Mapping quantitative trait loci for larval disease resistance in honeybees (*Apis mellifera* L.) using haploid drones

D i s s e r t a t i o n

zur Erlangung des
Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

der

Naturwissenschaftlichen Fakultät I – Biowissenschaften –

der Martin-Luther-Universität
Halle-Wittenberg,

von

Herrn Dieter Behrens

geb. am 04.10.1980 in Hamburg

Gutachter / innen

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Halle (Saale), den 13.03.2014

Promotionsgesuch eingereicht am: 19.11.2013

Tag der öffentlichen Verteidigung: 12.03.2014

“Understanding the laws of nature does not mean that we are immune to their operations.”

David Gerrold (*1944)

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Abbreviation list

A. cerana	*Apis cerana*
A. mellifera	*Apis mellifera*
AFB	American Foulbrood
ANOVA	analysis of variance
ATCC	American type culture collection
BSA	bulk segregant analysis
CCD	colony collapse disorder
cfu	colony forming units
chitA	chitinase A
cM	centiMorgan
COI	cytochrome oxidase subunit I
df	degrees of freedom
DNA	deoxyribonucleic acid
ERIC	enterobacterial repetitive intergenic consensus
Kbp	kilo base pairs
LD	lethal dose
LOD	logarithm of the odds
M-W-U test	Mann-Whitney *U* test
MAS	marker-assisted selection
Mb	megabase
n	sampling size
NGS	next-generation-sequencing
p	probability
PCR	polymerase chain reaction
P. larvae	*Paenibacillus larvae*
qPCR	quantitative real-time PCR
QTL	quantitative trait locus
RAD	restriction-site associated DNA (-sequencing)
RNA	ribonucleic acid
SE	standard error
SNP	single nucleotide polymorphism
V. destructor	*Varroa destructor*

General introduction

Honeybees (*Apis mellifera* L.) are eusocial insects belonging to the order Hymenoptera. Their significance for mankind results partly from the variety of apicultural products they collect and produce (honey, wax, pollen, propolis, royal jelly), but also from the economical value of many agricultural plants they pollinate (e.g. rape, maize, almonds, many fruit). The pollination services of honeybees do not only result in immediate economical benefit, but also play an important ecological role in conserving the integrity of global ecosystems (Brown and Paxton 2009).
Honeybees have furthermore become model organisms in a number of scientific fields including the evolution of sociality, task allocation, swarm intelligence, learning and pathology.

Honeybee pathology includes both applied perspectives for diagnosis, control and disease prevention, as well as evolutionary questions on how pathogens and hosts coevolve in an eusocial context, where selection takes place on an additional level, i.e. the colony level. This additional level is of great scientific interest, given that sociality is an extremely successful evolutionary concept (Wilson 1971) and pathogens are regarded to be key drivers in evolution (Thompson 1994). Over the past decades honeybees emerged as an insect model organism for pathology, since they do not only share the advantages of many insect species, such as the availability of a large number of related individuals in short time spans, low maintenance costs, space requirements and ethical constrains as well as an available genome sequence, but in addition their haplodiploid sex determination and the exceedingly high recombination rate of 22 centiMorgan per Megabase (cM/Mb) (Solignac *et al.* 2007) make them superior among the eusocial hymenoptera especially for genetic studies.
As a consequence of these features and their economical importance, the field of honeybee pathology has recently seen remarkable growth. Honeybees, and eusocial colonial insects in general, are exposed to a larger pathogen pressure compared to solitary insects, since their colonies offer favourable conditions for many parasites, e.g. protection and a large amount of food resources (honey, pollen, wax, larvae) (Schmid-Hempel 1998). Moreover, the accurate regulation of temperature and humidity as well as the presence of many closely related and regularly interacting individuals in the colony provide ideal conditions for many microbial pathogens. Consequently numerous parasites and pathogens are known from honeybees and

have been studied in greater detail than in other eusocial insects (ants, bumblebees, wasps, termites). These pests span a huge organismal variety of taxa including pathogenic bacteria (e.g. *Paenibacillus larvae* (Genersch 2010); *Melissococcus plutonius* (Forsgren 2010); *Spiroplasma apis* (Mouches *et al.* 1983)), protozoans (*Crithidia mellificae*; *Malpighamoeba mellificae*), fungi (*Nosema sp.* (Fries 2010); *Ascosphaera apis* (Aronstein and Murray 2010)) and viruses (de Miranda *et al.* 2010; de Miranda and Genersch 2010). The macroparasites of honeybees include parasitic mites (*Varroa destructor* (Rosenkranz *et al.* 2009); *Acarapis woodi*; *Tropilaelaps sp.*), predatory insects and nest parasites (e.g. the small hive beetle *Aethina tumida* (Neumann and Ellis 2008); the wax moth *Galleria mellonella*), the phorid fly *Apocephalus borealis* (Core *et al.* 2012) and even other honeybee subspecies as social nest parasites (*Apis mellifera capensis*) (Moritz *et al.* 2011). Some of these macroparasites additionally act as vectors for viruses and bacteria.

The unusually high losses of honeybee colonies in the USA from 2006 to 2010, which were called 'colony collapse disorder' (CCD), and subsequent reports of high over-winter colony losses in Europe and Asia alerted scientists and politicians worldwide and drew the public attention towards the economical and ecological importance of honeybees. As a consequence several actions of research facilities were launched on national and international scale (Genersch *et al.* 2010; Neumann and Carreck 2010; Pettis and Delaplane 2010). The elevated levels of over-winter colony losses could not be explained by a single factor, but seemed to be most likely caused by the interaction of many factors including pathogen (co-)infections (mainly *Varroa*, *Nosema*, viruses) (Dainat *et al.* 2011; 2012), pesticides and poor nutrition (Bromenshenk *et al.* 2010; van Engelsdorp *et al.* 2009). Since pathogen infections are a key driver not only in honeybees but also in worldwide pollinator declines (Potts *et al.* 2010), strategies to fight these diseases are highly desirable. Chemical treatments have been found to be problematic since residues may end up in the apicultural products, endanger the consumer's health (Wilson 1974) and may negatively effect the bees (Boncristiani *et al.* 2012; Gill *et al.* 2012; Pettis *et al.* 2012). Furthermore, several pathogens have developed resistances to specific chemicals (e.g. antibiotics, acaricides (Evans 2003)) limiting the effectiveness of these treatments. Therefore the preferred strategy is the development of sustainable beekeeping techniques and the production of tolerant honeybee strains.

Breeding for disease tolerance is usually very time-consuming since phenotypes need to be measured in every generation and experimental crosses need to be done in large numbers.

Nevertheless, great effort has been put into breeding disease tolerant honeybee lines, especially against the *Varroa* mite (Bourgeois and Rinderer 2009; Danka *et al.* 2011; Ibrahim *et al.* 2007; Rinderer *et al.* 2010) and into the production of *Varroa* tolerant populations by natural selection (Fries *et al.* 2006; Le Conte *et al.* 2007). These breeding efforts could be much more efficient if genes or genomic regions that influence the desired phenotype would already be known. By using a marker-assisted approach this process could be accelerated since only individuals with the desired allele combinations would need to be crossed and phenotypes would only need to be measured in order to verify the outcome of the selective crosses (Lande and Thompson 1990).

One way to identify genomic regions that control the desired phenotype, is genetic mapping or mapping of quantitative trait loci (QTL), which is widely used in humans (Altshuler *et al.* 2008), domesticated plants (Hall *et al.* 2010), and livestock (Goddard and Hayes 2009). In this technique the genomes of individuals with known phenotypes are searched for nucleotide sequence differences and those genomic elements (e.g. genes) that influence the investigated trait are identified. For this purpose usually the allele frequencies of evenly distributed and selectively neutral markers, e.g. single nucleotide polymorphisms (SNPs), enzyme restriction sites or repetitive elements (microsatellites), are used. Those regions in which a biased distribution of the marker alleles correlates with phenotypic differences are suggested to control the phenotype.

In honeybees QTL-mapping has been used up to date to identify genomic regions influencing a number of behavioural, physiological and morphological traits (Ammons and Hunt 2008; Arechavaleta-Velasco and Hunt 2004; Graham *et al.* 2011; Guzmán-Novoa *et al.* 2002; Hunt *et al.* 1995; 1998; 1999; Lapidge *et al.* 2002; Lattorff *et al.* 2007; Linksvayer *et al.* 2009; Mougel *et al.* 2012; Oxley *et al.* 2008; 2010; Page *et al.* 2000; Rüppell *et al.* 2004; Rueppell *et al.* 2011).
So far only few QTL-studies have used haploid drones in honeybees (Chandra *et al.* 2001; Rueppell *et al.* 2006) as well as in other hymenoptera (Gadau *et al.* 2002; Pannebakker *et al.* 2004; Rütten *et al.* 2004; Wilfert *et al.* 2007), probably because drones are usually less abundant and seasonally restricted in the colony and do not express many behavioural traits that are of interest in an economical context (e.g. foraging, brood care). However, regarding pathological questions drones often do produce measurable phenotypes and also act on the epidemiology, as they do become infected by most honeybee pests. Furthermore, haploid

drones offer the huge advantage for QTL-mapping studies in honeybees (and other hymenoptera) that a genetically very simple mapping population may be composed of the haploid drone offspring of a single honeybee queen. Thereby the number of alleles for each marker is restricted to only two. The haploid genome of the individuals also results in potentially very distinct phenotypes since all interactions between homologous alleles are excluded. With this approach it is therefore possible to detect QTL with a higher precision and a lower number of individuals than in mapping populations consisting of diploid individuals.

The principle of QTL-mapping of disease resistance in honeybees using drones is illustrated in Figure 1. To enhance the phenotypic segregation of the mapping population, a heterozygous honeybee queen is useful (Figure 1), ideally resulting from a cross between a susceptible and a resistant honeybee line. Such a queen will produce haploid drone offspring at equal rates carrying either the susceptible (red) or the resistant (blue) allele. In a first selective step, the so called bulk segregant analysis (BSA), DNA of individuals of a common resistance phenotype will be pooled. The DNA pools will then be screened for differences in the frequency ratio of the neutral (in our case microsatellite) markers. Markers that show a biased (and opposed) allele distribution in the pools (markers **A**, **B**, **C** and **D**) must be linked to a gene (or QTL) influencing the resistance phenotype. Due to crossover events more distant markers (markers **A** and **D**) will show a decreasing bias with increasing distance to the QTL. Markers that appear at equal frequencies in both pools have to be unlinked to any QTL (marker **X**). In subsequent steps candidate regions will need to be confirmed by individual genotyping and higher resolved by fine mapping using additional markers in these regions. If the marker map is sufficiently dense and the genome sequence is known, it is possible to identify the gene(s) that control the observed phenotype. The size of the effect can be taken as a measure for the relevance of each QTL for the investigated trait.

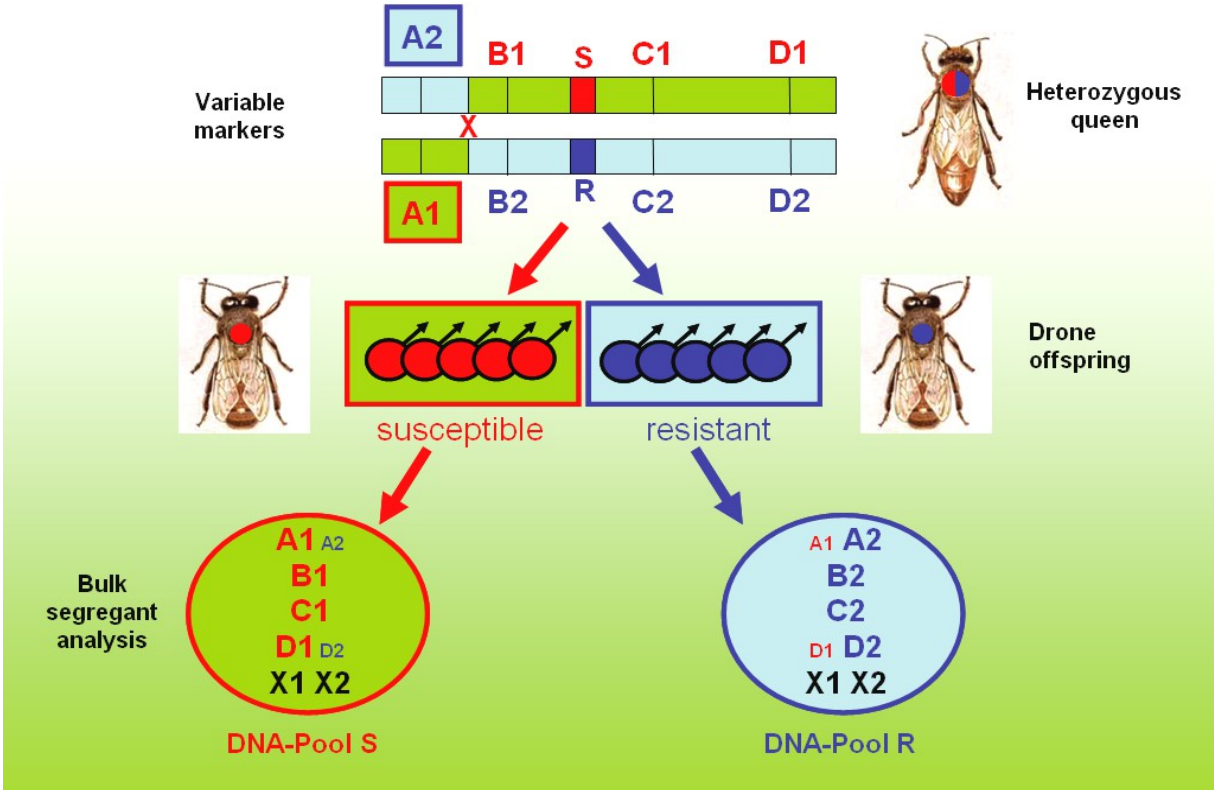

Figure 1: (from Behrens and Moritz 2011) Bulk Segregant Analysis (BSA): A heterozygous queen, carrying alleles for susceptibility (**S**) and resistance (**R**), produces haploid drone offspring of both phenotypes at equal frequencies. The microsatellite marker alleles, linked to a major gene (QTL) influencing the resistance phenotype, will cosegregate with the phenotype and appear at unequal frequencies in the DNA pools. Alleles of closely linked markers (**B** and **C**) will cosegregate completely, whereas allele frequencies of more distant markers (**A** and **D**) will approach an equal distribution between DNA pools with increasing distance due to recombination events (**x**). Unlinked markers (**X1 X2**) will always appear at equal frequencies in both pools.

This thesis aims to identify QTL influencing individual larval disease resistance using haploid drones focussing on two of the most detrimental pests of the honeybee which also cause the greatest damage to apiculture. The first one is the bacterium *Paenibacillus larvae*, which causes the highly contagious brood disease American Foulbrood (AFB), leading to the death of subsequent generations of larvae, and thereby unlike most other honeybee diseases ultimately to the death of the whole colony. The second is the ectoparasitic mite *Varroa destructor*, which is the most prominent and best studied parasite in the honeybee and has developed its devastating effect after a host switch from the Eastern honeybee (*Apis cerana*), where it only reproduces in the numerically and temporarily limited drone brood. In the Western honeybee (*Apis mellifera*) it also reproduces in worker brood thereby reaching much

higher population sizes within the honeybee colony. While the *Varroa* mite does not directly kill its host by sucking hemolymph from the infested larvae, it acts as vector for many viruses which may reduce the life span of adults decreasing the colony strength, and thereby leading especially in temperate climates over winter to the death of entire colonies.

Aims of this thesis

The aims of this thesis are to

1. evaluate haploid drones as study organisms for QTL-studies on disease tolerance in honeybees and
2. identify major QTL affecting individual larval resistance against two major pests of honeybees, namely *Paenibacillus larvae* and the *Varroa* mite.

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Chapter 1: Lethal infection thresholds of *Paenibacillus larvae* for honeybee drone and worker larvae (*Apis mellifera*)

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Summary

We compared the mortality of honeybee (*Apis mellifera*) drone and worker larvae from a single queen under controlled *in-vitro* conditions following infection with *Paenibacillus larvae*, a bacterium causing the brood disease American Foulbrood (AFB). We also determined absolute *P. larvae* cell numbers and lethal titres in deceased individuals of both sexes up to eight days post infection using quantitative real-time PCR (qPCR). Our results show that in drones the onset of infection induced mortality is delayed by one day, the cumulative mortality is reduced by 10% and *P. larvae* cell numbers are higher than in worker larvae. Since differences in bacterial cell titres between sexes can be explained by differences in body size, larval size appears to be a key parameter for a lethal threshold in AFB tolerance. Both means and variances for lethal thresholds are similar for drone and worker larvae suggesting that drone resistance phenotypes resemble those of related workers.

Environmental Microbiology (2010) 12 (10), 2838-2845.
(Impact factor 2012: 5,756)
Received: 27 January 2010; Accepted: 09 April 2010; Published online: 01 June 2010
Full version online at http://onlinelibrary.wiley.com.

Chapter 2: QTL-mapping of individual resistance against American Foulbrood in haploid honeybee drone larvae (*Apis mellifera*)

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Abstract - American Foulbrood (AFB) is a severe brood disease in honeybees. Since sustainable treatment is not available, selection of genetically resistant honeybee stock is highly desirable. Using a set of 291 heterozygous microsatellite markers in a bulk segregant analysis with subsequent finemapping of haploid drone offspring from a single honeybee queen we identified one significant and three suggestive quantitative trait loci (QTL) as well as one significant epistatic interaction influencing prepupal survival after AFB infection. While we were not able to verify specific genes responsible for tolerance we suggest that developmental genes may have played an important role. The identified markers can be used as regions of interest in future mapping or expression studies. In order to use them for marker assisted selection in breeding programmes for AFB resistant honeybee stock it will be required to evaluate these loci more extensively under variable experimental conditions.

Apidologie (2013) (*published online*), doi: 10.1007/s13592-013-0255-0.
(Impact factor 2012: 2,155)
Received: 01 March 2013; Accepted: 31 October 2013; Published online: 29 November 2013
Full version online at http://link.springer.com.

Chapter 3: Three QTL in the honey bee *Apis mellifera* L. suppress reproduction of the parasitic mite *Varroa destructor*

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Ecology and Evolution (2011) 1 (4), 451-458.

(Impact factor 2012: 1,184)

Received: 01 June 2011; Accepted: 19 July 2011; Published online: 01 November 2011

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Abstract

Varroa destructor is a highly virulent ectoparasitic mite of the honey bee *Apis mellifera* and a major cause of colony losses for global apiculture. Typically, chemical treatment is essential to control the parasite population in the honey bee colony. Nevertheless a few honey bee populations survive mite infestation without any treatment. We used one such *Varroa* mite tolerant honey bee lineage from the island of Gotland, Sweden, to identify Quantitative Trait Loci (QTL) controlling reduced mite reproduction. We crossed a queen from this tolerant population with drones from susceptible colonies to rear hybrid queens. Two hybrid queens were used to produce a mapping population of haploid drones. We discriminated drone pupae with and without mite reproduction, and screened the genome for potential QTL using a total of 216 heterozygous microsatellite markers in a bulk segregant analysis. Subsequently we fine mapped three candidate target regions on chromosomes 4, 7 and 9. Although the individual effect of these three QTL was found to be relatively small, the set of all three had significant impact on suppression of *V. destructor* reproduction by epistasis. Although it is in principle possible to use these loci for marker assisted selection (MAS), the strong epistatic effects between the three loci complicates selective breeding programs with the Gotland *Varroa* tolerant honey bee stock.

Summary

The first aim of this thesis was to evaluate haploid drones as study organisms for QTL studies on disease tolerance in honeybees. Their simple genetic makeup predisposes them as preferred study objects for basically any study where a reduced complexity on the individual and / or the populational level is desired. In this regard honeybee drones would also function as models for genetic studies in other hymenoptera. Therefore it needed to be tested whether or to which extent haploid individuals represent the situation in their diploid conspecifics.

In the first chapter we therefore compared honeybee drones and workers in their prepupal survival after an infection with the bacterial pathogen *Paenibacillus larvae* causing American Foulbrood (AFB). We concluded that honeybee drones may replace workers in studies on individual AFB resistance and provided an estimation of lethal bacterial cell numbers in both worker and drone larvae, as well as a new method to measure the bacterial titres at which larvae succumb to the disease. However, we did only test individuals of one genetic origin and not whether drones and workers of a common origin resemble one another more closely than unrelated individuals do. Therefore our study can be taken only as preliminary result for the suitability of drones to assess AFB resistance in workers. Also their suitability for research on other features of workers (e.g. other traits, disease resistances) will need to be verified independently. However, since the advantages of drones in theory are immense, we would like to encourage the usage of haploid drones as study organisms in future studies on functional genomics in hymenoptera where possible. This is especially true for mapping studies, since QTL may be identified using comparably few individuals, as indicated by the results from chapters two and three. It would be interesting to quantify the advantage of a mapping population constructed of haploid individuals, e.g. in terms of required number of individuals and QTL detection limits, compared to one composed of diploid individuals by using a mathematical model.

The bacterial *P. larvae* titres measured in the first chapter were found to be variable among individuals which suggests the existence of a genetically determined lethal threshold. We proposed that this trait was normally distributed among the offspring of a common genetic background, and may be used as tolerance parameter in AFB studies. Since the onset of infection-induced mortality correlated with developmental differences in drone and worker larvae and the observed differences were dependent on body size, we concluded that mortality after AFB infection is possibly triggered by a certain concentration of *P. larvae* cells in the

host. As alive but infected larvae often appear asymptomatic, a quorum sensing process, in which the bacteria produce the toxic compounds only at a given bacterial concentration in the host's midgut, seems possible. In that case the timing of mortality and larval peritrophic matrix degradation (Garcia-Gonzalez and Genersch 2013) would be mainly dependent on the bacterial versus the larval growth rates, which would concern developmental rather than immunological properties of the host. These findings contribute a new concept to AFB pathology and AFB resistance that would be worthwhile to test in more detail (e.g. the parameter space).

The second aim of this thesis was to use haploid drones to identify major loci affecting larval resistance against two major pests of honeybees, namely AFB and the *Varroa* mite.
In the case of larval AFB resistance we identified one significant and three suggestive QTL as well as one significant epistatic interaction, together explaining 41 % of the observed phenotypic variance in larval survival after AFB infection. While classical immune genes (e.g. antimicrobial peptides) were not among the candidate genes, a remarkably high number of developmental genes (transcription factors, homeobox genes) was found within the identified QTL regions. This led to the conclusion that larval development may play an important role in AFB resistance, supporting the results from the first chapter.
However, before these QTL regions should be used as targets in selective breeding programmes, future studies will need to confirm the identified loci using different genetic backgrounds of hosts and pathogens and re-evaluate their significance on this larger scale taking the mean effect of these QTL into account. Since we used natural variation and not selected extremes in our mapping population it will be also necessary to explore the allelic range of these QTL (and identify additional ones) by screening other honeybee lines or races. It will then be possible to advice beekeepers, which honeybee lines possess the highest individual AFB resistance, and / or to cross resistance alleles specifically into existing honeybee lines using a marker-assisted approach.
In the case of larval *Varroa* resistance we identified one significant and two suggestive QTL for larval suppression of mite reproduction. Since strong epistatic interactions of the three loci were found in our mapping population, we concluded that breeding attempts using these loci might be complicated. It would nevertheless be interesting to verify these loci in further *Varroa* tolerant lines (Bourgeois and Rinderer 2009; Kefuss *et al.* 2004; Le Conte *et al.* 2007) and investigate if or how these loci influence the host-finding process and oogenesis of the *Varroa* mite, which has been attributed to compounds of the larval cuticle (Frey *et al.* 2013).

It has further been shown that suppression of mite reproduction but not hygienic behaviour (Locke and Fries 2011) has been selected for as *Varroa* tolerance mechanism in two naturally selected populations in Sweden and France (Locke *et al.* 2012). As this route has been taken by natural selection in two independent populations, it seems to be less costly for the fitness of the host. Future breeding programmes should therefore focus on the selection of a reduced mite reproduction rather than on an improved general hygienic behaviour, as mainly done in the past (Boecking and Spivak 1999; Ibrahim *et al.* 2007; Rinderer *et al.* 2010; Spivak and Reuter 2001a; b). The selection of a trait which is energetically less costly for the honeybee colony will most likely also result in a greater economical benefit for the beekeeper.

In both of our cases the identification of a few phenotypically responsible genes was not possible although some promising candidates were found. While for *Varroa* resistance one of the candidate genes was supported by expression data (Navajas *et al.* 2008), a recent study for expression after AFB infection (Cornman *et al.* 2013) does not support any of the candidate genes in our QTL regions. Although expression data may provide strong support for a specific candidate gene, the opposite case does not invalidate an identified QTL-region since these techniques aim at different groups of genes. QTL-mapping primarily identifies *qualitative* differences between alleles of genes upstream those gene cascades that control the investigated trait. These genes potentially generate a large phenotypic variance and are therefore the most interesting ones in the context of breeding. In contrast, expression studies rather measure *quantitative* (expression) differences of the corresponding downstream genes. While it is possible that also upstream genes may alter their expression patterns in response to an infection, it is not required to generate a large phenotypic effect.

In order to reduce the size of our QTL regions and thereby the number of candidate genes, an increased marker density and a larger number of individuals would be needed. Both would also reduce the probability of a non-detection of QTL. Most likely upcoming studies will therefore not rely on microsatellite markers but on the more abundant SNPs (Arechavaleta-Velasco *et al.* 2012; Holloway *et al.* 2012; Tsuruda *et al.* 2012). Additionally, the higher resolving next-generation-sequencing (NGS) techniques (e.g. restriction-site associated DNA (RAD)-sequencing (Baxter *et al.* 2011)) will allow for a higher accuracy in the determination of the allele frequencies in the DNA pools. These techniques will also make the finemapping and the genotyping of individuals obsolete and only require a single-step analysis of bulked DNA samples. Once specific genes have been identified to cause the resistance phenotypes,

the sequencing of different alleles may then allow to study the nature of the resistance trait on a functional molecular level.
However, the detection by QTL-mapping is not a functional proof. Therefore bioassays will need to verify the outcome of any marker-assisted breeding programme. Alternatively the change of allele frequencies after a selection pressure and the speed at which specific alleles become fixated could be measured by consecutive sampling of populations under selection. These three techniques together - QTL-mapping, whole transcriptome analyses (e.g. RNA-sequencing) and the record of the temporal course of allele frequencies in populations under selection (e.g. the Gotland population) - will allow to evaluate the importance of the identified QTL and use them efficiently in an applied context in apiculture.

The availability of the honeybee genome (Weinstock *et al.* 2006), a growing number of available genomes of honeybee pests (Chan *et al.* 2011; Chen *et al.* 2013; Cornman *et al.* 2009; 2010; Lanzi *et al.* 2006; Nguyen and Le 2013; Okumura *et al.* 2011; Ongus *et al.* 2004; Qin *et al.* 2006) and of honeybee cell lines (Goblirsch *et al.* 2013; Kitagishi *et al.* 2011) indicate that honeybee pathology has clearly entered the molecular stage. The identification of disease resistance genes in the honeybee as well as of virulence factors, e.g. in *P. larvae* (Antúnez *et al.* 2011a; b; Fünfhaus *et al.* 2013; Poppinga *et al.* 2012), will allow to study gene-to-gene interactions in this economically important host-parasite system and target new questions such as, how coadaptations have shaped the affected genomes and how selection pressures change the allele frequencies within populations over time. The molecular level will thereby enable scientists by using the honeybee as a model organism to study pathological as well as evolutionary questions integrally.

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Zusammenfassung

Das erste Ziel dieser Arbeit war es, zu beurteilen, ob haploide Drohnenlarven als Studienorganismen für QTL-Studien zur Krankheitstoleranz bei Honigbienen geeignet sind. Ihr einfacher genetischer Aufbau macht sie zum bevorzugten Studienobjekt für praktisch jede Art von Studie, in der eine reduzierte Komplexität auf individueller und / oder Populationsebene erwünscht ist. In dieser Hinsicht würden Honigbienendrohnen auch als Modell für genetische Studien an anderen Hymenopteren dienen. Dafür musste getestet werden, ob und in welchem Ausmaß die Merkmalsausprägungen haploider Individuen denen ihrer diploiden Artgenossen entsprechen.

Im ersten Kapitel haben wir daher Honigbienendrohnen mit -arbeiterinnen verglichen, hinsichtlich ihres Überlebens während des Vorpuppenstadiums nach Infektion mit dem bakteriellen Pathogen *Paenibacillus larvae*, das Amerikanische Faulbrut (AFB) verursacht. Wir schlussfolgerten, dass Honigbienendrohnen Arbeiterinnen in Studien zur individuellen AFB-Resistenz ablösen können, ermittelten eine Abschätzung der letalen Bakterienzellzahlen sowohl für Arbeiterinnen als auch für Drohnen und entwickelten eine neue Methode, um den Bakterientiter zu messen, bei dem Larven an der Krankheit sterben. Da wir hingegen nur Individuen einer einzelnen genetischen Herkunft untersucht haben, und nicht, ob Drohnen und Arbeiterinnen einer gemeinsamen Herkunft einander ähnlicher sind als unverwandten Individuen, kann unsere Studie nur eine vorläufige Aussage über die Eignung von Drohnen als Indikator der AFB-Resistenz von Arbeiterinnen treffen. Ebenso muss ihre Eignung für die Untersuchung anderer Eigenschaften von Arbeiterinnen (z.B. weitere Merkmale, Krankheitsresistenzen) separat überprüft werden. Da die theoretischen Vorteile von Drohnen jedoch immens sind, möchten wir die Verwendung von haploiden Drohnen in künftigen Studien zur funktionellen Genomik in Hymenopteren wo möglich empfehlen. Dies trifft insbesondere auf Kartierungsstudien zu, da hier QTL unter Verwendung von vergleichsweise wenig Individuen identifiziert werden können, wie anhand der Ergebnisse in Kapitel zwei und drei gezeigt wurde. Es wäre interessant, den Vorteil, den eine Kartierungspopulation aus haploiden Individuen, z.B. hinsichtlich der benötigten Individuenzahl und der QTL-Detektionsgrenzen, gegenüber einer aus diploiden Individuen besitzt, mittels eines mathematischen Modells zu quantifizieren.

Die im ersten Kapitel gemessen bakteriellen *P. larvae*-Titer variierten zwischen Individuen, was die Existenz eines genetisch bestimmten letalen Grenzwerts nahe legt. Wir postulierten,

dass dieser Wert innerhalb der Nachkommenschaft eines gemeinsamen genetischen Ursprungs normal verteilt ist und als Toleranzparameter in AFB-Studien verwendet werden kann. Da der Beginn der durch Infektion hervorgerufenen Sterblichkeit mit Entwicklungsunterschieden zwischen Drohnen und Arbeiterinnen korrelierte und die beobachteten Unterschiede von der Körpermasse abhingen, schlussfolgerten wir, dass die Sterblichkeit nach AFB-Infektion möglicherweise durch eine bestimmte Konzentration an *P. larvae*-Zellen im Wirt ausgelöst wird. Da lebendige, infizierte Larven oft asymptomatisch erscheinen, ist der Ablauf eines Quorum-Sensing-Prozesses möglich, bei dem die Bakterien die toxischen Substanzen erst nach Erreichen einer bestimmten bakteriellen Konzentration im Mitteldarm des Wirtes produzieren. In diesem Fall hinge der Zeitpunkt des Todes und der Degradation der larvalen, peritrophen Matrix (Garcia-Gonzalez und Genersch 2013) hauptsächlich von den larvalen und bakteriellen Wachstumsraten ab, was also eher Eigenschaften der Entwicklung als der Immunologie des Wirtes betreffen würde. Dieses Ergebnis fügt ein neues Konzept zu AFB-Pathologie und AFB-Resistenz hinzu, das sich lohnen würde, detaillierter zu untersuchen (z.B. den Parameterraum).

Das zweite Ziel dieser Arbeit war, haploide Drohnen zu nutzen, um Hauptloci für larvale Resistenz gegen zwei der Hauptschädlinge der Honigbiene, nämlich AFB und die *Varroa* Milbe, zu identifizieren.

Im Fall der larvalen AFB-Resistenz identifizierten wir einen signifikanten und drei suggestive QTL, sowie eine signifikante epistatische Interaktion, die zusammen 41 % der beobachteten phänotypischen Varianz im larvalen Überleben nach AFB-Infektion erklärten. Während sich keine klassischen Immungene (z.B. antimikrobielle Peptide) unter den Kandidatengenen befanden, lag eine bemerkenswert hohe Anzahl Entwicklungsgene (Transkriptionsfaktoren, Homeobox-Gene) innerhalb der identifizierten QTL-Regionen. Dies führte zu der Vermutung, dass die larvale Entwicklung eine wichtige Rolle bei der AFB-Resistenz spielen könnte, was die Ergebnisse des ersten Kapitels unterstützen würde.

Bevor diese QTL-Regionen jedoch als Ziel in selektiven Zuchtprogrammen benutzt werden, sollten künftige Studien zunächst die identifizierten Loci in Wirten und Pathogenen von weiteren genetischen Herkünften bestätigen und die Signifikanz dieser QTL auf dieser größeren Skala unter Berücksichtigung des mittleren Effekts neu bewerten. Da wir in unserer Kartierungspopulation natürliche Varianz und nicht selektierte Extreme benutzt haben, wäre es auch notwendig, weitere Honigbienenlinien und -rassen zu untersuchen, um den allelischen Umfang dieser QTL zu erforschen (und zusätzliche zu finden). Dadurch wäre es möglich,

Imker gezielt zu beraten, welche Honigbienenlinien die höchste individuelle AFB-Resistenz besitzen, sowie mithilfe von Markern spezifisch Resistenzallele in bestehende Honigbienenlinien einzukreuzen.

Im Fall der larvalen *Varroa*-Resistenz identifizierten wir einen signifikanten sowie zwei suggestive QTL für die larvale Unterdrückung der Milbenreproduktion. Da in unserer Kartierungspopulation starke epistatische Interaktionen der drei Loci gemessen wurden, schlussfolgerten wir, dass Zuchtversuche mit diesen Loci stark erschwert wären. Es wäre dennoch interessant, diese Loci in weiteren *Varroa*-toleranten Populationen (Bourgeois und Rinderer 2009; Kefuss *et al.* 2004; Le Conte *et al.* 2007) zu überprüfen und zu untersuchen, ob und wie diese Loci den Wirtfindungsprozess und die Oogenese der *Varroa*-Milbe beeinflussen, die durch Bestandteile der larvalen Kutikula gesteuert werden (Frey *et al.* 2013). Die Unterdrückung der Milbenreproduktion wurde in zwei natürlich selektierten Populationen in Schweden und Frankreich als *Varroa*-Toleranzmechanismus ausgebildet (Locke *et al.* 2012) und nicht hygienisches Verhalten (Locke und Fries 2011). Da diese Route von der natürlichen Selektion in zwei voneinander unabhängigen Populationen gewählt wurde, scheint sie für die Fitness des Wirtes günstiger zu sein. Künftige Zuchtprogramme sollten sich daher auf die Selektion einer reduzierten Milbenreproduktion konzentrieren statt auf eine erhöhtes hygienisches Verhalten, wie es bisher hauptsächlich getan wurde (Boecking und Spivak 1999; Ibrahim *et al.* 2007; Rinderer *et al.* 2010; Spivak und Reuter 2001a; b). Die Wahl eines Merkmals, das für die Honigbienenkolonie energetisch günstiger ist, würde höchstwahrscheinlich auch zu einem größeren ökonomischen Nutzen des Imkers führen.

In beiden unserer Fälle war die Identifikation von einzelnen phänotypisch verantwortlichen Genen nicht möglich, obwohl einige gute Kandidaten gefunden wurden. Während bei der *Varroa*-Resistenz eines der Kandidatengene durch Expressionsdaten unterstützt wurde (Navajas *et al.* 2008), unterstützt eine neue Studie zur Expression nach AFB-Infektion (Cornman *et al.* 2013) keines der Kandidatengene in unseren QTL-Regionen. Obwohl Expressionsdaten ein spezifisches Kandidatengen durchaus deutlich stützen können, wird eine identifizierte QTL-Region im gegenteiligen Fall nicht entwertet, da diese Techniken auf unterschiedliche Gruppen von Genen zielen. QTL-Kartierung identifiziert vorrangig *qualitative* Unterschiede zwischen Allelen von Genen zu Beginn jener Genkaskaden, die das untersuchte Merkmal kontrollieren. Diese Gene erzeugen potentiell die größte phänotypische Varianz und sind daher für Zuchtzwecke auch am interessantesten. Im Gegensatz dazu messen Expressionsstudien vorrangig *quantitative* (Expressions-) Unterschiede der Gene am

Ende der entsprechenden Genkaskaden. Während es zwar möglich ist, dass auch Gene am Beginn von Kaskaden ihre Expressionsmuster in Reaktion auf eine Infektion ändern, ist es jedoch nicht erforderlich, um einen großen phänotypischen Effekt zu verursachen.

Um die Größe unserer QTL-Regionen und damit die Anzahl von Kandidatengenen zu reduzieren, wäre eine erhöhte Markerdichte und eine höhere Individuenzahl nötig. Beides würde ebenfalls die Wahrscheinlichkeit einer Nicht-Detektion von QTL verringern. Höchstwahrscheinlich werden kommende Studien daher nicht Mikrosatelliten, sondern die in größeren Mengen vorkommenden SNPs nutzen (Arechavaleta-Velasco *et al.* 2012; Holloway *et al.* 2012; Tsuruda *et al.* 2012). Zusätzlich werden die höher auflösenden Next-Generation-Sequenzierungs- (NGS) Techniken (z.B. Restriktionsseiten-assoziierte-DNA-(RAD) Sequenzierung (Baxter *et al.* 2011)) eine höhere Genauigkeit bei der Bestimmung der Allelfrequenzen in den DNA-Pools ermöglichen. Diese Techniken werden auch die Feinkartierung und die Genotypisierung von Individuen überflüssig machen und lediglich eine Ein-Schritt-Analyse von Massen-DNA-Proben erfordern. Sobald einzelne Gene identifiziert wurden, die den Resistenzphänotyp verursachen, wird die Sequenzierung verschiedener Allele dann evtl. erlauben, die Art der Resistenz auf funktional-molekularer Ebene zu untersuchen.
Da die Detektion durch eine QTL-Kartierung kein funktionaler Beweis ist, werden die Ergebnisse der Marker-unterstützten Zuchtprogramme durch Bioassays überprüft werden müssen. Alternativ könnte auch die Veränderung von Allelfrequenzen durch einen Selektionsdruck und die Geschwindigkeit, mit der bestimmte Allele fixiert werden, durch wiederholte Beprobung von Populationen unter Selektion gemessen werden. Diese drei Techniken zusammen - QTL-Kartierung, Transkriptom-Analysen (z.B. RNA-Sequenzierung) und die Aufzeichnung des zeitlichen Verlaufs der Allelfrequenzen in Populationen unter Selektion (z.B. der Gotland Population) - werden erlauben, die Bedeutung der identifizierten QTL zu bewerten und sie in einem angewandten Kontext effizient in der Apikultur zu nutzen.

Die Verfügbarkeit des Honigbienengenoms (Weinstock *et al.* 2006), einer wachsenden Zahl von Genomen von Honigbienenschädlingen (Chan *et al.* 2011; Chen *et al.* 2013; Cornman *et al.* 2009; 2010; Lanzi *et al.* 2006; Nguyen und Le 2013; Okumura *et al.* 2011; Ongus *et al.* 2004; Qin *et al.* 2006) und von Zelllinien von Honigbienen (Goblirsch *et al.* 2013; Kitagishi *et al.* 2011) zeigt deutlich an, dass die Honigbienen-Pathologie das molekulare Stadium erreicht hat. Die Identifikation von Krankheitsresistenzgenen der Honigbiene und

Virulenzfaktoren z.B. in *P. larvae* (Antúnez *et al.* 2011a; b; Fünfhaus *et al.* 2013; Poppinga *et al.* 2012) wird ermöglichen, Gen-Gen-Interaktionen in diesem ökonomisch wichtigen Wirt-Parasiten-System zu untersuchen und neue Fragen zu bearbeiten, wie z.B. wie Koadaptionen die betroffenen Genome geformt haben und wie Selektionsdrücke die Allelfrequenzen innerhalb von Populationen ändern. Die molekulare Ebene wird es dabei den Wissenschaftlern ermöglichen, unter Verwendung der Honigbiene als Modellorganismus sowohl pathologische als auch evolutionäre Fragestellungen ganzheitlich zu untersuchen.

Acknowledgements

As in bees an individual is nothing without a healthy social environment. Therefore, I would like to thank my supervisor Prof. Dr. Robin F. A. Moritz for setting me onto this topic by hiring me in the BEE SHOP and BEE DOC networks, thereby introducing me to the international scientific community, for his help, encouragement and council along the way.
Furthermore, I thank my co-authors Eva Forsgren, Ingemar Fries, Conny Geßner, Qiang Huang, Eva Frey, Peter Rosenkranz, Barbara Locke-Grandér and F. Bernd Kraus for their contributions to the articles, as well as a total of seven editors and nine anonymous reviewers for their helpful comments on the manuscripts.
I am furthermore grateful to many international colleagues, especially Joachim De Miranda, Peter Neumann, Robert Paxton, Michel Solignac and Jay Evans, for being encouraging and friendly on conferences and network meetings.
From the work group „Molecular Ecology“ at the University Halle-Wittenberg I would like to express my thanks to Petra Leibe and Denise Kleber for technical assistance in the lab and Holger Scharpenberg for help with bees and computers. I thank my office mates Rodolfo Jaffé, Stephan Wolf and Mario Popp for fun and coffee breaks, further Antje Jarosch, Silvio Erler, Eckart Stolle, Stephan Härtel and H. Michael G. Lattorff for advice in methodological concerns and fruitful discussions, and finally the whole work group for the pleasant working atmosphere and all the time we spent.
I am thankful to Volker Tanke and Farina Herrmann for proof-reading the introduction and summary of this thesis and their comments for improvement.
I thank the European Commission and the German Ministry for Education and Science (BMBF) for spending public money on funding this work as a part of the BEE SHOP and BEE DOC networks and the FUGATO-plus project FUGAPIS.

Finally, I thank my family and friends for diversion, their interest and social support, and most of all Undine for all her love, patience and trust and Vico for taking my alleles to the next generation.

Appendix

A. Declaration on the author contributions

Chapter 1:

Behrens D., Forsgren E., Fries I., Moritz R.F.A. (2010) Lethal infection thresholds of *Paenibacillus larvae* for honeybee drone and worker larvae (*Apis mellifera*), Environmental Microbiology 12 (10), 2838–2845.

I participated in the design of the project, performed the experimental work and data analyses and wrote the paper. Eva Forsgren and Ingemar Fries provided helpful discussions and participated in the writing of the paper. Robin F. A. Moritz participated in the design of the project and writing of the paper and supervised the work.

Chapter 2:

Behrens D. & Moritz R.F.A. (2013) QTL-mapping of individual resistance against American Foulbrood in haploid honeybee drone larvae (*Apis mellifera*), Apidologie, doi: 10.1007/s13592-013-0255-0.

I participated in the design of the project, performed the experimental work and data analyses and wrote the paper. Robin F. A. Moritz participated in the design of the project and writing of the paper and supervised the work.

Chapter 3:

Behrens D., Huang Q., Geßner C., Rosenkranz P., Frey E., Locke B., Moritz R.F.A., Kraus F.B. (2011) Three QTL in the honeybee *Apis mellifera* L. suppress reproduction of the parasitic mite *Varroa destructor*, Ecology and Evolution 1 (4), 451-458.

I participated in the design of the project, the experimental work, data analyses and writing of the paper. Conny Geßner, Qiang Huang and Eva Frey participated in the experimental work and data analyses. Peter Rosenkranz participated in the design of the project. Barbara Locke and F. Bernhard Kraus participated in the writing of the paper. Robin F. A. Moritz participated in the design of the project and writing of the paper and supervised the work.

B. CURRICULUM VITAE

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Personal information

Date of birth	04.10.1980
Place of birth	Hamburg
Academic degree	Dipl. Biol.
Nationality	German

School Education

1986 - 1990	Elementary school Mulsum
1990 - 1992	Orientational school Fredenbeck
1992 - 1999	Gymnasium Athenaeum Stade Exam: Abitur

Civil service

1999 - 2000	Gemeinnützige Gesellschaft für soziale Dienste e.V. in Stade

Higher education

2000 - 2003	Basic course in biology at the University of Hamburg
2003 - 2006	Main courses in zoology, genetics, immunology and geology at the Martin-Luther-University Halle-Wittenberg Exam: Diploma in biology
Jul - Nov 2004	DAAD scholarship at the TUCS University of Pretoria, South Africa
2006	Diploma thesis “Measuring mortality of drone larvae of two honey bee lines (*Apis mellifera*) after infection with American Foulbrood“

Research positions

2006 - 2009	PhD-position in the EU-network „BEE SHOP“ in the work group „Molecular Ecology“ at the Martin-Luther-University Halle-Wittenberg
2010 - 2011	PhD-position in the EU-network „BEE DOC“ in the work group „Molecular Ecology“ at the Martin-Luther-University Halle-Wittenberg

C. Publication list

Peer-reviewed articles

Behrens D., Moritz R.F.A. (2013) QTL-mapping of individual resistance against American Foulbrood in haploid honeybee drone larvae (*Apis mellifera*), Apidologie, doi: 10.1007/s13592-013-0255-0.

Crailsheim K., Brodschneider R., Aupinel P., **Behrens D.**, Genersch E. Vollmann J., Riessberger-Gallé U. (2013) Standard methods for artificial rearing of *Apis mellifera* larvae. In: Dietemann V., Ellis J.D., Neumann P. (Eds) The COLOSS BEEBOOK, Volume I: Standard methods for *Apis mellifera* research. Journal of Apicultural Research **52** (1): http://dx.doi.org/10.3896/IBRA.1.52.1.05.

Behrens D., Huang Q., Geßner C., Rosenkranz P., Frey E., Locke B., Moritz R.F.A., Kraus F.B. (2011) Three QTL in the honeybee *Apis mellifera* L. suppress reproduction of the parasitic mite *Varroa destructor*, Ecology and Evolution **1** (4), 451-458.

Behrens D., Forsgren E., Fries I., Moritz R.F.A. (2010) Lethal infection thresholds of *Paenibacillus larvae* for honeybee drone and worker larvae (*Apis mellifera*), Environmental Microbiology **12** (10), 2838-2845.

Behrens D., Forsgren E., Fries I., Moritz R.F.A. (2007) Infection of drone larvae (*Apis mellifera*) with American Foulbrood, Apidologie **38** (3), 281-288.

Books and book chapters

Behrens D., Mougel F., L'Anthoëne V., Merlin I., Jaffé R., Roussel E., Titera D., Giurfa M., Solignac M., Moritz R.F.A. (2011) Beekeeping without chemical treatment?, in **Behrens D.** & Moritz R.F.A. (Eds) Bees in Europe and Sustainable Honey Production (BEE SHOP): Results of a pan-European research network, Hauppauge NY, Nova Science Publishers.

Eidesstattliche Erklärung

Berlin, den 18. November 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.

Dieter Behrens