Honey bee fungal pathogen, *Ascosphaera apis*; current understanding of host-pathogen interactions and host mechanisms of resistance

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This review was inspired by the rapid development of genomic tools in the fields of honey bee immunity and disease pathology [1, 2]. Our aim in this chapter is to provide an overview of the profound knowledge accumulated in recent years from genome and transcriptome-wide attempts to determine host immune responses to honey bee fungal diseases and to identify quantitative trait loci (QTLs) that underline host mechanisms of resistance [3]. Considering the huge economic impact honey bees have on crop production and particularly on the pollination of specialty crops, a substantial part of this chapter will be dedicated to reviewing efforts to improve the health and survival of the honey bee.

We start with a brief discussion of *Ascosphaeara apis* biology and disease pathology to introduce the reader to key concepts of host-pathogen interactions, *A. apis* virulence factors and development of marker-assisted selection programs are discussed in the remainder of the chapter. We will look at examples of specific gene targets, both in the host and the pathogen to allow development of novel anti-microbial strategies, and to enhance bee survival by improving genetic lines of bees for resistance to microbial pathogens [5-7]. The latest achievements in basic honey bee research, disease detection methods [8, 9] and pathology provide a better understanding of current and future directions in this field of research to breed a better bee and to develop novel anti-microbial drugs protecting bees from invasive mycoses.

Keywords: Honey bee, *Ascosphaera apis*; fungal pathogen; genome; transcriptome; QTL

1. Introduction

Honey bee health can be affected by a number of infectious pathogens including bacteria, fungi and microsporidia. All of these pathogens are highly contagious; however some of them (e.g., *Paenibacillus larvae*) lead to the death of an entire colony and others (such as *Ascosphaera apis)* would normally kill only a sub-set of the colony population, never-the-less significantly affecting colony health, productivity, and the ability to pollinate agricultural crops. Although chalkbrood disease was described in the early 1900s [10], the causative agent of this disease, *A. apis* was not identified until much later [11]. Since then the disease has been intensively studied, including pathogen morphology and biology, and symptoms of the disease. However, until very recently [2], there was very little known about the *A. apis* genome. The lack of genomic data was the biggest impediment to molecular research efforts for many decades. A recent release of genome sequences, both for the host and the pathogen [2, 12], led to an explosion of new investigations that have already produced new insights into fungal reproduction [5], transcriptional regulation of fungal pathogenesis [7], and host transcriptional regulation during fungal invasion [6]. Beyond development of molecular diagnostics tools [8], these new data can be used for the development of anti-fungal drugs, using both traditional and gene silencing approaches. Significantly, a very recent research effort identified a quantitative trait locus (QTL) that may potentially underlie host resistance to *A. apis* infection and may lead to the development of new and improved stocks of bees. Research in this area has never been more exciting.

In this chapter, the reader will find a brief overview of fungal biology and disease pathogenesis (section 2); an extended review on this subject can be found in [4]. The main focus of this chapter is therefore new genomic data, including transcription, SNP and QTL studies [3, 7, 13] that opened potentials for deciphering the mechanism of host–pathogen interactions (section 3).

2. Disease pathology and effects of environmental factors on bee health

Honey bees can be viewed, in a way, as a bio-indicator of environmental health. Honey bee colonies rapidly respond to poor environmental conditions by losing brood and adult populations, colony productivity, and show increased loads of pathogens and parasites. A number of various biotic and abiotic stresses can inhibit honey bee physiological responses making them more vulnerable to infections. Recent reports show that an increase in the incidence of chalkbrood potentially relates to elevated stress of bee colonies. Stress may result from numerous environmental circumstances including the use of agricultural pesticides and interactions between pesticides and fungicides (e.g., tau-fluvalinate, coumaphos and fenpyroximate vs fungicide prochloraz, [14]). Although in-hive pesticides are strictly regulated to prevent overexposure of honey bees to chemicals, in practice, bees are often exposed to a wide variety of agriculturally applied chemicals that are later found in honey bee wax and other hive products that build up over time. Also, foraging bees are frequently exposed to a large variety of agrochemicals, often at exceedingly high concentrations. Pollen

contaminated with agrochemicals, even at sub-lethal doses, may have a direct effect on honey bee health and longevity. In addition, interactions between pathogens (e.g., *Nosema* spp. and viruses) have been shown to induce higher honey bee mortality than any of these pathogens could have done alone. For example, the co-infection of *A. apis* with a closely related fungus *A. atra,* found in bee pollen and previously not known to infect honey bees, showed a rapid increase in bee mortality [15].

Chalkbrood infection spreads within the colony via food contaminated with fungal spores. Dissemination of fungal infection among colonies of the same apiary, and long distance, is most likely assisted by poor beekeeping practices such as contaminated tools and hive materials. Moving brood frames from one colony to another is one of the practices routinely used by the beekeepers that aids in the rapid spread of infectious diseases. This problem is aggravated by the fact that after decades of testing anti-fungal agents, there are still no chemicals approved for the control of chalkbrood in bee colonies. In addition to synthetic chemicals, natural plant products had also been tested against chalkbrood [16, 17], some showing anti-fungal activity *in vitro*. However none of them have been developed commercially. Therefore, increased efforts are currently underway (ARS-USDA laboratory, Baton Rouge, LA) to counter the lack of the disease control by developing new tools for selection of chalkbrood resistant bee stocks.

2.1. Chalkbrood pathology

Most frequently, chalkbrood appears in early spring during cool and humid weather conditions effecting exclusively larval stages of the honey bee. This loss of brood substantially reduces colony population. Infected larvae normally die prior to pupation, but the disease rarely kills an entire colony. It is worth noting that some *A. apis* isolates show several fold differences in the level of host virulence and may infect honey bees at a much higher rate than the others [18]. The disease incidence and prevalence decrease during summer as the temperature improves, but can reappear in the same colonies the following season, possibly assisted by transmission of fungal spores through contaminated pollen and other hive stores.

Infection occurs by fungal spores (ascospores) contaminating larval food. Sexually produced ascospores are the only known source of infection. To date, asexual reproduction has not been described in this fungus [4]. Also, *in vivo* challenge experiments showed that mycelium fed to larvae was not able to induce chalkbrood infection (Aronstein, not published). This observation suggests that both fungal idiomorphs (see reproduction biology, section 2.2) have to be present in the environment to cause fungal infection in bee larvae.

Ascospores germinate in the larval midgut and the fungus invades all internal organs causing chronic mycoses. Prior to sexual reproduction, mycelia exit the cadaver to form aerial hyphae on the skin surface. *In vivo* bioassays showed that the length of time from the initial inoculation of larvae until fungal mycelia become visible on the surface of the cadaver is about 72 hours. Figure 1 shows honey bee larvae inoculated with *A. apis* spores 72 hours post inoculation (1a) and 78 hours post inoculation (1b).

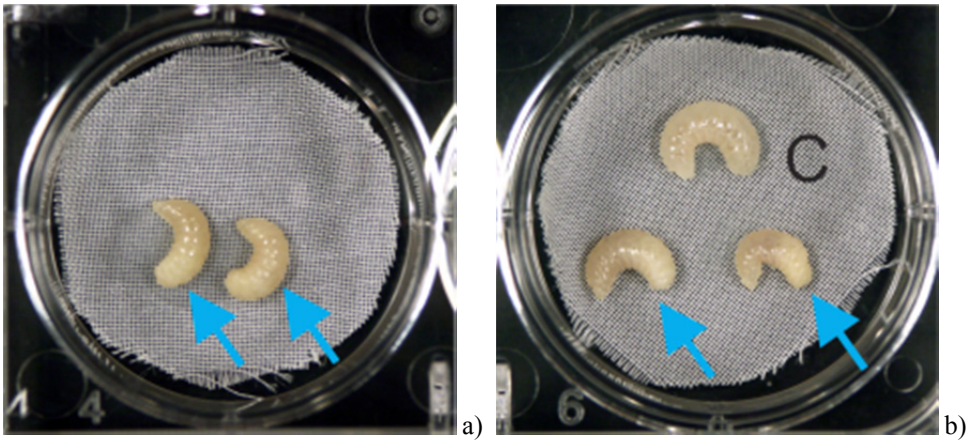

Fig. 1 Figure showing honey bee larvae: a) 72 hr post inoculation with *A. apis* using 10^6 spores per larva; b) 78 hr post inoculation. Blue arrows are pointing to white mycelia growing at the posterior end of the larva; C- control uninfected larva.

Large white masses of mycelia become visible under the semi-transparent skin of infected larvae even before it breaks out of the posterior end of cadaver. As the infection progresses, aerial hyphae cover the entire cadaver growing from the posterior end towards the head region. Figure 2 (a) shows bee cadavers covered with the white fungal mycelia that are the clinical sign of the disease.

When environmental conditions permit, the fungus sporulates forming dark-brown fruiting bodies (ascomata). This gives the cadaver a black appearance. If the weather becomes too hot and dry, the fungus may not be able to complete its reproductive cycle and the dry, chalk-like cadavers will remain white in appearance, hence the name “chalkbrood” mummies. The fact that chalkbrood mummies are found of two different colours, white and black, had for a long time

puzzled researchers and beekeepers alike. Our experiments demonstrated that white mummies, as well as, black mummies resulted from infection caused by the two mating type strains (see reproduction biology, section 2.2). Incubating white mummies in humid and warm conditions would stimulate mating leading to production of ascomata and changing the mummy's appearance from white to black (Fig 2 b).

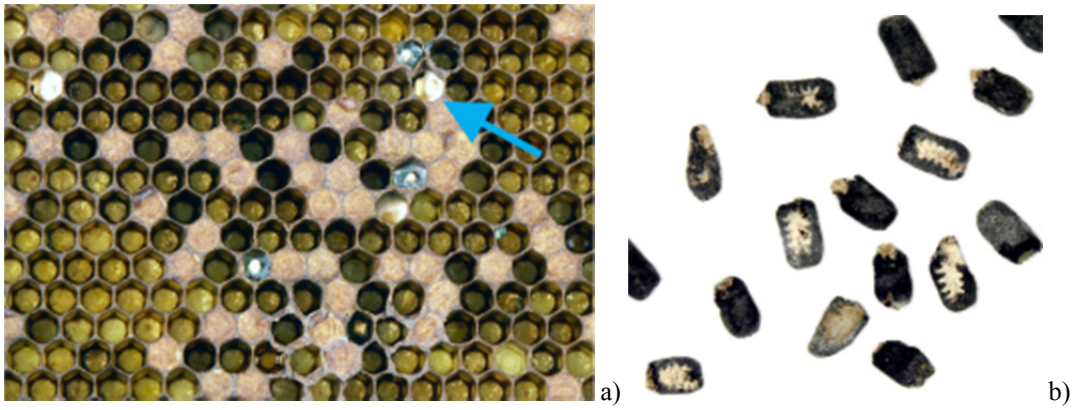

Fig. 2 Figure showing: a) brood frame infected with chalkbrood fungus (blue arrow); b) chalkbrood mummies collected from the colony infected with *A. apis*.

2.2. Fungal reproduction and culture

A. apis is a filamentous fungus that belongs to the heterothallic class of Ascomycetes. Sexual reproduction occurs between morphologically identical haploid mating type idiomorphs. At the molecular level, mating is controlled by a single mating type locus (MAT) represented by two different alleles, MAT1-1 and MAT1-2 (ARSEF 7405, 7406) [5, 7]. The size of the MAT locus was estimated from the genomic sequence at about 3.6 kb, including regions flanking *mat1-1-1* and *mat1-2-1* genes. Each of these genes encodes a functional transcription factor; *mat1-1-1* encodes a protein containing an alpha box domain and *mat1-2-1* encodes a protein containing a high-mobility-group domain (HMG-box) [5]. Both of these transcription factors control expression of the pheromone and pheromone receptor genes involved in mating, fertilization and development of the fungus.

The genomic organization of the two MAT alleles differ in that one of the flanking genes, *Sla2,* had not been found in the same genomic location in MAT1-1 as it was found in MAT1-2, potentially indicating either the asymmetric gene loss in MAT1-1 or gene linkage on the same side. This is not an unusual genomic organization; synteny analysis revealed similar features in the organisation of MAT loci in other Ascomycota fungi [19]. Since mating in pathogenic fungi may be associated with virulence, a better understanding of the genomic organization of the MAT loci may help to elucidate regulation of host pathogenicity.

Figure 3 (a) shows sexual reproductive structures (ascomata) produced by the fungus. These are large round structures brown to black colour and measuring in the range of 47–140 microns in diameter. They contain smaller structures called spore balls (asci) containing ascospores (Fig 3 b), that are the primary source of infection.

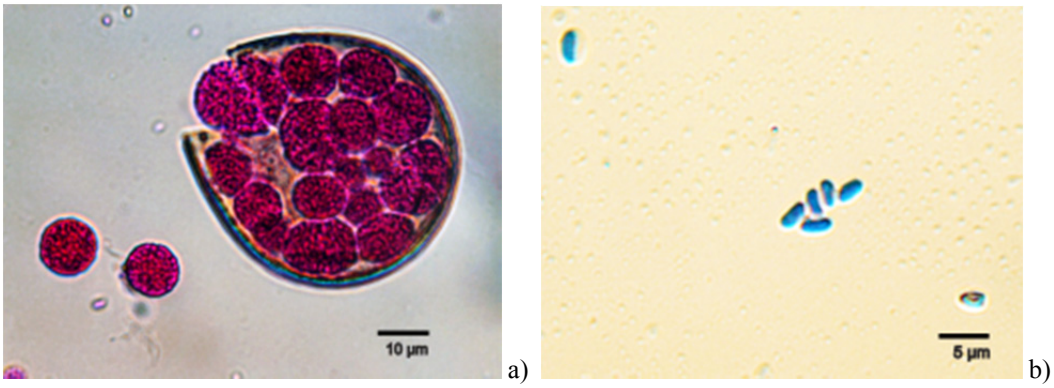

Fig. 3 Figure showing: a) *A. apis* spore cyst (ascoma) and spore balls (asci); b) mature ascospores

In culture, *A. apis* can be propagated using different types of media [4], however, it is most commonly grown on a solid culture media, Yeast–Glucose–Starch agar (YGPSA) (1% yeast extract, 0.2% glucose, 0.1 M KH2PO4, 1% soluble starch, 2% agar). Yeast extract in this media supports abundant vegetative growth and sexual reproduction of the fungus. In culture, fungus can grow at different temperature conditions, ranging from 25^{o} C to 35^{o} C with the

optimum growth at 30° C to 33° C. In addition, 6% CO_2 is routinely used to stimulate spore germination. However, we found that *A. apis* will successfully grow and reproduce without the supplemental CO_2. Well-defined colonies can be observed 2 to 3 days after plating spores. Mating takes about 5 days of culture and becomes visible as a well-defined black line of spore cysts formed in between two colonies representing different MAT idiomorphs. Ascospores are relatively stable in the environment. However, their viability depends largely on storage conditions. *A. apis* spores can remain viable for a number of years when stored as chalkbrood mummies at room temperature or in bee colonies exposed to ever changing environmental conditions. However, purified spores kept at 4° C rapidly lose their viability. Storage at -20° C improves viability with the best option of freezing cultured spores at -80° C in 20% glycerol/ YGPSA liquid media (Aronstein, not published). Storage of single idiomorphs presents even more challenges. Unless they are continually propagated in culture, freezing mycelia at -80° C in 20% glycerol/ YGPSA provides suitable alternative.

3. Genomic studies

3.1. Maintenance of genetic diversity

Genetic diversity in honey bee populations is the key for maintaining normal colony functions that directly correlate with overall colony health and performance. Genetic diversity in honey bees is based on genetic heterogeneity of multiply mated queens. Due to the extreme polyandry resulting from multiple mating, a single bee colony consists of sub-families or patrilines of super- sisters that have same father. The relationship between workers with different fathers is that of half-sisters. This complex genetic structure of the colony provides significant variation among patrilines. In addition to improving the genetic variance of a honey bee colony, patriline variations serve as a primary resource for selection of disease-resistant bee stocks [3, 18, 20, 21]. Furthermore, highly inbred honey bee stocks, with the reduced frequency of sex alleles or complementary sex determiner (*csd)* alleles may predispose colonies to contracting infectious diseases [22].

The most immediate impact of the honey bee genome and genome–wide transcriptome analyses conducted in recent years [6, 12] is the potential for the development of breeding programs to improve honey bee resistance to diseases and parasites. Such breeding programs are already well established in a number of laboratories in the United States (USDA-ARS, Baton Rouge, LA; Purdue University, IN; University of Minnesota), Canada (University of Guelph, Ontario; University of Manitoba, Winnipeg) and several Labs in the EU. In the sub-sections below we will discuss genomic studies as they relate to the honey bee mechanisms of resistance to diseases, breeding or virulence mechanisms identified in the fungal pathogen.

3.2. Honey bee immune defences against pathogenic fungi

There is a very fine balance between the host's ability to resist infectious attack and the pathogen's ability to cause infection. Therefore, it is essential to clearly understand factors contributing to a sustained fungal infection. The genetic background of honey bees seems to play a key role in honey bee resistance to infectious diseases, including chalkbrood. Similarly, different strains of *A. apis* showed substantial differences in host virulence. Vojvodic and colleagues (2011) found significant genetic variations between strains of *A. apis* collected in the EU and the USA. Importantly, these genetic variations well correlated with the honey bee resistance to fungal infection.

Honey bee larvae challenged with *A. apis* respond in an age-dependant fashion. Very young larvae, 1-2 day old (6 mg, live weight), stop feeding soon after consuming spore inoculum, thereby not gaining much weight post inoculation. They may survive up to 36-48 hours, but usually dye long before the appearance of visible signs of the disease. Older larvae, 3-4 days old (25 mg-93 mg, live weight), continue feeding post inoculation, although at a reduced rate compared to controls. Mycelial growth on the larval skin typically becomes visible at about 72 hr post inoculation. By that time larvae are not responsive to any stimuli.

Honey bees display a unique (to social organisms) ability to defend themselves against invading pathogens. A multi-leveled approach evolutionarily developed in social insects provides: 1) colony level protection based on social immunity (e.g., hygienic behaviour and anti-microbial hive products), and 2) individual level protection based on innate immune responses. Although hygienic behaviour is critical for colony survival, in this chapter we are focusing on the physiological responses to diseases including cell mediated and cell-free immune responses.

Similar to other invertebrates, the most common anti-fungal responses in the honey bee are phagocytosis and encapsulation. As it was first demonstrated in 1884 by Metchnikoff in fresh-water crustacean, fungal spores are rapidly attacked by the phagocytes. Cellular reactions are typically followed by humoral immune responses. For example, septic injury of honey bee pupae with *A. apis* spores triggered a rapid development of melanin deposits at the site of injury (~1hr post injury), indicating activation of the phenoloxidase cascade (Fig 4a). However, the level of melanin production observed in response to *A. apis* was several magnitudes lower than that in response to gram positive bacteria, *Paenibacillus larvae* (Fig 4b).

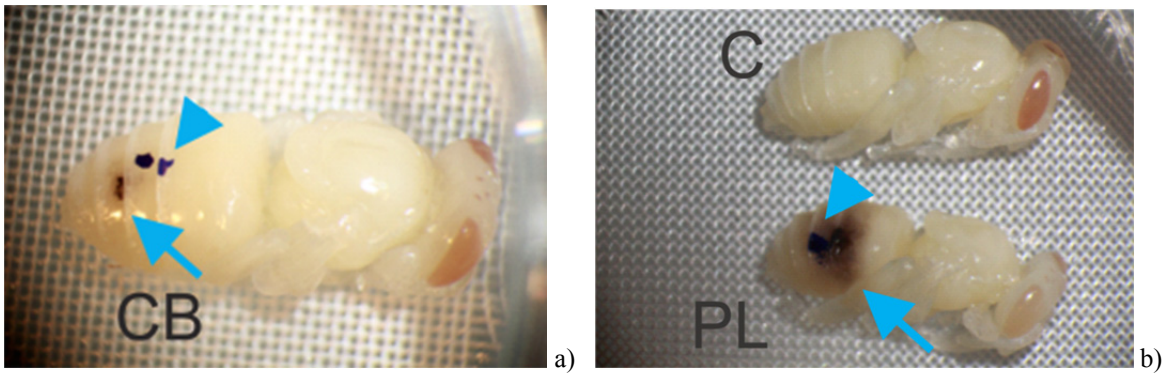

Fig. 4 Figure showing melanin deposition at the site of septic injury in honey bee pupae: a) using fungal *A. apis* spores (CB); b) using bacterial *P. larvae* spores (PL); C) control pupae injected with sterile water. Blue arrows pointing to melanin depositions; blue arrow heads pointing to a black marker, next to the site of injury.

The insect's humoral immunity discriminates between different types of pathogens (Gram positive and negative bacteria, fungi and viruses) and responds to infection by induction of a wide repertoire of humoral factors, including a large number of inducible anti-microbial peptides (AMPs); some of them showed specific anti-fungal activities (De Lucca, and Walsh, 1999). For example, cecropins of the giant silk moth (*Hyalopora cecropia*) showed fungicidal activity against *Aspergillus* and *Fusarium* species*; Drosophila* drosomycin was effective against *F. oxysporum* isolates. The anti-fungal peptide holotricin 3, purified from larvae of coleopteran insect, *Holotrichia diomphalia*, inhibited growth of *Candida albicans*. Thanatin, a 21-residue peptide with bactericidal and fungicidal activity produced by the spined soldier bug, *Podisus maculiventris*, showed activity against *F. oxysporum* and *A. fumigatus*. Thaumatins-like polypeptides found in aphids and beetles were shown to inhibit spore germination of the filamentous fungi, including *Beauveria bassiana* and *F. culmorum* [23].

The APMs' expression is regulated by two principal NF-κB/Rel immune signalling pathways, Toll and IMD. , According to *Drosophila* model, the anti-fungal responses are mostly controlled by the Toll pathway. Drosophila Toll mutants showed an increased susceptibility to fungal infection and were unable to induce the anti-fungal AMP, drosomycin [24]. However in addition to fungi, the same pathway can process signals activated by gram positive bacteria. Significantly, activation of Toll dependant AMPs persisted in Toll mutants, indicating some level of compensation by the second NF-κB signalling pathway. It is now apparent that the two signalling pathways, Toll and IMD, are not independent of each other and may work synergistically in response to microbial infections. Recent studies provide new evidence that there is cross-talk between the two complementary pathways and the activation of one of them, Toll or IMD, can branch to the activation of other immune related signalling pathways, JNK and/or JAK/STAT [25]. As a result, the anti-microbial responses involve a wide range of effector molecules (e.g., thioester-containing proteins, TEPs, C-type lectins) that target microbial cells for the cell mediated destruction, trigger degradation of damaged proteins or damaged host cells [26]. Furthermore, a recent study by Tsuzuki and colleagues (2012) provided new evidence that insect AMPs are also induced under non-infectious stress when larvae are physically or environmentally stimulated (e.g., aseptic wounding, elevated or low body temperature) by activating growth-blocking peptide (GBP)-JNK signalling [27].

In the honey bee, the core elements of immune related pathways have been described by Evans et al. (2006). Although it is still not clear which AMPs are controlled strictly by the Toll or the IMD, it was determined that some of them, *abaecin* and *hymenoptaecin*, are mostly regulated by the IMD [28], whereas expression of bee *defensins 1* was not affected by Relish/IMD silencing. Honey bee defensins showed a wide range of anti-microbial activities. It is speculated that the multiplicity of the anti-microbial activity of bee defensins is due to genetic variations in gene structure, presence of multiple alleles, tissue specific level of gene expression and post-translational modifications leading to production of multiple isoforms [29].

Similar to other insects, the anti-fungal responses in the honey bee are thought to be controlled by the Toll pathway. However, the up-regulation of *abaecin and defensisn-1* [6] in response to fungal infection points to a more complex regulation of AMPs in the honey bee. To add to this complexity, it was demonstrated [1, 26], that the same AMPs can be activated in response to septic wounding with different types of pathogens, suggesting a potential cross-talk between immune signalling pathways. Similarly to the honey bee, *abaecin, defensin 1, hymenoptaecin* (IMD) and *basket* (JNK pathway) were differentially expressed in response to gram negative bacteria and wounding in another eusocial insect, *Bombus terrestris* [30]. In addition to classical immune pathways, insects can activate a targeted anti-fungal activity, including production of the anti-fungal chitinases (*AgChi-1, Cht8* and *AmelCht*) described in mosquitoes, red flour beetle and the honey bee [6, 31, 32].

As described above, the status of host immune competence is often assessed by measuring the level of gene expression. In that sense, researchers are using the level of gene regulation as a molecular marker for a quick assessment of the insects' immune competence. However, it is now apparent that there may be no simple correlation

between the up- or down-regulation of immune-related markers and immune status of the animal. The relationship between the level of host resistance, immune competence and gene expression appears to be more complex. For example, worker bees infected with *Metarhizium anisopliae* showed activation of all major (Toll, IMD/JNK and JAK/START) immune pathways [33] with the AMPs (*abaecin, defensin-2, hymenoptaecin*) up-regulated as much as 16 to 64 fold in comparison to non-infected bees. However, these bees were highly susceptible to the fungus. In contrast, infected foragers had much lower mortality with fewer immune genes activated in response to fungal infection. Bull and colleagues (2012) speculate that the high level of immune gene regulation seems to have no significant effect on bee survival.

These results impose a more general question: how well can the status of immune competence of the honey bee be predicted by monitoring the expression levels of immune related genes, and can a subset of genes serve as markers for determining the immune status of the animal?

3.2.1. Functional groups differentially regulated in honey bee larvae during *A. apis* pathogenesis

During the host invasion, *A. apis* penetrates the larval mid gut by employing hyphal pressure and possibly enzymatic degradation of the peritrophic membrane. After crossing protective barriers and reaching the body cavity, the fungus invades internal organs producing large masses of mycelia, and typically kills larvae within the first 72 hr post infection.

Transcriptional responses in bee larvae infected with chalkbrood fungus were analysed by Aronstein and colleagues (2010) using a genome-wide approach. The study identified a wide range of differentially-regulated transcripts; some of them were previously implicated in general mechanisms of stress adaptation, immune signalling and anti-fungal activity. Others had not been previously associated with any known immune functions and could be involved in general mechanisms controlling removal of damaged proteins, protein transport, hypoxic stress and apoptosis.

Genes potentially involved in immune signalling included trypsin-like serine protease (*Tryp_SPc*) that was highly up-regulated in infected larvae. The up-regulation of the CLIP domain-containing serine protease suggests its potential involvement in immune responses, possibly leading to activation of NF-κB signalling or prophenoloxidase (PPO) activity. In addition, we detected components of NF-κB signalling, including *MyD88*, the universal adopter of Toll, and selected AMPs (*abaecin and defensin 1*), all of them significantly up-regulated 36 hr post inoculation. Interestingly, the honey bee C-type lysozyme-1 showed a 2-fold increase in transcription 24 h post inoculation that would correspond temporally with germination of the fungal spores and the onset of invasion of the peritrophic membrane.

A putative chitinase-encoding glycosyl hydrolase 18 (*AmelCht*) transcript found in this study could also be among those that directly target invading fungi. The potential anti-fungal activity of insect chitinases has been well documented using *in vitro* assays. This group of glycosyl hydrolases has the ability to degrade chitin leading to breakdown of the fungal cell walls, and therefore could be important for biocontrol applications. Gene sequence analysis of the honey bee chitinase *AmelCht* showed that it is highly similar to gut-specific chitinases identified in *Locusta migratoria manilensis, Anopheles gambiae* and *Tribolium castaneum* [6]. The *AmelCht* transcript was significantly down-regulated 36 hr post inoculation, indicating possible inhibition by the fungus. Once the anti-fungal activity of *AmelCht* is tested *in vivo*, a purified chitinase can be tested for the ability to protect honey bees from fungal diseases. The enchantment of the honey bee immune related gene regulation could be one of the alternatives to the use of synthetic drugs.

3.3. Breeding for resistance to *A. apis*

Honey bee queens mate with multiple drones. Worker progeny can be segregated into partilines based on genetic markers particularly at the *csd* gene which must be maintained in a heterozygous status for viability of individual brood (reviewed in [34]). Importantly, the required genetic diversity at *csd* results in diversity throughout the genome, thus leading to broad phenotypic diversity within and among colonies. Based on the differential chalkbrood incidences observed among honey bee colonies [35] and the well-established genetic diversity in those colonies, it is expected that a genetic mechanism functions to control chalkbrood infections if appropriate alleles exist in a population. A recent study [3] has generated a mapping population of Russian honey bees in which individual larvae segregate for resistance versus susceptibility when inoculated with *A. apis* spores. A quantitative trait loci (QTL) analysis identified a single genomic interval on chromosome 11 that significantly associated with the phenotype (Figure 5).

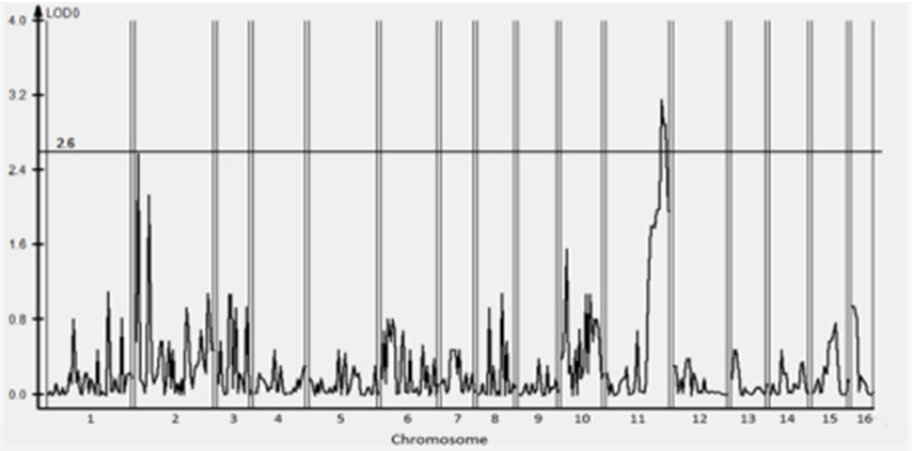

Fig. 5 Whole genome view of Single Marker Association QTL mapping of larval-mediated chalkbrood resistance. The significant interval (peak LOD score of 3.15) that suggests a genetic mechanism of resistance is located on chromosome 11.

The genetic interval that likely confers chalkbrood resistance is currently being investigated to identify specific mechanisms of importance. Fine mapping has resolved the interval to contain only two protein-coding genes: single Ig IL-related receptor-like, and a putative juvenile hormone-binding protein. In *Drosophila*, both genes are likely involved in the Toll immunity pathway by altering the expression of the AMP drosomycin (reviewed in [36]). While both genes are expressed (quantitative Real-time PCR) at appropriate susceptible developmental life stages and are involved in a physiological pathway related to disease resistance, it remains unclear which gene (if either) is causative for the resistance phenotype observed in larvae [37]. The presence of multiple genes within the larval-mediated chalkbrood resistance interval requires additional functional studies to determine the specific physiological basis of resistance to this fungus in the honey bees. Further genomic characterization for promoter and coding sequence differences, modified predicted gene annotations, siRNA regulators expressed from the QTL, or other contributing features of the interval will enable a more defined mechanism of resistance to be determined. Interestingly, the resistance mechanism mapped in the QTL study may not be the only genetic source of chalkbrood resistance. While not significant in the original QTL study, a minor association found on chromosome 2 (Figure 5) may prove synergistic with that on chromosome 11 if able to be studied in a separate mapping population. Analysis of additional segregating populations may identify other independently acting means of resistance.

QTL studies pave the way for the development of marker assisted selection (MAS) programs that increase the frequencies of positive alleles, and therefore desirable phenotypes, within populations. MAS programs have been widely successful in agriculture, particularly in cattle breeding to reduce recessive defects and increase milk production during heat stress [38]. Successful mapping of larval-mediated chalkbrood resistance [3, 37]; potentiates a selection program in honey bees for chalkbrood resistance. Importantly, a broad spectrum fungal- or microbial-resistance may be increased in honey bee populations if the responsible genetic mechanism is not specific to chalkbrood, thus allowing increased resistance to fungal, bacterial, or microsporidian infections. While the use of the current single genetic target in MAS will help to overcome the inherently complexity in honey bee breeding due to haplo-diploidy and the *csd* gene, long range goals for honey bee breeding may incorporate into a single honey bee population several economically important traits such as chalkbrood and other disease resistance, desirable behavioural characteristics, pollination efficiency, or honey production.

3.4. Functional groups differentially regulated in the fungus, *A. apis* during host invasion

The ability of fungi to infect its host depends on the coordinated activity of virulence factors and degrading enzymes that together may inhibit host immune defences and allow pathogens to penetrate the insect's protective barriers.

Following the *A. apis* genome project [2, 5], a comprehensive analysis of the fungal transcriptome has been recently published by Cornman and colleagues (2012) predicting 6,992 gene models in the ~24 Mb *A. apis* genome. Sequence analysis using computational and manual annotations revealed functional categories of genes involved in fungal reproduction, intracellular signal transduction, stress response, and a diverse group of transcripts potentially involved in host virulence and escape mechanisms. Among them are a large number of gene transcripts encoding enzymes, chitinases, proteases and esterases. The list of differentially expressed transcripts includes an extracellular glucoamylase, 3 chitinases, 16 amidases, 30 esterases, 42 proteases, and 24 lipases.

The identification of fungal chitinase (GH18) presents special interest since this group of chitinases is highly sensitive to allosamidin (β-D-allopyranoside), and therefore could become a potential target for fungal control.

We have also identified a number of transcripts of well-known toxins and a diverse family of enzymes involved in toxin biosynthesis. Some of them are involved in the Aflotoxin (AF)-Sterigmatocystin (ST) biosynthesis pathway (*AflR,*

StcU, Nor-1, SteW, OmtA, OrdA) and HC-toxin biosynthesis (*Tox A, ToxG, ToxD, ToxF and Hts1*). Analysis of these transcripts may help to better understand the genetics of *A. apis* virulence, and therefore, identify the most promising targets for gene–specific therapeutics as well as gene silencing (RNAi) targets.

Although the RNAi pathway has been lost in various fungal species and lineages, the core components of RNAi were identified in most filamentous fungi examined to date. Our genome analysis [7] showed that *A. apis* contains most of the core RNAi components, including two Dicer genes (*Dcl-1* and *Dcl-2*), one Argonaute (*Ago1*) and RdRP. Therefore, presence of the core RNAi components in the *A. apis* genome and new molecular targets identified in recent studies can be exploited for the development of an RNAi-based control strategy of this major bee pathogen.

In conclusion, the recent advancements in understanding chalkbrood disease place *A. apis* in a precarious situation. Research into the biology and infective process of chalkbrood, as well as the molecular understanding of honey bee resistance to the pathogen suggests that *A. apis* infections may soon be on the brink of a multi-faceted ambush. Future selective breeding in honey bee populations may increase larval resistance. Additionally, our better understanding of the host-pathogen interaction may shed light on chemical and physiological means of control.

References

[1] Aronstein K, Saldivar E. Characterization of a honey bee Toll related receptor gene *Am18w* and its potential involvement in antimicrobial immune defense. *Apidologie*. 2005;36:3.

[2] Qin X, Evans JD, Aronstein K, Murray KD, Weinstock GM. Genome sequences of the honey bee pathogens Paenibacillus larvae and Ascosphaera apis. *Insect molecular biology*. 2006;15:715.

[3] Holloway BH, Sylvester A, Bourgeois L, Rinderer TE. Association of single nucleotide polymorphisms to resistance to chalkbrood in *Apis mellifera*. *Journal of Apicultural Research*. 2012;51:154.

[4] Aronstein K, Murray KD. Chalkbrood disease in honey bees. *Journal of invertebrate pathology*. 2010;103:20.

[5] Aronstein KA, Murray KD, de Leon J, Qin X, Weinstock G. High mobility group (HMG-box) genes in the honey bee fungal pathogen *Ascosphaera apis*. *Mycologia*. 2007;99:553.

[6] Aronstein KA, Murray KD, Saldivar E. Transcriptional responses in honey bee larvae infected with chalkbrood fungus. *BMC genomics*. 2010;11.

[7] Cornman RS, Bennett A, K., Murray KD, Evans JD, Christine Elsik C, Aronstein KA. Transcriptome analysis of the Honey Bee fungal pathogen, Ascosphaera apis: implications for host pathogenesis. *BMC genomics*. 2012;13:285.

[8] Murray KD, Aronstein KA, Jones WA. A molecular diagnostic method for selected *Ascosphaera* species using PCR amplification of internal transcribed spacer regions of rDNA. *Journal of Apicultural Research*. 2005;44:61.

[9] Jensen AB, Aronstein KA, José Manuel Flores JM, Vojvodic S, Palacio MA, Spivak M. Standard methods for fungal brood disease research. *Journal of Apicultural Research*. 2013;52.

[10] Maassen A. Weitere Mitteilungen uber der seuchenhaften Brutkrankheiten der Bienen [Further communication on the epidemic brood disease of bees]. *Mitteilungen aus der Kaiserlichen Biologischen Anstalt fur Land- und Forstwirtscshaft*. 1913;14:48.

[11] Spiltoir CF, Olive LS. A reclassification of the genus Pericystis betts. *Mycologia*. 1955;**47**:238.

[12] Consortium THGS. Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature*. 2006;443:931.

[13] Invernizzi C, Peñagaricano F, Tomasco IH. Intracolonial genetic variability in honeybee larval resistance to the chalkbrood and American foulbrood parasites. *Insectes Sociaux*. 2009;56:233.

[14] Johnson RM, Dahlgren L, Siegfried BD, Ellis MD. Acaricide, Fungicide and Drug Interactions in Honey Bees (*Apis mellifera*). *PLoS One*. 2013 8:e54092.

[15] Vojvodic S, Boomsma JJ, Eilenberg J, Jensen AB. Virulence of mixed fungal infections in honey bee brood. *Frontiers in Zoology*. 2012;9.

[16] Aronstein K, Hayes G. Antimicrobial activity of allicin against honeybee pathogens. *Journal of Apicultural Research*. 2004;43:57.

[17] Sahinler N, Kurt S. A Study on Antifungal Activity of Formic Acid and Propolis Extract Against Chalkbrood Disease Pathogen Ascosphera apis. *Journal of Animal and Veterinary Advances*. 2004;3:554.

[18] Vojvodic S, Jensen AB, Markussen B, Eilenberg J, Boomsma JJ. Genetic Variation in Virulence among Chalkbrood Strains Infecting Honeybees. *PLoS One*. 2011;6:e25035.

[19] Li W, Metin B, White TC, Heitman J. Organization and Evolutionary Trajectory of the Mating Type (*MAT*) Locus in Dermatophyte and Dimorphic Fungal Pathogens. *Eukaryotic cell*. 2010;9:46.

[20] Tarpy DR. Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. *Proceedings of the Royal Society B: Biological Sciences*. 2002;Ser. B:99.

[21] Tarpy DR. Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. *Proceedings of the Royal Society B: Biological Sciences*. 2003 270:99.

[22] Cobey SW, Shepard WS, Tarpy DR. *Status of Breeding. Practices and Genetic Diversity in Domestic U.S. Honey Bees*. In: Samataro D, Yoder JA, editors. Honey Bee Colony Health: Challenges and Sustainable solutions: CRP Press, Taylor and Francis, LLC; 2011, p. 25.

[23] Gerardo NM, Altincicek B, Anselme C, Atamian H, Barribeau SM, Vos Md, Duncan EJ, Evans JD, Gabaldón T, Ghanim M, Heddi A, Kaloshian I, Latorre A, Moya A, Nakabachi A, Parker1 BJ, Pérez-Brocal V, Pignatelli M, Rahbé Y, Ramsey JS, Spragg CJ, Tamames J, Tamarit D, Tamborindeguy C, Vincent-Monegat C, Vilcinskas A. Immunity and other defenses in pea aphids, *Acyrthosiphon pisum*. *Genome Biology*. 2010;11:R21.

[24] Alarco A-M, Marcil A, Chen J, Suter B, Thomas D, Whiteway M. Immune-Deficient Drosophila melanogaster: A Model for the Innate Immune Response to Human Fungal Pathogens. *The Journal of Immunology*. 2004 172 5622.

[25] Kurz L, Ewbank JJ. *Caenorhabditis elegans*: an emerging genetic model for the study of innate immunity. *Nature Reviews Genetics*. 2003;4:380.
[26] Wang Q, Liu Y, He H-J, Zhao X-F, Wang J-X. Immune responses of *Helicoverpa armigera* to different kinds of pathogens. *BMC Immunology*. 2010;11:9.
[27] Tsuzuki S, Ochiai M, Matsumoto H, Kurata S, Ohnishi A, Hayakawa Y. Drosophila growth-blocking peptide-like factor mediates acute immune reactions during infectious and non-infectious stress. *Scientific Reports*. 2012 2:210.
[28] Schlüns H, Crozier RH. Relish regulates expression of antimicrobial peptide genes in the honeybee, *Apis mellifera*, shown by RNA interference. *Insect molecular biology*. 2007;16:753.
[29] Ilyasov R, Gaifullina L, Saltykova E, Poskryakov A, Nikolenko A. Review of the Expression of Antimicrobial Peptide Defensin in Honey Bees *Apis Mellifera* L. *Journal of Apicultural Science*. 2012;56:115.
[30] Erler S, Popp M, Lattorff HMG. Dynamics of Immune System Gene Expression upon Bacterial Challenge and Wounding in a Social Insect *(Bombus terrestris)*. *PLoS One*. 2011;6:e18126.
[31] Zhu Q, Arakane Y, Banerjee D, Beeman RW, Kramer KJ, Muthukrishnan. S. Domain organization and phylogenetic analysis of the chitinase-like family of proteins in three species of insects. *Insect Biochem Mol Biol*. 2008;38:452.
[32] Zhu Q, Arakane Y, Beeman RW, Kramer KJ, Muthukrishnan S. Functional specialization among insect chitinase family genes revealed by RNA interference. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105:6650.
[33] Bull JC, Ryabov EV, Prince G, Mead AA, Zhang C, Baxter LA, Pell JK, Osborne JL, Chandler D. A strong immune response in young adult honeybees masks their increased susceptibility to infection compared to older bees. *PLoS pathogens*. 2012;8:e1003083.
[34] Palmer KA, Oldroyd BP. Evolution of multiple mating in the genus *Apis*. *Apidologie*. 2000;31:235.
[35] Jensen AB, James RR, Eilenberg J. Long-term storage of *Ascosphaera aggregata* and A. apis, pathogens of the leafcutting bee *(Megachile rotundata*) and the honey bee (*Apis mellifera*). *Journal of invertebrate pathology*. 2009;101:157.
[36] Zhang Z-T, Zhu S-Y. Drosomycin, an essential component of antifungal defence in Drosophila. *Insect molecular biology*. 2009;18:549.
[37] Holloway B, Tarver MR, Rinderer TE. Fine mapping identifies significantly associating markers for resistance to the honey bee brood fungal disease, Chalkbrood. *Journal of Apicultural Research*. 2013;52:In Press.
[38] Hayes BJ, Bowman PJ, Chamberlain AC, Verbyla K, Goddard ME. Accuracy of genomic breeding values in multi-breed dairy cattle populations. *Genetics, Selection, Evolution*. 2009;41:51.

