"Evolution of innate and social immunity in eusocial bees"

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''The spread of infections between bees is limited by an innate immunity of individuals during much of their lives, by their short life-span and replacement with healthy individuals, and by events that decrease the chance of contact between pathogens and susceptible healthy individuals. Pathogens that spread contagiously between live bees are especially hindered when the normal activities of colonies, particularly foraging, are intense.''

L. Bailey & B. V. Ball, 1991

(Honey bee pathology)

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Chapter 1 - Introduction

1.1. Eusocial bees

Eusocial (Greek, *eu*: 'good/true' + 'social') groups of individuals are the highest level of social organization in a hierarchical classification of animal societies. Such societies are characterized by adult individuals living together in groups, sharing care of offspring, sharing breeding sites, alloparental brood care (adults care for brood that is not their own) and the presence of different castes. Eusocial insects (e.g. ants, bees, wasps and termites) were thought to have much more sophisticated characteristics related to sociality than other social societies. Therefore, the term eusociality coined to define species with the following traits: reproductive division of labor, overlap of generations, and cooperative brood care (Batra, 1966; Michener, 1974; Wilson, 1971).

The evolutionary transition from solitary individuals to eusocial groups presents one of the major evolutionary transitions in evolution (Maynard Smith & Szathmáry, 1995). Two different routes describe the path to eusociality, 1) the sub-social (female offspring forego their own reproduction and stay at the nest to help their mother e.g. *Halictus*, *Lasioglossum*) or 2) para-social route (aggregation of females establish dominance hierarchies for reproduction e.g. *Polistes* wasps), both ending in highly (advanced) and primitively (less advanced) eusocial societies (Michener, 1958). Distinct differences in morphology for reproductive (queens) and non-reproductive (workers) individuals are the major characteristic for highly eusocial organisms whereas primitively eusocial species have a lack of major morphological differences between queens and workers beyond size. Morphological specialization even within the non-reproductive individuals can also be observed (e.g. different castes of army or leaf-cutter ants; Hölldobler & Wilson, 1990). Primitively eusocial organisms do not show such phenotypic variation, termed caste dimorphism or polymorphism.

Eusociality independently evolved several times in animal societies (Andersson, 1984). Currently, we know two species of eusocial mammals (Damaraland and naked mole rat), several snapping shrimp (*Synalpheus* sp.), Australian gall thrips (*Kladothrips* sp.), various aphids and all termite species; and a single eusocial ambrosia beetle (*Austroplatypus incompertus*). Hymenoptera are not only one of the largest order of insects with more than 150,000 species, they are also the most numerous group of animals (regarding biomass and number of individuals) globally, with single super-colonies of billions of individuals (e.g., Argentine ant *Linepithema humile*) (Wilson, 1990). This enormous dominance in abundance, especially of eusocial Hymenoptera (ants, bees, wasps), might have been driven by the sophisticated mechanisms associated with eusociality. Across the Hymenoptera, evolutionary transitions to eusociality probably evolved 7 times (other say at least 10 times) independently (Hölldobler & Wilson, 1990; Wilson, 1971). Comparing the three major groups of eusocial Hymenoptera, it came clear that all Formidae, multiple Vespidae species and some Apoidae are eusocial, with bees showing the most extreme diversity of sociality (Fig. 1.1).

Colonies of eusocial bees are headed by single queens, mated either singly (monandrous, e.g. bumble bees and stingless bees) or multiply (polyandrous, e.g. honeybees). Facultative and obligate primitively eusocial bees are further characterized by small colonies with one or more workers (Fig. 1.1). One trait to distinguish primitively and highly eusocial Hymenoptera, especially for the Apoidae, is the annual (bumble bees) or perennial (honey and stingless bees) existence of the colony. A key aspect that differs between an annual and perennial colony is the flow of nutrients (nectar and pollen) into the colony and the level of food storage (Judd, 2011). Theoretical approaches predict that the evolution of primitively and highly eusocial behavior involved innovation, changes and regulatory flexibility of genes and gene networks to create morphological and task-specialized reproductive (queens and drones) and non-reproductive (workers) individuals (Gadau et al., 2012; Simola et al., 2013).

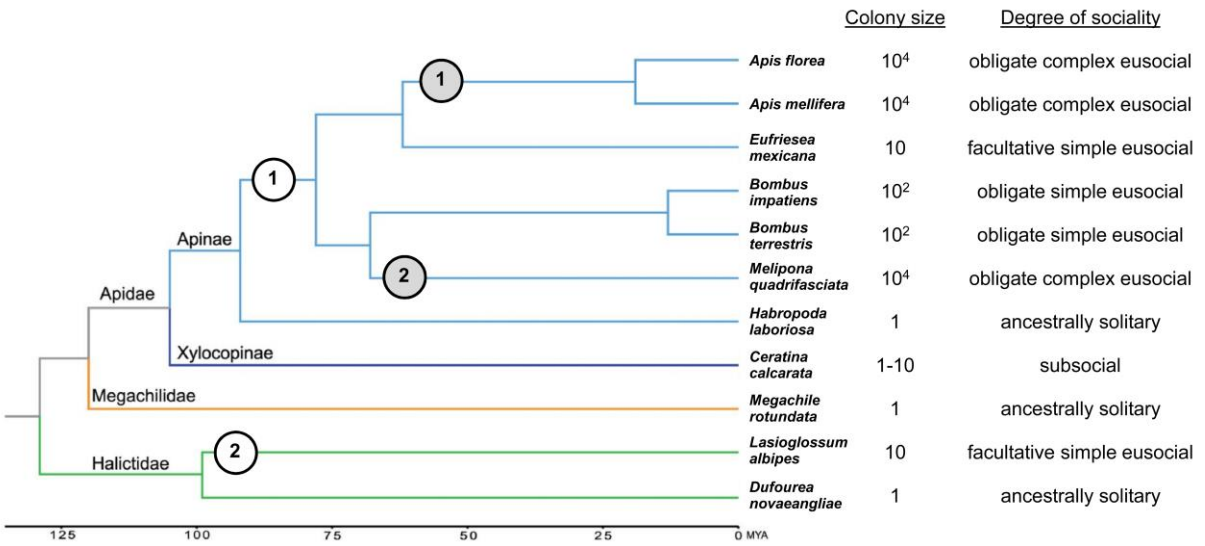

Fig. 1.1 Phylogeny and divergence times (Cardinal & Danforth, 2013; Rehan & Schwarz, 2015) of all whole genome sequenced bees; with two independent origins of primitive eusociality from a solitary ancestor, one each in Apidae (white circle 1) and Halictidae (white circle 2), and two independent elaborations of complex eusociality in honeybees (gray circle 1) and stingless bees (gray circle 2). The social biology of *E. mexicana* is unknown, but is representative of the facultative primitive eusocial life history (Cardinal & Danforth, 2011). (MYA: millions of years ago; modified from Kapheim et al., 2015 and Rehan et al., 2016)

The first step towards understanding the genomic organization of eusocial bees and social behavior was taken in 2006 by sequencing the honeybee (*Apis mellifera ligustica*) genome (Honeybee Genome Sequencing Consortium, 2006). More than a decade later, the automated generation and analysis of insect genomes has become less expensive, faster, and easier in data handling. In 2013, the first socially polymorphic bee (*Lasioglossum albipes*) genome became available to study the evolution of social behavior by comparing solitary and social female individuals (Kocher et al., 2013). However, only six genes were identified that diverged more rapidly between social forms, including a putative odorant receptor and a cuticular protein (Kocher et al., 2013). Such a low number of genes was not expected to drive the major transition in evolution. Gene loss and gain, development of specific pathways and genome modifications were mechanisms expected to drive this transition.

I participated in the Bumble bee Genome consortium in 2015 (Sadd et al., 2015) by annotating the genomes of two key bumble bee model species (*Bombus impatiens*, *Bombus terrestris*) as representatives of primitively eusocial bees, to allow for greater comparative sociogenomic analyses. We could show that most genomic features related to advanced eusociality (e.g. depauperate complements of xenobiotic detoxification and immune genes) are present and highly conserved in both primitively and advanced eusocial bees (honey and bumble bees), indicating an earlier evolution in the bee lineage (Sadd et al., 2015). Key differences include a bias in bumble bee chemoreception towards gustation rather than olfaction, and striking differences in microRNAs, potentially responsible for gene regulation underlying social and other traits (Sadd et al., 2015).

The most recent comparative sequencing efforts of ten bee species from three families (Apidae, Megachilidae and Halictidae) included two independent origins of eusociality and two independent elaborations of simple to complex eusociality (Fig. 1.1; Kapheim et al., 2015). The study suggested that there is no single road map to eusociality, without any correlation of independent transitions and genetic underpinning (Kapheim et al., 2015). Important genes related to sociality (e.g. regulation of transcription, RNA splicing, ribosomal structure and regulation of translation) showed evidence of neutral evolution caused by relaxed selection with increasing social complexity (Kapheim et al., 2015). In summary, sociogenomic studies showed that Apoidae eusociality may have arisen through different mechanisms each time, but always involved an increase in the complexity of gene networks (Kapheim et al., 2015). Decoding social Hymenoptera genomes further showed that there are

common features between genomes of the same genus (lineage-specific genetic changes related to independent origin of eusociality) but also adaptations regarding eusociality in both bees and ants, including different sets of genes showing caste-biased expression across species (Kapheim et al., 2015). The story continues with the newly published genome of the sub-social (organisms with reproductive division of labor and overlapping generations but no cooperative brood care) small carpenter bee *Ceratina calcarata*, which highlights mechanisms associated with DNA methylation and nutrition as candidate targets of evolutionary changes to more complex social societies (Rehan et al., 2016). The black box containing the secret mechanisms for the evolution of sociality and social behavior has been opened and includes nowadays 12 different bee species genomes of extreme social diversity. Nevertheless, functional studies are needed to understand which mechanisms control the transition from solitary individuals to (eu-)social societies.

In addition to comparative genome projects across the Apoidae, several recent publications have given a more precise insight into the genome of *Apis mellifera*, with genomes from several subspecies (*A. m. intermissa*, *A. m. sinisxinyuan*, *A. m. syriaca*, 9 sub-species across populations; Chen et al., 2016; Haddad et al., 2015, 2016; Wallberg et al., 2014) and sister-species (*Apis cerana*, *Apis florea*; Kapheim et al., 2015; Park et al. 2015) offering to provide a greater understanding of the evolution of local adaptation, climate change and resistance to parasites and pathogens. A comparative analysis for the sister-species *Apis florea* and *Apis dorsata* is in the pipeline (Rueppel et al., in prep.) and might help to develop the panorama of social behavior at a molecular level in eusocial honeybees.

1.2. The downside of eusociality

Colonies of eusocial bees are usually characterized by a high density of individuals of various ages and sexes (if overlapping), extremely frequent social interactions (intra-colonial communication and food transmission) and high relatedness (depending on type of mating: single or multiple). This environment of a high probability for transmission, with near-constant within-nest climatic conditions and almost endless food resources, provides ideal requirements for the spread of parasites and pathogens (Schmid-Hempel 1998). Social insect colonies and individuals are host organisms for many kinds of parasite and pathogen (e.g. viruses, bacteria, fungi, protozoa, nematodes, parasitic Diptera / Hymenoptera / Lepidoptera and mites) (Schmid-Hempel 1998).

Social insects, as well as all other animals on earth, are by no means defenseless against parasite and disease attack. To fight infections, they use a highly efficient suite of cellular (e.g. phagocytosis, nodulation and encapsulation mediated *via* hemocytes) and humoral immune defense mechanisms (Beckage et al., 2008). The humoral tool kit includes the secretion of antimicrobial peptides, proteasome-dependent degradation, phagocytosis, melanization, enzymatic degradation of pathogens and apoptosis (Evans et al., 2006). Four non-autonomous immune pathways with a highly conserved overall architecture, namely Toll, Imd, JAK/STAT and JNK, control the inducible humoral defense system (Fig. 1.2) (Beckage et al., 2008; Evans et al., 2006). By comparing the gene composition of honeybees with solitary insects (e.g. *Drosophila melanogaster*, *Anopheles gambiae*), it became clear that the honeybee had only one-third of the genes related to innate immunity and defense (Evans et al., 2006). With rising number of insect genomes, it was shown that a low number of immune genes is the rule rather than the exception for social Hymenoptera, at least for bees and ants (Barribeau et al., 2015; Gadau et al., 2012; Xu and James, 2009).

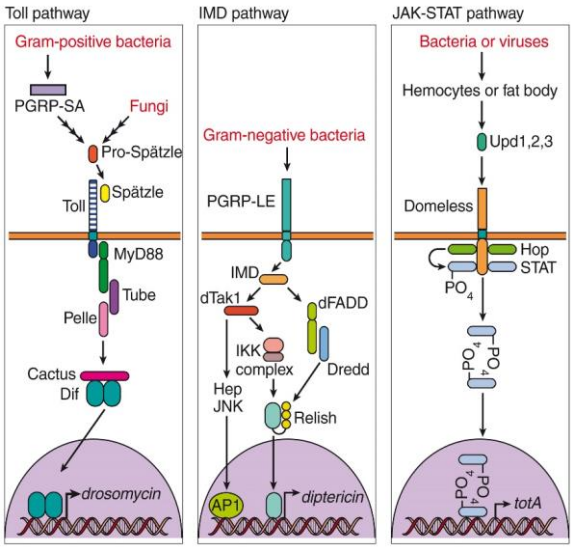

Fig. 1.2 Overview of defensive pathways in *Drosophila* with simplified schemes of the Toll, IMD (incl. JNK) and JAK-STAT immune signaling pathways. The Toll signaling pathway mediates the response to many Gram-positive bacteria and fungal pathogens, which in many cases are recognized when secreted PGRPs (peptidoglycan recognition proteins) initiate an extracellular proteolytic cascade. In the IMD pathway, Gram-negative bacteria are detected by a transmembrane PGRP (PGRP-LE), which signals via the cytoplasmic protein IMD. The other branch emanating from dTak1 activates MAPKKs in the JNK pathway. JNK (also known as Bsk in *Drosophila*) activation eventuates in activation of the AP1 transcription factor. The JAK-STAT pathway: infection of flies with bacteria or viruses leads to the production of signals such as the Unpaired (Upd) ligands, which bind and activate the Domeless receptor (modified from Bier & Guichard, 2012).

The observed reduction in immune flexibility by gene loss or reduced gene duplication might be compensated by the evolution of behavioral defense mechanisms ('social immunity', Cremer et al., 2007; Cotter & Kilner, 2010), which includes prophylactic and adjustable mechanisms on demand to protect the individual organism and finally the whole colony. Across the social Hymenoptera, several behavioral defense mechanism are well known; for example: grooming behavior, avoidance of sickened individuals, waste and corpse management, or other behaviors increasing nest hygiene and reducing the impact of pathogenic/parasitic micro- and macro-organisms. All behavioral, physiological and organizational mechanisms defined within 'social immunity' should prevent disease up-/intake, establishment and spread/transmission (Cremer et al., 2007). The development of such non-innate immune system defense tools may decrease the selective pressure on the individuals' immune system. However, a recent study showed that this is not the case, as there is no evidence for relaxed selection in bees and ants on their innate immune genes, which would otherwise be expected if 'social immunity' reduced selection pressures (Roux et al., 2014).

1.3. Immune system evolution in bumble bees

The Asian bumble bee *Bombus ignitus*, the North American bumble bee *B. impatiens*, and the European bumble bee *B. terrestris* are since decades essential natural and commercial pollinators but also key model species for studying host-parasite interaction, social behavior and the evolution of sociality (Goulson et al., 2010). All species can be artificially bred, genetically manipulated (e.g. instrumental insemination, RNAi) and housed in the laboratory in cages for controlled infection or other experimental manipulations (Baer & Schmid-Hempel, 2000; Deshwal & Mallon, 2014; Velthuis & van Doorn, 2006). Bumble bees are of further interest as they have recently been used to study the causes of pollinator decline, which might be driven by anthropogenic disturbances (e.g. habitat destruction and fragmentation), pesticides, parasites and pathogens (Goulson et al., 2015) (for more details, see paragraph 1.5).

The bumble bee innate immune system can be activated by bacterial and parasitic challenges or simply by cuticular wounding (Erler et al., 2011; Riddell et al., 2009). Even social context, for instance crowding, can lead to prophylactic up-regulation of the immune system (Richter et al., 2012). Adaptive immunity, following parasite/pathogen challenge, can even be transmitted to following generations (mother *via* eggs to offspring). This mechanism, known as trans-generational immune priming, increases the success of the offspring in fighting against a known parasite and disease (Sadd & Schmid-Hempel 2006, 2007). Today we have a rather comprehensive understanding of the architecture of the innate immune system of bees (particularly bumble bees) in relation to other insects. We used the recently sequenced genomes of *B. impatiens* and *B. terrestris* to explore patterns of innate immune system evolution across a social gradient (chapter 2). To do so, we compared the immune repertoire and sequences of immune genes (across 27 immune-related gene families or pathways, Fig. 1.2) with those of two species of highly eusocial honeybees (*A. florea*, *A. mellifera*), the solitary leaf-cutting bee *Megachile rotundata* and four solitary non-bee insect species (*Anopheles gambiae*, *Drosophila melanogaster*, *Nasonia vitripennis* and *Tribolium castaneum*) (chapter 2).

Antimicrobial peptides, the effector molecules of the innate immune system, are activated upon bacterial and fungal infections, and wounding (Erler et al., 2011; Evans et al., 2006; Riddell et al., 2009). Their rather unspecific broadband activity against parasites and pathogens implies that they are not specific to co-adapted pathogens. Their main function for social insects, living in relatively clean environments, might be protecting them from saprophytes, omnipresent microorganisms, but also disease associated microorganisms (Evans et al., 2006; Hultmark, 2003).

Host-parasite interactions and adaptations on the molecular level of the innate immune system have mainly been studied in animal hosts and their micro- and macro-parasites. Bumble bees provide the unique opportunity to study the interaction inherent to host-parasite systems where hosts and parasites are closely related (Cameron et al., 2007). This very specific type of obligatory parasitism is known as social parasitism or brood parasitism, where host bumble bees and their social parasites (called cuckoo bumble bees) share similar life history traits (Alford, 1975; van Honk, 1981). Host and social parasites forage on the same flowers and most importantly they live in the same environmental conditions, as the social parasite queen takes over the colony from the host queen (Alford, 1975; van Honk, 1981). As a consequence, closely related host-social parasite couples, living in the same colonies, also have the same parasite pressure (Erler et al., 2012; Popp et al., 2012), driving evolutionary adaptation by positive selection of the hosts' (host and social parasite) immune system. We tested whether parasite or pathogen-driven evolutionary adaptations (parallel evolution of antimicrobial peptide genes) can be observed in six specialist host-social parasite couples by determining the mode of selection for three antimicrobial peptide genes (e.g. *abaecin*, *defensin-1* and *hymenoptaecin*), both within and between host and their respective social parasite species (chapter 3).

Host-parasite co-evolutionary arms races are modulated by the effective population sizes (N_e) of hosts and the parasites. Larger N_e allows faster evolutionary rates, pushing hosts into strategies that maximize genetic responses to their parasitic enemies (Bousjein et al., 2016). Social parasitism is the only case where the parasite has a smaller N_e than its host, because the social parasite cannot reproduce outside the colony of the host. The potentially biased N_e of host-social parasite couples ($N_{e\ \text{Social parasite}} < N_{e\ \text{Host}}$; Erler & Lattorff, 2010) may gave a distorted view on the evolution of antimicrobial peptide genes for most of the bumble bee species. Comparing evolutionary changes between host species with known effective population sizes is essential to unveil the relationship between both (N_e and immune gene evolution). We therefore use the two most common Central European bumble bees, *B. lapidarius* and *B. terrestris*, for comparative analysis (chapter 4). Both species are similar regarding their ecological niches and general biology (e.g. colony size, annual life cycle, parasite prevalence for *Crithidia bombi*) (Alford, 1975; Erler et al., 2012; Goulson et al., 2010; Popp et al., 2012). Current census (colony number) and short term effective population sizes (number of reproductives) of both species were estimated using a large scale microsatellite study. The impact of population size on immune system evolution was estimated by measuring selection pressures acting on the key antimicrobial peptide gene *hymenoptaecin* (chapter 4).

1.4. Social immunity in honeybees

In the evolutionary arms race between hosts and their parasite, physiological responses (including innate immunity) are not the sole defence mechanism of the hosts. Social immunity resulting from behavioral adaptations is often most efficiently used to reduce the effects of pathogen infections or even to avoid them altogether (Cremer et al., 2007; see 1.2.). These behavioral traits reduce infection probability ('prophylactic self-medication') and reduce pathogen burden once infected ('therapeutic self-medication') (Hart, 1990). Therapeutic self-medication has been reported for many invertebrates, mainly insects (reviewed by Parker et al., 2011). It seems inevitable that the mechanisms of self-medication that we know from solitary insects (e.g. consumption of non-nutritional/toxic plant compounds to support self or offspring of butterflies and moths) may provide a much more efficient and probably play a very fundamental role in 'social immunity' and colony health as a whole in eusocial insects.

However, it is known that self-medication mechanisms of solitary insects can also be integrated into the social interactions of a eusocial insect colony, becoming a major feature of 'social immunity'. For example, the wood ants *Formica paralugubris* incorporate pieces of solidified conifer resin into their nests, which inhibits growth of bacteria and fungi (Castella et al., 2008; Chapuisat et al., 2007; Christe et al., 2003). Also honeybees collect resin from trees, whose secondary plant metabolites have strong antimicrobial and antifungal functions, preventing pathogen infections and decreasing pathogen growth (Simone et al., 2009; Simone-Finstrom & Spivak, 2012; reviewed in Simone-Finstrom & Spivak, 2010). Honeybees use resin to seal and cover the nest cavity. Hence resin collection does not have a direct health benefit for the individual bee collecting the material but rather acts as an overall colony level defence.

Many bee products are long known to have potent antimicrobial properties (Dustmann, 1979; Gilliam et al., 1988; Molan, 1992a, 1992b; Viuda-Martos et al., 2008). However, they have rarely, if ever, been studied in the context of active self-medication agents. Whereas honey, propolis, royal jelly and even bee venom are widely used as treatments for human diseases (Efem et al., 1992; Lusby et al., 2005; Mandal & Mandal, 2011; reviewed in Ratcliffe et al., 2011), their effects on bees themselves are much less studied. Especially for honey, the evolutionary background seems clear: floral nectar contains many secondary plant metabolites to prevent bacterial fermentation of the sugars and keep the flower attractive for pollinators (Fig. 1.3). These antibiotic compounds of nectar can also be highly effective against pathogens of pollinators (Cowan, 1999; Rhoades & Bergdahl, 1981).

A  **B** 

Fig. 1.3 A) Honeybee foraging for nectar and B) Worker honeybees storing, sharing and consuming nectar and pollen (photo A) by Maciej A. Czyzewski, from Wikipedia).

A timely and comprehensive review, summarizing the impact of both self-produced gland secretions and foraged hive products on colony health, is given in chapter 5. Self-produced gland products are cuticular hydrocarbons, wax, venom and food jelly (e.g. drone, worker and royal jelly) (Fig. 1.4), whereas foraged hive products include raw and processed materials that are resin and propolis, pollen and bee bread, and honey (Fig. 1.3). This review mainly focusses on the honeybee *Apis mellifera*, but also contains data on other honeybee species, bumble and stingless bees. Comparing the different studies for their relevance for pollinator health, we have to stress the fact that the specific bee health-enhancing and antibiotic/anti-parasitic activities

of bee products have clearly to be distinguished from the effects of an intact nutrition ensuring the basic immune competence of bees. Many studies do not differentiate between these aspects, and it remains unclear if observed effects were caused by the antibiotic potential of the tested bee product, or simply due to malnutrition (Erler & Moritz, 2016). Much more rigorous controls are needed in future experiments to rule out effects of malnutrition and others influencing the host response upon parasite and pathogen infection.

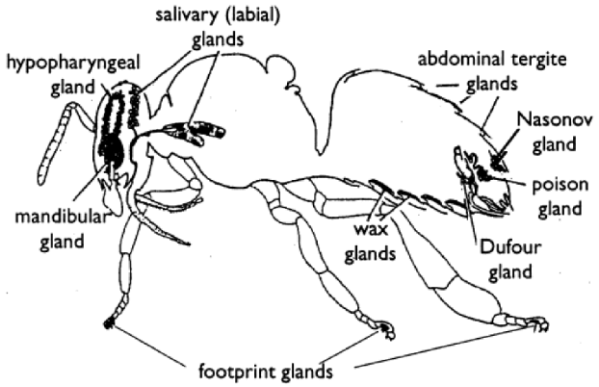

Fig. 1.4 Exocrine glands of the honeybee (modified from Michener, 1974).

Honey has a central position among the foraged and consumable hive products as it provides food to all individuals, including both brood and adults (Winston, 1987) (Fig. 1.3). This makes honey a prime candidate as a self-medication agent in honeybee colonies to prevent or decrease infections. Secondary plant metabolites in floral nectar and consequently also in honey are well known for their antimicrobial effects (Adler, 2000; Cowan, 1999). We analysed the antimicrobial potential of honey and its secondary metabolites with respect to their plant specific floral origin of nectar foraged by bees (chapter 6). Bacteria used to characterize this activity were causal agents and strains associated with bacterial brood diseases of the honeybee, namely American foulbrood - AFB (bacterium: *Paenibacillus larvae*; Genersch et al., 2006) and European foulbrood - EFB (bacteria: *Melissococcus plutonius, Bacillus pumilus, Brevibacillus laterosporus, Enterococcus faecalis, Paenibacillus alvei*; Forsgren, 2010). The specificity as well as intensity of antimicrobial activity were assessed for monofloral and polyfloral honeys to see how important diverse honey stores might be for colony health (chapter 6).

The suite of pathogens that bees encounter in nature is highly diverse (Schmid-Hempel 1998). Thus it would be highly adaptive for a colony's 'social immunity' if honeybees would preferentially forage for a specific nectar, pollen or resin to prophylactically inhibit or reduce pathogen infection, and therapeutically to cure the disease or at least reduce harming effects of the pathogen. In order to ensure the healing potential of foraged plant products, honeys need to be stored and available in the bee colony upon pathogen infection. Following nectar flow in the colony, nectar will be stored in specific comb regions and filled cells are sealed before the next nectar flow is available (Seeley & Morse, 1976; Seeley et al., 1991; Winston, 1987). Hence, different types of honey are available for the worker bees at any given time of year, if beekeepers do not interrupt this storage system by harvesting the honey for marketing. Using honeybee nurse bees infected with the microsporidian gut parasite *Nosema ceranae* (Fries, 2010), we test if in-hive bees could choose among different types of honey stored in the colony based on their own health status (chapter 7). Different honey types were offered to healthy and diseased nurse bees in a simultaneous choice test to estimate preference behavior as a sign of therapeutic medication, which might be relevant at both the individual and the colony level.

Beekeepers not only harvest high amounts of honey, pollen and propolis for marketing and thereby significantly reducing the natural diversity of food supply in the honeybee colony, they also treat colonies against pathogens and parasites. Treatments, mainly against the parasitic mite *Varroa destructor*, mean the application of chemical pesticides and antibiotics that may harm honeybees as well (Eisenstein, 2015; Staveley et al., 2014). However, for some diseases (e.g. AFB-*P. larvae, N. ceranae, V. destructor*), it has been shown that

natural products can be applied as well as chemicals (Antúnez et al., 2008; Damiani et al., 2014; Porrini et al., 2011). Plant and bee product extracts, primarily secondary metabolites, harbour high antibiotic activities and nearly unlimited resources to prophylactically and therapeutically treat honeybee colonies, improving their health status. In recent years, plant extracts came into the focus of alternative bee treatment research as natural antibiotics against bacteria, fungi and mites (Damiani et al., 2014). Here, we tested whether antimicrobial plant extracts may also be active against honeybee virus diseases. Antiviral treatments of commercial honeybee colonies are completely unknown for beekeepers so far. Using a naturally infected Black queen cell virus - forager honeybee system for initial screening, the high activity of *Laurus nobilis* leaf extracts could be demonstrated in comparison to *Artemisia absinthium* and European propolis extracts (Aurori et al. 2015). In a subsequent study (chapter 8), several concentration of *L. nobilis* leaf ethanolic extracts were tested for their antiviral potential to elucidate if plant secondary metabolites can reduce virus loads and virus replication in diseased honeybees.

1.5. Winter survival and honeybee decline

Honeybees can be infected by various types of parasite and pathogen many of which have been claimed to have contributed to or caused colony declines – including 'Colony Collapse Disorder' (CCD) (Core et al., 2012; Cox-Foster et al., 2007; Neumann & Carreck, 2010; Oldroyd, 2007; vanEngelsdorp et al., 2009). Microsporidia (*Nosema ceranae*) and *Varroa destructor* and their viruses (particularly DWV) have been suggested to be the main culprits triggering this phenomenon (Cox-Foster et al., 2007; Dainat et al., 2012; Genersch et al., 2010; McMahon et al., 2016). Both parasites have been introduced to *A. mellifera* apiculture in the last century, spilling over from the Asian honeybee *Apis cerana* (reviewed in Fries, 2010; Rosenkranz et al., 2010). One reason for their disastrous impact on global apiculture may be the lack of evolutionary adaptations in *A. mellifera*. Current attempts to reduce disease risk include changes in honeybee management and breeding for resistant or tolerant honeybees (Evans & Spivak, 2010).

In addition to pathogens, mainly pesticides and interactions between both pathogens and pesticides, climate change, landscape alteration, agricultural intensification and non-native (invasive) species have been accused to substantially contribute to the losses of honeybee colonies (González-Varo et al., 2013; Goulson et al., 2015; Kluser et al., 2010; Le Conte et al., 2012; Martin et al., 2012; Potts et al., 2010a). All these factor are not only relevant for honeybee declines but also for the decline of other wild and managed pollinators (e.g. bumble bees, butterflies, flies etc.) (Biesmeijer et al., 2006; Goulson et al., 2015; Potts et al., 2016). Recent metadata analysis showed that colony losses can be regional extremely variable, rarely exceeding 30% at the national scale (Potts et al., 2010b). By reporting country specific regional colony losses, agencies and researcher take almost exclusively data on winter colony losses, which means the number of colonies that died between autumn and spring the following year. Winter and summer bees differ not only significantly in their total lifespan but also in their general physiology and tasks performed for the colony (Winston, 1987). The number of winter colony losses is however less relevant than the number of existing colonies throughout the pollination season from a societal or ecological perspective.

Nowadays, there is still going debate on why winter honeybees live much longer (up to six times) in comparison to summer bees (Amdam & Omholt, 2002). Colonies not declining during winter may have a stronger immunological background or better health status *per se*. Long-living winter honeybees (syn. *diutinus* bee) stay with the queen, forming a winter cluster, without brood, and full stores of highly antibiotic honey to survive the cold season. The main activities of *diutinus* worker bees is heating and thermoregulation instead of brood rearing and foraging (Winston, 1987). As mentioned earlier, the high density of workers and high nest temperature provide ideal conditions for both transmission and growth of pathogens (Schmid-Hempel, 1998). Several theories of aging have been discussed explaining the observed aging plasticity in honeybees. In chapter 9, we report on the importance of innate immunity, the antioxidant machinery and an aging-specific pathway (insulin/insulin-like growth factor signalling - IIS) in protecting winter honeybees from fast aging and potential

disease associated colony failure. A more adaptive immune system of winter bees would be highly beneficial to start colony growth in spring with healthy and vital worker bees instead of sick bees spreading diseases when foraging for food. In brief, we used early and late winter bees that were both healthy and experimentally bacteria-infected, and compared their gene expression levels for target genes of innate immunity, antioxidative enzymes and the insulin/insulin-like growth factor signalling-pathway.

Chapter 2 - A depauperate immune repertoire precedes evolution of sociality in bees

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Abstract

Background: Sociality has many rewards, but can also be dangerous, as high population density and low genetic diversity, common in social insects, is ideal for parasite transmission. Despite this risk, honeybees and other sequenced social insects have far fewer canonical immune genes relative to solitary insects. Social protection from infection, including behavioral responses, may explain this depauperate immune repertoire. Here, based on full genome sequences, we describe the immune repertoire of two ecologically and commercially important bumblebee species that diverged approximately 18 million years ago, the North American *Bombus impatiens* and European *Bombus terrestris*.

Results: We find that the immune systems of these bumblebees, two species of honeybee, and a solitary leafcutting bee, are strikingly similar. Transcriptional assays confirm the expression of many of these genes in an immunological context and more strongly in young queens than males, affirming Bateman's principle of greater investment in female immunity. We find evidence of positive selection in genes encoding antiviral responses, components of the Toll and JAK/STAT pathways, and serine protease inhibitors in both social and solitary bees. Finally, we detect many genes across pathways that differ in selection between bumblebees and honeybees, or between the social and solitary clades.

Conclusions: The similarity in immune complement across a gradient of sociality suggests that a reduced immune repertoire predates the evolution of sociality in bees. The differences in selection on immune genes likely reflect divergent pressures exerted by parasites across social contexts.

Chapter 3 - Rapid evolution of antimicrobial peptide genes in an insect host-social parasite system

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Abstract

Selection, as a major driver for evolution in host-parasite interactions, may act on two levels; the virulence of the pathogen, and the hosts' defence system. Effectors of the host defence system might evolve faster than other genes e.g. those involved in adaptation to changes in life history or environmental fluctuations. Host-parasite interactions at the level of hosts and their specific social parasites, present a special setting for evolutionarily driven selection, as both share the same environmental conditions and pathogen pressures.
Here, we study the evolution of antimicrobial peptide (AMP) genes, in six host bumblebee and their socially parasitic cuckoo bumblebee species. The selected AMP genes evolved much faster than non-immune genes, but only *defensin-1* showed significant differences between host and social parasite. Nucleotide diversity and codon-by-codon analyses confirmed that purifying selection is the main selective force acting on bumblebee defence genes.

Keywords: social insect, co-evolution, innate immunity, bumblebee, Bombus, host-parasite

Chapter 4 - Effective population size as a driver for divergence of an antimicrobial peptide (Hymenoptaecin) in two common European bumblebee species

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Abstract

Social insects are the target of numerous pathogens. This is because the high density of closely-related individuals frequently interacting with each other enhances the transmission and establishment of pathogens. This high selective pressure results in the rapid evolution of immune genes, which might be counteracted by a reduced effective population size (N_e) lowering the effectiveness of selection. We tested the effect of N_e on the evolutionary rate of an important immune gene for the antimicrobial peptide Hymenoptaecin in two common central European bumblebee species: *Bombus terrestris* and *Bombus lapidarius*. Both species are similar in their biology and are expected to be under similar selective pressures because pathogen prevalence does not differ between species. However, previous studies indicated a higher N_e in *B. terrestris* compared to *B. lapidarius*. We found high intraspecific variability in the coding sequence but low variability for silent polymorphisms in *B. lapidarius*. Estimates of long- and short-term N_e were three- to four-fold higher N_e in *B. terrestris*, although the species did not differ in census population sizes. The difference in N_e might result in less efficient selection and suboptimal adaptation of immune genes (e.g. *hymenoptaecin*) in *B. lapidarius*, and thus this species might become less resistant and more tolerant, turning into a superspreader of diseases.

Keywords: antimicrobial peptide, *Bombus lapidarius*, *Bombus terrestris*, effective population size, *hymenoptaecin*, innate immunity

Chapter 5 - Pharmacophagy and pharmacophory: mechanisms of self-medication and disease prevention in the honeybee colony (*Apis mellifera*)

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***Apidologie*, 2016, 47(3), 389-411. DOI: 10.1007/s13592-015-0400-z - Review article**

Abstract

Apitherapy promises cures for diseases in human folk medicine, but the effects of honeybee produced and foraged compounds on bee health are less known. Yet, hive products should chiefly facilitate medication and sanitation of the honeybees themselves rather than other organisms. We here review the impact of both self-produced gland secretions and foraged hive products (pharmacognosy) on colony health. Although foraged plant-derived compounds vary highly in antibiotic activity depending on the floral and regional origins, secondary plant metabolites in honey, pollen and propolis are important for the antibiotic activity against pathogens and parasites. However, specific bee health-enhancing activities of bee products should clearly be distinguished from the effects of an intact nutrition ensuring the basic immune competence of bees. Further unravelling the interactions among groups of active substances or individual compounds used in concert with specific behavioural adaptations will deepen our understanding of the natural potential of honeybees to maintain colony health.

Keywords: honey, propolis, pollen, bee bread, royal jelly, antimicrobial activity, self-medication, host-parasite interaction

Chapter 6 - Diversity of honey stores and their impact on pathogenic bacteria of the honeybee, *Apis mellifera*

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Abstract

Honeybee colonies offer an excellent environment for microbial pathogen development. The highest virulent, colony killing, bacterial agents are *Paenibacillus larvae* causing American foulbrood (AFB), and European foulbrood (EFB) associated bacteria. Besides the innate immune defense, honeybees evolved behavioral defenses to combat infections. Foraging of antimicrobial plant compounds plays a key role for this "social immunity" behavior. Secondary plant metabolites in floral nectar are known for their antimicrobial effects. Yet, these compounds are highly plant specific, and the effects on bee health will depend on the floral origin of the honey produced. As worker bees not only feed themselves, but also the larvae and other colony members, honey is a prime candidate acting as self-medication agent in honeybee colonies to prevent or decrease infections. Here, we test eight AFB and EFB bacterial strains and the growth inhibitory activity of three honey types. Using a high-throughput cell growth assay, we show that all honeys have high growth inhibitory activity and the two monofloral honeys appeared to be strain specific. The specificity of the monofloral honeys and the strong antimicrobial potential of the polyfloral honey suggest that the diversity of honeys in the honey stores of a colony may be highly adaptive for its "social immunity" against the highly diverse suite of pathogens encountered in nature. This ecological diversity may therefore operate similar to the well-known effects of host genetic variance in the arms race between host and parasite.

Keywords: American foulbrood, antimicrobial activity, disease ecology, European foulbrood, host-parasite interaction, Paenibacillus larvae, self-medication

Chapter 7 - Pathogen-associated self-medication behavior in the honeybee *Apis mellifera*

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Abstract

Honeybees, *Apis mellifera*, have several prophylactic disease defense strategies, including the foraging of antibiotic, antifungal, and antiviral compounds of plant products. Hence, honey and pollen contain many compounds that prevent fungal and bacterial growth and inhibit viral replication. Since these compounds are also fed to the larvae by nurse bees, they play a central role for colony health inside the hive. Here, we show that honeybee nurse bees, infected with the microsporidian gut parasite *Nosema ceranae*, show different preferences for various types of honeys in a simultaneous choice test. Infected workers preferred honeys with a higher antibiotic activity that reduced the microsporidian infection after the consumption of the honey. Since nurse bees feed not only the larvae but also other colony members, this behavior might be a highly adaptive form of therapeutic medication at both the individual and the colony level.

Keywords: honeybee, honey, antimicrobial activity, therapeutic self-medication, *Nosema ceranae*, social immunity

Chapter 8 - Bay laurel (*Laurus nobilis*) as potential antiviral treatment in naturally BQCV infected honeybees

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Abstract

Viral diseases are one of the multiple factors associated with honeybee colony losses. Apart from their innate immune system, including the RNAi machinery, honeybees can use secondary plant metabolites to reduce or fully cure pathogen infections. Here, we tested the antiviral potential of *Laurus nobilis* leaf ethanolic extracts on forager honeybees naturally infected with BQCV (Black queen cell virus). Total viral loads were reduced even at the lowest concentration tested (1 mg/ml). Higher extract concentrations (≥5 mg/ml) significantly reduced virus replication. Measuring *vitellogenin* gene expression as an indicator for transcript homeostasis revealed constant RNA levels before and after treatment, suggesting that its expression was not impacted by the *L. nobilis* treatment. In conclusion, plant secondary metabolites can reduce virus loads and virus replication in naturally infected honeybees.

Keywords: BQCV, Honeybee, Antiviral activity, Plant secondary metabolite, Polyphenol, Flavonoid

Chapter 9 - What is the main driver of ageing in long-lived winter honeybees: antioxidant enzymes, innate immunity, or vitellogenin?

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Abstract

To date five different theories compete in explaining the biological mechanisms of senescence or ageing in invertebrates. Physiological, genetical, and environmental mechanisms form the image of ageing in individuals and groups. Social insects, especially the honeybee *Apis mellifera*, present exceptional model systems to study developmentally related ageing. The extremely high phenotypic plasticity for life expectancy resulting from the female caste system provides a most useful system to study open questions with respect to ageing. Here, we used long-lived winter worker honeybees and measured transcriptional changes of 14 antioxidative enzyme, immunity, and ageing-related (insulin/insulin-like growth factor signaling pathway) genes at two time points during hibernation. Additionally, worker bees were challenged with a bacterial infection to test ageing- and infection-associated immunity changes. Gene expression levels for each group of target genes revealed that ageing had a much higher impact than the bacterial challenge, notably for immunity-related genes. Antimicrobial peptide and antioxidative enzyme genes were significantly upregulated in aged worker honeybees independent of bacterial infections. The known ageing markers *vitellogenin* and *IIP-1* were opposed regulated with decreasing *vitellogenin* levels during ageing. The increased antioxidative enzyme and antimicrobial peptide gene expression may contribute to a retardation of senescence in long-lived hibernating worker honeybees.

Keywords: *Apis mellifera*, antioxidative enzymes, innate immunity, social insect, senescence

Chapter 10 - Synopsis

10.1. Immune system evolution in bumble bees

10.1.1. The whole genome perspective

Comparing the full genomes of three genera of Hymenoptera, *Apis*, *Bombus* and *Megachile*, in particular genes involved with innate immunity, revealed that bumble bees and the other bees have a highly conserved suite of the canonical innate immune pathways, though with only a small number of genes required to guarantee their functionality (chapter 2). However, we found no evidence that there is any relationship between the evolution of sociality and the total number of immune genes (Barribeau et al., 2015, chapter 2). These results were confirmed by the comparative '10 bee genomes' study of Kapheim et al. (2015). Both studies are in line with the first publication of the immune gene set of the honeybee (Evans et al., 2006), which stated that the honeybee has a depauperate innate immune system in terms of its total number of immune genes in comparison to *Anopheles* and *Drosophila* (Evans et al., 2006). Nowadays we know this phenomenon is not unique to eusocial Hymenoptera but more likely a feature of all Hymenopteran species, including ants and wasps, all of which show a lower number of immune genes compared to non-Hymenopteran species (Gadau et al., 2012; Werren et al., 2010; Xu and James, 2009). Some non-Hymenoptera genomes (pea aphid *Acyrthosiphon pisum*, tsetse fly *Glossina morsitans*) are as well known to have a quite low number of immune genes or are even lacking several key immune system genes (Gerardo et al., 2010; International *Glossina* Genome Initiative, 2014). This case of secondary loss was discussed to be associated with obligate microbial symbionts (Gerardo et al., 2010; International *Glossina* Genome Initiative, 2014). Even within the Hymenoptera, more specifically the genus *Apis*, high variance can be observed. For example, *A. ceranae* lacks the genes *FADD*, *dredd*, *kenny* (Imd pathway) and *pelle* (Toll pathway) in comparison to *A. mellifera* (Park et al., 2015). The authors speculate that this reduction might be compensated by the uniqueness of strong behavioral defense of *A. cerana* (e.g. hygienic and grooming behavior; Park et al., 2015). However, it seems not plausible that key genes of the two major immune pathways are missing, which would mean that they are inactive or at least inefficient in defending the organism. Using reference RNA sequences and transcriptome shotgun assembly, as implemented in NCBI BLAST with standard settings, I found homologues of *dredd* (aka *caspase*-8, XM_017059605), *kenny* (XM_017061996) and *pelle* (XM_017063311) in *Apis cerana*. This means that these genes are not missing in *A. ceranae* and it has a fully functional innate immune system, as have all other *Apis* species for which data exist.

Fischman et al. (2011) were first to claim that we do not observe a reduction in total gene number in Hymenoptera but an expansion of the immune gene repertoire in flies and mosquitoes. This means that the small number of immune genes would be ancestral to the Hymenoptera. The recently published genome of the house fly *Musca domestica* confirms this assumption. In the genome of this species, several different classes of immune-related genes (notably pathogen recognition or pathogen killing) are duplicated at a significantly accelerated rate relative to other Dipterans (e.g. mosquito, *Glossina*, *Drosophila*) (Sackton et al., 2017). The fly's lifestyle in septic environments might explain the elevated diversity of immune-related genes. So far it is not clear if the *M. domestica* genome shows increased rates of gene duplication, decreased rates of gene loss, or both (Sackton et al., 2017). This observation is not unique to flies. The increase in immunity-related genes was observed likewise in the Asian tiger mosquito (*Aedes albopictus*) and the southern house mosquito (*Culex quinquefasciatus*) in comparison to other mosquito species (Arensburger et al., 2010; Chen et al., 2015). The expansion of immune genes seems to be a Diptera-specific phenomenon; conversely, the lower number for Hymenoptera seems to be ancestral.

The total number of immune-related genes, as estimated recently, may actually be an inappropriate parameter in comparative genomic studies. High-throughput RNAseq in combination with reference genome assembly appears to be a more powerful way of annotating unknown genomes (Kapheim et al., 2015). On the other side, there is still a huge number of annotated genes lost through *de novo* assembly and revision of genomes via automatic gene annotation, particularly if such assemblies are not supported by additional empirical evidence

(e.g. RNAseq, qPCR). For the honeybee, thousands of sequences were not retained in the OGS (official gene set) v1.0 to OGSv3.2 as they were not at that time supported by empirical evidence (McAfee et al., 2016; Trapp et al., 2017). High-resolution mass spectrometry, including (nano)LC-MS/MS, revealed tissue-specific peptide and protein sequences with 8% of all identified honeybee peptides matching sequences found only in OGSv1.0 (McAfee et al., 2016; Trapp et al., 2017). Not only many proteins were deleted from the OGSv1.0 by *de novo* annotation of the *A. mellifera* genome but also approx. 500 coding sequences are not present in either OGSv1.0 or OGSv3.2 (McAfee et al., 2016; Trapp et al., 2017). By adding the newly identified sequence entries to the current number of honeybee genes, we can see an increase from 15314 in OGSv3.2 to the current 17372. (McAfee et al., 2016). Future studies investigating bee biology (host defense), origin and consequences of eusociality, caste determination and social behavior will use targeted gene manipulation techniques (e.g. RNAi, CRISPR-Cas9; Trapp et al., 2017), which eventually will reveal both the number and functions of genes that are mandatory to ensure the survival of bees.

10.1.2. Gene-specific selection between species

We know that immune genes evolve much faster than other genes in bumble bees and their social-parasites (cuckoo bumble bees) (Erler et al., 2014; chapter 3). However, there is no indication for parallel evolution between hosts and social parasites, which might be expected as both host and social parasite share the same environment and hence also pathogen pressure. Comparing host and social parasite, only a single gene, *defensin-1*, showed significant differences between both, with hosts' genes evolving faster than the social parasite (Erler et al., 2014; chapter 3). In general, we observed that selection based differences are detectable at very low frequency. This observation might be explained by the fact that purifying selection is the main selective force acting on bumble bee antimicrobial peptide genes.

Whole immune system gene scans found evidence of positive selection in genes encoding antiviral responses, components of the Toll and JAK/STAT pathways, and serine protease inhibitors in both social and solitary bees (Barribeau et al., 2015; chapter 2). Both studies revealed that antimicrobial peptide genes, those which I also selected to study host and social-parasite bumble bees, are possibly much more conserved than genes for antiviral response and receptor/recognition genes. Direct evidence for this variance in conservation was found for the genes *argonaute 2*, *armitage* and *maelstrom* (Helbing & Lattorff, 2016). All these genes involved in antiviral defense evolve much faster in social than in social-parasitic bumble bees. Genes directly interacting with viruses showed the highest rates of molecular evolution (Helbing & Lattorff, 2016). In particular RNAi genes seem to be permanently exposed to selection pressure imposed by viruses, leading to faster evolution than non-RNAi genes and even faster than other innate immune genes (Helbing & Lattorff, 2016). Once again, comparing antiviral RNAi gene evolution between host and social parasite species or the bumble bee's geographic origin did not shown any general pattern of parallel evolution (Helbing & Lattorff, 2016).

Similar results have been found for candidate genes involved in division of labor (so called social effect genes; e.g. *foraging*, *salivary gland secretion 3* and *vitellogenin*) (Fouks & Lattorff, 2016). The social effect genes showed no general evolutionary trend. However, by comparing social hosts and their social-parasite species, signatures of higher selection coefficients could be detected in social species (Fouks & Lattorff, 2016).

10.1.3. Gene evolution and effective population size

All these case studies (Barribeau et al., 2015; Erler et al., 2014; Fouks & Lattorff, 2016 and Helbing & Lattorff, 2016) suggest that the observed pattern of immune gene evolution has resulted from a small effective population size (N_e) of the socially parasitic cuckoo bumble bee species. The latter are assumed to show higher rates of protein evolution due to relaxed selective constraints, as a consequence of a stronger reduction in N_e (see chapter 3 and 4 for details). Relaxed means that the selective constrain is partially removed, for example along a gene with different functional regions, allowing accumulation of non-synonymous substitutions and leading to faster gene evolution.

The effects observed for host and cuckoo bumble bees might mainly be driven by differences in N_e but may also reflect phylogenetic constrains. Two closely related bumble bee host species, sharing a similar biology and potential selective pressure due to same parasite prevalence make them an ideal study system because it avoids the issue of phylogenetic constrains driven by the monophyletic group of the cuckoo bumble bees. A population based study comparing the two most-common bumble bee species in Europe, *B. terrestris* and *B. lapidarius*, across five sampling sites confirmed that purifying selection is the major force acting on *hymenoptaecin* (Lattorff et al., 2016; chapter 4). The higher intraspecific variability in the coding sequence but low variability for silent polymorphisms in *B. lapidarius*, resulting in less efficient selection and suboptimal adaptation of immune genes, was explained by differences in N_e. Reduced parasite resistance and higher tolerance might be a result of the variability in this particular antimicrobial peptide gene. This study was the first estimating long- and short-term N_e from gene-based data for both species. Three- to four-fold higher N_e was estimated for *B. terrestris*, although the species did not differ in census population sizes. The estimates for N_e did not substantially change with the recent estimation of the mutation rate for the bumble bee *B. terrestris* (3.6×10^{-9}, 95% confidence intervals: 2.38×10^{-9} and 5.37×10^{-9}) (Liu et al., 2017). Using the *B. terrestris* specific mutation rate instead of the *Drosophila* mutation rate (5.8×10^{-9}) increased the long-term N_e 1.6 times than previously estimated (chapter 4). The estimates of N_e for other bumble bees are still vague as species-specific mutation rates for other social bumble bees and the social-parasitic cuckoo bumble bees are still missing.

Summing up, variation in immune gene evolution (including both, purifying and positive selection) is obvious between the highly eusocial *Apis* clade and the primitively eusocial *Bombus* clade, and between the solitary *Megachile* and both eusocial clades (Barribeau et al., 2015; chapter 2). Within-genus comparison of species sharing the same habitat and parasite pressure revealed that these species (social and socially-parasitic) have in common that local adaptation to bacteria and fungi might be less extreme and primarily occur in recognition and effector genes (Barribeau et al., 2015; Erler et al., 2014; chapter 2, 3). Population-wide studies on bumble bees as well as on *Drosophila melanogaster* showed no overall trend of recent rapid adaptation in immune genes across populations (this thesis – chapter 2-4 and Early et al., 2017). This means for most of the classical genes used for studying the evolution of immunity, local adaptation might be measurable with great difficulty, if at all. The only classes of immune genes suitable to study local adaptation are those involved in the antiviral response (RNAi). They evolve rapidly in eusocial and non-social insects, with signs of elevated selection (Early et al., 2017; Helbing & Lattorff, 2016; Obbard et al., 2009).

For social Hymenoptera, a potential strategy to combat and survive pathogen and parasite infections with the lower number of immune genes (chapter 2) and mainly purifying selection acting on effector genes (chapter 3, 4) can be the potentiating interaction with defense molecules. Two recent studies gave evidence that this strategy, in combination with behavioral defense ('social immunity' and self-medication;, Cremer et al., 2007; chapter 5), is sufficient to develop resistance against bacterial and protozoan infections (Marxer et al., 2016; Rahnamaeian et al., 2015). Combinations of the bumble bee's antimicrobial peptides Abaecin, Defensin and Hymenoptaecin were more effective than the use of a single antimicrobial peptide alone against Gram-negative bacterial pathogens and different strains of the gut parasite *C. bombi*. Not only lower amounts of each peptide are more effective in combination, even high strain-specificity was detected (Marxer et al., 2016). Such specificity drives the evolutionary arms-race between hosts and parasites. Strain-specificity can be good for the host and bad for the parasite, but only on a short-term scale. Under natural conditions, alternative parasite strains will resist the host's specific peptide combination against a specific parasite strain; consequently, new strains survive better and become as virulent as a previously widespread strain.

10.2. Social immunity in honeybees

Many bee species have been shown to use self-produced (gland secretions) and foraged substances to enhance individual and colony health. All these hive products facilitate medication and sanitation of the honeybees and their colony environment in addition to individual innate immunity (Erler & Moritz 2016; chapter 5). Self-

produced antibiotic substances (e.g. cuticular hydrocarbons, venom, wax and food jelly) mainly vary across species and lesser within species; in the latter case, variation in response is driven by the quality of food and genetic background, whereas foraged plant-derived products and substances (e.g. resin - propolis, pollen – bee bread and honey) vary vastly in their antibiotic activity depending on floral and regional origin (reviewed in Erler & Moritz, 2016; chapter 5). The main active ingredients of foraged hive products with antibiotic activity are plant secondary metabolites. However, all health enhancing and parasite/pathogen reducing activities have to be distinguished from effects of malnutrition during experiments, so as to differentiate cases of suppressed or elevated non-basic immune competence of the tested individuals. Hive products can be used as prophylactic and therapeutic self-medicating agents. Honey is the only continuously foraged product throughout the season which is foraged and stored *en mass*. The in-hive pharmacy comprising many different monofloral and polyfloral honeys provides excellent conditions for colony medication.

10.2.1. Self-medication using diverse hive products

Worker honeybees infected with *N. ceranae* preferentially selected highly antibiotic honey instead of honey with lower antibiotic activity in a choice assay (Gherman et al., 2014; chapter 7). This behavior might be a form of highly adaptive medication to prevent disease spread and transmission within the colony e.g. by feeding larvae and worker bees to prevent further infections. In addition to honey, increased foraging for propolis has been observed in fungi-infected bee colonies, leading to reduced infection intensities (Simone-Finstrom & Spivak, 2012). On the other hand, stored honey could be prophylactically used in honeybee defense as there is high specificity of different monofloral honeys against different bacterial honeybee pathogens (Erler et al., 2014; chapter 6). Monofloral and polyfloral honeys are extremely active and specific against bacterial brood diseases (American and European foulbrood). Consequently, the diverse honey stores in the colony may be highly adaptive for prophylactic and therapeutic self-medication against parasites and pathogens (Erler el al., 2014; chapter 6). A non-*Apis* insect study confirmed the high self-medication potential of honey. Greater wax moth (*Galleria mellonella*) larvae injected with human-pathogenic bacteria pretreated with honey survived significantly better than larvae injected with non-pretreated bacteria (da Silva et al., 2016). The honey treatment weakened the virulence of the bacteria, but as sugar controls were missing from the experiment, nothing is known on the non-osmotic effect of the honey.

Other hive products with prophylactic antibiotic activity are pollen and royal jelly; both are mixed with honey as worker and drone jelly to feed the bee brood. Recently, a major antibiotic compound of royal jelly, in addition to the fatty acid 10-HDA, was discovered. Using bacteria causing and otherwise associated with European foulbrood, Vezeteu et al. (2017) showed that major royal jelly protein 1, the main royal jelly protein, adds to the antibacterial activity of royal jelly in addition to 10-HDA. In combination with major royal jelly protein 2 and bee defensin-1, other self-produced antimicrobials, proteins are of great importance in the bee colony. Not only royal, worker and drone jelly contain these three proteins (MRJP 1, 2 and defensin-1) but also honey, the carbohydrate rich energy food produced from nectar or non-*Apis* insect secretions, as well as bee bread (stored fermented pollen) (reviewed in Buttstedt et al., 2014). The major role of these bee peptides in honey and pollen, beyond their potential antibiotic activity, are mostly unknown.

Diversity matters not only for the antibiotic activity of foraged hive products (Erler & Moritz, 2016), it is also a major determinant of nutritional quality, preventing malnutrition. Especially pollen, the protein source for brood rearing, has to be highly variable in floral taxon diversity and high in nutritional quality. Confirming this, a recent example from France showed that bee health is reduced mainly by pollen depletion and low quality pollen, as generated by intensive monoculture (Di Pasquale et al., 2016). Such effect can be compensated by feeding taxonomically diverse pollen mixes. The composition may change throughout season with varying flower availability, thus without significant changes on honeybee health. The only exception are months with high amount of poor quality maize pollen, leading to poor survival and poor brood nursing (Di Pasquale et al., 2016). Pollen itself has a mainly nutritional function in the colony, but also an antiviral activity (DeGrandi-Hoffman et al., 2010), and again diverse pollen has higher antiviral activity than monofloral pollen (Antúnez et al., 2015). A rare example of pollen type specific effects on bee health and survival was discovered recently. Bees foraging for a specific type of pollen were less frequently parasitized by parasitoid wasps, and survival of the parasitic wasp larvae was reduced as well (Spear et al., 2016). All these examples show that adequate

nutrition with high quality carbohydrate and peptide sources are important for healthy colonies and can help reduce parasites and diseases. Malnutrition may also be a key factor in colony losses. Experiments comparing protein supplements with naturally foraged protein revealed that bees fed an artificial diet had higher virus levels and *Nosema* loads and higher queen losses (DeGrandi-Hoffman et al., 2016). This means that high quality food may also improve the overwintering success of queens and long-living winter worker bees.

10.2.2. Self-medication using single plant compounds

Plant secondary metabolites are highly active against bee disease-causing bacteria, fungi and eukaryote parasites (Erler & Moritz, 2016; and references therein). Most of the literature available on this topic covers propolis/resin and pollen extracts (e.g. using the solvents: ethanol, methanol and acetone), as demonstrated by antibiotic activity assays. Flavones/flavonols and flavanones/dihydroflavonols are candidate groups within propolis compounds that are thought to confer antibacterial activity (Mihai et al., 2012). Notably not only dihydroflavonols are antibacterial, they are also antifungal. Especially pinobanksin-3-octanoate and five other 3-acyl-dihydroflavonols showed highly efficient antibiotic activity, with longer acyl groups exhibiting increased activity against *Paenibacillus larvae* and shorter acyl groups exhibiting increased activity against *Ascosphaera apis* (Wilson et al., 2017).

Propolis is the major hive product used in human apitherapy, a discipline still controversially debated and with scientifically mixed merit. In contrast to research into the use of hive products for human medical applications, the activity and identification of biologically active substances from honey and nectar against bee pathogens and parasites is nearly completely neglected. Only a few substances (compound classes: alkaloids, terpenoids and iridoid glycosides) originating from plant nectar, mostly consumed by bumble bees, were shown to have parasite (*Crithidia bombi*) inhibitory activity *in vivo*, but without rescuing hosts survival (Baracchi et al., 2015; Manson et al., 2010; Richardson et al. 2015). A potential role of floral compounds in reducing parasite transmission within and between colonies was discussed but not tested (Richardson et al., 2015). For some substances like anabasine, dose-dependent effects on reducing or clearing parasite loads are known, though only for concentrations higher than natural range levels (Anthony et al., 2015). Most recently, several studies using the bumble bee gut parasite *C. bombi* for *in vitro* assays and the aforementioned plant metabolites, primarily thymol and eugenol, showed high variance in phytochemical resistance (> 3-fold) across different parasite genotypes (Palmer-Young et al., 2016; Palmer-Young et al., 2017a; b). At least for thymol the natural range in *Thymus vulgaris* nectar was similar to inhibitory concentrations from the *in vitro* assay (Palmer-Young et al., 2016). Using an experimental evolution approach, Palmer-Young et al. (2017a), demonstrated that resistance to phytochemicals (single substances or in combination) increased over time, with final inhibitory concentrations exceeding concentrations in floral nectar and concentrations initially applied in the assay. This means repeated phytochemical exposure, possibly due to intensive monoculture or excessive therapeutic treatment of managed hives, may cause rapid parasite evolution, leading to phytochemical resistance (Palmer-Young et al., 2016; Palmer-Young et al., 2017a). Depending on the substances tested, synergistic (eugenol and thymol; Palmer-Young et al., 2017b) and potential antagonistic (nicotine and anabasine; Thorburn et al., 2015) interactions against *C. bombi* were observed. Both studies nicely show that phytochemical interactions vary across tested parasite strains, experiments, tested substances and environmental variables. Thus all plant secondary metabolites might be either toxic or medicinal depending on context (Thorburn et al., 2015). Last but not least, field experiments verified that *C. bombi* infected bumble bees, but not bees infected with parasitoid flies, foraged longer at flowers with higher antibiotic phytochemical concentrations (Richardson et al., 2016).

These examples support the valuable self-medication potential of foraged plant nectar for bumble bees infected with the protozoan gut parasite *C. bombi*. As already mentioned, previous studies on honeybees showed that processed nectar (honey) has prophylactic and therapeutic antibiotic activities against the intracellular protozoan parasite *N. ceranae* and bacteria causing American and European foulbrood (Erler et al., 2014; Gherman et al., 2014; and chapters 6, 7). Future studies are needed to identify the biologically active substances of honey and nectar against honeybee and bumble bee pathogens and parasites that might reduce the probability of individual death and whole colony loss. These substances, or plants producing high quantities of them, could be used to reduce local parasite/pathogen loads, increase pollinator health and, consequently, pollination efficiency.

Substance concentration, identity and pathogen/parasite species are not the only parameters affecting growth inhibitory activities of nectar/honey. In particular, microbes colonizing plant nectar may reduce concentrations of phytochemicals, reduce deterrent effects of toxic nectar to pollinators and can add antimicrobial substances to the nectar/honey (Butler et al., 2013; Olofsson and Vásquez 2008; Vannette & Fukami, 2016). A bee's intestinal microbial community possesses the potential to detoxify compounds toxic to the bee as well as harmless substances. These same microbes, might develop resistance to antimicrobials and may modify phytochemicals on their way from the honey crop to the rectum. This bacterial trait might drive pollinator diversification and coevolution with nectar-secreting plants (with novel allelochemicals), summarized as the 'gut microbial facilitation hypothesis' (reviewed in Hammer & Bowers, 2015). Bacteria species involved in above mentioned modifications, mechanisms and traits belong to the core gut microbiota of the honeybee, including Gram-negative Proteobacteria (*Gilliamella apicola*, *Snodgrassella alvi*), Gram-positive Firmicutes (*Lactobacillus* Firm-4, *Lactobacillus* Firm-5 clade) and Actinobacteria (*Bifidobacterium* species cluster) (reviewed in Kwong & Moran 2016).

At least for the phytochemical nicotine, we know now that it is metabolized within 24 h on its way through the digestive tract of the honeybee. Nicotine titers decrease constantly, whereas 4-hydroxy-4-(3-pyridyl) butanoic acid (the main metabolite of nicotine) can be increasingly detected on the way to the rectum (du Rand et al., 2017). As a consequence, observed antibiotic activities of nicotine (Anthony et al., 2015; Baracchi et al., 2015; Richardson et al. 2015; Thorburn et al., 2015) might not be directly linked to nicotine itself but more likely to one of its metabolites.

10.2.3. Potential self-medication against viruses using plant extracts

Honeybee virus research currently aims to identify infection mechanisms, transmission routes (within and between species) and how the host organism fights virus infections. RNAi, pathogen-associated molecular pattern (PAMP) triggered signal transduction cascades, and reactive oxygen species generation are key parameter of the honeybee's antiviral defense mechanisms (Brutscher et al., 2015). Within ongoing research strategies, the complete antiviral mechanisms against the more than 20 honeybee viruses will undoubtedly be soon revealed. However, this knowledge alone will not help curing virus infected colonies. Pollen itself and its diversity are of great importance in fighting viral diseases (Antúnez et al., 2015; DeGrandi-Hoffman et al., 2010). Nevertheless it is clearly not feasible to harvest and store tons of highly specific antiviral pollen to prophylactically and therapeutically feed virus infected honeybee colonies. The only economically realistic solution might be feeding antiviral substances of natural origin that can be synthetically produced in relevant amounts. The antiviral activity of ethanolic extracts of European propolis, *Artemisia absinthium* leaf extracts, and pinocembrin, as the major propolis flavonoid, against BQCV (black queen cell virus) are weak or totally absent (Aurori et al., 2014). The same authors found evidence that ethanolic *Laurus nobilis* leaf extracts and substances therein are candidates for future in-field antiviral colony treatments (Aurori et al., 2015; chapter 8). Bay laurel extracts not only reduced total virus load but also virus replication without harming the bee. *L. nobilis* extracts are not only active against BQCV, they are also active against non-*Apis* viruses as well (Ertürk et al., 2000; Loizzo et al., 2008). Most bee viruses are single-stranded RNA viruses belonging to the picornavirus superfamily, which includes the *Dicistroviridae* and Iflavirus family (Aubert et al., 2008). BQCV belongs to the *Dicistroviridae* family, together with ABPV and KBV. The replication and assembly machinery of the *Dicistroviridae* might be comparable for other viruses. Therefore, *L. nobilis* plant secondary metabolites may inhibit replication and reduces virus loads for all members of this family by a comparable process. The mechanism of this is currently unknown, but we know that phytochemicals interfere with virus receptors, virus replication, and virion assembly (Jassim and Naji, 2003). Future in-field colony tests are needed to fully confirm the antiviral activity of this particular plant extract and others, including single candidate substances.

10.2.4. Self-medication and innate immunity

Only few studies have demonstrated an interaction between innate immunity and self-medication in eusocial bees, in particular honeybees (Alaux et al., 2010; Johnson et al., 2012; Simone et al., 2009; Borba et al., 2015). Non-infected honeybees reduced their innate immune response and upregulated detoxification genes upon exposure to honey, pollen and propolis extracts (Johnson et al., 2012; Simone et al., 2009). However, methanolic and ethanolic extracts do not reflect the real situation in the hive. A year-long study on honeybee

colonies having a natural propolis envelop confirmed previous results of reduced and more uniform baseline expression of innate immune genes (mainly effector genes) in bees during summer and autumn (Borba et al., 2015). In the same study, no differences were found for bacteria, *Varroa*, *Nosema* and virus loads between treatment groups (with and without propolis envelop). The authors interpreted the reduction in expression of the immune system in the presence of propolis with the bees' reduction in activation of physiologically costly humoral immune responses (Borba et al., 2015). As direct mechanisms and interaction pathways are so far not known, the main function of propolis continues to be considered as an antimicrobial layer covering the inner wall of the beehive.

10.3. Winter survival and honeybee decline

10.3.1. Winter bee ageing and immunity

In general, differences in gene expression between honeybee phenotypes (e.g. summer and winter worker bees) are driven by the process of ageing *per se*, potential infections, environmental conditions and variation in food availability across the seasons. Here, we could show that ageing has a much higher impact on gene expression levels (for antioxidative enzymes, innate immune system genes, and insulin/insulin-like growth factor signaling pathway genes) than bacterial infections in aged winter honeybees (Aurori et al., 2014; chapter 9). Exclusively immune response genes were upregulated following bacterial infection. Nevertheless, antimicrobial peptide and antioxidative enzyme genes were significantly upregulated in aged worker bees independent of bacterial infection, without an impact of ageing on infection response. Both increased antioxidative enzyme and antimicrobial peptide gene expression may contribute synergistically to retarding of senescence in long-living (hibernating) worker honeybees (Aurori et al., 2014; chapter 9). This may also lead to a lower susceptibility to parasite infections of long-lived bees, as shown by Bull and colleagues (2012). Therefore immunocompetence of honeybees is not independent of ageing. However, higher disease resistance of aged honeybees might be a fungi-specific phenomenon. The two studies demonstrating the theory of enhanced disease resistance in older bees used *Metarhizium anisopliae* and *Nosema ceranae* with young and old summer bees for their infection experiments (Bull et al., 2012; Roberts & Hughes, 2014). The latter study also demonstrated that older infected individuals survived longer than younger bees, with the consequence of more intense infections (pathogen load) and a lower baseline immunocompetence. All these studies showed that young and old summer and winter honeybees differ significantly for their humoral and cellular immune response, and that immune function correlated with age. Hence results for cage and in-hive experiments conducted with summer worker bees cannot directly be transferred to the retarded ageing process of long-living winter bees. Nonetheless, we can say that phenoloxidase levels seem to be higher in younger bees, whereas antimicrobial peptides are highest in older bees (Aurori et al., 2014; Roberts & Hughes, 2014; Steinmann et al., 2015).

But if bees are already infected when starting overwintering (e.g. high DWV loads), the expression of genes involved in cellular immune response and physiological activity are reduced (Steinmann et al., 2015). The same study confirmed that winter bees show high expression of humoral immune genes (mainly antibacterial defense - *defensin-1* and *hymenoptaecin*) for aged winter bees compared with summer bees. However, with the current results we still do not know if immune senescence in honeybees occurs mainly on the cellular and/or humoral immune system or if bees simply respond to cold stress and/or nutritional/physiological changes accompanying overwintering. In summary, stress, of whichever nature, is a critical factor for individual bee and colony survival and health. A recent study could show that early life stress has greater impact on survival than late life stress exposure (Rueppell et al., 2017). The same study further demonstrates that *Varroa* mite stress during development consistently reduced mortality.

10.3.2. Beekeeping and colony decline

Beekeeping practices are significantly linked to increased colony mortality, with migratory bees suffering higher mortality than stationary bees (Simone-Finstrom et al., 2016). Moving honeybee colonies for several months repeatedly to large monocultures of low nutritional value and constantly treating them against disease using

chemicals decreases colony health. Beekeepers are one of the key factors when talking about (winter) colony losses. A multi-year study across > 10 European countries recently showed that a beekeeper's background and apicultural practices are directly linked with overwinter colony losses (range: 2 - 32%), and that high summer losses are likely to follow high losses during winter (Jacques et al., 2017). Less experienced hobbyist beekeepers with small apiaries had twice the winter colony mortality when compared to professional beekeepers. The same pattern can be seen for colony disease loads. Professional beekeeper apiaries had much lower signs of bacterial infections and heavy *Varroa* infestation than the hobbyists (Jacques et al., 2017).

European-wide generally acceptable and sustainable recommendations are needed for hobbyist and professional beekeepers to reduce winter colony losses. Recently published management practice guidelines included suggestions such as enhancing colony strength and food stores in autumn, improving queen quality and protecting bees from *Varroa* mites, pathogens and pesticides (Döke et al., 2015). In addition, more research is needed to take advantage of natural selection e.g. in disease resistance, as a sustainable solution for apiculture. Natural selection seems to have been much more important than selective breeding over the last centuries in improving the health of honeybees (Neumann & Blacquière, 2017).

In conclusion, we can say that the highly active innate immune system of bees acts very well under natural conditions in natural environments with locally adapted parasites and pathogens, driving evolutionary adaptations in the host-parasite arms race. The major pitfall for bee survival and health is nowadays the beekeeper and others involved in the pollination industry. Moving bees all over the world facilitated disease transmission and host switches for many parasites (e.g. *Varroa* and *Nosema*). Education of hobbyist and professional beekeepers, and restricting global bee trade and migratory beekeeping, are needed to prevent, or at least reduce, adverse effects of pathogen dispersal and spill-over into other bee species (Owen, 2017). However, long-term studies are needed to test if the beekeeper effect is Europe and USA-specific, as observed by Jacques et al. (2017) and Seitz et al. (2015), or if this can be claimed a global phenomenon. For most regions of the world, the number of winter colony losses is poorly, or at least not consistently, documented, in contrast to the numbers of managed colonies that have officially been documented for more than 100 countries since 1961 in the global database managed by the FAO (Food and Agriculture Organization of the United Nations) (Potts et al., 2010b). To estimate long-term effects on colony health, we undertook a meta-analysis (1961-2013) to reveal potentially causal reasons for regional colony decline, including the major factors: parasites and pathogens, pesticides and human society itself (chapter 11). Our meta-dataset included the total honeybee colony number, national honey production, and import and export of marketed honey, as economic parameter based on the FAO data base.

Chapter 11 - Lost colonies found in a data mine: Global honey trade but not pests or pesticides as a major cause of regional honeybee colony declines

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Abstract

Recent losses of honeybee (*Apis mellifera*) colonies have been linked to several non-exclusive factors; such as pests, parasites, pesticides (e.g., neonicotinoids) and other toxins. Whereas these losses pose a threat to apiculture, the number of globally managed colonies appeared to be less affected because beekeepers replace lost colonies. From a socioeconomic and ecological perspective the number of managed colonies is arguably more relevant when addressing the issue of apiculture and pollination services provided by honeybees. We here use the FAO data base to dissect the interactions between the global honey market and the number of colonies. Global scale analyses do not show a general colony decline. Whereas Western Europe and the US show suffer colony declines, other regions show considerable increase. We could not find any link between the colony dynamics and the occurrence of specific pathogens or the use of pesticides. In contrast, changes in the political and socioeconomic system show strong effects on apiculture. In addition, many countries show a tight negative correlation between honey import and the number of managed colonies. For some countries, the amount of honey produced per colony is highly positively correlated with the amount of honey imports, and we cannot exclude large scale relabeling of imported to nationally produced honey. It is very clear that honey trade is a dominating factor for the number of managed colonies since countries with a strong import and export ratio are those suffering most strongly from colony declines.

Keywords: Socioeconomics, Honey, Global trade, Colony losses, Beekeeping,

Chapter 12 - Summary

Eusocial bees are prone to parasites and pathogens by living together at high densities and providing a constant climatic and nutritional environment inside the colony. However, this does not mean that they are defenseless and more susceptible to diseases than organisms living alone. Bees and all other Hymenoptera harbor a highly conserved set of innate immune system pathways, though without many duplicated genes that non-Hymenopteran insects (chapter 2) possess. These genes, especially antimicrobial effector genes and genes involved in antiviral RNAi machinery, evolve much fast than non-immune genes, leading to faster adaptation, at least to local disease pressure (mainly viruses). However, similar parasite pressure does not automatically lead to parallel evolution of immune genes, as shown for host bumble bees and their social parasites (chapter 3). Phylogenetic constrains seem to be more important than locally resembling parasite diversity. On the species level, even if closely related, effective population size is the driving force for the overall observed purifying selection acting on immune system genes (chapter 4).

Antimicrobial immune defense is completed by behavioral immune defense. In particular, foraging of antibiotic plant products and the production of antibiotic gland secretions represent the second important defense wall against disease infections and spread of diseases (chapter 5). Highly diverse pollen and nectar/honeys are used for therapeutic and prophylactic self-medication (chapter 6, 7). The combination of both systems, innate immunity and self-medication, with other behavioral defense mechanisms gives bees a sufficient tool box to survive variable environmental conditions and for example seasonal varying parasite prevalence.

However, winter survival is the most critical step in the life cycle of the bee. The immune system and antioxidative enzymes are increasingly active during winter in honeybees (chapter 9). This helps survive the cold period and makes them stronger against potential infections in spring, if colonies are not stressed by previous infections. Viral infections, in combination with high *Varroa* infestation rates, are the top candidates for reduced winter survival. Antiviral treatment of natural origin might be a forward-looking method to treat honeybee colonies in autumn so as to reduce their virus loads. Reduced virus loads and virus replication was recently observed in a pilot study for BQCV infected honeybees (chapter 8). Future studies are needed to understand enhanced disease resistance and defense mechanisms by combining innate immunity with self-medication and study of how self-medication works at the molecular level.

Nonetheless, all obtained knowledge on bee immunity, evolution, behavioral defense and antibiotic secondary plant metabolites does not help to improve bee health if the beekeeper is the main parameter for colony loss. Sustainable beekeeping, supported by local governments, is needed to maintain and increase colony numbers needed for natural and commercial pollination (chapter 11). The semi-domestication of the honeybee in the northern hemisphere and usage of chemicals to kill mites and bacteria-causing diseases was rather a step backward than forward in the selection of naturally disease resistant or tolerant honeybee colonies. Beekeeper education is the most critical mission for the following years to breed naturally resistant and healthy colonies, supported by naturally occurring antibiotic resources found in diverse flowering foraging sites. Yet the potential of the pharmacy beehive is currently underestimated by most beekeepers, though it provides the biggest potential for natural disease management.

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Appendix

A. Publication list

Peer-reviewed

[30] Dezmirean DS, Mărghitaş LA, Chirilă F, Copaciu F, Bobiş O, **Erler S** (2017) Influence of geographic origin, plant material and polyphenolic substances on antibacterial properties of propolis against human and bee pathogens. **Journal of Apicultural Research**, (*under review*).

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- Radio interviews, 'World affairs (e.g. politics, socioeconomics and global trade) influence bee decline more than pests, pathogens and pesticides' for coloRadio, detektor.fm, MDR JUMP, MDR KULTUR, radio CORAX, SWR2

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- Radio interview, 'Beekeepers complain bee death' for MDR INFO
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B. Curriculum Vitae

Name	Silvio Erler
Nationality	german
Date of birth	08.09.1983
Place of birth	Karl-Marx-Stadt, Saxony, Germany
Marital status	married

Professional career

Since 05/2013	**Postdoctoral researcher / lecturer**, Martin-Luther-University Halle-Wittenberg, Institute of Biology-Zoology, Molecular Ecology, Germany – lab of Prof. R.F.A. Moritz, own research group with DFG funding
01/2012 – 04/2013	**Postdoctoral researcher**, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Department of Apiculture and Sericulture, Romania (ERDF-Project 'RoBeeTech')
2008 - 2011	**Research assistant**, Martin-Luther-University Halle-Wittenberg, Institute of Biology-Zoology, Molecular Ecology, Germany (BMBF-Project (FUGATO-plus) 'FUGABEE'and BMELV-Project 'FIT BEE')

Higher Education

04/2008 – 02/2012	**Ph. D. student – postgraduate studies,** Martin-Luther-University Halle-W. **PhD thesis**: Advisor - PD Dr. H. Michael G. Lattorff Title: 'Molecular Analysis of Host-Parasite Interaction in the Bumblebee *Bombus terrestris* (Linnaeus, 1758) ' Degree: Dr. rer. nat. (Grade: *magna cum laude*)
10/2003 - 03/2008	**Studies of Biology,** Martin-Luther-University Halle-Wittenberg Major: zoology, Minors: genetics, immunology, cell-biochemistry **Diploma thesis**: Advisor - PD Dr. H.-H. Kaatz Title: 'Analysis of the mitochondrial genome of *Schistocerca gregaria gregaria* (Forskåhl, 1775)' Degree: Diploma (Grade: 1.2, 'very good')
Civilian service	10/2002 – 05/2003, Botanical garden Chemnitz (as substitute for military service)

School Education

1994 - 2002	Secondary school - Gottfried-Leibniz, Chemnitz Degree: Abitur (Grade: 2.5, 'good')
1990 - 1994	Elementary school - Dr.-Salvador-Allende, Chemnitz

Halle (Saale), 26.06.2017

Erklärung

Halle (Saale), den 26.06.2017

Hiermit erkläre ich an Eides statt, dass diese Habilitationsschrift selbständig und ohne fremde Hilfe verfasst wurde. Andere als die angegebenen Quellen und Hilfsmittel wurden nicht benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen wurden als solche kenntlich gemacht.

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Dr. Silvio Erler