Nutrition facts of pollen: nutritional quality and how it affects reception and perception in bees

Nährwertinformationen von Pollen: Nährstoffzusammensetzung und wie diese sich auf Rezeption und Perzeption von Bienen auswirkt

Doctoral thesis for a doctoral degree at the Graduate School of Life Sciences, Julius-Maximilians-Universität Würzburg, Section Integrative Biology

submitted by

Fabian Rüdenauer

from

Bad Mergentheim

Würzburg, 25.05.2020

Submitted on: ……..

Office stamp

Members of the *Promotionskomitee*:

Chairperson:

Primary Supervisor: Dr. Sara Leonhardt

Supervisor (Second): Dr. Johannes Spaethe

Supervisor (Third): Prof. Dr. Klaus Lunau

Date of Public Defense: ……

Date of Receipt of Certificates: ……

Affidavit

I hereby confirm that my thesis entitled “Putting nutrition facts labels on pollen: nutritional analyses of pollen and nutrient receptional and perceptional usage by bees” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

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

Würzburg, 25.05.2020

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Pollen mit Nährwertinformationen ausstatten: Nährstoffanalysen von Pollen und wie Bienen Nährstoffe rezeptiv und perzeptiv verwenden“ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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

Würzburg, 25.05.2020

Ort, Datum Unterschrift

“A bee is an exquisite chemist”

- Royal bee keeper to Charles II.

“Not a single bee has ever sent you an invoice. And that is part of the problem - because most of what comes to us from nature is free, because it is not invoiced, because it is not priced, because it is not traded in markets, we tend to ignore it.”

- Pavan Sukhdev, United Nations report, The Economics of Ecosystems and Biodiversity.

Acknowledgements

First off, I am highly grateful to my supervisors Dr. Sara Leonhardt, Dr. Johannes Spaethe and Prof. Dr. Klaus Lunau for the opportunity to work with them and pursue a PhD for the past three years. While giving me the freedom to work independently, they were also constantly supporting and inspiring me and I am looking forward to continue working with them in a new chapter and adventure in our lives.

I also would like to thank all of the other members and ex-members of the Leonhardt group and the rest of the Zoology III department for the nice working environment not only throughout the years of my PhD time, but also in the years before that have had a huge impact on my PhD time. Out of the Leonhardt group, I would like to especially thank Daniela Beierlein, Nils Grund-Müller, Lisa Noack, Birte Peters, Stefan Sachs, David Sydow, Gemma Villagomez and Christine Wöhrle for collaboration, friendship and fun. Another special thanks goes to Prof. Dr. Thomas Schmitt and Doris Waffler and the other staff of the department's technical and secretary team.

Many thanks go to our collaborators and co-authors: Prof. Dr. David Raubenheimer (WP6), Dr. Casper J van der Kooi (WP1), Dr. Robert R. Junker (WP2) and Dr. Alexander Keller (WP2) for contributing their expertise to improve the studies conducted in and papers written for this thesis to a new level. Dr. Martin Strube-Bloss for useful comments on the experimental setup and helpful discussions of the results of WP5. Andrea Hilpert for the amino acid analyses in WP2, 4 & 6. Dr. Adrian Brückner and Prof. Dr. Bill Collins for developing and helping with

modifying the fatty acid analysis method conducted in WP2, 4 & 6. Dr. Leonie Lichtenstein for advice on the experimental setup and the handling of bees and Dirk Ahrens-Lagast for keeping and managing the honeybee colonies in WP3. Katja Tschirner for help with plant species identification in WP2.

I thank the Deutsche Forschungsgemeinschaft (DFG) for financing the studies in this thesis and paying my salary.

Additional thanks go to the Zoology II department and the team of the Graduate School of Life Sciences.

Thank you René Brachvogel, Katharina & Nils Grund-Müller, and Gemma Villagomez for proofreading the draft of this thesis.

A very special thanks goes to my (other) friends and family who always supported me and especially to René Brachvogel simply for making my life so much better.

Summary

Nutrients belong to the key elements enabling life and influencing an organism's fitness. The intake of nutrients in the right amounts and ratios can increase fitness; strong deviations from the optimal intake target can decrease fitness. Hence, the ability to assess the nutritional profile of food would benefit animals. To achieve this, they need the according nutrient receptors, the ability to interpret the receptor information via perceptive mechanisms, and the ability to adjust their foraging behavior accordingly. Additionally, eventually existing correlations between the nutrient groups and single nutrient compounds in food could help them to achieve this adjustment. A prominent interaction between food and consumer is the interaction between flowering plants (angiosperms) and animal pollinators. Usually both of the interacting partners benefit from this mutualistic interaction. Plants are pollinated while pollinators get a (most of the times) nutritional reward in form of nectar and/or pollen. As similar interactions between plants and animals seem to have existed even before the emergence of angiosperms, these interactions between insects and angiosperms very likely have co-evolved right from their evolutionary origin. Therefore, insect pollinators with the ability to assess the nutritional profile may have shaped the nutritional profile of plant species depending on them for their reproduction via selection pressure. In Chapter I of this thesis the pollen nutritional profile of many plant species was analyzed in the context of their phylogeny and their dependence on insect pollinators. In addition, correlations between the nutrients were investigated. While the impact of phylogeny on the pollen protein content was little, the mutual outcome of both of the studies included in this chapter is that protein

content of pollen is mostly influenced by the plant's dependence on insect pollinators. Several correlations found between nutrients within and between the nutrient groups could additionally help the pollinators to assess the nutrient profile of pollen. An important prerequisite for this assessment would be that the pollinators are able to differentiate between pollen of different plant species. Therefore, in Chapter II it was investigated whether bees have this ability. Specifically, it was investigated whether honeybees are able to differentiate between pollen of two different, but closely related plant species and whether bumblebees prefer one out of three pollen mixes, when they were fed with only one of them as larvae. Honeybees indeed were able to differentiate between the pollen species and bumblebees preferred one of the pollen mixes to the pollen mix they were fed as larvae, possibly due to its nutritional content. Therefore, the basis for pollen nutrient assessment is given in bees. However, there also was a slight preference for the pollen fed as larvae compared to another non-preferred pollen mix, at least hinting at the retention of larval memory in adult bumblebees. Chapter III looks into nutrient perception of bumblebees more in detail. Here it was shown that they are principally able to perceive amino acids and differentiate between them as well as different concentrations of the same amino acid. However, they do not seem to be able to assess the amino acid content in pollen or do not focus on it, but instead seem to focus on fatty acids, for which they could not only perceive concentration differences, but also were able to differentiate between. These findings were supported by feeding experiments in which the bumblebees did not prefer any of the pollen diets containing less or more amino acids but preferred pollen with less fatty acids. In no choice feeding experiments, bumblebees receiving a diet with high fatty acid content accepted undereating other nutrients instead of overeating fat, leading to increased mortality and the inability to reproduce. Hence, the importance of fat in pollen needs to be looked into

further. In conclusion, this thesis shows that the co-evolution of flowering plants and pollinating insects could be even more pronounced than thought before. Insects do not only pressure the plants to produce high quality nectar, but also pressure those plants depending on insect pollination to produce high quality pollen. The reason could be the insects' ability to receive and perceive certain nutrients, which enables them to forage selectively leading to a higher reproductive success of plants with a pollinator-suitable nutritional pollen profile.

Zusammenfassung

Nährstoffe gehören zu den zentralen Elementen, die das Leben an sich ermöglichen und die Fitness eines Organismus beeinflussen können. Nährstoffaufnahme in den richtigen Mengen und Verhältnissen kann die Fitness verbessern, starke Abweichungen von der optimalen Aufnahme können sie verschlechtern. Deshalb könnten Tiere von der Fähigkeit profitieren das Nährstoffprofil von Nahrung bewerten zu können. Dafür benötigten sie jedoch die passenden Nährstoffrezeptoren, die Fähigkeit die Rezeptorinformationen durch perzeptive Mechanismen zu interpretieren und ihr Sammelverhalten daran anzupassen. Eine zusätzliche Hilfe dabei könnten Korrelationen zwischen sowohl den Nährstoffgruppen als auch einzelnen Nährstoffen bieten. Eine bekannte Interaktion zwischen Nahrung und Konsument ist die zwischen Blühpflanzen (Angiospermen) und tierischen Bestäubern. Normalerweise profitieren beide Interaktionspartner von dieser mutualistischen Interaktion. Pflanzen werden bestäubt, während die Bestäuber eine (zumeist) nahrhafte Belohnung in Form von Nektar und/oder Pollen erhalten. Da ähnliche Interaktionen zwischen Pflanzen und Tieren vermutlich schon vor dem Auftreten der Angiospermen existierten, könnte sich diese Interaktion, im Speziellen mit Insekten, direkt vom evolutiven Startpunkt der Angiospermen aus koevolviert haben. Deshalb ist es möglich, dass Bestäuber mit der Fähigkeit das Nährstoffprofil von Pollen bewerten zu können, dieses bei von ihnen abhängigen Pflanzen durch Selektionsdruck formen konnten. Im Kapitel I dieser Thesis wurde das Nährstoffprofil von Pollen vieler Pflanzenarten im Kontext ihrer Phylogenie und ihrer Abhängigkeit von Insekten als Bestäubern analysiert. Außerdem wurden Korrelationen zwischen den

Nährstoffen untersucht. Während die Phylogenie nur einen geringen Einfluss auf den Proteingehalt von Pollen haben könnte, ist der gemeinsame Nenner der beiden Studien in diesem Kapitel, dass der Proteingehalt des Pollens hauptsächlich von der Abhängigkeit der Pflanzen von Bestäubern bestimmt wird. Es wurden zudem einige Korrelationen sowohl in als auch zwischen den Nährstoffgruppen gefunden, die den Bestäubern helfen könnten das Nährstoffprofil von Pollen bewerten zu können. Eine wichtige Grundvoraussetzung für diese Bewertung wäre, dass die Bestäuber überhaupt dazu in der Lage sind zwischen Pollen von unterschiedlichen Pflanzenarten zu unterscheiden. Dies wird in Kapitel II behandelt, in dem untersucht wurde ob Honigbienen in der Lage sind zwischen Pollen zweier nah verwandter Pflanzenarten zu unterscheiden und ob Hummeln eine von drei Pollenmischungen bevorzugen, wenn sie nur mit einer davon als Larve in Kontakt kamen. Honigbienen war es tatsächlich möglich zwischen den Pollenarten zu unterscheiden und Hummeln bevorzugten eine bestimmte Pollenmischung gegenüber der, die sie als Larve erhalten hatten, möglicherweise aufgrund eines vorteilhaften Nährstoffprofils. Die Grundlage zur Nährstoffbewertung scheint bei Bienen also gegeben zu sein. Allerdings hatten die Hummeln auch eine leichte Präferenz für die Pollenmischung, die sie als Larve erhalten hatten gegenüber der dritten, nicht bevorzugten Pollenmischung, was zumindest darauf hindeuten könnte, dass Larvenerinnerungen bei erwachsenen Hummeln erhalten bleiben könnten. Kapitel III beschäftigt sich tiefergehend mit der Nährstoffwahrnehmung von Hummeln. Es wurde gezeigt, dass diese prinzipiell befähigt sind Aminosäuren wahrzunehmen als auch zwischen ihnen und verschiedenen Konzentrationen der gleichen Aminosäure zu unterscheiden. Allerdings scheinen sie entweder nicht in der Lage zu sein oder sich zumindest nicht darauf zu fokussieren den Aminosäuregehalt von Pollen zu bewerten, sondern sich eher auf Fettsäuren zu konzentrieren. Von diesen konnten sie nicht nur

Konzentrationsunterschiede feststellen, sondern auch zwischen verschiedenen Fettsäuren im Pollen unterscheiden. Diese Ergebnisse wurden von denen in Fütterungsexperimenten gestützt, in denen die Hummeln gleiche Mengen von Pollen mit mehr oder weniger Aminosäuren aufnahmen, aber Pollen mit weniger Fettsäuren bevorzugten. In Experimenten, in denen die Hummeln keine Wahl hatten, nahmen die Hummeln mit einer Diät, die eine hohe Fettsäurekonzentration hatte, lieber in Kauf, dass sie zu wenig von den anderen Nährstoffen aufnahmen, als zu viel Fett, was zu einer erhöhten Mortalitätsrate und der Unfähigkeit sich zu reproduzieren führte. Deshalb sollten zukünftige Studien sich eingehender mit dem Fettsäuregehalt von Pollen beschäftigen. Zusammenfassend zeigt diese Thesis, dass die Koevolution von Pflanzen und bestäubenden Insekten ausgeprägter sein könnte, als bisher angenommen. Insekten setzen die Pflanzen nicht nur unter Druck qualitativ hochwertigen Nektar zu produzieren, sondern setzen vor allem auch die Pflanzen unter Druck, die von ihrer Bestäubung abhängig sind, qualitativ hochwertigen Pollen zu produzieren. Der Grund dafür könnte die Fähigkeit der Insekten sein, bestimmte Nährstoffe zu rezipieren und perzipieren und dann ihr Sammelverhalten so anzupassen, dass Pflanzen mit einem passenden Nährstoffprofil einen höheren Reproduktionserfolg haben.

Table of contents

Part I

Introduction

Ecological interactions

Our environment and ecological communities are shaped by intra- and interspecific interactions between organisms (Jones et al. 1996). One prominent example are interactions between animals and plants (Berenbaum et al. 1986; Bernays 1989). These interactions can be antagonistic (e.g. between plants and herbivores (Barbosa and Letourneau 1988; Crawley 1983) or between carnivorous plants and their prey (Merbach et al. 2002)) benefitting only one of the interaction partners. In addition, they can be mutualistic (e.g. between plants and pollinators (Faegri and Van der Pijl 2013) or seed dispersers (Waser 2006)), benefitting both interaction partners. Hence, plants try to either deter or attract these interaction partners. For example, as a defense against herbivores some plants produce toxic, distasteful or indigestible compounds (Swain 1977). On the other hand, many plants attract mutualistic interaction partners by yielding rewards. These rewards can be nonnutritive, e.g. provision of a sleeping place or mating site, heat, nest material or sexual attractants (Simpson and Neff 1981). As an interesting example for such an interaction with non-nutritive rewards pose the odors of flowers visited by male tropical euglossine orchid bees (Euglossini) that pollinate while collecting a perfume of different odors to attract females (reviewed by Cameron 2004). However, most of these types of rewards are nutritional (Simpson and Neff 1981), with pollen and nectar as the most prominent examples. Nevertheless, there are other types of nutritional rewards as well (Simpson and Neff 1981). For example, the nutritional composition of elaiosomes, seed appendages rich in fat and protein, are more nutritious than the seeds they are attached to (Fischer et al. 2008). They serve as a reward for ants, which disperse the seeds in return for consuming the elaiosomes (Fischer et al. 2008).

Nutrition and food

The reason why most of the rewards are nutritional is that food is playing a vital role to make life possible. For humans it became even more than just a necessity. It has become part of our cultural and social life. However, the primary function of eating is to provide nutrients to maintain the body's metabolism. These nutrients can be classified as macronutrients and micronutrients (Simpson and Raubenheimer 2012) (Figure I.1).

Protein, carbohydrates and fat belong to the macronutrients, which are needed in high amounts (Biesalski 2017). Protein is needed to provide amino acids for the consumer's own protein synthesis, which is required for muscle growth (Phillips and Van Loon 2011), enzyme and hormone and carrier protein production (Cooper 2000) and bolsters immune health (Li et al. 2007). Additionally, the amino acid glycine and the amino acid metabolites aspartate, glutamate, and taurine also can act as neuromodulators (Bicker 1991; Siegel et al. 1999). Carbohydrates mostly provide energy (Biesalski 2017). Fat is also mainly providing and storing energy, but is also needed as an essential part of membranes and as carrier for fat-soluble vitamins A and E (Kono and Arai 2015).

Vitamins and dietary minerals are classified as micronutrients (Biesalski 2017; Higdon and Drake 2011). These are needed for a plethora of different tasks, including energy regulation, being part of enzymes and hormones and immune system functions (Higdon and Drake 2011).

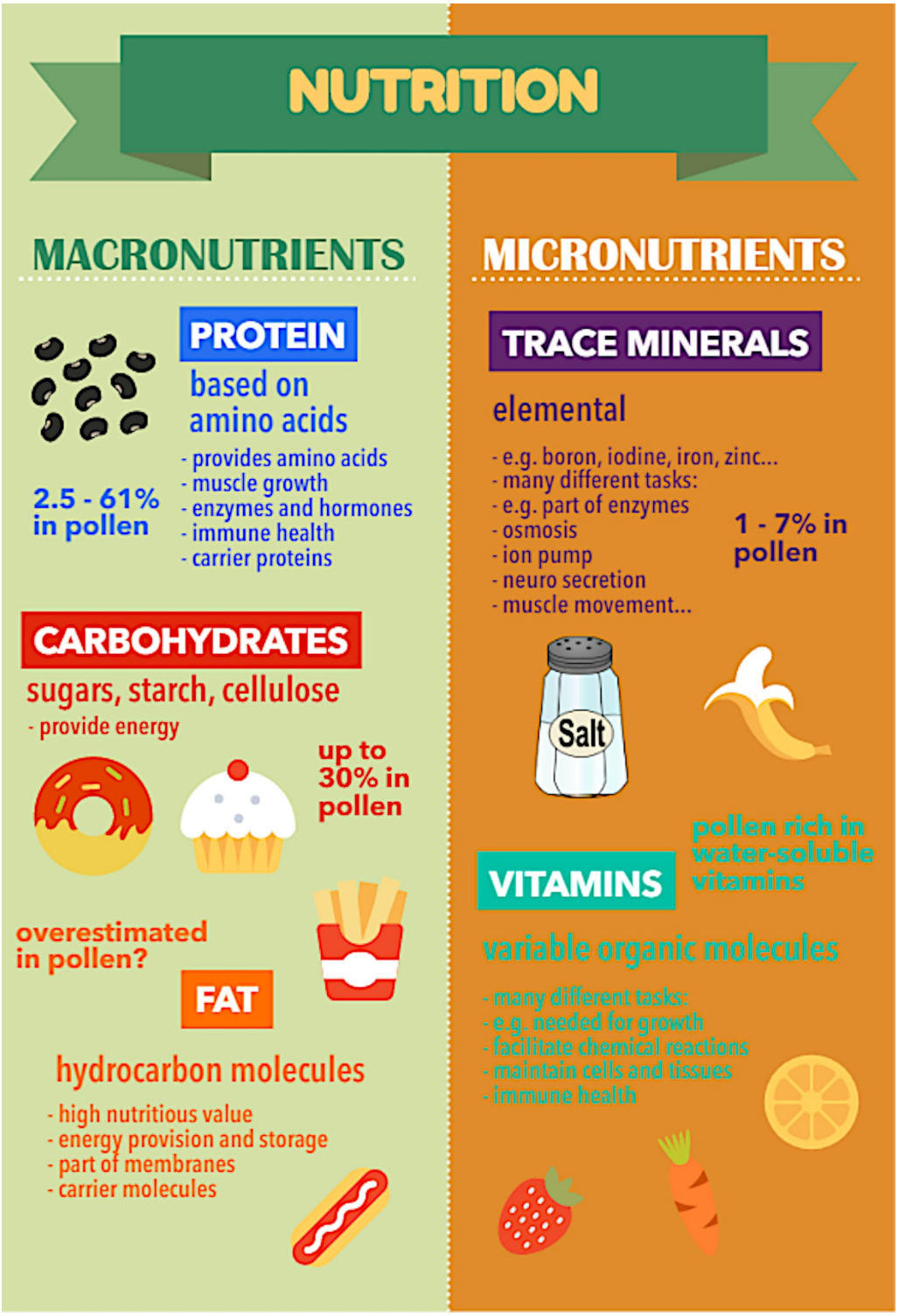


Figure I.1 Overview of the macro- and micronutrients and their functions in animals.

The requirements of both, macro- and micronutrients vary not only between but also within the same species (Behmer 2009b; Simpson and Raubenheimer 2012). Individuals of different ages, sex and fertility state can have different nutrient requirements (Behmer 2009b; Biesalski

2017; Maklakov et al. 2008). This is, for example, also true for humans, where children have a higher need for fat, protein and the micronutrients calcium and zinc for growth. And especially during menstruation and pregnancy the nutrient requirements for women differ significantly from men's (Biesalski 2017).

Fitness consequences of malnutrition

Malnutrition, i.e., strong deviations from the so-called "nutrient target" (the optimal intake of nutrient amounts) can lead to severe fitness consequences (Simpson and Raubenheimer 2012), like a weakened immune system, poor growth and a reduced to non-existent ability to reproduce (Behmer 2009a; Kropàcovà et al. 1968; Maklakov et al. 2008; Roulston and Cane 2000a). In general, a trade-off between lifespan and reproductive success is considered to be driven by the intake ratio between protein and carbohydrates (P:C-ratio) (Fanson et al. 2009; Lee et al. 2008). While a protein-rich diet increases reproductive success, it also decreases lifespan in locusts (Raubenheimer and Simpson 1993) and *Drosophila* fruit flies (Fanson et al. 2009; Lee et al. 2008). Excess amounts of protein can even lead to a complete colony collapse in ants (Dussutour and Simpson 2012).

However, the quantity of nutrients is not the only factor influencing the impact of nutrients. The composition of each nutrient group, e.g., the amounts of each single amino acid in the protein content or fatty acid in the fat content, can have a strong effect on nutrient intake (Altaye et al. 2010), reception and perception (Ruedenauer et al. 2017; Ruedenauer et al. 2015) and fitness (Grandison et al. 2009). For example, the brain development of mammals can be severely hindered if essential fatty acids (EFAs) are not consumed in appropriate amounts (Uauy and Dangour 2006) and if food contains a lot of essential amino acids (EAA), the amino acid demand is satisfied faster (Huether 2013). The amino acid methionine alone

had the same positive effect on *Drosophila* fertility as a full protein diet but did not decrease longevity, as other amino acids do (Grandison et al. 2009). Hence, specific molecules of each nutrient group may have different effects, rendering nutrient composition another important aspect of nutrition. Nonetheless, quantity is important, as only sufficient amounts of nutrients can ensure sufficient supply. For instance, bumblebee colonies with high amounts of low quality pollen perform much better than colonies with low amounts of high quality pollen (Kämper et al. 2016).

Malnutrition is also a problem in humans, causing economic costs of ca. US$ 3.5 trillion per year worldwide (Food and Agriculture Organization of the United Nations 2013). It is resulting in health issues, due to overconsumption (e.g. obesity, cardiovascular diseases and diabetes (Belanger et al. 1978; Colditz et al. 1997)) as well as underconsumption (e.g. diarrhea, dystrophy and autoimmune diseases (Stratton et al. 2003)). While macronutrient malnutrition is often due to a shortage or surplus of food, micronutrient deficiencies can be complemented e.g. by supplements, like iodine in table salt.

However, as naturally occurring food differs quite strongly in the ratios between different nutrients (Biesalski 2017), animals eventually have to trade off overeating certain nutrients against undereating other nutrients (Simpson and Raubenheimer 2012). Perceiving and assessing the nutrient content of food would enable animals to weigh up these two options against each other. There are several possibilities on how animals could achieve this task. One possibility is that they increase their receptor responsiveness towards their current needs. For example, the responsiveness of the receptors of locusts increases towards amino acids, when they were deprived of protein in advance (Simpson et al. 1991). Another possibility is that the brain remembers subconsciously the nutrients in food eaten before and sends

hunger signals for that specific food. Such a mechanism is proposed for humans (Biesalski 2017). However, it is hard to imagine that short-lived animals with a constantly changing food environment, like e.g. honeybees, can use such a mechanism. On the other hand, solitary bees, which usually emerge around the same time every year, could find the same food provisioning plants (Schenk et al. 2018) and social bees might also find at least some of the plants they were feeding on as larvae (Park and Nieh 2017). However, the nutrient content of the same food resource (e.g. pollen of the same plant species) might still differ, e.g. due to different weather conditions or soil nutrient availability (Somerville 2001). Therefore, a third possibility would be the most challenging but also the most reliable method. Animals could constantly assess the nutrient amounts and ratios of their food and compare them to their current needs. While regulating the intake of all nutrients with this method would be even more reliable, this might be too challenging. Hence, most of the animals investigated so far, mostly regulated the intake of either one nutrient or the ratio between two nutrients (Simpson and Raubenheimer 2012). Correlations between nutrient groups and single nutrient compounds could help to assess the whole (or a big part of the) nutrient profile by only using a few key substances. Anyway, no matter how many nutrients are regulated, to be able to do so animals would need the receptive and perceptive abilities to detect and differentiate between nutrient concentrations. Research on these abilities represents a big part of this thesis (WP3 – 6).

Senses and sensory information and memory connected to nutrition

In this thesis, the term reception will only refer to the processes at the receptor level, hence the binding of receptor proteins. Perception is defined as in Meister (2015) as “the internal state of the sensory system at the stage where discrimination decisions are made” and hence

as the individual sensation of the animal produced by higher order processing levels (e.g. the brain) after the signal produced by the receptors reached the perceptive centers there. How perception is achieved exactly is not known, but it seems to integrate the input of all affected receptor signals to create a percept (Gregory 2004). This means that even signals from receptors not directly related to the actual stimulus can interfere with perception. For example, the taste of wine seems to be influenced by background music (North 2012). Hence, the receptor signals can be modified and even eliminated on their way to the higher order processing units (Eltz and Lunau 2005) and therefore perception can differ severely from reception.

As nutrients are usually big molecules, they are non-volatile. Therefore, firstly, if an organism is able to receive nutrients it needs the according contact chemoreceptors (i.e. taste receptors in humans). Not much is known about nutrient taste reception. Taste receptors in mammals usually are divided into sweet, bitter, sour, salty and umami (hearty) receptors (Lindemann 1996). Most of the work on nutrient reception focused on sugar receptors in insects, bees and *Drosophila* in particular (de Brito Sanchez 2011; Kent and Robertson 2009; Slone et al. 2007). These receptor types are mainly tuned to sweet reception, i.e., sugars. Most animals like and/or respond to sugary, sweet substances (Nguyen et al. 2003; Scheiner et al. 2004). Hence, they do not only receive, but also perceive sugars. In addition, many animals can easily distinguish between different concentrations of sugar (Nowlis and Kessen 1976; Scheiner et al. 2004). Other nutrient receptors may not be as specialized as e.g. amino acids seem to trigger receptors of different receptor groups in humans, as they have subjective taste attributes from different receptor types (i.e. some taste sweet, others sour or bitter; Schiffman et al. 1981).

While humans can receive some peptides, whole proteins do not taste on their own, most likely because they are too large to fit into the receptors, but only contribute to the taste via participating to the texture (Solms 1969) or via modifying receptor properties, like the taste-modifying protein miraculin, which makes sour taste appear sweet (Kurihara and Beidler 1968; Kurihara and Beidler 1969). Therefore, more studies focused on amino acid reception, which also appear in food in their free, non-protein-bound form. Amino acid reception is best studied in fish, which seem to have specialized amino acid receptors (Mullin et al. 1994). However, as molecular transfer is essentially different in water, it is difficult to transfer this knowledge to land living animals. For instance, amino acids can be solved in water and therefore they can surround the fish just like volatiles in the air. In the air, they are, however, not volatile, as mentioned before. In mammals, a heterodimer of two gustatory receptors (T1R1+3) is able to receive most of the proteinogenic amino acids and therefore, most likely in combination with co-receptors, seems to be a/the most specialized receptor for amino acids in this animal group (Nelson et al. 2002). In *Drosophila*, the ionotropic receptor IR76b seems to have a similar function, as it is also co-activated with different co-receptors by different amino acids (Croset et al. 2016). Additionally, single sensillum recordings and electroantennographical measurements (EAG), both measuring receptor potentials after stimulus presentation, revealed distinctive signals for different amino acids in both the ground beetle *Pterostichus oblongopunctatus* (Merivee et al. 2008) and the bumblebee *Bombus terrestris* (Rüdenauer 2016).

Even less is known about amino acid perception. While for humans each amino acid (and even the two different isomers) has a unique taste characteristic (Schiffman and Dackis 1975; Schiffman et al. 1981), the determination of whether animals are able to perceive or differentiate a substance is not as easy. Hence, mostly behavioral approaches, like learning or

preference experiments, have to be used to determine it. For example, *Drosophila* larvae seem to perceive all 20 proteinogenic amino acids and prefer different ones, depending on their larval stage (Kudow et al. 2017).

Lipids, like proteins, are likely too large to fit into receptors. Therefore, it was thought for a long time that fat would only contribute to texture of food. However, fatty acids are supposedly received via taste receptors or receptor-like proteins (Laugerette et al. 2007; Running et al. 2015). In rats and mice the receptor-like protein CD36 seems to be the fat taste receptor (Laugerette et al. 2005). In humans, the perception of fat seems to be rather different between individuals (Bartoshuk et al. 2006). Individuals sensitive to the bitter tasting substance 6-n-propylthiouracil (PROP) (Tepper and Nurse 1997) and individuals with some variants of the gene for CD36 (Keller et al. 2012) are also more sensitive to differences in fat content. Such individual differences in perception are eventually linked to diseases caused by malnutrition, such as obesity (Bartoshuk et al. 2006; Keller et al. 2012).

Reception and perception of micronutrients has virtually not been studied at all, even though we know that humans are able to taste vitamins (Schiffman and Dackis 1975) and several taste abnormalities for micronutrients are leading to diseases (Gershoff 1977). Besides their important role and essentiality in food, the concentrations might be too low to be perceived in the complex mixture of substances food usually provides and their signal overlaid by macronutrients and other tasting substances provided in high amounts.

Methods for investigating perception

While electrophysiological studies like electroantennography (Ruedenauer et al. 2017) and sensillum recordings (Merivee et al. 2008) can be used to investigate the receptor level, behavioral paradigms, like learning experiments, feeding experiments or surveys (in humans)

need to be used to reveal the information processed on the perceptive level. A method used in this thesis is the conditioning of the Proboscis Extension Response (PER). This method can be used in insects, especially bees, for experiments associated with learning (Shiraiwa and Carlson 2007; Takeda 1961; Vareschi 1971) but can also be used to assess whether individuals can differentiate between two stimuli perceptively (Ruedenauer et al. 2015). The method utilizes some insects' (like flies or bees) extension of the proboscis in response to a sugar solution, similar to nectar, making contact to their chemotactile receptors on the antennae, mouthparts or tarsi (de Brito Sanchez et al. 2007). In terms of classical Pavlovian conditioning (Pavlov 1927) this represents the unconditioned stimulus (US) (Matsumoto et al. 2012). Chemotactile (Ruedenauer et al. 2015), olfactory (Giurfa 2007; Hammer and Menzel 1995; Hannaford et al. 2013), tactile (Erber et al. 1998; Scheiner et al. 1999), thermal (Hammer et al. 2009) or visual (Hori et al. 2006; Lichtenstein et al. 2015) stimuli can be used as the stimulus that is supposed to be learned, the so-called conditioned stimulus (CS). If CS and US are presented in combination the insect learns the connection between the stimuli and extends the proboscis without the need of being presented with the US (Bitterman et al. 1983; Matsumoto et al. 2012). If two CS are used, and one of them is reinforced by the US (CS+), while the second one is not (CS-), this differential conditioning method can be used to reveal whether insects are able to perceive nutrients (Ruedenauer et al. 2015). For example, one of the stimuli can be food while the second one can be the same food supplemented with an additional nutrient. If the insect responds only to the CS+ after some training, and therefore can differentiate between the two stimuli, identical except for the additional nutrient, it has to be able to perceive this nutrient.

Another approach on the perceptive level going one step further than simply investigating the ability to perceive certain nutrients are feeding experiments (Crocker et al. 1993;

Ruedenauer et al. 2016). These experiments can reveal foraging preferences of animals in two-choice experiments (i.e., when the animals have the choice between two different diets, Albert and Parisella 1988; Chen and Henderson 1996) or fitness consequences of a diet in no-choice experiments (i.e., when the animals are forced to eat one particular diet, Paul et al. 2006). Varying the concentrations of one particular nutrient or nutrient group can uncover whether an animal regulates the intake of this nutrient in choice experiments (Buchsbaum et al. 1984; Toth and Pavia 2002; Vaudo et al. 2016a) and the fitness consequences of this particular nutrient when over- or undereaten in the no-choice experiments (Rodriguez et al. 1993). For example, the caterpillar of the African cotton leaf worm (*Spodoptera littoralis*) composes a diet higher in protein than usual when infected with an entomopathogen to increase its resistance towards the virus (Lee et al. 2006) and in the wood-eating termite, *Coptotermes formosanus,* several types of wood lead to 100% mortality rates in just three months (Morales-Ramos and Rojas 2001). However, while performing such feeding assays, the experimenter always has to keep in mind, that the results may be context dependent and different in a different food subject/nutrient mix. For instance, in eastern spruce budworm caterpillars (*Choristoneura fumiferana*) the amino acid serine induced higher feeding rates compared to some other amino acids when fed purely but acted deterrently when fed in combination with sugar (Albert and Parisella 1988).

Experience and memory influence foraging decisions

Foraging decisions are not only based on a current snapshot of reception and perception, but are also based on experience and memory (Hirvonen et al. 1999; Regular et al. 2013). In humans and tufted capuchin monkeys (*Cebus apella*) alike, during their first weeks of eating, babies prefer novel food with high sugar content (Addessi et al. 2004). However, the more

experienced they get, the young capuchins tend to prefer food that maximizes their net gain of energy instead (Addessi et al. 2004). In rats experience and long-term memory even seems to outweigh recent experiences. If they receive food that is safe only once, they have a "learned safety" towards this food type, even after they had received the same food with non-lethal amounts of poison (Kalat and Rozin 1973). Not only information on the food itself can help to optimize foraging, time and space are important as well. In bison (*Bison bison*) their memory about location, timing of grass growth and quality of meadows helps them to maximize their energy intake as well (Merkle et al. 2014).

Holometabolous insects represent a special case in terms of memory, as at least some species seem to be able to retain their larval memory after metamorphosis (Caubet et al. 1992; Gandolfi et al. 2003; Thorpe and Imms 1939), despite the complete restructuring of their whole body including their receptive organs (Keil 1997). This so-called preimaginal learning seems to be important for e.g. nestmate recognition in social insects (Kukuk et al. 1977; Pfennig et al. 1983). Foraging for food on the knowledge that the individual itself was able to grow up and emerge on as an adult, would be a relatively simple method to ensure a properly provisioned brood. How such retained memories play a role in nutrition and foraging behavior is widely unknown. However, adult *Drosophila* prefer menthol scented food, which usually acts aversively, when they were fed with it as larvae (Barron and Corbet 1999), hinting at a possible role of larval memory in foraging decisions, which was investigated in bumblebees in WP4.

Nutrients in nectar and pollen

A particularly well studied plant-insect interaction is the (mostly) mutualistic interaction between flowering plants and pollinators (Waser 2006). The nectar and pollen provided by

these plants represent the sole source of nutrients for many pollinator species (Faegri and Van der Pijl 2013; Haydak 1970). While nectar mainly provides carbohydrates and also contains low amounts of proteins, free amino acids, lipids and phytochemicals (Baker 1977; Nicolson and Thornburg 2007), pollen provides all other macro- and micronutrients for pollinators (Baidya et al. 1993; DeGroot 1953; Keller et al. 2005; Roulston and Cane 2000b). However, in both of them the nutrient composition is known to vary a lot (Petanidou et al. 2006; Roulston and Cane 2000b; Somerville 2001; Weiner et al. 2010).

In nectar, the sugar concentration can range between less than 10% (Nicolson and Nepi 2005) and almost 70% (Langenberger and Davis 2002). While it is clear that the sugar in nectar has evolved as a reward for pollinators, the function of the other constituents of nectar are most likely more complex. Amino acid concentrations and profiles are discussed to also have evolved partly as a reward, as they seem to be shaped by the according pollinator groups (Petanidou et al. 2006). However, as free amino acids are also known to shorten lifespan (Huang et al. 2011) and can act as both either phagostimulant or deterrent (Bell et al. 1996), they could also play a role in deterring unwanted visitors. Also, some amino acids, like hydroxyproline are part of the plant cell walls (Nicolson and Thornburg 2007) and may therefore simply be leaking into the nectar. Proteins, called nectarins as part of nectar, mostly fulfill tasks in chemical reactions like sucrose hydrolysis (Beutler 1953; Heil et al. 2005), pH-balance the nectar (Carter and Thornburg 2004), and act as defense against microbes (Peumans et al. 1997). As lipids are very nutritious, and nectar of plants in regions where pollination is energy demanding is rich in lipids, it is likely that lipids are also mostly produced as a reward for pollinators (Bernardello et al. 1999; Forcone et al. 1997). Hence, it seems as if pollinators shaped the nutritional profile evolutionarily by means of natural selection processes (Petanidou et al. 2006).

Pollen is more chemically complex than nectar (Palmer-Young et al. 2019a; Roulston and Cane 2000b; Todd and Bretherick 1942). Besides containing all of the macronutrients, it also contains minerals (M. Loper et al. 1980), sterols (Kvanta 1968; Standifer et al. 1968), vitamins (Togasawa et al. 1967a), and phytochemicals (Palmer-Young et al. 2019a; Palmer-Young et al. 2017b). Pollen carbohydrates are either used directly for energy or converted into other molecules, but mainly stored as starch (Baker and Baker 1979; Pacini and Franchi 1983; Todd and Bretherick 1942). Starch content ranges between 0-22%, with most plants having a content of less than 1% (Roulston and Buchmann 2000). Starch in pollen is most likely used as energy storage, needed for pollen tube growth (Baker and Baker 1979). Baker and Baker (1979) and Grayum (1985) have postulated there is a significantly higher starch content in anemophilous compared to zoophilous plants. However, this was most likely a statistical artifact revealed by Roulston and Buchmann (2000), who found that starch content was unusually high explicitly in three species-rich anemophilous clades of the Baker and Baker (1979), which likely biased the data. When they applied a statistical test corrected for phylogeny, they did not find such an effect anymore (Roulston and Buchmann 2000). Also, pollinators do neither seem attracted nor averse to starch (Roulston and Buchmann 2000) and pollinators usually get most of their carbohydrates from nectar anyway (Brodschneider and Crailsheim 2010). Hence, it is unlikely that the carbohydrate profile underlies the selection pressure by pollinators.

The protein content of pollen ranges between 2.5 and 61% of dry mass among all plant species (Figure 1, Roulston et al. 2000). Even though most of the nutrients for the fertilization process are provided by the mother plant (Labarca and Loewus 1973), the proteins in pollen are mostly enzymes supporting pollen tube growth (Roulston et al. 2000; Stanley and Linskens

1974). Proteins are discussed to be the most important nutrient for pollinators and hence could be one of the nutrients formed by potential selection processes.

However, the importance of other nutrients in pollen (e.g., fat) for pollinators is becoming clearer in more recent research (Manning 2006). Pollen lipids mainly consist of fat and sterols (Roulston and Cane 2000b). Fat in pollen is present in two different primary sites, the exine (the pollen's outer layer) and the intine (the pollen's cytoplasm) (Stanley and Linskens 1974). In the exine lipids are present in the so-called pollenkitt, a layer surrounding the pollen grains to make them adhesive (Pacini and Hesse 2005) and possibly is antimicrobial (Manning 2001). It is also discussed that pollinators could be attracted by the scent or taste of pollenkitt (Dobson 1988). As it mainly consists of non-volatile lipids, the latter would be likelier. However, if this is the case, pollenkitt lipid profiles of zoophilous plants may also be shaped by pollinator selection. The intine lipids seem to be more abundant, as ground pollen contains a much larger fraction of substances in petroleum ether extracts than intact pollen (Ibrahim 1974). This extraction method was used by most of the studies on pollen fat content (e.g., Andrikopoulos et al. 1985; Čeksterytė et al. 2016; Human and Nicolson 2006). However, it is very inaccurate as it does not only measure fat, but also contains vitamins, pigments, higher alcohols, waxes, sterols and saturated hydrocarbons (Solberg and Remedios 1980). Hence, it overestimates the fat content to an unknown extent (Figure 1). Therefore, one goal of this thesis was establishing a new method for pollen fat analysis (WP1 & 2). A special case of lipids is sterols. They cannot be synthesized by insects (Hobson 1935) and therefore by the largest group of pollinators (Faegri and Van der Pijl 2013). Hence, the sterol profiles could possibly also be influenced by pollinator selection.

Trace mineral content is relatively high in pollen with 1-7% (Lunden 1954). Their tasks are numerous. For example, boron increases the success of pollen germination, most likely because it is involved in carbohydrate transport (Wang et al. 2003) and pollen deficient in copper is completely sterile (Dell 1981). Molybdenum- and manganese-deficient plants produce fewer and smaller pollen grains, indicating that it is involved in pollen production (Agarwala et al. 1979; Sharma et al. 1991). Zinc from pollen seems to be transferred during fertilization, as isotope marked zinc from pollen can still be found in the seeds (Polar 1975). Little is known about the function of vitamins in pollen. Most of the vitamins in pollen are water-soluble, while the content of fat-soluble vitamins is low (Roulston and Cane 2000b).

As seen from the differences in the functions of the nutrients, unlike nectar, pollen did not evolve as a reward for pollinators, but for the plants' own reproductive process (Roulston et al. 2000). Hence, the collection and consumption of pollen by pollinators usually is not benefiting the plant. Therefore, on the first glance it seems unlikely that pollinators could have shaped the nutritional profile of pollen. However, many plants are completely dependent on animal pollination. Therefore, if pollinators assess the nutrient content and collect the pollen according to their nutritional needs, they would apply selection pressure onto the plants, by increasing the reproductive success of those plants with pollen closer to their needs.

Correlations between the different nutrient groups as well as between different molecules within the same nutrient group could help pollinators to assess the overall nutrient content much easier by assessing only a few nutrients. Such a correlation is present within the amino acids ($r \geq 0.5$, $P < 0.01$ for the dataset composed by Weiner et al. 2010), but remains to be shown for the other nutrient groups as well as between them.

Nutrition and senses in bees

Bees represent one of the key groups in pollination (Klein et al. 2007). Almost all of the bee species exclusively rely on pollen and nectar as food (Brodschneider and Crailsheim 2010; Haydak 1970; Keller et al. 2005; Loper and Berdel 1980). Hence, they are particularly prone to malnutrition, especially in regions with high land use (Klein et al. 2017), which is often connected to little plant diversity and therefore little nutrient diversity (Filipiak et al. 2017). Hence, the current bee-decline is thought to be partly blamed on malnutrition (Biesmeijer et al. 2006; Goulson and Darvill 2004; Klein et al. 2017). However, not much is known about the nutritional needs of bees, even though this could be crucial for bee conservation (Filipiak 2018).

Bees cover a wide spectrum between food generalists and specialists (Strickler 1979). While specialists usually are more effective in exploiting the food resources they are specialized on (MacArthur and Pianka 1966; Schoener 1971; Thostesen and Olesen 1996), they are also more prone than generalists to local (Suhonen et al. 2014) and global extinction (Ferrer and Negro 2004; Roberts et al. 2011), if their food resources disappear. Additionally, specialists should be adapted to the nutritional profile of their food resources and/or have shaped them via co-evolution, while generalists simply can switch and mix their food resources according to their needs (Eckhardt et al. 2014; Strickler 1979).

Another reason why bees are interesting for nutritional research are the different levels of sociality they show. While a solitary bee only has to take care about its own nutrition and the provision of its offspring, social bee foragers have to supply a whole colony with food. Social bee colonies are often described as a superorganism (Seeley 1989) and therefore many nutritional rules and mechanisms discussed before can be applied to colonies as a whole.

However, different individuals in the colonies have different nutritional needs and the foragers have to satisfy all of them (Altaye et al. 2010). For example, larvae and queens have a higher need for protein for growth and egg production than foragers (Human et al. 2007). Therefore, bee foragers would benefit from being able to receive and perceive nutrients for quality assessment (Ruedenauer et al. 2017; Ruedenauer et al. 2015; Ruedenauer et al. 2016).

Taste receptors in bees can be found in the antennae, mouth parts and tarsi, similar to other insects (de Brito Sanchez 2011). In this thesis, the focus is mainly on perception mechanisms via antennal receptors. Bee antennae comprise olfactory, gustatory, auditory, tactile and temperature-sensitive receptors (Slifer and Sekhon 1961). The taste receptors are integrated into sensory hairs or peg like structures, the taste sensillae (Esslen and Kaissling 1976). These sensillae are mostly present at the bee's antennal tip (Haupt 2004; Whitehead and Larsen 1976), the part of the antenna making first and most contact with gustatory stimuli. The gustatory sensillae comprise an aperture at their apex through which molecules can enter before binding to adequate receptors (de Brito Sanchez et al. 2007) and generating action potentials sent along the antennal nerve. Mechanoreceptors often are also present within this sensillum type (de Brito Sanchez 2011; de Brito Sanchez et al. 2007). Hence, gustatory and tactile reception and perception can hardly be separated in bees, which is why this thesis refers to chemotactile reception and perception as combined sensory information of both (Ruedenauer et al. 2015).

Honeybees and bumblebees represent two of the most important groups of pollinators, in nature and commercially. While honeybees need to be kept in hives, bumblebees can also be purchased easily and kept in the laboratory without problems. Besides not learning as fast and as good as honeybees, bumblebees can be used excellently for PER experiments (Laloi

and Pham-Delegue 2004; Ruedenauer et al. 2015; Sommerlandt et al. 2014). Additionally, bumblebees can be used very well for long-time feeding experiments, as they can be kept easily in queenless microcolonies, which are comparable to big colonies (Larrere and Couillaud 1993).

In previous experiments with chemotactile EAG I could already show that bumblebees already differentiate between two different pollen types on the receptor level (Ruedenauer et al. 2017). This translates into perception, as they were also able to differentiate between the same two pollen types in PER experiments (Ruedenauer et al. 2015). Also, amino acids solved in water did produce separating EAG responses, hinting that bumblebees posess amino acid receptors (Rüdenauer 2016). Bumblebees are also able to differentiate between different pollen qualities, shown in PER experiments with diluted and undiluted pollen (Ruedenauer et al. 2015) and use this ability to forage for high quality over low quality pollen in microcolonies (Ruedenauer et al. 2016). Additionally, they are able to quickly adjust their foraging behavior quickly as soon as pollen quality changes (Ruedenauer et al. 2016). Moreover, the two bumblebee species *Bombus impatiens* and *B. terrestris* collect pollen according to a species-specific stable protein:lipid-ratio (P:L-ratio) by mixing pollen of different P:L-ratios (Vaudo et al. 2016a).

Objectives

This thesis aims to analyze what governs the nutrient content of pollen and reveal more about the food and nutrient perception mechanisms of honeybees and bumblebees as well as the mechanisms of bumblebee foraging decisions and therefore nutrient assessment. This was done in six working packages (WP):

(WP1) It was tested whether pollen nutritional content is influenced by relatedness between plant species or their dependence on insect pollination and therefore shaped via evolutionary selection processes. This was done with a meta-analysis covering the nutritional content of pollen of 387 plant species.

(WP2) As very different analysis methods were used in the meta-analysis, which could potentially have an impact on the results, another dataset of pollen of 139 plant species was compiled. The pollen was analyzed with standardized methods with the same question as in WP1. Information from single substance identification was used to look into the data in more detail than with overall contents of nutrient groups.

(WP3) A prerequisite for pollen nutritional assessment is the ability to differentiate between pollen of different plant species. Hence, in this WP it was tested whether honeybees are able to differentiate between apple and almond pollen via chemotactile PER.

(WP4) The ability of pollen differentiation could lead to preimaginal learning, which could overwrite the preference for high quality pollen. Therefore, in this WP bumblebee larvae were reared on a specific pollen type and after emergence as imagoes tested for whether they would prefer the pollen they were reared on or pollen with higher quality.

(WP5) In this WP it was tested whether bumblebees are able to perceive and differentiate amino acids solved in water via chemotactile PER. This could show that bumblebees have an amino acid receptor and could potentially use amino acids for nutritional assessment in nectar and possibly in pollen.

(WP6) As perception is context dependent, the perception of nutrients in pollen could differ substantially from perception in nectar. Bumblebees were tested for their ability to perceive amino acids and fatty acids in pollen via chemotactile PER. In two-choice feeding

experiments they were tested for their foraging decisions when offered pollen with different concentrations of amino acids or fatty acids. In no-choice feeding experiments the fitness consequences on adult survival and reproductive success of these different diets were tested as well.

Part II

Publications

Main findings of the publications

Chapter I: Pollen biochemical analyses

This chapter contains a meta-analysis (WP1) and analysis of a self-collected dataset (WP2) on pollen nutrients. The main findings of both analyses are that pollen of plants depending on insect pollination have a higher protein content, possibly as a reward for pollinators, and that nutrient contents of some of the different nutrient groups correlate, facilitating nutrient assessment for insect pollinators. While a phylogenetic signal (i.e. that nutrient content seems to correlate with relatedness between plants) was found for the protein content in WP1, no phylogenetic signal was found in WP2. Possible reasons are discussed in WP2. Additionally, a single compound (i.e. single nutrient compounds instead of total nutrient group content as a sum of single compounds) correlation analysis was done in WP2. Many of the single compound nutrients correlated within and between the different nutrient groups, possibly caused by shared biosynthetic pathways and/or selection pressure by pollinators.

Chapter II: Differentiation and preferences of bees of pollen from different plant species

This chapter deals with whether honeybees (WP3) and bumblebees (WP4) are able to differentiate between pollen of different plant species with two different behavioral experiments. In WP3, honeybees were able to differentiate between two different pollen types, a prerequisite for pollen nutritional assessment and especially for sharing information within the hive. In feeding experiments in WP4 bumblebees did not only prefer one of the pollen mixes offered, possibly due to its nutrient content (probably its lower fat content regarding the results of WP6), but also showed signs of preimaginal learning, as they also slightly preferred the pollen mix of their larval diet.

Chapter III: Nutrient perception and its fitness consequences for bumblebees

In this chapter, it was tested whether bumblebees are able to perceive amino acids in water (WP5) or pollen (WP6) and fatty acids in pollen (WP6). While they were able to perceive some of the amino acids (the ones with an additional chemical functional group) and differentiate between them and different concentrations of the same amino acid in water (and therefore eventually in nectar), they were not able to do so in pollen, probably due to its chemical complexity. However, they were able to perceive all of the tested fatty acids and differentiated between them and different concentrations of the same fatty acid. Fitting these results, they also ate similar amounts of pollen containing different concentrations of amino acids without suffering any fitness consequences. However, they tried to avoid pollen containing more fatty acids than the natural pollen mix, which probably led to them undereating other nutrients. This resulted in higher mortality and lower reproduction, severely degrading these individuals' fitness. Therefore, bumblebees most likely regulate mostly their fat intake.

Chapter I: Pollen biochemical analyses

WP1 Pollinator or pedigree: which factors determine the evolution of pollen nutrients?

Authors:

Fabian A. Ruedenauer*, Johannes Spaethe, Casper J. van der Kooi, Sara D. Leonhardt

*first author

published: 2019

Journal: Oecologia

Volume: 191 Issue: 2 Pages: 349-358

Abstract WP1

A prime example of plant–animal interactions is the interaction between plants and pollinators, which typically receive nectar and/or pollen as reward for their pollination service. While nectar provides mostly carbohydrates, pollen represents the main source of protein and lipids for many pollinators. However, the main function of pollen is to carry nutrients for pollen tube growth and thus fertilization. It is unclear whether pollinator attraction exerts a sufficiently strong selective pressure to alter the nutritional profile of pollen, e.g., through increasing its crude protein content or protein-to-lipid ratio, which both strongly affect bee foraging. Pollen nutritional quality may also be merely determined by phylogenetic relatedness, with pollen of closely related plants showing similar nutritional profiles due to shared biosynthetic pathways or floral morphologies. Here, we present a meta-analysis of studies on pollen nutrients to test whether differences in pollen nutrient contents and ratios correlated with plant insect pollinator dependence and/or phylogenetic relatedness. We hypothesized that if pollen nutritional content was affected by pollinator attraction, it should be different (e.g., higher) in highly pollinator-dependent plants, independent of phylogenetic relatedness. We found that crude protein and the protein-to-lipid ratio in pollen strongly correlated with phylogeny. Moreover, pollen protein content was higher in plants depending mostly or exclusively on insect pollination. Pollen nutritional quality thus correlated with both phylogenetic relatedness and pollinator dependency, indicating that, besides producing pollen with sufficient nutrients for reproduction, the nutrient profile of zoophilous plants may have been shaped by their pollinators' nutritional needs.

Introduction WP1

Interactions between organisms shape our environment and ecological communities, and drive ecosystem functions (Jones et al. 1996). One prominent example are interactions between animals and plants, which are typically driven by resource use, as many animal species rely on plants for meeting their nutritional and/or protective needs (Berenbaum et al. 1986; Bernays 1989). Some plants provide chemically attractive rewards to attract partners, such as pollinators or seed dispersers (Waser 2006). In addition to non-nutritional floral factors like color and scent (McCall and Primack 1992; van der Kooi et al. 2019b), reward nutritional quality can strongly affect the community of flower-visiting animals (Petanidou et al. 2006). For example, bees (Camazine and Sneyd 1991; Nicholls and Hempel de Ibarra 2016; Ruedenauer et al. 2016; Somme et al. 2015), butterflies (Lewis 1986) and hummingbirds (Stiles 1976), appear to select plant species based on differences in the nutritional quality of rewards.

Pollen is a nutritionally diverse and highly valuable reward for pollinators, because it provides protein, fat, carbohydrates, sterols and various micronutrients (Roulston and Cane 2000b). Due to its valuable nutritional quality, some pollinators, such as bees, exclusively rely on pollen for reproduction and survival (e.g. Baidya et al. 1993; Haydak 1970; Loper and Berdel 1980; Saffari et al. 2010). These pollinators consequently need to find pollen that meet their nutritional needs, which may result in foraging choices and thus visitation patterns that are strongly, if not exclusively, determined by pollen nutritional quality. For example, bumblebees can assess the nutritional quality of pollen and preferentially forage on plants with pollen of high protein (and low lipid) content, while other (combinations of) pollen nutrients reduce the number of certain flower visitors (Kitaoka and Nieh 2009; Leonhardt and Blüthgen 2012;

Ruedenauer et al. 2015; Ruedenauer et al. 2016; Somme et al. 2015; Vaudo et al. 2016b). Thus, akin to nectar, pollen nutritional quality can significantly affect the spectrum of flower visitors. Pollinator foraging choices may in turn shape the nutritional composition of pollen of those plants that are highly dependent on animal pollination. Such plants may benefit from increased visitation – and potentially pollination – through presenting pollen with nutrients that meet the visitors' preferences. In contrast, plants that depend little or not on pollinators, e.g. wind- and self-pollinated plants, are unlikely subject to pollinator-mediated selection for pollen nutrients (Baker and Baker 1979). The nutritional content of their pollen may thus differ from pollen of plants that do depend on animals for pollination. Indeed, the large differences often observed for relative amounts of nutrients (e.g. protein content which can range between 2.5 and 61%; Buchmann, 2000) may be explained by different levels of insect pollinator dependence.

However, from the plant's perspective, the primary function of pollen is reproduction. In this regard, closely related plant species likely require similar amounts of nutrients or nutrient ratios to ensure efficient fertilization due to common metabolic pathways and similar floral morphologies. Although most of the nutrients for fertilization are provided by the mother plant (Labarca and Loewus 1973), pollen needs to carry additional nutrients to remain fertile over the transfer period and to initiate the fertilization process. For example, pollen protein and sugar content play an important role for pollen tube growth (Labarca and Loewus 1973). Similarly, pollen lipids - typically stored in the pollen intine - most likely act as energy storage (Ibrahim 1974). Plants with long styles thus require more protein and energy in form of sugars or lipids to grow sufficiently long pollen tubes (Roulston et al. 2000). Moreover, the collection and use of pollen as nutrient source by many flower-visiting animal species (Haydak 1970; Roulston and Cane 2000b; Stanley and Linskens 1974) is often of no benefit or even a cost to

the plant - particularly if the plant does not rely on animals for pollination, such as wind-pollinated plants - because pollen consumed by animals is lost and cannot be used for fertilization. Besides producing overall larger amounts of pollen to increase chances of fertilization (Friedman and Barrett 2009), the exclusion of pollinators could thus be a reason for the low pollen nutrient amounts typically found in wind-pollinated plants. Differences in pollen nutritional content may therefore be largely (or solely) determined by plant species-specific requirements for pollen fertility and thus phylogenetic relatedness, e.g., due to family- or genera-specific metabolic pathways or similar floral morphologies (Hanley et al. 2008; Roulston et al. 2000). The nutritional composition of pollen thus appears to be subject to two different and potentially conflicting selective pressures: the preferences of pollinators and the plant's own fertility which is largely determined by phylogenetic relatedness. These selective pressures may also explain why some animal pollinated plant species even present two types of stamen: stamen with pollen that specifically serves as reward for pollinators, and stamen with pollen for ovule fertilization (heteranthery: (Vallejo-Marín et al. 2009)).

It is still largely unclear to which extent pollen nutrient content is driven by pollinator needs and/or phylogenetic relatedness. Although pollen did not primarily evolve as reward for pollinators, the plants' dependence on animals for pollination may have altered its nutritional profile over the course of evolution. The few previous studies comparing pollen nutritional content across plant species provided inconsistent results. For example, the ground breaking work by Roulston et al. (2000) found a phylogenetic signal for pollen protein content, but did not find any influence of the pollination system. By contrast, Hanley et al. (2008) found a significant correlation between pollen protein content and pollination strategy, but did not specifically test for a phylogenetic signal.

Protein was, until recently, considered the main quality feature of pollen for pollinators, especially for bees (DeGroot 1953; Herbert et al. 1977), as it is the most abundant nutrient in pollen (Roulston and Cane 2000b) and is considered the most important nutrient for bee larval growth (DeGroot 1953). Recent studies, however, highlight the importance of other pollen nutrients, e.g., lipids, fatty acids (Manning et al. 2007) and sterols (Vanderplanck et al. 2011), as well as the significance of specific ratios between different nutrients (Raubenheimer and Simpson 1999; Vaudo et al. 2016a; Vaudo et al. 2016b). While a lack of nutrients may simply be compensated by eating more, an unbalanced ratio will automatically lead to over- or undereating at least some nutrients (Raubenheimer and Simpson 1999; Vaudo et al. 2016a; Vaudo et al. 2016b). Nutrient amounts differ strongly between plant species, though very little is known about differences in nutrient ratios between plant species.

Only few studies compared pollen nutritional content across plant species (e.g. Auclair and Jamieson 1948; Baker and Baker 1979; Somerville 2001; Todd and Bretherick 1942; Weiner et al. 2010), and most of these studies focused on either one or few plant species and analyzed only one or a subset of nutrients, while studies analyzing a broader spectrum of plant species and nutritional components remain scarce (e.g. Somerville 2001; Todd and Bretherick 1942). Moreover, many previous studies analyzed bee-collected pollen, which usually contains salivary secretions added by bees, i.e., regurgitated nectar to facilitate pollen handling (Winston 1991), which contain nutrients and may alter the nutrient composition of the analyzed pollen (Roulston and Cane 2000b). More importantly, bee-collected pollen by definition reflects bee preferences and may thus not be highly representative for the flowering plant community. Hence, the analysis of hand-collected pollen would provide a more accurate picture of pollen nutrients.

Here, we conducted a meta-analysis on the datasets published in 70 studies (Supplementary Material S1) to better understand which factors (phylogenetic relatedness and/or pollinator attraction) are associated with (i) pollen nutritional content and (ii) nutrient ratios. We tested for a phylogenetic signal and an effect of insect dependence (i.e., full, high, low or no insect pollinator dependence) on various nutrients as well as their ratios. We expected (1) a phylogenetic signal in pollen nutritional content as a consequence of phylogenetic relatedness. Due to the strong effect of pollen nutritional quality on pollinator fitness and thus flower choice behavior, we further hypothesized that insect dependence is correlated with pollen nutrient content and the dietary requirements of their (main) pollinators. If so, we expected (2) this to result in differences in nutritional content between different levels of insect pollinator dependence, which are independent of the plants' phylogenetic relatedness. Notably, there is no common quality parameter that serves all pollinators. Instead, nutritional requirements may depend on different pollinator species/groups. Unfortunately, very little is known on nutritional requirements of different pollinators. As our dataset is largely confined to insect-pollinated plants, we primarily took into account nutritional requirements of insect herbivores, many of which were found to regulate protein intake (Simpson and Raubenheimer 2012). As protein and fat further appear to be the most important and most abundant nutrients in pollen and to affect the foraging behavior of bees (Leonhardt and Blüthgen 2012; Roulston and Cane 2000b; Vaudo et al. 2016a; Vaudo et al. 2016b), we hypothesized to find differences in contents and ratios particularly for these two nutrients.

Materials and Methods WP1

We compiled data from literature on the nutritional content of one or more pollen nutrients. In total, our study includes 387 different plant species belonging to 229 plant genera in 75 different families (Fig. WP1.1, Supplementary Material, Table WP1.S1). We included all studies, which provided data on sugar content, crude protein content, polypeptide content, free amino acid content and/or lipid content of pollen as well as information about whether the pollen was bee-collected (58.6% of the dataset) or hand-collected (41.4%) (Supplementary Material, Table WP1.S1). As most of the studies on carbohydrate content solely included sugars, while some others included all carbohydrates or both, we included only the sugar content (but still refer to “protein to carbohydrate” ratio, which is the common term used in most studies). For the units (w/w or percentages of dry weight) in which nutrient contents were reported varied between studies, we only used studies with clearly defined units and converted all units into percentages of total pollen dry weight. We further calculated the protein to carbohydrate (P:C)-ratio and the protein to lipid (P:L)-ratio based on nutrient percentages.

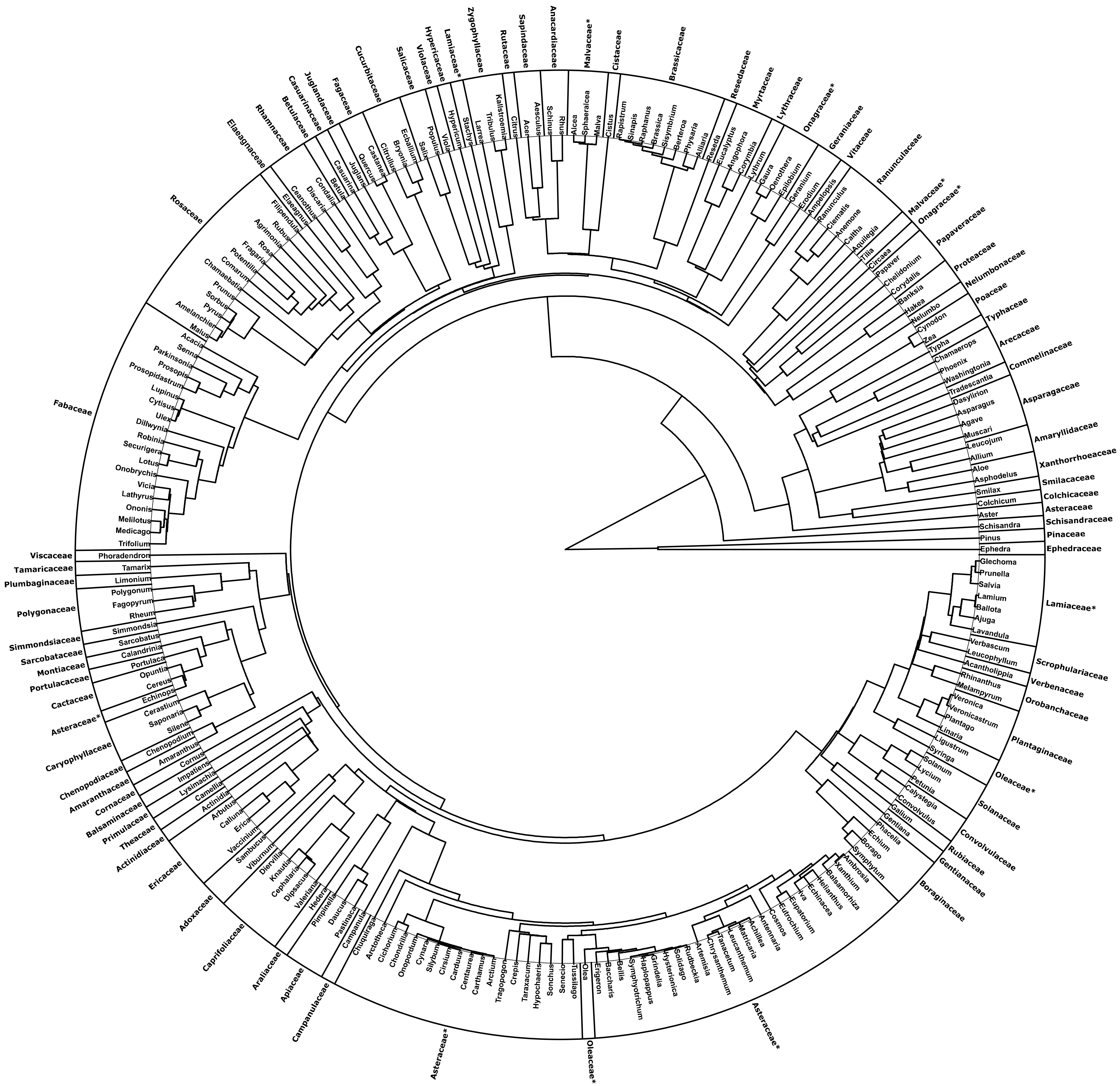


Figure WP1.1 Phylogenetic tree of the plant genera included in this meta-analysis. Plant families are indicated along the outer circle of the tree. The tree was generated based on the molecular phylogeny of Zanne et al. (2014) using the *pez* package in R. Asterisks behind family names indicate families that appear at least twice in the generated phylogeny. Isolated single genera that were not placed in the correct family were excluded from subsequent phylogenetic analyses.

As insects are typically the most abundant and most common pollinators (Faegri and Van der Pijl 2013) and insect pollination is considered the oldest form of pollination (Labandeira and Currano 2013), the majority of animal-pollinated plants in our study was found to be pollinated by insects with only few bird- and mammal-pollinated species (e.g., Australian *Banksia* species (Hopper 1980)). We thus included only insect-pollinated plants in our analyses, as the degree of dependence on animal pollination is hardly known for other plants.

Where available (~ 70% of plants in the dataset), we assigned the level of insect pollinator dependence to each plant species based on information provided by the BiolFlor database v 1.1 (http://www2.ufz.de/biolflor/index.jsp, accessed in April 2019). We classified plants as (I) fully dependent on insect pollinators when they need insects for pollination and are self-incompatible (N = 103, ca. 43% of the dataset), (II) highly dependent on insect pollinators when they are mostly insect pollinated, but can self-pollinate and are self-compatible (N = 76, ca. 32%), (III) little dependent on insect pollinators when they are mainly wind- or self-pollinated, but can also be pollinated by insects (N = 36, ca. 15%), and (IV) independent of insect pollinators when they are exclusively wind- or self-pollinated (N = 24, ca. 10%). Classifications III and IV were combined in one category (i.e. low to none dependence on insect pollinators) in our statistical analyses.

All analyses were performed at the plant species level. Initial data screening with generalized linear models (GLMs, McCullagh 2018) revealed significant interactions between nutrient content and collection method (i.e., whether pollen was bee- or hand-collected, Table WP1.1). To assess whether the effect of collection method was due to differences in nutrient contents or to differences in the selection of plant species in each dataset, we restricted the dataset to those plant species that were found in both original datasets (N = 29)

and performed separate t-tests to test for differences in nutrient contents between collection methods. For this dataset, we found differences in the sugar content of pollen of the same plant species (Table WP1.1). Interestingly, the mean relative lipid content was also higher in hand-collected than in bee-collected plants. We therefore treated both datasets separately in subsequent analyzes.

Table WP1.1 Results of general linear models (GLMs) (*F* and *P (>F)*) testing for a significant interaction between nutrient content and collection method (for the entire dataset), and t-tests (*t* and *P (>t)*) testing for differences in the relative contents (% dry weight) of the three main macronutrients as well as polypeptides and free amino acids between the two different collection methods (hand- or bee-collected, for a dataset reduced to 29 plant species that were included in both collection methods). Shown are the standardized means of each group and *t*- and *P*-values (significance level: $P < 0.05$). Significant *P*-values are marked in bold.

Nutrients	Mean bee-collected	Mean hand-collected	*F*	*P (>F)*	*t*	*P (>t)*
Crude protein (N = 29)	0.932	1.019	1.814	0.079	-1.029	0.312
Polypeptides (N = 27)	1.051	1.000	2.737	**0.004**	0.282	0.782
Free amino acids (N = 21)	2.296	1.639	1.484	0.169	0.780	0.449
Lipids (N= 18)	0.888	2.190	2.037	0.161	-1.221	0.285
Sugars (N = 9)	1.697	0.807	5.340	**0.033**	2.854	**0.028**

We then tested for a phylogenetic signal in nutritional content according to Junker et al. (2017). We used Blomberg's *K* to test whether nutrient contents correlated with phylogeny and were therefore likely influenced by the evolutionary history of a plant species. Blomberg's *K* depicts the variance between phylogenetic clades in relation to the variance within clades. The underlying phylogenetic tree was based on the recent molecular phylogeny of Zanne et al. (2014) restricted to those plant genera that were included in our analysis (Fig. WP1.1). The tree was constructed using the R-package *pez* (Pearse et al. 2015). Missing species were bound, and terminal branches pruned to produce a cladogram.

For the bee-collected pollen dataset, we subsequently performed phylogenetic analyzes of variance (phyl-ANOVA) for each nutrient group and ratio to test for differences between the

different pollination strategies independent of plant relatedness (using the R package *phytools*). Post-hoc pairwise comparisons were corrected with False Discovery Rate (FDR, Benjamini and Hochberg 1995). Unfortunately, the small sample size for the hand-collected dataset precluded equivalent statistical tests, and we therefore present the data on hand-collected samples only graphically (Figure S1). To further assess whether contents of different nutrients were correlated, e.g., due to linked biochemical pathways, we used phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation (using the R package *nlme*). *P*-values were corrected for multiple testing using FDR. All statistical tests were performed in R v3.5.1 (R Core Team 2018).

Results WP1

Pollen crude protein content and the P:L-ratio showed a clear phylogenetic signal (Blomberg's *K*) for both the bee- and hand-collected pollen dataset (Table WP1.2). Polypeptides showed a phylogenetic signal only for hand-collected pollen (Table WP1.2). Contents and ratios of all other nutrients were not correlated with plant phylogenetic relatedness (Table WP1.2).

Table WP1.2 Results of Blomberg's *K*-test testing for a phylogenetic signal for each nutrient for the bee- and hand-collected dataset. Shown are Blomberg's *K* and *P*-values. Significant *P*-values after Benjamini Hochberg correction (FDR) are marked in bold.

Nutrients	Collection method	*K*	*P*
Crude protein (N = 179)	Bee	0.199	**0.001**
	Hand	0.237	**0.016**
Polypeptides (N = 167)	Bee	0.029	0.968
	Hand	0.828	**0.001**
Free amino acids (N = 79)	Bee	0.541	0.391
	Hand	0.134	0.509
Lipids (N = 67)	Bee	0.054	0.538
	Hand	0.416	0.114
Sugars (N = 38)	Bee	0.165	0.216
	Hand	0.082	0.849
Protein:carbohydrate ratio (N = 38)	Bee	0.077	0.739
	Hand	0.605	0.101
Protein:lipid ratio (N = 59)	Bee	0.208	**0.012**
	Hand	0.776	**0.029**

In bee-collected pollen, the crude protein content was further higher in plants that are fully or highly depending on insect pollinators, independent of their phylogenetic relatedness (Table WP1.3, Fig. WP1.2A). The protein to lipids ratio also tended to be higher in plants that are fully or highly depending on insect pollinators, independent of their phylogenetic relatedness (Table WP1.3, Fig. WP1.2G). Free amino acids, polypeptides and lipids, the protein to carbohydrate ratio as well as pollen sugar content (Table WP1.3, Fig. WP1.2 C, E) did not significantly differ between different degrees of insect pollinator dependencies.

Table WP1.3 Results of phylogenetic ANOVAs testing for differences in the relative contents (% dry weight) and ratios of the four main macronutrients between different degrees of insect pollinator dependence for the bee-collected pollen dataset. Shown are *F*- and *P*-values. The number of plant species (N) for each nutrient is indicated in brackets behind each nutrient. *P*-values indicating significant differences after Benjamini Hochberg correction (FDR) between pollination strategies are marked in bold.

Nutrients	***F***	***P***
Crude protein (N = 87)	14.345	**0.001**
Polypeptides (N = 25)	1.3891	0.275
Free amino acids (N = 17)	0.820	0.314
Lipids (N = 41)	0.652	0.473
Sugars (N = 23)	0.664	0.525
Protein:carbohydrate ratio (N = 23)	0.063	0.940
Protein:lipid ratio (N = 34)	2.094	0.095

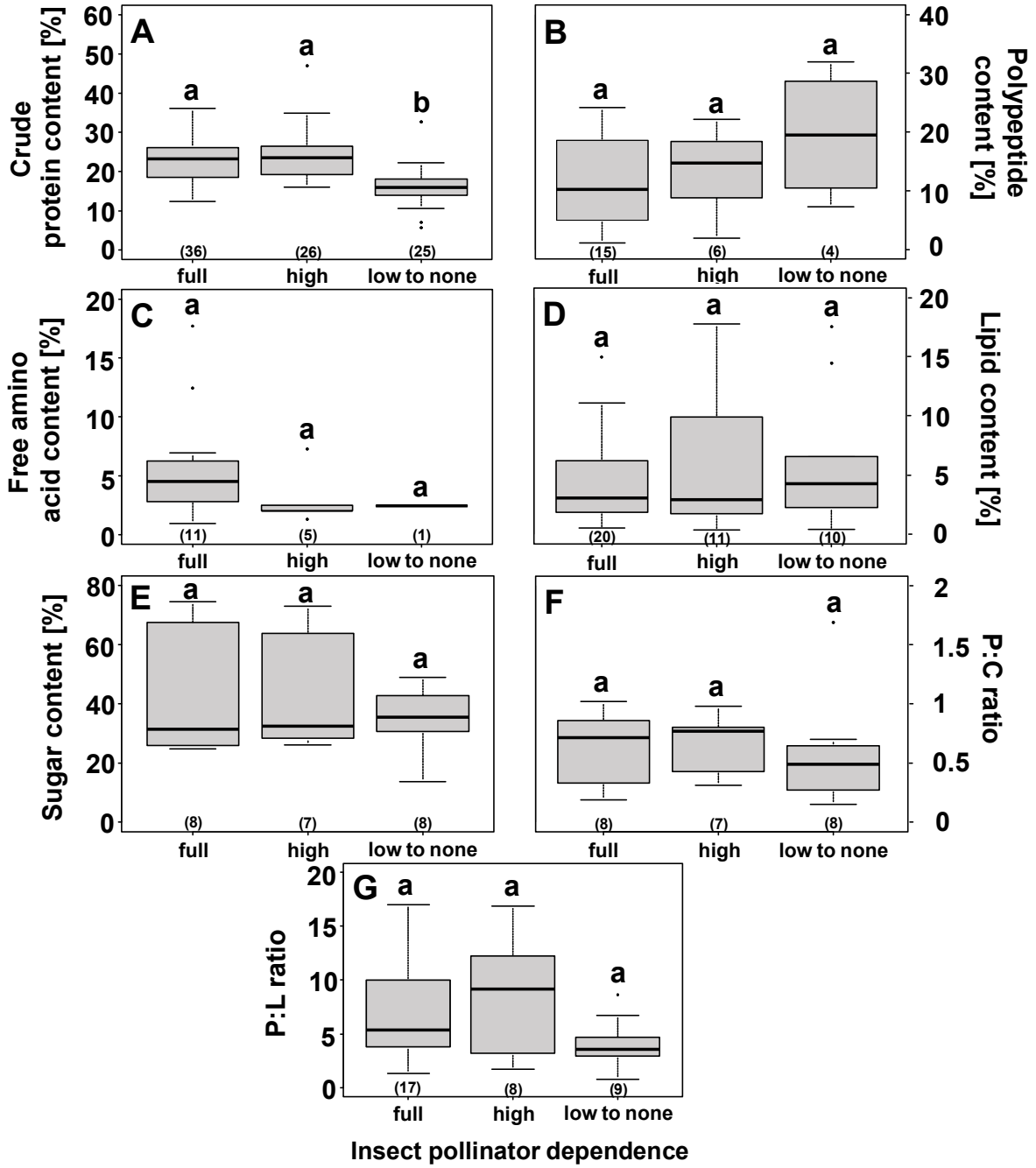


Figure WP1.2 Differences in the relative content (% pollen dry weight) of crude protein (A), polypeptides (B), free amino acids (C), lipid (D) and sugars (E), as well as the protein to carbohydrate-ratio (P:C-ratio, F) and protein to lipid-ratio (P:L-ratio, G) of plants differing in the degree of insect pollinator dependence for bee-collected pollen. Numbers in brackets below boxplots give the numbers of plant species included in each group. Different letters above boxes indicate significant differences between different degrees of insect pollinator dependence (following pairwise comparisons of the phylogenetic ANOVA posthoc tests corrected for multiple testing). Boxplots represent the median (central mark), the 25th and 75th percentiles (edges of the boxes), the most extreme data points (whiskers). Outliers (outside of the range of 1.5 x IQR) are plotted individually (dots).

For bee-collected pollen, contents of crude protein, free amino acids and polypeptides were correlated (Table WP1.4), and the relative amount of crude protein was correlated with the

relative amounts of lipids and sugars (Table WP1.4). Additionally, the relative amount of lipids was correlated with the relative amount of polypeptides (Table WP1.4). For the hand-collected dataset, only the relative amount of crude protein was correlated with the relative amount of free amino acids and polypeptides (Table WP1.5).

Table WP1.4 Phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between the four main macronutrients and free amino acids in bee-collected pollen. Given are *t*- and *P*-values. Significant *P*-values after Benjamini Hochberg correction (FDR) are marked in bold. N gives the number of plant species included in each correlation analysis.

Nutrients	**Polypeptides**	**Free amino acids**	**Lipids**	**Sugar**
Crude protein	*t* = 3.185 ***P* = 0.002** (N = 25)	*t* = 3.434 ***P* < 0.001** (N = 20)	*t* = 5.712 ***P* < 0.001** (N = 76)	*t* = 2.888 ***P* = 0.007** (N = 29)
Polypeptides	-	*t* = -16.245 ***P* < 0.001** (N = 23)	*t* = 4.033 ***P* = 0.002** (N = 14)	*t* = -1.007 *P* = 0.335 (N = 13)
Free amino acids	-	-	*t* = -0.217 *P* = 0.833 (N = 13)	*t* = 1.813 *P* = 0.103 (N = 11)
Lipids	-	-	-	*t* = -0.815 *P* = 0.421 (N = 33)

Table WP1.5 Phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between the four main macronutrients and free amino acids for the hand-collected pollen. Given are *t*- and *P*-values. Significant *P*-values after Benjamini Hochberg correction (FDR) are marked in bold. N gives the number of plant species included in each correlation analysis.

Nutrients	**Polypeptides**	**Free amino acids**	**Lipids**	**Sugar**
Crude protein	*t* = 3.402 ***P* = 0.006** (N = 13)	*t* = 3.272 ***P* = 0.010** (N = 11)	*t* = 1.008 *P* = 0.328 (N = 18)	*t* = -0.312 *P* = 0.760 (N = 17)
Polypeptides	-	*t* = 0.605 *P* = 0.546 (N = 133)	*t* = 0.536 *P* = 0.620 (N = 6)	*t* = -0.682 *P* = 0.544 (N = 5)
Free amino acids	-	-	*t* = -0.822 *P* = 0.497 (N = 4)	*t* = -2.348 *P* = 0.143 (N = 4)
Lipids	-	-	-	*t* = 0.085 *P* = 0.933 (N = 15)

Discussion WP1

In this meta-analysis, we show that the degree of a plant species' dependence on insects for pollination correlated with differences in the crude protein content of pollen, independent of the plants' phylogeny. In line with our hypothesis of pollinator-mediated selection, pollen protein content increased with increasing dependence on insect pollinators (Fig. WP1.2A). Our study also revealed a significant phylogenetic signal for crude protein and the P:L-ratio of pollen. Variation in the relative content of other nutrients did not clearly correlate with phylogenetic relatedness, indicating that phylogenetic relatedness alone may not always explain variation in overall pollen nutritional content, as has recently also been shown for pollenkitt lipids (Chichiriccò et al. 2019). Notably, detecting phylogenetic signals with the help of statistical tools is a useful measure of pattern, but cannot be interpreted as evidence of acting evolutionary processes (Revell et al. 2008).

Alternatively, pollen nutritional variation may be explained by biotic or abiotic factors, such as pollinator requirements, soil quality (Lau and Stephenson 1993), surrounding plant species (Sargent et al. 2011), temperature (van der Kooi et al. 2019a) as well as plant species-specific traits. For example, an increased style length may require the pollen grain to contain higher amounts of nutrients involved in pollen tube growth, which might result in significant differences in macronutrient (i.e. protein, fat and carbohydrate) content.

Our finding that pollen protein content was considerably higher in plants that fully or highly depended on insect pollinators compared to plants with low insect dependence, independent of phylogenetic relatedness, suggests that the importance of pollen protein could exert a considerable selective pressure on animal-pollinated plants. Indeed, bumblebees prefer plants with pollen of comparatively high protein content or a high P:L-ratio (Leonhardt and

Blüthgen 2012; Ruedenauer et al. 2016; Somme et al. 2015; Vaudo et al. 2016b), which may be due to the importance of pollen nutritional quality for insect larval development (Haydak 1970; Herbert et al. 1977; Raubenheimer and Simpson 1999). As a consequence, plants that highly depend on animals for pollination appear to produce pollen of a comparatively higher protein content compared to plants with low or no dependence on insect pollinators (Figure 2A). Plants that do not or weakly rely on animals for pollination clearly also produce some pollen nutrients, because those nutrients are necessary for pollen germination and/or pollen tube growth. Pollinators are known to collect pollen also from these plants (Saunders 2018) and likely transfer pollen in the process, which seemingly contradicts the apparent preference of pollinators for pollen of high protein content. However, this behavior may be explained by the pollinators' need to mix pollen of different plant species to e.g. dilute toxic pollen or adjust nutrient ratios (Simpson and Raubenheimer 2012) or because these plants may, at times, simply be the only nutrient sources available (Ackerman 2000).

For both the bee- and hand-collected dataset, we further found crude protein, free amino acids and polypeptides to be correlated, which may be due to shared biochemical pathways (Külheim et al. 2009). Additionally, lipids were correlated with crude protein, sugar and polypeptides at least in the bee-collected dataset. The ratio of protein to lipids was found to strongly affect bumblebee foraging preferences with lipid intake being more strongly regulated than protein intake (Vaudo et al. 2016b). From the bees' perspective, a correlation between these two nutrient groups would enable them to more easily assess and potentially even regulate both macronutrients and their ratio simultaneously, e.g. by mixing pollen from different plants (Kriesell et al. 2017). After all, pollen is a complex mixture of a relatively high number of different substances, potentially rendering it challenging for pollinators to assess them all simultaneously. Moreover, amino acids correlate with each other (Weiner et al.

2010) as well as with the total protein content across plants. Bees do therefore not need to perceive all amino acids, because it suffices to perceive some amino acids in order to make inferences on overall amino acid/protein content (Ruedenauer et al. 2019a).

Interestingly, earlier studies on pollen protein content revealed partly contrasting results. While Roulston et al. (2000) also found a phylogenetic signal for crude pollen protein, they did not find differences between different pollination strategies as found in our analysis and by Hanley et al. (2008). This discrepancy could be due to different statistical approaches. Also, a potential limitation of meta-analyses on pollen nutrients is that extraction protocols and analytical methods applied usually differ among studies, which increases overall variance. For example, in our dataset, highly different analytical approaches were used to analyze pollen lipid content, some being highly lipid specific, others also extracting additional non-polar substances besides lipids. We could, unfortunately, not restrict our dataset to studies which performed more specific analyses due to the overall small sample size of studies which have analyzed pollen lipids. Future studies using standardized analytical methods for different nutrients and high numbers of plant species should provide more robust datasets.

In conclusion, our results suggest that variation in pollen nutritional content is not only determined by phylogenetic relatedness, but also other factors, e.g. selection imposed by pollinator preferences or by plant species' traits demanding high pollen nutrient contents. In plant species that are fully or highly dependent on insect pollinators, pollen nutrient contents and ratios might even have co-evolved with the needs of their insect pollinator partners.

Supplementary material WP1

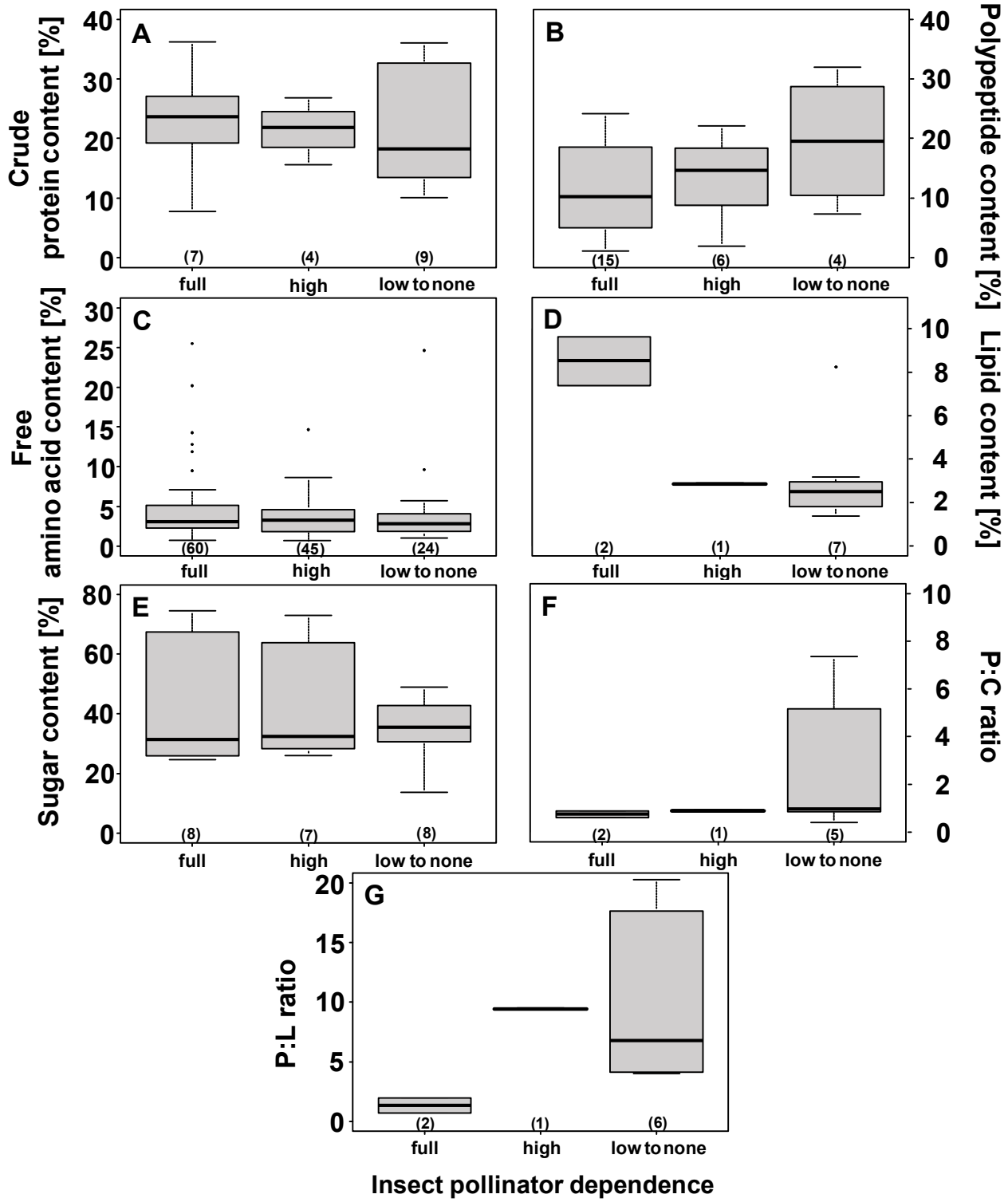


Figure WP1.S1 Differences in the relative crude protein (A), polypeptide (B), free amino acid (C), lipid (D) and sugar (E) content (% pollen dry weight), the protein to carbohydrate-ratio (P:C-ratio, F) and protein to lipid-ratio (P:L-ratio, G) of plant genera with different degrees of insect pollinator dependence for hand-collected pollen. Numbers in brackets below boxplots give the numbers of plant species included in each group.
Boxplots represent the median (central mark), the 25th and 75th percentiles (edges of the boxes), the most extreme data points (whiskers). Outliers (outside of the range of 1.5 x IQR) are plotted individually (dots).

Table WP1.S1 List of the plant species compiled and used for the meta-analysis including their families, the degree of insect pollinator dependence (see Material and Methods, based on the Biolflor database: http://www2.ufz.de/biolflor/index.jsp) as well as the reference studies from which the data was obtained. NA indicates that no data was available.

Family	Genus	Species	Insect pollinator dependence (Biolflor database)	References
Actinidiaceae	*Actinidia*	*chinensis*	low	Liolios et al., 2016
		deliciosa		Clark & Lintas, 1992, Vanderplanck et al., 2014
Adoxaceae	*Sambucus*	*nigra*	low	Weiner et al., 2010
	Viburnum	*lantana*		Weiner et al., 2010
Amaranthaceae	*Amaranthus*	*palmeri*	none	Schmidt et al., 1987
	Chenopodium	*album*		Liolios et al., 2016
Amaryllidaceae	*Allium*	*cepa*	high	Weiner et al., 2010
		ursinum	low	Weiner et al., 2010
	Leucojum	*vernum*	full	Weiner et al., 2010
Anacardiaceae	*Rhus*	*lancea*	full	Schmidt et al., 1987
	Schinus	*fasciculatus*	NA	Andrada & Tellería, 2005
Apiaceae	*Daucus*	*carota*	high	Weiner et al., 2010
	Pastinaca	*sativa*		Weiner et al., 2010, Liolios et al., 2016
	Pimpinella	*peregrina*		Liolios et al., 2016
Araliaceae	*Hedera*	*helix*	full	Weiner et al., 2010, Vanderplanck et al., 2014
Arecaceae	*Chamaerops*	*humilis*	NA	Liolios et al., 2016
	Phoenix	*dactylifera*		Todd & Bretherick, 1947
Asparagaceae	*Agave*	*palmeri*	NA	Schmidt et al., 1987
	Asparagus	*officinalis*	high	Todd & Bretherick, 1966
	Dasylirion	*wheeleri*	NA	Schmidt et al., 1987
	Muscari	*comosum*	high	Weiner et al., 2010
Asteraceae	*Achillea*	*millefolium*	full	Weiner et al., 2010
	Antennaria	*dioica*	full	Weiner et al., 2010
	Arctium	*lappa*	low	Pernal & Currie, 2001
		minus		Weiner et al., 2010
		tomentosum		Weiner et al., 2010
	Arctotheca	*calendula*	NA	Somerville, 2001
	Aster	*tripolium*	high	Vanderplanck et al., 2014
	Baccharis	*sarothroides*	NA	Schmidt, 1982, Schmidt et al., 1987
	Bellis	*perennis*	low	Weiner et al., 2010
	Carduus	*acanthoides*	low	Somerville, 2001
		nutans		Weiner et al., 2010
	Carthamus	*lanatus*	none	Somerville, 2001
Asteraceae	*Centaurea*	*cyanus*	full	Weiner et al., 2010
		jacea		Weiner et al., 2010
		solstitialis		Todd & Bretherick, 1958, Somerville, 2001, Andrada & Tellería, 2005
	Chondrilla	*juncea*	full	Somerville, 2001

	Chrysanthemum	*indicum*	NA	Yang et al., 2013
	Chuquiraga	*erinacea*	NA	Andrada & Tellería, 2005
	Cichorium	*intybus*	full	Pernal & Currie, 2001, Weiner et al., 2010, Liolios et al., 2016
	Cirsium	*arvense*	full	Pernal & Currie, 2001, Weiner et al., 2010
		oleraceum		Weiner et al., 2010
		palustre		Somme et al., 2015
		vulgare		Somerville, 2001, Weiner et al., 2010
	Cosmos	*bipinnatus*	full	Singh et al., 1999
	Crepis	*biennis*	high	Weiner et al., 2010
	Echinacea	*purpurea*	NA	Vaudo et al., 2016
	Echinops	*sphaerocephalus*	high	Weiner et al., 2010
	Erigeron	*annuus*	high	Weiner et al., 2010
	Eupatorium	*perfoliatum*	full	Vaudo et al., 2016
	Eutrochium	*purpureum*	NA	Vaudo et al., 2016
	Grindelia	*squarrosa*	NA	Levin & Bohart, 1955, McCaughey et al., 1980
		tehuelches		Andrada & Tellería, 2005
	Haplopappus	*laricifolius*	NA	Schmidt et al., 1987
		tenuisectus	NA	McCaughey et al., 1980
	Helianthus	*annuus*	full	Singh et al., 1999, Pernal & Currie, 2000, Somerville, 2001, Weiner et al., 2010, Nicolson & Human, 2013, Yang et al., 2013, Vanderplanck et al., 2014
	Hypochaeris	*radicata*	full	Somerville, 2001, Weiner et al., 2010
	Hysterionica	*jasionoides*	NA	Andrada & Tellería, 2005
	Iva	*axillaris*	none	Levin & Bohart, 1955
	Leucanthemum	*ircutianum*	high	Weiner et al., 2010
		vulgare		Weiner et al., 2010
	Matricaria	*recutita*	high	Weiner et al., 2010
	Onopordum	*acanthium*	high	Andrada & Tellería, 2005
	Rudbeckia	*fulgida*	full	Weiner et al., 2010
	Senecio	*erucifolius*	full	Weiner et al., 2010
		fuchsii		Weiner et al., 2010
		jacobaea		Weiner et al., 2010
		madagascariensis		Somerville, 2001
	Silybum	*marianum*	high	Todd & Bretherick, 1955, Liolios et al., 2016
	Solidago	*canadensis*	full	Pernal & Currie, 2001
	Sonchus	*arvensis*	full	Pernal & Currie, 2001
	Symphyotrichum	*novae-angliae*	NA	Vaudo et al., 2016
	Tanacetum	*vulgare*	high	Weiner et al., 2010
	Taraxacum	*officinale*	none	Loper & Berdell, 1980, McCaughey et al., 1980, Schmidt et al., 1987, Weiner et al., 2010, Liolios et al., 2016
		vulgare	none	Todd & Bretherick, 1949
	Tragopogon	*pratensis*	high	Weiner et al., 2010
	Tussilago	*farfara*	high	Weiner et al., 2010
	Xanthium	*strumarium*	none	Pernal & Currie, 2001
Balsaminaceae	*Impatiens*	*glandulifera*	full	Weiner et al., 2010, Vanderplanck et al., 2014
		parviflora	low	Weiner et al., 2010

Betulaceae	*Betula*	*pendula*	low	Weiner et al., 2010
Boraginaceae	*Borago*	*officinalis*	full	Weiner et al., 2010
	Echium	*vulgare*	high	Somerville, 2001, Weiner et al., 2010
	Phacelia	*tanacetifolia*		Pernal & Currie, 2000
	Symphytum	*officinale*		Weiner et al., 2010
Brassicaceae	*Alliaria*	*petiolata*	low	Weiner et al., 2010
	Berteroa	*incana*	high	Weiner et al., 2010
	Brassica	*kaber*	high	Todd & Bretherick, 1961
		napus		Evans et al., 1991, Pernal & Currie, 2001, Somerville, 2001, Weiner et al., 2010, Yang et al., 2013
		nigra	full	Levin & Bohart, 1955, Todd & Bretherick, 1959
		rapa		Todd & Bretherick, 1965, Singh et al., 1999, Pernal & Currie, 2000
	Physaria	*gordoni*	NA	Loper & Berdell, 1980
	Raphanus	*sativus*	high	Singh et al., 1999
	Rapistrum	*rugosum*	full	Somerville, 2001
	Sinapis	*alba*	full	Pernal & Currie, 2001, Szczêsna, 2006
		arvensis		Szczêsna, 2006
	Sisymbrium	*irio*	low	Loper & Berdell, 1980, Liolios et al., 2016
		officinale		Somerville, 2001
Cactaceae	*Cereus*	*aethiops*	NA	Andrada & Tellería, 2005
		gigantea		Loper & Berdell, 1980, McCaughey et al., 1980, Schmidt, 1982, Schmidt et al., 1987
Campanulaceae	*Campanula*	*glomerata*	full	Weiner et al., 2010
		patula		Szczêsna, 2006, Weiner et al., 2010
		rapuncoloides		Weiner et al., 2010
		trachelium		Weiner et al., 2010
Caprifoliaceae	*Cephalaria*	*transsylvanica*	NA	Liolios et al., 2016
	Diervilla	*lonicera*		Pernal & Currie, 2001
	Valeriana	*officinalis*	full	Weiner et al., 2010
		repens		Somme et al., 2015
Caryophyllaceae	*Cerastium*	*arvense*	full	Weiner et al., 2010
	Saponaria	*officinalis*	low	Weiner et al., 2010
	Silene	*dioica*	full	Weiner et al., 2010
		latifolia		Weiner et al., 2010
Casuarinaceae	*Casuarina*	*littoralis*	NA	Somerville, 2001
Colchicaceae	*Colchicum*	*autumnale*	full	Weiner et al., 2010
Commelinaceae	*Tradescantia*	*ohiensis*	NA	Vaudo et al., 2016
Convolvulaceae	*Calystegia*	*sepium*	high	Weiner et al., 2010
	Convolvulus	*arvensis*	full	Weiner et al., 2010
Cornaceae	*Cornus*	*stolonifera*	high	Pernal & Currie, 2001
Cucurbitaceae	*Bryonia*	*dioica*	full	Weiner et al., 2010
	Citrullus	*lanatus*	NA	Loper & Berdell, 1980, Yang et al., 2013
	Dipsacus	*fullonum*	high	Weiner et al., 2010
	Ecballium	*elaterium*	NA	Liolios et al., 2016
	Knautia	*arvensis*	high	Weiner et al., 2010
Elaeagnaceae	*Elaeagnus*	*angustifolia*	high	Liolios et al., 2016

Ephedraceae	*Ephedra*	*trifurca*	NA	Schmidt et al., 1987
Ericaceae	*Arbutus*	*unedo*	NA	Rasmont et al., 2005
	Calluna	*vulgaris*	high	Vanderplanck et al., 2014
	Erica	*manipuliflora*		Liolios et al., 2016
	Vaccinium	*angustifolium*		Pernal & Currie, 2001
Fabaceae	*Acacia*	*auriculaeformis*	NA	Agarwal & Nair, 1989
		baileyana		Rayner & Langridge, 1985
		cunninghamii		Kleinschmidt & Kondos, 1976
		dealbata		Muss, 1987
		greggi		McCaughey et al., 1980
		implexa		Kleinschmidt & Kondos, 1976
		ixiophylla		Kleinschmidt & Kondos, 1976
		melanoxylon		Kleinschmidt & Kondos, 1976
		polybotrya		Kleinschmidt & Kondos, 1976
		pycnantha		Muss, 1987
	Cytisus	*scoparius*	full	Vanderplanck et al., 2014
	Lathyrus	*pratensis*	full	Weiner et al., 2010
	Lotus	*corniculatus*	full	Pernal & Currie, 2001, Weiner et al., 2010
	Lupinus	*angustifolius*	high	Somerville, 2001
		polyphyllus		Weiner et al., 2010
	Medicago	*falcata*	full	Weiner et al., 2010
		minima	none	Andrada & Tellería, 2005
		sativa	full	Levin & Bohart, 1955, McCaughey et al., 1980, Weiner et al., 2010, Vanderplanck et al., 2014
	Melilotus	*albus*	low	Pernal & Currie, 2001
		officinalis	full	Pernal & Currie, 2000, Pernal & Currie, 2001
	Onobrychis	*viciifolia*	full	Weiner et al., 2010
	Ononis	*spinosa*	high	Weiner et al., 2010
	Parkinsonia	*aculeata*	NA	McCaughey et al., 1980
	Prosopidastrum	*globosum*	NA	Andrada & Tellería, 2005
	Prosopis	*velutina*	NA	McCaughey et al., 1980, Schmidt et al., 1987
	Robinia	*pseudoacacia*	high	McCaughey et al., 1980, Liolios et al., 2016
	Securigera	*varia*	full	Weiner et al., 2010
	Senna	*hebecarpa*	NA	Vaudo et al., 2016
	Trifolium	*balansae*	full	Pernal & Currie, 2001
		hybridum		Pernal & Currie, 2001
		medium		Weiner et al., 2010
		pratense		McCaughey et al., 1980, Weiner et al., 2010, Somme et al., 2015
		repens		Todd & Bretherick, 1964, Pernal & Currie, 2001, Somerville, 2001
	Ulex	*europaeus*	high	Somerville, 2001
	Vicia	*cracca*	high	Pernal & Currie, 2001
		faba		Somerville, 2001, Yang et al., 2013
		sepium	low	Weiner et al., 2010
Fagaceae	*Castanea*	*sativa*	none	Vanderplanck et al., 2014, Conte et al., 2016
	Quercus	*kelloggii*	none	Todd & Bretherick, 1954

Gentianaceae	*Gentiana*	*lutea*	full	Weiner et al., 2010
Geraniaceae	*Erodium*	*cicutarium*	low	Andrada & Tellería, 2005
	Geranium	*pratense*	full	Weiner et al., 2010
		pyrenaicum	high	Weiner et al., 2010
		sylvaticum	full	Weiner et al., 2010
Hypericaceae	*Hypericum*	*perforatum*	high	Todd & Bretherick, 1971, Weiner et al., 2010
Juglandaceae	*Juglans*	*nigra*	none	Todd & Bretherick, 1946, Liolios et al., 2016
Lamiaceae	*Ajuga*	*reptans*	high	Weiner et al., 2010
	Ballota	*nigra*	high	Weiner et al., 2010
	Glechoma	*hederacea*	full	Weiner et al., 2010
	Lamium	*album*	high	Weiner et al., 2010
		galeobdolon		Weiner et al., 2010
		maculatum		Weiner et al., 2010
		purpureum		Weiner et al., 2010
	Prunella	*vulgaris*	high	Weiner et al., 2010
	Salvia	*pratensis*	high	Weiner et al., 2010
		verbenaca		Liolios et al., 2016
	Stachys	*recta*	full	Weiner et al., 2010
		sylvatica	high	Weiner et al., 2010
Lythraceae	*Lythrum*	*salicaria*	full	Weiner et al., 2010
Malvaceae	*Alcea*	*rosea*	high	Weiner et al., 2010
	Malva	*alcea*	full	Weiner et al., 2010
		moschata		Weiner et al., 2010
		neglecta	high	Weiner et al., 2010
		sylvestris	full	Weiner et al., 2010
	Sphaeralcea	*australis*	NA	Andrada & Tellería, 2005
	Tilia	*americana*	high	Liolios et al., 2016
		cordata		Weiner et al., 2010
		intermedia		Pernal & Currie, 2001
Montiaceae	*Calandrinia*	*ciliata*	NA	Todd & Bretherick, 1953
Myrtaceae	*Angophora*	*floribunda*	NA	Somerville, 2001
	Corymbia	*calophylla*	NA	Somerville, 2001, Manning, 2006
		gummifera	NA	Somerville, 2001
		maculata	NA	Manning, 2006
	Eucalyptus	*accedens*	NA	Somerville, 2001
		albens		Somerville, 2001
		blakelyi		Somerville, 2001
		bridgesiana		Somerville, 2001
		camaldulensis		Somerville, 2001
		delegatensis		Somerville, 2001
		diversicolor		Somerville, 2001
		dumosa		Somerville, 2001
		fibrosa		Somerville, 2001
		globoidea		Somerville, 2001

		globulus		Somerville, 2001
		longifolia		Somerville, 2001
Myrtaceae	*Eucalyptus*	*macrorhyncha*	NA	Somerville, 2001
		mannifera		Somerville, 2001
		marginata		Somerville, 2001
		microcarpa		Somerville, 2001
		patens		Somerville, 2001
		polyanthemos		Somerville, 2001
		punctata		Somerville, 2001
		robusta		Todd & Bretherick, 1968
		saligna		Manning, 2006
		sclerophylla		Manning, 2006
		socialis		Manning, 2006
		viminalis		Manning, 2006
		wandoo		Manning, 2006
Nelumbonaceae	*Nelumbo*	*nucifera*	NA	Yang et al., 2013
Oleaceae	*Ligustrum*	*lucidum*	low	Liolios et al., 2016
	Olea	*europaea*	none	Todd & Bretherick, 1952, Liolios et al., 2016
Onagraceae	*Circaea*	*lutetiana*	low	Weiner et al., 2010
	Epilobium	*angustifolium*	high	Weiner et al., 2010
		hirsutum	low	Weiner et al., 2010
	Gaura	*lindheimeri*	full	Weiner et al., 2010
	Oenothera	*biennis*	low	Weiner et al., 2010
Orobanchaceae	*Melampyrum*	*pratense*	low	Weiner et al., 2010
	Rhinanthus	*alectorolophus*	high	Weiner et al., 2010
Papaveraceae	*Chelidonium*	*majus*	high	Szczêsna, 2006, Weiner et al., 2010
	Corydalis	*cava*	full	Weiner et al., 2010
	Papaver	*rhoeas*	full	Weiner et al., 2010, Yang et al., 2013, Liolios et al., 2016
Pinaceae	*Pinus*	*banksiana*	none	Pernal & Currie, 2000
		contorta		Todd & Bretherick, 1948
		halepensis		Schmidt, 1982, Andrikopoulus et al., 1985, Liolios et al., 2016
		radiata		Todd & Bretherick, 1943
		sabiniana		Todd & Bretherick, 1942
		taeda		Scott & Strohl, 1962
Plantaginaceae	*Linaria*	*vulgaris*	full	Weiner et al., 2010
	Plantago	*lanceolata*	low	Weiner et al., 2010
		media		Weiner et al., 2010
	Veronica	*chamaedrys*	high	Weiner et al., 2010
Plumbaginaceae	*Limonium*	*cornarianum*	full	Liolios et al., 2016
Poaceae	*Cynodon*	*dactylon*	none	Todd & Bretherick, 1956
	Zea	*mays*	none	Todd & Bretherick, 1945, Pernal & Currie, 2001, Somerville, 2001, Höcherl et al., 2012, Yang et al., 2013, Liolios et al., 2016
Polygonaceae	*Fagopyrum*	*esculentum*	full	Somerville, 2001, Yang et al., 2013
	Polygonum	*aviculare*	low	Liolios et al., 2016
		bistorta		Szczêsna, 2006

Portulacaceae	*Portulaca*	*oleracea*	low	Liolios et al., 2016
Primulaceae	*Lysimachia*	*nummularia*	full	Weiner et al., 2010
		punctata		Weiner et al., 2010
Primulaceae	*Lysimachia*	*vulgaris*	full	Weiner et al., 2010
Proteaceae	*Banksia*	*ericifolia*	full	Somerville, 2001
		ornata		Somerville, 2001
		serrata		Somerville, 2001
	Hakea	*sericea*	NA	Somerville, 2001
Ranunculaceae	*Anemone*	*ranunculoides*	high	Weiner et al., 2010
	Aquilegia	*vulgaris*	high	Weiner et al., 2010
	Caltha	*palustris*	full	Weiner et al., 2010
	Clematis	*vitalba*	high	Weiner et al., 2010
	Ranunculus	*acris*	full	Weiner et al., 2010
		bulbosus		Weiner et al., 2010
		lanuginosus		Weiner et al., 2010
		repens		Weiner et al., 2010
Resedaceae	*Reseda*	*lutea*	full	Weiner et al., 2010
Rhamnaceae	*Ceanothus*	*crassifolius*	NA	Todd & Bretherick, 1967
	Ceanothus	*integerrimus*		Todd & Bretherick, 1973
	Condalia	*macrophylla*		Andrada & Tellería, 2005
	Discaria	*americana*		Andrada & Tellería, 2005
Rosaceae	*Agrimonia*	*eupatoria*	low	Weiner et al., 2010
	Amelanchier	*humilis*	high	Pernal & Currie, 2001
		lamarckii	low	Weiner et al., 2010
	Chamaebatia	*foliolosa*	NA	Todd & Bretherick, 1963
	Comarum	*palustre*	NA	Somme et al., 2015
	Filipendula	*ulmaria*	low	Weiner et al., 2010
	Fragaria	*x_ananassa*	low	Grünfeld et al., 1989
	Malus	*domestica*	full	McCaughey et al., 1980, Pernal & Currie, 2000
	Potentilla	*fruticosa*	high	Pernal & Currie, 2001
		anserina	full	Weiner et al., 2010
		reptans	high	Weiner et al., 2010
	Prunus	*communis*	NA	Todd & Bretherick, 1972
		dulcis		Loper & Berdell, 1980, McCaughey et al., 1980, Schmidt, 1982, Schmidt et al., 1987, Somerville, 2001
		persica	high	Todd & Bretherick, 1969
		serotina		McCaughey et al., 1980
		spinosa		Weiner et al., 2010
	Pyrus	*communis*	full	Somerville, 2001
		pyraster		Liolios et al., 2016
	Rosa	*acicularis*	high	Pernal & Currie, 2001
		rugosa		Yang et al., 2013
	Rubus	*idaeus*	full	Liolios et al., 2016
		ulmifolius	high	Pernal & Currie, 2001
		fruticosus	NA	Weiner et al., 2010

	Sorbus	*aucuparia*	full	Vanderplanck et al., 2014
Rubiaceae	*Galium*	*album*	high	Weiner et al., 2010
Salicaceae	*Populus*	*deltoides*	none	Loper & Berdell, 1980, McCaughey et al., 1980
		fremontii		Schmidt, 1982, Schmidt et al., 1987
Salicaceae	*Populus*	*nigra*	none	McCaughey et al., 1980
	Salix	*alba*	low	Conte et al., 2016
		babylonica		Somerville, 2001
		caprea		Vanderplanck et al., 2011, Vanderplanck et al., 2014
		cinerea		Weiner et al., 2010
		dasyclados		Weiner et al., 2010
		discolor		Somerville, 2001
		nigra		Todd & Bretherick, 1960
		triandra		Weiner et al., 2010
		viminalis		Weiner et al., 2010
Santalaceae	*Phoradendron*	*californicum*	NA	Schmidt et al., 1987
Sapindaceae	*Acer*	*grandidentatum*	full	Weiner et al., 2010
		platanoides		Schmidt, 1982
	Aesculus	*hippocastanum*	full	Weiner et al., 2010, Liolios et al., 2016
Sarcobataceae	*Sarcobatus*	*vermiculatus*	NA	Levin & Bohart, 1955
Schisandraceae	*Schisandra*	*chinensis*	NA	Yang et al., 2013
Scrophulariaceae	*Leucophyllum*	*frutescens*	NA	Schmidt et al., 1987
	Verbascum	*pulverulentum*	low	Weiner et al., 2010
		thapsus	high	Weiner et al., 2010
	Veronicastrum	*virginicum*	NA	Vaudo et al., 2016
Simmondsiaceae	*Simmondsia*	*chinensis*	NA	Schmidt et al., 1987
Solanaceae	*Lycium*	*chinense*	full	Andrada & Tellería, 2005
	Petunia	*hybrida*	NA	Singh et al., 1999
	Solanum	*dulcamara*	high	Weiner et al., 2010
Theaceae	*Camellia*	*japonica*	NA	Yang et al., 2013
Typhaceae	*Typha*	*latifolia*	none	Todd & Bretherick, 1944, Schmidt et al., 1987
Verbenaceae	*Acantholippia*	*seriphoides*	NA	Andrada & Tellería, 2005
Violaceae	*Viola*	*reichenbachiana*	low	Weiner et al., 2010
Xanthorrhoeaceae	*Aloe*	*greatheadii*	NA	Human & Nicolson, 2006
	Asphodelus	*fistulosus*	NA	Somerville, 2001
Zygophyllaceae	*Kallstroemia*	*grandiflora*	NA	Schmidt et al., 1987
	Larrea	*divaricata*	NA	Loper & Berdell, 1980b
		tridentata	NA	McCaughey et al., 1980, Schmidt, 1982, Schmidt et al., 1987
	Tribulus	*terrestris*	NA	Liolios et al., 2016

WP2 Pollen nutrient composition correlates with plant dependence on insect pollinators

Authors:

Fabian A. Ruedenauer*, Stefan S. Sachs, Casper J. van der Kooi, Alexander Keller, Johannes Spaethe, Sara D. Leonhardt

*first author

published:

Journal:

Volume: Pages:

Abstract WP2

Background and aims

The (mutualistic) interaction between flowering plants and their insect pollinators has resulted in numerous co-adaptations in both plants and insects. Pollen plays an intriguing role, because it is subject to partly contrasting interests between the two mutualistic partners, i.e. reproduction in plants and food provision in some pollinators. It is still unclear to what degree nutritional requirements of pollen consuming insects, such as bees, have shaped the pollen nutrient profile of pollinator dependent plants. The goal of our study was to assess how this conflict of interest may have affected pollen nutrient profiles.

Methods

We chemically analyzed and compared pollen nutrient profiles (i.e. amino acids, fatty acids and sterols) of 139 plant species and tested, if nutrient profiles differed between plants with different levels of pollinator dependency. We specifically focused on possible correlations among different nutrient groups.

Key results

We could show that variation in pollen nutrient profiles, in particular protein content and protein to lipid ratio, were better explained by the plants' pollinator dependence than by phylogeny. We also revealed that correlations e.g. between single amino acids were twice as common in insect pollinated plants than in plants that do not depend on insect pollinators.

Conclusions

Our findings suggest that plants depending on pollinators may not only invest more energy into producing pollen of higher protein content, but also ensure similar ratios between different nutrients to facilitate (rapid) assessment of pollen nutritional quality by their insect pollinators. Correlations indicate stable ratios between specific nutrients and would enable pollinators to rapidly obtain critical information on complex pollen nutrient profiles based on only one or a few nutritional cues. Specific nutrient ratios have recently been shown to strongly affect pollinator foraging behavior and may therefore represent an efficient strategy for pollinator-dependent plants to increase pollinator visitation rate and fidelity.

Introduction WP2

As part of the mutualistic interaction between pollinators and flowering plants, nectar, pollen or other floral resources are often offered as food reward in exchange for pollination services. For many pollinating species, e.g. bees or butterflies, these resources are the major source of nutrients (Faegri and Van der Pijl 2013; Haydak 1970). The nutritional composition (henceforth referred to as nutritional quality) of floral rewards offered by species that strongly depend on their mutualistic partner is therefore expected to (at least partly) match the nutritional preferences of these partners. In fact, bees and other insect pollinators show distinct nutritional preferences and select plants to forage on accordingly (Camazine and Sneyd 1991; Nicholls and Hempel de Ibarra 2016; Ruedenauer et al. 2016; Somme et al. 2015). Bumble bees, for instance, preferentially collect pollen of high protein and low lipid content, which favors their survival and colony growth (Leonhardt and Blüthgen 2012; Ruedenauer et al. 2020; Somme et al. 2015; Vaudo et al. 2016b).

However, amounts and composition of nutrients in pollen often vary strongly between plant species (Hanley et al. 2008; Roulston et al. 2000; Roulston and Cane 2000b; Ruedenauer et al.

2019b). For example, pollen protein content can vary between 2.5% and 61% (Roulston et al. 2000) and pollen lipid content between 1% and 20% (Roulston and Cane 2000b) dry mass. Pollinators and other flower visiting animals therefore need to assess the nutritional profile of pollen of different plant species in order to assess its appropriateness. The sheer number of different nutritional compounds in pollen (e.g. > 25 amino acids, > 55 fatty acids, different sugars, micronutrients, plant secondary metabolites) is however likely too high to be fully and adequately assessed within the short timeframe of a pollinator visit. Instead, to facilitate efficient food collection, insects may confine assessment to a restricted set of nutrients to save time and energy. Indeed, recent work on nutritional perception revealed that *Bombus terrestris* workers restricted perception mostly to fatty acids and seemingly ignored amino acids when offered pollen of different nutritional quality (Ruedenauer et al. 2020), although they were able to detect and perceive amino acids, when offered in isolation (Ruedenauer et al. 2019a). Such restricted nutrient perception may be particularly beneficial, when different classes of nutrients were correlated, resulting in relatively stable ratios between nutrients. In theory, correlations between nutrients would enable bees and other pollen consumers to snapshot the whole nutrient profile by restricting perception to a few nutrients, thereby simplifying and thus speeding up nutritional quality assessment.

From the perspective of the plant, however, the primary function of pollen is to carry the male gamete. Pollen nutrients are mainly needed for initiating pollen tube growth, thereby directly supporting the plants' reproductive fitness (Roulston et al. 2000; Stanley and Linskens 1974). Nutritional interests of plants and pollinators are thus likely to differ, which creates a conflict of interest on pollen chemistry. In particular, those plant species that depend on pollinators for pollen transfer and offer pollen as reward face the dilemma of simultaneously needing to meet their own (reproductive) needs and the nutritional needs of their pollinators. It is still

largely unclear, however, to what degree nutritional requirements of pollen consuming insects, such as bees, have shaped the pollen nutrient profile of pollinator dependent plants.

Pollen is the main source of protein, fat and micronutrients and thus essential for offspring production, particularly for bee pollinators (Roulston and Cane 2000b; Todd and Bretherick 1942). Previous analyses of pollen nutrient composition were mostly restricted to few plant species or represented meta-analyses which used datasets with different analytical methods and often restricted to overall (protein, fat) content, thus lacking information on individual compounds, such as individual amino acids or fatty acids. To reduce variation induced by different methodologies and to enable detailed analyses with individual nutrients, we created a dataset on pollen nutrient profiles of 139 plant species using standardized analytical methods.

Our aim was to investigate (i) whether plant phylogenetic relatedness or pollinator dependency better explained variation in pollen nutritional profiles; (ii) whether nutrients showed correlations within and among specific nutrient classes and (iii) whether such potential correlations were coupled to the plant's dependence on pollinators.

We hypothesized that pollen protein, protein-related polypeptides and amino acids, as well as the protein to lipid-ratio (P:L-ratio), which has been shown to be highly important for bees (Kraus et al. 2019; Vaudo et al. 2016b; Vaudo et al. 2020), and increase with increasing insect pollinator dependence. We also predicted correlations between total contents of nutrient classes as well as individual nutrient compounds within and between nutrient classes. We expected a higher proportion of such correlations and thus comparatively more stable nutrient ratios in pollen of plants that are highly dependent on insect pollination and/or in plants specifically collected by bees (see Supplementary Material 1).

Materials and Methods WP2

Pollen collection

We collected flowers from 139 plant species (Table 1), including non-native species, in semi-natural, park and garden areas in and around Würzburg, Germany, and cultivated from seed in the University greenhouse in Groningen, The Netherlands. After air-drying flowers for 3-7 days (depending on the species), we either used a pollen extractor (180 µm or 150 µm mesh width, depending on the pollen size of the plant species; Pollen Extractor Shop, La Charité-sur-Loire, France) to extract pollen, or we removed pollen directly from the anthers using forceps. Pollen was weighed and stored at -18°C in microcentrifuge tubes. Nutritional analyses were limited by the final pollen amounts, because specific minimum quantities were required for each nutritional analysis (i.e. a minimum of 0.1 mg for fatty acids, 5 mg for amino acids, and 20 mg for sterols). We only analyzed fatty acids and/or amino acids when less than 28 mg pollen could be collected (Table 1).

Table 1 Overview of the 139 collected plant species, their level of insect dependence, whether or not they were found in bee-collected pollen (Y = Yes, X = Not available) and nutritional analyses (amino acids (N = 79), fatty acids (N = 115) and sterols (N = 38) in pollen) performed. NA indicates that there was no data available on the plant's insect dependence. Lines denote plants that were only identified to genus level, preventing the determination of their level of insect dependence. Green checkmarks indicate successfully conducted analyses for each nutrient class, red crosses indicate that there was not enough pollen to perform the analysis.

Species	Family	Insect dependence	Collected by bees (Table S1.1)?	Amino acids	Fatty acids	Sterols
Acer negundo	Sapindaceae	none	X	✓	✓	✓
Achillea millefolium	Asteraceae	full	Y	✗	✓	✗
Aesculus hippocastanum	Sapindaceae	full	X	✓	✓	✗
Agrimonia procera	Rosaceae	low	X	✗	✓	✗
Alliaria petiolata	Brassicaceae	low	Y	✓	✓	✗
Allium ursinum	Amaryllidaceae	low	Y	✓	✓	✓

Anemone nemorosa	Ranunculaceae	full	X	✓	✓	✓
Anthriscus sylvestris	Apiaceae	low	Y	✓	✓	✓
Arabis sp.	Brassicaceae	-	X	✗	✓	✗
Arctium sp.	Asteraceae	-	X	✗	✓	✗
Artemisia vulgaris	Asteraceae	none	X	✓	✓	✓
Aster laevis	Asteraceae	full	X	✓	✗	✗
Barbarea vulgaris	Brassicaceae	high	Y	✗	✓	✗
Bellis perennis	Asteraceae	none	Y	✓	✓	✗
Berberis aquifolium	Berberidaceae	high	Y	✓	✓	✗
Berberis vulgaris	Berberidaceae	high	Y	✓	✓	✓
Bergenia cordifolia	Saxifragaceae	NA	X	✓	✗	✗
Betula pendula	Betulaceae	none	X	✓	✗	✗
Bidens aristosa	Asteraceae	NA	X	✓	✗	✗
Brassica napus	Brassicaceae	high	X	✓	✓	✓
Bunias orientalis	Brassicaceae	high	Y	✓	✓	✗
Caltha palustris	Ranunculaceae	full	X	✓	✓	✗
Campanula medium	Campanulaceae	full	X	✗	✓	✗
Capsella bursa-pastoris	Brassicaceae	low	Y	✗	✓	✗
Cardamine pratensis	Brassicaceae	full	X	✓	✓	✗
Centaurea jacea	Asteraceae	full	Y	✓	✓	✓
Centranthus ruber	Caprifoliaceae	high	X	✓	✓	✗
Cichorium intybus	Asteraceae	high	Y	✓	✓	✓
Cirsium vulgare	Asteraceae	high	Y	✓	✓	✓
Clematis recta	Ranunculaceae	high	X	✓	✓	✗
Conium maculatum	Apiaceae	high	Y	✓	✓	✗
Cornus mas	Cornaceae	high	X	✓	✗	✗
Corydalis solida	Papaveraceae	full	X	✗	✓	✗
Corylus avellana	Betulaceae	none	X	✓	✓	✗
Cosmos bipinnatus	Asteraceae	full	X	✓	✗	✗
Cyanus segetum	Asteraceae	full	X	✓	✓	✓
Dactylis glomerata	Poaceae	none	Y	✗	✓	✗
Doronicum orientale	Asteraceae	NA	X	✗	✓	✗
Erodium cicutarium	Geraniaceae	low	X	✗	✓	✗
Euphorbia amygdaloides	Euphorbiaceae	high	X	✓	✓	✗
Euphorbia cyparissias	Euphorbiaceae	high	X	✗	✓	✗
Ficaria verna	Ranunculaceae	high	X	✓	✓	✓
Forsythia x intermedia	Oleaceae	full	X	✓	✗	✗
Fragaria vesca	Rosaceae	high	X	✓	✗	✗
Galium odoratum	Rubiaceae	high	X	✗	✓	✗
Galium verum	Rubiaceae	high	X	✗	✓	✗
Geranium pratense	Geraniaceae	high	X	✓	✗	✗
Geranium pyrenaicum	Geraniaceae	high	X	✗	✓	✗
Geranium sanguineum	Geraniaceae	high	X	✓	✓	✓
Geum rivale	Rosaceae	high	X	✓	✓	✓

Geum urbanum	Rosaceae	low	X	✓	✓	✓
Glechoma hederacea	Lamiaceae	high	X	✓	✓	✓
Helenium flexuosum	Asteraceae	NA	X	✓	✗	✗
Helianthus tuberosus	Asteraceae	full	X	✗	✓	✗
Helleborus foetidus	Ranunculaceae	high	X	✗	✓	✗
Hepatica nobilis	Ranunculaceae	high	X	✗	✓	✗
Hibiscus sp.	Malvaceae	-	X	✗	✓	✗
Hieracium sp.	Asteraceae	-	X	✓	✓	✓
Hydrangea macrophylla	Hydrangeaceae	NA	X	✗	✓	✗
Hypericum perforatum	Hypericaceae	low	X	✓	✓	✗
Iberis sempervirens	Brassicaceae	NA	X	✗	✓	✗
Jacobaea aquatica	Asteraceae	NA	X	✗	✓	✗
Knautia arvensis	Caprifoliaceae	high	X	✓	✓	✓
Knautia macedonica	Caprifoliaceae	NA	X	✓	✗	✗
Laburnum anagyroides	Fabaceae	full	X	✓	✓	✓
Lamium album	Lamiaceae	high	X	✗	✓	✗
Lamium galeobdolon	Lamiaceae	high	X	✗	✓	✗
Lamium maculatum	Lamiaceae	high	X	✗	✓	✗
Lamium purpureum	Lamiaceae	high	X	✓	✓	✓
Lathrea squamaria	Orobanchaceae	low	X	✓	✓	✗
Lavandula angustifolia	Lamiaceae	high	X	✗	✓	✗
Leucanthemum vulgare	Asteraceae	low	X	✓	✗	✗
Ligustrum vulgare	Oleaceae	low	Y	✓	✓	✓
Lobularia maritima	Brassicaceae	high	X	✓	✓	✗
Lonicera nitida	Caprifoliaceae	NA	X	✓	✓	✓
Lonicera tatarica	Caprifoliacea	low	X	✗	✓	✗
Lotus corniculatus	Fabaceae	full	Y	✓	✓	✗
Lunaria annua	Brassicaceae	high	X	✗	✓	✗
Lysimachia vulgaris	Primulaceae	full	X	✗	✓	✗
Magnolia sp.	Magnoliaceae	-	X	✗	✓	✗
Malus domestica	Rosaceae	full	X	✓	✓	✓
Malva sylvestris	Malvaceae	full	X	✗	✓	✗
Melampyrum arvense	Orobanchaceae	high	X	✗	✓	✗
Melilotus officinalis	Fabaceae	full	X	✓	✓	✗
Nepeta cataria	Lamiaceae	high	Y	✗	✓	✗
Oenothera biennis	Onagraceae	low	X	✓	✓	✓
Ornithogalum nutans	Asparagaceae	high	X	✓	✓	✓
Papaver rhoeas	Papaveraceae	full	Y	✓	✓	✓
Petunia axilliaris	Solanaceae	none	X	✓	✓	✗
Pinus mugo	Pinaceae	none	X	✓	✓	✓
Plantago lanceolata	Plantaginaceae	low	X	✓	✓	✓
Potentilla fruticosa	Rosaceae	low	X	✗	✓	✗
Prunus cerasus	Rosaceae	high	X	✗	✓	✗
Prunus domestica	Rosaceae	high	X	✓	✓	✓

Prunus laurocerasus	Rosaceae	NA	X	✗	✓	✗
Prunus padus	Rosaceae	high	X	✗	✓	✗
Ranunculus acris	Ranunculaceae	full	X	✓	✓	✓
Ranunculus bulbosus	Ranunculaceae	full	Y	✓	✗	✗
Ranunculus repens	Ranunculaceae	full	X	✓	✓	✓
Ribes rubrum	Grossulariaceae	high	X	✓	✗	✗
Ribes sanguineum	Grossulariaceae	high	X	✗	✓	✗
Rosa canina	Rosaceae	high	X	✗	✓	✗
Rudbeckia hirta	Asteraceae	full	X	✓	✗	✗
Rumex acetosa	Polygonaceae	none	X	✓	✓	✗
Rumex obtusifolius	Polygonaceae	none	X	✓	✗	✗
Salix alba	Salicaceae	full	X	✓	✓	✓
Salix caprea	Salicaceae	full	X	✓	✗	✗
Salvia pratensis	Lamiaceae	high	X	✗	✓	✗
Sambucus ebulus	Adoxaceae	high	X	✓	✓	✗
Sambucus nigra	Adoxaceae	low	Y	✓	✓	✓
Saponaria ocymoides	Caryophyllaceae	low	X	✓	✓	✗
Saponaria officinalis	Caryophyllaceae	low	X	✗	✓	✗
Scabiosa columbaria	Caprifoliaceae	high	X	✓	✗	✗
Scabiosa ochroleuca	Caprifoliaceae	high	X	✓	✓	✓
Securigera varia	Fabaceae	full	Y	✗	✓	✗
Sedum acre	Crassulaceae	low	X	✗	✓	✗
Sedum album	Crassulaceae	high	X	✗	✓	✗
Sinapis arvensis	Brassicaceae	full	X	✓	✓	✓
Smyrnium perfoliatum	Apiaceae	low	X	✓	✓	✗
Solidago canadensis	Asteraceae	full	X	✗	✓	✗
Sorbus aucuparia	Rosaceae	full	X	✗	✓	✗
Spiraea sp.	Rosaceae	-	X	✗	✓	✗
Taraxacum officinale	Asteraceae	none	X	✓	✗	✗
Telekia speciosa	Asteraceae	high	X	✗	✓	✗
Tilia platyphyllos	Malvaceae	high	Y	✓	✓	✓
Trifolium incarnatum	Fabaceae	high	X	✓	✓	✗
Trifolium pratense	Fabaceae	full	X	✓	✓	✓
Tussilago farfara	Asteraceae	high	X	✗	✓	✗
Verbascum densiflorum	Scrophulariaceae	full	X	✓	✗	✗
Verbascum sp.	Scrophulariaceae	-	X	✗	✓	✗
Veronica longifolia	Plantaginaceae	NA	X	✗	✓	✗
Veronica teucrium	Plantaginaceae	low	X	✗	✓	✗
Viburnum lantana	Adoxaceae	low	X	✓	✓	✓
Viburnum opulus	Adoxaceae	low	X	✗	✓	✗
Viburnum rhytidophyllum	Adoxaceae	NA	X	✗	✓	✗
Vicia cracca	Fabaceae	high	X	✓	✗	✗
Vinca minor	Apocynaceae	full	X	✗	✓	✗
Viola cornuta	Violaceae	NA	X	✗	✓	✗

Nutritional analyses

(i) Amino acid analysis

Pollen amino acids were analyzed for 79 plant species (Table 1) following Leonhardt and Blüthgen (2012) and Kriesell et al. (2017). We analyzed free and protein-bound amino acids separately via ion exchange chromatography (IEC: Biochrom 20 plus amino acid analyzer, Laborservice Onken, Gründau, Germany).

To analyze free amino acids, 5 – 10 mg of collected pollen was extracted for 30 min in an ultrasonic bath (Emmi 20HC, EMAG, Mörfelden-Walldorf, Germany) in 100 µl of de-ionized water. The extract was then refrigerated for 60 min, before centrifuging and membrane filtering for 10 min. The residue was kept for protein-bound amino acid analysis. The supernatant was boiled for 2 min at 100°C. After cooling down to room temperature on ice, it was centrifuged again for 5 min. The supernatant was mixed with 10 µl of 12.5% 5-sulfosalicylic acid (Merck, Darmstadt, Germany), refrigerated for 30 min and centrifuged for 10 min. Then, 50 µl of the supernatant was mixed with 50 µl of thinning buffer (Laborservice Onken), transferred into a membrane filter (Vecta Spin) and centrifuged for 5 min. The filtrate was used for IEC analysis.

To analyze protein-bound amino acids (polypeptides) the residue of the membrane filtration in step 1 was mixed with 200 µl of 6 N HCl. The mixture was boiled at 100°C for 4 h, cooled down to room temperature and centrifuged for 10 min. Water was evaporated from the supernatant at 100°C and the sample was re-dissolved in 200 ml fresh water thrice and centrifuged again. Then, 100 µl of the supernatant was mixed with 20 µl of 12.5% sulfosalicylic acid and extracted at 5°C for 30 min. The sample was mixed briefly, centrifuged again for 10 min and 100 µl of the supernatant was mixed with 100 µl of sample rarefaction buffer. The

mixture was filtered by membrane filtering in the centrifuge for 5 min. The filtrate was used for the IEC analysis.

The IEC was equipped with a high-pressure PEEK column and an autosampler. Lithiumcitrate buffers (Laborservice Onken) with different pH values were used as eluents. After elution, amino acids were stained with ninhydrin (Laborservice Onken) and measured in a photometer. For amino acid quantification an external standard (physiological calibration standard, Laborservice Onken) was used, containing all proteinogenic amino acids besides glutamine und asparagine, which were added manually prior to running standards and samples. Tryptophan is destroyed in HCl and could therefore not be analyzed with this method. Total protein content was calculated as the sum of all free amino acids and polypeptides.

(ii) Fatty acid analysis

Pollen fatty acids were analyzed for 115 plant species (Table 1) adapted from Brückner et al. (2017), as detailed below. To analyze the fatty acid content, 0.3 – 8.8 mg of pollen was extracted in 1 ml of a chloroform:methanol 2:1 (V/V) mixture (both Sigma-Aldrich, Taufkirchen, Germany) in a thermomixer (Thermomixer Compact, Eppendorf, Hamburg, Germany) at 60°C for 24 h. After adding 20 µl of nonadecanoic acid in methanol (0.2 mg/ml, both Sigma-Aldrich) as internal standard, fatty acids extracts were purified and fractionated on 3 ml SiOH columns (Macherey Nagel, Düren, Germany), which were pre-conditioned with two column equivalents of hexane (Merck). After loading the column, the triglyceride fraction was eluted with 4 ml isooctane:ethyl acetate (10:1, both Sigma-Aldrich), the diglyceride fraction with 5 ml isooctane:ethylacetate (3:1), and the free fatty acids with 6 ml of isooctane:ethylacetate:acetic acid (75:25:2). All three fractions were pooled to yield the

overall pollen fatty acid content. All solvents were removed under CO_2 and the fatty acid residue was dissolved in 250 µl of dichloromethane:methanol (2:1, both Merck). The mixture was transferred into a 2.5 ml analytical vial containing a 300 µl microvolume insert and the solvent was removed under CO_2. The residue was derivatized (chemically modified) with 20 µl of a 0.25 M trimethyl sulfonium hydroxide (TMSH) solution in methanol (Sigma Aldrich) to form fatty acid methyl esters (FAME), which were then analyzed with gas chromatography/mass spectrometry (GCMS: 5975C inert XL MSD, Agilent Technologies, Santa Clara, USA). One µl of the sample was injected in splitless mode. Helium was used as carrier gas. Injection temperature was 60°C, which was held for 1 min before heating at a rate of 15°C/minute to 150°C, which was held for 10 min. Afterwards, temperature was increased at 10°C/minute to 320°C, which was held for 10 min. Electron ionization mass spectra were recorded from m/z 40 to 650. Ion source and transfer line temperature were constant at 250°C. FAME Mix, C4-C24 (Supelco, Bellefonte, USA) and the NIST MS Search 2.0 library were used to identify the FAMEs. MSD ChemStation F.01.00.1903 was used to manually integrate FAME peaks. If peaks of two fatty acids could not be separated (i.e. oleic and linolenic acid), they were integrated as one peak. The integrated area of the internal standard (nonadecanoic acid) was used to calculate the concentrations of identified fatty acids. Total fat content was calculated by summing up amounts of all fatty acids.

(iii) Sterol analysis

Pollen sterols were analyzed for 38 plant species (Table 1) following Vanderplanck et al. (2011). At least 20 mg of pollen was weighed into a round bottom flask (250 ml) and saponified in 2.5 ml of a 2M methanolic (Carl Roth, Karlsruhe, Germany) KOH (Honeywell Riedel-de Haën, Seelze, Germany) solution for 1 h in a water bath at 80°C with an attached

reflux condenser of 15°C. After cooling to room temperature for 10 min, 4 ml Betulin (Sigma-Aldrich) in EtOH (Merck) (0.1 mg/ml) and 10 ml de-ionized water were added and the solution was transferred into a separating funnel and shaken. The condenser and flask were rinsed with 10 ml diethyl ether (AppliChem, Darmstadt, Germany), which was then also added to the separating funnel. After shaking and separation of the ether and water phase in the funnel, the ether phase was collected in a new flask, while the water phase was re-collected and washed two more times with 10 ml of diethyl ether, before being discarded. The three ether phases were combined in one flask, washed three times in the separating funnel with 10 ml of de-ionized water, dried over anhydrous sodium sulfate (Merck) and transferred into a new flask. The ether was removed under CO_2 and the residue was solved in 2 ml hexane. The solution was transferred into a microcentrifuge tube and the solvent evaporated under CO_2. The extracted sterols were silylized with 100 µl anhydrous pyridine (Merck) and 100 µl N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Sigma-Aldrich) in a thermomixer at 90°C and 1400 rpm for 30 min. Afterwards, the reagents were removed under CO_2. The residue was solved in 100 µl hexane, transferred into a 2.5 ml analytical vial containing a 300 µl microvolume insert and analyzed via GCMS. Except for the temperature program, the GCMS setup was identical to the one used for the fatty acid analysis.

After injection at 60°C, the oven was heated at 30°C/min to 290°C, which was held for 22 min. Then temperature was raised at 30°C/min to 325°C and held for another 5 min. Chromatograms and mass spectra were analyzed as described above for the fatty acid analysis. If peaks of two sterols could not be separated, they were integrated as one peak. The integrated area of the internal standard (betulin) was used to calculate the concentrations of the other sterols. Total sterol content was calculated as the sum of all sterol concentrations.

Statistical analyses

We could assign an insect pollinator dependence level to 117 plant species of the main dataset. The required information was extracted from the BiolFlor database v 1.1 (http://www2.ufz.de/biolflor/index.jsp, accessed in June 2019). Plant species were classified into four different categories: (i) fully dependent on insect pollinators when they implicitly need insects for pollination and are self-incompatible (N = 33, ca. 28% of the dataset), (ii) highly dependent on insect pollinators when they are mostly insect-pollinated, but can self-pollinate (N = 51, ca. 44%), (iii) little dependent on insect pollinators when they are mainly wind- or self-pollinated, albeit some degree of insect pollination can occur (N = 23, ca. 20%), and (iv) independent of insect pollinators when they are exclusively wind- or self-pollinated (N = 10, ca. 9%). We could not assign a pollinator dependence level to 22 plant species due to missing data entries. These plants were excluded from all analyses on the effect of pollinator dependence and were only considered for other analyses. All analyses were performed at plant species level.

All tests were performed for nutrient concentrations (w/w of the whole pollen weight) and relative proportions (within the nutrient class). The use of proportions enables analyses of the relative contributions of nutrients independent of concentration differences. To calculate relative proportions of e.g. amino acids, all amino acid concentrations were summed up and the concentration of each single amino acid was divided by this sum.

For each plant species, we also calculated the protein:lipid (P:L)-ratio as well as ratio of omega 3 and omega 6 fatty acids, which is known to affect the cognitive performance in bees (Arien et al. 2018; Arien et al. 2015).

(i) Testing for phylogenetic signals

In order to test whether the nutrient content was influenced by the level of relatedness between plant species and therefore its evolutionary history we tested for phylogenetic signal among variables. We used the molecular phylogeny of Zanne et al. (2014) for all phylogenetic analyses. For each nutrient class, we tested for a phylogenetic signal with Blomberg's K following Junker et al. (2017) and Ruedenauer et al. (2019b) to test whether the nutrient content was influenced by the level of relatedness between plant species and therefore its evolutionary history. Phylogenetic trees were generated with the R-package pez (Pearse et al. 2015). After pruning the terminal branches, we generated a cladogram to illustrate our dataset.

(ii) Testing for differences between different levels of pollinator dependence

Using the R-package phytools (Revell 2012), we performed phylogenetic analyses of variance (phyl-ANOVA) to test for differences in the content of each nutrient class and the P:L-ratio between the different insect pollination dependence levels. Subsequent Tukey post-hoc pairwise comparisons were corrected via false discovery rate for multiple testing (FDR, Benjamini and Hochberg 1995).

(iii) Testing for nutrient correlations

Nutrient correlations between the total content of each nutrient class (e.g. total fat vs. total amino acid content) and individual nutrient compounds within (e.g. amino acid 1 vs. amino acid 2) and between (e.g. amino acid 1 vs. fatty acid 1) nutrient classes were tested via phylogenetic generalized least squares (PGLS) models fit with maximum likelihood with Brownian correlation (using the R package nlme) for both concentrations and proportions.

Resulting p-values were corrected for multiple testing using FDR before they were used in subsequent analyses (below).

Because pollen is often targeted by bee pollinators, which rely on pollen for offspring production and represent the main pollinator group in Europe, it is likely that bees adapt to specific plant species rather than plant species adapt to pollinators. In this case, bees might target plant species for pollen collection which are more likely to provide stable nutrient ratios. We therefore additionally tested whether nutrient correlations were more common in pollen of plant species specifically collected by bees (Supplementary Material 1).

(iv) Testing for differences in nutrient correlations between plant groups

To test for differences in nutrient correlations with regard to insect pollinator dependence, separate correlation tests were performed for all plant species that are highly or fully dependent on insect pollination (levels I and II), and all plant species that are little or not dependent on insect pollination (levels III and IV). Correlation results were then classified as "0" for non-significant correlations ($p > 0.05$) and "1" for significant correlations ($p < 0.05$). We then tested for differences in the number of significant nutrient correlations between the two plant groups (i.e. full/high and low to no insect pollinator dependence) as well as among the different nutrients using phylogenetic generalized linear mixed models (PGLMMs) with a binomial distribution.

Results WP2

Phylogenetic signals and effect of pollinator dependence

We did not detect a phylogenetic signal for any of the nutrients tested (Table 2). The total content of all three protein-related nutrients (protein, polypeptides and amino acids) and the

P:L-ratio were significantly higher in plants which fully and highly depend on insect pollinators than in plants that do not at all depend on insect pollination, independent of phylogenetic relationship (Figure 1 A-C & F, Table 3). Total fat and sterol contents did not differ between plants with different levels of insect pollinator dependences, independent of phylogeny (Figure 1 D&E, Table 3).

Table 2 Results of Blomberg's *K*-test testing for a phylogenetic signal in the total content of protein-related nutrients (protein, polypeptides and free amino acids), fat and sterol content and the protein to lipid (P:L) ratio. Note that here and in the following tables and figures, protein represents the sum of all amino acids. Shown are Blomberg's *K* and *P*-values.

Nutrients	*K*	*P*
Protein (N = 79)	0.074	0.287
Polypeptides (N = 79)	0.083	0.213
Free amino acids (N = 79)	0.077	0.329
Lipids (N = 115)	0.015	0.907
Sterols (N = 38)	0.081	0.222
Protein:lipid ratio (N = 57)	0.106	0.145

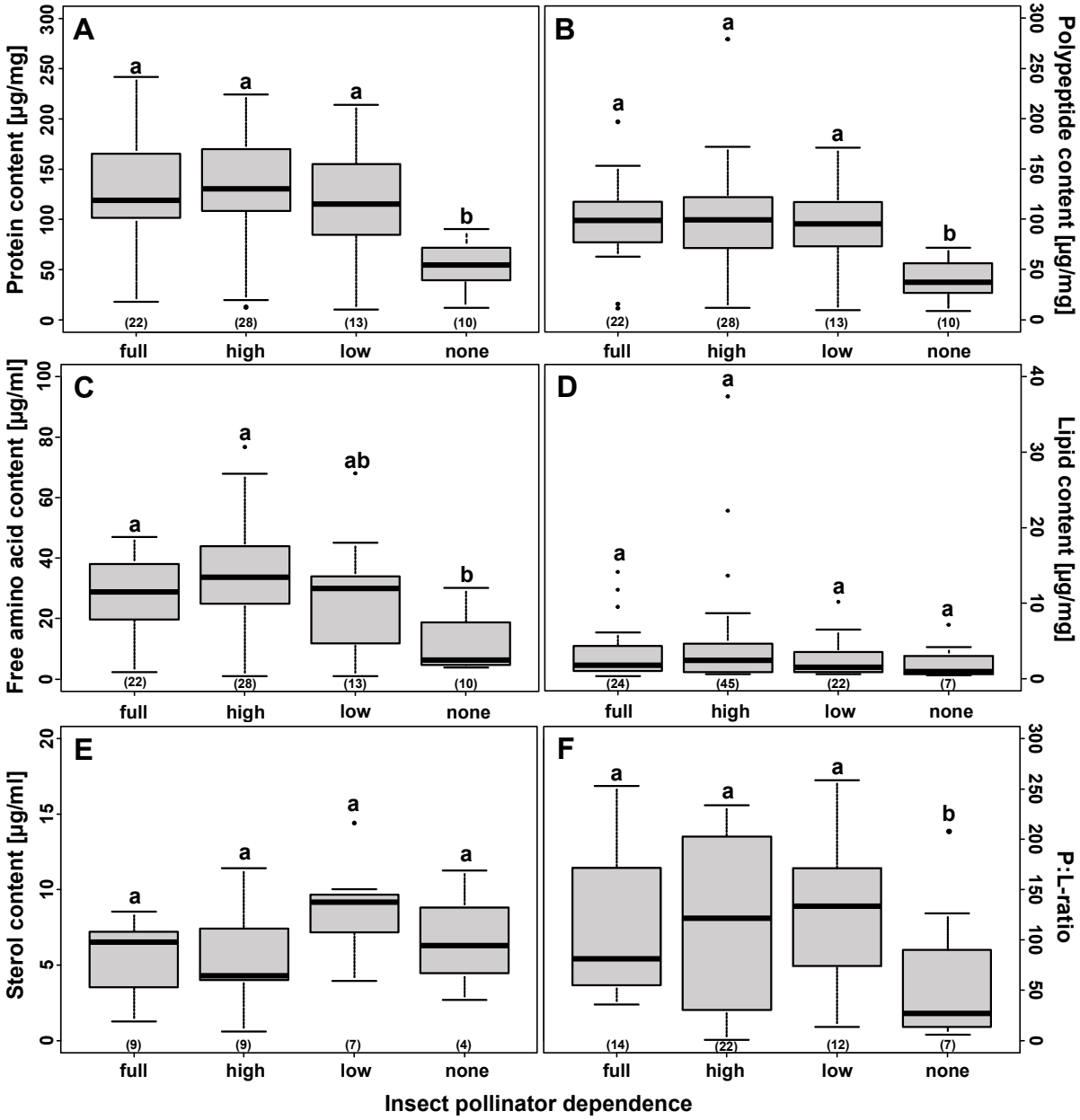


Figure 1 Differences in the total content (pollen fresh weight) of protein (A), polypeptides (B), free amino acids (C), lipids (D) and sterols (E), as well as the protein to lipid ratio (P:L-ratio, F) of plants differing in the level of insect pollinator dependence. Numbers in brackets below boxplots give the numbers of plant species included in each group. Different letters above boxes indicate significant differences between different levels of insect pollinator dependence (according to pairwise comparisons following the phylogenetic ANOVA corrected for multiple testing). Boxplots represent the median (central line), the 25th and 75th percentiles (edges of boxes), and the most extreme data points (whiskers). Outliers (outside of the range of 1.5 x IQR) are plotted as individual dots.

Table 3 Results of phylogenetic ANOVAs testing for differences in the relative contents (% fresh weight) of total protein-related nutrients, fat and sterol content and the protein to lipid (P:L) ratio between different levels of insect pollinator dependence. Shown are *F*- and *P*-values. The number of plant species (N) for each nutrient is indicated in brackets behind each nutrient. *P*-values indicating significant differences between pollination strategies are marked in bold.

Nutrients	*F*	*P*
Protein (N = 79)	4.985	**0.009**
Polypeptides (N = 79)	3.396	**0.045**
Free amino acids (N = 79)	5.003	**0.008**
Fatty acids (N = 115)	0.463	0.793
Sterols (N = 38)	1.681	0.236
P:L ratio (N = 57)	2.984	**0.048**

Nutrient correlations

Nine out of ten correlations among nutrient classes were significantly positive, with total fat and sterols being the only non-significant pair (Table 4). All correlations between the total contents of protein-related nutrients were also positive (Table 4). Significant correlations between individual nutrients in pollen were also common both within (12-42% of all correlations for concentrations (Figure 2A), 10-41% of all correlations for proportions (Figure 2B)) and among (14-54% and 17-60% (Figure S2.1)) nutrient classes; 90% of the concentration correlations were positive. Significant correlations between proportions of individual nutrients were also mostly positive (66 % of all correlations), but partly also negative, meaning that if one of the compound's concentrations increases, the concentration of the other compound decreases. Additionally, the concentrations of omega 3 and omega 6 fatty acids were positively correlated (PGLS: coeff: 0.36, $P < 0.001$). Their average ratio was 1.14 ± 1.41 for the entire dataset and was similar across plant species with different levels of insect pollinator dependence (high dependence: 1.16 ± 1.42, low dependence: 1.10 ± 1.41).

Table 4 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation describing relationships between concentrations of total contents of the three protein-related nutrients, fat and sterols. Coefficients with significant *P*-values (**p*<0.05, ***p*<0.01 and *** *p*<0.001) after Benjamini Hochberg correction (FDR) are marked in bold. N gives the number of plant species included in each correlation analysis. All correlations were positive.

Nutrients	Polypeptides	Free amino acids	Fatty acids	Sterols
Protein	**1.486***** (N = 80)	**3.097***** (N = 80)	**15.753***** (N = 58)	**7.144**** (N = 38)
Polypeptides	-	**2.989***** (N = 80)	**11.542***** (N = 58)	**4.519*** (N = 38)
Free amino acids	-	-	**3.766**** (N = 58)	**2.626***** (N = 38)
Fatty acids	-	-	-	0.029 (N = 38)

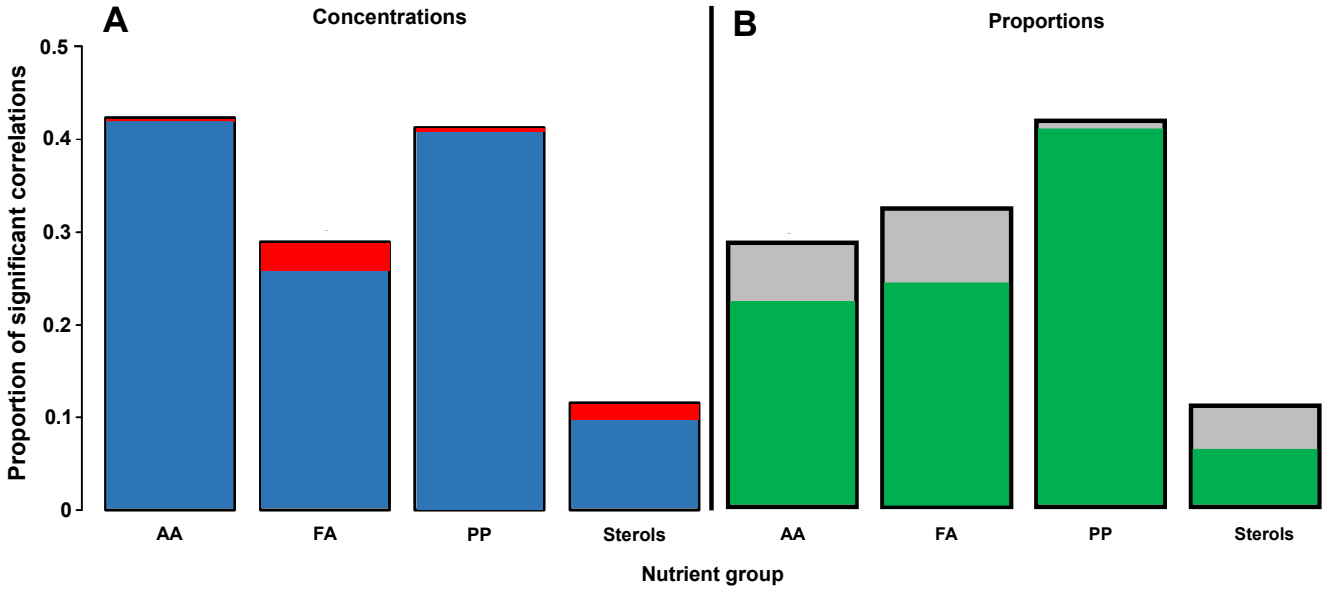


Figure 2 Differences in the proportions of significant correlations between all individual nutrients within the same nutrient class (e.g. between all individual amino acids) for concentrations (A) and proportions (B) of all amino acids (AA), fatty acids (FA), polypeptides (PP) and sterols. Blue and green represent positive correlations, red and grey negative correlations. Significant results were found for concentrations (*PLR* = 7.834, *P* < 0.001) and proportions (*PLR* = 6.967, *P* < 0.001).

Effect of pollinator dependence on nutrient correlations

Pollinator dependence was an important predictor for correlations among classes of nutrients. Significant correlations between polypeptides (concentration) were 1.5 times more common in species with low pollinator dependence than in plant species with high dependence (Figure S2.1A). By contrast, significant correlations between different free amino acids (concentration) were twice as common in plants with high pollinator dependence as in plants with low to no pollinator dependence (Figure S2.1B, Table S2.2). Proportions of significant correlations did not differ between plant groups for fatty acid (Figure S2.1C) and

sterol concentrations (Figure S2.1D). We also found no differences between plant groups for any nutrient class when testing proportions (Figure S2.2).

When testing for correlations between individual nutrients of different classes using concentrations, correlations were in general significantly more common in plants with high pollinator dependence (Figure 3). The same increase with pollinator dependence was found for correlations between individual nutrients of different nutrient classes using proportions, except for correlations between fatty acids and sterols, which did not significantly differ between the two groups (Figure 4). Correlations were also pronounced in plant species visited by pollen collecting bees (Supplementary Material 1). In this data set, fatty acids were significantly correlated with sterols, while they showed no significant correlations in the full dataset (Table S1.2).

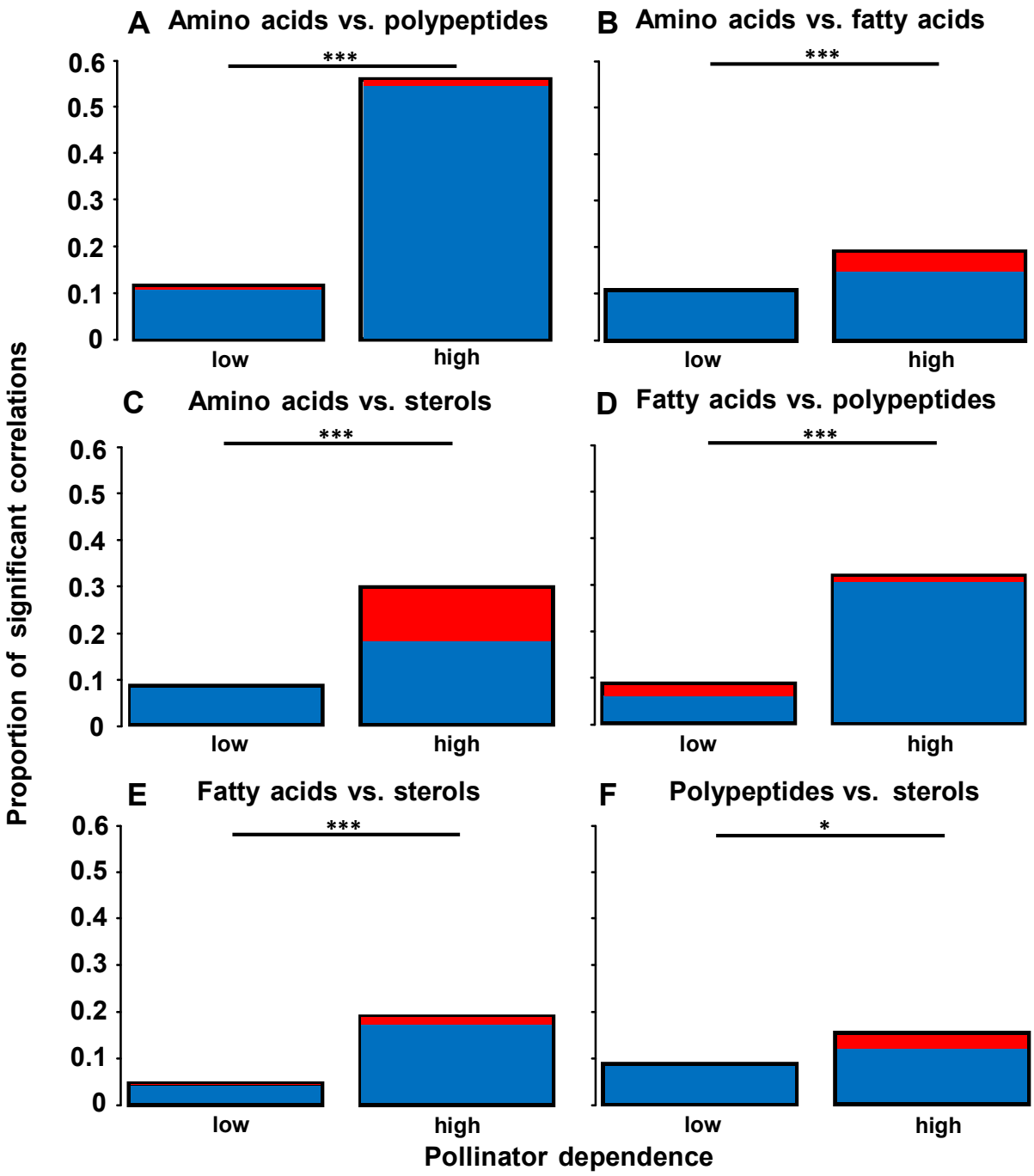


Figure 3 Differences in the proportions of significant correlations between all individual nutrients of different nutrient classes (concentrations) between different levels of pollinator dependence (low = none & low, high = high & full). Blue indicates positive correlations, red negative correlations. Significant differences were found for all nutrient class pairs: amino acids & polypeptides (A, (χ^2 = 236.9, P < 0.001)), amino acids & fatty acids (B, (χ^2 = 37.243, P < 0.001)), amino acids & sterols (C, (χ^2 = 41.396, P < 0.001)), fatty acids & polypeptides (D, (χ^2 = 188.43, P < 0.001)), fatty acids & sterols (E, (χ^2 = 61.811, P < 0.001)) and polypeptides & sterols (F, (χ^2 = 4.643, P = 0.031)). Asterisks above the bars indicate the level of significance (n.s. P > 0.05, * P < 0.05, *** P < 0.001).

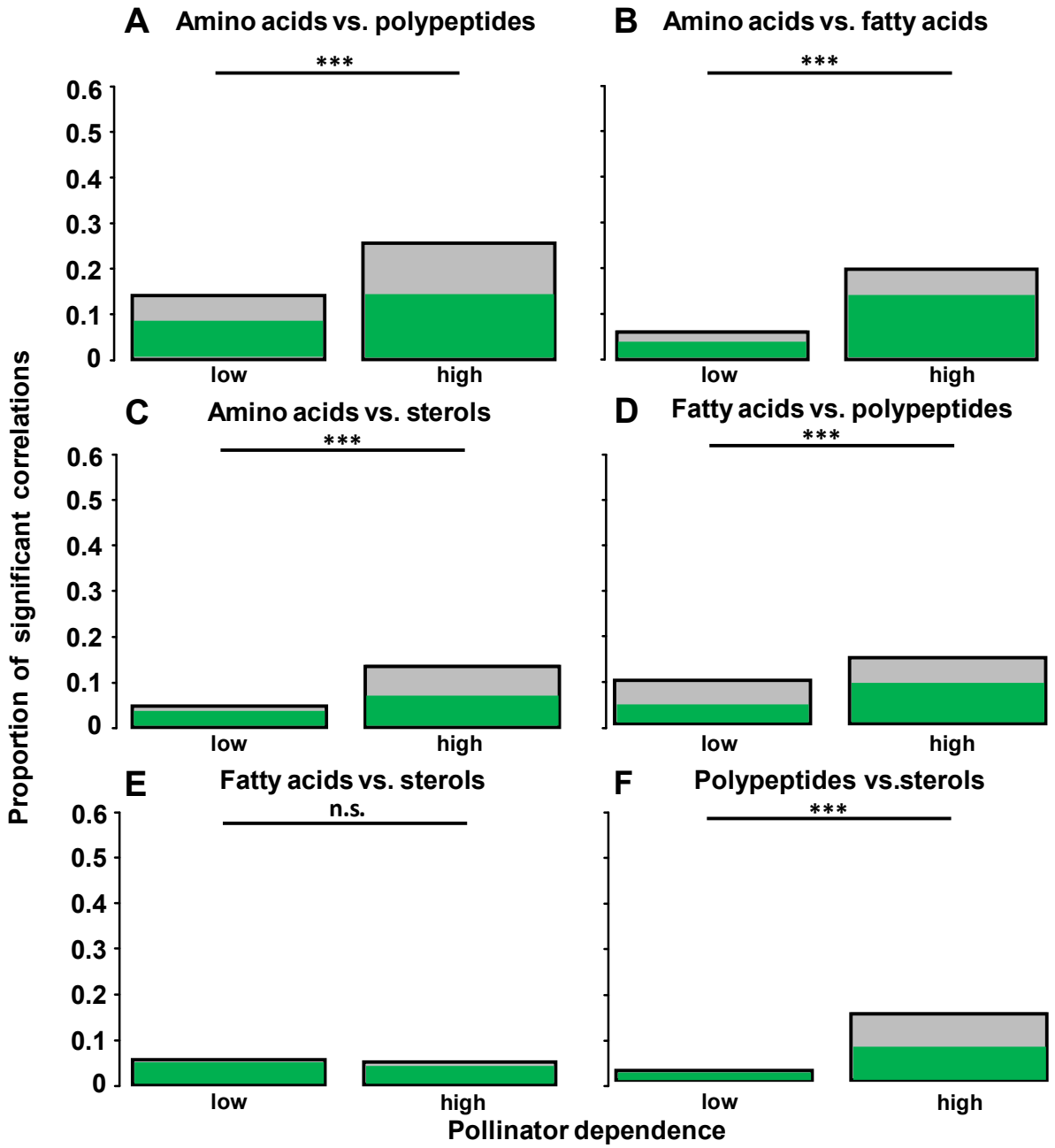


Figure 4 Differences in the proportions of significant correlations between all individual nutrients of different nutrient classes (proportions) between different levels of pollinator dependence (low = none & low, high = high & full). Green indicates positive correlations, grey negative correlations. Significant differences were found for all nutrient class pairs, besides fatty acids and sterols (E, (χ^2 = 0.35, P = 0.554)): amino acids & polypeptides (A, (χ^2 = 21.769, P < 0.001)), amino acids & fatty acids (B, (χ^2 = 119.56, P < 0.001)), amino acids & sterols (C, (χ^2 = 14.053, P < 0.001)), fatty acids & polypeptides (D, (χ^2 = 14.096, P < 0.001)) and polypeptides & sterols (F, (χ^2 = 41.764, P < 0.001)). Asterisks above the bars indicate the level of significance (n.s. P > 0.05, *** P < 0.001).

Discussion WP2

Pollen nutrient content relates to pollinator dependence more strongly than to relatedness

Our study provides new insights into pollen nutritional chemistry and its potential role in the plant-insect pollinator mutualism. Specifically, we reveal a higher amount of protein, a higher protein to lipid (P:L) ratio as well as a higher proportion of significant nutrient correlations in pollen of plant species that depend fully or highly on insects for pollination. These observations appear to be independent of plant phylogenetic relatedness.

Interestingly, we did not find a phylogenetic signal for any pollen nutrient, while we did for protein in our meta-analysis study (Ruedenauer et al. 2019b), and as did Roulston et al. (2000) in their study. Notably, the lack of a phylogenetic signal itself does not fully rule out an effect of relatedness (Revell et al. 2008). It does, however, indicate that pollen nutrient chemistry is mostly determined by other factors, such as species-specific needs for plant fertilization and/or selection imposed by pollinators.

Compared to our previous meta-analysis of pollen compounds (Ruedenauer et al. 2019b), we confirmed that crude protein (i.e. total amino acid) content as well as contents of protein-related nutrients (i.e. free amino acids and protein-bound amino acids) were at least two times higher in pollen of plant species depending on insect pollination than in species that do not depend on insect pollinators. Protein is crucial for larval development in insects (Haydak 1970; Herbert et al. 1977; Raubenheimer and Simpson 1999), which may explain why some insect pollinators prefer pollen with relatively high protein content (Kriesell et al. 2017; Leonhardt and Blüthgen 2012). This preference for protein-rich pollen seems to exert a sufficiently strong selective pressure on plant species that depend largely on insects for pollination and offering pollen as reward, resulting in an increased protein content (beyond

what is needed for fertilization) and thus increased attractiveness to pollinators. Future research should investigate how fast pollen nutrient content changed upon a transition in pollination system; for example the transition between vertebrate (with nectar offered as only reward) and insect pollinated plant systems.

Nutrient correlations

In line with our hypothesis, nutrient correlations were found both within and among nutrients. Moreover, an increase in the content of one nutrient class generally indicated an increase in the content of another nutrient class (in 9 out of 10 correlations). Highest proportions (48%) of (mostly positive) correlations of individual nutrients were found within and among protein-related nutrients, such as between individual amino acids. Moreover, nutrient correlations between different free amino acids were two times more likely for pollen of plant species, which fully or highly depend on insects for pollination, hinting at a possible role of this nutrient class in nutrient quality assessment of at least some pollinators. On average 91% of the correlations between concentrations of individual compounds within nutrient classes were positive. This may be a consequence of shared biosynthetic pathways and functions (Külheim et al. 2009; Piironen et al. 2000). However, if shared biosynthetic pathways were responsible for the positive correlations between (total) contents of nutrients of different nutrient classes this would likely result in a phylogenetic signal. Alternatively, these correlations may be a consequence of plant species-specific requirements for specific ratios between nutrients that best support reproduction and/or selection by insect pollinators.

From the insects' perspective, nutrient correlations indicate specific ratios between two nutrients or nutrient classes, which remain relatively stable across plant species. Stable ratios

could facilitate rapid assessment of pollen nutritional quality. For example, a higher content of one amino acid generally indicates a higher amount of other amino acids. Likewise, a low stearic or palmitic acid content indicates high protein content. In fact, similar nutrient ratios across plant species may explain why bumble bees can afford to prioritize fatty acid perception and 'seemingly ignore' amino acid concentrations in pollen (Ruedenauer et al. 2020), although they are principally able to detect differences between concentrations of some amino acids in water and therefore likely in nectar (Ruedenauer et al. 2019b). Previous behavioral research and our current nutrient analyses suggest that bees do not assess all individual nutritional compound in pollen, but instead focus on specific key compounds, i.e. fatty acids. Correlations between nutrients, e.g. protein and fat as shown here particularly for plant species depending on insect pollinators and targeted by bees (Supplementary Material 1), would then allow them to simultaneously obtain information on contents of different nutrients and overall nutrient contents within a relatively short timeframe.

Do lipid content and ratios fit the needs of (bee) pollinators?

Interestingly, proportions between polypeptides and fatty acids showed the highest proportion (60 %) of correlations. Moreover, while on average 90% of nutritional correlations were positive, some nutrients showed predominantly negative correlations, e.g. several fatty acids with protein-related nutrients. The two saturated fatty acids palmitic and stearic acid (which can both be perceived by bumble bees (Ruedenauer et al. 2020)) made up for an average of 64 ± 18% of the whole fatty acid profile in our dataset and significantly decreased with increasing protein content, resulting in high protein to low saturated fatty acid ratios for pollen of high protein content. These two fatty acids are known to reduce survival and reproduction in honey bees and bumble bees (Manning 2001; Manning 2006; Vaudo et al.

2016b), which may explain why *Bombus terrestris* avoids pollen with high fatty acid content (Ruedenauer et al. 2020). Further, bumble bees (*Bombus terrestris* and *B. impatiens*) were found to specifically target diets and preferentially collect pollen with high P:L ratios or low fat content (Kraus et al. 2019; Ruedenauer et al. 2020; Vaudo et al. 2016b; Vaudo et al. 2020). By focusing perception on fatty acids and subsequently avoiding them, bees can increase protein intake while simultaneously reducing fat intake due to the negative correlation between both nutrient classes. Prioritized perception and nutrient correlations may explain how insects manage to efficiently target specific nutrient ratios.

Total fat and sterol content were the only two nutrient classes whose total contents did not correlate with each other for the full dataset. However, some individual fatty acids were positively correlated with sterols and fatty acids were significantly correlated with sterols in pollen of plant species specifically targeted by bees (Supplementary Material 1). Insects cannot synthesize sterols themselves (Hobson 1935) and therefore have to rely on plant resources, e.g. pollen, as a source of sterols. It is not known whether bees and other pollinators can perceive sterols in pollen (pre- or postdigestively). However, the positive correlation between (some) fatty acids and sterols would allow them to infer the sterol content of pollen by assessing contents of specific fatty acids (e.g. caprylic or linoleic acid), specifically for plants that are attracting bees. However, if bees use such a strategy needs to be tested in future studies.

In conclusion, our species-wide analysis of pollen nutrients provides further evidence for insect pollinators as agents of selection on the nutrient profiles of pollen in plant species fully or highly depending on insects for pollination. Our findings further suggest that plants that depend on insect pollination both invest more nutrients (especially protein) into pollen and

maintain similar ratios between different nutrients to increase attractiveness and thus efficient pollen transfer. This strategy can decrease amounts of pollen required for pollination (compared to e.g. wind-pollinated plants (Friedman and Barrett 2009)) and thus compensate for the additional investment into nutrients per pollen grain. The observed nutrient correlations within and among nutrient classes on the other hand allows pollinators to easily and rapidly assess the pollen nutrient profile by prioritizing perception of just one or few nutrients.

Supplementary Material WP2

Supplementary Material 1

ITS2 meta-barcoding to determine plants used for pollen collection by bees

Bees, the most common pollinator group to many plant species in our study area (Klein et al. 2007), do not exclusively collect pollen from plants that depend on animal pollination, including wind-pollinated species (Saunders 2018). Moreover, some bee species (e.g. Apis mellifera, Bombus terrestris and B. impatiens) are known to target specific nutrient ratios (Arien et al. 2018; Pirk et al. 2010; Vaudo et al. 2016a; Vaudo et al. 2016b). At least B. terrestris also prioritizes nutrient perception (i.e. focuses on fatty acids, (Ruedenauer et al. 2020)), suggesting that likely all bees benefit from nutritional correlations.

Therefore, correlations in bee-collected plant species also are not exclusively connected to pollinator dependence. Instead, they could have evolved from bees' preferences to collect pollen from plants with specific correlations or bees could exclusively collect pollen from plants with these specific correlations. Hence, to investigate whether nutrient correlations are more likely in pollen specifically targeted by bees for pollen collection, we re-analyzed

four previously published metabarcoding datasets for pollen collections of bees in our study area, where sequencing data has been made publicly available at the European Nucleotide Archive (https://www.ebi.ac.uk/ena). Accession IDs were for Apis mellifera: PRJEB15870 (Danner et al. 2017) and PRJEB32797 (Nürnberger et al. 2019) and for solitary bees PRJEB31610 (Voulgari-Kokota et al. 2019) and PRJEB8640 (Sickel et al. 2015). All datasets have been equally generated with a MiSeq sequencing device. We added twelve samples of Bombus terrestris (PRJEB38233), which have been produced with the same laboratory procedure (Sickel et al. 2015). We merged forward and reverse reads and quality filtered the dataset (Q20) using USEARCH v11 (Edgar 2010). For taxonomy assignments we denoised and dereplicated sequences to amplicon sequence variants (ASVs). Taxonomic matching was performed with USEARCH v11 (Edgar et al. 2011) and global alignments (0.97% identity) against a custom reference database representing locally known plants as used in Keller et al. (2015) and created by the tool BCdatabaser (Keller et al. 2019). This list was matched against the plants collected for nutritional analyses to generate a subset of plants known to be used as pollen sources by bees in our sample region (meta-barcoding dataset, Table S1.1). All of the total contents of the different nutrient classes correlated in this dataset (Table S1.2). Interestingly, even sterols and fatty acids, which were not correlated for the whole dataset were correlated in the bee dataset, making it likely that bees could prefer pollen with such correlations. Additionally, pollen collected by bees is also close to the ratio of 1 between omega 3 and 6 fatty acids (1.13 ± 1.41).

Table S1.1 Relative abundance of plant species in pollen samples collected by Apis mellifera (N = 295), Bombus terrestris (N = 12), Heriades truncorum (N = 164), Megachile ligniseca (N = 8), Megachile rotunda (N = 20), Megachile versicolor (N = 4), Osmia bicornis (N = 325), Osmia caerulescens (N = 8) and Osmia leaiana (N = 4). Given are the plant and bee species names, the mean and median abundance of the plant species in the pollen samples and the minimum (Min) and maximum (Max) abundance of the plant species in the pollen samples. Data taken from Sickel et al. (2015), Danner et al. (2017), Voulgari-Kokota et al. (2019) and Nürnberger et al. (2019).

Plant	Bee	Mean	Median	Min	Max
Achillea millefolium	*Apis mellifera*	0.00059	0	0	0.05115
Alliaria petiolata	*Apis mellifera*	0.00028	0	0	0.03321
Allium ursinum	*Apis mellifera*	0.00012	0	0	0.03172
Anthriscus sylvestris	*Apis mellifera*	0.00019	0	0	0.05196
Barbarea vulgaris	*Apis mellifera*	0.0004	0	0	0.06311
Bellis perennis	*Apis mellifera*	0.00002	0	0	0.00205
Berberis aquifolium	*Apis mellifera*	0.00109	0	0	0.31108
Berberis vulgaris	*Apis mellifera*	0.00008	0	0	0.01316
Bunias orientalis	*Apis mellifera*	0.00686	0	0	0.38647
Capsella bursa-pastoris	*Apis mellifera*	0.00032	0	0	0.06977
Centaurea cyanus	*Apis mellifera*	0.00059	0	0	0.11827
Cichorium intybus	*Apis mellifera*	0.01536	0.00041	0	0.39478
Cirsium vulgare	*Apis mellifera*	0.0002	0	0	0.00988
Conium maculatum	*Apis mellifera*	0	0	0	0.00029
Dactylis glomerata	*Apis mellifera*	0.00091	0	0	0.11826
Ligustrum vulgare	*Apis mellifera*	0.00015	0	0	0.02326
Lotus corniculatus	*Apis mellifera*	0.00284	0	0	0.26985
Nepeta cataria	*Apis mellifera*	0.00081	0	0	0.07827
Papaver rhoeas	*Apis mellifera*	0.02383	0.00014	0	0.80792
Prunus avium	*Apis mellifera*	0.00174	0	0	0.204
Ranunculus bulbosus	*Apis mellifera*	0.01646	0.00003	0	0.7381
Sambucus nigra	*Apis mellifera*	0.00049	0	0	0.07683
Securigera varia	*Apis mellifera*	0.00002	0	0	0.00145

Tilia platyphyllos	*Apis mellifera*	0.00016	0	0	0.02326
Achillea millefolium	*Bombus terrestris*	0.00001	0	0	0.00008
Anthriscus sylvestris	*Bombus terrestris*	0.00033	0	0	0.00372
Bellis perennis	*Bombus terrestris*	0.00009	0	0	0.00054
Bunias orientalis	*Bombus terrestris*	0.00129	0	0	0.0093
Capsella bursa-pastoris	*Bombus terrestris*	0	0	0	0.00003
Centaurea cyanus	*Bombus terrestris*	0.0056	0.00006	0	0.06589
Cichorium intybus	*Bombus terrestris*	0.00141	0.0001	0	0.01462
Cirsium vulgare	*Bombus terrestris*	0.00002	0	0	0.0001
Conium maculatum	*Bombus terrestris*	0.01619	0.00001	0	0.19344
Dactylis glomerata	*Bombus terrestris*	0.00001	0	0	0.0001
Ligustrum vulgare	*Bombus terrestris*	0.00037	0	0	0.00145
Lotus corniculatus	*Bombus terrestris*	0.0013	0.00019	0	0.00951
Nepeta cataria	*Bombus terrestris*	0.00263	0.00007	0	0.0213
Papaver rhoeas	*Bombus terrestris*	0.07197	0.02048	0	0.48923
Ranunculus bulbosus	*Bombus terrestris*	0.00031	0.0001	0	0.00207
Sambucus nigra	*Bombus terrestris*	0.00414	0.00027	0	0.02193
Securigera varia	*Bombus terrestris*	0.03472	0.00853	0	0.15875
Tilia platyphyllos	*Bombus terrestris*	0.06518	0.03966	0	0.27986
Achillea millefolium	*Heriades truncorum*	0.00656	0.00029	0	0.12663
Alliaria petiolata	*Heriades truncorum*	0	0	0	0.00016
Allium ursinum	*Heriades truncorum*	0.00003	0	0	0.00392
Anthriscus sylvestris	*Heriades truncorum*	0.00001	0	0	0.00028
Bellis perennis	*Heriades truncorum*	0.00001	0	0	0.00058
Berberis aquifolium	*Heriades truncorum*	0	0	0	0.00003
Berberis vulgaris	*Heriades truncorum*	0.00001	0	0	0.00094
Bunias orientalis	*Heriades truncorum*	0.00027	0	0	0.02532
Capsella bursa-pastoris	*Heriades truncorum*	0.00004	0	0	0.00504

Centaurea cyanus	*Heriades truncorum*	0.00004	0	0	0.00394
Cichorium intybus	*Heriades truncorum*	0.01106	0.00025	0	0.25424
Cirsium vulgare	*Heriades truncorum*	0.00662	0	0	0.42386
Conium maculatum	*Heriades truncorum*	0.00002	0	0	0.00272
Dactylis glomerata	*Heriades truncorum*	0.00064	0	0	0.10408
Ligustrum vulgare	*Heriades truncorum*	0	0	0	0.00033
Lotus corniculatus	*Heriades truncorum*	0.00035	0	0	0.03007
Nepeta cataria	*Heriades truncorum*	0.00044	0	0	0.04656
Papaver rhoeas	*Heriades truncorum*	0.00104	0.0001	0	0.04776
Ranunculus bulbosus	*Heriades truncorum*	0.00441	0.00066	0	0.30391
Sambucus nigra	*Heriades truncorum*	0.00007	0	0	0.00547
Securigera varia	*Heriades truncorum*	0.00009	0	0	0.00572
Tilia platyphyllos	*Heriades truncorum*	0.00001	0	0	0.00109
Achillea millefolium	*Megachile ligniseca*	0.00063	0	0	0.00488
Anthriscus sylvestris	*Megachile ligniseca*	0.00006	0	0	0.00029
Bellis perennis	*Megachile ligniseca*	0.00001	0	0	0.00008
Capsella bursa-pastoris	*Megachile ligniseca*	0.00001	0	0	0.00009
Centaurea cyanus	*Megachile ligniseca*	0.00018	0	0	0.00141
Cichorium intybus	*Megachile ligniseca*	0.0109	0.00007	0	0.0841
Cirsium vulgare	*Megachile ligniseca*	0.20191	0.10661	0	0.58049
Lotus corniculatus	*Megachile ligniseca*	0.00021	0	0	0.00113
Nepeta cataria	*Megachile ligniseca*	0.00014	0	0	0.00106
Papaver rhoeas	*Megachile ligniseca*	0.00013	0	0	0.00057
Ranunculus bulbosus	*Megachile ligniseca*	0.00134	0.00028	0	0.00548
Sambucus nigra	*Megachile ligniseca*	0.00443	0.0025	0	0.01414
Securigera varia	*Megachile ligniseca*	0.00044	0.00016	0	0.00214
Achillea millefolium	*Megachile rotundata*	0.00265	0.00003	0	0.02519
Anthriscus sylvestris	*Megachile rotundata*	0.00003	0	0	0.00019

Bunias orientalis	*Megachile rotundata*	0.0018	0	0	0.01386
Capsella bursa-pastoris	*Megachile rotundata*	0.00011	0	0	0.00131
Centaurea cyanus	*Megachile rotundata*	0.00034	0	0	0.00193
Cichorium intybus	*Megachile rotundata*	0.00311	0	0	0.02947
Cirsium vulgare	*Megachile rotundata*	0.00332	0	0	0.04514
Dactylis glomerata	*Megachile rotundata*	0.00067	0	0	0.00411
Ligustrum vulgare	*Megachile rotundata*	0.00001	0	0	0.00017
Lotus corniculatus	*Megachile rotundata*	0.00698	0.00049	0	0.10625
Nepeta cataria	*Megachile rotundata*	0.00006	0	0	0.00068
Papaver rhoeas	*Megachile rotundata*	0.12389	0	0	0.54177
Ranunculus bulbosus	*Megachile rotundata*	0.02604	0.00363	0	0.39918
Sambucus nigra	*Megachile rotundata*	0.00009	0	0	0.00125
Securigera varia	*Megachile rotundata*	0.00781	0	0	0.15198
Achillea millefolium	*Megachile versicolor*	0.00032	0.00027	0.00015	0.00057
Centaurea cyanus	*Megachile versicolor*	0.12714	0.00554	0	0.49748
Cichorium intybus	*Megachile versicolor*	0.00494	0	0	0.01978
Cirsium vulgare	*Megachile versicolor*	0.00052	0.00004	0	0.002
Lotus corniculatus	*Megachile versicolor*	0.04025	0.03767	0.01855	0.06711
Nepeta cataria	*Megachile versicolor*	0.01105	0.00167	0	0.04088
Papaver rhoeas	*Megachile versicolor*	0.16641	0.17061	0.00092	0.32348
Ranunculus bulbosus	*Megachile versicolor*	0.00701	0.00871	0	0.0106
Sambucus nigra	*Megachile versicolor*	0.00065	0.00019	0	0.0022
Securigera varia	*Megachile versicolor*	0.03174	0.00525	0	0.11645
Achillea millefolium	*Osmia bicornis*	0.00027	0	0	0.03759
Alliaria petiolata	*Osmia bicornis*	0.00106	0	0	0.13928
Allium ursinum	*Osmia bicornis*	0.00408	0	0	0.53279
Anthriscus sylvestris	*Osmia bicornis*	0.00058	0	0	0.02261
Bellis perennis	*Osmia bicornis*	0.00154	0	0	0.18486

Berberis aquifolium	*Osmia bicornis*	0.00001	0	0	0.00097
Berberis vulgaris	*Osmia bicornis*	0.00149	0	0	0.22891
Bunias orientalis	*Osmia bicornis*	0.002	0	0	0.06667
Capsella bursa-pastoris	*Osmia bicornis*	0.00031	0	0	0.01583
Centaurea cyanus	*Osmia bicornis*	0.00004	0	0	0.00339
Cichorium intybus	*Osmia bicornis*	0.00215	0	0	0.10227
Cirsium vulgare	*Osmia bicornis*	0.00009	0	0	0.00756
Dactylis glomerata	*Osmia bicornis*	0.00075	0	0	0.20708
Ligustrum vulgare	*Osmia bicornis*	0.0002	0	0	0.05619
Lotus corniculatus	*Osmia bicornis*	0.00032	0	0	0.01234
Nepeta cataria	*Osmia bicornis*	0.00006	0	0	0.00552
Papaver rhoeas	*Osmia bicornis*	0.04709	0.00034	0	0.93572
Ranunculus bulbosus	*Osmia bicornis*	0.15641	0.02369	0	0.97068
Sambucus nigra	*Osmia bicornis*	0.00079	0	0	0.05221
Securigera varia	*Osmia bicornis*	0.00007	0	0	0.00722
Tilia platyphyllos	*Osmia bicornis*	0.0004	0	0	0.11937
Achillea millefolium	*Osmia caerulescens*	0.00005	0	0	0.00021
Allium ursinum	*Osmia caerulescens*	0.00001	0	0	0.00006
Bellis perennis	*Osmia caerulescens*	0.00001	0	0	0.0001
Bunias orientalis	*Osmia caerulescens*	0.00304	0.00021	0	0.01732
Capsella bursa-pastoris	*Osmia caerulescens*	0.00026	0.00003	0	0.00162
Centaurea cyanus	*Osmia caerulescens*	0.0001	0	0	0.00081
Cirsium vulgare	*Osmia caerulescens*	0.00011	0.00006	0	0.00044
Dactylis glomerata	*Osmia caerulescens*	0.00003	0	0	0.00016
Lotus corniculatus	*Osmia caerulescens*	0.37044	0.34862	0.25905	0.53253
Papaver rhoeas	*Osmia caerulescens*	0.00014	0.0001	0	0.00043
Ranunculus bulbosus	*Osmia caerulescens*	0.00372	0.00172	0.00032	0.01067
Sambucus nigra	*Osmia caerulescens*	0.0002	0.00013	0	0.00048

Securigera varia	*Osmia caerulescens*	0.00319	0.00066	0	0.01328
Achillea millefolium	*Osmia leaiana*	0.00365	0.00314	0.0006	0.00773
Centaurea cyanus	*Osmia leaiana*	0.00006	0	0	0.00022
Cichorium intybus	*Osmia leaiana*	0.089	0.01597	0.00037	0.32371
Cirsium vulgare	*Osmia leaiana*	0.00005	0.00003	0	0.00015
Lotus corniculatus	*Osmia leaiana*	0.00068	0.00073	0.00007	0.00119
Nepeta cataria	*Osmia leaiana*	0.00002	0	0	0.00006
Papaver rhoeas	*Osmia leaiana*	0.00007	0.00007	0	0.00015
Ranunculus bulbosus	*Osmia leaiana*	0.0002	0.0001	0	0.0006

Table S1.2 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation describing relationships between concentrations of total contents of the three protein-related nutrients, fat and sterols for pollen specifically collected by bees. Coefficients with significant *P*-values (**p*<0.05, ***p*<0.01 and *** *p*<0.001) after Benjamini Hochberg correction (FDR) are marked in bold. N gives the number of plant species included in each correlation analysis.

Nutrients	**Polypeptides**	**Free amino acids**	**Fatty acids**	**Sterols**
Protein	**0.720***** (N = 34)	**0.280***** (N = 34)	**0.016***** (N = 25)	**0.054***** (N = 13)
Polypeptides	-	**0.234***** (N = 34)	**0.022***** (N = 25)	**0.068***** (N = 13)
Free amino acids	-	-	**0.052***** (N = 25)	**0.217***** (N = 13)
Fatty acids	-	-	-	**0.134*** (N = 38)

Supplementary Material 2

Supplementary Material Tables S2.1 – S2.20 in Excel file in the digital version of the thesis:

Table S2.1 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between concentrations of individual free amino acids of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.2 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between concentrations of individual polypeptide amino acids of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.3 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between concentrations of individual fatty acids of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.4 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between concentrations of individual sterols of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.5 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between concentrations of individual free amino acids and polypeptide amino acids of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.6 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between concentrations of individual free amino acids and fatty acids of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.7 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between concentrations of individual free amino acids and sterols of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.8 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between concentrations of individual polypeptide amino acids and fatty acids of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.9 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between concentrations of individual polypeptide amino acids and sterols of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.10 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between concentrations of individual fatty acids and sterols of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.11 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between proportions of individual free amino acids of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.12 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between proportions of individual polypeptide amino acids of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.13 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between proportions of individual fatty acids of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.14 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between proportions of individual sterols of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.15 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between proportions of individual free amino acids and polypeptide amino acids of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.16 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between proportions of individual free amino acids and fatty acids of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.17 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between proportions of individual free amino acids and sterols of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.18 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between proportions of individual polypeptide amino acids and fatty acids of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.19 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between proportions of individual polypeptide amino acids and sterols of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

Table S2.20 Correlation coefficients of phylogenetic generalized least squares (PGLS) models fit by maximum likelihood with Brownian correlation denoting relationships between proportions of individual fatty acids and sterols of (A) the complete dataset, (B) plant species depending little on or are independent of insect pollination or (C) plant species highly or fully dependent of insect pollination. Positive coefficients with significant *p*-values after Benjamini Hochberg correction (FDR) are marked in green, negative ones in blue.

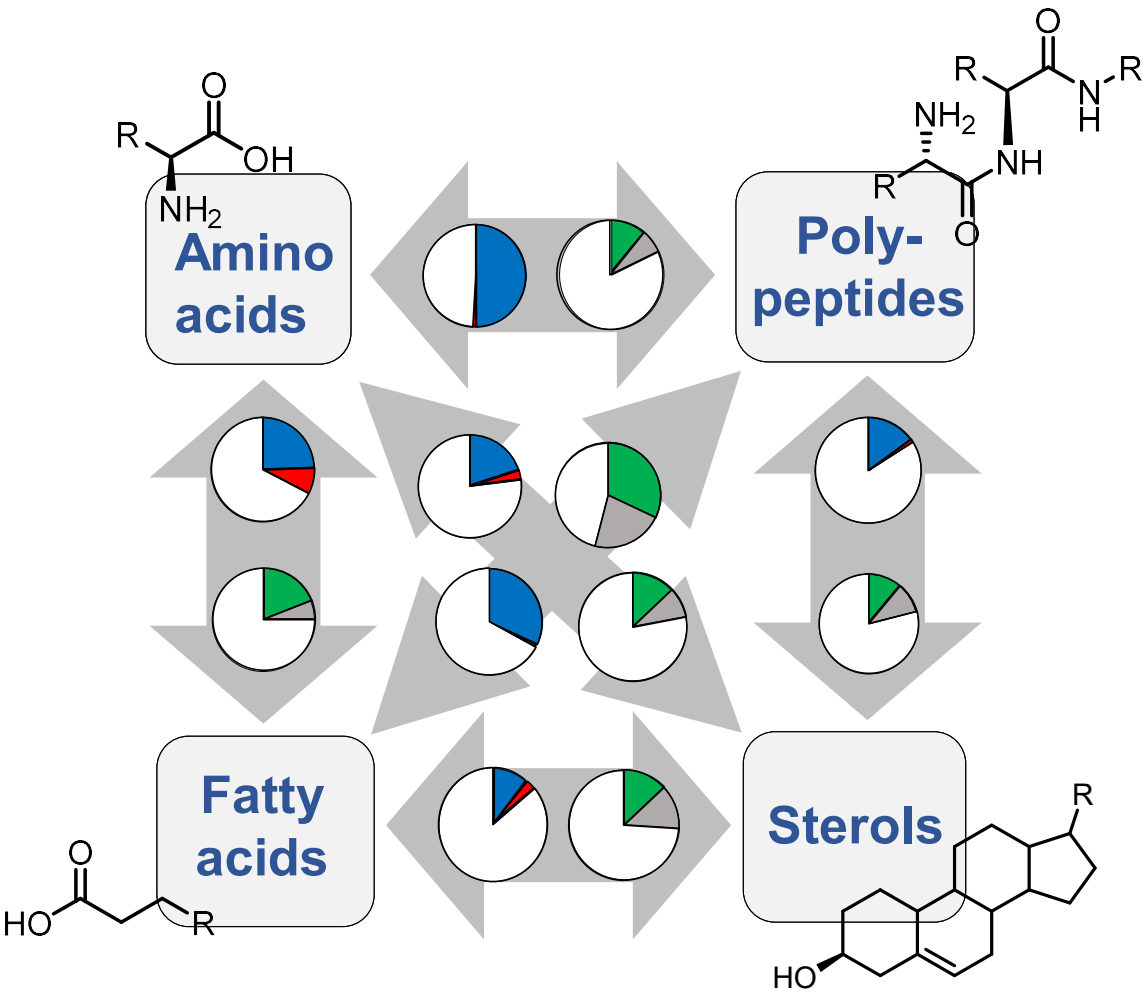


Figure S2.1 Proportions of significant correlations between all individual nutrient compound concentrations (blue and red) and proportions (green and grey) between the different nutrient classes (amino acids, polypeptides, fatty acids and sterols) in pollen of 139 plant species. Blue and green represent positive correlations, red and grey negative correlations.

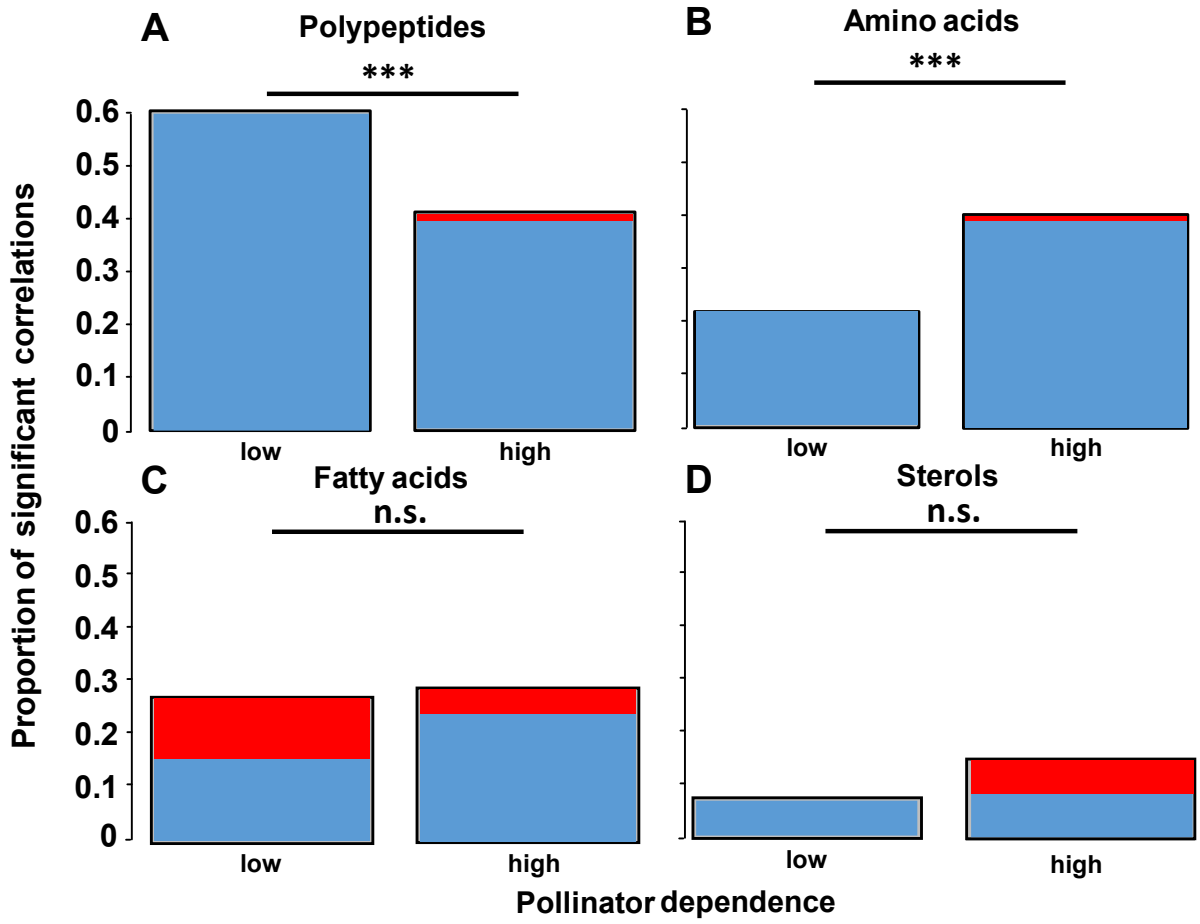


Figure S2.2 Differences in the proportions of significant correlations between all individual nutrient compounds within the same nutrient class (concentrations) between different levels of pollinator dependence (low = none & low, high = high & full). Blue represents positive correlations, red negative correlations. Differences were found for polypeptides (A, (χ^2 = 19.428, P < 0.001)) and amino acids (B, (χ^2 = 36.756, P < 0.001)). No differences were found for fatty acids (C, (χ^2 = 1.728, P = 0.189)) and sterols (D, (χ^2 = 2.89, P = 0.089)). Asterisks above the bars indicate the level of significance (n.s. P > 0.05, *** P < 0.001).

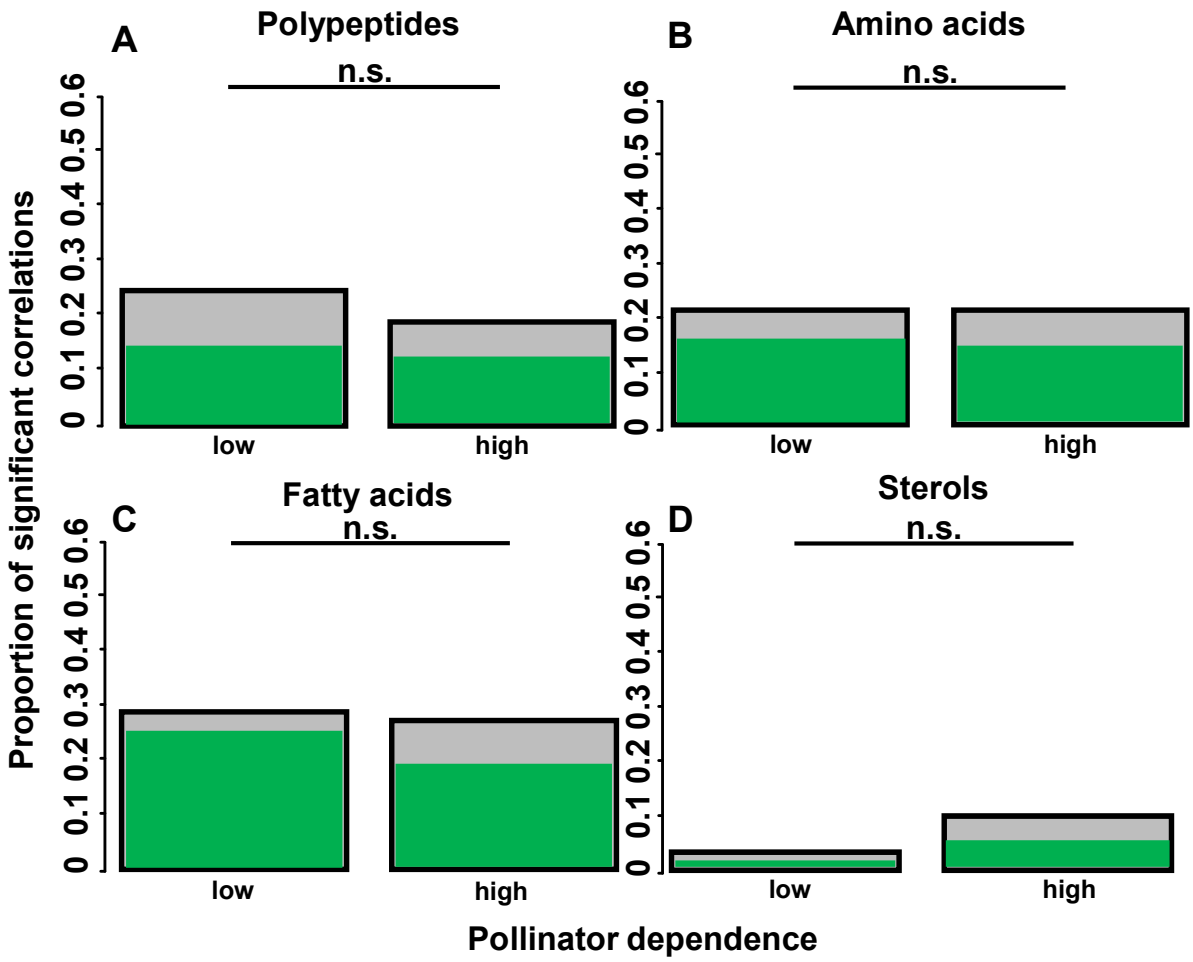


Figure S2.3 Differences in the proportions of significant correlations between all individual nutrient compounds within the same nutrient class (proportions) between different levels of pollinator dependence (low = none & low, high = high & full). Green represents positive correlations, grey negative correlations. No significant differences were found for any nutrient class: polypeptides (A, (χ^2 = 2.768, P = 0.096)), amino acids (B, (χ^2 = 0.002, P = 0.959)), fatty acids (C, (χ^2 = 1.663, P = 0.197)) and sterols (D, (χ^2 = 3.687, P = 0.055)), but a trend can be seen for sterols.

Chapter II: Differentiation and preferences of bees of pollen from different plant species

WP3 Do honeybees (*Apis mellifera*) differentiate between different pollen types?

Authors:

Fabian A. Ruedenauer*, Christine Wöhrle*, Johannes Spaethe, Sara D. Leonhardt

*shared first authorship

published: 2018

Journal: PLoS ONE

Volume: 13 Issue: 11 Pages: e0205821

Abstract WP3

Bees receive nectar and pollen as reward for pollinating plants. Pollen of different plant species varies widely in nutritional composition. In order to select pollen of appropriate nutritional quality, bees would benefit if they could distinguish different pollen types. Whether they rely on visual, olfactory and/or chemotactile cues to distinguish between different pollen types, has however been little studied. In this study, we examined whether and how *Apis mellifera* workers differentiate between almond and apple pollen. We used differential proboscis extension response conditioning with olfactory and chemotactile stimulation, in light and darkness, and in summer and winter bees. We found that honeybees were only able to differentiate between different pollen types, when they could use both chemotactile and olfactory cues. Visual cues further improved learning performance. Summer bees learned faster than winter bees. Our results thus highlight the importance of multisensory information for pollen discrimination.

Introduction WP3

Social bees collect nectar and pollen from flowers to nourish their colony and simultaneously transfer pollen between flowers, which is crucial for the reproduction and conservation of about 80% of all flowering plant species worldwide (Ollerton et al. 2011; Wang et al. 2013). The nutrient content of pollen differs strongly between different plant species (Roulston and Cane 2000b) and is directly linked to bee health (Alaux et al. 2010; Brodschneider and Crailsheim 2010; Di Pasquale et al. 2013; Roger et al. 2017). In fact, imbalanced diets may play a significant role in the observed decline of honeybee colonies (Naug 2009), because the nutritional state of a colony strongly affects its health and fitness (Archer et al. 2014; Brodschneider and Crailsheim 2010; Potts et al. 2010). Consequently, a nutritionally balanced

diet strengthens the entire colony, and well-nourished honeybees are generally more resistant to pathogen infections or other stressors (Alaux et al. 2010; Archer et al. 2014; Potts et al. 2010; Szymas and Jedruszuk 2003). While, for honeybees, nutritional requirements have been well defined (e.g. Altaye et al. 2010; Archer et al. 2014; Brodschneider and Crailsheim 2010; DeGroot 1953; Di Pasquale et al. 2013), sensory modalities involved in resource selection based on nutritional criteria have received less attention, which is particularly true for pollen foraging (Nicholls and Hempel de Ibarra 2016). While nectar provides predominantly carbohydrates, pollen supplies both larvae and adult bees additionally with essential macro- and micro-nutrients (Roulston and Cane 2000b), including proteins (DeGroot 1953), lipids (Almeida-Muradian et al. 2005; Katsumata et al. 1975; Manning 2001), inorganic compounds (Togasawa et al. 1967b) and vitamins (Togasawa et al. 1967a). Pollen consequently represents a very complex mixture of different chemical substance classes (also including non-nutritional plant secondary metabolites). It is typically collected from a large spectrum of different plant species (Kevan and Baker 1983), with pollen nutrient content strongly differing between different species (Roulston and Cane 2000b). Colonies would therefore benefit if foragers assessed pollen nutritional composition (henceforth referred to as pollen quality) at flowers and distinguished between different pollen types differing in nutrient content (Alaux et al. 2010; Pernal and Currie 2002; Ruedenauer et al. 2016). However, the chemical complexity of pollen renders this task challenging, as it confronts bees with a large variety of different chemical cues, which could (in theory) be used to infer quality and thus for differentiation. Pollen foragers are further likely influenced by additional (non-nutrient related) factors, such as the current provisional state of the colony (Fewell and Winston 1992), weather conditions or season (McCall and Primack 1992; Riessberger and Crailsheim 1997), which may affect their nutritional target and thus choice of chemical cue

used for differentiation. Although most nutrients are inaccessibly stored within the pollen cell walls, some nutrients, such as amino acids and lipids, can easily be accessed without digestion (Pacini and Hesse 2005) and may therefore represent promising cue candidates for differentiation.

Bees likely rely on floral and/or pollen color (i.e. vision), floral and/or pollen odor (i.e. olfaction) and/or pollen taste (i.e. their sensitivity to chemotactile cues) as cues to distinguish between different types of pollen (Nicholls and Hempel de Ibarra 2016; Pernal and Currie 2002; Ruedenauer et al. 2017; Ruedenauer et al. 2015). Honeybees (*Apis mellifera*) can learn floral patterns, shapes and colors of different plant species and foraging decisions are often based on such visual cues (Dyer et al. 2008; Muth et al. 2015; Nicholls and Hempel de Ibarra 2016). Honeybees can also discriminate between many different odors (Dietz and Humphreys 1971; Schwarz 1955; von Frisch 1921) and thus various floral scents (Chittka et al. 1999; Reinhard et al. 2004), for example field-bean (*Vicia faba*, Fabaceae) and oilseed-rape (*Brassica napus*, Brassicaceae) pollen scent (Cook et al. 2005). They may also use taste/gustatory receptors on the distal segment of their antennae, their mouthparts and the tarsi of their forelegs to perceive water, sugars, salt and possibly other nutrients (de Brito Sanchez 2011; Ruedenauer et al. 2017). Because it is still unknown whether taste reception via touch is primarily chemical, tactile or a mix of both, we generally refer to taste or gustatory cues as chemotactile cues. Notably, the use of such chemotactile cues for pollen differentiation has as yet not been studied in honeybees (de Brito Sanchez 2011). This is surprising given that resource nutritional quality can only be directly inferred from taste perception, which is a prerequisite for selecting e.g. the currently “best” pollen type directly in the field. Moreover, several studies (reviewed in Nicholls and Hempel de Ibarra 2016) indicate that honeybees are,

just like bumblebees (Ruedenauer et al. 2017; Ruedenauer et al. 2015), able to assess pollen nutritional quality.

In this study, we investigated the contribution of the major senses to pollen type differentiation using differential conditioning of the proboscis extension response (PER), a behavior which relies on the bees' innate response to sugar water and is used in many studies investigating learning and memory formation in (honey)bees (e.g. Bitterman et al. 1983; Takeda 1961; Vareschi 1971). We tested whether honeybees can discriminate between two pollen types using (i) only olfactory cues, (ii) olfactory and chemotactile cues and (iii) olfactory, chemotactile and visual cues. Experiments were further conducted in two different seasons (summer and winter) to account for possible effects between these two groups on learning performance. Based on the previous work by Cook et al. (2005), we expected that honeybees were able to distinguish between the two different pollen types offered (i.e. almond (*Prunus dulcis*, Rosaceae) and apple (*Malus domestica*, Rosaceae) pollen) based on olfactory cues alone. We further hypothesized that access to both chemotactile and visual cues would improve their differentiation ability, as discrimination is improved by using several interrelated cues (Nicholls and Hempel de Ibarra 2016). We finally assumed that bees tested in summer would show better performance in pollen differentiation than winter bees, because they are more experienced with the task of differentiating between different pollen types and differ physiologically from winter bees (Fluri et al. 1982).

Materials and Methods WP3

Study animals and test substances

Experiments were performed with the western honeybee (*Apis mellifera carnica*) at different times of the year. Honeybee colonies were kept at the bee-station at the Biocenter of the

University of Würzburg, Germany. In the first and third test period, conducted in August 2016 and May 2018, respectively, leaving forager bees were caught randomly from the entrance of five different hives placed outside in the field (henceforth referred to as summer bees). In the second period, conducted in October and November 2016, honeybees were collected in the same way, but from two hives kept in a heated greenhouse, where they could forage on bee-collected pollen (Naturwaren Niederrhein GmBH, Goch Asperden, Germany), over the winter months (henceforth referred to as winter bees).

Hand-collected apple (*Malus domestica*, Rosaceae) and almond (*Prunus dulcis*, Rosaceae) (anther) pollen (obtained from Firman Pollen, Yakima, WA, USA) were used to investigate the contribution of olfactory, visual and chemotactile cues used for pollen type differentiation. Both pollen types were most likely new to our bees, as both almond and apple pollen was collected from plants grown in the United States. Also, apple flowers in spring. Summer bees in May and August thus hardly encounter apple pollen, unless it was stored for a prolonged period and then also processed and mixed with other pollen. Such pollen most likely strongly differs from the fresh non-processed pollen used in our experiments. Pollen was placed on a wet filter paper to test olfactory cues, and pollen was mixed with de-ionized water (60 ml apple pollen + 55 ml water, 60 ml almond pollen + 60 ml water; different amounts of water were added to reach a similar consistency) to create a paste, which could be applied to the plates for testing chemotactile cues (Ruedenauer et al. 2015, see below).

The amino acid contents of both pollen types were analyzed using ion exchange chromatography (IEC) (for a detailed method description see Ruedenauer et al. 2015).

Experimental setup

The following restraining procedure was adapted from Bitterman et al. (1983). Upon capture from hives, foragers (between ten and 20 individuals per container) were chilled on ice for about 10 min to reduce their activity. They were then harnessed in plastic tubes (25x10 mm) made from pipette tips and fixed with two crepe tape strips (Hartenstein, Würzburg, Germany). A broad strip (10 mm) was wrapped around the tube horizontally to prevent honeybees from moving their abdomen, while a smaller strip (1 mm) was placed between the bee's head and thorax and allowed free movement of the antennae, mandibles and proboscis (Bitterman et al. 1983). All restrained individuals were fed 4 µl of 30 % w/w sucrose solution with a micropipette and finally kept for 3 h in a climate chamber at 25 °C at a relative humidity of 50 %.

All experiments were conducted in a temperature constant room (≈ 22 °C) at the University of Würzburg, Germany. The experimenter always wore latex gloves to avoid interference of pollen odors and the smell of human skin. After three hours starvation time, each bee was tested for a proper PER by presenting 30 % w/w sucrose solution with a toothpick to their antennae. We used only those bees that extended their proboscis upon this gustatory stimulation (about 80% of the bees) for the following experiments, while all other bees were discarded. All individuals were used in one experiment only.

Differential PER conditioning

All conditioning experiments were adapted from Ruedenauer et al. (2015). For differential conditioning, we used two conditioned stimuli (pollen types) and an unconditioned stimulus (US: sucrose) as reward. However, in contrast to classical PER conditioning, where the CS is neutral at the beginning and bees usually do not respond upon presentation, almost all

honeybees spontaneously extended their proboscis after they received the pollen odor (see below). Consequently, the learning curves in our experiments usually started at high response levels and decreased in the course of the experiment, when the bees had learned that the non-rewarded stimulus was not rewarded with sucrose. We therefore refer to all tested stimuli as S instead of CS, because CS typically defines a neutral stimulus, which, as turned out, was not the case in our learning experiments (see below). These experiments should therefore not be compared to classical PER conditioning, and the percentage of bees showing a PER should not be seen as learning performance, as is usually is the case in classical PER conditioning. Based on our results, we can however make inferences on whether bees can differentiate between the two pollen types.

To test whether bees were able to distinguish the two different pollen types, one pollen type (S+) was rewarded with an unconditioned stimulus (US) (i.e. sucrose solution) (as for CS in Bitterman et al. 1983). The US was presented with a toothpick covered with 30 % w/w sucrose solution, touching one of the bees' antennae, and the bee was allowed to lick the toothpick. The second pollen type remained unrewarded (S-). If bees were able to discriminate S+ and S-, they should only show a PER when the S+ was presented in anticipation of the associated US. Both pollen types were used as S+ and S-, respectively, with a similar number of bees tested.

For all conditioning experiments, we used a standard protocol established for bees by Bitterman et al. (1983). Each individual went through 20 trials (10 S+ and 10 S- trials) presented in a pseudo-randomized order, with an inter-trial interval (ITI) of 8 min. In the first 15 s of each trial, the individual bee was allowed to rest and habituate to the setup. Then, the S was presented for 6 s. In the case of a rewarded trial, the US was offered in addition to the

S+ by briefly touching one antenna with sucrose solution 3s after the S+ presentation started. The bee was then allowed to lick the reward as soon as it extended its proboscis. In an unrewarded trial, only the S- was presented for 6 s. Finally, the trial ended with a period of another 15 s resting time before the bee was replaced by the next individual.

To test whether olfactory cues were sufficient to enable honeybees to distinguish between apple and almond pollen, 10 mg, 50 mg and 300 mg apple or almond pollen were placed on a wet filter paper inside a 20 ml syringe. Different amounts were used to test whether pollen (and thus odor) amount affected learning. Even though equally large pollen amounts are clearly not found at flowers, bees may still encounter large amounts of pollen stored in their nests.

The used filter paper equaled the size of the diameter of the syringe to avoid spillage of pollen. To prevent the plunger from touching the pollen while pressing, a pin was pierced through the syringe at its 4 ml mark. For presentation of the S, the syringe was at maximum filled with air and the plunger pressed slowly downwards until the pin stopped it. The so produced airstream was directed at the bees' antennae forcing bees to rely on pollen odor only to distinguish between pollen types. Overall, 120 bees were tested during olfactory conditioning (ten bees per experimental round, only summer bees captured in May).

To test for the importance of chemotactile cues, additional experiments were performed using cupreous sticks with a small plate (3x4 mm) at one end (Scheiner et al. 1999). For the S, 50 mg of the pollen pastes were applied to the plate, which was then moved towards one of the bees' antennae by means of a micromanipulator and touched the antenna for 6 s. After the trial, all plates were cleaned in 70 % ethanol (Ruedenauer et al. 2015). Overall 96 bees

were tested in chemotactile conditioning, 32 summer bees and 64 winter bees (eight bees per experimental round).

To test for the importance of visual cues, chemotactile conditioning was performed under both, red light conditions with a spectrum larger than 640 nm (*N* = 32 individuals), which they cannot perceive (Menzel and Blakers 1976; Peitsch et al. 1992), and daylight conditions (i.e. light from outside plus fluorescent tubes in the laboratory, *N* = 32 individuals) where bees could not only touch, but also see the pollen pastes tested and thus may use visual cues in addition to chemotactile (and olfactory) cues. Here, only winter bees were tested.

Unrewarded PER experiment

Our experiments revealed that honeybees did not differentiate between the two different pollen types when only olfactory cues were presented (see below), likely because pollen odor alone evoked a spontaneous PER, similarly to the innate PER shown in response to sugar solution. This spontaneous response had also been observed in previous studies (Nicholls and Hempel de Ibarra 2016; Scheiner et al. 2004). In contrast, honeybees were able to differentiate between the different pollen types when (additional) chemotactile cues were accessible (see below), likely because they now either suppressed the spontaneous PER in response to the S- or maintained high levels of PER across all trials in response to S+. In order to differentiate between the two possibilities, we performed an additional experiment in April 2017 by repeating the chemotactile conditioning experiment in the dark (to exclude visual cues). Now, half of the tested individuals did not receive a (sugar) reward (but both of the S, i.e. both pollen types) over the entire experiment, while the other half was differentially conditioned as before (see above), with individuals of both groups being tested simultaneously. The order of pollen types (or S+ and S-) was the same as described above. For

non-rewarded individuals, we therefore refer to the two different pollen types as pseudo-S+ and pseudo-S-. We assumed that if differentiation in the rewarded experiment was due to suppressing the spontaneous PER, the spontaneous response should be maintained throughout the 10 trials in the unrewarded experiment. Alternatively, if differentiation in the rewarded experiment was caused by keeping the PER response to the S+ high, the spontaneous response should gradually drop in the unrewarded experiment.

Statistical analysis

All statistical tests were conducted using R v 3.3.2. For olfactory and chemotactile differential conditioning experiments, the number of PER to each S were summed up and used as response variable, ranging between 0 and 10 for each bee (see Ruedenauer et al. 2015). For all conditioning experiments, generalized linear mixed effect models (GLMMs) with Poisson distribution were used (*glmmML* package) with bee individuals as random factor to account for repeated testing of the same bee (and thus data dependency). We first tested whether the interaction between stimulus (i.e. S+, S-) and pollen type (apple, almond) significantly affected the number of PER. We found no significant effect for this interaction neither for olfactory (z_{73} = -0.554, P = 0.579, Fig.WP3.S1) nor chemotactile trials (z_{187} = 1.791, P = 0.073, Fig. WP3.S2), indicating that the type of pollen used for S+ and S- did not affect the bees' learning performance (Ruedenauer et al. 2015). Therefore, only stimulus (S+, S-) was tested for a significant effect on PER numbers in a second set of models.

We additionally tested for a significant effect of the interaction between stimulus and illumination (light, dark) and the interaction between stimulus and season, as chemotactile differential conditioning experiments were further conducted at two different time periods. Both season and illumination significantly interacted with stimulus type in affecting PER

numbers (see results). Therefore, separate GLMMs were performed for each group (i.e. summer and winter bees, and bees tested in light and dark) to test for the effect of stimulus on PER numbers in each group. Note that the same group of winter bees (i.e. 32 individuals) tested in chemotactile conditioning in light was included twice in our models (for the summer – winter and light – dark comparison). Because of multiple testing of the same dataset, we finally performed a *P*-value adjustment using Bonferroni correction. All *P*-values remained significant after correction.

To test whether bees tested at different times showed differences in their general response behavior prior to conditioning, we compared the number of spontaneous responses in the first trial across all three experiments using a Chi-squared homogeneity test. We found no significant differences in the first responses between groups ($x^2_2 = 0.82$, $P = 0.663$, Fig. WP3.S3), indicating that all study bees shared the same initial response behavior.

To compare the amino acid profiles of both pollen types we also used a Chi-squared homogeneity test.

In the unrewarded PER experiment, we applied generalized linear models (GLM) with Poisson distribution to compare differences between pollen types and (pseudo-) S+ and S- followed by a Tukey test for multiple comparisons.

Results WP3

Differential PER conditioning of olfactory cues

Honeybees were not able to distinguish between apple and almond pollen, based on olfactory cues alone ($z_{237} = 0.145$, $P = 0.885$; Fig. WP3.1), independent of pollen amount. Interestingly, the majority of all tested individuals (>90 %) showed a spontaneous PER to pollen odors

immediately after the first presentation of the S (pollen) and thus before the US (sugar solution) was provided. Moreover, the average response rate to both S+ and S- remained high over all ten conditioning trials (i.e. above 85 %, Fig. WP3.1).

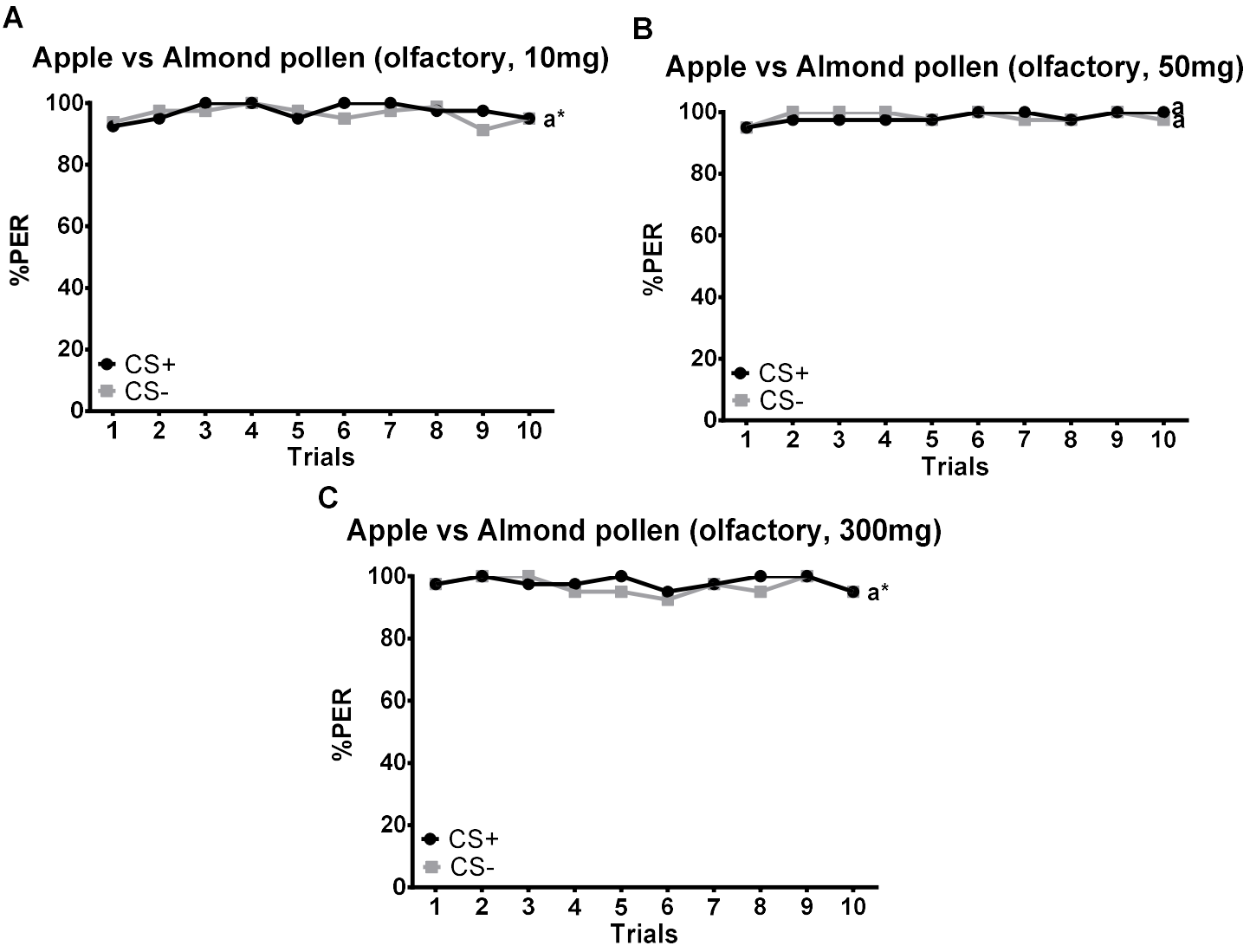


Figure WP3.1 Percentage of proboscis extension responses (%PER) shown by *Apis mellifera* individuals (*N* = 120) in differential olfactory conditioning to the odor of (A) 10 mg (N = 40), (B) 50 mg (N=40) and (C) 300 mg (N = 40) of apple versus almond pollen over 10 trials. S+ (black) represents the rewarded conditioned stimulus, S- (grey) the unrewarded conditioned stimulus. Both, apple and almond pollen were used as S+ and S-. As there was no significant difference in learning performance between apple and almond pollen odor used as S+ or S- (z_{227} = 0, *P* = 1, Fig. WP3.S1), both groups were summarized into one. Similar letters next to each line indicate no significant difference between stimuli (*P* > 0.05). Asterisks indicate overlapping letters.

Differential PER conditioning of chemotactile cues

When honeybees were allowed to touch the pollen paste with their antennae, they were able to distinguish between apple and almond pollen (z_{189} = 14.34, P < 0.001, Fig. WP3.2). Again, a high proportion of individuals showed a spontaneous PER in the very first trial (84-91 %), independent of season or setup (Fig. WP3.S3), but the number of PER responses towards S- decreased in subsequent trials, unlike those towards the S+ (Fig. WP3.2).

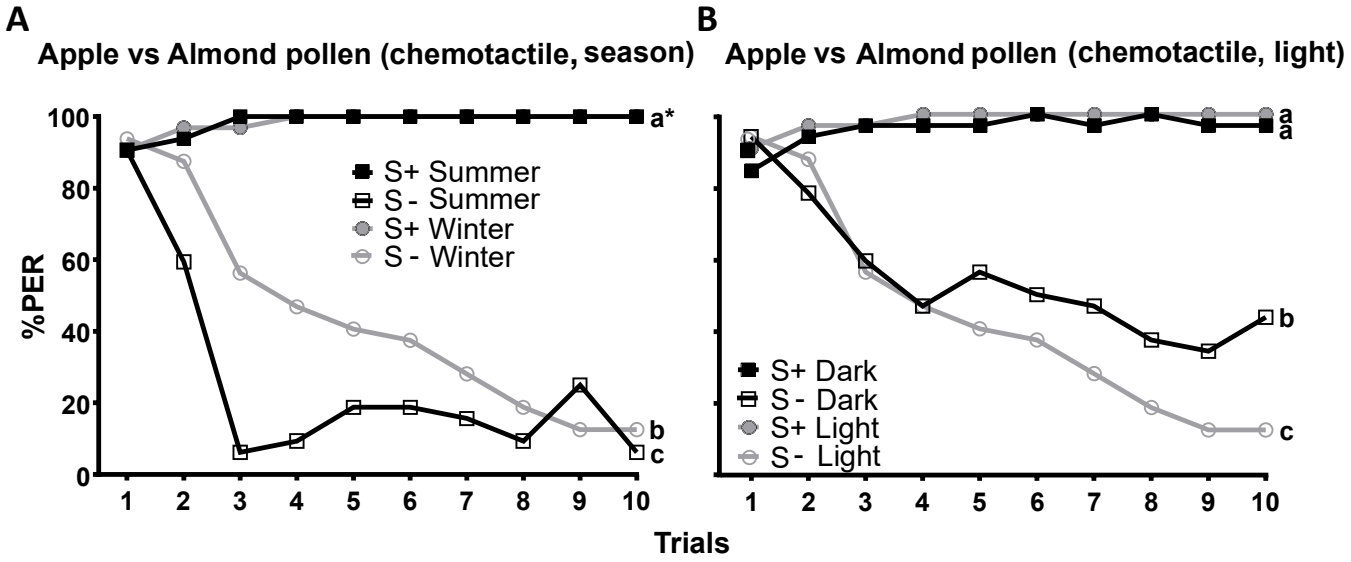


Figure WP3.2 Percentage of proboscis extension responses (%PER) shown by *Apis mellifera* individuals in differential chemotactile conditioning to the taste of apple versus almond pollen over ten trials tested in (A) different seasons (*N* = 64) and (B) with and without the availability of visual cues (*N* = 64). S+ represents the rewarded conditioned stimulus, S- the unrewarded conditioned stimulus. Both apple and almond pollen were used as S+ and S-. As there was no significant difference in learning performance between the taste of apple and almond pollen used as S+ or S- (z_{187} = 1.791, P = 0.073, Fig. WP3.S2), both groups were summarized into one. (A) Differential conditioning of chemotactile cues in summer (square, N = 32) and winter (circle, N = 32). (B) Differential conditioning of chemotactile cues in winter in light (circle, N = 32) and darkness (square, N = 32). Different letters next to the lines indicate significant differences between groups (Tukey test for the models comparing S+ and S-, P < 0.001 for all differences). An asterisk indicates the same letter for two overlaying curves.

Both summer- and winter bees were able to distinguish the two pollen types (summer bees: z_{61} = 10.335, P < 0.001; winter bees: z_{125} = 9.874, P < 0.001), but winter bees required more trials to reach the same differentiation level as summer bees (significant interaction: z_{187} = -4.597, P < 0.001; Fig. WP3.2A, Fig. WP3.S4).

Likewise, bees tested in light and in darkness were both able to distinguish the two pollen types (light: $z_{125} = 13.36$, $P < 0.001$; darkness: $z_{61} = 5.934$, $P < 0.001$; Fig. WP3.2B), but bees tested in light showed an overall higher learning performance (significant interaction: $z_{187} = 3.919$, $P < 0.001$; Fig. WP3.2B).

Unrewarded PER experiment

In the unrewarded PER experiment, no difference was found for the PER rates towards the two different pollen types ($z_{89} = -0.753$, $P = 0.451$). However, responses to the four stimulus types (i.e. S+, S-, pseudo-S+ and pseudo-S-) differed significantly ($z_{89} = 2.874$, $P = 0.027$; Fig. WP3.3). The two pseudo-S rates were similar, but differed from both conditioned S, while the differences between rewarded S+ and S- remained significant (Fig. WP3.3).

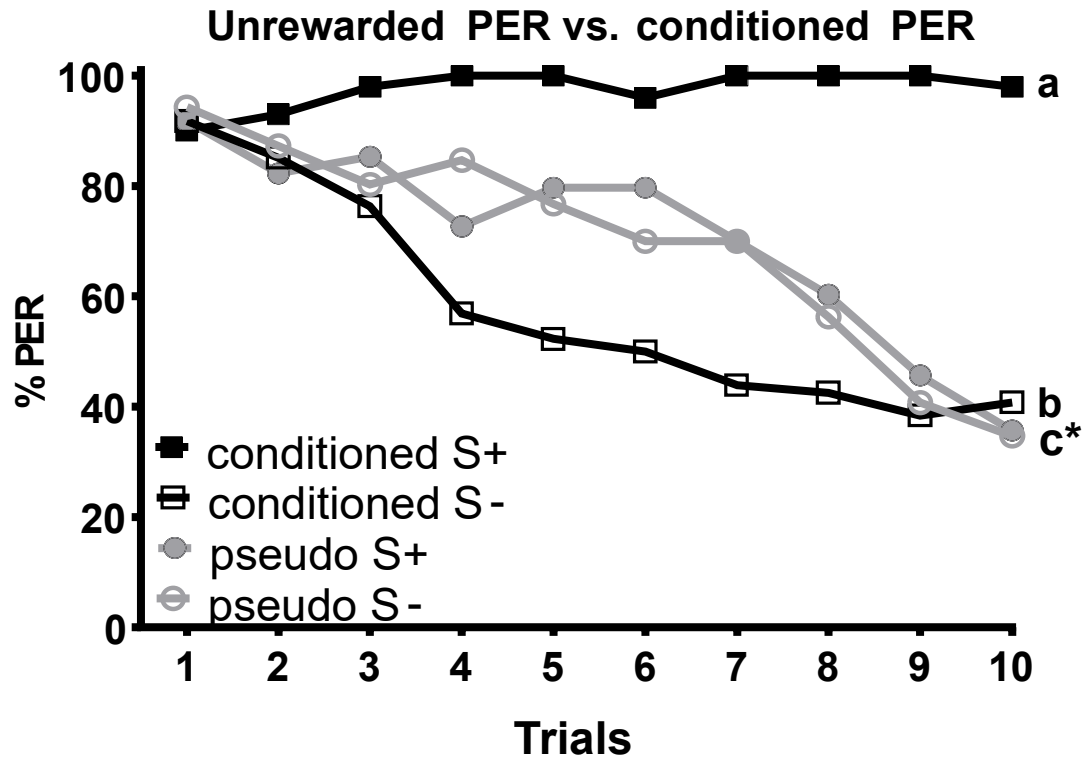


Figure WP3.3 Percentage of proboscis extension responses (%PER) shown by *Apis mellifera* individuals in the unrewarded trials and differential chemotactile conditioning in the dark to the taste of apple versus almond pollen. Bees of the four groups were tested within the same experimental series. As before, the conditioned S+ and S- (*N* = 20) represent the rewarded and unrewarded conditioned stimulus. In contrast, both pseudo-S+ and S- represent unrewarded stimuli presented to a second group of individuals (*N* = 20). Both apple and almond pollen were used as (pseudo) S+ and S-. As there was no significant difference in PER rate between the taste of apple and almond pollen used as (pseudo) S+ or S- (z_{87} = 0.608*, P* = 0.543), both groups were summarized into one. Different letters next to the lines indicate significant differences between groups. An asterisk indicates the same letter for two overlaying curves.

Discussion WP3

The ability to discriminate different pollen types would be highly beneficial for bees for optimizing their nutritional intake (Alaux et al. 2010; Pernal and Currie 2002; Ruedenauer et al. 2015). Contrary to our hypothesis and unlike bumblebees (Ruedenauer et al. 2015), honeybees were not able to distinguish between apple and almond pollen based on olfactory cues alone, but needed (additional) chemotactile cues. However, the bees clearly perceived the presented pollen scents as demonstrated by the high rate of spontaneous PER for both pollen odors at the beginning of the conditioning experiments (above 90 %; Fig. WP3.1).

The differences between our findings and the results of Cook et al. (2005) may be explained by the different pollen types tested. While Cook et al. (2005) selected pollen from different plant families (Fabaceae and Brassicaceae), we used apple and almond pollen, which both belong to the Rosaceae. We can thus not rule out family-specific similarities in odor composition, which may have rendered distinction between the odor of apple and almond pollen more difficult for honeybees (Deisig et al. 2002; Wright et al. 2005; Wright et al. 2002; Wright and Smith 2004b). In fact, bees can discriminate similar odors worse than dissimilar odors (Laska et al. 1999), but can improve discrimination upon repeated exposure (Laska et al. 1999; Smith et al. 1991; Wright and Smith 2004a). Alternatively (or additionally) different results may be explained by different experimental setups. While Cook et al. (2005) used glass wool (which may filter out components that elicit a spontaneous PER), we placed pollen on a humidified filter paper, which may have dissolved additional pollen odor compounds and thereby have provided different cues eliciting spontaneous responses. Cook et al. (2005) further used bee-collected pollen in their differential experiments, which could contain additional volatile substances not found in pure pollen as used in our study. The pollen used by Cook and Sandoz may however be comparable to pollen stored in hives and their bees' responses thus represent in-hive situations, whereas our results rather represent foraging decisions in the field.

It cannot be entirely ruled out that honeybees were able to distinguish between the odor of apple and almond pollen but this was masked by the high rate of spontaneous PER. In fact, olfactory cues alone may not have been sufficient for suppressing their spontaneous proboscis extension response. This may be one reason why honeybees showed similarly high response rates for S+ and S-, whereas bumblebees (which did not respond spontaneously) were able to distinguish almond from apple pollen by odor cues alone (Ruedenauer et al.

2015). One explanation for why bumblebees did not respond spontaneously may be their overall lower motivation to extend their proboscis (Laloi et al. 1999). Alternatively, bumblebees may rely on different components of the presented pollen odors for decision-making. Moreover, bumblebees assessed pollen quality and selected pollen of higher quality in a choice experiment (Ruedenauer et al. 2015; Ruedenauer et al. 2016), whereas comparable studies on honeybees did not find any preferences for high-quality pollen (Pernal and Currie 2001; Pernal and Currie 2002). Unlike honeybees with their mass recruiting dance language, individual bumblebee foragers also tended to rely more on "personal information" than on "social information" (Leadbeater and Florent 2014) and do receive little feedback from larvae or nest-mates (Goulson 2003). In turn, individual (recruited) honeybees may not themselves assess pollen quality, but rather rely on feedback from nest-mates (Pernal and Currie 2002), reducing the need for nutrient selective foraging.

Contrary to the olfactory conditioning experiment where honeybees showed similar response rates to S+ and S- (Fig. WP3.1), they clearly differentiated between apple and almond pollen in chemotactile experiments (Fig. WP3.2), indicating that honeybees were able to suppress their (spontaneous) proboscis extension reaction to the S- when chemotactile cues were available in addition to olfactory cues. Thus, chemotactile cues appear to enable honeybees to overcome their spontaneous PER response and to build an association between the S+ and the reward. In fact, the differences between pseudo-S and conditioned S- in the unrewarded PER experiment suggest that conditioned bees suppress their spontaneous PER at least to some extent. Additionally, after ten trials, both pseudo-S as well as the conditioned S- resulted in a lower response level (about 40%) than the S+ (>90%), further indicating that the PER to the conditioned S+ is maintained by the reward. Consequently, differentiation in the

chemotactile conditioning experiments was due to both suppressing the PER to the S- and maintaining the PER to the S+.

We suggest that the ability of honeybees to differentiate between the two pollen types was largely based on perceiving differences in the nutritional profile of the two pollen types. Protein (i.e. amino acids), fat (i.e. fatty acids) and sugars are the most common nutrients in pollen (Roulston and Cane 2000b). Moreover, the amino acid profiles of the two pollen types significantly differ (see Table WP3.S1), rendering amino acids suitable cues for discrimination. However, other non-volatile cues, e.g. sugars, fatty acids and secondary substances, such as flavonoids, may also serve as alternative or additional cues.

Moreover, when we prevented honeybees from perceiving visual cues and thus from using the visual differences between (red-orange) apple and (yellowish) almond pollen in chemotactile experiments, they were still able to differentiate the two pollen types, but learning performance was significantly lower (i.e. 44 % of bees tested in darkness still responded to the S- by the end of ten trials compared to only 13% of bees tested in light), which would provide further evidence for the importance of visual information in supporting differential learning and thus foraging decisions in bees (Chittka and Waser 1997; Kevan et al. 2001; Muth et al. 2015; Waser 1986). Alternatively, the bees tested in light and darkness may have differed in their internal state and therefore motivation in their sensory sensitivity towards the stimuli. However, because response rates in the first trials of our experiments did not differ between winter bees tested in light and bees tested in darkness (Fig. WP3.S3), we consider light induced differences in motivation or sensory sensitivity rather unlikely.

We thus suggest that the combination of several (i.e. olfactory, chemotactile and visual) cues, a situation which more closely resembles natural conditions, likely facilitates successful

distinction between different rewards. The combination of sensory cues provided by a potential resource (e.g. pollen) likely conveys important information used by bees to assess its properties, which can then be used to learn differences between different resources (e.g. pollen types) and thus to make foraging decisions based on different resource qualities. However, multimodal cues can also reduce the ability of the bees to make the correct choices (Muth et al. 2017).

With regard to seasonal effects, winter bees needed more trials to reach a similar learning performance than summer bees (conditioned in August, Fig. WP3.S4), which agrees with our expectation and with Scheiner et al. (2003), who also observed overall higher learning performances in bees tested in August. This finding may be explained by the summer bees' experience in foraging on flowers in the field, whereas the winter bees were confined to a strongly impoverished foraging environment in the glass house. In fact, summer and winter bees kept under constant conditions in the laboratory without access to floral resources showed no difference in learning performance (Ray and Ferneyhough 1997). Differences in learning performances may further be influenced by differences in juvenile hormone titers (Huang and Robinson 1995) known to affect learning (Pham-Delegue et al. 1990; Ray and Ferneyhough 1999), associated inactivity and entailed changes in the organization of mushroom bodies (Huang and Robinson 1995; Scheiner et al. 2003; Withers et al. 1993). However, we cannot rule out that the differences found resulted from physiological characteristics specific for the 2016 winter and summer cohort. As we found no differences when comparing the number of bees spontaneously responding to the stimulus in the first trial between cohorts, we can at least presume that the summer and winter cohorts shared the same response behavior prior to conditioning (see Fig. WP3.S3).

Also note that collected pollen can be stored in the nest over prolonged periods, which can alter its chemical composition following microbial processing (Estevinho et al. 2012; Standifer et al. 1980) or enrichment with bee salivary compounds (Human and Nicolson 2006). How such chemical modification affects interactions between bees and pollen is largely unknown.

In summary, we conclude that honeybees rely on several sensory cues (i.e. olfactory, chemotactile and visual stimuli) to most effectively differentiate between different pollen types, which likely represents the most natural condition, as foraging resources typically provide more than one sensory cue. Under natural conditions, individual honeybee foragers are therefore able to differentiate different pollen types and potentially select those pollen types, which best support an optimal diet.

Supplementary material WP3

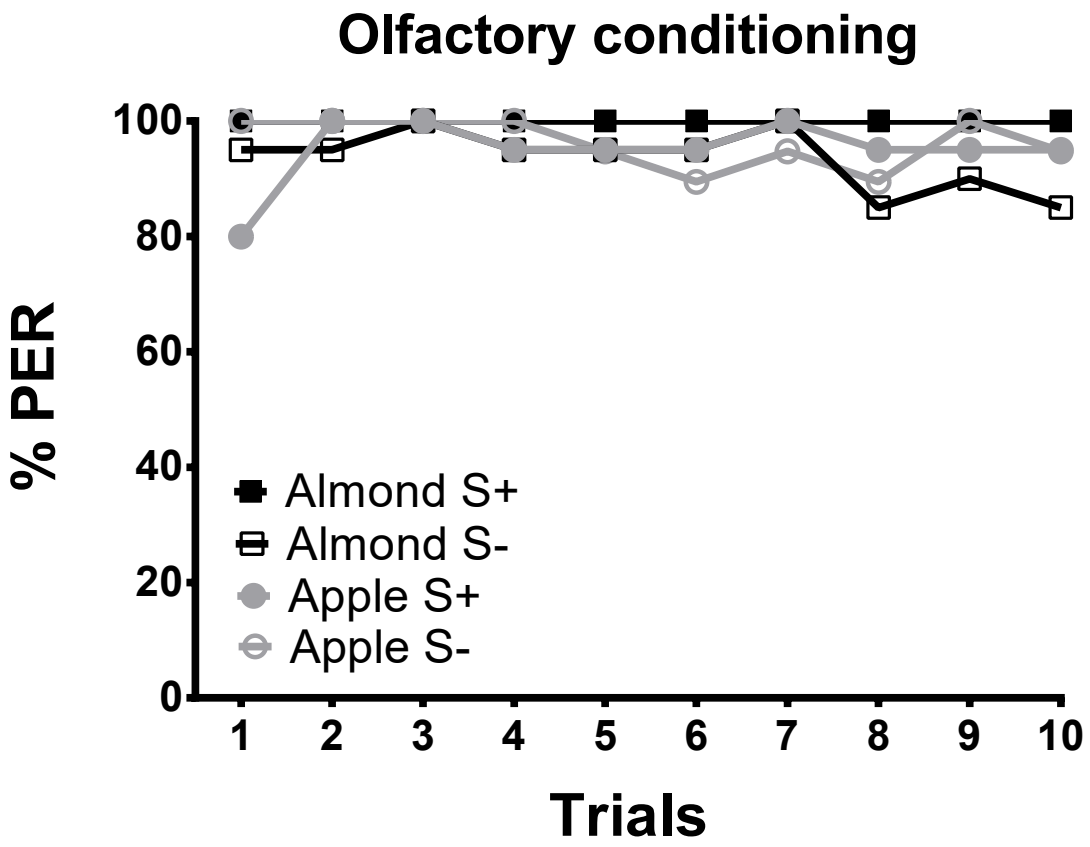


Figure WP3.S1 Percentage of proboscis extension responses (%PER) shown by *Apis mellifera* individuals (*N* = 39) in differential olfactory conditioning to the odor of apple versus almond pollen over 10 trials with separate lines for rewarded (S+, filled symbols) and unrewarded (S-, clear symbols) stimuli. Both, apple (grey) and almond (black) pollen were used as S+ and S-. There was no significant difference in learning performance between apple and almond pollen odor used as S+ or S- (z_{73} = -0.554, *P* = 0.579).

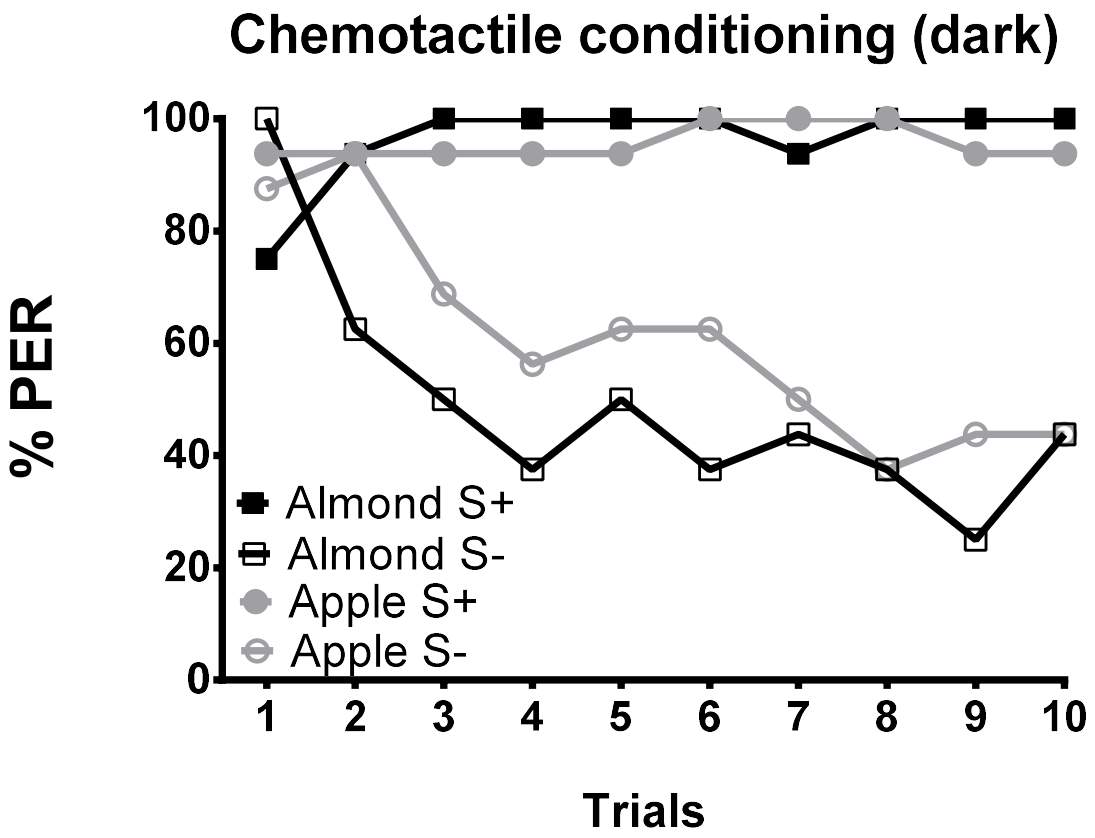


Figure WP3.S2 Percentage of proboscis extension responses (%PER) shown by *Apis mellifera* individuals (*N* = 64) in differential chemotactile conditioning in the dark to the taste of apple versus almond pollen over 10 trials with all stimuli separated. S+ (filled) represents the rewarded conditioned stimulus, S- (clear) the unrewarded conditioned stimulus. Both, apple (grey) and almond (black) pollen were used as S+ and S-. There was no significant difference in learning performance between apple and almond pollen odor used as S+ or S- (z_{187} = 1.791, *P* = 0.073).

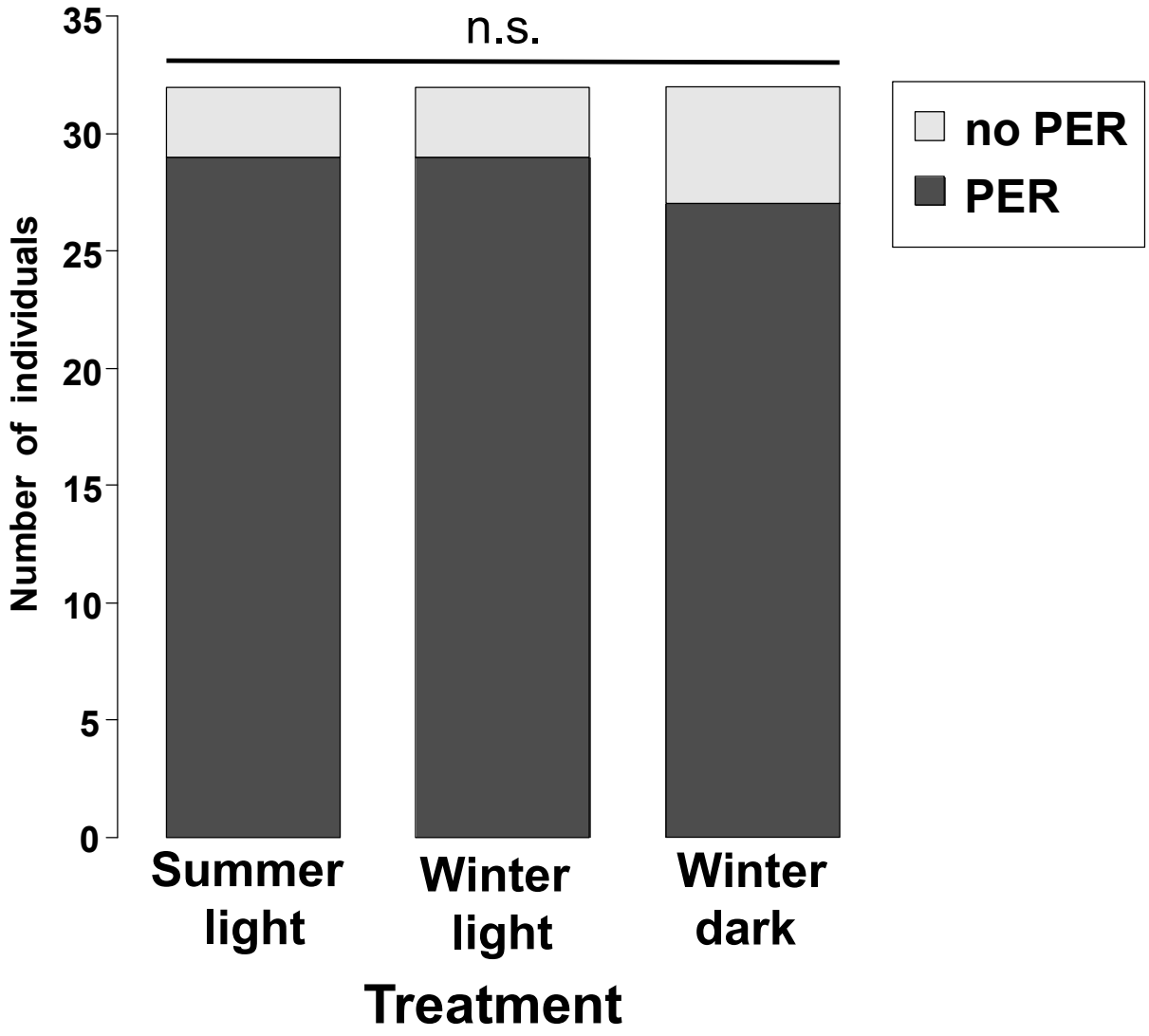


Figure WP3.S3 Number of individuals showing a spontaneous proboscis extension response (PER) to pollen chemotactile stimulation (dark grey) and not showing a PER (light grey) in the first trial of all experiments performed (N = 96). There were no significant differences (n.s.) between different seasons or light conditions ($X^2_2 = 0.82$, $P = 0.663$).

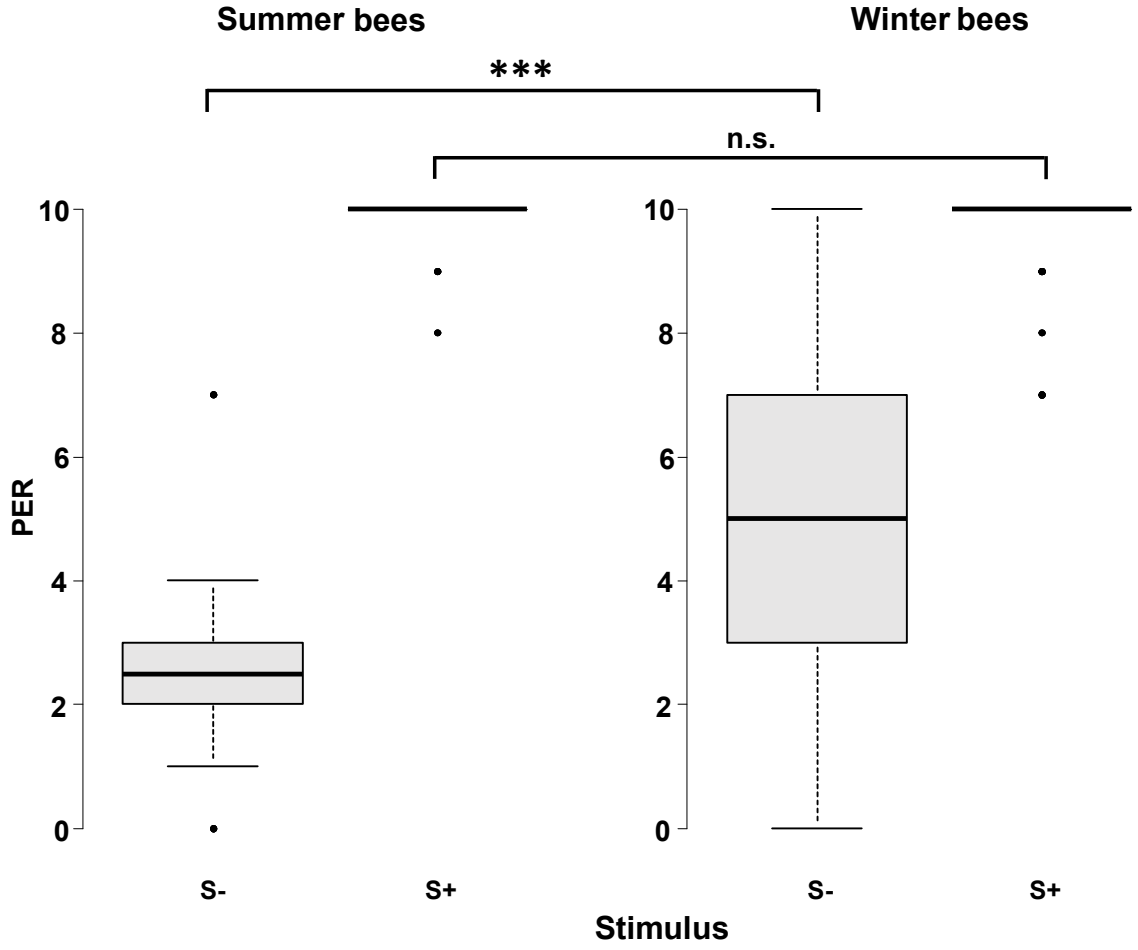


Figure WP3.S4 Number of proboscis extension responses (PER) shown by *Apis mellifera* individuals (*N* = 64) in differential chemotactile conditioning of summer (N = 32, left) and winter (N = 32, right) bees to the taste of apple versus almond pollen. Boxplots display responses to S+ and S-. S+ represents the rewarded stimulus, S- the unrewarded stimulus. Both, apple and almond pollen were used as S+ and S-. While there was no difference between the S+ between summer and winter bees (GLMM: z_{93} = -0.185, P = 0.853), summer bees responded significantly less to the S- (GLMM: z_{93} = 4.969, P < 0.001).

Table WP3.S1 Amino acid content (in µmol/g dry weight) of apple and almond pollen used in the PER experiments: determined via ion exchange chromatography (see (Ruedenauer et al. 2015)). In addition to concentrations of 20 protein-coding amino acids, concentrations for gamma-Aminobutyric acid (GABA) and hydroxyproline are provided. The amino acid contents of both pollen types differ significantly (chi squared test: $X^2_{17} = 83.379$, $P > 0.001$).

Amino acid	Apple µmol/g	Almond µmol/g
Asparagine	275.80	116.32
Hydroxyproline	1.69	6.18
Threonine	23.29	26.12
Serine	45.73	48.96
Asparagine	0.00	0.00
Glutamic acid	67.19	57.59
Glutamine	0.00	0.00
Proline	77.93	131.78
Glycine	53.04	64.36
Alanine	66.11	64.39
Valine	18.04	18.25
Cysteine	0.00	0.00
Methionine	6.59	7.34
Isoleucine	13.14	12.37
Leucine	36.26	40.74
Tyrosine	8.24	9.45
Phenylalanine	20.86	30.67
GABA	12.99	8.43
Lysine	36.94	42.07
Histidine	9.15	9.64
Tryptophane	0.00	0.00
Arginine	15.29	17.02

WP4 Young bumble bees may rely on both direct pollen cues and larval experience when foraging

Authors:

Fabian A. Ruedenauer*, David Sydow, Johannes Spaethe, Sara D. Leonhardt

*first author

Submitted to: Proceedings of the Royal Society B Biological Sciences

Abstract WP4

An adequate supply of macro- and micronutrients determines health and reproductive success in most animals. Many bee species, for example, collect nectar and pollen to satisfy their demands for carbohydrates, protein and fat, respectively. Bees can assess the quality of pollen by feeding on it, but also pre-digestively by means of chemotactile assessment. If they additionally use larval nutritional experience, as has been shown for *Drosophila melanogaster* and *Bombyx mori*, is unknown. In this study, we tested whether pollen selection of bumble bee foragers is affected by nutritional experience during their larval stage. Bumble bee larvae were fed with one out of three different pollen blends ("*Cistus*", "*Prunus*" and "*Salix*"). As adults, they were offered all three blends when they started foraging for the first time. Bees from all three treatment groups preferred the "*Salix*" blend. This blend provided the highest nutritional quality and increased the bees' lifespan, as shown by feeding studies with microcolonies. Besides, bees also chose the pollen blend fed during their larval stage more often than expected, indicating a significant effect of the "larval memory" on adult pollen foraging behavior. The combination of both direct pollen quality assessment and larval/early imaginal experience seems to allow foraging bumble bees to efficiently select the most suitable pollen for their colony.

Introduction WP4

Dietary intake is important for individual fitness as well as the fitness of a group or colony (Purves et al. 2006; Raubenheimer and Simpson 1993; Raubenheimer and Simpson 1999; Simpson and Raubenheimer 2012). All animals therefore need to find or compose nutritionally appropriate food. Foraging decisions are typically based on nutritional quality (i.e. nutrient content and digestibility), previous experience or associated cues (e.g. odor,

taste). Some species prefer familiar food, e.g. food that was eaten during the juvenile stage. For instance, newly hatched sepias of the common cuttlefish (*Sepia officinalis*) innately prefer "shrimp-like prey" throughout the first weeks of their life, but they do prefer crabs as prey, when they were exposed to crabs for at least 90 min directly after hatching (Wells 1958). Similar examples can be found in insects, where adult foraging preferences can be primed at the larval stage. *Drosophila melanogaster* flies, for instance, which received peppermint-scented food as larvae, also preferred this food as adults (Barron and Corbet 1999; Caubet et al. 1992). In contrast, adult flies avoided peppermint-scented food when fed with standard food as larvae (Thorpe and Imms 1939). Larvae of the moth *Bombyx hesperus* even completely refused feeding and starved on their naturally preferred host plant when they were raised on a different plant species as larvae (Bernays 1989). Unlike more freely moving larvae, larvae of bees or wasps do not have any choice of food and have to feed on what is provided by foraging adults. This leaves the adults to find nutritionally appropriate food for their larvae.

Within insects, bees (Apidae) represent an interesting model system to investigate food choice behavior, because they obtain nutrients mainly from pollen and nectar, which can vary strongly in chemical composition among habitats and time of the year (Michener 2000). Nectar is the main source of carbohydrates for bees. In contrast, pollen supplies the macronutrients protein and fat, and micronutrients, such as sterols and vitamins (Dobson and Peng 1997; Haydak 1970; Roulston and Cane 2000b; Togasawa et al. 1967a), and thus represents a complex mixture of different chemical substance classes (also including potentially toxic, non-nutritional plant secondary metabolites) (Roulston et al. 2000; Roulston and Cane 2000b), whose concentrations and composition differ between plant species (Palmer-Young et al. 2019a; Roulston et al. 2000; Roulston and Cane 2000b). Generalist bee species therefore typically collect pollen from a large spectrum of different plant species to

reach nutrient targets and dilute potentially toxic substances (Kevan and Baker 1983; Roulston and Cane 2000b).

Individual bumble bee foragers (*Bombus terrestris*) were shown to differentiate between different pollen types and to choose "high-quality" (i.e. non-diluted) over "low-quality" (diluted) pollen based on chemotactile cues (Kitaoka and Nieh 2009; Ruedenauer et al. 2015; Ruedenauer et al. 2016). While they do not seem to perceive protein content itself in pollen, bumble bees seem to assess fat content and protein:lipid- (P:L-) ratio (Ruedenauer et al. 2020; Vaudo et al. 2016a; Vaudo et al. 2016b). Hence, foraging choices of bumble bees are influenced by the nutrient content and ratios that pollen provides. Whether it may also be influenced by larval nutritional experience, e.g. through feeding on specific pollen types as larvae, as shown for *Drosophila* and *Bombyx*, remains unknown (Barron and Corbet 1999; Blackiston et al. 2008).

In the present study, we tested whether adults of *B. terrestris* relied on larval experience when making (naïve) pollen foraging decisions or whether their food choice was solely driven by an innate preference for a specific nutritional quality.

As *B. terrestris* and most other bumble bee species are floral generalists and known to prefer pollen with specific nutritional quality parameters (i.e. low fat content and/or high protein:lipid-ratio, Ruedenauer et al. 2016; Vaudo et al. 2016b), we expected them to only weakly rely on larval experience for their foraging decisions. We hypothesized that, instead, they prefer pollen of high nutritional quality, i.e. with the lowest fat content and the highest protein to lipid (P:L)-ratio (Ruedenauer et al. 2020; Vaudo et al. 2016b), independent on the pollen blend which they received as larvae.

Materials and Methods WP4

Preparation of colonies

Three queenright colonies of *Bombus terrestris* were obtained from Koppert B.V. (Berkel en Rodenrijs, Netherlands). Each colony, including the queen, workers and eggs, was transferred into a two-chambered wooden box (21.0 x 19.5 x 10.8 cm). One chamber served as nesting chamber and the other as foraging area. We discarded larvae, pupae and pollen pots present in the colony at delivery to ensure that all newly hatched bees were only fed pollen provided by us. Colonies had *ad libitum* access to 2 M sucrose solution and were additionally fed *ad libitum* with one out of three different pollen blends. Pollen blends were freshly prepared every day and provided in petri dishes. We used three honeybee-collected pollen blends, referred to as *Salix*, *Cistus* and *Prunus* blend according to the manufacturer (Aristée/Pollenergie, St Hilaire de Lusignan, France). Please note that these blends did not present pure pollen, but mixtures of pollen from at least five different plant species (as found through microscopic analyses, Figure S1). We did use pollen blends instead of single species pollen, since it is more easily available in the amounts needed for the experiment and single species pollen may lack essential nutrients necessary for the development of the larvae. We only used one bag per mixture for the whole experiment, to prevent nutritional differences between bags affecting our results. We additionally analyzed the nutritional composition of each blend (see below).

Diet regime experiments

Young workers were caught from mother colonies within one day after hatching, marked with differently colored and individually numbered, small plates (Holtermann, Brockel, Germany), and transferred to microcolonies (two-chambered wooden boxes 14.5 x 13.0 x 10 cm). Each

microcolony consisted of ten individuals of each mother colony and thus overall 30 individuals. Like in the mother colony boxes, one chamber served as nesting chamber and one as foraging area. Microcolonies also had ad libitum access to a sucrose solution from two 15 ml centrifuge tubes (Hartenstein) with small holes. After three days of acclimatization, we started the experiments. Each microcolony was given the choice between 1 g of each of the three different pollen blends in small petri dishes (3.5 x 1 cm; Hartenstein). To prevent position learning we randomized the position of petri dishes every day (Fig. 1).

After positioning the dishes, we filmed the decisions and feeding behavior of each microcolony for four hours per day using a video camera (Sony HDR-CX405 B.CEN) for ten days. Videos were analyzed as follows: The first 10 min of each recording were used to determine the first four decisions and total time spent in dishes by each individual (detention time), and whether the chosen pollen was consumed or not.

Fitness experiments

To determine how the three pollen blends affected bumble bee fitness, we collected 135 workers (45 from each queenright colony) and split them into nine microcolonies, each containing 15 workers (5 workers from each queenright colony). All microcolonies had *ad libitum* access to sucrose solution and to one of the three different pollen blends offered in a petri dish (0.8 g freshly provided pollen per day, representing an *ad libitum* amount) and were observed for 20 days. After the first larvae appeared, pollen amounts were increased to 1.5 g/day. Amounts of pollen eaten/individual, number of egg clumps, larval cells, pupae, and male offspring (subsequently added to the total number of individuals in a microcolony) were recorded daily to determine the reproductive success of each colony. We additionally recorded survival rates of workers from all microcolonies.

Digestibility of pollen blends

To examine whether the pollen blends differed in their digestibility, we collected 15 pollen samples (five of each pollen blend, directly from the purchased bag) and 15 fecal samples (from the waste of the colonies used in the fitness experiment). Samples were mixed with water and photographed under a light microscope at 20x magnification (Microscope: Zeiss West, Axiphot; camera: Visitron Systems, Spot Pursuit). Pollen digestibility was estimated by counting whole grains and broken/digested (empty) grains in five randomly chosen areas (477 pixel diameter) of the photograph per sample and comparing the proportions of empty grains between pollen and feces using the program Fiji (Java, Version 1.5.1) (Figure S1).

Chemical analyses

To assess the nutritional quality (i.e. composition and ratio of nutrients) of pollen blends used in our experiment, we conducted three different nutritional analyses.

Protein/Amino acid analysis

Amino acid content was analyzed via ion exchange chromatography (IEC, Amino Acid Analyzer LC 3000, Eppendorf Biotronik, Hamburg, Germany) as described in Leonhardt and Blüthgen (2012). At first, 10mg of pollen was mixed with 200 µl of 6 N HCl and boiled at 100°C for 4 h to break down protein into amino acids. After cooling down to room temperature it was centrifuged for 10 min. Water was evaporated from the supernatant at 100°C and the sample was re-dissolved in 200 ml fresh water thrice and centrifuged. Then, 100 µl of the supernatant was mixed with 20 µl of 12.5 % sulfosalicylic acid and extracted in the refrigerator for 30 min. The sample was mixed shortly, centrifuged again for 10 min and 100 µl of the supernatant was mixed with 100 µl of sample rarefaction buffer. The mixture was filtered by membrane

filtering in the centrifuge for 5 min and the filtrate was used for IEC analysis. For amino acid quantification, an external standard (physiological calibration standard, Laborservice Onken GmbH, Gründau, Germany) was used, containing all proteinogenic amino acids besides glutamine und asparagine, which were added manually prior to running standards and samples. Tryptophan is destroyed in HCl and can therefore not be analyzed with this method. Protein content was expressed as total amino acid content and calculated for each sample by summing up all single amino acid contents.

Fat/Fatty acid analysis

To analyze fatty acid contents we modified the protocol of Brückner et al. (2017). At first, we extracted fatty acids from 5 mg of each pollen blend in 1 ml hexane for 24 h at 60°C using a thermomixer (Thermomixer Compact, Eppendorf). We added 20 µl nonadecanoic acid in methanol (0.2 mg/ml, both Sigma-Aldrich, Taufkirchen, Germany) as internal standard. Fatty acids were purified by loading resulting extracts on 3ml SiOH columns (Macherey Nagel, Düren, Germany), conditioned with hexane (Merck, Darmstadt, Germany) and ethylacetate (Acros Organics, Geel, Belgium) in an 80:1 ratio. Triglycerides were eluted from columns with 5 ml hexane:ethylacetate (20:1), diglycerides were eluted with 5 ml hexane:ethylacetate (3:1) and free fatty acids were eluted with 5 ml of a hexane:ethylacetate:acetic acid mixture of 75:25:2. All three fatty acid fractions were collected in one vial and filtered to remove coarse pollutants (membrane filter, Type 5 µm, Durapore membrane filters, Merck). Solvents were then removed with CO_2. The residue was dissolved in 250 µl of dichloromethane:methanol (2:1) (Sigma Aldrich) and transferred into a GC vial. The solvent was removed with CO_2 and the residue dissolved in 20 µl of a 0.25 M trimethyl sulfonium hydroxide (TMSH) solution in methanol (Sigma Aldrich) for derivatization to fatty acid methyl esters (FAMEs). Samples were

finally analyzed with a gas chromatograph coupled to a mass spectrometer (GCMS) (Agilent Technologies, 5975C inert XL MSD) using the temperature program suggested by Brückner et al. (2017). One µl of each sample was injected at splitless mode. Helium was used as carrier gas. Injection temperature was 60°C, which was held for 1 min before heating with a rate of 15°C/minute until 150°C was reached, which was held for 10 min. Afterwards, the oven was heated to 320°C at a rate of 10°C/minute, which was held for 10 min. Electron ionization mass spectra were recorded from m/z 40 to 650. Ion source and transfer line temperature was constant at 250°C. FAME Mix, C4-C24 (Supelco, Bellefonte, USA) and the NIST MS Search 2.0 library were used to identify the FAMEs. MSD ChemStation F.01.00.1903 was used to manually integrate peaks. If peaks of two fatty acids could not be separated, they were integrated as one peak. The integrated area of the internal standard (nonadecanoic acid) was used to calculate concentrations of all other identified fatty acids. Fat was expressed as total fatty acid content and calculated for each sample by summing up all single fatty acids. The protein to lipid ratio (P:L-ratio) was calculated by dividing the protein content by the fat content.

Sterol analysis

To analyze the sterol content of different pollen blends, we followed Vanderplanck et al. (2011). We mixed 20 mg of each pollen blend with 2 M methanolic KOH (Sigma Aldrich). The mixture was saponified at 80°C for 1 hour and then cooled for 15 min before adding 4 ml of betulin as internal standard (Sigma Aldrich) (0.4 mg betulin in 4 ml ethanol) and 5 ml of deionized water.

The mixture was filled into a separating funnel and washed thrice with 5 ml diethyl ether (AppliChem GmbH, Darmstadt, Germany), which was re-collected in a separate vial following

phase separation, while the aqueous phase was discarded. The ether phase was then washed three times with 5 ml deionized water and dried on sodium sulfate (Sigma Aldrich). After removing the ether with CO_2, the residue was resolved in 1 ml hexane and transferred into 2.5 ml micro test tubes (Hartenstein Laborbedarf, Würzburg, Germany). Hexane was subsequently evaporated under CO_2 and the residue resolved in 100µl anhydrous pyridine (Merck) and 100µl N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, Sigma Aldrich). The mixture was shaken at 90°C and 1400 rpm (Thermomixer Compact, Eppendorf) for 30 min, before removing the solvents under CO_2 and resolving the residue in 100 µl hexane. The sample was finally transferred into a GC vial and analyzed via GCMS, with the temperature protocol described in Vanderplanck et al. (2011). Besides the temperature program, the GCMS setup was identical to the fatty acid analysis described above. After injection at 60°C, the oven was heated with 30°C/min to 290°C, which was held for 22 min. Temperature was then raised with 30°C/min to 325°C, which was held for another 5 min. Chromatograms and mass spectra were analyzed as described in the fatty acid analysis. If peaks of two sterols could not be separated, they were integrated as one peak. The integrated area of the internal standard (betulin) was used to calculate the concentrations of the other sterols. Total sterol content was calculated for each sample by summing up all single sterol contents.

Statistical analyzes

Differences in the number of choices of a particular pollen blend in the first feeding choice (with detectable consumption of pollen) were analyzed using Laplace generalized linear mixed effect models (GLMMs) with binomial distribution and with microcolony included as random factor. Choices were counted as 0 if an individual did not chose a pollen blend or as 1 if the pollen blend was chosen. After testing for overdispersion, the models were analyzed

with a Wald χ^2 test. A Tukey test was used for subsequent pairwise comparisons between diet regimes. We further composed Laplace GLMMs with binomial distribution and microcolony as random factor to test for the influence of nutrient class (i.e. total protein, fat and sterols, with one model composed for each diet regime group) on bee decisions. We calculated and compared model R^2 values (MuMIn package) to determine which model explained most of the observed variance.

To analyze differences in the probability of switching between pollen blends, we performed χ^2 tests on the total number of switches. We tested whether pollen blends differed in the probability of bees switching to another blend after their first choice. Subsequent pairwise χ^2 tests (analyzing differences between two blends) were corrected with Bonferroni to account for multiple testing. We again tested whether the number of switches were affected by nutrient class through comparing R^2 values of GLMMs with Gaussian distribution and microcolony as random factor.

To analyze diet regime-specific differences in relative feeding time we used GLMMs with Gaussian distribution and microcolony as random factor. The same type of models were used for consumption and reproductive success in the fitness experiment. The models were followed by a Tukey test for post-hoc pairwise comparisons.

Finally, a Kaplan Meier survival test was applied to analyze diet regime-related differences in survival probabilities of individuals in the fitness experiment, with log-rank tests used for pairwise comparisons. *P*-values were corrected for multiple testing using Bonferroni.

Results WP4

The three pollen blends differed in their nutrient content as well as in their digestibility (Table 1). The *Salix* blend had the highest absolute protein content (more than 2% higher than the *Prunus* blend) and protein to lipid (P:L)-ratio (more than 35% higher than the *Prunus* blend). It also showed the lowest fat and sterol content (Table 1). The *Prunus* blend had the lowest protein content and P:L-ratio and a fat content similar to the *Cistus* blend, which had the highest sterol content (Table 1). The *Salix* blend could be digested most efficiently (85% of the pollen grains were broken), while the other two blends showed an approximately 10% lower proportion of digested pollen grains (73% and 79%, respectively; Table 1).

Table 1 Nutrient content and digestibility of the three pollen blends used in the experiments. Given are the protein (P) and fat (F) content in % wet mass, the protein:lipid-ratio (P:L-ratio), the sterol content in % wet mass as well as the digestibility [% of empty pollen grains in feces compared to total pollen grains].

Pollen blend	Protein content	Fat content	P:L-ratio	Sterol content	Digestibility
Cistus	11.67%	0.64%	18.2:1	1.66%	73.46%
Prunus	10.34%	0.62%	16.6:1	1.07%	78.93%
Salix	12.58%	0.55%	22.9:1	0.66%	85.37%

Bumble bees randomly picked any of the three pollen blends as first choice (χ^2 = 0.215, P = 0.975) as well as when making their first feeding decision (χ^2 = 2.870, P = 0.412) (Figure 1). Consequently, neither pollen diet regime nor nutritional quality affected the first choices (Table 2).

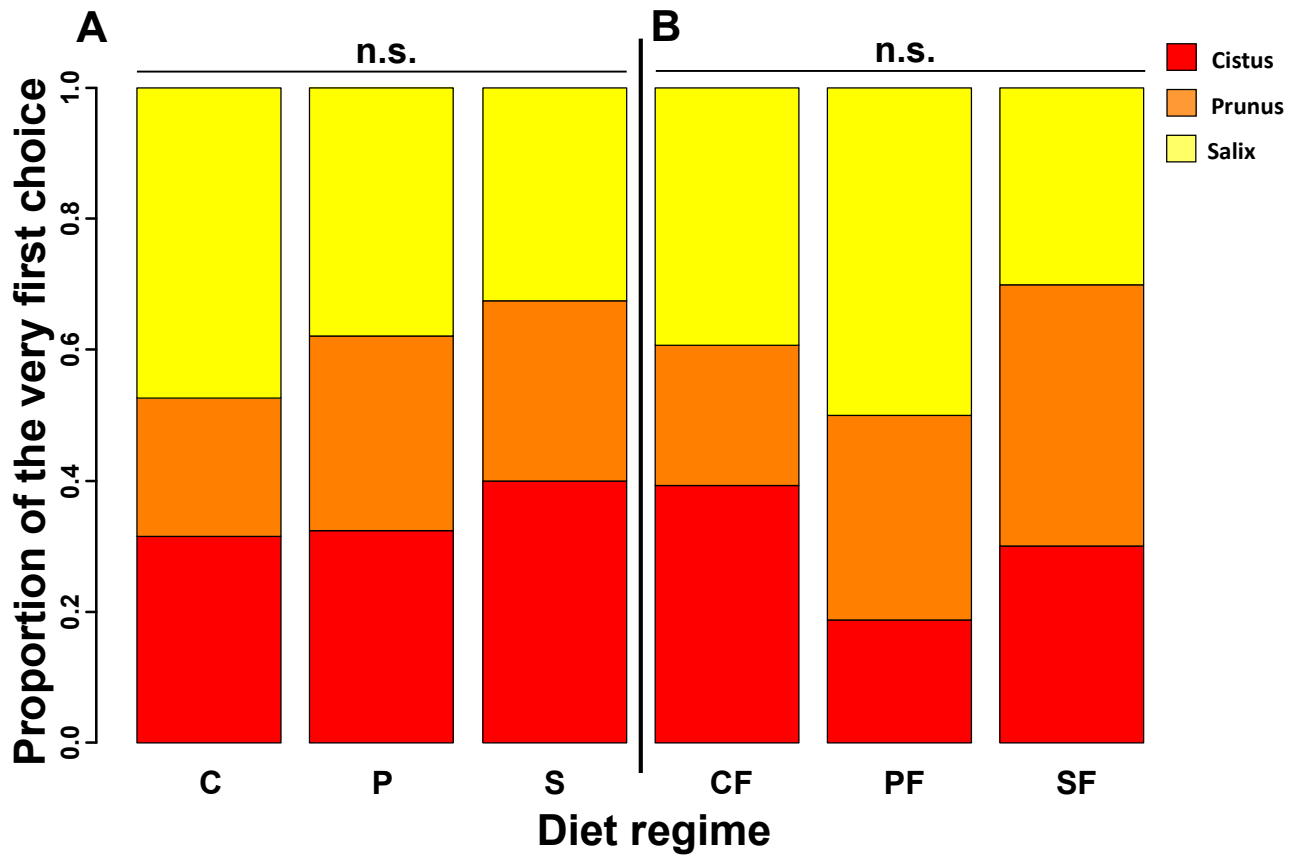


Figure 1 Proportion of (A) first pollen blend choices and (B) first feeding choices of *Bombus terrestris* individuals (N = 120) raised under three different diet regimes (4 microcolonies per regime). Bumble bees were raised on *Cistus* (C), *Prunus* (P) or *Salix* (S) pollen blends and were then offered a choice between the three blends (*Cistus* (black), *Prunus* (white) or *Salix* (grey)). Bumble bees chose randomly among the pollen blends in (A) as well as (B).

Table 2 Statistical results of generalized linear mixed effect models (GLMMs) testing for differences in the first feeding choices of *Bombus terrestris* individuals (N = 120, N microcolonies = 4) due to the different diet regimes for each pollen blend offered. Additionally, it was tested whether first choices of bees raised within the same diet regime were influenced in their choices by the protein, fat or sterol content and the protein:lipid-ratio (P:L-ratio) of pollen blends offered. "Microcolony" was used as random factor. Given are the conditional R^2 values of the models, as well as χ^2- and *P*-values.

Pollen blend	Diet regime	Protein content	Fat content	P:L-ratio	Sterol content
Cistus	**R^2 = 0.001** χ^2 = 0.026 *P* = 0.987	R^2 = 0.001 χ^2 = 0.026 *P* = 0.872	R^2 < 0.001 χ^2 = 0.012 *P* = 0.914	R^2 < 0.001 χ^2 = 0.006 *P* = 0.940	R^2 < 0.001 χ^2 = 0.002 *P* = 0.963
Prunus	R^2 = 0.028 χ^2 = 1.209 *P* = 0.546	R^2 = 0.017 χ^2 = 0.679 *P* = 0.410	R^2 = 0.028 χ^2 = 1.191 *P* = 0.275	**R^2 = 0.029** χ^2 = 1.234 *P* = 0.267	R^2 = 0.022 χ^2 = 0.860 *P* = 0.354
Salix	R^2 = 0.016 χ^2 = 0.784 *P* = 0.676	R^2 = 0.007 χ^2 = 0.357 *P* = 0.550	R^2 = 0.016 χ^2 = 0.784 *P* = 0.376	**R^2 = 0.017** χ^2 = 0.857 *P* = 0.355	R^2 = 0.013 χ^2 = 0.628 *P* = 0.428

However, bumble bees of all three experimental groups spent most time with the *Salix* pollen blend (Figure 2, $F = 21.346$, $P < 0.001$). This preference for the *Salix* blend was most pronounced for those bees that had been raised under the *Salix* diet regime. They spent more time with this blend than with any of the other two blends (Figure 2, Tukey: $F = 39.058$, $P < 0.001$). Bees raised on the *Cistus* and *Prunus* blend spent similar times with the pollen blend they experienced during their larval phase and the *Salix* blend, while the other blend, i.e. *Prunus* and *Cistus*, respectively, was less preferred (Figure 2). Therefore, in addition to the overall preference for the *Salix* blend, the diet regime significantly influenced the time bumble bees spent with any of the pollen blends (Table 3). Variation in feeding times furthermore correlated with the protein content and P:L-ratio of pollen blends (Table 3). The bees spent most time with the pollen blend containing the highest P:L ratio (i.e. the *Salix* blend, Figure 2) and the least with the blend with the lowest P:L-ratio (i.e. the *Prunus* blend, Figure 2). The P:L ratio of pollen blends explained almost 31 % of the variation observed in feeding times of bees raised under the *Salix* diet regime (Table 3).

Table 3 Statistical results of generalized linear mixed effect models (GLMMs) testing for differences in the relative feeding times of *Bombus terrestris* individuals (N = 120, N microcolonies = 4) due to the different diet regimes for each pollen blend offered. Additionally it was tested whether feeding times of bees raised within the same diet regime were influenced in their choices by protein, fat or sterol content or the protein:lipid-ratio (P:L-ratio) of pollen blends offered. "Microcolony" was used as random factor. Given are the conditional R^2 values of the models, as well as *F*- and *P*-values. Significant *P*-values are marked in bold.

Pollen blend	Diet regime	Protein content	Fat content	P:L-ratio	Sterol content
Cistus	**R^2 = 0.166**	R^2 = 0.125	R^2 = 0.068	R^2 = 0.156	R^2 = 0.082
	F = 6.403	F = 7.232	F = 0.045	F = 9.361	F = 1.496
	P = 0.002	**P = 0.008**	P = 0.831	**P = 0.003**	P = 0.224
Prunus	**R^2 = 0.134**	R^2 = 0.132	R^2 = 0.119	R^2 = 0.038	R^2 = 0.093
	F = 3.778	F = 7.068	F = 5.472	F = 4.370	F = 2.303
	P = 0.026	**P = 0.009**	**P = 0.021**	**P = 0.039**	P = 0.132
Salix	R^2 = 0.167	R^2 = 0.165	R^2 = 0.103	**R^2 = 0.309**	R^2 = 0.034
	F = 11.398	F = 22.486	F = 13.026	F = 33.893	F = 4.045
	P < 0.001	**P < 0.001**	**P < 0.001**	**P < 0.001**	**P = 0.047**

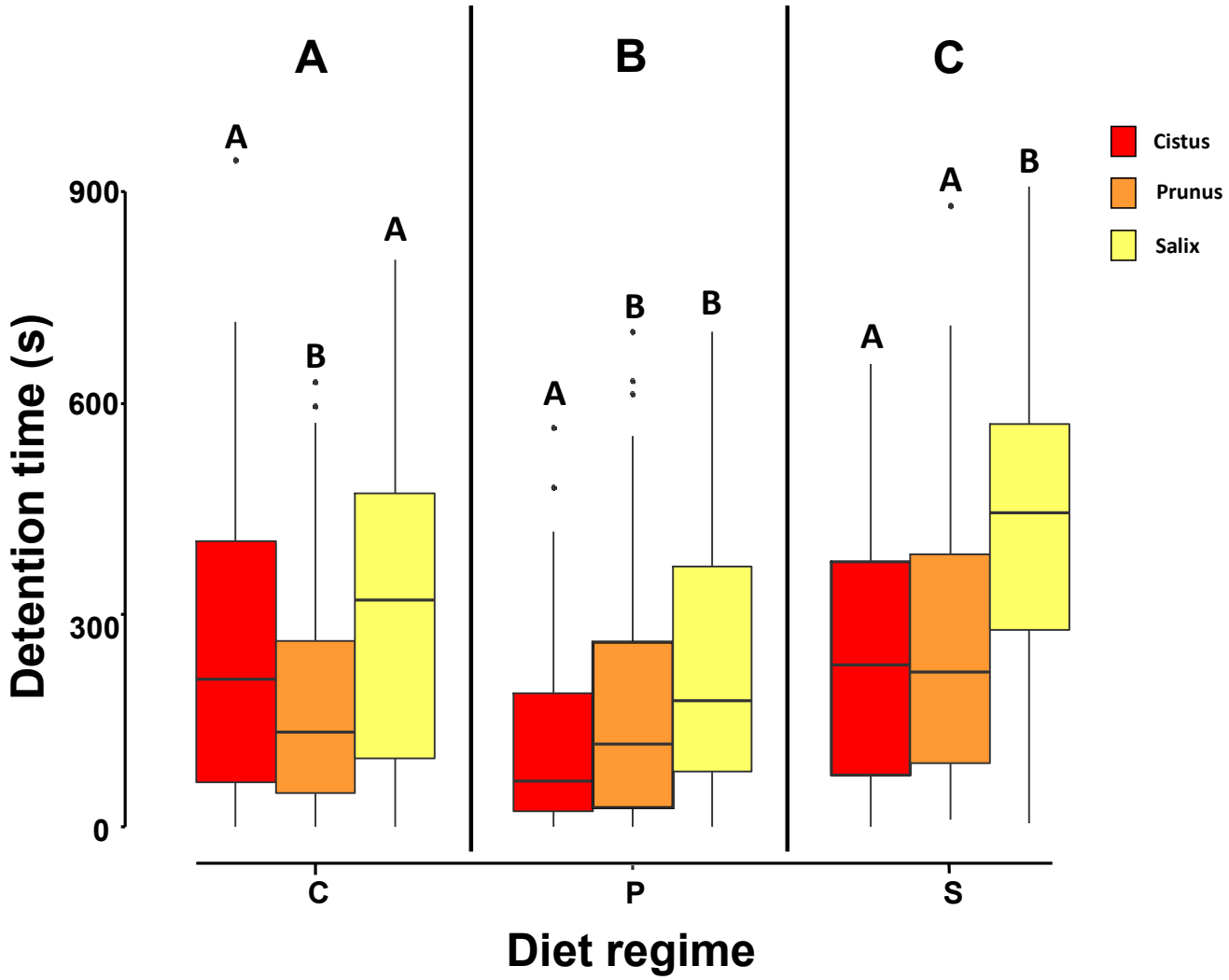


Figure 2 Relative time *Bombus terrestris* individuals (N = 120), which were fed different diets as larvae (*Cistus* (C), *Prunus* (P) or *Salix* (S)), spent with the three different pollen blends when offered simultaneously. Different letters above boxes indicate significant differences between feeding times within one diet regime group (Tukey post hoc test).

The probability of a switch was affected by the pollen type which bees chose first (χ^2 = 16.582, *P* = 0.002). After feeding on the first pollen blend, most bees switched or returned to the *Salix* blend (Figure 3, Table 4). There was a more than 21% higher probability to stay with the *Salix* blend than with any of the other two blends. Also, the probability of a switch from one pollen blend to another was not influenced by diet regime, but by protein content and P:L-ratio (Table 5).

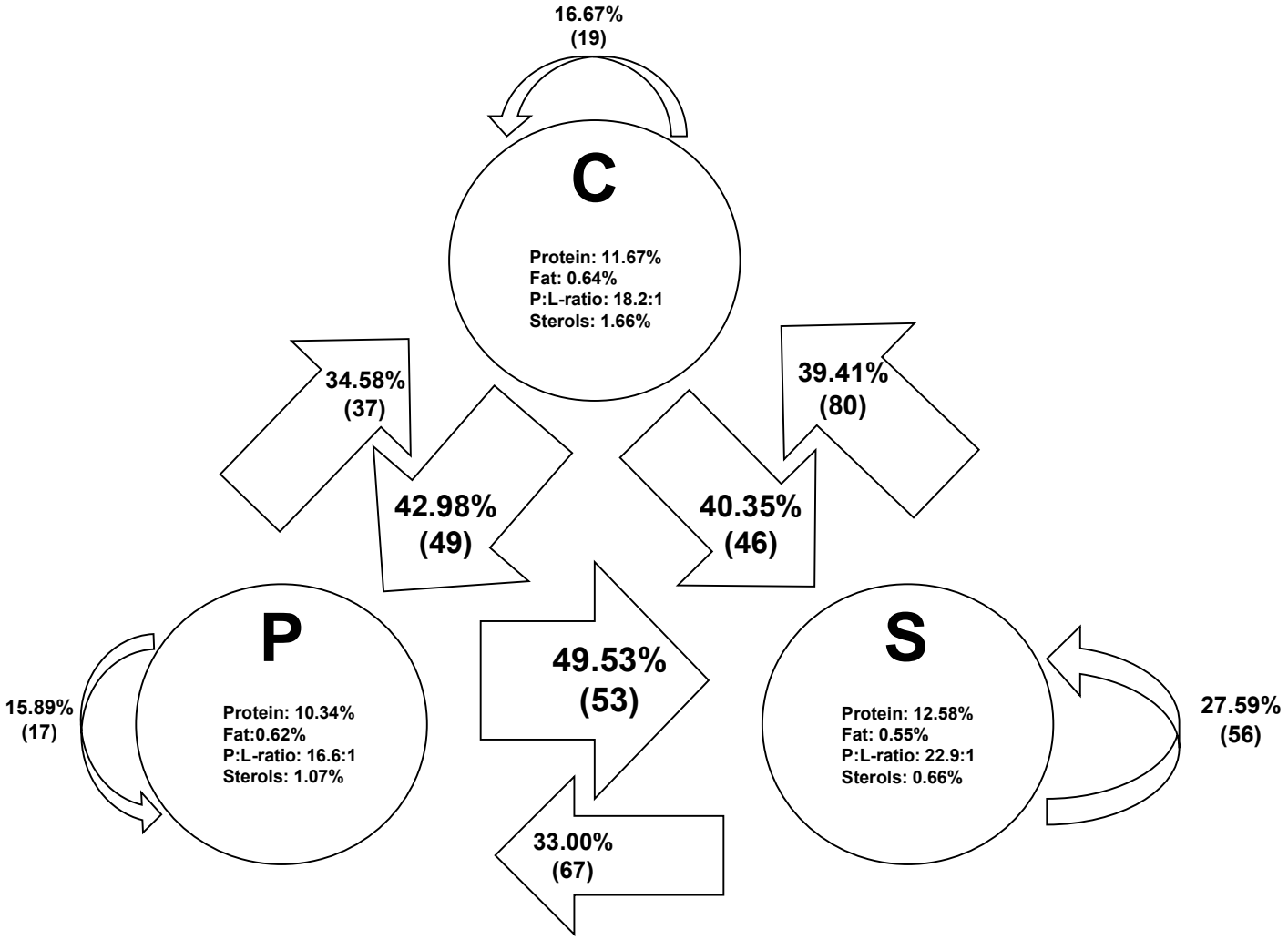


Figure 3 Percentage of switches between pollen diets after the first feeding activity as shown by *Bombus terrestris* individuals (N = 120) raised under three different diet regimes (four microcolonies per diet regime, pooled for all three diet regimes). Bumble bees were feeding one of three pollen blends (*Cistus* (C), *Prunus* (P) or *Salix* (S)) and then switched to another one or returned to the same pollen blend. Numbers in brackets give the absolute numbers of switches. Nutritional parameters (i.e. the protein, fat and sterol content as well as the protein to lipid (P:L) ratio are given in the circles denoting the different pollen blends. Most bumble bees switched after feeding, but the probability of staying with the same pollen blend was different between pollen blends. Fewer bees switched when they were already feeding on the *Salix* blend (Table 4, not significant after Bonferroni correction).

Table 4 Statistical results of chi-squared tests testing for differences in switching probabilities after the first feeding choice of *Bombus terrestris* individuals (N = 120, 4 microcolonies per regime). Given are *P*-values. Significant *P*-values after FDR correction for multiple testing are marked in bold.

	Cistus	***Prunus***
Prunus	***P* = 0.001**	-
Salix	***P* = 0.022**	***P* = 0.029**

Table 5 Statistical results of binomial Laplace generalized linear mixed effect models (GLMMs) testing for differences in switching probabilities after first feeding of *Bombus terrestris* individuals raised under different diet regimes (N = 120, 4 microcolonies per regime) as well as whether switches correlated with protein, fat or sterol content or the protein:lipid-ratio (P:L-ratio). "Microcolony" was used as random factor. Given are the conditional R^2 values of the models, as well as χ^2- and *P*-values. Significant *P*-values are marked in bold.

	Diet regime	Protein content	Fat content	P:L-ratio	Sterol content
Switch	**R^2 = 0.080**	R^2 = 0.077	R^2 = 0.019	R^2 = 0.051	R^2 = 0.001
	χ^2 = 4.856	χ^2 = 4.155	χ^2 = 0.582	χ^2 = 2.297	χ^2 = 0.025
	P = 0.088	***P* = 0.042**	*P* = 0.446	*P* = 0.130	*P* = 0.874

In the fitness experiment, where bees were forced to feed on only one pollen blend, they consumed more than double the amount of *Salix* than *Prunus* blend (χ^2 = 6.489, *P* = 0.039), while the *Cistus* blend was in between. They were also more than 20% less likely to survive on the *Prunus* blend than on any of the other two blends (Figure 5, Table 6, χ^2 = 13.5, *P* = 0.001). Pollen blend did not affect the reproductive success of microcolonies (Table 7).

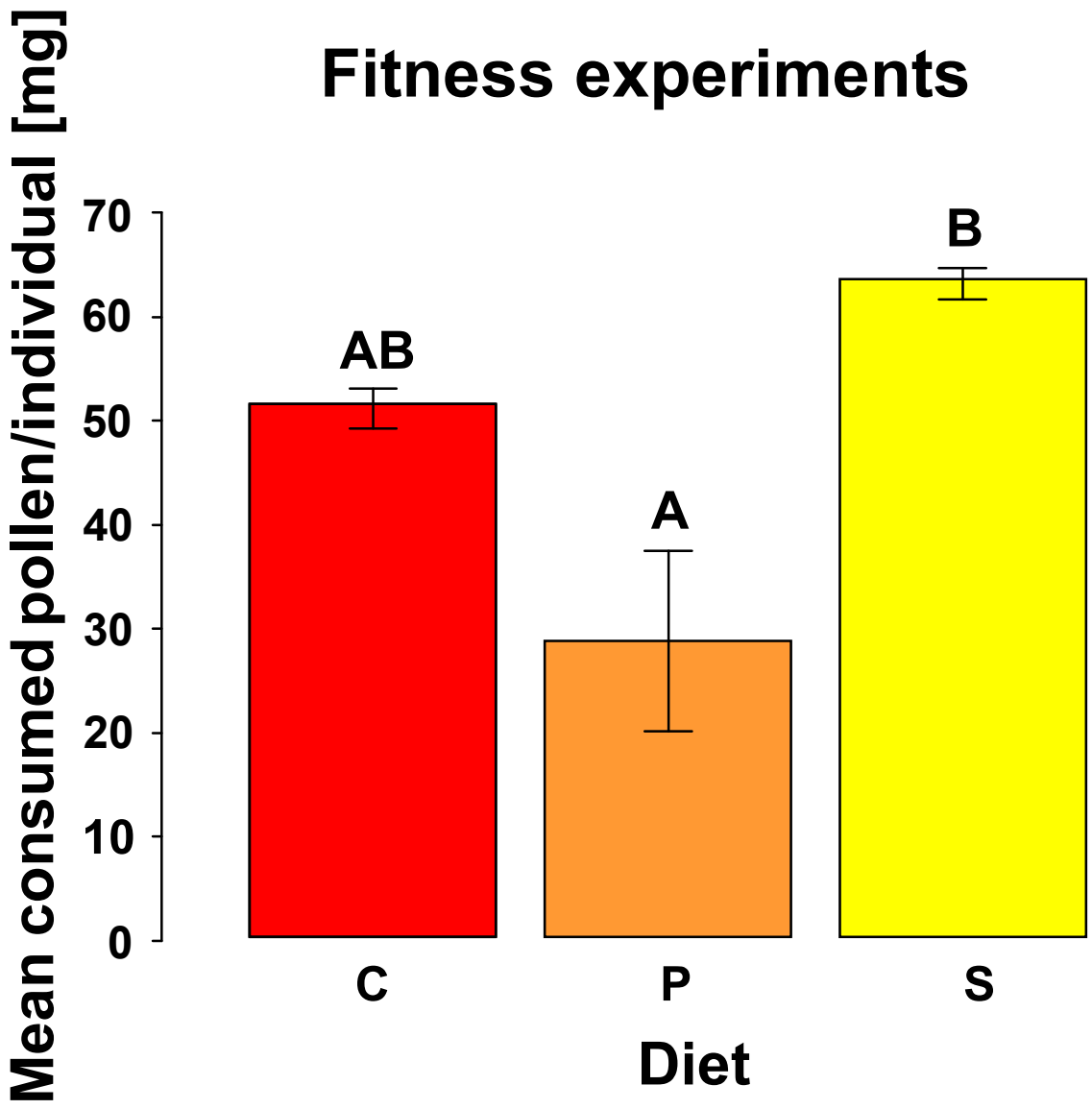


Figure 4 Mean amounts of pollen consumed [mean mg of food collected/individual and day ± SD] by *Bombus terrestris* individuals kept in microcolonies (N = 9) with 15 workers per colony (fitness experiment). Bumble bees were fed only one of the three pollen blends (i.e. *Cistus* (C), *Prunus* (P) or *Salix* (S)). Bees consumed higher amounts of the *Salix* than the *Prunus* blend. Different letters above bars indicate significant differences (Tukey post hoc test).

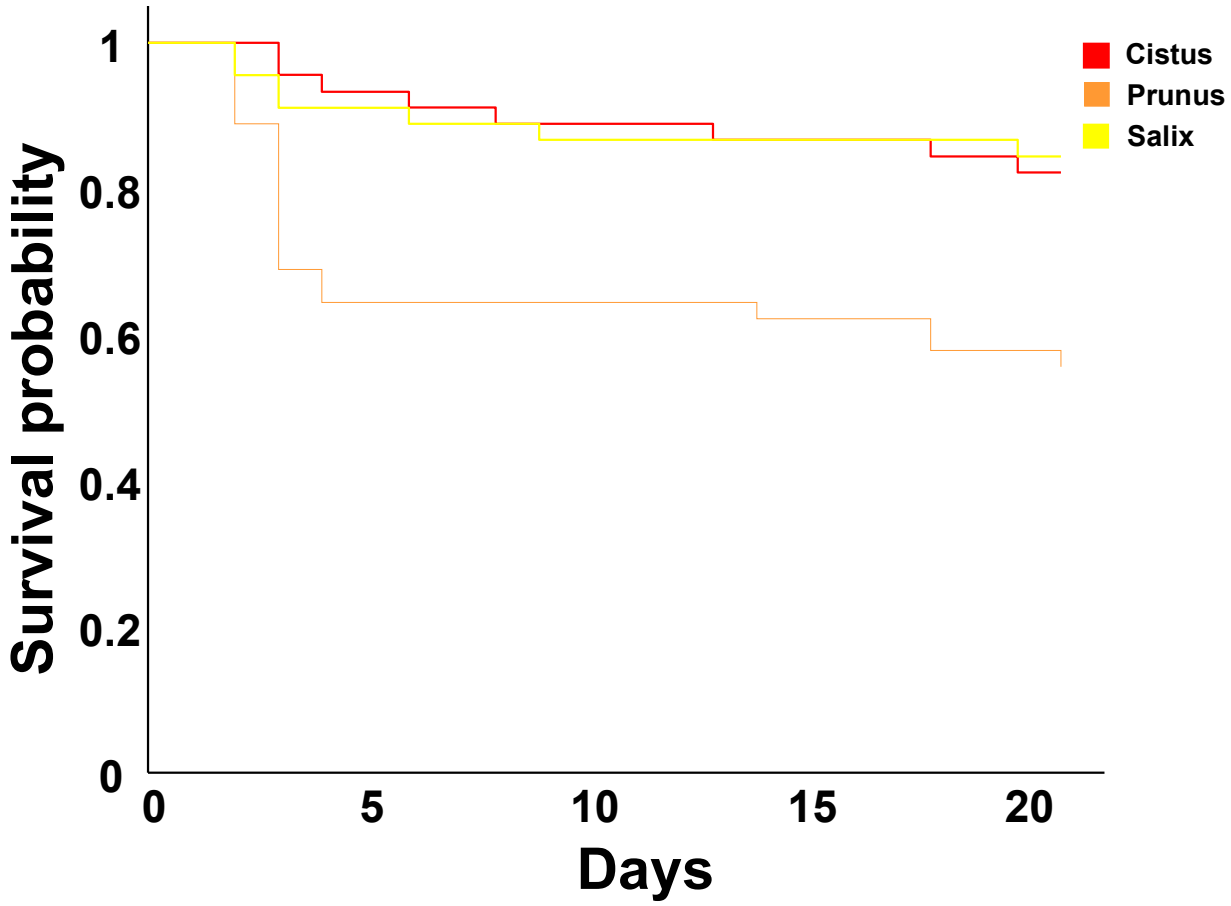


Figure 5 Survival probability of *Bombus terrestris* individuals offered either the *Cistus* (black line, N = 45), *Prunus* (dotted line, N = 45) or *Salix* (grey line, N = 45) pollen blend. The survival probabilities differed between the groups, with bumble bees fed *Prunus* pollen showing the lowest survival probability (Table 6). Different letters next to the survival curves indicate significant differences between groups.

Table 6 Statistical results of post-hoc pairwise Kaplan Meier survival tests analyzing differences in survival of *Bombus terrestris* individuals fed only one of three different pollen blends (i.e. *Cistus*, *Prunus* or *Salix*). Given are χ^2- and *P*-values for post-hoc comparisons with log-rank tests. *P*-values were corrected with Bonferroni for multiple testing. The significance level after correction was $P = 0.017$. *P*-values that were significant after correction are marked in bold.

	Cistus	***Prunus***
Prunus	$\chi^2 = 8.3$ **$P = 0.004$**	-
Salix	$\chi^2 = 0.1$ $P = 0.823$	$\chi^2 = 8.9$ **$P = 0.003$**

Table 7 Statistical results of Poisson distributed Laplace generalized linear mixed effect models (GLMMs) testing for differences in the reproductive success (i.e. number of egg clumps, larval cells, pupae and males produced, Table S1) of microcolonies fed only one of three different pollen blends (*Cistus, Prunus, Salix*, 3 microcolonies per blend). "Microcolony" was used as random factor. Given are the χ^2- and *P*-values.

	Egg clumps	Larval cells	Pupae	Males
Reproductive success	$\chi^2 = 0.119$ $P = 0.942$	$\chi^2 = 0.130$ $P = 0.937$	$\chi^2 = 0.544$ $P = 0.762$	$\chi^2 = 1.807$ $P = 0.179$

Discussion WP4

In some insect species, the feeding behavior of adult individuals is affected by food which they had experienced as larvae (Barron and Corbet 1999; Caubet et al. 1992), indicating that some kind of larval experience can be retained during metamorphosis. Since we did not catch the bees immediately after hatching, early imaginal experience could additionally have had impact on the bees' decisions. Our study suggests that foraging choices of bumble bee workers are partly affected by their preimaginal/early imaginal experience, even though they are also driven by the food's nutritional quality.

Interestingly, the bees' first feeding choice depended neither on familiarity nor on nutritional quality. Instead, bees chose randomly among blends. This may have been a consequence of having been (pollen) starved for several days, which could have resulted in the bees readily accepting the first pollen blend encountered. Alternatively, such random foraging may represent an innate strategy, which allows bees to probe several pollen types before making a final decision on its appropriateness based on chemotactile cues (Ruedenauer et al. 2020; Ruedenauer et al. 2015; Ruedenauer et al. 2016). In fact, we did find that, after probing, adult bees spent more time with familiar pollen, i.e. pollen they had been fed as larvae, than with unfamiliar pollen blends (Fig. 2).

A prerequisite for benefitting from larval/early experience with regard to food is that bees can find the same or similar resources when they became foragers. Such food resource continuity will only be provided by plants that flower and hence provide food resources over prolonged periods. This is the case for several plants, e.g. *Taraxacum* species, *Verbena officinalis, Bellis perennis* or *Trifolium* species. which is especially important for wild bees (Westphal et al. 2003). In fact, such long flowering plant species might even be more efficiently located and exploited by bees relying on larval/early experience during their first foraging flights compared to completely naïve bees. Moreover, specialist bee species, which forage on a restricted set of flowering species could, in theory, completely rely on their preimaginal experience; but, to the best of our knowledge, this has not yet been tested.

Keeping in mind, that our experiments were laboratory experiments under controlled conditions, there certainly are other factors that under natural conditions influence the foraging decisions of bees (e.g. foraging distance, a wider range of choices…). These factors may, in addition to direct pollen cues, contribute to further diluting the effects of larval/early experience. Also, we cannot entirely rule out a colony-specific effect for our results. However, in addition to the fact that actually all three colonies preferred the *Salix* mixture, we consider it highly unlikely that all three colonies had a colony-specific preference for the pollen they were fed.

However, the effect of larval experience was accompanied by a strong preference for the *Salix* pollen blend, which could be largely explained by its favorable nutritional composition. *Salix* had the highest proportion of protein, the highest P:L-ratio and the lowest proportion of fat and sterols. Even though we do not know which nutrients the bees used in our experiments for making their foraging choices, recent evidence strongly suggests that bumble bees prefer

pollen with high P:L-ratios (Vaudo et al. 2016b), as shown for *B. terrestris* and *B. impatiens*, most likely because they perceive, regulate and try to avoid overeating lipids, including fatty acids (Ruedenauer et al. 2020; Vaudo et al. 2016a; Vaudo et al. 2016b). In our study, 31% of the variation in time bees, which were raised under the *Salix* regime, spent with each pollen blend were explained by the blends' P:L-ratios. Even though the nutritional differences between the pollen types may not seem especially big, the P:L-ratio in particular hence could be an influential factor for the bees' decisions in the experiments. Also, we do not know yet, how finely tuned bees' perception of such nutritional differences really is. Therefore, bees could have preferred the *Salix* blend due to its nutritional quality.

Besides its nutritional quality, the *Salix* blend may additionally provide highly attractive cues (e.g. olfactory, color) or a low concentration of phytochemicals (Palmer-Young et al. 2017a; Palmer-Young et al. 2016; Palmer-Young et al. 2017b), which could also have affected the bees' preferences. For example, *Salix* pollen may contain more volatiles than other pollen types, since *Salix* flowers do not have petals that could emit volatiles. Moreover, the *Salix* blend was also most digestible, suggesting that bees could efficiently extract nutrients from this blend.

In our fitness experiment, bumble bees consumed lower amounts of the *Prunus* and *Cistus* than of the *Salix* blend. They also died earlier on the *Prunus* blend than on the *Salix* blend. This decrease in survival might be explained by either undereating or an overall less appropriate nutritional quality of the *Prunus* blend (Schmidt et al. 1987). *Bombus terrestris* was shown to avoid pollen with high fat content and to likely undereat other nutrients to avoid overeating fat (Ruedenauer et al. 2020). The relatively higher fat content and lower P:L-

ratio of the *Prunus* blend (Table 1) might have reduced consumption (Figure 4), which likely resulted in undereating other nutrients.

In all treatment groups, most bees switched to a different pollen blend after first feeding, which agrees with the often observed tendency of foraging bumble bees to keep on probing novel food sources (Free 1970). This tendency may be explained by exploratory behavior and/or the need for mixing pollen to complement nutrients and dilute toxic substances (Eckhardt et al. 2014). However, despite the high tendency to switch after first feeding, a considerably higher proportion of bees in our experiment stayed with the *Salix* blend compared to the other two blends. Moreover, more bees switched to the *Salix* blend than to any other blend (see Figure 3), which further highlights the high attractiveness of this blend. Please note that the observed preference for the *Salix* blend did not necessarily indicate a preference for *Salix* pollen per se, as the blend used in our experiment only contained ca. 56 ± 2% of pure *Salix* pollen (personal observation, microscopic analysis).

In conclusion, pollen foraging behavior of bumble bees seems to be affected by their feeding experience as larvae/early imagines, which might be achieved by retaining larval memory to the adult stage (but see Blackiston et al. (2008) and discussion therein for alternative mechanisms, e.g. chemical legacy, residues of chemical substances remaining in the insect haemolymph or outside the pupal case). However, other (most likely) nutritional factors also play a significant role in guiding the adult bees' foraging behavior, which enables them to flexibly respond to the ever changing spectrum of available food sources.

Supplementary material WP4

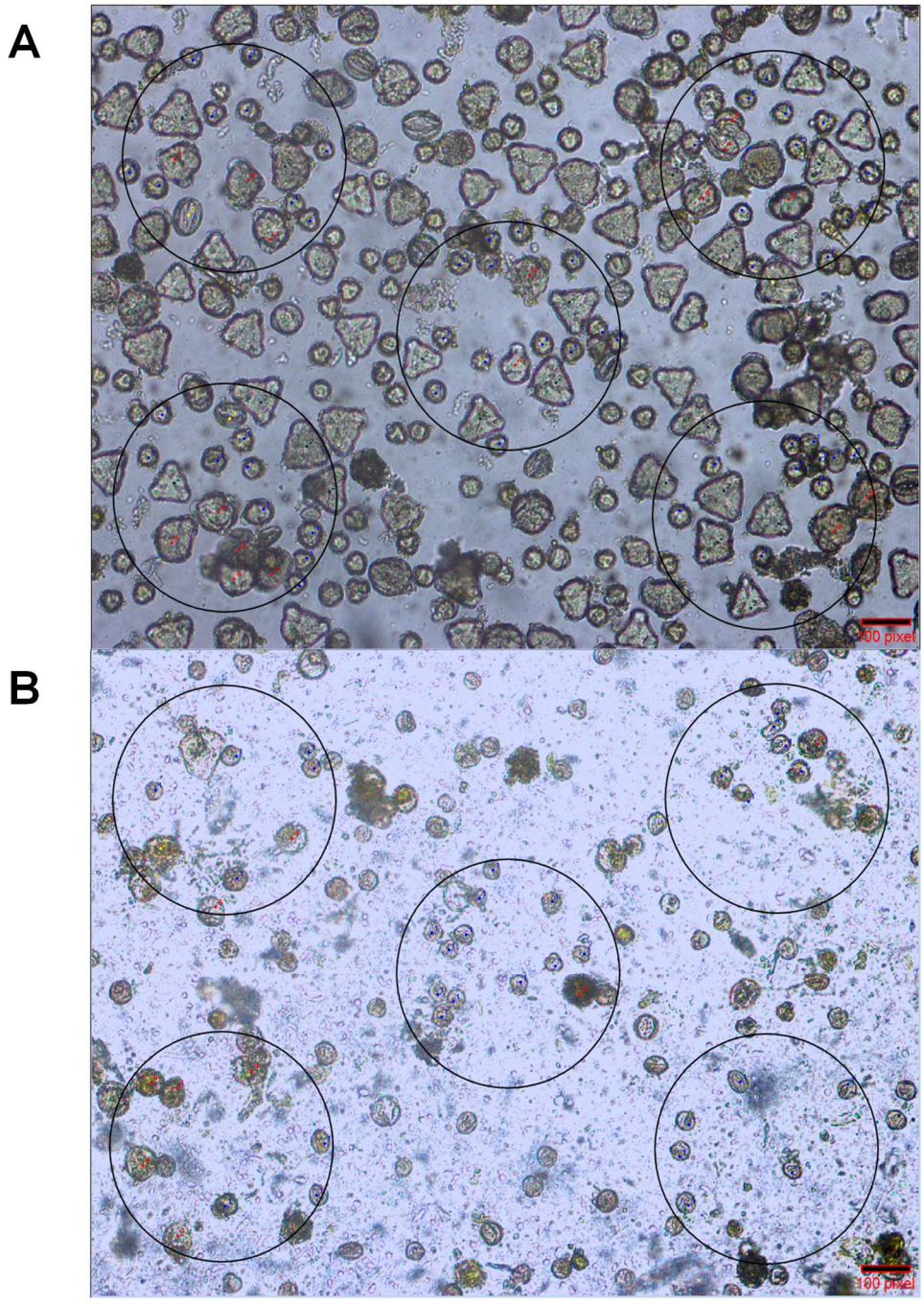


Figure S1 Pollen (A) and feces (B) sample of the *Salix* blend under the light microscope at 20x times magnification. A variety of different pollen types can be seen in the blend. Black circles represent the areas that were used for intact and empty grain counts. Differently colored dots represent counts for different pollen types.

Table S1 Mean reproductive success [±SD] per day of nine *Bombus terrestris* microcolonies fed with one pollen blend (*Cistus*, *Prunus* or *Salix*). Given are the number of egg clumps, larval cells, pupae and drones produced in microcolonies (N = 9) receiving one of the pollen blends to feed on.

Pollen	*N* egg clumps	*N* larval cells	*N* pupae	*N* drones
Cistus	1.34 ± 2.39	0.86 ± 1.68	0.11 ± 0.43	0.04 ± 0.07
Prunus	1.23 ± 3.11	0.91 ± 1.65	0.13 ± 0.33	0.05 ± 0.07
Salix	1.30 ± 2.98	0.92 ± 1.76	0.15 ± 0.34	0.04 ± 0.07

Chapter III: Nutrient perception and its fitness consequences for bumblebees

WP5 Bumblebees are able to perceive amino acids via chemotactile antennal stimulation

Authors:

Fabian A. Ruedenauer*, Sara D. Leonhardt, Klaus Lunau, Johannes Spaethe

*first author

published: 2019

Journal: Journal of Comparative Physiology A

Volume: 205 Issue: 3 Pages: 321-331

Abstract WP5

Like all animals, bees need to consume essential amino acids to maintain their body's protein synthesis. Perception and discrimination of amino acids is, however, still poorly understood in bees (and insects in general). We used chemotactile conditioning of the proboscis extension response (PER) to examine (1) whether *Bombus terrestris* workers are able to perceive amino acids by means of their antennae and (if so) which ones, (2) whether they are able to differentiate between different amino acids and (3) whether they are able to differentiate between different concentrations of the same amino acid. We found that workers were able to perceive asparagine, cysteine, hydroxyproline, glutamic acid, lysine, phenylalanine and serine, but not alanine, leucine, proline or valine by means of their antennae. Surprisingly, they were unable to differentiate between different (perceivable) amino acids, but they were able to distinguish between different concentrations of lysine. Consequently, bumblebees seem to possess amino acid receptors at the tip of their antennae, which enable a general perception of those solute amino acids that have an additional functional group (besides the common amino and carboxylic groups). They could thus have the ability to assess the overall amino acid content of pollen and nectar prior to ingestion.

Introduction WP5

Proteins and specifically essential amino acids are important nutrients for all animals to maintain their own protein synthesis. Under- or overeating protein damages health by e.g. weakening the immune system, reducing growth or impeding reproduction (Behmer 2009b; Kropàcovà et al. 1968; Roche et al. 2011; Simpson and Raubenheimer 2012). For example, excess amounts of protein (DeGroot 1953; Dourmad et al. 1994; Helm et al. 2017; Herbert et al. 1977; Iwasaki et al. 1988; Pirk et al. 2010; Standifer et al. 1960) or of certain amino acids

(Huang et al. 2011; Simpson and Raubenheimer 2009; Vrzal et al. 2010; Wu 2013) can shorten the lifespan. Consequently, animals would benefit from assessing the protein content of potential food resources, because this information would enable them to compose diets which best support their current metabolic needs.

As amino acids are hardly volatile substances, even though some degradation products may be volatile (Linander et al. 2012), assessment of their amount and composition requires chemotactile sensation, an inseparable combination of taste and tactile sensation (Ruedenauer et al. 2015). However, compared to olfaction, much less is known on chemotactile sensation, particularly in invertebrates (however see Amrein and Thorne 2005).

We here investigate chemotactile perception of amino acids via the antennae in a social bee, *Bombus terrestris* (Apidae), to better understand the sensory mechanisms underlying the perception of these essential nutrients in insects. Bees obtain all amino acids from floral resources, i.e. pollen and nectar, which are known to vary in the composition and particularly in the amount of amino acids (Petanidou et al. 2006; Roulston and Cane 2000b; Somerville 2001; Weiner et al. 2010). Bees are known to detect differences in the nutritional composition of pollen and nectar and respond accordingly, e.g. through changing foraging patterns or reproductive investment (Brodschneider and Crailsheim 2010; Hendriksma and Shafir 2016; Muth et al. 2016; Ruedenauer et al. 2015; Ruedenauer et al. 2016; Somme et al. 2015; Zarchin et al. 2017). The sensory mechanisms underlying such nutrient assessment in bees have so far only been elucidated for carbohydrates, i.e. sugars, in nectar, which are detected via specific sugar receptors on the antenna (Jung et al. 2015; Slone et al. 2007). In contrast, the mechanisms underlying amino acid perception remain largely unclear.

In *Drosophila,* ionotropic receptors (IR) appear to be involved in amino acid reception (Benton et al. 2009; Croset et al. 2016; Ganguly et al. 2017). This recently described new receptor gene family most likely evolved from ionotropic glutamate receptors (iGluR), a large and conserved family of synaptic ligand-gated ion channels (Rytz et al. 2013). However, it remains unclear whether IRs are specialized on particular amino acids allowing to differentiate between them or if they act as general receptors sensitive to a subset (or all) amino acids (Croset et al. 2016; Kudow et al. 2017).

To investigate amino acid perception in *B. terrestris*, we used conditioning of the proboscis extension response (PER) shown upon perception of e.g. an olfactory (e.g. Laloi et al. 1999; Matsumoto et al. 2012) or gustatory/chemotactile (Ruedenauer et al. 2015) cue associated with a sugar reward. A PER in the context of classical conditioning (Pavlov 1927) is typically shown when touching the bees' antennae, tarsi or parts of the mouth with a sugar solution (de Brito Sanchez et al. 2007). In a learning experiment, this so-called unconditioned stimulus (US) is evoked shortly after presenting a conditioned stimulus (CS, e.g. an olfactory or chemotactile cue) in order to associate both stimuli (i.e. US and CS). If the bees are able to learn this association, they will, after repeated exposure, extend their proboscis at the mere presentation of the CS (Bitterman et al. 1983; Matsumoto et al. 2012). Moreover, rewarding only one out of two different stimuli (i.e. differential conditioning) allows testing whether bees can differentiate between two stimuli. In insects, gustatory/chemotactile receptors are distributed over several body parts, including the antennae (Amrein and Thorne 2005; de Brito Sanchez 2011). PER conditioning can thus be used to test whether bees can not only perceive, but also differentiate between different non-volatile cues by touching the antennae.

Using chemotactile PER conditioning, we tested whether bumblebees can perceive and differentiate between different amino acids as well as between different amounts/concentrations of the same amino acid. Given their nutritional importance, we hypothesized that bumblebees can perceive amino acids and differentiate between different amino acids and concentrations. We especially expected the essential amino acids (i.e. arginine, histidine, lysine, phenylalanine, tryptophan, leucine, isoleucine, methionine, threonine and valine (DeGroot 1953)) as well as proline to be perceived by bumblebees. The latter is important as energy source of the flight muscles (Micheu et al. 2000) and seems to support colony growth in *Bombus terrestris* (Kämper et al. 2016).

Materials and Methods WP5

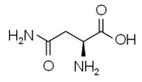
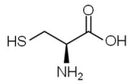
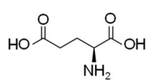
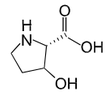
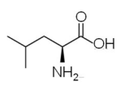
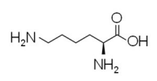
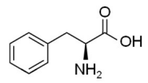
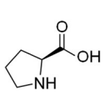
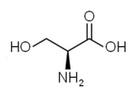
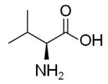
Study animals and test substances

Bombus terrestris colonies were purchased (Behr, Kampen, Germany) and kept in two-chambered wooden boxes (240×210×110 mm per chamber) in a climate chamber (25°C, 50% humidity, 12/12 h light/dark-cycle) for ca. three weeks before starting the behavioral experiment. Bumblebees had *ad libitum* access to Apiinvert (a mixture of sucrose, fructose, and glucose; Südzucker AG, Mannheim, Germany) and bee-collected polyfloral pollen (Naturwaren Niederrhein GmBH, Goch-Asperden, Germany). For all learning experiments, individual workers were captured and chilled on ice for 15 min. As the size of a bumblebee determines its antennal sensitivity and differently sized workers may carry out different tasks (Spaethe et al. 2007), we randomly selected bumblebees of different sizes to cover the full size spectrum.

We tested the following proteinogenic essential and non-essential L-amino acids: alanine, asparagine, cysteine, glutamic acid, leucine, lysine, phenylalanine, proline, serine and valine

as well as the non-proteinogenic hydroxyproline (Table WP5.1, all Sigma-Aldrich, Taufkirchen, Germany). For stimulus presentation, all amino acids were solved in de-ionized water (henceforth only referred to as water) at a concentration of ~ 10 g/l, which was the highest possible concentration of the least water soluble amino acid, i.e. glutamic acid. This concentration is higher than concentrations reported for single amino acids in pollen or nectar of several plant species (Ruedenauer et al. 2015; Weiner et al. 2010) and should therefore fall within the perception range of bees.

Table WP5.1 Characteristics of the eleven amino acids used in the experiments. The molecular mass is rounded to two digits. The amino acid group is determined by chemical characteristics. Essentiality of an amino acid depends on whether it can be synthesized by the organism or needs to be taken up via food. Essentiality of amino acids for bees was determined by DeGroot (1953). Water solubility of amino acids is influenced by polarity, charge and hydrophobicity. The acidity is influenced by additional functional groups that may be at the end of the atom. The taste to humans was determined by Schiffman et al. (1981). Amino acids that were differentiated from water are marked with a hashtag and in bold.

Amino acid											
	Alanine	***Asparagine#***	***Cysteine#***	***Glutamic acid#***	***Hydroxyproline#***	***Leucine***	***Lysine#***	***Phenylalanine***	***Proline***	***Serine***	***Valine***
Molecular mass	89.10	132.12	121.16	147.13	131.13	131.17	146.19	165.19	115.12	105.09	117.15
Essential	no	no	no	no	no	yes	yes	yes	no/needed for flight	no	yes
Polarity	nonpolar	polar	polar	polar	polar	nonpolar	polar	nonpolar	nonpolar	polar	nonpolar
Charge	uncharged	negative	neutral	negative	uncharged	uncharged	positive	uncharged	uncharged	uncharged	uncharged
Water solubility	high	low	high	low	high	low	high	low	high	high	high
Acidity	neutral	neutral	neutral	acidic	neutral	neutral	alkaline	neutral	neutral	neutral	neutral
Additional functional group	none	Amino	Thiol	Carboxyl	Hydroxyl	none	Amino	Ring	none	Hydroxyl	none
Form	forked	T	forked	T	Ring	forked	T	Ring + forked	Ring	forked	forked
Taste (human)	sweet	bitter	obnoxious	umami	NA	bitter	complex/ bitter	bitter	sweet	sweet	weak

Experimental setup

The setup of the PER experiments followed Sommerlandt et al. (2014) and Ruedenauer et al. (2015). The chilled bumblebees were placed in a plastic tube (7 mm diameter, 35 mm long) and fixed with a "yoke" made of a paper clip, which allowed free movement of the bumblebees' head and forelegs, thus enabling a proper PER. The fixed animals were fed *ad libitum* with a 0.5 M sucrose solution before being placed in a climate cabinet for 25 h, at 20°C and 70% relative air humidity.

Prior to the actual experiment, we tested whether the animals showed PERs by touching their antennae with a toothpick soaked with a 0.5 M sucrose solution. Only bumblebees that properly responded to this stimulus by extending their proboscis were used for the learning experiments. We used a standard PER protocol for differential conditioning, which was established for honeybees (e.g. Bitterman et al. 1983; Laloi et al. 1999) and allows testing whether bees can differentiate between two stimuli, one rewarded (CS+) with sucrose solution (US) and the second one unrewarded (CS-). Each test animal was placed in a rack and allowed to rest for 15 s. For stimulus presentation, 5 µl of the solution was pipetted on a small filter paper, which was then placed on a copper plate and moved towards an antenna using a micromanipulator. The bee could then freely touch the stimulus with the tip of its left antenna. After the first touch, the stimulus was presented for 6 s. Three seconds after stimulus onset, a toothpick was presented to the right antenna, either as US (soaked with sucrose solution) or plain to equalize visual cues for the CS+ and CS- presentation. If the bumblebee extended its proboscis it was allowed to lick on the toothpick. The US was removed together with CS offset after 3 s. Subsequently, the bumblebee was allowed to rest for another 15 s before being replaced by the next one. The time between trials (inter trial interval, ITI) was 8 min. The number of trials was 20 per

individual (ten for CS+ and ten for CS-) presented in a pseudorandomized order (CS+, CS−, CS+, CS+, CS−, CS+, CS−, CS−, CS+, CS−, CS+, CS−, CS+, CS+, CS−, CS−, CS+, CS−, CS−, CS+). Each stimulus was tested once as CS+ and once as CS- (reversed meaning), and each animal was tested in one experiment only.

The plates were cleaned with 70% ethanol after each usage.

We performed three different experiments:

1. To determine whether bumblebees can perceive the amino acids, each amino acid (10 g/l water) was tested against pure water. Note that bumblebees, unlike honeybees (Page et al. 1998), do not show a PER to pure water. This experiment revealed that bumblebees could differentiate some amino acids (e.g. lysine, cysteine, hydroxyproline), but not others (e.g. alanine, leucine, proline) from water (Fig. WP5.1 & WP5.2).

2. To determine whether the bumblebees can further differentiate between different amino acids, we subsequently tested amino acids that could be differentiated from water against each other and amino acids that could not be differentiated from water against each other. We finally tested all amino acids that could be differentiated from water against one or two of the amino acids that could not be differentiated from water.

3. To finally determine whether the bumblebees can also differentiate between different concentrations of the same amino acid, we presented the bumblebees with three different concentrations (i.e. 1 g/l, 10 g/l and 20 g/l) of lysine (which the bumblebees could differentiate from water) and proline (which was not differentiated from water). These two amino acids were chosen because of their

high water solubility, which allowed testing of higher and lower concentrations than the one previously used (i.e. 10 g/l).

Statistical analysis

The numbers of responses to each CS+ and each CS- were counted for each animal (e.g. an animal may respond nine times to CS+ and one time to CS-) and used as response variable. To determine whether the bumblebees' response behavior and thus learning performance depended on the type of rewarded substance, we first composed a Laplace generalized linear mixed effect model (GLMM) with Poisson distribution and individual included as random effect and tested for a significant effect of the interaction between stimulus type (i.e. rewarded (CS+) or unrewarded (CS-)) and substance (i.e. amino acids, water, different concentrations). When the interaction was not significant (Table WP5.S1 – WP5.S3), we only tested for a significant effect of stimulus type. When the interaction was significant (i.e. in the case of asparagine), we tested for a significant effect of stimulus for both substance groups separately.

All statistical tests were performed using R v3.1.2 (R Foundation for Statistical Computing, Vienna, Austria). For data visualization GraphPad Prism v6.01 was used.

Results WP5

Differential conditioning of amino acids vs water

Bumblebees differentiated asparagine, cysteine, glutamic acid, hydroxyproline, lysine, phenylalanine and serine from water (Fig. WP5.1 & WP5.2, Table WP5.2). In case of asparagine, bumblebees only differentiated between asparagine used as CS+ and water used as CS-, but not vice versa (Fig. WP5.2, Table WP5.2). Learning performance did not depend on

the type of rewarded substance for any of the other perceived amino acids (Table WP5.S1). However, bumblebees did not differentiate alanine, leucine, proline or valine from water (Fig. WP5.3, Table WP5.2).

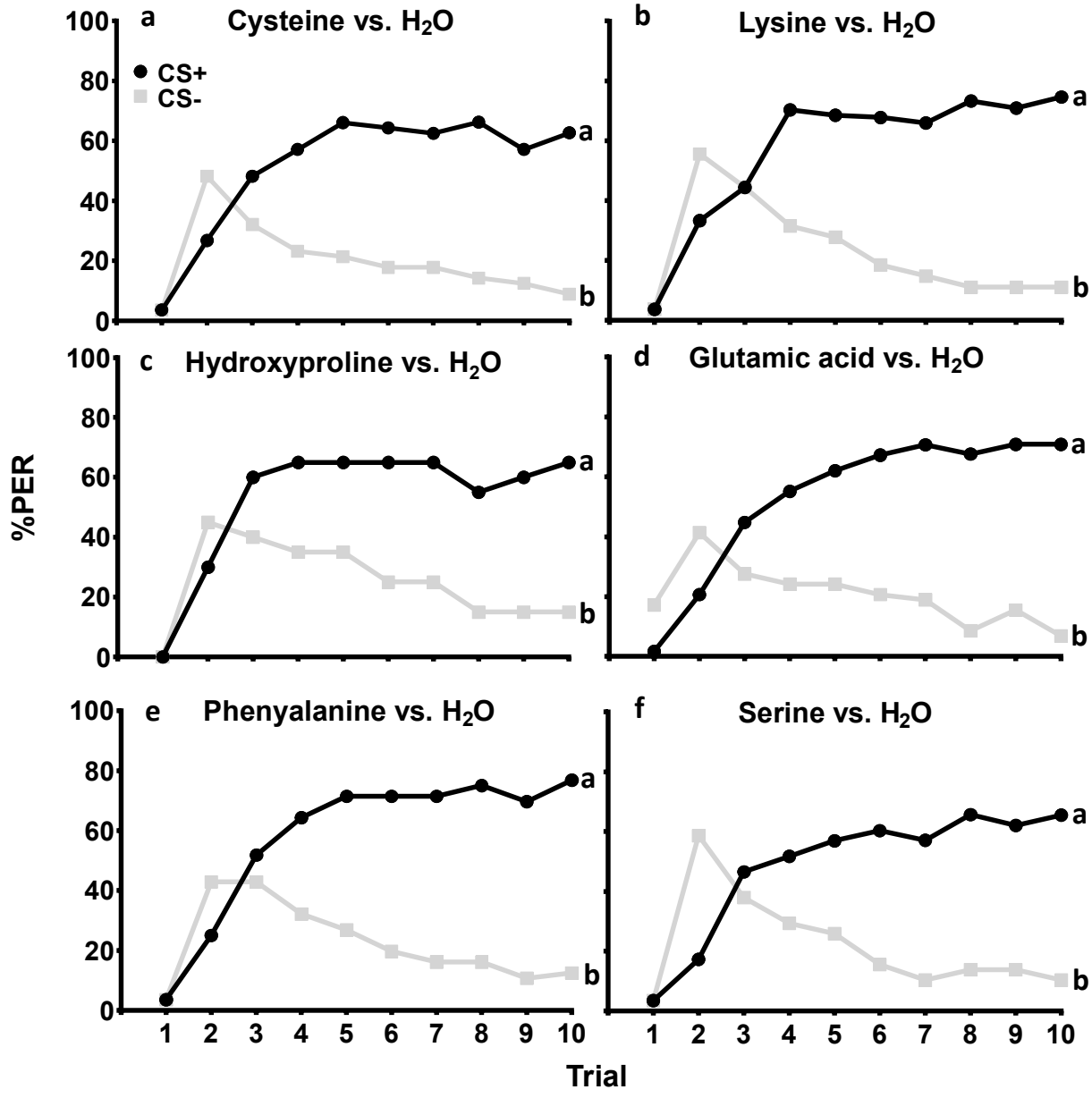


Figure WP5.1 Proportions of proboscis extension responses (PER) shown by *Bombus terrestris* towards amino acids (all 10 mg/ml water), which were differentiated from water (H_2O) in chemotactile conditioning: (a) cysteine, (b) lysine , (c) hydroxyproline, (d) glutamic acid, (e) phenylalanine and (f) serine. CS+ represents the rewarded conditioned stimulus, CS− the unrewarded conditioned stimulus. Both substances (i.e. amino acid and water) were used as CS+ and CS− with no significant differences between the two stimulus groups (see Supplementary Material, Table WP5.S1). Different letters to the right of the learning curves indicate significant differences in learning performance between groups (Table WP5.2).

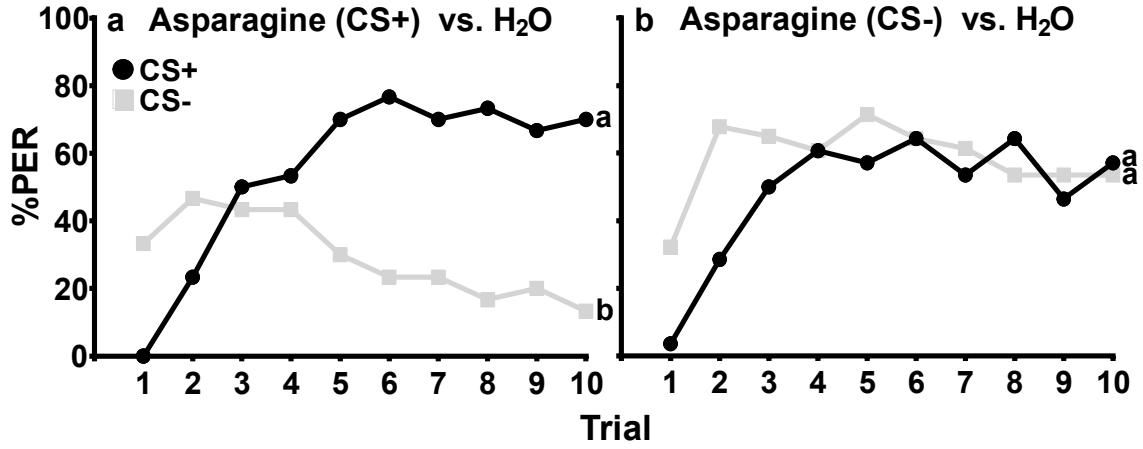


Figure WP5.2 Proportion of proboscis extension responses (PER) shown by Bombus terrestris towards asparagine (10 mg/ml water) vs water (H2O): (a) asparagine used as CS+, (b) asparagine used as CS-. CS+ represents the rewarded conditioned stimulus, CS− the unrewarded conditioned stimulus. There was a significant difference between the two stimulus groups (Supplementary Material Table WP5.S1), preventing pooling of the two groups. Different letters to the right of the learning curves indicate significant differences in learning performance between groups (Table WP5.2).

Table WP5.2 Statistical results (X^2- and *P*-values) of Laplace generalized linear mixed effect models (GLMMs) with Poisson distribution analyzing differences between the rewarded (CS+) and unrewarded stimulus (CS−) presented in differential chemotactile conditioning of amino acids vs. water. The table shows the amino acids used as stimulus, the number of individuals tested (N) and the results of the GLMM. Significant *P*-values (<0.05) are marked in bold.

Amino acid	N	X^2	*P*
Alanine	58	1.232	0.267
Asparagine CS+	30	19.015	<0.001
Asparagine CS-	28	0.282	0.595
Cysteine	56	64.880	<0.001
Glutamic acid	59	88.108	<0.001
Hydroxyproline	60	26.808	<0.001
Leucine	59	2.330	0.127
Lysine	54	76.627	<0.001
Phenylalanine	56	76.114	<0.001
Proline	56	1.357	0.295
Serine	58	58.762	<0.001
Valine	59	3.616	0.057

Differential conditioning between different amino acids

Bumblebees did not differentiate between amino acids that could be differentiated from water, or between amino acids that could not be differentiated from water (Fig. WP5.4,

Table WP5.3). They were, however, able to differentiate between amino acids that could be differentiated from water and amino acids that could not be differentiated from water (Fig. WP5.5, Table WP5.3).

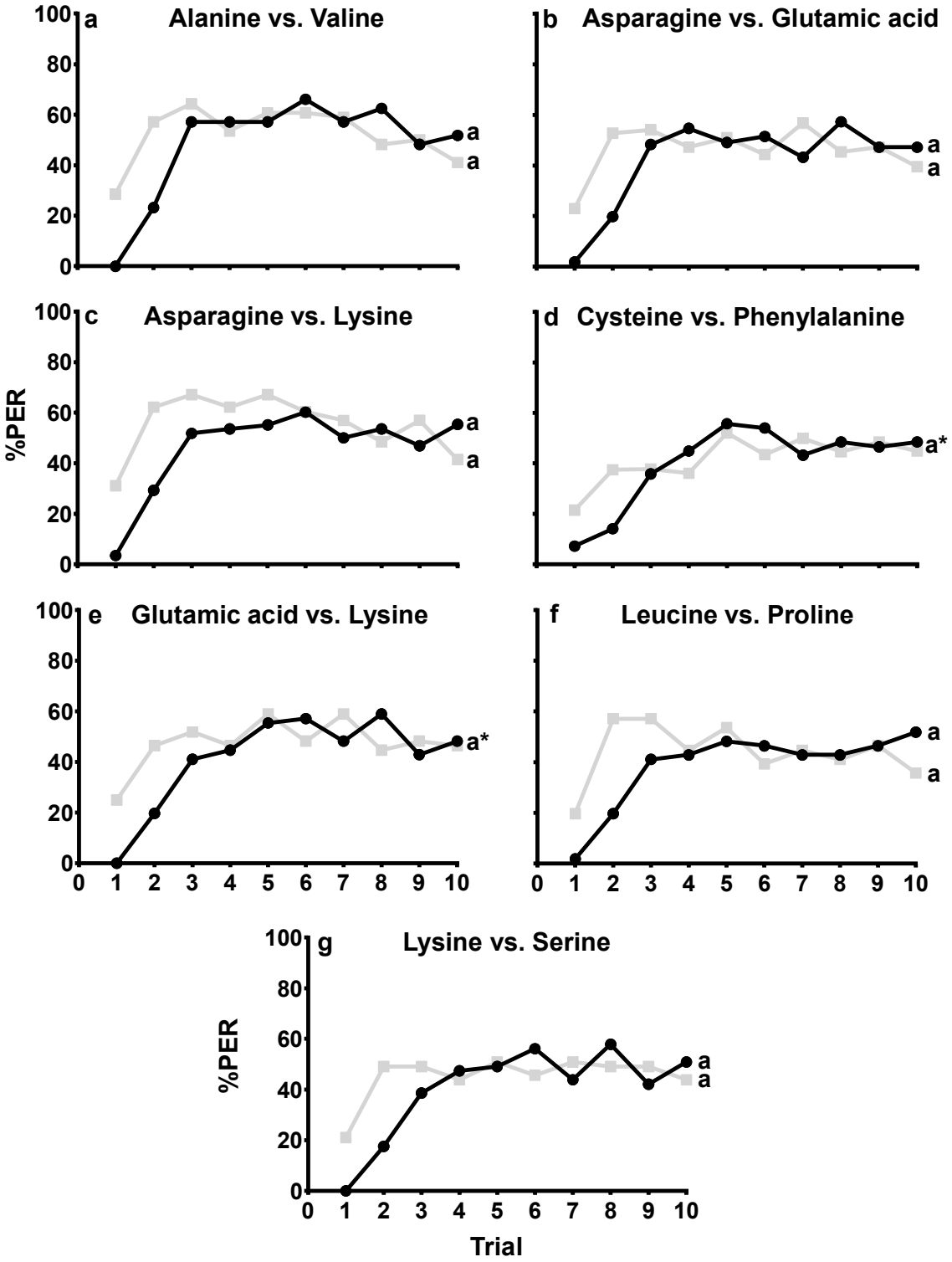


Figure WP5.4 PER of *Bombus terrestris* towards different amino acids (all 10 mg/ml water), which were or were not differentiated from water, tested against amino acids from the same group: (a) alanine vs valine, (b) asparagine vs. glutamic acid, (c) asparagine vs. lysine (d) cysteine vs. phenylalanine, (e) glutamic acid vs. lysine, (f) leucine vs. proline and (g) lysine vs. serine. CS+ represents the rewarded conditioned stimulus, CS− the unrewarded conditioned stimulus. Both amino acids were used as CS+ and CS− with no significant differences between groups (Supplementary Material Table WP5.S2). Amino acids that were differentiated from water are marked with a hashtag. The same letters to the right of the learning curves indicate no significant differences in learning performance between groups (Table WP5.3); letters with an asterisk indicate that the two (overlapping) curves had the same letter.

Table WP5.3 Statistical results (X^2- and *P*-values) of Laplace generalized linear mixed effect models (GLMMs) with Poisson distribution analyzing differences between the rewarded (CS+) and unrewarded stimulus (CS−) presented in differential chemotactile conditioning of different amino acids against each other. The table shows the amino acids used as stimuli, the number of individuals tested (N) and the results of the GLMM. Amino acids that were differentiated from water are marked with a hashtag. Significant *P*-values (<0.05) are marked in bold.

Amino acids	N	X^2	*P*
Alanine vs. Phenylalanine#	57	8.894	0.035
Alanine vs. Valine	56	0.114	0.736
Asparagine# vs. Glutamic acid#	56	0.703	0.402
Asparagine# vs. Lysine#	58	0.083	0.773
Cysteine# vs. Phenylalanine#	56	0	1
Glutamic acid# vs. Lysine#	56	2.1576	0.142
Glutamic acid# vs. Proline	58	57.191	<0.001
Leucine vs. Proline	56	2.033	0.154
Lysine# vs. Serine#	57	1.605	0.205

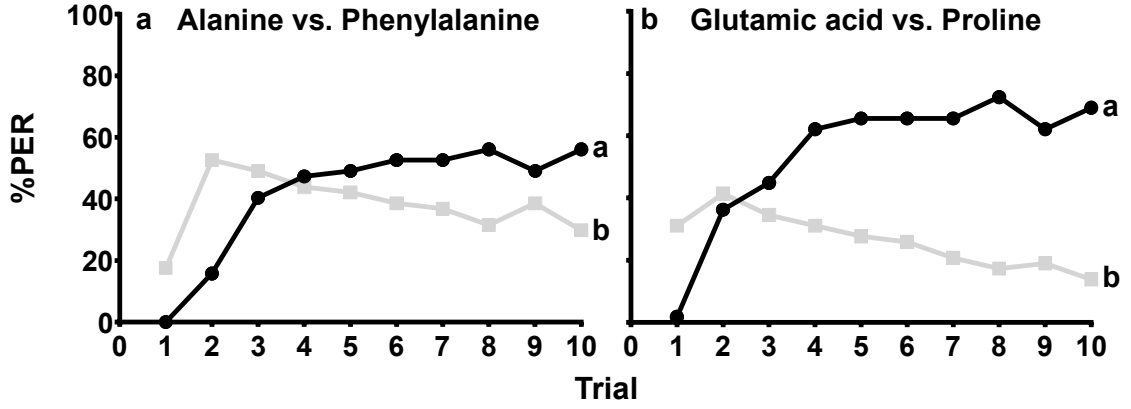


Figure WP5.5 Proportion of proboscis extension responses (PER) shown by Bombus terrestris towards amino acids (all 10 mg/ml water), which were or were not differentiated from water tested against amino acids from the other group: (a) alanine vs phenylalanine and (b) glutamic acid vs. proline. CS+ represents the rewarded conditioned stimulus, CS− the unrewarded conditioned stimulus. Both amino acids were used as CS+ and CS− with no significant differences between groups (Supplementary Material Table WP5.S2). Amino acids that were differentiated from water are marked with a hashtag. Different letters to the right of the learning curves indicate significant differences in learning performance between groups (Table WP5.3).

Differential conditioning between different concentrations of the same amino acid

Bumblebees differentiated between different concentrations of lysine (which could be differentiated from water), but not between different concentrations of proline (which could also not be differentiated from water) (Fig. WP5.6, Table WP5.4).

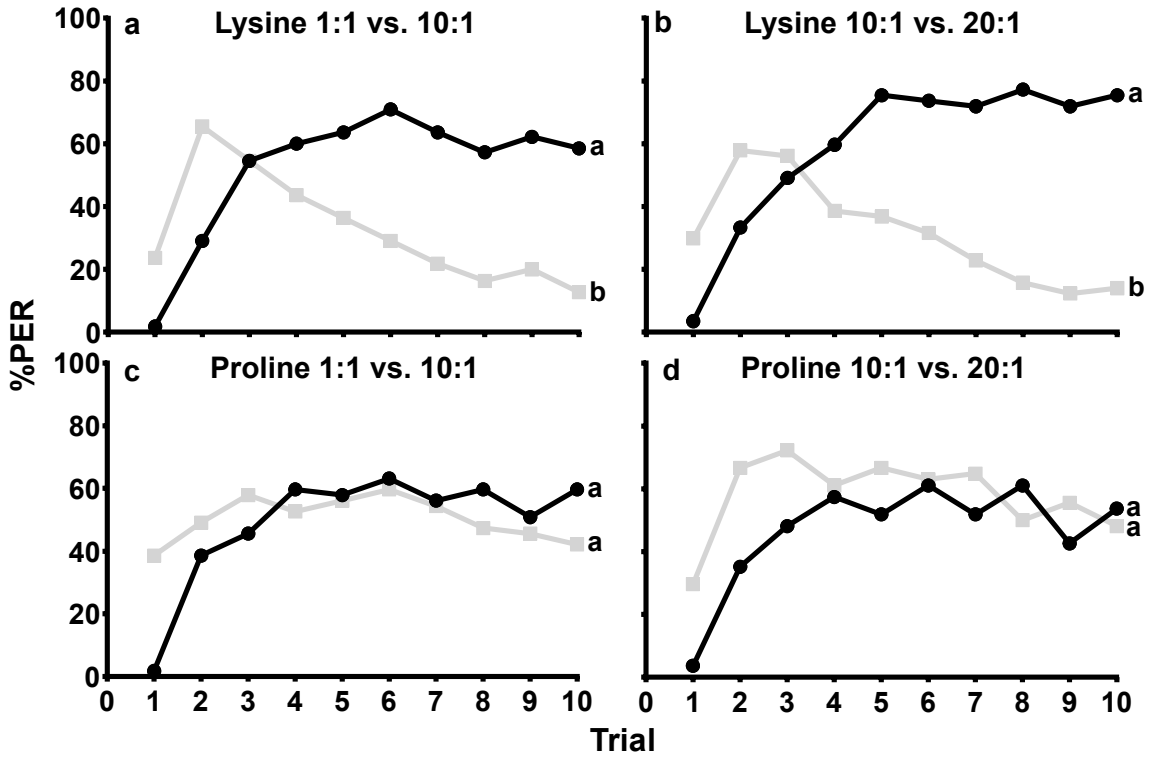


Figure WP5.6 Proportion of proboscis extension responses (PER) shown by *Bombus terrestris* towards different concentrations of amino acids which were or were not differentiated from water (concentrations given in mg/ml water): (a) lysine 1:1 vs. 10:1, (b) lysine 10:1 vs. 20:1, (c) proline 1:1 vs 10:1 and (d) proline 10:1 vs. 20:1. CS+ represents the rewarded conditioned stimulus, CS− the unrewarded conditioned stimulus. Both concentrations were used as CS+ and CS− with no significant differences between groups (Supplementary Material Table WP5.S3). Amino acids that were differentiated from water are marked with a hashtag. Different letters to the right of the learning curves indicate significant differences in learning performance between groups (Table WP5.4).

Table 4 Statistical results (X^2- and *P*-values) of Laplace generalized linear mixed effect models (GLMMs) with Poisson distribution analyzing differences between the rewarded (CS+) and unrewarded stimulus (CS−) presented in differential chemotactile conditioning of different concentrations of the amino acids lysine and proline. The table shows the amino acids and concentrations used as stimuli, the number of individuals tested (N) and the results of the GLMM. Amino acids that were differentiated from water are marked with a hashtag. Significant *P*-values (<0.05) are marked in bold.

Amino acids	N	X^2	*P*
Lysine# 10:1 vs. 1:1	55	30.831	<0.001
Lysine# 20:1 vs. 10:1	57	47.844	<0.001
Proline 10:1 vs. 1:1	57	0.053	0.819
Proline 20:1 vs. 10:1	54	0.622	0.430

Discussion WP5

Bumblebees can perceive some amino acids

Our results clearly show that, with access to chemotactile information by means of the antennae, bumblebee workers can differentiate some amino acids (i.e. asparagine, cysteine, glutamic acid, hydroxyproline, lysine, phenylalanine and serine) from water, but not others (i.e. alanine, leucine, proline and valine). Moreover, while bumblebees were perfectly able to differentiate different concentrations of the same ('perceivable') amino acid, they could not differentiate between different amino acids of the same group ('perceivable' or 'non-perceivable'). These results were quite unexpected and contrast our expectations that all amino acids could be differentiated from water and against each other.

Interestingly, perception was not confined to those amino acids that are essential to bees (DeGroot 1953) (Table WP5.1). Neither was perception related to polarity, charge, acidity or the (subjective) human taste impression (participants were asked to eat amino acids and describe the taste, Schiffman et al. 1981) (Table WP5.1). The only consistent difference between the two groups was that 'perceivable' amino acids had a terminal functional group in addition to the amino acid-characteristic amino- and carboxyl groups (Table WP5.1). This

difference is especially apparent for proline and hydroxyproline, which only differ in the terminal (hydroxyl) group (Table WP5.1). While hydroxyproline was perceived by the bumblebees, proline was not, indicating that this difference was sufficient to enable perception.

Bumblebee non-specific amino acid reception is different to other animals

The rather non-specific perception of amino acids with additional terminal functional groups contrasts with amino acid perception in vertebrates. For example, humans are able to differentiate between different amino acids and even between different enantiomers, i.e. D- and L-forms (Schiffman and Dackis 1975; Schiffman et al. 1981), which are received by different receptor types (as reviewed by Chandrashekar et al. 2006). Likewise, fish show different levels of attraction by different amino acids, suggesting that they also may be able to differentiate between different amino acids (Kasumyan and Morsi 1996; Sutterlin 1975), most likely by means of specific chemoreceptors (Marui and Kiyohara 1987; Mullin et al. 1994).

Interestingly, the proboscis of the hoverfly *Eristalis tenax* is sensitive to proline as a component of pollen (Wacht et al. 2000) and honeybees prefer nectar containing higher concentrations of proline (Carter et al. 2006). These findings suggest that bumblebees might also be able to perceive more amino acids and differentiate between them by means of receptors that are located on body parts other than the antennae, e.g. the proboscis, or after ingestion.

The lack of antennal differentiation in *B. terrestris* suggests that bumblebees possess generalistic receptors on their antennae, which are activated non-specifically by amino acids that possess an additional functional group. Such a reception system may resemble the

mammalian amino acid receptor heteromer (T1R1+3) or the *Drosophila* ionotropic co-receptor IR76b, which both respond to a broad spectrum of L-amino acids (Ganguly et al. 2017; Nelson et al. 2002), including amino acids that could not be perceived by bumblebees. Moreover, IR76b is highly conserved among insects and also present in *Apis mellifera* (Croset et al. 2010), rendering it a potential amino acid co-receptor also in *B. terrestris*. In fact, the IR76b receptor gene is present in the *B. terrestris* genome, but it is distinct from the *A. mellifera* IR76b in its amino acid sequence (Sadd et al. 2015).

Essentiality of amino acids is not important for perception

Interestingly, some essential amino acids (i.e. leucine and valine) were not perceived, indicating that antennal amino acid perception in *Bombus terrestris* does not reflect the importance of a particular amino acid or its effect on the bumblebees' health. However, essential amino acids seem to be present in every pollen species and proline is in most cases the dominating amino acid in bee resources (Weiner et al. 2010). Consequently, these amino acids either do not need to be assessed specifically, are perceived via receptors on other body parts or are perceived only post-ingestive.

Amino acids information could be influencing foraging decisions

Although bumblebees cannot differentiate between different amino acids and are therefore not able to assess qualitative differences in amino acid profiles of nectar and pollen by means of their antennae, they can use antennal perception to infer concentration differences of specific amino acids. In both pollen and nectar, proportions of different amino acids correlate with each other and with overall amino acid content ($r \geq 0.5$, $P < 0.01$ for the dataset composed by Weiner et al. 2010). Therefore, bumblebees could easily infer the overall quantity based on the content of only some amino acids and use this information for their

foraging decisions. This would provide them with sufficient information on the amino acid content of floral resources. It would further enable bumblebees to avoid high concentrations of (free) amino acids potentially detrimental to bees (Huang et al. 2011).

It remains open why our tested bumblebees could only differentiate between asparagine and water when asparagine was presented as CS+. One possible explanation for this finding is that asparagine needs reinforcement by the reward (CS+) in order to be learned, whereas it cannot be learned when it is not rewarded (CS-).

Conclusion

To conclude, our experiments suggest that at least one type of amino acid-specific receptor is expressed in the antennae of *B. terrestris*, which selectively responds to amino acids with an additional functional group. However, unlike in vertebrates and *Drosophila* (Croset et al. 2016; Ganguly et al. 2017; Nelson et al. 2002), this antennal receptor obviously does not allow to differentiate between different amino acids. Alternatively, the additional functional groups may be received non-specifically by non-amino acid specific receptors responding to functional groups in general.

Summarizing our results, we suggest that antennal perception of dissolved amino acids enables bumblebees to assess the free amino acid content of floral resources and thus to assess their overall amino acid content, but most likely not their qualitative composition. Moreover, the overall protein content of pollen could be easily inferred via this free amino acid assessment, as free amino acids seem to be positively correlated with overall protein (i.e. the sum of protein-bound and free amino acids) in pollen (Weiner et al. 2010). Although most nutrients in pollen are located inside the grain and therefore inaccessible to bees (Stanley and Linskens 1974), nutritional information could still be obtained from small nutrients (e.g. free

amino acids) leaking through pores onto the pollen surface or through occasionally damaged pollen grains. Following antennal perception of overall amino acid content, more precise information on amino acid composition might be obtained via receptor on other body parts or post-ingestive. In future studies, electrophysiological and molecular methods as well as tests for proboscis or internal (via feeding) perception of amino acids will help to further elucidate the mechanisms underlying amino acid reception and perception in bees.

Supplementary Material WP5

Table WP5.S1 Statistical results (*z*- and *P*-values) for the interaction of stimulus type (i.e. CS+ and CS-) and amino acid tested in differential chemotactile conditioning of amino acids vs. water and analyzed with a Laplace generalized linear mixed effect model (GLMM) with Poisson distribution. The table shows the amino acids used as stimulus, the number of individuals tested (N) and the results of the GLMM. Significant *P*-values (<0.05) are indicated in bold.

Amino acid	N	*z*	*P*
Alanine	58	1.110	0.267
Asparagine	58	-2.409	0.016
Cysteine	56	0.129	0.897
Glutamic acid	59	-0.584	0.560
Hydroxyproline	60	-0.020	0.984
Leucine	59	1.318	0.187
Lysine	54	0.162	0.871
Phenylalanine	56	0.533	0.594
Proline	56	0.793	0.394
Serine	58	1.207	0.227
Valine	59	0.476	0.634

Table WP5.S2 Statistical results (*z*- and *P*-values) for the interaction of stimulus type (CS+ and CS-) and amino acids tested in differential chemotactile conditioning of amino acid vs. amino acid and analyzed with a Laplace generalized linear mixed effect model (GLMM) with Poisson distribution. The table shows the amino acids used as stimulus, the number of individuals tested (N) and the results of the GLMM. Amino acids that were differentiated from water are marked with a hashtag.

Amino acids	N	*z*	*P*
Alanine vs. Phenylalanine#	57	-0.980	0.327
Alanine vs. Valine	56	1.420	0.156
Asparagine# vs. Glutamic acid#	56	0.838	0.402
Asparagine# vs. Lysine#	58	0.735	0.462
Cysteine# vs. Phenylalanine#	56	-1.324	0.185
Glutamic acid# vs. Lysine#	56	-0.007	0.994
Glutamic acid# vs. Proline	58	-1.287	0.198
Leucine vs. Proline	56	-0.687	0.492
Lysine# vs. Serine#	57	0.425	0.671

Table WP5.S3 Statistical results (*z*- and *P*-values) for the interaction of stimulus type (CS+ and CS-) and amino acid tested in differential chemotactile conditioning of different amino acid concentrations and analyzed with a Laplace generalized linear mixed effect model (GLMM) with Poisson distribution. The table shows the amino acids used as stimulus, the number of individuals tested (N) and the results of the GLMM. Amino acids that were differentiated from water are marked with a hashtag.

Amino acids	N	*z*	*P*
Lysine# 10:1 vs. 1:1	55	-0.007	0.994
Lysine# 20:1 vs. 10:1	57	0.827	0.408
Proline 10:1 vs. 1:1	57	0.470	0.638
Proline 20:1 vs. 10:1	54	0.789	0.430

WP6 Best be(e) on low fat: linking nutrient perception, regulation and fitness

Authors:

Fabian A. Ruedenauer*, David Raubenheimer, Daniela Kessner-Beierlein,

Nils Grund-Mueller, Lisa Noack, Johannes Spaethe, Sara D. Leonhardt

*first author

published: 2020

Journal: Ecology Letters

Volume: 23 Issue: 3 Pages: 545-554

Abstract WP6

Preventing malnutrition through consuming nutritionally appropriate resources represents a challenge for foraging animals. This is due to often high variation in the nutritional quality of available resources. Foragers consequently need to evaluate different food sources. However, even the same food source can provide a plethora of nutritional and non-nutritional cues, which could serve for quality assessment. We show that bumble bees, Bombus terrestris, overcome this challenge by relying on lipids as nutritional cue when selecting pollen. The bees 'prioritized' lipid perception in learning experiments and avoided lipid consumption in feeding experiments, which supported survival and reproduction. In contrast, survival and reproduction were severely reduced by increased lipid contents. Our study highlights the importance of fat regulation for pollen foraging bumble bees. It also reveals that nutrient perception, nutrient regulation and reproductive fitness can be linked, which represents an effective strategy enabling quick foraging decisions that prevent malnutrition and maximize fitness.

Introduction WP6

Malnutrition resulting from the consumption of inadequate or nutritionally inappropriate food resources has severe health, performance and fitness consequences for most organisms (Arganda et al. 2017; Lee et al. 2008; Simpson and Raubenheimer 2012; Su and Gao 2007; Vaudo et al. 2018). In fact, malnutrition may contribute to or enhance widespread population declines, such as currently observed in insects in general and bees in particular (Goulson et al. 2015; Hallmann et al. 2017; Naug 2009; Potts et al. 2010; Seibold et al. 2019). Whether or not a resource is appropriate for an organism largely depends on species-specific requirements and thus on the nutritional quality of food, i.e. the composition and quantity of

nutrients. For example, high intake of fat and deviations from ideal fatty acid (FA) ratios (Simopoulos 2002) can impair learning (Arien et al. 2015) and shorten lifespans in honeybees (Haddad et al. 2007; Manning et al. 2007). Selecting and consuming appropriate food resources through behavioral and physiological adaptations (e.g. differentiation by taste, memorization of valuable resource patches, nutrient-selective foraging) can strongly increase individual health and reproductive fitness.

The intake of ideal nutrient ratios is however challenged by the high degree of variation in the amounts and ratios of different micro- and macronutrients in different food resources (Biesalski 2017; Simpson and Raubenheimer 2009; Simpson and Raubenheimer 2012). Avoiding malnutrition and obtaining nutritionally appropriate diets consequently requires nutrient-sensitive foraging and/or consumption (Jensen et al. 2011; Mayntz et al. 2005; Raubenheimer and Simpson 1993; Raubenheimer and Simpson 1999). In fact, many animals, e.g. bumble bees (*Bombus terrestris*) (Ruedenauer et al. 2016) or trap-jaw ants (*Odontomachus hastatus*) (Bazazi et al. 2016), rapidly adapt their foraging behavior and resource intake to changes in the nutritional quality of food. These prompt behavioral responses indicate that these species are capable of sensing nutritional differences between different food sources likely by means of specific taste receptors (Abisgold and Simpson 1988; Simpson et al. 1991). However, different food sources vary in the composition of a plethora of nutritional cues and signals, not all of which are meaningful in each context. This may explain why many animal species regulate the intake of one specific macronutrient more strongly than the intake of others (Simpson and Raubenheimer 2012). As a consequence, animals readily consume too low or excessive amounts of other nutrients in order to reach the intake target of the most strongly regulated nutrient (Simpson and Raubenheimer 2012). For example, some omnivores (e.g. humans) and herbivores (e.g. herbivorous primates and

insects (e.g. locusts, caterpillars)) most strongly regulate protein intake, while many predators (e.g. minks, carabid beetles) predominantly regulate carbohydrate and/or fat intake (Bray et al. 2012; Gosby et al. 2011; Simpson and Raubenheimer 2012). Nutritional quality is thus largely defined by the content of this most strongly regulated nutrient. Interestingly, we hardly know the perceptional mechanisms underlying nutritional quality assessment (Abisgold and Simpson 1988; Simpson et al. 1991; Simpson and Raubenheimer 2012). For example, it is unknown which nutritional cues are perceived or whether they are linked to regulated nutrients.

In this study, we elucidate nutritional quality assessment and perception and determine whether it can be linked to nutritional target regulation in the bumble bee *Bombus terrestris*. We studied bees, because they are important pollinators, obtain most nutrients from nectar and pollen as main food sources and appear to regulate protein intake (like other herbivores) (Pirk et al. 2010; Vaudo et al. 2016b). While nectar is mainly a source for carbohydrates, pollen contains most other required nutrients (protein, fat, minerals and vitamins (Roulston and Cane 2000b)). Besides nutrients, pollen additionally contains several other compounds such as secondary metabolites (Palmer-Young et al. 2019a; Palmer-Young et al. 2019b) and odors (Dobson et al. 1999; Dobson et al. 1996). Pollen thus represents a complex chemical mixture with many different volatile (e.g. terpenoids and benzenoids (Dobson et al. 1999)) and non-volatile (e.g. nutrients and plant secondary metabolites) cues. Among these cues, bumble bees appear to use non-volatile nutritional cues for nutritional quality assessment, as they can differentiate between two pollen types differing in nutritional quality only when they can taste pollen (Ruedenauer et al. 2015). Besides their own individual perception, bumble bees, like other social insects, could additionally rely on behavioral or chemical feedback from

relatives, which process (e.g. nurse bees) or consume (e.g. offspring) allocated food (Dussutour and Simpson 2008; Grüter et al. 2013; Ruedenauer et al. 2016).

Moreover, the nutritional quality of pollen and in particular its protein content seems to largely determine bumble bee colony development (Kämper et al. 2016; Moerman et al. 2017; Roger et al. 2017) as well as bumble bee immune defence (Brunner et al. 2014; Di Pasquale et al. 2013; Roger et al. 2017). Pollen protein content and amino acid profiles furthermore correlate with foraging preferences (Kitaoka and Nieh 2009; Kriesell et al. 2017; Leonhardt and Blüthgen 2012). However, more recent studies suggest that, in addition to protein, fat also plays an important role in nutrient regulation for bumble bees (Vaudo et al. 2016a; Vaudo et al. 2016b).

We performed a range of behavioral experiments with *B. terrestris* to determine (i) the nutritional cues perceived and thus potentially used for nutritional quality assessment, (ii) whether these cues were linked to nutrient regulation and (iii) whether these cues affected *B. terrestris* health and reproductive fitness. We focused on protein and fat, the two main pollen macronutrients apparently regulated by bumble bees (Pirk et al. 2010; Vaudo et al. 2016b). However, most protein and fat molecules are likely too large for taste receptors (Solms 1969), rendering smaller molecules, e.g. amino acids (AAs) and fatty acids (FAs), more likely candidates for reception and thus perception. In fact, the content of free AAs positively correlates with the total protein content of pollen ($r = 0.40$, $P < 0.001$; data obtained from Weiner et al. 2010) and negatively with its fat content (for bee-collected pollen, Ruedenauer et al. 2019b). We consequently predicted that bees would use both AAs and FAs in pollen as nutritional cues to obtain information on the content of their regulated macronutrient protein. Because bees were suggested to avoid excessive protein/AA intake (Helm et al.

2017), we further expected bumble bees to reduce collection of pollen enriched with AAs. As animals mostly regulate only one nutrient group and because bees were suggested to be particularly sensitive to pollen protein content, we predicted that they should show no differences in collection between pure pollen and pollen enriched with FAs. We finally hypothesized that colonies fed pollen enriched with excessive amounts of AAs or FAs would show reduced survival and reproduction, as overconsumption of nutrients is generally toxic (Pirk et al. 2010; Simpson and Raubenheimer 2009).

Materials and Methods WP6

Bee colonies

We purchased 24 Bombus terrestris colonies from a commercial supplier (Behr, Kampen, Germany) between February 2017 and April 2018. Six of these colonies were transferred into two-chambered wooden boxes (240×210×110 mm per chamber, where one chamber served as brood chamber and the other chamber as foraging chamber). These colonies were used for learning experiments. The other 18 colonies kept in the original boxes (270×240×200 mm) provided by the supplier and were used as source colonies for the feeding experiments. All animals were kept in a climate chamber (25°C, 50% humidity, 12/12 h light/dark-cycle) and fed ad libitum Apiinvert (Südzucker AG, Mannheim, Germany; a mixture of sucrose, fructose, and glucose, delivered with the colonies) and honey bee-collected pollen (Naturwaren Niederrhein GmBH, Goch-Asperden, Germany).

Preparation of pollen diets

For all experiments, pollen was prepared in large quantities by mixing and grinding the same bee-collected pollen (as fed to the source colonies) with a coffee mill (CM 800, Graef,

Arnsberg, Germany). The bee-collected pollen was relatively diverse in colors (personal observation) and can comprise pollen from up to 15 different genera (Ruedenauer et al. 2016). For each diet, we mixed 48 g of the resulting powder with 11 ml (for pure pollen) or 13 ml (for pollen subsequently 'enriched' with AAs and FAs) of de-ionized water to create a paste that sticks to the copper plates used in the learning experiments (Fig. S1). We subsequently added powdery AAs or FAs to 'nutritionally enrich' the latter pollen-water mixture. The slightly different amounts of water were used to achieve a similar pollen paste texture across diets. Prepared pollen diets were kept frozen until usage. Consequently, all diets of one experimental series were produced from the same batch of pollen and differed only in the amount and type of admixture added. Note that bee-collected pollen is mixed with regurgitated nectar by honey bees, which does not, however, elicit a spontaneous PER in unconditioned bumble bees.

We tested pure bee-collected pollen and bee-collected pollen enriched with 0.5, 5 or 10x the natural mean concentrations of (a) eleven AAs (0.5x/5x/10x AA) (Table S1, means taken from the dataset of Weiner et al. (2010)) or (b) seven FAs (0.5x/5x/10x FA) (Table S2, taken from Manning (2006)) in learning and feeding experiments. We therefore tested AA and FA concentrations, which are typical for natural pollen, except for the 10x concentration, which exceeds the upper limits of known concentrations (Manning 2006; Roulston and Cane 2000b; Weiner et al. 2010). AA and FA contents of the bee-collected pollen used in our experiments were analyzed as described in Methods S1. Contents were within the natural range as calculated for hand-collected pollen (Table S1&2).

Note that we did not include oleic acid and linoleic acid in the FA mixture, because they modify the pollen paste texture in a way that prevents testing. However, as oleic and linoleic acid are

both beneficial to bees and were found to support cognitive performance in bees (Arien et al. 2018; Arien et al. 2015; Muth et al. 2018), we tested them separately in the learning experiments (see below).

Learning experiments

We used a recently established technique, chemotactile differential conditioning of the proboscis extension response (PER) (Ruedenauer et al. 2015), to test whether Bombus terrestris workers can differentiate pollen differing in its AA and FA content. The PER learning assay is based on classical conditioning (Pavlov 1927) and allows testing whether or not bees can learn to differentiate between two sensory cues (e.g. food with two different concentrations of the same nutrient). While other methods, such as electro-antennographic measurements or single-sensillum recordings investigate cue processing at the receptor level, PER conditioning ultimately tests perception and thus the effect of specific cues on actual behavioral responses. In contrast, signals measured at the receptor level can be modified or even nullified on their way from receptors to processing centers in the brain (Eltz and Lunau 2005) and thus have no or little impact on an animal's behavior. PER conditioning thus enabled us to test the bees' ability for pre-ingestive perception by means of the antennae and consequently the bees' assessment of pollen nutritional quality based on specific nutrients.

The experimental setup of the PER experiments was based on Sommerlandt et al. (2014) and Ruedenauer et al. (2015). Bumble bees covering the full size spectrum from small to large workers were caught from the foraging chamber and chilled on ice for 15 min. Thereafter, they were placed inside tubes made of plastic pipette tips (Hartenstein, Würzburg, Germany) and fixed with "yokes" made of paper clips (Ruedenauer et al. 2015; Sommerlandt et al. 2014). Head and forelegs were unfixed to allow movement and a proper PER. The bees were then

placed in a rack equipped with damp cloths and fed with a 0.5 M sucrose solution. The rack was placed in a climate chamber (see above) for 25 h. On the next day, each individual was tested for a proper PER by holding a toothpick soaked with 0.5 M sucrose solution to its antennae. Only bumble bees showing a proper PER were used for the experiments. We used a standardized PER protocol (e.g. Bitterman et al. 1983; Laloi et al. 1999) with differential chemotactile conditioning (Ruedenauer et al. 2015). This method uses two different conditioned stimuli (CS), one rewarded (CS+) with sucrose solution (unconditioned stimulus, US) and one unrewarded (CS-). Bees were placed in a test rack and allowed to rest for 15 s, before a CS was presented to the antenna for 6 s using a copper stick mounted on a micromanipulator (Ruedenauer et al. 2015). As soon as the bee touched the CS, the 6 s time interval was started to test for a PER. In case of the CS+, a tooth pick soaked with sugar water (US) was held to the antenna for the last 3 s of the CS presentation, and the bee was allowed to lick. When a CS- was presented a blank tooth pick was held near the antenna to account for a possible bias due to the tooth pick movement and thus prevent bees from using visual cues for learning. After stimulus presentation, the bee was allowed to rest for another 15 s and the next bee was tested. The inter trial interval (ITI), the time between trials of the same individual, was 8 min (Bitterman et al. 1983). One experimental series consisted of 20 trials presenting CS+ and CS- in the following order: CS+, CS−, CS+, CS+, CS−, CS+, CS−, CS−, CS+, CS−, CS+, CS−, CS+, CS+, CS−, CS−, CS+, CS−, CS−, CS+. This order does not allow for any inferences on the next stimulus and remains stable across trials. Each individual was only tested in one experimental series. Each stimulus was tested as both CS+ and CS- in two different experimental series (referred to as “reversed meaning”).

For presentation of the CS, a wet filter paper was placed on the copper sticks and about 50 mg of pollen paste was applied to this paper. The pollen and filter paper were renewed after every stimulation (each bee was stimulated with fresh pollen and filter paper). The plates were cleaned in 99% ethanol (Hartenstein, Würzburg, Germany) after each stimulation.

At first, we tested pure bee-collected pollen (PP) (henceforth referred to as pollen) against the same pollen enriched with (a) 10x AA or (b) 10x FA. Note that, despite adjusting water amounts, pollen paste texture was practically identical for pollen enriched with different amounts of FAs and only slightly different for pure pollen. To nevertheless ensure that the bees actually learned differences in nutrient concentrations and not in texture, we tested pollen enriched with 0.5x the natural mean concentrations of the seven FA (0.5x FA) against the 10x FA. We additionally tested pollen enriched with different concentrations of only linoleic and only oleic acid separately (0.5x against 10x), because these two FAs are abundant in pollen and beneficial for bees (Manning 2006), but could not be included in the mixture (see above). To finally determine whether differentiation was based on all or only specific FAs out of the nine tested FAs, we additionally tested each of the nine FAs against pure pollen.

The addition of AAs could have exceeded the natural detection/perception range of bumble bees. To account for this possibility, we repeated the AA experiment using pollen which was first diluted with cellulose (pollen:cellulose 1:10) before adding AAs (0.5x and 10x AA) and testing each concentration against diluted pollen. The pollen types used thus contained overall lower AA concentrations which were still within the natural concentration range of pollen and provided similar relative differences as tested above. Cellulose can neither be smelled nor tasted by bumble bees and should therefore not affect perception (Mapalad et al. 2008; Ruedenauer et al. 2015).

Feeding experiments

We additionally tested for the effect of pollen enriched with AAs or FAs on consumption and thus changes in bee foraging behavior post-ingestion using feeding experiments with microcolonies (Ruedenauer et al. 2016; Vaudo et al. 2016b). These queenless colonies, which consist exclusively of workers, have been proven to be fully comparable to queenright colonies for measuring nutritional requirements and fitness effects (Génissel et al. 2002; Tasei and Aupinel 2008).

The experimental approach followed Ruedenauer et al. (2016). Based on 18 mother colonies, we prepared overall 336 queenless microcolonies, each consisting of 20 randomly selected Bombus terrestris workers all obtained from the same mother colony. This resulted in ~19 microcolonies per mother colony (Table S3). Microcolonies were kept in small two-chambered wooden boxes (14.5 × 13 × 10 cm per chamber) covered with clear acrylic glass. Food was provided in one chamber (foraging chamber) and the second chamber was used for nesting (nest chamber). The boxes were kept in the laboratory in a 12/12 h light/dark cycle at 25°C. Bees had ad libitum access to a 2 M sucrose solution.

We performed two types of feeding experiments: choice and no choice diet experiments (Table S3). In no choice diet experiments, the bees were provided with one pollen type/one diet only, which allowed us to compare the effect of each diet on the worker bees' survival and reproduction, which we used as measure for fitness. In choice diet experiments, bees were offered two different diets (pure pollen and enriched pollen) simultaneously to measure differences in consumption and thus foraging choices between the two diets. The choice diet experiment consequently provided information on whether or not bees discriminated between pure and enriched pollen when they could probe and ingest the pollen. In contrast,

the PER learning experiments only provided information on pre-ingestive discrimination. To finally determine whether the workers relied on larval feedback for their foraging decisions, we performed each choice diet experiment with half of the microcolonies being allowed to rear brood (brood treatment) and brood removed from the other half (no brood treatment). Larval feedback could, in theory, interfere with individual worker assessment as requirements can differ between larvae and adults, resulting in different resource intake between colonies with and without brood. As microcolonies were queenless, they only produced male offspring.

Fresh pollen was provided daily in small petri dishes placed in the center of the foraging chamber containing either one diet only (no choice diet experiments) or two different diets (choice diet experiments). For the choice diet experiment, the position of the two petri dishes was randomized across days. Each day, the dishes were weighed to quantify the food uptake from each diet by each microcolony. Evaporation was taken into account by calculating the weight loss of dishes (containing the same diets) placed outside the colonies and correcting weights of experimental dishes accordingly. As bees died over the course of the experiments and the number of individuals varied between colonies, we always divided overall food collection by the number of individuals present in each microcolony per day. We determined the effect of pollen enriched in AA or FA content on the longevity and reproductive success of bumble bees in microcolonies in the no choice diet experiments. For the survival analysis, dead individuals were recorded each day. To analyze differences in reproductive success, we recorded the number of egg clumps, larval cells, pupae and hatched drones per day.

A complete overview of the feeding experiments, treatments (no choice or choice diet, pollen type(s) offered and brood or non-brood) and numbers of microcolonies tested in each setup

is shown in Table S3. In order to produce robust results, we repeated each feeding experiment at least twice between February 2017 and April 2018.

Data analysis

To analyze differences in learning performance (PER experiments), we used the number of positive responses (i.e. proboscis extension reactions) to each conditioned stimulus. First, we checked whether response numbers depended on whether a substance was used as CS+ or CS- (reversed meanings) using a Mann-Whitney U test (because data were not normally distributed). As we found no differences in any of the experiments (Table S4), data were combined following standard PER conditioning procedures (Laloi et al. 1999; Sommerlandt et al. 2014). For the comparison of responses towards the CS+ and CS- we used paired U tests to account for the fact that CS+ and CS- values came from the same individual.

For each feeding experiment, we always tested first for significant effects of the "experimental period" and the "mother colony" by including these factors as random effects in a generalized linear mixed effect model (GLMM) and comparing the GLMM to a generalized linear models (GLM) without random effects (both with Gaussian distribution), following Zuur et al. (2009). When both or one of the random factors explained a significant proportion of the observed variance, as assessed through a likelihood ratio test for the model comparison (Table S9), we performed a GLMM (lme4 package (Bates et al. 2015)) with the respective random factor(s). When there was no significant effect of either one of the random factors, we performed a GLM (lme4 package). We always tested for the effect of diet (fixed effect) on the mean pollen consumed per individual and microcolony over the whole experiment, either within one treatment (choice diet) or between treatments (no choice diet).

Differences in reproductive success between microcolonies offered different pollen diets in the no choice diet experiment were analyzed either with a GLMM or a GLM (see above) testing for differences in the number of egg clumps, larval and pupal cells (newly produced or removed in relation to the previous day) between diets (fixed effect).

Differences in the survival rate of bees in the no choice diet experiment were analyzed with Kaplan-Meyer survival statistics by comparing median survival times between each diet pair using log-rank tests (survival (Therneau and Grambsch 2013) & KMsurv package (Klein and Moeschberger 2006)). As this involved multiple testing, we adjusted the α-level using Bonferroni.

All statistical analyses were performed using the program R v3.5.0 (R Core Team 2018).

Results WP6

Learning experiments

Bumble bees differentiated between pure pollen and pollen enriched with FAs already after the first three CS+ and three CS- trials (10x FA, Fig. 1B, Table S4), while they did not learn to differentiate between pure pollen and pollen enriched with AAs even after 20 trials (Fig. 1A, Table S4). Bumble bees were also not able to differentiate between diluted pollen and diluted pollen enriched with AAs (Fig. S2). However, pollen enriched with the 0.5x FA and 10x FA mixtures as well as with linoleic and oleic acid (0.5x and 10x) was also clearly differentiated by the bumble bees after six CS+ and six CS- trials (Fig. S3, Table S4). We further showed that bumble bees could also discriminate between pure pollen and pollen enriched with each one out of the nine FAs separately at similar levels as found for pollen enriched with all FAs (Fig. S4, Table S4).

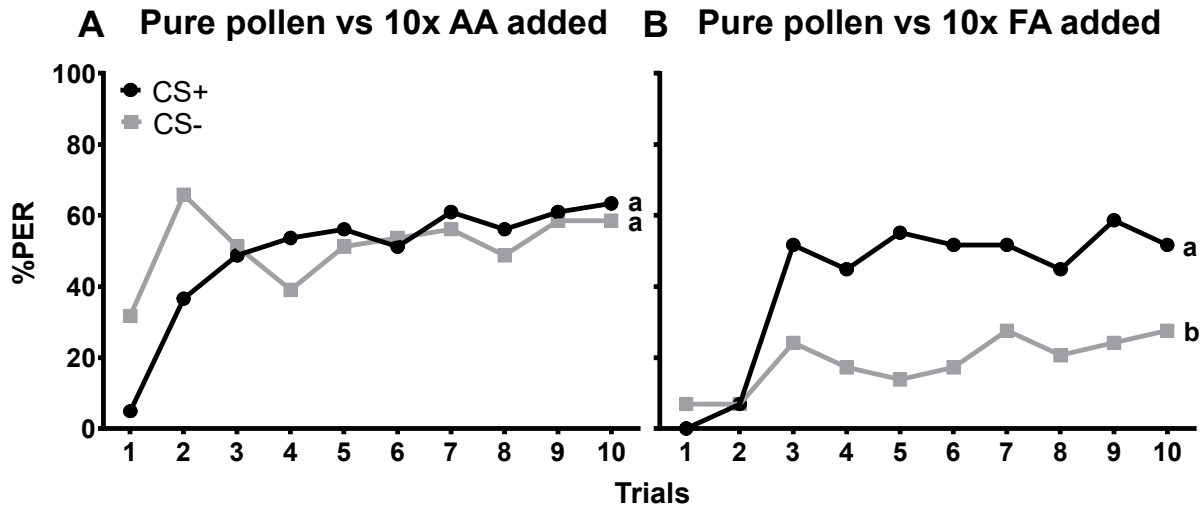


Fig. 1 Percentage of proboscis extension responses (%PER) shown by *Bombus terrestris* individuals (N = 100) in differential chemotactile conditioning to bee-collected pollen enriched with (A) 10x the natural concentration of amino acids (N = 41) and (B) 10x the natural concentration of fatty acids (N = 58). CS+ (black) represents the rewarded conditioned stimulus, CS- (grey) the unrewarded conditioned stimulus. Both stimuli were used as CS+ and CS-. As there was no significant difference in learning performance between these reversed meanings (Table S4), both groups were combined. Different letters next to each line indicate a significant difference between stimuli ($P < 0.05$).

Feeding experiments

Adding different concentrations of AAs did not affect overall pollen consumption of microcolonies, neither in the choice diet (Fig. S5, Table S5) nor in the no choice diet experiment (Fig. 2A, Table S5). Bumble bees in all treatments consumed on average between 11 mg (± 3 mg) and 17 mg (± 3 mg) per individual and day. Consequently, bumble bees consumed more AAs in the high AA no choice diet experiments (Fig. 2C, Table S6). However, this did not affect their reproduction (Table S7) or survival (Fig. 2E, Table S8).

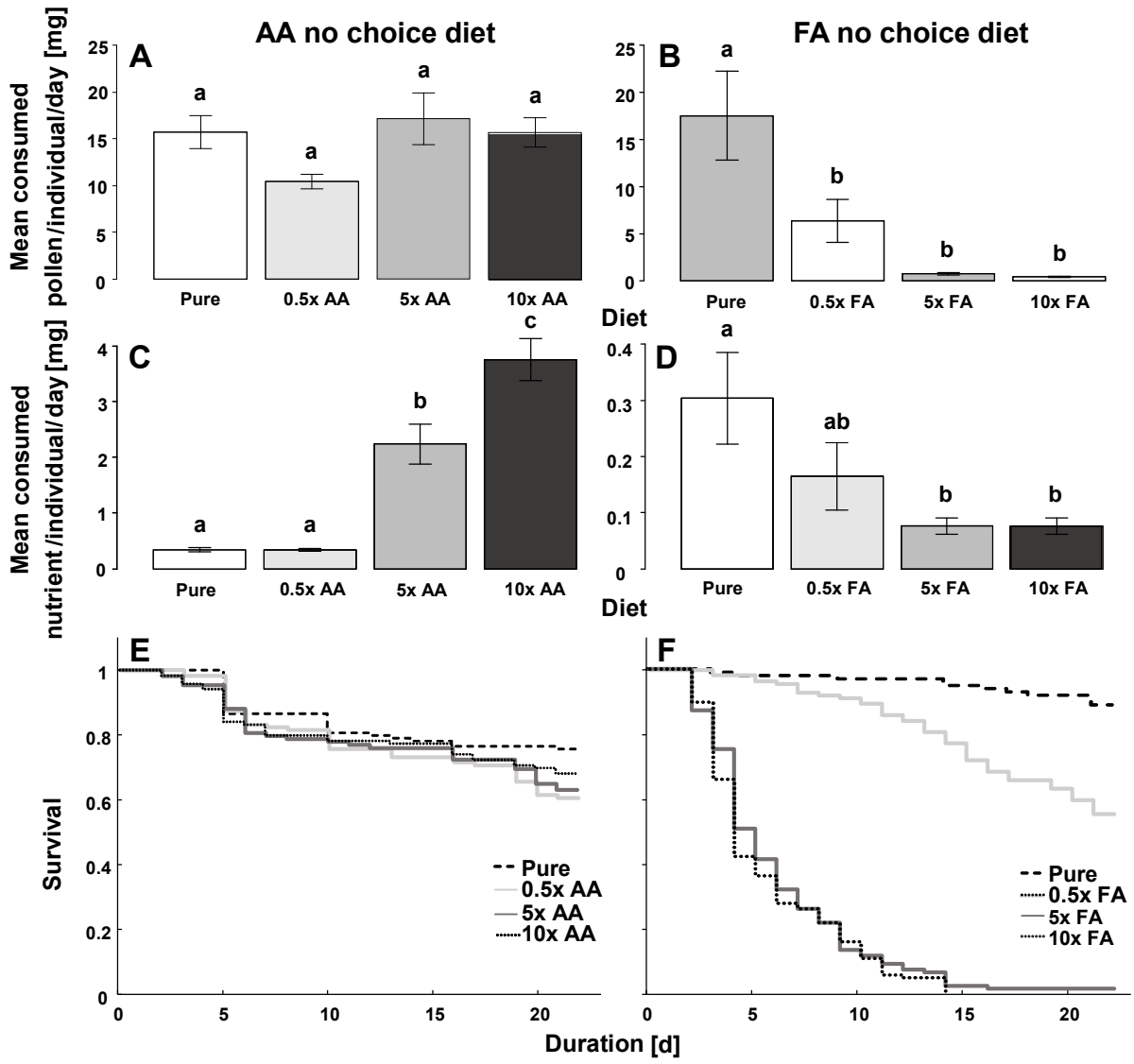


Fig. 2 Consumption of pollen and nutrients and survival of *Bombus terrestris* in six microcolonies in no choice diet experiments (N = 48). Average daily (A, B) food and (C, D) nutrient collection [µg/individual ± SD] of different pollen diets. Bees were offered pollen enriched with (A, C, E) different concentrations of amino acids (AAs) or (B, D, F) different concentrations of fatty acids (FAs). Different letters above the bars indicate significant differences ($P < 0.05$) between pollen diet/nutrient consumption according to Tukey post-hoc pairwise comparisons (see Tables S5 & S6). (E, F) Average survival probability of *Bombus terrestris* individuals. There was no difference in the survival of (E) individuals fed with different AA diets (Table S9, S10), but (F) individuals fed with 0.5x, 5x and 10x FA diets died faster compared to individuals fed pure pollen, and individuals fed with 5x and 10x FA diets died faster compared to individuals fed 0.5x FA diets (Table S8).

In contrast, bumble bees showed clear preferences for pure pollen over pollen enriched with FAs at ecologically relevant (low) concentrations in the choice diet experiment, independently of the presence of brood (Fig. 3, Table S5). While they consumed on average 18 mg (± 5 mg) of pure pollen, they only consumed on average 7 mg (± 3 mg) per individual and day of pollen enriched with FAs. Consequently, consumption of FAs decreased (to less than 2 mg (± 0.5 mg)

per individual and day) with increasing pollen FA content (Fig. 2D, Table S6). Pollen enriched with FAs also significantly reduced reproduction (Table S7) and survival (Fig. 2F, Table S8) by 80% in the high FA treatment compared to pure pollen colonies on the day the last microcolony of the high FA treatment died. Colonies in the middle and high FA treatments were only able to produce four egg clumps in total, none of which developed into larvae.

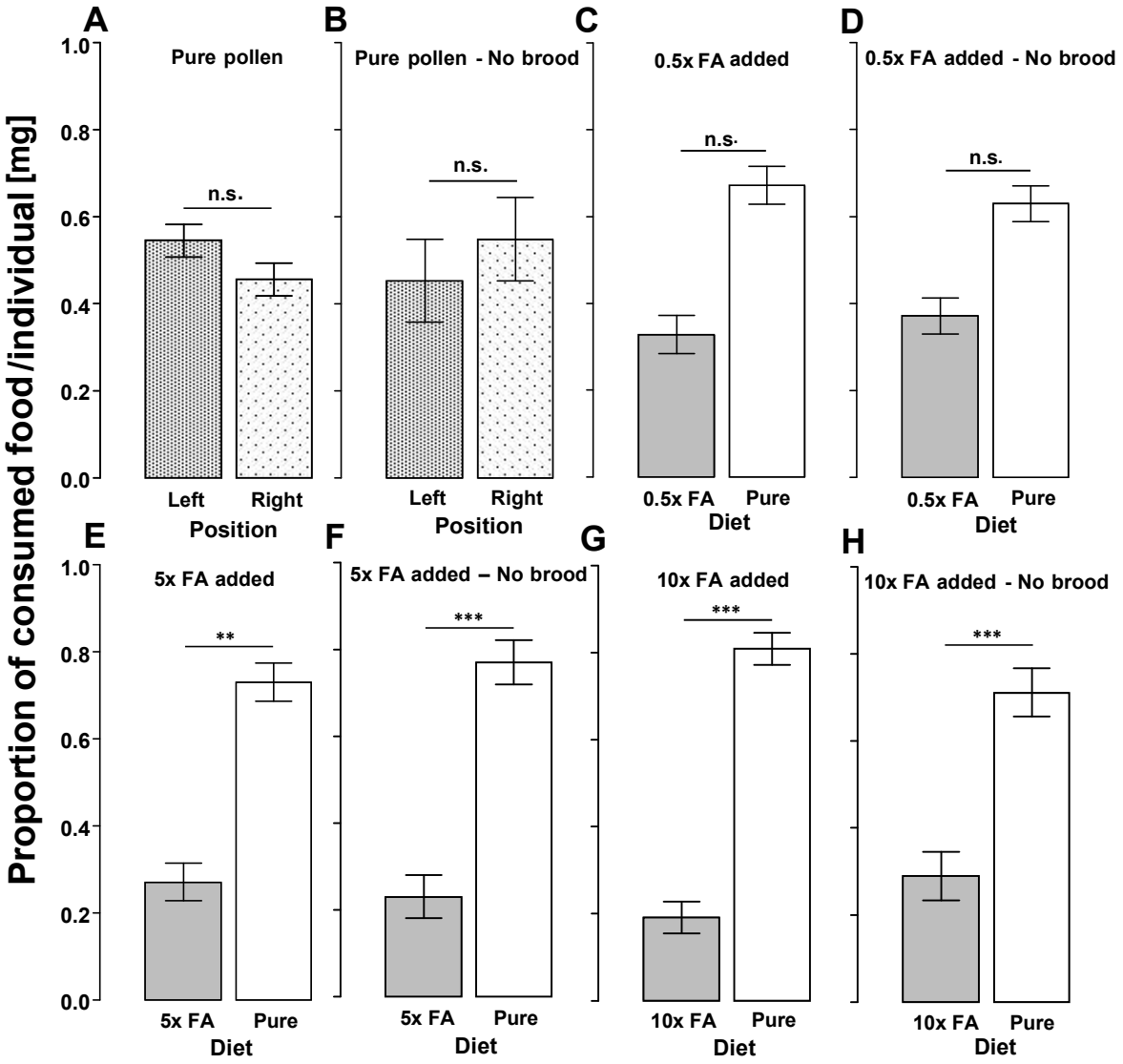


Fig. 3 Proportion of daily food collected [mean proportion food collected/individual ± standard deviation (SD)] from each of two different pollen diets offered to individuals of 12 *Bombus terrestris* microcolonies in choice diet feeding experiments (N = 96). Bees were offered a choice between (A, B) two pure pollen diets as control, (C, D) pure pollen and pollen enriched with 0.5x the natural concentration of fatty acids (FAs), (E, F) pure pollen and pollen enriched with 5x the natural FA concentrations and (G, H) pure pollen and pollen enriched with 10x the natural FA concentration. In half of the treatments, colonies were allowed to raise brood, while the in the other half of the treatments, egg clumps were removed daily (No brood). Significance levels: n.s. = not significant, ** $P < 0.01$, *** $P < 0.001$.

Discussion WP6

Our results demonstrate that *Bombus terrestris* workers focus perception on and thus learn one particular nutrient group, fatty acids (FAs), while ignoring others, e.g. amino acids (AAs), when assessing pollen nutritional quality. Moreover, while FAs are essential for bees (Annoscia et al. 2017; Arien et al. 2015), increased FA concentrations in pollen had a more detrimental effect on survival and reproductive fitness than AAs. Our results consequently suggest that, when assessing pollen nutritional quality, *B. terrestris*, and potentially also other bees, 'prioritize' perception of one particular nutritional cue, which also appears to be the nutrient with the strongest fitness consequences.

Links between nutrition and fitness, here defined as individual and microcolony survival and reproduction, have been repeatedly demonstrated in several insects, including bees (i.e. Alaux et al. 2010; Archer et al. 2014; Brodschneider and Crailsheim 2010; Keller et al. 2005; Roger et al. 2017). However, none of these studies investigated the role of nutrient perception or how perception may be linked to nutrient regulation and individual/colony fitness.

Queenright colonies and queenless microcolonies (as used in our feeding experiments) are comparable in terms of nutritional intake and reproductive behavior (Génissel et al. 2002; Tasei and Aupinel 2008). Moreover, unlike different castes in honey bees, bumble bee workers, queens and drones receive food of equal nutritional composition (Pereboom 2000), which suggest that they have the same (or at least similar) nutritional requirements and are therefore similarly affected by food of inappropriate quality (e.g. of high fat content). Fitness effects (i.e. effects on worker reproduction and survival) observed in microcolonies do consequently most likely apply to queenright colonies and to the reproduction of virgin

queens and drones (Génissel et al. 2002; Tasei and Aupinel 2008). Moreover, workers are largely responsible for provisioning the colony. If they avoid specific pollen sources in the field or die faster as a consequence of inappropriate quality (e.g. high fat content), the colony as a whole will be affected and potentially starve. We are therefore confident that the negative fitness consequences found in our experiments with microcolonies fed pollen enriched with FAs can be directly related to queenright colonies.

Similar negative effects of FAs on survival were also shown for honeybees (*Apis mellifera*) by Manning et al. (2007). In fact, high FA concentrations in food can limit the uptake rate of FAs by midgut cells (as reviewed by Canavoso et al. 2001), which can subsequently damage cell membranes (Haddad et al. 2007) and may explain why *B. terrestris* workers strongly avoided consuming pollen enriched with FAs (Fig. 2B and 2D). Consequently, the observed negative survival and fitness effect of FAs in pollen were most likely due to a combination of both intoxication with excessive FA amounts (Canavoso et al. 2001; Haddad et al. 2007; Manning et al. 2007) and a lack of sufficient other essential nutrients as a consequence of reduced overall pollen consumption (Rodriguez et al. 1993; Simpson and Raubenheimer 2012) due to fat avoidance. Both intoxication and a lack of nutrients will ultimately impact on reproduction (Human et al. 2007; Pirk et al. 2010) and reduce survival.

Our findings seem to contradict the frequently discussed importance of pollen protein and AAs for bumble bee foraging, and suggest an at least equally important role of fat/FAs. However, most previous studies only considered protein/AA content but rarely fat/FA content. It is possible that contents of both macro-nutrients are naturally correlated in pollen, e.g. due to linked biosynthesis pathways. For example, a negative correlation between protein and fat, as found by Ruedenauer et al. (2019b) for pollen collected by bees, might

enable bees to select a high P:L ratio (Vaudo et al. 2016b) through focusing on a reduced fat/FA intake.

Notably, bumble bees are capable of receiving and perceiving specific AAs and of learning differences in AA concentrations, at least when AAs are dissolved in water and not in pollen (Ruedenauer et al. 2019b). Likewise, honeybees appear to use AAs to select nectar rich in (essential) AAs, which they prefer over nectar poor in (essential) AAs (Alm et al. 1990; Hendriksma et al. 2014). Such context- or food resource-dependent cue perception suggests that bees are not only sensitive to the nutritional quality of collected food, but also adjust their sensory perception to the nutritional profile and dietary role of specific food resources. In fact, different types of food (e.g. pollen vs. nectar) appear to be subject to different nutritional quality measures (e.g. pollen quality may be mostly assessed by its fat, nectar quality by its sugar and AA content), likely because they have different dietary roles (e.g. pollen provides protein, fat and micro-nutrients, while nectar is the main sugar and thus energy source). When regulated nutrients occur in combination with other nutrients, as is e.g. the case for fat(ty acids) in pollen, perception of cues directly related to regulated nutrients and reproductive fitness seems to be 'prioritized'. Reception of other nutrients, e.g. AAs, at the receptor level may however still take place. A simple mechanism explaining this 'perceptional prioritization' would be that the FA input, as soon as present, is overlaying the AA input, either through receptive reinforcement (Abisgold and Simpson 1988; Simpson et al. 1991) or through adaptations of the received information at the brain level (Eltz and Lunau 2005), which may lead to a modification or even full extinction of "non-relevant" (nutritional) cues. Such processing would enable a specific and context dependent nutritional quality assessment, as observed in our study.

Our results are thus in contrast with our expectations and with assumptions of previous studies suggesting that bees, like many other herbivores, regulate protein intake when collecting pollen. We suggest that, instead, *Bombus terrestris* workers, and potentially also other bees, focus on fat regulation when collecting pollen and use FAs as major nutritional cue for nutritional quality assessment. Moreover, we show, for the first time in insects, that perception, nutrient regulation and fitness can be linked for a specific resource (Fig. S6). 'Prioritized perception' of nutritional cues/nutrients, which are most closely linked to fitness, may represent a most valuable, highly efficient and evolutionary beneficial strategy for foraging animals.

Supplementary Material WP6

Methods S1 Nutritional analyses of pollen blend

Amino acids

The amount and composition of amino acids (AAs) in the pollen blend used was analyzed by ion exchange chromatography (IEC: Biochrom 20 *plus* amino acid analyser) following Kriesell et al. (2017). Each sample (12.3 ± 8.4 mg) was mixed with 200 ml of 6N HCl, boiled for 4 h at 100 ◦C, cooled down to room temperature and centrifuged (10 min). The supernatant was transferred into a fresh tube. Water was evaporated at 100 ◦C before the sample was thrice re-dissolved in 200 ml fresh water and centrifuged again. Then, 100 µl were mixed with 12.5 % sulphosalicylic acid and extracted for 30 min at ~5 °C, before short mixing and centrifuging (10 min) again. Finally, 100 µL of the supernatant were mixed with 100 µL sample rarefaction buffer in a fresh microcentrifuge tube, filtered and centrifuged, and analyzed by IEC. We used an external standard (physiological calibration standard, Laborservice Onken GmbH, Gründau, Germany) for AA quantification. This standard comprises all AAs, except for

glutamine and asparagine, which quickly deteriorate and were therefore manually added prior to running standard and samples. Note that our analytical approach destroys tryptophan, which is therefore not considered in our analysis.

Fatty acids

The amount and composition of fatty acids (FAs) in the pollen blend used was analyzed in triplicate following the protocol of Brückner et al. (2017) modified as described as follows. First, FAs of 5 mg of each pollen blend were extracted in 1 ml hexane for 24 h at 60°C using a thermomixer (Eppendorf, Thermomixer Compact). Then, 20 µl of nonadecanoic acid in methanol (0.2 mg/ml, both Sigma-Aldrich, Taufkirchen, Germany) was added as internal standard. FAs were purified by fractionating extracts on 3 ml SiOH columns (Macherey Nagel, Düren, Germany), conditioned with 2 column equivalents (CE) hexane (Merck, Darmstadt, Germany) and ethylacetate (Acros Organics, Geel, Belgium) in an 80:1 ratio. Triglycerides were eluted with 5 ml hexane:ethylacetate (20:1), diglycerides were eluted with 5 ml hexane:ethylacetate (3:1) and free FAs were eluted with 5 ml of a hexane:ethylacetate:acetic acid mixture of 75:25:2. All three FA fractions were combined in one vial and filtered to remove coarse pollutants (membrane filter, Type 5 µm, Durapore membrane filters, Merck, Germany). Solvents were then removed with CO_2 and the residue was dissolved in 250 µl of dichloromethane:methanol (2:1) (Sigma Aldrich) and transferred into a 2.5 ml analytical vial. The solvent was removed with CO_2 and the residue derivatized with 20 µl of a 0.25M trimethyl sulfonium hydroxide (TMSH) solution in methanol (Sigma Aldrich). Samples were finally analyzed with a gas chromatograph coupled to a mass spectrometer (GCMS) (Agilent Technologies, 5975C inert XL MSD). We used the temperature protocol as suggested by Brückner et al. (2017).

Fig. S1 Photograph of a mounted *Bombus terrestris* worker showing a proboscis extension response (PER) after her antenna touched a copper plate with a stimulus. Photo credit: Dieter Mahsberg

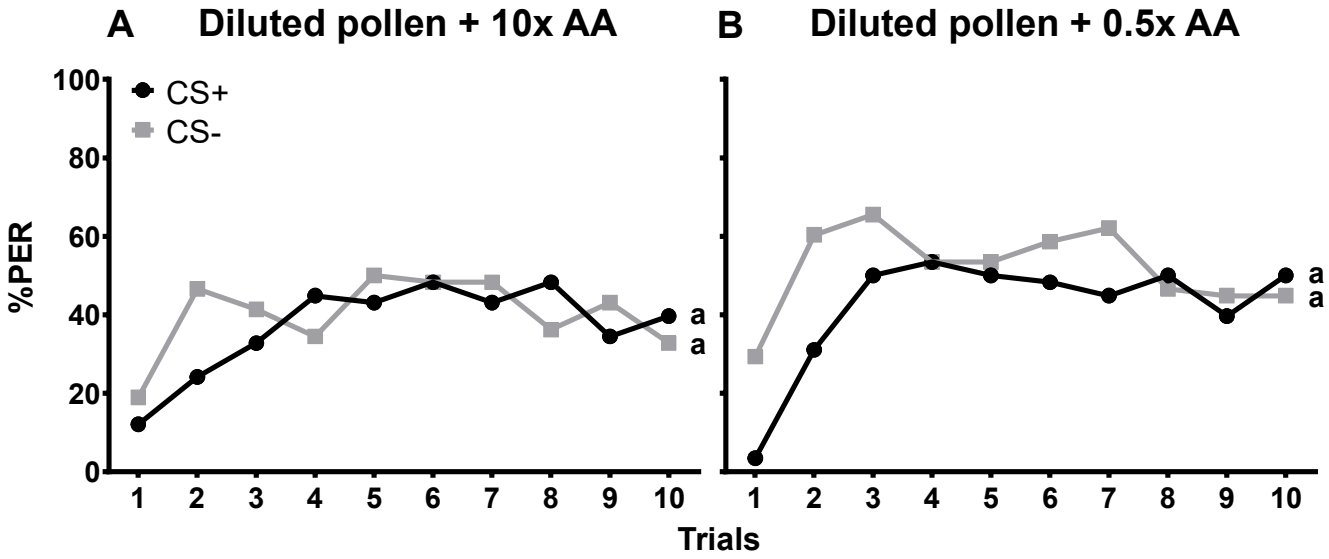


Fig. S2 Percentage of proboscis extension responses (%PER) shown by *Bombus terrestris* individuals (N = 116) in differential chemotactile conditioning to bee-collected pollen diluted 1:10 with cellulose against the same diluted pollen with (A) 10x (N = 58) or (B) 0.5x (N = 58) the natural concentrations of amino acids. CS+ (black) represents the rewarded conditioned stimulus, CS- (grey) the unrewarded conditioned stimulus. Both stimuli were used as CS+ and CS-. As there was no significant difference in learning performance between these reversed meanings (Table S4), both groups were combined. Same letters next to each line indicate no significant difference between stimuli ($P > 0.05$).

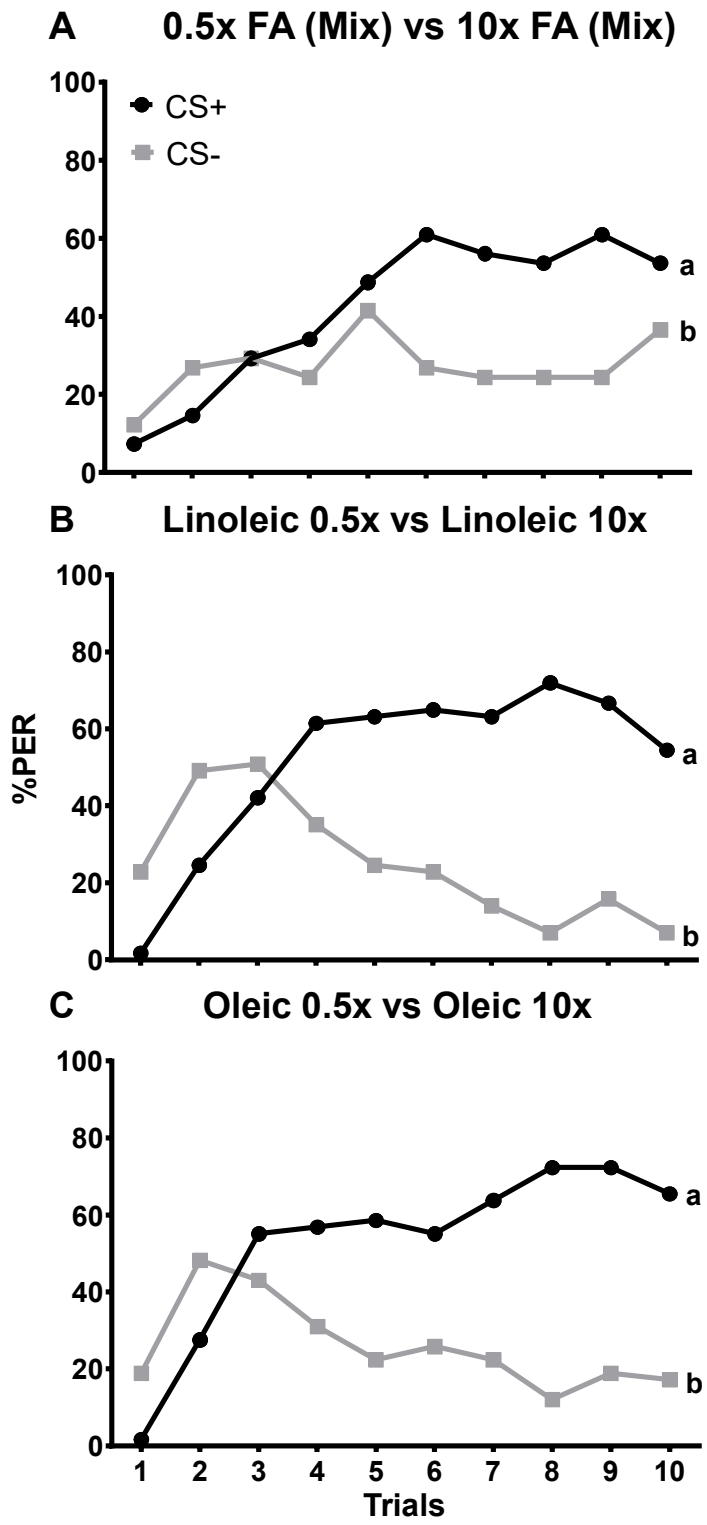


Fig. S3 Percentage of proboscis extension responses (%PER) shown by *Bombus terrestris* individuals (N = 31) in differential chemotactile conditioning to bee-collected pollen enriched with 0.5x the natural concentration of (A) the mixture of seven fatty acids, (B) linoleic acid and (C) oleic acid against the same pollen with 10x the natural concentrations of the same fatty acids. CS+ (black) represents the rewarded conditioned stimulus, CS- (grey) the unrewarded conditioned stimulus. Both stimuli were used as CS+ and CS-. As there was no significant difference in learning performance between these reversed meanings (Table S4), both groups were combined. Different letters next to each line indicate a significant difference between stimuli ($P < 0.05$).

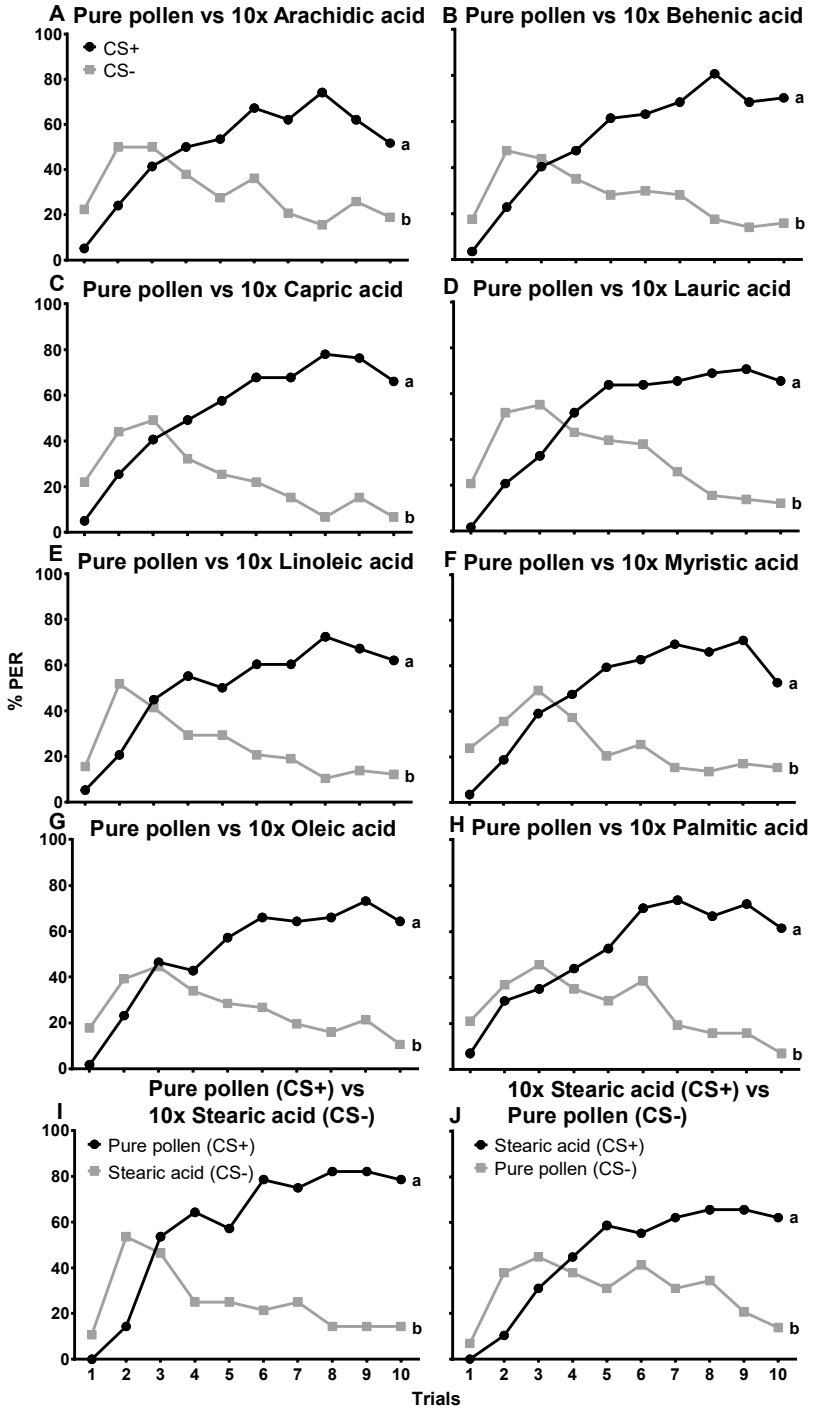


Fig. S4 Percentage of proboscis extension responses (%PER) shown by *Bombus terrestris* individuals (N = 519) in differential chemotactile conditioning to bee-collected pollen enriched with 10x the natural concentration of (A) arachidic acid (N = 58), (B) behenic acid (N = 57), (C) capric acid (N = 59), (D) lauric acid (N = 58), (E) linoleic acid (N = 58), (F) myristic acid (N = 59), (G) oleic acid (N = 56), (H) palmitic acid (N = 57), (I) stearic acid as CS- (N = 28) and (J) stearic acid as CS+ (N = 29) against the same pure pollen. CS+ (black) represents the rewarded conditioned stimulus, CS- (grey) the unrewarded conditioned stimulus. Both stimuli were used as CS+ and CS-. As there was a significant difference in learning performance between 10x stearic acid pollen and pure pollen used as CS+ or CS- (Table S4), both groups were analyzed separately. This was not the case for the other substances, where the reversed meanings of CS+ and CS- were combined. Different letters next to each line indicate a significant difference between stimuli ($P < 0.05$).

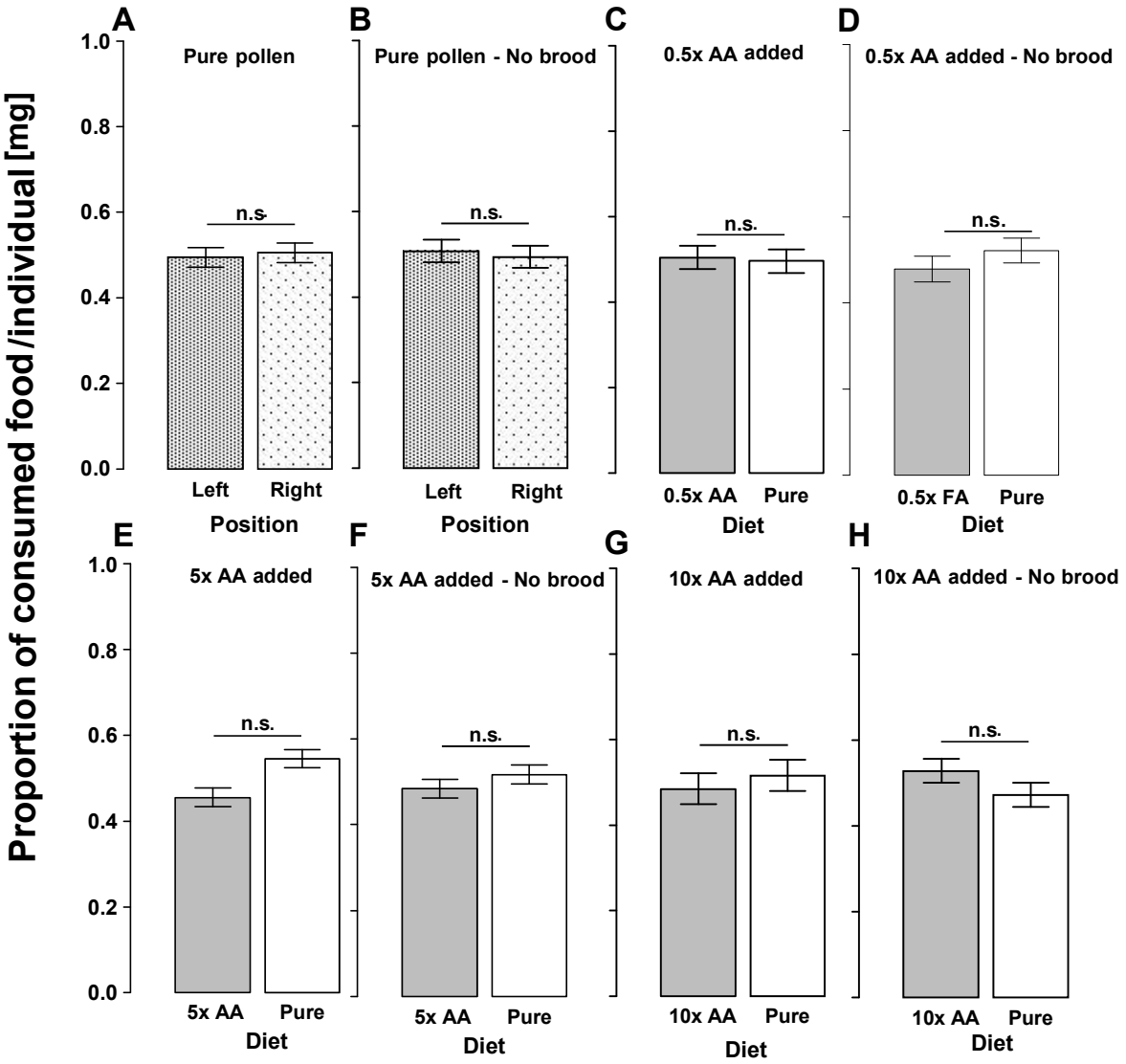


Fig. S5 Proportion of daily food collected [mean proportion food collected/individual ± standard deviation (SD)] from each of two different pollen diets offered to individuals of 18 *Bombus terrestris* microcolonies in choice diet feeding experiments (N = 144). Bees were offered a choice between (A, B) two pure pollen diets as control, (C, D) pure pollen and pollen enriched with 0.5x the natural concentration of amino acids (AAs), (E, F) pure pollen and pollen enriched with 5x the natural AA concentrations and (G, H) pure pollen and pollen enriched with 10x the natural AA concentration. In half of the treatments, colonies were allowed to raise brood, while the in the other half of the treatments, egg clumps were removed daily (No brood). Significance level: n.s. = not significant).

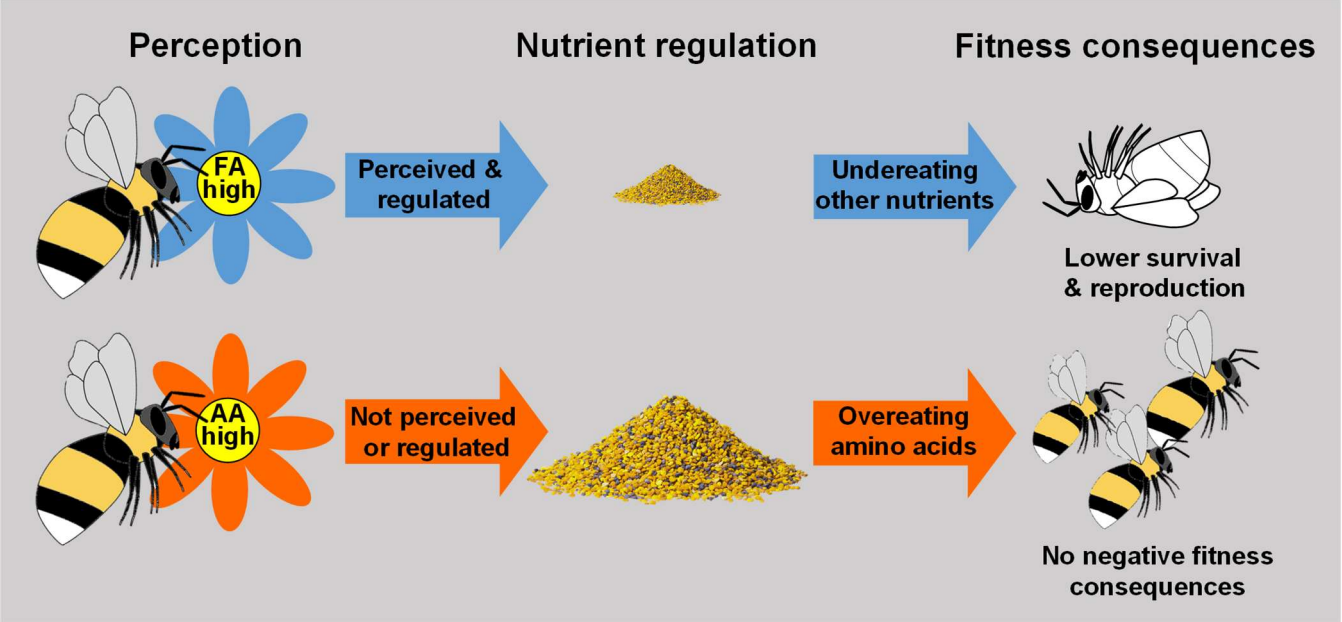


Fig. S6 Perception, nutrient regulation and fitness consequences are linked in *Bombus terrestris*. Bumble bees perceive pollen fatty acid content in pollen and regulate fat intake through reduced pollen collection. Overall less pollen is consumed, which results in undereating of other nutrients with negative fitness effects, i.e. lower survival and reproduction. In contrast, amino acid content in pollen is not perceived or regulated, with no negative fitness effects, even when amino acids are overeaten.

Tab. S1 Mean natural concentrations of the eleven amino acids used in our experiments as found in natural pollen, the pollen used in our experiment and the final concentrations of the enriched pollen diets used in our treatments (i.e. 0.5x, 5x, 10x the mean natural concentration added). Natural concentrations were taken from the dataset of 142 plant species compiled by Weiner et al. (2010) and averaged to obtain mean natural concentrations. Amino acids of the pollen used in the experiments were analyzed as described in Methods S1 and in Kriesell et al. (2017). All amino acids were purchased from Sigma-Aldrich, Taufkirchen, Germany.

Amino acid	Concentration natural [mg/g]	Concentration pollen [mg/g]	Concentration 0.5x [mg/g]	Concentration 5x [mg/g]	Concentration 10x [mg/g]
Alanine	1.353	1.153	1.830	7.918	14.683
Asparagine	0.846	0.868	1.291	5.098	9.328
Cysteine	0.036	0.002	0.020	0.182	0.362
Glutamic acid	1.037	1.359	1.878	6.544	11.729
Hydroxyproline	1.473	0.115	0.852	7.480	14.845
Leucine	0.797	0.588	0.987	4.573	8.558
Lysine	1.007	1.112	1.616	6.147	11.182
Phenylalanine	0.502	0.582	0.833	3.092	5.602
Proline	12.975	12.992	19.480	77.867	142.742
Serine	1.001	1.246	1.747	6.251	11.256
Valine	0.705	0.614	0.967	4.139	7.664

Tab. S2 Mean natural concentrations of the seven fatty acids used in our experiments as found in natural pollen, the pollen used in our experiment and the final concentrations of the enriched pollen diets used in our treatments (i.e. 0.5x, 5x, 10x the natural concentration added). Natural concentrations were taken from the dataset of 45 plant species compiled by Manning (2006) and averaged to obtain mean natural concentrations. Analysis of the fatty acids is described in Methods S1. Capric acid was purchased from Carl Roth, Karlsruhe, Germany. All other fatty acids were purchased from Sigma-Aldrich, Taufkirchen, Germany.

Fatty acid	Concentration natural [mg/g]	Concentration pollen [mg/g]	Concentration 0.5x [mg/g]	Concentration 5x [mg/g]	Concentration 10x [mg/g]
Arachidic acid	0.506	0.552	0.805	3.082	5.612
Behenic acid	0.807	0.219	0.623	4.254	8.289
Capric acid	0.611	0.583	0.889	3.638	6.693
Lauric acid	0.706	0.787	1.140	4.317	7.847
Myristic acid	2.793	2.037	3.434	16.002	29.967
Palmitic acid	9.460	8.721	13.451	56.021	103.321
Stearic acid	2.461	3.005	4.236	15.310	27.615

Tab. S3 Overview of the treatments used in the feeding experiments. Microcolonies were offered only one diet enriched with either different concentrations (0.5x, 5x or 10x natural concentration) of amino acids (AAs) or fatty acids (FAs) in no choice diet experiments, and could choose between pure and enriched pollen in choice diet experiments. *Bombus terrestris* workers were allowed to raise their brood (Y), or egg clumps were removed each day (N). No choice diet experiments were repeated two and choice diet experiments three times.

Treatment	Date	Brood	Diet	Number of microcolonies
AA no choice diet	February – March 2017 & January – February 2018	Y	Pure	6
			0.5x AA	6
			5x AA	6
			10x AA	6
		N	Pure	6
			0.5x AA	6
			5x AA	6
			10x AA	6
AA choice diet	February – March 2017, June-July 2017 & November – December 2017	Y	Pure	18
			0.5x AA	18
			5x AA	18
			10x AA	18
		N	Pure	18
			0.5x AA	18
			5x AA	18
			10x AA	18
FA no choice diet	November – December 2017 & March – April 2018	Y	Pure	6
			0.5x FA	6
			5x FA	6
			10x FA	6
		N	Pure	6
			0.5x FA	6
			5x FA	6
			10x FA	6
FA choice diet	February – March 2017, November – December 2017 & March – April 2018	Y	Pure	12
			0.5x FA	12
			5x FA	12
			10x FA	12
		N	Pure	12
			0.5x FA	12
			5x FA	12
			10x FA	12

Tab. S4 Results of the Mann-Whitney-U tests and paired Mann-Whitney-U tests testing for differences in learning performance between different pollen mixtures in learning experiments. Either pure pollen (Pure) was tested against pollen enriched with a mixture of amino acids (AAs) or fatty acids (FAs) in 10x natural concentration, pollen with 0.5x natural concentration was tested against the 10x FA pollen, pure pollen was tested against single FA added in 10x natural concentration or pollen enriched with 0.5x natural concentration of single FA was tested against pollen enriched with the same FA in 10x natural concentration. Given are the *W*- (unpaired) or *V*- values (paired) and *P*-values for comparisons between rewarded and unrewarded stimulus for each of the reversed meanings of the CS and for comparisons between CS+ and CS- in the combined datasets. Significant *P*-values and the corresponding stimuli are marked in bold. 'Paired' indicates whether a paired test was used (Y) or not (N). The "CS+ vs CS-"comparisons are reflected in the corresponding Figures (given in brackets).

Pollen	Stimuli	Paired	*W*	*V*	*P*
Pure vs 10x AA	CS+ vs CS+	N	237.5	-	0.253
	CS- vs CS-	N	151	-	0.226
	CS+ vs CS- (Fig. 2A)	Y	-	294	0.814
Diluted vs Diluted 10x AA	CS+ vs CS+	N	376	-	0.486
	CS- vs CS-	N	420.5	-	1
	CS+ vs CS- (Fig. S2A)	Y	-	491.5	0.827
Diluted vs Diluted 0.5x AA	CS+ vs CS+	N	334	-	0.180
	CS- vs CS-	N	491.5	-	0.265
	CS+ vs CS- (Fig. S2B)	Y	-	758.5	0.147
Pure vs 10x FA	CS+ vs CS+	N	113.5	-	0.618
	CS- vs CS	N	66	-	0.104
	CS+ vs CS- (Fig. 2B)	Y	-	10	**<0.001**
0.5x FA vs 10x FA	CS+ vs CS+	N	236.5	-	0.494
	CS- vs CS-	N	232.5	-	0.558
	CS+ vs CS- (Fig. S3A)	Y	-	65	**0.002**
Linoleic0.5x vs 10x Linoleic	CS+ vs CS+	N	505.5	-	0.180
	CS- vs CS-	N	402.5	-	0.788
	CS+ vs CS- (Fig. S3B)	Y	-	282.5	**<0.001**
Oleic0.5x vs 10x Oleic	CS+ vs CS+	N	451	-	0.474
	CS- vs CS-	N	334	-	0.245
	CS+ vs CS- (Fig. S3C)	Y	-	217.5	**<0.001**
Pure vs 10x Arachidic	CS+ vs CS+	N	637.5	-	0.404
	CS- vs CS-	N	571	-	0.995
	CS+ vs CS- (Fig. S4A)	Y	-	394.5	**<0.001**
Pure vs 10x Behenic	CS+ vs CS+	N	384	-	0.729
	CS- vs CS-	N	393	-	0.840
	CS+ vs CS- (Fig. S4B)	Y	-	307.5	**<0.001**
Pure vs 10x Capric	CS+ vs CS+	N	414	-	0.754
	CS- vs CS-	N	502.5	-	0.303
	CS+ vs CS- (Fig. S4C)	Y	-	163.5	**<0.001**
Pure vs 10x Lauric	CS+ vs CS+	N	443	-	0.724
	CS- vs CS-	N	409.5	-	0.875
	CS+ vs CS- (Fig. S4D)	Y	-	342	**0.002**
Pure vs 10x Linoleic	CS+ vs CS+	N	467	-	0.471
	CS- vs CS-	N	366.5	-	0.398
	CS+ vs CS- (Fig. S4E)	Y	-	282.5	**<0.001**
Pure vs 10x Myristic	CS+ vs CS+	N	400	-	0.597
	CS- vs CS-	N	472.5	-	0.568
	CS+ vs CS- (Fig. S4F)	Y	-	187.5	**<0.001**

Pure vs 10x Oleic	CS+ vs CS+	N	343.5	-	0.426
	CS- vs CS-	N	384.5	-	0.907
	CS+ vs CS- (Fig. S4G)	Y	-	351.5	**<0.001**
Pure vs 10x Palmitic	CS+ vs CS+	N	329.5	-	0.227
	CS- vs CS-	N	386.5	-	0.771
	CS+ vs CS- (Fig. S4H)	Y	-	311.5	**<0.001**
Pure vs 10x Stearic	**CS+ vs CS+**	N	532.5	-	**0.042**
	CS- vs CS-	N	443	-	0.556
	CS+ (Pure) vs CS- (Stearic) (Fig. S4I)	Y	-	60	**0.010**
	CS+ (Stearic) vs CS- (Pure) (Fig. S4J)	Y	-	12.5	**<0.001**

Tab. S5 Results of the generalized linear models (GLMs) or generalized linear mixed effect models (GLMMs) testing for differences in pollen consumption per individual in the feeding experiments. Microcolonies were offered only one diet enriched with either different concentrations (0.5x, 5x or 10x natural concentration) of amino acids (AAs) or fatty acids (FAs) in no choice diet experiments and were able to choose between pure pollen and enriched pollen in the choice diet experiments. Given are the experiment, diet(s) tested, the random factor (where necessary, requiring a GLMM) and the *F*- and *P*-values of the model. Significant *P*-values are marked in bold. Brood indicates if colonies were allowed to rear brood (Y) or egg clumps were daily removed (N). In the case of the no choice FA diet experiment, consumption differed significantly between diets. We therefore conducted a Tukey test for post-hoc comparisons, with *z*- and *P*-values given.

Experiment	Diet(s)	Brood	Random factor	*F*	*z*	*P*
AA no choice	No choice	Y	-	2.108	-	0.131
AA choice	Pure	Y	-	0.117	-	0.735
		N	-	0.024	-	0.878
	0.5x AA	Y	Experimental period	0.010	-	0.919
		N	Experimental period	0.204	-	0.654
	5x AA	Y	-	0.298	-	0.589
		N	Experimental period	0.538	-	0.469
	10x AA	Y	Experimental period	0.156	-	0.696
		N	Experimental period	0.132	-	0.719
FA no choice	No choice	Y	-	8.642	-	**<0.001**
	Pure vs 0.5x FA	Y	-	-	2.947	**0.017**
	Pure vs 5x FA	Y	-	-	4.425	**<0.001**
	Pure vs 10x FA	Y	-	-	4.513	**<0.001**
	0.5x FA vs 5x FA	Y	-	-	-1.550	0.407
	0.5x FA vs 10x FA	Y	-	-	-1.643	0.354
	5x FA vs. 10x FA	Y	-	-	-0.092	1
FA choice	Pure	Y	Mother colony	0.035	-	0.856
		N	-	0.053	-	0.822
	0.5x FA	Y	Mother colony	1.859	-	0.231
		N	Mother colony	14.716	-	**0.012**
	5x FA	Y	-	5.953	-	**0.035**
		N	-	6.362	-	**0.030**
	10x FA	Y	-	8.952	-	**0.014**
		N	-	5.606	-	**0.039**

Tab. S6 Results of the generalized linear mixed effect models (GLMMs) testing for differences in nutrient intake per individual in the feeding experiments. Microcolonies were offered only one diet enriched with either different concentrations (0.5x, 5x or 10x natural concentration) of amino acids (AAs) or fatty acids (FAs). Given are the experiment, the diets and the *F*- and *P*-values of the corresponding model. Significant *P*-values are marked in bold. A Tukey test was performed for post-hoc comparisons, with *z*- and *P*-values given.

Experiment	Diet(s)	*F*	*z*	*P*
AA no choice	No choice	33.465	-	**<0.001**
	Pure vs 0.5x AA	-	-0.004	1
	Pure vs 5x AA	-	4.690	**<0.001**
	Pure vs 10x AA	-	8.444	**<0.001**
	0.5x AA vs 5x AA	-	4.695	**<0.001**
	0.5x AA vs 10x AA	-	8.448	**<0.001**
	5x AA vs 10x AA	-	3.753	**<0.001**
FA no choice	No choice	3.759	-	**0.028**
	Pure vs 0.5x FA	-	1.793	0.277
	Pure vs 5x FA	-	2.936	**0.017**
	Pure vs 10x FA	-	2.935	**0.017**
	0.5x FA vs 5x FA	-	-1.198	0.628
	0.5x FA vs 10x FA	-	-1.197	0.629
	5x FA vs. 10x FA	-	-0.001	1

Tab. S7 Results of the generalized linear models (GLMs) or generalized linear mixed effect models (GLMMs) testing for differences in reproduction between colonies fed different diets in no choice diet feeding experiments. Given are the nutrient (amino acids (AAs) or fatty acids (FAs)) added, the brood parameter analyzed, the diet fed and the random factor included in GLMMs (where necessary) as well as the *F*- and *P*-values of the corresponding models. Significant *P*-values and are marked in bold. A significant difference between diets was found only for the no choice diet FA treatments. Tukey post-hoc comparisons were subsequently performed, with *P*-values given.

Added nutrient	Brood parameter	Diets	Random factor	*F*	*P*
AA	Egg clumps	-	-	0.796	0.497
	Larval cells	-	-	0.598	0.617
	Pupae	-	-	0.434	0.729
FA	Egg clumps	-	Mother colony	11.961	**<0.001**
		Pure vs 0.5x FA	-	-	**0.007**
		Pure vs 5x FA	-	-	**<0.001**
		Pure vs 10x FA	-	-	**<0.001**
		0.5x FA vs 5x FA	-	-	**0.048**
		0.5x FA vs 10x FA	-	-	0.069
		5x FA vs 10x FA	-	-	1
	Larval cells	-	-	5.919	**<0.001**
		Pure vs 0.5x FA	-	-	**0.010**
		Pure vs 5x FA	-	-	**0.001**
		Pure vs 10x FA	-	-	0.201
		0.5x FA vs 5x FA	-	-	0.518
		0.5x FA vs 10x FA	-	-	0.901
		5x FA vs 10x FA	-	-	1
	Pupae	-	-	3.244	0.051

Tab. S8 Results of log-rank tests on differences in survival probability of bumble bees in no choice diet feeding experiments. Given are the added nutrient (amino acids (AAs) or fatty acids (FAs)), diet comparison, χ^2- and *P*-values for each diet. For the no choice diet experiment with FAs, there was a significant difference between all diets. We therefore tested each diet pair separately and corrected for multiple testing with Bonferroni. The new significance level after Bonferroni correction was $P = 0.008$. Significant *P*-values and diet combinations are marked in bold.

Added nutrient	Diets	χ^2	*P*
AA	All	6.1	0.108
FA	**All**	435	**<0.001**
	Pure vs 0.5x FA	29.2	**<0.001**
	Pure vs 5x FA	216	**<0.001**
	Pure vs 10x FA	231	**<0.001**
	0.5x FA vs 5x FA	188	**<0.001**
	0.5x FA vs 10x FA	213	**<0.001**
	5x FA vs 10x FA	0.8	0.367

Tab. S9 Results of model comparisons performed to determine whether or not the "experimental period" and/or "mother colony" had to be used as random factor in the statistical models testing for differences in consumption per individual in the different feeding experiments. Given are the likelihood ratio and the *P*-value for the comparison of two models, one with and the other without the random factor. Significant *P*-values and the corresponding random factor are marked in bold. For the choice experiments, the concentrations (0.5x, 5x or 10x natural concentration added) of amino acids (AAs) or fatty acids (FAs) added to pollen are indicated. Brood indicates whether colonies were allowed to rear brood (Y) or egg clumps were daily removed (N).

Experiment	Diet	Brood	Random factor	Likelihood ratio	*P*
AA no choice diet	No choice	Y	Experimental period	$3.718e^{-8}$	0.998
			Mother colony	$3.901e^{-8}$	1
AA choice diet	Pure	Y	Experimental period	2.619	0.106
			Mother colony	2.042	0.153
		N	Experimental period	1.387	0.239
			Mother colony	0.142	0.706
	0.5x AA	Y	**Experimental period**	6.978	**0.008**
			Mother colony	2.258	0.133
		N	**Experimental period**	7.925	**0.005**
			Mother colony	$6.988e^{-8}$	1
	5x AA	Y	Experimental period	0.057	0.811
			Mother colony	0.310	0.578
		N	Experimental period	8.399	0.004
			Mother colony	$6.305e^{-8}$	1
	10x AA	Y	**Experimental period**	13.111	**< 0.001**
			Mother colony	0.0345	0.853
		N	**Experimental period**	4.122	**0.042**
			Mother colony	1.200	0.273
FA no choice diet	No choice	Y	Experimental period	$3.786e^{-8}$	1
			Mother colony	$5.684e^{-14}$	1
FA choice diet	Pure	Y	Experimental period	0.254	0.615
			Mother colony	5.212	**0.022**
		N	Experimental period	$1.831e^{-8}$	1
			Mother colony	$1.572e^{-8}$	1
	0.5x FA	Y	Experimental period	$2.539e^{-6}$	0.999
			Mother colony	0.784	0.376
		N	Experimental period	$4.401e^{-8}$	1
			Mother colony	0.261	0.610
	5x FA	Y	Experimental period	$2.754e^{-8}$	1
			Mother colony	2.563	0.109
		N	Experimental period	0.456	0.499
			Mother colony	1.665	0.197
	10x FA	Y	Experimental period	0.306	0.580
			Mother colony	$2.576e^{-8}$	1
		N	Experimental period	0.183	0.669
			Mother colony	0.008	0.927

Tab. S10 Results of model comparisons performed to determine whether or not the "experimental period" and/or "mother colony" had to be used as random factor in the statistical models testing for differences in reproduction in the different no choice feeding experiments with amino acids (AAs) and fatty acids (FAs). Given are the likelihood ratio and the *P*-value for the comparison of two models, one with and the other without the random factor. Significant *P*-values and the corresponding random factor are marked in bold. Brood type indicates which developmental stage was tested.

Experiment	Brood type	Random factor	Likelihood ratio	*P*
AA no choice diet	Egg clumps	Experimental period	$3.847e^{-8}$	1
		Mother colony	$4.471e^{-7}$	1
	Larval cells	Experimental period	$1.881e^{-7}$	0.999
		Mother colony	$2.887e^{-7}$	1
	Pupae	Experimental period	$4.874e^{-7}$	1
		Mother colony	$1.437e^{-7}$	1
FA no choice diet	Egg clumps	Experimental period	0.093	0.760
		Mother colony	4.091	**0.043**
	Larval cells	Experimental period	0.004	0.948
		Mother colony	3.107	0.078
	Pupae	Experimental period	$1.790e^{-8}$	1
		Mother colony	$1.769e^{-8}$	1

Part III

Synopsis

Overview

The studies presented in Chapter I (WP1 and WP2) have analyzed the nutrient content of pollen to show that crude protein may be the only nutrient influenced by the relatedness between plant species. Additionally, protein is, together with its related nutrients polypeptides and free amino acids, influenced by the plants' pollinator dependence. Many correlations between and within the nutrient groups were found.

Chapter II has shown that honeybees are able to differentiate between pollen of different plant species (WP3) and that bumblebees, for which this ability has been shown before (Ruedenauer et al. 2015), prefer one of the pollen mixes (WP4), possibly due to its low fat content. In addition, they have shown a preference for pollen they were fed as larvae, hinting to bumblebees retaining their larval memory.

The studies in Chapter III have looked into bumblebees' abilities to perceive nutrients. They have shown that bumblebees are principally able to perceive amino acids (WP5), but do not seem to do so in pollen (WP6). Instead, fat seems to be the key nutrient in pollen nutrient assessment. This became clear in the feeding experiments, in which bumblebees regulated their fat consumption so far that they suffered decreased fitness due to undereating other nutrients.

From plant investment to fitness consequences for pollinators

This thesis is covering the nutrient cycle from what plants invest into their pollen, how their pollinators receive and perceive this pollen and how this influences their foraging choices to whether this has fitness consequences for these pollinators (Figure D.1). While plants usually would try to minimize their nutrient investment in pollen to the lowest level needed for their own reproduction, many of the flowering plants may be forced by their dependence on

pollinators to invest more. The abilities to receive and perceive certain nutrients to prevent negative fitness consequences could have enabled the pollinators to forage selectively on pollen suiting their nutritional needs and therefore shape the pollen nutritional profile. As bumblebees seem to mostly regulate their fat intake, possibly due to the negative impact of taking in too much fat (WP6), especially the various correlations between fat and most of the other nutrients (WP1 & 2) could help them to co-regulate their complete nutrient intake. The absence of the correlation between total fat and sterol content (WP2) on the other hand could mean that either sterol intake is regulated separately or only some of the sterols are important.

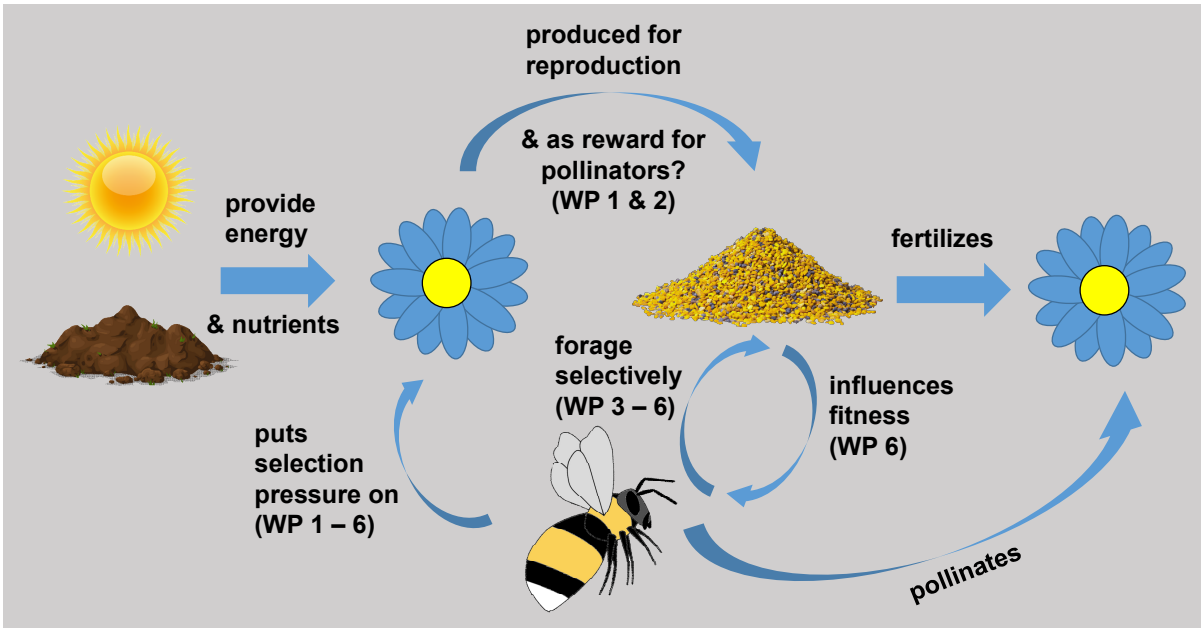


Figure D.1 Relationships between plants, pollen and pollinators and their coverage in the WPs. Sun and soil provide energy (for nutrient synthesis) and nutrients for plants. These are used to produce pollen, containing nutrients needed for reproduction. Whether plants also adjust their pollen nutrient profile to make pollen act as a reward for pollinators was discussed in WP1 & 2. Pollinators, like bees, then forage selectively on pollen. How these foraging choices are influenced by pollen type, preimaginal memory and pollen nutrient profiles was investigated throughout WP3 – 6. The pollinators then pollinate other plants, where the pollen they apply to the stigma fertilizes the plant. The pollen pollinators consume themselves (or feed to their offspring) influences the pollinators' fitness, which was investigated in WP6. All of these interactions most likely pressure the plants to produce pollen with a nutritional profile fitting their own reproduction as well as the pollinator needs.

Evolution of insect pollination

In this thesis, it was shown that pollinators very likely shape the nutrient content of plants that depend on them (WP1 & 2). This is probably possible due to the strong connection between insect pollinators and flowering plants (angiosperms) right from the beginning of the development of angiosperms during the late Cretaceous (99.6 to 65.5 mya, Proctor et al. 1996). Insect pollination may very likely even be older (possibly 300 mya) than angiosperms themselves (Crepet 1979; Ren et al. 2009). The mutualistic relationship between these two partners could have started by insects feeding on the pollination drop of early plants, which probably evolved into nectar as a reward for pollination later on (Nepi et al. 2009). While it is unclear, when insects started feeding on pollen as well, considering the long time (more than 200 myr) in between, it is likely that this has already happened before the start of the development of angiosperms. Hence, insects would have applied selection pressure to the nutrient profile of flowering plants depending on insect pollination right from the start of their evolutionary history.

Insect nutrient reception

The basis for this evolutionary theory and the nutrient assessment of insect pollinators investigated in this thesis (WP 3 – 6) is, however, that they have the appropriate receptors, enabling them to perceive nutrients. As already mentioned in the introduction, taste receptors in insects can be found in different locations on the insect body and are connected to tactile reception (de Brito Sanchez 2011). In general, they seem to be much less specialized than olfactory receptors (Harrison et al. 2012). Not much is known about which nutrient receptors insects possess. There are different types of receptors or receptor-like structures that can receive chemotactile stimuli in insects: classical gustatory receptors (GRs, as

reviewed by Hallem et al. 2006), ionotropic receptors (IRs, Croset et al. 2010; Lee et al. 2017; Rytz et al. 2013) and receptor-like proteins.

Sugar receptors (SRs) in insects seem to be only consisting of gustatory receptors (Slone et al. 2007). While most *Drosophila* species and the mosquito *Anopheles gambiae* have eight SRs and some other insects like the beetle *Tribolium castaneum* even up to 16 SRs responding to several different sugars (e.g. sucrose, trehalose, glucose…), only two were identified for the parasitoid jewel wasp *Nasonia vitripennis* (Kent and Robertson 2009), the honeybee *Apis mellifera* (Robertson and Wanner 2006), and the bumblebee *Bombus terrestris* (Sadd et al. 2015). In general, 23 GR genes have been identified in *B. terrestris* (Sadd et al. 2015), while only ten GR genes have been identified for *A. mellifera*, leading to a discussion about an impoverished gustatory perception of the honeybee (Robertson and Wanner 2006). However, besides alternative splicing, leading to more than one receptor type per GR gene and the fact that one receptor type can receive a broader variety of substances (de Brito Sanchez 2011), receptors can either work on their own, or together as co-receptors or heterodimers. This later has been shown for the honeybee sugar receptors, where one of the two receptors (AmGR2) is not responsive to sugars itself, but increases the variability of sugars the other receptor (AmGR1) is able to receive (Jung et al. 2015). In addition to that, the other two types of chemotactile receptors (IRs and receptor-like proteins) could help to enrich the gustatory repertoire further.

Ionotropic receptors (IRs) in insects seem to be responsible for amino acid reception (Croset et al. 2010; Croset et al. 2016; Rytz et al. 2013). IR76b, acting as a salt receptor when activated on its own, has been found to be involved as co-receptor of other IRs for amino acids in *Drosophila melanogaster* (Ganguly et al. 2017; Rytz et al. 2013). Its function as salt receptor

is blocked when co-activated with IR20a, although this is not enough for both receptors to function as the only amino acid receptors (Ganguly et al. 2017). Hence, more co-receptors are likely involved (Ganguly et al. 2017). IR76b is highly conserved among insects (Croset et al. 2010) and has been shown to have the same functions in *A. gambiae* (Ganguly et al. 2017). It is also present in bees (*A. mellifera*: Croset et al. 2010; *B. terrestris*: Sadd et al. 2015) and therefore very likely also acts as amino acid receptor (WP5).

While in mammals a receptor-like protein seems to be responsible for fat reception (Keller et al. 2012; Laugerette et al. 2005), in *Drosophila* a gustatory receptor (DmGR64e), usually acting as a sugar receptor, is working together with the phospholipase C pathway for fat sensing (Kim et al. 2018; Masek and Keene 2016). Therefore, the sugar receptors GR1 & 2 of *A. mellifera* and *B. terrestris*, which are closely related to DmGR64e (Sadd et al. 2015), could act in a similar manner (WP6). Additionally, IR76b and other IRs also seem to play a role in fatty acid perception in *Drosophila* (Ahn et al. 2017) and therefore likely in other insects as well.

The impact of humans on the nutrient assessment system

Even though at least some pollinators seem to be able to assess the quality of their food, potentially helping them to increase their fitness, they are still in decline in recent years. The reasons for this current pollinator decline are likely caused by humans (Biesmeijer et al. 2006; Potts et al. 2010; Vanbergen and the Insect Pollinators Initiative 2013). The monocultures and use of herbicides in today's agriculture, as well as the consequences of man-made climate change (e.g. draughts) can cause a lower flower diversity (Biesmeijer et al. 2006; Persson and Smith 2013). Additionally, it has been shown that the bees may also even prefer pollen containing neonicotinoids (Kessler et al. 2015), a group of insecticides, some of them labelled as bee-friendly, but still toxic to them in higher amounts. Furthermore, as insecticides usually

act as neurotoxins (Costa et al. 2008) they could damage the receptor system of insects. Another impact of humans is the introduction of alien species, not only leading to the co-introduction of new diseases and parasites for pollinators as well as their plants but possibly also to higher competition (Potts et al. 2010; Stout and Morales 2009). This could undermine the region-specific co-evolution of plants and their pollinators (WP1 & 2). All of these impacts could force pollinators to forage outside of their preferred nutritional range, causing negative fitness consequences (as seen in WP6) and therefore adding indirect negative effects to the impacts' direct effects.

Amino acid vs. fatty acid metabolism

As already shortly discussed in Discussion WP6, the reason why bumblebees did try to avoid overeating fatty acids could be their inability to cope with excess amounts of them compared to amino acids. Excess amounts of amino acids can simply be deaminated by insects producing carbohydrates and ammonium, which then can be excreted (Harrison et al. 2012). Fatty acids in insects, however, usually are converted into *sn*-1,2-diacylglycerol (DAG), which then is converted into triacylglycerol (TAG), serving as the fatty acid reservoir (Canavoso et al. 2001). Besides the fast adsorption rates in the insect midgut and the high conversion rate into TAG, overeating fatty acids could still accumulate DAG and free fatty acids in the haemolymph, which both can be toxic at high concentrations (Canavoso et al. 2001). Therefore, the level at which overeating fatty acids gets detrimental could be much lower than the level at which overeating amino acids does.

Outlook

While the work of this thesis contributed to a better understanding of the interaction between pollinators (especially bees) and plants as well as the sensory mechanisms underlying their foraging decisions, many questions remain unsolved.

For example, the importance of other plant traits (e.g. color, corolla shape, odor…) compared to pollen nutrient assessment was not integrated into the studies of this thesis. These traits most likely interact with nutrient assessment, mostly acting as attractants for the insects from further away and just like the pollen nutritional profile, very likely co-evolved with their pollinators (Schiestl and Johnson 2013). The question whether one or several of these other traits or nutrient assessment are more important or whether they are tuned to fit each other would be a next step in understanding the relationship between plant and pollinator. I would assume that the other traits first attract the pollinator, which then decides whether to collect the pollen.

Additionally, non-nutrient compounds of pollen, like phytochemicals (Palmer-Young et al. 2019a; Palmer-Young et al. 2017b) could also influence foraging choices, since they were not only shown to be potentially toxic, but also, in the right amounts, can have antibacterial and antifungal properties as well as act against parasites (Palmer-Young et al. 2017a; Palmer-Young et al. 2016). Therefore, they could also interact with nutrient assessment, especially in infected bee colonies.

Another unanswered question is whether all of the results of this thesis are similar in other bee species. As there were already differences in the PER responses for pollen between honeybees and bumblebees (honeybees extended their proboscis after being presented with pollen odor, WP3) more differences between the two species could be possible. Hence,

looking into whether honeybees can perceive the same nutrients as bumblebees could be interesting. Additionally, for both species, sterols could be another candidate nutrient group they could be able to perceive and which could even play a special role, since bees are not able to synthesize them themselves (Hobson 1935). Moreover, especially for solitary bees, with a different lifestyle compared to the social bee species used in the experiments in this thesis, mechanisms could be substantially different. I expect solitary bees to be even more precise in assessing pollen quality, since they only provide their larvae with food once. However, attempts to perform PER experiments with solitary bees, like *Osmia* species, were not yet successful (Vorel and Pitts-Singer 2010). Therefore, developing methods for learning experiments with solitary bees would be a valuable tool for behavioral scientists.

As seen in humans, which eat more sugar and fat than they should, food preferences do not always meet the real nutritional requirements. Hence, applying a mixture of stoichiometric (Filipiak et al. 2017; Filipiak and Weiner 2017) and genetic (Paoloni-Giacobino et al. 2003) analyses could help to find the nutritional requirements for bees, which would help to specify future studies on bee nutrition.

While the studies revealed that bumblebees are able to perceive certain nutrients (WP5 & 6), the according receptors remain undetected. Molecular tools could help to find the ligands to receptors of bees as well, with the help of what is known for the *Drosophila* model. After receptor identification the neuronal pathway to the brain and perception centers could be visualized, receptor mutants could be used for behavioral experiments and the influence of pesticides on nutrient reception and perception could be further investigated. Hence, the discovery of the receptors could prove quite important for future research.

Bibliography

Abisgold JD, Simpson SJ (1988) The effect of dietary protein levels and haemolymph composition on the sensitivity of the maxillary palp chemoreceptors of locusts. J. Exp. Biol. 135:215-229

Ackerman JD (2000) Abiotic pollen and pollination: Ecological, functional, and evolutionary perspectives. Plant Syst. Evol. 222:167-185. doi: 10.1007/bf00984101

Addessi E, Galloway AT, Birch L, Visalberghi E (2004) Taste perception and food choices in capuchin monkeys and human children. Primatologie 6:101-128

Agarwala SC, Chatterjee C, Sharma PN, Sharma CP, Nautiyal N (1979) Pollen development in maize plants subjected to molybdenum deficiency. Canadian Journal of Botany 57:1946-1950. doi: 10.1139/b79-244

Ahn J-E, Chen Y, Amrein H (2017) Molecular basis of fatty acid taste in *Drosophila*. eLife 6:e30115. doi: 10.7554/eLife.30115

Alaux C, Ducloz F, Crauser D, Le Conte Y (2010) Diet effects on honeybee immunocompetence. Biology Letters 6:562-565. doi: 10.1098/rsbl.2009.0986

Albert PJ, Parisella S (1988) Feeding preferences of eastern spruce budworm larvae in two-choice tests with extracts of mature foliage and with pure amino acids. J Chem Ecol 14:1649-1656. doi: 10.1007/bf01014549

Alm J, Ohnmeiss TE, Lanza J, Vriesenga L (1990) Preference of cabbage white butterflies and honey bees for nectar that contains amino acids. Oecologia 84:53-57. doi: 10.1007/bf00665594

Almeida-Muradian LB, Pamplona LC, Coimbra Sl, Barth OM (2005) Chemical composition and botanical evaluation of dried bee pollen pellets. Journal of Food Composition and Analysis 18:105-111. doi: 10.1016/j.jfca.2003.10.008

Altaye SZ, Pirk CWW, Crewe RM, Nicolson SW (2010) Convergence of carbohydrate-biased intake targets in caged worker honeybees fed different protein sources. J. Exp. Biol. 213:3311-3318. doi: 10.1242/jeb.046953

Amrein H, Thorne N (2005) Gustatory perception and behavior in *Drosophila melanogaster*. Curr. Biol. 15:R673-R684. doi: 10.1016/j.cub.2005.08.021

Andrikopoulos NK, Siafaka-Kapadai A, Demopoulos CA, Kapoulas VM (1985) Lipids of *Pinus halepensis* pollen. Phytochemistry 24:2953-2957. doi: 10.1016/0031-9422(85)80034-0

Annoscia D et al. (2017) Elucidating the mechanisms underlying the beneficial health effects of dietary pollen on honey bees (*Apis mellifera*) infested by *Varroa* mite ectoparasites. Sci Rep 7:6258. doi: 10.1038/s41598-017-06488-2

Archer CR, Pirk CWW, Wright GA, Nicolson SW (2014) Nutrition affects survival in African honeybees exposed to interacting stressors. Funct. Ecol. 28:913-923. doi: 10.1111/1365-2435.12226

Arganda S et al. (2017) Parsing the life-shortening effects of dietary protein: effects of individual amino acids. Proc Roy Soc B-Biol Sci 284. doi: 10.1098/rspb.2016.2052

Arien Y, Dag A, Shafir S (2018) Omega-6:3 ratio more than absolute lipid level in diet affects associative learning in honey bees. Frontiers in Psychology 9. doi: 10.3389/fpsyg.2018.01001

Arien Y, Dag A, Zarchin S, Masci T, Shafir S (2015) Omega-3 deficiency impairs honey bee learning. PNAS 112:15761-15766. doi: 10.1073/pnas.1517375112

Auclair JL, Jamieson CA (1948) A qualitative analysis of amino acids in pollen collected by bees. Science 108:357-358. doi: 10.1126/science.108.2805.357

Baidya DK, Sasaki M, Matsuka M (1993) Effect of pollen-substitute feeding site on brood rearing in honeybee colonies. Appl. Entomol. Zoolog. 28:590-592

Baker H, G. (1977) Non-sugar chemical constituents of nectar. Apidologie 8:349-356

Baker HG, Baker I (1979) Starch in angiosperm pollen grains and its evolutionary significance. American Journal of Botany 66:591-600

Barbosa P, Letourneau DK (1988) Novel aspects of insect-plant interactions. John Wiley & Sons, New York

Barron AB, Corbet SA (1999) Preimaginal conditioning in *Drosophila* revisited. Anim. Behav. 58:621-628. doi: 10.1006/anbe.1999.1169

Bartoshuk LM, Duffy VB, Hayes JE, Moskowitz HR, Snyder DJ (2006) Psychophysics of sweet and fat perception in obesity: problems, solutions and new perspectives. Philosophical Transactions of the Royal Society B: Biological Sciences 361:1137-1148. doi: 10.1098/rstb.2006.1853

Bates D, Mächler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. Journal of Statistical Software 67:1-48

Bazazi S, Arganda S, Moreau M, Jeanson R, Dussutour A (2016) Responses to nutritional challenges in ant colonies. Anim. Behav. 111:235-249. doi: 10.1016/j.anbehav.2015.10.021

Behmer ST (2009a) Animal behaviour: feeding the superorganism. Curr. Biol. 19:R366-R368. doi: 10.1016/j.cub.2009.03.033

Behmer ST (2009b) Insect herbivore nutrient regulation. Annu. Rev. Entomol. 54:165-187. doi: 10.1146/annurev.ento.54.110807.090537

Belanger CF, Hennekens CH, Rosner B, Speizer FE (1978) The nurses' health study. AJN The American Journal of Nursing 78:1039-1040

Bell AE, Perera CKPW, Nunn PB, Simmonds MSJ, Blaney WM (1996) Non-protein amino acids of *Lathyrus latifolius* as feeding deterrents and phagostimulants in *Spodoptera littoralis*. Phytochemistry 43:1003-1007. doi: 10.1016/S0031-9422(96)00425-6

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal statistical society: series B (Methodological) 57:289-300

Benton R, Vannice KS, Gomez-Diaz C, Vosshall LB (2009) Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. Cell 136:149-162. doi: 10.1016/j.cell.2008.12.001

Berenbaum M et al. (1986) Insect-plant interactions. Springer Science & Business Media, New York

Bernardello G, Galetto L, Forcone A (1999) Floral nectar chemical composition of some species from Patagonia. II. Biochemical Systematics and Ecology 27:779-790. doi: 10.1016/S0305-1978(99)00029-0

Bernays EA (1989) Insect-plant interactions. CRC Press, Boca Raton

Beutler R (1953) Nectar. Bee World 34:106-116. doi: 10.1080/0005772X.1953.11094798

Bicker G (1991) Taurine-like immunoreactivity in photoreceptor cells and mushroom bodies: a comparison of the chemical architecture of insect nervous systems. Brain Research 560:201-206. doi: 10.1016/0006-8993(91)91233-Q

Biesalski HK (2017) Unsere Ernährungsbiografie: Wer sie kennt, lebt gesünder. Albrecht Knaus Verlag, München

Biesmeijer JC et al. (2006) Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science 313:351-354. doi: 10.1126/science.1127863

Bitterman ME, Menzel R, Fietz A, Schäfer S (1983) Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). Journal of comparative psychology 97:107-119

Blackiston DJ, Silva Casey E, Weiss MR (2008) Retention of memory through metamorphosis: can a moth remember what it learned as a caterpillar? PLOS ONE 3:e1736. doi: 10.1371/journal.pone.0001736

Bray GA, Smith SR, de Jonge L, et al. (2012) Effect of dietary protein content on weight gain, energy expenditure, and body composition during overeating: A randomized controlled trial. JAMA 307:47-55. doi: 10.1001/jama.2011.1918

Brodschneider R, Crailsheim K (2010) Nutrition and health in honey bees. Apidologie 41:278-294. doi: 10.1051/apido/2010012

Brückner A, Hilpert A, Heethoff M (2017) Biomarker function and nutritional stoichiometry of neutral lipid fatty acids and amino acids in oribatid mites. Soil Biol Biochem 115:35-43. doi: 10.1016/j.soilbio.2017.07.020

Brunner FS, Schmid-Hempel P, Barribeau SM (2014) Protein-poor diet reduces host-specific immune gene expression in *Bombus terrestris*. Proceedings of the Royal Society B: Biological Sciences 281

Buchsbaum R, Valiela I, Swain T (1984) The role of phenolic compounds and other plant constituents in feeding by Canada geese in a coastal marsh. Oecologia 63:343-349. doi: 10.1007/bf00390663

Camazine S, Sneyd J (1991) A model of collective nectar source selection by honey bees: Self-organization through simple rules. Journal of Theoretical Biology 149:547-571. doi: 10.1016/S0022-5193(05)80098-0

Cameron SA (2004) Phylogeny and biology of neotropical orchid bees (Euglossini). Annu. Rev. Entomol. 49:377-404. doi: 10.1146/annurev.ento.49.072103.115855

Canavoso LE, Jouni ZE, Karnas KJ, Pennington JE, Wells MA (2001) Fat metabolism in insects. Annual review of nutrition 21:23-46. doi: 10.1146/annurev.nutr.21.1.23

Carter C, Shafir S, Yehonatan L, Palmer RG, Thornburg R (2006) A novel role for proline in plant floral nectars. Naturwissenschaften 93:72-79. doi: 10.1007/s00114-005-0062-1

Carter CJ, Thornburg RW (2004) Tobacco Nectarin III is a bifunctional enzyme with monodehydroascorbate reductase and carbonic anhydrase activities. Plant Molecular Biology 54:415-425. doi: 10.1023/b:plan.0000036373.84579.13

Caubet Y, Jaisson P, Lenoir A (1992) Preimaginal induction of adult behaviour in insects. Q J Exp Psychol-B 44:165-178. doi: 10.1080/02724999208250610

Čeksterytė V et al. (2016) Fatty acid profiles of monofloral clover beebread and pollen and proteomics of red clover (*Trifolium pratense*) pollen. Bioscience, Biotechnology, and Biochemistry:1-9. doi: 10.1080/09168451.2016.1204218

Chandrashekar J, Hoon MA, Ryba NJP, Zuker CS (2006) The receptors and cells for mammalian taste. Nature 444:288. doi: 10.1038/nature05401

Chen J, Henderson G (1996) Determination of feeding preference of Formosan subterranean termite (*Coptotermes formosanus* Shiraki) for some amino acid additives. J Chem Ecol 22:2359. doi: 10.1007/bf02029552

Chichiriccò G, Pacini E, Lanza B (2019) Pollenkitt of some monocotiledons: lipid composition and implications in pollen germination. Plant Biology (online first). doi: 10.1111/plb.12998

Chittka L, Thomson JD, Waser NM (1999) Flower constancy, insect psychology, and plant evolution. Naturwissenschaften 86:361-377. doi: 10.1007/s001140050636

Chittka L, Waser NM (1997) Why red flowers are not invisible to bees. Israel Journal of Plant Sciences 45:169-183. doi: 10.1080/07929978.1997.10676682

Colditz GA, Manson JE, Hankinson SE (1997) The nurses' health study: 20-Year contribution to the understanding of health among women. Journal of Women's Health 6:49-62. doi: 10.1089/jwh.1997.6.49

Cook SM, Sandoz JC, Martin AP, Murray DA, Poppy GM, Williams IH (2005) Could learning of pollen odours by honey bees (*Apis mellifera*) play a role in their foraging behaviour? Physiol. Entomol. 30:164-174. doi: 10.1111/j.1365-3032.2005.00445.x

Cooper G (2000) The cell: A molecular approach. 2nd edition. Sinauer Associates, Sunderland (MA)

Costa LG, Giordano G, Guizzetti M, Vitalone A (2008) Neurotoxicity of pesticides: a brief review Front Biosci, vol. 13, pp 1240-1249

Crawley MJ (1983) Herbivory. The dynamics of animal-plant interactions. University of California Press, Berkeley

Crepet WL (1979) Insect pollination: a paleontological perspective. BioScience 29:102-108

Crocker DR, Scanlon CB, Perry SM (1993) Repellency and choice: feeding responses of wild rats (*Rattus norvegicus*) to cinnamic acid derivatives. Applied Animal Behaviour Science 38:61-66. doi: 10.1016/0168-1591(93)90042-N

Croset V et al. (2010) Ancient protostome origin of chemosensory ionotropic glutamate receptors and the evolution of insect taste and olfaction. PLOS Genetics 6:e1001064. doi: 10.1371/journal.pgen.1001064

Croset V, Schleyer M, Arguello JR, Gerber B, Benton R (2016) A molecular and neuronal basis for amino acid sensing in the *Drosophila* larva. Sci Rep 6:34871. doi: 10.1038/srep34871

Danner N, Keller A, Härtel S, Steffan-Dewenter I (2017) Honey bee foraging ecology: Season but not landscape diversity shapes the amount and diversity of collected pollen. PLOS ONE 12:e0183716. doi: 10.1371/journal.pone.0183716

de Brito Sanchez MG (2011) Taste perception in honey bees. Chemical Senses 36:675-692. doi: 10.1093/chemse/bjr040

de Brito Sanchez MG, Ortigao-Farias JR, Gauthier M, Liu FL, Giurfa M (2007) Taste perception in honeybees: just a taste of honey? Arthropod-Plant Interact. 1:69-76. doi: 10.1007/s11829-007-9012-5

DeGroot AP (1953) Protein and amino acid requirements of the honey bee (*Apis mellifica* L.). Physiologia comparata et oecologia 3:197-285

Deisig N, Lachnit H, Giurfa M (2002) The effect of similarity between elemental stimuli and compounds in olfactory patterning discriminations. Learning & Memory 9:112-121. doi: 10.1101/lm.41002

Dell B (1981) Male sterility and anther wall structure in copper-deficient plants. Annals of Botany 48:599-608. doi: 10.1093/oxfordjournals.aob.a086168

Di Pasquale G et al. (2013) Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter? Plos One 8:e72016. doi: 10.1371/journal.pone.0072016

Dietz A, Humphreys WJ (1971) Scanning electron microscopic studies of antennal receptors of the worker honey bee, including Sensilla campaniformia. Ann Entomol Soc Am 64:919-925. doi: 10.1093/aesa/64.4.919

Dobson HEM (1988) Survey of pollen and pollenkitt lipids – chemical cues to flower visitors? American Journal of Botany 75:170-182. doi: 10.1002/j.1537-2197.1988.tb13429.x

Dobson HEM, Danielson EM, Wesep IDV (1999) Pollen odor chemicals as modulators of bumble bee foraging on *Rosa rugosa* Thunb. (Rosaceae). Plant Species Biology 14:153-166. doi: doi:10.1046/j.1442-1984.1999.00020.x

Dobson HEM, Groth I, Bergstrom G (1996) Pollen advertisement: chemical contrasts between whole-flower and pollen odors. American Journal of Botany 83:877-885. doi: 10.2307/2446264

Dobson HEM, Peng YS (1997) Digestion of pollen components by larvae of the flower-specialist bee *Chelostoma florisomne* (Hymenoptera: Megachilidae). J. Insect Physiol. 43:89-100. doi: 10.1016/s0022-1910(96)00024-8

Dourmad JY, Etienne M, Prunier A, Noblet J (1994) The effect of energy and protein intake of sows on their longevity: a review. Livestock Production Science 40:87-97. doi: 10.1016/0301-6226(94)90039-6

Dussutour A, Simpson SJ (2008) Carbohydrate regulation in relation to colony growth in ants. J. Exp. Biol. 211:2224-2232. doi: 10.1242/jeb.017509

Dussutour A, Simpson SJ (2012) Ant workers die young and colonies collapse when fed a high-protein diet. Proc. R. Soc. B-Biol. Sci. 279:2402-2408. doi: 10.1098/rspb.2012.0051

Dyer AG, Spaethe J, Prack S (2008) Comparative psychophysics of bumblebee and honeybee colour discrimination and object detection. Journal of Comparative Physiology A 194:617. doi: 10.1007/s00359-008-0335-1

Eckhardt M, Haider M, Dorn S, Müller A (2014) Pollen mixing in pollen generalist solitary bees: a possible strategy to complement or mitigate unfavourable pollen properties? J Anim Ecol 83:588-597. doi: 10.1111/1365-2656.12168

Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460-2461. doi: 10.1093/bioinformatics/btq461

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194-2200. doi: 10.1093/bioinformatics/btr381

Eltz T, Lunau K (2005) Antennal response to fragrance compounds in male orchid bees. Chemoecology 15:135-138. doi: 10.1007/s00049-005-0303-y

Erber J, Kierzek S, Sander E, Grandy K (1998) Tactile learning in the honeybee. J. Comp. Physiol. A-Sens. Neural Behav. Physiol. 183:737-744. doi: 10.1007/s003590050296

Esslen J, Kaissling K-E (1976) Zahl und Verteilung antennaler Sensillen bei der Honigbiene (*Apis mellifera* L.). Zoomorphologie 83:227-251. doi: 10.1007/BF00993511

Estevinho LM, Rodrigues S, Pereira AP, Feás X (2012) Portuguese bee pollen: palynological study, nutritional and microbiological evaluation. International Journal of Food Science & Technology 47:429-435. doi: 10.1111/j.1365-2621.2011.02859.x

Faegri K, Van der Pijl L (2013) Principles of pollination ecology. Elsevier, Amsterdam

Fanson BG, Weldon CW, Perez-Staples D, Simpson SJ, Taylor PW (2009) Nutrients, not caloric restriction, extend lifespan in Queensland fruit flies (*Bactrocera tryoni*). Aging Cell 8:514-523. doi: 10.1111/j.1474-9726.2009.00497.x

Ferrer M, Negro JJ (2004) The near extinction of two large European predators: super specialists pay a price. Conservation Biology 18:344-349. doi: 10.1111/j.1523-1739.2004.00096.x

Fewell JH, Winston ML (1992) Colony state and regulation of pollen foraging in the honey bee, *Apis mellifera* L. Behav. Ecol. Sociobiol. 30:387-393

Filipiak M (2018) A better understanding of bee nutritional ecology is needed to optimize conservation strategies for wild bees—The application of ecological stoichiometry. Insects 9:85

Filipiak M et al. (2017) Ecological stoichiometry of the honeybee: Pollen diversity and adequate species composition are needed to mitigate limitations imposed on the growth and development of bees by pollen quality. PLOS ONE 12:e0183236. doi: 10.1371/journal.pone.0183236

Filipiak M, Weiner J (2017) Plant–insect interactions: the role of ecological stoichiometry. Acta agrobotanica 70

Fischer RC, Richter A, Hadacek F, Mayer V (2008) Chemical differences between seeds and elaiosomes indicate an adaptation to nutritional needs of ants. Oecologia 155:539-547. doi: 10.1007/s00442-007-0931-8

Fluri P, Lüscher M, Wille H, Gerig L (1982) Changes in weight of the pharyngeal gland and haemolymph titres of juvenile hormone, protein and vitellogenin in worker honey bees. J. Insect Physiol. 28:61-68. doi: 10.1016/0022-1910(82)90023-3

Food and Agriculture Organization of the United Nations (2013) The state of food and agriculture 2013: Food systems for better nutrition. Food Agriculture Organization (FAO), Rome

Forcone A, Galetto L, Bernardello L (1997) Floral nectar chemical composition of some species from Patagonia. Biochemical Systematics and Ecology 25:395-402. doi: 10.1016/S0305-1978(97)00030-6

Free JB (1970) The flower constancy of bumblebees. J Anim Ecol 39:395-402. doi: 10.2307/2978

Friedman J, Barrett SCH (2009) Wind of change: new insights on the ecology and evolution of pollination and mating in wind-pollinated plants. Annals of Botany 103:1515-1527. doi: 10.1093/aob/mcp035

Gandolfi M, Mattiacci L, Dorn S (2003) Preimaginal learning determines adult response to chemical stimuli in a parasitic wasp. Proc Roy Soc B-Biol Sci 270:2623-2629. doi: 10.1098/rspb.2003.2541

Ganguly A et al. (2017) A molecular and cellular context-dependent role for Ir76b in detection of amino acid taste. Cell Reports 18:737-750. doi: 10.1016/j.celrep.2016.12.071

Génissel A, Aupinel P, Bressac C, Tasei JN, Chevrier C (2002) Influence of pollen origin on performance of *Bombus terrestris* micro-colonies. Entomol. Exp. Appl. 104:329-336. doi: 10.1046/j.1570-7458.2002.01019.x

Gershoff SN (1977) The role of vitamins and minerals in taste. The Chemical Senses and Nutrition. Academic Press New York, pp 201-210

Giurfa M (2007) Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. J. Comp. Physiol. A -Neuroethol. Sens. Neural Behav. Physiol. 193:801-824. doi: 10.1007/s00359-007-0235-9

Gosby AK et al. (2011) Testing protein leverage in lean humans: a randomised controlled experimental study. PLOS ONE 6:e25929. doi: 10.1371/journal.pone.0025929

Goulson D (2003) Bumblebees: their behaviour and ecology. Oxford University Press

Goulson D, Darvill B (2004) Niche overlap and diet breadth in bumblebees; are rare species more specialized in their choice of flowers? Apidologie 35:55-63. doi: 10.1051/apido:2003062

Goulson D, Nicholls E, Botías C, Rotheray EL (2015) Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science 347. doi: 10.1126/science.1255957

Grandison RC, Piper MDW, Partridge L (2009) Amino-acid imbalance explains extension of lifespan by dietary restriction in *Drosophila*. Nature 462:1061-1065. doi: 10.1038/nature08619

Grayum MH (1985) Evolutionary and ecological significance of starch storage in pollen of the Araceae. American Journal of Botany 72:1565-1577. doi: 10.1002/j.1537-2197.1985.tb08420.x

Gregory RL (2004) The Oxford companion to the mind. Oxford University Press, Oxford

Grüter C, Segers FHID, Ratnieks FLW (2013) Social learning strategies in honeybee foragers: do the costs of using private information affect the use of social information? Anim. Behav. 85:1443-1449. doi: 10.1016/j.anbehav.2013.03.041

Haddad LS, Kelbert L, Hulbert AJ (2007) Extended longevity of queen honey bees compared to workers is associated with peroxidation-resistant membranes. Exp. Gerontol. 42:601-609. doi: 10.1016/j.exger.2007.02.008

Hallem EA, Dahanukar A, Carlson JR (2006) Insect odor and taste receptors. Annu. Rev. Entomol. 51:113-135. doi: 10.1146/annurev.ento.51.051705.113646

Hallmann CA et al. (2017) More than 75 percent decline over 27 years in total flying insect biomass in protected areas. PLOS ONE 12:e0185809. doi: 10.1371/journal.pone.0185809

Hammer M, Menzel R (1995) Learning and memory in the honeybee. J. Neurosci. 15:1617-1630

Hammer TJ, Hata C, Nieh JC (2009) Thermal learning in the honeybee, *Apis mellifera*. J. Exp. Biol. 212:3928-3934. doi: 10.1242/jeb.034140

Hanley ME, Franco M, Pichon S, Darvill B, Goulson D (2008) Breeding system, pollinator choice and variation in pollen quality in British herbaceous plants. Funct. Ecol. 22:592-598. doi: 10.1111/j.1365-2435.2008.01415.x

Hannaford S, Sattler A, Siegel J, Foster RL (2013) Task specialization and odor effects on proboscis extension conditioning in bumblebees (*Bombus huntii*). J. Insect Behav. 26:762-779. doi: 10.1007/s10905-013-9389-1

Harrison JF, Woods HA, Roberts SP (2012) Ecological and environmental physiology of insects. Oxford University Press, Oxford

Haupt SS (2004) Antennal sucrose perception in the honey bee (*Apis mellifera* L.): behaviour and electrophysiology. Journal of Comparative Physiology A 190:735-745. doi: 10.1007/s00359-004-0532-5

Haydak MH (1970) Honey bee nutrition. Annu. Rev. Entomol. 15:143-156

Heil M, Rattke J, Boland W (2005) Postsecretory hydrolysis of nectar sucrose and specialization in ant/plant mutualism. Science 308:560-563. doi: 10.1126/science.1107536

Helm BR, Slater G, Rajamohan A, Yocum GD, Greenlee KJ, Bowsher JH (2017) The geometric framework for nutrition reveals interactions between protein and carbohydrate during larval growth in honey bees. Biology Open 6:872-880. doi: 10.1242/bio.022582

Hendriksma HP, Oxman KL, Shafir S (2014) Amino acid and carbohydrate tradeoffs by honey bee nectar foragers and their implications for plant–pollinator interactions. J. Insect Physiol. 69:56-64. doi: 10.1016/j.jinsphys.2014.05.025

Hendriksma HP, Shafir S (2016) Honey bee foragers balance colony nutritional deficiencies. Behav. Ecol. Sociobiol. 70:509-517. doi: 10.1007/s00265-016-2067-5

Herbert EW, Shimanuki H, Caron D (1977) Optimum protein levels required by honey bees (Hymenoptera, Apidae) to initiate and maintain brood rearing. Apidologie 8:141-146. doi: 10.1051/apido:19770204

Higdon J, Drake VJ (2011) Evidence-based approach to vitamins and minerals: health benefits and intake recommendations. Thieme, Stuttgart

Hirvonen H, Ranta E, Rita H, Peuhkuri N (1999) Significance of memory properties in prey choice decisions. Ecological Modelling 115:177-189. doi: 10.1016/S0304-3800(98)00191-4

Hobson RP (1935) On a fat-soluble growth factor required by blow-fly larvae. II. Identity of the growth factor with cholesterol 29:2023-2026. doi: 10.1042/bj0292023

Hopper S (1980) Bird and Mammal pollen vectors in *Banksia* communities at Cheyne Beach, Western Australia. Australian Journal of Botany 28:61-75. doi: 10.1071/BT9800061

Hori S et al. (2006) Associative visual learning, color discrimination, and chromatic adaptation in the harnessed honeybee *Apis mellifera* L. J. Comp. Physiol. A -Neuroethol. Sens. Neural Behav. Physiol. 192:691-700. doi: 10.1007/s00359-005-0091-4

Huang T, Jander G, de Vos M (2011) Non-protein amino acids in plant defense against insect herbivores: representative cases and opportunities for further functional analysis. Phytochemistry 72:1531-1537

Huang Z-Y, Robinson GE (1995) Seasonal changes in juvenile hormone titers and rates of biosynthesis in honey bees. Journal of Comparative Physiology B 165:18-28. doi: 10.1007/bf00264682

Huether G (2013) Amino acid availability and brain function in health and disease. Springer Berlin Heidelberg, Heidelberg

Human H, Nicolson SW (2006) Nutritional content of fresh, bee-collected and stored pollen of *Aloe greatheadii* var. *davyana* (Asphodelaceae). Phytochemistry 67:1486-1492. doi: 10.1016/j.phytochem.2006.05.023

Human H, Nicolson SW, Strauss K, Pirk CWW, Dietemann V (2007) Influence of pollen quality on ovarian development in honeybee workers (*Apis mellifera scutellata*). J. Insect Physiol. 53:649-655. doi: 10.1016/j.jinsphys.2007.04.002

Ibrahim SH (1974) Composition of pollen gathered by honeybees from some major sources. Albohouth Azziraiya 52:121-123

Iwasaki K, Gleiser CA, Masoro EJ, McMahan CA, Seo E-J, Yu BP (1988) The influence of dietary protein source on longevity and age-related disease processes of fischer rats. Journal of Gerontology 43:B5-B12. doi: 10.1093/geronj/43.1.B5

Jensen K, Mayntz D, Toft S, Raubenheimer D, Simpson SJ (2011) Nutrient regulation in a predator, the wolf spider *Pardosa prativaga*. Anim. Behav. 81:993-999. doi: 10.1016/j.anbehav.2011.01.035

Jones CG, Lawton JH, Shachak M (1996) Organisms as ecosystem engineers. Ecosystem Management: Selected Readings. Springer New York, New York, pp 130-147

Jung JW, Park KW, Ahn Y-J, Kwon HW (2015) Functional characterization of sugar receptors in the western honeybee, *Apis mellifera*. Journal of Asia-Pacific Entomology 18:19-26. doi: 10.1016/j.aspen.2014.10.011

Junker RR et al. (2017) Covariation and phenotypic integration in chemical communication displays: biosynthetic constraints and eco-evolutionary implications. New Phytologist. doi: 10.1111/nph.14505

Kalat JW, Rozin P (1973) "Learned safety" as a mechanism in long-delay taste-aversion learning in rats. Journal of Comparative and Physiological Psychology 83:198-207. doi: 10.1037/h0034424

Kämper W et al. (2016) How landscape, pollen intake and pollen quality affect colony growth in *Bombus terrestris*. Landscape Ecology:1-14. doi: 10.1007/s10980-016-0395-5

Kasumyan A, Morsi AK (1996) Taste sensitivity of common carp *Cyprinus carpio* to free amino acids and classical taste substances. Journal of Ichthyology 36:391-403

Katsumata T, Ishikawa T, Naito C, Togasawa Y (1975) Biochemical studies on pollen part 9 phospho lipids from pollen. Journal of the Faculty of Agriculture Iwate University 12:275-292

Keil TA (1997) Comparative morphogenesis of sensilla: A review. International Journal of Insect Morphology and Embryology 26:151-160. doi: 10.1016/S0020-7322(97)00017-2

Keller A et al. (2015) Evaluating multiplexed next-generation sequencing as a method in palynology for mixed pollen samples. Plant Biology 17:558-566. doi: 10.1111/plb.12251

Keller A, Hohlfeld S, Kolter A, Schultz J, Gemeinholzer B, Ankenbrand M (2019) BCdatabaser: on-the-fly reference database creation for (meta-) barcoding. EcoEvoRxiv Preprints

Keller I, Fluri P, Imdorf A (2005) Pollen nutrition and colony development in honey bees: part 1. Bee World 86:3-10. doi: 10.1080/0005772X.2005.11099641

Keller KL et al. (2012) Common variants in the CD36 gene are associated with oral fat perception, fat preferences, and obesity in African Americans. Obesity 20:1066-1073. doi: 10.1038/oby.2011.374

Kent LB, Robertson HM (2009) Evolution of the sugar receptors in insects. BMC Evolutionary Biology 9:41. doi: 10.1186/1471-2148-9-41

Kessler SC et al. (2015) Bees prefer foods containing neonicotinoid pesticides. Nature 521:74. doi: 10.1038/nature14414

Kevan P, Baker H (1983) Insects as flower visitors and pollinators. Annu. Rev. Entomol. 28:407-453

Kevan PG, Chittka L, Dyer AG (2001) Limits to the salience of ultraviolet: lessons from colour vision in bees and birds. J. Exp. Biol. 204:2571-2580

Kim H, Kim H, Kwon JY, Seo JT, Shin DM, Moon SJ (2018) *Drosophila* Gr64e mediates fatty acid sensing via the phospholipase C pathway. PLOS Genetics 14:e1007229. doi: 10.1371/journal.pgen.1007229

Kitaoka TK, Nieh JC (2009) Bumble bee pollen foraging regulation: role of pollen quality, storage levels, and odor. Behav. Ecol. Sociobiol. 63:501-510. doi: 10.1007/s00265-008-0684-3

Klein A-M et al. (2007) Importance of pollinators in changing landscapes for world crops. Proc Roy Soc B-Biol Sci 274:303-313. doi: 10.1098/rspb.2006.3721

Klein JP, Moeschberger ML (2006) Survival analysis: techniques for censored and truncated data. Springer New York, New York

Klein S, Cabirol A, Devaud J-M, Barron AB, Lihoreau M (2017) Why bees are so vulnerable to environmental stressors. Trends in Ecology & Evolution 32:268-278. doi: 10.1016/j.tree.2016.12.009

Kono N, Arai H (2015) Intracellular transport of fat-soluble Vitamins A and E. Traffic 16:19-34. doi: 10.1111/tra.12231

Kraus S, Gómez-Moracho T, Pasquaretta C, Latil G, Dussutour A, Lihoreau M (2019) Bumblebees adjust protein and lipid collection rules to the presence of brood. Current Zoology 65:437-446. doi: 10.1093/cz/zoz026

Kriesell L, Hilpert A, Leonhardt SD (2017) Different but the same: bumblebee species collect pollen of different plant sources but similar amino acid profiles. Apidologie 48:102-116

Kropàcovà S, Haslbachova H, Novàk V (1968) Development of honeybee ovaries as affected by narcosis and injections of certain substances. Sborn. Vys. zemed. v. Brne 16:537-543

Kudow N, Miura D, Schleyer M, Toshima N, Gerber B, Tanimura T (2017) Preference for and learning of amino acids in larval *Drosophila*. Biology Open 6:365-369. doi: 10.1242/bio.020412

Kukuk PF, Breed MD, Sobti A, Bell WJ (1977) The contributions of kinship and conditioning to nest recognition and colony member recognition in a primitively eusocial bee, *Lasioglossum zephyrum* (Hymenoptera: Halictidae). Behav. Ecol. Sociobiol.:319-327

Külheim C, Hui Yeoh S, Maintz J, Foley WJ, Moran GF (2009) Comparative SNP diversity among four *Eucalyptus* species for genes from secondary metabolite biosynthetic pathways. BMC Genomics 10:452. doi: 10.1186/1471-2164-10-452

Kurihara K, Beidler LM (1968) Taste-modifying protein from miracle fruit. Science 161:1241-1243. doi: 10.1126/science.161.3847.1241

Kurihara K, Beidler LM (1969) Mechanism of the action of taste-modifying protein. Nature 222:1176-1179. doi: 10.1038/2221176a0

Kvanta E (1968) Sterols in pollen. Acta Chemica Scandinavica 22

Labandeira CC, Currano ED (2013) The fossil record of plant-insect dynamics. Annual Review of Earth and Planetary Sciences 41:287-311. doi: 10.1146/annurev-earth-050212-124139

Labarca C, Loewus F (1973) The nutritional role of pistil exudate in pollen tube wall formation in *Lilium longiflorum* II. Production and utilization of exudate from stigma and stylar canal. Plant physiology 52:87-92

Laloi D, Pham-Delegue MH (2004) Bumble bees show asymmetrical discrimination between two odors in a classical conditioning procedure. J. Insect Behav. 17:385-396. doi: 10.1023/B:JOIR.0000031538.15346.e1

Laloi D, Sandoz JC, Picard-Nizou AL, Pham-Delegue MH (1999) Olfactory conditioning of the proboscis extension reflex in the bumble bee *Bombus terrestris*. Ann. Soc. Entomol. Fr. 35:154-158

Langenberger MW, Davis AR (2002) Temporal changes in floral nectar production, reabsorption, and composition associated with dichogamy in annual caraway (*Carum carvi*; Apiaceae). American Journal of Botany 89:1588-1598. doi: 10.3732/ajb.89.10.1588

Larrere M, Couillaud F (1993) Role of juvenile hormone biosynthesis in dominance status and reproduction of the bumblebee, *Bombus terrestris*. Behav. Ecol. Sociobiol. 33:335-338. doi: 10.1007/bf00172932

Laska M, Galizia CG, Giurfa M, Menzel R (1999) Olfactory discrimination ability and odor structure–activity relationships in honeybees. Chemical Senses 24:429-438. doi: 10.1093/chemse/24.4.429

Lau TC, Stephenson AG (1993) Effects of soil nitrogen on pollen production, pollen grain size, and pollen performance in *Cucurbita pepo* (Cucurbitaceae). American Journal of Botany 80:763-768

Laugerette F, Gaillard D, Passilly-Degrace P, Niot I, Besnard P (2007) Do we taste fat? Biochimie 89:265-269. doi: 10.1016/j.biochi.2006.10.011

Laugerette F et al. (2005) CD36 involvement in orosensory detection of dietary lipids, spontaneous fat preference, and digestive secretions. The Journal of Clinical Investigation 115:3177-3184. doi: 10.1172/JCI25299

Leadbeater E, Florent C (2014) Foraging bumblebees do not rate social information above personal experience. Behav. Ecol. Sociobiol. 68:1145-1150. doi: 10.1007/s00265-014-1725-8

Lee KP, Cory JS, Wilson K, Raubenheimer D, Simpson SJ (2006) Flexible diet choice offsets protein costs of pathogen resistance in a caterpillar. Proc Roy Soc B-Biol Sci 273:823-829. doi: 10.1098/rspb.2005.3385

Lee KP et al. (2008) Lifespan and reproduction in *Drosophila*: New insights from nutritional geometry. Proceedings of the National Academy of Sciences of the United States of America 105:2498-2503. doi: 10.1073/pnas.0710787105

Lee MJ et al. (2017) Ionotropic receptor 76b is required for gustatory aversion to excessive Na+ in *Drosophila*. Mol. Cells 40:787-795

Leonhardt SD, Blüthgen N (2012) The same, but different: pollen foraging in honeybee and bumblebee colonies. Apidologie 43:449-464. doi: 10.1007/s13592-011-0112-y

Lewis AC (1986) Memory constraints and flower choice in *Pieris rapae*. Science 232:863-865. doi: 10.1126/science.232.4752.863

Li P, Yin Y-L, Li D, Woo Kim S, Wu G (2007) Amino acids and immune function. British Journal of Nutrition 98:237-252. doi: 10.1017/S000711450769936X

Lichtenstein L, Sommerlandt FMJ, Spaethe J (2015) Dumb and lazy? A comparison of color learning and memory retrieval in drones and workers of the buff-tailed bumblebee, *Bombus terrestris*, by means of PER conditioning. PLOS ONE 10:e0134248. doi: 10.1371/journal.pone.0134248

Linander N, de Ibarra NH, Laska M (2012) Olfactory detectability of L-amino acids in the European honeybee (*Apis mellifera*). Chemical Senses 37:631-638. doi: 10.1093/chemse/bjs044

Lindemann B (1996) Taste reception. Physiological Reviews 76:719-766. doi: 10.1152/physrev.1996.76.3.719

Loper GM, Berdel RL (1980) A nutritional bioassay of honeybee brood-rearing potential. Apidologie 11:181-189

Lunden R (1954) A short introduction to the literature on pollen chemistry. Svensk Dem. Tidskr 66:201-213

M. Loper G, N. Standifer L, J. Thompson M, Gilliam M (1980) Biochemistry and microbiology of bee-collected almond (*Prunus dulcis*) pollen and bee bread. I- Fatty Acids, sterols, vitamins and minerals. Apidologie 11:63-73

MacArthur RH, Pianka ER (1966) On optimal use of a patchy environment. The American Naturalist 100:603-609. doi: 10.1086/282454

Maklakov AA et al. (2008) Sex-specific fitness effects of nutrient intake on reproduction and lifespan. Curr. Biol. 18:1062-1066

Manning R (2001) Fatty acids in pollen: a review of their importance for honey bees. Bee World 82:60-75. doi: 10.1080/0005772X.2001.11099504

Manning R (2006) Fatty acid composition of pollen and the effect of two dominant fatty acids (linoleic and oleic) in pollen and flour diets on longevity and nutritional composition of honey bees (*Apis mellifera*). PhD PhD thesis, Murdoch University

Manning R, Rutkay A, Eaton L, Dell B (2007) Lipid-enhanced pollen and lipid-reduced flour diets and their effect on the longevity of honey bees (*Apis mellifera* L.). Australian Journal of Entomology 46:251-257. doi: 10.1111/j.1440-6055.2007.00598.x

Mapalad KS, Leu D, Nieh JC (2008) Bumble bees heat up for high quality pollen. J. Exp. Biol. 211:2239-2242. doi: 10.1242/jeb.016642

Marui T, Kiyohara S (1987) Structure–activity relationships and response features for amino acids in fish taste. Chemical Senses 12:265-275. doi: 10.1093/chemse/12.2.265

Masek P, Keene AC (2016) Gustatory processing and taste memory in *Drosophila*. Journal of Neurogenetics 30:112-121. doi: 10.1080/01677063.2016.1185104

Matsumoto Y, Menzel R, Sandoz JC, Giurfa M (2012) Revisiting olfactory classical conditioning of the proboscis extension response in honey bees: A step toward standardized procedures. Journal of Neuroscience Methods 211:159-167. doi: 10.1016/j.jneumeth.2012.08.018

Mayntz D, Raubenheimer D, Salomon M, Toft S, Simpson SJ (2005) Nutrient-specific foraging in invertebrate predators. Science 307:111-113. doi: 10.1126/science.1105493

McCall C, Primack RB (1992) Influence of flower characteristics, weather, time of day, and season on insect visitation rates in three plant communities. American Journal of Botany 79:434-442. doi: 10.2307/2445156

McCullagh P (2018) Generalized linear models. CRC Press, Boca Raton

Meister M (2015) On the dimensionality of odor space. eLife 4:e07865. doi: 10.7554/eLife.07865

Menzel R, Blakers M (1976) Colour receptors in the bee eye — Morphology and spectral sensitivity. Journal of comparative physiology 108:11-13. doi: 10.1007/bf00625437

Merbach MA, Merbach DJ, Maschwitz U, Booth WE, Fiala B, Zizka G (2002) Mass march of termites into the deadly trap. Nature 415:36-37. doi: 10.1038/415036a

Merivee E, Märtmann H, Must A, Milius M, Williams I, Mänd M (2008) Electrophysiological responses from neurons of antennal taste sensilla in the polyphagous predatory ground beetle *Pterostichus oblongopunctatus* (Fabricius 1787) to plant sugars and amino acids. J. Insect Physiol. 54:1213-1219. doi: 10.1016/j.jinsphys.2008.05.002

Merkle JA, Fortin D, Morales JM (2014) A memory-based foraging tactic reveals an adaptive mechanism for restricted space use. Ecol Lett 17:924-931. doi: 10.1111/ele.12294

Michener CD (2000) The bees of the world. JHU Press

Micheu S, Crailsheim K, Leonhard B (2000) Importance of proline and other amino acids during honeybee flight. Amino Acids 18:157-175. doi: 10.1007/s007260050014

Moerman R, Vanderplanck M, Fournier D, Jacquemart A-L, Michez D (2017) Pollen nutrients better explain bumblebee colony development than pollen diversity. Insect Conservation and Diversity:n/a-n/a. doi: 10.1111/icad.12213

Morales-Ramos JA, Rojas MG (2001) Nutritional ecology of the formosan subterranean termite (Isoptera: Rhinotermitidae) - Feeding response to commercial wood species. J Econ Entomol 94:516-523. doi: 10.1603/0022-0493-94.2.516

Mullin CA, Chyb S, Eichenseer H, Hollister B, Frazier JL (1994) Neuroreceptor mechanisms in insect gustation: a pharmacological approach. J. Insect Physiol. 40:913-931. doi: 10.1016/0022-1910(94)90130-9

Muth F, Breslow PR, Masek P, Leonard AS (2018) A pollen fatty acid enhances learning and survival in bumblebees. Behav. Ecol. 29:1371–1379. doi: 10.1093/beheco/ary111

Muth F, Francis JS, Leonard AS (2016) Bees use the taste of pollen to determine which flowers to visit. Biology Letters 12. doi: 10.1098/rsbl.2016.0356

Muth F, Papaj DR, Leonard AS (2015) Colour learning when foraging for nectar and pollen: bees learn two colours at once. Biology Letters 11:20150628. doi: 10.1098/rsbl.2015.0628

Muth F, Papaj DR, Leonard AS (2017) Multiple rewards have asymmetric effects on learning in bumblebees. Anim. Behav. 126:123-133. doi: 10.1016/j.anbehav.2017.01.010

Naug D (2009) Nutritional stress due to habitat loss may explain recent honeybee colony collapses. Biol. Conserv. 142:2369-2372. doi: 10.1016/j.biocon.2009.04.007

Nelson G et al. (2002) An amino-acid taste receptor. Nature 416:199-202

Nepi M, von Aderkas P, Wagner R, Mugnaini S, Coulter A, Pacini E (2009) Nectar and pollination drops: how different are they? Annals of Botany 104:205-219. doi: 10.1093/aob/mcp124

Nguyen TX, Cole AM, Lehrer RI (2003) Evolution of primate θ-defensins: a serpentine path to a sweet tooth. Peptides 24:1647-1654. doi: 10.1016/j.peptides.2003.07.023

Nicholls E, Hempel de Ibarra N (2016) Assessment of pollen rewards by foraging bees. Funct. Ecol. doi: 10.1111/1365-2435.12778

Nicolson SW, Nepi M (2005) Dilute nectar in dry atmospheres: nectar secretion patterns in *Aloe castanea* (Asphodelaceae). International Journal of Plant Sciences 166:227-233. doi: 10.1086/427616

Nicolson SW, Thornburg RW (2007) Nectar chemistry. Nectaries and nectar. Springer, pp 215-264

North AC (2012) The effect of background music on the taste of wine. British Journal of Psychology 103:293-301. doi: 10.1111/j.2044-8295.2011.02072.x

Nowlis G, Kessen W (1976) Human newborns differentiate differing concentrations of sucrose and glucose. Science 191:865-866. doi: 10.1126/science.1251200

Nürnberger F, Keller A, Härtel S, Steffan-Dewenter I (2019) Honey bee waggle dance communication increases diversity of pollen diets in intensively managed agricultural landscapes. Molecular Ecology 28:3602-3611. doi: 10.1111/mec.15156

Ollerton J, Winfree R, Tarrant S (2011) How many flowering plants are pollinated by animals? Oikos 120:321-326. doi: 10.1111/j.1600-0706.2010.18644.x

Pacini E, Franchi G (1983) Pollen grain development in *Smilax aspera* L. and possible functions of the loculus. Pollen: biology and implications for plant breeding:183-190

Pacini E, Hesse M (2005) Pollenkitt – its composition, forms and functions. Flora - Morphology, Distribution, Functional Ecology of Plants 200:399-415. doi: 10.1016/j.flora.2005.02.006

Page RE, Erber J, Fondrk MK (1998) The effect of genotype on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L.). Journal of Comparative Physiology A 182:489-500. doi: 10.1007/s003590050196

Palmer-Young EC et al. (2019a) Secondary metabolites from nectar and pollen: a resource for ecological and evolutionary studies. Ecology 100:e02621. doi: 10.1002/ecy.2621

Palmer-Young EC et al. (2019b) Chemistry of floral rewards: intra- and interspecific variability of nectar and pollen secondary metabolites across taxa. Ecol Monogr 89:e01335. doi: 10.1002/ecm.1335

Palmer-Young EC, Sadd BM, Irwin RE, Adler LS (2017a) Synergistic effects of floral phytochemicals against a bumble bee parasite. Ecol Evol 7:1836-1849. doi: 10.1002/ece3.2794

Palmer-Young EC, Sadd BM, Stevenson PC, Irwin RE, Adler LS (2016) Bumble bee parasite strains vary in resistance to phytochemicals. Sci Rep 6:37087. doi: 10.1038/srep37087

Palmer-Young EC et al. (2017b) Nectar and pollen phytochemicals stimulate honey bee (Hymenoptera: Apidae) immunity to viral infection. J Econ Entomol 110:1959-1972. doi: 10.1093/jee/tox193

Paoloni-Giacobino A, Grimble R, Pichard C (2003) Genetics and nutrition. Clinical Nutrition 22:429-435. doi: 10.1016/S0261-5614(03)00064-5

Park B, Nieh JC (2017) Seasonal trends in honey bee pollen foraging revealed through DNA barcoding of bee-collected pollen. Ins. Soc:1-13. doi: 10.1007/s00040-017-0565-8

Paul NA, de Nys R, Steinberg PD (2006) Seaweed-herbivore interactions at a small scale: direct tests of feeding deterrence by filamentous algae. Marine Ecology Progress Series 323:1-9

Pavlov IP (1927) Conditioned reflexes. Oxford University Press, Oxford

Pearse WD et al. (2015) pez: phylogenetics for the environmental sciences. Bioinformatics 31:2888-2890. doi: 10.1093/bioinformatics/btv277

Peitsch D, Fietz A, Hertel H, de Souza J, Ventura DF, Menzel R (1992) The spectral input systems of hymenopteran insects and their receptor-based colour vision. Journal of Comparative Physiology A 170:23-40. doi: 10.1007/bf00190398

Pereboom JJM (2000) The composition of larval food and the significance of exocrine secretions in the bumblebee Bombus terrestris. Ins. Soc 47:11-20. doi: 10.1007/s000400050003

Pernal SF, Currie RW (2001) The influence of pollen quality on foraging behavior in honeybees (*Apis mellifera* L.). Behav. Ecol. Sociobiol. 51:53-68. doi: 10.1007/s002650100412

Pernal SF, Currie RW (2002) Discrimination and preferences for pollen-based cues by foraging honeybees, *Apis mellifera* L. Anim. Behav. 63:369-390

Persson AS, Smith HG (2013) Seasonal persistence of bumblebee populations is affected by landscape context. Agriculture Ecosystems & Environment 165:201-209. doi: 10.1016/j.agee.2012.12.008

Petanidou T, Van Laere A, Ellis WN, Smets E (2006) What shapes amino acid and sugar composition in Mediterranean floral nectars? Oikos 115:155-169. doi: 10.1111/j.2006.0030-1299.14487.x

Peumans WJ, Smeets K, Van Nerum K, Van Leuven F, Van Damme EJM (1997) Lectin and alliinase are the predominant proteins in nectar from leek (*Allium porrum* L.) flowers. Planta 201:298-302. doi: 10.1007/s004250050070

Pfennig DW, Reeve HK, Shellman JS (1983) Learned component of nestmate discrimination in workers of a social wasp, *Polistes fuscatus* (Hymenoptera: Vespidae). Anim. Behav. 31:412-416. doi: 10.1016/S0003-3472(83)80060-8

Pham-Delegue M-H, De Jong R, Masson C (1990) Effect de l'âge sur la réponse conditionnée d'extension du proboscis chez l'abeille domestique. Comptes rendus de l'Académie des sciences. Série 3, Sciences de la vie 310:527-532

Phillips SM, Van Loon LJC (2011) Dietary protein for athletes: from requirements to optimum adaptation. Journal of Sports Sciences 29:S29-S38. doi: 10.1080/02640414.2011.619204

Piironen V, Lindsay DG, Miettinen TA, Toivo J, Lampi A-M (2000) Plant sterols: biosynthesis, biological function and their importance to human nutrition. Journal of the Science of Food and Agriculture 80:939-966. doi: 10.1002/(sici)1097-0010(20000515)80:7<939::aid-jsfa644>3.0.co;2-c

Pirk CWW, Boodhoo C, Human H, Nicolson S (2010) The importance of protein type and protein to carbohydrate ratio for survival and ovarian activation of caged honeybees (*Apis mellifera scutellata*). Apidologie 41:62-72. doi: 10.1051/apido/2009055

Polar E (1975) Zinc in pollen and its incorporation into seeds. Planta 123:97-103. doi: 10.1007/bf00388064

Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O, Kunin WE (2010) Global pollinator declines: trends, impacts and drivers. Trends in Ecology & Evolution 25:345-353. doi: 10.1016/j.tree.2010.01.007

Proctor M, Yeo P, Lack A (1996) The natural history of pollination. Timber Press, Portland

Purves WK, Sadava D, Orians GH, Heller HC (2006) Biologie. Spektrum Akademischer Verlag

R Core Team (2018) R: A language and environment for statistical computing R Foundation for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria

Raubenheimer D, Simpson SJ (1993) The geometry of compensatory feeding in the locust. Anim. Behav. 45:953-964. doi: 10.1006/anbe.1993.1114

Raubenheimer D, Simpson SJ (1999) Integrating nutrition: a geometrical approach. Entomol. Exp. Appl. 91:67-82. doi: 10.1046/j.1570-7458.1999.00467.x

Ray S, Ferneyhough B (1997) Seasonal variation of proboscis extension reflex conditioning in the honey bee (*Apis mellifera*). Journal of Apicultural Research 36:108-110. doi: 10.1080/00218839.1997.11100936

Ray S, Ferneyhough B (1999) Behavioral development and olfactory learning in the honeybee (*Apis mellifera*). Developmental Psychobiology 34:21-27. doi: 10.1002/(SICI)1098-2302(199901)34:1<21::AID-DEV4>3.0.CO;2-K

Regular PM, Hedd A, Montevecchi WA (2013) Must marine predators always follow scaling laws? Memory guides the foraging decisions of a pursuit-diving seabird. Anim. Behav. 86:545-552. doi: 10.1016/j.anbehav.2013.06.008

Reinhard J, Srinivasan MV, Guez D, Zhang SW (2004) Floral scents induce recall of navigational and visual memories in honeybees. J. Exp. Biol. 207:4371-4381. doi: 10.1242/jeb.01306

Ren D et al. (2009) A probable pollination mode before angiosperms: eurasian, long-proboscid scorpionflies. Science 326:840-847. doi: 10.1126/science.1178338

Revell LJ (2012) phytools: an R package for phylogenetic comparative biology (and other things). Methods in Ecology and Evolution 3:217-223. doi: 10.1111/j.2041-210X.2011.00169.x

Revell LJ, Harmon LJ, Collar DC (2008) Phylogenetic signal, evolutionary process, and rate. Systematic Biology 57:591-601. doi: 10.1080/10635150802302427

Riessberger U, Crailsheim K (1997) Short-term effect of different weather conditions upon the behaviour of forager and nurse honey bees (*Apis mellifera carnica* Pollmann). Apidologie 28:411-426

Roberts S, Potts S, Biesmeijer K, Kuhlmann M, Kunin W, Ohlemüller R (2011) Assessing continental-scale risks for generalist and specialist pollinating bee species under climate change. BioRisk 6:1

Robertson HM, Wanner KW (2006) The chemoreceptor superfamily in the honey bee, *Apis mellifera*: Expansion of the odorant, but not gustatory, receptor family. Genome Research 16:1395-1403. doi: 10.1101/gr.5057506

Roche J, Burke C, Meier S, Walker C (2011) Nutrition x reproduction interaction in pasture-based systems: is nutrition a factor in reproductive failure? Animal Production Science 51:1045-1066

Rodriguez AE, Tingey WM, Mutschler MA (1993) Acylsugars of *Lycopersicon pennellii* deter settling and feeding of the green peach aphid (Homoptera: Aphididae). J Econ Entomol 86:34-39. doi: 10.1093/jee/86.1.34

Roger N, Michez D, Wattiez R, Sheridan C, Vanderplanck M (2017) Diet effects on bumblebee health. J. Insect Physiol. doi: 10.1016/j.jinsphys.2016.11.002

Roulston TaH, Buchmann SL (2000) A phylogenetic reconsideration of the pollen starch–pollination correlation. Evolutionary Ecology Research 2:627-643

Roulston TaH, Cane JH (2000a) The effect of diet breadth and nesting ecology on body size variation in bees (Apiformes). Journal of the Kansas Entomological Society 73:129-142

Roulston TaH, Cane JH, Buchmann SL (2000) What governs protein content of pollen: pollinator preferences, pollen-pistil interactions, or phylogeny? Ecol Monogr 70:617-643. doi: 10.1890/0012-9615(2000)070[0617:WGPCOP]2.0.CO;2

Roulston TH, Cane JH (2000b) Pollen nutritional content and digestibility for animals. Plant Syst. Evol. 222:187-209. doi: 10.1007/bf00984102

Rüdenauer F (2016) Amino acid perception in the bumblebee *Bombus terrestris*. M. Sc. Master thesis, University of Würzburg

Ruedenauer FA, Leonhardt SD, Lunau K, Spaethe J (2019a) Bumblebees are able to perceive amino acids via chemotactile antennal stimulation. Journal of Comparative Physiology A 205:321-331. doi: 10.1007/s00359-019-01321-9

Ruedenauer FA, Leonhardt SD, Schmalz F, Rössler W, Strube-Bloss MF (2017) Separation of different pollen types by chemotactile sensing in *Bombus terrestris*. J Exp Biol 220:1435-1442. doi: 10.1242/jeb.153122

Ruedenauer FA et al. (2020) Best be(e) on low fat: linking nutrient perception, regulation and fitness. Ecol Lett 23:545-554. doi: 10.1111/ele.13454

Ruedenauer FA, Spaethe J, Leonhardt SD (2015) How to know which food is good for you: bumblebees use taste to discriminate between different concentrations of food differing in nutrient content. J. Exp. Biol. 218:2233-2240. doi: 10.1242/jeb.118554

Ruedenauer FA, Spaethe J, Leonhardt SD (2016) Hungry for quality—individual bumblebees forage flexibly to collect high-quality pollen. Behav. Ecol. Sociobiol. 70:1209-1217. doi: 10.1007/s00265-016-2129-8

Ruedenauer FA, Spaethe J, van der Kooi CJ, Leonhardt SD (2019b) Pollinator or pedigree: which factors determine the evolution of pollen nutrients? Oecologia 191:349-358. doi: 10.1007/s00442-019-04494-x

Ruedenauer FA, Wöhrle C, Spaethe J, Leonhardt SD (2018) Do honeybees (*Apis mellifera*) differentiate between different pollen types? PLoS ONE 13:e0205821. doi: 10.1371/journal.pone.0205821

Running CA, Craig BA, Mattes RD (2015) Oleogustus: The unique taste of fat. Chemical Senses 40:507-516. doi: 10.1093/chemse/bjv036

Rytz R, Croset V, Benton R (2013) Ionotropic Receptors (IRs): Chemosensory ionotropic glutamate receptors in *Drosophila* and beyond. Insect Biochemistry and Molecular Biology 43:888-897. doi: 10.1016/j.ibmb.2013.02.007

Sadd BM et al. (2015) The genomes of two key bumblebee species with primitive eusocial organization. Genome Biology 16:1-32

Saffari A, Kevan PG, Atkinson JL (2010) Palatability and consumption of patty-formulated pollen and pollen substitutes and their effects on honeybee colony performance. Journal of Apicultural Science 54:63-71

Sargent RD, Kembel SW, Emery NC, Forrestel EJ, Ackerly DD (2011) Effect of local community phylogenetic structure on pollen limitation in an obligately insect-pollinated plant. American journal of botany 98:283-289

Saunders ME (2018) Insect pollinators collect pollen from wind-pollinated plants: implications for pollination ecology and sustainable agriculture. Insect Conservation and Diversity 11:13-31. doi: 10.1111/icad.12243

Scheiner R, Barnert M, Erber J (2003) Variation in water and sucrose responsiveness during the foraging season affects proboscis extension learning in honey bees. Apidologie 34:67-72

Scheiner R, Erber J, Page RE (1999) Tactile learning and the individual evaluation of the reward in honey bees (*Apis mellifera* L.). J. Comp. Physiol. A-Sens. Neural Behav. Physiol. 185:1-10. doi: 10.1007/s003590050360

Scheiner R, Page R, Erber J (2004) Sucrose responsiveness and behavioral plasticity in honey bees (*Apis mellifera*). Apidologie 35:133-142

Schenk M, Krauss J, Holzschuh A (2018) Desynchronizations in bee–plant interactions cause severe fitness losses in solitary bees. J Anim Ecol 87:139-149. doi: 10.1111/1365-2656.12694

Schiestl FP, Johnson SD (2013) Pollinator-mediated evolution of floral signals. Trends in Ecology & Evolution 28:307-315. doi: 10.1016/j.tree.2013.01.019

Schiffman SS, Dackis C (1975) Taste of nutrients: Amino acids, vitamins, and fatty acids. Perception & Psychophysics 17:140-146. doi: 10.3758/bf03203878

Schiffman SS, Sennewald K, Gagnon J (1981) Comparison of taste qualities and thresholds of D- and L-amino acids. Physiology & Behavior 27:51-59. doi: 10.1016/0031-9384(81)90298-5

Schmidt JO, Thoenes SC, Levin MD (1987) Survival of honey bees, *Apis mellifera* (Hymenoptera: Apidae), fed various pollen sources. Ann Entomol Soc Am 80:176-183

Schoener TW (1971) Theory of feeding strategies. Annual review of ecology and systematics 2:369-404

Schwarz R (1955) Über die Riechschärfe der Honigbiene. Z. Vergl. Physiol. 37:180-210. doi: 10.1007/bf00298308

Seeley TD (1989) The honey bee colony as a superorganism. American Scientist 77:546-553

Seibold S et al. (2019) Arthropod decline in grasslands and forests is associated with landscape-level drivers. Nature 574:671-674. doi: 10.1038/s41586-019-1684-3

Sharma CP, Sharma PN, Chatterjee C, Agarwala SC (1991) Manganese deficiency in maize affects pollen viability. Plant and Soil 138:139-142. doi: 10.1007/bf00011816

Shiraiwa T, Carlson JR (2007) Proboscis Extension Response (PER) assay in *Drosophila*. JoVE:e193. doi: 10.3791/193

Sickel W et al. (2015) Increased efficiency in identifying mixed pollen samples by meta-barcoding with a dual-indexing approach. BMC Ecology 15:20. doi: 10.1186/s12898-015-0051-y

Siegel GJ, Agranoff BW, Albers RW, Fisher SK, Uhler MD (1999) Basic neurochemistry. Elsevier, Amsterdam

Simopoulos AP (2002) The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomedicine & Pharmacotherapy 56:365-379. doi: 10.1016/S0753-3322(02)00253-6

Simpson BB, Neff JL (1981) Floral rewards: alternatives to pollen and nectar. Annals of the Missouri Botanical Garden 68:301-322. doi: 10.2307/2398800

Simpson SJ, James S, Simmonds MSJ, Blaney WM (1991) Variation in chemosensitivity and the control of dietary selection behaviour in the locust. Appetite 17:141-154. doi: 10.1016/0195-6663(91)90069-5

Simpson SJ, Raubenheimer D (2009) Macronutrient balance and lifespan. Aging-US 1:875-880

Simpson SJ, Raubenheimer D (2012) The nature of nutrition: a unifying framework from animal adaptation to human obesity. Princeton University Press, Princeton

Slifer EH, Sekhon SS (1961) Fine structure of the sense organs on the antennal flagellum of the honey bee, *Apis mellifera* Linnaeus. Journal of Morphology 109:351-381. doi: 10.1002/jmor.1051090308

Slone J, Daniels J, Amrein H (2007) Sugar receptors in *Drosophila*. Curr. Biol. 17:1809-1816

Smith BH, Abramson CI, Tobin TR (1991) Conditional withholding of proboscis extension in honeybees (*Apis mellifera*) during discriminative punishment. Journal of Comparative Psychology 105:345

Solberg Y, Remedios G (1980) Chemical composition of pure and bee-collected pollen. Meldinger fra Norges Landbrukshoegskole 59:2-12

Solms J (1969) Taste of amino acids, peptides, and proteins. Journal of Agricultural and Food Chemistry 17:686-688

Somerville D (2001) Nutritional value of bee collected pollens - A report for the Rural Industries Research and Development Corporation. In: Corporation RIRaD (ed)

Somme L et al. (2015) Pollen and nectar quality drive the major and minor floral choices of bumble bees. Apidologie 46:92-106. doi: 10.1007/s13592-014-0307-0

Sommerlandt FMJ, Rossler W, Spaethe J (2014) Elemental and non-elemental olfactory learning using PER conditioning in the bumblebee, *Bombus terrestris*. Apidologie 45:106-115. doi: 10.1007/s13592-013-0227-4

Spaethe J, Brockmann A, Halbig C, Tautz J (2007) Size determines antennal sensitivity and behavioral threshold to odors in bumblebee workers. Naturwissenschaften 94:733-739. doi: 10.1007/s00114-007-0251-1

Standifer L, McCaughey W, Dixon S, Gilliam M, Loper G (1980) Biochemistry and microbiology of pollen collected by honey bees (*Apis mellifera* L.) from almond, *Prunus dulcis*. II. Protein, amino acids and enzymes. Apidologie 11:163-171

Standifer LN, Devys M, Barbier M (1968) Pollen sterols—a mass spectrographic survey. Phytochemistry 7:1361-1365. doi: 10.1016/S0031-9422(00)85638-1

Standifer LN, McCaughey WF, Todd FE, Kemmerer AR (1960) Relative availability of various proteins to the honey bee. Ann Entomol Soc Am 53:618-625

Stanley RG, Linskens HF (1974) Pollen: Biology Biochemistry Management, Springer Verlag

Stiles FG (1976) Taste preferences, color preferences, and flower choice in hummingbirds. The Condor 78:10-26. doi: 10.2307/1366912

Stout J, C., Morales C, L. (2009) Ecological impacts of invasive alien species on bees. Apidologie 40:388-409

Stratton RJ, Green CJ, Elia M (2003) Disease-related malnutrition: An evidence-based approach to treatment. CABI Publishing, Wallingford

Strickler K (1979) Specialization and foraging efficiency of solitary bees. Ecology 60:998-1009. doi: 10.2307/1936868

Su JW, Gao F (2007) Growth rate and anticipated longevity of *Chrysopa phyllochroma* Wesmael (Neuroptera : Chrysopidae) larvae fed on *Aphis gossypii* Glover (Homoptera : Aphididae). Appl. Entomol. Zoolog. 42:41-47. doi: 10.1303/aez.2007.41

Suhonen J, Korkeamäki E, Salmela J, Kuitunen M (2014) Risk of local extinction of Odonata freshwater habitat generalists and specialists. Conservation Biology 28:783-789. doi: 10.1111/cobi.12231

Sukhdev P (2008) The economics of ecosystems and biodiversity, United Nations Report

Sutterlin AM (1975) Chemical attraction of some marine fish in their natural habitat. Journal of the Fisheries Research Board of Canada 32:729-738. doi: 10.1139/f75-095

Swain T (1977) Secondary compounds as protective agents. Annual review of plant physiology 28:479-501

Szymas B, Jedruszuk A (2003) The influence of different diets on haemocytes of adult worker honey bees, *Apis mellifera*. Apidologie 34:97-102. doi: 10.1051/apido:2003012

Takeda K (1961) Classical conditioned response in the honey bee. J. Insect Physiol. 6:168-179. doi: 10.1016/0022-1910(61)90060-9

Tasei J-N, Aupinel P (2008) Validation of a method using queenless *Bombus terrestris* micro-colonies for testing the nutritive value of commercial pollen mixes by comparison with queenright colonies. J Econ Entomol 101:1737-1742. doi: 10.1603/0022-0493-101.6.1737

Tepper BJ, Nurse RJ (1997) Fat perception is related to PROP taster status. Physiology & Behavior 61:949-954. doi: 10.1016/S0031-9384(96)00608-7

Therneau TM, Grambsch PM (2013) Modeling Survival Data: Extending the Cox Model. Springer New York, New York

Thorpe WH, Imms AD (1939) Further studies on pre-imaginal olfactory conditioning in insects. Proc Roy Soc B-Biol Sci 127:424-433. doi: 10.1098/rspb.1939.0032

Thostesen AM, Olesen JM (1996) Pollen removal and deposition by specialist and generalist bumblebees in *Aconitum septentrionale*. Oikos 77:77-84

Todd FE, Bretherick O (1942) The composition of pollens. J Econ Entomol 35:312-317. doi: 10.1093/jee/35.3.312

Togasawa Y, Katsumata T, Fukada M, Motoi T (1967a) Biochemical studies on pollen: Part VII. Vitamins of pollen. Nippon Nogeik Kaishi 41:184-188. doi: 10.1271/nogeikagaku1924.41.5_184

Togasawa Y, Katsumata T, Ota T (1967b) Biochemical studies on pollen: Part VI. Inorganic components and phosphorus compounds of pollen. Nippon Nogeik Kaishi 41:178-183

Toth GB, Pavia H (2002) Intraplant habitat and feeding preference of two gastropod herbivores inhabiting the kelp *Laminaria hyperborea*. Journal of the Marine Biological Association of the United Kingdom 82:243-247. doi: 10.1017/S0025315402005416

Uauy R, Dangour AD (2006) Nutrition in brain development and aging: role of essential fatty acids. Nutrition Reviews 64:24-33. doi: 10.1111/j.1753-4887.2006.tb00242.x

Vallejo-Marín M, Manson JS, Thomson JD, Barrett SCH (2009) Division of labour within flowers: heteranthery, a floral strategy to reconcile contrasting pollen fates. J. Evol. Biol. 22:828-839. doi: 10.1111/j.1420-9101.2009.01693.x

van der Kooi C, Kevan P, Koski M (2019a) The thermal ecology of flowers. Annals of Botany. doi: 10.1093/aob/mcz073

van der Kooi CJ, Dyer AG, Kevan PG, Lunau K (2019b) Functional significance of the optical properties of flowers for visual signalling. Annals of Botany 123:263-276. doi: 10.1093/aob/mcy119

Vanbergen AJ, the Insect Pollinators Initiative (2013) Threats to an ecosystem service: pressures on pollinators. Frontiers in Ecology and the Environment 11:251-259. doi: 10.1890/120126

Vanderplanck M, Michez D, Vancraenenbroeck S, Lognay G (2011) Micro-quantitative method for analysis of sterol levels in honeybees and their pollen loads. Anal Lett 44:1807-1820. doi: 10.1080/00032719.2010.526271

Vareschi E (1971) Odor discrimination in the honey bee - single cell and behavioral response. Z. Vergl. Physiol. 75:143-173

Vaudo AD, Farrell LM, Patch HM, Grozinger CM, Tooker JF (2018) Consistent pollen nutritional intake drives bumble bee (*Bombus impatiens*) colony growth and reproduction across different habitats. Ecol Evol 8:5765-5776. doi: 10.1002/ece3.4115

Vaudo AD, Patch HM, Mortensen DA, Tooker JF, Grozinger CM (2016a) Macronutrient ratios in pollen shape bumble bee (*Bombus impatiens*) foraging strategies and floral preferences. PNAS 113:E4035-E4042. doi: 10.1073/pnas.1606101113

Vaudo AD, Stabler D, Patch HM, Tooker JF, Grozinger CM, Wright GA (2016b) Bumble bees regulate their intake of the essential protein and lipid pollen macronutrients. J Exp Biol 219:3962-3970. doi: 10.1242/jeb.140772

Vaudo AD et al. (2020) Pollen protein: lipid macronutrient ratios may guide broad patterns of bee species floral preferences. Insects 11:132

von Frisch K (1921) Über den Sitz des Geruchssinnes bei Insekten. Zoologischer Jahresbericht 38:1–68

Vorel CA, Pitts-Singer TL (2010) The proboscis extension reflex not elicited in megachilid bees. Journal of the Kansas Entomological Society 83:80-83

Voulgari-Kokota A, Ankenbrand MJ, Grimmer G, Steffan-Dewenter I, Keller A (2019) Linking pollen foraging of megachilid bees to their nest bacterial microbiota. Ecol Evol Early View. doi: 10.1002/ece3.5599

Vrzal EM, Allan SA, Hahn DA (2010) Amino acids in nectar enhance longevity of female *Culex quinquefasciatus* mosquitoes. J. Insect Physiol. 56:1659-1664. doi: 10.1016/j.jinsphys.2010.06.011

Wacht S, Lunau K, Hansen K (2000) Chemosensory control of pollen ingestion in the hoverfly *Eristalis tenax* by labellar taste hairs. Journal of Comparative Physiology A 186:193-203. doi: 10.1007/s003590050019

Wang Q, Lu L, Wu X, Li Y, Lin J (2003) Boron influences pollen germination and pollen tube growth in *Picea meyeri*. Tree Physiology 23:345-351. doi: 10.1093/treephys/23.5.345

Wang TH, Jian CH, Hsieh YK, Wang FN, Wang CF (2013) Spatial distributions of inorganic elements in honeybees (*Apis mellifera* L.) and possible relationships to dietary habits and surrounding environmental pollutants. Journal of Agricultural and Food Chemistry 61:5009-5015. doi: 10.1021/jf400695w

Waser NM (1986) Flower constancy: definition, cause, and measurement. The American Naturalist 127:593-603. doi: doi:10.1086/284507

Waser NM (2006) Plant-pollinator interactions: from specialization to generalization. University of Chicago Press, Chicago

Weiner CN, Hilpert A, Werner M, Linsenmair KE, Blüthgen N (2010) Pollen amino acids and flower specialisation in solitary bees. Apidologie 41:476-487. doi: 10.1051/apido/2009083

Wells MJ (1958) Factors affecting reactions to *Mysis* by newly hatched *Sepia*. Behaviour 13:96-111. doi: 10.1163/156853958X00055

Westphal C, Steffan-Dewenter I, Tscharntke T (2003) Mass flowering crops enhance pollinator densities at a landscape scale. Ecol Lett 6:961-965. doi: 10.1046/j.1461-0248.2003.00523.x

Whitehead AT, Larsen JR (1976) Ultrastructure of the contact chemoreceptors of *Apis mellifera* L. (Hymenoptera : Apidae). International Journal of Insect Morphology and Embryology 5:301-315. doi: 10.1016/0020-7322(76)90030-1

Winston ML (1991) The biology of the honey bee. Harvard University Press, Cambridge

Withers GS, Fahrbach SE, Robinson GE (1993) Selective neuroanatomical plasticity and division of labour in the honeybee. Nature 364:238-240

Wright GA, Lutmerding A, Dudareva N, Smith BH (2005) Intensity and the ratios of compounds in the scent of snapdragon flowers affect scent discrimination by honeybees (*Apis mellifera*). Journal of Comparative Physiology A 191:105-114. doi: 10.1007/s00359-004-0576-6

Wright GA, Skinner BD, Smith BH (2002) Ability of honeybee, *Apis mellifera*, to detect and discriminate odors of varieties of canola (*Brassica rapa* and *Brassica napus*) and snapdragon flowers (*Antirrhinum majus*). J Chem Ecol 28:721-740. doi: 10.1023/a:1015232608858

Wright GA, Smith BH (2004a) Different thresholds for detection and discrimination of odors in the honey bee (*Apis mellifera*). Chemical Senses 29:127-135. doi: 10.1093/chemse/bjh016

Wright GA, Smith BH (2004b) Variation in complex olfactory stimuli and its influence on odour recognition. Proc Roy Soc B-Biol Sci 271:147-152. doi: 10.1098/rspb.2003.2590

Wu G (2013) Functional amino acids in nutrition and health. Amino Acids 45:407-411. doi: 10.1007/s00726-013-1500-6

Zanne AE et al. (2014) Three keys to the radiation of angiosperms into freezing environments. Nature 506:89-92. doi: 10.1038/nature12872

Zarchin S, Dag A, Salomon M, Hendriksma HP, Shafir S (2017) Honey bees dance faster for pollen that complements colony essential fatty acid deficiency. Behav. Ecol. Sociobiol. 71:172. doi: 10.1007/s00265-017-2394-1

Zuur AF, Ieno EN, Walker N, Saveliev AA, Smith GM (2009) Mixed Effects Models and Extensions in Ecology with R. Springer Science & Business Media, Berlin

Abbreviations

AA	amino acid
BSTFA	*N,O*-Bis(trimethylsilyl)trifluoroacetamide
CD36	cluster of differentiation 36
CE	column equivalent
CS	conditioned stimulus
CS+	rewarded conditioned stimulus
CS-	unrewarded conditioned stimulus
DAG	sn-1,2-diaglycerol
EAA	essential amino acid
EAG	electroantennogram
EFA	essential fatty acid
FA	fatty acid
FAME	fatty acid methyl ester
FDR	false discovery rate
GABA	γ-aminobutyric acid
GCMS	gas chromatograph coupled with mass spectrometer
GLM	generalized linear model
GLMM	generalized linear mixed model
GR	gustatory receptor
IEC	ion exchange chromatograph
iGluR	ionotropic glutamate receptor
IR	ionotropic receptor
ITI	intertrial interval
mya	million years ago
myr	million years
P:C-ratio	protein to carbohydrate-ratio
PER	proboscis extension response
PGLS	phylogenetic generalized least squares
phyl-ANOVA	phylogenetic analysis of variance
P:L-ratio	protein to lipid-ratio
PP	polypeptide
PROP	6-n-propylthiouracil
S	stimulus
S+	rewarded stimulus
S-	unrewarded stimulus
SD	standard deviation
SR	sugar receptor
T1R1+3	taste receptor type 1 member 1 and 3
TAG	triacyl glycerol
TMSH	trimethyl sulfonium hydroxide
US	unconditioned stimulus
WP	work package

"Dissertation Based on Several Published Manuscripts"

Statement of individual author contributions and of legal second publication rights

(if required please use more than one sheet)

Publication (complete reference): **Ruedenauer FA, Wöhrle C, Spaethe J, Leonhardt SD (2018) Do honeybees (*Apis mellifera*) differentiate between different pollen types? PLoS ONE 13(11): e0205821.**					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	SDL/JS FAR/JS	FAR SDL			
Data Collection	CW	FAR			
Data Analysis and Interpretation	FAR	CW	SDL		
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	 FAR FAR FAR FAR	 CW CW CW CW	 SDL SDL SDL	 JS JS JS	

Explanations (if applicable):

Publication (complete reference): **Ruedenauer FA, Leonhardt SD, Lunau K, Spaethe J (2019) Bumblebees are able to perceive amino acids via chemotactile antennal stimulation. Journal of Comparative Physiology A 205(3): 321-331.**					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	JS/SDL FAR/JS	FAR SDL	KL		
Data Collection	FAR				
Data Analysis and Interpretation	FAR	SDL			
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	 FAR FAR FAR FAR	 SDL SDL SDL	 JS JS JS	 KL KL KL	

Explanations (if applicable):

Publication (complete reference): **Ruedenauer FA, Spaethe J, van der Kooi CJ, Leonhardt SD (2019) Pollinator or pedigree: which factors determine the evolution of pollen nutrients? Oecologia 191(2): 349-358.**					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	SDL/JS SDL	FAR FAR			
Data Collection	FAR				
Data Analysis and Interpretation	FAR	SDL			
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	FAR FAR FAR FAR FAR	SDL SDL SDL SDL	JS JS JS JS	CJvdK CJvdK CJvdK	

Explanations (if applicable): Meta-analysis, therefore data collection here was literature research for pollen nutritional data.

Publication (complete reference): **Ruedenauer FA, Raubenheimer D, Kessner-Beierlein D, Grund-Mueller N, Noack L, Spaethe J, Leonhardt SD (2020) Best be(e) on low fat: linking nutrient perception, regulation and fitness. Ecology Letters, 23(3): 545-554.**					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	SDL/JS FAR/SDL/JS	FAR	DR		
Data Collection	FAR/DKB/NGM/LN				
Data Analysis and Interpretation	FAR	DKB/NGM/LN			
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	FAR FAR FAR FAR FAR	SDL SDL SDL SDL	JS JS JS JS	DR DR DR	

Publication (complete reference): **Ruedenauer FA, Sydow D, Spaethe J, Leonhardt SD (2020) Bumblebees rely on direct pollen cues more than on preimaginal memory. Proceedings of the Royal Society B, under review**					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	FAR FAR	JS/SDL JS/SDL			
Data Collection	DS				
Data Analysis and Interpretation	FAR	DS	SDL		
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	FAR FAR FAR FAR FAR	SDL SDL SDL SDL DS	DS DS DS DS	JS JS JS JS	

Publication (complete reference): **Ruedenauer FA, Sachs SS, van der Kooi CJ, Keller A, Spaethe J, Leonhardt SD (2020) Nutrient intake simplified: correlations between pollen nutrients allow for rapid quality assessment. Submitted to Annals of Botany**					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design	SDL	FAR/JS	CJvdK/AK		
Methods Development	SDL	FAR/SSS	JS		
Data Collection	FAR/SSS	AK			
Data Analysis and Interpretation	FAR	SDL	AK		
Manuscript Writing	FAR	SDL	JS	CJvdK/AK	
Writing of Introduction	FAR	SDL	JS	CJvdK/AK	
Writing of Materials & Methods	FAR	SDL	JS	CJvdK/AK	
Writing of Discussion	FAR	SDL	JS	CJvdK/AK	
Writing of First Draft	FAR				

The doctoral researcher confirms that she/he has obtained permission from both the publishers and the co-authors for legal second publication.

The doctoral researcher and the primary supervisor confirm the correctness of the above mentioned assessment.

Doctoral Researcher's Name	19.5.20 Freising		
	Date	Place	Signature

Primary Supervisor's Name	19.5.20 Freising		
	Date	Place	Signature

"Dissertation Based on Several Published Manuscripts"

Statement of individual author contributions to figures/tables/chapters included in the manuscripts

(If required please use more than one sheet)

Publication (complete reference): **Ruedenauer FA, Wöhrle C, Spaethe J, Leonhardt SD (2018) Do honeybees (*Apis mellifera*) differentiate between different pollen types? PLoS ONE 13(11): e0205821.**					
Figure	**Author Initials,** Responsibility decreasing from left to right				
1	FAR	CW			
2	FAR	CW			
3	FAR				
S1	FAR	CW			
S2	FAR	CW			
S3	FAR	CW			
S4	FAR	CW			
Table S1	FAR				

Explanations (if applicable):

Publication (complete reference): **Ruedenauer FA, Leonhardt SD, Lunau K, Spaethe J (2019) Bumblebees are able to perceive amino acids via chemotactile antennal stimulation Journal of Comparative Physiology A 205(3): 321-331**					
Figure	**Author Initials,** Responsibility decreasing from left to right				
1	JS	FAR			
Table 1	FAR				
2	FAR				
3	FAR				
Table 2	FAR				
4	FAR				
5	FAR				
Table 3	FAR				
6	FAR				
7	FAR				
Table 4	FAR				
Table S1	FAR				
Table S2	FAR				
Table S3	FAR				

Explanations (if applicable):

Publication (complete reference): **Ruedenauer FA, Spaethe J, van der Kooi CJ, Leonhardt SD (2019) Pollinator or pedigree: which factors determine the evolution of pollen nutrients? Oecologia 191(2): 349-358.**					
Figure	**Author Initials,** Responsibility decreasing from left to right				
1	FAR				
2	FAR				
Table 1	FAR				
Table 2	FAR				
Table 3	FAR				
Table 4	FAR				
Table 5	FAR				
S1	FAR				
Table S1	FAR				

Explanations (if applicable):

Publication (complete reference): **Ruedenauer FA, Raubenheimer D, Kessner-Beierlein D, Grund-Mueller N, Noack L, Spaethe J, Leonhardt SD (2020) Best be(e) on low fat: linking nutrient perception, regulation and fitness. Ecology Letters, 23(3): 545-554.**					
Figure	**Author Initials,** Responsibility decreasing from left to right				
1	FAR	DKB			
2	FAR	NGM/LN			
3	FAR/DKB				
Table 1	FAR	NGM/LN			
S1	FAR	DKB			
S2	FAR				
S3	FAR				
S4	FAR				
S5	FAR	NGM/LN			
S6	FAR	NGM/LN			
Table S1	FAR	NGM			
Table S2	FAR	LN			
Table S3	FAR	NGM/LN			
Table S4	FAR	NGM/LN			
Table S5	FAR	NGM/LN			
Table S6	FAR	NGM/LN			
Table S7	FAR	NGM/LN			
Table S8	FAR	NGM/LN			
Table S9	FAR	NGM/LN			

Publication (complete reference): **Ruedenauer FA, Sydow D, Spaethe J, Leonhardt SD (2020) Bumblebees rely on direct pollen cues more than on preimaginal memory. Proceedings of the Royal Society B, under review**					
Figure	**Author Initials,** Responsibility decreasing from left to right				
1	DS	FAR			
2	DS	FAR			
3	FAR	DS			
4	DS	FAR			
5	DS	FAR			
Table 1	FAR	DS			
Table 2	FAR	DS			
Table 3	FAR	DS			
Table 4	FAR	DS			
Table 5	FAR	DS			
Table 6	FAR	DS			
Table 7	FAR	DS			
Figure S1	DS	FAR			
Table S1	FAR	DS			

Publication (complete reference): **Ruedenauer FA, Sachs SS, van der Kooi CJ, Keller A, Spaethe J, Leonhardt SD (2020) Correlations between pollen nutrients increase with plant dependence of insect pollinators, submitted to Annals of Botany**					
Figure	**Author Initials,** Responsibility decreasing from left to right				
1	FAR				
2	FAR				
3	FAR				
4	FAR				
Table 1	FAR				
Table 2	FAR				
Table 3	FAR				
Table 4	FAR				
Figure S1	FAR	CJvdK			
Figure S2	FAR				
Figure S3	FAR				
Table S1.1	FAR				
Table S1.2	FAR				
Table S2.1	FAR				
Table S2.2	FAR				
Table S2.3	FAR				
Table S2.4	FAR				
Table S2.5	FAR				
Table S2.6	FAR				
Table S2.7	FAR				
Table S2.8	FAR				

Table S2.9	FAR				
Table S2.10	FAR				
Table S2.11	FAR				
Table S2.12	FAR				
Table S2.13	FAR				
Table S2.14	FAR				
Table S2.15	FAR				
Table S2.16	FAR				
Table S2.17	FAR				
Table S2.18	FAR				
Table S2.19	FAR				
Table S2.20	FAR				

I also confirm my primary supervisor's acceptance.

Doctoral Researcher's Name	Date	Place	Signature
	19.5.20	Freising	