Plant resin - an underestimated resource for bees:

How honeybees (*Apis mellifera* L.) and stingless bees (Apidae: Meliponini) benefit from a diversity of resin sources

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Sie legen Tränentau der Narzisse
Und Klebstoff der Rinde
Für die Waben als ersten Grund
Und von oben dann bauen
Zähes Wachs sie daran.

Vergil, aus "Georgica", 20 v. Chr.

Contents

Summary

Social insects like honeybees (*Apis mellifera*) and stingless bees (Apidae: Meliponini) face a relatively high risk to be attacked by pests and pathogens. To decrease the risk of infection, in addition to an innate immune system, these species have evolved various cooperative defense mechanisms such as hygienic behavior or allo-grooming, which contribute to the overall health of the colonies and are therefore also referred to as social immunity. The collection and use of plant resin is another important strategy of social immunity. Resin is a sticky, often aromatic substance with antimicrobial and deterrent properties secreted by plants for protection of the vegetative tissue. However, honeybees and stingless bees have managed to take advantage of these properties by using resins for nest construction (often mixtures of resin and wax called "propolis" or "cerumen"), to generate a sterile nest atmosphere, and as defense against pests and pathogens. Plant resins, thus, play a crucial role for the ecology of these species and are an important resource for them. Nevertheless, how bees exploit available resin sources and if resin collection can protect colonies from diseases received comparatively little attention in the past. Therefore the aim of this thesis is to provide new insights into the plant origin (**chapter II**) and significance (**chapter III**) as well as the influence of resin resource diversity (**chapter IV**) on bee colony health.

Resource use and availability form fundamental prerequisites, having decisive influence on the viability of individuals and maintenance of populations. Information on the resources required by a species is thus important to effectively promote and preserve it. For honeybees in temperate regions, precise information about which resin sources they use is largely lacking. Therefore, the second chapter focuses on the resin collection behavior of the Western honeybees (*A. mellifera* L.). By chemical comparing bee-collected resins and tree resins, I traced back the resin sources used by individual bees. Results show that honeybees collect distinct resin types that are related to different tree species. Accordingly, bees in the study area collect resin from trees of several poplar species (*Populus balsamifera*, *P. xcanadensis*), birch (*Betula alba*), horse-chestnut (*Aesculus hippocastanum*), and coniferous trees. With this study I provided the first evidence, that *A. mellifera* in temperate regions use a variety of different tree species as resin sources and, moreover, show preferences for specific resin sources.

Maintenance of colony health is probably one of the major purposes of resin collection. Nevertheless, studies investigating the benefits of resins at the colony level are rare and there

are only few evidences on the effects of raw propolis (unlike commonly used ethanol extracts) on colony health. For this reason, I conducted an experimental field study in which I investigated whether propolis, as it is naturally deposited in the nests, can protect honeybee colonies against some of the most important pathogens (the ectoparasitic mite, *Varroa destructor* and the Deformed Wing Virus, DWV). While propolis had no effect on *V. destructor* survival and infestation, I could show that, in relation to mite load, viral titers increased significantly less in propolis-rich colonies than in colonies without propolis. The results of this study showed that propolis in (semi-) natural conditions can increase the disease resistance of honeybee colonies, underscoring the importance of resins for honeybee health.

Resin collection by stingless bees is comparatively well studied and it is known that these species commonly forage on a variety of different plant species. To increase knowledge on whether and how bees may profit from a diversity of resin resources, I tested how the protective function of a resin varied among different sources (and their mixtures) and various potential aggressors (predators, parasites and pathogens). The results of this study revealed that resins from different trees vary in their effectivity against different target organisms. Moreover, resin blends were more effective than some of the individual resins, suggesting that bees can benefit from a variety of resin resources.

In summary, honeybees in temperate regions, similar to tropical stingless bees, use a variety of different tree species as resin sources. Because resins from different tree species varied in their protective function, this indicates that bees can profit from a variety of different resins/resin sources by improving the defense against diverse pests and pathogens. Conversely, the lack of resin had a negative impact on the disease resistance of colonies. Consequently, availability as well as the variety of suitable resin sources is of great importance for the health of bees. In addition to nectar and pollen, resin, as a further important resource, should therefore find more attention in beekeeping. Resin collection as the natural disease defense of bees should find more respect in beekeeping praxis and should be more strongly included in future consideration on how to promote bee colony health.

Zusammenfassung

Soziale Insekten wie Honigbienen (*Apis mellifera*) und stachellose Bienen (Apidae: Meliponini) haben ein relativ großes Risiko von Schädlingen und Krankheitserregern befallen zu werden. Um das Infektionsrisiko zu verringern haben diese Arten verschiedene kooperative Abwehrmechanismen (z. b. Hygieneverhalten oder gegenseitiges Putzen) entwickelt, die zur allgemeinen Gesundheit der Kolonien beitragen und daher auch als soziale Immunität bezeichnet werden. Das Sammeln und die Verwendung von Pflanzenharz ist eine weitere wichtige Strategie der sozialen Immunität. Harz ist eine klebrige, oft aromatische Substanz mit antimikrobiellen und abwehrenden Eigenschaften, welche von Pflanzen zum Schutz des vegetativen Gewebes abgesondert wird. Honigbienen und stachellose Bienen haben es jedoch geschafft diese Eigenschaften zunutze zu machen, indem sie Harze für den Nestbau (oft als Mischungen aus Harz und Wachs, genannt "Propolis" oder "Cerumen") sowie für die Abwehr von Schädlingen und Krankheitserregern verwenden. Pflanzenharz ist folglich eine essentielle Ressource für diese Bienen. Wie Bienen verfügbare Harzquellen nutzen und welche möglichen Konsequenzen sich daraus für die Gesundheit der Kolonie ergeben, erhielt in der Vergangenheit jedoch vergleichsweise wenig Aufmerksamkeit. Ziel dieser Arbeit war es daher, neue Erkenntnisse über den pflanzlichen Ursprung (**Kapitel II**) und die Bedeutung (**Kapitel III**), sowie den Einfluss der Harzressourcenvielfalt (**Kapitel IV**) auf die Koloniegesundheit von Honigbienen und stachellosen Bienen zu erlangen.

Ressourcennutzung und -verfügbarkeit bilden grundlegende Voraussetzungen die einen entscheidenden Einfluss auf die Lebensfähigkeit von Individuen und den Erhalt von Populationen haben. Kenntnisse über die von einer Art benötigten Ressourcen können daher wichtig sein, um diese effektiv zu fördern und erhalten zu können. Genaue Informationen darüber, welche Harzquellen von Honigbienen in gemäßigten Regionen verwendet werden, fehlen bisher weitgehend. Daher ist das erste Kapitel dieser Arbeit dem Harzsammelverhalten der Westliche Honigbiene (*A. mellifera* L.) gewidmet. Mittels chemischer Vergleiche der von Bienen-gesammelten Harzen und von Baumharzen habe ich die Harzressourcennutzung einzelner Bienen zurückverfolgt und konnte nachweisen, dass Honigbienen unterschiedliche Harz-chemotypen sammeln, die verschiedenen Baumarten zugeordnet werden können. Demnach sammeln Bienen im Untersuchungsgebiet Harze von Bäumen verschiedener Pappelarten (*Populus balsamifera, P. xcanadensis*), Birken (*Betula alba*), Rosskastanien (*Aesculus hippocastanum*) und Nadelbäumen. Mit dieser Studie habe ich den ersten eindeutigen Nachweis erbracht, dass *A. mellifera* in gemäßigten Regionen eine Vielzahl

verschiedener Baumarten als Harzquellen verwendet und darüber hinaus Präferenzen für bestimmte Harzquellen zeigt.

Einer der Hauptgründe für das Sammeln von Harz ist sehr wahrscheinlich die Reduktion pathogener Erreger in der Nestumgebung und damit der Erhalt der Kolonie Gesundheit. Dennoch gibt es nur wenige Studien die den Nutzen von Harzen auf Kolonieebene untersuchen, und darüber hinaus auch fast keine Nachweise darüber wie sich Propolis in seiner natürlichen Form (im Gegensatz zu häufig verwendeten Ethanol-Extrakten) auf die Gesundheit von Kolonien auswirkt. In einer experimentellen Feldstudie habe ich daher den Harzeintrag von Honigbienenvölkern manipuliert um zu untersuchen, ob Propolis - so wie es natürlicherweise in den Nestern abgelagert wird - Schutz vor einigen der wichtigsten Krankheitserreger (die ektoparasitäre Milbe, *Varroa destructor* und den von ihr übertragenen Deformed Wing Virus, DWV) bietet. Während Propolis keinen Einfluss auf das Überleben und den Befallsgrad von *V. destructor* hatte, konnte ich zeigen, dass die Virustiter im Vergleich zur Milbenbelastung in Propolis-reichen Kolonien signifikant weniger zunahmen als in Kolonien ohne Propolis. Die Ergebnisse dieser Studie haben gezeigt, dass Propolis unter natürlichen Bedingungen die Krankheitsresistenz von Honigbienenvölkern erhöhen kann, was die Bedeutung von Harzen für die Gesundheit von Honigbienenvölkern unterstreicht.

Für stachellose Bienen ist das Sammeln von Harzen vergleichsweise gut untersucht und es ist bekannt, dass diese Arten häufig eine Vielzahl von verschiedenen Pflanzenarten nutzen. Um zu untersuchen ob und inwiefern Bienen von einer Vielfalt von Harzressourcen profitieren können, habe ich getestet wie die Schutzfunktion von Harz zwischen verschiedenen Quellen (und deren Mischungen) und unterschiedlichen potentiellen Schadorganismen (Prädatoren, Parasiten und Pathogenen) variiert. Die Ergebnisse dieser Studie haben gezeigt, dass sich Harz von verschiedenen Bäumen in seiner Wirksamkeit gegen unterschiedliche Zielorganismen unterscheidet. Darüber hinaus waren Harzmischungen wirksamer als einige der einzelnen Harze, was nahelegt, dass Bienen von einer Vielfalt von Harzressourcen profitieren können.

Zusammenfassend lässt sich sage, das Honigbienen in gemäßigten Regionen, ähnlich wie tropische stachellose Bienen, eine Vielzahl von verschiedenen Baumarten als Harzquellen nutzen. Da Harze verschiedener Baumarten in ihrer Schutzfunktion variierten, weist dies darauf hin, dass Bienen von einer Vielzahl unterschiedlicher Harz- / Harzquellen profitieren können, indem sie die Abwehr gegen verschiedene Schädlinge und Krankheitserreger

verbessern. Umgekehrt hatte das Fehlen von Harz einen negativen Einfluss auf die Krankheitsresistenz der Kolonien. Folglich ist die Verfügbarkeit ebenso wie die Vielfalt geeigneter Harzquellen von großer Bedeutung für die Gesundheit von Bienen. Neben Nektar und Pollen sollte Harz, als weitere wichtige Ressource, daher mehr Beachtung in der Bienenhaltung finden. Darüber hinaus sollte die Nutzung von Harz als die natürliche Krankheitsabwehr der Bienen stärker in Überlegungen zur Förderung der Honigbienengesundheit einbezogen werden.

Pro-polis – "in front of the city"

"When the hive has been delivered to them [honey bees] clean and empty, they build their waxen cells, bringing in the juice of all kinds of flowers and the "tears" or exuding sap of trees, such as willows and elms and such others as are particularly given to the exudation of gum. With this material they besmear the groundwork, to provide against attacks of other creatures; the bee-keepers call this stuff "stop-wax". They also with the same material narrow by side-building the entrances to the hive if they are too wide."

Aristotle about the use of propolis by bees in his book *Historia Animalium* from the 4th century BC Chr. (book IX; translated in: Smith and Ross 1910).

"The instinct, highly developed in the majority of breeds, to smear the whole interior of the hive with propolis, is one of the most unpleasant, ugly qualities of the bee. This completely unnecessary operation also makes the work of the beekeeper considerably more difficult."

Bruder Adam (a Benedictine monk and founder of the Buckfast bees breeding line) about the use of propolis by bees in his lecture on modern bee breeding (published in: Deutsche Bienenwirtschaft 1953)

Chapter I

General introduction

1. Background

Plants and insects have coexisted over several hundred millions of years and therefore evolved diverse interactions. A large proportion of these interactions rely on the provision of food sources (e.g. foliage or nectar and pollen) and have formed diverse relationships, ranging from antagonistic (e.g. herbivory) to mutually beneficial interactions (e.g. pollination). Besides nutritional purposes, plants or plant parts can also serve insects as resource for e.g. nesting or nest construction (e.g. leave cutting bees: (Zillikens and Steiner 2004; Cane et al. 2007)) and/or defensive purposes (e.g. secondary plant compounds (Nishida 2002), plant resins (Chapuisat et al. 2007)). To defend themselves against diverse pests and pathogens, plants produce a range of secondary compounds with often toxic or deterrent properties (Schoonhoven et al. 2005). However, some insects such as several Lepidoptera species (Nishida 2002), managed to take advantage of these substances by digesting and accumulating toxic compounds which provide them protection against antagonists (reviewed e.g. in Mello and Silva-Filho 2002; Nishida 2014). Collection of antimicrobial plant resin for nest construction and as defense against pests and pathogens, is another strategy which is known from e.g. some ant (Chapuisat et al. 2007; Castella et al. 2008a) and bee species (Roubik 1989; Cane 1996; Simone-Finstrom and Spivak 2010).

Highly eusocial bees such as honeybees (*Apis mellifera*) and stingless bees (Apidae: Meliponini) collect partly considerable amounts of resins which have an important meaning for colony-level disease defense (Roubik 1989; Lehmberg et al. 2008; Leonhardt and Blüthgen 2009; Simone-Finstrom and Spivak 2010; Borba et al. 2015; Borba and Spivak 2017; Drescher et al. 2017). Bees are among the most important pollinator species, as they play a vital role for the reproduction of many wild- and crop plants perform key ecosystem functions and directly contribute to human well- being (Klein et al. 2007; Potts et al. 2010; da Silva et al. 2017; Meléndez Ramírez et al. 2018). In Europe about 85% of the cultivated crops depend at least to some extend on biotic pollination (Williams 1994) and 35% of the global food production results from pollinator depended crops (Klein et al. 2007). Since the diversity and abundance of many wild pollinator species decreases (Kluser and Peduzzi 2007; Kosior et

al. 2007; Goulson et al. 2008; Goulson et al. 2015) and there is a growing demand for pollinator dependent agricultural products (Aizen and Harder 2009), the systematic use of managed bees such as honeybees (*Apis mellifera*), become increasingly important to secure sufficient yields. However, especially the Western honeybee (*Apis mellifera* L.) suffers from the growing pressure resulting from their commercial use on the one hand and several environmental impacts (e.g. limited floral resources, pesticides and an increasing number of pests and parasites (see reviews of Moritz et al. 2010; Goulson et al. 2015)) on the other hand and their increased susceptibility to diseases as well as the observed loss of managed colonies in several regions in Europe and the US, raises concerns worldwide (Ellis et al. 2010; Neumann and Carreck 2010; Goulson et al. 2015). In this context improving bee's natural defenses such as hygiene behavior or allo-grooming is one strategy to enhance the disease resistance of colonies (Spivak and Reuter 2001; Ibrahim et al. 2007). However, resin collection, another crucial defense mechanism, rarely received any attention until Simone-Finstrom et al. (2010) and research on this only increased recently. Therefore this dissertation focuses on the collection and use of plant resins as an important but often neglected resource for honeybees and stingless bees.

1.1 General and nesting biology of honeybees and stingless bees

Honeybees (Apidae: Apini) and the tropical stingless (honey-) bees (Apidae: Meliponini) represent two closely related groups within the monophyletic group of the corbiculate bees (Apinae) (Michener 1997; Michener 2007). Among bees, they are the only highly eusocial species and therefor share several similarities (Michener 2007). Both, honeybees and stingless bees build large perennial colonies characterized by a high degree of social organization. They can be thought of as multigenerational family aggregations organized by division of labor and diverse social behaviors such as cooperative brood care, communication or cooperative defenses (Michener 2007). Colonies typically consist of one reproducing queen and a few dozen to several thousands of non-reproductive worker bees (Winston 1987; Roubik 1989; Seeley 1995). New colonies are established collaboratively by groups or swarms after a new queen is raised (Roubik 1989; Seeley 1995).

Because of their permanent food demand and to prevent supply gaps e.g. during winter or rainy seasons, honeybees and stingless bees build up large a stock of nectar and pollen and hence are the only honey-producing bees (Michener 2007).

The **Western honeybee,** *Apis mellifera* Linnaeus, is probably the most prominent honeybee species. With up to 30,000 and more bees they build large colonies (Seeley 1995) that nest in various kinds of hollow spaces (e.g. hollow tree trunks, cavities in rocks or human buildings), large enough for their complex nest structures (Seeley and Morse 1976). Nests typically consist of vertical hanging comb structure with the food combs surrounding the brood combs (Seeley 1995). *A. mellifera* are kept and intensively managed by humans for the provision of valuable products such as honey and wax since millennia and, more recently, also for the provision of pollination service (vanEngelsdorp and Meixner 2010). While originally native to large parts of Europe, Africa and the Middle East (Ruttner 1988; Han et al. 2012), *A. mellifera* have been distributed all over the world by human activities and today is largely maintained in managed populations (Michener 2007; Moritz et al. 2007).

Stingless bees (Apidae: Meliponini), in contrast to honeybees, are a large monophyletic group comprising of 61 described genera with at least 600 different species (honeybees comprise less than 12 species) that occur exclusively in tropical or subtropical areas (Michener 2007). Colonies usually consist of a few dozen, up to several thousand worker bees (Roubik 1989; Michener 2007). Nests are built in the soil, burrows of other animals as well as cavities and crevices in tree trunks, rocks or artificial buildings (Roubik 1989; Siqueira et al. 2012; Layek and Karmakar 2018). In contrast to *A. mellifera* nests of stingless bees represent considerable proportions of additive compounds such as mud and/or clay, feces (Roubik 2006; Michener 2007). While the nest construction can largely vary among species, the inner part commonly displays a circle shaped structure, with the inner brood cells surrounded by food storage pods and the whole nest coated by a thick layer of batumen (resin layer surfacing the nest) (Roubik 2006). In contrast to *A. mellifera* these species are less or not at all managed and usually native to their environment. In many tropical areas, Meliponini are the most common bees and are thus considered crucial pollinators for diverse plants (Roubik 1989; Corlett 2004; Slaa et al. 2006).

1.2 Resources use by honeybees and stingless bees

The main resources collected by honeybees and stingless bees are nectar and pollen (Fig.1a - b) (Roubik 1989; Seeley 1995). In addition, they also collect a variety of non-floral resources such as water, honeydew, oils, mud or soil, rotten wood, urine, feces, blood, gums, and plant resins, that can serve various purposes (e.g. nutrition or nest construction; Fig.1b - d) (Roubik 1989). Nectar serves primarily for provision of energy but is also used to stick pollen grains together and form pellets that can be attached to the hind legs for the transport (Roubik 1989;

Harano et al. 2014). Pollen is the main source of proteins, but also provides lipids, vitamins, and minerals to bees and is essential especially for larval rearing and development of young bees (Haydak 1970; Roulston and Cane 2000; Brodschneider and Crailsheim 2010). Forager bees are permanent or temporarily specialized to either nectar, pollen or resin (Sommeijer et al. 1983; Seeley 1995) and can have flight ranges of up to 3km (honeybees 10km and more), depending on their body size (Roubik 1989; Seeley 1995; Abou-Shaara 2014). Colonies usually show a huge food demand (honeybees collect up to 20 kg Pollen und 120 kg Nectar per year) (Seeley 1995) which requires an efficient use of available resources. Recruiting strategies, thus the communicating profitable foraging sites (e.g. the "waggle dance" of honeybees), allows bees to rapidly find and efficiently exploit available resources (von Frisch 1965; Seeley 1986; Wenner et al. 1991; Nieh 2004; Barth et al. 2008).

Figure 1. Ressource use by honeybees and stingless bees. *Apis mellifera* gathering a) pollen; b) nectar; c) Resin forager bee of *Tetragonula carbconaria* collecting resin from a tree of *Eucalyptus racemosa* rests; *d) Apis mellifera* collecting water

As generalists they are further known to exploit a wide variety of plant species to meet their nutritional needs (Engel and Dingemans-Bakels 1980; Sommeijer et al. 1983) and generally profit from diverse environments due to a spatial and temporal abundant supply of floral resources (Visscher and Seeley 1982; Michener 2007; Alaux et al. 2010; Requier et al. 2015; Donkersley et al. 2017; Kaluza et al. 2017). Moreover, nectar (Pacini et al. 2003) and pollen (Roulston and Cane 2000) can vary in their nutritional composition among different plant species and can also contain compounds toxic to bees (Johnson 2015).

Therefore the availability (Haydak 1970; Donkersley et al. 2014; Smart et al. 2016) as well as quality (Di Pasquale et al. 2013; Frias et al. 2016)and diversity (Alaux et al. 2010; Di Pasquale et al. 2013; Donkersley et al. 2017) of these resources play an important role for a balanced diet and thus of colony health.

1.3 Plant resin- composition and ecological role

Resin is a sticky often aromatic secretion which is produced by plants predominantly for protection of vegetative parts (Langenheim 2003). Resin production occurs mostly among conifers and tropical angiosperms (mostly in *Dipterocarpaceae*, *Burseraceae*, and *Leguminosae*) (Langenheim 1969; Langenheim 2003). In temperate regions diversity of resin producing plants is less diverse and is known mainly from several tree species of a few genera (e.g. *Salicaceae, Betulaceae*, *Sapindaceae*) (Langenheim 2003). To separate resin from various other plant exudates Langenheim (2003) gave a definition wherefore "…plant resin is defined operationally as primarily a lipid soluble mixture of volatile and nonvolatile terpenoid and/or phenolic secondary compounds that are (1) usually secreted in specialized structures located either internally or on the surface of the plant and (2) of potential significance in ecological interactions." Internal resins are usually secreted in response to injury and serves as a wound sealant to prevent ingress of moisture or pathogens (Shrimpton and Whitney 1968; Langenheim 2003). In comparison, constitutive (surface) resins, regularly coating surfaces of delicate plant parts (e.g. sprouts, leaves or tree buds) serve as protection against detrimental impacts and various antagonists such as herbivores, pathogens and also likely environmental impacts such as drought and UV light (see Langenheim 2003). For example, *Populus deltoides* accumulates resin within buds in late summer, which is secreted over expanding leaves in the following spring to protect them from leaf beetles (Curtis and Lersten 1974) and gypsy moths (Meyer and Montgomery 1987; Langenheim 2003). Functional properties (toxic, deterrent, antibacterial, and fungicide) of resin derive from a complex and highly variable mixture of various volatile and non-volatile substances, including terpenes or terpenoids

(terpenes with a hydroxyl, carbonyl or carboxyl function) as well as various phenolic compounds (e.g. flavonoids, aromatic acids, and their esters). Volatiles, mainly mono- and some sesquiterpenes as well as some phenolic components, are largely responsible for plants fragrance (Maffei 2010) and can be involved in direct and indirect defenses by e.g. repelling herbivores (Smith 1961), inhibiting fungal growth (Shrimpton and Whitney 1968), and/or attracting enemies of herbivores (Kessler and Baldwin 2001). Furthermore, they contribute to the fluidity of resin and thereby facilitating e.g. the transport of higher molecular weight, non-volatile compounds such as di-and triterpenes or phenolic compounds which are more viscous and harden faster (Langenheim 2003). Non-volatile compounds have often toxic or deterrent properties and thus play a role e.g. for defense against herbivores (Reichardt et al. 1984) and/or interfere with the growth of bacteria and fungi (Ahmed et al. 2010; Mokoka et al. 2013). Chemical blends can vary among species, individuals, plant parts (Langenheim 2003), and in response to herbivory (Bryant and Kuropat 1980; Rousi et al. 1989). Chemical diversity as well as variability (qualitative and quantitative) of biological active compounds provides plants flexibility to react effectively to the various and constantly evolving pests and pathogens. (Witham 1983; Delorme and Lieutier 1990; Langenheim 2003). Moreover variability in the defense of host population makes adaptation by pests and pathogens more difficult (Witham 1983).

Despite its toxic and deterrent properties some animals have managed to take advantage of resins by using them for their own purposes. For example larvae of the sawfly *Neodiprion sertifer* were found to sequester resin of *Pinus sylvestris* to deter predators (Eisner et al. 1974), whereas species of pemphredonine wasps use pine resin to seal their nests (Mudd and Corbet 1975). Wood ants (*Formica paralugubris*) collect resins from coniferous trees that are incorporated into their nests and partly mixed with endogenous chemicals to prevent growth of bacteria and fungi (Christe et al. 2003; Chapuisat et al. 2007; Brütsch et al. 2017) and serves as prophylaxes that decreases the inducible part of the immune system (Castella et al. 2008a; Castella et al. 2008b).

1.4 Resin collection and use

Bees collect resins from wounded tree trunks (Roubik 1989; Leonhardt and Blüthgen 2009), buds, young sprouts, and leaves (Alfonsus 1933; Drescher et al. 2019) as well as from a few tropical flowers (Armbruster 1984; Kumazawa et al. 2003) and seed capsule (Wallace and Lee 2010) of various plant species depending on the geographical location. Tropical stingless bees are known to exploit a wide variety of resin sources (Leonhardt et al. 2009; Wallace and

Lee 2010), whereas honeybees in temperate regions are thought to collect resins mainly from buds and young sprouts of several tree species (see chapter I). While it is widely accepted that bees in Europe and North America collect resins from different species of poplar (Greenaway et al. 1990; Bankova et al. 2000; Wilson et al. 2013; Drescher et al. 2019), aspen, birch, alder, horse-chestnut, pine, elm, oak, and willow are further suggestions for potential resin sources in the temperate zone of the Northern Hemisphere (Ghisalberti 1979; Popravko and Sokolov 1980; König 1985; Greenaway et al. 1988; Bankova et al. 1992).

Figure 2. Resin collection of honeybees and stingless bees. a) *Apis mellifera* collecting resin from a tree bud of *populus spp*.; b) *Tetragonula carbonaria* gathering resin from a tree woud of *Syncarpia glomerulifera* by attaching resin droplets to the hindlegs; c) Resinous tree bud of *Betula alba*, a potential resin sources in temperate regions; d) Unloading process of a returning resin forager bee (*A. mellifera*).

For stingless bees it was shown that species collect partly large amounts of resins (Leonhardt and Blüthgen 2009). Collection of resin usually accounts for 20 – 30% of all foragers and often several species can be observed simultaneously at a single site (Roubik 2006; Leonhardt

and Blüthgen 2009). In contrast, in honeybee colonies only a relatively small proportion (approximately 1%) of foragers gather resins (Nakamura and Seeley 2006) which is also likely one reason why this activity is seldom seen in honeybees. Conversely to stingless bees in tropical regions, honeybees collect resin usually during late summer (June – October) (Huber 1814; Meyer and Ulrich 1956), when trees start to prepare young buds for winter dormancy by building a protective resin coat (Curtis and Lersten 1974; Langenheim 2003).

Foraging for resin is conducted predominantly during warm days (>18°C) (Meyer and Ulrich 1956; Drescher et al. 2019) when resin is softer and therefor likely easier to handle for bees. Due to its sticky characteristic the collection of resin is a challenging and relatively time consuming task (Meyer and Ulrich 1956; Nakamura and Seeley 2006). Bees bite off small pieces of resin (or extract resin by fragmenting leaves) with their mandibles, which are then chewed until forming small droplets that can be transferred to the hind legs (Fig.2a-b) (Huber 1814; Alfonsus 1933; Roubik 1989). Similar to pollen, resin is transported at the corbicula of the hind legs (Huber 1814; Roubik 1989). Once back in the nest, resin foragers need the assistance of one or two other bees to remove the sticky resin load from their legs (Fig.2d), which can take 15 to 30 minutes or more (Nakamura and Seeley 2006). Following the collection, resins are deposited within the nest (Fig.3a) where they are sometimes mixed with wax (then called “propolis” in honeybees or “cerumen” in stingless bees) (Ghisalberti 1979; Roubik 2006).

Propolis or pure resin is used as a building material for nest construction e.g. to strengthen the comb structure, line cell rims (Fig.3d) or to seal small holes and cracks and thereby prevent air flow and the ingress of moisture (Huber 1814; Seeley and Morse 1976). From feral colonies under natural conditions it is further known that they use resin to coat the entire nest interior with a thin antimicrobial layer (also referred to as “propolis envelope”). Propolis envelope is used to cover and smoothen the inner nest walls, which often consist of rotten wood, and thereby prevent pathogen growth and limit the loss of water to maintain constant humidity inside the nest (Seeley and Morse 1976; Simone-Finstrom and Spivak 2010). Stingless bees use resin generally in a similar way, whereby these bees often use large amounts of resins that are also attached to the outer surface of nest entrance (Fig.3c) or used for construction of the, sometimes, long nest entrance tubes (Roubik 2006; Leonhardt and Blüthgen 2009). Besides its functional properties for nest construction, resin serves primarily as defense barrier against various pests (Lehmberg et al. 2008), parasites (Greco et al. 2010; Drescher et al. 2014) and pathogens (Simone-Finstrom et al. 2009; Simone-Finstrom and

Spivak 2012; Borba and Spivak 2017; Drescher et al. 2017) and thus plays an important role for colony health. Moreover, attacks from predators (Leonhardt and Blüthgen 2009) and pathogens (Simone-Finstrom and Spivak 2012) can cause an increase in collection of resin, indicating that resin serves not only as prophylaxes but probably also for self-medicating purposes.

Figure 3. Use of resin for nest construction by honeybees (*Apis mellifera*) and stinless bees. a) Worker bee processing resin deposits within the hive; b) Beehive with a thin resin layer coating the inner hivewalls; *Apis mellifera* worker bee with resin load; c) Hive entrace of *Tetragonula carbonaria* colony sourounded by a resin layer; d) Wax comb of honeybees with each cell rim showing a thin propolis film; parasitic mite *Varroa destructor* without its host.

1.5 Bee decline - main drivers and factors affecting bee health

Both, wild and managed bees suffer from impacts of multiple and partly interacting stressors, such as the use of pesticides, nutritional deficiencies and a growing number of pathogens (including viruses, bacteria, etc.) and parasites (Moritz et al. 2010; vanEngelsdorp and Meixner 2010; Mutinelli 2011; Smith et al. 2013; Gisder and Genersch 2015; Moritz and Erler 2016; Traynor et al. 2016). Pesticides use can have lethal and sub-lethal effects and cause substantial harm to bees (Goulson et al. 2015).

Chronical exposure to cocktails of pesticides, neonicotinoides and fungicides can increase toxicity of single pesticides due to synergistic and cumulative effects, and affect bees by e.g. reducing learning capacity, foraging behavior or homing ability (Goulson et al. 2015). Furthermore, nutritional stress resulting from reduced availability or monotonous floral resources can affect individual bee health and colony fitness by reducing longevity and immune function of bees and thus decreasing resistance or tolerance to pests and pathogens.

1.6 Honeybee health - pests and diseases

The Western honeybee (*A. mellifera* L.) has to deal with a growing number of pests and pathogens, facilitated by the worldwide trade of bees and bee products (Allen and Ball 1996; Ellis and Munn 2005; Klee et al. 2007; Genersch, Elke et al. 2010; Mutinelli 2011; Mondet et al. 2014). There are at least 23 described viruses that can affect honeybees (Gisder and Genersch 2015). Moreover, bacteria, such as *Paenibacillus larvae*, the causative agent of American Foulbrood, fungi such as *Ascosphaera apis* (causing Chalkbrood) and parasites such as *Nosema* spec. and *Varroa destructor* are further important pathogens (Genersch, Elke et al. 2010). Among these, *Varroa destructor* (Anderson and Trueman), an ectoparasitic mite that feeds on the hemolymph of emerging and mature bees (Fig. 4a) and functions as vector for various pathogens, is considered one of the most challenging threats. (Ball and Allen 1988; Chen et al. 2006; Yang and Cox-Foster 2007; Le Conte et al. 2010; Rosenkranz et al. 2010; Dainat et al. 2012). *V. destructor* was introduced through the trade of bees after it switched from its natural host (*Apis cerana*) to the less resistant *Apis mellifera* (Oldroyd 1999). While *A. cerana* have effective strategies to reduce mite infestation, the Western honeybee is not (yet) successful in fighting this parasite (Boecking and Spivak 1999; Oldroyd 1999; Le Conte et al. 2007; Le Conte et al. 2010). Damages to bees resulting mostly from mites' detrimental interaction with a virus called Deformed Wing Virus (DWV) (Yang and Cox-Foster 2005; Nazzi et al. 2015). By functioning as vector, *V. destructor* plays an

important role for viral transmission (Bowen-Walker et al. 1999). Moreover, feeding on the hemolymph can harm bees by reducing their body weight and suppressing immune function (Yang and Cox-Foster 2005; Yang and Cox-Foster 2007), which likely encourages viral replication and thus causes symptomatic disease outbreak (Nazzi et al. 2015). Correspondingly, DWV viral titers typically increase relatively to mite infestation from early summer to autumn (Tentcheva et al. 2004; Di Prisco et al. 2011). DWV (mostly in association with varroa) can provoke deformation of wings (Fig.4b), truncated abdomen and reduced life span of bees and thereby causes lasting impairment to colonies often leading to their collapse (reviewed in Le Conte et al. 2010; and Rosenkranz et al. 2010).

Consequently, *V. destructor* in correlation with DWV is considered a major driver for the partly unsustainable high winter losses observed in temperate regions, while other suggested pathogens such as the *Nosema* spec. and *Paenibacillus larvae*, and several viruses were found to be less relevant (Genersch, E. et al. 2010; Neumann and Carreck 2010).

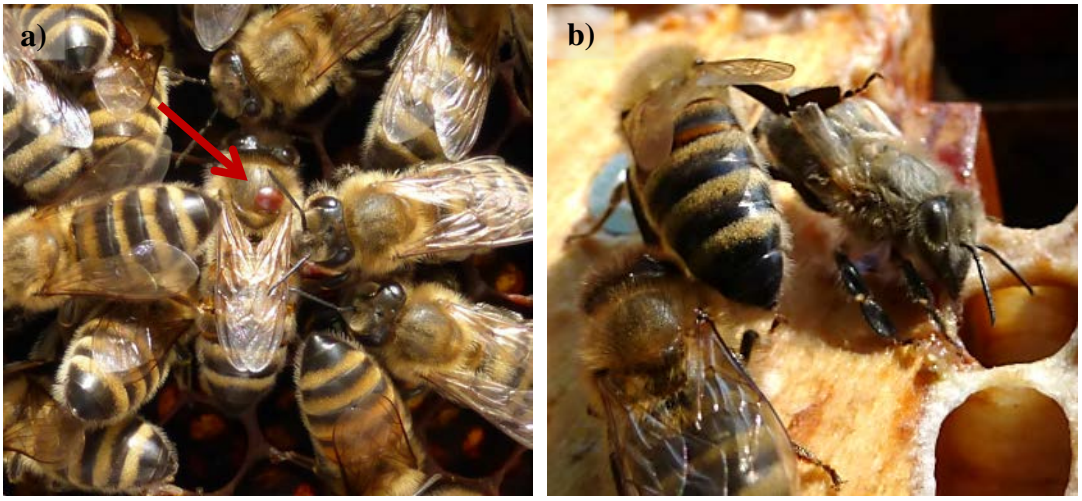

Figure 4. Honeybee (*Apis mellifera*) parasites and pathogens. a) Parasitic mite *Varroa destructor* attacking an adult *A. mellifera* worker bee; b) Adult bee showing typical disease symptoms (e.g. deformity of wings) of infection with the Deformed Wing Virus (DWV).

1.7 Resin collection and social immunity

Cooperative living in social Insects provides several benefits such as enhanced brood care or higher efficiency in foraging but also holds an increased risk of diseases. High numbers of closely related individuals on a relatively small spatial room and a fairly homeostatic environment are characteristic traits that favor the spread of diseases and makes them attractive to pests and pathogens (reviewed in Cremer et al. 2007). To counter this increased risk, besides an innate immune system (comprising humoral and cellular immune responses) (Evans et al. 2006), honeybees have further developed social defenses (Evans and Spivak 2010) such as hygienic behaviors, allo-grooming, undertaking, or so called "social fever", (whereby many bees simultaneously raise their body temperature to heat-kill bacteria) (Boecking and Spivak 1999; Starks et al. 2000; Spivak and Reuter 2001; reviewed in Evans and Spivak 2010), that contribute to overall health of colonies and therefore are also referred to as "social immunity" (Cremer et al. 2007).

Because, honeybees have evolved a relatively low individual immune response compared to other dipteran species (e.g. *Drosophila melanogaster*, *Anopheles gambiae*) (Evans et al. 2006) social immunity plays an vital role for colony health (Cremer et al. 2007; Evans and Spivak 2010). The collection and use of antimicrobial plant resins is another social defense strategy of honeybees. Since the collection of resins is a rather costly undertaking that bees follow up besides foraging for nutritional resources, it can be assumed to have an important meaning for them. In fact, it was shown that painting the inner walls of bee hives with resin/propolis ethanol extract lowers bacterial loads and investment in individual immune function (Simone-Finstrom et al. 2009). Propolis ethanol extract were further shown to be effective against Paenibacillus larvae (Bastos et al. 2008; Wilson et al. 2015), *Varroa destructor* (Garedew et al. 2002; Damiani et al. 2010) in-vitro and against Ascosphaera apis (Simone-Finstrom and Spivak 2012) and Paenibacillus larvae (Antunez et al. 2008) under field conditions. However, effects of ethanol extracts do not reflect how resins act under natural conditions. Therefore more recent works have started to investigate the effectivity of natural collected resins and could show that larger amounts of resin deposits within the nest atmosphere can support bees against P. larvae (Borba and Spivak 2017) and the interaction among *V. destructor* and DWV (Drescher et al. 2017).

2 Research scope and main research questions

Resin collection is an important part of the natural defense of honeybees (*Apis mellifera*) and stingless bees (Apidae: Meliponini) wherefore it can be assumed that the availability of appropriate resin sources has a crucial influence on the resilience and health of colonies (Fig. 5). Investigating the resin resource use and the significance of resins for colony health may thus provide important information to better promote and preserve these bees.

From stingless bees it is known that they exploit a wide variety of different resin sources. However, which plant species are used by honeybees in temperate regions and how they exploit available resources was hitherto largely unknown and research on this topic increased only recently (see chapter II). Moreover, it is unknown if bees might profit from a wider variety of resin sources by e.g. collecting more different resins, containing a wider variety of bio-active compounds and thereby enhancing protective function.

Resin/propolis is known as a natural remedy since ancient times and numerous studies have demonstrated wide range of antimicrobial, anti-fungicide, antiviral and immune enhancing properties with regard to human health (Marcucci 1995; Banskota et al. 2001; Sforcin 2007; Schnitzler et al. 2010; dos Santos et al. 2017). How resin/propolis benefits bees themselves is, however less well studied and research on this topic increased only recently. Therefore the general goal of this thesis was to provide new insight into the resin resource use and significance as well as the influence of resin diversity on colony health of honeybees and stingless bees, wherefore I have concentrated on the following questions:

- **Chapter II**:
  Resin resource use of honeybees *(Apis mellifera*)
  - Which tree species does the Western honeybees *(Apis mellifera* L.) in temperate regions use as resin sources?
  - How do they exploit available resources?
- **Chapter III**:
  Benefit of resins to honeybee health
  - Which influence has the availability of resin sources on the health of colony?
  - Can resin protect colonies from parasites and pathogens?
- **Chapter IV**:
  Influence of resin sources diversity
  - Do bees profit from a diversity of resin sources?
  - Does a greater diversity of resin resources/ resins enhance protective functions?

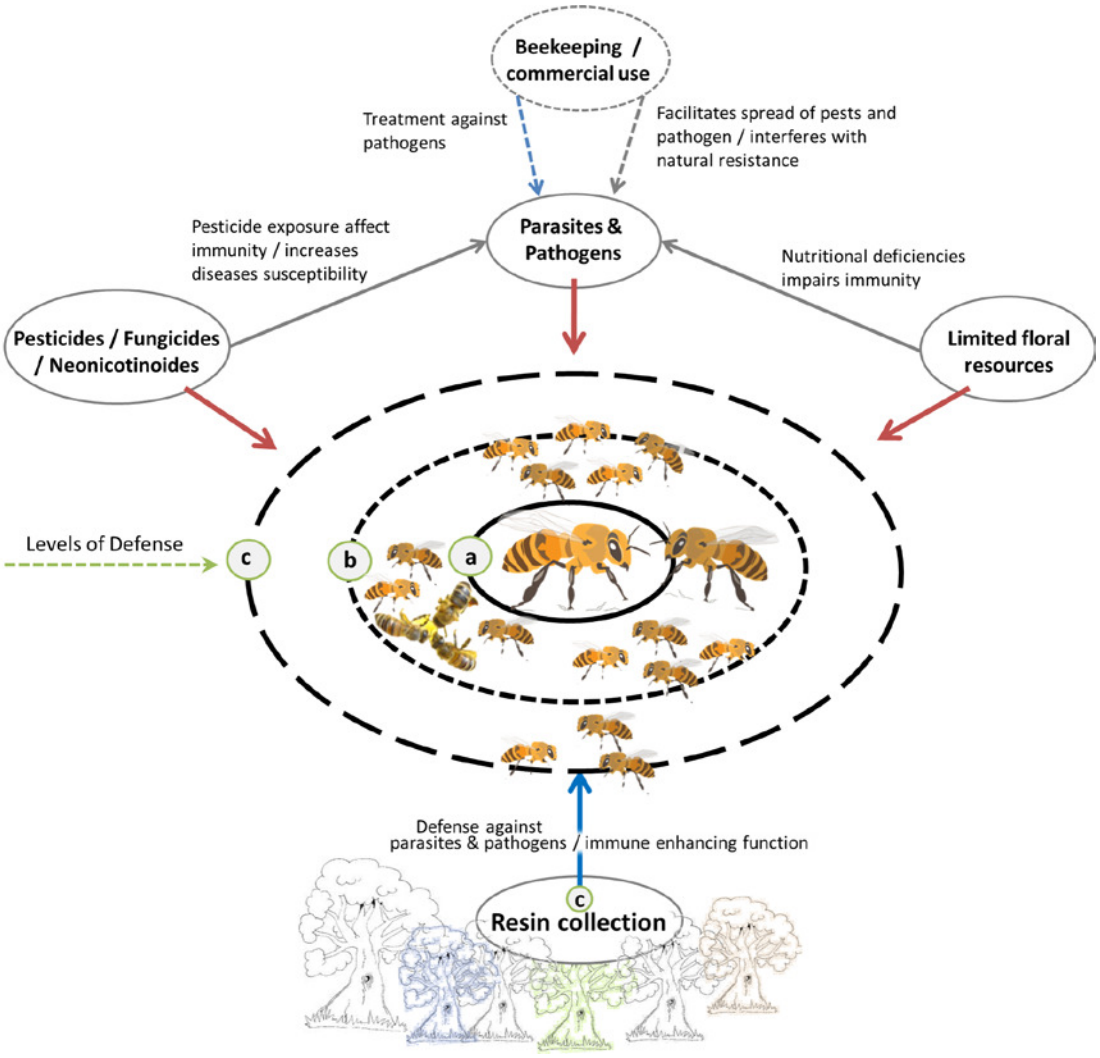

Figure 5. Resin collection as part of the natural defense of highly social bees.

Both, wild and managed bees suffer from impacts of multiple and partly interacting stressors. Sub-lethal effects from chronical exposure to cocktails of agrochemicals as well as nutritional stress resulting from limited or monotonous floral resources affecting bees by decreasing health of individuals and fitness of colonies, leading to an increased susceptibility to diseases. Additionally, bees are affected by increasing numbers of parasites and pathogens, whose single and combined effects can cause sustainable harm to the health of colonies. In managed honeybees, beekeepers support bees by conducting pest control. However, commercial use and beekeeping practice also facilitates rapid spread of pests and pathogen and interferes with adaptation to local pathogens. To protect themselves from various pests and pathogens highly social bees like honeybees (*Apis mellifera*) and stingless bees (Apidae: Meliponini) evolved several levels of defense: **(a)** Individual defenses **(b)** group level defenses (including allo-grooming, task differentiation and controlling entry of infectious agents and **(c)** colony level defenses such as the collection of antimicrobial plant resins (figure of defense levels adapted according to Evans and Spivak 2010). By reducing pathogen loads in the nest and increasing individual immunity, resin functions as social immune barrier and plays a vital role for the health of colonies. Similar to nectar and pollen bees use various resin sources and limited excess to appropriate sources might impact colony health. Knowledge on the sources used for resin collection as well as the importance of resin resource diversity can thus, provide information, important to enhance the natural defenses of colonies and thereby help them to encounter the increasing environmental pressure.

3 Thesis outline

I. *Resin resource use of the Western honeybee (Apis mellifera L.) in European temperate regions*

To study resin resources used by the Western honeybee (*A. mellifera* L.), I conducted field observations on the resin intake of colonies and took additional samples for identification of the botanical origin of collected resins. Field observation and sampling took place between June and October of 2012 and 2013 in Lower Saxony, in northwestern Germany. To identify the plant origin of bee-collected resin, I collected resin loads of returning resin foragers and compared it to samples I collected from different tree species from the surroundings of the apiary. Samples were chemically analyzed by GC/MS (gas chromatography coupled with mass spectrometer) and manually evaluated. To further investigate how bees exploit available resources, I observed resin intake of 28 *A. mellifera* colonies at seven different study sites. Different resin types collected by bees were categorized according to their color. To assess site-specific differences in the spectra of resins gathered by the colonies I further recorded the number of resin foragers carrying a particular color type within 15 minute time intervals.

I found that honeybees within the study area collected seven distinct resins types that are related to tree species of poplar (*Populus balsamifera*, *P. xcanadensis*), birch (*Betula alba*), horse chestnut (*Aesculus hippocastanum*), and coniferous trees (either *Picea abies* or *Pinus sylvestris*). Bees used multiple resin sources irrespectively from the study site, whereas relative proportions of different resins varied among sites. Moreover, bees showed clear preferences for three of the seven resin types.

II. *Benefit of resin/propolis to honeybee colony health*

To examine the effect of naturally collected resins on honeybee colony health I conducted an experimental field study between June and September 2013 in Lower Saxony, Germany. By manipulating resin intake of ten colonies by either adding or removing freshly gathered resins I investigated whether resin, as naturally deposited in the nests, can protect honeybees against the ectoparasitic mite, *Varroa destructor*, and associated viruses. Therefor I periodically (once per month) estimated mite infestation rates of colonies as well as colony strength. Additionally, I took samples of bees from each colony to analyze viral titers of four different viruses (Black Queen Cell Virus, Deformed Wing Virus, Israeli Acute Paralysis Virus and Sacbrood Virus) using reverse transcription and quantitative PCR.

I further conducted a laboratory assay to test possible effects of resin/propolis volatiles on *V. destructor* mite survival. Therefor I exposed 10 to 14 mites to 6g of grounded propolis and observed their survival over a time period of 36h.

In this study I found no effects of raw propolis on *V. destructor* mite survival and infestation levels. However, in relation to *V. destructor*, titers of the Deformed Wing Virus increased significantly less in colonies with added resin than in colonies where resin was removed.

III. *Influence of resin resource diversity*

To investigate whether and how bees may benefit from a diversity of different resins I performed several behavioral and microbiological assays that were conducted between February and April 2013 in Southern Queensland, Australia. I used resin extract from different tree species (all of which are known to be frequently used by stingless bees native to this region) to test how the protective function of resin varies among different sources and various target organisms (predators, parasites, and pathogens). To further estimate possible synergistic effects, I compared the effectivity of resins from different plant genera against a mixture of single resins. I used agar well diffusions technique to test for potential variances in bioactivity of the different resins against three categories of microorganisms (two gram-positive bacteria, three gram-negative bacteria, and one fungi). Further I conducted behavioral assays with three potential predatory species, two ants (green-head ants, *Rhytidoponera metallica*, and meat ants, *Iridomyrmex purpureus*) and one beetle (the small hive beetle, *Aethina tumida*), a common parasite of honeybees and stingless bees.

With this study I could demonstrate that resins from different trees can vary in their effectivity against different target organisms. Moreover, mixtures from different resins were more effective than some of the single resins, of different plant species can act synergistically when combined.

4 General discussion

The results of this thesis provide new insights into the resin resource use of the Western honeybee (*Apis mellifera* L.) and benefits of resin/resin diversity for honeybee and stingless bee health. With an experimental field study I could show that natural collected resin interferes with the destructive dynamic between *V. destructor* mites and DWV virus, further highlighting the great importance of resin/propolis for honeybee colony health. By following relatively new approaches I was able to prove for the first time that honeybees in European temperate regions use various trees from several genera as resin sources. Moreover, resin from different plant genera varied in their protective function against different target organisms, suggesting that bees can profit from a greater diversity of resin sources by collecting a broader range of resins and thereby enhancing their resilience against a variety of different pests and pathogens.

4.1 Resin resource use and influence of resin diversity (Chapter II. and IV)

Bees play a key role within most terrestrial ecosystems and the current decline in diversity and abundance of these species causes major concerns. Alteration of landscapes due to intensive agriculture and urbanization and the resulting loss of biodiversity including essential resources such as appropriate food plants and nesting space, are among the major drivers for this trend (Kremen et al. 2002; Winfree et al. 2009; Le Feon et al. 2010; Goulson et al. 2015; Papanikolaou et al. 2017). Resource use and availability determine some aspects crucial for survival of individuals and the maintenance of populations. Understanding which resources are required by a species and how available resources are exploited can thus provide important information to effectively promote and preserve it. As generalists honeybees (*Apis mellifera*) and stingless bees (Apidae: Meliponini) are known to exploit a wide variety of plant species to meet their nutritional needs and thus generally profit from a diversity of floral resources (Engel and Dingemans-Bakels 1980; Michener 2007; Alaux et al. 2010; Requier et al. 2015; Donkersley et al. 2017; Kaluza et al. 2017)

How honeybees and stingless bees exploit available resin sources and whether they profit from a diversity of resin producing plants, is, however comparatively less studied and received attention only recently. To better understand resin resource use and possible benefits arising from a diversity of resin producing plants, I conducted two different studies. In the first study (Chapter II) I focused on the resin resource use of the Western honeybee (*Apis*

mellifera L.) and in the second (Chapter IV) I investigated how different resins, as well as their mixtures vary in their effectivity against several potential antagonists.

4.1.1 Resin resource use of honeybees (Chapter II)

By tracking resin collection behavior of individual *A. mellifera* forager, I could show that this species uses a variety of different tree species as resin sources, but prefer some sources over others. Bees from the study hives did not necessarily collect resins from the closest resin source but gathered resins with specific chemical pattern and chose even among closely related tree species, indicating a highly selective forage strategy. Reasons for the preferences of specific resin types are so far unknown. Wilson et al. (2013) and Borba (2015) could show that resin from different source plants varied in vitro in their efficacy against *Paenibacillus larvae* (Wilson et al. 2013; Borba 2015) and *Aspergillus apis* (Borba 2015), but found no evidance that bees forage specifically for those resins with highest effectivity. Choice for a specific resin may, at least partly, be related to volatile components (e.g. mono-and sesquiterpenes) that are relevant to finding appropriate sources such as it is known from stingless bees (Leonhardt 2010). In fact, resin types frequently collected by bees contained striking amounts of the volatile benzoic acid. Benzoic acid occurred in resin of some poplar trees, but was lacking in other individuals of the same or closely related species, which could suggest that this substance play a role for choosing resin.

To the best of my knowledge hitherto there are only two further studies from north America (Wilson et al. 2013; Borba 2015) that analyzed resin collection of honeybees, using chemical analysis to track collection behavior of individual resin foragers. My results largely agreed with those of Wilson et al. (2013) and Borba (2015). However, in contrast to Wilson et al. (2013), who state that bees collect resins from *Populus balsamifera* and *P. deltoides* but not from the numerous hybrids, I found that bees collect resin from *Populus* hybrids as well. This is in accordance with the observations made by Borba (2015). Because, the landscape within the study area is strongly altered by human activities, this might be related to lacking of non-hybrid and/or native *Populus* species. For example occurrence of the native poplar species, *P. nigra* have become rare due to habitat loss (Cagelli and Lefèvre 1995; Smulders et al. 2008) and were likely not available to bees within the study region.

Moreover matches between bee-collected resin and tree resin revealed that bees use not only populous but also several further tree species as resin source. However, for one frequently collected resin type (ocher) I found no concordance to any of the sampled tree species and the source of this type remained unknown. Similar observations were made by Borba (2015), who

found evidences that bees in North America collect resin from several *Populus* species and from three further tree species that she could not identify. Interestingly I found almost no exact matches between bee-collected resin and tree resin but only significant similarities.

By observing resin intake of different colonies I found that the spectrum of resin types collected by single colonies ranged from three to six different types, suggesting that bees profit from the availability of different resin sources. Besides, chemical analysis revealed a striking compositional chemical diversity within and among different resin types indicating that bees may not only profit from a diversity of resin producing plants species, but also from a chemical diversity.

With this study I generated new information on which plant species honeybee's use as resin sources and how they exploit available resources. However, there are still several unexplained facts such as the lacking of concordance between two of bee-collected resin types ("ocher" and "yellow") and any of the sampled tree species, or the compositional discrepancies between bee-collected resin and tree resin. Understanding of which botanical sources are used and preferred by bees, could be important to choose or create environments that promote health of these animals by supporting resin collection. Therefore future studies should focus on the causes underlying the choices and the observed variability in resin intake.

4.1.2 Influence of resource diversity (Chapter IV)

With the second study I could show that, based on their chemistry, resins of different plant genera vary in their efficacy against different potential aggressors (predators, pests, and pathogens) and can act synergistically when combined. This indicates that mixing resins from different sources may benefit bees by enhancing the protective function of propolis/batumen against multiple antagonists.

Both, stingless bees and honeybees collect resins from a variety of different plant species and mix them within their nests (Leonhardt and Blüthgen 2009; Isidorov et al. 2016; Kaluza et al. 2017; Drescher et al. 2019). As both strongly rely on the protective function of resins (Seeley and Morse 1976; Roubik 1989; Lehmberg et al. 2008; Leonhardt et al. 2010; Borba et al. 2015; Borba and Spivak 2017; Drescher et al. 2017), availability of a variety of different resin sources may be essential for them.

For stingless bees it was shown, that diversity of resin intake increases with tree diversity and abundance suggesting that bees maximize resource diversity intake in habitats with high diversity (Kaluza et al. 2017). However, due to their commercial use for pollination of crop

plants, honeybees and stingless bees are frequently placed in rather unfavorable landscapes (e.g. large monocultures). Compared to natural or semi-natural habitats, landscapes intensively managed for agricultural use often comprise of lower plant species richness (Hostetler and McIntyre 2001; McKinney 2008; Decourtye et al. 2010) and thus usually provide less diverse resources. Because functional properties of resources (e.g. nutritional content of pollen or protective functions of resin) can vary among different sources (Roulston and Cane 2000; Pacini et al. 2003; Drescher et al. 2014), the diversity (Alaux et al. 2010; Donkersley et al. 2017) and abundance (Brodschneider and Crailsheim 2010; Di Pasquale et al. 2013) of resources can critically influence bees health. Consequently bees may not only suffer from lack of nutrition but also from appropriate resin sources when brought to intensively managed agricultural lands. Honeybees, similar to stingless bees, were shown to be selective in their choice of resins and use only specific tree species (Leonhardt and Blüthgen 2009; Leonhardt et al. 2011; Wilson et al. 2013; Drescher et al. 2019). Compared to stingless bees, honeybees collect resins mainly from buds or young sprouts of a comparatively low number of tree species (Wilson et al. 2013; Drescher et al. 2019). Thus, the strategy honeybees in temperate regions follow for resin collection might differ from that of tropical stingless bees. Consequently, honeybees may be especially susceptible to a reduced availability of preferred resin resources and the availability of appropriate resin sources should thus find more attention in (commercial) bee-keeping praxis.

4.2 Benefits of resin to colony health (Chapter III)

Honeybee (*Apis mellifera*) health became a widely discussed topic in recent years. As important pollinator of many wild and crop plants *A. mellifera* perform a vitally important ecosystem function and play a crucial role for human food security. Much research effort has been conducted to identify parasites and pests to improve the health of honeybees. In this context, the parasitic mite *Varroa destructor* in association with viruses has received particular attention due to its substantial role for high colony losses especially over winter. The detrimental interaction among *V. destructor* and the Deformed wing virus (DWV) is considered as one of the major cause for the observed losses (Ghisalberti 1979; Genersch, E. et al. 2010; Dainat et al. 2011; Dainat et al. 2012). Therefor I conducted an experimental field study to analyze if resin collection, as the natural defense of honeybees, can protect colonies from the impact of *V. destructor* mites in combination with DWV.

The results of this study showed that DWV viral titers were lower in relation to *V. destructor* mite loads, when colonies had large amounts of resin deposits (propolis), indicating that resin

collection can interfere with the destructive dynamic between these two pathogens. By affecting development of healthy winter bees, which play a critical role for colony winter survival in temperate regions, *V. destructor* in combination with DWV can cause substantial damage that (without human intervention) often leads to a total collapse of colonies (Genersch, E. et al. 2010; Dainat et al. 2011; Dainat et al. 2012). In my study colonies viral titers of DWV were not reduced due to the treatment, but increased significantly less in relation to *V. destructor*, when colonies were equipped with large amounts of propolis, suggesting that it can support bees to overcome infections. This could be due to the known antiviral properties of propolis (Amoros et al. 1992; Gekker et al. 2005; Schnitzler et al. 2010) but probably also due to its immune enhancing function (reviewed in Marcucci 1995; Burdock 1998). However, I found no evidence that natural propolis can interfere with *V. destructor* survival or colony infestation, which stands in contrast to the findings of previous studies that state lethal and narcotic effects resulting from propolis ethanol extracts (Garedew et al. 2002; Damiani et al. 2010). To the best of my knowledge, so far, there are only two other studies (Borba et al. 2015 and 2017) analyzing colony level effects of natural propolis. In both a slightly different method was used, where half of the colonies were provided with propolis traps in order to encourage them to build a propolis envelope. Using this method Borba (Borba et al. 2015) could show a lower investment in immune function, indicating a reduced need to activate the physiologically costly production of humoral immune responses. Further Borba and Spivak (2017) found that the presence of a natural propolis envelope is effective in reducing infection with *Paenibacillus larvae* (causative agent of AFB), but, however, does not completely prevent clinical symptoms of AFB.

The results of this study are comparable with my findings in so far as that it shows that even though natural resin deposits cannot prevent occurrence of diseases, it helps bees to overcome pathogenic challenges. By enhancing individual immune competence on the one hand (Simone-Finstrom et al. 2009; Borba et al. 2015) and affecting pathogenic growth on the other (Simone-Finstrom and Spivak 2012; Borba and Spivak 2017; Drescher et al. 2017), resin has a high potential to interfere with the destructive dynamic resulting from interactions of multiple stressors, emphasizing its crucial role for the health and resilience of colonies.

With this study I could show for the first time that naturally collected resins can enhance disease resistance of colonies by interfering with the damaging interaction between two pathogenic stressors, *V. destructor* and DWV virus. Thereby my findings shed light on some new aspects regarding to the functional properties of resin as a colony level defense, further

highlighting its substantial role for honeybee health. However, further studies under field conditions are necessary to better understand the functional role of resin as well as the mechanisms involved in their collection and use. Moreover, discrepancies between results of field and laboratory assays underline the necessity of studies under more realistic conditions.

4.3 Conclusion

Plant resins serve honeybees and stingless bees for various purposes, including nest construction and sealing as well as the defense against pests and pathogens (Seeley and Morse 1976; Roubik 2006; Simone-Finstrom et al. 2009; Leonhardt et al. 2010; Borba and Spivak 2017; Drescher et al. 2017). Despite its remarkable and diverse functions, as well as its significance for colony health, the use of resin receives comparatively little attention in beekeeping practice. In contrast, (honeybee) beekeeper often dislike the collection and use of resin/propolis, as it sticks e.g. the hive frames together and thus makes handling of bees more difficult and time intensive. Accordingly, it was a long time (and is probably still) regarded as an “unpleasant, ugly” quality (see page 7, breeding instructions (Bruder Adam 1953)) and breeding of resin-free honeybees as well as missing of appropriate resin sources due to human alteration of landscapes, could have negatively impacted the resilience of colonies. Both, wild and managed bees suffer from single and combined effects of multiple stresses such as nutritional deficiencies, pests and parasites and pesticides (reviewed in Goulson et al. 2015). Thereby, multiple interacting stressors can lead to the impairment of immune function and detoxification mechanisms and increase susceptibility to diseases (Boncristiani et al. 2012; Di Prisco et al. 2013; DeGrandi-Hoffman and Chen 2015; McMenamin et al. 2016). By enhancing individual immunity of honeybees on the one hand (Simone-Finstrom et al. 2009; Mao et al. 2013; Borba et al. 2015) and improving disease resistance of colonies on the other (Simone-Finstrom and Spivak 2012; Borba and Spivak 2017; Drescher et al. 2017), resin/propolis functions as a kind of external immune defense and thus play an important role in this context (Fig. 5).

Consequently, resin is an essential resource for honeybees and stingless bees and promoting resin collection as their natural defense could be a sustainable way to improve disease resistance and the resilience of the colonies. However, further research is needed to better understand the mechanisms and targets underlying resin collection and find appropriate ways that support resin intake and encourage the use of resins within bee hives.

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6 Article overview

Title of the PhD thesis:

„ Plant resin - an underestimated resource for bees: How honeybees (*Apis mellifera* L.) and stingless bees (Apidae: Meliponini) benefit from a diversity of resin sources“

Papers included

I. Drescher Nora, Klein Alexandra-Maria, Schmitt Thomas, Leonhardt Sara Diana (2019) A clue on bee glue: New insight into the sources and factors driving resin intake in honeybees. Published in: PLoS ONE 14(2):1-20, DOI: https://doi.org/10.1371/journal.pone.0210594

II. Drescher Nora, Klein Alexandra-Maria, Neumann Peter, Yañez Orlando, Leonhardt Sara Diana (2017) Inside honeybee hives: Impact of natural propolis on the ectoparasitic mite *Varroa destructor* and viruses. Published in: Insects 8(15): 1-18, DOI: https://doi.org/10.3390/insects8010015

III. Drescher Nora, Wallace Helen M., Katouli Mohammad, Massaro Carmelina F., Leonhardt Sara Diana (2014) Diversity matters: How bees benefit from different resin sources. Published in: Oecologia 4(176): 943-953, DOI: https://doi.org/10.1007/s00442-014-3070-z

Co-authored paper (not included)

Kaluza Benjamin F., Wallace Helen, Keller Alexander, Heard Tim A., Jeffers Bradley, Drescher Nora, Blüthgen Nico, Leonhardt Sara D.(2017) Generalist social bees maximize diversity intake in plant species-rich and resource-abundant environments. Published in: Ecosphere 8(3):1-19, DOI: https://doi.org/10.1002/ecs2.1758

Declaration are in accordance with the guidelines for cumulative dissertations in Sustainability Science [February 2011], in the following referred to as “the guideline”.

Authors' contribution to articles and article publication status, according to §16 of the guideline.

Article	Short title Publication	Specific contributions of all authors*	Author status	Weighing Factor**	Publication status	Conference Contributions
# I	Sources and factors driving resin intake in honeybees	ND: Data collection, ND, TS, SL: Data analysis, ND, TS, SL: Research question and design, ND: Writing – original draft SL, TS, AM: Writing – review & editing	Co-author with pre-dominant contribution	1.0	2019 Published in: PLoS ONE 14(2): IF 2.76	
# II	Impact of natural propolis on *V. destructor* and viruses	ND: Data collection, ND, OY, SL, PN: Data analysis, ND, SL, AK ,PN: Research question and design, ND: Writing – original draft SL, OY, AK, PN: Writing – review & editing	Co-author with pre-dominant contribution	1.0	2017 Published in: Insects 8(15), IF 1.81	
# III	How bees benefit from different resin sources	ND: Data collection, ND, SL, CM, MK: Data analysis, SL: Research question and design, ND: Writing – original draft SL, CM, MK, HW: Writing – review & editing	Co-author with equal contribution	1.0	2014 Published in: Oecologia 176(4), IF 3.13	
			Sum	**3.0**		

*Specific contributions of all authors

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MK	Mohammad Katouli

**Publication status

IF ISI Web of Science - Impact Factor respectively in the year of publication

Author status

According to §12b of the guideline:

Single author (Allein-Autorenschaft): Own contribution amounts to 100%.

- Co-author with predominant contribution (überwiegender Anteil): Own contribution is greater than the individual share of all other co-authors and is at least 35%.
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- Co-author with important contribution (wichtiger Anteil): own contribution is at least 25%, but is insufficient to qualify as single authorship, predominant or equal contribution.
- Co-author with small contribution (geringer Anteil): own contribution is less than 20%.

Weighting factor

According to §14 of the guideline:

- Single author (Allein-Autorenschaft) 1.0
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- Co-author with equal contribution (gleicher Anteil) 1.0
- Co-author with important contribution (wichtiger Anteil) 0.5
- Co-author with small contribution (geringer Anteil) 0

Chapter II

A clue on bee glue: New insight into the sources and factors driving resin intake in honeybees (*Apis mellifera*)

Nora Drescher, Alexandra-Maria Klein, Thomas Schmitt and Sara Diana Leonhardt

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Abstract

Honeybees (*Apis mellifera*) are threatened by numerous pathogens and parasites. To prevent infections they apply cooperative behavioral defenses, such as allo-grooming and hygiene, or they use antimicrobial plant resin. Resin is a chemically complex and highly variable mixture of many bioactive compounds. Bees collect the sticky material from different plant species and use it for nest construction and protection. Despite its importance for colony health, comparatively little is known about the precise origins and variability in resin spectra collected by honeybees. To identify the botanical resin sources of *A. mellifera* in Western Europe we chemically compared resin loads of individual foragers and tree resins. We further examined the resin intake of 25 colonies from five different apiaries to assess the effect of location on variation in the spectra of collected resin. Across all colonies and apiaries, seven distinct resin types were categorized according to their color and chemical composition. Matches between bee-collected resin and tree resin indicated that bees used poplar (*Populus balsamifera, P. x canadensis*), birch (*Betula alba*), horse chestnut (*Aesculus hippocastanum*) and coniferous trees (either *Picea abies* or *Pinus sylvestris*) as resin sources. Our data reveal that honeybees collect a comparatively broad and variable spectrum of resin sources, thus assuring protection against a variety of antagonists sensitive to different resins and/or compounds. We further unravel distinct preferences for specific resins and resin chemotypes, indicating that honeybees selectively search for bioactive resin compounds.

Introduction

Honeybees (*Apis mellifera*) are threatened by numerous pests and pathogens (Moritz et al. 2010; vanEngelsdorp and Meixner 2010; Gisder and Genersch 2015). They further have to deal with multiple environmental stressors caused by agricultural intensification, such as pesticides, alteration of foraging landscapes, reduced resource diversity and rapidly spreading new pests and pathogens (Klee et al. 2007; Alaux et al. 2010; Johnson et al. 2010; Jose Orantes-Bermejo et al. 2010; DeGrandi-Hoffman and Chen 2015; Donkersley et al. 2017). Impacts of single or combined stressors can have devastating consequences for honeybee health and can even lead to the total collapse of colonies as seen e.g. in the high winter losses in Europe and North America (reviewed in (Goulson et al. 2015)). To increase the resilience of colonies, instead of only addressing the symptoms, a sustainable strategy is to promote the bees' natural defenses (Spivak 1996; Spivak and Reuter 2001; Ibrahim et al. 2007; Niño and Cameron 2015). Honeybees perform several collaborative behaviors, such as allo-grooming, nest hygiene or the collection and use of antimicrobial plant resins, which are referred to as social or external immunity and play an essential role for colony health (Breed et al. 2004; Cremer et al. 2007; Evans and Spivak 2010; Otti et al. 2014). While much attention has been paid to hygienic behaviors, the use and role of plant resins has been largely neglected until recently (Simone-Finstrom et al. 2009; Simone-Finstrom et al. 2017).

Plant resin is a sticky, water insoluble substance which plants secrete primarily to protect injured tissue, young sprouts or leaf buds from herbivore and/or pathogen attack (Langenheim 2003). Resin comprises a chemically complex mixture of often more than 300 substances (mostly phenolic compounds such as flavonoids, aromatic carboxylic acids, and benzopyranes, and terpenoids), many of which show antimicrobial and/or repellent properties (Langenheim 2003). The chemical composition of resin is typically plant species-specific, but can vary greatly - both qualitatively and quantitatively - within and between plant families and even among closely related plant species (Langenheim 2003).

Apis mellifera as well as some tropical stingless bees (Apidae: Meliponini) use resins for nests construction and as defense against pests and pathogens (Roubik 2006; Duangphakdee et al. 2009; Leonhardt and Blüthgen 2009; Simone-Finstrom et al. 2009). Resin is often mixed with various amounts of wax, resulting in a tough, sticky matter which is called propolis or bee glue (Ghisalberti 1979). Non-managed, feral colonies of honeybees coat their entire nest interior with a thin propolis layer referred to as "propolis envelope" (Seeley and Morse 1976). Such a "propolis envelope" can reduce the load of microbes and decrease the expression of (at least two) immune-related genes (Simone-Finstrom et al. 2009). Moreover, several studies demonstrated that propolis reduced the growth of important microbial bee pathogens, i.e. *Paenibacillus larvae*, the causative agent of

American Foulbrood (Bastos et al. 2008; Wilson et al. 2015; Borba and Spivak 2017), and *Ascophaera apis*, a fungal parasite that causes chalkbrood (Simone-Finstrom and Spivak 2012; Wilson et al. 2015). Resin/propolis further enhanced the immune activity of individual bees, which enabled them to more effectively fight pathogenic challenges (Simone-Finstrom et al. 2009; Nicodemo et al. 2013; Borba et al. 2015; Drescher et al. 2017; Simone-Finstrom 2017). Consequently, resin plays a crucial role for colony health and represents an essential resource for honeybees.

Despite its obvious importance, we still know relatively little about resin collection in honeybees. While tropical stingless bees were shown to collect resins from a wide range of plant species (Leonhardt and Blüthgen 2009; Wallace and Lee 2010), precise information on the plant sources used by *Apis mellifera* is still scarce and largely restricted to sporadic observations (e.g. of foragers on hybrid *Populus* spp., France (Huber 1814), and coniferous trees, North America (Alfonsus 1933)), few chemical comparisons (e.g. for *Baccharis dracunculifolia,* Brasil (Teixeira et al. 2005), and *Populus deltoides*, *P. balsamifera* and hybrid *Populus* spp., North America (Wilson et al. 2013; Borba 2015)) and inferences from major chemical compounds of propolis (Bankova et al. 2000; Bankova et al. 2006; Isidorov et al. 2016). However, propolis is typically produced by mixing different resins (and wax), which renders inferences on single original resin sources or on variation among different resin sources difficult, as some compounds (e.g. camphene, *alpha*- and *beta*-pinene, limonene and myrcene) occur in resins of numerous plant taxa (Langenheim 2003). Moreover, identifying botanical sources based on observations is challenging, because the actual foraging process is difficult to observe as it is usually performed by a relatively low number of bees (Simone-Finstrom and Spivak 2012) and as it can occur high up in the trees (Crane 1988).

Based on the above listed studies, poplar species (hybrid *Populus* spp.), horse chestnut (*Aesculus hippocastanum*), alder (*Alnus* spp.), birch (*Betula* spp.), willow (*Salix* spp.) and some others are currently considered the main resin sources for honeybees in temperate regions (Ghisalberti 1979; Greenaway et al. 1990; Bankova et al. 2006). However, most plant species have not yet been confirmed as actual resin source through thorough chemical comparison (but see (Wilson et al. 2013)), and information on possible preferences as well as variation in the collection of different resin sources over space and time is largely missing.

Such information can only be inferred by tracing the collection behavior of single resin foragers, e.g. by collecting and chemically analyzing resin loads from hind legs of returning foragers. To the best of our knowledge, there are only two studies from North America that chemically analyzed loads from individual resin foragers (Wilson et al. 2013; Borba 2015). However, both studies investigated resin collection of bees for only one apiary/site and do therefore not provide information on variation between sites and over time.

Following up on the pioneering results of (Wilson et al. 2013), our study aimed to identify botanical sources of resins collected by *Apis mellifera* in Western Europe through comparative chemical analysis at the level of individual foragers. We further examined the resin intake of 28 different colonies from seven different apiaries placed at locations differing in the surrounding landscape structure and thus composition and/or relative abundance of tree species to assess the effect of location on variation in the collected resin spectra.

Material & methods

Study sites and honeybee colonies

Observations of resin collection behavior and sampling of resin from bees and trees were conducted at seven different sites in Lower Saxony, in northwestern Germany, between June and October 2012/2013. Distances between all sites ranged from minimum 1.3 km to maximum 38 km and, for sites included in the analysis of site specific differences, from 4.6 km to 29.5 km, respectively (S1 Fig). The habitat surrounding apiaries was characterized by either an agricultural landscape with croplands (dominated by corn maize, rapeseed and potato), pastures and mixed forests (N=5sites) or an urban landscape with parks, small gardens or allotments and roadside trees (N=2sites; Table 1). In general, this region is dominated by a continental climate with typical forest assemblages comprising oak (*Quercus robur /petraea*), beech (*Fagus sylvatica*), pine (*Pinus sylvestris*) and spruce (*Picea abies*). Other common occurring deciduous tree species belong to the genera birch (*Betula),* poplar (*Populus*), alder (*Alnus*), maple (*Acer*), ash (*Fraxinus*) and linden (*Tilia*). Our study region thus comprised several tree species assumed to be resin sources of *Apis mellifera*, i.e. *Betula spp.*, *Aesculus hippocastanum, Alnus spp.* and several species within the genus *Populus* (i.e. several unknown hybrids of *P. xcanadensi* of the section *Aigeiros*, *P. balsamifera* of the section *Tacamahaca,* and *P. tremula* of the section *Populus*). Individuals from all species were available within the flight range of each apiary, except for *P. balsamifera* which occurred only at two sites and *A. hippocastanum* which was missing at one site.

Observation of resin intake

Resin intake of 28 *A. mellifera* colonies of seven different apiaries was observed by opening each bee colony and searching for resin foragers on the top of the frames for 15 minutes. Because unloading of resin loads frequently occurs on the top of the frames with the help of one or two other bees (personal observation) here, resin foragers can be easily identified and captured. Different resins collected by bees were categorized according to their color (Kaluza et al. 2017), resulting in seven distinct resin color types, i.e. “orange”, “red”, “ocher”, “green-brown”, “light clear”, “yellow” and “whitish” (all also including varieties of the respective color, e.g. light-ocher or dark-orange, see Fig 1). Categories were then confirmed chemically (see below). Data from all 28 colonies was used to capture the whole spectrum of resins collected by bees in the study area. All resin color types were

further sampled for chemical analysis as described below. To avoid multiple counts of the same bee individual, resin foragers were either kept in plastic tubes until the end of the observation or their resin loads were taken before releasing them again. Colonies were observed three to eight times (16 colonies) or, due to time constrains, only one to two times (12 colonies). To assess site-specific differences in the spectra and diversity of resins gathered by colonies we further recorded the number of returning resin foragers carrying a particular color type within time intervals of 15 minutes. Analysis of site-specific differences was restricted to colonies that were observed at three or more different days (Table 1). Each of these 16 colonies was observed at 6 ± 3 (mean ± standard deviation (SD)) days between 12 and 6 pm (Table 1). Finally, temperature was recorded for each observation interval to assess possible influences on the resin intake

Table 1. Overview of the study sites and honeybee colonies.

a) Different sites are described by abbreviations (i.e. "Bb" Bienenbüttel, "Et" Ebstorf, "Ez" Eitzen, "Gr" Grünewald, "Lg" Lüneburg, "Ml" Melbeck, "Ol" Oldershausen). b) Total number of resin collecting bees observed per colony. c) Apiaries were located either in agricultural or urban environments. The surrounding environment differed between apiaries (described in brackets). For each colony (colony ID) number (N) of observation days, total number (N) of observed resin foragers and whether (x) or not (no entry) propolis samples were taken is given. In total, resin intake was observed in 28 Apis mellifera colonies of seven different apiaries between 12 and 6 pm.

Site[a]	Colony ID	N of observations	N of resin foragers[b]	Propolis sample	Environment[c]
Bb	1	1	5		agricultural (mixed forest, pastures)
Bb	2	2	18		agricultural (mixed forest, pastures)
Bb	3	1	3		agricultural (mixed forest, pastures)
Bb	4	1	5		agricultural (mixed forest, pastures)
Bb	5	2	19		agricultural (mixed forest, pastures)
Et	1	1	5		agricultural (coniferous forest)
Et	5	1	5		agricultural (coniferous forest)
Ez	1	1	7	x	agricultural (mixed forest, pastures)
Ez	2	1	19		agricultural (mixed forest, pastures)
Gr	1	2	4		agricultural (cropland, patches of mixed forest)
Gr	2	3	27		agricultural (cropland, patches of mixed forest)
Gr	3	6	49		agricultural (cropland, patches of mixed forest)
Gr	5	3	11		agricultural (cropland, patches of mixed forest)
Gr	7	6	48	x	agricultural (cropland, patches of mixed forest)
Gr	9	2	29	x	agricultural (cropland, patches of mixed forest)
Lg	4	8	15		urban (gardens, small forest)
Lg	5	11	43		urban (gardens, small forest)
Lg	6	12	36		urban (gardens, small forest)
Lg	11	10	24	x	urban (gardens, small forest)
Lg	12	9	22	x	urban (gardens, small forest)
Ml	2	4	34	x	urban (gardens, small forest)
Ml	4	3	23		urban (gardens, small forest)
Ol	1	4	21		agricultural (pastures, groves)
Ol	2	5	25		agricultural (pastures, groves)
Ol	3	2	17		agricultural (pastures, groves)
Ol	7	5	24		agricultural (pastures, groves)
Ol	8	6	18		agricultural (pastures, groves)
Ol	9	8	88		agricultural (pastures, groves)

Fig 1. Resin foragers carrying different resin types on their hind legs.
Different resin (color-) types (A-F) were used for visual determination of different resin types: A) "ocher", B-C) "orange", D) "clear", E) "yellow", F) "brown".

Identification of resin sources – sampling of bees and trees

To identify the plant sources of resins collected by bees we compared the chemical composition of resin loads of individual foragers with resin collected from locally occurring tree species. Resin loads were sampled from returning foragers by capturing them in a plastic tube with a grid attached at one side (as commonly used to mark honeybee queens) and carefully removing resin loads from the corbicula of their hind legs using forceps. Tree resins were collected from individual trees within the close surrounding (approximately 0.5 - 2 km flight distance) of our study apiaries by gently scraping natural resin secretions of buds or, in case of *Picea abis* and *Pinus sylvestris*, from fresh wounds. Samples of three buds or multiple sprouts were pooled per tree, and, if possible, a minimum of three individuals per tree species were sampled and analyzed to account for variation between individuals. We collected resins from eight different tree species: *Aesculus hippocastanum* (3 samples/trees), *Alnus glutinosa* (3), *Betula alba* (4), *Populus balsamifera* (1), *Populus tremula* (3), *Populus x canadensis* (9), *Picea abis* (2) and *Pinus sylvestris* (3).

Overall 37 samples of bees and 28 samples of trees were collected at seven different apiaries to account for possible variation between sites. All samples (of bees and trees) were immediately immersed in hexane and cooled (-18 C°) until chemical analysis. We additionally took samples of propolis from two individual colonies per site. Propolis was obtained by placing commercial plastic grids on top of the frames from June to September and collected once in September. For chemical analysis 0.3 g of ground propolis from each grid were extracted in hexane.

Chemical analysis

Hexane extracts of propolis and resin samples were analyzed by gas chromatography coupled with mass spectrometry (GC-MS) using a Hewlett Packard HP 6890 Series Gas Chromatographic System coupled to a Hewlett Packard HP 5973 Mass Selective Detector (Agilent Technologies, Böblingen, Germany) and a Shimadzu QP2010 Ultra GC/MS (Shimadzu, Duisburg, Germany). The following tree resin samples, i.e. POL1 (poplar Px2), POL2 (poplar Px2), LG109 (spruce) and bee collected resins: LG111 (spruce), OL126 (orange), BB204 (whitish), OL131 (ocker), OL128 (orange), BB206 (brown), were analyzed by the Hewlett Packard HP 5973 Mass Selective Detector (Agilent Technologies, Böblingen, Germany), while all other samples were analyzed with Shimadzu QP2010 Ultra GC/MS (Shimadzu, Duisburg, Germany). The GCs were equipped with a J & W, DB-5 fused silica capillary column (30 m×0.25 mm ID; df=0.25 μm; J & W, Folsom, CA, USA). Temperature was programmed from 60°C to 300°C with a 5°C/min heating rate and was held at 300°C for 10 min. Helium was used as carrier gas with a constant flow of 1 ml/min. Injection was carried out at 250°C in the splitless mode for 1 min. Electron impact mass spectra (EI-MS) were recorded at an ionization voltage of 70 eV and a source temperature of 230°C. We used the Windows version of the ChemStation software package (Agilent Technologies, Böblingen, Germany) and the GCMSsolution Vers. 2.7 for data acquisition. Sample chromatograms were analyzed manually by characterizing each peak of a chromatogram and comparing them across samples/chromatograms. Peaks were characterized by their retention times and mass spectra. A personal library was used to match peaks of different chromatograms, where we considered peaks with the same retention times and mass spectra the same substances (including both identified and unidentified substances). We then calculated relative peak areas for each compound by dividing the integrated area of each peak by the total area of all peaks. We additionally ran synthetic alkanes (Sigma-Aldrich, Munich, Germany) to confirm identification and to calculate Kovats indices. To finally identify different substance classes and, where possible, substances, if possible, we used three commercially available mass spectra libraries (Wiley 275, NIST 98 and Adams EO library 2205) and
diagnostic ions and Kovats indices to verify our characterization.

Statistical analysis

To analyze the effect of the surrounding habitat (site effects) on the spectrum of resin collected by individual colonies we performed permutation tests based on relative frequencies of different resin color types (Adonis command, R package vegan (Oksanen et al. 2017)). Differences in resin diversity were assessed by comparing resin type richness (pooled across colonies) between apiaries/sites using a Pearson's chi^2-test. Kruskal-Wallis ANOVA was used to investigate site-specific differences in the proportion of single resin types collected. Possible correlations between the intake of different resin types and between temperature and the total number of resin foragers were analyzed using Kendall's rank correlation tau test (R Core Team 2017). Prior to analyses data was checked for normality and homogeneity of variances using Shapiro-Wilk test and Fligner-Killeen test.

To identify the botanical sources of bee-collected resins we compared the chemical composition of bee-collected resins and tree resins. Prior to analysis trace compounds accounting for less than 0.05% (resin samples) or 0.1% (propolis samples) of the total peak area had been removed. Thresholds for resin and propolis samples were different to account for the higher concentration of propolis samples compared to bee and tree resin samples. All remaining (identified and unknown) substances were displayed by their relative peak areas (see above), which were used for all subsequent statistical analyses. We first investigated differences in the chemical composition of bee-collected resins between different color types. Second, we analyzed inter- and intra-specific differences in the chemical composition of tree resins. We then analyzed similarities of bee-collected resins and tree resins. All comparisons ((1) resin color types, (2) tree resins, (3) bee-collected resins/tree resins) were performed by permutation tests (Adonis command, R package vegan, 10000 runs) based on Bray-Curtis distances between compounds. Two-dimensional NMDS (non-metric dimensional scaling) based on Bray-Curtis distances (Oksanen et al. 2017), was used to generate ordination figures. Additionally, differences in the chemical composition of propolis samples from different colonies and different sites were analyzed as described above but excluding all alkanes and alkenes as potentially bee-/ wax-derived compounds. All analyses were performed in R statistical software version 3.4.1 (R Core Team 2017). Due to multiple testing the same data sets we only considered p-values < 0.01 significant.

Results

Resin intake

Overall, we observed 661 returning resin foragers from 28 colonies of seven apiaries located at seven different sites (Fig 2). The average number of resin foragers (calculated as the average total number of resin foragers observed across all colonies and sites for each month) was lowest in June (mean number of bees [± SD]: 4 ± 5), increased in July (6 ±5) and August (6 ± 6) and decreased again in September / October (5 ± 5). The total number

of resin foragers further increased with increasing temperature (Kendall's rank correlation tau: $z = 2.42$, $P < 0.015$, $tau = 0.16$). Across colonies and apiaries, we categorized seven distinct resin types according to their color and chemical composition, i.e. „ocher", orange-brown (type "orange"), red-brown (type "red"), green-brown (type "brown"), "yellow", "clear" and "whitish", with "ocher" (51% of all recorded resin foragers) and "orange" (32%) most frequently observed (Fig 2).

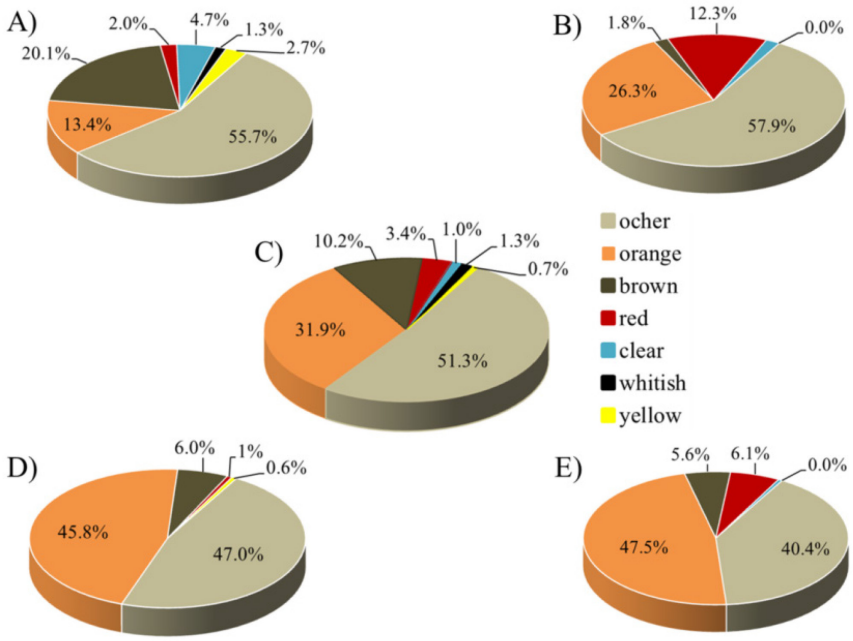

Fig 2. Percentages of different resins types collected by resin foragers from seven apiaries in Lower Saxony, Germany.
C) total of foragers (N=661), A) – E) four exemplary sites (A) site Lg (N=149); B) site Ml (N=57); D) site Gr (N=168) and E) site Ol (N=198)). For meaning of abbreviations and site descriptions see Table 1. Different resins types were characterized by color (i.e. "ocher", "orange", "brown", "red", "yellow" "whitish", and "clear") during field observations and subsequently verified by chemical analysis (via gas chromatography and mass spectrometry, GC-MS).

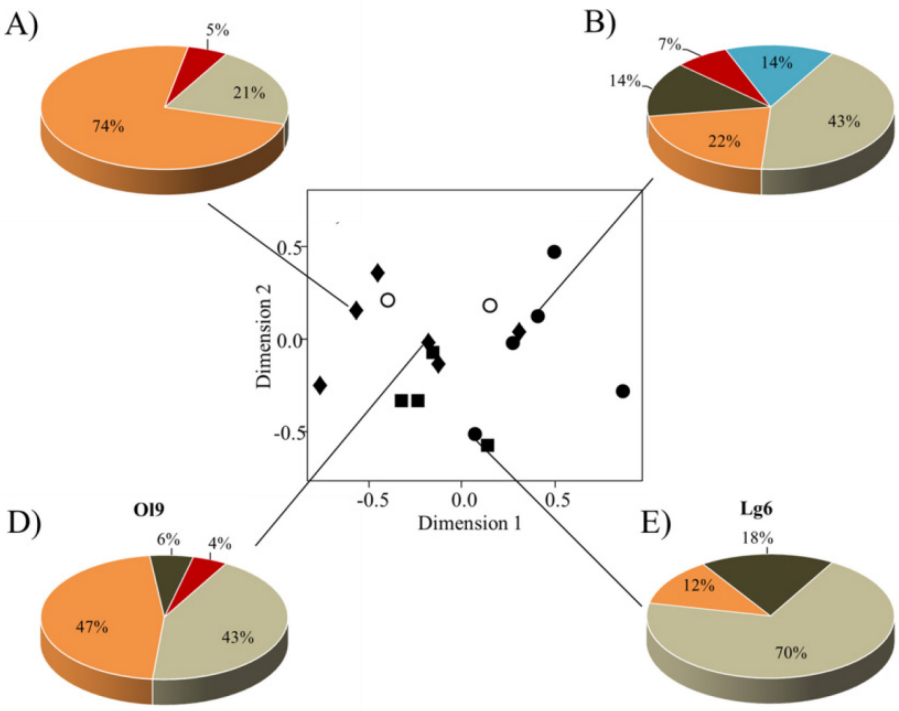

Fig 3. Variation in resin spectra collected by 16 colonies from four different apiaries/sites.

Bees collected varying amounts of up to five different types of resin ("ocher", "orange", "brown", "red", "clear"). Ordination figure (C) shows similarities among colonies from different sites based on Bray-Curtis dissimilarities between samples (stress value = 0.06). Sites are represented by different symbols: closed circle, "Lg"; closed diamond, "Ol"; closed square, "Gr"; open circle, "Ml" with each symbol representing a single colony (for meaning of abbreviations see Table 1). Circular charts (A – B) and C - D) display proportions of resin types collected by four examplary colonies from two different sites (site "Ol": A) colony ID 8: N resin samples = 19, and B) ID 9: N = 88; site "Lg": D) ID 4: N = 14 and E) ID 6: N = 40).

Our study colonies collected varying amounts of three to five different resin types out of the seven we observed in total (Fig 3). The resin spectra collected differed only marginally between study sites (Adonis: R^2 = 0.47, P = 0.047), but varied strongly between some (even neighboring) colonies (Fig 3). While "ocher" resin was collected by all colonies irrespective of the location (Kruskal-Wallis test: H = 3.62, P = 0.31, Figs 2. and 3A-E), proportion of foragers collecting "orange" varied significantly between study sites (Kruskal-Wallis-test: type "orange": H = 17.82, P = <0.009) and was negatively correlated with "brown" resin (Kendall's rank correlation tau: z = -3.05, P = 0.002, tau = -0.23). Other resin types were collected at comparatively low numbers and only at specific sites (Fig 3). Resin diversity (i.e. resin type richness) did not differ between apiaries/sites (Pearson's Chi-squared test: x^2 = 1.12, P = 0.77).

Chemical analysis - identification of resin sources

Bee-collected resins

Overall, resin samples from 39 bees and 28 individual trees of at least eight distinct species from eight different sites were analyzed by GC-MS, comprising overall 846 different compounds, of which 116 were identified

based on their retention and Kovats indices (S1 and S2 Tables).

The chemical analysis of bee collected resins revealed seven distinct types (Adonis: R^2 = 0.27, P = 0.001; Fig 4) correspondent to the visually assigned categories described above (Fig 4). The chemical composition of resin samples of the same type showed distinct patterns in the relative proportions of different substance classes (Table 2, Fig 4A), but also varied qualitatively and/or quantitatively within and between different apiaries/sites (Table 3, Figs 1, 4B and C). Variability within types was most pronounced within classes of terpenes, particularly sesquiterpenes. Resin types "ocher" and "orange" varied strongly among samples of individual foragers from the same and different sites and showed partly little overlap among each other and with other resin types (Table 3, Fig 4B).

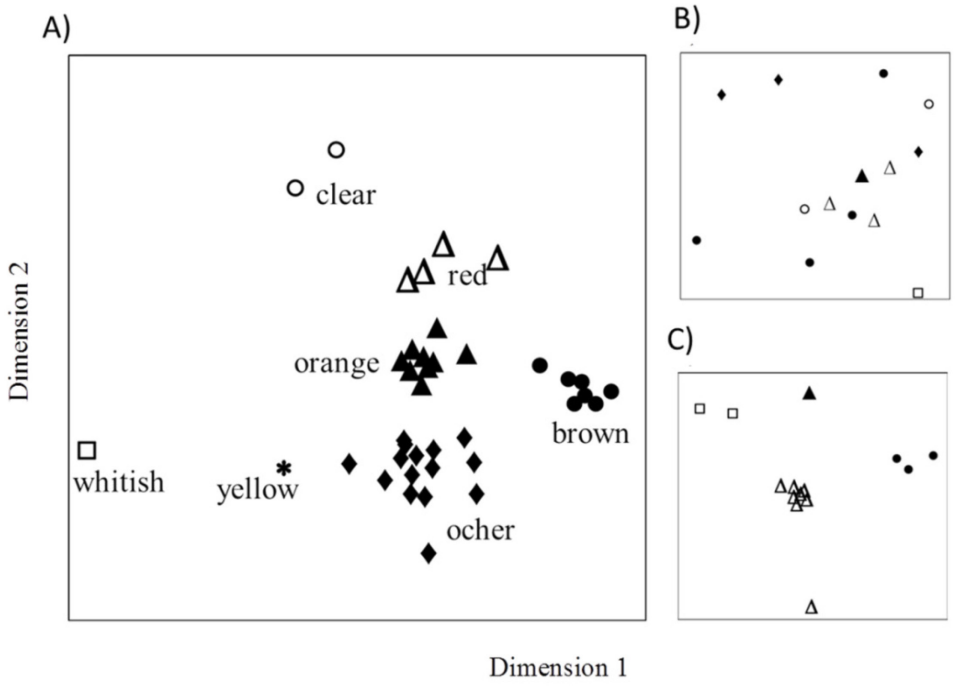

Fig 4. Chemical similarity within bee-collected resin samples.

Chemical similarity of A) all resin samples collected by returning resin foragers (n = 39), B) only "ocher" samples (n = 14) and C) only "orange" and "red" samples (n = 15); ordination Figure from non-metrical three-dimensional scaling (NMDS) based on Bray-Curtis dissimilarities (stress value = 0.15). Different symbols represent different resin types (closed circle, "brown"; closed diamond, "ocher"; closed triangle, "orange"; open triangle, "red"; star, "yellow"; open circle, "clear"; open square, "whitish") in A) and different sampling sites (closed circle, "Ez"; closed diamond, "Et"; closed triangle, "Lg"; open triangle, "Gr"; open circle, "Ml"; open square, "Ol"; for meaning of abbreviations in B and C see Table 1).

Both types were characterized by a large content of benzoic and phenolic acids as well as diverse sesquiterpenes and phenolic compounds (Table 2). In all samples of type "ocher" and "orange", benzoic acid (m/z 77, 122; Kovats-Indx: 1171) was the most abundant component, with type "ocher" comprising 15.9 – 47.7 % (mean 32.1 % of the

relative peak area) and type "orange" 0.8 – 23.3 % benzoic acid (mean: 12.1 % of the relative peak area). "Ocher" and "orange" resin further shared one flavonoid component (retention time (RT) 39.0 min) and about six sesquiterpenes (e.g. alpha-humulene, E-isoeugenol, alpha-guaiene and caryophyllene/oxide). In contrast to "ocher" and "orange" resin, variability in chemical composition was relatively low among "brown" resin samples from different sites (Fig 4, Table 3). They contained mainly sesquiterpenes and other terpenoids, only few phenolic compounds and almost no benzoic or phenolic acids (Table 2).

Three sesquiterpenes (Z-beta-farnesene, and two unidentified sesquiterpenes (retention time 21.58 min and 24.78 min) were most abundant (comprising 33.3 - 54.5 %, mean: 46.4% of the total peak area). They occurred exclusively in all samples of type "brown" and were further found in all propolis samples. Interestingly, these sesquiterpenes where present only in resins of B. alba tree species that were used by bees but not in resins of species not collected by bees (Fig 4).

Table 2. Prevalence of specific substance classes in different resin types.

Table shows mean [± standard deviation] number of compounds from specific substance classes and their mean relative proportions in the different resin types. The Table refers to all compounds, which have been identified at least to substance class, i.e. 762 out of 846 compounds in total. For each type, the number of compounds (N) within a substance class was counted. The mean relative proportion of each substance class (%) in a specific resin type was calculated by summing up relative peak areas of all compounds of a specific substance class and dividing the sum by the number of samples containing this substance class.

Substance class	Orange		Ocher		Red		Brown		Clear		Yellow		Whitish	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Aliphatic acids/ester	8 [3]	4 [7]	8 [6]	< 5	13 [0]	11 [2]	2 [2]	< 5	10 [1]	5 [0]	2	< 5	3	0
Phenolic acids/ester	6 [2]	2 [1]	4 [2]	12 [14]	9 [2]	8 [1]	2 [4]	< 5	3 [1]	< 5	0	0	0	0
Benzoic acid/ester	6 [1]	13 [9]	6 [3]	41 [9]	9 [1]	39 [5]	1 [1]	< 5	0	0	0	0	0	0
Phenolic compounds	5 [1]	< 5	6 [2]	5 [4]	5 [2]	< 5	3 [2]	< 5	7 [1]	< 5	3	< 5	4	< 5
Flavone/Flavonoids	5 [1]	10 [4]	1	< 5	6 [4]	8 [4]	3 [2]	< 5	1	0	0	0	0	0
Terpenoids	7 [1]	10 [7]	2 [1]	0	3 [1]	< 5	5 [14]	< 5	6 [0]	6 [1]	0	0	5	5
Monoterpenes/terpenoids	1	0	1	< 5	0	0	0	0	0	0	7	42	17	73
Sesquterpenes/terpenoids	18 [6]	40 [20]	17 [9]	27 [16]	6 [1]	< 5	33 [16]	77 [5]	0	0	2	9	3	0
Diterpenes/terpenoids	1	0	0	0	3 [0]	< 5	5 [3]	< 5	2 [0]	< 5	13	23	33	15
Triterpenes/terpenoids	0	0	2 [1]	0	2 [0]	< 5	2 [1]	< 5	22 [4]	37 [11]	0	0	0	0
Alcohols/aldehyds/ketons	5 [3]	< 5	3 [2]	< 5	7 [2]	< 5	4 [2]	< 5	10 [2]	< 5	1	0	0	0

Table 3. Compositional variability among samples of the same resin types.

[a)] Number of samples of this type. [b)] Mean number [± standard deviation] of total components. [c)] Mean proportional area overlap of all compounds shared among all samples of this type. [d)] Proportional area overlap [± standard deviation] of compounds shared by all samples of this type from a specific study site, the number of study sites where this type occurred (N study sites). Area overlap basically corresponds to compound overlap, but weighs more common compounds (large area) more than less common ones (small area). [e)] Number [± standard deviation] of compounds shared with propolis from the same apiary (matches with propolis for two propolis samples per site). [f)] n.a. = overlap was not calculated when there were less than two samples to compare.

Chemotype	N shared samples[a]	N components[b]	Area overlap [%][c]	Area overlap [%] per site[d]	N study sites	Matches with propolis[e]
Ocher	13	49 [20]	1.6	32.1 [9.6]	5	16 [5]
Orange	8	71 [17]	17.3	93.9 [4.6]	3	19 [2]
Red	3	80 [10]	78.8	78.8 [n.a.]f	1	16 [3]
Brown	7	67 [7]	62.9	92.5 [0.1]	4	15 [2]
Clear	2	79 [4]	67.6	n.a.	2	12 [2]
Whitish	1	74	n.a.	n.a.	1	0
Yellow	1	38	n.a.	n.a.	1	6 [1]

Tree resins

Tree resins were chemically highly diverse with up to 300 and more different compounds (including trace compounds) in a single sample (mean number of compounds per sample [± standard deviation] = 230 ± 133 compounds). Highest diversity was found for bud resins of *P. x canadensis* (Fig 5D). The chemical composition varied between different tree species (Adonis: $R^2 = 0.60$, $P = 0.009$; Fig 6D). Poplar displayed the comparatively greatest intra-specific variability (Figs 6 C, D, E and Figs 5A - D).

Chemical composition further varied between *P. x canadensis* individuals from different sites, but also between neighboring trees (Fig 5D), displaying three distinct *P. x canadensis* (chemo)types (henceforth referred to as *P. x canadensis* type1, type2 and type3) of which two were similar to the resin type "orange" (Fig 5D). Resin samples of *P. x canadensis* type1 and type2 as well as *P. balsamifera* were characterized by comparatively large amounts of benzoic and phenolic acids (proportion of the total peak area [± standard deviation]: 26.3 ± 14.3 %) and low quantities of monoterpenes (proportion: 0. ± 0.3), whereas samples of *P. x canadensis* type3 showed the opposite trend (proportion of benzoic and phenolic acids: 0.2 ± 0.2; proportion of monoterpenes: 3.9 ± 1.3). For *B. alba*, we found two different resin (chemo)types (each with two individuals) at overall three different sites, with two neighboring individuals from the same site belonging to different (chemo)types (Fig 6D).

The following bee-collected resin types chemically matched specific tree species: type "orange" chemically matched *P. x canadensis* type1 and type2, type "brown" matched *B.*

alba type1, type "red" matched *P. balsamifera*, type "clear" matched *A. hippocastanum* and type "whitish" matched the two sampled conifers (*Picea abis* / *Pinus sylvestris*). No clear matches were found for types "ocher" and "yellow" (Fig 5). Thus, honeybees collected resins from tree buds of *P. x canadensis*, *P. balsamifera*, *B. alba*, *A. hippocastanum* and from wounds of coniferous tree species (e.g. *P. abis* or *P. sylvestris*) (Fig 6). None of the bee collected resins showed chemically similarity to resin samples collected from *A. glutinosa*, *B. alba* type2 and *P. x canadensis* type3 (Fig 6). While resin type "ocher" did not unambiguously correspond to any tree resin, it contained up to nine compounds that were exclusively found in poplar resin samples. Likewise, resin type "yellow" showed some overlap with type "ocher", but further comprised compounds (e.g. D-limonene and 3-carene) which were primarily found in coniferous resins.

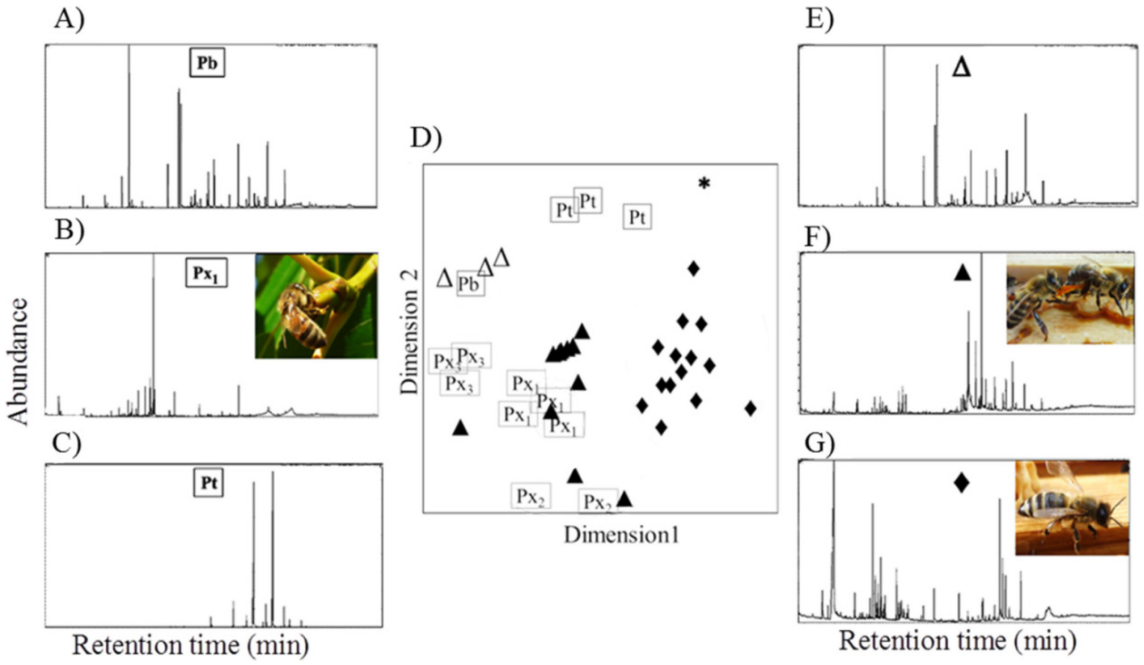

Fig 5. Similarity in chemical composition of tree bud resin from poplar tree species and bee-collected resins ("ocher", "orange", "red" and "yellow").

Bee-collected resins did not unambiguously correspond to any of the other sampled tree species. Ordination Figure (D) is based on Bray-Curtis dissimilarities between samples (stress value = 0.18). Different letters indicate different tree species: Pb: *Populus balsamifera*; Pt: *Populus tremula*; Px1-Px3: three different chemotypes of *Populus* x *canadensis*. Different symbols represent different resin types: closed diamond: "ocher"; closed triangle: "orange"; open triangle: "red"; star: "yellow". A – C and E – G) Exemplary chromatograms of hexane extracts from tree buds (left) and bee-collected resins (right): A) *P. balsamifera*; B) *P.* x *canadensis* (chemotype Px1); C) *P. tremula* (chemotype Px3); E) "red" bee-collected resin; F) "orange" bee-collected resin; G) "ocher" bee-collected resin; chromatograms display retention times in minutes on the x-axis and mass current (mc) on the y-axis; pictures in chromatograms show examples of resin foragers carrying the respective resin chemotype

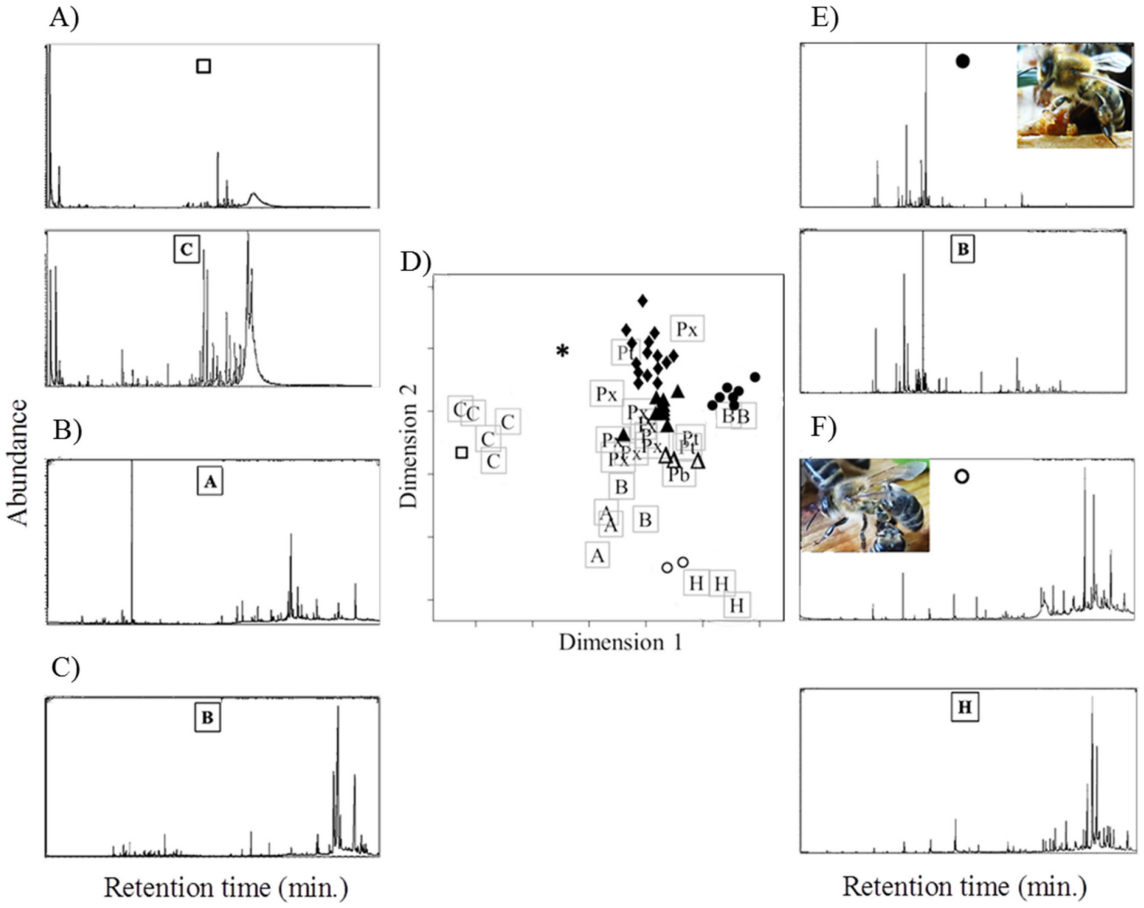

Fig 6. Similarity in chemical composition of tree bud resins and bee-collected resins. Tree bud resins (letters in ordination Figure; n = 30) and bee-collected resins (symbols; n = 39) from 17 colonies at seven different apiaries/sites. Ordination Figure (D) is based on Bray-Curtis dissimilarities between samples (stress value = 0.20). Different letters indicate different tree species: C, conifers (*Picea abis / Pinus sylvestris*); Pb, *Populus balsamifera*; Px, *Populus* x *canadensis*; Pt, *Populus tremula*; B, *Betula alba*; A, *Alnus glutinosa*; H, *Aesculus hippocastanum*. Different symbols represent different resin types: closed circle: "brown"; closed diamond: "ocher"; closed triangle: "orange"; open triangle: "red"; star: "yellow"; open circle: "clear"; open square: "whitish". A) - F) Exemplary chromatograms of hexane extracts from tree bud resins and bee-collected resins: A) top: corbicula resin "whitish", bottom: *P. abis*; B) *A. glutinosa*; C) B. alba; E) top: corbicula resin "brown", bottom: *B. alba*; F) top: corbicula resin "clear", bottom: *A hippocastanum*; chromatograms display retention times in minutes on the x-axis and mass current (mc) on the y-axis; pictures in chromatograms show examples of resin foragers carrying the respective resin type.

Finally, propolis samples from 10 colonies of five apiaries differed in their chemical composition between colonies of both the same site and different apiaries (Fig 7). Compared to single bee-collected resins, propolis samples were more similar to each other than to any of the single resins types (Fig 7). Moreover, we found only relatively few compound matches between bee-collected resins and propolis samples (Table 2).

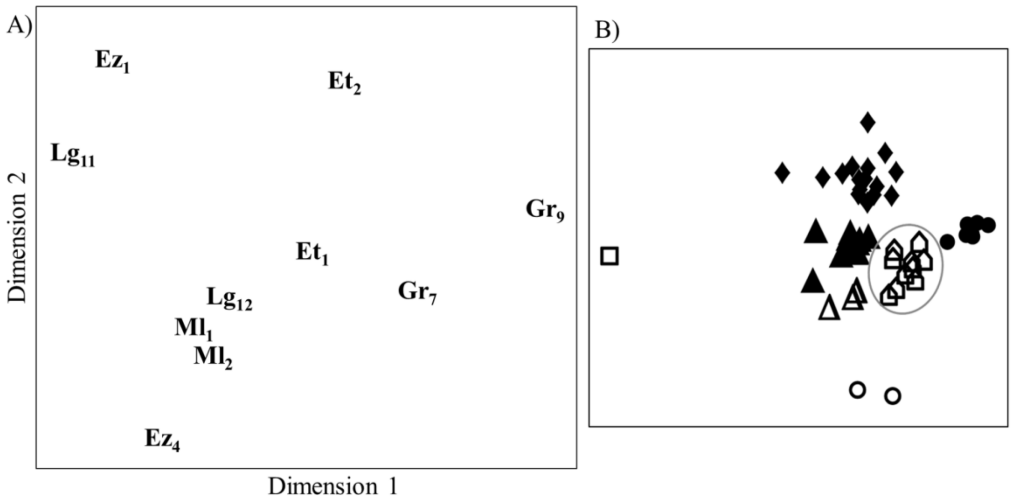

Fig 7. Similarity in chemical composition of propolis samples from 10 colonies of five different apiaries/sites (A) and propolis samples in relation to bee-collected resin types (B).

Letters indicate different apiaries/sites (for meanings of abbreviations see Table 1), numbers mark different colonies. Ordination Figures are based on Bray-Curtis dissimilarities between samples (stress value = 0.07). Different symbols in B) represent different resin types: closed circle: "brown"; closed diamond: "ocher"; closed triangle: "orange"; open triangle: "red"; open circle: "clear"; open square: "whitish".

Discussion

Honeybees collect plant resins for various purposes, such as defense against pests and pathogens and for nest construction and sealing (Huber 1814; Simone-Finstrom and Spivak 2010). Despite its importance, very little is known on precise resin sources as well as variation in the spectra and diversity of resins collected by honeybees.

Resin sources used by honeybees

We chemically compared resin collected from resin foragers and resin sampled from tree buds. Our results reveal that bees from our study colonies collected resin not only from several poplar species (*P. balsamifera*, *P. x canadensis*), but also from birch (*B. alba*), horse chestnut (*A. hippocastanum*) and coniferous trees (either *P. abis* or *P. sylvestris*). While the use of poplar resins had already been confirmed by previous studies (Greenaway et al. 1990; Bankova et al. 2002; Wilson et al. 2013), our study provides first evidence for the exploitation of a broader spectrum of resin sources, including poplar species, birch, horse-chestnut and conifers.

However, despite clear compositional similarity, we hardly found close matches between bee-collected resins and plant samples, which may partly be explained by the high variability within and among different plant taxa. Intra-specific variability in resin chemistry was particularly pronounced among tree individuals of poplar and birch, both known to build a large variety of hybrids

which are often difficult to distinguish based on morphological traits (Johnsson 1945; Walters 1968; Gardiner and Pearce 1978; Smith and Sytsma 1990; Atkinson 1992; Ceulemans et al. 1992; Howland et al. 1995; Eckenwalder 1996; Cronk 2005; Migalina et al. 2010; Thompson et al. 2010; Lindtke et al. 2013; Isidorov et al. 2014), but can strongly differ in resin chemical composition (Wilson et al. 2013; Isidorov et al. 2014).

Interestingly, we could not unambiguously identify the precise plant source of the frequently collected "ocher" resin type. Although this type contained a few compounds (up to nine) also found in *P. x canadensis* and *P. tremula* resins, its chemical composition did not fully overlap with that of the sampled poplar bud resins. In fact, at least 45 compounds (mostly sesquiterpenes) detected in *P. x canadensis* resins were missing in the "ocher" resin type.

Missing or incomplete matches between bee-collected resins and bud resin samples of potential source plants may indicate that bees either collected from source trees or tree parts (e.g. crown) which we did not sample and which were chemically different from our tree samples. Alternatively, honeybee resin foragers may somehow alter resins during the collection process, e.g. by enzymatically degrading specific compounds or changing the composition of compounds typically produced by the tree.

Moreover, bee-collected samples of a specific resin type (e.g. "orange" and "brown") from colonies of different apiaries were occasionally very similar (48.3 - 62.9 % area overlap, see Table 3 and Figs 5 and 6), suggesting that bees select specific (chemo)types independent of location and recruit foragers to these plants (Nakamura and Seeley 2006). In fact, bees did not necessarily collect resins from the closest sources available, but were highly selective and made distinct choices for specific (chemo)types even among closely related and partly neighboring tree species/individuals which strongly differed in their bud resin chemistry. Likewise, honeybees in North America collected resins from *P. deltoides* and *P. balsamifera*, but not from their numerous hybrids which were shown to produce bud resins with chemical profiles that were intermediate to the distinct profiles of their parental species (Wilson et al. 2013).

A functional role of benzoic acid and derivatives?

Both the "ocher" and "orange" resin type contained notable amounts of benzoic acid (i.e. 32.1 ± 9.6% in "ocher" and 12.1 ± 8.2% in "orange"), which was characteristic to all samples of both types. Benzoic acid also occurred in resins of some, but not all *P. x canadensis* and *P. tremula* individuals, although in much lower concentrations (i.e. 1.6 ± 2.4 %). Benzoic acid is an aromatic compound commonly used for food preservation by humans due to its antimicrobial and antimycotic activity (del Olmo et al. 2017). In plants, benzoic acids and their derivatives play a central role in attracting beneficial insects, e.g. pollinators, and in defense, e.g. against herbivores or other pests (Arimura et al. 2009; Maffei 2010; Widhalm

and Dudareva 2015). For example, in woody plants, increased production of benzoic acid in fruits takes place when they are infected with a fungus causing "tree cancer" (*Nectria galligena*) (Brown and Swinburne 1971). Consequently, benzoic acid has biological properties that may be beneficial to bees. Moreover, in contrast to most other chemical compounds commonly thought to be responsible for the biological activity of propolis (e.g. phenolic compounds or terpenes), benzoic acid is water-soluble. It is also regularly found in honey (Kaškonienė and Venskutonis 2010) and likely contributes to the preservability of honey. Given its prevalence in resin, benzoic acid in honey may actually be (at least partly) derived from resin storages (propolis). In fact, relative amounts of benzoic acid were much lower in propolis samples than in freshly gathered resin samples, suggesting that it evaporates in bee colonies (and e.g. into honey). Similarly, three other typical constituents of poplar bud resin (pinobanksin, pinobanksin 5-methyl ether, and pinocembrin, which induce detoxifying CYP9Q enzymes) were also found to be accumulated in honey (Mao et al. 2013). Due to its volatility, benzoic acid may further help honeybees to locate resin sources and thus function as chemical cue. Relatively large amounts of another benzoic acid derivate, benzyl benzoate (Kovats Index 1762), were also found in some bee collected resins of types "red" and "ocher" as well as in *P. balsamifera* (S2 Table). It is used as pharmaceutical acaricide e.g. against scabis and could thus protect bees against varroa mites (*Varroa destructor*).

May honeybees induce secretion of preferred resin types?

As defense responses in plants can generally lead to an accumulation of defensive compounds such as benzoic acid (Fürstenberg-Hägg et al. 2013), the preferential collection of distinct chemotypes (e.g. rich in benzoic acid) by our study bees may even indicate that bees particularly prefer resins produced as defense against stressors. The plant's defensive response may further increase overall resin secretion (e.g. in aspen which had comparatively low amounts of bud resin within the study region, personal observation), as known for birch and poplar when browsed by snowshoe hares (Reichardt et al. 1984; Reichardt et al. 1990; Bryant et al. 1992). Besides mechanical wounding, chemical substances (e.g. enzymes) from oral secretions and insect-associated microbes can trigger defense responses and thus induce or maintain the secretion and/or alter their chemical composition (Delorme and Lieutier 1990; Halitschke et al. 2001; Sugio et al. 2015; Unsicker et al. 2015). In fact, resin foragers occasionally injure vegetative parts (e.g. buds or leaves) of plants (Huber 1814; Teixeira et al. 2005) which may induce/increase resin secretion. In doing so they may transfer microbial symbionts (associated with the bees' saliva) or pathogenic microbes (see (Singh et al. 2010; Anderson et al. 2013; Corby-Harris et al. 2014)) and thus potentially trigger the production of preferred resin chemotypes. If this hypothesis holds true, this may further explain the missing overlap in chemical similarity between our tree and bee-collected samples.

Resin intake, variability and diversity

Our study honeybee colonies clearly preferred two chemically distinct resin types, "ocher" (unknown source, likely poplar) and "orange" (*P. x canadensis*), over all other sources. A strong preference for some resins over others agrees with previous studies (Bankova et al. 2006; Wilson et al. 2013). However, most of our colonies collected three or more different resin types, indicating that bees targeted a variety of different resins. Collected resin spectra in turn varied between apiaries/sites and colonies, indicating colony-specific site-independent collection targets. However, propolis samples from our colonies were chemically more similar to each other than to the bee-collected resin types, suggesting that colonies compile specific resin blends from a variety of different resins. Different resins show different functional properties, e.g. repel different antagonists (Drescher et al. 2014). Compiling resin from different sources thus increases the diversity of potentially bio-active compounds and may consequently better protect colonies against a variety of enemies (Drescher et al. 2014). In fact, our chemical analysis revealed a striking compositional chemical diversity within and among different resin types/sources. Thus, in addition to collecting resins from a comparatively broad spectrum of different plant species (as also shown for stingless bees: (Kaluza et al. 2017)), bees may further benefit from the large chemical intra-specific variability as found in resin from specific plant species.

Conclusion

Our data shows that honeybees collect a comparatively broad and highly variable spectrum of resin sources and make distinct choices by preferring some resins (e.g. specific poplar species and chemotypes) over others. This finding corresponds to observations made for their tropical relatives (Apidae: Meliponini) (Leonhardt and Blüthgen 2009; Leonhardt et al. 2010), and likely insures that bees can combat a variety of antagonists sensitive to different resin sources and/or compounds (see (Drescher et al. 2014)). Notably, the environment in our study area is shaped by intensive human impact, resulting in an altered assemblage of available plant species. For example, formerly occurring poplar species, such as *Populus nigra*, are now extremely rare and most probably not available to any of our apiaries. Instead, several planted and natural occurring hybrids of *P. nigra* and other poplar species are available in our study region. In an unaltered, intact environment, the bee's preferences may be shifted to other (or even to more diverse) resources to compose the resin blends that best meet their current needs. However, the high chemical variability found among resin (chemo)types collected by our study bees may alternatively indicate that bees target and benefit from chemical rather than tree species biodiversity.

Future studies should attempt to unravel the causes underlying the choices and variability in resin intake observed in honeybees. Such knowledge would not only provide new insight into self-medication and external immunity in

insects, but also provide important information for apicultural praxis.

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Ethics statement: We confirm that all land owners and bee keepers gave permission where necessary. Resin samples of trees where obtained from trees that were located at public sites where no specific permission was required. No endangered or protected species where involved in the study. We further confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Chapter III

Inside honeybee hives: Impact of natural propolis on the ectoparasitic mite *Varroa destructor* and viruses

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Abstract

Social immunity is a key factor for honeybee health, including behavioral defense strategies such as the collective use of antimicrobial plant resins (propolis). While laboratory data repeatedly show significant propolis effects, field data are scarce, especially at the colony level. Here, we investigated whether propolis, as naturally deposited in the nests, can protect honeybees against ectoparasitic mites *Varroa destructor* and associated viruses, which are currently considered the most serious biological threat to European honeybee subspecies, Apis mellifera, globally. Propolis intake of ten field colonies was manipulated by either reducing or adding freshly collected resin. Mite infestations, titers of Deformed wing virus (DWV) and Sacbrood virus (SBV), resin intake as well as colony strength were recorded monthly from July to September 2013. We additionally examined the effect of raw propolis volatiles on mite survival in laboratory assays. Our results showed no significant effects of adding or removing propolis on mite survival and infestation levels. However, in relation to *V. destructor*, DWV titers increased significantly less in colonies with added propolis than in propolis-removed colonies, whereas SBV titers were similar. Colonies with added propolis were also significantly stronger than propolis-removed colonies. These findings indicate that propolis may interfere with the dynamics of *V. destructor*-transmitted viruses, thereby further emphasizing the importance of propolis for honeybee health.

Introduction

Western honeybees, *Apis mellifera*, struggle with multiple environmental impacts, including numerous pests and pathogens. Particularly managed colonies of the European derived subspecies continue to suffer high (winter) losses, which is observed with increasing attention and concern (Ellis et al. 2010; Neumann and Carreck 2010; Potts, Simon G. et al. 2010; VanEngelsdorp et al. 2011). Honeybees are effective pollinators of various crops, and managed honeybees become increasingly important in securing yields, particularly with decreasing diversity and abundance of many wild pollinator species (Kosior et al. 2007; Potts, S. G. et al. 2010) and with increasing demand for agricultural products (Klein et al. 2007; Aizen et al. 2008).

The reasons for the observed elevated mortalities of managed honeybee colonies are still subject to debate (Martin 2001; Frazier et al. 2008; Le Conte et al. 2010; Moritz et al. 2010; Orantes-Bermejo et al. 2010; Smith et al. 2013; Pohorecka et al. 2014). Honeybees have been kept by humans for centuries, and it is likely that beekeeping practices (incl. breeding), which focus primarily on traits such as bee handling and productivity, conflict with the bees' local adaptions (Jones 2004; Meixner et al. 2010; De la Rua et al. 2013). Moreover, the commercial use and international trade of bees and bee products likely promotes the dispersal of diseases and pests (Klee et al. 2007; Mutinelli 2011; Smith et al. 2013; Neumann et al. 2016). Consequently, honeybees worldwide suffer from various pests and pathogens (Ellis and Munn 2005).

Among these, *Varroa destructor*, an ectoparasitic mite that feeds on the hemolymph of pupae and adult bees, is considered one of the most challenging threats (Ball and Allen 1988; Yang and Cox-Foster 2007; Le Conte et al. 2010; Rosenkranz et al. 2010; Dainat et al. 2012). Since these mites have shifted their host from the Eastern honeybee *A. cerana* to *A. mellifera*, *V. destructor* is spreading rapidly on almost every continent (Ellis and Munn 2005; Neumann and Carreck 2010; Rosenkranz et al. 2010). It has become a ubiquitous pest which commonly leads to colony collapse within 2-3 years, unless they are treated by beekeepers (Fries et al. 2006; Rosenkranz et al. 2010), because *V. destructor* vectors several honeybee viruses, which generate a fatal disease epidemic within the colony (Martin 2001; Dainat et al. 2011; Neumann et al. 2012; Francis et al. 2013; Nazzi et al. 2015).

At least seven viruses have been detected in *V. destructor* mites (i.e. Acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), Israeli acute bee paralysis virus (IAPV),

Chronic bee paralysis virus (CBPV), Sacbrood virus (SBV), Deformed wing virus (DWV) and *Varroa destructor* virus-1 (VDV-1) (Ball and Allen 1988; Ongus et al. 2004; Tentcheva et al. 2004; Di Prisco et al. 2011a), supporting its putative role in virus transmission. A particularly strong link is known for *V. destructor* and DWV. Several field and laboratory experiments demonstrated a positive correlation between mite infestations and virus

titers (Bowen-Walker et al. 1999; Shen et al. 2005b; Genersch et al. 2010; Dainat et al. 2011; Di Prisco et al. 2011b; Francis et al. 2013). Besides promoting horizontal transmission, these mites likely play a crucial role in activating latent virus infections (Shen et al. 2005b; Yang and Cox-Foster 2005). While DWV is of generally low virulence and causes rather asymptomatic infections, it became one of the most prevalent viruses in apiaries and is highly pathogenic in association with *V. destructor* (Martin 2001; Tentcheva et al. 2004; Nazzi et al. 2015). In contrast to DWV, other *V. destructor* associated viruses, such as SBV, mostly occur less frequently and in rather moderate amounts in mites (Tentcheva et al. 2004; Mondet et al. 2014). Unlike DWV, SBV titers are not directly correlated with *V. destructor* infestations, but are highest in spring and decrease towards late summer (Tentcheva et al. 2004; de Miranda et al. 2013). DWV loads typically increase relative to *V. destructor* infestation from spring to late summer (Dainat et al. 2011), thereby affecting the development of the long-living winter bees, which are essential for colony survival in temperate regions (Dainat et al. 2011; Dainat et al. 2012). Thus, *V. destructor* and associated DWV can cause substantial damage to colonies and most likely play a key role for colony losses during winter (Genersch et al. 2010; Moritz et al. 2010; Neumann et al. 2012).

Because dense aggregation of hosts in colonies can facilitate the spread of diseases, honeybees and other eusocial insects have evolved social immunity (Neumann and Härtel 2004; Evans and Spivak 2010), including hygienic behavior (i.e. nest hygiene and allogrooming) (Boecking and Spivak 1999; Spivak and Reuter 2001; Duangphakdee et al. 2009), and social analogues of immune functions, such as social fever (Starks et al. 2000), encapsulation (Neumann et al. 2001) and apoptosis (Page et al. 2016) to combat pathogens. Honeybees (Simone-Finstrom and Spivak 2010), stingless bees (Roubik 2006; Leonhardt and Blüthgen 2009) and some ant species (Chapuisat et al. 2007; Castella et al. 2008) also collect plant resin, a sticky substrate secreted by plants to protect young sprouts and leave buds (Langenheim 2003), which is used for nest construction and defense against pests and pathogens (reviewed in Simone-Finstrom and Spivak 2010).

Propolis, the apicultural term for a mixture from various plant resins and wax, has long been known for its antibiotic properties and been used by humans in traditional medicine for centuries (reviewed in Ghisalberti 1979 and; Burdock 1998). Chemically, it is a complex and highly variable mixture with up to 300 different substances, whose composition depends on the available plant resources (Greenaway et al. 1990; Marcucci 1995; Bankova et al. 2000). The functional properties of propolis derive mainly from a variety of water insoluble compounds, such as various phenolic constitutions (flavonoids, aromatic acids and benzopyranes) and terpenoids (Kujumgiev et al. 1993; Bonvehi et al. 1994; Cushnie and Lamb 2005; Melliou et al. 2007; Popova et al. 2009; Massaro et al. 2015). Volatile substances generally represent only a

small fraction (1 – 3%) of the entire bouquet, but significantly contribute to typical propolis characteristics, such as its distinctive aroma and its biological activity (Bankova et al. 1994; Bankova et al. 2014). The biological activity and pharmacological properties of propolis have been intensively investigated (Marcucci 1995) with a wide range of antimicrobial (Vardar-Unlu et al. 2008; Bonvehi and Gutierrez 2012), antifungal (Kujumgiev et al. 1999; Silici et al. 2005), antiviral (Kujumgiev et al. 1999; Gekker et al. 2005) and immunomodulatory (Dimov et al. 1991) properties described. However, while numerous studies examined the pharmacological value of propolis for humans, only few investigated how propolis benefits the bees themselves. For example, Simone-Finstrom et al. (2009) showed that treating hives with propolis extract can reduce overall bacterial loads and immune activity in individual bees. Propolis was further found to be active against the causative agent of American foulbrood (*Paenibacillus larvae*) (Bastos et al. 2008; Wilson et al. 2015), as well as of chalkbrood (*Ascosphaera apis*) (Simone-Finstrom and Spivak 2012). Propolis extracts can also have narcotic and lethal effects on *V. destructor* depending on the dosage used (Garedew et al. 2002; Damiani et al. 2010a). Consequently, propolis appears to be active against a range of honeybee pests and pathogens and can be considered a social immune defense mechanism, which is likely to be important for maintaining colony health (Simone-Finstrom et al. 2009; Nicodemo, D. et al. 2013; Nicodemo, Daniel et al. 2013; Borba et al. 2015). However, the majority of studies which investigated the effect of propolis on honeybee pests used propolis ethanolic extracts, and therefore do not inevitably reflect the functional properties of propolis in its natural (solid) form as placed in hives by bees. Moreover, testing effects of propolis on certain pathogens in laboratory assays may not necessarily implicate colony level effects (Retschnig et al. 2015).

In this study, we therefore investigated the effect of raw propolis on four common honeybee viruses and on *V. destructor* mite infestations. We manipulated the amount of resin present within a hive by either removing or adding resin deposited above the brood nest. Due to the known antiviral properties of propolis (Amoros et al. 1992; Gekker et al. 2005; Shimizu et al. 2008; Schnitzler et al. 2010), we hypothesized that viral titers would be reduced in colonies treated with extra propolis compared to propolis-removed colonies. We further expected that *V. destructor* infestation would be reduced due to the possible acaricide activity of propolis.

Material and methods

Bioassay to test for effects of propolis on virus infections and mite loads

Experimental site and set-up

The experimental site was located in an agricultural landscape close to Lüneburg, lower Saxony, Germany, comprising comparatively small arable fields with varying crops (such as rapeseed, grain, sugar beet,

potato) and pastures as well as some scattered trees and mixed forest patches. The experiment was conducted between June and October 2013 (local summer/early autumn). Ten honeybee colonies with young, naturally mated queens were established in May 2013 in clean propolis-free wooden Dadant boxes consisting of a single brood chamber. Experimental colonies where maintained by splitting colonies of a local stock of *A. m. carnica*, which were all reared in the same apiary and provided with three brood frames as well as honey and pollen storage. Source colonies had routinely been treated against ectoparasitic mites *V. destructor* with organic acids in the previous winter (November 2012), and none of them showed any obvious clinical disease symptoms during visual inspections.

Experimental manipulations of propolis were conducted between June, when bees initiated substantial resin collection (noticed by the deposits in propolis traps and/or inner hive parts), and September 2013. All ten hives were placed in pairs of two neighboring colonies (high and low propolis treatment) with a distance of 0.25 m and placed in a row with a distance of 1.5 m between the pairs. Five colonies were provided with additional propolis - in the following called "high" (propolis) treatment - that was removed from the other half - "low" (propolis) treatment. A commercial plastic propolis trap (Logar trade d.o.o., Slovenia) was placed on the top of the frames to stimulate resin deposition. We assumed that bees deposit a considerable amount of the gathered resin into the grids, but some resin was clearly also deposited elsewhere within the hive. To remove as much of the gathered resins as possible from the "low" treatment group, propolis grids were removed from the low propolis colonies once per week in June and every three days between July to September 2013. Grids were stored in a freezer and propolis deposits on grids were collected by flexing the frozen grids to break off propolis. Propolis from frames and inner hive walls was further removed by scratching it off with an apiary chisel. Following the collection, propolis was mixed, ground, weighed and evenly distributed across the five "high" propolis treatment colonies by placing it on top of the hive frames the latest one day after removal from the "low" treatment colonies. Each portion of grounded propolis was compressed and gently pressed on the frames to ensure that propolis remains on the frames. Overall, each "high" propolis treatment colony received 16 g (± SD 0.5) of extra propolis, which represented between 9 % and 70% (median 12%, ± SD 26) of the amount of propolis deposited in their own propolis grids.

Data collection and sampling

Quantitative resin intake per colony was quantified by weighing all grids each time propolis was removed. As the amount of resin collected by the bees varied between colonies and often exceeded the amount of propolis which was used for the "high" propolis treatment, we additionally tested for possible correlations between pathogen loads and propolis collection (see below). The status of all colonies was controlled by estimating

colony strength (see below) and verifying queen-state once per month between the end of June and the beginning of September 2013. Further, winter survival of all colonies was controlled in March 2014.

Bee sampling and determination of mite infestation levels

The sampling protocol for assessing virus (de Miranda et al. 2013) and mite infestation (Dietemann, Vincent et al. 2013) followed the COLOSS guidelines for *A. mellifera* pest and pathogen research. Once per month (July, August, September), ~300 adult workers were sampled from three brood free frames located next to the brood of each colony (de Miranda et al. 2013), placed immediately on ice and subsequently stored at -80° C until analyses. Phoretic mite infestation levels were investigated for each colony by the alcohol washing method described in Dietemann et al.(2013) and by counting dead mites which had fallen on bottom boards (i.e. natural mite fall). Therefore, bottom boards were placed underneath each hive and covered with a thick (0.3 cm) layer of aroma free petrolatum, to prevent wind or predators (e.g. ants) from removing mites and thus biasing mite quantification ("sticky board method") (Dietemann, V. et al. 2013; Simone-Finstrom et al. 2017). For moderate infestation levels, mite fall, i.e. natural mite mortality, is known to be directly correlated with mite population size in a colony and can therefore be used as a proxy for overall mite infestation (Branco et al. 2006). Dead mites which had fallen on bottom boards were counted every 24 hours over a time period of 10 days each month. Total numbers of mites were divided by the total number of days to calculate daily mite fall. Daily mite fall was further divided by the colony strength to estimate relative mite infestation rates for each colony. Thus, mite infestation is hitherto presented as infestation per 1000 bees. Mite infestation rates as calculated by both methods (alcohol washing and natural mite fall) were highly correlated (Kendall's rank correlation: $z = 3.05$, $p = 0.002$), and we therefore used only natural mite mortality in the statistical analyses.

Parallel to bee sampling, colony strength was recorded as the number of adult bees and brood cells according to the "Liebefelder-method" (Delaplane et al. 2013). We recorded colony size early (before sunrise) in the morning, before flight activity started. For each side of every frame within a hive, the proportion occupied by bees was visually assessed. Proportions were then summed up for all frames within a hive to obtain the total number of frames occupied by bees. We finally calculated the total number of bees for each hive by multiplying the number of frames fully occupied by bees with the number of bees, which fit onto one side of a frame (i.e., ~1400 bees per 1130 cm^2 for Dadant hives).

Brood cells were quantified using a grid with 1.5 × 1 × 0.5 dm^2 square fields to estimate the total area of brood on each side of a frame. The total brood area (in dm^2) was then converted into brood cells by multiplying brood area with the average number of cells occupying 1 dm^2 (i.e. 400 cells for Dadant hives).

Determination of viral loads

Pooled samples of ~300 adult workers per colony were used for the detection and quantification of four common honeybee viruses: 1) Black queen cell virus (BQCV), 2) Deformed wing virus (DWV), 3) Israeli acute paralysis virus (IAPV) and 4) Sacbrood virus (SBV). Virus analyses were performed using reverse transcription and quantitative PCR following (de Miranda et al. 2013; Evans et al. 2013). In brief, each sample was homogenized in TN buffer (10 mM Tris– 10 mM NaCl, pH 7.6) using MACS M tubes with a Dispomix® Drive homogenizer (Medic tools). RNA was then extracted using the RNA II NucleoSpin kit (Macherey Nagel, Germany) and eluted in 50 µl of RNAse-free water following the manufacturer's instructions. Reverse transcription was performed using the M-LV RT enzyme (Promega) with 1 µg of extracted RNA and 100 µM of random hexamers in a 25 µl final volume following the manufacturer's recommendations. Viral loads were determined by a relative quantification method using, as standard curves, serial dilutions of purified PCR products of known concentration. The qPCR reaction for each sample was performed in duplicate using Kapa SYBR® Fast Master Mix (Kapa Biosystems). Individual reactions contained 10 µl master mix, 3 µl cDNA template, 0.4 µl forward and reverse target primers (10 mM; Table 1) and 6.2 µl of Milli-Q water. Reactions were performed in an EcoTM Real-Time PCR System (Illumina) thermocycler. The following cycling conditions were used: 3 min at 95°C, followed by 40 cycles of 95°C for 3 sec and 55°C for 30 sec, during which fluorescence measurements were taken. A melting curve (95°C for 15 sec, 55°C for 15 sec and 95°C for 15 sec) was performed at the end of each run to ascertain the amplification of the target.

Bioassay to test for effect of propolis on V. destructor mite survival

Whether raw propolis affected the survival of adult *V. destructor* mites, was tested in a laboratory assay, in June 2014 and again in August 2014. Propolis for the assay was obtained from two honeybee colonies near Lüneburg, Lower Saxony, Germany between June and August 2013, using a commercial propolis grid (see above). In order to test for a cofounding effect of a thymol based acaricide treatment, one colony had previously been treated with thymol (propolis B), whereas the other one had not received any thymol treatment (propolis A). Propolis collected from each colony was grounded separately using an electric coffee mill (Severin, typ3871) and stored at -18° C. *V. destructor* mites were collected prior to the experiment from infested brood of three colonies placed in the same apiary.

Collection of V. destructor mites and performance of bioassays

In order to obtain mites, sealed brood was removed from brood combs and transferred to the laboratory where we opened the wax cap of each brood cell, removed pupae and collected attendant mites with forceps. Only the larger female mites with dark color were used for the assays, while those that appeared to have recently malted (pale color) or did not move

were discarded. Prior to testing, mites were kept in petri dishes together with a bee pupa as described in Delaplane et al. (2013) to prevent starvation.

For bioassays, 6 g of grounded propolis were placed in the lid of a plastic container (160 ml). Between 10 and 14 mites were placed in the bottom of each container with a small piece of moist tissue. Propolis and mites were separated by a nylon net which prevented direct contact, but allowed free evaporation of volatiles. A container without any propolis was used as control. We performed five repeats for each propolis sample and the control. Fresh mites were used for all tests. All containers were kept in an incubator at 34° C for 36 h. Following the protocol of

Garedew et al. (2002) and Damiani et al. (2010b), mite survival was monitored under a microscope 12, 18, 24 and 36 h after the assay had started. Mites were classified as "mobile" when they were still active (i.e. able to move extremities), "immobile" when they were inactive, but still alive (as validated by careful touching them with a needle) or "dead" when they showed no movement after three subsequent needle stimulations.

Table 1 Primers, sequences and references used for quantification of the honeybee viruses Black queen cell virus (BQCV), Deformed wing virus (DWV), Israeli acute paralysis virus (IAPV) and Sacbrood virus (SBV). Target virus genes were quantified in relation to a non-regulated *Apis mellifera* (A. m.) reference gene (β-actin analyzed in parallel in each sample).

Assay	Primers	Sequence (5' - 3')	Reference
BQCV	BQCV-qF7893	AGTGGCGGAGATGTATGC	Locke *et al*. (2012)
	BQCV-qB8150	GGAGGTGAAGTGGCTATATC	
DWV	DWV-F8668	TTCATTAAAGCCACCTGGAACATC	Forsgren *et al*. (2008)
	DWV-B8757	TTTCCTCATTAACTGTGTCGTTGA	
IAPV	IAPV-F6627	CCATGCCTGGCGATTCAC	de Miranda *et al*. (2010)
	KIABPV-B6707	CTGAATAATACTGTGCGTATC	
SBV	SBV-qF3164	TTGGAACTACGCATTCTCTG	Locke *et al*. (2012)
	SBV-qB3461	GCTCTAACCTCGCATCAAC	
β-actin (*A.m.*)	Am-actin2-qF	CGTGCCGATAGTATTCTTG	Locke *et al*. (2012)
	Am-actin2-qB	CTTCGTCACCAACATAGG	

The target gene (Cq) was quantified in relation to a non-regulated reference gene (β-actin analyzed in parallel in each sample). We calculated efficiency corrected ratio values according to Pfaff to account for differences in PCR efficiency (Pfaffl 2001):

$Ratio: \frac{E_{target} \wedge -Cq\ Virus[sample]}{E_{ref} \wedge -Cq\ Actin[sample]}$, with Etarget= real time PCR efficiency of target gene transcript, Eref.= real time PCR efficiency of reference gene transcript and Cq= quantification cycles.

Statistical analyses

Generalized linear mixed effect models (GLMM, glmer function in lme4 package) were used to analyze the effect of propolis treatment on honeybee worker viral loads (normalized fold ratios). Data of viral loads were log2-transformed to achieve normality. For the SBV load, only data from August and September were used, as SBV titers peaked before, or approximately with the beginning of the experiment, which may interfere with possible treatment effects. Due to the known positive correlation between *V. destructor* infestation and the degree of viral infection as described in Shen et al. (2005b), Dainat et al. (2011) and Francis et al. (2013), differences in *V. destructor* infestation between colonies may have masked treatment effects. Therefore, initial models for each virus included "treatment", "*V. destructor* infestation rate" and their interaction as explanatory variables. To account for possible colony specific differences in initial viral loads and viral fluctuations over the season, "colony" and "sampling time" were included as random factors in all models. We used backward elimination and maximum likelihood ratio estimators to fit the most parsimonious model. Models were ranked based on their Akaike's information criterion (AIC) values and compared to the null model (including only the random variables colony and sampling time) using likelihood ratio tests (anova command in the lme4 package). We additionally compared the ratio of viral titers and mite infestation (ratio = virus titer / *V. destructor* mite infestation) between the two treatment groups using Wilcoxon rank sum tests to confirm GLMM results. Ratios were calculated for each colony and each sampling point. We further tested for a possible correlation between quantitative resin collection and pathogen loads (*V. destructor* mite, viral infection) using Kendall's rank correlation tests. Correlations were tested for all colonies pooled as well as for each treatment group separately.

We composed and compared additional GLMMs to examine whether *V. destructor* infestation, virus loads, or colony size best explained the amount of resin collected by colonies. Here, initial models included the explanatory variables treatment and either *V. destructor* infestation rate, or viral load, or the number of adult bees. Models were fitted as described above with "colony" and "sampling time" included as random factors in all models, and the variances explained by each model were compared by marginal (R^2m) and conditional R (R^2c) squared values following Nakagawa and Schielzeth (2013) (Multi-model inference, r.squaredGLMM function in MuMIn

package). A Wilcoxon rank sum test was further used to compare quantitative resin collection between the two treatment groups.

In order to assess the survival of *V. destructor* mites exposed to propolis and to test for differences between propolis samples and the control, the Kaplan Meier estimator (corrected for multiple testing with Bonferroni) was used (surv function in survival package). For survival analyses, we combined data for the categories "immobile" and "dead" as both would lead to dropping of mites from bees and thus prevent them from causing further harm to bees. Therefore numbers of immobile and dead mites were summed up and compared to the number of mobile mites for each record. All statistical analyses were performed in R (R Development Core Team 2014).

Results

Resin intake and colony strength

After bees initiated resin intake in June, it was low for the first 20 days (mean amount of resin ± SD: 0.9 g/colony and day ± 0.5) and increased from the second half of July to August (2.8 g/ colony and day ± 1.9). Resin intake decreased again from late August (0.75 g/ colony and day ± 0.2) to September (0.6 g/ colony and day ± 0.3). The total amount of resin collected over the experimental period ranged from 28 g to 205 g (mean ± SD: 131.2 g ± 71.3) per colony and was positively correlated with colony strength (Kendall's rank correlation: $z = 3.52$, $r = 0.46$, $p < 0.001$). Colony strength was further higher in colonies which received additional propolis (Wilcoxon rank sum test: $W = 63$, $p = 0.041$, Fig. 1a).

Effects of propolis manipulation on virus infections and mite loads

Varroa destructor infestation generally increased from June to September 2013 in all experimental colonies (Fig. 1b), but mite infestation levels strongly varied among colonies (Fig. 1b) and were higher in stronger colonies (Kendall's rank correlation: $z = 3.43$, $p = 0.001$). We found no significant effect of propolis treatment on mite infestation (Wilcoxon rank sum test: $W = 97$, $p = 0.534$). Of the four viruses assessed in the study, only SBV and DWV were detected.

Viral titers differed between the colonies, but generally increased (DWV, Fig. 1d) or decreased (SBV, Fig. 1c) from July to August. Increase of DWV was interrupted after, at the beginning of September, all colonies received a thymol based acaricide treatment.

Overall, DWV titers were best explained by an interaction between propolis treatment and *V. destructor* infestation (Table 2). In colonies that received additional propolis, DWV titers increased less in relation to *V. destructor* infestation than in propolis-removed colonies, and the ratio of DWV and *V. destructor* was significantly lower (Wilcoxon rank sum test: $W = 141$, $p = 0.046$, Fig. 2a). In contrast, none of our variables explained a significant proportion of the variance in SBV titers (Table 2), and the ratio between SBV and *V. destructor* did not differ between treatments (Wilcoxon rank sum test: $W = 56$, $p = 0.113$, Fig. 2b). All colonies survived the winter.

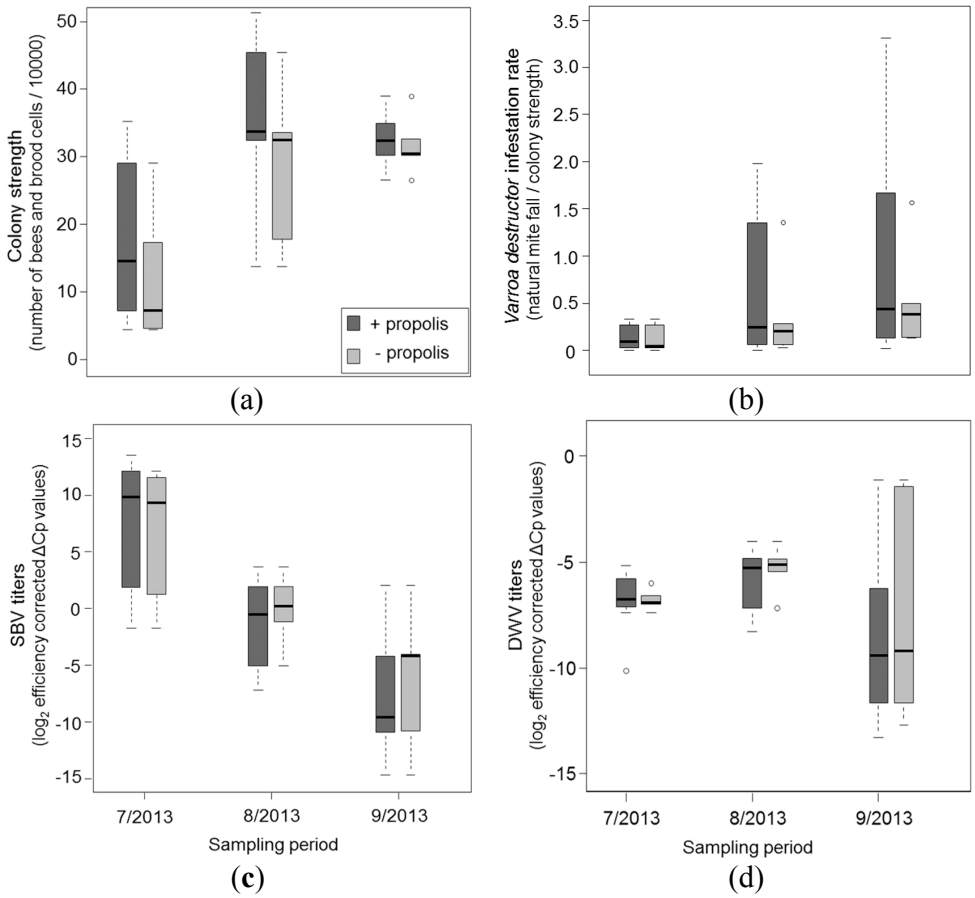

Figure 1 (a-d) Effects of the high (dark grey) and low (light grey) propolis treatment on the development of a) colony strength, b) *Varroa destructor* infestation rates (as assessed via natural mite fall), c) Sacbrood virus titers (SBV) and d) Deformed wing virus titers (DWV) over the course of the experiment from July to September 2013. Viral titers are expressed as log2 transformed, efficiency corrected ΔCq values (Cq = quantification cycles). Each boxplot represents median values of both treatment groups (N= 5) per month with default ranges for boxes (75th and 25th percentile), whiskers (+/- 1.5) and outliers (dots).

Table 2 Results of different mixed-effects models testing for effects of high and low propolis treatment ("Treatment") and *V. destructor* infestation ("Varroa") with (Treatment x Varroa) and without (Treatment + Varroa) interactions included (= explanatory variables) on titers of the Deformed wing virus (DWV) and the Sacbrood virus (SBV) (= response variables) over three months (number of samples N = 30, 3 repeats per colony). In all models, colony and month were included as random factors to take into account colony-specific differences and repeatedly measuring the same colony. Table shows degrees of freedom (df) and p-values (P) for comparing all models presented against the null model (i.e. a model including only colony and sampling date as random factors). Significant p-value is marked in bold.

Response variables	**Explanatory variables**	**df**	***P***
DWV	Treatment x Varroa	7	**0.027**
	Treatment + Varroa	6	0.108
	Treatment	5	0.263
	Varroa	5	0.282
SBV	Treatment x Varroa	7	0.065
	Treatment + Varroa	6	0.219
	Treatment	5	0.086
	Varroa	5	0.623

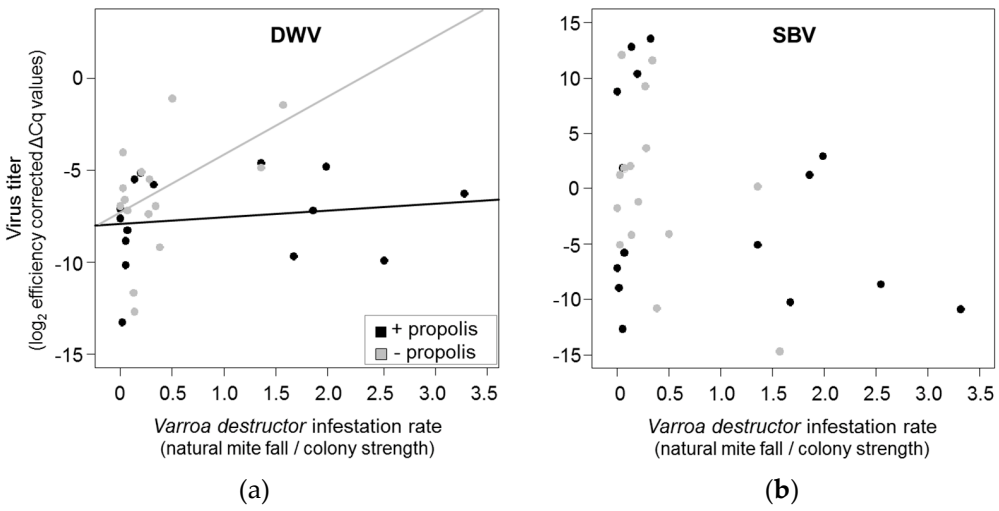

Figure 2 (a, b) Effect of the high (black) and low propolis (grey) treatment on the correlation between viral loads and the *V. destructor* mite infestations of a) Deformed wing virus (DWV) and b) Sacbrood virus (SBV). Each dot represents data from one colony for one month. Virus titers and *V. destructor* infestation were measured for each colony once per month from July to September 2013. Lines represent linear regressions between virus titers (log2 efficiency corrected fold-change relative to housekeeping gene, Cq = quantification cycles) and *V. destructor* infestation rates (natural mite fall / colony strength) for each treatment group.

Correlation between resin intake and pathogen loads

Resin intake was generally higher in colonies with added propolis than in propolis-removed colonies (Wilcoxon rank sum test: $W = 58$, $p = 0.025$), and positively correlated with *V. destructor* infestation (Kendall's rank correlation: $z = 3.43$, $r = 0.45$, $p < 0.001$), DWV titers (Kendall's rank correlation: $z = 1.95$, $r = 0.25$, $p = 0.026$, Fig. 3a) and colony strength (Kendall's rank correlation: $z = 2.34$, $r = 0.30$, $p = 0.01$), but not with SBV titers (Kendall's rank correlation: $z = 0.70$ $r = 0.09$, $p = 0.243$, Fig. 3b). However, variance in the amount of resin collected by bees was best explained by the interaction of DWV and treatment (R^2m= 32%, R^2c = 72%, Fig. 3a, Table 3). The positive correlations between resin collection and pathogen loads were highly significant for colonies with additional propolis (Kendall's rank correlation: *V. destructor*: $z = 2.43$, $r = 0.47$, $p = 0.008$; DWV: $z = 2.58$, $r = 0.5$, $p = 0.005$), but weaker (Kendall's rank correlation: *V. destructor*: $z = 1.89$, $r = 0.37$, $p = 0.029$) or not significant (Kendall's rank correlation: DWV: $z = 1$, $r = 0.20$, $p = 0.16$) for propolis removed colonies (Fig. 3a). No significant correlations were found for colony strength and resin collection, when treatment groups were tested separately (Kendall's rank correlation: low treatment: $z = 0.90$, $r = 0.18$, $p = 0.190$; high treatment: $z = 1.49$, $r = 0.29$, $p = 0.069$).

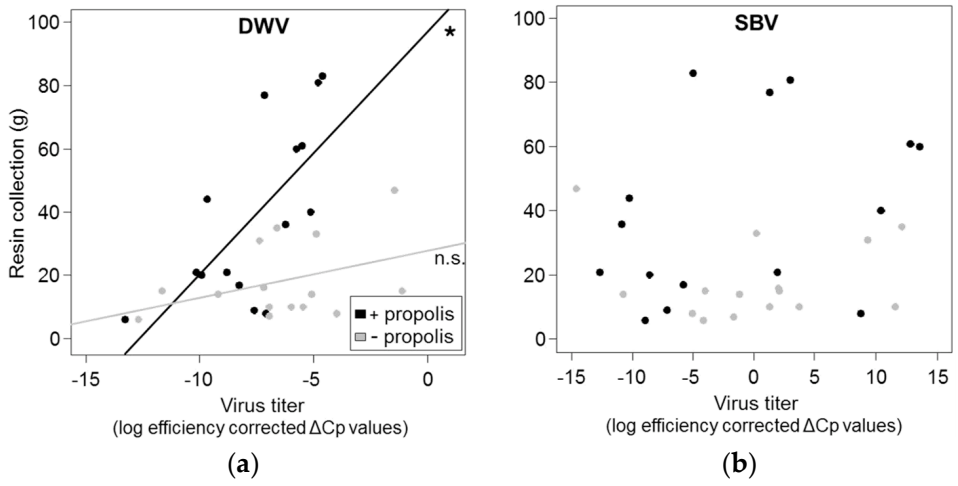

Figure 3 (a, b) Effect of viral infection with a) Deformed wing virus (DWV) and b) Sacbrood virus (SBV) on the amount of resin (g) collected by bees. Each dot represents data from one colony for one month. Lines represent linear regression between resin collection and virus titers (log efficiency corrected fold-change relative to housekeeping gene) for each treatment group (black = "high propolis treatment", gray = "low propolis treatment") with * indicating a significant correlation with $p < 0.05$ and n.s. a non-significant correlation.

Table 3 Results of mixed-effects models testing for effects of *V. destructor* infestation ("Varroa"), DWV infection (DWV) and treatment ("Treatment") as well as their interactions (indicated with x) on the quantity of resin collected by each colony over three months (number of samples N = 30, 3 repeats per colony). In all models, colony and month were included as random factors to take into account colony-specific differences and repeatedly measuring the same colony. Table shows p-values (P) and R2-values (i.e. percentage explained variance) for comparing all models presented against the null model (i.e. a model including only colony and sampling date as random factors), marginal R squared values (R2m: representing the explanatory power of the fixed effects only) and conditional R squared values (R2c: representing the explanatory power of the whole model, including the random effects). Significant p-value is marked in bold.

Explanatory variables	***P***	**R^2m (%)**	**R^2c (%)**
Treatment x DWV	0.022	32	72
DWV	0.203	2	76
Treatment x Varroa	0.176	24	71
Varroa	0.706	0.5	68
Treatment x Colony strength	0.475	18	72
Colony strength	0.127	< 0.01	72
Treatment	0.886	17	71

Effect of propolis on V. destructor mite survival

Survival of *V. destructor* mites differed between propolis samples and the control (χ^2 = 103, p < 0.0001; Fig. 4). Mites that were exposed to volatiles of propolis not treated with thymol (propolis A) lived about as long as the control group which had not been exposed to propolis (survival test: χ^2 = 1.5, p = 0.23; propolis A: median survival time ± SE = 36 hours ± 0.03, control: 36 hours ± 0.02; Fig. 4). In contrast, propolis obtained from colonies treated with thymol significantly reduced mite survival (24 hours ± 0.03) when compared with propolis A (χ^2 = 75.9, p < 0.0001) and the control (χ^2 = 59.2 p < *0.0001*; Fig. 4). After 36 hours, on average, 96% of all mites had died irrespective of the treatment.

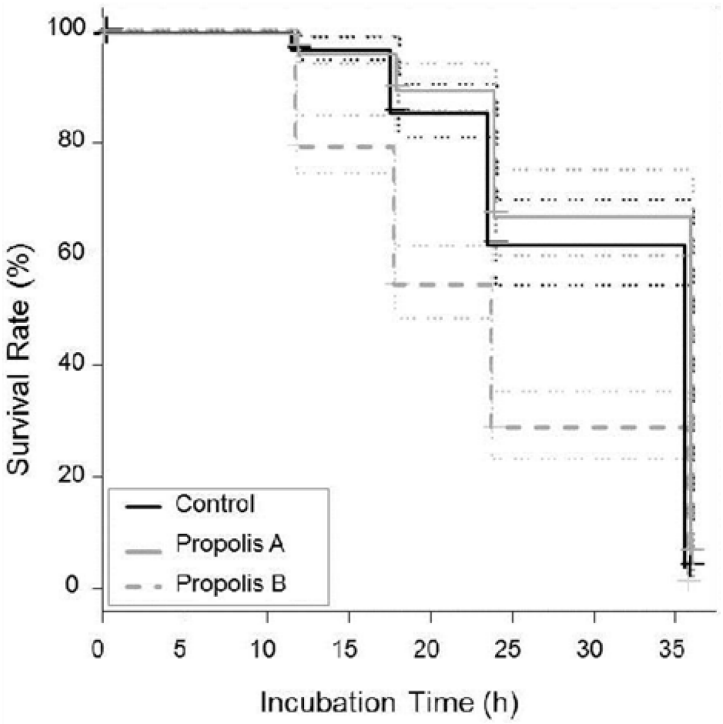

Figure 4 Kaplan-Meyer survival curves showing survival rates of *Varroa destructor* mites when exposed to propolis obtained from colonies treated with thymol (Propolis B = dashed line N = 58), not treated with thymol (Propolis A = gray line, N = 58) or not exposed to propolis (Control = black line, N = 58) under laboratory conditions. Dotted lines mark 95 % confidence intervals.

Discussion

Our results show that natural propolis can reduce DWV viral loads in relation to infestations with *V. destructor*, which further supports the key role of social immunity for eusocial insect health (Cremer et al. 2007; Masri and Cremer 2014; Meunier 2015).

Effects of propolis on virus infections and mite loads

Both viruses detected in our study, i.e. the Deformed wing virus (DWV) and the Sacbrood virus (SBV), are likely to be transmitted by *V. destructor* (Tentcheva et al. 2004; Shen et al. 2005a), but only DWV is known to be highly correlated with mite loads (Dainat et al. 2011; Francis et al. 2013) and therefore usually increase from early summer to autumn (Tentcheva et al. 2004; Di Prisco et al. 2011b). Accordingly, DWV titers varied proportionally with *V. destructor* infestation in all of our colonies, whereas SBV titers decreased continuously from June to September, irrespective of mite infestation. Such seasonal dynamics observed for SBV confirm earlier studies (e.g. Tentcheva et al. 2004). However, in colonies provided with additional propolis, DWV titers increased significantly less with *V. destructor* infestation compared to propolis-removed colonies. This finding suggests that the long known antiviral effect of propolis (Amoros et al. 1992; Gekker et al. 2005; Shimizu et al. 2008; Schnitzler et

al. 2010) may also account at the level of the honeybee colony. However, the effect of added propolis on DWV titers was relatively low and depended on *V. destructor* mite infestation. Thus, alternatively, but not mutually exclusive, bees in colonies with higher amounts of propolis may better cope with DWV infections due to a positive effect of propolis on their immune system (Borba et al. 2015). Such a positive effect on colony health is further supported by significantly higher colony strength in colonies with added propolis. Likewise, Nicodemo et al. (2013) found increased brood viability and higher worker longevity in Africanized honeybees that were bred for high propolis production. Propolis may thus benefit honeybee colonies by increasing general vitality (Simone-Finstrom et al. 2009; Simone-Finstrom and Spivak 2012). This increase in vitality may also explain why colonies that were provided with multiple grids to encourage formation of a "natural propolis envelope" were more likely to survive winter and were stronger in spring than colonies without envelope in the study of Borba et al (2015). Our own study did not see an effect of propolis enrichment on survival over winter, but this may be due to the smaller sample size of six colonies (compared to twelve in Borba et al. (2015)). Unlike our study, Borba et al. (2015) found no difference in DWV infection between colonies with "natural propolis envelope" and colonies without. This discrepancy may be explained by differences in overall propolis amounts and thus DWV infection dynamics between studies. Moreover, Borba et al. (2015) did not actively manipulate propolis amounts, quantify resin intake of colonies or analyze the interaction between mites and DWV, which renders a direct comparison between studies difficult.

In contrast to DWV, we found no significant effect of propolis on SBV infection. This finding may be explained by the fact that SBV infection peaked approximately with the beginning of our experiment and then decreased in all colonies. Thus, differences in SBV infection between treatments may have been masked by the virus' general demise.

Although antiviral activity of various propolis extracts against envelope and non-envelope viruses was demonstrated by several studies, the exact mode of action is still not completely understood (Amoros et al. 1992; Gekker et al. 2005). Reduction of viral replication tends to be highest when extracts were applied prior to or at the time of infection, suggesting that propolis derived compounds interfere with the binding or entering of viruses at host cells (Amoros et al. 1992; Schnitzler et al. 2010). However, propolis is a complex mixture of various substances with a broad therapeutic spectrum, including immunomodulatory activity resulting primarily from stimulation of macrophage activity (Amoros et al. 1992; Schnitzler et al. 2010; Otti et al. 2014). It was further shown to enhance the recognition of pathogens and support the initial steps of the immune response by up-regulating the expression of two toll-like receptors (Orsatti et al. 2010). In honeybees, viral defense is mediated mainly by RNA interference (RNAi), but also by immune-related mechanisms (i.e.

the pathogen-associated molecular pattern (PAMP) and induced toll pathways) (Brutscher et al. 2015). Thus, resistance to viral infections may be improved by propolis compounds through enhancing the non-specific immune defense, while simultaneously reducing the general investment in the cost intensive activation of humoral immune responses. In fact, the presence of propolis within bee hives was found to reduce the expression of immune related genes without affecting levels of pathogens and parasites (Borba et al. 2015). Other studies showed however that resin/propolis further reduced bacterial loads in the nests (Castella et al. 2008; Simone-Finstrom et al. 2009).

Constant removal of propolis may in turn increase physiological stress, which results in increased immune activity and thus reduced individual fitness. Such immune system weakening effects can play a critical role for viral infections such as with DWV (Yang and Cox-Foster 2005; Di Prisco et al. 2011b; Locke et al. 2012). While bees are generally able to combat DWV infections, physiological stress through mite pressure can enhance virus expansion and lead to a destabilized parasite-host relationship (Yang and Cox-Foster 2007; Nazzi et al. 2015). In fact, Nazzi et al. (2015) found 19 immune related genes to be down-regulated in *V. destructor*-infested colonies, indicating that mites not only function as vector for viruses, but also enhance viral infections by actively suppressing the immune function of bees. This finding was further confirmed by Ryabov et al. (2014) who revealed that mite pressure increased replication of a virulent DWV strain, which was itself not detected in mites.

Our results suggest that the immune suppressive effect caused by mites may be (partly) compensated by the immune supportive effect of propolis, which can have important implications for colony health, as the level of viral titers are known to be critical for the development of clinical disease symptoms (Yang and Cox-Foster 2005). Contrary to our expectations, our results showed no impact of propolis on *V. destructor* itself, which agrees with previous studies investigating the efficacy of natural propolis (Genersch et al. 2010; Nicodemo, D. et al. 2013). Survival of mites, in our study, was only reduced by raw propolis obtained from colonies that have previously been treated with thymol which is known to accumulate in propolis (Miguel et al. 2013).

In contrast, other studies showed that direct exposure of mites to ethanolic propolis extracts can cause mortality and narcotic effects (Garedew et al. 2002; Damiani et al. 2010b), suggesting that propolis can have a detrimental influence on mites. However, lethal effects were mainly observed for extracts with high alcohol content (70%) and less (or not) for extracts with lower alcohol content (40%), which may be explained by a comparable lower content of bioactive compounds (Garedew et al. 2002). Under natural conditions, mites rarely get in direct contact with high concentrations of non-volatile propolis compounds as found in 70% alcohol extracts (Garedew et al. 2002), but may be exposed to volatiles evaporating from propolis

deposited in varying areas of the hive (including cell rims). Our results thus indicate that such propolis volatiles do not affect mite survival (at least not for the samples from our study area).

However, the chemical composition of propolis is highly variable and depends largely on the plant sources used by bees (Greenaway et al. 1990; Teixeira et al. 2005). Bees may actually learn or evolve to shift their resin collection towards sources with acaricide activity as suggested by the study of Popova et al. (2014) who found a higher content of three biologically active compounds in propolis from *V. destructor* resistant colonies (for a review see Johnson et al. 2012). In fact, the existence of several *V. destructor* mite-surviving *A. mellifera* populations indicates that natural selection can lead to a stable parasite-host relationship, as e.g. seen in African and Africanized honeybees which occur in large wild *V. destructor* mite resistant populations (Hepburn and Radloff 1998). Compared to the European subspecies, African and Africanized honeybees generally collect larger amounts of propolis and have a better resistance against most of the common honeybee diseases (Pirk et al. 2014), which further supports the beneficial role of propolis for *A. mellifera* colony health.

Correlation between resin intake and pathogen loads

Resin intake was comparatively higher in colonies with added propolis which may be explained by the observed differences in colony size between the two treatment groups. Interestingly, the quantity of resin collected by each colony was overall positively correlated with levels of DWV infection, suggesting that bees actively respond to viral pressure by increasing resin collection. Such "self-medication" was also described by Simone-Finstrom and Spivak (2012), who showed that colonies increased resin foraging rates after having been challenged with the fungal parasite *Ascosphaera apis* (causative agent of chalkbrood).

However, we observed no such correlation between resin collection and SBV titers. As the collection of resins depends on its availability, which increases towards the end of summer (Langenheim 2003), the apparent lack of response towards SBV infection may also be related to the earlier peak of SBV infection.

Conclusions

In conclusion, our study provides new insights into the functional properties of propolis as a colony level defense mechanism and thereby further supports its substantial role for honeybee colony health. We have shown that propolis can naturally benefit honeybee field colonies by reducing DWV loads in relation to *V. destructor* infestation, suggesting that it may be vital for bees to overcome this pathogen challenge. However, future studies (ideally with more colonies) should verify this interaction and follow colony strength, health and viral titers over winter to better understand whether and (if so) how reduced DWV loads actually impact colony survival. Further studies including additional health parameters are also needed in order to better understand the actual mode of action of propolis. Our

result on the activity of propolis against *V. destructor* contradicts previous studies which used ethanolic extracts and stresses the general need for more studies conducted under natural conditions at the colony level to obtain more biologically relevant data (Retschnig et al. 2015). We further suggest that missing resin sources or the removal of propolis may have negative implications for honeybee colonies. Resin collection, as natural defense of honeybees, should therefore find more consideration in practical beekeeping, particularly as many bee keepers constantly remove propolis from bee hives during routine control.

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Chapter IV

Diversity matters: How bees benefit from different resin sources

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Abstract

Biodiverse environments provide a variety of resources that can be exploited by consumers. While many studies revealed a positive correlation between biodiversity and consumer biomass and richness, only few studies have investigated *how* resource diversity affects single consumers. To better understand whether a single consumer species benefits from diverse resources, we tested how the protective function of a defensive plant resource (i.e., resin exploited by social bees) varied among different sources and target organisms (predators, parasites and pathogens). To assess synergistic effects, resins from different plant genera were tested separately and in combination. We found that resin diversity is beneficial for bees, with its functional properties depending on the target organisms, type and composition of resin. Different resins showed different effects, and mixtures were more effective than some of the single resins (functional complementarity). We conclude that resins of different plant species target different organisms and act synergistically where combined. Bees that rely on resin for protection benefit more when they have access to diverse resin sources. Loss of biodiversity may in turn destabilize consumer populations due to restricted access to a variety of resources.

Introduction

Biodiversity is crucial for the functioning and stability of ecosystems (biodiversity ecosystem functioning (BEF): Tilman 1999; Loreau et al. 2001; Balvanera et al. 2006) . Biodiverse ecosystems also provide a higher diversity of resources compared to less diverse environments, resulting in more resources that can be exploited and consumed by its inhabitants (Duffy et al. 2007). Whilst the diversity of resources is beneficial to overall consumer diversity (Siemann 1998; De Deyn et al. 2004; Gamfeldt et al. 2005; Ives et al. 2005), few studies have investigated the effect of resource diversity on single consumers. Moreover, the precise mechanisms by which single consumers/organisms may benefit from an increased diversity of resources in a biodiverse environment have received little attention.

In theory, three mechanisms can explain how resource diversity increases the well-being, and ultimately fitness of organisms. Firstly, a variety of resources implies that more resources are available throughout the year, and shortages in one resource are compensated by others (functional redundancy; Rosenfeld 2002; Wohl et al. 2004). For instance, when one prey species declines in abundance, it can be replaced by an alternative prey species. Likewise, if one pollinator goes extinct, a plant can still be pollinated by another pollinator (Blüthgen and Klein 2011). Secondly, a variety of resources also implies that organisms can optimize the composition of resources, and compensate for the effects of toxic compounds, or the lack of nutrients or other important compounds that are present in some resources but not in others (e.g., in insect nutrition, Behmer 2009; Eckhardt et al. 2014). Mixing resources can also ensure that several functions can be simultaneously fulfilled due to different properties of its components. We refer to such benefits of a resource mixture as “functional balance”, thereby coining a new term that has, to our best knowledge, not been used in this context before. Thirdly, a variety of resources can increase a specific function (functional complementarity; Hooper 1998; Loreau and Hector 2001; Petchey 2003; Yachi and Loreau 2007; Finke and Snyder 2008), i.e. mixed resource diets can accelerate growth and/or development or improve immune functions (as e.g. shown in honeybees: Alaux et al. 2010; Höcherl et al. 2012; Eckhardt et al. 2014).

To differentiate between these three mechanisms, we tested how resource diversity can affect functional properties that are beneficial for a specific consumer, using stingless bees collecting different plant resins as a model. Stingless bees are a group of highly social bees with a pan-tropical distribution (Michener 2007). Like honeybees (*Apis mellifera*), they collect pollen and nectar to nourish their colonies and are highly important pollinators for their environments (Roubik 1989). They also collect large amounts of plant resins which they use to defend their colonies against predators and parasites and to build their nests (Roubik 1989; 2006; Leonhardt and Blüthgen 2009). The inclusion of foreign material, such as resin, in

the nest (material) is considered a key innovation in the evolution and diversification of bees in general (Litman et al. 2011). It may further have facilitated the evolution of sociality in stingless bees as well as their successful diversification in tropical ecosystems (Roubik 1989).

Moreover, stingless bees also use resin to extract chemical compounds that are included in the bees' cuticular chemical profiles (Leonhardt et al. 2009; Leonhardt et al. 2011a) where they protect bees against predation (Wenzel 2011) and affect interspecific aggressive behaviour (Leonhardt et al. 2010). Hence, for bees, resin represents a crucial resource that has been largely neglected in ecological studies, although it is essential for their colonies' fitness (Simone et al. 2009; Simone-Finstrom and Spivak 2010; Nicodemo et al. 2013) and has shaped their evolutionary phylogeny (Leonhardt et al. 2013). While the general importance of resin for bees has by now received some attention (Simone et al. 2009; Simone-Finstrom and Spivak 2010), virtually nothing is known on the importance of resin diversity.

To investigate whether and how stingless bees benefit from a diversity of plant resins, we compared the effectiveness of up to four different single resins (obtained from different plant genera) and their mixture to evaluate their protective functions. Therefore, we tested their effect in repelling predators (i.e., ants) and a common parasite of bee colonies (i.e., the small hive beetle, *Aethina tumida*) and in inhibiting the growth of four bacterial species and a single cell fungus.

Resin is secreted by many plant families following wounding (Langenheim 2003) or produced in flowers or seeds to attract pollinators or seed dispersers (Armbruster 1984; Wallace and Trueman 1995). Therefore, various resins (except for those produced in flowers and seeds) may be available all year in ecosystems with a high plant diversity (Leonhardt and Blüthgen 2009). In contrast, in agriculturally intensified landscapes with a predominance of monocultures (e.g. pine plantations), bees may have limited access to diverse resin sources. If bees live in environments with various resin plants, they have permanent access to many resins, some of which are similar in their functional properties (e.g. resins from plants of the same genus) and could replace each other (functional redundancy). That is to say, resin of one plant species which is collected and placed around the entrance (Leonhardt and Blüthgen 2009) could be as repellent to predatory ants as resin of a different plant species. Bees could thus collect either one or the other to repel ant predators. However, not all resins collected and accumulated outside or within nests are equally effective in protecting bees against antagonists. Therefore we postulated that resins from different plant genera differentially affect different organisms. For instance, resin of one plant species may have particularly strong antimicrobial properties and thus inhibit potential pathogens, but show little effect against larger organisms, such as predatory ant species. In contrast, the resin of a different

plant species may strongly repel ants, but show no antimicrobial effect. Such differential effects render the collection and mixture of various resins the most efficient solution (functional balance). Finally, we hypothesized that the effectiveness of resin mixtures is higher than the effectiveness of most single resins (functional complementarity). That is to say, mixing different resins may increase the effectiveness (e.g. antimicrobial and/or repellent effect) compared to single resins. We finally tested whether the effect of mixtures was additive (i.e., as strong as the sum of the effects of single resins) or synergistic (i.e., stronger than the sum of the effects of single resins).

Methods

Study site, species and resin sampling

The study was conducted in Southern Queensland, Australia, between February and April 2013. Two genera of stingless bees are found here, *Austroplebeia* and *Tetragonula* (Dollin et al. 1997; Walker 2010). *Tetragonula carbonaria* and *Austroplebeia australis* are the most common species in our study area, both of which collect resin from various plant species (Leonhardt et al., unpublished data; and Leonhardt and Wallace, personal observations).

Resin for behavioural and microbiological assays were obtained from four different plant species belonging to four different genera and three different families, all of which are known to be major resin sources (i.e., make up for 10 – 20 % of resin collected) of *T. carbonaria* (Wallace and Lee 2010; Leonhardt et al. 2011b; Leonhardt et al., unpublished data): *Corymbia torelliana* (Myrtaceae), *Syncarpia glomulifera* (Myrtaceae), a non-native pinus hybrid (*Pinus caribaea* × *Pinus elliotii*, Pinaceae), and *Araucaria cunninghamii* (Araucariaceae). Resin was collected by wounding the trees' trunks with a small knife and attaching a clean clear plastic bag beneath the wound to capture all resin secreted over a period of 5 to 20 hours following wound infliction. Bees also collect resin from wounds of our study tree species (all authors, personal observation). Because resin of *S. glomulifera* could only be sampled in small quantities using this method, this resin was only used for the microbiological assays. *Corymbia torelliana* resin was obtained from resin storages of *T. carbonaria* colonies, because it is produced in minute quantities in the trees' seed capsules where it is collected by bees (Wallace and Trueman 1995). As *C. torelliana* trees were fruiting prior to and during the study period, *T. carbonaria* colonies were collecting and storing ample amounts of its seed resin in their nests, which could be easily recognized based on colour and smell. These resin storages were shown to contain nearly pure *C. torelliana* capsule resin (Massaro et al. 2014).

Extract preparation

Freshly collected resins were transferred to the laboratory and kept in a freezer (-18°C) until extract preparation. For extraction, resins were weighed into clean 250 ml glass vials and dissolved in either hexane (for behavioural assays) or 70% ethanol (for microbial assays).

We used the non-polar solvent hexane for the behavioural assays to ensure extraction of volatile compounds, such as terpenoids. In doing so, some of the highly polar compounds were not extracted, rendering a direct comparison with the results of the microbial assays (where ethanol was used) problematic. The bottles with the solutions were covered with tin foil (to protect them from light) and stored in the fridge (~5°C) for extraction under periodical manual shaking for one week.

For behavioural assays, 20 g of each single resin was dissolved in 100 ml hexane (analytical grade, Sigma-Aldrich, Castle Hill, NSW, Australia) to achieve a stock solution of 20% (w/v). The extract was then transferred to a clean vial to separate the extract from the insoluble fraction. Different concentrations were obtained by adding 3 and 5 ml hexane to 1 ml of the stock solution, resulting in concentrations of 5% and 1.6% (w/v), respectively. The resin mixture was prepared by combining equal amounts of the 20% single resin stock solutions.

For microbiological assays, 6 g of each resin was extracted in 60 ml 70% ethanol (10% w/v). Extracts were filtered through filter paper twice using two different filters (Whatman No 1001055, Millipore 0.45 Typ HNWP, Sigma-Aldrich, Australia). To obtain exact concentrations the solvent was rotary evaporated in a water bath at 37°C and the resulting suspension was freeze-dried at -80°C for 24 hours. For stock solutions, 0.3 g of the dry extract was resolved in 10 ml ethanol (3% w/v). The mixture was prepared by combining equal amounts (0.075g) of each single dry resin extract and dissolving it in 10 ml ethanol. From each stock solution a serial dilution was made by adding equal amounts of the solvent (70% ethanol) to obtain a concentration range of [A] 3%, [B] 1.5%, [C] 0.75% and [D] 0.375% w/v.

Behavioural assays with predators and parasites

Behavioural assays were conducted with two Australian predatory ant species (green-head ants, *Rhytidoponera metallica*, and meat ants, *Iridomyrmex purpureus*) and the small hive beetle (SHB, *Aethina tumida*), a common parasite of honeybee and stingless bee colonies. Green-head ants are known to prey on stingless bees (Wenzel 2011), while meat ants are commonly found in habitats of stingless bees and prey on a large variety of insects (Burwell 2007).

Ants were sampled from three different colonies in Buderim (green-head ants) and Bundaberg (meat ants) by picking up foragers from the ground with forceps and transferring them to a clean plastic bowl. Small hive beetles were collected from three hives of infested honeybee colonies located in Buderim, Queensland, Australia, using an insect aspirator. Ants and beetles were kept in bowls for no longer than 24 hours before they were tested in the behavioural assays. When the test animals were kept in bowls for more than 5 hours, they were supplied with a moist tissue to prevent desiccation and with a sugar solution for nutrition. Each animal was tested only once where possible. However, as we did

not always find sufficient numbers of ants and beetles, they were occasionally tested more than once, with at least 1 hour in between each test.

Behavioural assays were conducted in an open arena (clean clear plastic, 25 cm in diameter, 5 cm depth) which was large enough to allow for free movement of individual ants/beetles. The bottom of the plastic arena was covered with a filter paper that was divided into four equally sized fields. A few drops (about 2 ml) resin extract were equally distributed across each of two opposing fields (extract fields), while the other two fields were covered with equal amounts of hexane (control fields). Once the solvent was evaporated, 10 ants or beetles were placed in the centre of the arena and thus exposed to both volatile and non-volatile resin compounds in the extract fields. After 2 min of habituation, the number of individuals in the extract and control fields was recorded every 30 sec. The plate was gradually rotated during the observation period to avoid a site bias. The trial was terminated after 6 minutes to ensure that highly volatile resin compounds had not yet completely evaporated. After each trial, the filter paper was replaced and the plastic arena was cleaned with hexane.

If predators were repelled by resin compounds, we expected them to avoid the extract fields of the filter papers and instead spend substantially more time in the control fields. If the animals were not affected by the resin compounds, they should show no difference in the time spent in extract or control fields. If they were actually attracted by resin, they should spent more time in extract than control fields.

First tests were performed using an extract concentration of 5% resin. To test whether behavioural responses were also found for lower extract concentrations, we further tested concentrations of 1.6% in meat ants and SHBs. Because green-head ants positioned themselves equally among extract and control fields when the 5% concentration was used, we additionally tested the stock concentration of 20% of the single resin extract to ensure that a lack of response was not due to too low resin concentrations. The 5% *P. caribaea*×*P. elliotii* resin extract as well as the 5% extract mixture were not tested in meat ants due to insufficient amounts of extract.

We performed 6 trials per extract and concentration, resulting in a total of 48 trials and about 480 animals tested for SHBs and green-head ants, and a total of 36 trials and about 360 animals tested in meat ants.

Microbiological assays

Antimicrobial assays were conducted using the agar well diffusions technique on a 64 well plate (27.9 x 27.9 x 1cm). To test for potential differences in bioactivity of the different resins against various pathogens, three categories of microorganisms were selected and tested. These included two type-culture strains of Gram-positive bacteria, i.e. *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (ATCC 11788), three strains of Gram-negative bacteria, i.e. *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 13311) and *Pseudosomonas aeroginosa* (ATCC 27853),

and one laboratory strain of unicellular fungus, i.e. *Candida albicans*. We chose to perform our experiments with those standard laboratory microbes, because, in contrast to honeybees, no specific pathogenic microbes have been described for stingless bees, which could have been used in our microbiological assays.

All bacterial strains had been obtained from the American Type Culture Collection (ATCC) and had been preserved at -20°C in nutrient broth and 15% glycerol. Working culture of the bacterial strains was prepared on tryptone soy agar (TSA). *Candida albicans* was a laboratory strain isolated from a clinical case.

For susceptibility tests, 400 ml Mueller Hinton agar (growth media) was inoculated with 4 ml of microbial suspension. The inoculum was prepared by suspending previously subcultured microorganisms in phosphate buffered saline (PBS, pH 7.2) and adjusting the solution to McFarland standard no. 1 (cell density of 3 x 10^8 colony-forming units (CFU) per ml). Molten agar was cooled to 45°C and - depending on the volume of agar used - bacterial suspension was added to give a final concentration of approximately 3 x 10^7 CFU/ml. Agar with bacteria was poured onto plates and let to solidify before punching 64 holes using a stainless steel cylinder (9 mm diameter).

Wells were randomly filled to their upper margin with 150 µl of each extract of all concentrations prepared plus controls (70% ethanol). Consequently, for each extract the effective amount of resin per well was [A] 4.5 mg, [B] 2.25 mg, [C] 1.125 mg and [D] 0.56 mg. Due to diminished extract solutions towards the end of the experiment, the amount of extract filled into wells was reduced to 100 µl, resulting in [A] 3.0 mg, [B] 1.75 mg, [C] 0.75 mg and [D] 0.375 mg resin per well in the second repeat. Plates were then incubated at 36.5°C (± 5°C) for 20 hours. To quantify antimicrobial activity the diameter of growth inhibition around each hole was measured in two directions using a digital calliper. For analyses, the mean value was calculated with the hole diameter subtracted. All extracts plus controls were tested in triplets on one plate and experiments were repeated twice. Except for the plate with *S. aureus*, extracts (particularly those of the 3.0 and 0.75 mg/well concentration) tended to overflow and cause distorted inhibition zones in the second repeat, which is why we did not include these data points in our analysis.

Statistical analyses

For each behavioural trial *i*, we calculated a single value (*b(i)*), the standardized mean difference in the number of animals in the 6 min observation period:

$$b(i) = \frac{\text{number of animals in control fields - number of animals in extract fields}}{\text{total number of animals tested}}.$$

We considered the extract attractant if $-1 \leq b(i) < 0$ and $b(i)$ was significantly different from 0 (using a Students t-test), neutral if $b(i)$ was not significantly different from 0, and repellent if $0 < b(i) \leq 1$ and $b(i)$ was significantly different from 0. Differences between the different extracts were compared using ANOVA followed by Tukey's posthoc tests. Likewise, for microbiological assays, we compared the inhibition zones between different extracts for each concentration and microorganism using ANOVAs and Tukey's post hoc tests.

To determine whether the effect of resin mixtures was additive, less than additive or synergistic, we summed up the values for all single resins for the 1.6% concentration in the behavioural assays with small hive beetles (SHB), and for the 0.75 and 1.13 mg/well in the microbiological assays, and compared this value to the values for the 5% and 3.0 as well as 4.5 mg/well mixture concentration, respectively, using t-tests. We considered the mixture effects in a purely statistical sense and refer to "additive" if the mixture values were not significantly different from the summed values of single resins, "less than additive" if they were significantly lower and "synergistic" if they were significantly higher. Our definition of "additive" or "synergistic" is therefore much less complex than the definition typically used in microbial research (Chou 2010) where the entire response (e.g. ranging from no response to maximum response) is the subject of comparison to determine additive or synergistic effects.

Where necessary, data was log- or square root transformed to meet the assumptions of normality and homogeneity of variances. All statistical analyses were performed in R statistical software (R Development Core Team 2013). All experiments comply with the current laws of Australia.

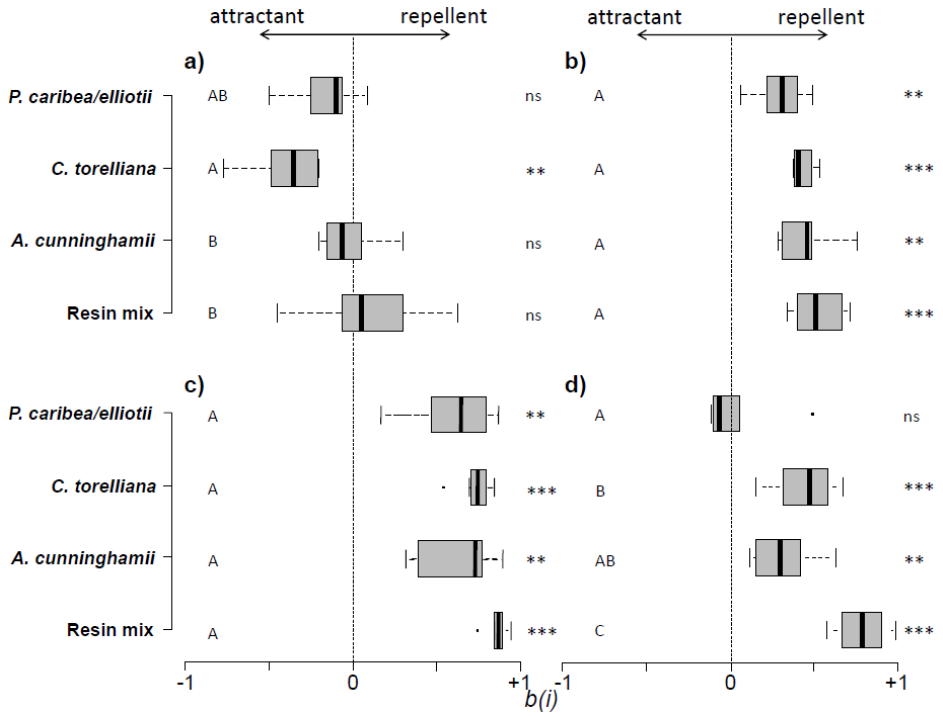

Fig. 1. Attractant and repellent effect (b(i)) of extracts of single resins and resin mixtures on (a) green-head ants (*Rhytidoponera metallica*) tested against 5% extract concentrations, (b) meat ants (*Iridomyrmex purpureus*) tested against 1.6% extract concentrations and small hive beetle (*Aethina tumida*) tested against (c) 5% and (d) 1.6% extract concentrations. The significance of attractant or repellent effect (b(i)) of resin extracts is indicated as follows: ns: not significantly different from 0 (neutral), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Resin extracts with different letters indicate significantly different b(i)-values according to Tukey's HSD.

Results

Behavioural assays with predators and parasites

The two predatory ant species responded differently to the resin and mixed extracts (Fig. 1). While meat ants were equally repelled by all single extracts and the extract mixture at low concentrations (Fig. 1b), green-head ants did not differentiate between *A. cunninghamii* and *P. caribaea*×*P. elliotii* resin, the resin mixture and hexane, but were attracted by *C. torelliana* resin (Fig. 1a). The exact same behaviour was shown when the 20% extract concentration was used (data not shown). In contrast, small hive beetles were equally repelled by all single resin extracts and the extract mixture when tested against the 5% concentration (Fig. 1c). However, when the 1.6% extract concentration was used, the mixed extract had the strongest repellent effect, while *P. caribaea*×*P. elliotii* resin had no effect and *A. cunninghamii* and *C. torelliana* were intermediate in their repellent effect (Fig. 1d).

Microbiological assays

The four resin extracts and the resin mixture differently affected the growth of the microorganisms tested (Fig. 2, Table 1). *Corymbia torelliana* resin had the strongest inhibitory effect of all resins and was generally

more effective than the resin mixture (Fig. 2, Table 1). *P. caribaea*×*P. elliotii* and *A. cunninghamii* resin had a relatively low inhibitory effect and did not inhibit the growth of *P. aeroginosa* and *S. typhimurium* (Fig. 2, Table 1). Neither single resins nor resin mixtures inhibited the growth of *E. coli* within the concentration range used.

Additive or synergistic effect?

We found both additive and less than additive, but no synergistic effects of the resin mixture compared to the sum of all single resin extracts (Table 2). While the repellent effect against the small hive beetle and the growth inhibitory effect against *P. aeroginosa* and *S. typhimurium* were additive, growth inhibition of *S. aureus*, *B. cereus* and *C. albicans* was significantly lower for the resin mixture than for the sum of all single resins (Table 2).

Table 2. Additive, less than additive (less) and synergistic effects (mean b(i) ± SD and mean growth inhibition range [mm] ± SD) of summed effects of single resins and resin mixture on small hive beetles (SHB), *Bacillus cereus* (Bc), *Candida albicans* (Ca), *Pseudosomonas aeroginosa* (Pa), *Salmonella typhimurium* (St) and *Staphylococcus aureus* (Sa). For microorganisms, effects were assessed for two different concentration combinations (I: 3.0 and 0.75 mg/well, II: 4.5 and 1.13 mg/well). Significance levels are as follows: ns: not significant, * P < 0.05, ** P < 0.01, *** P < 0.001.

Study organism	single resins	resin mix	*t*	*P*	Effect
SHB	0.82 ± 0.34	0.84 ± 0.08	0.11	ns	Additive
Bc I	-	12.07 ± 1.01	-	-	-
Bc II	46.03 ± 0.04	13.02 ± 0.78	-73.5	***	Less
Ca I	-	3.15 ± 0.67	-	-	-
Ca II	7.50 ± 1.15	4.15 ± 0.22	-4.94	*	Less
Pa I	-	-	-	-	-
Pa II	3.18 ± 1.14	1.78 ± 0.25	-2.08	ns	Additive
St I	-	2.62 ± 0.88	-	-	-
St II	6.67 ± 1.37	4.57 ± 1.58	-1.74	ns	Additive
Sa I	51.00 ± 1.13	12.60 ± 0.45	-37.51	***	Less
Sa II	-	-	-	-	-

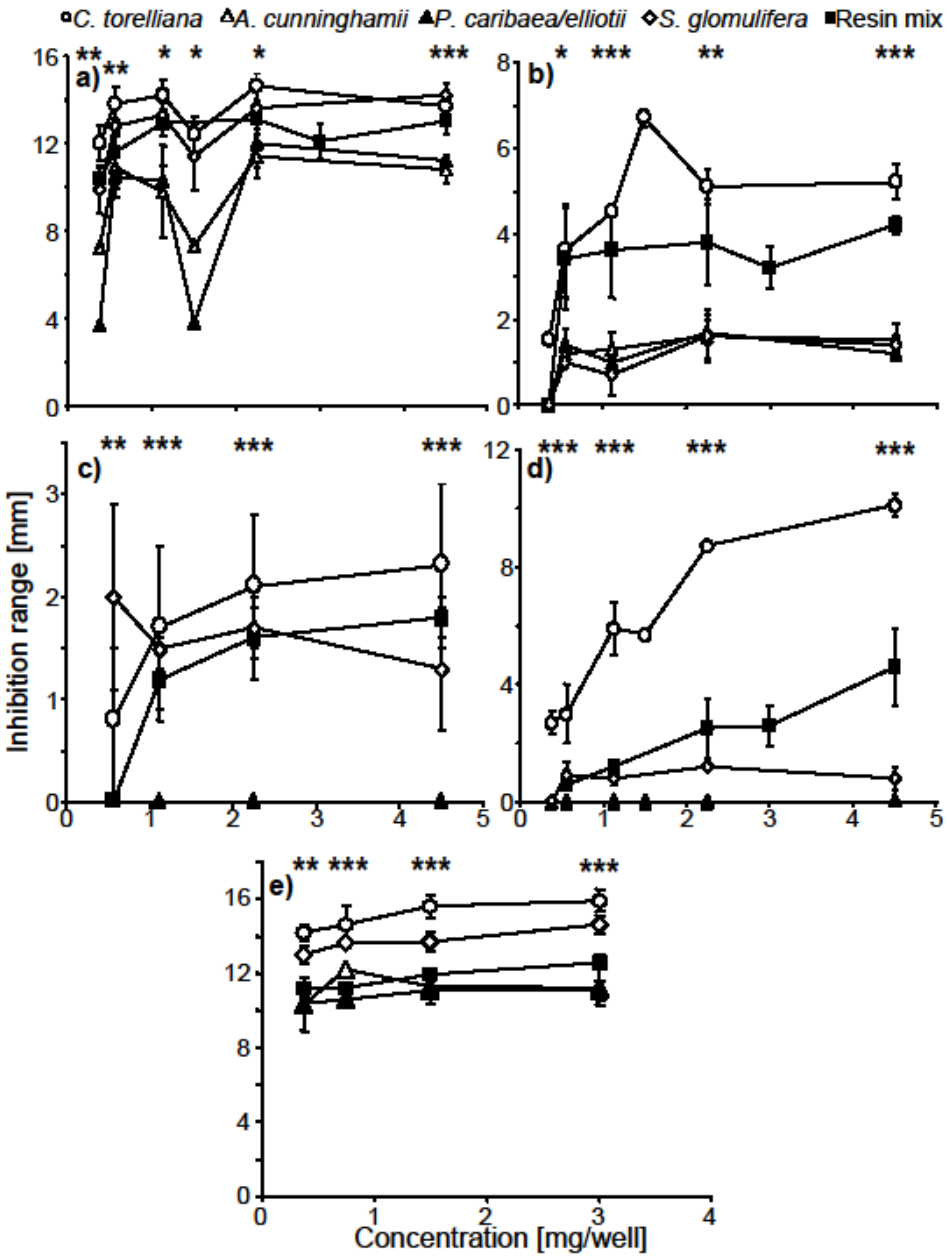

Fig. 2. Growth inhibitory effect of single resin and mixed extracts (applied in up to eight different concentrations [mg resin/well]) on a) *Bacillus cereus*, b) *Candida albicans*, c) *Pseudosomonas aeroginosa*, d) *Salmonella typhimurium* and e) *Staphylococcus aureus*. The control ethanol is not displayed as it did not show any growth inhibition. Stars indicate significant differences between the different extracts: Significance levels are as follows: ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Note that y-axis scales differ between graphs to allow for a better visual representation of the results.

Table 1. Tukey posthoc test results of comparisons of growth inhibition effects between resin extracts from *Corymbia torelliana* (Ct), *Araucaria cunninghamii* (Ac), *Pinus caribea× P. elliotii* (Pce), *Syncarpia glomerulifera* (Sg) and resin mixture (Mix) on five microorganisms; dashes indicate concentrations that were not tested. Significant P-values are marked in bold.

	Bacillus cereus								***Candida albicans***						
Concentration	4.50	3.00	2.25	1.50	1.13	0.75	0.56	0.38	4.50	3.00	2.25	1.50	1.13	0.75	0.56
Ct-Ac	**<0.001**	-	**0.012**	**0.028**	**0.023**	-	**0.027**	**0.006**	**<0.001**	-	**0.005**	-	**0.012**	-	0.064
Mix-Ac	**0.005**	-	0.268	-	0.118	-	0.883	**0.047**	**<0.001**	-	**0.047**	-	0.063	-	0.097
Pce-Ac	0.927	-	0.937	1.00	0.993	-	0.981	1.000	0.797	-	1.000	-	0.976	-	1.000
Sg-Ac	**<0.001**	-	0.102	0.063	0.079	-	0.200	0.093	0.969	-	1.000	-	0.587	-	0.995
Mix-Ct	0.576	-	0.311	-	0.791	-	0.113	0.336	**0.016**	-	0.585	-	0.824	-	0.999
Pce-Ct	**0.002**	-	**0.039**	0.083	0.077	-	**0.012**	**0.024**	**<0.001**	-	**0.006**	-	**0.005**	-	0.085
Sg-Ct	0.876	-	0.651	0.780	0.909	-	0.695	0.163	**<0.001**	-	**0.004**	-	**0.001**	-	**0.036**
Pce-Mix	**0.017**	-	0.639	-	0.313	-	0.610	0.146	**<0.001**	-	0.055	-	**0.026**	-	0.127
Sg-Mix	0.178	-	0.959	-	0.998	-	0.610	0.971	**<0.001**	-	**0.040**	-	**0.006**	-	0.054
Sg-Pce	**<0.001**	-	0.302	0.171	0.226	-	0.090	0.247	0.986	-	0.999	-	0.882	-	0.979

Table 1. continued.

	Pseudosomonas aeroginosa								*Salmonella typhimurium*						
Concentration	4.50	3.00	2.25	1.50	1.13	0.75	0.56	0.38	4.50	3.00	2.25	1.50	1.13	0.75	0.56
Ct-Ac	**<0.001**	-	**<0.001**	-	**0.002**	-	0.257	-	**<0.001**	-	**<0.001**	-	**<0.001**	-	**<0.001**
Mix-Ac	**0.001**	-	**<0.001**	-	**0.009**	-	1.000	-	**<0.001**	-	**<0.001**	-	**<0.001**	-	0.118
Pce-Ac	1.000	-	1.000	-	1.000	-	1.000	-	1.000	-	1.000	-	1.000	-	1.000
Sg-Ac	**0.008**	-	**<0.001**	-	**0.003**	-	**0.006**	-	**0.016**	-	**<0.001**	-	**0.001**	-	**0.040**
Mix-Ct	0.920	-	0.848	-	0.853	-	0.257	-	**0.004**	-	**<0.001**	-	**<0.001**	-	**0.003**
Pce-Ct	**<0.001**	-	**<0.001**	-	**0.002**	-	0.257	-	**<0.001**	-	**<0.001**	-	**<0.001**	-	**<0.001**
Sg-Ct	0.296	-	0.940	-	0.998	-	0.182	-	**<0.001**	-	**<0.001**	-	**<0.001**	-	**0.008**
Pce-Mix	**0.001**	-	**<0.001**	-	**0.009**	-	1.000	-	**<0.001**	-	**<0.001**	-	**<0.001**	-	0.118
Sg-Mix	0.713	-	0.999	-	0.948	-	**0.006**	-	**<0.001**	-	**0.044**	-	0.202	-	0.951
Sg-Pce	**0.008**	-	**<0.001**	-	**0.003**	-	**0.006**	-	**0.016**	-	**<0.001**	-	**0.001**	-	**0.040**

Table 1. continued.

	Staphylococcus aureus							
Concentration	4.50	3.00	2.25	1.50	1.13	0.75	0.56	0.38
Ct-Ac	-	**<0.001**	-	**0.001**	-	**0.005**	-	**0.003**
Mix-Ac	-	0.153	-	0.908	-	0.213	-	0.781
Pce-Ac	-	1.000	-	0.995	-	**0.023**	-	1.000
Sg-Ac	-	**0.002**	-	**0.031**	-	0.099	-	**0.028**
Mix-Ct	-	**0.005**	-	**0.002**	-	**<0.001**	-	**0.014**
Pce-Ct	-	**<0.001**	-	**<0.001**	-	**<0.001**	-	**0.003**
Sg-Ct	-	0.409	-	0.119	-	0.375	-	0.520
Pce-Mix	-	0.123	-	0.741	-	0.603	-	0.803
Sg-Mix	-	0.077	-	0.114	-	**0.003**	-	0.163
Sg-Pce	-	**0.001**	-	**0.017**	-	**<0.001**	-	**0.030**

Discussion

Using single resins and their mixture as a model and assessing the impact of resin diversity on stingless bees, we show that a single consumer species benefits more when it has access to diverse resources. Moreover, resource diversity can differentially affect functional (i.e., protective) properties. The protective effect of each resin tested in our study depended on the target organisms as well as the type and amount of resource, a characteristic of resource diversity that we termed "functional balance" and that differs from "functional complementarity" in that it comprises more than one functional property (e.g., repellence of ants *and* inhibition of microbial growth).

All of our tested resins strongly repelled meat ants, indicating that any of these resins can protect bee colonies against this predator species with none being superior over another (functional redundancy). These ant repellent properties observed in our laboratory trials agree with observations in the field: ants approaching a stingless bee colony are often repelled by resin that is typically placed around the colony's nest entrance (Duangphakdee et al. 2005; 2009; Leonhardt and Blüthgen 2009). The repellent effect of resin can be explained by the presence of terpenoids, which are frequently found in resin (Langenheim 2003; Gershenzon and Dudareva 2007), and particularly the more volatile mono- and sesquiterpenes are known ant repellents (Junker and Blüthgen 2008; Junker et al. 2011). In addition to being repellent, resin can be very sticky, which does further deter ants (Seeley et al. 1982). However, none of the single resins tested in our study nor the resin mixture repelled green-head ants, suggesting that these ants may be repelled by resins from plants other than the ones we used. Alternatively, green-head ants may not be repelled by resin at all. As they were attracted by *C. torelliana* resin, they instead tended to get stuck and die when this resin was offered (Drescher, personal observation), thereby representing a potentially different mode of protection.

Unlike ants, small hive beetles (SHB) clearly differentiated between different resins and the resin mixture when they were offered at low extract concentrations. Here, the resin mixture showed the strongest effect (functional complementarity). *Corymbia torelliana* and *A. cunninghamii* resin were similar in their repellent properties (functional redundancy) and *P. caribaea*×*P. elliotii* resin had no repellent effect on SHBs. However, the differential response towards different resins disappeared when higher extract concentrations were used, indicating that functional properties of resin were concentration-dependent. SHBs are virtually mummified alive by a resinous coating when they intrude into nests of stingless bees (Greco et al. 2010; Halcroft et al. 2011). The repellent effect of resin extracts shown here may further suggest that the beetles avoid colonies that have accumulated large resin deposits inside and/or outside their nests.

Different resins as well as the resin mixture also differed in their inhibition of the growth of several microorganisms. Whereas *A. cunninghamii* and *P. caribaea×P. elliotii* resin resulted in little to no microbial growth inhibition, *S. glomulifera* and *C. torelliana* were highly inhibitory (except for *E. coli*). Note that we recorded a relatively high variation in growth inhibition between the first and second repeat (see Fig. 2) likely due to the use of different extract concentrations. However, microbe-specific differences in growth inhibition induced by different resin extracts followed the same pattern across repeats, rendering our results for the different resins and the resin mixture comparable. In contrast to honeybees, we know of no pathogenic microbes that affect stingless bees. We could therefore not use bee pathogenic microbes in our microbiological assays, and our results cannot directly be linked to stingless bee health or fitness. However, given the consistently strong growth inhibitory effect of some resins (e.g., *Corymbia torelliana*), it is possible that this resin also affects pathogenic microbes associated with stingless bees.

In accordance with our hypothesis, the resin mixture was more effective in repelling SHBs (when applied in low concentrations) and in inhibiting microbial growth compared to some of the single resins (functional complementarity). However, with regard to growth inhibition, the resin mixture was generally less effective than an equally concentrated extract of *C. torelliana* resin. In fact, we found the complementarity effect to be either additive or even less than the summed effects of all single resins, suggesting that, at least with regard to (resin) resource diversity, diversity functions may be predominantly additive and rarely, if at all, synergistic. By contrast, synergistic effects of biodiversity have been described for functions other than those based on resource diversity, e.g., litter decomposition (reviewed by Hättenschwiler et al. 2005), microbial biomass production and respiration (Wardle and Nicholson 1996), the biocontrol of herbivores by natural enemies (Cardinale et al. 2003), or pollination success (Brittain et al. 2013).

The different activities observed for the different resins in our assays most likely correlate with differences in their chemical compositions. While coniferous resins (i.e., *A. cunninghamii* and *P. caribaea×P. elliotii*) are known to comprise mainly volatile terpenoids, such as monoterpenes and diterpenic acids (Langenheim 2003), *C. torelliana* fruit resin comprises a variety of phloroglucinols and flavonoids (Massaro et al. 2014; Massaro CF, unpublished data), similar to resin of other myrtaceous species including *S. glomerulifera* (Leonhardt, unpublished data). Flavonoids and phloroglucinols are well-known for their antimicrobial properties (Ghisalberti 1996; 2011), whereas terpenoids (e.g. mono- and sesquiterpenes found in floral scent bouquets) have been found to repel ants and other arthropods (Junker and Blüthgen 2008; 2010). Hence, based on their chemistry, the resins of the different plant genera used in our study can target different organisms (functional balance) and act synergistically when combined (functional complementarity).

Having access to a variety of different resin sources can thus be essential for animals relying on resin for protection and other functions (e.g. nest construction), such as stingless bees or honeybees. A similar benefit of mixing resources has been observed in herbivores (Hägele and Rowell-Rahier 1999; Unsicker et al. 2008) and even solitary bees (Eckhardt et al. 2014) and can be explained by either the dilution of toxins or the composition of a nutritionally more balanced diet (Behmer 2009; Simpson and Raubenheimer 2012). However, given that a strong effect (e.g., antimicrobial) of one resource (e.g., *C. torelliana* resin) can also be reduced by mixing this resource with other resources (e.g., resins) that are inferior in this property and thus diluting its effect, mixing resources can also have negative consequences for animals, at least for one particular property.

In honeybees, the use of resin is further considered an important component of the colony's social immune system because honeybees lack many of the immune system related genes typically found in other insects (reviewed by Simone-Finstrom and Spivak 2010). Instead, resin has become an important protective agent (Simone-Finstrom and Spivak 2010), benefiting the bees' immune system (Simone et al. 2009), protecting them against pests and pathogens (Garedew et al. 2002; 2004; Mihai et al. 2012) and increasing colony performance and health (Nicodemo et al. 2013). Chemically, the resin mixture composed by honeybees (known as propolis) is highly complex comprising various chemical compounds and several substance classes (Bankova et al. 2000). Propolis samples from various geographical locations that differ in their chemical composition showed nevertheless similar biological properties (Kujumgiev et al. 1999; Popova et al. 2007; Damiani et al. 2010), indicating redundant effects of different chemical compounds (Mihai et al. 2012) and resin sources. However, except for the ground breaking work of Wilson and colleagues (2013), surprisingly little is known about the botanical sources actually used by honeybees for resin collection due to a lack of thorough chemical comparisons between resin loads obtained from corbiculae of returning foragers and resin samples obtained from potential plant sources. Even less is known about the potential properties of different resins and their synergistic effects. Our results on the functional role of resin diversity for stingless bees most likely also apply to honeybees, thereby stressing the importance of not only resin collection itself but also the availability of various resin sources within the foraging range of bee colonies, which is likely reduced in the highly agricultural areas of many industrialized countries.

Given the beneficial functional properties of a variety of resources, a general lack of resource diversity (with regard to any resources), as typically found in environments with reduced biodiversity, may destabilize consumer populations due to reduced compensatory and/or synergistic mechanisms. This effect on consumer populations may further explain the negative correlation between biodiversity loss and reduced consumer diversity.

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Appendix

- **Supplement material**

  A) A clue on bee glue

  B) Inside honeybee hives

- **Eidesstattliche Erklärung**

Declaration (according to §16 of the guideline)

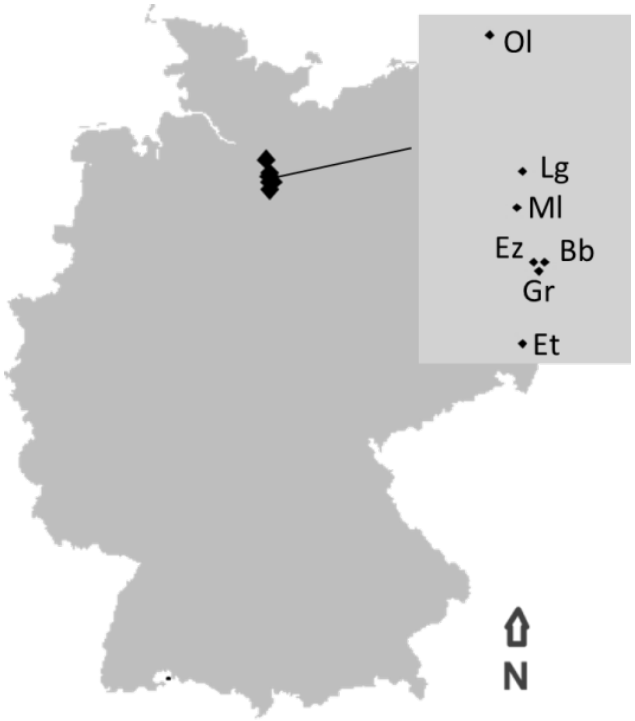
I declare that all information given in this appendix is true in each instance and overall.

Nora Ulrike Drescher

Supplement material

A) Sources and factors driving resin collection in honeybees

- **A1 Fig.** Location of the study apiaries. (please note: Figure A1 corresponds to **S1 Fig** referenced in chapter II)
- **A1 Table.** List of substance classes and the respective numbers of compounds (Total N) identified from resin samples of tree buds, returning honeybee (*Apis mellifera*) foragers and from propolis samples (please note: A1 Figure corresponds to **S1 Table** referenced in chapter I)
- **A2 Fig.** Exemplary chromatograms of resin hexane extracts (please note: Figure A1 corresponds to **S2 Fig.** referenced in chapter I)
- **A2 Table.** List of compound classes and the mean values of relative amounts [%] of components found in resin samples from tree buds, returning honeybee (*Apis mellifera*) resin foragers and propolis samples (please note: A 2 Table corresponds to **S1 Table** referenced in chapter I. Table A1 is included only in the electronic version (Supplement material TableA1, page 116-128).



A1 Fig. Location of the study apiaries. Study apiaries were located at seven different sites in Lower Saxony, Germany. Distances among study sites are between 1.3 km (Ez and Gr) and 38 km (Ol and Et).

A1 Table. List of substance classes and the respective numbers of compounds (Total N) identified from resin samples of tree buds, returning honeybee (*Apis mellifera*) foragers and from propolis samples.

Individual components are displayed with their retention time (Ret. Time),Kovats retention index (KI) and occurrence in different tree species, bee-collected resins and propolis samples (given are numbers (N) of bee and propolis samples containing compound). Tree species as follows: Pb, *Populus balsamifera*; Pt, *Populus tremula*; Px1 - Px3, three different (chemo)types of unknown *Populus xcanadensis* hybrids; B – B2, two different (chemo)types of unknown *Betula alba* hybrids; A, *Alnus glutinosa*; C, conifers (*Picea abis* / *Pinus sylvestris*); H, *Aesculus hippocastanum*.

Substance Classes	Total N	Constituents identified	Ret. time	KI	Tree species	N bees	N propolis
Acids and derivatives	167						
- Aliphatic acids /ester		Hexanoic acid	4.88	967	Px1	2	0
(total:97)		Hexadecanoic acid methyl ester	27.68	1927	Px3	5	0
		Linoleic acid	31.47	2123	Px2	6	0
- Phenolic acids /ester		Benzoic acid	9.69	1171	Px1;Px2; Pt	23	10
(total:70)		Methyl salicylate	10.18	1190	Px1; Pb	7	1
		benzyl benzoate	24.33	1762	Pb; Pt	21	10
		2-Ethylhexyl salicylate	25.13	1807	B2	1	0
		Benzyl salicylate	26.43	1864	Px2; Pb	15	9
Alcohols /aldehydes / ketones	53						
		Benzyl alcohol	6.26	1026	Px1;Pb; Pt	17	1
		Myristaldehyde (Tetradecanal)	20.92	1611	H	3	0
		Vanillin (4-Hydroxy-3 methoxybenzaldehyde)	15.59	1393	Pt	12	3
Other aliphatic compounds	73						
Phenolic compounds / phenylpropanoids	70						
		Chrysin	40.25	2649	-	0	3
		Coumaran (2,3-dihydro-Benzofuran)	10.85	1219	-	1	7

A1 Table continued

Compound Classes	Total N	Constituents identified	Ret. time	KI	Tree species	N bees	N propolis
Terpenes / terpenoids	398						
- Monoterpenes and terpenoids (total:24)		*alpha*-Pinene	4.24	932	Px1;Px3;C	2	0
		beta-Pinene	5.08	974	Px1;C	2	0
		beta-Myrcene	5.28	988	Px1;C	2	0
		3-Carene	5.74	1008	Px3;C	2	0
		D-Limonene	6.19	1031	C	2	0
		beta.-Phellandrene	6.23	1025	C	1	0
		Eucalyptol	6.24	1033	Px3;C	1	0
		trans-beta-Ocimene	6.56	1044	Px3;C	0	0
		gamma-Terpinene	6.87	1054	Px3;Pt;C	0	0
		Terpinolene	7.54	1086	C	1	0
		beta-Linalool	7.90	1095	Px1	7	0
		alpha-Campholene aldehyd	8.51	1125	-	1	0
		Sabinol	8.90	1137	C	1	0
		trans-Verbenol	9.00	1140	C	1	0
		Terpinen-4-ol	9.99	1174	Px3	1	0
		L-*alpha*-Terpineol	10.23	1186	-	1	0
		Estragole	10.43	1195	C	0	0
		Bornyl acetate	12.78	1282	C	1	0
		Geraniol	11.84	1249	Px1	0	0
		Thymol	12.93	1289	-	1	5
		Eugenol	14.51	1356	Px3	12	1
		E-Isoeugenol	16.97	1447	Px2	15	2
- Sesquiterpenes / terpenoids (total:176)		*gamma*-Elemene	14.04	1335	A	0	0
		alpha-Cubebene	14.42	1345	Px1;Pt;A	1	0
		alpha-Ylangene	14.98	1373	Px1;Px3;Pt;B	2	
		Copaene	15.16	1376	Px1;Px2;Pt;B;A	5	0
		beta-Bourbonene	15.25	1384	B;A;C	1	0

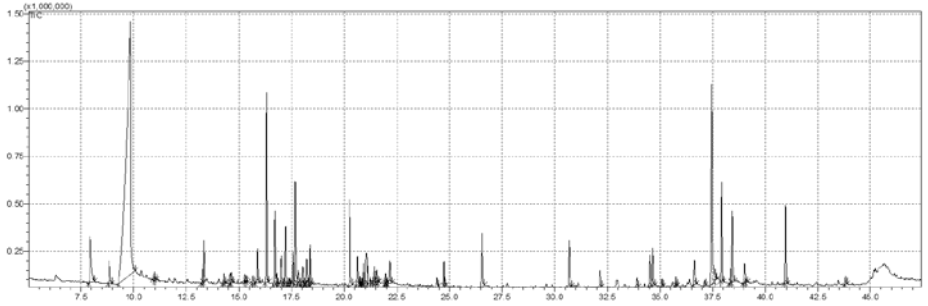
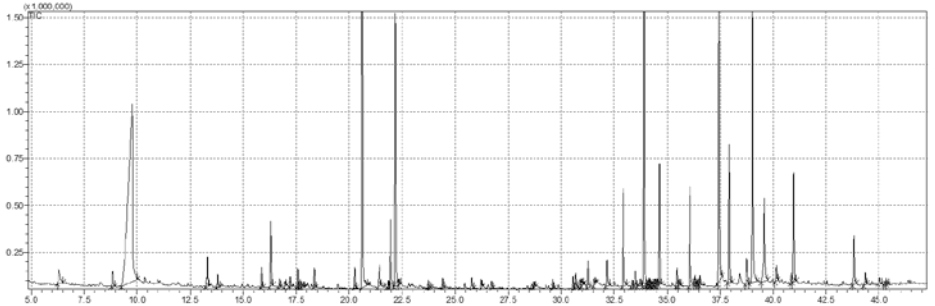
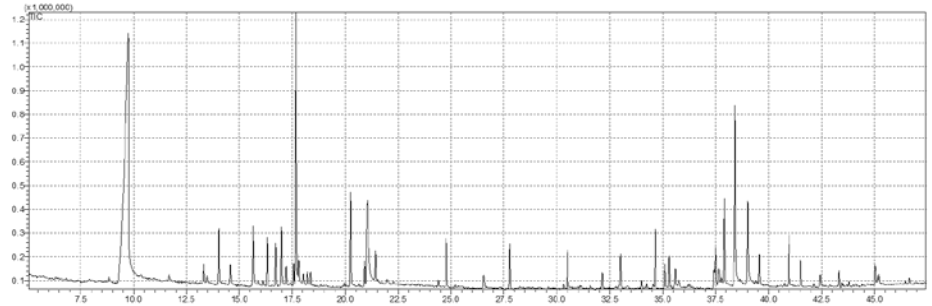
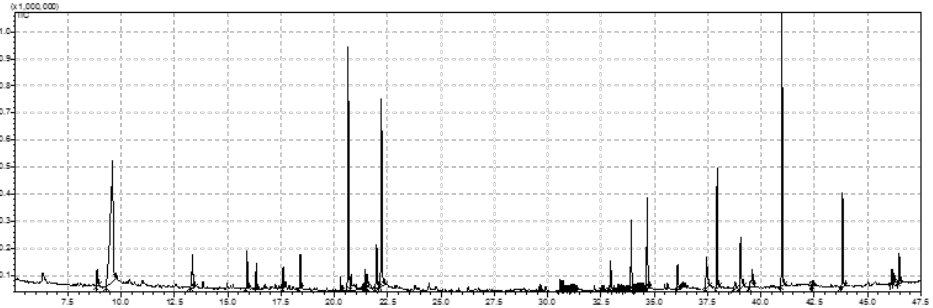
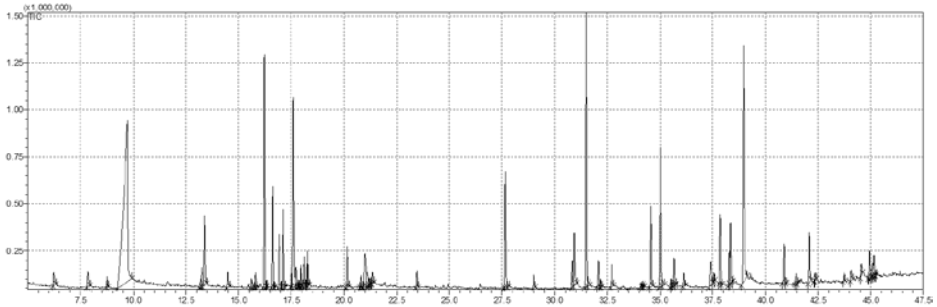
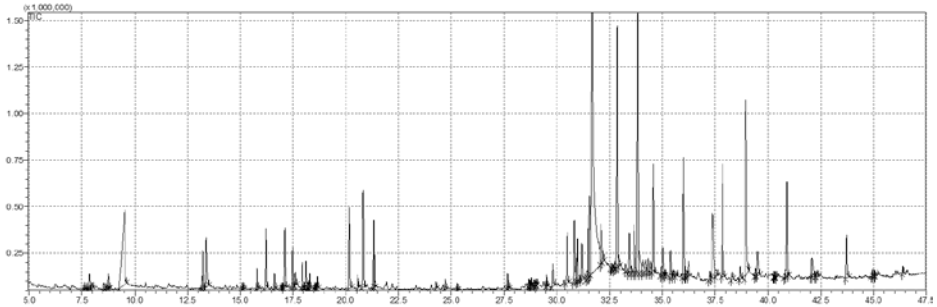
A1 Table continued

Compound Classes	Total N	Constituents identified	Ret. time	KI	Tree species	N bees	N propolis
		Caryophyllene	15.93	1408	B	1	0
		Longifolene	16.03	1407	B	0	0
		beta-Ylangene	16.15	1419	Px1;B;A	0	0
		Caryophyllene <E>	16.27	1417	Px1;Px2;Px3;Pt; B;C	28	1
		alpha.-Bergamotene	16.03	1411	Px2	1	0
		beta-Copaene	16.53	1430	Px1;B;A	1	0
		alpha-Guaiene	16.65	1437	Px3	18	0
		trans-alpha-Bergamotene	16.53	1432	Px2	2	0
		Z-*beta*-Farnesene	16.77	1443	B	10	5
		alpha-Humulene	17.17	1452	Px1;Px2;Px3;Pt; B;C	25	0
		Allo-aromadendrene	17.29	1461	Px1;Pt;B	4	0
		gamma-Muurolene	17.66	1477	Px1;Px3;Pt;B;C	2	0
		Ar-Curcumene	17.73	1479	Px2;C	2	0
		Germacene D	17.79	1480	Px3;A;C	1	0
		beta-Selinene	18.09	1485	-	2	0
		alpha-Selinene	18.17	1498	Px1;Px2;Px3;Pb	18	0
		alpha-Muurolene	18.33	1500	Px1	5	0
		alpha-Bulnesene	18.40	1505	-	8	0
		alpha-Farnesene	18.39	1508	Pb	4	0
		beta-Bisabolene	18.38	1509	Px2	2	0
		gamma-Cadinene	18.60	1513	Px1;Px2;Pb;B;A	7	0
		trans-calamenene	18.84	1521	Px1;Px3	1	0
		beta-Sesquiphellandrene	18.83	1521	Px2	2	0
		delta-Cadinene	18.75	1522	Px1;Px2;Px3;B; A;C	8	0

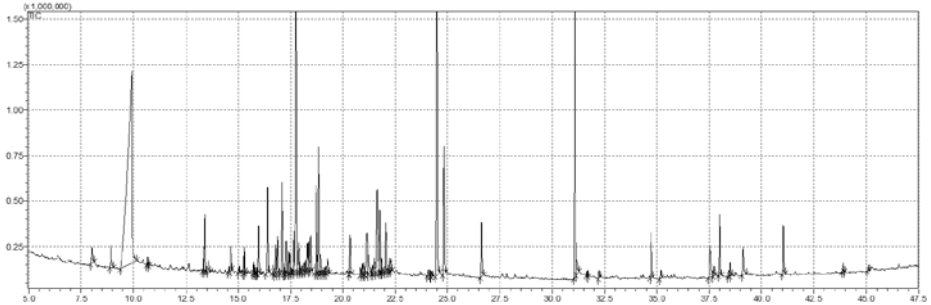
A1 Table continued

Compound Classes	Total N	Constituents identified	Ret. time	KI	Tree species	N bees	N propolis
		alpha-Copaene-11-ol	19.12	1539	Px2;Px3	2	0
		alpha-Calacorene	19.27	1544	Px1;Px3	1	0
		Selina-3,7(11)-diene	19.28	1545	Px3	0	0
		Elemol	19.56	1548	Px3	0	0
		Germacene D-4-ol	20.11	1574	B2;C	0	0
		Caryophyllene oxide	20.24	1582	Px1;Px2;Px3;B;C	30	7
		Gleenol	20.43	1586	Px1	0	0
		gamma-Eudesmol	21.42	1630	Px2;Px3	4	1
		alpha-Acorenol	21.45	1632	Px2;Px3	6	0
		Hinesol	21.58	1640	Px3	5	0
		T-Cadinol	21.70	1638	Px1	4	1
		delta-Cadinol	21.74	1644	Px1	4	0
		T-Muurolol	21.74	1640	Px1	4	0
		alpha-Cadinol	21.94	1652	Px1;C	8	3
		Eudesmol	22.01	1652	Px2;Px3	8	1
		Bulnesol	22.17	1666	Px3	8	1
		Geranyl linalool<(Z,E)>	27.21	1898	C	0	0
- Diterpenes/ terpenoids		Rosa-5,15-diene	27.73	1933	C	1	0
(total:94)		Cembrene	28.06	1942	C	2	0
		Kaur-15-ene	29.06	1997	C	1	0
		Geranyl linalool <(E,E)>	29.59	2026	C	0	0
		13-Epi-Manool	30.12	2059	C	0	0
- Triterpenes/ terpenoids (total: 67)							
- other terpenoids (total: 37)							
Unknown	85						
Total number	**846**						

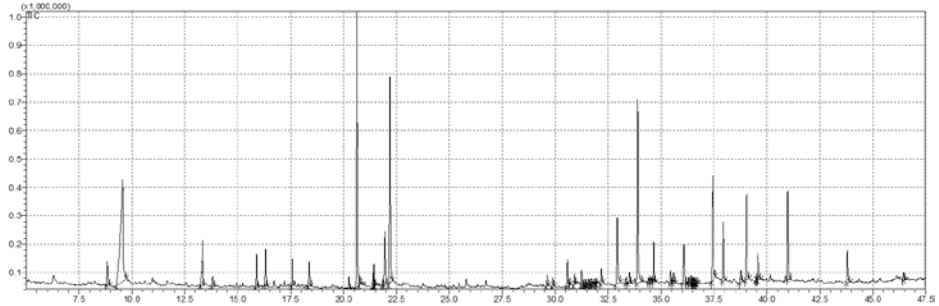
A2 Fig. Exemplary chromatograms

Ocher	Orange
	
1a) GR7-8.6.13	4a) GR9-6.5.13
	
1b) GR1-17.7.13	4b) GR9-8. 6.13
	
1c) GR3-16.8.13	4c) GR3-16.8.13

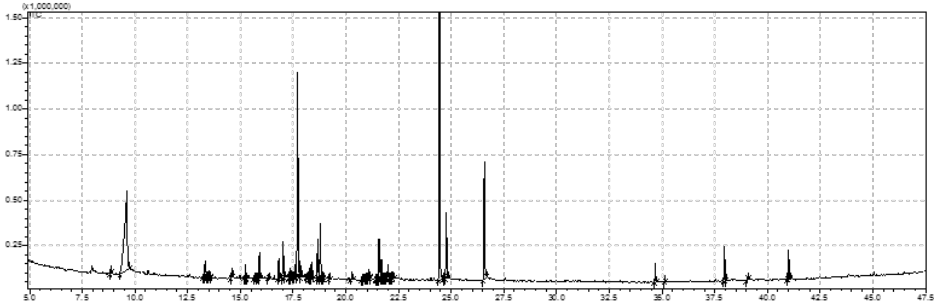
A2 Fig. continued



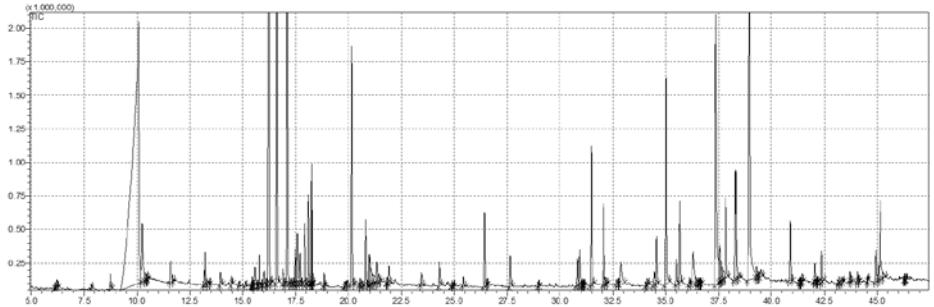
1d) ET5-12.7.13



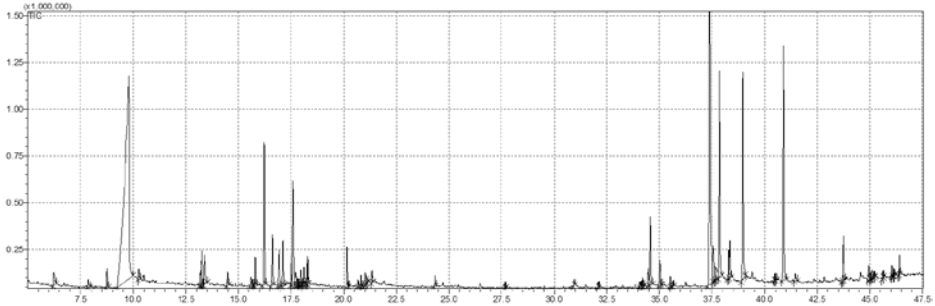
4d) GR7-8.6.13



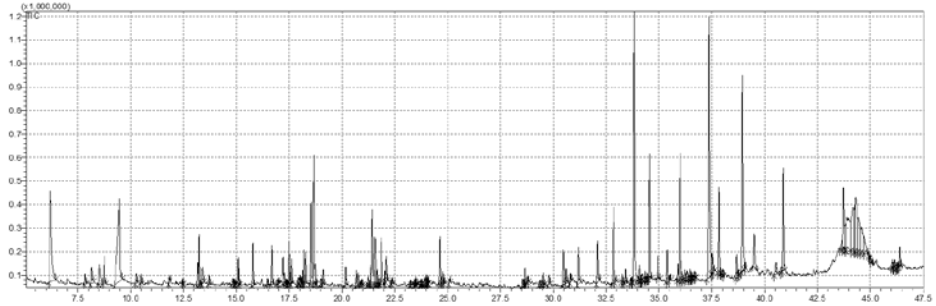
1e) ET5-12.7.13



4e) ET1-12.7.13

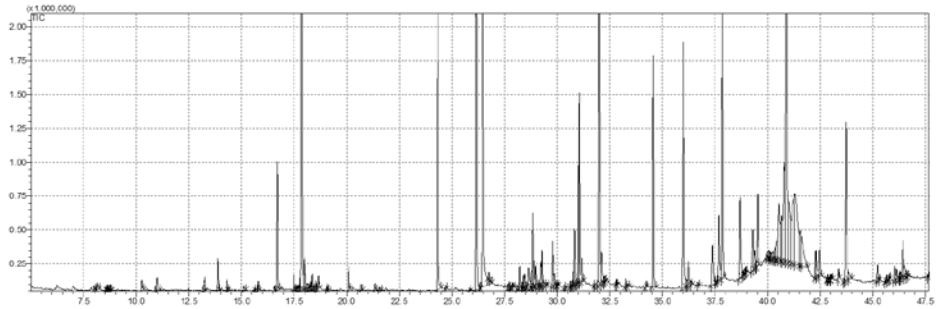
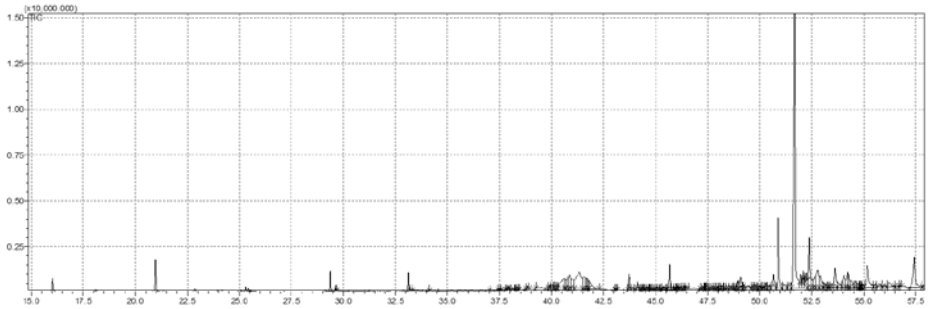
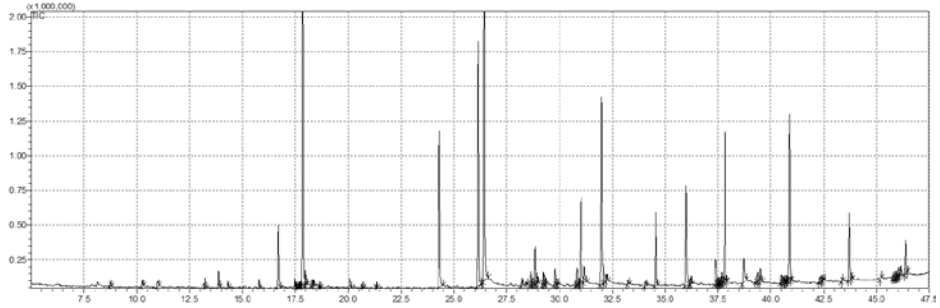
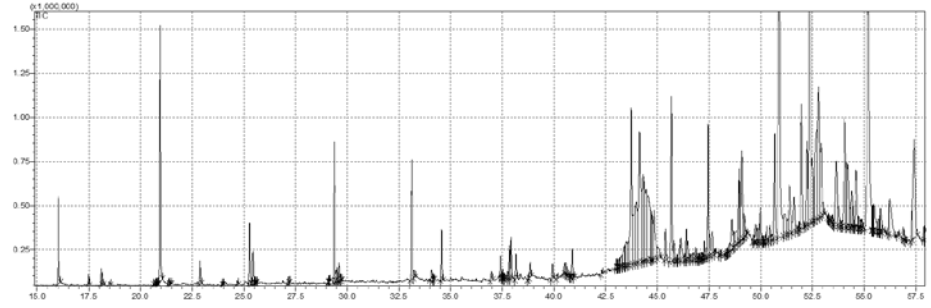


1f) LG6-16.7.13



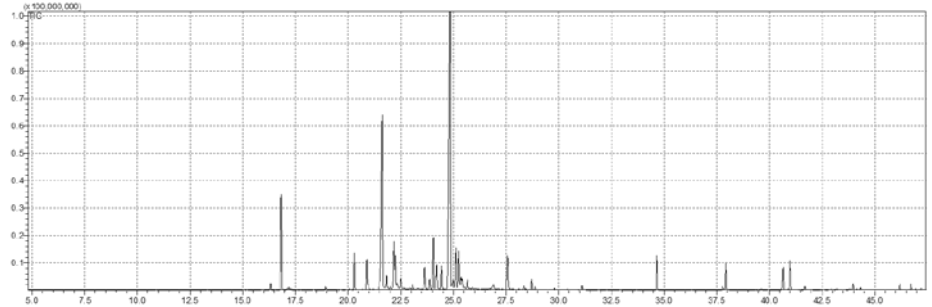
4f) LG6-16.7.13

A2 Fig. continued

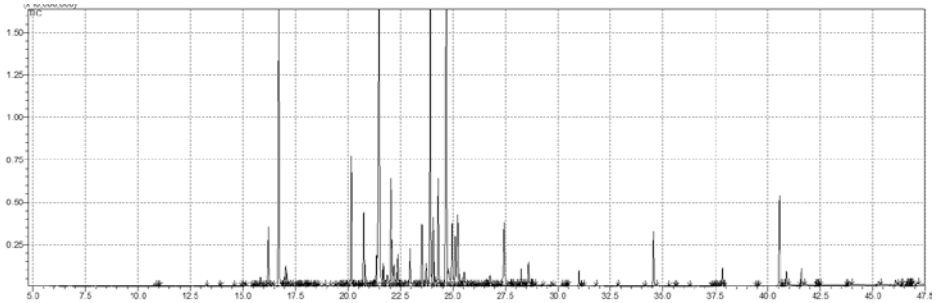
Red	Clear
	
2a) ML2-9.7.13	5a) ML4-2.10.13
	
2b) ML2-5.8.13	5b) GR1-3.10.13

A2 Fig. continued

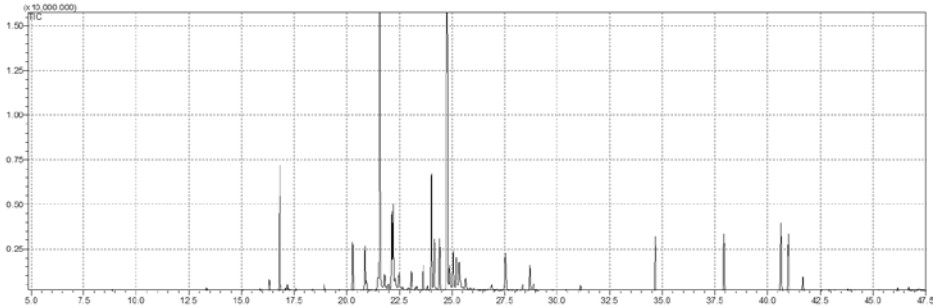
Brown



3a) GR3-8.6.13

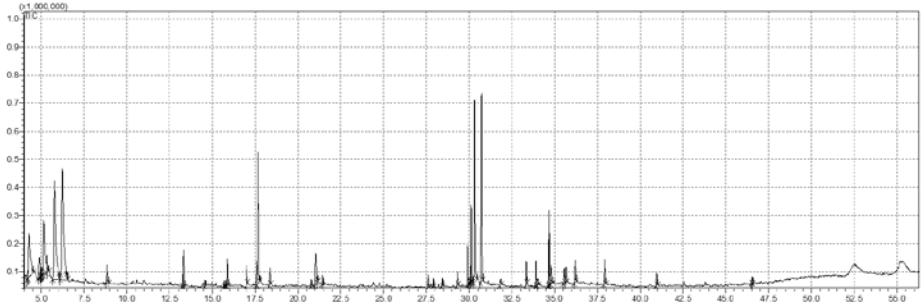


3b) GR5-3.10.13



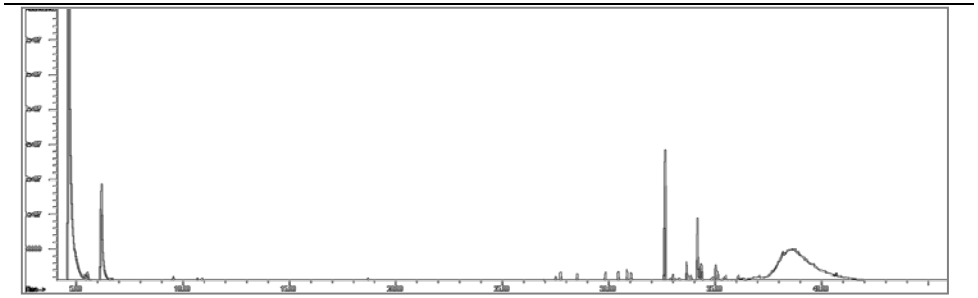
3c) LG12-8.6.13

Yellow



6a) LG11-7.6.13

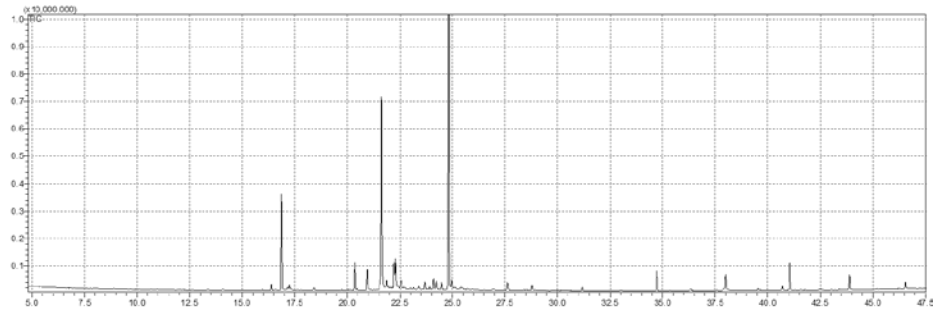
Whitish



7a) BB2-3.9.12

A2 Fig. continued

Brown



3d) ET5-12.7.13

S2 Fig. Exemplary chromatograms of hexane extracts from different bee-collected resins. Chromatograms 1a) - 7a) represent different resin color types (orange, red, ocher, brown, yellow, clear, whitish) collected by single resin foragers between June and October 2013 at five different apiaries in Lower Saxony, Germany. Each peak represents a specific substance, where the peak area reflects the relative amount of a substance in a given sample. Capital letters give site name of the apiary (i.e. "Bb" Bienenbüttel, "Et" Ebstorf, "Gr" Grünewald, "Lg" Lüneburg, "Ml" Melbeck) with subsequent numbers reflecting colony IDs and sampling date. 1a) – f) resin type ocher (a) GR7-8.6.13, b) GR1-17.7.13, c) GR3-16.8.1, d) ET5-12.7.1, e) ET5-12.7.13, f) LG6-16.7.13); 2a) – b) type red (a) ML2-9.7.13, b) ML2-5.8.13); 3a) - d) type brown (a) GR3-8.6.13, b) GR5-3.10.13, c) LG12-8.6.13, d) ET5-12.7.13); 4a) – f) type orange (a) GR9-6.5.13, b) GR9-8. 6.13, c) GR3-16.8.13, d) GR7-8.6.13, e) ET1-12.7.13, f) LG6-16.7.13); 5a) – b) type clear (a) ML4-2.10.13, b) GR1-3.10.13); 6a) type yellow (a) LG11-7.6.13); 7a) type whitish (a) BB2-3.9.12). Resin samples were obtained by trapping returning resin foragers and removing their resin load from the corbicula of their hind legs with forceps. Hexane extracts of resin samples were analyzed by gas chromatography coupled with mass spectrometry (GC-MS).

A2 Table. List of substance classes and the mean values of relative amounts [%] of all components identified from resin samples of tree buds, returning honeybee (*Apis mellifera*) foragers and propolis.

See separate file

B) Impact of natural propolis on *V. destructor* and viruses

Table B1.*Varroa destructor* infestation rate and resin intake of colonies. Numbers in "Colony" represent colony identity (colonies 1 - 10); Propolis treatment: high = added propolis; low = propolis removed; *V. destructor* infestation rate I: Data obtained by the natural mite fall method (counting dead mites on the bottom board of hives); *V. destructor* infestation rate II: Data obtained by the alcohol washing method (samples of 300 bees/colony were used to estimate colony infestation rate); Resin intake: Amount of resin collected by colonies. Resin quantity was measured by weighing propolis grids which were subsequent removed in the "low propolis" treatment group; NA: Alcohol washing was not performed because colonies received a thymol based acaricide treatment in the beginning of September.

Colony	Month	Propolis treatment	Natural mite fall/day	V. *destructor* infestation rate I (daily mite fall/colony strength)	*V. destructor* infestation rate II (alcohol washing; mites/100 bees)	Resin intake (g)
2	7/2013	high	4.20	0.14	1.00	61
3	7/2013	high	5.60	0.32	1.33	60
8	7/2013	high	0.60	0.05	0.00	21
9	7/2013	high	5.00	0.19	1.00	40
10	7/2013	high	0.00	0.01	0.00	8
1	7/2013	low	0.60	0.04	0.00	35
4	7/2013	low	0.40	0.02	0.00	7
5	7/2013	low	2.00	0.34	2.33	10
6	7/2013	low	0.40	0.03	0.00	10
7	7/2013	low	6.00	0.27	2.00	31
2	8/2013	high	51.88	1.98	0.33	81
3	8/2013	high	33.25	1.36	2.33	83
8	8/2013	high	1.38	0.06	0.00	17
9	8/2013	high	54.75	1.86	3.00	77
10	8/2013	high	0.20	0.01	0.00	9
1	8/2013	low	1.63	0.06	0.00	16
4	8/2013	low	0.38	0.02	4.67	8
9	8/2013	high	54.75	1.86	3.00	77
10	8/2013	high	0.20	0.01	0.00	9
1	8/2013	low	1.63	0.06	0.00	16
4	8/2013	low	0.38	0.02	4.67	8
5	8/2013	low	4.88	0.28	0.33	10
6	8/2013	low	4.75	0.20	0.00	14
7	8/2013	low	36.38	1.35	2.67	33

Table B1 continued

Colony	Month	Propolis treatment	Natural mite fall/day	V. *destructor* infestation rate I (daily mite fall/colony strength)	*V. destructor* infestation rate II (alcohol washing; mites/100 bees)	Resin collection (g)
2	9/2013	high	86.71	3.31	NA	36
3	9/2013	high	40.86	1.67	NA	44
8	9/2013	high	1.00	0.05	NA	21
9	9/2013	high	75.00	2.54	NA	20
10	9/2013	high	0.43	0.02	NA	6
1	9/2013	low	3.43	0.14	NA	6
4	9/2013	low	2.00	0.13	NA	15
5	9/2013	low	8.57	0.50	NA	15
6	9/2013	low	9.00	0.38	NA	14
7	9/2013	low	42.00	1.56	NA	47

Eidesstattliche Erklärung

Eigenständigkeitserklärung

Ich versichere, dass ich die eingereichte Dissertation mit dem Titel „ Plant resin - an underestimated resource for bees: How honeybees (*Apis mellifera* L.) and stingless bees (Apidae: Meliponini) benefit from a diversity of resin sources“ selbstständig und ohne unerlaubte Hilfe verfasst habe. Anderer als der von mir angegebenen Hilfsmittel und Schriften habe ich mich nicht bedient. Alle wörtlich oder sinngemäß anderen Schriften entnommenen Stellen habe ich kenntlich gemacht.

Ort, Datum Nora Ulrike Drescher