



The identification of botanical and zoological origins of different honeydew honeys

Dissertation

for the purpose of obtaining a Doctorate
in the Faculty of Mathematics and Natural Sciences
of the University of Wuppertal

in the work group of
Molecular Plant Research/Plant Biochemistry (Botany)

submitted by

Basel Shaaban

Wuppertal, July 2020

Reviewer: Prof 'in Dr. Gertrud Lohaus, Bergische Universität Wuppertal

Co-Reviewer: Prof 'in Dr. Gela Preisfeld, Bergische Universität Wuppertal

The PhD thesis can be quoted as follows:

urn:nbn:de:hbz:468-20200921-104750-2

[<http://nbn-resolving.de/urn/resolver.pl?urn=urn%3Anbn%3Ade%3A468-20200921-104750-2>]

DOI: 10.25926/mesk-c384

[<https://doi.org/10.25926/mesk-c384>]

*For Mayada Mirza and Mohamad Shaaban
Everything started with you.....*

Contents

Contents.....	i
List of figures.....	i
List of tables	ii
Abstract	1
Zusammenfassung.....	2
1. Introduction.....	3
1.1. Host plants.....	4
1.2. Honeydew producers	5
1.3. Honeybees.....	7
1.4. Honeydew honey	8
1.5. Honey compounds	9
1.6. Aims of this work	12
2. Material and Methods	14
2.1. Chemicals and equipment	14
2.2. Plant material	14
2.2.1. Collecting of plants material	14
2.2.2. Collection of phloem exudates	14
2.3. Honeydew material.....	15
2.4. Enzyme activity in whole-body homogenates of aphids.....	15
2.5. Enzyme activity in the abdomen of honeybees	15
2.6. Honeydew honey material	16
2.7. Extraction of sugar, amino acids and ions from plant tissue by chloroform-methanol extraction.....	18
2.8. Protein extraction for honey.....	18
2.9. High-Performance-Liquid-Chromatography (HPLC).....	20
2.9.1. Analysis of sugars.....	20
2.9.2. Analysis of free amino acids	20
2.9.3. Analysis of inorganic ions.....	22
2.10. Statistic	22
2.10.1. Analyses of variance.....	22
2.10.2. Non-metric multidimensional scaling (NMDS)	23
2.10.3. Principle component analysis (PCA).....	23
2.10.4. Permutational analysis of variance (PERMANOVA)	23
2.10.5. Permutational Analysis of Multivariate Dispersions (PERMDISP).....	24
2.10.6. Redundancy analysis (RDA).....	24

2.11. Bioinformatics	25
3. Results.....	26
3.1. Section I: Honeydew composition of six different hemipteran species feeding on <i>Abies alba</i> and <i>Picea abies</i>	27
3.2. Section II: Effects of different temperatures on melezitose production in aphid species.....	64
3.3. Section III: The ability of honeybees <i>A. mellifera</i> to digest melezitose	67
3.4. Section IV: Honeydew honey profiling of fir, spruce and pine honey	70
3.5. Section V: Nectar, honeydew and honey profiling of lime tree <i>Tilia sp.</i>	78
4. Discussion	83
4.1. From phloem sap to honeydew by conifers	83
4.2. The enzymatic formation and cleavage of melezitose	85
4.3. From honeydew to honeydew honey by conifers	87
4.4. Nectar, honeydew and honey profiling of lime tree <i>Tilia sp.</i>	91
5. References.....	92
Appendix	100
Acknowledgement	104
Declaration	105

List of figures

Fig 1. 1. Some honeydew producers while feeding on host plants	7
Fig 1. 2. The production of honeydew honey	8
Fig 3. 1. Melezitose proportion of <i>C. pilicornis</i> and <i>C. pectinatae</i> honeydew.....	64
Fig 3. 2. Melezitose production in whole-body homogenates of <i>C. pilicornis</i> and <i>C. pectinatae</i>	66
Fig 3. 3. Sugar compositions in “honey”, produced by bees after 21 days.	68
Fig 3. 4. Sugar proportions in abdomen homogenates of <i>A. mellifera</i>	69
Fig 3. 5. Redundancy Analysis (RDA) plots.	76
Fig 3. 6 Redundancy Analysis (RDA) plots.	77
Fig 3. 7 Sugar proportion in nectar, honeydew and honey of <i>Tilia sp.</i>	80
Fig 3. 8. Sugar concentration in whole-body homogenates of <i>Eucallipterus tiliae</i>	82

List of tables

Table 1. 1. Honeydew producers and their host plants	6
Table 1. 2. Honey oligosaccharides	10
Table 2. 1. The geographic coordinates for the collection stands of honeydew samples.....	16
Table 2. 2. The geographic coordinates for the collection stands of honeydew honey samples.....	17
Table 2. 3. Setting for the pulse measurements on the amperometer.....	20
Table 2. 4. Solution for pre-column derivatization.....	21
Table 2. 5. Eluent for the analysis of free amino acids	21
Table 2. 6. A list of used software	25
Table 3. 1. Sugar concentration from whole aphid's body	65
Table 3. 2. Sugar contents in four groups of honeydew honey.....	71
Table 3. 3. Amino acids contents in four groups of honeydew honey.	72
Table 3. 4. Inorganic ions contents in four groups of honeydew honey.....	73
Table 3. 5. Protein contents in four groups of honeydew honey	73
Table 3. 6. Results of the RDA.....	74
Table 3. 7. <i>P</i> -values of pairwise comparisons for each group of honeydew honey.	74
Table 3. 8. Results of the RDA.....	77
Table 3. 9. <i>P</i> -values of pairwise comparisons for each group of honeydew honey.....	77
Table 3. 10. Sugar proportions in nectar, honeydew and honey of <i>Tilia sp.</i>	79
Table 3. 11. Amino acids proportions in nectar, honeydew and honey of <i>Tilia sp.</i>	81
Table 3. 12. Inorganic ions proportions in nectar, honeydew and honey of <i>Tilia sp.</i>	82

Abstract

Honeydew honey is produced by bees from excretions – the honeydew – of plant-sucking insects. Honeydew can be produced by different insect-species, such as the genera *Cinara* and *Physokermes* on conifers and the genera *Eucallipterus* on lime trees. Therefore, honeydew honey can stem from different botanical as well as different zoological origins. In order to investigate the process from phloem sap to honeydew, honeydew samples from different Hemipteran species were collected and the sugar, amino acid, and inorganic ion profiles were determined. Honeydew from all species contains different proportions of hexoses, sucrose, melezitose, erlose, and trehalose, whereas the phloem exudates of the host trees contain no trisaccharide. That was confirmed by incubating whole-body homogenates of different aphid species with sucrose, the outcome of which was melezitose and erlose. Additionally, the classification of honeydew samples on the basis of their sugar profiles showed that the proportions of sugars differed significantly between different hemipteran species feeding on the same tree species. Moreover, statistical analyses reveal that the sugar composition of honeydew is determined more by the hemipteran species than by the host plant.

In order to identify the botanical and zoological origin of honeydew honey, fir, spruce, and pine honey samples were collected and analyzed. Fir and spruce samples were collected in different locations in south Germany, pine honey samples were collected in Turkey. Pine honeys can be separated from fir and spruce honey because of their high contents of (undef 3) sugar and inorganic ions. In addition, fir and spruce honey can be divided in three groups: *Physokermes*/spruce, *Cinara*/spruce, and *Cinara*/fir. *Physokermes*/spruce honey also has a significantly higher content of phosphate and (undef 6) sugar than the other two honeys. To reliably distinguish between fir/*Cinara* and spruce/*Cinara* honey, however, no chemical marker was found within the analyzed compounds.

In order to clarify the origin of linden honey, sugars, amino acids, and inorganic ions, profiles for honeydew, nectar, and honey from *Tilia* sp. were determined. Melezitose and erlose were determined in the honeydew and in the honey, but not in the nectar. In addition, the incubation of whole-body homogenates of different aphid species with sucrose, resulting in melezitose and erlose, confirmed our results. Finally, the ability of honeybees' cleavage enzymes to digest melezitose was also investigated. Honeybees' abdomen enzymes are able to cleave melezitose and produce glucose and fructose; this process, however, is not as efficient as the cleavage of sucrose.

Zusammenfassung

Honigtau Honig wird von Bienen aus den Ausscheidungen – dem Honigtau – von pflanzensaugenden Insekten hergestellt. Honigtau wird von verschiedenen Insektenarten produziert, z.B. von den Gattungen *Cinara* und *Physokermes* auf Nadelbäumen. Honigtau Honig kann daher sowohl von verschiedenen botanischen als auch verschiedenen zoologischen Herkünften stammen. Um die Umwandlungsprozesse von Phloemsaft zu Honigtau zu untersuchen, wurden Honigtau Proben von verschiedenen Hemipteren-Arten gesammelt und die Zucker-, Aminosäure- und anorganischen Ionenprofile erstellt. Honigtau von allen Arten enthielt unterschiedliche Anteile an Hexosen, Saccharose, Melezitose, Erlöse und Trehalose, während die Phloem-Exsudate der Wirtsbäume keine Trisaccharide enthielten. Dies wurde durch die Inkubation von Ganzkörperhomogenaten verschiedener Blattlausarten mit Saccharose und den daraus resultierenden Trisacchariden Melezitose und Erlöse bestätigt. Zusätzlich wurden Honigtau Proben aufgrund ihrer Zuckerprofile klassifiziert. Die Zuckeranteile unterschieden sich signifikant zwischen unterschiedlichen Lausarten, die sich von der gleichen Baumart ernährten. Darüber hinaus zeigen statistische Analysen, dass die Zuckerzusammensetzung von Honigtau eher durch die Lausarten als durch die Wirtspflanze bestimmt wird.

Um die botanische und zoologische Herkunft des Honigtau Honigs zu identifizieren, wurden Tannen-, Fichten- und Kiefern Honigproben gesammelt und analysiert. Tannen- und Fichten Honigproben wurden an verschiedenen Orten in Süddeutschland gesammelt, Kiefern Honigproben wurde in der Türkei gesammelt. Kiefern Honige konnten wegen ihres hohen Gehalts an (undef 3) Zucker und anorganischen Ionen von Tannen- und Fichten Honig unterschieden werden. Darüber hinaus konnten Tannen- und Fichten Honig in drei Gruppen unterteilt werden: *Physokermes*/Fichte, *Cinara*/Fichte und *Cinara*/Tanne. *Physokermes*/Fichten Honig weist einen signifikant höheren Gehalt an Phosphat und (undef 6) Zucker als die anderen beiden Honige auf. Jedoch wurde kein chemischer Marker innerhalb der Kategorien der analysierten Verbindungen gefunden um verlässlich zwischen Tanne/*Cinara* und Fichte/*Cinara*-Honig unterscheiden zu können.

Zusätzlich wurde die Melezitose-Verdaulichkeit der Spalt-Enzyme der Honigbienen untersucht. Die Enzyme im Abdomen der Honigbiene sind in der Lage, Melezitose in Glukose und Fruktose zu spalten, dies ist aber nicht so effizient wie die Spaltung von Saccharose.

1. Introduction

Honey is a naturally sweet substance produced by the honeybee *Apis mellifera* from the nectar of flowers or the secretion left behind by plant-sucking insects (honeydew). Honey can be distinguished in different ways. It can be declared either by botanical, zoological or geographical origin. The most common distinction according to botanical origin is between blossom honey and honeydew honey. Nowadays, honey is the most important primary product of beekeeping and is studied in the most detail with regard to its effectiveness and composition. The quality of honey is defined by various standards in the European food law. The high value placed on honey as a product is also reflected in its economic interest. About 1.5 million tons of honey are produced per year. In Germany alone, about 100,000 tons are consumed per year, with a per capita consumption of about 1.4 kg. Therefore, the Germans register the largest average consumption world-wide (Beckmann 2008).

Native honeydew honeys, known as forest honeys, are among the most desirable honeys in Germany due to their spicy and malty taste. In addition, spruce honeys and fir honeys are the most produced honeydew honeys in Germany (Kunkel and Kloft 1985). As pure fir honeys are considered preferable among other forest honeys and they are rare due to the small number of fir stands, they can be sold particularly profitable (BMEL 2016). To date, fir honey can only be distinguished from spruce honey by sensory means. However, chemical or physical parameters which can be specifically assigned to fir or spruce are still missing (Bertoncelj *et al.* 2011). Therefore, the local or regional origin, the botanical origin (fir or spruce) and the zoological species (honeydew producers) can currently not be clearly controlled either by the official food control authorities or by scientists using chemical analyses and parameters in order to protect the consumer from deception.

In order to identify the botanical (host tree), zoological (honeydew producers) and geographical origin of honeydew honeys, the project BoogIH was established (<https://boogih.uni-hohenheim.de/startseite>). Several analytical methods are necessary to clarify these claims.

Apart from the honeydew honeys' economic importance, there is also a relevance for the health and stability of honeybees' populations. Honeydew can contain melezitose, which can lead to up to 30% bee population losses in cases where the population is wintering with more than 20 % melezitose proportions (Bogdanov 1985). Therefore, the determination of the melezitose proportion in honeydew and the identification of the factors that influence its proportion is remarkably important for the health, the spread and the prosperity of honeybees' populations.

1.1. Host plants

Honeydew-producing plants include mainly conifers such as the genus *Abies sp.*, *Picea sp.* and *Pinus sp.* and deciduous trees of the genus *Quercus sp.*, *Castanea sp.*, *Ulmus sp.* and *Fraxinus sp.* as well as *Fagus sylvatica L.* and *Corylus avellana L.* (Diez *et al.* 2004; von der Ohe *et al.* 2004). All of them have vascular vessels to transport water and assimilates throughout the plant. Transporting elements within vascular plants are the phloem, which transports mainly assimilates, and the xylem, which transports water and minerals.

Phloem sap is generally dominated by sucrose, with concentrations ranging from 0.7 to 1.5M (Fink *et al.* 2018; Lohaus and Moellers 2000 and Woodring *et al.* 2004). However, some tree species also translocate oligosaccharides of the raffinose family like members of the Oleaceae (Öner-Sieben and Lohaus 2014). Additionally, members of the Rosaceae also transport some sugar alcohols in the phloem sap (Nadwodnik and Lohaus 2008).

Phloem sap also contains amino acids with a concentration of 50 to 200 mM. Although some amino acids, like GLU, GLN, ASP, and ASN, are dominant in different plant species (Lohaus and Moellers 2000 and Woodring *et al.* 2004), all amino acids were found in the phloem sap (Sandström and Moran 2001). Ions were also determined in the phloem.

Despite the fact that all amino acids were found and the high sugar concentrations, phloem sap is not an ideal diet for insects. This is because of its high osmotic pressure, the low ratio of amino acids compared to sugars, and the ratio of essential-to-non-essential amino acid, which is lower in phloem sap than in the insect protein (Douglas 2006).

Spruce (*Picea abies* (L.) H. Kast.) belongs to the Pinaceae, genus of *Picea*. It occurs mainly in Central, Eastern and Northern Europe. Spruce translocates mainly sucrose in the phloem sap (Ziegler and Mittler 1959).

Fir (*Abies alba* Mill.) belongs to the Pinaceae, genus of *Abies*. It is common in humid locations in Central, South and Southeast Europe. Fir is characterized by its deep root system, which makes it a stabilizing element in stepped forests created by natural regeneration (Kutschera and Lichtenegger 2002).

The lime trees (*Tilia sp.*) belongs to the Malvaceae. The genus *Tilia* contain almost 45 different species. The winter linden (*Tilia cordata* Mill.) and the large-leaved linden (*Tilia platyphyllos* Scop.) are the most common ones in Europa. The linden flowers differently depending on its species, for example the large-leaved linden flowers in June.

1.2. Honeydew producers

Honeydew producers belong to the insect order Hemiptera. Important groups within this order are the animals of the order Aphidinea (aphids), Coccinea (scale insects, especially Lecanidae) and Auchenorrhyncha (cicadas). They live on leaves, branches and twigs (Kloft *et al.* 1985). They use their bristles to directly pierce the sieve tubes of herbs, shrubs, and trees. The uptake into the feeding tube is either passive due to the turgor pressure that exists in the plant or active by sucking (Nabors and Scheibe 2007). The honeydew excreted by the hemipteran species differs significantly from the phloem sap ingested, and this is due to the enzymes added by the hemipteran species, such as invertases (Auclair 1958; Auclair 1959). Since it was already subject to enzymatic changes in the intestines of honeydew producers as well as through microorganisms living in the intestine, significantly higher amounts of oligosaccharides such as maltose, erlose and melezitose are found in honeydew (Doner 1977). The amount of honeydew excreted per hour can exceed the body weight of some species. However, there is a big variation from species to species (Mittler 1957; Mittler 1958). Freshly excreted honeydew is a clear colorless liquid with a dry matter content of 5-18% and it increases to 35-50% by evaporation.

Honeydew is mainly composed of sugars: there are different monosaccharides (glucose, fructose), disaccharides (e.g. sucrose, trehalose, maltose) and trisaccharides (e.g. melezitose, erlose, raffinose) (Hendrix *et al.* 1992, Bacon and Dickinson 1957 and Liebig 1979). However, inorganic ions, amino acids, proteins and other compounds were also found in honeydew (Auclair 1963; Leroy *et al.* 2011; Sabri *et al.* 2013; Völkl *et al.* 1999).

The honeydew content is mainly determined by the honeydew producers. However, host plant species, seasonal changes, and environmental conditions can also affect the composition of honeydew (Sandström and Maron 2001; Fischer *et al.* 2002 and Hendrix *et al.* 1992). Moreover, samples from the same aphid species feeding on the same host plant differ strongly among each other (Fischer and Shingleton 2001).

Different aphid species feeding on different host trees Fig 1. 1, Table 1. 1. *Cinara pectinatae* (Nördlinger, 1880), *Cinara confinis* (Koch, 1856) feeding on fir, *Cinara pilicornis* (Hartig, 1841), *Cinara piceae* (Panzer, 1801), *Physokermes piceae* (Schrank, 1801), and *Physokermes hemicryphus* (Dalman, 1826) feeding on spruce, and *Eucallipterus tiliae* (Linnaeus, 1758) feeding on lime trees.

Table 1. 1. Honeydew producers and their host plants

Host plant	Honeydew producers
<i>Picea abies</i>	<i>Cinara pilicornis</i> <i>Cinara piceae</i> <i>Physokermes piceae</i> <i>Physokermes hemicryphus</i>
<i>Abies alba</i>	<i>Cinara pectinatae</i> <i>Cinara confinis</i>
<i>Tilia sp.</i>	<i>Eucallipterus tiliae</i>

The Lachniden (*Cinara sp.*) are mobile through their life-times. They can produce more than one generation per year. Their sucking place is almost always unprotected, for example against weather influences. Therefore, they are very dependent on the weather conditions. The honeydew is usually dropped by *Cinara sp.*, so that it can be found further away from honeydew producers. On the contrary, Lecania (*Physokermes sp.*) are immobile for most of their lives, therefore they do not change their suction point. The suction point lies hidden under the whisking shed, thus, they can develop without remarkable impact of the weather (Kloft and Kunkel 1985).



Physokermes piceae
Picea abies



Physokermes hemicryphus
Picea abies



Cinara piceae
Picea abies



Cinara pilicornis
Picea abies



Cinara pectinatae
Abies alba



Cinara confinis
Abies alba

Fig 1. 1. Some honeydew producers while feeding on host plants (<https://boogih.uni-hohenheim.de/startseite>)

1.3. Honeybees

Honeybees (*Apis mellifera*) belong to the social insects that live together collectively in a beehive. They usually live in colonies consisting of one queen, 10,000 - 30,000 female worker bees and zero to several thousand male drones. Worker bees perform all tasks connected with colonial life, such as building combs and collecting pollen, nectar or honeydew. The queen is the only reproductive animal in the colony and is therefore responsible for its preservation. Worker bees and queen bees of a colony have very different lifetimes. While the former live only three to six weeks in spring and summer and up to four months in winter, the latter can

live two to three years. However, queens usually live less than one year in hives used for commercial honey production (Page and Peng 2001). Workers produce honey; they gather nectar or honeydew in their honey stomach, and then they stock it in honeycombs. In addition, cervical gland enzymes are added to the sweet liquid so that the honey is transformed for example catalyzing the cleavage of sucrose into glucose and fructose (Kubota *et al.* 2004).

1.4. Honeydew honey

Honeydew honey is a natural sweet substance produced by *Apis mellifera* from secretions of living parts of plants or from excretions of plant-sucking insects (Directive 2014/63/EU of the European Parliament and of the Council amending Council Directive 2001/110/EC relating to honey). Therefore, honeydew honey has one botanical origin and two zoological origins (honeydew producers and honeybee) Fig 1.2.

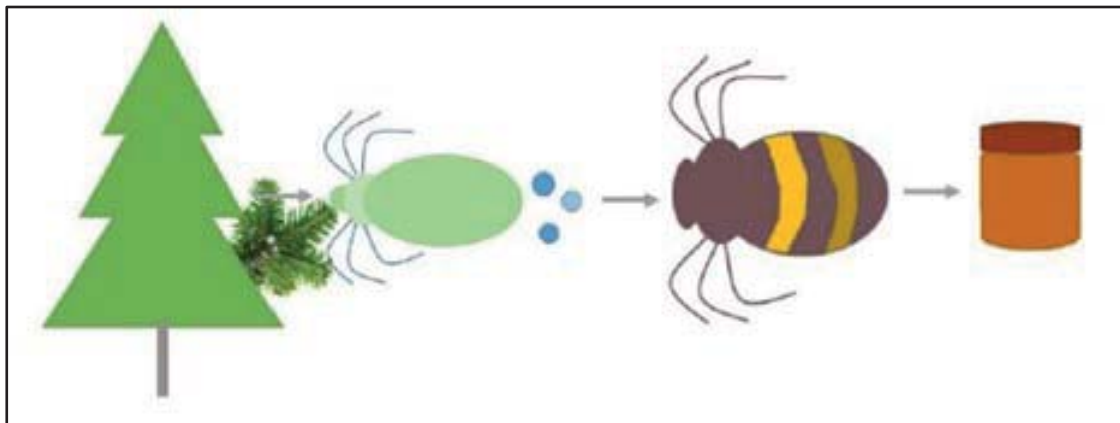


Fig 1. 2. The production of honeydew honey (Source: Gertrud Lohaus).

Honeydew honeys are also called the forest honeys and can be classified according to their botanical origins: spruce, fir or pine honey. Compared to blossom honeys, honeydew honeys have a rather spicy, malty or woody taste and a darker color (Belitz *et al.* 2008). Honeydew honeys can be characterized because of their honeydew elements (HDE) such as fungal spores, soot elements, wax wool and tubes and crystalline mass. These are components of the natural surface of trees or leaves. They found their way to honeydew honey during the collection of honeydew (Kloft and Kunkel 1985). In addition, according to the European Legislation (Council Directive 2001/110/EC) honeydew honeys must have electrical conductivity values $\geq 0.8 \text{ mS cm}^{-1}$, while flower honeys (with some exceptions) must have electrical conductivity values $\leq 0.8 \text{ mS cm}^{-1}$.

1.5. Honey compounds

Honey is a very concentrated and complex nutrition because it contains various components, such as carbohydrates, proteins, amino acids, inorganic ions, organic acids, aromatic and phenolic components, and vitamins. These components define the sensory properties of honey, such as taste, color, and odor. They also determine the physical and chemical properties of honey, like viscosity and pH. Furthermore, because many of these components are introduced to the honey from different botanical, zoological and geographical origins, they can be used to determine the various origins of honey (Bertoncelj *et al.* 2011; Pita-Calvo and Vázquez 2018).

Carbohydrates are the quantitatively most important ingredients in honey and they are responsible for the high viscosity as well as for possible crystallization processes. The main component of honey are carbohydrates, they account for about 80 g per 100 g honey; therefore, honey has a good storage stability. The exact composition of a given honey is influenced by the botanical, geographical and zoological origin (Ruoff *et al.* 2007). However, the two main sugars are always glucose and fructose. Together, their content makes about 70 g per 100 g honey (da Silva *et al.* 2016, Pita-Calvo and Vázquez 2018). In addition, honey contains a large number of di- and trisaccharides like turanose, maltose, isomaltose, raffinose, melezitose and erlose. Table 1.2 summarizes the oligosaccharides that can be found in honey. Interestingly, the oligosaccharides content is much higher in honeydew honey than in blossom honey (Doner 1977; Ruoff *et al.* 2007; and Bogdanov *et al.* 2008). The trisaccharide melezitose is considered to be a feature of honeydew honey (Doner 1977). As some honeydew producers feed on phloem sap, the conversion of sucrose to melezitose can occur because of the transglucosylase activity in their stomach.

The second essential component of honey is water, the average content of which is between 16 and 20%. The water content is critically important for the determination of the consistency and durability of the honey. The higher the water and the lower the sugar content, the shorter the durability of honey.

Table 1. 2. Honey oligosaccharides (Doner 1977)

Common name	Structure
Disaccharides	
Trehalose	1- α -D- glucopyranosyl-1 α -glucopyranosid
Nigrose	O- α -D- glucopyranosyl- (1 \rightarrow 3) -D- glucopyranose
Maltose	O- α -D- glucopyranosyl- (1 \rightarrow 4) -D- glucopyranose
Isomaltose	O- α -D- glucopyranosyl- (1 \rightarrow 6) -D- glucopyranose
Melibiose	O- α -D- galactopyranosyl- (1 \rightarrow 6) -D- glucopyranose
Sucrose	α -D-glucopyranosyl- (1 \rightarrow 2)- β -D-fructofuranoside
Turanose	O- α -D- glucopyranosyl- (1 \rightarrow 3)-D-fructose
Palatinose	O- α -D- glucopyranosyl- (1 \rightarrow 6)-D-fructose
Maltulose	O- β -D- glucopyranosyl- (1 \rightarrow 4)-D-fructose
Kojibiose	α -D-glucopyranosyl-(1 \rightarrow 2)-D-glucose
Gentiobiose	β -D-glucopyranosyl-(1 \rightarrow 6)-D-glucopyranose
Trisaccharides	
Maltotriose	O- α -D- glucopyranosyl- (1 \rightarrow 4) - O- α -D- glucopyranosyl- (1 \rightarrow 4) - D- glucopyranose
Isomaltotriose	O- α -D- glucopyranosyl- (1 \rightarrow 4) - D- glucopyranosyl- (1 \rightarrow 6) -D- glucopyranose
Melezitose	O- α -D- glucopyranosyl- (1 \rightarrow 3)-O- β -D-fructofuranosyl- (2 \rightarrow 1) α -D- glucopyranose
1-Kestose	O- α -D- glucopyranosyl- (1 \rightarrow 2)- β -D-fructofuranosyl- (1 \rightarrow 2) β -D- fructofuranoside
Erlose	O- α -D- glucopyranosyl- (1 \rightarrow 4)- α -D-glucopyranosyl β -D- fructofuranoside
Raffinose	α -D- galactopyranosyl- (1 \rightarrow 6) -D- glucopyranose-(1 \rightarrow 2) β -D- fructofuranoside
Tetrasaccharide	
Stachyose	α -D- galactopyranosyl- (1 \rightarrow 6) - α -D- galactopyranosyl- (1 \rightarrow 6) -D- glucopyranose-(1 \rightarrow 2) β -D-fructofuranoside

Honey contains also 0.2-0.7% proteins, which mainly stem from the glandular secretions of bees (Kubota *et al.* 2004; Mohammed *et al.* 2012). Additionally, a smaller part comes from the nectar, the honeydew, and the pollen. Therefore, the protein content varies according to the botanical, geographical and zoological origin; thus, the concentration of different proteins is a possibly useful indicator for the geographical and botanical origin. In addition, the protein content can be influenced by several other factors, such as the origin and the condition of the bee colony or the season. Further, prolonged storage, heat and exposure to light can decrease the protein concentration (Mohammed *et al.* 2012). The main protein in honey is Major Royal Jelly Protein 1 (MRJP1), which makes up about 23% of the total protein content. Up to nine important royal jelly proteins from different botanical and geographical origins were determined in honey (Escuredo *et al.* 2013).

Honey also contains enzymes, that are secreted mainly by bees from the sap and salivary glands, or from microorganisms (Kubota *et al.* 2004). Enzymes are sensitive to light, heat and other energy sources, and their activity is considered a quality parameter and indicator for the storage and processing of honey. The most important honey enzymes are invertase, disastase and glucose oxidase (Belitz *et al.* 2008). Invertase makes up about 50% of the proteins secreted from the glands, while disastase and glucose oxidase make up only about 2-3%. They all come from honeybees, although invertase can occur as a result of microbial activities (Kubota *et al.* 2004). Further, three different types of the invertase α -glucosidase were determined in honey: α -glucosidase I, II and III, and more than 18 isomers of them were discovered in honeybees (Kubota *et al.* 2004). They split the α -glycosidic bond of sucrose and other oligosaccharides, releasing glucose and fructose. The disastase is capable of splitting starch and higher sugars. Glucose oxidase is a particularly important enzyme as it plays a significant role in honey maturation. The enzyme catalyzes the reaction of glucose to glucono- δ -lactone, which further reacts to gluconic acid and hydrogen peroxide. This is responsible for the low pH values in honey and, thus, associated with the antimicrobial properties of honey (Flanjak *et al.* 2016).

Honey generally contains about 0.3 to 2.0 g kg⁻¹ free amino acids, mainly proline (Cotte *et al.* 2004). Amino acids found their way into the honey primarily from honeybee's proteins in honey (Ball 2007). In addition, nectar, honeydew, and pollen are also involved in the determination of the amino acids contents; therefore, their proportions and contents can be influenced by botanical and zoological origins of the honey (Sing and Sing 1996). For example, amino acids were used to identify the geographical or botanical origin of honey, however, without highly conclusive results (Iglesias *et al.* 2004 and Cotte *et al.* 2004).

The total mineral content in honey varies dramatically between the different botanical and geographical origins of honey. The inorganic ion-content in honey is a result of various factors such as nature of the soil and plant species. Further, anthropogenic factors, like environmental pollution from industry and urban areas, also influence the composition of the metal ions in the honey (Fermo *et al.* 2013). Honeydew honey has high amounts of Potassium and the total mineral content is about 1%, while it is only 0.1-0.2% in flower honey (Castro-Vázquez *et al.* 2006, Escuredo *et al.* 2012). Further, the mineral content was also used to distinguish honey on the basis of their botanical origins (Jovetić *et al.* 2017).

The organic acids are present in the honey at 0.5% and contribute to its organoleptic and physico-chemical properties such as pH, acidity and electrical conductivity. Despite the higher acidity, the pH of honeydew honey is higher than that of nectar honey, which is due to the better buffering effect of the minerals and salts contained in honeydew honey (Mato *et al.* 2007).

The different aromas and taste qualities can be used to differentiate between honeydew honey and nectar honey. Compared to the blossom honey, honeydew honey has a stronger, more powerful, malty-spicy aroma. The sensory analysis is a commonly used method to differentiate between different types of honey (BMEL 2004).

The phenolic compounds are one of the largest group of plant secondary substances that protect the plant from biotic, abiotic and oxidative stress. Among them are phenolic carboxylic acids and flavonoids, which can be present in different concentrations. The most common compounds are aromatic and aryl-aliphatic carboxylic acids, as well as hydroxyl and methoxy derivatives of benzoic acid and cinnamic acid. Some studies consider that dark honeys, especially honeydew honey, tend to contain higher concentrations of phenolic compounds (Seraglio *et al.* 2016).

1.6. Aims of this work

This work is a part of the BoogIH project and aims to clarify the processing of phloem sap to honeydew by the honeydew producer and of honeydew to honey by the honeybee. Further, the occurrence and the proportion of melezitose in honeydew was investigated. Moreover, this work aims to identify distinguishing features for the botanical (host tree) and zoological (honeydew producers) origins of honeydew honeys and associate them with different honeydew producers and plant species. Thus, the variety of "honeydew honeys" can be extended and controlled by means of reliable declarations and by means of honeybee protection, which is of great economic importance, and ecological importance worldwide.

Honeydew samples from different honeydew producers feeding on different trees and phloem exudates and plants extraction from host plants were analyzed using many HPLC approaches. In particular, the sugars, amino acids, and inorganic ions were determined for each sample. Then various statistical methods were utilized to distinguish between honeydew samples and to classify samples according to their botanical or zoological origins.

Then, enzyme activities of hole bodies of different honeydew producers were examined to investigate which factors (honeydew producers or temperature) contribute more to the production process of melezitose in honeydew. After that, the ability of honeybee's abdomen enzymes to catalyze melezitose was examine and compared to sucrose.

In the next step, sugar, amino acid and inorganic ion profiles of honeydew honeys from four botanical origins (fir, spruce, linden and pine) were established and statistical model was created to distinguish between them. In addition, fir and spruce honeys were also analyzed according to their zoological origins to examine if the zoological origins contribute more than the botanical origins of the honey component.

To summarize, this work aims to clarify the following questions:

1. Does the sugar, amino acid and inorganic ion content of honeydew differ significantly between aphid species and are they related to the phloem sap of host tree? Additionally, which factors contribute more to the components of honeydew (botanical or zoological origins)?
2. Why is the melezitose content inconsistent in honeydew, and which factors can influence the presence/absence and the proportion of melezitose in honeydew?
3. Are the honeybees able to digest melezitose effectively?
4. Can fir, spruce, and pine honey be distinguish on the basis of sugar, amino acid, and inorganic ion contents? Can spruce and fir honey be classified according to their zoological origins?
5. Can the origin (nectar or honeydew) of linden honey be identified on the basis of sugar, amino acid, and inorganic ion contents?

2. Material and Methods

2.1. Chemicals and equipment

A list of the chemicals used with sources of supply and laboratory equipment with manufacturer's details can be found in the appendix. The chemicals used in the tests were always purchased in the highest possible degree of purity.

2.2. Plant material

Within this work five different tree species were investigated. Fir (*Abies alba*), spruce (*Picea abies*), linden (*Tilia sp.*), beech (*fagus sylvatica*) and oak (*Quercus robur*). All trees were obtained as 3-year-old seedlings from the Selders (Haan, Germany). Trees were kept in a greenhouse. The roof was made of UV-transparent Plexiglas and the roof windows were open wide enough to prevent rain from entering and to accumulate heat. The plants were watered regularly. In addition, some plant samples were collected in five spruce (*Picea abies*) or fir (*Abies alba*) stands of Baden-Wuerttemberg (Germany). The geographic coordinates for the stands are 48° 66'41"N, 8°32'54"E; 48°48'19"N, 8°37'13"E; 48°31'56"N, 8°47'04"E; 48°95'58"N, 8°70'60"E; and 49°00'40"N, 10°07'07"E. The samples were collected in plastic tubes, immediately frozen and stored at -80°C until analysis.

2.2.1. Collecting of plants material

Needles, leaves, bark and wood samples were collected in spring from all tree species. Samples were then immediately stored in plastic tubes at -80°C until analysis. Further, on June when linden flowers are opened, nectar was gathered from the flower using a micropipette or by placing flowers in in a 0.5 ml plastic tube with a small hole at its bottom and centrifuge them at 5000 rpm for 20 min at 4°C. Nectar samples were stored at -80°C until analysis.

2.2.2. Collection of phloem exudates

Phloem exudate of bark from all tree species was collected in spring and early summer (between 11 am and 3 pm). For each tree species, six samples of phloem exudate were prepared. roughly 2 cm long pieces of bark were placed in a 0.5 ml plastic tube with a small hole at its bottom. This tube was placed inside a 2 ml plastic tube. The samples were then centrifuged at 5000 rpm for 20 min at 4°C (Hijaz and Killiny 2014). The samples were stored -80°C until analysis.

2.3. Honeydew material

The honeydew samples were collected in five stands of Baden-Württemberg (Germany) between 2016 and 2020. Honeydew of *Cinara pectinatae* (Nördlinger, 1880), *Cinara confinis* (Koch, 1856), *Cinara pilicornis* (Hartig, 1841), *Cinara piceae* (Panzer, 1801), *Physokermes piceae* (Schrank, 1801), *Physokermes hemicryphus* (Dalman, 1826) and *Eucallipterus tiliae* (Linnaeus, 1758) was collected with micropipettes and pooled up to a volume of at least 1 µL per sample and immediately frozen and stored at -80°C until analysis. All geographic coordinates for the collection stands of honeydew samples are summarized in table 2.1. Honeydew samples were diluted with water (1:100) and then analyzed via HPLC.

2.4. Enzyme activity in whole-body homogenates of aphids

Individuals of two aphid species were collected from the host plant using a paintbrush, namely the species *C. pilicornis* feeding on *P. abies*, *C. pectinatae* feeding on *A. alba* and *E. tiliae* feeding on *T. cordata*. After collection, 20 mg aphid-material was homogenized with the help of a pestle and 300 µL sucrose solution (10%, pH 7) was added to the homogenate. The homogenate-sucrose-mixture was then incubated at three different incubation temperatures namely 25°, 30° and 35° C. Of the differently incubated samples, aliquots (50 µL) were taken after 0, 30, 60, and 120 minutes and immediately put into 50 µL NaOH (200 mM) to inactivate the enzyme activities. Finally, the solution was centrifuged (13,000 x g, 30 sec) and the supernatants were taken for sugar analysis by HPLC. The experiment was performed at least three times for each sugar solution

2.5. Enzyme activity in the abdomen of honeybees

The enzyme activities were analyzed in the abdomen of *A. mellifera*. The abdomens of worker bees were separated with a razor blade and homogenized in a cooled plastic tube with the help of a pestle. 300 µL sugar solution (20%, pH 7) were added to 200 mg abdomen homogenate and incubated at 37°C. The sugar solutions were either 10% sucrose/10% melezitose, 20% melezitose or 20% sucrose. After 0, 30, 60, and 120 minutes, aliquots (50 µL) were taken and 50 µL NaOH (200 mM) were added to inactivate any enzyme activities. The solution was then centrifuged (13,000 xg, 30 sec) and the supernatants were taken for sugar analysis by HPLC. The experiment was performed at least three times for each sugar solution.

Table 2. 1. The geographic coordinates for the collection stands of honeydew samples.

Location	GPS
Hohenheim	48°42'33.1"N 9°12'40.3"E
Göbrichen	48°57'313"N 008°42'4940"E
Tiefenbronn	48°49'57"N 008°48'17"E
Oberentersbach	48°53'29.274"N 009°50'51.021"E
Eschach	48°53'35"N 009°50'20"E
Alfdorf	48°53'29.274"N 009°50'51.021"E
Fluorn-Winzeln	48°17'30.4"N 8°27'09.5"E
Eisenbach	47°57'32.4"N 8°19'45.2"E
Jagstzell	49°01'07.5"N 10°06'53.8"E
Bartholomä	48°44'05.7"N 9°58'53.3"E
Schömberg	48°49'12.2"N 8°45'56.8"E
Langenbrand	48°48'19.1"N 8°37'13.3"E
Schömberg	48°49'12.2"N 8°45'56.8"E
Langenbrand	48.7022, 008.3624
Bretzfeld	49.1849, 009.3779
Vogt	47.7745, 009.7255
Höchenschwand	47.6987, 008.2218
Böhmenkirch	48.7087, 009.9426
Michelfeld	49.0981, 009.6162
Forbach	48.6571, 008.3301
Raumünzach	48.6368, 008.3438
Schwabbach	49.1819, 009.4080
Pfedelbach	49.1460, 009.5238
Birkach	48.7208, 009.2113
Herrenwies	48.6556, 008.2907
Hundsbach	48.6451, 008.2213
Untersmatt	48.6138, 008.1918

2.6. Honeydew honey material

The Apicultural State Institute, University of Hohenheim (Stuttgart, Germany) obtained, between 2016 and 2019, fir, spruce and linden honey from selected beekeepers. The samples were marked by beekeepers and classified by the botanical and zoological origin, indicated by

the location where the beehives were positioned and the Apicultural State Institute, University of Hohenheim (Stuttgart, Germany), has verified the honeydew producers. All locations are showed in Table 2.2. In addition, Intertek Food Services GmbH, Bremen, obtained pine honey from Turkey. Furthermore, sensory evaluation was carried out to confirm the honeydew nature of all honey samples. All honeydew honey samples had electrical conductivity values ≥ 0.8 mS cm⁻¹. Honeydew honey samples were diluted with water (1:40), centrifuged (5,000 rpm, 10 min) to separate pollen and then analyzed using HPLC.

Table 2. 2.The geographic coordinates for the collection stands of honeydew honey samples.

Location	GPS
Bühlerzell	49°00'30.12"N 009°59'17.12"E
Zwerenberg	48°37'40.4"N 008°35'47.0"E
Schenkenzell	48°18'32.6"N 8°20'54.9"E
Dautmergen-Teufental	48°14'46.9"N 8°44'36.9"E
Buchenbach	47°57'04.2"N 8°00'49.0"E
Freudenstadt	48°27'17.1"N 8°23'52.5"E
Neubulach	48°39'22.4"N 8°42'45.4"E
Eschach-Kemnaten	47°42'27.9"N 10°11'24.1"E
Winzeln	48.2823, 008.4636
Schömberg	48.7847, 8.6216
Bretzfeld	49.1862, 9.3752
Hechingen-Stetten	48°21'25.0"N 8°59'45.4"E
Bodelshausen	48°24'33.05"N 8°58'24.46"E
Seuversholz	48°57'31.7"N 11°10'02.0"E
Dornhan	48°21'04.8"N 8°31'23.7"E
Forbach Raumünzach	48.6641, 008.3247
Albershausen	48.6893, 009.5506
Forbach	48.6365, 008.3444
Alfdorf	48.9022, 9.7094
Fischerbach	48.1828, 8.0656
Schenkenzell	48°42'47.6"N 9°56'26.8"E
Schiltach	48°18'44.64"N 8°19'16.24"E
Zell am Harmersbach	48°20'49.6"N 8°03'15.1"E
Loßburg	48°24'40.5"N 8°25'46.2"E
Fluorn-Winzeln	48°17'22.1"N 8°27'38.6"E

2.7. Extraction of sugar, amino acids and ions from plant tissue by chloroform-methanol extraction

For the analysis of metabolites from plant tissue a chloroform-methanol extraction was performed. Transfer 200 mg of grained plant tissue (wood, park or leaf) into a centrifuge tube and note the exact weight of the sample, so that the concentrations determined by HPLC can be converted later into per gram fresh weight (FG/FW). Then 5 ml chloroform/methanol (1,5:3,5; v/v) was added and incubated from ice for 30 min. After the addition of 3 ml water, the mixture was vortexed and centrifuged (4500 rpm, 1 min). The aqueous upper phase is then transferred to a round bottom flask and the extraction step is repeated with 2 ml water. The aqueous phases from the extraction steps are dried in a rotary evaporator. The metabolites were then dissolved back in 1 ml water for leaves, park and wood. Then, the dissolved metabolites were collected by means of a 1 ml syringe and transferred through a syringe filter (nylon membrane with 0.2 µm pore size) into a 1.5 ml reaction vessel. They were stored at -20°C until further use.

2.8. Protein extraction for honey

Protein concentration was determination according to Lowry, therefore, a sample preparation in the form of a protein extraction with methanol and chloroform was carried out. From the honeydew honey samples, 0.5 g were first dissolved in 1 mL ultrapure water. Due to the possibility of losing proteins, pollen and other components were not removed by centrifugation. For the extraction 50 µL of this solution were mixed with 200 µL methanol, vortexed and centrifuged for 10 sec at 9000 x g, then 50 µL chloroform was added, vortexed and centrifuged for 10 sec at 9000g. lastly 150 µL ultrapure water were added, vortexed and centrifuged for 1 min at 9000g. To generate a protein pellet, the upper phase was discarded and the lower phase, which contained the proteins after extraction, was mixed with 150 µL methanol, vortexed and centrifuged at 9000g for 2 min. The protein pellet was freed from the liquid, dried at room temperature and then absorbed into 10 µL ultrapure water. The pellets were stored at -20°C until protein determination (Wessel 1984).

Protein determination according to Lowry

The protein determination is based on two reactions. In the first step, a biuret reaction is carried out with formation of a blue-violet complex, a reaction between the peptide bonds of the protein and the copper(II) ions of an alkaline copper(II) solution. The copper(II) ions are reduced to copper(I) ions in this reaction step. In the second step, the sample solution is mixed with a

Folin-Ciocalteu reagent consisting of sodium tungstate, sodium molybdate, hydrochloric and phosphoric acid. The blue-violet biuret complex reduces the yellow Folin-Ciocalteu dye (molybdenum and tungsten heteropoly acids). The reduced Folin reagent then forms a deep blue chelate complex with the copper(I) ions, which is used for the quantitative determination of the protein concentration by photometry at a wavelength of 750 nm (Lowry 1951).

the protein determination was performed in 96 well plates with a triple determination. First, 40 μL of the sample solution and 40 μL of the BSA calibration solutions were added to each well. To determine the blank value and thus the self-absorption of the 96-well plates and the reagent solutions, 40 μL ultrapure water was added to one chamber of each plate instead of the sample or the calibration solution. Next, 200 μL of solution D was added to each chamber, swiveled for 30 sec and incubated under cover for 10 min at room temperature. After the addition of 30 μL of the Folin-Ciocalteu solution (2M) diluted 1:2, it was swiveled and covered for 30 min at room temperature. The absorbance measurement was performed directly afterwards at a wavelength of 750 nm.

By photometric measurement of the BSA calibration series, the protein content of honeydew honey samples can be determined relative to the reference protein. The calibration line corresponds to a linear function, which results in the equation:

$$(1) \quad y = mx + b$$

Adapted to the present provision, the following equation results. To determine the protein content of the sample, the equation is changed to c.

$$(2) \quad E = mc * + b$$

$$(3) \quad c \left[\frac{\mu\text{g}}{\text{ml}} \right] = \frac{E - b}{m \left[\frac{1}{\mu\text{g}/\text{ml}} \right]}$$

With: E = absorbance

m = slope of the calibration line [$1/(\mu\text{g}/\text{ml})$].

b = y-intercept

c = concentration [$(\mu\text{g})/\text{ml}$]

2.9. High-Performance-Liquid-Chromatography (HPLC)

2.9.1. Analysis of sugars

The determination of sugars from honey, honeydew, plant tissue extracts and phloem exudate was carried out using high-performance liquid chromatography (HPLC) according to Nadwodnik and Lohaus (2008). The extracts and phloem exudate were purified with syringe filters (0.2 μm pore size, Roth). All samples were diluted with H_2O to such an extent that the concentration of sugars was within the linear detection range (50 -500 μM) of the pulse amperometric detector (cell: #5040, amperometer: Coulochem II, model 5200, ESA). The pulse settings are listed in Table 2.3.

Table 2. 3.Setting for the pulse measurements on the amperometer

Duration	Mode	Voltage
500 ms	Measurement	50 ms
540 ms	cleaning	700 ms
540 ms	Regeneration	-800 ms

The anion exchange column (precolumn: CarboPac PA10 Guard; main column: CarboPac PA10, Dionex) was loaded using an autosampler (2157, LKB Pharmacia), which injected the samples into the column's eluent supply (100 mM NaOH) via a valve. A two-piston high performance pump was used to achieve flow rate of 0.8 ml/min at approx. 130 bar (LC-9A; Shimadzu company). Separation was performed by the anion binding capacity of the stationary phase and the number of negative charges on the sugar molecules in the mobile phase. For each sample run, a standard curve for the determination of the concentration was generated with sugar concentrations of 50, 100, 250 and 500 μM . The collected chromatographic data were analyzed on a computer with the software PeakNet (version 5.1, Fa. Dionex).

2.9.2. Analysis of free amino acids

Free amino acids in honey, honeydew, extracted plant tissue and phloem exudate were analysed by HPLC according to Lohaus and Schwerdtfeger (2014). The HPLC system used is a Thermo Fisher Scientific Dionex Ultimate 3000 system. For amino acids with a primary amino group a precolumn derivatization with o-phthaldialdehyde (OPA) was used (Table 2.4). However, no amino acid with a secondary amino group (e.g. proline) could be detected with this method. Therefore, a precolumn derivatisation with fluorenylmethyloxycarbonyl chloride (FMOC-CL)

instead of OPA was used for the detection of proline (Table 4.5). In addition, honey samples were diluted with 100mM NaOH solution instead of water to adjust the pH near to 10; thus, the derivatisation with fluorenylmethyloxycarbonyl chloride is optimized. The separation of the derivatives was performed using a reversed-phase chromatography column (precolumn: LiChrospher 100 RP-18 endcapped 5 µm LichroCART 4-4; main column: Superspher 100 RP-18 endcapped LichroCART 125-4, Merck) with an acetonitrile gradient (Table 2.5) at a flow rate of 0.9 ml/min.

Table 2. 4. Solution for pre-column derivatization

Solution	Substance	Amount
OPA-stock solution	OPA	5-8 mg
	Methanol	1 ml
	potassium borate (1 M, pH 10,4)	125µl
	β-Mercaptoethanol	12,5µl
OPA-work solution	potassium borate (1 M, pH 10,4)	1 ml
	OPA-stock solution	0,5ml
Fmoc-work solution	Fmoc-CL	3,8 mg
	Aceton	5 ml

Table 2. 5. Eluent for the analysis of free amino acids

Solution	Substance	Amount
HPLC-solution	Ultrapure water	2,5 L
	Phosphoric acid	3 ml
	EDTA	1,25 g
	pH 7,06-7,1 adjusted with (25%NaOH)	
Eluent A	HPLC-solution	95%
	Acetonitrile	5%
Eluent B	HPLC-solution	50%
	Acetonitrile	50%
Eluent C	Acetonitrile	70%
	Ultrapure water	30%

After separation, the derivatives were detected by fluorescence (FLD-3100, Dionex). For derivatisation, detection was performed at an excitation of 330 nm and an emission of 408 nm, whereas for derivatization with FMOC-Cl an excitation of 265 nm and an emission of 305 nm was used. The external calibration was performed with amino acid standards measured in parallel. The collected chromatographic data were evaluated with the integration program Chromeleon (Version 7.2, Fa. Dionex).

2.9.3. Analysis of inorganic ions

The analysis of inorganic anions and cations was performed separately by HPLC according to Lohaus *et al.* (2001). An anion exchange column (IonPac™ AS11 4 x 250 mm, Dionex) was used to separate inorganic anions by isocratic elution (20 mM Na₂CO₃). Inorganic cations could be separated with a cation exchange column (CS 12A, 4 x 250 mm, Dionex) by isocratic elution (20 mM H₂SO₄). Sensitivity was increased by using a suppressor (ASRS Ultra II 4mm, Dionex), which increases the peak intensity and reduces baseline noise by decreasing the ground conductivity of the eluent. With an electronic conductivity detector the inorganic ions and organic acids could be quantified. Standards of the ions were determined in parallel for external calibration. The collected chromatographic data were evaluated with the integration program Peaknet (Version 5.1, Fa. Dionex).

2.10. Statistic

2.10.1. Analyses of variance

When comparing mean values of two groups, student's t-tests were carried out for mean values from five or more samples, because in these cases it is possible to test for normality and homogeneity of the data. In cases of smaller numbers of samples ($n < 5$) a nonparametric test (Mann Whitney U) was carried out. When comparing mean values of more than two groups, analyses of variance (ANOVA) were performed. Skewness and kurtosis were calculated to capture the distribution of the dataset; normal distribution was assumed if skewness values were less than 2 and kurtosis values were less than 7 (West *et al.* 1995). Moreover, Levene's tests were applied to test for homogeneity of variances for the data. When data conformed to the normality assumption but failed on homogeneity of variances, analysis of variance was performed using the Welch's test followed by post-hoc test (Games-Howell test). For the cases where both normality and homogeneity assumptions were confirmed, a one-way ANOVA was performed. Subsequently, post-hoc tests (Tukey's HSD) were carried out ($p\text{-value} < 0.05$).

2.10.2. Non-metric multidimensional scaling (NMDS)

NMDS is used to visualize the similarity between samples in datasets by reducing its number of dimensions to a two- or three-dimensional. It considers a non-parametric monotonic relationship between the distance in the sample-sample matrix and the Euclidean distances between items. However, unlike the multidimensional scaling (MDS), it also takes into account the location of each sample in the low-dimensional space (Mead 1992).

First, the number of dimensions (N) is determined and the distance metric is set. Then, a distance matrix between samples is calculated. After that, samples are distributed in the dimensions according to the initial configuration and the stress value is calculated, according to this distribution. Stress is the mismatch between the rank order of distances in the data and the rank order of distances in the ordination. In order to decrease the stress, samples are moved slightly. The movement of samples is repeated until the stress reaches the lowest value.

2.10.3. Principle component analysis (PCA)

Principal Component Analysis (PCA) is a classical ordination method for raw data based on eigenvalue decomposition of a covariance of the data set. It creates components that summarize the original information of the data in a reduced space. More accurately, the first step is to calculate a covariance matrix of all possible pairs of the samples. This matrix summarizes the correlations between all the possible pairs of variables. Then, the eigenvectors and eigenvalues of the covariance matrix is computed to identify the principal components. Principal components represent the direction of the data that explain a maximal amount of variance. In addition, the number of principal components resembles the number of dimensions of the data set. Lastly, the data is reoriented to the axes that represents the principal components and then plotted against the first two or three components.

2.10.4. Permutational analysis of variance (PERMANOVA)

Multivariate analyses like NMDS or PCA demonstrate visual separations of samples; however, further tests, such as permutational analysis of variance (PERMANOVA, Anderson 2001), are required to test whether the separations is significant,. This is a non-parametric multivariate test that can be used to test for the significance between groups. First, a matrix of distances between each pair of samples is calculated. Then, a pseudo *F*-ratio based on the sum of squares between groups is computed. Then, multiple permutations of the data are set by shuffling the samples

between groups and a permutation F ratio is calculated for each permutation. Finally, a P -value is calculated by:

$$P = \frac{(\text{count } F_p > F) + 1}{(\text{total count } F_p) + 1}$$

Where F_p is the permutation F and F is the pseudo- F . The P -value is low if pseudo F is almost always smaller than permutations F .

2.10.5. Permutational Analysis of Multivariate Dispersions (PERMDISP)

Permutational analyses of multivariate dispersions (PERMDISP, Anderson 2006) is an analogy to the Levene's test. In other words, it is a test for homogeneity of multivariate variation. Significant results in PERMANOVA are primarily caused by dispersion in the data set. However, in case of heterogeneity of the data, a significant PERMANOVA's results can occur due to location effect of the data set (Anderson *et al.* 2008). Therefore, Permutational analyses of multivariate dispersions (PERMDISP) is performed to test for the homogeneity of multivariate dispersions and to distinguish between location and dispersion effects in case of significant PERMANOVA values (Anderson 2006).

2.10.6. Redundancy analysis (RDA)

Redundancy analysis (RDA) is a method to summarize the variations in a set of response variables that can be explained by a set of explanatory variables. It decomposes the explained and unexplained variation from the data and interprets the significant effects in a single ordination (Herve' *et al.* 2018). First, models should be created where data are identified according to one or more controlled variable. Then, multivariate linear regression (MLR) between samples and controlled variable is fit. More precisely, a bi-multivariate redundancy statistic R^2 (Miller and Farr 1971 and Peres-Neto *et al.* 2006) was calculated, which describes the total percentage of the variation explained by the controlled variable. In addition, permutation F -test based on R^2 was carried out to investigate the significance of the controlled variable (Legendre and Legendre 2012). Further, in case of more than one controlled variable, pairwise composition permutation F -test was performed to test the significance of differences between each controlled variable.

After that, two PCAs were performed. The ‘constrained PCA’ is applied on the fitted values of the multivariate linear regression. This PCA summarizes the variation of data that can be explained by the controlled variables. The second PCA, ‘unconstrained PCA’ is applied on the residuals of the multivariate linear regression, thus it explains the variation in the data that cannot be related to the controlled variables.

2.11. Bioinformatics

Various programs and software packages were utilized for the computer-based work. Table 5 summarized them.

Table 2. 6. A list of used software

Name	Version	Function	Resource
Programs			
Peaknet	5.1	Chromatography Data Software	Dionex
Chromeleon	7.2	Chromatography Data Software	Dionex
R	3.5.1	statistic	www.r-project.org
SPSS	24.0	statistic	IBM, Cooperation
R packages			
Vegan		<i>metaMDS</i> <i>betadisper</i> <i>Adonis</i> <i>rda</i>	https://CRAN.R-project.org/package=vegan
RVAideMmoire		<i>MVA.synt</i> <i>MVA.anova</i> <i>pairwise.factorfit</i> <i>MVA.plot</i>	https://CRAN.R-project.org/package=RVAideMmoire

3. Results

The results are presented in the following structure:

Section I: Honeydew composition of six different hemipteran species feeding on *Abies alba* and *Picea abies*

Section II: Effects of different temperatures on melezitose production in aphid species

Section III: The ability of honeybee *A. mellifera* to digest melezitose

Section IV: Honeydew honey profiling of fir, spruce and pine honey

Section V: Nectar, honeydew and honey profiling of lime tree *Tilia sp.*

3.1. Section I: Honeydew composition of six different hemipteran species feeding on *Abies alba* and *Picea abies*

To investigate if the hemipteran species or the host plant influence the honeydew composition, honeydew samples from many hemipteran species feeding on *A. alba* and *P. abies* were collected and analyzed. Further, phloem exudates from host plants were also obtained and analyzed. Then, all honeydew samples and phloem exudates values were compared and statistically verified. The results were published in the article “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): e0228171. <https://doi.org/10.1371/journal.pone.0228171>).

In this paper, honeydew samples from six different hemipteran species were collected from five stands of Baden-Wuerttemberg (Germany). In particular, honeydew droplets directly from two Coccidae species (*Physokermes piceae* and *Physokermes hemicryphus*) and from two Lachninae species (*Cinara pilicornis* and *Cinara piceae*) located on spruce (*Picea abies* (L.) H. Karst.) and also from two Lachninae species (*Cinara pectinatae* and *Cinara confinis*) located on fir (*Abies alba* Mill.) were gathered and the sugars, amino acids and inorganic ions profiles were determined via various HPLC approaches. Further, sugars, amino acids and inorganic ions composition of phloem exudates were also determined and compared to honeydew samples. Then, non metric multidimensional scaling (NMDS) followed by Permutational Multivariate Analysis of Variance (PERMANOVA) and Permutational Analysis of Multivariate Dispersions (PERMDISP) was performed to identify the relative importance of the variables hemipteran species and tree species on the honeydew composition. Lastly, the enzyme activity in whole-body homogenates of two aphid species were tested and statistically verified.

Phloem exudates contain mainly sucrose, but honeydew of all species contain different proportions of glucose, fructose, sucrose, trehalose, melezitose, erlose and further Oligosaccharides. In addition, the proportion of sugars differ significantly among aphids species hosting two different plant species or the same plant species. Additionally, visual separation in NMDS plot of different groups of honeydew sugar profiles were obtained, and the significance of this separation is verified by further statistic tests. As conclusion, honeydew sugars proportions is significantly related to hemipteran species more than host plants.

The total contents of amino acids in the honeydew were much lower than the sugar content. In addition, all amino acids were found in phloem exudates and honeydew samples. Further, the

proportion of amino acids in phloem exudates and honeydew is not the same. However, glutamine and glutamate were predominant amino acids in the honeydew of all six hemipteran species and also in the phloem exudates of both tree species. Finally, no visual separation in NMDS plot of different groups of honeydew amino acids profiles was obtained and no significant differences.

The total contents of inorganic ions in the honeydew were much lower than the sugar content. Potassium was the dominant inorganic ion in all honeydew samples and also in the phloem exudate. Further, *Physokermes sp.* seems to have higher phosphate proportions than *Cinara sp.*

The whole-body homogenates of aphids experiment shows that sucrose is hydrolyzed and oligosaccharides are formed. Moreover, honeydew producers mainly influence the type of the produced oligosaccharides.

Sugar, amino acid and inorganic ion profiling of the honeydew from different hemipteran species feeding on *Abies alba* and *Picea abies*

Basel Shaaban¹, Victoria Seeburger², Annette Schroeder², Gertrud Lohaus^{1*}

¹Molecular Plant Science / Plant Biochemistry, University of Wuppertal, Wuppertal, Germany

²Apicultural State Institute, University of Hohenheim, Stuttgart, Germany

*Corresponding author

E-mail: lohaus@uni-wuppertal.de

PLOS ONE

Abstract

Several hemipteran species feed on the phloem sap of plants and produce large amounts of honeydew that is collected by bees to produce honeydew honey. Therefore, it is important to know whether it is predominantly the hemipteran species or the host plant to influence the honeydew composition. This is particularly relevant for those botanical and zoological species from which the majority of honeydew honey originates. To investigate this issue, honeydew from two *Cinara* species located on *Abies alba* as well as from two *Cinara* and two *Physokermes* species located on *Picea abies* were collected. Phloem exudates of the host plants were also analyzed. Honeydew of all species contained different proportions of hexoses, sucrose, melezitose, erlose, and further di- and trisaccharides, whereas the phloem exudates of the host trees contained no trisaccharides. Moreover, the proportions of sugars differed significantly between hemipteran species feeding on the same tree species. Sucrose hydrolysis and oligosaccharide formation was shown in whole-body homogenates of aphids. The type of the produced oligosaccharides in the aphid-extracts correlated with the oligosaccharide composition in the honeydew of the different aphid species. The total contents of amino acids and inorganic ions in the honeydew were much lower than the sugar content. Glutamine and glutamate were predominant amino acids in the honeydew of all six hemipteran species and also in the phloem exudates of both tree species. Potassium was the dominant inorganic ion in all honeydew samples and also in the phloem exudate. Statistical analyses reveal that the sugar composition of honeydew is determined more by the hemipteran species than by the host plant. Consequently, it can be assumed that the sugar composition of honeydew honey is also more influenced by the hemipteran species than by the host tree.

Introduction

Many insects of the order Hemiptera, including most aphids and coccids, feed on the phloem sap of their respective host plants [1]. Phloem sap is generally dominated by sucrose, with concentrations ranging from 0.7 to 1.5 M [2,3,4]. Some plant species also translocate oligosaccharides of the raffinose family, such as members of the Oleaceae, or sugar alcohols, such as members of the Rosaceae, in addition to sucrose [5,6].

Phloem sap also contains amino acids with a concentration of 50 to 200 mM. In several plant species, especially GLU, GLN, ASP, and ASN are dominant amino acids [3,4,7,8]. For several insects, nine amino acids are essential (HIS, ILE, LEU, LYS, MET, PHE, THR, TRP, VAL) [1]. Although all amino acids were found in the phloem sap [3,9], it is not an ideal diet for insects because of its high osmotic pressure, the low ratio of amino acids compared to sugars, and the ratio of essential-to-non-essential amino acid, which is lower in phloem sap than in the insect protein [1]. Therefore, phloem feeders ingest phloem sugars in quantities exceeding their carbon requirement to fulfil their metabolic need, and high concentrations of sugars in modified composition are egested as honeydew [10,11].

Honeydew is mainly composed of sugars, but it also contains inorganic ions, amino acids, proteins and other compounds [12,13,14,15]. The honeydew composition varies between different insect species; it can also be influenced by different host plant species, seasonal changes or different environmental conditions [9,16,17]. In addition, the honeydew composition may be influenced by variation in ant-aphid interaction [18]. Moreover, there is a considerable variation in the composition within samples from a particular aphid species [18].

Different monosaccharides (glucose, fructose), disaccharides (e.g. sucrose, trehalose, maltose) and trisaccharides (e.g. melezitose, erlose, raffinose) were found in honeydew [15,17,19,20] but not in the phloem sap of several tree species, where only sucrose was present [2,21]. In willow trees, for example, sucrose was the only sugar found in the phloem sap,

whereas the honeydew of a phloem feeding willow aphid (*Tuberolachnus salignus*) contained different mono-, di-, and trisaccharides [21]. Moreover, large variations of the sugar composition in honeydew of three aphid species feeding on the same tree species (*Populus tremula*) were observed [18].

For the osmotic regulation of the phloem feeding insects, it is important that they form oligosaccharides from the sucrose ingested with the phloem sap [10,22,23]. A positive relationship between the dietary sucrose concentration and the oligosaccharide content in the honeydew was shown for different aphid species [10,22,23]. The oligosaccharides are probably synthesized by several enzymes in the gut of the aphids [10,24,25].

Associated bacteria may also be involved in the nutrient metabolism in the insects [1]. One of the best-studied symbioses of this type are the symbioses of *Buchnera* and aphids, where the microorganisms are involved in the provision of essential amino acids for aphids [26,27,28,29]. In the case of oligosaccharides, the aphids rather than the associated microbiota mediate the synthesis of these sugars [10].

When floral nectar is scarce, bees often collect honeydew that has fallen onto plants. Therefore, it is important for the honey production industry to know its composition. The production of large amounts of honeydew is known for insects feeding on conifers, i.e. fir, spruce, or pine and also on deciduous trees, such as oak or lime [30]. Honeydew honey is also called forest, spruce or fir honey. Forest honey contains more di- and oligosaccharides than flower honey [31]. Nottbohm and Lucius [32] found melezitose in honeydew honey, of which Hudson and Sherwood [33] already knew that it was responsible for the crystallization of honey in the combs. This can have negative effects on the honey production process. There are some, mainly older, publications about the proportion of melezitose in honeydew of hemipteran feeding aphids on conifers, but the data are partly inconsistent [34]. Liebig [20] reports a share of 15% melezitose of the total sugar content in honeydew of *Cinara pectinatae* feeding on *Abies*

alba, whereas in other studies, no melezitose was detected [35]. The quality of honey depends, in addition to several other factors, also on the quantity of melezitose and other oligosaccharides. Therefore, it is important to know which insect species or plant species could be responsible for high melezitose contents in honeydew. This is particularly important for hemipteran species and conifer species which are associated with honeydew honey.

The aim of this study was to determine the proportions of melezitose and other sugars, as well as amino acids and further ions in the honeydew of different hemipteran species in order to examine whether the honeydew compositions differ among hemipteran species and/or among conifer species. Therefore, we collected honeydew droplets directly from two Coccidae species (*Physokermes piceae* and *Physokermes hemicryphus*) and from two Lachninae species (*Cinara pilicornis* and *Cinara piceae*) located on spruce (*Picea abies* (L.) H. Kast.) and also from two Lachninae species (*Cinara pectinatae* and *Cinara confinis*) located on fir (*Abies alba* Mill.). The analyzed hemipteran species are important producers of honeydew on conifers in Germany and other countries of Central Europe [30]. We determined the sugars, amino acids, and inorganic ions in the honeydew and examined the formation of oligosaccharides in different *Cinara* species. The results were compared to the corresponding proportions of the different compounds in phloem exudates of the tree species.

Material and Methods

Plant species, hemipteran species, and collection of honeydew

The honeydew and plant samples were collected in five spruce (*Picea abies*) or fir (*Abies alba*) stands of Baden-Wuerttemberg (Germany). The geographic coordinates for the stands are 48°66'41''N, 8°32'54''E; 48°48'19''N, 8°37'13''E; 48°31'56''N, 8°47'04''E; 48°95'58''N, 8°70'60''E; and 49°00'40''N, 10°07'07''E. Honeydew of *Cinara pectinatae* (Nördlinger, 1880) and *Cinara confinis* (Koch, 1856) located on *A. alba* (Mill.) and of *Cinara pilicornis* (Hartig, 1841), *Cinara piceae* (Panzer, 1801), *Physokermes piceae* (Schrank, 1801)

and *Physokermes hemicryphus* (Dalman, 1826) located on *P. abies* ((L.) H. Kast.) was collected. The taller trees were about 20-30 years old. Honeydew was collected from the lower or overhanging branches. Field experiments were carried out from May to July 2016 and also 2017. To minimize diurnal influences, all samples were collected at mid-day (between 11 am and 3 pm). For each species, at least 15 honeydew samples were collected from 15 colonies feeding on different tree individuals. The honeydew from different individuals in one colony was directly collected with micropipettes and pooled up to a volume of at least 1 μ L per sample. All droplets were still liquid, their age, meaning the time of release by the insect, however, was unknown. The samples were collected in plastic tubes, immediately frozen and stored at -80°C until analysis.

Collection of phloem exudates

Phloem exudate of bark from *A. alba* and *P. abies* was collected parallel to the honeydew sampling from May to July 2016 and also 2017 at mid-day (between 11 am and 3 pm). For each tree species, six samples of phloem exudate were prepared. According to the method of Hijaz and Killiny [36], roughly 2 cm long pieces of bark were placed in a 0.5 ml plastic tube with a small hole at its bottom. This tube was placed inside a 2 ml plastic tube. The samples were then centrifuged at 5000 rpm for 20 min at 4°C . This exudate consists mainly of phloem sap, but there may also be small amounts of xylem and other cell sap from the wound surface during the cutting process. The samples were stored -80°C until analysis.

Ethics statement

As per the authors' institutions' guidelines as well as applicable national regulations, no ethics approval was required or obtained for the present study. This study was carried out in spruce (*P. abies*) or fir (*A. alba*) stands in Baden-Wuerttemberg (Germany). No specific permissions were required for these locations. We collected honeydew from aphids and scale

insects, as well as material of *P. abies* and *A. alba*. Neither the insects nor the plants are protected by German law and no endangered or protected species were involved in this study.

HPLC analyses of sugars, amino acids, and inorganic ions

Sugars, amino acids, and inorganic ions were analyzed via different HPLC systems. Honeydew and phloem exudates were measured directly. Sugar standards (0-500 μM), amino acid standards (0-20 μM), and standards for inorganic ions (0-1000 μM) were measured in parallel. A calibration curve was created for each sugar, amino acid, or inorganic ion. The peak areas in the measured chromatograms were evaluated with an integration program (Chromeleon 7.2, Dionex Corp, Sunnyvale, CA, United States). The concentrations of sugars, amino acids, or inorganic ions were determined with the help of the calibration curves for each of the different substances. In order to make the results of the different hemipteran species or biological origin (honeydew or phloem exudate) comparable, the proportion of each sugar, amino acid or ions of the total sugar, as well as the amino acid or ion concentration was calculated.

Sugar analyses

The sugars in honeydew and bark exudates were analyzed according to Lohaus and Schwerdtfeger [8]. Therefore, an anion exchange column and pulse amperometric detection were used. Standards (glucose, fructose, sucrose, trehalose, melibiose, maltose, isomaltose, maltulose, isomaltulose, melezitose, erlose, raffinose, 1-kestose, isomaltotriose, maltotriose, nidrose, stachyose) were measured in parallel. The identification of each sugar was based on the comparison of the retention time of the different peaks with that of the standards. Furthermore, the obtained results were checked regularly with the standard addition method. The co-elution of sugars in the samples with known standards confirmed our assumption. Long-chain oligosaccharides (degree of polymerization (DP) ≥ 5) were analyzed with the same system, with the difference that the anion exchange column was eluted isocratically with 200

mM NaOH instead of 80 mM NaOH [37]. For the non-availability of higher oligosaccharide standards, long-chain oligosaccharides were quantified against verbascose (DP5) standard and presented as verbascose equivalents.

Amino acid analyses

The analysis of free amino acids was performed according to Göttlinger *et al.* [38]. Amino acids with a primary amino group were processed by precolumn derivatization with o-phthalaldehyde, amino acids with a secondary amino group (e.g. proline) with fluorenylmethyloxycarbonyl. The derivatives were detected by fluorescence.

Analyses of inorganic ions

Anions and cations were analyzed separately according to Göttlinger *et al.* [38]. The ions were detected by their electronic conductivity.

Enzyme activity in whole-body homogenates of aphids

The enzyme activities were analyzed in *C. pectinatae* feeding on *A. alba* and *C. pilicornis* feeding on *P. abies*. About five *C. pectinatae*-individuals and about ten *C. pilicornis*-individuals, were collected from the host plant using a paintbrush. After collection, they were homogenized in a cooled plastic tube with the help of a pestle. 300 μ L sucrose solution (10%, pH 7) were added to 20 mg aphid homogenate and incubated at 30°C. After 0, 30, 60, and 120 minutes, aliquots (50 μ L) were taken and 50 μ L NaOH (200 mM) were added to inactivate any enzymes or microbe-activity. The solution was then centrifuged (13,000 xg , 30 sec) and the supernatants were taken for sugar analysis by HPLC (as described above). The experiment was performed at least three times for each aphid species.

Statistical analyses

Data for sugar, amino acids, or ion proportions in honeydew are shown as means (\pm SD). The means of each of the sugars, amino acids, or ions in the honeydew of the six hemipteran species were compared separately to check for significant differences. Skewness and kurtosis were calculated to capture the distribution of the dataset; normal distribution was assumed if skewness values were less than 2 and kurtosis values were less than 7 [39]. Moreover, Levene's tests were applied to test for homogeneity of variances for the data of each metabolite or ion. When data conformed to the normality assumption but failed on homogeneity of variances, analysis of variance was performed using the Welch's test followed by post-hoc test (Games-Howell test). If both normality and homogeneity assumptions were confirmed, a one-way ANOVA was performed. Subsequently, post-hoc tests (Tukey's HSD) were carried out (p -value ≤ 0.05).

For the enzyme activities in the *Cinara* species a nonparametric test (Mann Whitney U) was carried out to test for significant differences in the mean values.

To conduct a non-metric multidimensional scaling (NMDS) for sugar, amino acid, and ion proportions of honeydew samples for all hemipteran species, data were reconstructed into similarity matrices using the Euclidean distance [40]. They were analyzed with the help of NMDS ordiplots. The fit of the ordination compared to the original sample ranking was assessed using the stress function. The ordination represents the data when the stress value is less than 0.2 [41]. The analysis was performed using the 'Vegan' package with the *metaMDS* routine of the program "R" [42].

Permutational Multivariate Analysis of Variance (PERMANOVA, [43]) was performed to identify the relative importance of the variables hemipteran species and tree species on the honeydew composition. Furthermore, Permutational Analysis of Multivariate Dispersions

(PERMDISP, [44]) was performed to test the homogeneity of multivariate dispersions and to distinguish between location and dispersion effects in case of significant PERMANOVA values. Both analyses are based on Euclidean distance measures, they were performed using the ‘Vegan’ package with the *betadisper* routine for PERMDISP and *adonis* routine for PERMANOVA of the program “R”. For the PERMANOVA, 999 permutations were applied.

All statistical analyses were performed using R (version 3.5.1, www.r-project.org) and SPSS (version 24.0, IBM, Cooperation).

Results

Sugars, amino acids, and inorganic ions in honeydew

Honeydew, produced by *C. pectinatae* and *C. confinis* on *A. alba* and by *C. piceae*, *C. pilicornis*, *P. piceae* and *P. hemicryphus* located on *P. abies* were analyzed for sugars, amino acids, and inorganic ions. The monosaccharides glucose and fructose, as well as the disaccharide sucrose, were found in all samples (Table 1). In general, the proportion of fructose was higher than the proportion of glucose. The trisaccharides, melezitose and erlose represented also major components of honeydew. There were, however, significant differences between the hemipteran species (Fig 1). Honeydew, produced by *C. piceae* located on spruce, revealed the highest proportion of melezitose (mean \pm SD; 48 ± 13 %), followed by *C. pilicornis* (mean \pm SD; 36 ± 8 %), whereas honeydew produced by *P. piceae* and *P. hemicryphus* located on spruce and by the Lachninae species *C. pectinatae* and *C. confinis* on fir showed much lower proportions of melezitose. A reverse picture emerges when analyzing the proportion of erlose: high proportions of erlose were found in honeydew of *C. pectinatae* and *C. confinis*, medium in *P. piceae* and *P. hemicryphus*, and low in *C. pilicornis* and *C. piceae* (Fig 1).

Table 1. Sugar composition of the honeydew of six hemipteran species feeding on *Abies alba* or *Picea abies*.

Sugar [%]	<i>Abies alba</i>		<i>Picea abies</i>			
	<i>Cinara pectinatae</i>	<i>Cinara confinis</i>	<i>Cinara pilicornis</i>	<i>Cinara piceae</i>	<i>Physokermes piceae</i>	<i>Physokermes hemicryphus</i>
Glucose (glu)	12 ± 8 ^a	6 ± 6 ^{a,b}	7 ± 10 ^{a,b}	4 ± 3 ^b	10 ± 4 ^{a,b}	14 ± 10 ^a
Fructose (fru)	13 ± 4 ^a	49 ± 12 ^d	25 ± 6 ^{b,c}	34 ± 8 ^c	30 ± 10 ^c	19 ± 12 ^{a,b}
Sucrose (suc)	51 ± 13 ^a	24 ± 16 ^{b,c}	30 ± 13 ^b	11 ± 8 ^c	35 ± 16 ^{a,b}	48 ± 22 ^a
Trehalose (tre)	2 ± 2 ^{a,b}	2 ± 1 ^{a,b}	0 ± 0 ^a	2 ± 1 ^b	3 ± 2 ^b	2 ± 3 ^b
Maltose (mal)	0 ± 0 ^a	2 ± 3 ^{a,b}	1 ± 2 ^a	0 ± 0 ^a	0 ± 0 ^a	4 ± 4 ^b
Further disaccharides (fds)	0 ± 0 ^{a,b}	1 ± 1 ^b	0 ± 0 ^a	0 ± 0 ^a	0 ± 0 ^{a,b}	0 ± 0 ^{a,b}
Melezitose (mel)	2 ± 2 ^a	2 ± 2 ^a	36 ± 8 ^c	48 ± 13 ^d	13 ± 9 ^b	2 ± 5 ^a
Erllose (erl)	15 ± 4 ^a	14 ± 7 ^a	1 ± 2 ^c	1 ± 2 ^c	7 ± 4 ^{b,c}	9 ± 10 ^{a,b}
1-Kestose* (kes)	4 ± 5 ^a	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	1 ± 2 ^b	2 ± 2 ^{a,b}
Further trisaccharides (fts)	1 ± 1 ^a	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b	0 ± 0 ^b
Further oligosaccharides	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1

All values are mean proportions (%) of n = 15 independent measurements ± SD.

Different letters represent significant differences between the sugar proportion in honeydew of the different hemipteran species.

Further disaccharides: isomaltose, isomaltulose, maltulose, melibiose, and turanose.

Further trisaccharides: isomaltotriose, maltotriose, and raffinose. *peak of kestose was not completely separated from nigerose and stachyose peak.

Further oligosaccharides: oligosaccharides with a degree of polymerization ≥ 4.

The disaccharides trehalose and maltose occurred in minor proportions in most honeydew samples (Table 1). Several honeydew samples of *C. pectinatae*, *P. piceae* and *P. hemicryphus* also showed a minor peak in the chromatogram with the retention time of 1-kestose. However, this peak could also represent nigerose and stachyose, because they could not be separated in the chromatogram. Melibiose, isomaltose, turanose, maltulose, isomaltulose, maltotriose, isomaltotriose, raffinose were found not at all or only in single

samples, but these sugars never constituted more than 1–2% of the total sugar content. The proportions of long-chain oligosaccharides ($DP \geq 5$) were below 1% in all honeydew samples.

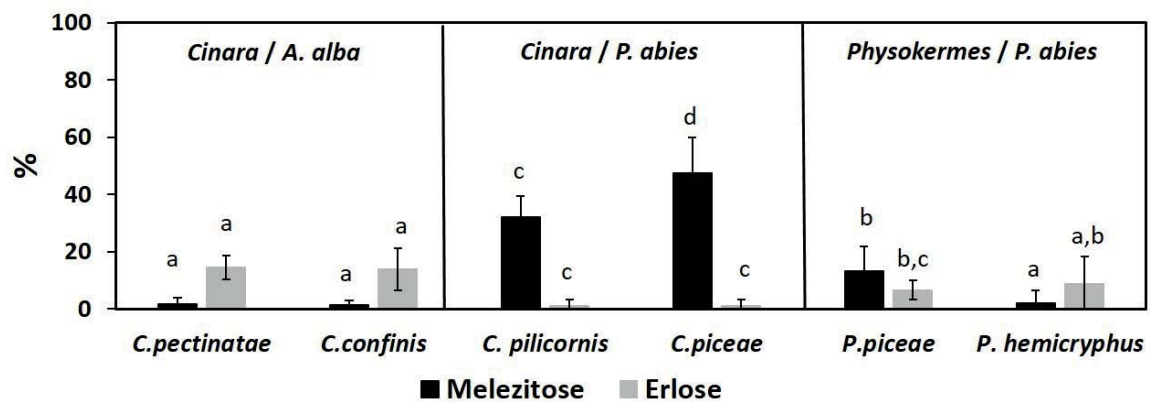


Fig 1. Melezitose and erlose proportion in the honeydew of six hemipteran species feeding on *A. alba* and *P. abies*.

All values are mean proportions (%) of $n=15$ independent measurements \pm SD. Data were taken from Table 1. Different letters represent significant differences in melezitose and erlose proportion between different hemipteran species (Tukey's HSD; $p < 0.05$).

Despite the very low amino acids concentration in honeydew, all proteinogenic amino acids and some further amino compounds could be detected (Table 2). The main amino acids were GLN, GLU, PRO, and the essential amino acid HIS. The proportions of the other essential amino acids were low. No significant differences could be detected for most of the amino acids in the honeydew of the different hemipteran species,

Table 2. Amino acid composition of the honeydew of six hemipteran species feeding on *Abies alba* or *Picea abies*.

Amino acid [%]	<i>Abies alba</i>		<i>Picea abies</i>			
	<i>Cinara pectinatae</i>	<i>Cinara confinis</i>	<i>Cinara pilicornis</i>	<i>Cinara piceae</i>	<i>Physokermes piceae</i>	<i>Physokermes hemicryphus</i>
Glutamate (GLU)	16 ± 17 ^a	6 ± 7 ^a	17 ± 17 ^a	15 ± 15 ^a	7 ± 8 ^a	10 ± 16 ^a
Glutamine (GLN)	23 ± 21 ^a	23 ± 30 ^a	26 ± 29 ^a	17 ± 21 ^a	37 ± 26 ^a	17 ± 21 ^a
Aspartate (ASP)	15 ± 15 ^a	4 ± 4 ^b	7 ± 6 ^b	3 ± 2 ^b	3 ± 5 ^b	9 ± 12 ^b
Asparagine (ASN)	10 ± 11 ^a	3 ± 3 ^a	3 ± 4 ^a	3 ± 4 ^a	4 ± 5 ^a	7 ± 12 ^a
Proline (PRO)	10 ± 6 ^a	20 ± 25 ^a	8 ± 12 ^a	6 ± 8 ^a	5 ± 6 ^a	14 ± 12 ^a
Glycine (GLY)	6 ± 9 ^a	1 ± 2 ^a	5 ± 8 ^a	8 ± 15 ^a	12 ± 14 ^a	5 ± 12 ^a
Serine (SER)	4 ± 3 ^a	3 ± 3 ^a	4 ± 5 ^a	8 ± 12 ^a	4 ± 8 ^a	2 ± 4 ^a
Alanine (ALA)	6 ± 11 ^a	2 ± 4 ^{a,b}	4 ± 6 ^{a,b}	6 ± 5 ^b	5 ± 4 ^{a,b}	2 ± 3 ^{a,b}
Tyrosine (TYR)	0 ± 1 ^a	0 ± 1 ^a	3 ± 4 ^{a,b}	2 ± 2 ^{a,b}	4 ± 6 ^b	2 ± 4 ^{a,b}
Arginine (ARG)	1 ± 4 ^a	1 ± 1 ^a	1 ± 2 ^a	2 ± 3 ^a	1 ± 1 ^a	0 ± 0 ^a
Histidine (HIS)	4 ± 5 ^a	27 ± 26 ^b	5 ± 6 ^a	14 ± 19 ^a	4 ± 5 ^a	20 ± 21 ^a
Lysine (LYS)	2 ± 6 ^a	0 ± 1 ^a	1 ± 2 ^a	5 ± 13 ^a	0 ± 1 ^a	3 ± 10 ^a
Threonine (THR)	2 ± 6 ^a	1 ± 2 ^a	2 ± 4 ^a	2 ± 4 ^a	2 ± 4 ^a	1 ± 2 ^a
Valine (VAL)	1 ± 1 ^a	2 ± 4 ^a	3 ± 3 ^a	3 ± 3 ^a	1 ± 2 ^a	1 ± 2 ^a
Isoleucine (ILE)	0 ± 1 ^a	1 ± 1 ^{a,b}	1 ± 1 ^{a,b}	1 ± 1 ^b	1 ± 1 ^{a,b}	0 ± 1 ^{a,b}
Leucine (LEU)	0 ± 0 ^a	1 ± 1 ^{a,b}	2 ± 4 ^b	1 ± 1 ^{a,b}	1 ± 1 ^{a,b}	1 ± 1 ^{a,b}
Phenylalanine (PHE)	0 ± 0 ^a	2 ± 3 ^b	1 ± 1 ^a	1 ± 2 ^a	1 ± 2 ^{a,b}	0 ± 1 ^a
Tryptophan (TRP)	0 ± 0 ^a	0 ± 1 ^a	2 ± 3 ^a	1 ± 2 ^a	1 ± 2 ^a	1 ± 2 ^a
Methionine (MET)	0 ± 0 ^a	0 ± 0 ^a	0 ± 0 ^a	0 ± 0 ^a	0 ± 0 ^a	0 ± 0 ^a
Non-proteinogenic amino acids (NP)	4 ± 9 ^a	3 ± 1 ^a	3 ± 1 ^a	3 ± 1 ^a	6 ± 1 ^a	3 ± 1 ^a

All values are mean proportions (%) of n = 15 independent measurements ± SD.

Different letters represent significant differences between the amino acid proportion in honeydew of the different hemipteran species.

Non-proteinogenic amino acids: β-alanine, γ-amino-butyric acid, homoserine, phosphoserine, ornithine, and taurine.

The main anions in honeydew were phosphate (PO₄³⁻) and chloride (Cl⁻), and potassium (K⁺) was the most abundant cation (Table 3). Significant differences between the hemipteran species

were mainly detected for chloride, phosphate, and ammonium (NH₄⁺). The proportion of phosphate was particularly high in the honeydew of *Physokermes* species.

Table 3. Inorganic cation and anion composition of the honeydew of six hemipteran species feeding on *Abies alba* or *Picea abies*.

Ion [%]	<i>Abies alba</i>		<i>Picea abies</i>			
	<i>Cinara pectinatae</i>	<i>Cinara confinis</i>	<i>Cinara pilicornis</i>	<i>Cinara piceae</i>	<i>Physokermes piceae</i>	<i>Physokermes hemicryphus</i>
Cations						
Potassium (K ⁺)	49 ± 10 ^a	81 ± 10 ^b	72 ± 18 ^c	69 ± 15 ^d	77 ± 14 ^e	82 ± 8 ^f
Sodium (Na ⁺)	9 ± 11 ^{a,b}	10 ± 7 ^{a,b}	8 ± 6 ^b	2 ± 1 ^a	2 ± 1 ^a	7 ± 8 ^{a,b}
Ammonium (NH ₄ ⁺)	35 ± 29 ^a	2 ± 3 ^c	12 ± 16 ^{b,c}	24 ± 15 ^{a,b}	16 ± 15 ^{a,b}	5 ± 5 ^c
Magnesium (Mg ²⁺)	2 ± 1 ^a	4 ± 1 ^a	4 ± 3 ^a	2 ± 2 ^a	3 ± 2 ^a	5 ± 4 ^a
Calcium (Ca ²⁺)	2 ± 1 ^{a,b}	2 ± 1 ^{a,b}	4 ± 4 ^b	3 ± 3 ^{a,b}	1 ± 1 ^a	1 ± 1 ^a
Anions						
Chloride (Cl ⁻)	49 ± 21 ^{a,b}	39 ± 20 ^{a,b,c}	31 ± 18 ^{b,c,d}	57 ± 14 ^a	20 ± 11 ^{c,d}	17 ± 17 ^d
Phosphate (PO ₄ ³⁻)	37 ± 20 ^a	32 ± 18 ^a	44 ± 14 ^{a,b}	14 ± 10 ^d	59 ± 25 ^b	75 ± 20 ^c
Sulfate (SO ₄ ²⁻)	10 ± 5 ^{a,b}	25 ± 12 ^{c,d}	23 ± 7 ^{c,d}	25 ± 7 ^d	20 ± 15 ^{b,c}	6 ± 5 ^a
Nitrate (NO ₃ ⁻)	4 ± 5 ^a	4 ± 3 ^a	2 ± 2 ^a	4 ± 9 ^a	0 ± 1 ^a	2 ± 4 ^a

All values are mean proportions (%) of n = 15 independent measurements ± SD. Different letters represent significant differences between the inorganic ion proportion in honeydew of the different hemipteran species.

Comparison of honeydew and phloem exudates

Phloem exudates of bark tissues from *A. alba* and *P. abies* were dominated by sucrose (about 60 %), but also contained larger proportions of glucose and fructose (about 40 %) (S1 Table). No other di- or trisaccharides were detected. In contrast, different proportions of further disaccharides in addition to sucrose as well as different proportions of trisaccharides (mainly melezitose and erlose) were found in all honeydew samples (Table 1, Fig 1).

All proteinogenic amino acids and some further amino compounds were detected in phloem exudates and in honeydew (Fig 2, S2 Table). GLN and GLU were predominant amino acids in the honeydew of all six hemipteran species (Table 2) and also in the phloem exudates

of *A. alba* and *P. abies* (GLN about 18-19% and GLU 8-15%). In honeydew, there was additionally a considerable amount of PRO and HIS, the proportions of which were much lower in phloem exudates. On the other hand, the proportions of ARG and THR were much higher in the phloem exudate of both tree species (ARG 9-15%; THR 10-13%) than in the honeydew of all hemipteran species (ARG and THR each 1-2%); Table 2).

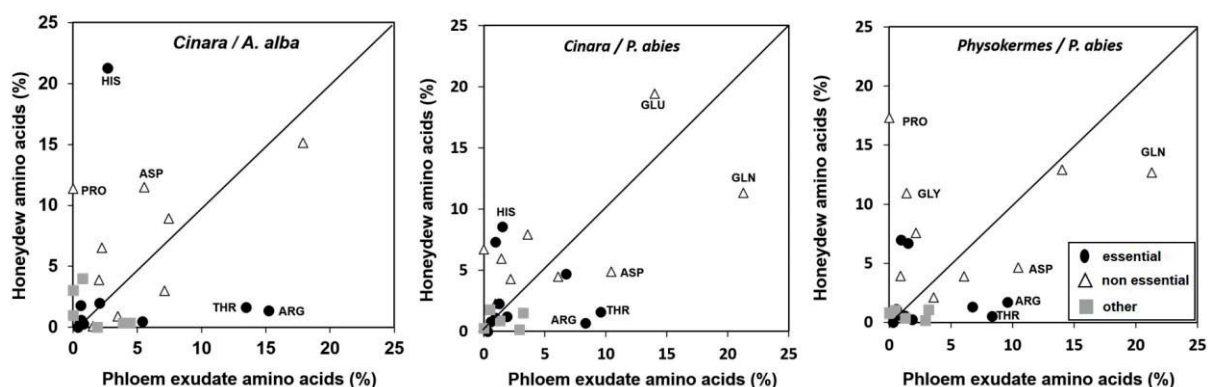


Fig 2. Amino acids in phloem exudate and honeydew, each expressed as percentage of the total amino acid concentration.

Cinara species feeding on *A. alba*, *Cinara* species feeding on *P. abies*, and *Physokermes* species feeding on *P. abies*. The data show means across both species of a hemipteran genus feeding on the same tree species for the proportion of amino acids in honeydew (data from Table 2). Grey squares: non-essential amino acids, black circles: essential amino acids, white triangles: other organic amino compounds. Points with the highest orthogonal distance to the bisecting line were noted.

Potassium was the most abundant inorganic ion in all honeydew samples and also in the phloem exudate (Fig 3, S3 Table). In honeydew, the most abundant anion was either chloride or phosphate (Table 3), whereas in phloem exudate, it was always chloride.

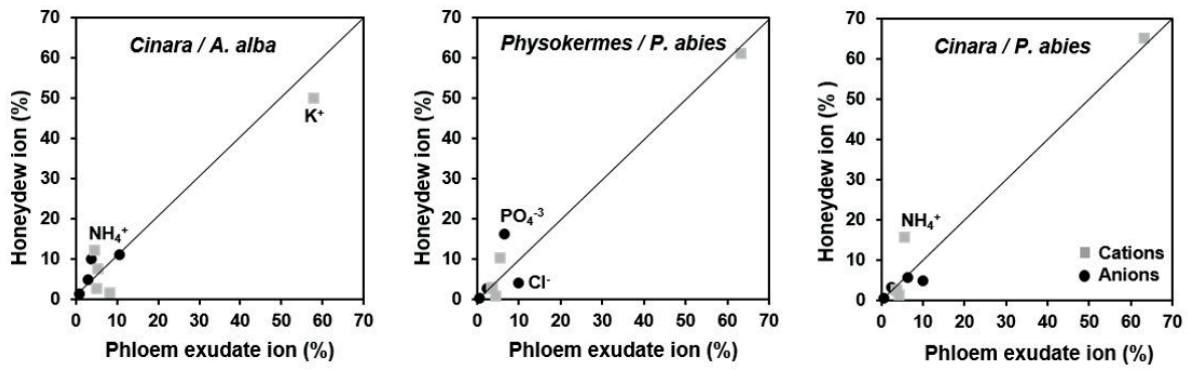


Fig 3. Inorganic ions in phloem exudate and honeydew, each expressed as percentage of the total inorganic ion concentration.

Cinara species feeding on *A. alba*, *Cinara* species feeding on *P. abies*, and *Physokermes* species feeding on *P. abies*. The data show means across both species of a hemipteran genus feeding on the same tree species for the proportion of inorganic ions in honeydew (data from Table 3). Points with the highest orthogonal distance to the bisecting line were noted. Grey squares: cations, black circles: anions.

Honeydew and phloem exudate also showed different ratios sum-of-sugars to sum-of-amino-acids and sum-of-sugars to sum-of-inorganic-anions (Table 4). The ratios were calculated from the total concentration of sugars, amino acids, or inorganic ions either in the honeydew of the different hemipteran species or in the phloem exudate of *A. alba* and *P. abies*. The ratio sum-of-sugars to sum-of-amino-acids was about 6-7 in phloem exudates of both plant species (Table 4). The corresponding ratio in the honeydew of the different hemipteran species was much higher (2.000-20.000, Table 4). This means that honeydew contains much more sugars in relation to amino acids than phloem exudate. The same applies to the inorganic ions. The ratio sum-of-sugars to sum-of-inorganic-cations was 30-90 and the ratio sum-of-sugars to sum-of-inorganic-anions was 90-900. In phloem exudate, they were about 1-2 and 6-9, respectively (Table 4).

Table 4. Ratios of different compounds in phloem exudates of *Abies alba* and *Picea abies* and in the honeydew of different hemipteran species feeding on *A. alba* and *P. abies*.

	Sugars/amino acids	Sugars/cations	Sugars/anions
<i>Abies alba</i>			
Phloem exudate	7 ± 1	2 ± 1	9 ± 3
<i>C. pectinatae</i> honeydew	4580 ± 3959 ^a	68 ± 45 ^{b,c}	274 ± 162 ^a
<i>C. confinis</i> honeydew	2163 ± 2999 ^a	55 ± 15 ^{a,b,c}	139 ± 146 ^a
<i>Picea abies</i>			
Phloem exudate	6 ± 2	1 ± 0	6 ± 2
<i>C. piceae</i> honeydew	19416 ± 32641 ^{a,b}	90 ± 50 ^c	919 ± 713 ^b
<i>C. pilicornis</i> honeydew	3503 ± 3765 ^a	69 ± 44 ^c	319 ± 137 ^a
<i>P. piceae</i> honeydew	7152 ± 7151 ^{a,b}	28 ± 12 ^a	175 ± 95 ^a
<i>P. hemicyphus</i> honeydew	24107 ± 28870 ^b	33 ± 24 ^{a,b}	94 ± 90 ^a

Values for the ratios in honeydew are means of n = 15 independent measurements ± SD.

Values for the ratios in phloem exudate are means of n = 6 independent measurements ± SD.

Data are calculated from the concentrations of sugars, amino acids, inorganic cations and anions in phloem exudates and honeydew.

Different letters represent significant differences in sugar-to-amino acids, sugar-to-anions, and sugar-to-cations ratios in honeydew of the different hemipteran species.

Honeydew composition in relation to hemipteran species and tree species

In order to reduce the amount and complexity of the data, several NMDS analyses were performed. The scatterplot of analyzed sugars is shown in Fig 4A. There is a visual separation between the samples of *C. piceae* and *C. pilicornis*, and other hemipteran species samples as well as a visual separation between *C. pectinatae* and other hemipteran species.

Fig 4B shows the loading plot of the analyzed sugars in honeydew. The different sugars contribute differently to the separation of the samples. Melezitose is a major contributor to the separation of *C. piceae* and *C. pilicornis*, whereas sucrose and erlose are major contributors to the separation of *C. pectinatae*.

The scatterplot of the amino acids is shown in Fig 4C. In this case, no separation of the hemipteran species was found. The corresponding loading plot of the analyzed amino acids in honeydew is shown in Fig 4D.

The scatterplot of inorganic ions is shown in Fig 4E. There is a visual separation between *P. hemicryphus* and the other hemipteran species (Fig 4E). The loading plot of the inorganic ions shows that phosphate is a major contributor to the separation of *P. hemicryphus* (Fig 4F).

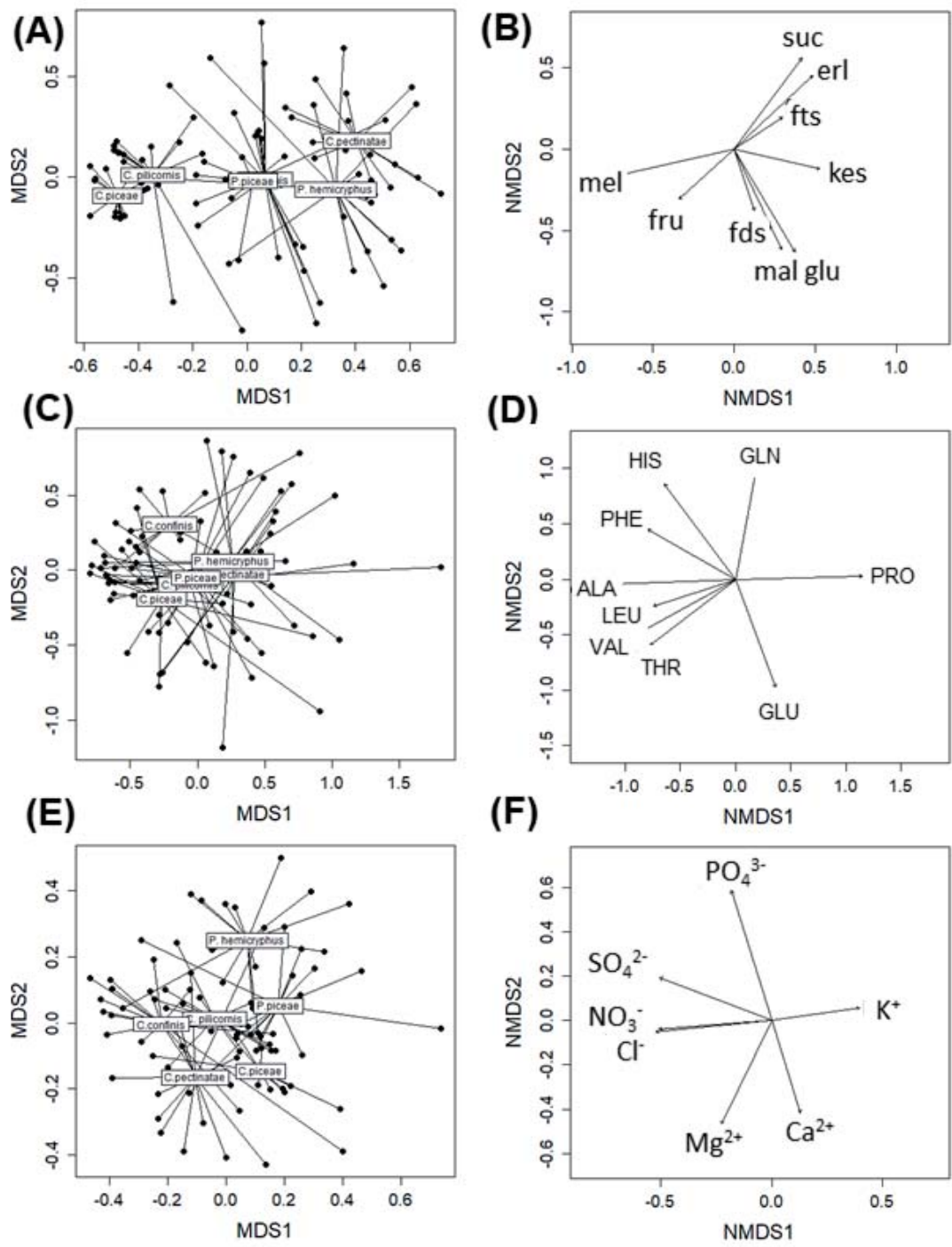


Fig 4. Scatterplots and loadings of NMDS

(A,C,E) Scatterplots of NMCS for (A) sugars, (C) amino acids, and (E) inorganic ions (stress values A=0.18 C=0.19, E= 0.19). Samples of each of the hemipteran species are connected with the centroids of the corresponding convex hulls using the function ‘ordispider’ (package Vegan). (B,D,F) Loading plots, which illustrate the original variables (B sugars, D amino acids,

F inorganic ions) loaded as vectors in NMDS space. The analyses are based on the proportions of sugars, amino acids, and inorganic ions in honeydew.

To support the graphical evaluation, a PERMANOVA and PERMDISP was performed with the same honeydew data using hemipteran species and tree species as categorical variables (Table 5). When considering sugars, there is a high significance for the category of hemipteran species ($p < 0.001$) with 47.8 % of the data variation being explained by the hemipteran species and only 13.69% of the data variation being explained by the tree species ($p < 0.001$). In addition, the non-significant values of PERMDISP for sugars indicate that the separations of hemipteran species and tree species in PERMANOVA is caused only by location and not by different dispersion. Considering the amino acids, only 10.7 % of the variance is explained by the hemipteran species ($p < 0.001$) and 4.12% by tree species ($p < 0.001$). For inorganic ions only 21.76 % of the data variance is influenced by the hemipteran species and 8.85% by tree species ($p < 0.001$). However, the significant values of ions and amino acids in PERMDISP test indicate that there were effects of different dispersions of hemipteran species separation and tree species separation in PERMANOVA. In other words, the significant values in PERMDISP assume that there is an unbalanced group paired with heterogeneity of variance, which makes PERMANOVA very sensitive and promotes type I errors [45].

Table 5. Results of multivariate statistical tests PERMANOVA/PERMDISP based on the Euclidean distance matrix of proportions of metabolites and ions in honeydew samples.

	PERMANOVA		PERMDISP	
	Pseudo-F	<i>p</i> -value	F	<i>p</i> -value
Hemipteran species				
Sugars	47.8	0.001	1.65	0.15
Amino acids	10.7	0.001	4.86	>0.001
Ions	21.76	0.001	4.05	0.002
Tree species				
Sugars	13.69	0.001	0.285	0.59
Amino acids	4.12	0.001	21.87	>0.001
Ions	8.85	0.001	7.59	0.007

Hemipteran species: N (permutations) = 999; $df = 5$

Tree Species: N (permutations) = 999; $df = 1$

Oligosaccharide formation in aphid whole-body homogenates

Sucrose was converted to monosaccharides and oligosaccharides in the whole-body homogenates of both *Cinara* species (Fig 5). In the extracts of *C. pectinatae*, more erlose than melezitose was produced, whereas in the extracts of *C. pilicornis*, more melezitose than erlose was produced.

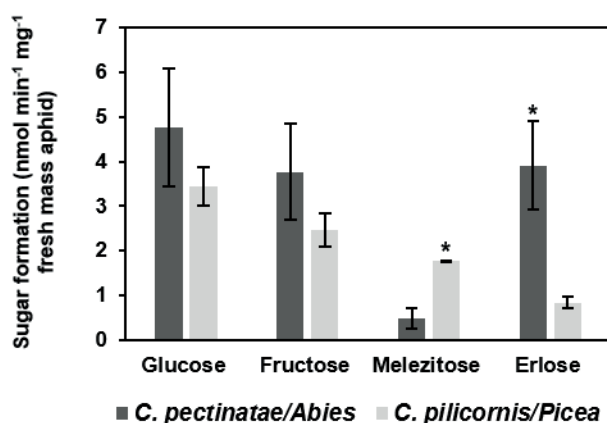


Fig 5. Sugar formation in whole-body homogenates of *C. pectinatae* and *C. pilicornis*.

The aphid homogenates were incubated with 10% sucrose solution. All values are means of $n=3$ independent measurements \pm SD. Mann-Whitney U tests comparing means of each sugar production rate between different *Cinara* species (* $p < 0.05$) were performed.

Discussion

Honeydew of hemipteran species feeding on conifers like spruce or fir is often the basis for honeydew honey; therefore, the quality of it is also influenced by the chemical composition of the honeydew [30]. For beekeepers and for the honey industry it is important to know which factors influence the chemical composition of honeydew, be it the hemipteran species, the host trees, and/or environmental factors.

Origin of sugars in honeydew

Phloem exudates of bark tissues from *A. alba* and *P. abies* contained sucrose, glucose and fructose. In contrast, pure phloem sap normally does not contain glucose and fructose [2]. Ziegler and Mittler [46] collected phloem sap from *P. abies* with the help of aphid stylectomy and found that sucrose was the only sugar in the phloem. Hexoses in the phloem exudate stem mainly from artificially hydrolyzed sucrose by the activity of sucrose cleavage enzymes from the wounded surface of the bark [2]. This may also be assumed for *A. alba*, because the bark exudate also contained sucrose and glucose and fructose, but no other di- or trisaccharides.

In contrast, honeydew of the different hemipteran species feeding on fir or spruce contained hexoses like glucose and fructose as well as a wide variety of oligosaccharides which do not typically occur in the phloem sap (Table 1). This corresponds to the results of Mittler [21], who showed that glucose, fructose, sucrose, and melezitose were present in the honeydew of the aphids *Tuberolachnus salignus* feeding on willow, but not in the phloem sap of willow, where only sucrose was present. Therefore, sucrose ingested by the phloem-feeding insects is hydrolyzed into glucose and fructose in the insects' digestive tract. Fisher *et al.* [22] suggested that, after hydrolyzation of sucrose, a large portion of the glucose is transformed by the aphid into glucose-containing oligomers for osmoregulation of ingested phloem sap. The observed predominance of fructose over glucose and the presence of large quantities of glucose-containing oligosaccharides in honeydew (Table 1) supports that. High sucrase activity, an α -

glucosidase which probably also shows transglucosidation activity, was found in pea aphids [25,26,47]. Furthermore, crushed aphids incubated with sucrose solution produced glucose, fructose, melezitose, erlose, and other oligosaccharides [19], with melezitose being a biosynthetic end product in aphid carbohydrate metabolism [25]. The enzymatic hydrolysis and oligosaccharide-forming transglycosidation reactions show considerable specificity for sucrose, whereas other sugars remain unaffected [25]. However, the enzymatic level of transformations of dietary sucrose or other sugars in the insect gut is not yet completely understood [1].

Origin of amino acids in honeydew

The ratios sugar-to-amino-acids were much lower in the phloem exudate of the host plants than in the honeydew of the different hemipteran species (Table 4). Hemipterans are very efficient in absorbing amino acids from the ingested phloem sap, as only 1-3% of the amount ingested was found in honeydew of different aphid species [9].

Weibull *et al.* [48] showed that the amino acid composition of phloem samples taken from leaves via excised aphid stylets and that of exudates from cut leaves were highly correlated. Thus, the exudate technique offers a proper alternative to the aphid stylet technique for studying the composition of phloem sap [48]. Although all proteinogenic amino acids were found in phloem exudates and honeydew, the composition was different (Fig 2). Most essential amino acids were found in lower proportions in honeydew compared to phloem exudate; the proportion of HIS, however, was increased. This may be an indication for an active regulation mechanism in the insects and varying uptakes of individual amino acids, probably as a result of variable supply and demand [11].

In general, the main nitrogen source of phloem-feeding hemipterans are free amino acids of the phloem sap [1]. In addition, these insects also produce amino acids from sucrose

carbon [11] or from the carbon of dietary amino acids [49]. Symbiotic microorganisms, several species of *Buchnera*, are known to be involved in the synthesis of amino acids, including some essential amino acids [1,50].

Origin of inorganic ions in honeydew

The composition of inorganic ions in honeydew of the different hemipteran species roughly reflect that composition in the phloem exudate of *A. alba* and *P. abies*. The main cation in the honeydew of all analyzed hemipteran species was potassium (50-80% of the total cation content), whereas the proportion of sodium was much lower (Table 3). Similar ratios of potassium-to-sodium were found in the honeydew of *Megoura viciae* feeding on *Vicia faba* [51] or in the honeydew of *Myzus persicae* feeding on *Aster tripolium* [52]. This is probably caused by the diet of the insects. It was proposed that there is also a correlation between the diet of an insect and the ratio of potassium-to-sodium in haemolymph: in carnivorous insects, this ratio is lower and in herbivorous insects it is higher [53].

Honeydew honey contains more inorganic cations and anions than blossom (nectar) honey [54]. It could be assumed that this difference is caused by different ratios of sugars-to-inorganic ions in honeydew and floral nectar, assuming that they are lower in nectar. However, the sugar-to-cation and the sugar-to-anion ratios (Table 4) were similar or even higher in honeydew than in floral nectar of other plant species [38,55]. Therefore, differences in the content of inorganic ions in both types of honey must have further and other reasons.

Sugar composition is influenced by the hemipteran species

The analysis of the data shows that the hemipteran species has a much higher influence on the sugar composition of the honeydew than the tree species (Table 5). In the case of the amino acids or ions, the variance of the data cannot be elucidated by either the hemipteran species or the conifer species. This raises the question whether there are models or selective agents beyond this to predict the honeydew composition. The honeydew composition may also

depend on several environmental factors, like weather conditions or ant tending as well as the developmental stage of the host plant or the insect [9,18].

It has been shown that melezitose has negative effects on the quality of honeydew honey, and the so-called “cement honey” is associated with the melezitose content [56]. Furthermore, melezitose can have negative effects on overwintering honey bees [57]. The results of the present study show that the sucking hemipteran and not the conifer species is primarily responsible for the diversity of the oligosaccharides, especially melezitose and erlose, in honeydew (Table 5). This corresponds with the results of other hemipteran and host plant species. Bacon and Dickinson [19] found that the honeydew of the aphid *Eucallipterus tiliae* (L.) feeding on lime tree contained appreciable amounts of melezitose, whereas in the honeydew of the scale insect *Eulecanium coryli* (L.) feeding also on lime tree, no melezitose could be detected. A wide variation in the proportions of erlose and melezitose in honeydew was also observed for eight aphid species feeding on *Tanacetum vulgare* [4].

Moreover, incubation of sucrose with whole-body homogenates of *C. pectinatae* and *C. pilicornis* resulted in similar oligosaccharide patterns as were found in honeydew samples (Fig 5). This indicates that different aphid species probably have different enzymatic activities, which lead to different oligosaccharide compositions in honeydew. The total rate of sucrose hydrolyses was similar in both aphid species (about 1.5 mg sucrose mg⁻¹ fresh weight of aphid day⁻¹) and with that corresponds to the rates of sucrose hydrolyses reported by Bacon and Dickinson ([19]; about 1 mg sucrose mg⁻¹ fresh weight of aphid day⁻¹).

Liebig [20] found higher proportions of melezitose (up to 20%) in the honeydew of *C. pectinatae* on *A. alba* than was shown in this study (2%). Reasons for this difference may be the high variability between samples, even if taken from the same hemipteran species and the same host plant [18], or different environmental conditions during sample collection. Unfortunately, in the study by Liebig [20], the proportion of the other important trisaccharide,

erlose, was not shown. Therefore, it cannot be excluded that the two trisaccharides were not separated.

The proportion of melezitose in honeydew was related to the presence or absence of ants [18]. Woodring *et al.* [4] proposed that ants are attracted to melezitose not because it is itself a valuable food source, but because it is associated with a very sugar-rich honeydew. In the present study, ant-tending was not analyzed in detail. Differences in ant-tending among the *Cinara* species are known from the literature; *C. piceae* (on spruce) and *C. confinis* (on fir) are more often tended by ants while *C. pilicornis* (on spruce) and *C. pectinatae* (on fir) are rarely tended by ants [30]. Considering these general data, there is no correlation between ant-tending and melezitose content in honeydew of the analyzed *Cinara* species. However, the variation in honeydew composition within the hemipteran species may in part reflect variation in ant-hemipteran interaction.

Conclusion

In conclusion, the sucking hemipteran and not the host plant is primarily responsible for the diversity of the sugars, especially the oligosaccharides in honeydew. In the case of inorganic ions, mainly the proportions of chloride, phosphate, and ammonium showed significant differences between the honeydews of different hemipteran species. In contrast, the composition of amino acids in honeydew was rather similar across the hemipteran species.

Acknowledgements

The authors like to thank Rosi Ritter for critical reading of the manuscript and the reviewer for helpful comments.

Funding

The project is supported by funds of the Federal Ministry of Food and Agriculture (BMEL) based on the decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program to G.L. and A.S. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. There was no additional external funding received for this study.

References

1. Douglas AE. Phloem-sap feeding by animals: problems and solutions. *J Exp Bot.* 2006; 57: 747-754.
2. Fink D, Dobbstein E, Barbian A, Lohaus G. 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.* 2018; 248: 661-673.
3. Lohaus G, Moellers C. Phloem transport of amino acids in two *Brassica napus* L. genotypes and one *B. carinata* genotype in relation to their seed protein content. *Planta.* 2000; 211: 833–840.
4. Woodring J, Wiedemann R, Fischer M K, Hoffmann K H, Völkl W. 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.
5. Nadwodnik J, Lohaus G. Subcellular concentrations of sugar alcohols and sugars in relation to phloem translocation in *Plantago major*, *Plantago maritima*, *Prunus persica*, and *Apium graveolens*. *Planta.* 2008; 227: 1079–1089.
6. Öner-Sieben S, Lohaus G. Apoplastic and symplastic phloem loading in *Quercus robur* and *Fraxinus excelsior*. *J Exp Bot.* 2014; 65: 1905-1916.
7. Karley AJ, Douglas AE, Parker WE. Amino acid composition and nutritional quality of potato leaf phloem sap for aphids. *J Exp Biol.* 2002; 205: 3009-3018.
8. 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): e87689.
9. Sandström JP, Moran NA. Amino acid budgets in three aphid species using the same host plant. *Physiol Entomol.* 2001; 26: 202-211.

10. Wilkinson TL, Ashford DA, Pritchard J, Douglas AE. Honeydew sugars and osmoregulation in the pea aphid *Acyrtosiphon pisum*. *J Exp Biol* 1997; 200: 2137-2143.
11. Febvay G, Rahbé Y, Rynkiewicz M, Guillaud J, Bonnot G. Fate of dietary sucrose and neosynthesis of amino acids in the pea aphid, *Acyrtosiphon pisum*, reared on different diets. *J Exp Biol*. 1999; 202: 2639-2652.
12. Auclair J. Aphid feeding and nutrition. *Ann Rev Entomol*. 1963; 8: 439-490.
13. Leroy P, Wathelet B, Sabri A, Francis F, Verheggen F, Capella Q, Thonart P, Haubruge E. Aphid-host plant interactions: does aphid honeydew exactly reflect the host plant amino acid composition? *Arthropod-Plant Interact*. 2011; 5: 193-199.
14. Sabri A, Vandermoten S, Leroy PD, Haubruge E, Hance T, Thonart P, de Pauw E, Francis, F. Proteomic investigation of aphid honeydew reveals an unexpected diversity of proteins. *PloS ONE*. 2013; 8: e74656.
15. Völkl W, Woodring J, Fischer M, Lorenz MW, Hoffmann KH. Ant-aphid mutualisms: the impact of honeydew production and honeydew sugar composition on ant preferences. *Oecologia*. 1999; 118: 483-491.
16. Fischer MK, Völkl W, Schopf R, Hoffmann KH. Age-specific pattern in honeydew production and honeydew composition in the aphid *Metopeurum fuscoviride*: implications for ant-attendance. *J Insect Physiol*. 2002; 48: 319-326.
17. Hendrix DL, Wei Y, Legget JE. Homopteran honeydew is determined by both the insect and the plant species. *Comp Biochem Physiol*. 1992; 101: 23-27.
18. Fischer MK, Shingleton AW. Host plant and ants influence the honeydew sugar composition of aphids. *Funct Ecol*. 2001; 15: 544-550.
19. Bacon JSD, Dickinson B. The origin of melezitose: a biochemical relationship between the lime tree (*Tilia* spp.) and an aphid (*Eucallipterus tiliae* L.). *Biochem*. 1957; 66: 289-297.

20. Liebig G. Gaschromatographische und enzymatische Untersuchungen des Zuckerspektrums des Honigtaus von *Buchneria pectinatae* (Nördl.) Apidologie. 1979; 10: 213-225.
21. Mittler TE. Studies on the feeding and nutrition of *Tuberolachnus salignus* (Gmelin) (Homoptera, Aphididae). II. The nitrogen and sugar composition of ingested phloem sap and excreted honeydew. J Exp Biol. 1958; 35: 74-84.
22. Fisher DB, Wright JP, Mittler TE. Osmoregulation by the aphid *Myzus persicae*: a physiological role for honeydew oligosaccharides. J Insect Physiol. 1984; 30: 387-393.
23. Rhodes JD, Croghan PC, Dixon AFG. Dietary sucrose and oligosaccharide synthesis in relation to osmoregulation in the pea aphid, *Acythosiphon pisum*. Physiol Entomol. 1997; 22: 373-379.
24. Ashford DA, Smith WA, Douglas AE. Living on a high sugar diet: the fate of sucrose ingested by a phloem feeding insect, the pea aphid *Acyrtosiphon pisum*. J Insect Physiol. 2000; 46: 335-341.
25. Walters FS, Mullin CA. Sucrose-dependent increase in the oligosaccharide production and associated glycosidase activities in the potato aphid *Macrosiphum euphorbiae* (Thomas). Arch Insect Biochem Physiol. 1988; 9: 35-46.
26. Braendle C, Miura T, Bickel R, Shingleton AW, Kamphampati S, Stern DL. Developmental origin and evolution of bacteriocytes in the aphid-*Buchnera* symbiosis. PLoS Biol. 2003; 1: e1.
27. Prosser WA, Douglas AE. A test of the hypothesis that nitrogen is upgraded and recycled in an aphid (*Acythosiphon pisum*) symbiosis. J Insect Physiol. 1992; 38: 93-99.
28. Sasaki T, Ishikawa H. Production of essential amino acids from glutamate by mycetocyte symbionts of the pea aphid, *Acythosiphon pisum* maintained on artificial diets. J Insect Physiol. 1995; 37: 749-756.

29. Wilkinson TL, Adams D, Minto LB, Douglas AE. The impact of host plant on the abundance and function of symbiotic bacteria in an aphid. *J Exp Biol.* 2001; 204: 3027-3028.
30. Kunkel H, Kloft, W. J. Die Honigtau-Erzeuger des Waldes, in: Kloft, W.J. and Kunkel, H. (Eds.), *Waldtracht und Waldhonig in der Imkerei.* Ehrenwirth, Munich. 1985; pp. 48-265.
31. Ruoff K, Luginbühl W, Kilchenmann V, Bosset JO., von der Ohe K, von der Ohe W, Amadò R. Authentication of the botanical origin of honey using profiles of classical measurands and discriminant analysis. *Apidologie.* 2007; 38: 438-452.
32. Nottbohm FE, Lucius F. Melezitose im Honigtau von der Linde. *Zeitschrift für Untersuchungen der Lebensmittel.* 1929; 57: 549-558.
33. Hudson CS, Sherwood SF. The occurrence of melezitose in honey. *J Am Chem Soc.* 1920; 42: 116–125.
34. Gölz H. Der Melezitosegehalt im Honigtau verschiedener Lachnidenarten. *Apidologie.* 1982; 13: 89-91.
35. Zoebelein G. Die Rolle des Waldhonigtaus im Nahrungshaushalt forstlich nützlicher Insekten. *Forstw Centralbl.* 1957; 76: 24-34.
36. Hijaz F, Killiny N. Collection and chemical composition of phloem sap from *Citrus sinensis* L. Osbeck (Sweet Orange). *PLoS ONE.* 2014 9(7): e101830.
37. Findling S, Zanger K, Krueger S, Lohaus G. Subcellular distribution of raffinose oligosaccharides and other metabolites in summer and winter leaves of *Ajuga reptans* (Lamiaceae). *Planta.* 2015; 241: 229-241.
38. Göttliger T, Schwerdtfeger M, Tiedge K, Lohaus G. What do nectarivorous bats like? Nectar composition in Bromeliaceae with special emphasis on bat-pollinated species. *Front Plant Sci.* 2019; 10: 205.

39. West SG, Finch JF, Curran PJ. Structural equation models with nonnormal variables: problems and remedies. In: Hoyle RH, editor. Structural equation modeling: Concepts, issues and applications. Newbury Park, CA: Sage; 1995. p56-75.
40. Hervé MR, Nicolè F, Lê Cao KA. Multivariate analysis of multiple datasets: a practical guide for chemical ecology. *J Chem Ecol*. 2018; 44: 215-234.
41. Ramette A. Multivariate analyses in microbial ecology. *FEMS Microbiol Ecol*. 2007; 62: 142–160.
42. Oksanen J, Kindt R, Legendre P, O’Hara B, Henry M, Stevens H. The vegan package. Community ecology package. R package version 2.5-3 [online]. 2007; <https://CRAN.R-project.org/package=vegan> (accessed on 07 October 2019).
43. Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecology*. 2001; 26: 32–46.
44. Anderson MJ. Distance-based tests for homogeneity of multivariate dispersions. *Biometrics*. 2006; 62: 245–253.
45. Anderson MJ, Walsh DCI. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: what null hypothesis are you testing? *Ecol Monogr*. 2013; 83: 557–574.
46. Ziegler H, Mittler TE. Über den Zuckergehalt der Siebröhren- bzw- Siebzellensäfte von *Heracleum mantegazzianum* und *Picea abies* (L.) Karst *Z Naturforschung*. 1959; 14 B: 278-281.
47. Cristofolletti PT, Ribeiro AF, Deraison C, Rahbe Y, Terra WR. Midgut adaption and digestive enzyme distribution in a phloem feeding insect, the pea aphid *Acyrtosiphon pisum*. *J Insect Physiol* 2003; 49: 11-24.
48. Weibull J, Ronquist F, Brishammar, S. Free amino acid composition of leaf exudates and phloem sap. *Plant Physiol*. 1990; 92: 222-226.

49. Haribal M, Jander, G. Stable isotope studies reveal pathways for the incorporation of non-essential amino acids in *Acyrtosiphon pisum* (pea aphids). *J Exp Biol.* 2015; 218: 3797-3806.
50. Sasaki T, Fukuchi N, Ishikawa H. Amino acid flow through aphid and its symbiont: studies with ¹⁵N-labeled glutamine. *Zool Sci.* 1993; 10: 787-791.
51. Ehrhardt P. Die anorganischen Bestandteile des Honigtaues von *Megoura viciae* Buckt.. *Experientia.* 1965; XXI/8: 472-473.
52. Downing N. The regulation of sodium, potassium and chloride in an aphid subjected to ionic stress. *J Exp Biol.* 1980; 87: 343-349.
53. Wyatt GR. The biochemistry of insect haemolymph. *Ann Review Entomol.* 1961; 6: 75-102.
54. Fermo P, Beretta G, Facino RM, Gelmini F. Ionic profile of honey as a potential indicator of botanical origin and global environmental pollution. *Environ Pollution.* 2013; 178: 173-181.
55. Tiedge K, Lohaus G. Nectar sugars and amino acids in day- and night-flowering *Nicotiana* species are more strongly shaped by pollinators' preferences than organic acids and inorganic ions. *PLoS ONE.* 2017; 12(5): e0176865.
56. Schmelz H, Gall H, Fehlinger GF, Heider E, Rösch J. Alle Jahre wieder Ratlosigkeit – oder was tun bei Melezitosestracht. *ADIZ.* 2002; 1: 23-24
57. Imdorf, A, Bogdanov S, Kilchenmann V. Zementhonig im Honig- und Brutraum – was dann? *Schweizer Zentrum für Bienenforschung.* 2002; 1: 1–16.

Supporting information

S1 Table. Sugar composition in phloem exudates of *Abies alba* and *Picea abies*. All values are mean proportions (%) of n = 6 independent measurements \pm SD.

Sugar [%]	<i>Abies alba</i>	<i>Picea abies</i>
Glucose (glu)	19 \pm 4	15 \pm 2
Fructose (fru)	24 \pm 4	27 \pm 2
Sucrose (suc)	57 \pm 8	58 \pm 3

S2 Table. Amino acid composition in phloem exudates of *Abies alba* and *Picea abies*. All values are mean proportions (%) of n = 6 independent measurements \pm SD.

Amino acid [%]	<i>Abies alba</i>	<i>Picea abies</i>
Glutamate (GLU)	7.6 \pm 1.2	15.0 \pm 2.3
Glutamine (GLN)	18.1 \pm 4.6	19.4 \pm 9.4
Aspartate (ASP)	5.6 \pm 0.8	11.7 \pm 3.7
Asparagine (ASN)	2.2 \pm 0.7	1.9 \pm 1.5
Proline (PRO)	0.1 \pm 0.0	0.1 \pm 0.1
Glycine (GLY)	2.0 \pm 0.6	1.5 \pm 0.6
Serine (SER)	7.2 \pm 0.9	5.9 \pm 1.8
Alanine (ALA)	3.6 \pm 2.2	3.8 \pm 1.1
Tyrosine (TYR)	1.5 \pm 0.2	0.8 \pm 0.5
Arginine (ARG)	14.9 \pm 3.1	8.8 \pm 5.2
Histidine (HIS)	2.6 \pm 0.6	1.5 \pm 0.2
Lysine (LYS)	2.0 \pm 0.2	0.9 \pm 0.2
Threonine (THR)	13.2 \pm 2.4	10.0 \pm 5.2
Valine (VAL)	5.4 \pm 0.3	6.4 \pm 1.7
Isoleucine (ILE)	0.6 \pm 0.1	1.5 \pm 1.3
Leucine (LEU)	0.7 \pm 0.3	1.1 \pm 0.7
Phenylalanine (PHE)	0.6 \pm 0.1	0.5 \pm 0.2
Tryptophan (TRP)	0.8 \pm 0.3	0.9 \pm 0.4
Methionine (MET)	0.4 \pm 0.2	0.2 \pm 0.2
Non-proteinogenic amino acids (NP)	10.9 \pm 3.0	8.1 \pm 2.7

S3 Table. Inorganic cation and anion composition in phloem exudates of *Abies alba* and *Picea abies*. All values are mean proportions (%) of n = 6 independent measurements \pm SD.

Ion [%]	<i>Abies alba</i>	<i>Picea abies</i>
Cations		
Potassium (K ⁺)	71.3 \pm 3.6	77.4 \pm 3.0
Sodium (Na ⁺)	5.9 \pm 3.0	5.3 \pm 0.7
Ammonium (NH ₄ ⁺)	7.2 \pm 3.5	5.8 \pm 1.9
Magnesium (Mg ²⁺)	5.9 \pm 1.5	5.0 \pm 1.1
Calcium (Ca ²⁺)	9.7 \pm 2.6	6.5 \pm 2.3
Anions		
Chloride (Cl ⁻)	59.6 \pm 10.1	52.7 \pm 5.7
Phosphate (PO ₄ ³⁻)	22.1 \pm 13.1	29.0 \pm 6.7
Sulfate (SO ₄ ²⁻)	13.5 \pm 6.0	14.1 \pm 2.0
Nitrate (NO ₃ ⁻)	4.9 \pm 2.3	4.2 \pm 2.2

3.2. Section II: Effects of different temperatures on melezitose production in aphid species

Environmental factors like temperature and humidity can influence melezitose content in honeydew. Aphids belong to the poikilothermic animals, i.e. their body temperature is variable and dependent on ambient temperature. Therefore, two aphid species were investigated to study the effect of temperature on the production of melezitose and other sugars in their body. First, melezitose proportions were determined in honeydew samples of *C. pilicornis* and *C. pectinatae* in two different years 2016 and 2017. In addition, whole body of *C. pilicornis* and *C. pectinatae* were homogenized and incubated with 10% sucrose at three different temperature (25, 30, and 35°C) for 120 minutes and the sugar content were then determined via HPLC.

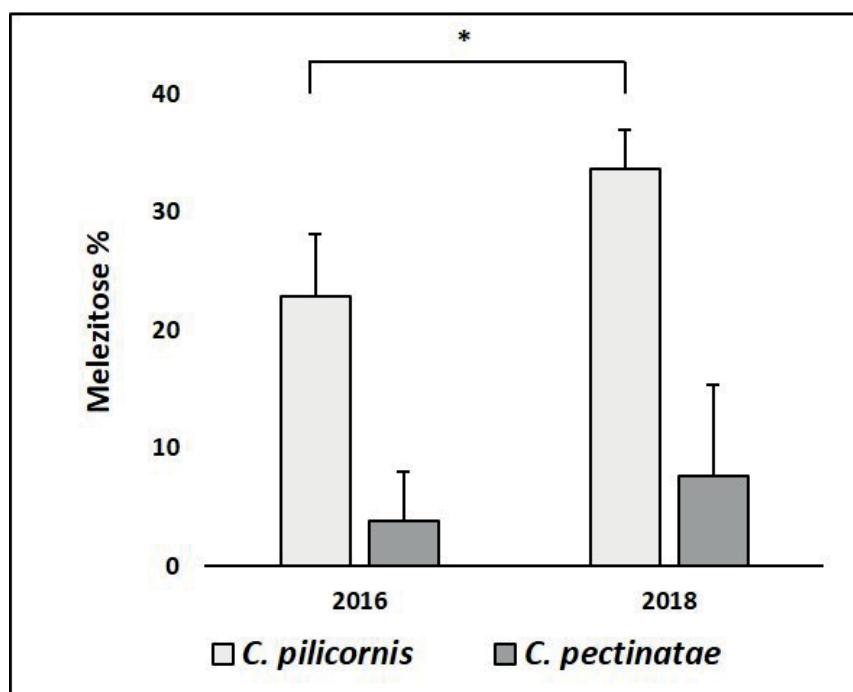


Fig 3. 1. Melezitose proportion of *C. pilicornis* and *C. pectinatae* honeydew in year 2016 and 2017. The aphid homogenates were incubated with 10% sucrose solutions. All values are means of n=10 independent measurements \pm SD. T-tests comparing means of melezitose proportions for each *Cinara* species (*p < 0.05, ** p < 0.01, *** p < 0.001).

Melezitose proportions for honeydew of *C. pilicornis* collected in summer 2017 is significantly higher than it is in 2016 (Fig 3.1). Further, the proportion of melezitose increased also in *C. pectinatae* honeydew but no significant differences were obtained. In addition, in both years melezitose proportions were higher in the honeydew of *C. pilicornis* compared to *C. pectinatae*.

Both aphids were able to digest sucrose to glucose, fructose, trehalose, melezitose and erlose at all tested temperature (Table 3.1). Moreover, glucose and fructose have the highest concentration among other sugars and significant differences in their concentration were obtained between 25 and 35°C. Trehalose concentration was almost stable in both aphid species and at all tested temperatures.

Table 3. 1. Sugar concentration from whole aphid's body incubated with 10% sucrose solution for 120 min at three different temperatures.

Sugars (mmol/L)	<i>C. pilicornis</i>			<i>C. pectinatae</i>		
	25° C	30° C	35° C	25° C	30° C	35° C
Glucose	11 ± 5	11 ± 2	26 ± 4**	8 ± 2	15 ± 5	18 ± 7**
Fructose	9 ± 3	8 ± 2	19 ± 4**	7 ± 2	11 ± 1	15 ± 5**
Trehalose	3 ± 1	2 ± 0	2 ± 0	1 ± 0	1 ± 0	2 ± 0
Melezitose	6 ± 5	6 ± 0	11 ± 2**	1 ± 0	1 ± 1	4 ± 1*
Erlose	0 ± 1	2 ± 1	1 ± 1	4 ± 2	9 ± 2	14 ± 5**
Further sugars	1 ± 1	1 ± 0	1 ± 1	0 ± 0	1 ± 1	3 ± 2

All values are means of n=3 independent measurements ± SD. Dunn's tests comparing means of sugars productions concentration at different temperatures for each *Cinara* species (*p < 0.05, ** p < 0.01, *** p < 0.001).

Additionally, the melezitose production was higher for *C. pilicornis* than for *C. pectinatae*, and the erlose production was higher for *C. pectinatae* than for *C. pilicornis*. Further, the production of melezitose and erlose of both *Cinara* species increased at higher temperature. This increase is significant between 25 and 35°C for both species (Fig 3.2).

In addition, glucose, fructose, erlose and melezitose concentrations in whole aphid's body increased in high temperature. Therefore, we can assume that they were produced because of enzymatic activity. However, enzymatic activity increases generally in high temperature. In addition, although more melezitose occurs at high temperatures in both species, the melezitose formation in the whole *C. pilicornis* body is remains significantly higher than in *C. pectinatae*. Therefore, we can assume that the honeydew producers is mainly influence the melezitose concentration in honeydew.

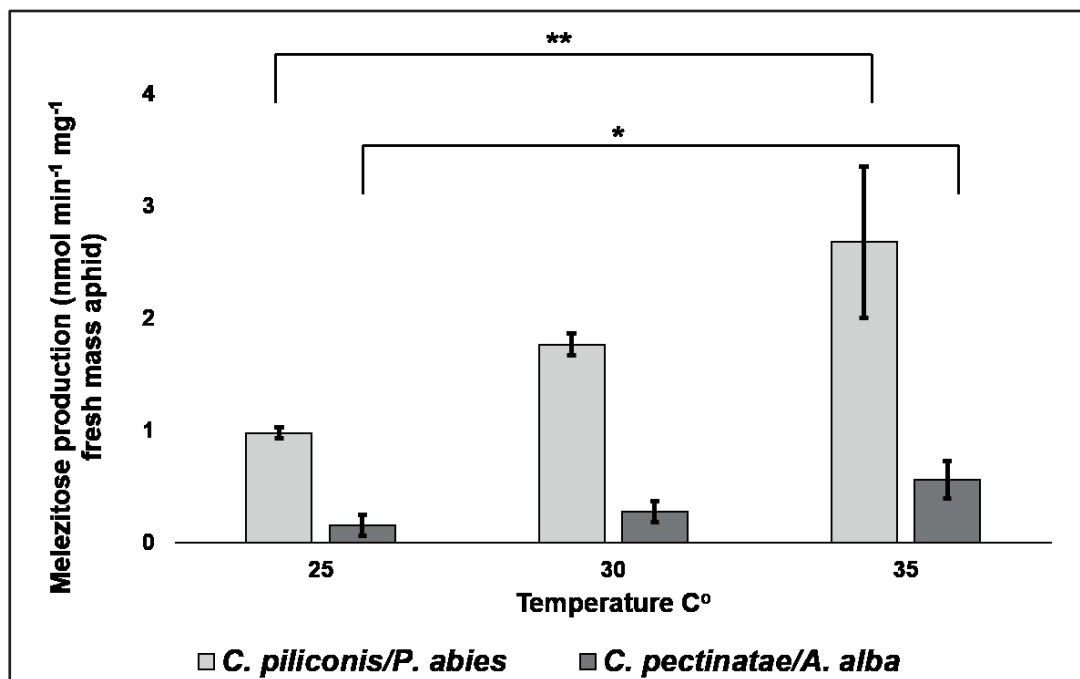


Fig 3. 2. Melezitose production in whole-body homogenates of *C. pilicornis* and *C. pectinatae* at three different temperatures. The aphid homogenates were incubated with 10% sucrose solutions. All values are means of n=3 independent measurements \pm SD. Dunn's tests comparing means of melezitose production rates at different temperatures for each *Cinara* species (*p < 0.05, ** p < 0.01, *** p < 0.001). The production of melezitose differs significantly between 25 and 35° C in both *Cinara* species.

3.3. Section III: The ability of honeybees *A. mellifera* to digest melezitose

Worker honeybees gather nectar or honeydew, stored in hives and produce honey. While nectar contains no melezitose, honeydew can contain melezitose. In addition, the ability of honeybees to digest melezitose is not completely understood. Therefore, two experiments were taken place to investigate this issue. In the first experiment, bees were fed with two different solutions (with melezitose and without melezitose) in order to understand if melezitose is digested by bees. Newly emerged bees were placed in hive's cages and the temperature was adjusted to 35°C. Then, bees were fed with two different solutions, without melezitose S1 (31% glucose, 39% fructose and 30% sucrose) and with melezitose S2 (15.5% glucose, 19.5% fructose, 15% sucrose and 50% melezitose). After 21 days, the cages contents were collected (This were done by Victoria Seeburger at the University of Hohenheim). Then, sugars composition were analyzed via HPLC.

At the second experiment, the ability of workers abdomen to digest melezitose is investigated. The abdomen of worker honeybees was homogenate and three sugar solutions were added for 120 minutes (S1: 10% melezitose and 10% sucrose, S2: 20% melezitose and S3: 20% sucrose).

3.3.1. Feed experiment of honeybees

Young bees were able to digest melezitose and sucrose. They also produced glucose and fructose (Fig 3.3). In both solutions, glucose and fructose proportions are increased and sucrose and melezitose proportions are decreased. In addition, fructose proportion was higher than glucose in both solutions after 21 days.

The proportion of sucrose in S1 solution decreased from 30% to less than 10%, while glucose and fructose proportions grow about 10% each. The proportion of melezitose decreased dramatically in S2 solution from 50% to approximately 19% after 21 days. In addition, the sucrose proportion in S2 solution was clearly decreased. Moreover, glucose and fructose proportions grew about 20%.

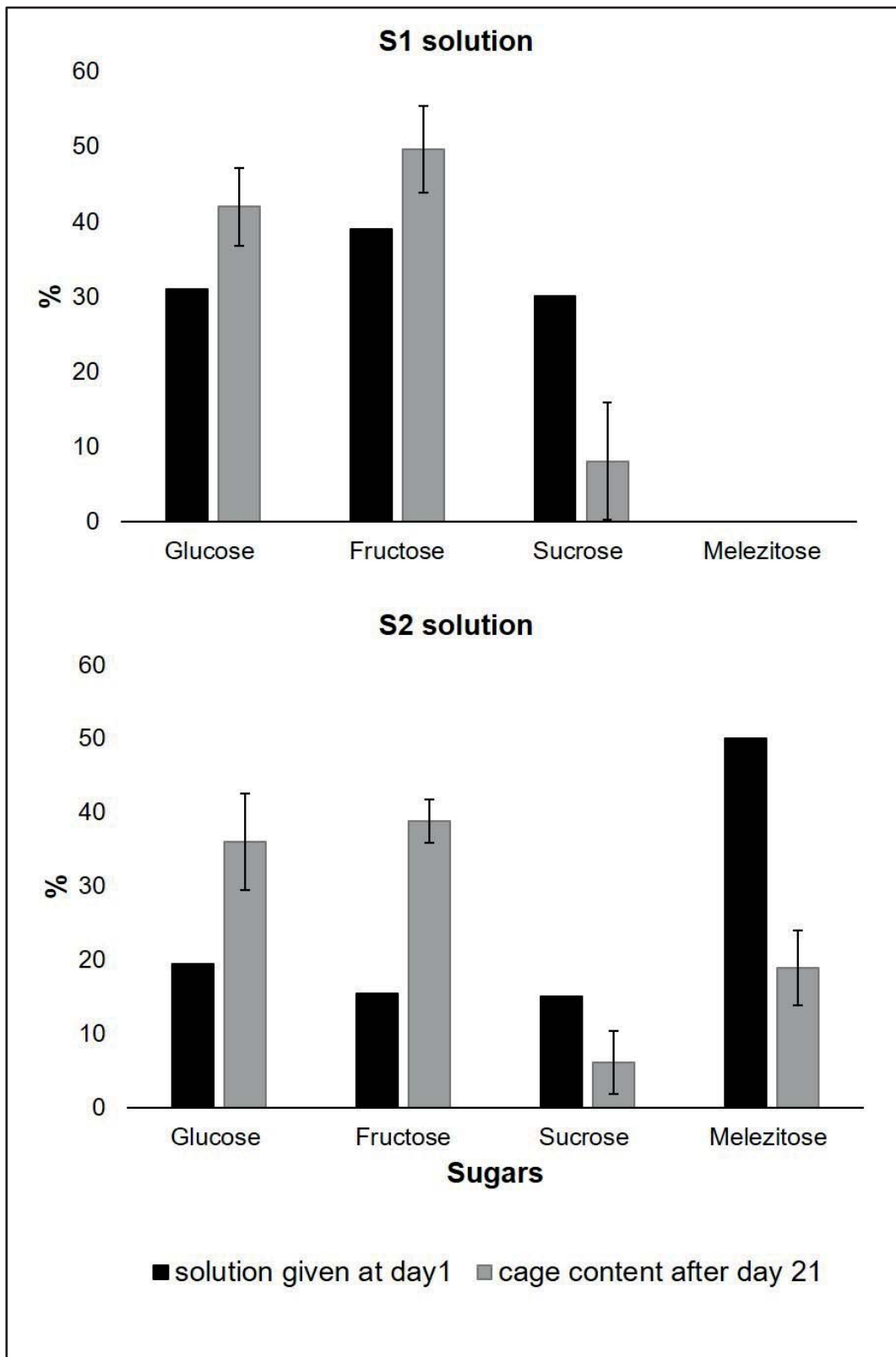


Fig 3. 3. Sugar compositions in “honey” produced by bees after 21 days fed on two different solutions. All values of day 21 are means of n=33 independent measurements \pm SD for S1 and means of n=68 independent measurements \pm SD for S2.

3.3.2. Sugar cleavage in abdomen homogenates of adult's *A. mellifera*

In S1 solution, sucrose was particularly cleavage after 60 minutes. Moreover, melezitose proportion decreased slowly. More precisely, after 120 minutes abdomen enzymes split less than 20% of the melezitose (Fig 3.4; S1). However, melezitose proportion in the solution differs significantly after 120 minutes. The same results were shown if only sucrose or melezitose was given.

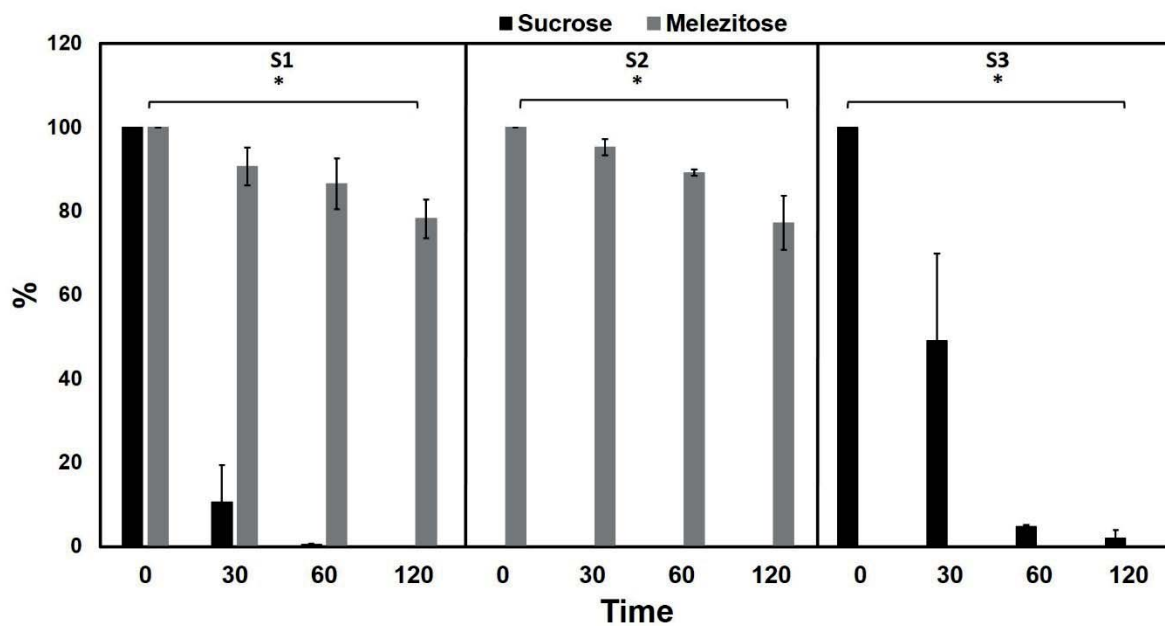


Fig 3. 4. Sugar proportions in abdomen homogenates of *A. mellifera* during the experiment. The honeybee abdomen homogenates were incubated with S1 (10% sucrose/10% melezitose), S2 (20% melezitose) and S3 (20% sucrose) solution. All values are means of $n = 3$ independent measurements \pm SD.

3.4. Section IV: Honeydew honey profiling of fir, spruce and pine honey

Honeydew honey should be characterized according to its botanical, zoological or geographical origins. Therefore, the ability of sugars, amino acids and inorganic ions to identify the botanical, zoological and geographical origin of honeydew honey was investigated.

Honeydew honey samples were collected from two geographical origins (Germany and Turkey) and three different botanical origins (pine, fir and spruce). However, the zoological origins of those samples were only identified by the German samples (fir/*Cinara*, spruce/*Cinara*, and spruce/*Physokermes*). Then, sugars, amino acids, and inorganic ions profiles of the honeydew honey samples were determined via HPLC. In addition, the total proteins content of all honey were also determined.

Sugar, amino acids and inorganic ions profiles for honeydew honey

The monosaccharides glucose and fructose were the dominant sugars in all samples (about 60g/100g honey), and the fructose content was always higher than that of glucose content (Table 3.2). No significant differences of glucose contents between the four groups of honey were found, but fructose was significantly higher in pine honey. Different disaccharides (sucrose, trehalose, isomaltose, maltose, turanose and kojibiose) were detected in all honeydew honey samples, with trehalose showed high contents in fir and spruce samples and a significantly lower content in pine honey. Further, isomaltose showing the highest contents in all samples, especially in pine honey. It must be stated, however, that the isomaltose peak was not separated from the maltulose peak. Melezitose and erlose were the dominant trisaccharides. Melezitose was the most abundant trisaccharide in fir and spruce *Cinara* honey and in pine honey, whereas the erlose content was significantly higher in spruce/*Physokermes* honey compared to the other honey samples. Several further sugars were detected in the honey samples, named as undefined 1 to 7. Undefined sugar 6 is probably an oligosaccharide and its content was significantly higher in spruce/*Physokermes* honey than in fir/- or spruce/*Cinara* or pine honey. Additionally, undefined sugar 3 can also be an oligosaccharide and the content was higher in pine samples comparing to other samples (Table 3.2).

Table 3. 2. Sugar contents in four groups of honeydew honey (*Abies alba*/*Cinara* spec., *Picea abies*/*Cinara* spec., *Picea abies*/*Physokermes* spec., and *Pinus* sp.).

	<i>Abies alba</i>	<i>Picea abies</i>		<i>Pinus</i> sp.
Sugars [g/100g]	<i>Cinara</i> sp.	<i>Cinara</i> sp.	<i>Physokermes</i> sp.	<i>n.d.</i> ****
Glucose (glu)	29.6 ± 2.5 ^a	28.3 ± 3.6 ^a	27.5 ± 3.9 ^a	26.1 ± 1.8 ^a
Fructose (fru)	29.9 ± 3.8 ^a	30.5 ± 3.7 ^{a,b}	28.4 ± 2.6 ^a	33.9 ± 3.2^b
Sucrose (suc)	1.09 ± 0.56 ^a	0.87 ± 0.52 ^a	1.15 ± 0.69 ^a	1.36 ± 0.5 ^a
Trehalose (tre)	1.92 ± 0.86 ^a	1.22 ± 0.75 ^a	1.75 ± 0.78 ^a	0.1 ± 0.05^b
Maltose (mal)	0.95 ± 0.49 ^a	0.87 ± 0.85 ^a	0.97 ± 0.74 ^a	1.11 ± 0.28 ^a
Isomaltose* (iso)	2.42 ± 1.30 ^a	2.00 ± 1.00 ^a	1.55 ± 0.44 ^a	3.78 ± 0.94^b
Kojibiose (koj)	1.09 ± 0.41 ^a	1.17 ± 0.71 ^a	1.34 ± 0.89 ^a	1.12 ± 0.33 ^a
Turanose** (tur)	1.39 ± 0.79 ^a	1.03 ± 0.51 ^a	0.94 ± 0.85 ^a	0.11 ± 0.07 ^a
Melezitose (mel)	3.29 ± 2.12 ^a	5.20 ± 4.37 ^a	3.36 ± 1.98 ^a	2.68 ± 1.67^b
Erlose (erl)	1.97 ± 1.73 ^a	1.31 ± 0.99 ^a	5.01 ± 3.35^b	0.28 ± 0.3 ^c
Raffinose (raf)	0.83 ± 0.79 ^a	0.53 ± 0.50 ^a	0.71 ± 0.63 ^a	0.24 ± 0.13 ^a
1-Kestose*** (kes)	0.34 ± 0.64 ^a	0.89 ± 1.23 ^a	1.36 ± 1.36 ^a	1.4 ± 0.41 ^a
Undef 1	0.06 ± 0.07 ^a	0.08 ± 0.09 ^a	0.08 ± 0.07 ^a	0.03 ± 0.03 ^a
Undef 2	0.06 ± 0.08 ^a	0.05 ± 0.04 ^a	0.09 ± 0.12 ^a	0.25 ± 0.09 ^a
Undef 3	0.00 ± 0.00 ^a	0.33 ± 0.78 ^a	0.12 ± 0.38 ^a	1.61 ± 0.49^b
Undef 4	0.93 ± 0.49 ^a	1.07 ± 0.33 ^a	1.11 ± 0.47 ^a	0.87 ± 0.56 ^a
Undef 5	0.07 ± 0.09 ^a	0.08 ± 0.11 ^a	0.15 ± 0.15 ^a	0.0 ± 0.0 ^a
Undef 6	0.81 ± 0.60 ^a	0.46 ± 0.56 ^a	1.71 ± 0.85^b	0.0 ± 0.0 ^c
Undef 7	0.10 ± 0.20 ^a	0.08 ± 0.18 ^a	0.21 ± 0.28 ^a	0.0 ± 0.0 ^a

All values are means of n = 12 independent measurements ± SD.

Different letters represent significant differences between the sugars in the tree groups of honeydew honey.

*peak of isomaltose was not completely separated from maltulose peak.

**peak of turanose was not completely separated from isomaltulose and gentiobiose peak.

***peak of kestose was not completely separated from nigerose and stachyose peak.

Undef. = undefined Peaks

****n.d. = not determined

All proteinogenic as well as further non-proteinogenic amino acids were found in the honey samples, but some amino acid contents differ significantly among honeydew honey groups. More precisely, the contents of proline, tyrosine, arginine, lysine, and isoleucine differed significantly between the four groups of honey. Proline was the dominant amino acid in all groups; its content was up to 90% of the total amino acids content. However, the total contents of all free amino acids were particularly low in all honey samples (less than 0.1g/100g honey; Table 3.3).

Table 3. 3. Amino acid contents in four groups of honeydew honey (*Abies alba*/*Cinara* spec., *Picea abies*/*Cinara* spec., *Picea abies*/*Physokermes* spec., and *Pinus* sp.).

	<i>Abies alba</i>	<i>Picea abies</i>		<i>Pinus</i> sp.
Amino acids [mg/100g]	<i>Cinara</i> sp.	<i>Cinara</i> sp.	<i>Physokermes</i> sp.	<i>n.d.</i> *
Glutamate (GLU)	1.25 ± 0.68 ^a	2.48 ± 2.48 ^a	1.54 ± 2.24 ^a	5.87 ± 2.47 ^a
Glutamine (GLN)	1.79 ± 1.86 ^a	3.83 ± 3.41 ^a	1.71 ± 2.62 ^a	5.8 ± 2.98 ^a
Aspartate (ASP)	0.56 ± 0.56 ^a	1.93 ± 2.12 ^a	1.51 ± 2.96 ^a	1.54 ± 0.54 ^a
Asparagine(ASN)	0.78 ± 0.67 ^a	1.61 ± 1.60 ^a	1.13 ± 2.93 ^a	1.09 ± 0.45 ^a
Proline (PRO)	60.0 ± 15.5 ^a	49.2 ± 21.6^{a,b}	37.1 ± 21.0^b	54.0 ± 15.1 ^a
Glycine (GLY)	0.25 ± 0.10 ^a	0.35 ± 0.22 ^a	0.24 ± 0.17 ^a	0.97 ± 0.37 ^a
Serine (SER)	0.35 ± 0.19 ^a	0.68 ± 0.59 ^a	0.40 ± 0.32 ^a	1.27 ± 0.46 ^a
Alanine (ALA)	0.68 ± 0.40 ^a	1.14 ± 0.89 ^a	0.98 ± 1.25 ^a	1.62 ± 0.47 ^a
Tyrosine (TYR)	0.31 ± 0.15 ^a	0.84 ± 0.44^b	0.77 ± 0.52^b	0.58 ± 0.32 ^a
Arginine (ARG)	0.20 ± 0.17 ^a	0.58 ± 0.39^b	0.30 ± 0.23^{a,b}	0.33 ± 0.11 ^a
Histidine (HIS)	0.11 ± 0.37 ^a	0.36 ± 0.54 ^a	0.54 ± 0.57 ^a	0.0 ± 0.0 ^a
Lysine (LYS)	0.16 ± 0.12 ^a	0.58 ± 0.55^b	0.25 ± 0.16^{a,b}	0.24 ± 0.13 ^a
Threonine (THR)	0.14 ± 0.06 ^a	0.30 ± 0.22 ^a	0.20 ± 0.19 ^a	0.18 ± 0.08 ^a
Valine (VAL)	0.27 ± 0.17 ^a	0.55 ± 0.36 ^a	0.38 ± 0.25 ^a	0.40 ± 0.14 ^a
Isoleucine (ILE)	0.16 ± 0.11 ^a	0.35 ± 0.23^b	0.21 ± 0.1^{a,b}	0.40 ± 0.68 ^a
Leucine (LEU)	0.18 ± 0.24 ^a	0.34 ± 0.28 ^a	0.38 ± 0.29 ^a	0.15 ± 0.08 ^a
Phenylalanine (PHE)	0.50 ± 0.48 ^a	1.57 ± 1.41^b	0.49 ± 0.34 ^a	0.82 ± 1.11 ^a
Tryptophan (TRP)	0.22 ± 0.13 ^a	0.19 ± 0.33 ^a	0.13 ± 0.11 ^a	0.0 ± 0.0 ^a
Methionine (MET)	0.04 ± 0.06 ^a	0.13 ± 0.15 ^a	0.03 ± 0.04 ^a	0.04 ± 0.03 ^a
β-Alanine (β-ALA)	0.48 ± 0.21 ^a	0.87 ± 0.56 ^a	0.45 ± 0.17 ^a	0.95 ± 0.33 ^a
γ-Amino-butyric acid (GABA)	0.17 ± 0.10 ^a	0.51 ± 0.33 ^a	0.36 ± 0.47 ^a	0.45 ± 0.20 ^a
Further non-proteinogenic amino acids (NP)	0,22 ± 0,17 ^a	0,70 ± 0,48 ^a	0,46 ± 0,43 ^a	1.10 ± 0.89 ^a

All values are means of n = 12 independent measurements ± SD.

Different letters represent significant differences between the sugars in the tree groups of honeydew honey.

Further non-proteinogenic amino acids: homoserine, phosphoserine, ornithine, and taurine.

*n.d. = not determined

Honeydew honey samples contained several inorganic ions and the total content of inorganic ions was about 0.5g/100g honey (Table 3.4). Potassium (K⁺) was the main cation and phosphate (PO₄³⁻) was the most abundant anion. Magnesium and phosphate differed significantly between

the four groups of honey. The phosphate content was about 2-fold higher in spruce/*Physokermes* honey than in *Cinara* honey and much lower in pine honey. Chloride was about 3-fold higher in pine honey (Turkey) comparing to fir and spruce honey (Germany).

Table 3. 4. Inorganic ions contents in four groups of honeydew honey (*Abies alba*/*Cinara* spec., *Picea abies*/*Cinara* spec., *Picea abies*/*Physokermes* spec., and *Pinus* sp.).

	<i>Abies alba</i>	<i>Picea abies</i>		<i>Pinus</i> sp.
Ion [mg/100g]	<i>Cinara</i> sp.	<i>Cinara</i> sp.	<i>Physokermes</i> sp.	<i>n.d.</i> *
Cations				
Potassium (K ⁺)	213 ± 69 ^a	185 ± 3 ^a	252 ± 103 ^a	318 ± 61^b
Sodium (Na ⁺)	2 ± 5 ^a	3 ± 4 ^a	4 ± 7 ^a	1 ± 1 ^b
Ammonium (NH ₄ ⁺)	9 ± 3 ^a	7 ± 4 ^a	6 ± 4 ^a	7 ± 4 ^a
Magnesium (Mg ²⁺)	12 ± 7 ^a	15 ± 9^{a,b}	22 ± 14^b	10 ± 3 ^a
Calcium (Ca ²⁺)	4 ± 2 ^a	4 ± 5 ^a	6 ± 8 ^a	5 ± 4 ^a
Anions				
Chloride (Cl ⁻)	26 ± 8 ^a	32 ± 19 ^a	43 ± 42 ^a	103 ± 44^b
Phosphate (PO ₄ ³⁻)	122 ± 55 ^a	109 ± 33 ^a	210 ± 48^b	32 ± 22^c
Sulfate (SO ₄ ²⁻)	20 ± 4 ^a	20 ± 6 ^a	28 ± 13 ^a	27 ± 10 ^a
Nitrate (NO ₃ ⁻)	2 ± 2 ^a	2 ± 2 ^a	2 ± 4 ^a	5 ± 3 ^a

All values are means of n = 12 independent measurements ± SD.

Different letters represent significant differences between the inorganic ion content in the tree groups of honeydew honey.

**n.d.* = not determined

Protein content was significantly high in pine honey comparing to fir and spruce honey, about 0.8 g/100 g honey (Table 3.5). In addition, protein content in fir and spruce honey was almost 4-fold lower than in pine honey.

Table 3. 5. Protein contents in four groups of honeydew honey (*Abies alba*/*Cinara* spec., *Picea abies*/*Cinara* spec., *Picea abies*/*Physokermes* spec., and *Pinus* sp.).

	<i>Abies alba</i>	<i>Picea abies</i>		<i>Pinus</i> sp.
	<i>Cinara</i> sp.	<i>Cinara</i> sp.	<i>Physokermes</i> sp.	
Protein [mg/100g]	175 ± 82 ^a	175 ± 94 ^a	209 ± 134 ^a	769 ± 190^b

Honey composition in relation to the botanical origin

In order to ascertain whether the differences in sugar, amino acid, and ion contents could be explained by the botanical origin of the fir, spruce and pine honey samples, several redundancy analysis (RDA) were performed. All sugars, amino acids, and ions were taken for the RDA and all metabolite values were standardized as g/100g. Almost 22% of sugar variation were explained by the botanical origins (Table 3.6). However, about 20% of amino acids and inorganic ions variations were explained by the botanical origins. This means that the remaining 80% of the total variation is caused by other factors. A permutation F-test based on the canonical R^2 was carried out to test for the significance of controlled variable explanation. Because all p -values were significant, the interpreting of the loading plots was meaningful (Table 3.6).

Table 3. 6. Results of the RDA: Degrees of freedom (df), variance (%), pseudo-F (F), and p -values.

		<i>df</i>	Variance (%)	F	<i>p-value</i>
Sugar	Botanical origin	2	21.6	6.197	0.001 ***
	Residual	45	78.4		
Amino acid	Botanical origin	2	19.19	5.344	0.001 ***
	Residual	45	80.81		
Inorganic ion	Botanical origin	2	19.52	5.457	0.001 ***
	Residual	45	80.48		

Permutation: free, Number of permutations: 999

Pairwise comparisons showed that the sugars, amino acids and inorganic ions values of pine honey differed significantly from fir and spruce honey (Table 3.7). However, sugars values had no significant difference between fir and spruce honey.

Table 3. 7. P -values of pairwise comparisons for each group of honeydew honey.

		Fir/pine	Fir/spruce	Spruce/pine
<i>p-value</i>	Sugar	0.001 ***	0.560	0.001 ***
	Amino acid	0.012 **	0.380 *	0.006 **
	Inorganic ion	0.030 **	0.360 *	0.038 *

Pine honey were visually separated from fir and spruce honey in all three RDA plots (Fig 3.6. A, C and E). Fructose, isomaltose, *undef3* and *undef2* sugars were responsible for the separation of pine honey samples (Fig 3.6. B). Moreover, GLY, GLU, SER, GLN, and PRO caused the separation of pine honey (Fig 3.6. D). Additionally, potassium, chloride and nitrate were responsible for the separation of pine honey (Fig 3.6. F).

Honey composition in relation to the zoological origin

Fir and spruce honey were not separated efficiency considering the botanical origins of the honey samples. However, fir and spruce honey have different zoological origins. In addition, honeydew components are mainly influenced by the honeydew producers. Therefore, fir and spruce honey were characterized in three different groups, fir/*Cinara*, spruce/*Cinara*, and spruce/*Physokermes*. Moreover, in order to ascertain whether the differences in sugar, amino acid, and ion contents could be explained by the zoological or botanical origin of the fir and spruce honey samples, a redundancy analysis (RDA) was performed. Only sugars, amino acids, and ions that showed significant differences in the analysis of variance were taken for the RDA. All metabolite values were standardized as g/100g. The percentage of variation explained by the honeydew producer (zoological origin = 28.94 %) was higher than that for the tree species (botanical origin = 10.51 %; Table 3.8). This also means that the remaining 60.55% of the total variation is caused by other factors, for example the honeybee population, the geographical location or environmental conditions. A permutation F-test based on the canonical R^2 was carried out to test for the significance of controlled variable explanation. Because both *p-values* were significant, the interpreting of the loading plots was meaningful (Table 3.8). It was possible to visually separate three groups of honey (fir/*Cinara*, spruce/*Cinara*, and spruce/*Physokermes*; Fig 3.7. A), with erlose, undefined sugar 6, magnesium, and phosphate being responsible for the separation of spruce/*Physokermes* honey samples (Fig 3.7. B). Additionally, proline was the reason for the separation of fir/*Cinara* honey from the other two, and tyrosine, arginine, lysine, isoleucine, and phenylalanine caused the separation of spruce/*Cinara* from other honeys (Fig 3.7. B). Pairwise comparisons showed that the values of each botanical or zoological origin differed significantly from each other (Table 3.9).

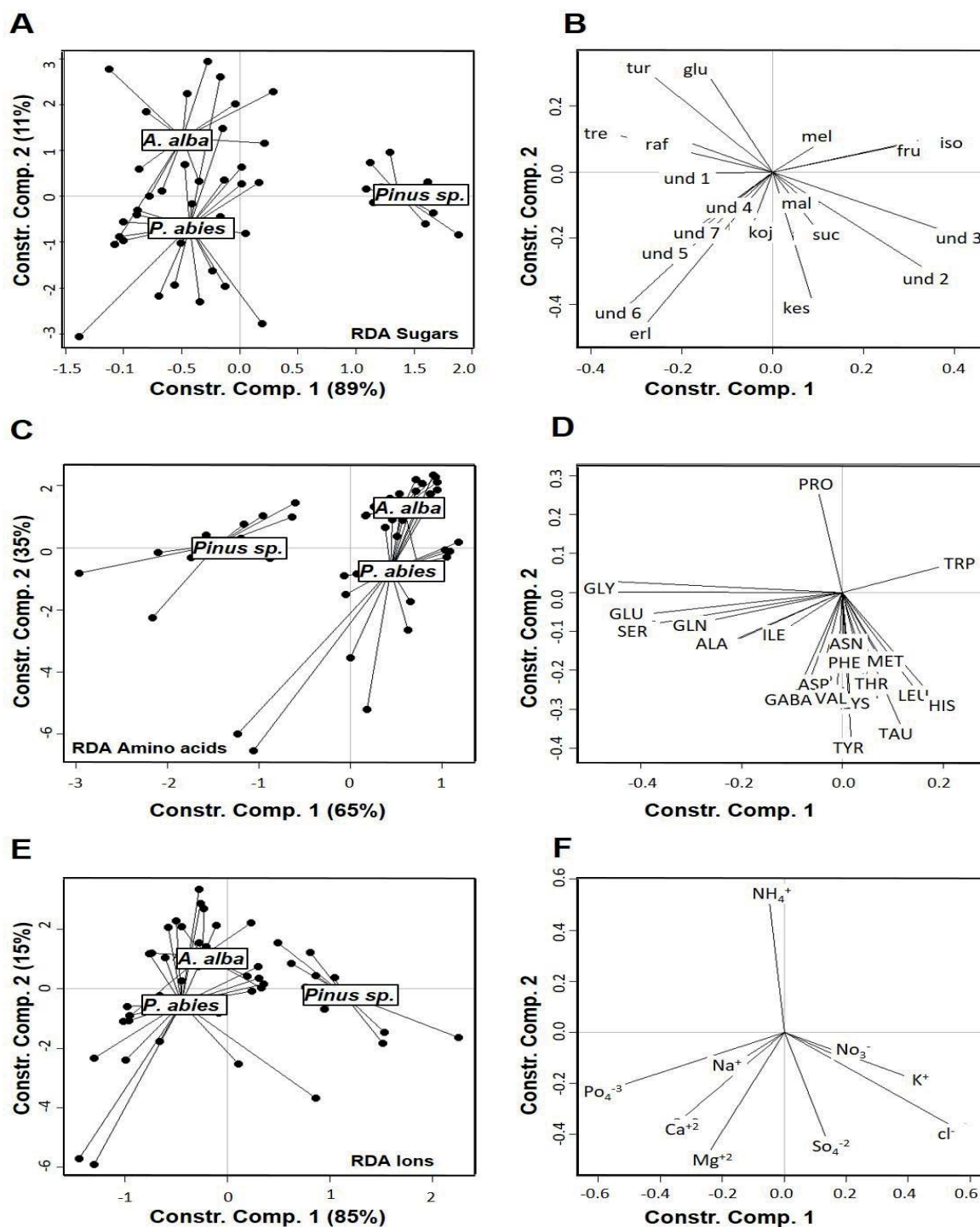


Fig 3. 5. Redundancy Analysis (RDA) plots (A, C and E) are score plots from the constrained Principal Component Analysis (PCA). Samples of each of the botanical origin are connected with the centroids of the corresponding convex hulls. (B, D and F) are loading plots, which illustrate the original variables loaded as vectors in PCA space. The analyses are based on the content of sugars, amino acids, and inorganic ions in honeydew honey.

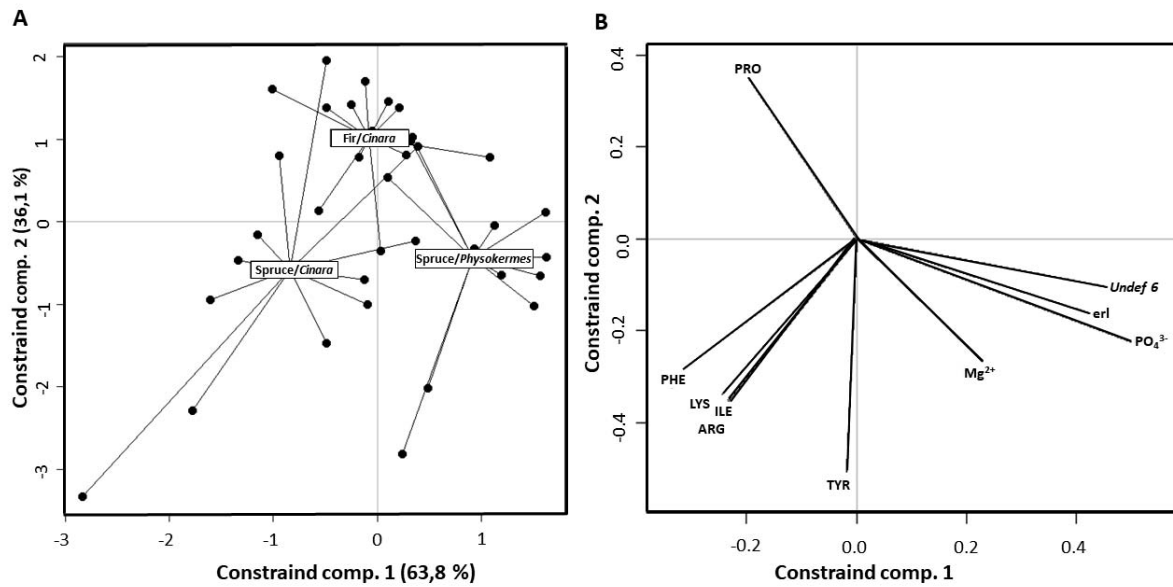


Fig 3. 6. Redundancy Analysis (RDA) plots. (A) Score plot from the constrained Principal Component Analysis (PCA). Samples of each of the honey groups are connected with the centroids of the corresponding convex hulls. (B) Loading plots, which illustrate the original variables loaded as vectors in PCA space.

Table 3. 8. Results of the RDA: Degrees of freedom (*df*), variance (%), pseudo-F (*F*), and *p*-values.

	<i>df</i>	Variance (%)	<i>F</i>	<i>p</i> -value
Botanical origin	1	10.51	3.99	0.005 **
Zoological origin	2	28.94	6.72	0.001 ***
Residual	32	60.55		

Permutation: free, Number of permutations: 999

Table 3. 9. *P*-values of pairwise comparisons for each group of honeydew honey.

	<i>Fir/Cinara</i>	<i>Spruce/Cinara</i>
<i>Spruce/Cinara</i>	0.0020 **	-
<i>Spruce/Physokermes</i>	0.0015 **	0.15

3.5. Section V: Nectar, honeydew and honey profiling of lime tree *Tilia sp.*

There are two possible kinds of honeys from lime tree. First, the blossom honey, which honeybees prepare from the nectar of the lime tree flowers, and the second lime honey occur when honeybees collect honeydew of hemipteran species, like *Eucallipterus tiliae*, feeding on lime trees. To investigate if differences between nectar and honeydew are reflected in lime honeys, samples from nectar, honeydew and honey of lime trees were collected and analyzed for sugars, amino acids and inorganic ions.

The main sugars in honey were glucose and fructose (Table 3.10 and Fig 3.7). Moreover, the proportion of fructose was slightly higher than that of glucose. Sucrose has the highest proportion in nectar and honeydew samples. Further, trehalose, melezitose and erlose were only found in honeydew and honeys. About 20% of honeydew sugars proportion was melezitose, but this proportion decreased to only 6% in honey. The same for erlose, its proportion decreased from 9% in honeydew to only 1% in honey samples. Additionally, trehalose proportions were only 1% in honeydew and honey samples and no trehalose were found in nectar. Furthermore, small amount of raffinose were determined in nectar, honeydew and honey. It was about 1% of all sugar proportions.

All proteinogenic, and some non-proteinogenic, amino acids were detected in nectar, honeydew and honey, but with different proportions (Table 3.11). Alanine, asparagine and glutamine were the dominant amino acids in nectar. In addition, glutamate, serine, aspartate and asparagine were found mostly in honeydew. Further, honey contain all amino acids that were detected in nectar and honeydew, but proline was clearly the dominant one with approximately 55% of the total amount of amino acids. On the contrary, proline proportions in nectar and honeydew were only about 1%.

Table 3. 10. Sugar proportions in nectar, honeydew and honey of *Tilia sp.*

Sugars [%]	Nectar <i>n</i> = 8	Honeydew <i>n</i> = 9	Honey <i>n</i> =12
Glucose (glu)	27 ± 2 ^a	10 ± 1 ^b	39 ± 3 ^c
Fructose (fru)	27 ± 2 ^a	15 ± 0 ^b	42 ± 3 ^c
Sucrose (suc)	44 ± 3 ^a	39 ± 4 ^a	2 ± 1^b
Trehalose (tre)	0 ± 0 ^a	1 ± 0 ^a	1 ± 0 ^a
Maltose (mal)	0 ± 0 ^a	2 ± 3^b	1 ± 0 ^{a,b}
Isomaltose* (iso)	0 ± 0 ^a	0 ± 0 ^a	2 ± 1 ^b
Kojibiose (koj)	0 ± 0 ^a	0 ± 0 ^a	2 ± 1 ^b
Turanose** (tur)	0 ± 0 ^a	0 ± 0 ^a	1 ± 0 ^b
Melezitose (mel)	0 ± 0 ^a	21 ± 3^b	6 ± 3^c
Erlose (erl)	0 ± 0 ^a	9 ± 3^b	1 ± 0 ^a
Raffinose (raf)	1 ± 1 ^a	1 ± 0 ^a	2 ± 1^b
1-Kestose*** (kes)	0 ± 0 ^a	0 ± 0 ^a	1 ± 0 ^a
Undef 1	0 ± 0 ^a	0 ± 0 ^a	0 ± 0 ^a
Undef 2	0 ± 0 ^a	0 ± 0 ^a	0 ± 0 ^a
Undef 3	0 ± 0 ^a	0 ± 0 ^a	0 ± 0 ^a
Undef 4	0 ± 0 ^a	0 ± 0 ^a	0 ± 0 ^a
Undef 5	0 ± 0 ^a	0 ± 0 ^a	0 ± 0 ^a
Undef 6	0 ± 0 ^a	0 ± 0 ^a	0 ± 0 ^a
Undef 7	0 ± 0 ^a	0 ± 0 ^a	0 ± 0 ^a
Total content	1764 ± 330 mmol/L	3271 ± 1003 mmol/L	77.62 ± 5.94 g/100g honey

All values are means of independent measurements ± SD.

Different letters represent significant differences between the sugars in nectar, honeydew and honey.

*peak of isomaltose was not completely separated from maltulose peak.

**peak of turanose was not completely separated from isomaltulose and gentiobiose peak.

***peak of kestose was not completely separated from nigerose and stachyose peak.

Undef. = undefined Peaks

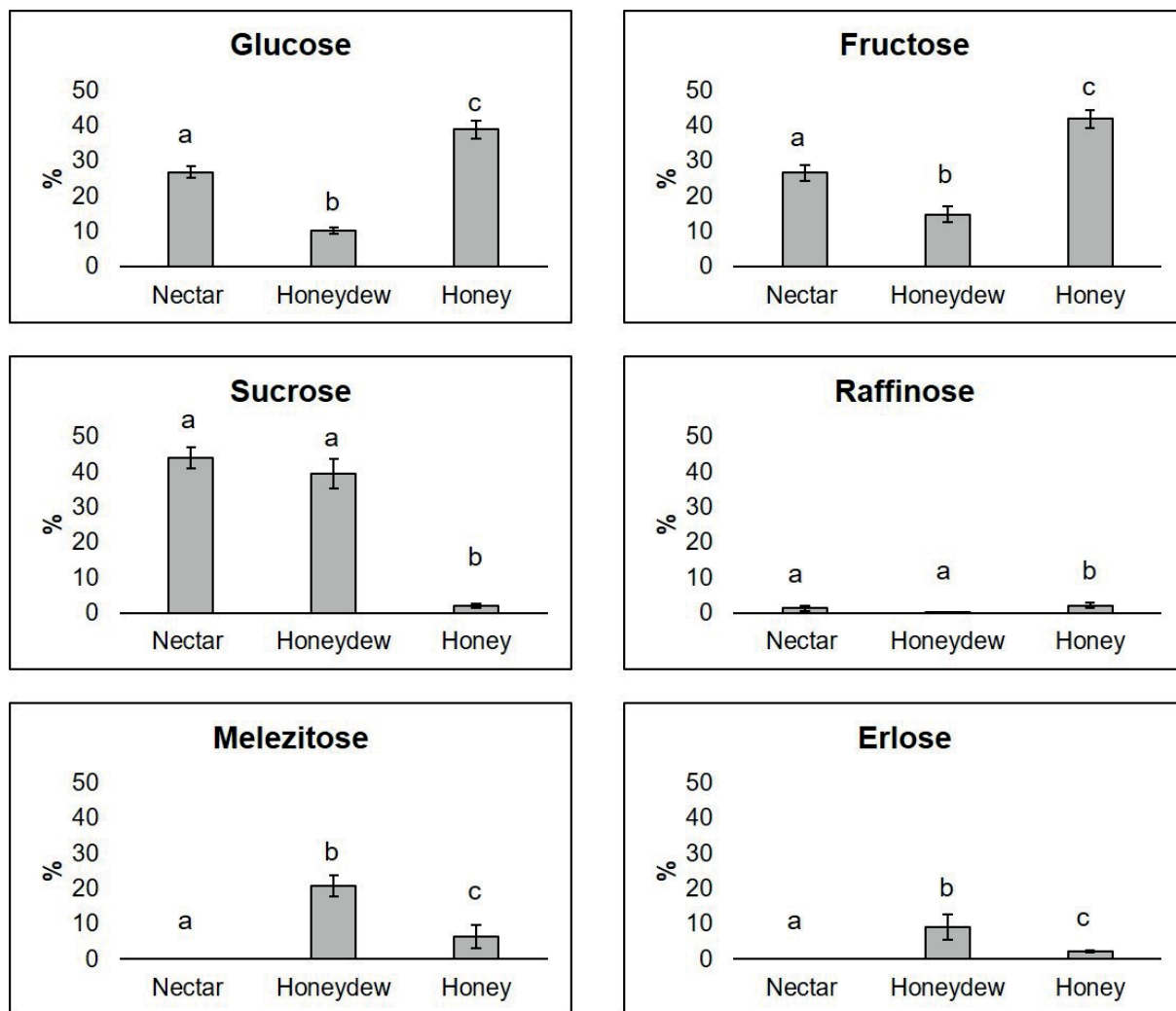


Fig 3. 7. Sugar proportion in nectar, honeydew and honey of *Tilia sp.* All values are mean proportions (%) independent measurements \pm SD. Data were taken from Table 3.10. Different letters represent significant differences in sugars proportion between nectar, honeydew and honey (Tukey's HSD; $p < 0.05$).

Potassium was the main cation and chloride was the main anion in all samples of phloem exudate, nectar, honeydew and honey (Table 3.12). Additionally, phloem exudate has a high proportion of phosphate, but low sulfate and nitrate proportions. Further, nectar showed significantly higher proportions of ammonium and sodium than honeydew or honey. Moreover, magnesium was significantly higher in honeydew than nectar or honey. Although all inorganic ions were found in honey, Potassium was clearly dominant with approximately 62 %.

Table 3. 11. Amino acids proportions in nectar, honeydew and honey of *Tilia sp.*

Amino acids [%]	Nectar <i>n</i> = 8	Honeydew <i>n</i> = 9	Honey <i>n</i> =12
Glutamate (GLU)	8 ± 2 ^a	26 ± 8 ^b	7 ± 3 ^a
Glutamine (GLN)	15 ± 4 ^a	8 ± 3 ^{a,b}	4 ± 2 ^b
Aspartate (ASP)	6 ± 2 ^a	17 ± 2 ^b	6 ± 3 ^a
Asparagine(ASN)	12 ± 7 ^a	18 ± 17 ^b	4 ± 3 ^a
Proline (PRO)	1 ± 0 ^a	1 ± 0 ^a	55 ± 12 ^b
Glycine (GLY)	2 ± 1 ^a	3 ± 1 ^a	1 ± 0 ^a
Serine (SER)	7 ± 1 ^a	11 ± 3 ^b	1 ± 0 ^c
Alanine (ALA)	17 ± 4 ^a	3 ± 2 ^b	2 ± 1 ^b
Tyrosine (TYR)	2 ± 0 ^a	0 ± 0 ^a	2 ± 0 ^a
Arginine (ARG)	2 ± 1 ^a	2 ± 1 ^a	2 ± 0 ^a
Histidine (HIS)	1 ± 1 ^a	1 ± 0 ^a	2 ± 0 ^a
Lysine (LYS)	1 ± 0 ^a	1 ± 0 ^a	3 ± 1 ^a
Threonine (THR)	3 ± 1 ^a	2 ± 1 ^a	1 ± 0 ^a
Valine (VAL)	5 ± 1 ^a	2 ± 0 ^b	2 ± 0 ^b
Isoleucine (ILE)	7 ± 1 ^a	1 ± 0 ^a	1 ± 0 ^a
Leucine (LEU)	3 ± 0 ^a	0 ± 0 ^a	1 ± 0 ^a
Phenylalanine (PHE)	0 ± 0 ^a	0 ± 0 ^a	4 ± 3 ^b
Tryptophan (TRP)	2 ± 1 ^a	0 ± 0 ^a	0 ± 0 ^a
Methionine (MET)	1 ± 1 ^a	0 ± 0 ^a	0 ± 0 ^a
β-Alanine (β-ALA)	0 ± 0 ^a	0 ± 0 ^a	2 ± 0 ^a
γ-Amino-butyric acid (GABA)	3 ± 0 ^a	0 ± 0 ^a	1 ± 0 ^a
Further non-proteinogenic amino acids (NP)	1 ± 1 ^a	3 ± 1 ^a	0 ± 0 ^a
Total content	7.1 ± 3 mmol/L	143 ± 88 μmol/L	91.90 ± 34.20 mg/100g honey

All values are means of independent measurements ± SD.

Different letters represent significant differences between the sugars in nectar, honeydew and honey.

Further non-proteinogenic amino acids: homoserine, phosphoserine, ornithine, and taurine.

Oligosaccharide formation in Eucallipterus tiliae whole-body homogenates

Sucrose was converted to monosaccharides and oligosaccharides in the whole-body homogenates of *Eucallipterus tiliae* (Fig 3.8). After 120 minutes glucose, fructose, trehalose, melezitose and erlose were formed. Glucose and fructose had the highest concentrations. In addition, melezitose concentration was higher than erlose.

Table 3. 12. Inorganic ions proportions in nectar, honeydew and honey of *Tilia sp.*

Ion [%]	Nectar <i>n</i> = 8	Honeydew <i>n</i> = 9	Honey <i>n</i> =12
Cations			
Potassium (K ⁺)	31 ± 19 ^a	60 ± 8 ^b	62 ± 10 ^b
Sodium (Na ⁺)	18 ± 8 ^a	2 ± 1 ^b	5 ± 3 ^b
Ammonium (NH ₄ ⁺)	12 ± 6 ^a	1 ± 1 ^b	2 ± 2 ^b
Magnesium (Mg ²⁺)	5 ± 3 ^a	10 ± 3 ^b	5 ± 4 ^a
Calcium (Ca ²⁺)	3 ± 1 ^a	3 ± 2 ^a	2 ± 1 ^a
Anions			
Chloride (Cl ⁻)	18 ± 8 ^a	19 ± 5 ^a	12 ± 7 ^a
Phosphate (PO ₄ ³⁻)	5 ± 2 ^a	3 ± 1 ^a	8 ± 4 ^b
Sulfate (SO ₄ ²⁻)	3 ± 1 ^a	2 ± 1 ^a	2 ± 1 ^a
Nitrate (NO ₃ ⁻)	4 ± 2 ^a	0 ± 0 ^a	1 ± 1 ^a
Total content	71.69 ± 42.44 mmol/L	585 ± 487 mmol/L	268 ± 199 mg/100g honey

All values are means of independent measurements ± SD.

Different letters represent significant differences between the sugars in nectar, honeydew and honey.

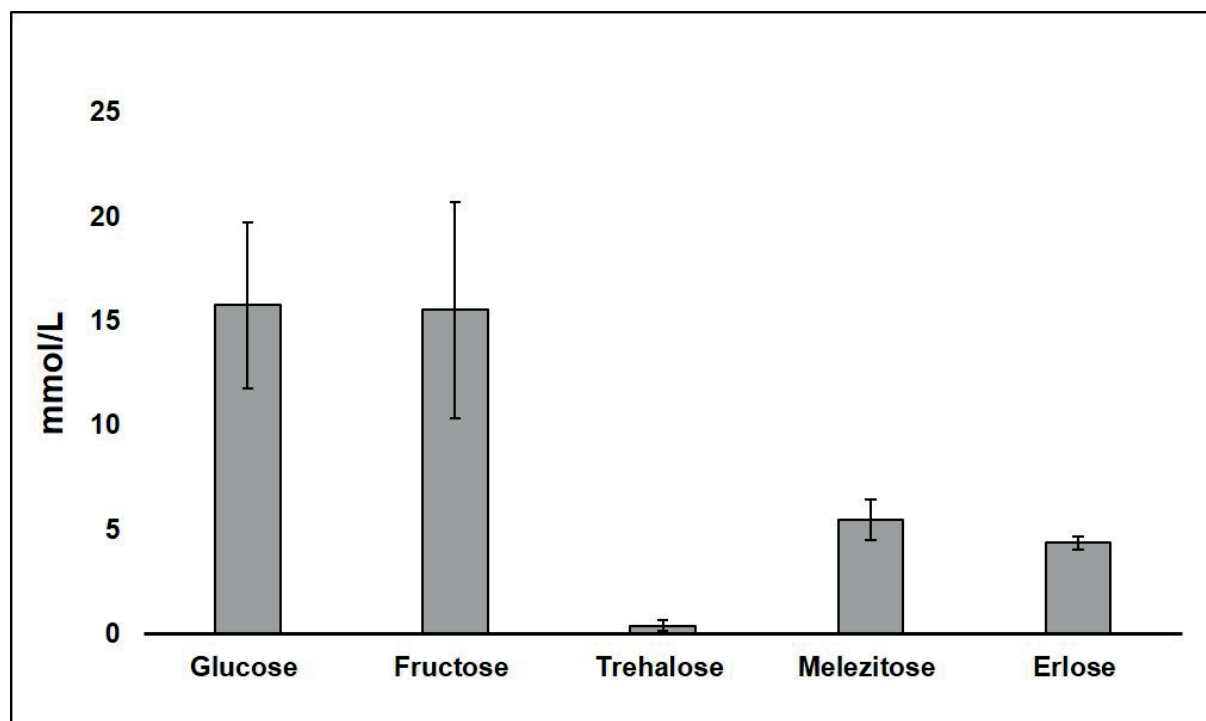


Fig 3. 8. Sugar concentration in whole-body homogenates of *Eucallipterus tiliae* after 120 minutes of incubation. The aphid homogenates were incubated with 10% sucrose solution. All values are means of *n*=3 independent measurements ± SD.

4. Discussion

Honeydew producers feeding on phloem sap of plants produce honeydew. In addition, honeybees collect the honeydew, and produce honeydew honey. This work aims to clarify the processing of phloem sap to honeydew by aphid species and of honeydew to honey by the honeybees. The second part of the thesis deals with the identification of distinguishing features of honeydew honeys according to their botanical and zoological origins. The results of this work are summarized in four main sections and discussed with regard to the research questions.

4.1. From phloem sap to honeydew by conifers

Sugars

Various hemipteran species feed on phloem sap and produce honeydew. Phloem exudates of bark tissues from *A. alba* and *P. abies* contained sucrose, glucose and fructose (see 3. Results: Section I: S1). However, glucose and fructose cannot be found normally in pure phloem sap (Ziegler and Mittler 1959; Fink *et al.* 2018). They probably stem from the wounded surface of the bark because of the activity of sucrose cleavage enzymes. In addition, no further oligosaccharides were found in phloem exudates of *A. alba* and *P. abies*.

Honeydew of different honeydew producers feeding on fir and spruce contains, comparable to the phloem exudates, glucose, fructose and sucrose. However, other oligosaccharides were also found in honeydew, especially melezitose and erlose (see 3. Results: Section I: table1). Oligosaccharides were also determined in honeydew of various other aphid species (Mittler 1958; Bacon and Dickinson 1957; Walters and Smith 1988; Braendle *et al.* 2003). It is likely that the presence of oligosaccharides in honeydew is due to the transformation of glucose into glucose-containing oligomers in order to reduce the osmotic stress caused by ingested phloem sap (Fisher *et al.* 1984).

The proportions of melezitose and erlose differ significantly between different aphid species. In addition, the classification of honeydew according to their zoological origins were visually and statistically verified (see 3. Results: Section I: Fig 3 and Table 4). Moreover, honeydew of different aphids feeding on the same host plants have different sugar profiles; thus, the zoological origins of honeydew influence honeydew sugar profile more than the botanical origin (Bacon and Dickinson 1957; Walters and Smith 1988).

Amino acids

Aphids rely on the free amino acids of the phloem sap as their main nitrogen source. However, they can synthesize some amino acids (Febvay *et al.* 1999) and some microorganisms, such as several species of Buchnera, can also be involved in the synthesis of some essential amino acids (Downing 1980). In addition, all essential and non-essential amino acids were found in the phloem exudates of the tree species (see 3. Results: Section I: Table 2). Further, all essential amino acids were also detected in honeydew, but with low proportions compared to the phloem exudates. Moreover, the ratios sugar-to-amino-acids were much lower in the phloem exudates of fir and spruce than in the honeydew of different aphid species feeding on them (see 3. Results: Section I: Table 4). Sandström and Moran (2001) showed that only 1-3% of absorbed amino acids from the phloem sap were found in honeydew. No visual separation and no significant differences between amino acid profiles of different honeydews could be obtained (see 3. Results: Section I: Fig 3 and Table 4). As a result, amino acids occur infrequently in honeydew, because of their highly important roles in the aphids' life circles. Therefore, they are not useful to distinguish between different honeydews. In other words, the variance of the amino acids data cannot be elucidated by either the hemipteran species or the host plant species.

Inorganic ions

The composition of cations in honeydew of the different hemipteran species roughly reflect that composition in the phloem exudate of *A. alba* and *P. abies*. Potassium was the main cation in the honeydew of all analyzed hemipteran species and the sodium proportion was lower (see 3. Results: Section I: Table 3 and Section VI: Table 17). Additionally, phloem exudates in all host plants also had similar ratios of potassium-to-sodium (see 3. Results: Section I: S3). Wyatt (1961) suggested a correlation between the diet of the insects and the ratio of potassium-to-sodium in haemolymph. That can explain our results; however, it makes the cation profiles a non-useful feature to classify honeydew according to their botanical or zoological origins.

The proportions of anion in honeydew differ between different hemipteran species. Further, while chloride is the dominant anion in the phloem exudate of *A. alba* and *P. abies*, honeydew of *Physokermes sp.* had higher phosphate than chloride proportions (see 3. Results: Section I: Table 3; S3). Moreover, the high phosphate proportion caused a visual separation of some hemipteran species (see 3. Results: Section I: Fig 4). This indicates that some hemiptera species influence the anion content of the honeydew.

In conclusion, the sucking hemipteran and not the host plant is primarily responsible for the diversity of the sugar contents, especially the oligosaccharides in honeydew. In the case of inorganic ions, mainly the proportions of phosphate showed significant differences between the honeydews of different hemipteran species. In contrast, the composition of amino acids in honeydew was rather similar across the hemipteran species.

4.2. The enzymatic formation and cleavage of melezitose

Oligosaccharides formations in whole-body homogenates aphids

Whole-body homogenates of *C. pectinatae* and *C. pilicornis* incubated at 30°C with sucrose solution produced glucose, fructose, melezitose and erlose (see 3. Results: Section I: Fig1). This corresponds to the results of Bacon *et al.* (1956), who showed that melezitose is formed in whole-body homogenates incubated with sucrose solutions. However, the proportions of formed sugars were not the same in all aphid species. More precisely, *C. pilicornis* produced more melezitose than erlose, but *C. pectinatae* produced more erlose than melezitose. In other words, the different aphid species produced different amounts of melezitose and erlose, which is reason to assume that they have different enzymatic activities.

In addition, the total rate of sucrose hydrolyses was similar in all aphid species (about 1.5 mg sucrose mg⁻¹ fresh weight of aphid day⁻¹). This indicates that the enzymatic level of transformation of dietary sucrose in the insect gut depends on the aphid species. As a conclusion, types and quantity of oligosaccharides in honeydew is influenced mainly by aphid species.

Effects of different temperatures on melezitose production in aphid species

The analysis of the data shows that the hemipteran species has a much higher influence on the sugar composition of the honeydew than the tree species (see 3. Results: Section I: Fig 3; Table 5). However, Liebig (1979) found higher proportions of melezitose (up to 20%) in the honeydew of *C. pectinatae* on *A. alba* than was shown in this study (about 2%). Further, the developmental stage of the host plant or the insect can also influence the honeydew composition (Fischer and Shingleton 2001). Moreover, Walters and Mullin (1988) found that enzymatic activities increased with high sucrose concentrations in the aphid diet. Additionally, the honeydew composition of the same honeydew producer may vary by different environmental

conditions during sample collection, like weather conditions (Liebig 1979). Therefore, the effect of different temperatures on the production of melezitose and erlose was tested. Melezitose and erlose were found in all the whole-body homogenates of *C. pectinatae* and *C. pilicornis* incubated with sucrose at three different temperatures (25°C, 30°C and 35°C) (see 3. Results: Section II: Fig 3.2 and Table 3.1). Further, melezitose and erlose formations increased significantly in both aphid species when they were incubated with high temperature. However, all sugar formations increased significantly with high temperature. Further, the ratio of melezitose to erlose did not change in high temperature. This means that the melezitose content in honeydew is mainly influenced by aphid species.

Melezitose cleavage in A. mellifera

Melezitose was detected in various types of honeydew. It was shown that melezitose can have negative effects on overwintering honeybees (Imdorf *et al.* 2002) as well as on the quality of honeydew honey (Schmelz *et al.* 2002). Moreover, bees feeding on melezitose showed elevated mortality and increased disease symptoms than bees feeding on sucrose (Seeburger *et al.* 2020). The ability of honeybee to digest melezitose in newly emerged bees and worker bees was investigated. Newly emerged bees were able to digest melezitose to glucose and fructose (see 3. Results: Section III: Fig 3.3). The melezitose proportion decreased almost 30%. In addition, homogenates worker honeybee's abdomen incubated with melezitose solution were also able to cleavage melezitose (see Section III: Fig 3.4). However, sucrose cleavage was quicker and more efficiently than melezitose. In order to digest oligosaccharides, honeybees organs secrete different types of α -glucosidases, (α -glucosidases I, II and III) (Kubota *et al.* 2004). Generally, they catalyze the splitting of an α -glucosyl residue from the non-reducing terminal side of the oligosaccharides having an α -glucosidic linkage to liberate α -glucose (Kubota *et al.* 2004). Their activities and locations in the honeybees' organs are not equally distributed. They were found in the ventriculus, the haemolymph and the hypopharyngeal gland, from which the enzymes may be secreted into the honeydew. Further, they were able to split maltose, sucrose, and turanose, albeit with different activity levels (Kubota *et al.* 2004). The decrease of melezitose in feed experiment and the homogenates worker honeybee's abdomen experiment allowed us to conclude, that some types of α -glucosidases in the honeybees' abdomen and also in the hypopharyngeal gland can digest melezitose, albeit not as efficiently as sucrose.

4.3. From honeydew to honeydew honey by conifers

Honeybees collect nectar and transform it to blossom honey, or nectar honey. However, at the end of summer when the nectar becomes sparser, honeybees gather honeydew and produce honeydew honey. In Germany, fir honey achieves a higher market value than spruce honey, but until now it is hardly possible to distinguish between the two types, e.g. on the basis of the flavonoid contents (Bertoncelj *et al.* 2011). Furthermore, honeydew honey not only has different botanical origins like blossom honey, but also different zoological origins, namely the honeydew producers. In this section, the results of sugars, amino acids, and inorganic ions of honeydew honey from different botanical origins (fir, spruce and pine) and from different zoological origins (*Cinara*/fir, *Cinara*/spruce and *Physokermes*/spruce) are summarized and discussed in order to examine whether these compounds are useful to distinguish between the different types of honeydew honey.

Sugars

Glucose and fructose make up about 60% (w/w) of fir, spruce, and pine honey and with that they are the dominant compounds (see Section IV: Table 3.2). Glucose and fructose contents were lower than that in blossom honey, but similar to honeydew honey (Ruoff *et al.* 2007, Pita-Calvo and Vázquez 2018). The monosaccharides in honeydew honey can originate either directly from the honeydew (see Section I: Table1) or from the honeybees after the cleavage of di- or oligosaccharides, probably by the activity of α -glucosidases (see Section III: Table 3.1, Kubota *et al.* 2004). The latter possibility is more likely as the proportion of sucrose is higher in honeydew than in honeydew honey of fir and spruce (see Section I: Table1). The trehalose content in the honeydew honey of fir and spruce is about 2% (w/w), which is higher than that of several flower honeys (Pita-Calvo *et al.* 2017). Further, pine honey contain only 0.1% (w/w) of the total sugar proportions. Trehalose was also found in the honeydew of hemipteran species feeding on fir or spruce (see Section I: Table 1). In insects, trehalose serves as a haemolymph-sugar and it is the major carbohydrate energy source used by insects to facilitate flight (Thompson 2003).

Fir, spruce and pine honeys contained up to 10% (w/w) oligosaccharides, mainly melezitose and erlose. In general, honeydew honey contains more di- and oligosaccharides than flower honey (Ruoff *et al.* 2007) and melezitose was reported to be a marker for honeydew honey (Maurizio 1962, Slddiqui and Purgala 1968). So far there is no evidence of melezitose formation in honeybees, but it was found in the honeydew of different hemipteran species (see Section I:

Table 1; Fischer and Shingleton 2001). Like melezitose, erlose was found in the honeydew of different hemipteran species (see Section I: Table 1), but this could also be produced by the action of honeybee invertase on sucrose (Doner 1977).

Two unknown oligosaccharides (*undef 3* and *undef 6*) were found and there was a significant difference in their contents between the groups of honeydew honey (see Section IV: Table 3.2). The content of *undef 6* was about 2 - 4-fold higher in spruce/*Physokermes* honey than other honeys. Von der Ohe and von der Ohe (1996) also reported the detection of a particular oligosaccharide (named L1) in *Physokermes* honey; the structure of this sugar, however, has not yet been identified. It can be assumed that *undef 6* and L1 are the same oligosaccharides, further analyses are necessary for a final clarification. The content of *undef 3* was about 10-fold higher in pine honey than other groups. Unfortunately, the zoological origin of the used pine honey samples is unknown. Blackman & Eastop (2000) listed about 170 species of aphids as feeding on pines. Therefore, further analyses are necessary to clarify the origin of this sugar. It was possible, however, to visually separate pine honey samples from fir and spruce honeys according to the sugar compositions (see Section IV: Fig 3.5 and Table 3.2). It was also possible to identify *Physokermes* honeys according to the sugar composition, which means that the sugar composition is useful to distinguish between honeys of different botanical as well as different zoological origins.

Amino acids

Although numerous amino acids were found in fir, spruce, and pine honeys, the total amino acid content was less than 0.1% (w/w). The amino acids in honeydew honeys can have different origins as well. Honeydew contains small amounts of amino acids (see Section I: Table 2; Douglas 2006). However, the sugar-to-amino acid ratio is higher in honeydew than in nectar, which means that for honeybees a honeydew diet is less rich in nitrogen than a nectar diet (Lohaus and Schwerdtfeger 2008, Tiedge and Lohaus 2017). The bees themselves can also contribute to the amino acids in honey, particularly to the amount of proline, and ripe honey should contain at least 180 mg proline kg⁻¹ honey (von der Ohe *et al.* 1991).

The content of some amino acids (proline, tyrosine, arginine, lysine, isoleucine and phenylalanine) differed significantly between the groups of honeydew honey (see Section IV: Table 3.3). Proline was the dominant amino acid in all groups. In addition, the content of proline was significantly higher, and the contents of the other five amino acids were significantly lower in fir/*Cinara* honey than in spruce/*Cinara* or in spruce/*Physokermes* honey. In former studies,

the proline and phenylalanine contents in honey were already used to characterize the botanical origin of the honey (Cotte *et al.* 2004). It was possible to identify samples of lavender honey on the basis of high phenylalanine contents, (Cotte *et al.* 2004). However, similarly high contents of phenylalanine were also detected in honeys of other Lamiaceae, like *Rosmarinus* or *Thymus* (Conte *et al.* 1998). One reason for this could be the high content of phenylalanine in the nectar of some Lamiaceae species (Petandiou *et al.* 2006), probably because phenylalanine has a phagostimulatory effect on potential pollinators, like honeybees (Inouye and Waller 1984). Moreover, the amino acid content in honey decreases with storage time and the decrease-rate is not identical among the individual amino acids (Iglesias *et al.* 2006). Therefore, the differentiation between different honeydew honeys solely on the basis of the amino acid composition appears to be insufficient (Cotte *et al.* 2004). Finally, a visual separation between pine honey and fir and spruce honey based on their amino acid profiles were obtained, but not between fir and spruce honey (see Section IV: Table 3.6; Table 3.7 and Fig 3.5).

Inorganic ions

There are more inorganic ions in honeydew honey than in flower honey (Fermo *et al.* 2013) and this can be a reason for the higher electrical conductivity of honeydew honey (Bogdanov *et al.* 2007; Silva *et al.* 2009). Potassium is the main inorganic ion in all honey samples (see Section IV: Table 3.4) and it is also the dominant ion in the honeydew of hemipteran species feeding on fir or spruce as well as in the phloem exudates of these tree species (see Section I: Table 3). Yet, not only potassium, but also the complete inorganic ion composition (with the exception of phosphate) in honey roughly reflects the composition in honeydew or phloem exudates. As inorganic ions are taken up from the soil by the plant's roots, their contents and compositions in honeys indirectly depend on the soil composition and the geographical area (Anklam 1998, Fermo *et al.* 2013). That can explain the high amount of potassium and chloride in the pine honeys, because they have a different geographical origin. The fir and spruce honeys used in this study, though, were all from the same region (Schwarzwald, Baden-Württemberg, Germany). Differences in the inorganic ion contents in honeys are also used to identify their botanical origin, but the major differences were found between flower and honeydew honey (Fernández-Torres *et al.* 2005, Fermo *et al.* 2013).

As for the inorganic ions of fir and spruce samples, only magnesium and phosphate differed significantly between the three groups of honeydew honeys (see Section IV: Table 3.4). The content of both ions is about 1.5 - 2-fold higher in spruce/*Physokermes* honey than in

spruce/*Cinara* or fir/*Cinara* honey. This corresponds to the results on the ion composition of honeydew, as the proportion of phosphate was particularly high in the honeydew of *Physokermes* species located on spruce compared to *Cinara* species located on spruce or fir (see Section I: Table 3). In addition, a visual separation between pine honey and fir and spruce honey on the basis of the inorganic ion contents were obtained, but not between fir and spruce honeys (see Section IV: Table 3.6; Table 3.7 and Fig 3.5). Therefore, the inorganic ion composition seems to be more useful for the determination of the honeys' zoological origin than its botanical origin.

Suitability of the sugar, amino acid, and inorganic ion composition to identify the zoological origin of fir and spruce honey

The contents of erlose, an undefined oligosaccharide, proline, tyrosine, arginine, lysine, isoleucine, phenylalanine, magnesium, and phosphate are significantly different for the three groups of honey. Therefore, they probably play a more important role for the identification of the honeys' origins than the other metabolites or ions. Based on these compounds, a partial separation of the three groups of honey (fir/*Cinara*, spruce/*Cinara*, spruce/*Physokermes*) was visible in the RDA (see Section IV: Fig 3.6). The analysis of the data shows that the honeydew producing species (zoological origin) has a higher influence on the honey composition than the tree species (botanical origins) (see Section IV: Table 3.8; Table 3.9). However, the largest portion of the variance of the data cannot be elucidated by either of the grouping options. Further factors may influence the honey's chemical composition, such as collection season, weather conditions, conditions of honey harvest and storage. This makes it difficult to find reliable chemical markers to identify botanical or zoological origins (Kaškonienė and Venskutonis 2010, Pita-Calvo and Vázquez 2018, Soares *et al.* 2017).

In summary, spruce/*Physokermes* honey can be separated from spruce/*Cinara* or fir/*Cinara* honey by its higher contents of phosphate and an undefined oligosaccharide (*undef 6*). However, no chemical marker has been found within the categories of the analyzed compounds to reliably distinguish between fir/*Cinara* and spruce/*Cinara* honey.

4.4. Nectar, honeydew and honey profiling of lime tree *Tilia sp.*

Honeydew of *E. tiliae* feeding on *Tilia sp.* contains glucose, fructose and sucrose. However, other oligosaccharides were also found in honeydew, especially melezitose and erlose. In addition, melezitose proportion was higher than erlose (see 3. Results: Section V: Table 3.10). Whole-body homogenates of *E. tilia* incubated at 30°C with sucrose solution produced glucose, fructose, melezitose and erlose (see 3. Results: Section V: Fig 3.8). No melezitose or erlose were found, neither in the nectar nor in the phloem exudates of *Tilia sp.* Glucose, fructose and sucrose were the main sugars (see 3. Results: Section V: Table 3.10 and Fig 3.7). Further, glucose and fructose had the same proportion and sucrose had the highest proportion. Additionally, a small amount of raffinose was also determined. Jacquemart *et al.* (2018) also determined raffinose in nectar of *Tilia sp.* In addition, some plant species transport raffinose in their phloem sap (Nadwodnik and Lohaus 2008; Öner-Sieben and Lohaus 2014). Raffinose synthesis in plants occur by transferring a galactosyl from galactinol to sucrose, thereby releasing myo-inositol. Lime honey samples contain melezitose and erlose (see 3. Results: Section V: Table 3.10). Therefore, we can assume that they have zoological origin.

All essential and non-essential amino acids were also found in honeydew and nectar of *Tilia sp.* (see Section V: Table 3.11). Furthermore, the amino acid concentrations in nectar differ significantly between different plant species. In addition, environmental factors and pollinators can also influence the amino acids composition in nectar (Göttlinger *et al.* 2019; Tiedge and Lohaus 2017).

All inorganic ions were determined in nectar, honeydew, and honey of *Tilia sp.* Potassium was the dominant ion and low amount of sodium was found in honeydew. In contrast to the honeydew, nectar of *Tilia sp.* had a significantly higher proportion of sodium; thus, there is a lower potassium-to-sodium ratio in nectar compared to honeydews (see 3. Results: Section V: Table 3.12).

5. References

1. Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecology*. 2001; 26: 32–46.
2. Anderson MJ. Distance-based tests for homogeneity of multivariate dispersions. *Biometrics*. 2006; 62: 245–253. <https://doi.org/10.1111/j.1541-0420.2005.00440.x> PMID: 16542252
3. Anderson MJ, Walsh DCI. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: what null hypothesis are you testing? *Ecol Monogr*. 2013; 83: 557–574.
4. Anderson MJ, Gorley RN, Clarke KR. PERMANOVA? For PRIMER: guide to software and statistical methods. PRIMER-E, Plymouth 2008.
5. Anklam, E. A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chem*. 1998, 63, 549–562.
6. Auclair J. Aphid feeding and nutrition. *Ann Rev Entomol*. 1963; 8: 439–490.
7. Bacon JSD, Dickinson B. The origin of melezitose: a biochemical relationship between the lime tree (*Tilia* spp.) and an aphid (*Eucallipterus tiliae* L.). *Biochem*. 1957; 66: 289–297.
8. Bailey L. The occurrence of chronic and acute bee paralysis viruses in bees outside Britain. *J Invertebr Pathol*. 1965; 7: 167–169.
9. Ball DW. The chemical composition of honey. *J Chem Educ*. 2007, 84:1643–1646.
10. Beekman, M.; Sumpter, D.J.T.; Seraphides, N.; Ratnieks, F.L.W. 2004: Comparing foraging behaviour of small and large honeybee colonies by decoding waggle dances made by foragers. In: *Functional Ecology* 18 (6), S. 829–835.
11. Beckmann. Entwicklung neuer analytischer Methoden zur Erweiterung der Qualitätssicherung von Honig [Dissertation]. Dresden: Technische Universität Dresden, 2008.
12. Belitz, H.-D.; Grosch, W.; Schieberle, P. (Hg.). *Lehrbuch der Lebensmittelchemie*. 6. Aufl. Berlin-Heidelberg: Springer-Verlag 2008.
13. Bertonec, J.; Polak, T.; Kropf, U.; Korošec, M.; Golob, T. LC-DAD-ESI/MS analysis of flavonoids and abscisic acid with chemometric approach for the classification of Slovenian honey. *Food Chem*. 2011, 127(1): 296–302.
14. Blackman R. L., Eastop V. F. *Aphids on the World's Crops: An Identification and Information Guide*, 2nd Edition 2000.

15. Bogdanov, S.; Jurendic, T.; Sieber, R.; Gallmann, P. Honey for nutrition and health: A review. In: *Journal of the American College of Nutrition*, 2008, 27 (6), S. 677–689.
16. Bogdanov, S.; Haldimann, M.; Luginbuhl, W.; Gallmann, P. Minerals in honey: environmental, geographical and botanical aspects. *J Apic Res* 2007, 46, 269–275.
17. Bundesministerium für Verbraucherschutz, Ernährung und Landwirtschaft. Honigverordnung: HonigV; 2004.
18. Bundesministerium für Verbraucherschutz, Ernährung und Landwirtschaft. Der Wald in Deutschland - ausgewählte Ergebnisse der dritten Bundeswaldinventur, 2016.
19. Castro-Vázquez, L.; Diaz-Maroto, M.C.; Pérez-Coello, MS. Volatile composition and contribution to the aroma of Spanish honeydew honeys. Identification of a new chemical marker. *J. Agric. Food Chem.* 2006, 54, 4809-4813.
20. Cotte, J.F.; Casabianca, H.; Giroud, B.; Albert, M.; Lheritier, J.; Grenier-Loustalot, M. Characterization of honey amino acid profiles using high-pressure liquid chromatography to control authenticity. *Anal Bioanal Chem.* 2004, 378, 1342-1350.
21. Conte, L.S.; Miorini, M.; Giomo, A.; Bertacco, G.; Zironi, R. Evaluation of some fixed components for unifloral honey characterization. *J. Agric. Food Chem.* 1998, 46, 1844-1849.
22. Da Silva, P. M.; Gauche, C.; Gonzaga, L. V.; Costa, A. C. O.; Fett, R. Honey: chemical composition, stability and authenticity. In: *Food Chemistry*, 2016, 196, S. 309–323.
23. Directive 2014/63/EU of the European Parliament and of the Council amending Council Directive 2001/110/EC relating to honey. *Official Journal of the European Communities*, 2014, L164: 1-5.
24. Doner, L. W. The sugars of honey - a review. *J. Sci. Food Agric.* 1977, 28 (5), 443–456.
25. Douglas, A. E. Phloem-sap feeding by animals: problems and solutions. *J. Exp. Bot.* 2006, 57, 747–754.
26. Dullely JR, Grieve PA. A simple technique for eliminating interference by detergents in the Lowry method of protein determination. *Analytical Biochemistry*; DOI: 10.1016/0003-2697(75)90415-7
27. Escuredo O, Míguez M, Fernández-González M *et al.* Nutritional value and antioxidant activity of honeys produced in a European Atlantic area. *Food Chemistry* 2013; (138): 851–856
28. Escuredo, O., Silva, L. R., Valentão, P., Seijo, M. C., & Andrade, P. B. Assessing *Rubus* honey value: Pollen and phenolic compounds content and antibacterial capacity. *Food Chemistry*, 2012, 130, 671–678.

29. Fermo, P.; Beretta, G.; Facino, R.M.; Gelmini, F.; Piazzalunga, A. Ionic profile of honey as a potential indicator of botanical origin and global environmental pollution. *Environ Pollution*. 2013, 178: 173–181.
30. Fernández-Torres, R.; Pérez-Bernal, J.L.; Bello-López, M.A.; Callejón-Mochón, M.; Jiménez-Sánchez, J.C.; Guiraúm-Pérez, A. Mineral content and botanical origin of Spanish honeys. *Talanta*. 2005, 65, 686-691.
31. Fink D, Dobbelstein E, Barbian A, Lohaus G. 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*. 2018; 248: 661–673. <https://doi.org/10.1007/s00425-018-2933-7> PMID: 29882156
32. Fischer MK, Völkl W, Schopf R, Hoffmann KH. Age-specific pattern in honeydew production and honeydew composition in the aphid *Metopeurum fuscoviride*: implications for ant-attendance. *J Insect Physiol*. 2002; 48: 319–326.
33. Fischer, M. K.; Shingleton, A.W. Host plant and ants influence the honeydew sugar composition of aphids. *Funct Ecol*. 2001, 15, 544-550.
34. Flanjak I, Strelec I, Kenjerić D *et al.* Croatian produced unifloral honey characterized according to the protein and proline content and enzyme activities. *Journal of Apicultural Science*; 2016.
35. Grant, W. D.; Beggs, J. R. Carbohydrate analysis of beech honeydew. In: *New Zealand Journal of Zoology*, 1989, 16 (3), S. 283–288.
36. Göttlinger, T.; Schwerdtfeger, M.; Tiedge, K.; Lohaus, G. What do nectarivorous bats like? Nectar composition in Bromeliaceae with special emphasis on bat-pollinated species. *Front Plant Sci*. 2019, 10, 205.
37. Hagler, J. R.; Mueller, S.; Teuber, L. R.; Machtley, S. A.; van Deynze, A. Foraging range of honey bees, *Apis mellifera*, in alfalfa seed production fields. In: *Journal of Insect Science* 11, 2011, (144), S. 1–12.
38. Herve´ MR, Nicolè F, Lê Cao KA. Multivariate analysis of multiple datasets: a practical guide for chemical ecology. *J Chem Ecol*. 2018; 44: 215–234.
39. Hendrix, D.L.; Wie, Y.; Legget, J.E. Homopteran honeydew is determined by both the insect and the plant species. *Comp Biochem Physiol*. 1992, 101, 23-27.
40. Hijaz F, Killiny N. Collection and chemical composition of phloem sap from *Citrus sinensis* L. Osbeck (Sweet Orange). *PLoS ONE*. 2014 9(7): e101830.
41. Horn H. Die Waldtrachtkrankheit der Honigbiene I. Der Einfluß des Mineralstoffgehaltes in Honigtauhonigen. *Apidologie*. 1985; 16: 139–156.

42. Hudson CS, Sherwood SF. The occurrence of melezitose in honey. *J Am Chem Soc.* 1919; 42: 116– 125.
43. Iglesias, M.T.; de Lorenzo, C.; Polo, M.C.; Martín-Álvarez, P.J.; Pueyo, E. Usefulness of amino acid composition to discriminate between honeydew and floral honeys. Application to honeys from a small geographic area. *J Agric Food Chem.* 2004, 52, 84-89.
44. Iglesias, M.T.; Martín-Álvarez, P.J.; Polo, M.C.; de Lorenzo, C.; González, M.; Pueyo, E. Changes in the free amino acid contents of honeys during storage at ambient temperature. *J Agric Food Chem.* 2006, 54, 9099–9104.
45. Inouye, D.W.; Waller, G.D. Responses of honeybees (*Apis mellifera*) to amino acid solutions mimicking floral nectars. *Ecology.* 1984, 65, 618-625.
46. Jovetić, M.; Trifković, J.; Stanković, D.; Manojlović, D.; Milojković-Opsenica, D. Mineral content as a tool for the assessment of honey authenticity. *Journal of AOAC International*, 2017, 100, 862–870.
47. Jacquemart, A., Moquet, L., Ouvrard, P. *et al.* *Tilia* trees: toxic or valuable resources for pollinators? *Apidologie*, 2018, 49, 538–550.
48. Kloft, W. J.; Kunkel, H. *Waldtracht und Waldhonig*. Unter Mitarbeit von A. Fossel, A. Maurizio und H. Pechhacker. 2. Aufl. München: Franz Ehrenwirth Verlag, 1985.
49. Kubota, M.; Tsuji, M.; Nishimoto, M.; Wongchawalit, J.; Okuyama, M.; Mori, H. Localization of α glucosidases I, II, and III in organs of European honeybees, *Apis mellifera* L., and the origin of α -glucosidase in honey. *Biosci Biotechnol Biochem.* 2004, 68: 2346–2352.
50. Kunkel, H; Kloft, W. J. Die Honigtau-Erzeuger des Waldes. In *Waldtracht und Waldhonig in der Imkerei*, Kloft, W.J. and Kunkel, H., Eds.; Ehrenwirth, Munich. 1985, pp. 48-265.
51. Kuś, P. M.; Jerković, I.; Marijanović, Z.; Tuberoso, C. I. G. Screening of Polish Fir Honeydew Honey Using GC/MS, HPLCDAD, and Physical-Chemical Parameters: Benzene Derivatives and Terpenes as Chemical Markers. *Chem. Biodiversity.* 2017, 14 (9), e1700179.
52. Kutschera, L.; Lichtenegger, E. *Wurzelatlas mitteleuropäischer Waldbäume und Sträucher*. Stocker, Graz 2002, ISBN 3-7020-0928-0.
53. Legendre, P; Legendre, L. *Numerical Ecology*. Elsevier, Amsterdam, 2012; 24.

54. Leroy P, Wathelet B, Sabri A, Francis F, Verheggen F, Capella Q, *et al.* Aphid-host plant interactions: does aphid honeydew exactly reflect the host plant amino acid composition? *Arthropod-Plant Interact.* 2011; 5: 193–199.
55. Liebig G. Gaschromatographische und enzymatische Untersuchungen des Zuckerspektrums des Honigtaus von *Buchneria pectinatae* (Nördl.) *Apidologie.* 1979; 10: 213–225.
56. Lohaus G, Moellers C. Phloem transport of amino acids in two *Brassica napus* L. genotypes and one *B. carinata* genotype in relation to their seed protein content. *Planta.* 2000; 211: 833–840.
57. 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): e87689.
58. Lowry OH. Protein measurement with the Folin Phenol Reagent. *The Journal of Biological Chemistry.* 1951, (193): 265–275.
59. Mato I, Huidobro JF, Simal-Lozano J *et al.* Analytical methods for the determination of organic acids in honey. *Crit. Rev Anal Chem* 2007; (36): 3–11.
60. Maurizio, A. From the raw material to the finished product: honey. *Bee World,* 1962, 43(3) 66-81.
61. Mead, A. Review of the Development of Multidimensional Scaling Methods. *Journal of the Royal Statistical Society. Series D (The Statistician).* 1992, 41 (1): 27–39
62. Miller, J.; Farr, S. Bimultivariate redundancy: a comprehensive measure of interbattery relationship. *Multivar Behav Res.* 1971, 6, 313–324.
63. Mohammed SEA, Azim MK. Characterisation of natural honey proteins: implications for the floral and geographical origin of honey. *International Journal of Food Science & Technology,* 2012.
64. Nadwodnik J, Lohaus G. Subcellular concentrations of sugar alcohols and sugars in relation to phloem translocation in *Plantago major*, *Plantago maritima*, *Prunus persica*, and *Apium graveolens*. *Planta.* 2008; 227: 1079–1089. <https://doi.org/10.1007/s00425-007-0682-0> PMID: 18188589
65. Öner-Sieben S, Lohaus G. Apoplastic and symplastic phloem loading in *Quercus robur* and *Fraxinus excelsior*. *J Exp Bot.* 2014; 65: 1905–1916. <https://doi.org/10.1093/jxb/eru066> PMID: 24591056
66. Oksanen, J.; Blanchet, G.; Friendly, M.; Kindt, R.; Legendre, P.; McGlenn, D.; Minchin, P. R.; O'Hara, R. B.; Simpson, G. L.; Solymos, P.; Stevens, M. H. H.; Szoecs, E.;

- Wagner, H. *vegan: Community Ecology Package*. 2019, R package version 2.5-6. <https://CRAN.R-project.org/package=vegan>.
67. Page, R. E.; Peng, C. Y.-S. (2001): Aging and development in social insects with emphasis on the honeybee, *Apis mellifera* L. In: *Experimental Gerontology* 36 (4-6), S. 695–711.
 68. Peres-Neto, P.R.; Legendre, P.; Dray, S.; Borcard, D. Variation partitioning of species data matrices: estimation and comparison of fractions. *Ecology*. 2006, 87, 2614–2625.
 69. Persano Oddo, L.; Piro, R. Main European unifloral honeys: descriptive sheets. *Apidologie*. 2004, 35 (Suppl. 1) S38-S81.
 70. Petanidou, T.; Van Laere, A.; Ellis, W. N.; Smets, E. What shapes amino acid and sugar composition in Mediterranean floral nectars? *Oikos*. 2006, 115, 155–169.
 71. Pita-Calvo, C.; Vázquez, M. Differences between honeydew and blossom honeys: A review. *Trends in Food Science & Technology*, 2017, 59, 79–87.
 72. Pita-Calvo, C.; Vázquez, M. Honeydew honeys: A review on the characterization and authentication of floral and geographical origins. *J Agric Food Chem*. 2018, 66, 2523–2537.
 73. Ruoff K, Luginbühl W, Kilchenmann V, Bosset JO., von der Ohe K, von der Ohe W, Amadò R. Authentication of the botanical origin of honey using profiles of classical measurands and discriminant analysis. *Apidologie*. 2007; 38: 438-452.
 74. Sabri A, Vandermoten S, Leroy PD, Haubruge E, Hance T, Thonart P, *et al*. Proteomic investigation of aphid honeydew reveals an unexpected diversity of proteins. *PloS ONE*. 2013; 8: e74656. <https://doi.org/10.1371/journal.pone.0074656> PMID: 24086359
 75. Sandström JP, Moran NA. Amino acid budgets in three aphid species using the same host plant. *Physiol Entomol*. 2001; 26: 202–211.
 76. Sanchez, M.D.; Huidobro, J.F.; Mato, I.; Muniategui, S.; Sancho, M.T. Correlation between proline content of honeys and botanical origin, *Dtsch. Lebensm. Rundsch*. 2001, 97, 171–175.
 77. Sanz, M. L.; Gonzalez, M.; De Lorenzo, C.; Sanz, J.; Martínez- Castro, I. A contribution to the differentiation between nectar honey and honeydew honey. *Food Chem*. 2005, 91 (2), 313–317.
 78. Seraglio SKT, Valse AC, Daguer H *et al*. Development and validation of a LC-ESI-MS/MS method for the determination of phenolic compounds in honeydew honeys with the diluted-and-shoot approach. *Food Res Int*; DOI: 10.1016/j.foodres.2016.06.019.

79. Simova, S.; Atanassov, A.; Shishiniova, M.; Bankova, V. A rapid differentiation between oak honeydew honey and nectar and other honeydew honeys by NMR spectroscopy. *Food Chem.* 2012, 134(3): 1706-1710.
80. Sing RP, Sing PN. Amino acid and lipid spectra of larvae of honeybee (*Apis cerana Fabr*) feeding on mustard pollen. *Apidologie*, (1996) 27:21–28.
81. Shaaban, B.; Seeburger, V.; Schroeder, A.; Lohaus, G. 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): e0228171.
82. Soares, S.; Amaral, J. S.; Oliveira, M. B. P. P.; Mafra, I. A comprehensive review on the main honey authentication issues: Production and origin. *COMPR REV FOOD SCI F.* 2017, 16, 1072–1100.
83. Spilioti, E.; Jaakkola, M.; Tolonen, M.; Lipponen, M.; Virtanen, V.; Chinou, I.; Kassi, E.; Karabournioti, S.; Moutsatsou, P. Phenolic acid composition, antiatherogenic and anticancer potential of honeys derived from various regions in Greece. *PLoS ONE*. 2014, 9(4): e94860.
84. Steffan-Dewenter, I.; Kuhn, A. (2003): Honeybee foraging in differentially structured landscapes. In: *Proceedings. Biological Sciences* 270 (1515), S. 569–575.
85. Tananaki, C.; Thrasyvoulou, A.; Giraudel, J. L.; Montury, M. Determination of volatile characteristics of Greek and Turkish pine honey samples and their classification by using Kohonen self organising maps. *Food Chem.* 2007, 101 (4), 1687–1693.
86. Thermo Fisher Scientific Inc. Modified Lowry Protein Assay Kit, Nr 23240 2011
87. Thompson, S. N. Trehalose – the insect ‘blood’ sugar. *ADV INSECT PHYSIOL.* 2003, 31: 205-285.
88. Tiedge, K; Lohaus, G. Nectar sugars and amino acids in day- and night-flowering *Nicotiana* species are more strongly shaped by pollinators’ preferences than organic acids and inorganic ions. *PLoS ONE*. 2017; 12(5): e0176865.
89. Van Den Wollenberg, A. L. Redundancy analysis an alternative for canonical correlation analysis. *Psychometrika.* 1977, 42, 207–219.
90. Vasić, V.; Gašić, U.; Stanković, D.; Lušić, D.; Lušić, V. D.; Opsenica, M. D.; Trifković, J. Towards better quality criteria of European honeydew honey: Phenolic profile and antioxidant capacity. *Food Chem.* 2019, 247, 629–641.

91. Vasić, V.; Durdića, S.; Tostia, T.; Radoičić, A.; Lušić, D.; Milojković-Opsenica, D.; Tešić, Z.; Trifkovića, J. Two aspects of honeydew honey authenticity: Application of advance analytical methods and chemometrics. *Food Chem.* 2020, 305, 125457.
92. Völkl W, Woodring J, Fischer M, Lorenz MW, Hoffmann KH. Ant-aphid mutualisms: the impact of honeydew production and honeydew sugar composition on ant preferences. *Oecologia.* 1999; 118: 483–491. <https://doi.org/10.1007/s004420050751> PMID: 28307416
93. Von der Ohe, W.; von der Ohe, K. 3. Charakterisierung von Honigtau Honig anhand spezifischer Saccharide. *Apidologie.* 1996, 27 (4), 270-272.
94. Von der Ohe, W.; Dustmann, J. H.; von der Ohe, K. Prolin als Kriterium der Reife des Honigs. *Dtsch. Lebensm. Rundsch.* 1991, 87, 383–386.
95. Waddington, K. D.; Herbert, T. J.; Visscher, P. K.; Richter, M. R. (1994): Comparisons of forager distributions from matched honeybee colonies in suburban environments. In: *Behavioral Ecology and Sociobiology* 35 (6), S. 423–429.
96. Walters FS, Mullin CA. Sucrose-dependent increase in the oligosaccharide production and associated glycosidase activities in the potato aphid *Macrosiphum euphorbiae* (Thomas). *Arch Insect Biochem Physiol.* 1988; 9: 35-46.
97. Woodring J, Wiedemann R, Fischer M K, Hoffmann K H, Völkl W. 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.
98. Wessel D. A Method for the Quantitative Recovery of Protein in Dilute Solution in the Presence of Detergents and Lipids. *Analytical Biochemistry* 1984; (138): 141–143
99. West SG, Finch JF, Curran PJ. Structural equation models with nonnormal variables: problems and remedies. In: Hoyle RH, editor. *Structural equation modeling: Concepts, issues and applications.* Newbery Park, CA: Sage; 1995. p56–75.
100. West, S.G.; Finch, J.F.; Curran, P.J. Structural equation models with nonnormal variables: problems and remedies. In: Hoyle RH, editor. *Structural equation modeling: Concepts, issues and applications.* Newbery Park, CA: Sage; 1995. p 56-75.
101. Ziegler H, Mittler TE. Über den Zuckergehalt der Siebröhren- bzw. Siebzellensäfte von *Heracleum mantegazzianum* und *Picea abies* (L.) Karst *Z Naturforschung.* 1959; 14 B: 278–281.

Appendix

List of used chemicals

Chemical	company
Acetonitrile	Roth
Acetic acid	Roth
Calcium Chloride (CaCl ₂)	Roth
Chloroform	VWR
Copper(II) sulphate (CuSO ₄)	Merck
Di-potassium hydrogen phosphate (K ₂ HPO ₄ · 3 H ₂ O)	Roth
Ethylenediaminetetraacetic acid (EDTA)	Roth
2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid	Sigma
Fluorenylmethyloxycarbonylchlorid (Fmoc-Cl)	Roth
Formaldehyde	Roth
Hydrochloric acid (HCl)	VWR
Isopropanol	Roth
magnesium chloride (MgCl ₂)	Roth
Methanol	Roth
β-Mercaptoethanol	Roth
Nitrogen, liquid	Linde
o-Phthaldialdehyd (OPA)	Roth
Potassium chloride (KCl)	Roth
Potassium hydroxide (KOH)	Roth
Potassium sodium tartrate Tetrahydrate	Roth
Phenol	Roth
Silver nitrate (AgNO ₃)	Chempur
Sodium carbonate (Na ₂ CO ₃)	Roth
Sodium chloride (NaCl)	Roth
Sodium hydroxide (NaOH)	Sigma
Sodium thiosulfate (Na ₂ S ₂ O ₃)	Roth
Tris(hydroxymethyl)-aminomethane (Tris)	Roth
Ultrapure water	Millipore Milli-Q® Anlage

List of used sugars

Common name	Structure	company
Glucose	D(+) Glucose	Merck
Fructose	D(+) Fructose	Roth
Trehalose	1- α -D- glucopyranosyl-1 α -glucopyranosid	Sigma
Nigrose	O- α -D- glucopyranosyl- (1 \rightarrow 3) -D- glucopyranose	Sigma
Maltose	O- α -D- glucopyranosyl- (1 \rightarrow 4) -D- glucopyranose	Roth
Isomaltose	O- α -D- glucopyranosyl- (1 \rightarrow 6) -D- glucopyranose	Sigma
Melibiose	O- α -D- galactopyranosyl- (1 \rightarrow 6) -D- glucopyranose	Sigma
Sucrose	α -D-glucopyranosyl- (1 \rightarrow 2)- β -D-fructofuranoside	Roth
Turanose	O- α -D- glucopyranosyl- (1 \rightarrow 3)-D-fructose	Sigma
Palatinose	O- α -D- glucopyranosyl- (1 \rightarrow 6)-D-fructose	Sigma
Maltulose	O- β -D- glucopyranosyl- (1 \rightarrow 4)-D-fructose	Sigma
Kojibiose	α -D-glucopyranosyl-(1 \rightarrow 2)-D-glucose	Sigma
Gentiobiose	β -D-glucopyranosyl-(1 \rightarrow 6)-D-glucopyranose	Sigma
Maltotriose	O- α -D- glucopyranosyl- (1 \rightarrow 4) - O- α -D- glucopyranosyl- (1 \rightarrow 4) -D- glucopyranose	Sigma
Isomaltotriose	O- α -D- glucopyranosyl- (1 \rightarrow 4) - D- glucopyranosyl- (1 \rightarrow 6) -D- glucopyranose	Sigma
Melezitose	O- α -D- glucopyranosyl- (1 \rightarrow 3)-O- β -D-fructofuranosyl- (2 \rightarrow 1) α -D- glucopyranose	Alfa Aesar
1-Kestose	O- α -D- glucopyranosyl- (1 \rightarrow 2)- β -D-fructofuranosyl- (1 \rightarrow) β -D- fructofuranoside	Sigma
Erlose	O- α -D- glucopyranosyl- (1 \rightarrow 4)- α -D-glucopyranosyl β -D- fructofuranoside	Sigma
Raffinose	α -D- galactopyranosyl- (1 \rightarrow 6) -D- glucopyranose-(1 \rightarrow 2) β -D- fructofuranoside	Roth
Stachyose	α -D- galactopyranosyl- (1 \rightarrow 6) - α -D- galactopyranosyl- (1 \rightarrow 6) -D- glucopyranose-(1 \rightarrow 2) β -D- fructofuranoside	Sigma

List of used laboratory equipment

Device	company
Autoclave	H+P Labortechnik
Binocular	Zeiss
Incubator	Binder
Drying cabinet	Binder, Memmert
Freezer -80°C	FRYKA
Heating block	Biometra
HPLC	Dionex, Thermo Fisher Scientific
Horizontal shaker	VWR
Magnetic stirrers	Phoenix Instrument
Microscope	Zeiss
Mini centrifuge	Roth
Mill ball	Retsch
Piston stroke pipette	Gilson Pipetman, VWR
pH meter	Mettler Toledo
Photometer	Thermo Scientific
Refrigerated centrifuge	Hettich, VWR
Rotary evaporator	VWR
Rough balance	Ohaus Corporation
Thermal shaker	Biometra, Grant-Bio
Table centrifuge	Eppendorf, Heraeus, VWR
Vortex shaker	VWR
Ultra balance	Ohaus Corporation

List of abbreviations

°C	Grad Celsius
EDTA	Ethylenediaminetetraacetate
<i>et al.</i>	and others (" <i>et alii</i> ")
Fig	figure
g	gram
HPLC	High Performance Liquid Chromatography
l	Liter
m	Milli
M	Molar
min	Minute
μ	Micro
n	Number of measurements
PH	negative decadic logarithm of the H ⁺ ion activity
rpm	rounds per minute
RT	Room temperature
w/w	weight per weight

Acknowledgement

The Federal Ministry of Food and Agriculture (BMEL) funded the present work.

First of all I would like to thank Prof'in Dr. Gertrud Lohaus for the opportunity to work on this interesting topic in her research group, the scientific and personal support, the patient and motivating supervision, the freedom to realize own ideas and the freedom to write publications and this work. I will never forget my time in your research group. I would also like to thank Prof'in Dr. Gela Preisfeld for taking over the second supervision.

I would also like to thank the working group of Molecular Plant Research/ Plant Biochemistry. The scientific and personal discussions and the great working atmosphere contributed a lot to the success of this work. You were like a family for me and I will never forget great moments I spend with you. To Thomas for the discussions concerning HPLC and for the many scientific discussions. To Kira for the training in the laboratory. To Elena for the perfect welcome in the work group. To Daniel for the friendship and the great time in Wuppertal. To Antje, Laura, and Wiebke for the nice discussions and great working atmosphere. To Tim for the help with the extractions and for the help in the daily laboratory routine. Also to Ralf for the help with the repair of the HPLC. To Anja for the help in all administrative matters and for the nice discussions. Very special thanks goes to Rosi for proofreading this work and the publications, some of them at short notice. I would like to thank the members of the two working groups Zoology and Molecular Cell Biology and Microbiology for their constant helpfulness.

15 years ago, when I started studying at the University of Damascus, I had a dream to get a PhD degree and to belong to the global scientific community by contributing to the scientific research and by publishing scientific papers. The dream was something intangible and far away, but I get all the support and understanding I needed from my family. Therefore, all my thanks to them; my father Mohamed; you have created trust and ambition in us, my mother Mayada; you made everything beautiful and possible, my sister Maha; for your care and love, my brother Saad; we shared the dream and the moments, and my new family, Rasha, you made everything meaningful and happy. This work wouldn't have been possible without you.

The journey took me to Germany and changed me in many ways I could rarely imagine. I was not alone, but with many friends that I want to thank them all for every minutes we lived together. Alaa, Sabine, Khaled, Mahmoud, Feras, Ahmad, Eyad, Lilas, Max and Kevin; I thank you for all joy and happiness you spread.

Declaration

I hereby declare that I have produced this work independently and that I have not used any aids or sources other than those indicated.

I also declare that this dissertation has not been submitted to any other faculty with the aim of obtaining an academic title.

Basel Shaaban

(Wuppertal, July 2020)

Hiermit erkläre ich, diese Arbeit selbständig angefertigt und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet zu haben.

Ich erkläre ebenso, dass diese Dissertation keiner anderen Fakultät mit dem Ziel vorgelegt worden ist, einen akademischen Titel zu erwerben.

Basel Shaaban

(Wuppertal, Juli 2020)