

Bombus vs. Crithidia:
Population Genetic Analysis of Parasite
Interactions with Multiple Hosts

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“Now, here, you see, it takes all the running you can do, to keep in the same place.”

Lewis Carroll, 1960
(Through the Looking-Glass and What Alice Found There)

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Chapter 1

General Introduction

The process of co-evolution is underlying adaptive, reciprocal genetic changes between all kinds of interacting species, meaning that changes in the gene frequencies as a result of selection acting on one population results, in turn, in selection for changes of gene frequencies in the other associated population. This phenomenon can occur in a variety of interacting populations such as prey / predator or plant / herbivore systems. In this context, host-parasite interactions are of special interest, because of their tightly linked association, which causes strong selective pressure on each other (Thompson, 1994).

Regarding, for example, the interaction of the parasite's infectivity on the one hand and, in turn, the host's resistance can be explained by an evolutionary arms race, also referred to as the Red Queen Hypothesis (Woolhouse *et al.*, 2002). This hypothesis states that organisms must constantly adapt and evolve to survive while competing with ever-evolving counterparts in a constantly changing environment (Bell, 1982).

As environment changes, selective pressures may also vary. If selection favors changes in allele frequencies, e.g. in the host, this surely will impose selection resulting in changes in allele frequencies in the parasite. Selection can act intraspecifically on both, the host and the parasite. If the host gains benefits from resistance, it will increase in the host population, but resistance will decrease when there are costs connected to it, favoring increased host susceptibility. The parasite on the other hand also has to deal with the boon and bane of its pathogenicity, resulting in either high or low variants of this trait according to whether there are benefits

or costs associated with it. Furthermore, selection also can act interspecifically as parasite pathogenicity can drive an increase in selection for higher host resistance and this therefore may then again lay the foundation for enhanced parasite infectivity and as a result higher pathogenicity. By contrast, low pathogenicity results in a reduced selection for host resistance. At last, a high degree of host susceptibility prevailing in the host population will favor reduced selection for parasite infectivity.

A co-evolutionary system like the host-parasite interaction should finally result in both a stable host as well as a stable parasite population with fluctuating changes in either host or parasite population density due to selection acting on both (Schmid-Hempel, 2011). Therefore, changes in parasite virulence would increase parasite transmission and its population while diminishing the density of the host. An evolutionary stable strategy (ESS) model presented by Frank (1992) on the evolution of virulence revealed that transmission increases with virulence, imposing a trade-off between a parasite's survival and transmission, resulting in a decrease in host recovery with increasing virulence. Another theoretical model by Frank (1996) also identified the mode of transmission governing the evolution of virulence. Therefore, predictions can be drawn to that extent that low virulence is a result of vertical transmission and high virulence is due to horizontal transmission.

Given a host individual being infected by more than one strain of a certain parasite due to accumulation of various parasite genotypes, relative to single infections,



Figure 1.1: Bumblebees foraging on sunflower. Photo reproduced by permission of Dr. Silvio Erler, Institut für Biologie, Molekulare Ökologie, Martin-Luther-Universität Halle-Wittenberg. Cover photo of *Ecology and Evolution*; Volume 2, Issue 5, first published online 10 May 2012.

infection by several competing parasite genotypes is expected to result in a suboptimal form of host exploitation due to competition among different strains within the host. Thus, multiple infections are predicted to be more virulent than single infections (Levin and Pimentel, 2001). However, depending on their relatedness, cooperation between parasite genotypes might occur, resulting in decreased virulence (Schjørring and Koella, 2003). Moreover, competition between parasite strains within the host could also lead to a decrease in virulence (Schjørring and Koella, 2003).

Transmission and virulence of parasites are crucial factors shaping both, the host- and the parasite adaptation. The life style of social insects may strongly enhance parasite adaptation. Social insects in particular are extremely prone to parasitic attacks, as the colony members are usually present in high densities, exhibit highly related genotypes and, consequently, facilitate high pathogen transmission rates.

Furthermore, maintenance of nest homeostasis provides a relatively uniform environment, additionally facilitating the invasion, spread and adaptation of pathogens (microorganisms, micro- and macro-parasites) (Schmid-Hempel, 1998).

As mentioned above, host-parasite interactions can be found everywhere in nature between all kinds of species (Clark, 1977; Day *et al.*, 1992; Read and Taylor, 2001; Schmid-Hempel and Reber-Funk, 2004). One special system is the interaction of the social insects and their numerous parasites. Hymenopterans like ants, wasps and bees are social insects, which in terms of diversity and biomass dominate almost all terrestrial habitats, are frequently exposed to an equal diversity of pathogens/parasites. All these species are eusocial, characterized by a continued care of the offspring, cooperative brood care, reproductive division of labour, consisting of colonies with at least two adult generations and egg-layers which are usually morphologically

differentiated (Wilson, 1971; Michener, 1969 & 1974; Starr, 1984). In general, only the queen is the only reproductive individual in the colony. Males in the Hymenopteran species are haploid (n), i.e. they possess just one set of chromosomes and develop from unfertilized eggs laid by the queen. In contrast, females (workers) develop out of fertilized eggs and are therefore diploid (2n) like the queen. This special sex determining system is called the Haplo-Diploidy System.

Individually, intruding parasites are attacked by the individual innate immune system (Hultmark, 1993; Siva-Jothy *et al.*, 2005) consisting of the cellular immune system with e.g. hemocytes and cellular responses to infection, phagocytosis, encapsulation and nodule formation. On the other hand, the humoral immune system consists of phenoloxidase and melanization reactions and the induction of antimicrobial peptides (antibacterial and antifungal) (Gillespie *et al.*, 1997). Both interact, mostly temporal staggered, to kill invading parasites.

Surface molecules of the parasite (e.g., antigenic peptides, carbohydrates and lipids) lead to an activation of the innate immune system by up- or down-regulation of immune related pathways like “Toll”, “Imd” (immuno deficiency), and “JAK/STAT” (janus kinase/signal transducers and activators of transcription). The “Toll” pathway is mainly triggered by infections with fungi and gram⁺ bacteria, whereas the “Imd” pathway is activated by gram⁻ bacteria. Both are mainly directing the production of antimicrobial peptides (AMPs). The less understood “JAK/STAT” pathway is thought to control cellular reactions (Schmid-Hempel, 2005) and anti-viral responses (Dostert *et al.* 2005).

It has been revealed that there is a marked reduction in the number of genes of the innate immune system from whole-genome studies on social insects like ants

(*Camponotus floridanus*, *Harpegnathos saltator*, *Pogonomyrmex barbatus* and *Linepithema humile*) (Bonasio *et al.*, 2010; Smith *et al.*, 2011a, b) and the honeybee (Honeybee Genome Sequencing Consortium, 2006) relative to non-social insects like the fruit fly *Drosophila melanogaster*, the mosquito *Anopheles gambiae* and the red flour beetle *Tribolium castaneum* (Adams *et al.*, 2000; Holt *et al.*, 2002; Tribolium Genome Sequencing Consortium, 2008).

A vast range of behavioral traits at the individual and/or colony level has been evolved in this group that might ameliorate their heightened exposure to pathogens and parasites e.g., “social immunity”, hygienic behavior, social behavior (brood care, grooming), “social fever”, foraging for antibiotic compounds like propolis or avoiding contaminated food sources (Cremer *et al.*, 2007; Simone *et al.*, 2009; Fouks and Lattorff, 2011; Schmid-Hempel, 2011). Also the increase of genetic diversity by multiple mating of the queen is an important and well described feature (e.g. Schmid-Hempel and Crozier, 1999; Tarpay, 2003; Hughes and Boomsma, 2004; Seeley and Tarpay, 2007). The existence of these traits manifests the importance of pathogens and parasites in shaping the evolution of social insect societies.

Thus, activation of the host’s immune system results in an anti-parasite response via melanization, proteasome-dependent degradation, apoptosis, expression of antimicrobial peptides and cytotoxic enzymes (Evans *et al.*, 2006), with various components of the hosts’ immune response acting as short-term clearance or as long-range protection.

Moreover, maternal or paternal immune priming is thought to increase the success of fighting against a known parasite. As invertebrates were long thought to lack an adaptive immune system compared to vertebrates, it was also thought that they

consequently lack the ability to produce lasting and specific immunity. Lasting immunity following an initial exposure that proves protection on a secondary exposure has been shown in several species of invertebrates. This phenomenon has become known as immune priming (Roth *et al.*, 2009; Sadd and Schmid-Hempel, 2007; Zanchi *et al.*, 2011).

Well, to choose and to study an appropriate model system for host-parasite interaction, both, the host and the parasite, have to fulfill several criteria to become suitable study objects. Most important, the host's as well as the parasite's life cycles have to be fully understood and studied to an extent, to make it easy for scientists to rear them in the lab or to have an opportunity to have them available elsewhere. Moreover, several previous studies have to reveal the special evolutionary ecology of the host-parasite system to obtain a basis to address further questions to, as parasites are selective factors for host life history, behavior and immune defense (Baer and Schmid-Hempel, 1999; Allander and Schmid-Hempel, 2000)

During the last decade the *Bombus-Crithidia* system has become such a well studied system of host-parasite interactions. Among other things, special emphasis was put on the genotype-genotype interactions between the parasite and its host (Imhoof and Schmid-Hempel, 1998; Brown *et al.*, 2000; Schmid-Hempel and Reber-Funk, 2004). Bumblebees are usually infected by more than one strain of the parasite and a remarkably high degree of genetic diversity among infections has been found in *Crithidia bombi* (Schmid-Hempel and Reber-Funk, 2004; Erler *et al.*, 2012; Popp *et al.*, 2012).

On the host side, our focal taxon of social insect, the bumblebees (*Bombus* spp.), are annual social insects which are headed by a single mated queen. The colony life cycle starts in spring and the colonies grow to a

size of approximately 20-400 individuals depending on the species (Alford, 1975). Towards the end of the season sexually reproductive individuals are produced, mating takes place and the resultant mated gynes hibernate (Alford, 1978). Hence, the life cycle of the bumblebee involves both a solitary and a social phase. The buff-tailed bumblebee, *B. terrestris*, has already been brought to the point, as colonies are commercially available for greenhouse pollination services and breeding protocols have been established for years (Plowright and Jay, 1966; Velthuis and van Doorn, 2006).

The monogenetic trypanosomatid parasites of the genus *Crithidia* parasitize insects by colonizing the mid- and hindgut of their hosts, where development takes place. These protozoan flagellates utilize their flagellum for interactions with the host's intestinal epithelium. One representative of this genus we put our main focus on, *Crithidia bombi*, is one of the major parasites in bumblebees, such as *Bombus terrestris*, one of the most common bumblebee species in Central Europe. Vertical transmission of *C. bombi* might be succeeded through nesting material or infected food as well as horizontal transmission is thought to happen in the infective feces through shared use of flowers (Durrer and Schmid-Hempel, 1994). Although infection is not fatal under normal conditions, infected spring queens are less successful in founding colonies, therefore producing smaller colonies with fewer males having a reduced overall fitness compared with uninfected queens (Brown *et al.*, 2003).

Studies of natural populations of bumblebees have shown that large numbers of distinct genotypes of *C. bombi* contribute to infections and that multiple infections are quite common in this host-parasite system (Schmid-Hempel and Reber-Funk, 2004; Salathé and Schmid-Hempel, 2011). These numerous strains accumulate within the host

individuals in the course of a season in terms of horizontal transmission between workers of one colony (intra-colonial) and also between workers of different colonies (inter-colonial) when workers forage on flowers already contaminated with infected faeces by other workers from other colonies (Durrer and Schmid-Hempel, 1994), although workers are able to recognise and avoid contaminated flowers to a certain extent (Fouks and Lattorff, 2011). In addition, vertical transmission also takes place from the queen to her offspring. The characteristic of one particular strain in revealing a faster growth rate than other competing strains could then be of great advantage when fighting for resources within the host. This strain could then propagate and outcompete others just by quantity. This strain would then have the greatest chances of being transmitted to other hosts.

In bumblebees, queens are usually single mated and hence all workers are full sisters (Schmid-Hempel & Schmid-Hempel, 2000) potentially facilitating parasite establishment within a colony, if the genotype of the host is important in susceptibility to a parasite. As a consequence, there is very little genetic variance within a colony. After the first batch of workers has emerged, colonies enter an ergonomic growth phase during spring and early summer. These workers then take over all tasks related to colony maintenance and the queen exclusively engages in oviposition behavior. This division of labour (egg-laying by the queen – brood care by workers) strongly enhances an ergonomic growth phase, i.e. rapid growth of the colony over a short period of time. During this time large numbers of new individuals enter the population serving as potential susceptible hosts, if they haven't been previously infected by the queen or other nest-mates. In late summer, the colony has reached its maximum number of individuals. This

population dynamic greatly enhances the spread of horizontally transmitted parasites like *C. bombi* within the colony (Schmid-Hempel, 1998).

Moreover, according to Ulrich *et al.* (2011) bumblebee queens are capable of filtering parasitic strains during the season. By doing so, the parasitic composition within the colonies varies drastically during one season. Strain filtering also plays a major role in reduction of the amount of parasite genotypes, hence lowering the amount of multiple infections and, as a consequence, diminishing infection intensity. Strain filtering is a very substantial process in the life cycle of a colony when it comes to producing young queens. This could select for only a few parasite genotypes being able to be transmitted to the next generation. At the end of the season, the whole colony, including the queen, dies. The only individuals surviving and hibernating are the young queens. After hibernation, they establish a new colony in the next season, while carrying some parasitic genotypes they "inherited" by their mother. This transition from one season to the next is a crucial time-point for the parasite in the colony life cycle of the host. This 'bottleneck' might complicate local adaptation of *C. bombi* to a host population.

On the one hand the social phase during the season facilitates the parasite transmission/spread but on the other hand the solitary phase represents a part in the life cycle of the host the parasite isn't able to cope with. During the season the parasite invested a lot in transmission strategies, fight for resources within the host etc. but in this solitary phase, it just has to reside within its host waiting for the beginning of the new season to be transmitted to the queen's offspring. During this period the parasite has to circumvent the host's innate immune system.

The induction of antimicrobial peptides (AMPs), as a part of the humoral immune system, is the first line of defense when it comes to protect the host against a vast range of bacteria and fungi. Therefore, they are one of the most important effectors of the innate immune system of invertebrates (Boman, 1995; Bulet *et al.*, 1999; Hoffmann *et al.*, 1999). The production of antimicrobial peptides is initiated via two distinct signaling pathways (“Toll” and “Imd”) (Schmid-Hempel, 2005; Ferrandon *et al.*, 2007). Activation of these two pathways is triggered by the recognition of bacteria and fungi. The amino acid sequences in regions important for AMP-translation or intra-cellular trafficking are conserved across a vast range of organisms, indicating common constraints on their production and delivery (Zasloff, 2002).

As AMPs act against a vast range of microbes, they are not specialized and represent a general mode of immune defense. This is made obvious when considering that this effect even then occurs when the insects are exposed to novel pathogens or parasites they may not have been previously exposed to in their usual natural habitat (Casteels *et al.*, 1993).

Hultmark (2003) raised the hypothesis stating that rather than acting specifically against certain pathogens and parasites, this high degree of conservation of the immune system pathways across numerous insect taxa could perhaps be maintained due to protection against saprophytes, which represent omnipresent microorganisms mainly decomposing dead organic material. As mentioned, AMPs are highly conserved across taxa and do not show elevated evolutionary rates between populations, but a number of studies on AMP evolution within social insects revealed that elevated rates of molecular evolution are indeed possible (Viljakainen *et al.*, 2009).

AMPs have been shown to be strongly up-regulated after artificial bacterial challenge of laboratory kept bumblebees (Erler *et al.*, 2011). The expression of AMPs is context-dependent and shows genotype-genotype interactions in so far, as it reveals significant differences in up-regulation, when distinct host genotypes are exposed to different *C. bombi* genotypes (Riddell *et al.*, 2009). Richter *et al.* (2012) demonstrated a strong impact of the social context on an individual’s AMP expression pattern. Individuals kept in a social context, i.e. with nestmates, showed a significantly stronger expression of AMPs compared to isolated individuals. One of these AMPs, *hymenoptaecin* (*Hym*), showed very strong effects in all of the former experiments. Its name is derived from the Hymenopterans and it has been reported in this order so far only. It acts broadly against gram⁺ and gram⁻ bacteria (Casteels *et al.*, 1993).

In conclusion, the *Bombus-Critithidia* system provides a powerful tool to study host-parasite interactions. Detailed studies on the host’s life cycle, genetics, innate immune system traits including their evolutionary pattern on the one hand and the parasite’s life history and reproduction, including new insights in the facultative sexual genetic exchange, the different types of transmission and the underlying genetics are the basis for the questions I have put my focus on:

1. A great diversity of *C. bombi* genotypes is found in natural populations. I will test here, whether these genotypic differences are associated with phenotypic differences that might help to explain differences in the distribution of *C. bombi* between individuals, colonies, populations and/or species. In order to test for such associations a new in vitro cultivation technique is developed that allows for a high-throughput phenotyping

approach under controlled laboratory conditions (Chapter 2).

2. The transmission of *C. bombi* might vary with the bumblebees' demographic changes throughout the season, potentially leading to an increase in prevalence of both, single and multiple infections, with the latter one increasing the competition between strains. I will test, whether and how changes in the transmission throughout the season affect the population structure of *C. bombi*. Changes in the population structure of the parasite might be due to differences in the phenotype of different parasite genotypes and might also be affected by competition of these parasites, when they co-exist in a host (Chapter 3).

3. *C. bombi* appears to be a multi-host parasite affecting several bumblebee species. Different genotypes of *C. bombi* might be locally adapted to certain host species (genetic adaptation). However, *C. bombi* faces drastic selective pressures

due to strain filtering (Ulrich *et al.* 2011) and transmission between seasons in hibernating queens resulting in strong impact on the distribution of genotypes of *C. bombi* between years (temporal adaptation). We test for these two types of adaptation and their effects on the distribution of genotypes of *C. bombi* by sampling several species over 2 subsequent years (Chapter 4).

4. The AMP *hymenoptaecin* shows strong effects with respect to gene expression levels upon pathogen exposure and seems to be involved in genotype by genotype interactions. Hence, we are studying the molecular evolutionary pattern of *hymenoptaecin* in two common species of bumblebees that differ in their prevalence for *C. bombi*. We study the degree of conservation of *hymenoptaecin* across and within bumblebee species, and infer the types of selection acting on *hymenoptaecin* (Chapter 5).

Chapter 2

A quantitative in vitro cultivation technique to determine cell number and growth rates in strains of *Crithidia bombi* (Trypanosomatidae), a parasite of bumblebees

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The protozoan parasite *Crithidia bombi* and its host, the bumblebee *Bombus terrestris* are used as a model system for the study of the evolutionary ecology of host-parasite interactions. In order to study these interactions we established a method for in vitro cultivation of single parasite strains. Additionally, a high-throughput method for determining cell numbers in cultures by means of optical density (OD) measurements is developed. The protocol for in vitro cultivation allowed for growing different strains on agar plates as well as in culture medium. A calibration curve for the relationship between cell number and OD has been developed. Subsequently, growth rates for different genotypes of *C. bombi* have been recorded. Significant differences in the growth rates and generation times between these genotypes were demonstrated. As this might be related to the virulence of the parasite, this relationship might be confirmed by in vivo growth rate determination. In comparison to conventional cell-counting the application of OD measurements allows for high-throughput experiments by reducing the time per sample 30 times. The in vitro cultivation method allows for controlled infection experiments in order to study host-parasite interactions.

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Supplementary material

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INTRODUCTION

The protozoan flagellates of the genus *Crithidia* parasitize exclusively arthropods, mainly insects. These monogenetic trypanosomatid parasites colonize the mid- and hindgut of their hosts, where development takes place. Interaction with the intestinal epithelium occurs using their flagellum. They display very low host-specificity and a single parasite can infect a large range of invertebrate hosts (Boulanger *et al.* 2001).

One special representative of this genus, *Crithidia bombi* (*C. bombi*) is one of the main parasites in bumblebees such as *Bombus terrestris* (*B. terrestris*). Bumblebees are annual social insects with one queen starting a colony after emergence from hibernation in spring. In early summer, the prevalence of *C. bombi* in field-populations can be as high as 80% (Shykoff & Schmid-Hempel 1991b). *C. bombi* might be transmitted horizontally as well as vertically through nesting material, infected food and through shared use of flowers from host to host in the infective feces (Durrer & Schmid-Hempel 1994). Infection is not fatal under normal conditions but a previous study by Brown *et al.* (2003) showed that infected spring queens are less successful at founding a colony, produce therefore smaller colonies with fewer males and have a reduced overall fitness when compared with uninfected queens. Generally, in infected bumblebees, ovaries are smaller, and the fat bodies are bigger than those of uninfected workers. The fat bodies play an important role in the immune-reaction of insects. In contrast to this, males show less infestation than their female nestmates because they leave the nest early soon after hatching and therefore do not participate in brood care (Shykoff & Schmid-Hempel 1991b).

Former studies from Wu (1994 as cited in Imhoof & Schmid-Hempel 1998) described the occurrence of more than one genotype of *C. bombi* in its host by enzyme electrophoresis. The parasite *C. bombi* and its host *Bombus* are

currently used as a model organism for analysis of the evolutionary ecology of host-parasite interactions. As parasites can act as selective factors for given traits like host life history, behavior and immune defense (Allander & Schmid-Hempel 2000; Baer & Schmid-Hempel 1999), research has focused on this interesting field of interaction, in particular the genotype-genotype interactions between the parasite and its host. The parasite strain as well as the origin of the host is two important factors for the success of infections with *C. bombi* (reviewed in Schmid-Hempel 2001). This was supported by a study of Schmid-Hempel *et al.* (1999) in which the genotypic composition of infections was directly assessed by microsatellite analysis. Recent work from Riddell *et al.* (2009) showed expression differences between different host genotypes of three different antimicrobial peptides (*hymenoptaecin*, *abaecin* and *defensin*) in response to four different strains of *C. bombi*.

Transmission experiments, for example, have demonstrated a strong genetic component to *Crithidia* susceptibility. These studies have shown that the spread of a *Crithidia* infection is slower in genetically heterogeneous groups (Shykoff & Schmid-Hempel 1991a,b). Similarly, the parasite loads, including *Crithidia*, for individual workers and entire colonies are significantly reduced in the field when the colony is genetically heterogeneous (Liersch & Schmid-Hempel 1998; Baer & Schmid-Hempel 1999, 2001). These results suggest strong genotype by genotype interactions.

We developed methods for isolation of *C. bombi* from naturally infected bumblebees and subsequent *in vitro* cultivation. Furthermore, we developed a fast and accurate method for determining cell numbers. We applied this method for determining growth rates of different parasite strains. Differences in growth rates are discussed in relation to differences in virulence as well as genotype-genotype interactions.

MATERIALS AND METHODS

Culture media

For cultivation we used brain-heart-infusion (BHI) broth (AppliChem, Darmstadt, Germany) according to Tetaud *et al.* (2001). 37 g BHI were dissolved in aqua bidest and autoclaved. Haemin (Roth, Karlsruhe, Germany) was added at a concentration of 3.5 µg/ml. Additionally, the medium was supplemented with an anti-contamination cocktail, consisting of penicillin G (60 µg/ml), kanamycin (100 µg/ml), flucytosin (50 µg/ml) and chloramphenicol (10 µg/ml) (Mäser *et al.* 2002). 2% of this mix was sufficient to prevent growth of bacteria. Agar plates were prepared using BHI-agar (Merck, Darmstadt, Germany) according to the manufactures instructions and additionally supplemented with haemin (3.5 µg/ml) and a mix of antibiotics (2%) (Mäser *et al.* 2002).

Isolation of *C. bombi* strains

As between July and August the prevalence of *C. bombi* can be as high as 80% in bumblebee workers (Shykoff & Schmid-Hempel 1991b), there was a good chance in detecting *Crithidia*. The abdomen of a *Bombus terrestris* worker (sampled in Heide Süd, Halle; July 2008) was chosen for isolating *C. bombi* strains and homogenized in 500 µl HPLC water. 10 µl of this homogenate were diluted to 10⁻¹ and 10⁻². Each dilution was plated on BHI-agar plates in three replicates. Plates were incubated at 27.7 °C in an incubator for 24 hours. Different colonies were isolated using an inoculating loop and transferred to seven ml BHI liquid medium and incubated at 27.7 °C for 24 hours. A sample of the inoculated medium was screened microscopically for the presence of *C. bombi* cells.

In vitro cultivation of *Crithidia bombi*

After the microscopic screening and the validation of the presence of pure *C. bombi* cells, 100 µl of this *C. bombi* solution were transferred into seven ml of liquid BHI-broth (AppliChem, Darmstadt, Germany), supplemented with 3.5 µg/µl haemin (Roth, Karlsruhe, Germany). Cultures were kept in the incubator at 27.7 °C for 24 hours. One ml of this culture was diluted (10⁻¹ and 10⁻²) and 10 µl plated on BHI-agar plates (Merck, Darmstadt, Germany) (supplemented with the same amount of haemin and a mix of antibiotics (2%)) and incubated at 27.7 °C for 24 h.

Genotyping *C. bombi* strains

10 µl of a homogenated abdomen of one bumblebee was diluted to 10⁻¹ and 10⁻² and each plated on agar plates three times. Altogether, 15 colonies were taken from these plates and each was put in seven ml BHI-medium. From each of the 15 cultures, one ml was centrifuged for 30 min at 4000* g. The supernatant was discarded and the pellets were resuspended in 500 µl deionized water. 200 µl of the suspension was transferred to a microtitre plate and centrifuged for 30 min at 4000* g. The supernatant was discarded and the DNA was extracted after standard protocols (Walsh *et al.* 1991). For genotyping we used four polymorphic microsatellite loci (Cri4, 2.F210, Cri1.B6 and 4G9 as described by Schmid-Hempel & Reber Funk 2004). All microsatellites were amplified in a single multiplex reaction in a 10 µl reaction volume containing one µl template DNA, 5 µl PCR Master Mix (Promega, Madison, USA), 0.3 µM (Cri4; 2.F10) and 0.15 µM (Cri1.B6; 4G9) of each forward and reverse primer (Metabion, Martinsried, Germany) and 2.2 µl of HPLC grade water. The following temperature conditions for PCR amplification were used: an initial denaturation step for four min at 95 °C, followed by 34 cycles of one min, 95 °C; 30 s,

53 °C; 30 s, 72 °C and a final elongation step at 72 °C for four minutes. All PCR reactions were done in a Perkin Elmer PE 9700 thermocycler. PCR products were diluted 1:1 with HPLC-grade water. Fragment lengths were detected using an automated capillary DNA sequencer (MegaBACE™ 1000 Sequencer (GE Healthcare, Munich, Germany)). For this purpose one µl diluted PCR product was mixed with 8.7 µl HPLC-grade water and 0.3 µl MegaBACE™ ET400-R Size Standard (Amersham Bioscience, Freiburg, Germany). Allele sizes were scored using the MegaBACE™ Fragment Profiler software.

OD (optical density)-measurement and cell-number

The *C. bombi* cultures grown on agar plates were again screened microscopically for the presence of pure *C. bombi* cells and then transferred to seven ml of liquid BHI-broth (AppliChem, Darmstadt, Germany) (supplemented with haemin and a mix of antibiotics as described above). We used four different strains each replicated three times. Each consisted of one ml *Crithidia*-medium in an Eppendorf tube. Dilutions of overnight cultures were prepared ranging from 10⁻¹ to 10⁻⁴ for all samples. For dilution 10⁻² *C. bombi* cells were counted using a Fuchs-Rosenthal counting chamber (Roth, Karlsruhe, Germany) according to standard protocols. Cell counts (for $V_{chamber} = 3.2 \text{ mm}^3 (= 3.2 \text{ µl})$) were extrapolated to the volume of cell suspension used for counting in order to assess total cell numbers. The optical density (OD) at 600 nm was measured using the NanoDrop 1000 (peqlab, Erlangen, Germany) for all dilutions and replicates. Each measurement was replicated three times. Means for replicate measurements were calculated. Cell numbers and OD measurements were used to set up a calibration curve. Statistical analyses were performed using Statistica 6.0 (StatSoft) and standard spreadsheet software (Excel).

Growth rate

For an overnight culture seven ml of BHI liquid medium including haemin and antibiotics were inoculated using two single colonies (strain M-A06 and M-A12) of *C. bombi* grown on BHI agar plates. The cultures were incubated for at least 24 h at 27 °C in an incubator. From each culture one ml was removed and diluted to 10⁻² and 10⁻³. 20 µl of each culture were placed in a Fuchs-Rosenthal counting chamber and cell number within those cultures was counted. Again, cell counts were extrapolated to the volume of cell suspension used for counting in order to assess total cell numbers as described above in the previous chapter. Then, these cultures were kept in the incubator at 27.7 °C for 24 h. For 26 hours the optical density was measured every hour using the NanoDrop 1000. In the end, we focused on the six hours linear growth of the parasites. Only during this particular growth phase, accurate analyses are possible (e.g. establishing a calibration curve). We missed adjusting the two strains to the same initial amount of cells, so we compared the growth rate of the dilution 10⁻³ from strain M-A12 and the dilution 10⁻² from strain M-A06. Initial cell number was similar (about 30,000 cells) for both strains. Using the calibration curve the measurements of OD₆₀₀ were transformed to cell numbers, which were used for further analysis.

Generation time was calculated according to the following formula:

$$t_{\text{gen}} = \frac{\log 2 \cdot dt}{\log N - \log N_0} \quad (1)$$

With dt = time interval during exponential growth phase, N = cell number at the end of exponential phase and N_0 = cell number at the beginning of exponential growth.

RESULTS

Isolation of different *C. bombi* clones out of bumblebees from natural populations

Six different *C. bombi*-clones could be isolated out of this one individual of *Bombus terrestris* as revealed by genotyping using 4 microsatellite markers (Table S2). Marker resolution is sensitive enough to detect low concentrations of different strains. Two of the isolated parasite strains were used for further studies (M-A06 and M-A12).

Cultivation and isolation of *C. bombi*

C. bombi clones could be cultivated in both, BHI-medium and on BHI-agar. Macroscopic inspections of cultures and plates suggested a contamination-free growth of *Crithidia*. Microscopic inspections also revealed the absence of contaminating bacteria and fungi (Fig. S1). Furthermore, microsatellites analyses (see above) gave clear results that colonies grown on BHI plates were *C. bombi*.

OD (optical density) measurement for determining cell-numbers

With our measurements of the optical density, we could establish a calibration curve for *C. bombi* (Fig. 2.1).

With the equation

$$y = 4.2835 \cdot 10^{-7} x + 0.0009 \quad (2)$$

with $x = \text{cells} \cdot \text{ml}^{-1}$ and $y = \text{abs}_{600 \text{ nm}}$ cell numbers can be calculated by resolving (2) for x

$$\text{cells} \cdot \text{ml}^{-1} = \frac{\text{abs}_{600 \text{ nm}} - 0.0009}{4.2835 \cdot 10^{-7}} \quad (3).$$

A variance component analysis showed that the main difference in the variance of all measurements is due to the difference in cell numbers (92.5%), whereas strains, and replicates, both biological and technical replicates (repeated measurements),

contribute 0.2%, 0.2% and 0.3% to the observed variance. The remaining variance is due to the sum of all interaction terms. Thus, the only significant contribution comes from the variation of cell numbers at different dilutions. Hence, the calibration curve is quite robust.

Robustness of this calibration curve can also be assessed by analyzing the confidence intervals and the error related to this. As equation (2) suggests, the lowest cell numbers to determine by means of optical density measurement is $100 \text{ cells} \cdot \text{ml}^{-1}$. A 95% confidence interval for the calibration curve was determined by jack-knifing over strains (Fig. S2). It already shows a narrow range, despite the fact that it has been determined by jack-knifing over only four strains. Hence, accuracy might be improved by increasing the number of replicates for determining a calibration curve.

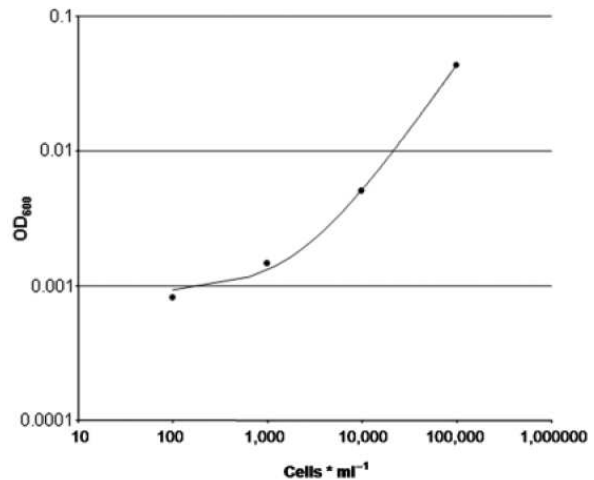


Figure 2.1 Correlation of optical density with cell number for *Crithidia bombi*. The calibration curve was calculated as the average of the four strains M-A06, M-A12, M-A91, and M-A161.

Growth rate

The growth rate of two different strains was determined using the optical density measurements of one ml solutions with an initial cell number of 30,000 cells. The two strains (M-A12 & M-A06) showed strong differences in their growth during the linear

phase. The two strains differ in their cell numbers due to different growth rates. Figure 2.2 shows that strain M-A12 is growing faster than strain M-A06 (for values see Table S1). Two hours after beginning of the linear phase strain M-A12 already has more than double the cell number of strain M-A06, even if initially cell number was slightly lower for strain M-A12. The generation time calculated according to equation (1) using the exponential phase (240 – 420 min) gives an estimate of 1.7 h for strain M-A06 and 3.0 h for strain M-A12.

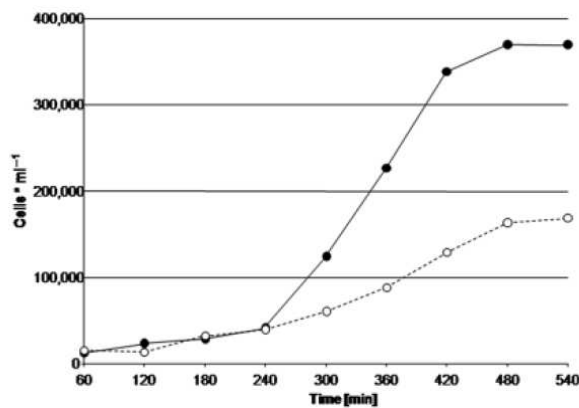


Figure 2.2 Comparison of the growth rate of the two strains of *Crithidia bombi*. Cells were counted at the beginning of the measurement and then the cell numbers were calculated with the equation for the calibration curve. Strain M-A12—filled circles, solid line; strain M-A06—open circles, dashed line.

This is somewhat lower than is known for other species of *Crithidia*, in which generation times of 4-5 h were recorded (Glassberg *et al.* 1985).

DISCUSSION

The *Bombus-Crithidia* model system for host-parasite interactions is very helpful to assess the full effects of parasites on their hosts, especially the genotype-genotype interactions between the parasite and its host (Brown *et al.* 2000; Imhoof & Schmid-Hempel 1998; Riddell *et al.* 2009; Schmid-Hempel & Reber Funk 2004). In order to fully exploit that system for large scale analysis on genotype-genotype

interactions some requirements for the experimental handling of the host and the parasite species have to be fulfilled. The host, *Bombus terrestris* is already brought to that point, as colonies are commercially available and breeding protocols are established for years (Plowright & Jay 1966; Velthuis & van Doorn 2006). Hence, the aim of this study was to facilitate the in vitro cultivation of *Crithidia bombi*. Former studies from Wu (1994) have already addressed the cultivation of this parasite. Here, the clones were grown in a different culture medium (Mattei medium, after Jenni *et al.* 1988), supplemented with a gradient of 1-10% gentamycin for suppressing fungal contamination. To obtain the requested cell count, the researcher let the parasites grow in medium and counted the resulting cell concentrations in regular intervals. However, counting only provided a rough estimate of the cell count and the growth rates of the used *Crithidia* clones.

The anti-contamination cocktail used for cultures and agar-plates consisting of four antibiotics, three of them against contaminating bacteria and one against fungi, eradicated any contamination. Wu (1994) only used gentamycin to suppress fungal growth and no antibiotics, which are important to obtain pure *C. bombi* solutions and to prevent bacterial growth. As *C. bombi* is a gut parasite of bumblebees the isolation from faeces or directly from the gut always will introduce a whole range of bacteria and yeasts into the culture as these constitute the typical gut fauna of bumblebees, e.g. *Alpha-*, *Beta-* and *Gammaproteobacteria*, *Bacteroidetes* and *Firmicutes*, which are also found in other bee species (*Apis mellifera carnica* and *Osmia bicornis*), as well as common bacterial phylotypes related to *Simonsiella*, *Serratia* and *Lactobacillus* (Mohr & Tebbe 2006). Cell-counting using microscopic techniques is quite time consuming. Moreover, it is not suitable for high-throughput analysis when lots of strains need to be determined. Using a spectrophotometric measurement of the

optical density, which is commonly used to determine cell numbers in bacteria, increased the speed of cell number determination considerably. We roughly estimated the time for cell counting for a single sample using a microscope and a counting-chamber including all cleaning steps that allow the processing of the next sample as 20 min. However, using the OD measurement we are able to determine the cell number for a single sample in triplicate measurement within 30 seconds. Additionally, data are electronically stored and hence immediately available for any sort of calculation or analysis. This allows for experiments that provide real time estimates for growth rates of a large number of strains processed parallel.

For the first time we could show that genotypically distinct strains of *C. bombi* show different growth rates. The two strains tested were cultivated under the same conditions starting with the same cell number. One strain grew double as fast as the other one, suggesting that there are differences in growth rate linked to the genotype. This genotypic difference in growth rate might be linked to the virulence of a parasite strain, as faster growing strains might outcompete other strains in case of co-occurrence. As multiple infections are quite common (Erler *et al.*, unpubl. data; Popp *et al.*, unpubl. data.) competition between different genotypes of *C. bombi* are not unusual. Hence, we provide the methodology to test the association of genotype and growth rate in a large number of strains, which will set the basis for determining the genes underlying this growth rate differences and their relationship to the virulence of the parasites.

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Chapter 3

Seasonal variability of prevalence and occurrence of multiple infections shapes the population structure of *Crithidia bombi*, an intestinal parasite of bumblebees (*Bombus* spp.)

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Ergonomic growth phases of annual social insect societies strongly influence horizontally transmitted parasites. Here, we focused on the impact of temporal changes in host demography on the population structure of a horizontally transmitted parasite. Seasonal fluctuations in prevalence and the occurrence of multiple infections of the gut parasite *Crithidia bombi* were analysed in repeatedly sampled populations of two common bumblebee (*Bombus* spp.) species. Prevalence of *C. bombi* was greatest in the middle of the foraging season and coincided with the maximal occurrence of multiple infections. Both decline later in the season. The genetic structure of the parasite population also showed strong seasonal fluctuations with a drastic decline in effective population size and an increase in linkage disequilibrium when infection rates were highest. These effects are mainly attributable to significant changes in parasite allele frequencies, leading to selection of specific alleles and increasing the frequency of homozygote genotypes in the middle of the season. Within host competition between parasite genotypes might explain the observed pattern leading to selection of these alleles and thus a boost of homozygote genotypes in the middle of the season. Towards the end of the season, selection appears to relax and we observed a recovery in linkage equilibrium as well as an increase in effective population size. This might be explained by genetic exchange in these trypanosomes in natural populations.

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INTRODUCTION

Colonies of social insects are infected by a wide range of parasites and pathogens. These colonies can be infected by more than one strain of a certain pathogen because the colony conditions facilitate accumulation of pathogens and parasites as the colony members are large in number and form long-lived, warm and resource-rich nests that provide ideal conditions for the spread of microorganisms (Schmid-Hempel, 1998). Indeed, multiple infections have been shown to be quite common in many host-parasite systems (Clark, 1977; Day *et al.*, 1992; Read and Taylor, 2001; Schmid-Hempel and Reber-Funk, 2004). Relative to single infections, infection by several competing parasite genotypes is expected to result in a suboptimal form of host exploitation due to competition among different strains within the host. Thus, multiple infections are predicted to be more virulent than single infections (Levin and Pimentel, 2001). However, depending on their relatedness, cooperation between parasite genotypes might occur, resulting in decreased virulence (Schjorring and Koella, 2003). Frank (1992) presented an evolutionary stable strategy (ESS) model for the evolution of virulence in which transmission increases with virulence, imposing a trade-off between a parasite's survival and transmission, resulting in a decrease in host recovery with increasing virulence.

As transmission rates are strongly dependent on the availability of susceptible hosts, the host population structure has a strong impact on the transmission rate (Schmid-Hempel *et al.*, 1999). Annual social insect colonies exhibit rapid growth phases after colony foundation by post-hibernation queens in spring. Once workers from the first brood emerge they take over all tasks related to colony maintenance and the queen exclusively engages in oviposition behavior.

This division of labour strongly enhances an ergonomic growth phase, i.e. rapid growth of the colony over a short period of time. During this time large numbers of new individuals enter the population serving as potential susceptible hosts, if they haven't been previously infected by the queen or other nest-mates. This population dynamic greatly enhances the transmission of horizontally transmitted parasites within the colony (Schmid-Hempel, 1998). Therefore, throughout the year it is expected that the transmission rate decreases as more and more individuals become infected and, thus, the number of available susceptible hosts also decreases. But still, the transmission rate becomes higher than the egg-laying rate of the queen during the season. Moreover, the increased rate of intra-colonial horizontal parasitic transmission also increases the probability of multiple infections per host (Schmid-Hempel, 1998).

Bumblebees are primitively social insects. As one characteristic, they exhibit an annual life cycle. Queens are usually single mated and hence all workers are full sisters (Schmid-Hempel & Schmid-Hempel, 2000). Once the first workers emerge, colonies enter an ergonomic growth phase during spring and early summer. Depending on the species, colonies vary in the number of workers per colony, e.g. *B. terrestris* and *B. lapidarius* colonies can contain up to 400 workers whereas those of *B. pascuorum*, *B. sylvarum*, *B. ruderarius* and *B. muscorum* are relatively small (20-100 workers) (Alford, 1975). Towards the end of the season, sexuals are produced, mating takes place and the resultant mated gynes hibernate (Alford, 1978).

A broad spectrum of parasites is known to infect bumblebees. One of the most common parasites is *Crithidia bombi*, a trypanosome gut parasite that has several subtle effects on its host (Lipa and Triggiani, 1980). Shykoff and Schmid-Hempel (1992) described

slower growth of infected colonies during the critical period early in the colony cycle and smaller ovary size of infected individuals. Nevertheless, it is highly virulent when residing in hibernated queens, as their success in founding colonies is dramatically reduced, thus lowering fitness by 40% and more (Brown *et al.*, 2003).

C. bombi infections have been studied in detail in both natural populations and *ex situ* experimental studies. Several tools have been developed for the study of *C. bombi* enhancing its detection and experimental manipulation. Microsatellite markers have been developed (Schmid-Hempel and Reber-Funk, 2004) allowing for detailed analyses of genotypes infecting bumblebees. Studies of natural populations have shown that large numbers of distinct genotypes contribute to infections and that multiple infections are quite common (Schmid-Hempel and Reber-Funk, 2004; Salathé and Schmid-Hempel, 2011). Furthermore, within a colony, individuals are parasitized by more than one strain of this gut parasite (Schmid-Hempel and Reber-Funk, 2004). Recently, it has been discovered experimentally that *C. bombi* strains show genetic exchange between genotypes in cases where multiple infections occur (Schmid-Hempel *et al.*, 2011). Moreover, Salathé and Schmid-Hempel (2011) found a striking diversity of infection in several bumblebee hosts in two ecologically different habitats over a time period of three years. In addition, their data strongly suggest that a mixture of both sexual and clonal reproduction occurs in natural populations of this trypanosome gut parasite. Also Erler *et al.* (2012) suggest, based on their data, that sexual reproduction is an alternative strategy in this parasite. They also detected high levels of multiple infections which might enhance this phenomenon.

Observed prevalences in field populations are typically around 10-30% in *Bombus*

workers within colonies, but this can reach up to 80% in early summer (Shykoff and Schmid-Hempel, 1991). Aside from seasonal differences, the genetic composition of the host population might also influence the prevalence of this parasite. In inbred populations, or populations with low levels of heterozygosity, the prevalence and abundance of *C. bombi* has been found to be higher than in more diverse populations (Whitehorn *et al.*, 2011).

C. bombi can be transmitted horizontally between colonies (inter-colonial) when workers forage on flowers already contaminated with infected faeces by other workers from other colonies (Durrer and Schmid-Hempel, 1994), although workers are able to recognise and avoid contaminated flowers to a certain extent (Fouks and Lattorff, 2011). This inter-colonial horizontal transmission might enhance the occurrence of multiple infections. Additionally, identical clones of *C. bombi* were found in different host species, also supporting horizontal transmission of this parasite and underpinning the lack of host-specific adaptation (Erler *et al.*, 2012). Furthermore Ruiz-González *et al.* (2012) stated that the evolution of *C. bombi* to a generalist parasite is due to the following: Short-scale specialization (host quality and transmission) is overridden by repeated bottlenecks, combined with the reproductive strategy of this parasite.

In addition, within a single colony, *C. bombi* is passed on among workers and is also transmitted horizontally to the daughter queens (intra-colonial), which represent the reproductive offspring. Interestingly, Ulrich *et al.* (2011) showed that most colonies are capable of filtering a circulating infection before it reaches the young queens and this represents a temporal shift in parasitic composition in the workers. In colonies, where workers harbour up to five parasitic strains after artificial infection, the daughter

queens were only singly or doubly infected. This study showed a dramatic change in the representation of the five strains used for infection from the day of infection to the production of new daughter queens in the colonies.

We aim to study the impact of temporal changes in host demography on the population structure of a horizontally transmitted parasite. Specifically, along a temporal gradient and in two of the most abundant host bumblebee species (*Bombus terrestris* and *Bombus lapidarius*), we investigate: i) the prevalence and intensity of *C. bombi* infection and the corresponding changes throughout the season as the demography of the host population changes along this temporal gradient, and ii) the population genetics of *C. bombi*, determining the frequency distribution of genotypes, the frequency of single versus multiple infections and the rate of intra- and interspecific transmission.

EXPERIMENTAL PROCEDURES

Sampling of bumblebees

Bumblebees were caught during foraging flights in the beginning of June, the middle of July and in early August 2009 in an urban, flower-rich park, in Halle (Saale), Germany (51° 29' 27.44" N; 11° 56' 11.44" E). The sampling area was 130 x 130 m. Depending on the sampling date, exclusively workers or workers and drones of *Bombus terrestris* (June: 27 workers; July: 36 workers, 16 drones; August: 12 workers) and *Bombus lapidarius* (June: 42 workers; July: 24 workers, 24 drones; August: 66 workers, 6 drones) were collected. Individuals were immediately anesthetized with acetic acid ethyl ester. Bumblebees were determined using the species identification key of Mauss (1994). The intestinal tracts were dissected

from the abdomen and the remaining material stored at -20°C for later genotyping.

DNA extraction and genotyping

The dissected intestinal tracts were homogenized in 500 µl deionised water. 200 µl of the homogenized tissue was transferred to a 96 well microtiter plate and centrifuged for 30 min at 3220 × g. The supernatant was discarded and the DNA was extracted after standard protocols (Walsh *et al.*, 1991; Popp and Lattorff, 2011). For genotyping the bumblebees, a single leg was cut and the same procedure for DNA extraction was conducted as described above. Genotyping and assignment to colonies is necessary to ensure that samples were continuously taken from the same population.

C. bombi strains were genotyped using four polymorphic microsatellite loci (Schmid-Hempel and Reber Funk, 2004). Amplification of microsatellites and genotyping was conducted according to Popp and Lattorff (2011). Currently, there is no information and, therefore, it is not known whether these loci are linked to genes which underlie selection (Schmid-Hempel and Reber Funk, 2004).

Bumblebees were genotyped using five highly variable microsatellite markers, originally developed for *Bombus terrestris* (Estoup *et al.*, 1995; 1996). Multiplex-amplification of PCR products and genotyping was conducted according to Erler and Lattorff (2010).

Sibship reconstruction of *Bombus* hosts

In order to assign individual worker and drone bumblebees to colonies we used the sibship reconstruction algorithm implemented in the software Colony v1.3 (Wang, 2004). It is a maximum likelihood algorithm that takes the population-wide (i.e. sample-wide) allele frequencies into account.

All individuals of one of the species were fed into one run of Colony. Twenty runs were performed using different random seed numbers. The assignment of individuals to colonies was recorded and from all twenty runs we analysed the recovery rate of colony assignments. Incorrect assignments were assumed to have a low recovery rate; those assignment groups having a recovery of more than 50 % have been chosen for further analysis.

Prevalence of *C. bombi*

The prevalence of *C. bombi* infections at each sampling date was determined as the relative frequency of infected individuals for each of the two bumblebee species. As sample size was restricted, confidence intervals based on a binomial distribution were calculated using a Java based tool available at <http://statpages.org/confint.html>.

The differentiation into infections caused by a single genotype or infections caused by multiple genotypes was done on the outcome of genotyping of *C. bombi*. Multiple infections can be definitely assigned when at least one locus has three or more alleles. *C. bombi* is usually diploid, although the ploidy number of the genus *Crithidia* is not generally known. The microsatellite study by Schmid-Hempel and Reber-Funk (2004) strongly indicates that *C. bombi* is diploid, too; therefore a single genotype shows a maximum of two alleles when heterozygous. Overall prevalence and the prevalence of multiple infections were analysed using a general linear model (GLM) with a binomial distribution and the logit link function. Species, sampling month, and their interaction were used as fixed explanatory factors.

Intensity of *Crithidia* infections

In order to quantify the intensity of infection, we used the peak height information of the microsatellites from the *C. bombi* genotyping from all single and multiple infected bumblebees. We assume higher numbers of *C. bombi* cells correspond with increased peak heights, i.e. a positive correlation between parasite cells and peak heights. This method is reliable as there is a positive correlation between increasing DNA concentration and the corresponding relative peak heights (Moritz *et al.* 2003).

Peak heights of all *Crithidia*-infections were obtained from genotyping profiles analysed with MegaBACE™ Fragment Profiler software (Amersham Biosciences, Freiburg, Germany). All peaks representing single alleles, excluding any stutter bands, were summated per locus and then per locus values summated across all loci. In order to fulfil criteria of normality, data were log-transformed. An ANOVA with species, type of infection (single or multiple) and sampling date as fixed factors was used to infer the main factors contributing to the variability in the intensity of infection. Statistical analyses were performed using STATISTICA 6.0 (StatSoft, Tulsa, OK, US).

Decomposition of multiple infections into contributing single genotypes

As multiple infections are composed of a combination of different genotypes these are not accessible for population genetic analysis. In order to include them into further analyses on the population structure of *C. bombi* the individual genotypes contributing to a certain multiple infection need to be reconstructed. An algorithm has been developed by Schmid-Hempel and Reber Funk (2004), but the approach is not described in sufficient detail to implement in this study.

Here, we develop a new algorithm based on the frequency of alleles within a multiple infection and the relative intensity of the genotypes contributing to a multiple infection. The allele frequency indicated by its relative peak height is composed by the within-genotype frequency and by the between-genotype frequency, the latter being strongly influenced by the ratio of the genotypes to each other. For every multiple infection allele sizes and their corresponding peak heights were extracted from the capillary sequencer electropherograms. Peak height was assumed to reflect the initial copy number of an allele (Fouks & Lattorff, unpublished data) and was used to calculate the allele frequency within a multiple infection for every locus. Thus, the allele frequency corresponds to the allele specific peak height divided by the sum of all peak heights of that locus. Reconstruction of single genotypes was initiated by randomly choosing one locus for which the lowest frequency (f_1) of an allele was assumed to represent the single occurrence of that allele, present in one of the genotypes only. Depending on the number of distinct alleles occurring at that locus, either a second allele was assigned with a similar frequency ($f_1 = f_2$) to the same genotype or the other alleles showed a frequency pattern suggesting it belongs to the alternative genotype ($1-f_1$). The remaining alleles were assigned to the alternative genotype ($f_3; f_4$). The ratio of alleles at one locus between the genotypes indicates the frequency distribution of the genotypes (genotype 1 = f_1 ; genotype 2 = f_3). This decomposition was continued for the remaining loci assuming a similar genotype frequency distribution. The procedure was verified using a different locus as starting point. A representative scheme is given in Fig. S2.

Intra- and interspecific transmission of parasitic genotypes

After decomposition of multiple infections into contributing single parasite genotypes, we recorded the occurrence of identical parasite strains within and between the two host species across sampling dates. Hence, the numbers of intra- and interspecific transmission events was detected throughout the season.

RESULTS

Sibship reconstruction of *Bombus* hosts

In total, we sampled 93 individuals of *B. terrestris* (both sexes only present in July) and 162 of *B. lapidarius*, where both sexes were present in July and August. These were assigned to 47 and 61 colonies, respectively. Sibship reconstruction, using workers and males, was run 20 times in order to test the rigor of colony assignment. The median recovery for every colony was high with 17 (25% quartile: 13, 75% quartile: 20) for *B. lapidarius* and 20 (18, 20) for *B. terrestris*. As the finite sample size influences the observed number of colonies, we used the truncated Poisson method as described in Goulson *et al.* (2010) to estimate the number of non-sampled colonies.

These were 8.12 for *B. terrestris* and 4.95 for *B. lapidarius*. An alternative approach to estimate the number of non-sampled colonies, developed by Cornuet and Aries (1980), was used resulting in 12.1 for *B. terrestris* and 5.8 for *B. lapidarius*. The congruous assignment of individuals to colonies indicated consistent sampling over the season with many colonies being re-sampled across months and only 10-25 % of the colonies were not present in the sample. For *B. lapidarius* 5, 4 and 14 unique colonies were found for the months June, July and August, respectively, and four shared

colonies recovered in June and July, nine in July and August and 15 in June and August. 10 colonies contributed individuals in every month sampled. For *B. terrestris* we found 10, 16 and 6 colonies exclusively for the months June, July and August. Twelve colonies were recovered in June and July and two in July and August, whereas there were no co-occurring colonies in June and August. One colony was sampled during all 3 months.

The microsatellites used for genotyping showed a high degree of polymorphism and low non-detection errors (Boomsma and Ratnieks, 1996): 5.79×10^{-5} for *B. lapidarius* and 3.48×10^{-5} for *B. terrestris*.

Prevalence of *C. bombi* infections

For *B. terrestris* a prevalence of 14.8 % was observed in June, increasing to 77.8 % in July and decreasing in August to 58.3 % (Fig. 3.1A). A similar situation was found for the *B. lapidarius* population with prevalences of 19.0 % in June, 64.6 % in July and 62.5 % in August (Fig. 3.1C). We used a generalized linear model (GLM) to test whether the general prevalence of the parasite is affected by the host species, sampling date (month) or by an interaction of both. The GLM showed no significant difference between the two host species for the general parasitic prevalence. In contrast, the infection with *C.*

bombi significantly differed between the three sampling events, whereas there was no significant interaction of host species and the three sampling points (Table 3.1).

When distinguishing the type of infection into single and multiple infections, we observed that none of the individuals of *B. terrestris* were infected by multiple strains of *C. bombi* in June, whereas in *B. lapidarius* the rate of multiple infections was 12.5 %. The highest prevalence of multiple infections in both species was observed in July, (90.5 % in *B. terrestris* and 54.8 % in *B. lapidarius*). In August, the proportion of multiply infected individuals decreased in both species (42.9 % in *B. terrestris* and 17.9 % in *B. lapidarius*) (Fig. 3.1B and 3.1D). A GLM was used to test, whether there are differences between species, sampling points and the interaction of these two parameters on the prevalence of multiple infections. Single and multiple infections differed significantly between months and there was also a significant interaction between host species and month (Table 3.1). Prevalence did not differ between sexes in months when both males and females are available (Mann-Whitney-U-Test; *B. lapidarius* $Z = -1.45$, $p = 0.15$, *B. terrestris* $Z = -1.07$, $p = 0.28$). For *B. terrestris*, both sexes were available in the samples from July, and for *B. lapidarius* both sexes were sampled in July and August.

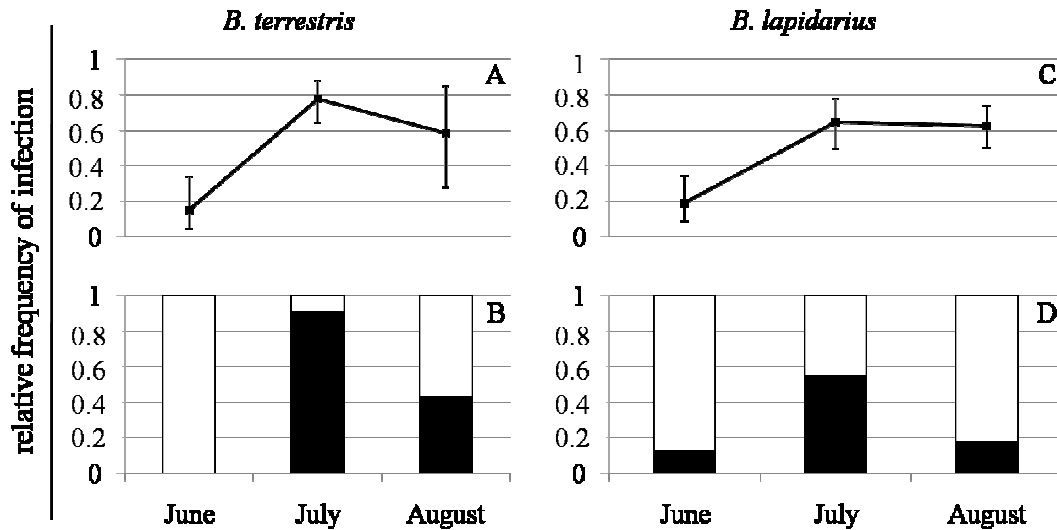


Figure 3.1 Prevalence of *C. bombi* infection in *B. terrestris* - (A) and *B. lapidarius* (C) individuals between June and August 2009; infections divided into single-infected (white) and multiple-infected (black) for *B. terrestris* (B) and *B. lapidarius* (D). Samples: *Bombus terrestris* (June: 27 workers; July: 36 workers, 16 drones; August: 12 workers) and *Bombus lapidarius* (June: 42 workers; July: 24 workers, 24 drones; August: 66 workers, 6 drones). Error bars denote 95% confidence intervals.

Table 3.1 Results of GLM (binomial distribution, logit link function) with backward stepwise removal of non-significant factors for the prevalence of *Crithidia bombi* and the distribution of single vs. multiple infections including host species and month as fixed factors. Only the final simplified best model is shown. Significant values are shown in bold.

Factor	df	Wald-Statistic	p-value
Prevalence of <i>C. bombi</i> month	2	42.906	<10 ⁻⁶
Single vs. multiple infection month	2	29.398	<10 ⁻⁶
host species x month	2	12.831	0.002

Intensity of *Crithidia*-infections

The intensity of parasitic infection did not differ significantly between species (ANOVA; $df = 1$; $F = 2.011$; $p = 0.159$), or the number of genotypes contributing to an infection

(ANOVA; $df = 1$; $F = 0.629$; $p = 0.429$). The intensity of infection did not differ between sexes in either species (ANOVA; *B. terrestris*: $df = 1$; $F = 0.209$; $p = 0.65$; *B. lapidarius*: $df = 1$; $F = 0.041$; $p = 0.839$), although there was an interaction between sex and infection type (ANOVA; $df = 1$; $F = 5.073$; $p = 0.027$) in *B. lapidarius*. Here, singly infected males have a higher intensity of infection than the multiply infected ones, whereas workers show a similar pattern when single and multiple infections are compared. Based on these results samples were pooled over species and a significant decrease in the intensity of infection was observed as the season progressed (ANOVA; $df = 2$; $F = 25.547$; $p < 0.01$) (Fig. S1).

Population genetics of *Crithidia bombi*

In total, we identified 193 parasitic genotypes across the whole season in the both bumblebee host species. 65 genotypes were recorded as single infections and 128 genotypes were found in multiple infections.

Amongst these, 171 genotypes were unique and only 12 were recorded more than once. As the number of observed genotypes was influenced by the finite sample size, we used two different approaches to correct the number of genotypes and estimate the number of non-sampled genotypes. Based on a binomial distribution and assuming an equal distribution for all genotypes, the method by Cornuet and Aries (1980) gave an upper estimate of a total of 777 genotypes. When assuming a Poisson distribution for the genotypes, the zero category of a fitted truncated Poisson distribution indicates the number of non-sampled genotypes. This serves as a lower estimate and results in a total number of 253 genotypes. As the observed distribution does not fit to a Poisson distribution, the expected number of genotypes present in this population is probably closer to the upper estimate.

Using an AMOVA (Arlequin v. 3.5.1.2), we tested for the factors influencing the variance in the parasitic genotypes. There was a significant difference of genotypic variance between the host species ($p = 0.04$) and also between the three sampling points ($p < 0.001$), but there were no significant differences between the host individuals within species and the sampling points ($p = 0.88$, Table 3.2). In addition, the variance of parasitic genotypes between all host individuals did not influence ($p = 0.31$) the overall variance in parasite distribution.

Genetic diversity within *C. bombi*

We used both heterozygosity (i.e. the presence of different alleles at one locus averaged over loci) and allelic richness (i.e. the number of alleles per locus corrected for sample size) as measures of the parasite populations' genetic diversity. Factors influencing the heterozygosity of *C. bombi* have been analysed using an ANOVA with species, locus and sampling month as fixed factors. Non-significant interaction effects have been removed by backward stepwise selection and simplified models tested against more complex models using an ANOVA. The final model included host species as a single factor ($p = 0.02$), with *C. bombi* extracted from *B. lapidarius* having higher levels of heterozygosity (H_E : 0.72 vs. 0.67) and a sampling time (month) by locus interaction ($p = 0.02$) (Fig. 3.2A). The strongest predictor was locus ($p < 0.001$), with two loci (Cri1.B6 and Cri4.G9) showing high levels of heterozygosity (0.79 and 0.76, respectively), whereas the remaining two (Cri2.F10 and Cri4) showed reduced levels of heterozygosity (H_E : 0.58 and 0.65). A Tukey HSD post hoc test revealed significant differences between each of the high heterozygosity and each of the low heterozygosity loci (Cri1.B6 – Cri2.F10 $p < 0.001$; Cri1.B6 – Cri4 $p = 0.002$; Cri4.G9 – Cri2.F10 $p < 0.001$; Cri4.G9 – Cri4 $p = 0.018$). No influence of the sampling time (month) was detected ($p = 0.86$), as the average heterozygosity did not change (June: 0.70, July: 0.69, August: 0.70). However, the locus Cri4 deviated from this pattern showing a drastic reduction in heterozygosity in July (Fig. 3.2A).

Table 3.2 Results of an AMOVA (weighted average over loci) of *Crithidia bombi* genotypes according to the species they were extracted from (host species, $df = 2$) and sampling date (months, $df = 3$). P-values were obtained from 1023 permutations. Significant values are shown in bold.

	Sum of squares	Variance components	Percentage variation	p-value
Among host species	13.250	0.035	2.354	0.04
Among months within host species	10.160	0.043	2.851	<10⁻⁵
Among individuals within host species & months	236.602	-0.035	-2.333	0.88
Within individuals	265.000	1.452	97.129	0.31
Total	525.012	1.494		

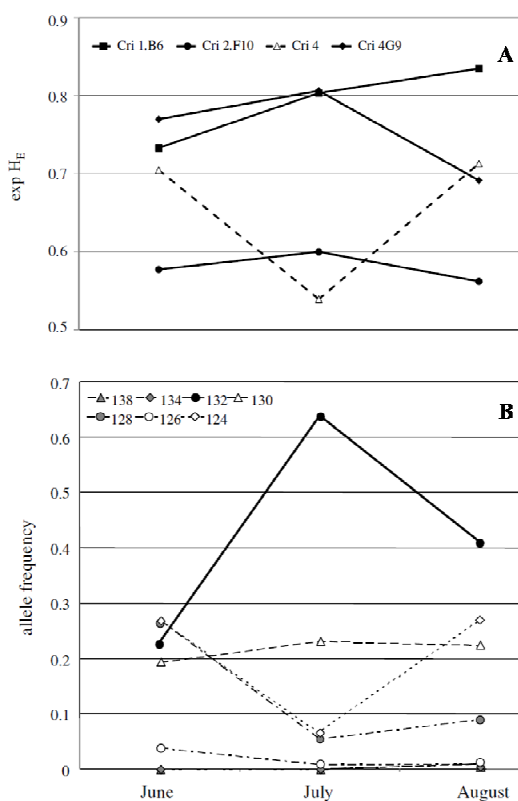


Figure 3.2 Measurements of the genetic diversity of *C. bombi* throughout the season. (A) Expected heterozygosity for all four microsatellite loci of *C. bombi* and (B) allele frequency distribution of alleles for the microsatellite locus Cri4.

This effect of reduction was more pronounced in *B. terrestris* (H_E : 0.45) than in *B. lapidarius* (H_E : 0.63), which showed in the other months levels of 0.67 and 0.74, respectively. This drastic decrease in heterozygosity was due to an increase of frequency of a single allele (132 bp) (Fig. 3.2B), that is virtually absent in June with 26 chromosomal sets being sampled allowing for the detection of alleles with a minimum frequency of 0.04. In July this allele shows a frequency of 0.64 resulting in 50% of all genotypes being homozygous for 132/132 (Fig. 3.2B).

This marked increase in frequency of a single allele resulted in strong linkage disequilibrium between all pair-wise comparisons involving Cri4 in July ($p = 0.003$). In contrast, no significant linkage disequilibrium was detected in June or August (Table S1). Selection for the allele 132 in July also led to a reduction of the effective population size in July. Using a point estimate method based on the linkage disequilibrium implemented in LDN_e (Araki *et al.*, 2007) values for N_e were 63.7, 22.0 and

59.6 for June, July and August, respectively (Table S2).

Intra- and interspecific transmission of parasitic genotypes (overlap of genotypes)

Our sampling design facilitated the observation of intraspecific (i.e. from one colony to another within one species) and interspecific (from one colony to another between the two host species) *C. bombi* transmission events. One intraspecific and also one interspecific transmission event was observed between June and July, five intraspecific transmissions in July and three interspecific ones between July and August. One intraspecific transmission of a parasitic genotype occurred in August and finally between June and August there was one genotype of *C. bombi* transmitted intraspecifically. The balanced number of intra- and interspecific transmission events represents additional evidence for *C. bombi* being a multi-host parasite (Salathé & Schmid-Hempel 2011; Erler *et al.* 2012)

DISCUSSION

Our data show that population structure of *Crithidia bombi*, a multi-host parasite of bumblebees, changed dramatically throughout the year. We observed two distinct phases which might be explained as follows: 1) a phase of competition and selection early in the year, during the ergonomic growth phase of the host; and 2) later on in the year, a phase of 'relaxation' possibly characterized by the occurrence of genetic exchange (Fig. 3.3) (Schmid-Hempel *et al.*, 2011; Salathé and Schmid-Hempel, 2011).

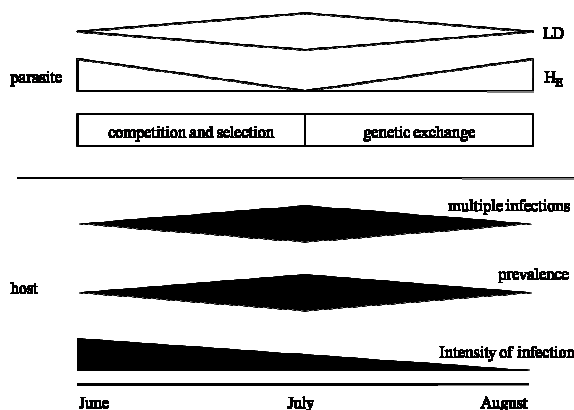


Figure 3.3 Scheme of the temporal changes in the *Bombus-Crithidia* host-parasite system over a season (June – August).

This pattern of infection prevalence being highest during the middle of the season and subsequent decline at the end of the season has been observed in other *Crithidia-Bombus* systems, but this is the first study to examine the population structure of both host and parasite (Gillespie, 2010; Shykoff and Schmid-Hempel, 1991). During the first phase the prevalence and rate of multiple infection of the parasite increases (Fig. 3.3), and selection at a gene closely linked to a molecular marker (Cri 4) used for the assessment of genetic diversity was detected. This suggests that Cri 4 might be involved in competition between clonal lineages of the parasite during multiple infections. This selective pressure leads to a dramatic decrease of the genetic diversity and the effective population size of the parasite population. During the second phase a large number of new genotypes occur and linkage disequilibrium, which accumulated during the first phase due to the selection for the gene linked to Cri 4, breaks down again and the initial genetic diversity of the population is restored (Fig. 3.3). Intensity of parasite infection continuously declines throughout the year (Fig. 3.3). This may be due to the enhanced competition between *C. bombi* genotypes during multiple infections, as indicated by similar infection intensities of

multiple and single infections or strain-filtering of the host itself (Ulrich *et al.*, 2011).

In this study, the prevalence and intensity of *C. bombi*-infections in local *in situ* populations of *Bombus terrestris* and *Bombus lapidarius* were studied within a single year. Similarly, Shykoff and Schmid-Hempel (1991) also investigated local natural populations of bumblebees in Switzerland and their parasite prevalence, including *C. bombi*. The authors data on the prevalence of *C. bombi* was limited to between late July and August, whereas our data encompasses the beginning of summer (June). Although Shykoff and Schmid-Hempel (1991) did not distinguish between sample dates, the observed field prevalence supports our findings concerning prevalence. Furthermore, due to the genetic analysis of the host populations and the high overlap of colonies between the sampling points in our study, differences in prevalence and parasite intensity of infection in our data are not biased as samples were taken from different colonies at the three sampling dates.

The prevalence of *C. bombi* in *B. lapidarius* and *B. terrestris* in this study was 64.6% and 77.8%, respectively. This is comparable to the 80.3% of infected *B. terrestris* reported by Shykoff and Schmid-Hempel (1991) in Switzerland and within the 0 – 82% range observed by Gillespie (2010) in N. American bumblebees. In addition to the general parasitic prevalence, we also focused on the incidence of infection with multiple strains of *C. bombi*. We detected no multiple infections in *B. terrestris* workers from June. This suggests that, up to this point in time, this host species may not have been exposed to a high frequency of *Crithidia*-contaminated flowers, as colony sizes are relatively small at this early part of the season and the spatial density of infected individuals is low. As a consequence, they do not encounter as many parasitic strains and later transfer it horizontally within their colonies. Correlated

to the enormous ergonomic growth phase of the host colonies and the increase in potential contacts between individuals, the relative frequency of multiple-infected bumblebee workers increases, reaching its highest level in July. This increase in multiple-infection appears to be caused by a growing number of infected colonies within populations, combined with a greater number of infected workers within each of these colonies that transmit the disease during visits to flowers (Durrer and Schmid-Hempel, 1994). Subsequently, the rate of multiple infections then decreases later in August. This may be due to: (1.) the survival of particular strains due to competition between the strains within the host. Therefore, some strains detected in early summer may become increasingly less frequent during the year; (2.) The immune system of the host itself: some strains might be attacked more than others by the immune system resulting in different and temporal variation of expression of antimicrobial peptides (Riddell *et al.*, 2009; 2011); (3.) Recombination of parasite strains: although *C. bombi* appears to reproduce primary clonally, Schmid-Hempel *et al.* (2011) showed that genetic exchange takes place in these trypanosomatids, which are closely related to *Leishmania spec.*, where recombination also occurs (Akopyants *et al.*, 2009). Due to these recombination events, certain new allele combinations have higher competitive abilities compared to their parental allele combinations, resulting in decreased multiple infections; (4.) the capability of colonies filtering parasitic strains during the season, so that the parasitic composition within the colonies varies drastically during one season (Ulrich *et al.*, 2011). Strain filtering results in reduction of the number of parasite genotypes, hence lowering the amount of multiple infections; and (5.) sampling effects could also be potential causes for lower

multiple infection rates later in the season, as we just randomly sampled a small, potentially non-representative, number of individuals. However, sibship reconstruction of the host populations indicates that 80-90 % of all present colonies were sampled.

Furthermore, the occurrence of intra- and interspecific transmission events may also explain the reappearance of certain parasitic genotypes within and between the two host species during the year. This was also supported statistically in our data (AMOVA), indicating that there is no difference in parasite genotypic variance between the individuals of the two host species. Additionally, by simple count of reappearances of parasitic genotypes, we found that these shared parasitic genotypes were not present at all three sample dates. Due to one intraspecific - and one interspecific transmission event, this was the case for two parasite genotypes present in June and July, but not in August. This could be due to recombination or simply non-detection. In July, three genotypes were the same as in August but were not detected in June. The reason may be due to sample size limitation and the probability of detecting rare strains when host population size is low. These three parasitic genotypes appeared in both time points, possibly due to interspecific transmission. Thus, both types of transmission occur in nearly equal frequency supporting the low genetic differentiation of the parasite populations between host species.

We did not observe any difference in the intensity of infection with *C. bombi* between species or types of infection, but the intensity of infection did decline over the course of the season despite the increasing density of host bumblebee populations as colony size expands. As competition between the different parasitic strains may occur, the decrease in the intensity of infection could increase selection for certain parasitic

genotypes (Schmid-Hempel *et al.*, 1999). Alternatively, Manson *et al.* (2009) found that the consumption of the nectar alkaloid gelsemine significantly reduces the intensity of *C. bombi* seven days after infection. Immune priming of the bumblebees might also explain the decrease of intensity later in the season, as infection with *Crithidia* can lead to the expression of immune-related genes in the bumblebee protecting against subsequent infections (Schlüns *et al.*, 2010).

The main factor influencing *C. bombi* genotypic variance is time. As the season progresses, parasite genotypes within the hosts dramatically change from June to August, whereas only slight levels of differentiation were observed between host species. This is also supported by the level of intra- vs. interspecific transmission. The dramatic loss of parasite heterozygosity in July and the related decline of allelic richness are due to an increase of one allele (132 bp) at the locus Cri4. The molecular marker itself is unlikely to be the target of selection, but it might be tightly linked to a gene that is under selection. As multiple infections of *C. bombi* are highest at that time, this marker may indicate a region that involved in within-host competition between strains. Negative frequency dependent selection might be the reason for later depletion of this allele. Yet another explanation for disappearance of this allele is genetic exchange demonstrated by Schmid-Hempel *et al.* (2011). As their study is restricted to laboratory conditions, we in turn could show that genetic exchange is one of the indications for sexual reproduction being an alternative reproductive strategy besides clonal reproduction in natural populations which also has been shown by Salathé and Schmid-Hempel (2011). In summary, our results demonstrate the genetic processes that underlie this complex dynamic system of multi-host parasite interaction, including host availability and susceptibility, virulence,

transmission and the resulting changes in the population genetic architecture of both the parasite and its host.

The trade-off between transmission and intensity of infection is dynamic and may be highly dependent upon the growth phase of host populations. In annual social insect societies, colonies are founded by a single individual, the queen, and then undergo a strong ergonomic growth rate, resulting in a drastic increase in number of workers over a short time period and, therefore, enhancing transmission leading to an increase in the proportion of infected hosts. Our data suggests that high transmission rates result in decreased intensity of infection in *C. bombi*, especially when competition occurs between strains.

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Chapter 4

Sex, horizontal transmission, and multiple hosts prevent local adaptation of *Crithidia bombi*, a parasite of bumblebees (*Bombus* spp.)

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Local adaptation within host-parasite systems can evolve by several nonexclusive drivers (e.g. host species - genetic adaptation; ecological conditions - ecological adaptation and time - temporal adaptation). Social insects, especially bumblebees, with an annual colony life-history not only provide an ideal system to test parasite transmission within and between different host colonies, but also parasite adaptation to specific host species and environments. Here we study local adaptation in a multiple-host parasite characterized by high levels of horizontal transmission. *Crithidia bombi* occurs as a gut parasite in several bumblebee species. Parasites were sampled from five different host species in two subsequent years. Population genetic tools were used to test for the several types of adaptation.

Although we found no evidence for local adaptation of the parasite towards host species, there was a slight temporal differentiation of the parasite populations which might have resulted from severe bottlenecks during queen hibernation. Parasite populations were in Hardy-Weinberg equilibrium and showed no signs of linkage disequilibrium suggesting that sexual reproduction is an alternative strategy in this otherwise clonal parasite. Moreover, high levels of multiple infections were found, which might facilitate sexual genetic exchange. The detection of identical clones in different host species suggested that horizontal transmission occurs between host species and underpins the lack of host specific adaptation.

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INTRODUCTION

Co-evolution might occur either in beneficiary relationships as mutualisms or symbioses, or in antagonistic relationships as host-parasite systems. Initially, a first step towards a co-evolutionary pattern occurs via local adaptation. Genotypes might adapt to their local environment, which might be either the abiotic or the biotic environment.

Transmission and virulence of parasites are crucial factors for any type of host-parasite adaptation. The evolution of parasite virulence, however, becomes more complex in social host species, especially eusocial insects. Colony structure, overlapping generations and colony life cycles may strongly affect parasite adaptation (Schmid-Hempel 1998). Social insect colonies typically provide stable abiotic and biotic conditions, as large amount of brood and food resources, which makes them prone to attacks by parasites and pathogens (Schmid-Hempel 1995). Moreover, the high density of individuals within a colony increases the rate of transmission of parasites between individuals (Schmid-Hempel 1998).

Transmission of pathogens in populations of social insect colonies occurs at three levels: 1) from the sexuals to the offspring (vertical); 2) among individuals, of the same and / or different sex or caste (horizontal / vertical) and 3) between colonies (horizontal). Here, especially the second point (among castes) may play an important role, as it is nearly exclusively found in social insects (Schmid-Hempel 1998).

The bumblebees *Bombus* spp. and the obligatory gut parasite *Crithidia bombi* (Lipa & Triggiani 1988) have been used as a model system for the study of host-parasite interactions (Schmid-Hempel 1998). Bumblebees are primitively eusocial insects with an annual colony life cycle. Mated queens hibernate and found new nests' in

spring. After the first batch of workers has emerged, the queen exclusively devotes herself to reproduction whereas workers maintain the nest, forage for food and care for the brood (Goulson 2010; Sladen 1912). At the end of the season, males and females are produced. After mating, queens enter hibernation. Usually, queens mate only once (Estoup *et al.* 1995; Schmid-Hempel & Schmid-Hempel 2000), which results in a high intra-colonial relatedness among workers of 75%, potentially facilitating parasite establishment within a colony, if the genotype of the host is important in susceptibility to a parasite.

C. bombi is a widespread, benign gut pathogen of bumblebees that, nevertheless, might reduce the fitness of bumblebee queens (Brown *et al.* 2003) drastically. The pathogen affects ovarian development as well as early colony development (Shykoff & Schmid-Hempel 1991a), but hibernating queens are not directly affected by *C. bombi* (Shykoff & Schmid-Hempel 1991b; Yourth *et al.* 2008). The main infection pathway is by ingestion of infectious cells. *C. bombi* typically reproduces clonally, but recent experimental studies showed that the parasite also reproduces sexually (Schmid-Hempel *et al.* 2011). In addition, foraging workers may acquire infections on flowers via horizontal transmission of *C. bombi* (Durrer & Schmid-Hempel 1994). Although bumblebees might differ in their ability to detect and avoid contaminated flowers, especially when encountering co-adapted pathogens (Fouks & Lattorff 2011). This horizontal transmission may result in a prevalence of *C. bombi* of 80% during the middle of the season (Popp *et al.* 2012). In contrast to the high degree of infection in workers, only 5-10% of the young queens are infected (Ulrich *et al.* 2011) and if they are, infections with multiple *Crithidia* strains are rare. This has been explained by a process of strain filtering in workers (Ulrich

et al. 2011) possibly accumulating strains due to subsequent infections. Both the low number of infected hibernating queens and the low frequency of multiple infections among these queens constitute a potentially severe bottleneck for *C. bombi* populations.

Several factors influence the transmission and establishment of *C. bombi* in bumblebee populations. Both intra- and inter-colonial transmission is strongly influenced by the number of individuals available as potential vectors, hence by the colony size. The forager frequency on commonly exploited flowers will affect the colony-to-colony dispersal within this foraging assemblage even across species. However, bumblebee species with different tongue length typically have a small niche overlap (Goulson & Darvill 2004) and horizontal infections across different species should primarily occur among those with similar preferences to food plants.

Besides the demographic and ecological factors, also genetic factors may play an important role. At the population level the prevalence of *C. bombi* has been shown to be associated with overall host heterozygosity (Whitehorn *et al.* 2010). Bumblebee populations exhibiting low genetic diversity, possibly due to inbreeding, show a higher prevalence of *C. bombi* than populations having high levels of heterozygosity (Whitehorn *et al.* 2010).

Hosts may vary in their ability to respond to *C. bombi* infection by activating their innate immune system and up-regulate effector genes (e.g. antimicrobial peptides (AMPs), lysozymes, serine proteases) (Riddell *et al.* 2011). AMPs are up-regulated within hours after parasite or pathogen exposure (Erler *et al.* 2011), but can be highly specific in their action with significant genotype by genotype interactions for the expression profile (Riddell *et al.* 2009). Although *C. bombi* can infect many bumblebee species little is known about genotypic interactions between multiple

hosts and multiple parasite infections. Under natural conditions parasites may be adapt to their hosts in broad or narrow ranges (Lajeunesse & Forbes 2002). Local adaptation between *B. terrestris* and *C. bombi* during one infection cycle has not been detected (Imhoof & Schmid-Hempel 1998). However, using mixed *C. bombi* strains from different colonies for serial infections of different bumblebee colonies, local *C. bombi* strain infections result in decreased cell numbers in bumblebees of the parasite alien colony (Yourth & Schmid-Hempel 2006). Thus local adaptation was observed under experimental conditions for related bumblebee colonies, which can be explained by genotype-genotype interaction between host and parasite.

Here, we test for the significance of genetic effects (host species) and temporal effects (years) on local host parasite adaptations by screening five different bumblebee species, in two subsequent years. This allowed us for assessing any local host parasite adaptations and also *C. bombi* prevalence with respect to ecological, genetic and demographic factors.

MATERIAL AND METHODS

Bumblebee samples

Workers and males (drones) from five different bumblebee species, *Bombus terrestris*, *B. hortorum*, *B. pascuorum*, *B. lapidarius* and the cuckoo bumblebee *Bombus (Psithyrus) vestalis*, were collected during foraging on flowers in Halle (Saale), Germany, in two similar sites, which served as replicates; the botanical garden (51°29'21.52" N; 11°57'37.36" E) and an urban flower rich park (51°29'27.55" N; 11°56'11.54" E) in 2008 and 2009 (overview see Table S1, Supporting information). Samples for both years were taken in the first week of July. Both sampling sites are

separated from each other by 2 km, thus well within the flight range of workers, males and queens (*B. terrestris*: Kraus *et al.* 2009; *B. terrestris* / *B. lapidarius*: Wolf *et al.* 2012). Bumblebee species were identified using the taxonomic key of Mauss (1994).

Bumblebee genotyping

Genomic DNA was extracted from a hind leg per individual using a modified Chelex protocol (Erlor & Lattorff 2010). The genotyping multiplex PCR primer set (fluorescent-labelled B10, B11, B124, B126 and B100) is based on Estoup *et al.* (1995, 1996). PCR amplification and analysis of fragment sizes followed the protocol from Erlor & Lattorff (2010) using a MegaBACE 1000 Sequencer (GE Healthcare, Munich, Germany) for DNA fragment separation and the MegaBACE Fragment Profiler software for genotyping.

DNA extraction of *C. bombi*

Nuclear DNA of the parasite *C. bombi* was extracted using a modified Chelex protocol (Walsh *et al.* 1991) by homogenizing the abdomen (samples of 2008) or the gut (samples of 2009) of each individual in 500 µl and 300 µl aqua dest., respectively. 200 µl of each homogenate was centrifuged at 3220 × g for 30 minutes. The supernatant was discarded and the remaining pellet was homogenized in 100 µl 5%-Chelex solution (Bio-Rad, Munich, Germany) and 5 µl 1% proteinase K was added. Samples were processed in a thermocycler using the following programme: 1 h at 55°C; 15 min at 99°C; 1 min at 37°C and a final step for 15 min at 99°C. DNA was stored at -20°C until further processing.

***Crithidia bombi* genotyping**

Fluorescence-labelled primers (Cri 4, Cri 1.B6, Cri 2.F10 and Cri 4G9) were used in a single multiplex PCR as in Schmid-Hempel & Reber Funk (2004) (4G9, R. Schmid-Hempel pers. comm.). A PCR reaction (10 µl) comprised 1 µl template DNA, 5 µl PCR Master Mix (Promega, Madison, USA), 2.2 µl aqua dest., 0.3 µM of each forward and reverse primer for Cri 4 and Cri 2.F10 and 0.15 µM of each forward and reverse primer for Cri 1.B6 and Cri 4G9. The PCR reactions were run in a Perkin Elmer PE 9700 thermocycler with the following program: 4 minutes denaturation at 95°C, then 35 cycles with 1 min, 95°C; 30 sec, 53°C; 30 sec, 72°C and final elongation at 72°C for 4 minutes. Allele sizes were analysed with MegaBACE 1000 Sequencer (GE Healthcare, Munich, Germany) and assigned using the software MegaBACE Fragment Profiler.

DATA ANALYSIS AND STATISTICS

Bumblebee sibship reconstruction

The software COLONY version 1.3 (Wang 2004) was used for sibship reconstruction of bumblebee samples. Based on a maximum likelihood approach this software uses both the individual genotypes (haploid and diploid) and the overall allele frequencies in the sample to infer the minimum number of putative natal colonies. In order to correct for the number of non-detected colonies due to finite sample sizes, the non-sampling error was estimated by fitting the observed distribution to a truncated Poisson distribution (Goulson *et al.* 2010; Kraus *et al.* 2009).

Analysed microsatellite parameters (e.g. allelic richness, observed and expected heterozygosity, fragment size ranges) for the five different host species were either based on the genotypes of collected bumblebees or on the inferred queen genotypes. Expected and observed heterozygosity was determined with GENEPOP version 4.0 (Raymond & Rousset 1995; Rousset 2008), allelic richness by using HP-RARE 1.0 (Kalinowski 2005).

A generalized linear model (binomial distribution, logit-link function) implemented in STATISTICA 8.0 (StatSoft, Tulsa, Oklahoma, USA) was used to estimate the impact of host species and sampling year on the prevalence of *C. bombi*. The same method was applied to test for a significant difference between the two sites.

Host-parasite co-evolution

A reconstruction of the genealogical relationships was used to analyse congruence between the host and the parasite genealogies. The reconstructed queen genotypes of the five different bumblebee host species were used to construct distance matrices using the microsatellite distance program Microsat.c version 1.5e (<http://hpgl.stanford.edu/projects/microsat/>). Similarly, the distance matrices for the parasites were constructed using an estimate of the allele frequencies of all *C. bombi* genotypes extracted from a certain host species. Distance matrices were calculated based on number of shared alleles. $-\ln$ (proportion of shared alleles) transformed distances were used in the Clustering Calculator software (<http://www2.biology.ualberta.ca/jbrzusto/cluster.php>) with the Saitou and Nei Neighbour Joining algorithm to reconstruct a genealogical tree. Congruence between the two trees was tested and visualized using

TreeMap version 1.0a (R.D.M. Page 1995, <http://taxonomy.zoology.gla.ac.uk/rod/tree-map.html>) using the default costs for evolutionary events (co-divergence = 0, host switching = 1, duplication = 1 and loss = 1).

As the previous analysis pools all host and parasite genotypes over years and sites additional data analysis was processed focussing on a finer scale to determine which factors influences parasite genotypic variance in host populations. As the allele frequencies in multiple infected hosts could not be unambiguously translated into individual genotypes, all further analyses were based on *C. bombi* genotypes from single *Crithidia*-infected individuals. Pairwise F_{ST} values were calculated for host species, sampling sites and sampling year, using GENEPOP version 4.0 (Raymond & Rousset 1995; Rousset 2008). Parasite populations extracted from the respective host populations were analysed similarly. Correlation analysis of corresponding F_{ST} values from host and parasite populations was done using STATISTICA 8.0.

In order to assess the population genetic structure of *C. bombi* populations' two different approaches were applied. The Bayesian clustering algorithm STRUCTURE (Pritchard *et al.* 2000) was used to reveal any hidden population structure, which might occur due to host genotypes or sampling year. We performed 10 replicate-runs with STRUCTURE varying the prior number of population to be expected ranging from $k = 1$ to $k = 10$. The number of populations represented by the *C. bombi* genotypes was analysed from visual inspections of the graphic representation of the results as well as using the likelihood estimator for the varying number of populations.

C. bombi genotypes from different host populations were used for an AMOVA (Arlequin version 3.0; Excoffier *et al.* 2005) in order to assess any host species specific

structure in the distribution of genotypes. This type of analysis was also used to identify any temporal pattern in the distribution of *C. bombi* genotypes. Testing for significant differences, as shown by Salathé & Schmid-Hempel (2011), between the two sites was also performed by using AMOVA. Finally, we tested three different scenarios: 1) separated into host species neglecting spatial and temporal effects, 2) temporal separation neglecting species and spatial effects and 3) spatial separation neglecting host species and temporal effects. As the experimental design is not hierarchical and not full-factorial a single analysis focusing on all effects was not possible. Bonferroni correction was used to correct of multiple tests adjusting the significance level to $p = 0.017$.

Determination of clonal genotypes and horizontal transmission

As only *C. bombi* genotypes from bumblebees infected with a single strain of *Crithidia* were taken into account, genotypes sampled several times from different host individuals were determined. To assess asexual propagation of *C. bombi* we used MLGsim (Stenberg *et al.* 2003) to determine the probability of a genotype sampled more than once to be clonal and not produced by sexual reproduction. Based on the sample size, the frequency of microsatellite alleles of the target genotype and the population wide allele frequencies MLGsim simulates populations and calculates probabilities for all clonal genotypes found during simulations, in order to determine a significance level. The distribution of the probabilities was used to calculate a critical probability, with values below this level are significant for a given nominal p-level (10^6 simulations were used with a nominal significance level of $p = 0.05$). For samples with missing data (locus Cri4 or locus

Cri4G9) the probability of the genotype was calculated from the remaining loci and simulations were run separately to determine the critical probability and the significance level. Finally, the genotypes occurring more than once were analysed with respect to the host species from which they were extracted in order to determine the level of horizontal transfer between host species.

RESULTS

Test for host-parasite local adaptation

Highly polymorphic microsatellite markers for the host (five, Table S2, Supporting information) and the parasite (four) populations allowed for the reconstruction of genealogical trees based on the allele frequency estimates. Pooled samples of bumblebees per species, years and sites and pooled samples of *C. bombi* populations (single and mixed infections) showed a similar topology for both trees (Fig. 4.1). The host species tree matched the expected phylogenetic clusters for the five different bumblebee species, separated in short-faced (*B. lapidarius*, *B. terrestris*) and long-faced bumblebees (*B. hortorum*, *B. pascuorum*, *B. vestalis*) (Cameron *et al.* 2007).

The parasite tree is characterised by short branch lengths between the five different *C. bombi* populations (Fig. 4.1). The *C. bombi* population from *B. vestalis* was placed between the *C. bombi* cluster of *B. hortorum* / *B. pascuorum* and the cluster *B. lapidarius* / *B. terrestris*.

Three methods were used to characterise population differences and to test for significant differences among the five different *C. bombi* populations, because the similar topology of the host-parasite tree might indicate co-evolution: (1) Pairwise F_{ST} for host and parasite populations; (2)

Bayesian clustering analysis and (3) analysis of genetic differentiation.

(1) Genetic distances calculated on single *Crithidia*-infections showed no sign for local adaptation. Pairwise F_{ST} values between host and parasite populations did not correlate ($r = -0.019$, $N = 91$, $p = 0.852$). Maximum F_{ST} values of 0.607 were found between host species, whereas between parasites populations F_{ST} a maximum of 0.189 was detected, but most values being close to zero (Table S3, Supporting information).

(2) Using the Bayesian clustering algorithm of STRUCTURE only slight evidence for a differentiation of the populations was found, with respect to the sampling years. Multiple runs of STRUCTURE varying the prior number of populations resulted in $k = 2$ populations (log-likelihood

= -1064.4). However, log-likelihood values for $k = 1$ to $k = 3$ populations are very close to each other and differentiation was not possible.

(3) Finally the genetic differentiation of *C. bombi* populations isolated from different host species was assessed using an AMOVA. Significant effects of differentiation were assessed for samples taken in two different years ($p = 0.004$). There were no significant effects with respect to host species or sites ($p > 0.05$). Results are combined in Table 4.1.

Summarizing these results, no significant correlation for host and parasite F_{ST} , no sign for population differentiation and no significant effects for species indicate that local adaptation may not occur for the five different bumblebee species and their respective trypanosome parasites.

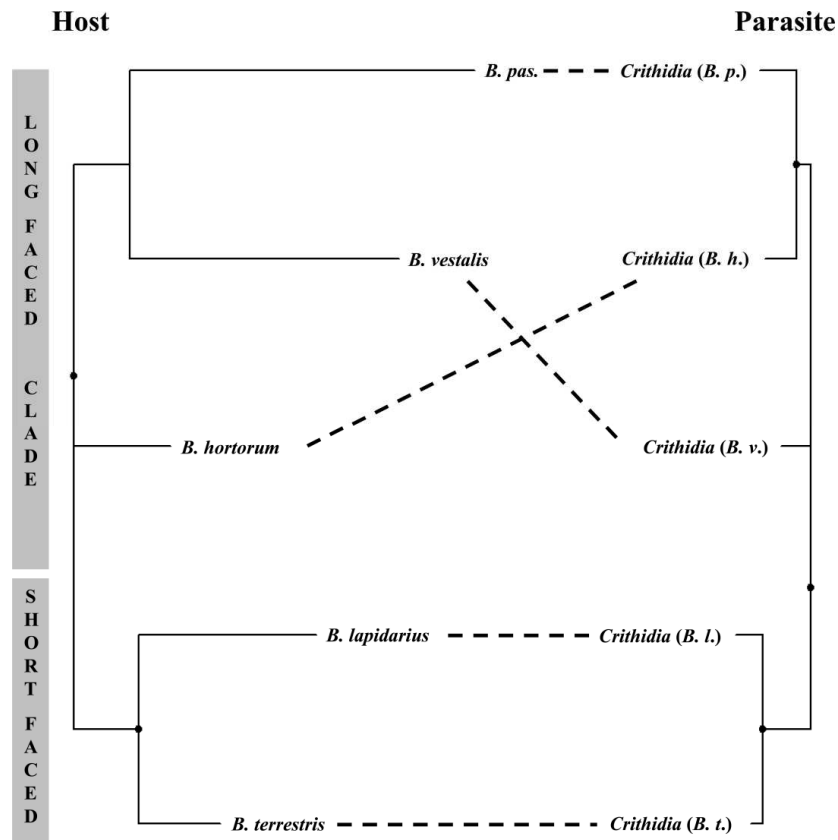


Figure 4.1 Genealogical analysis of potential genotype-genotype-associations between different *C. bombi* populations and its different host-populations. (*B. pas.* = *Bombus pascuorum*).

Table 4.1 AMOVA results for the effect of host species, year and site. P-values have been adjusted ($p = 0.017$) due to multiple testing by 1023 permutations. Significant values are in bold letters.

AMOVA for effect of host species			
Source	df	SS	p-value
Among host species	4	2.412	0.874 ± 0.010
Among populations within host species	9	7.653	0.076 ± 0.007
Among individuals within populations	89	58.557	0.953 ± 0.007
Within individuals	103	76.500	0.932 ± 0.008
Total	205	145.121	
AMOVA for effect of site			
Source	df	SS	p-value
Among sites	1	0.866	0.409 ± 0.018
Among populations within sites	12	9.198	0.142 ± 0.009
Among individuals within populations	89	58.557	0.954 ± 0.007
Within individuals	103	76.500	0.907 ± 0.010
Total	205	145.121	
AMOVA for effect of year			
Source	df	SS	p-value
Among years	1	2.582	0.004 ± 0.002
Among populations within years	12	7.482	0.572 ± 0.014
Among individuals within populations	89	58.557	0.948 ± 0.007
Within individuals	103	76.500	0.929 ± 0.008
Total	205	145.121	

Crithidia bombi reproduction and genotype dispersal

If the main form of reproduction in *C. bombi* were clonal, the parasite populations should be highly structured with clear deviations from Hardy-Weinberg-Equilibrium (HWE)

Table 4.2A, B Results of the statistical analysis for deviation from Hardy-Weinberg-equilibrium (A) and linkage disequilibrium (B), for the four different polymorphic loci of the trypanosome *C. bombi*. Values are p-values. Only parasite genotypes from single *Crithidia*-infected bumblebees were included.

A		
locus	2008	2009
Cri 1.B6	0.256	0.998
Cri 2.F10	0.518	0.911
Cri 4	0.152	0.243
Cri 4G9	0.065	0.376
B		
locus vs. locus	2008	2009
Cri 1.B6 / Cri 2.F10	0.742	0.653
Cri 1.B6 / Cri 4	0.998	0.077
Cri 2.F10 / Cri 4	0.976	0.661
Cri 1.B6 / Cri 4G9	0.145	0.246
Cri 2.F10 / Cri 4G9	0.43	0.261
Cri 4 / Cri 4G9	0.972	0.099

and strong linkage disequilibria (LD). However, a global analysis of all available *C. bombi* genotypes extracted from the singly *Crithidia*-infected individuals showed no significant deviations from HWE with p-values ranging from 0.065 - 0.518 (2008) and 0.243 - 0.998 (2009) (Table 4.2A).

Likewise, none of the pairwise comparisons of the four loci showed significant LD with p-values ranging from 0.145 - 0.998 (2008) and 0.077 - 0.661 (2009) (Table 4.2B).

A comparison of *C. bombi* genotypes showed a high diversity with 84 distinct genotypes to infer. Of those, 75 were singletons and 9 occurred more than once. Two different approaches were used to estimate the number of non-sampled genotypes. Since, the observed number of genotypes, which is restricted due to the finite sample size, might not represent the true number of genotypes circulating in the tested of bumblebee populations. Using the approach developed by Cornuet & Aries (1980), which assumes a binomial sampling distribution a total number of 242 (non-

sampling error, NSE = 158) genotypes was estimated, whereas a fitted truncated Poisson distribution revealed 119 (NSE = 35) genotypes to be present. Multiple occurring *C. bombi* genotypes appeared in 2 - 7 cases and were rarely restricted to a single species (2 out of 9) (Fig. 4.2). The seven remaining genotypes occurred in two or even three different species. Testing for the clonal nature of these genotypes (see Fig. 4.2); we found at least five out of the nine clonal

genotypes are real clones. Based on the allele frequency of those clones, a low probability was calculated, that excludes their co-occurrence by chance. Non-significant genotypes had either low sample size (2) or low number of microsatellite loci contributing to the multi-locus genotype (3 instead of 4) (Fig. 4.2). One genotype was exclusively found in 2008, five were exclusively found in 2009 and three genotypes were present in both years.

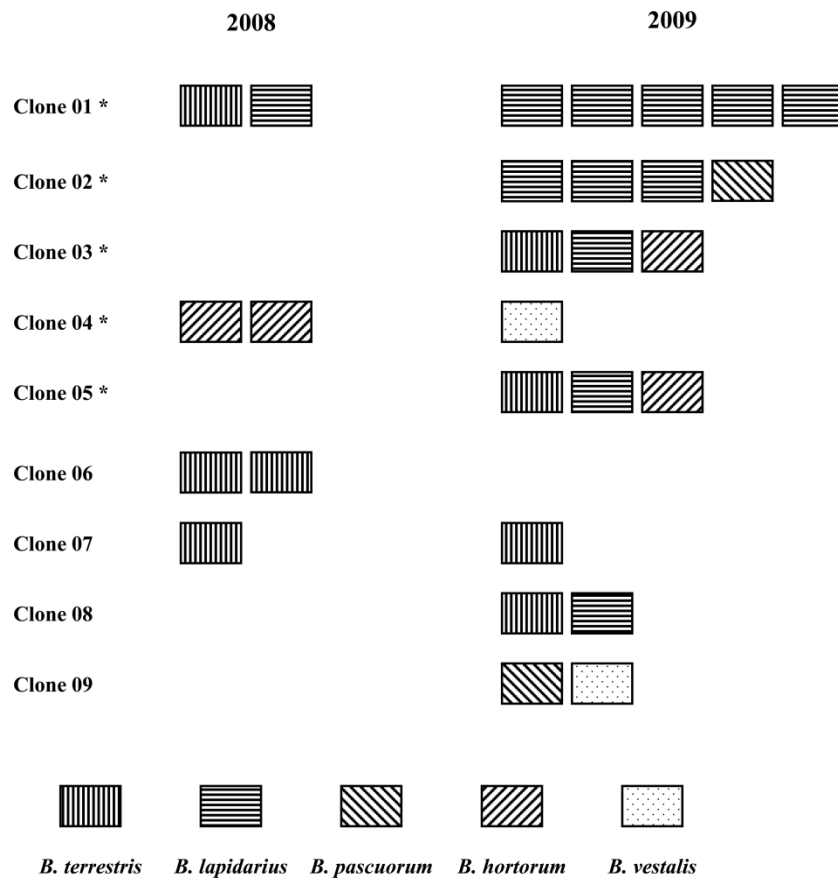


Figure 4.2 Association of multiple occurring clones within the five different host species and the two sampling years (2008, 2009). Each box represents one individual of the respective host species from which a parasite genotype has been extracted. Values for each single clone indicate the likelihood that the genotype represents a true clone, based on the allele frequencies of the multi-locus genotype: Clone 01: 4.8×10^{-15} ; Clone 02: 8.6×10^{-8} (locus Cri4G9 missing); Clone 03: 1.7×10^{-12} ; Clone 04: 6.4×10^{-10} ; Clone 05: 3.8×10^{-7} ; Clone 06: 9.4×10^{-4} (locus Cri4 missing); Clone 07: 3.8×10^{-5} ; Clone 08: 2.3×10^{-2} (locus Cri4G9 missing) and Clone 09: 3.5×10^{-2} (locus Cri4G9 missing). Asterisks indicate significant values for the likelihood that a genotype represents a clonal type.

Horizontal vs. vertical transmission: the impact of the environment

The analysis of matching topologies of the genealogical trees indicated a weak association of *C. bombi* genotypes and host species. Obviously, the host tree, based on genotypic markers, showed a separation of species with respect to ecologically important traits, such as tongue length. The prevalence of this parasite in different host species indicates a link between ecologically relevant factors that might influence the transmission of the parasite. A generalized linear model (GLM) showed that host species ($p < 0.001$) and sampling year ($p < 0.001$) had a highly significant impact on the prevalence of the parasite. In contrast, sampling sites ($p = 0.096$) no significant influence, as expected from experimental design and Salathé & Schmid-Hempel (2011). Using model reduction by means of stepwise

backward removal only host species and year remained inside the model, whereas sampling location was removed.

We selected a subset of biological markers (morphological data, foraging indices, etc.) for the respective bumblebee species from the literature (Goulson & Darvill 2004; Hagen & Aichhorn 2003) in order to evaluate an association of the parasite prevalence and species specific ecological covariates. We found a negative association between tongue length and prevalence as well as positive associations between prevalence and colony size and the diversity of flowers used for nectar collection (Simpson index of diversity, Goulson & Darvill 2004), respectively. The association of prevalence and flower diversity for pollen collection was less strong. However, none of these relationships appeared to be significant, most likely due to the restricted number of species available for analysis (Fig. 4.3).

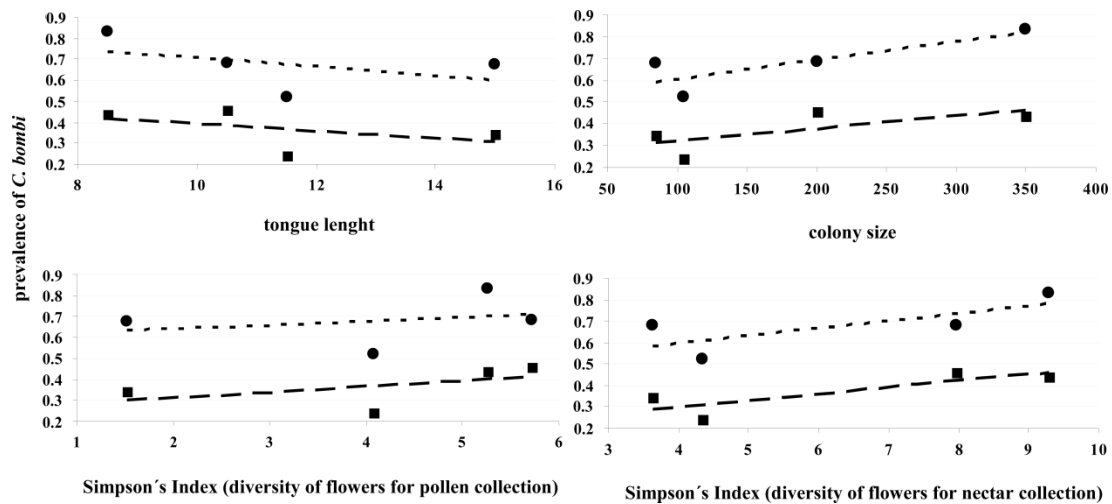


Figure 4.3 Correlation analysis of prevalence of *C. bombi* and several ecological and life history characters. Tongue length and colony sizes are adapted from von Hagen & Aichhorn (2003) and Simpson's indices were taken from Goulson & Darvill (2004). (filled circles: samples 2009, filled squares: samples 2008; from left to right - tongue length: *Bombus terrestris*, *B. lapidarius*, *B. pascuorum* and *B. hortorum*; - colony size: *B. hortorum*, *B. pascuorum*, *B. lapidarius* and *Bombus terrestris*; - Simpson's Index (pollen): *B. hortorum*, *B. pascuorum*, *Bombus terrestris* and *B. lapidarius*; - Simpson's Index (nectar): *B. hortorum*, *B. pascuorum*, *B. lapidarius* and *Bombus terrestris*; dashed lines: corresponding trend lines).

DISCUSSION

This study aims at determining local adaptation at different levels in a multi-host parasite system. The variation in genotypic distributions has been tested in order to predict factors that might favour one of the hypotheses. Temporal variation appeared to be the most important factor, whereas host species did not show any significant effect on parasite genotype distribution. Geographic locations with different habitat structure as influencing factor might be another source for variation in parasite genotype distribution as shown recently by Salathé & Schmid-Hempel (2011). There are three mutually non-exclusive explanations for this temporal effect: 1) the hibernation period of young queens represents a significant bottleneck for the *C. bombi* population, 2) the high mobility of males in late summer and autumn (Wolf *et al.* 2012) as well as the high mobility of young queens (Lepais *et al.* 2010) connects different populations and leads to a high migration rate of *C. bombi* genotypes and 3) differences in the selection pressures acting on different *C. bombi* genotypes throughout the year (Popp *et al.* 2012).

The prevalence of *C. bombi* changes throughout the year. Hibernated, colony founding queens revealed a prevalence level of approximately 10%, whereas the prevalence of *C. bombi* in workers and males steadily increases throughout the year (Gillespie 2010; Imhoof & Schmid-Hempel 1999; Popp *et al.* 2012). Flight ranges of males and queens have been estimated to range from 2.6 - 9.9 km for males of *Bombus terrestris* (Kraus *et al.* 2009) and up to 3 or 5 km for queens of *B. lapidarius* and *B. pascuorum*, respectively (Lepais *et al.* 2010). These ranges typically exceed those of workers (267.2 m ± 180.3 m, Wolf & Moritz 2008; reviewed in Goulson & Osborne, 2009) and hence may contribute to wide range of dispersal of *C. bombi* genotypes. Selection

pressures on *C. bombi* genotypes may change throughout the year, as they reside in queens exclusively during hibernation, whereas within the remaining season *C. bombi* reside in workers. Especially during multiple infections selective pressure might act on *C. bombi*, either due to strain filtering (Ulrich *et al.* 2011) or due to competition between parasites strains (Popp *et al.* 2012). Horizontal transmission on flowers (Durrer & Schmid-Hempel 1994) might increase throughout the year, as colonies grow under resource-rich conditions during spring and early summer, when contact rate on flowers increases due to an increase in abundance of foraging workers. *C. bombi* transmission is further enhanced in bumblebee species sharing similar plants for foraging. Pollen- and nectar-foragers with small tongue lengths (*B. terrestris*, *B. lapidarius*) share a broad spectrum of available flowers compared to bumblebee species having long tongue lengths (*B. hortorum*, *B. pascuorum*) (Goulson & Darvill 2004). Higher prevalence of *C. bombi* might be explained by increased access to *C. bombi* contaminated flowers as well as with high intra-colonial transmission in large colonies.

However, adaptation to different host species was not detectable when studying single *Crithidia*-infected bumblebees. Global analysis of all detected *C. bombi* genotypes and their hosts indicated slight association of parasite genotypes with their host species (Fig. 4.1). One switch on the side of the *C. bombi* populations occurred involving the *C. bombi* genotypes extracted from *B. (Psithyrus) vestalis*, the cuckoo bumblebee parasitizing *B. terrestris* (Loken 1984). As there might be more frequent contact between these species due to the shared nest, there might also be an increase in the inter-specific transmission of *C. bombi* genotypes between them.

Very strong support for a host-parasite association was detectable from a type of

multiple infections recovered in *B. lapidarius* and another one in *B. pascuorum*. Here, several host individuals show signs of a multiple infection of the very same type. Two *C. bombi* genotypes always occurred together. Similar patterns for multiple infections across different species are rather common and are fixed by ecological factors (Salathé & Schmid-Hempel 2011). An alternative explanation might be, that these are triploid clones, derived during genetic exchange, as this phenomenon is known from *Leishmania major* (Akopyants *et al.* 2009). Unfortunately, it was not possible to verify this from a marker-based analysis.

In midsummer not only the prevalence increases but also the degree of multiple infections (Popp *et al.* 2012), which might lead to competition between different *C. bombi* genotypes and eventually to sexual recombination. Asexual reproduction is assumed to be the main mode of propagation for the genus *Crithidia* (McGee & Cosgrove 1980), but signs of sexual reproduction in putatively asexual trypanosomatid species are known from *L. major*, *Trypanosoma brucei* and *T. cruzi* (Akopyants *et al.* 2009; Schurko *et al.* 2009). Recently, experimental tests in the laboratory have shown that genetic exchange also occurs in *C. bombi* (Schmid-Hempel *et al.* 2011). Our data indicate that sexual reproduction as an alternative reproductive strategy also occurs under natural conditions, as suggested by Salathé & Schmid-Hempel (2011) and Schmid-Hempel & Reber Funk (2004). The high diversity of genotypes present in the population (total number of circulating genotypes: 119 - 242), lack of deviation from Hardy-Weinberg equilibrium and no signs of linkage disequilibrium strongly suggest recurrent genetic exchange between strains.

However, some genotypes, independent of marker resolution, were recovered more than once clearly representing true clonal types. Two out of nine types were found only

within one host species, whereas all the others were found in at least two different species indicating that inter-specific transmission is rather common and thus, prevents local adaptation towards host species. Only three of the nine types were recovered in both years giving additional support for the strong effects of queen hibernation as already observed from the AMOVA analysis for the distribution of genotypes.

The main factor shaping the population structure of the parasite is a temporal effect. Frequent horizontal transmission between different host species might explain the lack of an adaptation of certain *C. bombi* genotypes towards specific host species. Additionally, genetic exchange between genotypes, when different strains are co-infecting a single host, reduces the effectiveness of adaptation. *C. bombi* might be transmitted from year to year via hibernating queens and possibly by nesting material, in case that old bumblebee nests will be reoccupied (Sladen 1912), although the vitality of *C. bombi* is drastically reduced outside of the host (Schmid-Hempel *et al.* 1999). Thus, the transmission via hibernating queens must be assumed to be the major pathway. Hibernating queens do show quite low levels of prevalence (10-30%, Shykoff & Schmid-Hempel 1991b) and might be infected by single strains only. From this it becomes evident that hibernating queens represent a strong bottleneck for the *C. bombi* population.

Most of the previous studies that focused on local adaptation between *C. bombi* and its host usually paid attention to a single host species, *Bombus terrestris*. The results that have been found so far did not allow for a full picture of host-parasite interaction. However, by analyzing a broad host spectrum we were able to show that local adaptation is very unlikely to occur within certain host species. Frequent horizontal

transmission may be the main driver preventing local adaptation with respect to the two proposed mechanisms (genetic and temporal). In this study we show that remarkable differences within the parasite population exist between years, but no genetic adaptation (distinct host species) was detectable.

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Chapter 5

Divergent patterns of selection on an antimicrobial peptide in common species of bumblebees

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Exposure to a wide range of pathogens leads to the production of antimicrobial peptides (AMPs), the major defense mechanism for animals lacking an adaptive immune system. Theory predicts that host-pathogen interactions between social insects and numerous pathogens drive an evolutionary arms race. Here, we studied the evolutionary pattern of an AMP, *hymenoptaecin*, in males of several bumblebee species and a focused study on two common species, *Bombus terrestris* and *B. lapidarius* along a 300 km transect within Germany.

Hymenoptaecin appears to be quite conserved over species as the majority of the gene is experiencing either neutral or purifying selection. Nevertheless, several codons are undergoing positive selection. The distribution of polymorphisms along the gene is significantly different between *B. lapidarius* and *B. terrestris*. Deviations from neutrality for the two exons of the gene differ between these two species with *B. terrestris* showing a lack of polymorphism in the mature peptide, while *B. lapidarius* shows an increased level of polymorphism in the mature peptide. Within the 5' region of the intron of *B. lapidarius*, high linkage disequilibrium and positive values for Tajima's D indicate balancing selection affecting a fragment of 174 bp with 12 tightly linked SNPs segregating as distinct alleles. Both, the polymorphism within the mature peptide and the haplotypes within the intron of *B. lapidarius*, are not associated with specific sampling sites or pathogen load, but may decrease the load with the bumblebee specific gut parasite *Crithidia bombi*.

Keywords:

AMPs, co-evolution, host-pathogen interaction, selection, polymorphis

Supplementary material:

(http://www.fugabee.uni-halle.de/fugabee_-_people/hymenoptaecin/)

INTRODUCTION

Host-pathogen co-evolution is based on antagonistic interactions. Co-evolution is characterized by a continuous challenge of host immunity and pathogenicity. The host's resistance to infection and, in turn, the mechanisms by which pathogens evade the host immune response can be explained as an evolutionary arms race with reciprocal, adaptive genetic change (Woolhouse *et al.*, 2002).

Invertebrates, especially insects, which in terms of diversity and biomass dominate almost all terrestrial habitats, are frequently exposed to an equal diversity of pathogens. Social insects in particular are extremely susceptible to pathogenic attacks, as the colony members are usually present in high densities, exhibit highly related genotypes and, consequently, facilitate high pathogen transmission rates. Furthermore, maintenance of nest homeostasis provides a relatively uniform environment, additionally facilitating the invasion and spread of pathogens (microorganisms, micro- and macro-parasites) (Schmid-Hempel, 1998).

These aspects of social insect biology therefore provide an ideal model system to test the theory of the evolutionary arms race. Specifically, we aim to examine the genetic components of social insect immunity in the context of host-pathogen co-evolution.

There is a marked reduction in the number of genes related to the innate immune system from whole-genome studies on social insects like ants (*Camponotus floridanus*, *Harpegnathos saltator*, *Pogonomyrmex barbatus* and *Linepithema humile*) (Bonasio *et al.*, 2010; Smith *et al.*, 2011a, b) and the honeybee (Honeybee Genome Sequencing Consortium, 2006; Lattorff and Moritz, 2013) relative to non-social insects like the fruit fly *Drosophila melanogaster*, the mosquito *Anopheles gambiae* and the red flour beetle *Tribolium*

castaneum (Adams *et al.*, 2000; Holt *et al.*, 2002; Tribolium Genome Sequencing Consortium, 2008).

How do social insects compensate this lack of immune related genes? A vast range of behavioral traits at the individual and/or group level has been observed in this group that might ameliorate their heightened exposure to pathogens and parasites e.g., 'social immunity' like hygienic behavior, social behavior (brood care, grooming), 'social fever', foraging for antibiotic compounds like propolis or avoiding contaminated food sources (Cremer *et al.*, 2007; Simone *et al.*, 2009; Fouks and Lattorff, 2011; Schmid-Hempel, 2011).

In recent years, declines of pollinator populations have been demonstrated (Biesmeijer *et al.*, 2006; Cameron *et al.*, 2011) as well as sudden losses of honeybee colonies known as "colony collapse disorder (CCD)" (Cox-Foster *et al.*, 2007). These declines have been attributed to a wide range of factors amongst them parasites and pathogens (Cameron *et al.*, 2011; Cox-Foster *et al.*, 2007; vanEngelsdorp *et al.*, 2009).

Antimicrobial peptides (AMPs), as a part of the humoral immune system, are one of the most important effectors of the innate immune system of invertebrates. Various AMPs are key elements of the insect immune system (Boman, 1995; Bulet *et al.*, 1999; Hoffmann *et al.*, 1999). The amino acid sequences in regions important for AMP-translation or intra-cellular trafficking are conserved across organisms, indicating common constraints on their production and delivery (Zasloff, 2002). In addition, AMPs show a very poor specificity and, therefore, act against a wide range of pathogens, including novel pathogens that an insect may not have been previously exposed to in its natural habitat (Casteels *et al.*, 1993).

This lack of specificity laid the foundation of a hypothesis by Hultmark (2003) whereby the immune system pathways and their high

degree of conservation across numerous taxa may be explained in terms of protecting insects from omnipresent saprophytes, (microorganisms, mainly decomposing dead organic material) rather than specificity to certain pathogens.

Our focal taxon, the bumblebees (*Bombus* spp.), are annual social insects which are headed by a single mated queen. The colony life cycle starts in spring and the colonies grow to a size of approximately 20-400 individuals depending on the species (Alford, 1975). Towards the end of the season sexually reproductive individuals are produced, mating takes place and the resultant mated gynes hibernate (Alford, 1978). Hence, the life cycle of the bumblebee involves both a solitary and a social phase.

In these organisms, the basic genes of the immune system pathways have been found by means of EST (Expressed Sequence Tag) library sequencing (Sadd *et al.*, 2010; Colgan *et al.*, 2011). In support of previous studies on social insect genomes, bumblebees also show a drastic reduction in the number of immune genes (Gadau *et al.*, 2012).

Here, we focus on one antimicrobial peptide (AMP) in bumblebees, *hymenoptaecin*. Thus far, *hymenoptaecin* has been reported from the order Hymenoptera only. It is a cationic peptide, enriched with glycine and it inhibits viability of gram⁻ and gram⁺ bacteria under a range of physiological conditions (Casteels *et al.*, 1993). *Hymenoptaecin* contains both a signal- and a pro-peptide sequence. After processing, it gives rise to the active AMP (Choi *et al.*, 2008; Xu *et al.*, 2009). *Hymenoptaecin* has been shown to be up-regulated after bacterial challenge (Erlor *et al.*, 2011). Moreover, it also is context-dependent and is involved in genotype-genotype interactions between host and pathogen. Riddell *et al.* (2009) conducted an infection assay where four different host strains were infected with four different

genotypes of their intestinal parasite *Crithidia bombi*. Depending on the host strain and the parasitic genotype, there were significant differences in the up-regulation of *hymenoptaecin*. With respect to the conservation of the social insects' immune system and the interaction of social immunity and individual immunity, Richter *et al.* (2012) demonstrated a strong impact of sociality on an individual's *hymenoptaecin* expression. Individuals kept in a social context (i.e. with nestmates) showed a significantly stronger expression of *hymenoptaecin* relative to isolated individuals.

To investigate the molecular evolution of AMPs in social insects, we sequenced *hymenoptaecin* from three bumblebee species (*Bombus terrestris*, *B. lapidarius*, *B. ruderarius*) and three cuckoo bumblebee species (sub-genus *Psithyrus*: *B. vestalis*, *B. bohemicus*, *B. rupestris*) sampled across a transect of various sampling sites in Germany and performed tests on selection to evaluate, whether evolution of this gene is connected with parasite and pathogen exposure or whether it is neutrally evolving and conserved over species.

MATERIALS AND METHODS

Sampling of bumblebees

Bumblebees were sampled during their foraging flights in July and August 2010, immediately anesthetized with acetic acid ethyl ester and either stored in 70% Ethanol or at -20 °C. 46 males representing six different species (17 *B. lapidarius*, 16 *B. terrestris*, 3 *B. ruderarius*, 6 *B. rupestris*, 2 *B. bohemicus* and 2 *B. vestalis*) were chosen for the study (Table 5.1, Fig. S5.1). Males are haploid and thus, they only carry one set of chromosomes. Hence, there is only one allele for *hymenoptaecin*, which ensures clear

Table 5.1: Sampling sites used in this study. Numbers indicate the sampled males from each species at each sub-site. *B. ter.* = *Bombus terrestris*, *B. lap.* = *B. lapidarius*, *B. rud.* = *B. ruderarius*, *B. rup.* = *B. rupestris*, *B. bohem.* = *B. bohemicus*, *B. vest.* = *B. vestalis*;

sub-site ID	geographic location	<i>B. ter.</i>	<i>B. rud.</i>	<i>B. rup.</i>	<i>B. bohem.</i>	<i>B. vest.</i>	<i>B. lap.</i>
1	51° 30' 31.72" N10° 1' 58.55" E			2	1		1
2	51° 35' 4" N 9° 49' 43" E	1			1		2
3	51° 29' 20" N 11° 57' 41" E					1	2
4	51° 28' 8.33" N 12° 1' 24.62" E	2					3
5	51° 33' 7" N 11° 55' 18" E	1	1	2			
6	51°34'38"N 11°52'36"E	3	2				
7	51° 20' 17" N 11° 57' 19" E	3					2
8	51° 19' 58" N 11° 55' 19" E	1		2		1	1
9	52° 21' 31" N 14° 3' 51" E	2					3
10	52° 19' 42" N 14° 6' 24" E	3					3

results after sequencing without having interference with an alternative allele. The first three species belong to the short-faced clade and the latter three ones to the long-faced clade (Cameron *et al.*, 2007). We sampled at five different sites, each with two sub-sites, across a transect of approximately 300 km. All individuals belonging to a certain species are referred to as a population as other studies suggest low genetic differentiation across a broad spatial scale in bumblebees (Estoup *et al.*, 2007; Lye *et al.*, 2011).

DNA extraction

DNA was extracted and purified from the flight muscles of individuals following the Spin-Column Protocol of the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany).

Primer design

The two exons of *hymenoptaecin* range from 1-87 bp (exon 1) and 989-1180 bp (exon 2), primers residing outside of the gene had to be designed to ensure complete sequencing of these regions. Therefore, genomic sequences of *B. terrestris* and *B. impatiens* containing the *hymenoptaecin* gene were aligned using Clustal W Multiple Alignment (Thompson *et al.*, 1994) as implemented in BioEdit (Hall, 1999). The program Primer3 (Rozen and Skaletsky, 2000) was used to develop primers based on the consensus sequence. Primer synthesis was performed by metabion international AG (Martinsried, Germany).

Amplification, electrophoretic separation and purification

The antimicrobial peptide *hymenoptaecin* was amplified in two separate PCRs (first PCR: primers 1.1fwd (5'-GCATCAGAGATCTTGTGTCA-3') and 1.1rev (5'-AAACATCGAACAGTTAAACG-3') for exon 1 and partial intronic sequence; second PCR: 2.5fwd (5'-CTTGTCAATTTGCTGAAGTT-3') and 2.4rev (5'-TACTACAGTCTCCTTGATCC-3') for the remaining part of the intron and exon 2) of a 10 µl volume containing 0.2 µl template DNA, 1 µl reaction buffer S and 0.5 U peqGOLD Taq-DNA-Polymerase (peqlab, Erlangen, Germany), 2 mM dNTPs, 0.3 µM of each forward and reverse primer (1.1fwd/1.1rev and 2.5fwd/2.4rev) and 7.9 µl HPLC-grade water. The following temperature conditions for PCR amplification were used: an initial denaturation step for 4 min at 95 °C, followed by 35 cycles of 40 s, 95 °C; 30 s, 55 °C; 2 min 20 s, 72 °C and a final elongation step at 72 °C for 10 minutes. All PCR reactions were conducted in a Perkin Elmer (Waltham, MA, USA) PE 9700 thermocycler. PCR efficiency and size of the PCR-products was verified by means of the capillary electrophoresis system QIAxcel using the High Resolution Cartridge (Qiagen, Hilden, Germany). PCR-products were cleaned using Sure Clean (Bioline, Luckenwalde, Germany) and the DNA-pellet resuspended in 25 µl HPLC-grade water (Diagonal, Münster, Germany). The quantity and quality of the purified PCR-products were measured using the NanoDrop ND-1000 (Peqlab, Erlangen, Germany) before sequencing.

Species differentiation

For species identification we used the 16S rRNA primer (Richter *et al.*, 2012). This product was amplified in a single PCR of a 10 µl volume containing 0.2 µl template DNA, 1

µl reaction buffer S and 0.5 U peqGOLD Taq-DNA-Polymerase (peqlab, Erlangen, Germany), 2 mM dNTPs, 0.2 µM of the forward and reverse primer and 8.1 µl HPLC-grade water. For PCR amplification, we chose an initial denaturation step for four min at 95 °C, followed by 35 cycles of 40 s, 95 °C; 30 s, 55 °C; 20 s, 72 °C and a final elongation step at 72 °C for 10 minutes. Measuring of quantity and quality, purification of PCR products etc. was done as described in the previous section.

DNA Sequencing

The 96-well plate sequencing was performed by LGC Genomics (Berlin, Germany), based on traditional Sanger sequencing. Each PCR product was sequenced with forward and reverse primers as described above. All sequences were trimmed to the same length, so that evolutionary analyses were performed on 1198 bp (including gaps) for each individual with exon 1 (1-87 bp), intron (88-1005 bp) and exon 2 (1006-1198 bp). All sequences were made accessible via NCBI GenBank (Accession Number XXX-XXX).

DNA Sequence Analysis

Sequence chromatograms were checked and edited manually using ContigExpress implemented in Vector NTI Advance 10.3.0 (Invitrogen, Karlsruhe, Germany). If possible, forward and reverse sequences were assembled to build contiguous sequences, also using ContigExpress. This resulted in two different contiguous sequence parts (contigs) (one covering exon 1 and the first part of the intron and one covering the corresponding second part of the intron and exon 2). As these resulting contigs did not have an overlapping sequence part, they were assembled by the help of CLC Genomics Workbench 5.5 (CLCbio, Århus, Denmark) to create consensus sequences using known

sequences from each species as reference genes. To verify homology, sequences were analyzed using the BLASTX tool of NCBI. Afterwards, multiple sequence alignment was performed using the algorithm MUSCLE implemented in Mega 5.05 (Tamura *et al.*, 2011).

Global analyses of selection

In order to test for neutral evolution or deviations from neutrality in the *hymenoptaecin* gene a HKA-test (Hudson–Kreitman–Aguade-test) as implemented in DnaSp v. 5.1 (Librado and Rozas, 2009) was used utilizing either exon 1 and the intron or exon 2 and the intron for comparisons. Here, the intron serves as a position matched control that is assumed to be evolving without selection. Furthermore, Tajima’s D test for neutrality was used on the full sequence, exon 1 and exon 2 separately as well as on the intron. Confidence intervals for Tajima’s D were computed using coalescent simulations using the observed number of segregating sites with 10000 replications and allowing for no recombination (as the most conservative setting) implemented in DnaSp v5.1 (Librado and Rozas, 2009). Additionally, a sliding window approach with 20 bp window size and 10 bp step size was used as implemented in DnaSp v. 5.1. For both tests all sequences of *B. lapidarius* and *B. terrestris* were used, as these species were represented by the highest number of sequences available, which allows for an estimation of the within species polymorphism frequency. Species represented by a lower number of sequences had been excluded, as Tajima’s D is very sensitive to sample size (Simonsen *et al.*, 1995; Larsson *et al.*, 2013).

Polymorphism Analyses

DNA polymorphism, in form of the nucleotide diversity, was analyzed for the whole gene per species as well as for exons and the intron separately. Furthermore, polymorphisms were determined for functionally different regions in the protein – signal peptide, pro- and mature peptide. Differences in polymorphism were analyzed between all *hymenoptaecin* sequences of *B. terrestris* and *B. lapidarius*, as in these species, due to the large sample size, even polymorphisms with a frequency below 0.1 are detectable. As *B. lapidarius*, in contrast to *B. terrestris* shows deletions in the intron, these regions, including the respective ones from *B. terrestris* had to be deleted in order to compare the sequences of the two species. Analyses of all sites were done with the software DnaSP v.5 (Librado and Rozas, 2009), using default settings. Statistical evaluation was performed with STATISTICA 7.0 (StatSoft, Tulsa, OK, US).

Linkage disequilibrium and balancing selection in intronic sequences

In *B. lapidarius* and *B. terrestris* all polymorphisms were checked for linkage disequilibrium (LD) using all pair-wise comparisons as implemented in DnaSp v. 5 (Librado and Rozas, 2009). Statistical significance was tested using Fisher’s Exact Test with Bonferroni corrections to adjust for multiple testing.

The SNPs along the complete gene were further subdivided into four subgroups and the number of significant pair-wise comparisons within and between subgroups was compared. The size of the subgroups was chosen according to the gene structure (exon/intron), gaps within the intron and to satisfy the demand for equally sized (with respect to size and polymorphism content) subgroups. Higher levels of within-subgroup

LD is an indicator for a significant association of SNPs forming an allele, which is independently re-tested by applying Tajima's D test (see above). Linkage disequilibrium has been visualized in comparison to Tajima's D using Textile Plot (Kumasaka *et al.*, 2010).

Positive selection at the codon level

As positive selection for complete genes is rarely observed, usually due to constraints in protein domains, we analyzed selection at the level of individual codons using a Bayesian approach implemented in the software Selecton v 2.4 (Stern *et al.*, 2007) and PAML 4.7 (codeml) (Yang, 2007). Alignments of the coding sequence produced with Clustal X 2.1 (Larkin *et al.*, 2007) were used. Each of the six sampled species was represented by a consensus sequence created by alignments using BioEdit (Hall, 1999). A tree (Fig. S2) representing the topology of the species' phylogenetic relationships was used to test, whether mutation rate in *hymenoptaecin* differs from the rate expected from the divergence of the species. For tree reconstruction consensus sequences of the 16S rRNA gene were extracted using BioEdit (Hall, 1999). Trees were constructed using the maximum likelihood algorithm implemented in MEGA 5.05 (Tamura *et al.*, 2011). In Selecton, the M8 model enabled for positive selection was used. In case of positively selected sites being detected by the algorithm, the model was compared to the null-model (M8a) that assumes no positive selection. Model comparison was done using a likelihood ratio test (LRT).

Ancestral state reconstruction of codons

Ancestral states of codons were reconstructed using the maximum likelihood algorithm implemented in MEGA version

5.05 (Tamura *et al.*, 2011). A phylogenetic tree serving as reference for the ancestral state reconstruction was built using the 16S rRNA sequences in combination with the maximum likelihood algorithm of MEGA and the appropriate nucleotide substitution model evaluated suiting the model selection tool in MEGA. Best model was chosen according to the lowest BIC (Bayesian information criterion) and for 16S rRNA, the GTR+G (general time reversible model including a discrete Gamma distribution) was chosen (Fig. S5.2).

Peptide Sequence Analyses

Peptide regions were defined as the following: signal peptide, pro-peptide and the mature peptide region by aligning the sequences using *B. ignitus* and *Apis cerana* as reference organisms (Choi *et al.*, 2008; Xu *et al.*, 2009). In accordance with the protocol of Ratzka *et al.* (2012), the sequences were checked for putative processing sites of the signal and pro-peptide.

RESULTS

Alignments of all sequences revealed large deletions in the intron of short-faced bumblebees with *B. lapidarius* experiencing most of the deletions resulting in a total sequence length of 947 bp. In comparison, *B. terrestris* has fewer deletions resulting in 1098 bp sequence length, similar to 1180 bp in the long-faced bumblebee clade. Differentiation between deletions and insertions was done according to the most parsimonious explanation including information about the phylogenetic relationship between species. Sequences of *B. terrestris* (short-faced clade) and all the species of the long-faced clade appear to be similar, but *B. lapidarius* (short-faced clade) shows large gaps for these parts, implying a

loss of these parts from the genome of the latter species.

Global analysis of selection

Two different tests for deviations from neutrality were used. The HKA-test, as a polymorphism vs. divergence ratio based test, showed opposing patterns of selection in the *hymenoptaecin* gene for the two focal species, *B. terrestris* and *B. lapidarius*. Exon 1 did not deviate from neutrality for *B. terrestris* ($p = 0.685$), but values for *B. lapidarius* were close to statistical significance ($p = 0.098$) with more substitutions than expected. By contrast, exon 2 shows the inverse pattern, whereby *B. lapidarius* shows no deviation from neutrality ($p = 0.980$), while *B. terrestris* shows a significant deviation ($p = 0.028$) due to a nearly complete lack of substitutions.

The Tajima's D statistic, an allele frequency based test, indicates no overall deviation from neutral evolution. However, this test statistic also suggests marked differences between the species. Overall, *B. terrestris* (as well as *B. rupestris*) had negative values (*B. terrestris*: $D = -0.93$; *B. rupestris*: $D = -1.16$, for confidence intervals see Table 5.2) indicating slight purifying selection (Table 5.2). Even when subdivided into exon/intron, the values for Tajima's D range between -0.91 and -1.29 for both species. However, values for *B. lapidarius* do not follow this pattern as the global value for $D = 0.18$. When analyzing exons and the intron separately, it appears that exon 1 ($D = -1.04$) is in the range of the other species, but the intron ($D = 0.42$) and exon 2 ($D = -0.16$) fall out of the range of the values obtained for the other species (Table 5.2). Although these values are well in the range of the 95% confidence intervals, this is most likely due to deficiency in sample size.

Polymorphism Analyses

Comparison of the whole *hymenoptaecin* sequences between *B. terrestris* and *B. lapidarius* revealed significant differences in the distribution of polymorphic sites (Wilcoxon matched pairs test; $T = 733.50$; $Z = 4.69$; $p < 0.001$). *B. terrestris* is more polymorphic than *B. lapidarius* over the majority of the analyzed sequence, but exon 2 in *B. terrestris* is completely lacking any polymorphism (Fig. 5.1).

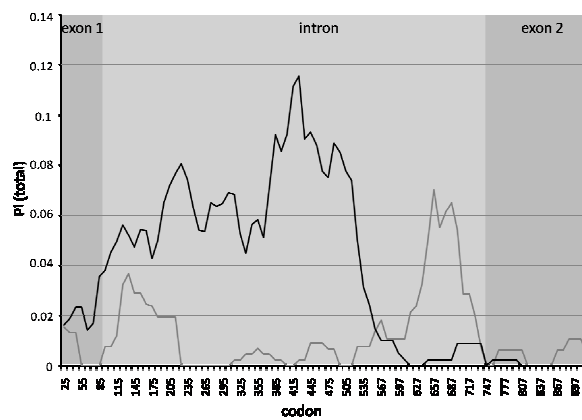


Figure 5.1: Differences in overall polymorphism (P_i (total)) of *hymenoptaecin* between *B. lapidarius* (grey line) and *B. terrestris* (black line). Data derived from a sliding window approach with a 50 bp window and a step size of 10 bp. X-axis indicates the midpoint of the 50 bp window.

In total, 158 SNPs were detected in *B. terrestris* compared to 37 in *B. lapidarius*. This considerable difference in the number of polymorphisms is additionally reflected by the occurrence of hypervariable sites (more than two nucleotides occur in a homologous position): Nine occur in *B. terrestris* and only one in *B. lapidarius*. An analysis of variation at silent sites indicates that the effective population size of *B. terrestris* is three times larger than that of *B. lapidarius* (K_s (JC-silent) = 0.12535; P_i (JC-silent) – *B. terrestris*: 0.03326; *B. lapidarius*: 0.01124).

The levels of nucleotide diversity differ between species, as well as between functionally different sections of the gene

Table 5.2: Polymorphism in three species of bumblebees (*B. ter.* = *Bombus terrestris*, *B. lap.* = *B. lapidarius*, *B. rup.* = *B. rufus*). Nucleotide diversity values are given for different regions of the gene (exon 1 & 2 and intron) as well as for different functional parts of the protein (signal-, pro- and mature peptide). Tajima's D, as a test statistic for deviation from neutral evolution, is estimated for different regions of the gene. Values in brackets indicate 95% confidence intervals derived from coalescent simulations.

	Nucleotide diversity (π)			Tajima's D		
	<i>B. ter.</i>	<i>B. lap.</i>	<i>B. rup.</i>	<i>B. ter.</i>	<i>B. lap.</i>	<i>B. rup.</i>
sample size (n)	16	17	6			
Total	0.0328	0.0120	0.0131	-0.934 (-1.764-1.667)	0.176 (-1.767-1.715)	-1.164 (-1.391-1.595)
Exon 1	0.0149	0.0090	0.0153	-0.962 (-1.798-1.830)	-1.044 (-1.843-1.922)	-1.295 (-1.295-1.799)
Intron	0.0422	0.0147	0.0149	-0.910 (-1.772-1.658)	0.422 (-1.778-1.712)	-1.102 (-1.443-1.597)
Exon 2	0.0006	0.0043	0.0034	-1.162 (-1.162-1.529)	-0.157 (-1.706-1.955)	-1.132 (-1.132-1.753)
Signal peptide	0.0160	0.0153	0.0261			
Pro-peptide	0.0085	0	0			
Mature peptide	0.0007	0.0049	0.0039			

(exon, intron), but also between functionally different parts of the protein (signal-, pro- and mature peptide) (Table 5.2).

Linkage disequilibrium and balancing selection in intronic sequences

In *B. terrestris* and *B. lapidarius* within-species sample sizes were sufficiently large to detect linkage disequilibrium between polymorphic sites. For both species a substantial number of sites were found to be in linkage disequilibrium. On average, there was a higher degree of linkage disequilibrium in *B. lapidarius* (11.7 % of all pair-wise comparisons) relative to *B. terrestris* (6.3 %). However, the polymorphic SNPs that are in linkage disequilibrium are not evenly distributed along the gene, but occur in clusters. This is especially obvious in

B. lapidarius, where a strong cluster of linked SNPs could be identified by means of a subsection analysis comparing the degree of significant LD within a section to the pair-wise LD between sections (Table 5.3). The 5' region of the intron contains a set of 12 SNPs in high LD. Within that section 48 % of all pair-wise comparisons are significant in contrast to 0-1.2 % of the between section comparisons. In four individuals all 12 SNPs contain the alternative allele (called the low frequency (*lf*) allele) compared to the remaining 13 individuals (high frequency (*hf*) allele). The *lf* allele shows an observed frequency of 0.24 with a 95% confidence interval (binomial distribution) of 0.07-0.50. These 12 SNPs are distributed over 174 bp (position 803 - 977). This region is characterized by highly positive values (up to 1.62 for interval 912 to 961) for Tajima's D (Fig. 5.2).

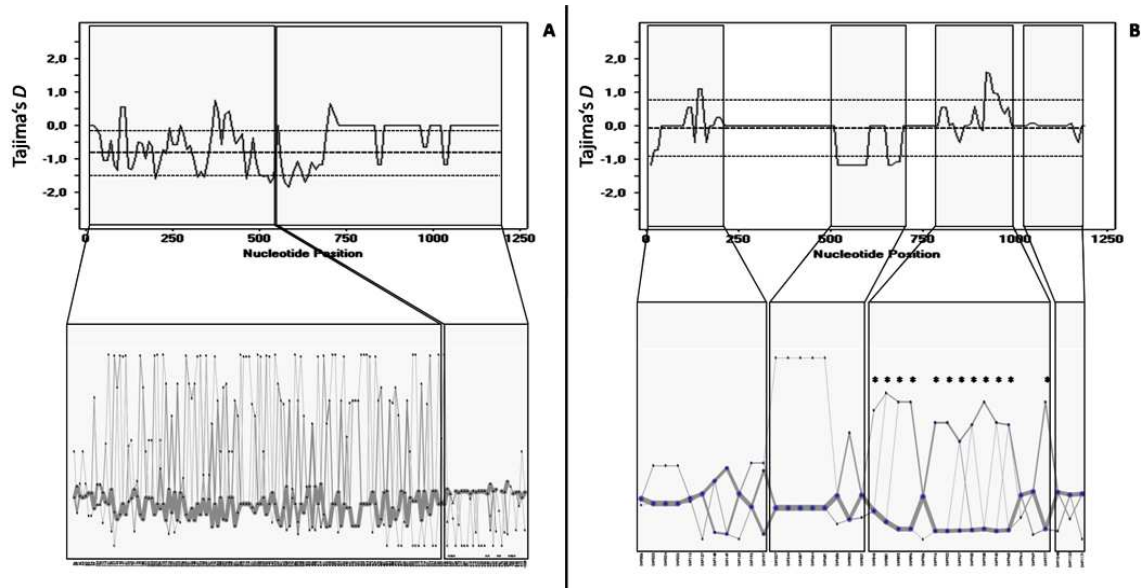


Figure 5.2: Comparative plot of Tajima’s D and the degree of linkage disequilibrium visualized as textile plot for (A) *B. terrestris* and (B) *B. lapidarius*. Tajima’s D was calculated along the gene using a sliding window approach with a window size of 20 bp and a step size of 10 bp. Thick dotted lines indicate the mean over the full length of the gene (\pm s.d. shown as thin dotted lines). In the textile plot of *B. lapidarius* the 12 SNPs contributing to the two distinct haplotypes are indicated by asterisks.

Table 5.3: Analysis of linkage disequilibrium within the *hymenoptaecin* gene of *B. lapidarius* and *B. terrestris* based on the relative numbers of significant to non-significant pair-wise comparisons (given a proportion in parentheses), both within and between sub-sections of the gene. Sections are numbered from S1-S4 and their positions are given in brackets.

<i>B. lapidarius</i>	within	between		
		S1	S2	S3
S1 (pos. 6-195)	5/45 (0.11)			
S2 (pos. 527-686)	0/15 (0)	0/60 (0)		
S3 (pos. 803-977)	65/136 (0.48)	2/170 (0.01)	0/102 (0)	
S4 (pos. 1048-1171)	0/3 (0)	2/30 (0.07)	0/18 (0)	0/51 (0)
<i>B. terrestris</i>		S1	S2	S3
S1 (pos. 36-197)	13/276 (0.05)			
S2 (pos. 202-502)	440/4005 (0.11)	114/2136 (0.05)		
S3 (pos. 513-685)	1/300 (0.003)	13/600 (0.02)	55/2225 (0.02)	
S4 (pos. 699-1038)	0/10 (0)	0/120 (0)	5/445 (0.01)	0/125 (0)

A re-sampling approach by means of jack-knifing over individual sequences revealed a binomial distribution for Tajima’s D with an increase of the frequency of the *lf* allele to 0.25 (one of the 13 *hf* alleles left out) results in highly significant values for Tajima’s D (1.81) whereas a decrease of the frequency of the *lf* allele to 0.19 (one of the *lf* alleles left out) results in non-significant values (1.13).

The occurrence of these two alternate haplotypes within this 174 bp intronic sequence coupled with highly positive values for Tajima’s D suggest the occurrence of balancing selection within this region maintaining high degrees of heterozygosity. This is supported by the fact that the nucleotide diversity within an allelic group is similar to another, similar sized intronic region (position 88-262) ($\pi_{lf\text{ allele}} = 0.009$; π_{hf}

allele = 0.017; $\pi_{lf\text{control}} = 0.009$; $\pi_{hf\text{control}} = 0.021$) but the divergence between those sequences is extremely high ($K_{\text{allele}} = 0.07$; $K_{\text{control}} = 0.02$). Individuals carrying the *lf*-allele were sampled throughout the whole range of the transect, as well as the individuals carrying the alternate *hf*-allele.

Positive selection at the codon level

Analysis of selection at the codon level revealed seven codons under significant positive selection. Codons 31, 37, 44, 47, 49, 72 and 75 provided K_a/K_s scores exceeding the level of 1 (Fig. 5.3), irrespective of the algorithm that was used (Selecton or codeml). Models including selection are significantly different from the null-model (disabling positive selection) in Selecton (ln M8 model: -492.996; ln M8a model: -496.472; LRT: $p = 0.008$), whereas these are close to significance with codeml (ln M8 model: -497.408; ln M7 model: -499.171; LRT: $p = 0.06$).

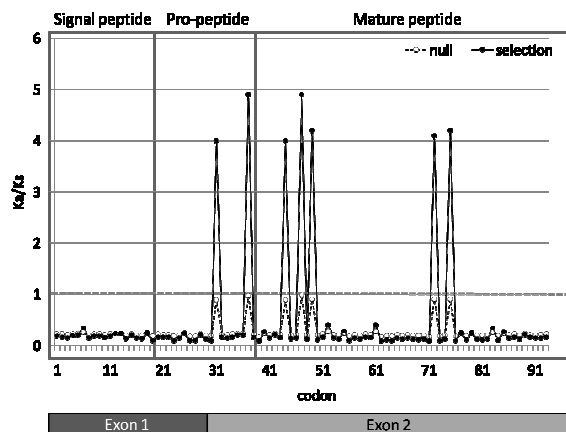


Figure 5.3: Bayesian analysis of per codon selection in the *hymenoptaecin* gene. The analysis is based on 93 codons of *hymenoptaecin* and a position matched control (intron), including all individuals. Significant K_a/K_s values exceed the level of 1 at positions 31, 37, 44, 47, 49, 72 and 75. The gene is divided into a signal peptide region (codon 1-17), a pro-region (codon 18-36) and a mature peptide region (codon 37-93).

Almost all species possess the ancestral triplet TCT (coding for serine) at codon 31

except for one representative of the long-faced bumblebee species, *B. ruderarius*, which owns TAT encoding for tyrosine. The next codon 37 which is lying under positive selection is related to the socially parasitic bumblebees, *B. rupestris* and *B. bohemicus*, except *B. vestalis*. Both cuckoo bumblebee species are fixed for the codon TAC, coding for tyrosine, differing from the ancestral codon CAC (histidine) in all other sampled species.

Codon 44 polymorphism segregates with respect to clades as well as within a species. All long-faced bumblebee species (*B. ruderarius*, *B. vestalis*, *B. rupestris*, *B. bohemicus*) possess an GTC coding for the amino acid valine. All short-faced bumblebee species (*B. terrestris*, *B. lapidarius*) as well as the reference sequence of *B. ignitus* show a CTC coding for leucine and three *B. lapidarius* individuals have an ATC coding for isoleucine. An analysis of linkage disequilibrium on all pair-wise combinations of polymorphisms within the gene and its intron did not reveal any significant association between alleles at codon 44 and any other polymorphic site within the *B. lapidarius* population suggesting that this is an evolutionary old polymorphism maintained within the population. This is further supported by the fact that three individuals showing the alternative ATC codon are sampled at localities 1, 3 and 9, thus spanning the full range of the sampled transect.

The following two positive selected codons 47 and 49 are exclusively found in *B. terrestris*. In this species, the triplets encode aspartic acid (# 47) and lysine (# 49) and therefore derive from the ancestral codons of all other species, which are CAG (glutamine) in both cases.

The next codon (# 72) under positive selection is again related to the socially parasitic bumblebees, especially *B. vestalis* and *B. rupestris*. The former species appears

to be fixed for the codon AAA (coding for lysine) and the latter is polymorphic for the ancestral AGA (coding for arginine) and the derived AAA.

B. lapidarius possesses the last codon # 75 under positive selection. The derived codon # 75 ATG is coding for methionine and therefore differs from the ancestral codon GTG (valine) in all other sampled species.

After using the more restrictive algorithm approach in codeml to test for positive selection, only three codons are remaining which seem to be positively selected (# 37, 44 and 47).

Peptide Sequence Analyses

Across all individuals, the amino acid sequence of this peptide was highly conserved across species. Overall, the signal peptide region does not vary at all within the genus *Bombus*, except for positions #8 and 9 in two *B. lapidarius* individuals (Fig. S3). Furthermore, we found an amino acid insertion (alanine) in the first processing site in *A. cerana* (QAELEP), which is not present in the *Bombus* species (Q-EIEP). In addition, regarding the processing site of *A. cerana*, the amino acid leucine (L) is replaced by isoleucine (I) in the genus *Bombus* (QAELEP → Q-EIEP). Within *Bombus* this processing site is strongly conserved across species, although two individuals of *B. terrestris* show a change into QEIDP, both individuals originate from different sampling locations: 4 and 9 (approx. 180 km apart). The second putative processing site of *B. ignitus* differs from all the other analyzed individuals (SR instead of RR).

DISCUSSION

Global analysis of selection

Hymenoptaecin was sequenced from 46 haploid bumblebee males originating from

six different species (three cuckoo bumblebee and three host species) sampled across a transect of approximately 300 km in Germany. It is obvious that this antimicrobial peptide is highly conserved across species and is relatively invariable among sampling sites. In general, the majority of the codons are under neutral or strong purifying selection due to the low Ka/Ks scores, supported by analyses over all species as well as *B. terrestris* and *B. lapidarius*, separately. Due to this high degree of conservation, the sequences do not exhibit a high evolutionary rate and do not reveal strong differences between species. Although Tajima's D statistics indicates no deviation from neutral evolution, there are significant differences between the species *B. lapidarius* (and *B. rupestris*) and *B. terrestris* when applying an HKA-test. Tajima's D test is an allele frequency based test, that is sensitive to low sample sizes, most likely due to improper estimation of the allele frequency. In contrast, the HKA-test is based on the polymorphism vs. divergence ratio and requires much less samples (Ochola *et al.*, 2010).

Peptide Sequence Analyses

By comparing the nucleotide and protein sequence from *B. ignitus* (GenBank Accession Number: EU411044.1 and ACA04900) (Choi *et al.*, 2008) and *A. cerana* (GenBank Accession Number: EU727297) (Xu *et al.*, 2009), we found striking similarities between the different *hymenoptaecin* amino acid sequences of the different genera. In the first putative cutting site, which is located between the signal- and the pro-region, in *A. cerana*, an insertion of alanine (A) is obvious after glutamine (Q), the first amino acid of this site, compared to the genus *Bombus*. QAELEP is present in almost all *Apis* species, except for *A. dorsata*, where the processing site is QEELEP. We decided not to include the

A. cerana sequence in our alignment as it would have altered the consecutive numbering of the amino acid sequences compared to the codon analyses, which were necessarily combined from some analyses in this study. Also the second cutting site, which was identified using the protocol of Ratzka *et al.* (2012), is also conserved throughout the genus *Bombus*, except for *B. ignitus*, which shows a single amino acid change (Fig. S3).

We sequenced several males from six different species and all their sequences are identical across species at this second cutting site, and in addition, in another sequence available for *B. ignitus* (GenBank Accession Number: EU411043.1), this processing site is RR. As this site is conserved across all Hymenoptera (Ratzka *et al.* 2012), we assume a sequencing error for *B. ignitus*.

Linkage disequilibrium and balancing selection in intronic sequences

We also used Tajima's D test (D_T) to test for balancing selection in the intronic sequences of *B. lapidarius*, as we had detected a segment that shows high levels of within-segment linkage disequilibrium, but appears to be in linkage equilibrium with other SNPs outside that segment. A special region in the intron of about 174 bp, containing 12 tightly linked SNPs, showed two alternate haplotypes (high and low frequency (*hf*; *lf*) allele) in this species. High values of D_T strongly suggest balancing selection is maintaining a high level of heterozygosity in this region. This approach was also used by (Hörger *et al.*, 2012) to address to a similar concern, where they detected two different sequence types of the *RCR3* gene, a target for pathogen-derived molecules which facilitates recognition pathogens via interaction with a resistance gene in the wild tomato *Solanum peruvianum*.

The values for D_T might be even higher, depending on the allele frequency of the *lf*

allele. A re-sampling approach indicated only slight increases in the allele frequency (up to 0.25) result in highly significant D_T . As the sample of the *B. lapidarius* population is finite, we can not exactly predict the allele frequency. However, binomial confidence intervals (95%) for the allele frequency (0.06-0.50) indicate a quite high probability of even higher allele frequencies in the total population.

Positive selection at the codon level

The nucleotide sequences show significant differences in overall polymorphism between the two most abundant species in this study, *B. terrestris* and *B. lapidarius*, with the former species having an effective population size three times larger than the latter. Despite this disparity in polymorphism between species, there are seven codons in exon 2 (# 31, 37, 44, 47, 49, 72 and # 75), which are prominent due to positive selection. These codons have been found by two different algorithms (implemented in Selecton (Stern *et al.*, 2007) and PAML (codeml) (Yang, 2007)), both based on a Bayesian approximation. However, the Naïve Empirical Bayes (NEB) method might unreliably calculate posterior probabilities, especially when data sets are small (Yang *et al.*, 2005). We have also applied a more advanced algorithm, the Bayes Empirical Bayes (BEB) method implemented in PAML (codeml), which especially deals with uncertainties in calculating posteriors (Yang *et al.*, 2005). When applying the BEB, codons 37, 44 and 47 are expected to be positively selected, but not significantly, with probabilities of 0.815; 0.821 and 0.845, respectively. As we do not know, for which codons there might be an overestimation by the NEB algorithm, we will not completely exclude it. Nevertheless, the codons that also show up under the restrictive BEB algorithm might be

considered as definitely being under positive selection.

For codon 31, only one representative of the long-faced bumblebee species, *B. ruderarius* is revealing a polymorphism at this site. Codon 44 shows polymorphism over all studied species. Interestingly, only three individuals of *B. lapidarius* differ in their allele (ATC) from the other samples of this species (CTC). Further research is needed to test whether this polymorphism is evolutionary ancestral with no connection to any other polymorphic site in this population. Due to the limited sample size it is not possible to infer, whether this polymorphism is selectively neutral or whether it is actively maintained by balancing selection. However, as this codon is under positive selection when analyzing the whole data set, it is most likely not a neutral polymorphism in the *B. lapidarius* population. Notably, two codons under positive selection (#37 and 72) only arise in the cuckoo bumblebees. Two other positively selected codons, 47 and 49, are exclusively found in *B. terrestris*. In *B. lapidarius*, codon 75 appears to be polymorphic for a non-synonymous substitution.

AMPs are essential components of the social insect immune system. Hence the high level of conservation of *hymenoptaecin* across species indicates that it acts against a broad spectrum of microbes as an immediate immune defense. Our findings of purifying selection in *B. terrestris* and the highly conserved peptide sequences across species also run counter to the theory of the evolutionary arms race of the social insects and their pathogens/parasites but support the theory of Hultmark (2003), postulating conservation of AMPs due to protection from omnipresent saprophytes, rather than specificity to certain pathogens. Supporting data (Parsche and Lattorff, unpublished data) show no connection between the polymorphism of this gene and the

prevalence of the intestinal parasite *Crithidia bombi* at various sub-sites (Mann-Whitney-U-Test, $U = 8$, $p = 0.56$), but polymorphism may be associated with a lower infection intensity (t-test, $t = 1.94$, $p = 0.09$).

Several recent studies demonstrate that genes of the innate immune system are less conserved than previously expected. Particularly, it has been suggested that polymorphisms maintained by balancing selection might play an essential role in maintaining immunity to a broad spectrum of pathogens, as they have been detected in innate immune genes in *Strongylocentrotus purpuratus* (Pespeni *et al.*, 2012) and also in the human genome (Casals *et al.*, 2011). Essential tests for the functionality of these polymorphisms are scarce, but some promising results have been found for an antimicrobial peptide (*defensin*) in great tits (*Parus major*). Different alleles of this peptide show differential activity towards various kinds of bacteria in an in vitro assay, whereby both alleles strongly reduced the growth of gram⁻ *E. coli*, but only one allele was also active against the gram⁺ *Staphylococcus aureus* (Hellgren *et al.*, 2010).

We found striking differences in the selection pressure on an antimicrobial peptide gene in two of the most common bumblebee species in Central Europe, *B. terrestris* and *B. lapidarius*. Although *B. terrestris* has a three times larger effective population size, we had sufficient power to detect a balancing polymorphism in *B. lapidarius*: one in the intron and one codon in the mature peptide. The mature peptide of *B. terrestris* is under very strong purifying selection as virtually no polymorphism was detected.

Recent studies on the decline of pollinators (Biesmeijer *et al.*, 2006) have identified parasites and pathogens as a major factor contributing to this decline (Cameron *et al.*, 2011). Thus, the study of adaptation within the immune system of pollinator

insects is essential for the understanding of potential mechanisms to resist pathogens in order to make predictions about the outcome of pathogen pressure on bee species. As the present study illustrates, differences in the adaptive variance of antimicrobial peptide genes, whereby higher levels of polymorphism can be maintained in species with lower population sizes, it might be misleading to claim species with low population sizes are less able to resist pathogens.

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Chapter 6

Synopsis

Besides numerous types of co-evolutionary systems, emphasis was put on host-parasite interactions in this present work. This special relationship is characterized by a tightly linked association and the great reciprocal impact of both, the host and the parasite, which causes strong selective pressure on each other (Anderson and May, 1979a and b, 1981; Thompson, 1994). Changes in allele frequencies of traits, like the parasite's virulence, may result in changes in allele frequencies for host resistance. This everlasting interplay can be explained by an evolutionary arms race, called the Red Queen Hypothesis (Woolhouse *et al.*, 2002). According to this hypothesis, both, parasites and their hosts as well, must constantly adapt and evolve to survive while competing with their ever-evolving counterparts in a constantly changing environment (Bell, 1982).

As a result in an increase in parasite virulence, transmission will increase thereafter. As Frank (1992) presented in his ESS (evolutionary stable strategy) model on the evolution of virulence, transmission will increase with virulence, imposing a trade-off between the parasites survival and its transmission, resulting in a decrease in host recovery with increasing virulence. This is true for a host infected by a single strain of a parasite.

Now consider a multi-host / multi-parasite system like the *Bombus/Crithidia* model system. Here,

various parasite strains can accumulate within the bumblebee hosts due to inter- and intraspecific / inter- and intracolony horizontal transmission in the course of a season via e.g. foraging on shared flowers previously visited by an infected individual (Durrer and Schmid-Hempel, 1994), resulting in what is referred to as multiple infections. The different parasite strains are expected to compete for resources within the host individuals. When competing within the host, strains growing faster than others may have a competitive advantage while fighting for resources. The slowly-growing strains are just outnumbered. Fast growth and outcompeting opponents is just logic following Charles Darwin's postulates for evolutionary processes (Darwin, 1859): Parasites vary in their phenotype (growth rate), proliferate to variable extents and therefore compete with each other. The ones growing faster are more successful in survival and reproduction. These traits will then be inherited to their offspring by clonal reproduction or to some extent by genetic exchange in cases where multiple infections occur (Schmid-Hempel *et al.*, 2011), therefore resulting in fewer allele combinations having higher competitive abilities than others. This reveals a steady change in the composition of parasite strains within the host during one season with strain-filtering of the queen also playing its part (Ulrich *et al.*, 2011).

Relative to infections by just one strain (single infection), infection by several competing parasite genotypes is expected to result in a suboptimal form of host exploitation due to competition among different strains within the host. Thus, Levin and Pimentel (2001) predicted multiple infections being more virulent than single infections. However, depending on their relatedness, cooperation between parasite genotypes might occur to a certain extent. The outcome would then be decreased virulence (Schjørring and Koella, 2003). Moreover, competition between parasite strains within the host could also lead to a decrease in virulence (Schjørring and Koella, 2003). In order to compete within the host different *C. bombi* strains are expected to show differences in their phenotypes like variation in growth rates. Indeed, as expected we found differences in the phenotype associated with differences in genotypes. Certain strains do show a growth rate two times as high as other genotypes (Chapter 2). This was only possible by a new method for isolating and propagating *C. bombi* in a cell culture. So far, this was the first time a suitable method was developed, although others have presented improved methods afterwards (Salathé *et al.* 2012).

The benign intestinal gut parasite shows a different connection of virulence and transmission. The circumstance of its high transmission is rather due to the high density of host individuals within a single colony and the high relatedness among them than high virulence. The ergonomic growth phase of the colonies favours high horizontal transmission

rates. Infections of bumblebee workers with *C. bombi* are not lethal under normal conditions, but might reduce the fitness of the queens (Brown *et al.*, 2003). During the season, the impact of temporal changes in host demography on the population structure of *C. bombi* is enormous. The parasite population is changing drastically. Parasite prevalence in general is highest in midsummer, which represents the middle of the foraging season, and coincides with the maximal occurrence of multiple infections, both declining later in the season (Chapter 3). Within the host, competition between parasite genotypes might explain the observed pattern leading to selection of a certain allele and thus a boost of homozygote genotypes in the middle of the season. Later on, as competition is declining, negative frequency dependent selection of this allele might lead to its depletion. In addition, recovery of heterozygosity and disappearance of linkage disequilibrium also gives strong evidence for genetic exchange in these trypanosomes in natural populations, as it is already known that this intestinal parasite shows evidence of genetic exchange as a facultative strategy to clonal reproduction under laboratory conditions (Schmid-Hempel *et al.*, 2011). The results of our study have been confirmed by another study showing that genetic exchange is taking place in natural populations in the case where co-infections of different strains occur (Tognazzo *et al.*, 2012).

The parasite's transmission and also its virulence are driving the adaptation of both, the host and the parasite. The social

insects' life cycle characterized by ergonomic growth rate, maintenance of nest homeostasis, high density of nest mates etc. should provide a relatively uniform environment, additionally facilitating the invasion, spread and adaptation of parasites (Schmid-Hempel, 1998). In contrast to this belief, we found no indication for local adaptation of *C. bombi* towards several bumblebee host species (*B. terrestris*, *B. hortorum*, *B. pascuorum*, *B. lapidarius* and the cuckoo bumblebee *Bombus (Psithyrus) vestalis*), although we found evidence for temporal differentiation of the parasite populations, which might have resulted from severe bottlenecks during queen hibernation. Moreover, representatives of identical clones were detected in different host species suggesting horizontal transmission taking place between species, underpinning the lack of host specific adaptation. Moreover, parasite populations were in Hardy-Weinberg equilibrium and showed no signs of linkage disequilibrium again suggesting that sexual reproduction is an alternative strategy. Occurrence of high levels of multiple infections also might facilitate this alternative type of reproduction (Chapter 4).

As the queen as the only individual is able to filter parasite strains (Ulrich et al., 2011), transition from one season to the next is a crucial time-point for the parasite in the colony life cycle of the host. This abovementioned 'bottleneck' complicates local adaptation of *C. bombi* to the host population. As young queens disperse while searching for a place to hibernate, they leave the population and new young queens might immigrate in

the population containing different *C. bombi* strains within them and establish their new colony after the winter. For *B. pascuorum* and *B. lapidarius* it is known that in these species young queens can disperse up to three to five km (Lepais *et al.*, 2010). Associated with that, the new bumblebee workers of the colonies sure will then serve as transmission factors for the parasite within the population over large spatial scales. Strains will again accumulate due to inter- and intraspecific transmission resulting again in drastic changes of parasite composition. The life cycle of the bumblebees surely is no active defense mechanism against the gut parasite, but it can be understood as a passive one backing the host's innate immune system to a certain extent. Dispersal of young queens serves as a strategy to maintain gene flow within host populations and also for the transmission of the generalist gut parasite *C. bombi*.

In addition, other factors can contribute to parasite's adaptation to its host, like adaptation to different host species. Although Erler *et al.* (2012) state that *C. bombi* is a generalist gut parasite due to the lack of host specific adaptation, Salathé and Schmid-Hempel (2011) demonstrated that the distribution of parasite genotypes is associated not only with host species, but also with niche overlap in flower choice. In regions with relatively high parasite prevalence, both factors contribute to the distribution of parasite strains, whereas in regions with lower parasite prevalence, niche overlap more strongly contributes to the observed distribution of parasite genotypes. This shows that

the quality of the host species is also an important factor for the adaptation of *C. bombi*. As Ruiz-Gonzalez *et al.* (2012) stated, variation in quality of the bumblebee host leads to variation of parasite growth within different species. Moreover, their findings coincide with Erler *et al.* (2012), when demonstrating variation in parasite population structure from one year to the next within individual host species at a given site due to the already mentioned 'bottlenecks'. In the end, host quality as well as transmission may lead to short-term host specialization by *C. bombi*, but repeated bottlenecking in combination with the parasite's clonal and sexual reproduction strategies results in the evolution of a generalist parasite of bumblebees.

As parasites can act as selective factors for given traits like host life history, behavior and immune defense (Allander & Schmid-Hempel 2000; Baer & Schmid-Hempel 1999), scientific focus was put on this interesting field of interaction, in particular the genotype-genotype interactions between the parasite and its host. The parasite strain as well as the origin of the host are two important factors for the success of infections with *C. bombi* (reviewed in Schmid-Hempel 2001). This was supported by a study of Schmid-Hempel *et al.* (1999) in which the genotypic composition of infections was directly assessed by microsatellite analysis. Riddell *et al.* (2009) showed differences in up-regulation of three different antimicrobial peptides (*hymenoptaecin*, *abaecin* and *defensin*) between different host genotypes in response to four different strains of *C. bombi*.

Moreover, transmission experiments have demonstrated a strong genetic component to *Crithidia* susceptibility. These studies have shown that the spread of a *Crithidia* infection is slower in genetically heterogeneous groups (Shykoff & Schmid-Hempel 1991a,b). Similarly, the parasite loads, including *Crithidia*, for individual workers and entire colonies are significantly reduced in the field when the colony is genetically heterogeneous (Liersch & Schmid-Hempel 1998; Baer & Schmid-Hempel 1999, 2001). These results suggest strong genotype by genotype interactions.

Recently, Martinson *et al.* (2011) but also Koch and Schmid-Hempel (2011a) detected a beneficial gut microbiota in honey- and bumblebees. In addition, Koch and Schmid-Hempel (2011b) could also demonstrate that manifestation of *C. bombi* is very much dependent on the presence or absence of this Gammaproteobacteria residing in the guts of *B. terrestris* workers. Interestingly, these Gammaproteobacteria are not shared with related solitary bee species. Transmission of these beneficial gut bacteria could therefore represent an important benefit of sociality and therefore boost social immunity. In contrast to the belief of the interactions between the host- and the parasite-genotype, other aspects seem to be responsible for the degree and the success of parasite manifestation. In 2012, Koch and Schmid-Hempel obtained striking results of their studies. They transplanted the gut microbiota between individuals of six different *B. terrestris*

colonies. The outcome revealed that the general infection load and the specific success of different *C. bombi* genotypes were mostly driven by the microbiota, rather than by worker genotype. Further, they stated that variation in gut microbiota can therefore be responsible for specific immune phenotypes and the evolution of gut parasites like *C. bombi* might be driven by interactions with 'microbiota types' as well as with host genotypes.

To that time when the parasite resides within the host queen, he has to circumvent the host's immune system. Therefore, the parasite has to evolve strategies to evade the innate immune system of the host, but also the host has to evolve strategies to defend itself from the parasite. Hence, this is what co-evolution is all about.

The antimicrobial peptides (AMPs) act against a broad range of parasites and pathogens and therefore are thought to be quite conserved and that this high degree of conservation of the immune system pathways across numerous insect taxa could perhaps be maintained due to protection against omnipresent saprophytes rather than acting specifically against certain pathogens and parasites (Hultmark, 2003). In order to test, whether selection is acting on AMPs, we are searching for evidence of positive and negative selection in those genes involved in host-parasite interactions. The argument is, if co-evolution as one possible factor is doing its bit to accelerate the accumulation of genetic variation, then positive selection should be more likely where co-evolution is acting, although this is not universally

accepted (Yang and Bielawski, 2000; Woolhouse *et al.*, 2002). On the other hand, if co-evolution does not accelerate accumulation of genetic variation, then negative or purifying selection should be more likely. One way to test for these kinds of selection is to look for deviations from neutral molecular evolution and to compare the ratios of non-synonymous (i.e. amino acid-changing) to synonymous (i.e. silent) nucleotide substitutions (Hudson *et al.*, 1987; Viljakainen *et al.*, 2009). Objective of investigation of molecular evolution was *hymenoptaecin* in two common bumblebee species in Central Europe, *B. terrestris* and *B. lapidarius*. The deviation from neutrality has been analysed using two different approaches. Using the HKA (Hudson-Kreitman-Aguade) test we found differences in the selective pressures and deviations from neutral evolution for *B. terrestris* in exon 2, which is under purifying selection, whereas *B. lapidarius'* exon 2 is not. In contrast, exon 1 of *B. lapidarius* shows higher levels of non-synonymous substitutions indicating more rapid evolution than in *B. terrestris*, but the HKA test was close to significance ($p = 0.09$). Another test, Tajimas's D did not reveal significant deviations from neutrality, which most likely is due to the vulnerability of the test to low sample sizes (Simonsen *et al.*, 1995; Larsson *et al.*, 2013). In addition, we could detect a balancing polymorphism in *B. lapidarius*: one in an intronic region of 174 bp and one codon (#44) in the mature peptide. Within the host species *B. lapidarius*, these polymorphisms of *hymenoptaecin* are spread widely within the population

across various sampling sub-sites within a transect of about 300 km in Germany. Therefore, they are not locally restricted to sub-populations of this bumblebee species. Hence, we found striking differences in the selection pressure on *hymenotaecin* in these two common bumblebee species, *B. terrestris* and *B. lapidarius*. After all, these findings finally suggest the assumption that the immune system and especially the AMPs may play an important role in the evolution of the bumblebees' immune defense towards specific parasite exposure. In cases where infections with *C. bombi* occur, supporting data (Parsche and Lattorff, unpublished data) show no connection between the polymorphism of this gene and the prevalence of this intestinal parasite of bumblebees at various sub-sites, but polymorphisms may be associated with lower infection intensity in the bumblebee host, at least in *B. lapidarius*.

Recent studies on the decline of pollinators (Biesmeijer *et al.*, 2006) have identified parasites and pathogens as a major factor contributing to this decline (Cameron *et al.*, 2011). Thus, the study of adaptation within the immune system of pollinator insects is essential for the understanding of potential mechanisms to resist parasites and pathogens in order to make predictions about the outcome of parasite / pathogen pressure on bee species.

In the end, we were able to show that different *C. bombi* strains show different phenotypes, i.e. growth rate. Successfully, we developed a method for cultivation of this bumblebee gut parasite in the laboratory to determine

growth rates of various different parasite strains. The effects of different growth rates were studied and discussed in the context of parasite infections in bumblebees of natural populations within one season and between two subsequent years. Molecular adaptation of an antimicrobial peptide was analyzed to increase the insight in the complex structure of *Bombus-Crithidia* co-evolution.

This newly obtained knowledge in the field of host-parasite interactions including parasite growth abilities, seasonal variability in parasitic prevalence (including multiple infections) in bumblebees within and between seasons, associated changes in parasite population and the molecular evolution of the host's innate immune system will help to expand our knowledge about systems of social insect pollinators serving as hosts for generalist (multiple-host) parasites.

Chapter 7

Zusammenfassung

Neben zahlreichen Arten co-evolutionärer Systeme wurde in der vorliegenden Arbeit der Fokus auf Wirt-Parasit Interaktionen gelegt. Diese besondere Beziehung ist gekennzeichnet durch eine eng verknüpfte Verbindung und den enormen wechselseitigen Einfluss beider, des Wirts und des Parasits, welcher einen starken gegenseitigen Selektionsdruck verursacht (Anderson and May, 1979a and b, 1981; Thompson, 1994). Diese Wechselwirkung beider kann durch ein evolutionäres Wettrüsten, auch bekannt als die „Red Queen Hypothese“ erklärt werden (Woolhouse *et al.*, 2002). Nach dieser Hypothese müssen sich Wirt und Parasit ständig anpassen und weiterentwickeln, während sie mit ihrem Gegenpart im Wettstreit stehen (Bell, 1982).

Im Speziellen wird hier auf das *Bombus/Crithidia* Modellsystem eingegangen, welches sich durch eine Multi-Parasit/Multi-Wirts Interaktion auszeichnet. Hier kommt es aufgrund intra- und interspezifischen Transmissionen zur Akkumulierung mehrerer Stämme des Darmparasiten der Hummel, *Crithidia bombi*, im Wirtsorganismus. Dieses Phänomen wird als multiple Infektion bezeichnet.

Es herrscht eine große Diversität an *C. bombi* Genotypen in natürlichen Hummelpopulationen vor. In der vorliegenden Arbeit wurde getestet, ob diese genotypischen Unterschiede mit unterschiedlichen phänotypischen Merkmalen (unterschiedliche Wachstumsraten verschiedener Genotypen) assoziiert sind, um die Unterschiede in der Verteilung von *C. bombi* zwischen Wirts-Individuen, -Kolonien, Populationen und/oder Arten zu erklären. Hier wurde eine

neue in vitro Kultivierungsmethode des Parasiten unter kontrollierten Laborbedingungen entwickelt, um eventuelle unterschiedliche Wachstumsraten verschiedenartiger *C. bombi* Genotypen zu ermitteln. Tatsächlich zeigen einzelne Parasitenstämme schnellere Wachstumsraten als andere Genotypen. Dies könnte vorteilhaft für den Parasit in seiner natürlichen Umgebung sein, wenn er mit anderen Genotypen um Ressourcen im Wirtskörper konkurriert.

Die Transmission von *C. bombi* könnte nun aber auch aufgrund demographischer Veränderungen des Wirtes über die Saison hinweg schwanken. Dies wiederum könnte zu einem Ansteigen der Prävalenz von Einzel- und multiplen Infektionen führen, wobei es bei der letzteren Art zu Konkurrenz der einzelnen Genotypen kommen könnte.

Während der Saison verändert sich die Parasitenpopulation drastisch. Generell ist das Parasitenvorkommen im Wirt im Hochsommer am größten. Dieser Zeitpunkt stellt den Zenit der Nahrungssuche in der Saison dar. *C. bombi* wird an Blüten von Individuum zu Individuum (intra- und interspezifisch) übertragen. Dieses Vorkommen deckt sich mit dem Vorhandensein multipler Infektionen. Beide Infektionsarten nehmen zum Ende der Saison hin ab. Konkurrenz innerhalb des Wirtes könnte dieses beobachtete Muster erklären. Dies wiederum führt zur Selektion eines bestimmten Allels eines Locus, in diesem Falle eines Mikrosatelliten-Locus, welcher höchstwahrscheinlich zu einem anderen wichtigen Gen gekoppelt ist. Diese Selektion eines Allels erhöht die Frequenz der homozygoten Genotypen dieses Locus. Wenig

später verringert sich der Konkurrenzkampf der verschiedenen Genotypen und negativ frequenzabhängige Selektion könnte zum Schwund dieses Allels führen. Darüber hinaus geben die Wiederherstellung der Heterozygotie und das Verschwinden des Kopplungsungleichgewichts einen deutlichen Hinweis auf genetischen Austausch in diesen, sonst sich klonal vermehrenden Trypanosomen.

Es gibt zahlreiche Hinweise darauf, dass *C. bombi* einen Parasit darstellt, welcher multiple Wirte befällt und somit mehrere Wirtshummelarten von ihm betroffen sind. Unterschiedliche Genotypen dieses Parasiten könnten lokal an bestimmte Wirtshummelarten angepasst sein (genetische Anpassung). Jedoch ist *C. bombi* starken Selektionsdrücken ausgesetzt. Das sog. „Strain filtering“ (Ulrich *et al.* 2011) und die Notwendigkeit der Übertragung in die nächste Saison durch die neuen Jungköniginnen stellen einen starken Einfluss auf die Ausbreitung der *C. bombi* Genotypen zwischen den Jahren dar (zeitliche Anpassung). Diese beiden Arten der Anpassung und deren Effekte wurden ebenfalls als mögliche Ursachen für die Ausbreitung der *C. bombi* Genotypen untersucht. Hierzu wurden mehrere Hummelarten in zwei aufeinander folgenden Jahren in ihren natürlichen Habitaten untersucht.

Zudem sollte die Lebensweise sozialer Insekten die Ausbreitung und Anpassung von Parasiten erheblich erleichtern. Wir fanden jedoch keine Anzeichen lokaler Anpassung der Parasiten an mehrere Wirtshummelarten (*B. terrestris*, *B. hortorum*, *B. pascuorum*, *B. lapidarius* and the cuckoo bumblebee *Bombus (Psithyrus) vestalis*). Jedoch fanden wir eine zeitliche Differenzierung der Parasitenpopulationen, welche durch die Jungköniginnen, als einzige Überträger der Parasiten in die nächste Saison („Flaschenhalswirkung“) zustande

gekommen sein könnte. Zudem wurden identische Klone derselben Genotypen in unterschiedlichen Hummelarten gefunden, was auf horizontale Transmission zwischen den einzelnen Hummelarten schließen lässt. Dies untermauert zusätzlich den Mangel an wirtsspezifischer Anpassung. Wie schon im vorigen Kapitel zu sehen war, befanden sich die Parasitenpopulationen im sog. Hardy-Weinberg Gleichgewicht und zeigten zusätzlich keine Anzeichen eines Kopplungsungleichgewichts. Dies deutet einmal mehr auf die Vermutung, dass sexuelle Reproduktion in *C. bombi* eine alternative Fortpflanzungsstrategie darstellt. Das zusätzliche Vorhandensein eines hohen Ausmaßes multipler Infektionen könnte außerdem diese alternative Reproduktionsstrategie erleichtern.

Während sich der Parasit im Wirtsorganismus befindet, ist er folglich dem Immunsystem des Wirtes ausgesetzt und muss versuchen, dieses zu umgehen. Somit muss der Parasit Strategien entwickeln, um dem angeborenen Immunsystem des Wirtes zu entkommen. Im gleichen Zuge muss auch der Wirt Strategien entwickeln, um sich vor dem Parasiten zu schützen oder ggf. zu wehren.

Antimikrobielle Peptide (AMPs) als Teil des angeborenen Immunsystems der Insekten wirken gegen ein breites Spektrum von Pathogenen und Parasiten und man nimmt an, dass sie daher ziemlich konserviert sind. Dieser hohe Grad an Konservierung der „Immune Pathways“ über zahlreiche Taxa hinweg könnte womöglich eher durch den Schutz vor allgegenwärtigen Saprophyten (Organismen, die ihren Nährstoffbedarf teilweise oder ganz aus toter organischer Substanz decken) aufrecht erhalten werden, als zur Abwehr von spezifischen Pathogenen und Parasiten (Hultmark, 2003). Um nun zu testen, welche Rolle AMPs bei der Pathogen-abwehr spielen, suchen wir sowohl nach Hinweisen

auf positive als auch negative Selektion in den Genen, welche in Wirts-Parasit Interaktionen involviert sind. *Hymenoptaecin* als ein Vertreter der antimikrobiellen Peptide zeigt starke Effekte bezüglich Genexpression nach bakterieller Infektion der Wirte (Erler *et al.* 2011), bei der Genotyp-Genotyp Wechselwirkung zwischen *C. bombi* und *B. terrestris* (Riddell *et al.* 2009) und scheint prophylaktisch angeschaltet zu werden, wenn erhöhte Infektionsgefahr besteht (Richter *et al.* 2012). Wir untersuchen, ob dieses AMP unterschiedliche molekulare Evolutionsmuster in zwei, in Mitteleuropa häufig vorkommenden Hummelarten (*B. terrestris* und *B. lapidarius*) aufweist. Die beiden untersuchten Hummelarten unterscheiden sich im Parasitenbefall mit *C. bombi*.

Hymenoptaecin scheint ziemlich konserviert über die untersuchten Hummelarten zu sein, da der Großteil dieses Gens entweder neutrale oder reinigende Selektion aufweist. Dennoch weisen einige Codons positive Selektion auf. Die Verteilung von Polymorphismen ist unterschiedlich zwischen den beiden Hummelarten. Wir stellen fest, dass der Polymorphismus in der reifen Peptidregion und der Haplotyp im Intron des Gens von *B. lapidarius* nicht auf einzelne lokale Fanggebiete begrenzt oder mit dem spezifischen Vorkommen von *C. bombi* assoziiert ist. Dennoch könnte ein Zusammenhang dieses Haplotypen mit geringerer Infektionsintensität von *C. bombi* in *B. lapidarius* bestehen.

Diese neugewonnenen Erkenntnisse im Bereich der Wirts-Parasit Interaktionen werden hilfreich sein, um unser Wissen über soziale Insekten in ihrer Rolle als Bestäuber und Wirte für generalistische Parasiten zu erweitern.

Chapter 8

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Mein aufrichtiger Dank gilt außerdem Herrn PD Dr. Michael H. G. Lattorff, der mir in den letzten Jahren als direkter Betreuer immer mit Rat und Tat zur Seite stand. Es war mir eine große Freude mit ihm zusammen beruflich, als auch privat die verschiedensten Aspekte, nicht nur im fachlichen Bereich, sondern auch die des alltäglichen Lebens zu diskutieren.

Herzlichen Dank auch an meine Co-Autoren!

Ich möchte auch nicht meine Kollegen der Arbeitsgruppe, sowohl ehemalige als auch die derzeitig anwesenden vergessen. Ich habe es sehr genossen, tagtäglich hierher zu kommen. Danke euch Antje, Dieter, Stephan, André, Sophie, Bertrand (Lord Handsome), Alexis, Ecki, John usw...

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Danke an die Jungs der Hall(e) Of Fame, meine anderen guten Freunde aus den Würzburg-Zeiten, die mich nicht vergessen haben. Ich freue mich immer wieder, euch zu sehen!

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Besonderer Dank geht auch an meine Familie, ohne die ich nicht so weit gekommen wäre!

Am meisten jedoch möchte ich meiner Frau Anne und meinen beiden Kindern Lorenz und seit Kurzem auch meiner kleinen Johanna danken, die immense Geduld mir gegenüber in den letzten Jahren aufgebracht haben. Anne, vielen Dank, dass Du meine oftmals schlechte Laune und mein mürrisches Wesen ausgehalten hast und große Geduld mit mir bewiesen hast, wenn's mal wieder nicht so lief, wie es denn hätte laufen sollen!!!

Appendix

A. Declaration of author contributions

Mario Popp and H. Michael G. Lattorff (2011) A quantitative in vitro cultivation technique to determine cell number and growth rates in strains of *Crithidia bombi* (Trypanosomatidae), a parasite of bumblebees.

Journal of Eukaryotic Microbiology **58(1)**: 7-10. (Erratum **58(1)**: 273).

I participated in the design of the project, developed all protocols for cultivation of the strains, performed the analyses and wrote the paper. H. M. G. Lattorff participated in the design of the project, the analyses, writing of the paper, supervised the work and provided helpful discussions.

Mario Popp, Silvio Erler and H. Michael G. Lattorff (2012) Seasonal variability of prevalence and occurrence of multiple infections shapes the population structure of *Crithidia bombi*, an intestinal parasite of bumblebees (*Bombus* spp.)

MicrobiologyOpen (2012) **1(4)**: 362–372.

I participated in the design of the project, collected and genotyped the bumblebee samples, performed the analyses and wrote the paper. Silvio Erler participated in the analyses and writing of the paper. H. Michael G. Lattorff participated in the design of the project, the analyses, writing of the paper, supervised the work and provided helpful discussions.

Silvio Erler, **Mario Popp**, Stephan Wolf, H. Michael G. Lattorff (2012) Sex, horizontal transmission, and multiple hosts prevent local adaptation of *Crithidia bombi*, a parasite of bumblebees (*Bombus* spp.)

Ecology & Evolution **2(5)**: 930-940

Silvio Erler participated in the design of the project, collected and genotyped the bumblebee samples, performed the analyses and wrote the paper. I participated in the analyses and writing of the paper. Stephan Wolf provided bumblebee samples including the genotypes and provided helpful comments on the manuscript. H. M. G. Lattorff participated in the design of the project, the analyses, writing of the paper, supervised the work and provided helpful discussions.

Mario Popp, Sophie Helbing, Susann Parsche, Silvio Erler and H. Michael G. Lattorff (2013) Divergent patterns of selection on an antimicrobial peptide in common species of bumblebees

I participated in the design of the project, genotyped the bumblebee samples, performed the analyses and wrote the paper. Sophie Helbing participated in the design and analyses. Susann Parsche collected the bumblebee samples and extracted the DNA. Silvio Erler helped with the first analyses and provided helpful comments on the manuscript. H. Michael G. Lattorff participated in the design of the project, the analyses, writing of the paper, supervised the work and provided helpful discussions.

B. Curriculum vitae

Personal Information

Date of Birth: March 19th 1981 in Schweinfurt (Bayern), Germany
Marital Status: married, one child
Languages: German: native speaker
 English: excellent knowledge written and spoken
 French: basic knowledge written and spoken

Professional Career

09/2011-present: PhD student/research assistant at the Martin-Luther-University, Halle-Wittenberg.
04/2008-09/2011: PhD student/research assistant at the Martin-Luther-University, Halle-Wittenberg.
 Dissertation Thesis concerned with "*Bombus* vs. *Crithidia*: Population Genetic Analysis of Parasite Interactions with Multiple Hosts" within the FUGABEE-Project (funded by BMBF)

Academic Education

09/2006-07/2007: Graduate thesis at the Department of Behavioral Physiology and Sociobiology (Zoologie II), AG Dr. Heike Feldhaar: "Establishing an Immunoassay for *Camponotus floridanus*"
10/2001-07/2007: Studies of Biology (Diploma) at the Julius-Maximilians-University, Würzburg
07/2007: Diploma (very good)
10/2003-07/2007: **Main Studies:**
Major subject: Behavioral Physiology/Sociobiology
Minor subjects: Ecology of Animals/Tropical Biology, Human Genetics, Genetics, Microbiology, Cell- and Developmental Biology (e.g. in Marine Organisms), Pathogen and Parasite Biology
09/2003: Pre-degree in Biology and Chemistry
09/2002: Pre-degree in Physics

School Career

09/1991-07/2000: Secondary school – Walther-Rathenau, Schweinfurt
 Degree: Abitur (good)
09/1987-07/1991: Elementary school, Schwanfeld

C. Publication list

Peer-reviewed articles

Mario Popp and H. Michael G. Lattorff (2011) A quantitative in vitro cultivation technique to determine cell number and growth rates in strains of *Crithidia bombi* (Trypanosomatidae), a parasite of bumblebees.
Journal of Eukaryotic Microbiology **58(1)**: 7-10. (Erratum **58(1)**: 273).

Silvio Erler, **Mario Popp** and H. Michael G. Lattorff (2011) Dynamics of immune system gene expression upon bacterial challenge and wounding in a social insect (*Bombus terrestris*).
PLoS ONE **6(3)**: e18126

Silvio Erler, **Mario Popp**, Stephan Wolf, H. Michael G. Lattorff (2012) Sex, horizontal transmission, and multiple hosts prevent local adaptation of *Crithidia bombi*, a parasite of bumblebees (*Bombus* spp.)
Ecology & Evolution **2(5)**: 930-940

Mario Popp, Silvio Erler and H. Michael G. Lattorff (2012) Seasonal variability of prevalence and occurrence of multiple infections shapes the population structure of *Crithidia bombi*, an intestinal parasite of bumblebees (*Bombus* spp.)
MicrobiologyOpen (2012) **1(4)**: 362–372.

Popular Articles

Mario Popp, Silvio Erler and H. Michael G. Lattorff (2009): FUGABEE: Funktionelle Analyse von Krankheitsresistenzgenen bei der Erdhummel (*Bombus terrestris*).
Genomexpress **4.09**: 7-10

D. Oral Presentations

- Mario Popp**, Sophie Helbing, Susann Parsche, Silvio Erler and H. Michael G. Lattorff: "Divergent patterns of selection on an antimicrobial peptide in two common bumblebees (*Bombus* spp.)."
3rd Central European Meeting of the International Union for the Study of Social Insects (IUSSI) (Cluj-Napoca, Romania - March 2013)
- Mario Popp**, Silvio Erler and H. Michael G. Lattorff: "Population structural changes of an intestinal parasite of bumblebees (*Bombus* spp.) in the course of one season."
5th European Conference of Apidology (Halle (Saale), Germany – September 2012)
- Mario Popp**, Silvio Erler and H. Michael G. Lattorff: "Seasonal variability of prevalence and the presence of multiple infections shape the population structure of an intestinal parasite of bumblebees (*Bombus* spp.)"
59. Annual Meeting of „Arbeitsgemeinschaft der Institute für Bienenforschung e.V.“ (Bonn, Germany - March 2012)
- Mario Popp**, Silvio Erler and H. Michael G. Lattorff: "Temporal variation of parasitism in bumblebees (*Bombus* spp.) by *C. bombi* (Trypanosomatidae) on a local scale".
2nd Central European Meeting of the International Union for the Study of Social Insects (IUSSI) (Papenburg, Germany - March 2011)
- Mario Popp** and H. Michael G. Lattorff: "*Crithidia bombi* - In vitro cultivation of a gut parasite of bumblebees".
1st Central European Meeting of the International Union for the Study of Social Insects (IUSSI) (Fraueninsel in Lake Chiemsee, Germany - October 2009)
- Mario Popp** and H. Michael G. Lattorff: "In vitro cultivation of *Crithidia bombi* - a visceral parasite of bumblebees"
56. Annual Meeting of „Arbeitsgemeinschaft der Institute für Bienenforschung e.V.“ (Schwerin, Germany - March 2009)

E. Poster Presentations

- Mario Popp**, Silvio Erler and H. Michael G. Lattorff: "Temporal variation of parasitism in bumblebees (*Bombus* spp.) by *C. bombi* (Trypanosomatidae) on a local scale".
XVI International Congress of the International Union for the Study of Social Insects (IUSSI) (Copenhagen, Denmark – August 2010)
- Mario Popp** and H. Michael G. Lattorff: "In vitro cultivation of the trypanosome *Crithidia bombi*, a parasite of bumble bees". 2. FUGATO-Statusseminar (Kassel, Germany - October 2009)
- Mario Popp** and H. Michael G. Lattorff: "In vitro cultivation of the trypanosome *Crithidia bombi*, a parasite of bumble bees". 102. Annual Meeting of „Deutsche Zoologische Gesellschaft“ (DZG) (Regensburg, Germany - September 2009)
- Heike Feldhaar and **Mario Popp**: "Immunity in a social context: Do infected ants signal that they are ill? "
ESF-FWF conference (Obergurgl, Austria – April 2007)

Erklärung

Halle (Saale), den 02. 10. 2013

Hiermit erkläre ich an Eides statt, dass diese Arbeit von mir bisher weder an der Naturwissenschaftlichen Fakultät I - Biowissenschaften der Martin-Luther-Universität Halle-Wittenberg noch einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion eingereicht wurde.

Ich erkläre weiterhin, dass ich mich bisher noch nicht um den Doktorgrad beworben habe. Ferner erkläre ich, dass ich diese Arbeit selbstständig und nur unter Zuhilfenahme der angegebenen Quellen und Hilfsmittel angefertigt habe.

Mario Popp