did not occur in bees fed with melezitose, whereas *Lactobacillus* Firm-5 increased and *Lactobacillus* Firm-4 decreased in bees fed with melezitose. This may be evidence to the key role of an adapted microbiota in the processing of oligosaccharides. Especially the core member species of the gut microbiota *Lactobacillus* Firm-4 has diverse capabilities for oligosaccharide metabolism (Olofsson et al. 2014) and may explain the increase in the case of a melezitose-rich diet.

4.2.1 Production of melezitose by hemipteran species

The occurrence of melezitose-rich honey was shown to be harmful for bees (Bailey 1965; Hudson and Sherwood 1919) and led to economic losses for beekeepers (Hudson and Sherwood 1919). This demonstrates the importance of the understanding which conditions enhances melezitose production. To evaluate the effect of seasonal changes on the melezitose production of hemipteran species, melezitose proportion of honeydew

samples were compared with meteorological and site-specific factors as well the date and time of the sample collection. Since it is known that hemipteran species have to produce oligosaccharides for osmoregulation, especially when the osmolality of the phloem sap of their host trees is high, a higher melezitose production under conditions with high air temperatures and low relative humidity levels could be expected.

The aphid species living on *Picea abies* produced the highest proportions of melezitose, as shown in Fig 9. This can be explained by the lack of hydration of the shallow-root system of the spruces (Nadezhdina et al. 2014), leading to an increase of osmotically active substances in the phloem sap (Woodruff 2014) which in turn can cause higher osmoregulation and melezitose production especially of the aphid species living on spruces. The scale insect species did not show a higher production of melezitose despite living on spruces with shallow-root systems and less access to water (Nadezhdina et al. 2014). One explanation of this effect could be the more phylogenetic distantly relation of the scale insect species compared to the aphid species. Here, an optimised adaptation to phloem sap with high osmolality could have replaced melezitose production in scale insect species.

Furthermore, it has been shown that variation of melezitose content in honeydew also occurs intraspecific (Fischer et al. 2002). The phenomenon of different melezitose proportions in honeydew of the same honeydew producing species is mainly explained by a mutualistic symbiosis of hemipteran species and ants (Banks 1962; Fischer and Shingleton 2001; Samways 1983; Vantaux et al. 2011; Woodring 2004). Despite the absence of ants during the honeydew sampling in this study, differences in melezitose production within the different hemipteran species could be shown. The results demonstrate the various impacts of meteorological factors on melezitose production. The trisaccharide melezitose was formed in higher proportions when the temperature was high and the relative humidity was low (Figs 6 and 7). These data indicate that meteorological conditions consistent with high air temperatures and low relative humidity levels indirectly affects melezitose production of hemipteran species. Furthermore, an increasing melezitose production with higher altitudes of the sampling sites could be shown (Fig 8). A lower access to water with higher altitudes can be assumed resulting in higher phloem sap osmolality as shown in previous literature (Lantemona et al. 2013).

The effect of a lower access to water of the host trees could also explain the positive influence of the natural area “Schwaebische Alb” on the occurrence of melezitose (Fig 10). The natural area is based on limestone which is karstificated (fissured stone) and highly water conductive (Gimbel et al. 2016) instead of the soil of the other four natural areas (Meynen et al. 1953-1962). The positive effect of the sampling years 2017 and 2019 on melezitose production (Fig 11) can be explained by the low precipitation sum in January. This is the month with the highest average precipitation rates before honeydew season and could provide the environment with groundwater. The precipitation rate in January was under 100 l/m² in 2017 and 2019 and over 100l/m² in 2016 and 2018 (Deutscher Wetterdienst 2019). Over the spring and summer period, a time when the groundwater level is usually decreasing, the trees were exposed to less water supply. As expected, with increasing time during the sampling period April and August, the melezitose proportion increased as well (Fig 12). The negative effect of the sampling time afternoon on melezitose production (Fig 13) agrees with previous literature, showing that the photoperiodic effect enhances melezitose production (Binazzi and Scheurer 2009; Liebig 1979)]. The hemipteran species inhabit the lower surface of the branches of their host trees (Binazzi and Scheurer 2009) and therefore may get the highest sunlight irradiance when the position of the sun is not directly above the trees. This direct irradiance occurs mostly in the afternoon hours and could explain its negative effect on melezitose production.

For a deeper insight, a view of the interaction effects between the hemipteran species and meteorological variables were estimated (Table 7). Interestingly, the scale insect species and three aphid species had a negative interaction effect with air temperature on the production of melezitose. Specifically, the scale insect species tend to be less affected by higher air temperatures. However, there was no significant interaction effect between the hemipteran species and relative humidity. A detailed analysis of interaction effects could be conducted with higher sample numbers and would be important for further research.

Table 7. Results of the extended model with interaction effects hemipteran species x air temperature, hemipteran species x relative humidity as well as hemipteran species and altitude for melezitose; logit and fractional logit analysis (*p < 0.05, ** p < 0.01, *** p < 0.001). The multiple McFadden R^2 of the logit analysis is 0.278 (Cox&Snell 0.272; Nagelkerke 0.399): χ^2-test: 196.52 on 36 and 619 DF, p-value: < 0.001, N=620; the multiple R^2 of the fractional logit analysis is 0.577, N = 460.

Coefficients	**Occurrence of melezitose in honeydew**	**Proportion of melezitose in honeydew**
Hemipteran species and air temperature		
Baseline effect (*Cinara confinis*)	0.500* (p = 0.012)	0.024*** (p < 0.001)
Scale insect species on *Picea abies*		
Physokermes piceae	-0.667** (p = 0.002)	-0.018** (p = 0.004)
Physokermes hemicryphus	-0.707** (p = 0.004)	-0.049** (p = 0.008)
Aphid species on *Picea abies*		
Cinara pilicornis	-0.164 (p = 0.513)	-0.022*** (p < 0.001)
Cinara piceae	-0.481 (p = 0.999)	-0.012 (p = 0.162)
Aphid species on *Abies alba*		
Cinara curvipes	-0.813 (p = 0.988)	-0.050*** (p < 0.001)
Cinara pectinatae	-0.426* (p = 0.036)	-0.013 (p = 0.080)

Hemipteran species and relative humidity		
Baseline effect (*Cinara confinis*)	-0.082 (p = 0.128)	0.001 (p = 0.724)
Scale insect species on *Picea abies*		
Physokermes piceae	0.072 (p = 0.425)	-0.004 (p = 0.461)
Physokermes hemicryphus	-0.206 (p = 0.089)	-0.019 (p = 0.333)
Aphid species on *Picea abies*		
Cinara pilicornis	0.038 (p = 0.646)	-0.002 (p = 0.499)
Cinara piceae	0.091 (p = 0.999)	-0.007 (p = 0.057)
Aphid species on *Abies alba*		
Cinara curvipes	-0.737 (p = 0.987)	-0.001 (p = 0.744)
Cinara pectinatae	0.061 (p = 0.279)	-0.003 (p = 0.373)
Hemipteran species and altitude		
Baseline effect (*Cinara confinis*)	0.009 (p = 0.101)	0.001 (p = 0.062)
Scale insect species on *Picea abies*		
Physokermes piceae	-0.965 (p = 0.991)	-0.001 (p = 0.226)
Physokermes hemicryphus	-0.016* (p = 0.017)	-0.001 (p = 0.110)

Aphid species on *Picea abies*		
Cinara pilicornis	0.001 (p = 0.964)	-0.001* (p = 0.031)
Cinara piceae	-0.965 (p = 0.991)	0.018* (p = 0.010)
Aphid species on *Abies alba*		
Cinara curvipes	0.035 (p = 0.999)	-0.001 (p = 0.964)
Cinara pectinatae	-0.007 (p = 0.183)	-0.001 (p = 0.099)

During the study I was challenged by environmental factors that affected the honeydew production of various hemipteran species differently. As I only collected the present honeydew, it resulted in asymmetric sample numbers of the different hemipteran species producing the honeydew and of the different natural areas where the hemipteran species lived (Table 8) and the interpretation for single species had to be cautious. I would therefore recommend conducting follow-up experiments with hemipteran species fed on different sucrose concentrations in the laboratory to observe the direct effect of osmotic pressure on melezitose production. These experiments would help to quantify the exact melezitose production for each species under certain environmental conditions. Nevertheless, the data I collected of melezitose proportions found in honeydew in the nature are crucial, as they show the complexity of factors influencing the production of melezitose.

Table 8. Crosstable with sample numbers of the collected honeydew droplets from the seven different hemipteran species (first column) and the five natural areas (first line) "Neckar- und Tauber-Gaeuplatten" (NTG), "Schwaebische Alb" (SA), "Schwaebisches Keuper-Lias Land" (SKLL), "Schwarzwald" (S) and "Voralpines Huegel- und Moorland" (VHM).

	NTG	**SA**	**SKLL**	**S**	**VHM**
Cinara confinis	40	0	5	3	0
Cinara curvipes	16	0	5	1	0
Cinara pectinatae	16	0	45	165	0
Cinara piceae	0	0	0	26	9
Cinara pilicornis	2	7	43	88	0
Physokermes hemicryphus	2	0	9	19	0
Physokermes piceae	14	13	60	0	32

A higher osmoregulation by hemipteran species could also be conducted by producing erlose instead of melezitose, the only other oligosaccharide found in honeydew (Shaaban et al. 2020). In this study, no meteorological effect could be shown to affect the production of erlose (Table 9). There was a negative effect of all hemipteran species on the production of erlose. A negative effect of the natural area "Schwarzwald" consisting of clayey red sandstone could be found and a positive effect of "Voralpines Huegel- and Moorland" with soil based on swamp, sand and graves (Meynen et al. 1953-1962), both natural areas with water reservoir capacities. Furthermore, a negative effect of the sampling year 2018 could be found, when the precipitation rate was higher and a positive effect of the sampling month June and August on the production of erlose (Table 9). The

results indicate that the hemipteran species rather produce melezitose for the osmoregulation of a higher osmolality in the phloem sap especially due to high air temperatures and low relative humidity levels. This is consistent with the literature showing that melezitose is the most dominant sugar in honeydew (Shaaban et al. 2020).

Table 9. Results of the model for erlose; logit and fractional logit analysis: Estimate and p-value of the constant, hemipteran species, air temperature, relative humidity, altitude, natural area, sampling year, month and time data (*p < 0.05, ** p < 0.01, *** p < 0.001). The multiple McFadden R^2 of the logit analysis is 0.246 (Cox&Snell 0.279; Nagelkerke 0.380): χ^2-test: 203.05 on 18 and 619 DF, p-value: < 0.001, N=620; the multiple R^2 of the fractional logit analysis is 0.359, N =384.

Coefficients	**Occurrence of erlose in honeydew**	**Proportion of erlose in honeydew**
[a] Constant	0.358 (p = 0.764)	-
Air temperature	0.035 (p = 0.227)	0.001 (p = 0.819)
Relative humidity	0.014 (p = 0.155)	0.000 (p = 0.877)
Altitude	0.001 (p = 0.450)	0.000 (p = 0.326)
Hemipteran species living on *Picea abies*		
Physokermes piceae	-1.354** (p = 0.032)	-0.093*** (p = 0.003)
Physokermes hemicryphus	0.106 (p = 0.890)	-0.078*** (p = 0.004)
Cinara pilicornis	-2.484*** (p < 0.001)	-0.075* (p = 0.072)
Cinara piceae	-4.609*** (p < 0.001)	-0.122*** (p = 0.006)
Hemipteran species living on *Abies alba*		
Cinara curvipes	-1.402** (p = 0.030)	-0.116*** (p < 0.001)
Cinara pectinatae	1.021* (p = 0.088)	-0.041*** (p = 0.003)
Natural areas		
Schwaebische Alb	0.580 (p = 0.391)	0.011 (p = 0.534)

Schwaebisches Keuper-Lias-Land	-0.301 (p = 0.492)	0.012 (p = 0.276)
Schwarzwald	0.934** (p = 0.034)	0.029** (p = 0.037)
Voralpines Huegel- u. Moorland	0.913 (p = 0.200)	0.061*** (p < 0.001)
Sampling year		
2017	-0.177 (p = 0.704)	0.004 (p = 0.906)
2018	-1.194** (p = 0.016)	0.007 (p = 0.433)
2019	-0.063 (p = 0.917)	0.076 (p = 0.168)
Sampling month		
May	-	0.086 (p = 0.131)
June	-	0.136** (p = 0.019)
July	-	0.098 (p = 0.105)
August	-	0.140** (p = 0.022)
Sampling time		
Afternoon	-0.324 (p = 0.205)	-0.009 (p = 0.260)
Evening	-0.101 (p = 0.744)	0.004 (p = 0.673)

The base categories for the logit model are the hemipteran species *Cinara confinis*, the natural area Neckar-Tauber-Gaeuplatten, the sampling year 2016, month April and time Morning (expected mean value of melezitose proportion when all explanatory variables are 0).

[a] The constant absorbs the baseline categories that are needed to interpret the coefficients as partial effects.

Since erlose as well as melezitose are built from the same molecules, the question arises, why hemipteran species could prefer to produce melezitose. One explanation could be the well-known trophobiosis with ants. Ants feed preferably on melezitose in honeydew and protect the aphid species in front of predators (Banks 1962; Vantaux et al. 2015). The fact that the scale insect species living on *Picea abies* did not produce significantly more melezitose for osmoregulation, as it is the case for the aphid species on *Picea abies*, could also be explained by the missing trophobiosis between ants and scale insect species. Scale insect species have a phylogenetic distantly relation to aphid species. An optimised adaptation to phloem sap with high osmolality could have replaced the melezitose production and trophobiosis in scale insect species.

Although it is well-known that most enzymes show increased activity with increasing temperature, these results are consistent with the literature finding that more honey with high proportions of melezitose occurs in dry seasons (Owen 1978). With the ongoing climate change, more regions will be confronted with higher temperatures and lower relative humidity levels (Pasqui and Di Giuseppe 2019) and thus favour melezitose production in hemipteran species. Effects of the natural areas of South Germany can be transferred to regions throughout Europe having similar temperature and humidity situations in the near future.

In conclusion, it can be assumed that less water access of the host trees increases the osmolality in the phloem sap and enhances the osmoregulation by melezitose production in hemipteran species. Consequently, honey bees process more melezitose-rich honeydew to melezitose-rich honey, with the subsequent known fatal consequences: Honey with high proportions of melezitose crystallise rapidly, leading to an obstruction of the combs (Hudson 1946) and high economic losses for the beekeepers. Another problem is the difficult food uptake for honey bees because of its crystallisation. Furthermore, it recently could be shown that melezitose leads to severe intestinal disease symptoms in honey bees. The complex interaction of the trisaccharide melezitose leading to negative impacts on bees and beekeepers (Seeburger et al. 2020) is a so far underestimated problem and strengthens the importance of broadening our knowledge about hemipteran biology. By understanding the conditions which are favourable for melezitose production, beekeepers can avoid melezitose-rich honey by timely removing their bee hives out of forest regions.

The comprehension of the phenomenon therefore provides a promising tool to develop an early warning system for beekeepers to avoid melezitose.

4.2.2 Impact of melezitose on honey bees

This study describes the multiple effects of melezitose in honey bees and indicates its key significance for the occurrence of the described honeydew flow disease. This disease already led to colony losses during winter (Horn 1985), which are usually noticed by beekeepers and therefore documented in beekeepers' journals (Imdorf et al. 1985). In three feeding experiments, bees fed with melezitose showed intestinal symptoms, and increased food uptake, gut weight and mortality.

In order to analyse the progress of uptake and digestion of melezitose, the relative proportion of melezitose in the feed and in the crops was measured. The melezitose proportion decreased from feed to crop (Table 6), suggesting that bees or their crop microbiota did metabolise melezitose. In social insects, the proventriculus provides the individual with the amount of food needed to cover their actual energy needs, leaving as much as possible in the crop (Roces and Blatt 1999). Nevertheless, honey bees are known to digest harmful food to conserve the health of the colony (Colin, M.E., Ball, B.V. Kilani, M. 1999). Thus, it can be assumed, that the individual honey bees foraging on honeydew will digest as much of the harmful melezitose as possible. This ensures that the remaining colony is provided with easily digestible food which is processed into honey.

Interestingly, bees fed with melezitose from day 10 had twice as much food uptake than control-fed bees (Fig 15). Both the doubled food uptake and the increase of melezitose can lead to an accumulation of food in the gut. The average gut-body weight ratio that was eight percent higher in bees fed with melezitose (Fig 16) also explains the morphological symptom of the swollen abdomen. These results lead us to the assumption that bees need more time to digest melezitose or are unable to digest the absorbed melezitose and thus the sugar content in the intestine increases. The rising amount of melezitose can lead to the severe symptoms that were observed in these feeding experiments. Concentrating on the life expectancy of bees, the symptoms of honeydew flow disease appeared with increasing age (first on day 10 after emergence). A gradual accumulation of melezitose with the lifetime of honey bees can be assumed. The shorter

lifespan of bees fed with melezitose compared to the control group could be explained by their digestive problems and their influence on the physiology of their abdomen (Fig 17).

Consequently, I expected changes in their intestinal microbiota and performed 16S-amplicon sequencing to check for microbial shifts. There was a significant shift in the lactic acid bacteria species: *Lactobacillus kunkeei* did not occur in the bees fed with melezitose (Fig 22) and the proportion of *Lactobacillus* Firm-4 decreased (Fig 23). Conversely, the proportion of *Lactobacillus* Firm-5 increased with feeding on melezitose (Fig 24). Lactobacillus species ferment sugars to produce lactic or acetic acid and are adapted to sugar-rich environments with high acidity (Anderson et al. 2011). They are known to be dominant in the crop and most abundant in the ileum and rectum (Martinson et al. 2012; Powell et al. 2014). Within the bee-associated Lactobacilli, *Lactobacillus mellifera* and *Lactobacillus mellis* form a distinct phylogenetic cluster referred as *Lactobacillus* Firm-4, and the species *Lactobacillus apis*, *Lactobacillus helsingborgensis*, *Lactobacillus kimbladii*, *Lactobacillus kullabergensis*, and *Lactobacillus melliventris* are referred as *Lactobacillus* Firm-5 (Olofsson et al. 2014). Both clusters are located within the hindgut, *Lactobacillus* Firm-4 in the rectum, *Lactobacillus* Firm-5 in the ileum and rectum (Olofsson et al. 2014; Zheng et al. 2018). While these two clusters are rarely detected outside the hindgut, *Lactobacillus kunkeei* is also found outside the honey bee body in the hive. *Lactobacillus mellifera*, which belongs to the Firm-4 cluster is only capable of producing acids from fructose, while the species of the Firm-5 cluster can also utilise the sugars galactose, mannose, sorbose and sucrose (Olofsson et al. 2014). The more diverse capabilities for oligosaccharide metabolism of *Lactobacillus* Firm-5 species may explain their increase within the melezitose-fed bees.

These findings point out the importance of lactic acid bacteria for the nutrition of their host. The bees that were fed with control diet first and from day 10 on with melezitose diet died earlier than those fed with melezitose from the fifth day onwards (Fig 17). This may be seen as further evidence for the key role of an adapted microbiota in the processing of oligosaccharides. Bees fed with melezitose from the fifth day may have grown an adapted intestinal microbiota capable of degrading the oligosaccharides at an increased rate. The change in diet from the control to melezitose diet on day 10 day may therefore

have led to a rapid accumulation of melezitose in the guts of unadapted bees with acute, often lethal effects.

Although it is known that honey bees produce the enzyme invertase (Oddo et al. 1998; Simpson et al. 1968) with which it is possible to break down melezitose (Detrain et al. 2010; White and Maher 1953), it could not be shown in this study, whether the bees digested parts of the melezitose. Despite this, it can be assumed that the honey bees could not digest all of the melezitose and that the trisaccharide accumulated in the guts, because the gut weight increased in these individuals. Honey bees are capable of breaking down melezitose in characteristic nectar monosaccharides, however they probably need more time and energy to realise that. Within this time, melezitose could accumulate and lead to the disease symptoms shown in the experiments. The melezitose-fed honey bees showed diverse symptoms in high numbers that are comparable with the symptoms of the honey flow disease, described in many beekeepers' journals (Bailey 1965; Horn 1984; Hudson and Sherwood 1919; Imdorf et al. 1985) and shown in Fig 14. It is essential to understand, how the accumulation of melezitose could have led to the disease symptoms. It was shown that the sugar did not crystallise within the guts, but they still can destroy the gut tissue while accumulating. Besides, the gain of weight could lead to the problems with movement in honey bees. The whole abdomen laid on the bottom of the cage and the bees could barely move.

The honeydew flow disease is described to appear especially in winter (Bailey 1965; Hudson and Sherwood 1919) and beekeepers journals described that entire colonies died within this time period (Imdorf et al. 1985). The arising question is, why the disease could accumulate during the cold days of the year. One explanation can be the hygienic behaviour of honey bees in general (Sparks et al. 2010). Usually, honey bees can excrete their gut content, which can prevent them from the shown disease symptoms. To obtain a hygienic hive, honey bees do not defecate their gut content within their hives, but when flying outside (Sparks et al. 2010). This is only possible for honey bees, when the outside temperatures are high enough (Sparks et al. 2010) and not given during most winters in the honeydew regions in Austria, South Germany and Switzerland. This leads to the assumption, that the bees cannot dispose the increasing amount of melezitose during a long time period which may strengthen the honeydew flow disease. Since the bees could

not defecate inside the feeding experiment cages, the experiment simulated the winter period and the same honeydew flow disease symptoms could be shown as in the field. Furthermore, even bees with the possibility to defecate melezitose could have intestinal problems. The oligosaccharide is a bigger molecule than the monosaccharides and could need more time to process from the crop into the gut and more time to defecate. Also, defecating the melezitose without digesting only means a loss of energy for the honey bees. Honey bees could also be not capable of defecating melezitose because of the large size of the molecule. Overall, the consumption of melezitose would not be as nutritious as the nectar characteristic sugars glucose, fructose and sucrose. During warm periods, the bees can fly out but they could still have problems with digesting or getting rid of melezitose. It is also important to mention, that winter bees live several months (Fukuda and Sekiguchi 1966) and that there is no new brood during that time. During honeydew flow periods in spring and summer, the so-called summer honey bees only live several weeks (Fukuda and Sekiguchi 1966). They can collect and feed on melezitose simultaneously and could show disease symptoms from the diet. However, the melezitose rich honeydew flow periods generally only last several weeks (Liebig 1999) and the short-living summer bees with disease symptoms can easier be replaced by new healthy brood. Whereas during winter, the bees feed on the honey reserves in the hives. The melezitose-rich honeydew flow can be so profitable, that whole hives are full of the melezitose-rich honey during winter, when no beekeeper is removing the honey. In this time, the bees have to feed on melezitose-rich honey and the amount of the trisaccharide increases rapidly within the bees' gut. This could be one more reason, why the disease is very common during winter (Bailey 1965; Hudson and Sherwood 1919) and even lead to colony losses (Imdorf et al. 1985).

Furthermore, the question arises, why honey bees in the field collect honeydew with melezitose at all. There are at least two explanations possible, why honey bees could collect melezitose even if the sugar is not the most nutritious one for bees. It is possible, that honey bees merely forage in forests, when no nectar is available. This can happen in times when the flowering plants are missing, especially in the late summer or within monocultural landscapes such as large-scale rapeseed fields. In addition, nowadays the number of beekeepers is increasing and therewith the number of bee hives. As a

consequence, the amount of nectar could not be sufficient for the number of bees living in the foraging area.

Based on the results of the present study, it can be recommended to avoid honeydew with high contents of melezitose. Beekeepers can remove their colonies from the forests when specific environmental conditions are given that are highlighted within this thesis. This provides an important advance in the knowledge of honey bee health and emphasises the consequences between different sugar diets on honey bees. Since foraging is one of the major issues to establish a healthy bee colony (Brodschneider and Crailsheim 2010), the knowledge about nutritious effects on honey bees is of high importance. Melezitose also crystallises quickly, obstructs the combs and leads to economic losses for the beekeepers that cannot harvest the honey by centrifugation (Hudson and Sherwood 1919). This study clarified the phenomenon of diverse melezitose proportions even of the honeydew within the same hemipteran species. Regarding the increase in melezitose production under circumstances similar to drought, it can be assumed that the high air temperatures and low relative humidity levels increase the molality in the phloem sap and with that the osmoregulation in hemipteran species. This leads to the high differences in the sugar compositions between honeydew and nectar. In the case beekeepers move their hives into forest regions to gain the high valuable honeydew honey (Fairchild et al. 2003; Persano Oddo and Piro 2004), bees are forced to forage on honeydew. When harvesting honeydew honey, beekeepers should be aware of the fact, that melezitose has a negative impact on honey bees and that they should observe the forests for melezitose conditions.

4.3 Outlook

This study lead to a deeper understanding of the importance of nutrition on honey bee health and the tasks of their gut microbiota in digesting and metabolising the sugars. Furthermore, this study provides the possibility to counteract the described honeydew flow disease, with the knowledge about the shown environmental factors enhancing melezitose production. In the future, beekeepers can pay attention when locating their hives into forest regions. By observing the abundance of certain hemipteran species, they have a warning sign for high melezitose conditions. Beekeepers can also observe the described environmental conditions, especially the temperature and relative humidity levels and remove their colonies when melezitose conditions are given. It is also possible

to remove the freshly sampled melezitose-rich honeydew and honey within the hive combs. Beekeepers can also give blossom honey to their bee hives to mix the two types of honey and with that decrease the proportion of melezitose.

In summary, within this study the factors that are enhancing the production of melezitose have been deciphered and the adverse impact of the trisaccharide on honey bees could be displayed. With this knowledge, beekeepers can pay attention and avoid the poorly digestible oligosaccharide while gaining the high valuable honeydew honey.

5 Summary

5.1 Summary

Honeydew honey is a honey type which is of high economic importance in Europe. Phloem sap feeding insects of the order *Hemiptera* (true bugs) excrete honeydew, the key component of honeydew honey. Beekeepers move their hives between forest regions so that their bees can process the honeydew into honey. In case of high osmolality in the phloem sap of the hemipterans' host trees, they counteract osmotic pressure by osmoregulation and produce oligosaccharides such as melezitose. Melezitose-rich honeydew honey is a major issue for beekeepers; it crystallises and obstructs the combs, leading to an economical loss. Nevertheless, precise analyses of the conditions of the occurrence of melezitose have not been realised. Furthermore, it is not known which impacts the trisaccharide has on honey bee health and the honeydew flow disease documented in beekeepers' journals can have one explanation in the nutrition on melezitose.

In order to determine influence factors for the emergence of melezitose, more than 600 honeydew droplets from defined honeydew producer species were collected under different environmental conditions (hemipteran species (host tree specific), natural area, air temperature, relative humidity, altitude, time of the year and of the day) between 2016 and 2019. The sugar spectra were analysed by high performance anion exchange chromatography with pulsed amperometric detection. To obtain the impact of melezitose on honey bee health, additional feeding experiments with daily evaluation of food uptake, gut-body weight ratio and mortality have been realised between 2017 and 2019. Additionally, comprehensive 16S rRNA Illumina sequencing of the gut microbial community has been performed.

Remarkable differences could be found in the amount of melezitose between honeydew samples collected from different honeydew producer species and according to different environmental conditions. Air temperature increases and decreases in relative humidity increased the melezitose production in honeydew by the observed seven hemipteran species. Both, scale insect species on *Picea abies* and aphid species on *Abies alba* produced significantly less honeydew containing melezitose than aphid species on *Picea*

abies. Additionally, honeydew with increased melezitose content was significantly more frequent collected in natural areas with limited water reservoir capacities, at higher altitudes and years with low precipitation. All results lead to the conclusion that hemipteran species produce more melezitose when the host trees have less access to water, increasing the osmolality of the phloem sap and indirectly enhancing the osmoregulation with producing melezitose by hemipteran species. Bees fed with melezitose showed increased food uptake and higher gut-body weight ratio than the control groups. Furthermore, melezitose feeding caused disease symptoms such as swollen abdomen, abdomen tipping and impaired movement and a significantly higher mortality than in control groups. Gut microbiota analyses indicated a shift of the bacterial species *Lactobacillus* Firm-4 and *Lactobacillus kunkeei* in favour of *Lactobacillus* Firm-5 in melezitose fed bees.

This PhD project provides the important knowledge about the indicators that point out an enhanced melezitose production. This is a valuable contribution to design a warning system for beekeepers that will help to prevent harmful nutrition for honey bees or crystallised honey in the future by timely removal of bee colonies from local regions at risk. Additionally, feeding experiments point out the high effort that is required for the degradation process of the large-molecule melezitose. This effort might lead to a higher uptake of food, heavier guts, shorter lifespan and a higher susceptibility to intestinal diseases. Finally, an evidence was presented that the lactic acid bacteria of the gut microbiota are significantly involved in the digestion of melezitose.

5.2 Zusammenfassung

Honigtauhonig ist eine Honigsorte die in Europa eine hohe ökonomische Rolle spielt. Insekten der Ordnung *Hemiptera* (Schnabelkerfe) ernähren sich von Phloemsaft und scheiden den daraus entstehenden Honigtau wieder aus, welcher wiederum einen wesentlichen Bestandteil des Honigtauhonigs ausmacht. Imker versetzen ihre Bienenvölker zwischen den Waldregionen, sodass ihre Bienen den Honigtau in Honig weiterverarbeiten können. Bei einer hohen Osmolalität des Phloemsaftes der Wirtsbäume der Hemipteren, wirken diese dem osmotischen Druck durch Osmoregulation entgegen und produzieren Oligosaccharide wie Melezitose. Honigtauhonig mit einem hohen Anteil an Melezitose stellt eine große Problematik für Imker dar, da dieser schnell auskristallisiert und die Waben blockiert, welches zu ökonomischen Verlusten führt. Trotzdem ist bisher nicht präzise analysiert worden, unter welchen Bedingungen Melezitose bevorzugt produziert wird. Außerdem ist nicht bekannt was für Auswirkungen das Trisaccharid auf die Gesundheit von Honigbienen hat. Die Waldtrachtkrankheit, welche oft in Imkerzeitschriften dokumentiert wird, könnte unter anderem durch die Ernährung mit Melezitose erklärt werden.

Um den Einfluss verschiedener Umweltfaktoren auf das Vorkommen von Melezitose zu ermitteln, wurden zwischen 2016 und 2019 mehr als 600 Honigtautropfen von bestimmten Honigtauerzeuger-Arten unter verschiedenen Umweltkonditionen gesammelt (*Hemiptera* Art (Wirtsbaum-spezifisch), Naturraum, Lufttemperatur, relative Luftfeuchte, Höhenmeter, Jahr, Monat und Tageszeit). Das Zuckerspektrum wurde mittels einer Ionenaustauschchromatographie mit pulsierter amperometrischer Detektion analysiert. Um die Auswirkungen von Melezitose auf die Gesundheit der Honigbiene zu testen, wurden zusätzlich zwischen 2017 und 2019 Fütterungsversuche durchgeführt, bei denen täglich die Futteraufnahme, das Darm-Körper-Gewichtsverhältnis und die Mortalität dokumentiert wurden. Zusätzlich wurden umfassende 16S rRNA Illumina Sequenzierungen der Darmbakteriengemeinschaft durchgeführt.

Zwischen den gesammelten Honigtauproben der verschiedenen Honigtauerzeugerarten gemäß den verschiedenen Umweltbedingungen konnten bemerkenswerte Unterschiede festgestellt werden. Lufttemperaturzunahmen und Abnahmen in der relativen Luftfeuchte erhöhten die Melezitoseproduktion im Honigtau durch die beobachteten sieben

Hemiptera Arten. Abhängig von den verschiedenen Umweltfaktoren konnten beachtliche Unterschiede des Melezitosegehaltes im Honigtau verschiedener Honigtauerzeuger festgestellt werden. Höhere Lufttemperaturen und niedrige relative Luftfeuchte führten zu höherer Melezitoseproduktion innerhalb der *Hemiptera* Arten. Sowohl die Schildlausarten auf *Picea abies* als auch die Rindenlausarten auf *Abies alba* produzierten signifikant weniger Honigtau welcher Melezitose enthielt, als die Rindenlausarten auf *Picea abies*. Außerdem wurde Honigtau mit erhöhtem Melezitosegehalt signifikant häufiger in Naturräumen mit limitierenden Wasserspeicherkapazitäten, in größeren Höhen und in Jahren mit geringerem Niederschlag gesammelt. Alle Ergebnisse führen zu der Annahme, dass *Hemiptera* Arten mehr Melezitose produzieren, wenn die Wirtsbäume weniger Zugang zu Wasser haben, welches die Osmolalität ihres Phloemsaftes erhöht und damit indirekt die Osmoregulation mit der Produktion von Melezitose durch die *Hemiptera* Arten begünstigt. Bienen, die mit Melezitose gefüttert wurden, zeigten eine höhere Futteraufnahme und ein höheres Darm-Körper Gewichtsverhältnis als die Kontrollgruppen. Des Weiteren führte das Füttern mit Melezitose zu Krankheitssymptomen wie geschwollenen Abdomen, Abdomen tippen, einer erschwerten Fortbewegung und einer signifikant höheren Mortalität als in den Kotrollgruppen. Die Darmbakterienanalysen ergaben eine Verschiebung der Bakterienart *Lactobacillus* Firm-4 und *Lactobacillus kunkeei* zugunsten von *Lactobacillus* Firm-5 in Bienen, die mit Melezitose gefüttert wurden.

Diese Dissertation liefert wichtige Information über die Indikatoren für eine begünstigte Melezitoseproduktion. Dies ist ein wertvoller Beitrag für den Aufbau eines Warnsystems für Imker, welches helfen wird, schädliche Ernährung für Honigbienen oder kristallisierten Honig zu vermeiden, indem die Bienenkolonien rechtzeitig von den lokalen Risikoregionen entfernt werden. Des Weiteren zeigen die Fütterungsversuche den hohen Aufwand, der von den Bienen benötigt wird, um das große Molekül Melezitose abzubauen. Dies kann zu einer höheren Futteraufnahme, schwereren Därmen, kürzeren Lebensdauer und einer höheren Anfälligkeit für Darmkrankheiten führen. Schlussendlich wurde der Nachweis erbracht, dass die Milchsäurebakterien der Darmmikrobiota signifikant an der Verdauung von Melezitose beteiligt sind.

6 References

Almeida, L. G. de, Moraes, L. A. B. de, Trigo, J. R., Omoto, C., Cônsoli, F. L. (2017) The gut microbiota of insecticide-resistant insects houses insecticide-degrading bacteria: A potential source for biotechnological exploitation. PLOS ONE **12**(3), e0174754, DOI: 10.1371/journal.pone.0174754

Anderson, K. E., Sheehan, T. H., Eckholm, B. J., Mott, B. M., DeGrandi-Hoffman, G. (2011) An emerging paradigm of colony health: microbial balance of the honey bee and hive (*Apis mellifera*). Insect Soc **58**, 431–444, DOI: 10.1007/s00040-011-0194-6

Ashford, D., Smith, W., Douglas, A. (2000) Living on a high sugar diet: the fate of sucrose ingested by a phloem-feeding insect, the pea aphid Acyrthosiphon pisum. Journal of insect physiology **46**(3), 335–341, DOI: 10.1016/S0022-1910(99)00186-9

Bailey, L. (1965) The occurence of chronic and acute bee paralysis viruses in bees outside britain. J Invertebr Pathol **7**(2), 167–169, DOI: 10.1016/0022-2011(65)90031-5

Baker, H. G., Baker, I. (1983) Floral nectar sugar constituents in relation to pollinator type. Handbook Exp Poll Biol **1**, 117–141, DOI: 10.2307/2443763

Banks, C. J. (1962) Effects of the ant Lasius niger (L.) on insects preying on small populations of Aphis fabae Scop. on bean plants. Ann Appl Biol **50**(4), 669–679, DOI: 10.1111/j.1744-7348.1962.tb06067.x

Binazzi, A., Scheurer, S., eds. (2009) Atlas of the honeydew producing conifer aphids of Europe. Aracne, Rom

Brodschneider, R., Crailsheim, K. (2010) Nutrition and health in honey bees. Apidologie **41**(3), 278–294, DOI: 10.1051/APIDO/2010012

Castro-Vázquez, L., Díaz-Maroto, M. C., Pérez-Coello, M. S. (2006) Volatile composition and contribution to the aroma of spanish honeydew honeys. Identification of a new chemical marker. J Agr Food Chem **54**(13), 4809–4813, DOI: 10.1021/jf0604384

Claesson, M. J., Clooney, A. G., O'Toole, P. W. (2017) A clinician's guide to microbiome analysis. Nature reviews. Gastroenterology & hepatology **14**(10), 585–595, DOI: 10.1038/nrgastro.2017.97

Colin, M.E., Ball, B.V. Kilani, M. (1999) Bacterial diseases. Bee Dis Diagnosis **25**, 31–59

Crane, E., Walker, P. (1985) Important Honeydew Sources and their Honeys. Bee World **66**(3), 105–112, DOI: 10.1080/0005772X.1985.11098832

Crotti, E., Sansonno, L., Prosdocimi, E. E., Vacchini, V., Hamdi, C., Cherif, A., Gonella, E., Marzorati, M., Balloi, A. (2013) Microbial symbionts of honeybees: a promising tool to improve honeybee health. New Biotechnol **30**(6), 716–722, DOI: 10.1016/j.nbt.2013.05.004

D'Alvise, P., Böhme, F., Codrea, M. C., Seitz, A., Nahnsen, S., Binzer, M., Rosenkranz, P., Hasselmann, M. (2018) The impact of winter feed type on intestinal microbiota and parasites in honey bees. Apidologie **49**, 252–264, DOI: 10.1007/s13592-017-0551-1

D'Alvise, P., Seeburger, V., Gihring, K., Kieboom, M., Hasselmann, M. (2019) Seasonal dynamics and co-occurrence patterns of honey bee pathogens revealed by high-throughput RT-qPCR analysis. Ecology and evolution **9**(18), 10241–10252, DOI: 10.1002/ece3.5544

Detrain, C., Verheggen, F. J., Diez, L., Wathelet, B., Haubruge, E. (2010) Aphid-ant mutualism: how honeydew sugars influence the behaviour of ant scouts. Physiol Entomol **35**(2), 168–174, DOI: 10.1111/j.1365-3032.2010.00730.x

Deutscher Wetterdienst (2019) Das interaktive Portal des Climate Data Centers Version 2.0 [online] ftp://opendata.dwd.de/climate_environment/CDC/regional_averages_DE/monthly/precipitation

Douglas, A. E. (2006) Phloem-sap feeding by animals: problems and solutions. J Exp Bot **57**(4), 747–754, DOI: 10.1093/jxb/erj067

Edgar, R. C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res **32**(5), 1792–1797, DOI: 10.1093/nar/gkh340

Edgar, R. C. (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics **26**(19), 2460–2461, DOI: 10.1093/bioinformatics/btq461

Edgar, R. C. (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods **10**, 996–998, DOI: 10.1038/nmeth.2604

Engel, P., Bartlett, K. D., Moran, N. A. (2015) The Bacterium Frischella perrara Causes Scab Formation in the Gut of its Honeybee Host. Am Soc Microbiol **6**(3), 1–8, DOI: 10.1128/mBio.00193-15

Engel, P., Martinson G., V., Moran, N. A. (2012) Functional diversity within the simple gut microbiota of the honey bee. Proc Natl Acad Sci USA **109**(27), 11002–11007, DOI: 10.1073/pnas.1202970109

Engel, P., Moran, N. A. (2013a) Functional and evolutionary insights into the simple yet specific gut microbiota of the honey bee from metagenomic analysis. Gut Microb **4**(1), 60–65, DOI: 10.4161/gmic.22517

Engel, P., Moran, N. A. (2013b) The gut microbiota of insects - diversity in structure and function. FEMS microbiology reviews **37**(5), 699–735, DOI: 10.1111/1574-6976.12025

Fairchild, G. F., Nichols, J. P., Capps, Oral, Jr. (2003) Observations on Economic Adulteration of High-Value Food Products: The Honey Case. Journal of Food Distribution Research **34**(2), 1–8

Fink, D., Dobbelstein, E., Barbian, A., Lohaus, G. (2018) Ratio of sugar concentrations in the phloem sap and the cytosol of mesophyll cells in different tree species as an indicator of the phloem loading mechanism. Planta **248**(3), 661–673, DOI: 10.1007/s00425-018-2933-7

Fischer, M. K., Shingleton, M. K. (2001) Host plant and ants influence the honeydew sugar composition of aphids. Funct Ecol **15**(4), 544–550, DOI: 10.1046/j.0269-8463.2001.00550.x

Fischer, M. K., Völkl, W., Schopf, R., Hoffmann, K. H. (2002) Age-specific pattern in honeydew production and honeydew composition in the aphid Metopeurum fuscoviride implications for ant-attendance. J Insect Physiol **48**(3), 319–326, DOI: 10.1016/s0022-1910(01)00179-2

Fisher, D. B., Wright, J.P., Mittler, T.E. (1984) Osmoregulation by the aphid Myzus persicae: A physiological role for honeydew oligosaccharides. J Insect Physiol **30**(5), 387–393, DOI: 10.1016/0022-1910(84)90096-9

Fukuda, H., Sekiguchi, K. (1966) Seasonal change of the honeybee worker longevity in Sapporo, North Japan, with notes on some factores affecting the life-span. Ecol Soc Japan **16**(5), 206–212, DOI: 10.18960/seitai.16.5_206

Gimbel, K. F., Puhlmann, H., Weiler, M. (2016) Does drought alter hydrological functions in forest soils? Hydrol Earth Syst Sci **20**, 1301–1317, DOI: 10.5194/hess-20-1301-2016

Grice, E. A., Segre, J. A. (2012) The human microbiome: our second genome. Annual review of genomics and human genetics **13**, 151–170, DOI: 10.1146/annurev-genom-090711-163814

Hesse, B. D., Goisser, M., Hartmann, H., Grams, T. E. E. (2019) Repeated summer drought delays sugar export from the leaf and impairs phloem transport in mature beech. Tree physiology **39**(2), 192–200, DOI: 10.1093/treephys/tpy122

Holuša, J., Lubojacký, J., Čurn, V., Tonka, T., Lukášová, K., Horák, J. (2018) Combined effects of drought stress and Armillaria infection on tree mortality in Norway spruce plantations. For Ecol Manage **427**, 434–445, DOI: 10.1016/j.foreco.2018.01.031

Horn, H. (1984) Ätiologie der Waldtrachtkrankheit

Horn, H. (1985) Die Waldtrachtkrankheit der Honigbiene I. Der Einfluß des Mineralstoffgehaltes in Honigtauhonigen. Apidologie **16**, 139–156, DOI: 10.1051/apido:19850204

Hudson, C. S. (1946) Melezitose and Turanose. Adv Carbohyd Chem **2**, 1–36, DOI: 10.1016/S0096-5332(08)60005-3

Hudson, C. S., Sherwood, S. F. (1919) The occurrence of melezitose in honey. J Am Chem Soc **42**(1), 116–125, DOI: 10.1021/ja01446a016

Hughes, R. L. (2019) A Review of the Role of the Gut Microbiome in Personalized Sports Nutrition. Frontiers in nutrition **6**, 191, DOI: 10.3389/fnut.2019.00191

Imdorf, A., Bogdanov, S., Kilchenmann, V. (1985) Zementhonig im Honig- und Brutraum – was dann? Schw Zent Bienenforschung **1**, 1–16

Kešnerová, L., Emery, O., Troilo, M., Liberti, J., Erkosar, B., Engel, P. (2020) Gut microbiota structure differs between honey bees in winter and summer. ISME J **14**, 801–814, DOI: 10.1101/703512

Kloft, W., Maurizio, A., Kaeser, W., eds. (1985) Waldtracht und Waldhonig in der Imkerei: Herkunft, Gewinnung und Eigenschaften des Waldhonigs. Ehrenwirth, München

Kwong, W. K., Moran, N. A. (2016) Gut microbial communities of social bees. Nat Rev Microbiol **14**(6), 374–384, DOI: 10.1038/nrmicro.2016.43

Lachman, J., Kolihová, D., Miholová, D., Košata, J., Titěra, D., Kult, K. (2007) Analysis of minority honey components: Possible use for the evaluation of honey quality. Food Chemistry **101**(3), 973–979, DOI: 10.1016/j.foodchem.2006.02.049

Lagkouvardos, I., Fischer, S., Kumar, N., Clavel, T. (2017) Rhea: a transparent and modular R pipeline for microbial profiling based on 16S rRNA gene amplicons. PeerJ **5**, 2836, DOI: 10.7717/peerj.2836

Lagkouvardos, I., Joseph, D., Kapfhammer, M., Giritli, S., Horn, M., Haller, D., Clavel, T. (2016) IMNGS: A comprehensive open resource of processed 16S rRNA microbial profiles for ecology and diversity studies. Sci Rep **6**, 1–9, DOI: 10.1038/srep33721

Lantemona, H., Abadi, A., Rachmansyah, A., Pontoh, J. (2013) Impact and Altitude and Seasons to Volume, Brix Content, and Chemical Composition of Aren Sap in North Sulawesi. IOSR-JESTFT **4**, 42–48, DOI: 10.9790/2402-0424248

Lee, J. C., Heimpel, G. E., Leibee, G. L. (2004) Comparing floral nectar and aphid honeydew diets on the longevity and nutrient levels of a parasitoid wasp. Entomologia Experimentalis et Applicata **111**(3), 189–199, DOI: 10.1111/j.0013-8703.2004.00165.x

Lee, W.-J., Hase, K. (2014) Gut microbiota-generated metabolites in animal health and disease. Nat Chem Biol **10**(6), 416–424, DOI: 10.1038/nchembio.1535

Liebig, G. (1979) Gaschromatographische und enzymatische Untersuchungen des Zuckerspektrums des Honigtaus von *Buchneria pectinatae* (Nördl.). Apidologie **10**(3), 213–225, DOI: 10.1051/apido:19790301

Liebig, G. (1999) Die Waldtracht Entstehung Beobachtung Prognose. Selbstverlag, Stuttgart

Liebig, G., Schlipf, U., Düwel, W. (1982) Witterungsverlauf und Massenwechsel der grünen Tannenhoniglaus *Cinara pectinatae* (nördl.) (Homoptera, Lachnidae) in den Jahren 1977-1981. Apidologie **13**(3), 275–295, DOI: 10.1051/apido:19820307

Lohaus, G., Moellers, C. (2000) Phloem transport of amino acids in two *Brassica napus* L. genotypes and one *B. carinata* genotype in relation to their seed protein content. Planta **211**(6), 833–840, DOI: 10.1007/s004250000349

Lohaus, G., Schwerdtfeger, M. (2014) Comparison of sugars, iridoid glycosides and amino acids in nectar and phloem sap of Maurandya barclayana, Lophospermum erubescens, and Brassica napus. PLOS ONE **9**(1), 1-8, DOI: 10.1371/journal.pone.0087689

LUBW (2010) Naturräume Baden-Württembergs. Landesanstalt für Umwelt, Messungen und, 1–73

Martinson, V. G., Moy, J. K., Moran, N. A. (2012) Establishment of characteristic gut bacteria during development of the honeybee worker. Appl Environ Microbiol **78**(8), 2830–2840, DOI: 10.1128/AEM.07810-11

Meynen, E., Gellert, J., Schmithüsen, J., Neef, E., Müller-Miny, H., Schultze, J., eds. (1953-1962) Handbuch der naturräumlichen Gliederung Deutschlands: Band 1. Bundesforschungsanstalt für Landeskunde und Raumforschung, Bad Godesberg

Moran, N. A. (2015) Genomics of the honey bee microbiome. Curr Opin Ins Sci **10**, 22–28, DOI: 10.1016/j.cois.2015.04.003

Nadezhdina, N., Urban, J., Čermák, J., Nadezhdin, V., Kantor, P. (2014) Comparative study of long-term water uptake of Norway spruce and Douglas-fir in Moravian upland. J Hydrol Hydromech **62**(1), 1–6, DOI: 10.2478/johh-2014-0001

Oddo, L. P., Piazza, M. G., Pulcini, P. (1998) Invertase activity in honey. Apidologie **30**(1), 57–65, DOI: 10.1051/apido:19990107

Olofsson, T. C., Alsterfjord, M., Nilson, B., Butler, E., Vásquez, A. (2014) *Lactobacillus apinorum* sp. nov., *Lactobacillus mellifer* sp. nov., *Lactobacillus mellis* sp. nov., *Lactobacillus melliventris* sp. nov., *Lactobacillus kimbladii* sp. nov., *Lactobacillus helsingborgensis* sp. nov. and *Lactobacillus kullabergensis* sp. nov., isolated from the honey stomach of the honeybee *Apis mellifera*. International journal of systematic and evolutionary microbiology **64**(9), 3109–3119, DOI: 10.1099/ijs.0.059600-0

Owen, D. F. (1978) Why do aphids synthesize melezitose? Nordic Society Oikos **31**(2), 264–267, DOI: 10.2307/3543572

Pasqui, M., Di Giuseppe, E. (2019) Climate change, future warming, and adaptation in Europe. Anim Front **9**, 6–11, DOI: 10.1093/af/vfy036

Pernice, M., Simpson, S. J., Ponton, F. (2014) Towards an integrated understanding of gut microbiota using insects as model systems. Journal of insect physiology **69**, 12–18, DOI: 10.1016/j.jinsphys.2014.05.016

Persano Oddo, L., Piro, R. (2004) Main European unifloral honeys: descriptive sheets. Apidologie **35**(1), 38-81, DOI: 10.1051/apido:2004049

Pohorecka, K., Skubida, P. (2004) Healthfullness of honeybee colonies (*Apis mellifera* L.) wintering on the stores with addition of honeydew honey. Bull Vet Inst Pulawy **48**, 409–413

Powell, E., Martinson, V. G., Urban-Mead, K., Moran, N. A. (2014) Routes of Acquisition of the Gut Microbiota of the Honey Bee *Apis mellifera*. Appl Environ Microbiol **80**(23), 7378–7387, DOI: 10.1128/AEM.01861-14

Price, D. R. G., Karley, A. J., Ashford, D. A., Isaacs, H. V., Pownall, M. E., Wilkinson, H. S., Gatehouse, J. A., Douglas, A. E. (2007) Molecular characterisation of a candidate gut sucrase in the pea aphid, Acyrthosiphon pisum. Insect Biochem Molec Biol **37**(4), 307–317, DOI: 10.1016/j.ibmb.2006.12.005

Ramalho, E. A., Ramalho, J. J., Murteira, J. M. (2011) Alternative estimating and testing empirical strategies for fractional regression models. Journal of Economic Surveys **25**(1), 19–68, DOI: 10.1111/j.1467-6419.2009.00602.x

Roces, F., Blatt, J. (1999) Haemolymph sugars and the control of the proventriculus in the honey bee *Apis mellifera*. J Ins Physiol **45**(3), 221–229, DOI: 10.1016/s0022-1910(98)00116-4

Samways, M. J. (1983) Interrelationship between an entomogenous fungus and two ant-homopteran (Hymenoptera: Formicidae-Hemiptera: Pseudococcidae & Aphididae) mutualisms on guava trees. Bull Entomol Res **73**(2), 321, DOI: 10.1017/S0007485300008919

Sanz, M. L., Gonzalez, M., Lorenzo, C. de, Sanz, J., Martínez-Castro, I. (2005) A contribution to the differentiation between nectar honey and honeydew honey. Food Chem **91**(2), 313–317, DOI: 10.1016/j.foodchem.2004.06.013

Seeburger, V. C., D'Alvise, P., Shaaban, B., Schweikert, K., Lohaus, G., Schroeder, A., Hasselmann, M. (2020) The trisaccharide melezitose impacts honey bees and their intestinal microbiota. PLOS ONE **15**(4), e0230871, DOI: 10.1371/journal.pone.0230871

Seeley, T., Camazine, S., Sneyd, J. (1991) Collective decision-making in honey bees: how colonies choose among nectar sources. Behav Ecol Sociobiol **28**(4), DOI: 10.1007/BF00175101

Shaaban, B., Seeburger, V., Schroeder, A., Lohaus, L. (2020) Sugar, amino acid and inorganic ion profiling of the honeydew from different hemipteran species feeding on *Abies alba* and *Picea abies*. PLOS ONE **15**(1), 1-17, DOI: 10.1371/journal.pone.0228171

Simpson, J., Riedel, I. B. M., Wilding, N. (1968) Invertase in the Hypopharyngeal Glands of the Honeybee. J Apic Res **7**(1), 29–36, DOI: 10.1080/00218839.1968.11100184

Sparks, T. H., Langowska, A., Glazaczow, A., Wilkaniec, Z., Bienkowska, M., Tryjanowski, P. (2010) Advances in the timing of spring cleaning by the honeybee *Apis mellifera* in Poland. Ecol Entomol **35**(6), 788–791, DOI: 10.1111/j.1365-2311.2010.01226.x

Urban, J., Holušová, K., Menšík, L., Čermák, J., Kantor, P. (2013) Tree allometry of Douglas fir and Norway spruce on a nutrient-poor and a nutrient-rich site. Trees **27**(1), 97–110, DOI: 10.1007/s00468-012-0771-y

Vantaux, A., Schillewaert, S., Parmentier, T., van den Ende, W., Billen, J., Wenseleers, T. (2015) The cost of ant attendance and melezitose secretion in the black bean aphid Aphis fabae. Ecol Entomol **40**, 511–517, DOI: 10.1111/een.12217

Vantaux, A., van den Ende, W., Billen, J., Wenseleers, T. (2011) Large interclone differences in melezitose secretion in the facultatively ant-tended black bean aphid Aphis fabae. J Ins Physiol **57**(12), 1614–1621, DOI: 10.1016/j.jinsphys.2011.08.014

Vasić, V., Gašić, U., Stanković, D., Lušić, D., Vukić-Lušić, D., Milojković-Opsenica, D., Tešić, Ž., Trifković, J. (2019) Towards better quality criteria of European honeydew honey: Phenolic profile and antioxidant capacity. Food Chem **274**, 629–641, DOI: 10.1016/j.foodchem.2018.09.045

Völkl, W., Woodring, J., Fischer, M., Lorenz, M. W., Hoffmann, K. H. (1999) Ant-aphid mutualisms: the impact of honeydew production and honeydew sugar composition on ant preferences. Oecologia **118**, 483-491, DOI: 10.1007/s004420050751

Wang, Q., Garrity, G. M., Tiedje, J. M., Cole, J. R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol **73**(16), 5261–5267, DOI: 10.1128/AEM.00062-07

White, J. W., Maher, J. (1953) Transglucosidation by Honey Invertase. Arch Biochem Biophys **42**(2), 360–367, DOI: 10.1016/0003-9861(53)90365-8

Wilkinson, T. L., Ashford, D. A., Pritchard, J., Douglas, A. E. (1997) Honeydew sugars and osmoregulation in the pea aphid Acyrthosiphon pisum. J Exp Biol **200**, 2137-2143

Woodring, J. (2004) Honeydew amino acids in relation to sugars and their role in the establishment of ant-attendance hierarchy in eight species of aphids feeding on tansy (Tanacetum vulgare). Physiol Entomol **29**(4), 311–319, DOI: 10.1111/j.0307-6962.2004.00386.x

Woodruff, D. R. (2014) The impacts of water stress on phloem transport in Douglas-fir trees. Tree physiology **34**(1), 5–14, DOI: 10.1093/treephys/tpt106

Zheng, H., Steele, M. I., Leonard, S. P., Motta, E. V. S., Moran, N. A. (2018) Honey bees as models for gut microbiota research. Lab animal **47**(11), 317–325, DOI: 10.1038/s41684-018-0173-x

Ziegelmann, B., Abele, E., Hannus, S., Beitzinger, M., Berg, S., Rosenkranz, P. (2018) Lithium chloride effectively kills the honey bee parasite Varroa destructor by a systemic mode of action. Sci Rep **8**(683), 1–9, DOI: 10.1038/s41598-017-19137-5

7 Appendix

Publication of the thesis

Seeburger VC, D'Alvise P, Shaaban B, Schweikert K, Lohaus G, Schroeder A, et al. (2020) The trisaccharide melezitose impacts honey bees and their intestinal microbiota. PLOS ONE 15(4): e0230871. https://doi.org/10.1371/journal.pone.0230871.

Abstract

In general, honey bees (*Apis mellifera* L.) feed on honey produced from collected nectar. In the absence of nectar, during certain times of the year or in monocultural landscapes, honey bees forage on honeydew. Honeydew is excreted by different herbivores of the order *Hemiptera* that consume phloem sap of plant species. In comparison to nectar, honeydew is composed of a higher variety of sugars and additional sugars with higher molecular weight, like the trisaccharide melezitose that can be a major constituent of honeydew. However, melezitose-containing honey is known to cause malnutrition in overwintering honey bees.

Following the hypothesis that melezitose may be the cause for the so called "honeydew flow disease", three independent feeding experiments with caged bees were conducted in consecutive years. Bees fed with melezitose showed increased food uptake, higher gut weights and elevated mortality compared to bees fed a control diet. Moreover, severe disease symptoms, such as swollen abdomen, abdomen tipping and impaired movement were observed in melezitose-fed bees. 16S-amplicon sequencing indicated that the melezitose diet changed the species composition of the lactic acid bacteria community within the gut microbiota.

Based on these results, it can be concluded that melezitose cannot be easily digested by the host and may accumulate in the hindgut. Within cages or during winter, when there is no opportunity for excretion, the accumulated melezitose can cause severe intestinal symptoms and death of the bees, probably as result of poor melezitose metabolism capabilities in the intestinal microbiota. These findings confirm the causal relation between the trisaccharide melezitose and the honeydew flow disease and indicate a possible mechanism of pathogenesis.

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RESEARCH ARTICLE

The trisaccharide melezitose impacts honey bees and their intestinal microbiota

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Abstract

In general, honey bees (*Apis mellifera* L.) feed on honey produced from collected nectar. In the absence of nectar, during certain times of the year or in monocultural landscapes, honey bees forage on honeydew. Honeydew is excreted by different herbivores of the order *Hemiptera* that consume phloem sap of plant species. In comparison to nectar, honeydew is composed of a higher variety of sugars and additional sugars with higher molecular weight, like the trisaccharide melezitose that can be a major constituent of honeydew. However, melezitose-containing honey is known to cause malnutrition in overwintering honey bees. Following the hypothesis that melezitose may be the cause for the so called 'honeydew flow disease', three independent feeding experiments with caged bees were conducted in consecutive years. Bees fed with melezitose showed increased food uptake, higher gut weights and elevated mortality compared to bees fed a control diet. Moreover, severe disease symptoms, such as swollen abdomen, abdomen tipping and impaired movement were observed in melezitose-fed bees. 16S-amplicon sequencing indicated that the melezitose diet changed the species composition of the lactic acid bacteria community within the gut microbiota. Based on these results, we conclude that melezitose cannot be easily digested by the host and may accumulate in the hindgut. Within cages or during winter, when there is no opportunity for excretion, the accumulated melezitose can cause severe intestinal symptoms and death of the bees, probably as result of poor melezitose metabolism capabilities in the intestinal microbiota. These findings confirm the causal relation between the trisaccharide melezitose and the honeydew flow disease and indicate a possible mechanism of pathogenesis.

Introduction

'Honeydew flow disease' is a common problem for managed honey bee colonies. The disease occurs, when honey bees feed on honeydew honey, especially during winter [1,2]. The clinical

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symptoms of this food toxicosis are diverse: a high number of bees remain at the hive entrance instead of foraging, bees experience massive loss of hair (which may indicate complication with virus infections) and changes their behavioural patterns [3]. Even necrotic appearances could be shown in the midgut of honey bees when fed with honeydew honey in tent experiments [3]. This condition can deteriorate to a point where colonies collapse within short time. This is known to beekeepers and discussed in beekeeping journals [4], but the precise reason has remained unknown.

Honey bees primarily forage on nectar. The three most common nectar sugars are the two monosaccharides glucose and fructose, and the disaccharide sucrose [5]. However, seasonally or locally nectar plants are lacking, and nectar is not available. In such situations honey bees tend to forage on honeydew, which is not produced from nectaries, but by herbivores of the order *Hemiptera* as waste product from phloem sap of plant species [1]. In addition to the nectar sugars, honeydew contains more disaccharides such as maltose and melibiose, and trisaccharides such as erlose and melezitose [6]. These sugars are produced from aphids with α-glucosidase/transglucosidase [7].

Honey bees process nectar into blossom honey and honeydew into honeydew honey. In comparison to blossom honey, the mineral content (aluminium, boron, copper, magnesium, manganese, nickel and zinc) of honeydew honeys is up to four times higher [8,9]. Moreover, honeydew honey contains more oligosaccharides than blossom honey [10]. Oligosaccharides are known to be poorly assimilated by honey bees and lead to increased losses of winter colonies fed on honeydew honey [11]. In earlier literature, the reason for these losses were discussed, and it has been assumed that this specific kind of honey impacts wintering colonies because of anatomical effects on the bee gut, microbial changes or restricted assimilation of nutrients, which leads to a higher mortality rate [11]. Following the hypothesis that the increased mineral contents of honeydew honeys could be the reason for the honeydew flow disease, feeding experiments with sugar solutions of different mineral contents were conducted [3]. However, the typical honeydew flow disease symptoms could not be produced, even though physiological damages in the midgut of bees fed with sugar solutions with higher mineral content occurred [3]. Despite these arising concerns about honeydew and the processed honey, the exact reason for this honeydew flow disease symptoms are not identified yet.

In the present study we followed the hypothesis that honeydew flow disease could be caused by the trisaccharide melezitose. Melezitose is the primary trisaccharide in honeydew, especially in the more common honeydew of aphids that live on spruces, where it can constitute up to 70% of the sugar fraction [6]. This trisaccharide is composed of two glucose and one fructose molecules [7]. It is not clear whether honey bees can digest melezitose; other hymenopteran species are known to have the ability to process melezitose. Many studies discuss the preference of ants for melezitose [12–17] and their capability for digesting melezitose is established [18]. Before it can pass the intestinal epithelium, melezitose has to be metabolised to hexose units by specific enzymes. The link between a fructose and a glucose unit can be broken down by invertase [18], which is commonly present in the lumen of the ant gut [19]. Also under laboratory conditions, it was observed that invertase can break down melezitose [20]. Invertase can be detected in varying concentrations in honey bees. The concentration of invertase increases with the age of the bees in summer bees and is constantly present in winter bees in high concentration [21]. Since honey bees are also known to possess invertase [21,22], it is possible, that they are capable of breaking down melezitose in the same way as ants. In experiments in which sucrose, maltose, melezitose and trehalose were fed to honey bees, their melezitose metabolites glucose and fructose appeared in the haemolymph. However, melezitose did also appear in the haemolymph as unmetabolised molecule, while sucrose and maltose were metabolised to a greater extent [23]. Caged bees prefer sucrose over all other sugars,

namely: arabinose, xylose, fructose, glucose, galactose, mannose, lactose, maltose, melibiose, trehalose, raffinose and melezitose [24].

Further knowledge on possible dietary and health effects can be gained by characterising the intestinal microbiota, which consists of only few species in honey bees [25–31]. Since mono- and oligosaccharides constitute the main energy source for honey bees [24,32], components of sugar uptake systems, such as phosphotransferase system family genes, are enriched in their gut microbiota [28]. Genes for sugar transport and carbohydrate breakdown are enriched specifically in the microbial species *Gilliamella apicola*, *Bifidobacterium* spp., and the *Lactobacillus* species clusters Firm-4 and Firm-5 [27,28,33]. Especially the carbohydrate-degrading enzymes found in *Gilliamella apicola*, *Lactobacillus* Firm-4, *Lactobacillus* Firm-5 and *Bifidobacterium* spp. are beneficial for breaking down nectar sugars to use them as energy sources [27]. The bacteria digest carbohydrates and produce short-chain fatty acids as fermentation products. Honey bees can utilise both the sugars and their fermentation products for energy metabolism [34]. The gut microbiota could be important for degradation of more complex sugars that may otherwise have toxic effects [27].

Honeydew with high contents of the trisaccharide melezitose could cause the severe clinical symptoms of the honeydew flow disease in honey bees due to poor melezitose metabolism capabilities of the intestinal microbiota. To understand the impact of melezitose on honey bee health, feeding experiments with melezitose were performed, during which physiological condition and behaviour of the bees were monitored, and the intestinal microbiota was analysed by 16S-amplicon sequencing.

Material and methods

Performance of the feeding experiments

Four feeding experiments were performed during summer of the years 2017, 2018 and 2019. European honey bees (*Apis mellifera*) were collected from the hives of the Apicultural State Institute (University of Hohenheim, Germany). For every experiment, six brood combs without adult bees were removed from three different donor colonies, caged and incubated at 33˚C for 24 h. Newly emerged bees (day 1) were collected and pooled. Out of the pooled bees, 50 bees were randomly collected and placed in each one of twelve experimental cages. Bees were held in cages, as previously described [35]. The cages were placed in a darkened climate chamber in randomised block design at the typical brood nest temperature of 35˚C. Melezitose does not crystallise at this temperature. The bees were fed *ad libitum* with control feed (39% (w/v) fructose, 31% glucose, 30% sucrose) or melezitose feed (50% melezitose, 19.5% fructose, 15.5% glucose), which mimicked the sugar spectrum of honeydew honey with high melezitose content.

Six cages were supplied with control feed and six cages with melezitose feed. Both sugar solutions were treated in the same way, dissolved in an ultrasonic bath that heats up in 30 min from 23˚C to 70˚C. 2 ml of the respective sugar solution was freshly prepared daily to ensure same viscosity and no effect on the degree of crystallisation. Solutions were administered simultaneously with vials in each cage until all bees had died.

Feeding experiment 1 –Sugar analyses of crop content. In the first cage experiment, all bees were frozen at day 21. This was necessary to for crop content analysis in order to prove the ability of all bees to collect and process the food solutions with different sugar compositions. The crop content, if present, was collected for sugar analysis (34 crops of control-fed bees and 69 crops of melezitose-fed bees). The sugars were analysed according to Lohaus and Schwerdtfeger [36].

Feeding experiment 2 –Sugar analyses of processed sugar solution. In the second cage experiment, bees of all six melezitose-fed cages collected the sugar solution in small honey combs that they built from the provided wax foundation rectangle. The sugar proportions of the collected, processed feed were analysed [36] for each cage (six processed sugar solutions).

Feeding experiment 3 –Analyses of processed sugar solution, gut microbiota and water supply. Again, the bees in two of the melezitose-fed cages collected the sugar solution in honey combs, and the sugar proportions were analysed [36] for each cage (two processed sugar solutions).

In the third cage experiment, we performed a preliminary gut microbiome analysis. At day 10, one live bee was collected from one control-fed cage, and two live bees from two different melezitose-fed cages. At day 15 and 20 this procedure was repeated. The collected bees (N = 9) were frozen immediately at -80˚C. Further description of the preliminary gut microbiota analysis is provided below.

In the third cage experiment, distilled water was supplied *ad libitum* in centrifuge tubes; in addition to the sugar feed solution.

Feeding experiment 4 –Analyses of gut microbiota. In the fourth cage experiment, gut microbiota analysis was implemented. The results of the preliminary microbiota analysis in cage experiment three indicated that for acquisition of a complete gut microbiota, the caged bees needed contact to nurse bees [37,38]. To ensure this, the experimental design was adjusted. 1'832 newly emerged bees from the donor colonies described above (day 1) were marked with liquid water-proof marker in a colour representing their colony (1, 2 and 3). Later, on the same day, the marked bees were placed back into their donor colonies. Four days later (day 5), 20 marked bees from each donor colony were placed in each of the nine experimental cages. Six cages were fed with the control solution and three cages with the melezitose solution. Moreover, from day 10 on, three of the six control-fed cages were changed to melezitose diet ("changed diet").

For the gut microbiota analysis, bees were collected at different time points. On day 5, six bees from each donor colony (3 donor colonies x 6 bees = 18 bees) were collected. On day 10, six bees were collected from each of the three control and three melezitose-fed cages. Constantly two of the collected bees per cage originated from donor colony 1, two from donor colony 2 and two from donor colony 3, as identified by their colour marks (2 treatment groups x 3 cages x 3 donor colonies x 2 bees = 36 bees). On day 15, six bees were collected from each cage (3 treatment groups x 3 cages x 3 donor colonies x 2 bees = 54 bees). The collected bees (N = 108) were frozen immediately at -80˚C. Further procedures in the gut microbiota analysis are described below.

Additional field experiments

Additionally, at five sampling sites in the black forest (Southern Germany), 100 bees from three bee colonies per sampling site were collected during honeydew season in 2017 and 2018. The crop contents from each hive were pooled for sugar analysis (5 sampling sites x 3 colonies x 2 years = 30 analyses).

Analysis of the aspects of honey bee health

In order to measure the aspects of bee health (see Table 1) in feeding experiments 2–4, food uptake per cage was recorded daily by weighing of the food [g]. The food uptake was then calculated difference to the food weight given the day before. Mortality was recorded by counting the dead bees exactly every 24 hours. The whole body weights without crop and the weights of the dissected guts of the first ten dead bees in each cage were recorded.

Table 1. Overview of the analyses as well as the number of bees and cages per treatment of the feeding experiments performed between 2017 and 2019.

Feeding experiment	Year	Sugar analyses	Microbiota analysis	Aspects of bee health	Treatment groups	Cages per treatment	Bees per cage
1	2017	Cf, Cc, Fw	/	/	Co, Me	6	50
2	2017	Cc, Fw	/	FU, GW, ST	Co, Me	6	50
3	2018	/	9	FU, GW, ST	Co, Me	6	50+3
4	2019	/	108	FU, GW, ST	Co, Me, M10	3	60+12

The sugar content was analysed in crops of bees in the field (Cf), crops of bees from the feeding experiments (Cc) and from feed the bees transported into the cells of the beeswax foundation rectangle in the cages (Fw). Aspects of bee health monitored by daily food uptake per cage (FU), gut-body weight ratio of dead bees (GW) and survival of all bees per cage (ST). The treatment groups were fed a control solution of sucrose, glucose and fructose (Co) or with a 1:1 solution of control and melezitose (Me), in 2019 control-fed bees were fed with melezitose from day 10 (M10). The extra bees for microbiota analysis were marked and put in the cages additionally (noted with +).

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Gut microbiota profiling

To profile the gut microbiota, DNA of nine bees from the feeding experiment in 2018, as well as from 108 bees from the feeding experiment in 2019 was extracted using a TRIzol protocol. Whole single bees were extracted using our standard protocol because DNA and RNA can be extracted simultaneously and be used for further experiments. The bees were placed in a 2 ml lysis tube with five 0.8 mm steel beads, roughly 50 μl 0.1 mm glass/zirconia beads and 0.5 ml TRIzol (Invitrogen). The bees were homogenised on a FastPrep24 (MP Bio) at 5.5 m/s for 50 s. After 5 min of incubation at RT, 100 μl chloroform was added and the contents were mixed by vigorous shaking, followed by 5 min of incubation at RT. The two phases were separated by 15 min centrifugation at 12.000 *g* and 4˚C. The aqueous phase was transferred to another tube for RNA extraction. 250 μl back extraction buffer (4 M guanidine thio-cyanate, 50 mM sodium citrate, 1 M TRIS base) was added to the rest of the homogenate and mixed by vigorous shaking. After 10 min of incubation at RT and centrifugation for 15 min at 12.000 *g* and 4˚C, the aqueous phase was transferred to a new tube with 200 μl isopropanol and mixed by repeated inverting. After 5 min of incubation at RT and 15 min of centrifugation (12.000 *g*, 4˚C), the supernatant was removed, the pellet was washed with 80% ethanol, dried for 10 min at RT and centrifuged again (12.000 *g*, 4˚C) for 5 min. The supernatant was removed, the pellet dried for 5 min at RT and redissolved in 50 μl 8mM NAOH. After another centrifugation for 10 min (12.000 *g*, RT) to remove the membrane lipids, the supernatant was transferred into 4.25 μl 0.1 M HEPES and 0.5 μl RNAse A (Amresco 10 mg/ml), mixed carefully and incubated for 1 h at 37˚C. DNA concentrations were determined using Qubit fluorometer (Thermo Fisher Scientific). The resulting DNA concentrations ranged between 10.1–94.6 ng/μl. Amplicons from the V3-V4 region of the 16S-rRNA-gene were generated and Illumina-sequenced using 20 ng template DNA (Eurofins Genomics, Ebersberg, Germany). The PCR conditions, library preparation, sequencing and initial data preparation were described previously [39]. After demultiplexing by demultiplexor_v3.pl (Perl 5.30) and initial quality filtering, OTU binning (97% identity) was done by USEARCH 8.0 [40], as well as quality filtering and Chimera filtering by UCHIME [41] (with RDP set 15 as a reference database). The sequencing data were analysed on the Integrated Microbial NGS platform [42], using a 0.1% total abundance threshold. This is a UPARSE based analysis pipeline reporting OTU sequences with ≤1% incorrect bases in artificial microbial community tests [43]. Primary taxonomic classification was done by RDP classifier version 2.11 training set 15 [44] and sequence alignment was done by MUSCLE [45]. The taxonomic classification was controlled and refined by BLAST-searching the representative OTU sequences in the NCBI database (https://blast.ncbi.nlm.nih.gov).

Normalisation, taxonomic binning, and statistical analyses were carried out using the RHEA scripts [46] on R studio version 1.1.456.

Ethics statement

In accordance with the guidelines of the authors' institutions' and the applicable regulations, no ethics approval was required or obtained for the present study. This study was carried out in Baden-Wuerttemberg, Germany. Honey bees are no subjects of the German Animal Protection law. Additionally, neither endangered nor protected species were involved in this study.

Statistical analyses

The daily food uptake per bee was calculated in consideration of the number of bees alive on the respective day. The gut-body weight ratio was calculated from the weight of the recorded bee bodies (without crop) and their removed guts. In order to visualise the results for both measures, box plots were created for each group in the respective year. A linear regression was used to estimate the group differences in daily food uptake controlling for the number of bees alive, daily and annual effects. Since the gut-body weight ratio range between 0 and 1, a fractional logit regression model was employed to estimate group differences controlling for year effects and the age of bees. Survival of bees was analysed in a Cox proportional hazards model. Differences between bacterial species in the treatment groups were analysed with the Rhea R pipeline [46]. All statistical tests were conducted and graphs were drawn using R version 3.5.2.

Table 2. Results of the linear regression model (daily food uptake), fractional logit regression model (gut-body weight ratio) and Cox proportional hazard model (survival).

Coefficients	Estimate	St. Error	Pr(>|t|)
Panel I: Daily food uptake			
Intercept	0.1162	0.0096	<0.001
Melezitose	0.0027	0.0029	0.947
Melezitose day +10	0.0184	0.0061	0.003
2018	0.0109	0.0030	<0.001
2019	0.0151	0.0040	<0.001
Days	-0.0018	0.0003	<0.001
Bees_alive	-0.0017	0.0001	<0.001
Panel II: Gut-body weight ratio			
Intercept	-0.0520	0.1099	0.636
Melezitose	0.0866	0.0154	<0.001
Melezitose day +10	0.0550	0.0260	0.034
2018	0.0157	0.0136	0.248
2019	0.0028	0.0142	0.842
Days	0.0012	0.0016	0.440
Panel III: Survival			
Melezitose	0.9319	0.0555	<0.001
Melezitose day +10	2.2514	0.1073	<0.001
2018	-0.1144	0.0584	0.050
2019	-0.8595	0.0771	<0.001

The multivariate R^2 for the linear regression in Panel I is 0.253 and the F-test for overall significance yields F(6.893) = 50.4 ($p < 0.001$). For the fractional logit regression in Panel II, we report average partial effects. The multivariate R^2 is 0.174 based on 330 observations. The log-rank test (4.1740) yields 628.1 ($p < 0.001$).

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Results

The bees fed with melezitose in the experiments showed disease symptoms related to their physiological condition and generic behaviour. Bees fed with the control diet stayed predominantly on the beeswax foundation rectangle (S1 Video) and bees fed with melezitose were observed to crawl mostly on the bottom of the cage (S2 Video). In fact, towards the end of the experiment, this behaviour was exhibited more frequently. Additionally, melezitose-fed bees also moved more often and faster than the bees in the control-fed cages. Swollen abdomens, abdomen tipping, impaired movement, twitching and terminal paralysis were observed during all feeding experiments in the melezitose-fed bees (S2 Video and S3 Video).

Following our hypothesis that melezitose affects the health of honey bees, we conducted several multivariate regression analyses investigating multiple aspects of honey bee health. Using daily food uptake per bee, gut-body weight ratio and survival time as dependent variables, we can show that melezitose has a highly significant negative effect on honey bee health. The results of our individual regression models are presented in Table 2.

Sugar analyses of crop contents in cage and field experiments and of processed feed

The results of the sugar spectrum analysis in the crops of the caged bees point out that the trisaccharide melezitose was taken up and degraded into the small molecule sugars trehalose, sucrose, glucose and fructose. While the melezitose proportion in the food was 50%, the mean value in the crops from 69 bees was 18.88%.

The sugar analyses of the contents from the field and laboratory experiments showed that the bees ingested melezitose. Presence of liquid in the crop indicated active feeding. The crops of the field-collected bees contained up to 10.8% melezitose. Besides melezitose, these crops contained (in ranking order) mainly glucose and fructose, less than 10% sucrose, trehalose, turanose, maltose and erlose. Furthermore, less than 1% consisted of melibiose, raffinose and stachyose.

The mean melezitose content in the processed feed in the combs formed from the beeswax foundation rectangles in the cages was 28.92% (8 processed sugar solutions of melezitose-fed cages) (Table 3).

Effects of melezitose feeding on water and food uptake

Bees did not take up more water when fed with melezitose feed, as compared to control solution and also did not show a significantly higher food uptake than control-fed bees (Fig 1). In contrast, the food uptake of bees fed with melezitose starting from day 10 was 20 mg higher ($p < 0.01$) than that of bees fed with the control solution and higher than that of bees fed with melezitose from the first day onwards ($p < 0.01$). To illustrate the relative increase in food uptake, we determined the average uptake per bee, which was 20 mg for control-fed bees. Calculating from this value, a change from control to melezitose diet caused the bees to approximately double their food intake (Fig 1).

Effects of melezitose feeding on viscosity and weight of guts

The proportion of gut weight of the respective bee bodies was 52% in control-fed bees, 60% in melezitose-fed bees and 56% in bees fed with melezitose from day 10 (Fig 2). We conducted a fractional logit regression ($R^2(5,330) = 0.174$) and found that both groups fed with melezitose had significantly higher gut-body weight ratios than the control group ($p < 0.001$ for bees fed with melezitose from day 1 and $p < 0.05$ for bees fed with melezitose from day 10). The effect

Table 3. Fructose, glucose, sucrose and melezitose proportion of the feed solution, crop content of honey bees collected from the first feeding experiment, field experiment and the processed melezitose feed.

Analysed solution	Fructose [%]	Glucose [%]	Sucrose [%]	Melezitose [%]
Food solution	19.50	15.50	15.00	50.00
Crop content of feeding experiment (N = 69)	38.82	36.04	6.10	18.88
Crop content of field experiment (N = 15)	45.30	35.50	4.60	3.70
Processed sugar solution (N = 8)	24.71	37.59	8.79	28.92

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of the higher gut-body weight ratio was numerically slightly weaker (six percentage-points increase of gut-body-weight ratio instead of nine percentage points increase) for bees fed with melezitose starting from day 10, which implies that a longer exposure to melezitose seems to enlarge the gut of bees.

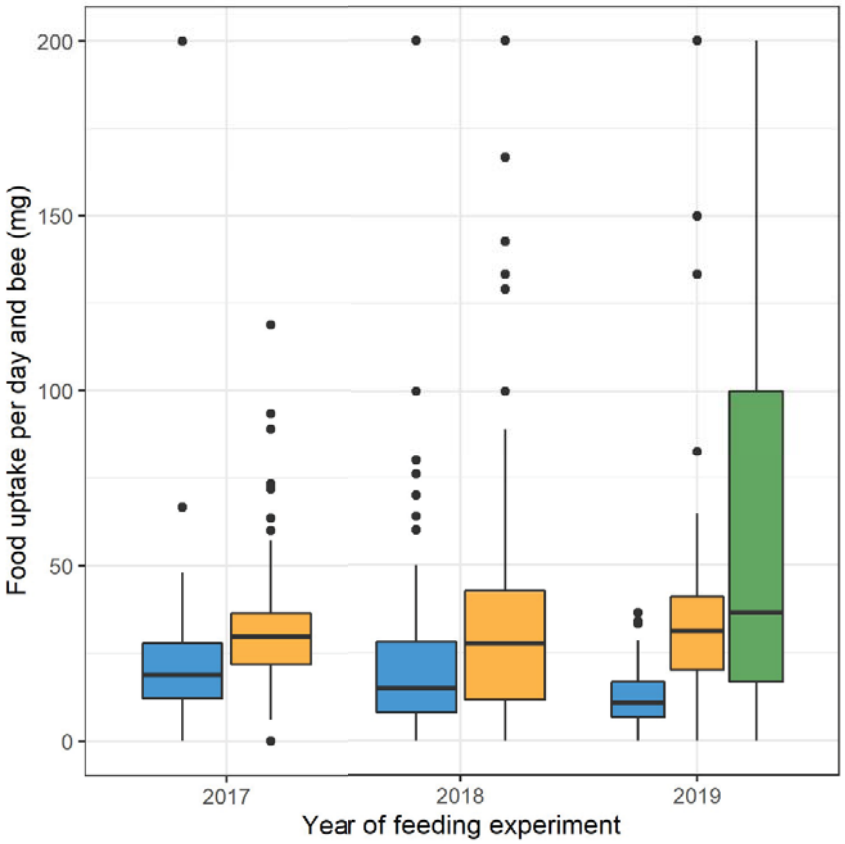

Fig 1. Boxplot of food uptake per day and bee in milligram (mg) in all cage experiments. The three treatment groups are highlighted with different colours. The mean value of daily food uptake per bee fed with control feed (blue) was 20 mg per day (n = 445) and 37 mg per day (n = 388) for bees fed with melezitose feed (yellow) and 70 mg per day (n = 67) for bees fed with melezitose feed from day 10 (green) of all years. The vertical boxplots depict the interquartile range (lower bound/ upper bound of the box correspond to the 25/75% quantile), the median (horizontal line in the box) and outlying observations (points outside the box).

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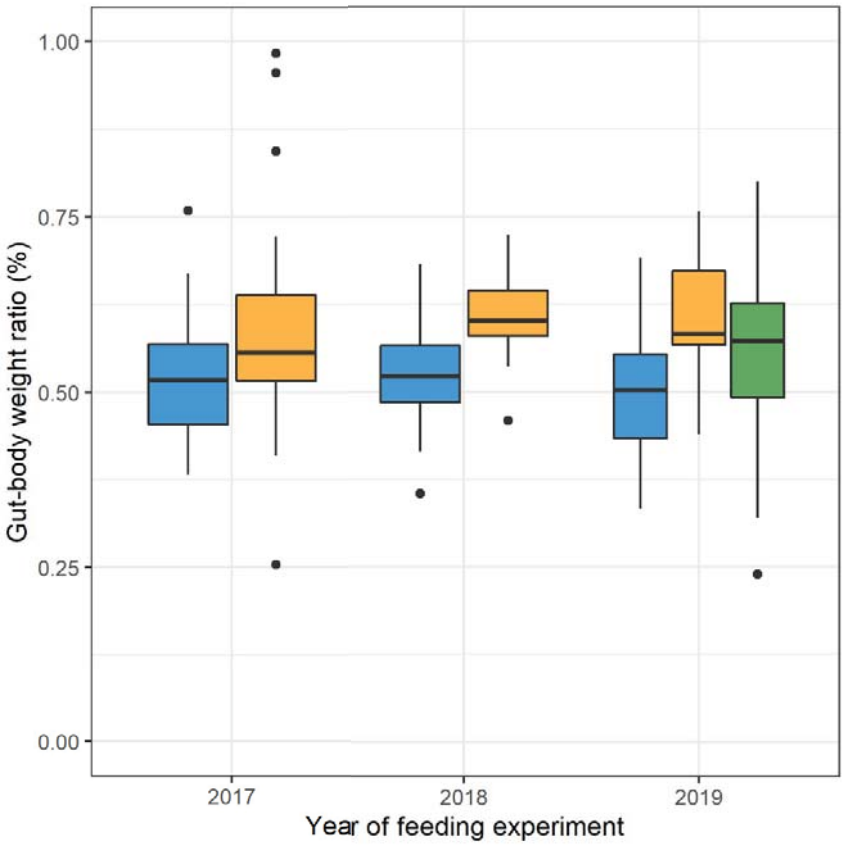

Fig 2. Boxplot of gut-body weight ratio of honey bees in all cage experiments. The three treatment groups are highlighted with different colours. The mean value of gut-body weight ratio per bee fed with control feed (blue) was 52% (n = 150) and 60% (n = 150) for bees fed with melezitose feed (yellow) and 56% (n = 30) for bees fed with melezitose feed from day 10 (green). The vertical boxplots depict the interquartile range (lower bound/ upper bound of the box correspond to the 25/75% quantile), the median (horizontal line in the box) and outlying observations (points outside the box).

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Effects of melezitose on survival

Estimated survival times for all feeding experiments are depicted in Fig 3. In general, the survival time of bees fed with melezitose was lower compared to the survival time of bees fed with the control solution in each year ($p < 0.001$). We found no significant differences between the feeding experiments in 2017 and 2018. However, the median survival rates in 2019 were significantly higher than those in 2017 and 2018 ($p < 0.001$). In the control group of the feeding experiment 2019, 50% of the bees had died after 29 days, while 50% of the melezitose-fed bees had died already after 25 days. Interestingly, the bees fed with melezitose starting from day 10 showed a more rapid inset of mortality than the bees fed with melezitose from the start of the experiment ($p < 0.001$). Their median survival time was only 17 days.

Effects of melezitose on the gut microbiota

A preliminary, small scale (N = 9) microbiota analysis was conducted in the second experiment, to determine if there were any effects of the treatments on the microbiota. Surprisingly,

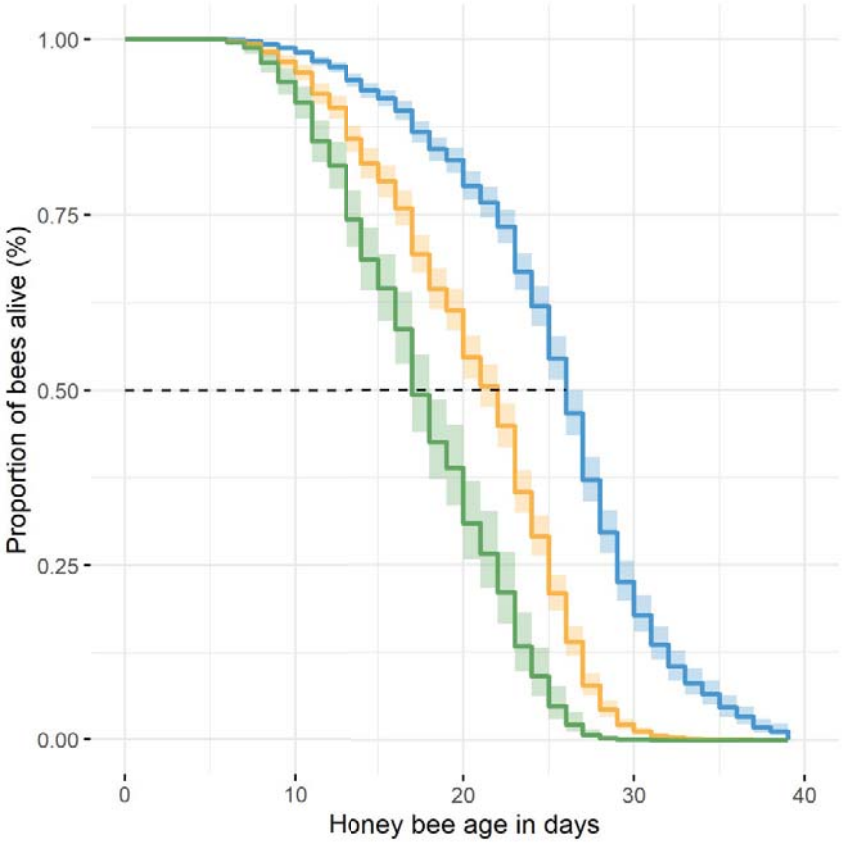

Fig 3. Overall survival probability plots in all cage experiments. The survival rate of the honey bees in the control (blue), in the melezitose group (yellow) and in the bees fed with melezitose from day 10 (green) (Cox regression, Log-rank (4,1740): 628.1, $p < 0.001$).

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we found that the core microbiota members *Gilliamella apicola* and *Snodgrassella alvi* were absent from all bees analysed (S1 Fig). This is remarkable, as they are usually, together with the *Lactobacillus* species, the most common and persistent bacteria in free-living adult bees. This absence of two core-microbiota bacteria was attributed to the circumstance that the bees used in the first three experiments had emerged from their cells and were put into the cages without contact to adult nest members. Therefore, it was decided to modify the experimental setup for the third experiment in a way that the age-standardised bees come into contact with their adult nest members for five days to allow natural development of the intestinal microbiota (S2 Fig). Diversity analyses of the 16S-amplicon sequence data revealed no significant differences in the community composition between the treatment groups of the feeding experiment (S3 Fig). In the bees from the fourth experiment (N = 108) a total of ten gut bacteria species could be detected. The five core members are *Snodgrassella alvi*, *Gilliamella apicola*, *Bifidobacterium* spp., *Lactobacillus* Firm-4 and *Lactobacillus* Firm-5, as well as bacteria from the species *Frischella perrara*, *Gluconacetobacter* spp., *Parasaccharibacter apium*, *Bartonella apis* and *Lactobacillus kunkeei* (S4 Fig).

At first glance, the microbial community appeared to be unaffected by the melezitose treatment. However, on closer examination of the OTU composition, we found that the

proportions of the lactic acid bacteria differed between the treatment groups. *Lactobacillus kunkeei* increased over time in bees fed with the control diet, but was not present at all in bees fed with melezitose, and it was very low in bees fed with melezitose from day 10 (F-statistic: 2.66 on 5 and 102 DF, $p = 0.03$). Also, the relative abundance of *Lactobacillus* Firm-4 increased in control-fed bees and decreased in bees fed with melezitose from day 10 (Fig 5; F-statistic: 4.245 on 5 and 102 DF, $p = 0.002$). In contrast, the relative abundance of *Lactobacillus* Firm-5 decreased in bees fed with the control diet, but increased significantly in bees fed with melezitose and bees fed with melezitose from day 10 (Fig 6; F-statistic: 7.048 on 5 and 102 DF, $p < 0.001$).

Discussion

This study describes the multiple effects of melezitose in honey bees and indicates its key significance for the occurrence of the described honeydew flow disease. This disease already led to colony losses during winter [3], which are usually noticed by beekeepers and therefore documented in beekeepers' journals [4]. In three feeding experiments, bees fed with melezitose showed intestinal symptoms, and increased food uptake, gut weight and mortality.

In order to analyse the progress of uptake and digestion of melezitose, the relative proportion of melezitose in the feed and in the crops was measured. The melezitose proportion decreased from feed to crop (Table 3), suggesting that bees or their crop microbiota did metabolise melezitose. In social insects, the proventriculus provides the individual with the amount of food needed to cover their actual energy needs, leaving as much as possible in the crop [47]. Nevertheless, honey bees are known to digest harmful food to conserve the health of the colony [48]. Thus, it can be assumed, that the individual honey bees foraging on honeydew will digest as much of the harmful melezitose as possible. This ensures that the remaining colony is provided with easily digestible food which is processed into honey.

Interestingly, we found that bees fed with melezitose from day 10 had twice as much food uptake than control-fed bees (Fig 1). Both the doubled food uptake and the increase of melezitose can lead to an accumulation of food in the gut. The average gut-body weight ratio that was eight percent higher in bees fed with melezitose (Fig 2) also explains the morphological symptom of the swollen abdomen. These results lead us to the assumption that bees need more time to digest melezitose or are unable to digest the absorbed melezitose and thus the sugar content in the intestine increases. The rising amount of melezitose can lead to the severe symptoms that were observed in these feeding experiments. Concentrating on the life expectancy of bees, the symptoms of honeydew flow disease appeared with increasing age (first on day 10 after emergence). A gradual accumulation of melezitose with the lifetime of honey bees can be assumed. The shorter lifespan of bees fed with melezitose compared to the control group could be explained by their digestive problems and their influence on the physiology of their abdomen (Fig 3).

Consequently, we expected changes in their intestinal microbiota and performed 16S-amplicon sequencing to check for microbial shifts. There was a significant shift in the lactic acid bacteria species: *Lactobacillus kunkeei* did not occur in the bees fed with melezitose (Fig 4) and the proportion of *Lactobacillus* Firm-4 decreased (Fig 5). Conversely, the proportion of *Lactobacillus* Firm-5 increased with feeding on melezitose (Fig 6). *Lactobacillus* species ferment sugars to produce lactic or acetic acid and are adapted to sugar-rich environments with high acidity [29]. They are known to be dominant in the crop and most abundant in the ileum and rectum [37,38]. Within the bee-associated *Lactobacilli*, *Lactobacillus mellifera* and *L. mellis* form a distinct phylogenetic cluster referred as *Lactobacillus* Firm-4, and the species *Lactobacillus apis*, *L helsingborgensis*, *L. kimbladii*, *L. kullabergensis*, and *L. melliventris* are referred as

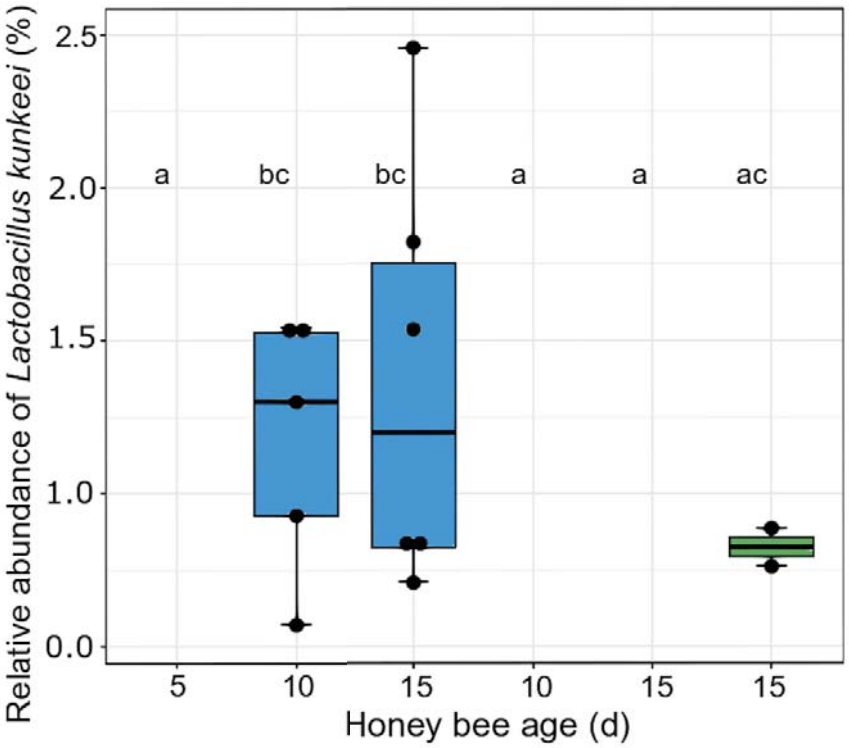

Fig 4. Relative abundance of *Lactobacillus kunkeei* in the gut microbiota. The three treatment groups are highlighted with different colours: control (blue), melezitose (not detected at all time points) and melezitose from day 10 (green). Significantly different groups are highlighted by the letters a, b and c (F-statistic: 2.66 on 5 and 102 DF, p = 0.027). For each treatment group and each bee age, 18 individuals were used for analysis. Not detected bacteria are marked with ND. The vertical boxplots depict the interquartile range (lower bound/ upper bound of the box correspond to the 25%/ 75% quantile), the median (horizontal line in the box) and outlying observations (points outside the box).

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Lactobacillus Firm-5 [49]. Both clusters are located within the hindgut, *Lactobacillus* Firm-4 in the rectum, *L.* Firm-5 in the ileum and rectum [49,50]. While these two clusters are rarely detected outside the hindgut, *Lactobacillus kunkeei* is also found outside the honey bee body in the hive. *L. mellifera*, which belongs to the Firm-4 cluster is only capable of producing acids from fructose, while the species of the Firm-5 cluster can also utilise the sugars galactose, mannose, sorbose and sucrose [49]. The more diverse capabilities for oligosaccharide metabolism of *Lactobacillus* Firm-5 species may explain their increase within the melezitose-fed bees.

These findings point out the importance of lactic acid bacteria for the nutrition of their host. The bees that were fed with control diet first and from day 10 on with melezitose diet died earlier than those fed with melezitose from the fifth day onwards (Fig 3). This may be seen as further evidence for the key role of an adapted microbiota in the processing of oligosaccharides. Bees fed with melezitose from the fifth day may have grown an adapted intestinal microbiota capable of degrading the oligosaccharides at an increased rate. The change in diet from the control to melezitose diet on day 10 day may therefore have led to a rapid accumulation of melezitose in the guts of unadapted bees with acute, often lethal effects.

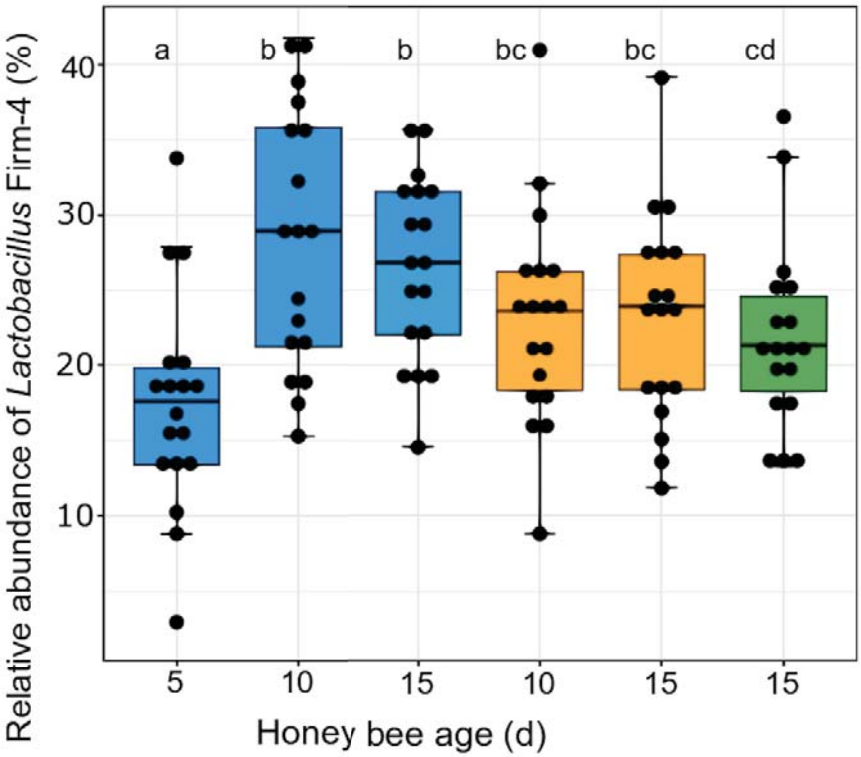

Fig 5. Relative abundance of *Lactobacillus* Firm-4 in the gut microbiota. The three treatment groups are highlighted with different colours: control (blue), melezitose (yellow) and melezitose from day 10 (green). Significantly different groups are highlighted by the letters a, b, c and d (F-statistic: 4.245 on 5 and 102 DF, p = 0.002). For each treatment group and each bee age, 18 individuals were used for analysis. The vertical boxplots depict the interquartile range (lower bound/ upper bound of the box correspond to the 25%/ 75% quantile), the median (horizontal line in the box) and outlying observations (points outside the box).

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Honey with high proportion of melezitose is well-known to crystallise rapidly, often already within the hives [1]. However, intestinal epithelial lesions, which might be caused by uptake of melezitose crystals, are an unlikely cause of the observed symptoms, since crystallisation did not occur under the experimental conditions. No crystals were found by microscopy during the examination of the affected intestine. Another possible effect of melezitose might be dehydration. However, the bees of the third experiment fed with melezitose diet did not ingest more water than the control group; therefore dehydration can be ruled out as major cause of these symptoms. It is well-known that oligosaccharides are poorly utilised by the gut microbiota of honey bees and beekeepers should therefore try to avoid them as food source for their colonies [33,51]. Here, we to show this effect for the oligosaccharide melezitose, which is very common in honeydew of spruce forests. The shift of the lactic acid bacteria in bees fed with melezitose provides evidence for the bees' struggle to digest melezitose. On the one hand, honey bees used to live in forests, so that it can be assumed that their bacteria have had sufficient evolution time for adapting to typical honeydew sugars. On the other hand, it should be acknowledged that natural forests with their higher botanical diversity used to provide more nectar than most forests today. The occurrence of melezitose at high concentrations in the colonies is a problem primarily caused by the beekeepers themselves. Bee colonies are, on

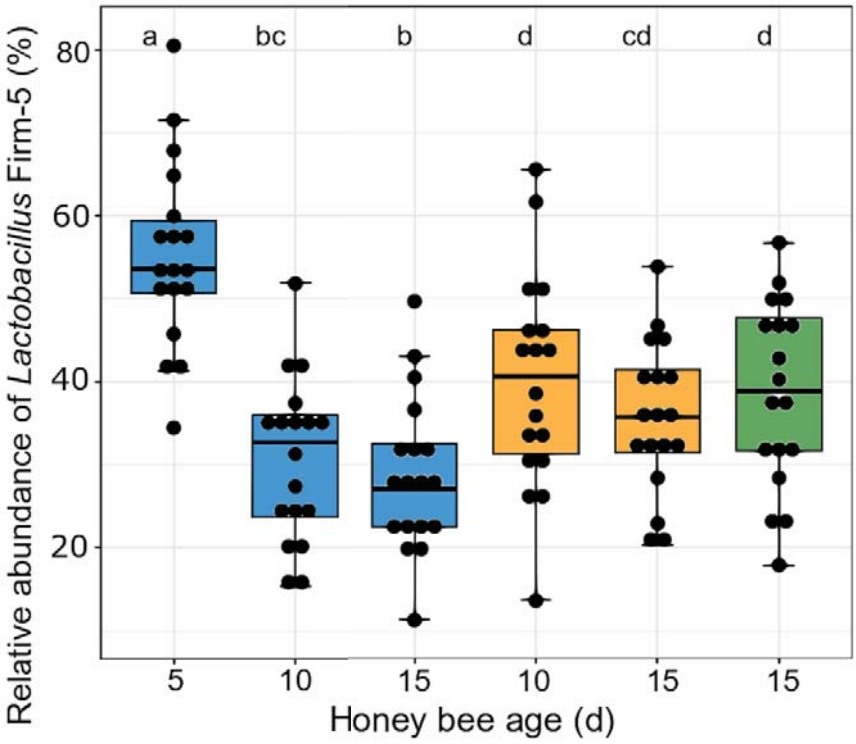

Fig 6. Relative abundance of *Lactobacillus* Firm-5 in the gut microbiota. The three treatment groups are highlighted with different colours: control (blue), melezitose (yellow) and melezitose from day 10 (green). Significantly different groups are highlighted by the letters a, b, c and d (F-statistic: 7.048 on 5 and 102 DF, $p < 0.001$). For each treatment group and bee age, 18 honey bee individuals were used for analysis. The vertical boxplots depict the interquartile range (lower bound/ upper bound of the box correspond to the 25%/ 75% quantile), the median (horizontal line in the box) and outlying observations (points outside the box).

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purpose, relocated to forests with lack of nectar plants, because honeydew honey with its strong malty-aromatic taste is very popular and has a high market value [9]. The situation of bees in the cages of the feeding experiment is similar to that of bees in winter that cannot leave the hive because of low outside air temperature and therefore cannot defecate [52]. Since bees have a well-studied hygienic behaviour [53] and only defecate outside of the colony, the melezitose inevitably remains in the hindgut. The gut microbiota of honey bees may be somewhat capable of processing melezitose, however this process may take time and melezitose may accumulate in the gut faster than it can be processed. Therefore, the melezitose remains in the bee and leads to the typical symptoms of the disease. This explains the occurrence of honeydew flow disease symptoms especially during winter. Since honey bees have a longer lifespan of several months in winter instead of several weeks [54], they are more dependent on effective digestion of the stored food, which may favour honeydew flow disease in winter.

Honeydew flow disease is a regional phenomenon, mainly noticed by beekeepers in Germany, Austria and Switzerland, which can be explained by the coincidence of sufficiently cold winters, large fir-dominated forests, and beekeepers aiming to produce honeydew honey. However, wild-living honey bee colonies that are not maintained by beekeepers can be found in forests all over Europe [55] and can come in contact with melezitose, too. It can be assumed,

that these wild honey bee colonies have adapted their behaviour to honeydew with high amounts of melezitose. They probably decrease the melezitose amount in their stored honey by intensely foraging for nectar. Apart from that, the proportion of melezitose in the excretion of honeydew producer species is variable [6] and there can be regional differences.

It should be noted that other oligosaccharides such as erlose and raffinose may probably cause similar problems as melezitose. Melezitose is the most common trisaccharide in the honeydew of spruces, as the spruce is the host tree of most honeydew producer species in Europe when pines are not present locally [56]. Nevertheless, the results of these experiments can be used as an example and be transferred to the effects of other oligosaccharides. Therefore, feeding experiments with other oligosaccharides would extend the knowledge gained from this study. Further experiments with several different sugar solutions in each cage could show the preference of bees, and a transfer of the experiments to the field would give deeper insights into the effects on colony level to qualitatively different nutrition. Additionally, it should be further investigated whether and how the bee's hindgut is physiologically affected by feeding with oligosaccharides.

From the experience gained during this study in respect of establishment of a complete, natural microbiota in experimental bees, we recommend to allow for contact between the newly emerged experimental bees and nurse bees. Gut microbiota bacteria are acquired by newly emerged bees by oral-oral transmission (especially lactic acid bacteria) and by faecal-oral transmission through contact with other worker bees or hive material [37,38].

Altogether, the results of this study lead to the conclusion that melezitose affects the health of honey bees. The trisaccharide may accumulate in the gut over time as the gut microbiota needs more time to metabolise melezitose than for simple sugars. The present results show that high amounts of melezitose play a key role in the occurrence of the honeydew flow disease in bee colonies. Additionally, it can be assumed that the honeydew flow disease can affect honey bees synergistically with pathogens, such as the paralysis viruses, which are most abundant during honeydew season [57]. Bee colonies are superorganisms and can compensate diseases through healthy new brood and maintenance of homeostasis [58]. However, if melezitose accumulates in the combs and is not removed by the beekeeper, the bees will face digestion problems during the winter when no brood can be reared. Based on the results of this present study, it can be recommended to avoid honeydew with high contents of melezitose. Beekeepers should therefore remove their colonies from the forests, when environmental conditions favour melezitose production.

Supporting information

S1 Fig. Overall gut microbiota of sequenced DNA of honey bees from the third feeding experiment (mean value of N = 9 analysed gut microbial communities). Honey bees were removed from their colonies directly after emerging and had no contact to nurse bees. Bacterial species are highlighted by colour and shown in the legend. Core-members of the honey bee gut microbiota are written in bold.
(TIF)

S2 Fig. Overall gut microbiota of sequenced DNA of honey bees from the fourth feeding experiment (mean value of analysed gut microbial communities of N = 108 bees). Honey bees lived in bee hive colonies with contact to nurse bees until day 5. Bacterial species are highlighted by colour and shown in the legend. Core-members of the honey bee gut microbiota are written in bold.
(TIF)

S3 Fig. Differences in α-diversity, i.e. Shannon effective number of species, in the gut microbiota of control (blue) and melezitose (yellow) fed bees and bees with a changed diet (green) based on 16S RNA gene amplicon sequencing. No significant differences between the groups were detected (Kruskal-Wallis chi-squared = 1.8162, df = 5, p-value = 0.8739). For each treatment group and honey bee age, 18 honey bee individuals were used for analysis. C = control-fed bees (blue), M = melezitose-fed bees (yellow), CM = bees first fed with control and from day 10 with melezitose (green); 5, 10 and 15 shows the honey bee age in days. The vertical boxplots depict the interquartile range (lower bound/ upper bound of the box correspond to the 25%/ 75% quantile) and the median (horizontal line in the box).
(TIFF)

S4 Fig. Absolute abundances of ten microbiota members monitored in the feeding experiment 2019. Absolute abundance of the ten monitored phylotypes: *Snodgrassella alvi*, *Gilliamella apicola*, *Bifidobacterium* spp., *Lactobacillus* Firm-4, *Lactobacillus* Firm-5, *Frischella perrara*, *Gluconacetobacter* spp., *Parasaccharibacter apium*, *Bartonella apis* and *Lactobacillus kunkeei*. The ten plots show the cumulative abundances for each bee. For each treatment group, 18 honey bee individuals were used for analysis. C = control-fed bees (blue), M = melezitose-fed bees (yellow), CM = bees first fed with control and from day 10 with melezitose (green); 5, 10 and 15 shows the honey bee age in days. Significant differences between the treatment groups could be shown for all *Lactobacillus* species and are demonstrated in Figs 4–6. The vertical boxplots depict the interquartile range (lower bound/ upper bound of the box correspond to the 25%/ 75% quantile), the median (horizontal line in the box) and outlying observations (points outside the box).
(TIFF)

S1 Video. Control-fed bees of one cage of feeding experiment three. The bees fed with control solution predominantly stayed on the beeswax foundation rectangle and moved only little.
(MP4)

S2 Video. Melezitose-fed bees of one cage of feeding experiment three. Bees fed with melezitose were observed to mostly crawl on the bottom of the cage and moved very often and fast. They displayed the disease symptoms: swollen abdomen, abdomen tipping, impaired movement, twitching and terminal paralysis.
(MP4)

S3 Video. Melezitose-fed bees of one cage at the end of feeding experiment three. Bees fed with melezitose showed swollen abdomens and impaired movements, which was more severe towards the ends of their live. Eventually, they are unable move and succumb to the disease.
(MP4)

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Writing – original draft: Victoria Charlotte Seeburger.

Writing – review & editing: Victoria Charlotte Seeburger, Paul D'Alvise, Basel Shaaban, Karsten Schweikert, Gertrud Lohaus, Annette Schroeder, Martin Hasselmann.

References

1. Hudson CS, Sherwood SF. The occurrence of melezitose in honey. J Am Chem Soc. 1919; 42: 116–125. https://doi.org/10.1021/ja01445a016
2. Bailey L. The occurrence of chronic and acute bee paralysis viruses in bees outside britain. J Invertebr Pathol. 1965; 7: 167–169. https://doi.org/10.1016/0022-2011(65)90031-5 PMID: 5827552
3. Horn H. Die Waldtrachtkrankheit der Honigbiene I. Der Einfluß des Mineralstoffgehaltes in Honigtauhonigen. Apidologie. 1985; 16: 139–156. https://doi.org/10.1051/apido:19850204
4. Imdorf A, Bogdanov S, Kilchenmann V. Zementhonig im Honig- und Brutraum–was dann. Schw Zent Bienenforschung. 1985; 1: 1–16.
5. Baker HG, Baker I. Floral nectar sugar constituents in relation to pollinator type. Handbook Exp Poll Biol. 1983; 1: 117–141. https://doi.org/10.2307/2443763
6. Shaaban B, Seeburger V, Schroeder A, Lohaus L. Sugar, amino acid and inorganic ion profiling of the honeydew from different hemipteran species feeding on Abies alba and Picea abies. PLoS ONE. 2020; 15: 1–17. https://doi.org/10.1371/journal.pone.0228171 PMID: 31978201
7. Price DRG, Karley AJ, Ashford DA, Isaacs HV, Pownall ME, Wilkinson HS, et al. Molecular characterisation of a candidate gut sucrase in the pea aphid, Acyrthosiphon pisum. Insect Biochem Molec Biol. 2007; 37: 307–317. https://doi.org/10.1016/j.ibmb.2006.12.005 PMID: 17368194
8. Lachman J, Kolihová D, Miholová D, Košata J, Titěra D, Kult K. Analysis of minority honey components: Possible use for the evaluation of honey quality. Food Chemistry. 2007; 101: 973–979. https://doi.org/10.1016/j.foodchem.2006.02.049
9. Castro-Vázquez L, Díaz-Maroto MC, Pérez-Coello MS. Volatile composition and contribution to the aroma of spanish honeydew honeys. Identification of a new chemical marker. J Agr Food Chem. 2006; 54: 4809–4813. https://doi.org/10.1021/jf0604384 PMID: 16787032
10. Sanz ML, Gonzalez M, Lorenzo C de, Sanz J, Martínez-Castro I. A contribution to the differentiation between nectar honey and honeydew honey. Food Chem. 2005; 91: 313–317. https://doi.org/10.1016/j.foodchem.2004.06.013
11. Pohorecka K., Skubida P. Healthfullness of honeybee colonies (Apis mellifera L.) wintering on the stores with addition of honeydew honey. Bull Vet Inst Pulawy. 2004; 48: 409–413.
12. Vantaux A, Schillewaert S, Parmentier T, van den Ende WIM, Billen J, Wenseleers TOM. The cost of ant attendance and melezitose secretion in the black bean aphid Aphis fabae. Ecol Entomol. 2015; 40: 511–517. https://doi.org/10.1111/een.12217
13. Banks CJ. Effects of the ant Lasius niger (L.) on insects preying on small populations of Aphis fabae Scop. on bean plants. Ann Appl Biol. 1962; 50: 669–679. https://doi.org/10.1111/j.1744-7348.1962.tb06067.x
14. Samways MJ. Interrelationship between an entomogenous fungus and two ant-homopteran (Hymenoptera: Formicidae-Hemiptera: Pseudococcidae & Aphididae) mutualisms on guava trees. Bull Entomol Res. 1983; 73: 321. https://doi.org/10.1017/S0007485300008919
15. Fischer MK, Shingleton A.W. Host plant and ants influence the honeydew sugar composition of aphids. Funct Ecol. 2001; 15: 544–550. https://doi.org/10.1046/j.0269-8463.2001.00550.x
16. Vantaux A, van den Ende W, Billen J, Wenseleers T. Large interclone differences in melezitose secretion in the facultatively ant-tended black bean aphid Aphis fabae. J Ins Physiol. 2011; 57: 1614–1621. https://doi.org/10.1016/j.jinsphys.2011.08.014 PMID: 21896277

17. Woodring J. Honeydew amino acids in relation to sugars and their role in the establishment of ant-attendance hierarchy in eight species of aphids feeding on tansy (Tanacetum vulgare). Physiol Entomol. 2004; 29: 311–319. https://doi.org/10.1111/j.0307-6962.2004.00386.x

18. Detrain C, Verheggen FJ, Diez L, Wathelet B, Haubruge E. Aphid-ant mutualism: how honeydew sugars influence the behaviour of ant scouts. Physiol Entomol. 2010; 35: 168–174. https://doi.org/10.1111/j.1365-3032.2010.00730.x

19. Heil M, Büchler R, Boland W. Quantification of Invertase Activity in Ants Under Field Conditions. J Chem Ecol. 2005; 31: 431–437. https://doi.org/10.1007/s10886-005-1352-y PMID: 15856794

20. White JW, Maher J. Transglucosidation by Honey Invertase. Arch Biochem Biophys. 1953; 42: 360–367. https://doi.org/10.1016/0003-9861(53)90365-8 PMID: 13031638

21. Simpson J, Riedel IBM, Wilding N. Invertase in the Hypopharyngeal Glands of the Honeybee. J Apic Res. 1968; 7: 29–36. https://doi.org/10.1080/00218839.1968.11100184

22. Oddo LP, Piazza MG, Pulcini P. Invertase activity in honey. Apidologie. 1998; 30: 57–65. https://doi.org/10.1051/apido:19990107

23. Maurizio A. Untersuchungen über das Zuckerbild der Hämolymphe der Honigbiene (Apis mellifica L.) - 1. Das Zuckerbild des Blutes erwachsener Bienen. J Ins Physiol. 1965; 11: 745–763. https://doi.org/10.1016/0022-1910(65)90155-1

24. Barker RJ, Lehner Y. Influence of diet on sugars found by thin-layer chromatography in thoraces of honey bees, Apis mellifera L. J Exp Zool. 1974; 188: 157–164. https://doi.org/10.1002/jez.1401880204 PMID: 4825425

25. Kwong WK, Moran NA. Gut microbial communities of social bees. Nat Rev Microbiol. 2016; 14: 374–384. https://doi.org/10.1038/nrmicro.2016.43 PMID: 27140688

26. Crotti E, Sansonno L, Prosdocimi EE, Vacchini V, Hamdi C, Cherif A, et al. Microbial symbionts of honeybees: a promising tool to improve honeybee health. New Biotechnol. 2013; 30: 716–722. https://doi.org/10.1016/j.nbt.2013.05.004 PMID: 23727340

27. Engel P, Martinson G. V, Moran NA. Functional diversity within the simple gut microbiota of the honey bee. Proc Natl Acad Sci USA. 2012; 109: 11002–11007. https://doi.org/10.1073/pnas.1202970109 PMID: 22711827

28. Engel P, Moran NA. Functional and evolutionary insights into the simple yet specific gut microbiota of the honey bee from metagenomic analysis. Gut Microb. 2013; 4: 60–65. https://doi.org/10.4161/gmic.22517 PMID: 23060052

29. Anderson KE, Sheehan TH, Eckholm BJ, Mott BM, DeGrandi-Hoffman G. An emerging paradigm of colony health: microbial balance of the honey bee and hive (Apis mellifera). Insect Soc. 2011; 58: 431–444. https://doi.org/10.1007/s00040-011-0194-6

30. Evans JD, Schwarz RS. Bees brought to their knees: microbes affecting honey bee health. Trends Microbiol. 2011; 19: 614–620. https://doi.org/10.1016/j.tim.2011.09.003 PMID: 22032828

31. Hamdi C, Balloi A, Essanaa J, Crotti E, Gonella E, Raddadi N, et al. Gut microbiome dysbiosis and honeybee health. J Appl Entomol. 2011; 135: 524–533. https://doi.org/10.1111/j.1439-0418.2010.01609.x

32. Barker RJ, Lehner Y. Acceptance and sustenance value of naturally occurring sugars fed to newly emerged adult workers of honey bees (Apis mellifera L.). J Exp Zool. 1974; 187: 277–286. https://doi.org/10.1002/jez.1401870211

33. Moran NA. Genomics of the honey bee microbiome. Curr Opin Ins Sci. 2015; 10: 22–28. https://doi.org/10.1016/j.cois.2015.04.003 PMID: 26140264

34. Lee W-J, Hase K. Gut microbiota-generated metabolites in animal health and disease. Nat Chem Biol. 2014; 10: 416–424. https://doi.org/10.1038/nchembio.1535 PMID: 24838170

35. Ziegelmann B, Abele E, Hannus S, Beitzinger M, Berg S, Rosenkranz P. Lithium chloride effectively kills the honey bee parasite Varroa destructor by a systemic mode of action. Sci Rep. 2018; 8: 1–9. https://doi.org/10.1038/s41598-017-17765-5

36. Lohaus G, Schwerdtfeger M. Comparison of sugars, iridoid glycosides and amino acids in nectar and phloem sap of Maurandya barclayana, Lophospermum erubescens, and Brassica napus. PLoS ONE. 2014; 9: 1–8. https://doi.org/10.1371/journal.pone.0087689 PMID: 24489951

37. Powell E, Martinson VG, Urban-Mead K, Moran NA. Routes of Acquisition of the Gut Microbiota of the Honey Bee Apis mellifera. Appl Environ Microbiol. 2014; 80: 7378–7387. https://doi.org/10.1128/AEM.01861-14 PMID: 25239900

38. Martinson VG, Moy JK, Moran NA. Establishment of characteristic gut bacteria during development of the honeybee worker. Appl Environ Microbiol. 2012; 78: 2830–2840. https://doi.org/10.1128/AEM.07810-11 PMID: 22307297

39. D'Alvise P, Böhme F, Codrea MC, Seitz A, Nahnsen S, Binzer M, et al. The impact of winter feed type on intestinal microbiota and parasites in honey bees. Apidologie. 2018; 49: 252–264. https://doi.org/10.1007/s13592-017-0551-1

40. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010; 26: 2460–2461. https://doi.org/10.1093/bioinformatics/btq461 PMID: 20709691

41. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011; 27: 2194–2200. https://doi.org/10.1093/bioinformatics/btr381 PMID: 21700674

42. Lagkouvardos I, Joseph D, Kapfhammer M, Giritli S, Horn M, Haller D, et al. IMNGS: A comprehensive open resource of processed 16S rRNA microbial profiles for ecology and diversity studies. Sci Rep. 2016; 6: 1–9. https://doi.org/10.1038/s41598-016-0001-8

43. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods. 2013; 10: 996–998. https://doi.org/10.1038/nmeth.2604 PMID: 23955772

44. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007; 73: 5261–5267. https://doi.org/10.1128/AEM.00062-07 PMID: 17586664

45. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004; 32: 1792–1797. https://doi.org/10.1093/nar/gkh340 PMID: 15034147

46. Lagkouvardos I, Fischer S, Kumar N, Clavel T. Rhea: a transparent and modular R pipeline for microbial profiling based on 16S rRNA gene amplicons. PeerJ. 2017; 5: 2836. https://doi.org/10.7717/peerj.2836 PMID: 28097056

47. Roces F, Blatt J. Haemolymph sugars and the control of the proventriculus in the honey bee Apis mellifera. J Ins Physiol. 1999; 45: 221–229. https://doi.org/10.1016/s0022-1910(98)00116-4

48. Colin M.E., Ball B.V. Kilani M. Bacterial diseases. Bee Dis Diagnosis. 1999; 25: 31–59.

49. Olofsson TC, Alsterfjord M, Nilson B, Butler E, Vásquez A. Lactobacillus apinorum sp. nov., Lactobacillus mellifer sp. nov., Lactobacillus mellis sp. nov., Lactobacillus melliventris sp. nov., Lactobacillus kimbladii sp. nov., Lactobacillus helsingborgensis sp. nov. and Lactobacillus kullabergensis sp. nov., isolated from the honey stomach of the honeybee Apis mellifera. Int J Syst Evol Microbiol. 2014; 64: 3109–3119. https://doi.org/10.1099/ijs.0.059600-0 PMID: 24944337

50. Zheng H, Steele MI, Leonard SP, Motta EVS, Moran NA. Honey bees as models for gut microbiota research. Lab Anim (NY). 2018; 47: 317–325. https://doi.org/10.1038/s41684-018-0173-x PMID: 30353179

51. Corby-Harris V, Maes P, Anderson KE. The bacterial communities associated with honey bee (Apis mellifera) foragers. PLoS ONE. 2014; 9: e95056. https://doi.org/10.1371/journal.pone.0095056 PMID: 24740297

52. Wratt EC. The Pollinating Activities of Bumble Bees and Honeybees in Relation to Temperature, Competing Forage Plants, and Competition from Other Foragers. J Apic Res. 1968; 7: 61–66. https://doi.org/10.1080/00218839.1968.11100190

53. Sparks TH, Langowska A, Glazaczow A, Wilkaniec Z, Bienkowska M, Tryjanowski P. Advances in the timing of spring cleaning by the honeybee Apis mellifera in Poland. Ecol Entomol. 2010; 35: 788–791. https://doi.org/10.1111/j.1365-2311.2010.01226.x

54. Fukuda H, Sekiguchi K. Seasonal change of the honeybee wirker longevity in Sapporo, North Janan, with notes on some factores affecting the life-span. Ecol Soc Japan. 1966; 16: 206–212. https://doi.org/10.18960/seitai.16.5_206

55. Requier F, Paillet Y, Laroche F, Rutschmann B, Zhang J, Lombardi F, et al. Contribution of European forests to safeguard wild honeybee populations. Conserv Lett. 2019; 62: 427. https://doi.org/10.1111/conl.12693

56. Binazzi A, Scheurer S. Atlas of the honeydew producing conifer aphids of Europe. 1st ed. Roma: Aracne; 2009.

57. D'Alvise P, Seeburger V, Gihring K, Kieboom M, Hasselmann M. Seasonal dynamics and co-occurrence patterns of honey bee pathogens revealed by high-throughput RT-qPCR analysis. Ecol Evol. 2019; 9: 10241–10252. https://doi.org/10.1002/ece3.5544 PMID: 31624548

58. Schmickl T, Crailsheim K. Inner nest homeostasis in a changing environment with special emphasis on honey bee brood nursing and pollen supply. Apidologie. 2004; 35: 249–263. https://doi.org/10.1051/apido:2004019

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9 Curriculum vitae

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CAREER

07/2017 – today	**PhD student at Faculty of Natural Sciences** University of Hohenheim, Stuttgart (DE) project management and administration; website maintenance; academic teaching; doctoral programme; education didactic
Teaching skills 2017 – 2019	Lecture with exercise and practical training: "*Apiculture and beekeeping"* and *"Social insects"*, master level, University of Hohenheim, Stuttgart (DE)
10/2016 – 06/2017	Research assistant at University of Hohenheim, Stuttgart (DE) Microbiota, phylogeny und pathology on bees
02/2016 – 06/2016	Research assistant University of Santa Catarina (BRA) Nutrition studies of the Cidade das Abelhas
07/2015 – 12/2015	Research assistant, University of Brisbane (AUS) Coral – algae Analysis at the Great Barrier Reef
04/2015 – 06/2015	Research assistant, Eurofins Agrosciences (DE) Neonicotinoid exposure analyses on honey bees
02/2015 – 04/2015	Research assistant, Ozeaneum Stralsund (DE) Development of a fish scale atlas

EDUCATION

10/2012 – 10/2014	Master of Science; Evolution and Ecology and Parasitology Eberhard Karls Universität Tübingen, Tübingen (DE)
10/2009 – 10/2012	Bachelor of Science; Biology Eberhard Karls Universität Tübingen, Tübingen (DE)
09/2000 – 06/2009	General higher education Fanny-Leicht-Gymnasium, Stuttgart (DE)

SKILLS AND FURTHER EDUCATION

Languages	English	C2
	Portuguese	B1
EDV	Microsoft Office	very good knowledge
	JMP, SPSS, R	advanced knowledge
	TYPO3, Citavi	
Certificates	FIT für die Lehre – higher education didactics; BaWü-Certificate	
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SOCIAL ENGAGEMENT AND HOBBIES

07/2010 – 11/2012	First aider with education; German Red Cross, Stuttgart (DE)
01/2009 – 12/2011	Leader of children's camps and cooperation in exhibitions Evangelische Jugend, Stuttgart (DE) Anne Frank Zentrum, Berlin (DE)
09/2011	Reproductive medicine and endocrinology Fertility centre, Praxis Villa Haag, Stuttgart (DE)
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Hobbies	beekeeping, literature, cultures and languages

LIST OF PUBLICATIONS

Seeburger VC, D'Alvise P, Shaaban B, Schweikert K, Lohaus G, Schroeder A, et al. (2020) The trisaccharide melezitose impacts honey bees and their intestinal microbiota. PLOS ONE 15(4): e0230871.

Shaaban B. Seeburger V. Schroeder A. Lohaus G. (2020) Sugar, amino acid and inorganic ion profiling of the honeydew from different hemipteran species feeding on Abies alba and Picea abies. PLoS ONE 15: 1.

D'Alvise P. Seeburger V.C. Gihring K. Kieboom M. Hasselmann, M. (2019) Seasonal dynamics and co-occurrence patterns of honey bee pathogens revealed by high-throughput RT-qPCR analysis. Ecology and Evolution 9: 10241–10252.

Michiels N.K. Seeburger V.C. Kalb N. Meadows M.G. Anthes N. Mailli A.A. Jack C.B. (2018) Controlled iris radiance in a diurnal fish looking at prey. Royal Society Open Science 5: 170838.

Shaaban B. Seeburger V. Schroeder A. Lohaus G. (accepted 2020) Suitability of sugar, amino acid, and inorganic ion compositions to distinguish fir and spruce honey.

D'Alvise P. Seeburger V. Sontheimer L. Ziegelmann B. Rosenkranz P. Hasselmann M. (submitted 2020) Morganella morganii is a pathogen of honey bees.

LIST OF CONFERENCE CONTRIBUTIONS

20.-22.03.2018, Koblenz, Germany; 65th Conference of the Association of the German Bee Research Institutes e.V. Seeburger V., Shaaban B., Schroeder A., Hasselmann M., Lohaus G.:
Sugar Spectrum in Honeydew – the Fingerprint of Hemipteran Species

18.-20.09.2018; Ghent, Belgium; 8th EUR BEE Conference Seeburger V., Shaaban B., Schroeder A., Lohaus G., Hasselmann M.
Formation of Melezitose in Honeydew and its Impact on Honey Bee Health

26.-28.03.2019; Frankfurt, Germany; 66th Conference of the Association of the German Bee Research Institutes e.V. Seeburger V., Shaaban B., Schroeder A., Lohaus G., Hasselmann M.
Cement Honey – Melezitose Solely is Sufficient to Affect Honey Bee Health

08.-13.09.2019; Montréal, Canada; 46th Apimondia International Apicultural Congress, Seeburger V., Shaaban B., D'Alvise P., Schroeder, S. Hasselmann M.
Formation of Melezitose in Honeydew and its Impact on Honey Bee Health

12.-13.10.2020; eConference, worldwide; 16th COLOSS eConference, Seeburger V., Shaaban B., D'Alvise, P., Schweikert, K., Schroeder A., Lohaus G., Hasselmann M.
Production of the trisaccharide melezitose in honeydew by *Hemiptera* and its impact on the health and intestinal microbiota of honey bees

Eichenau, 07.12.2020
Place and Date

Signature

10 Affidavit

according to Sec. 7(7) of the University of Hohenheim doctoral degree regulations for Dr. rer. nat.

1. For the dissertation submitted on the topic
   „The production of melezitose in honeydew and its impact on honey bees (*Apis mellifera* L.)“
   I hereby declare that I independently completed the work.
2. I only used the sources and aids documented and only made use of permissible assistance by third parties. In particular, I properly documented any contents which I used – either by directly quoting or paraphrasing – from other words.
3. I did not accept any assistance from a commercial doctoral agency or consulting firm.
4. I am aware of the meaning of this affidavit and the criminal penalties of an incorrect or incomplete affidavit.

I hereby confirm the correctness of the above declaration: I hereby affirm in lieu of oath that I have, to the best of my knowledge, declared nothing but the truth and have not omitted any information.

Eichenau, 07.12.2020 _____

Place and Date Signature