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

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Short title: QTL-mapping AFB resistance in honeybee drones

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.

Paenibacillus larvae **/ invertebrate pathology / disease resistance / quantitative trait loci / microsatellites**

1. INTRODUCTION

American Foulbrood (AFB) is a honeybee (*Apis mellifera*) specific brood disease which is caused by the spore forming bacterium *Paenibacillus larvae* (Genersch et al. 2006) and is notifiable in many countries. AFB is considered to be one of the most severe honeybee diseases since infections are often lethal on both the individual larval and the colony level, although sublethal infections do occur. It's classification, pathogenesis, transmission, control and economic significance has recently been reviewed (Genersch 2010). Only very young larvae succumb to the disease and are disintegrated to a brownish sticky mass or dried out scales, containing millions of highly contagious and persistent spores. Colonies showing clinical symptoms as well as contaminated hive material often need to be burnt, causing great economic losses to beekeepers and apiculture.

Since antibiotics have been shown to be often ineffective as treatment against AFB due to the selection of resistances in pathogens (Evans 2003), and may also end up as residues in honey (Wilson 1974), the use of resistant honeybee stock in apiculture is highly desirable.

Several host factors have been shown to influence AFB resistance at the colony level including hygienic behaviour (Rothenbuhler 1964), compounds of the brood food (Rose and Briggs 1969) and traits of the nurse bees (Thompson and Rothenbuhler 1957). Colony traits typically are very complex because large numbers of workers interact to perform a specific collective task. The interactions of many different genotypes and / or individual phenoptypes will eventually determine the overall colony phenotype. This complex colony phenotype poses a tremendous complication for any study aiming to

understand the genetic control of colony traits at the molecular level. However, honeybee lines are also known to differ in individual larval resistance (Palmer and Oldroyd 2003) and also the threshold of lethal infection titres has been shown to vary substantially among individual bees of the same colony (Behrens et al. 2010). Hence in order to assess any genetic variance for AFB resistance, we here focus on individual larval resistance phenotypes, which have been shown to play an important role in colony resistance (Evans and Pettis 2005).

The innate immune system and the immune genes that produce antimicrobial peptides are of central importance in the context of antibacterial competence of honeybees and have been studied in some detail (Evans 2006). Nevertheless, these genes seem to have a very limited capacity in controlling AFB in honeybee larvae and specific adaptations to *P. larvae* at the molecular level have not yet been identified. The number of immune related genes in honeybees has been reported to be reduced to one-third compared to other insects (*Drosophila*, *Anopheles*) (Evans et al. 2006; Weinstock et al. 2006) which seems surprising given the environmental conditions inside a honeybee colony (including 60% humidity, 35 °C and a high density of closely related hosts) pose ideal prerequisites for pathogen growth, resulting in a potentially high pathogen pressure. The low number of immune genes may be attributed to their rapid evolution, resulting in a low detection rate if identified by homology to genes in related taxa. Another possible explanation is that honeybees counterbalance a reduction in the number of immune genes by the evolution of "social" defences (social fever, swarming, collected antimicrobial plant compounds, hygienic behaviour). So the question arises whether the reduced number is real or whether many immune relevant genes have not been

identified in honeybees yet. Furthermore, an immune response to a host specific pathogen might be governed by highly specialized mechanisms as a result of an evolutionary arms race between host and pathogen. Such genes might be very different from the rather unspecific immune pathways common to all insects.

Quantitative trait locus (QTL)-mapping provides an excellent tool for the identification of functionally highly specific (e.g. immune) genes, since not only downstream regulated genes may be identified as by gene expression studies, but also regulatory genes, which are in control of entire pathways and act in a qualitative rather than in a quantitative way. Especially in the honeybee, QTL-mapping is an extremely effective tool due to the exceedingly high recombination rate of 22 cM / Mb (Solignac et al. 2007; 10 times higher as in most other higher eukaryotes), and the high density of microsatellite markers (Solignac et al. 2003; 2007), allowing for high-precision mapping with comparably few individuals. In addition, male haploidy of honeybees allows analyzing haploid offspring (drones), thus excluding any interaction of homologous alleles or dominance effects in the individual and thereby producing possibly more distinct phenotypes (Michelmore et al. 1991). By working with drone offspring from a single queen it is possible to reduce the number of alleles for any gene within the mapping population to only two, thereby producing a very simple genetic structure of the mapping population and massively reducing the number of interactions to be tested for epistatic effects. In particular for traits linked to individual disease resistance, males do produce measurable phenotypes and are therefore much more suitable for mapping studies than diploid workers are.

Nevertheless, to date QTL in honeybees have been primarily identified for worker specific traits including guarding (Arechavaleta-Velasco and Hunt 2004), stinging (Hunt et al. 1998; Guzmán-Novoa et al. 2002), foraging (Hunt et al. 1995; Page et al. 2000, Rüppell et al. 2004), hygienic behaviour (Oxley et al. 2010), worker-ovary size (Linksvayer et al. 2009; Rueppell et al. 2011; Graham et al. 2011), reproductive traits (Lattorff et al. 2007; Oxley et al. 2008), larval chalkbrood resistance (Holloway et al. 2012) as well as body composition and venom compounds (Mougel et al. 2012). We are aware of only three exceptions in which haploid drones have been used, which are on learning behaviour (Chandra et al. 2001), sucrose responsiveness (Rueppell et al. 2006) and larval *Varroa* tolerance (Behrens et al. 2011). We here embark on identifying QTL for individual larval resistance towards American foulbrood using the phenotypes of drone larvae.

2. MATERIAL AND METHODS

2.1. Infection assay and mapping population

Drone larvae younger than 24 hours (n = 507) from a single *A. mellifera carnica* queen were individually grafted from a full-size colony into wells of a microtitre plate and reared *in-vitro* as described in Behrens et al. (2007; 2010). In order to produce two very distinct resistance phenotypes, larvae were one-time infected orally with one of two dosages (500 or 1,000 colony forming units (cfu) per ml diet; n = 175 and 250 larvae respectively) of *P. larvae* spores of the type strain ATCC 9545 (ERIC1). A set of 82

larvae was reared as control on uninfected diet. Prepupal mortality was individually recorded under a stereomicroscope on a daily basis. Dead individuals were stored at -20 °C until DNA extraction. The experiments were terminated on day 8 and all surviving larvae were stored at -20 °C.

The *in-vitro* rearing system allowed for an analysis of individual larval AFB resistance under controlled conditions reducing environmental variance and excluding all factors related to nurse bee interactions and food compounds which are known to interfere with larval AFB resistance (Thompson and Rothenbuhler 1957; Rose and Briggs 1969). Since AFB resistant honeybee lines are not available for the production of hybrid queens, we relied on natural variation in AFB resistance, which had been found to be high even among the offspring of honeybee sister queens (Behrens et al. 2007).

2.2. Bulk segregant analysis (BSA) and fine mapping

Only the extreme phenotypes were considered for a selective genotyping approach aiming at a clear segregation of alleles (i.e. an accumulation of the responsible alleles in the DNA pools) and thereby a higher detection probability for the controlling QTL (Darvasi and Soller 1994). Larvae were defined as "susceptible" (n = 35) if they died between day 5 and 7 after infection with the low spore dose (500 cfu / ml; n = 175) and defined as "resistant" (n = 121) if they survived up to day 8 after infection with the higher dose (1,000 cfu / ml; n = 250) (Figure 1). The intermediate phenotypes and controls were excluded from the further analyses.

In a first selective step we screened pooled DNA samples of the susceptible and resistant drones in a bulk segregant analysis (BSA; Michelmore et al. 1991). This

procedure identified heterozygous loci where the two alternative queen alleles are present in inverted frequencies in the phenotypic DNA pools. DNA was extracted individually from homogenates of whole larvae using the DNeasy Blood & Tissue Kit (Qiagen, Cat. No.69506). DNA concentrations were measured using the Nano Drop ND 1000 Spectrophotometer (v 3.5.2) and equal amounts of DNA per individual were pooled according to the defined susceptible (n = 35) and resistant (n = 121) phenotypes. In this step a total of 573 fluorescent tagged microsatellite markers (Solignac et al. 2007) covering the entire genome were tested in multiplex PCRs (3 min 94 °C, 35 cycles of 30 sec 94 °C, 30 sec 55 °C and 1 min 72 °C, 10 min 72 °C, ∞ 4 °C; eight primer pairs per reaction) for different amplification patterns of the two phenotypic DNA pools, also including negative controls without DNA. PCR products were run on the MegaBACE 1000 capillary sequencer and analysed using the software Fragment Profiler Version 1.2 (Amersham Biosciences, © 2003). 291 loci were found to be heterozygous in our queen resulting in a resolution of about one informative marker every 14 cM or every 755 Kbp. The mean distance between markers was 12.1 ± 0.7 cM (SE), 56 % of the genome was less than 5 cM and 80 % less than 10 cM away from a heterozygous marker tested in the BSA. The marker coverage for each chromosome is illustrated in Figure S1.

In order to identify candidate marker loci that were linked to the resistance phenotype, the abundance of the two alternative alleles was quantified based on the measured fluorescent peak heights in the PCRs on the DNA pools. We determined the difference D in allelic abundance between the resistant and the susceptible pool as follows:

$$D = \left| \frac{R_1}{R_1 + R_2} - \frac{S_1}{S_1 + S_2} \right| + \left| \frac{R_2}{R_1 + R_2} - \frac{S_2}{S_1 + S_2} \right|$$

where

R_1 and R_2 = peak heights of the two alternative alleles in the resistant pool and

S_1 and S_2 = peak heights in the susceptible pool

Hence, D is the sum of the relative differences in allele abundance between the two pools, corrected for different PCR efficiencies. Loci with $D > 0.25$ (n = 103) were chosen for subsequent individual genotyping of all 35 susceptible and a subset of 50 resistant individuals. For all loci showing a significantly biased distribution of the segregating alleles between the resistant and susceptible larvae (Chi Square test, $p < 0.05$), additional neighbouring markers were genotyped to increase the marker density in these chromosomal regions.

2.3. Simple interval mapping and QTL modelling

The genotypes of all 85 genotyped individuals comprising 116 markers in 12 chromosomal candidate regions were entered into the R package R/qtl (R Development Core Team 2010; Broman *et al.* 2003) to conduct a simple interval mapping using a binary single QTL model, and to estimate the phenotypic variance explained by each QTL. Those of the "susceptible" phenotype were scored as "0" and individuals of the "resistant" phenotype as "1" as binary trait variable. Individuals were coded as doubled haploids. Marker distances were defined according to the genomic map (Amel_4.5, NCBI Map Viewer, www.ncbi.nlm.nih.gov). A two-QTL model was used to quantify

the epistatic effects and to construct a heat map illustrating the LOD score of each interaction using a colour-code. Significance thresholds (suggestive: p = 0.20; significant: p = 0.05) for the presence of a QTL were calculated using 10,000 permutations for both models seperately. This approach randomly permutates the trait values of the given data set among the individuals, thereby destroying any linkage between the phenotype and the genotype, and records the maximum genome-wide linkage score for the resulting data set.

Candidate regions were defined where the LOD score exceeded the significance thresholds. In addition we determined the impact of each QTL region over larval developmental time by separately analysing the deceased individuals of each day (day 5: n = 16; day 6: n = 12; day 7: n = 7) with all survivors (n = 50) in three data subsets. This allowed for visualizing the effect of a QTL on a specific day during larval development. Interval mapping and permutation tests were conducted separately on each subset. We then searched the honeybee genome (Amel_4.5) and corresponding data libraries from other organisms (Flybase, Kyoto Encyclopedia of Genes and Genomes) for annotated genes within the defined candidate regions to infer potential gene functions in search of potential candidate genes.

3. RESULTS

Onehundred and three of the 291 heterozygous loci tested in the BSA were above the fluorescent peak height ratio of D = 0.25, potentially informative, and were individually genotyped. Simple interval mapping using R/qtl identified one significant (LOD > 2.80)

QTL region on chromosome 1 for larval mortality on day 5 (Figure 2, grey line), which is the first day of infection induced mortality. The significant QTL region spans 233 Kilobases (Kbp) comprising 20 annotated genes. The phenotypic effect was smaller in the entire data set (Figure 2, black line) but still suggestive. The amount of phenotypic variance explained by this QTL in an one-locus-model was 15.3 % for the subset from day 5 and 7.3 % for the entire data set. A list of the 20 annotated genes located in the significant QTL region is given in Table S1.

In addition to the significant QTL on chromosome 1, three suggestive QTL regions (LOD > 2.00) were identified on chromosomes 1 and 16 (Figure 2). In all suggestive QTL regions the QTL effect was strongest on day 5 after infection compared to the effects on day 6 and 7 (Figure S2). When added to the model with the significant QTL the individual additive effects of the three suggestive loci explained 6.5 %, 4.8 % and 6.6 % of the phenotypic variance respectively using the entire data set. The amount of total variance explained by the single effects of all four QTL was 33.4 %.

In the two QTL model only one significant epistatic interaction (LOD score > 5.73; 5,000 permutations) was identified of a marker within the significant QTL region on chromosome 1 (UN328) with a marker on chromosome 11 (SV285) as illustrated in the heat map (Figure S3). This interaction explained 6.8 % of the total variance and together with the single effects of the four previous loci 40.7% of the overall variance (Table I).

4. DISCUSSION

We identified one significant and three suggestive QTL regions and one significant epistatic interaction that influenced larval survival of haploid drone offspring from a single honeybee queen after infection with the bacterial pathogen *P. larvae*.

Since all QTL regions contain many predicted genes (Tables S1 and S2), expression data for infected larva is still lacking, and the mechanism of larval AFB resistance is not yet understood, a meaningful selection of candidate genes is difficult at best at this stage. We will nevertheless in the following discuss the genes within our candidate regions based on the information available from other organisms.

Most of the twenty annotated genes within the significant QTL region on chromosome 1 are involved in processes presumably irrelevant for our phenotype, such as DNA repair (Table S1: Nr. 2, 3), ion transport (Nr. 4, 5), oogenesis (Nr. 6), energy metabolism (Nr. 7, 10, 11, 13 in Table S1), or encode tRNAs (Nr. 14) and hypothetical genes (Nr. 8, 9, 15, 16). Of the remaining ones four are involved in development (Nr. 1, 18, 19, 20), including two homologs to *plexin A*, which influences axon guidance during nervous system development, and one homolog of a *Patched-1 receptor*, which is part of the hedgehog signalling pathway. The only gene with potentially immunological function is a *C-type lectin 1* (Nr. 12) which has an attributed function in cell-cell adhesion and an immune response to pathogens.

While further genes with an immunological function are lacking in the suggestive QTL regions, numerous developmental genes are present. The second suggestive QTL region on chromosome 16 contains six genes which are involved in developmental processes (Table S2: Nr. 32, 33, 39, 46, 48, 49). The gene at the position with the highest LOD score is a *Lim3 homeobox* homolog (Nr. 46), which acts as a transcription factor in

larval morphogenesis in *Drosophila*. The remaining two suggestive QTL regions also contain two homeobox gene homologs (Table S2: Nr. 24 *proboscipedia*, Nr. 30 *sex combs reduced*) and are closely linked to four additional ones (*retinal homeobox* (GB19717), *orthopedia* (GB30099), *labial* (GB14027), *antennapedia* (GB18813)). Also further developmental genes are present in the suggestive regions (Table S2: Nr. 1, 20, 22, 23) including a *forkhead* transcription factor (Nr. 3), a homeobox corepressor (Nr. 5) and the Hox gene *deformed* (Nr. 34). Finally, the gene located at the position of the significant epistatic marker on chromosome 11 is *ecdysone-induced protein 75* (Table S1: Nr. 21), which influences the ecdysteroid titer during larval development.

Whereas we remain uncertain about the significance of these developmental genes, the high density of developmental genes is remarkable and an impact of these genes on AFB resistance in larvae appears plausible since it is in the developing larva where the pathogen strikes. Furthermore, developmental differences (growth rate) between worker and drone larvae have been shown to correlate with survival after an AFB infection (Behrens et al. 2010) and may therefore also influence AFB resistance phenotypes.

We would like to highlight that our findings only relate to prepupal survival after infection of larvae and that we cannot answer whether larvae of the "resistant" phenotype in this study would have developed into healthy adults. Any mortality during pupal development may be governed by different mechanisms and needs to be studied independently. Since we also did not look for further (sublethal) effects of the infection we cannot predict whether our identified QTL regions have the potential to cause a (complete) resistance of the host or rather an enhanced tolerance towards the pathogen.

In any case possible counter-adaptations of the pathogen might complicate breeding attempts for AFB resistant honeybee stock.

Clearly there are caveats due to the methodological constrains resulting from the bulk segregant analysis (false negative loci due to inaccurate measurements in the bulked sample), the limited marker coverage (Figure S1) and the general statistical limitations of QTL-mapping (Slate 2005), which include the occurrence of false positive signals, an underestimation of the number of QTL, and an overestimation of the effect sizes (known as the 'Beavis Effect'). Especially when using binary traits and a selective genotyping approach, false positive signals may occur (Manichaikul and Broman 2009). Also QTL effect sizes depend on the genetic background of the study population (allele differences, homozygous QTL are impossible to map, epistatic interactions) as well as on environmental conditions, which is why even similarly conducted mapping studies rarely discover the same QTL. It is therefore almost impossible not to miss major loci. In order to use the identified regions meaningfully in marker assisted breeding programmes, it is required to confirm and quantify the general impact of these regions on AFB resistance phenotypes in more detail using other mapping populations from different genetic backgrounds and honeybee subspecies as well as additional bacterial strains and resistance parameters. Furthermore, particularly in light of the detected epistatic interaction it will be important to test in which way these genes interact in the genetically more complex setting of diploid female larvae, and finally, how the results obtained under *in-vitro* conditions translate into those conditions within a colony.

Nevertheless, the positive signals are indications for the presence of major genes in the identified genomic regions that are relevant for AFB resistance. Whole transcriptome

expression studies (e.g. RNA-sequencing) may help to identify those candidate genes which actually are responsible for larval AFB resistance (Tables S1 and S2). The lack of immune genes in most of our QTL regions shows that genetic variance for *P. larvae* resistance can be driven through alternative physiological pathways. The candidate genes detected in this study may form an important basis to interpret gene cascades in expression studies and identify the regulatory genes that trigger the genetic immune response after AFB infection.

ACKNOWLEDGEMENTS

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FIGURE AND TABLE CAPTIONS

Fig. 1. Cumulative mortality in percent of drone larvae at two infection dosages: 500 cfu / ml (squares, n = 175) and 1,000 cfu / ml (circles, n = 250) (redrawn from Behrens *et al.*, 2010). The dash-lined boxes illustrate the definition of the phenotypes for the DNA pools (S = "susceptible" dead; R = "resistant" alive). Phenotypes outside the boxes were not concerned in this study.

Fig. 2. LOD scores in the significant and the three suggestive QTL candidate regions on chromosomes 1 and 16 for the entire data set (in black) and for the subset contrasting the survivors with the deceased individuals on day 5 after infection (in grey) using R/qtl. The horizontal lines indicate the respective QTL thresholds (significant = solid, suggestive = dashed; 10,000 permutations). The position of the microsatellite markers used is indicated by the vertical lines along the x-axis.

Table I. Description of QTL regions identified in this study. The position and length of each region in Kilo base pairs [Kbp], the number of annotated candidate genes, the name of the microsatellite marker with the highest LOD score, the LOD score at this marker, and the amount of variance explained by this marker in the five locus model is given for the significant (1) and suggestive (2-4) QTL regions and for the significant epistatic interaction (1:5).

TABLE

Table I

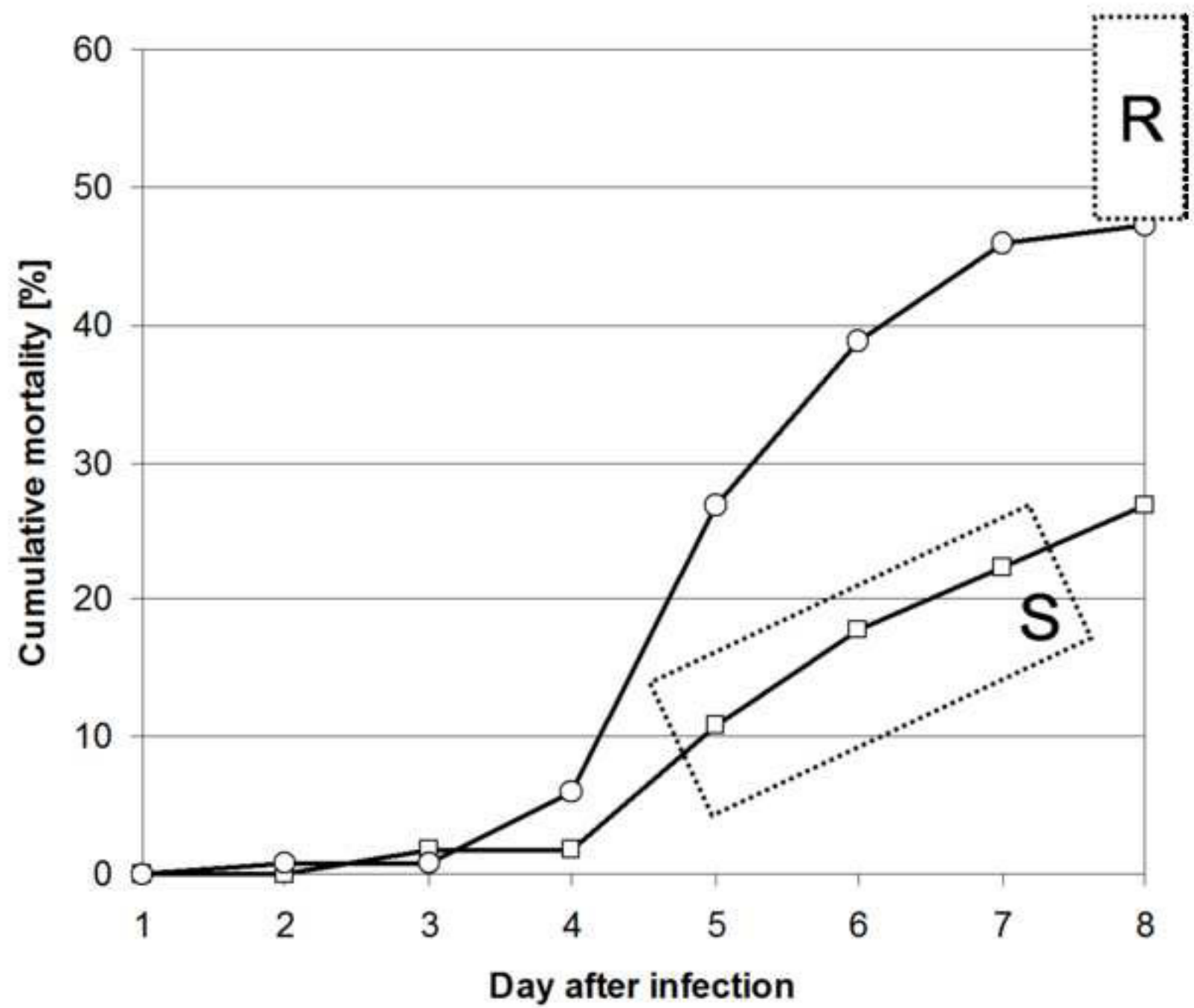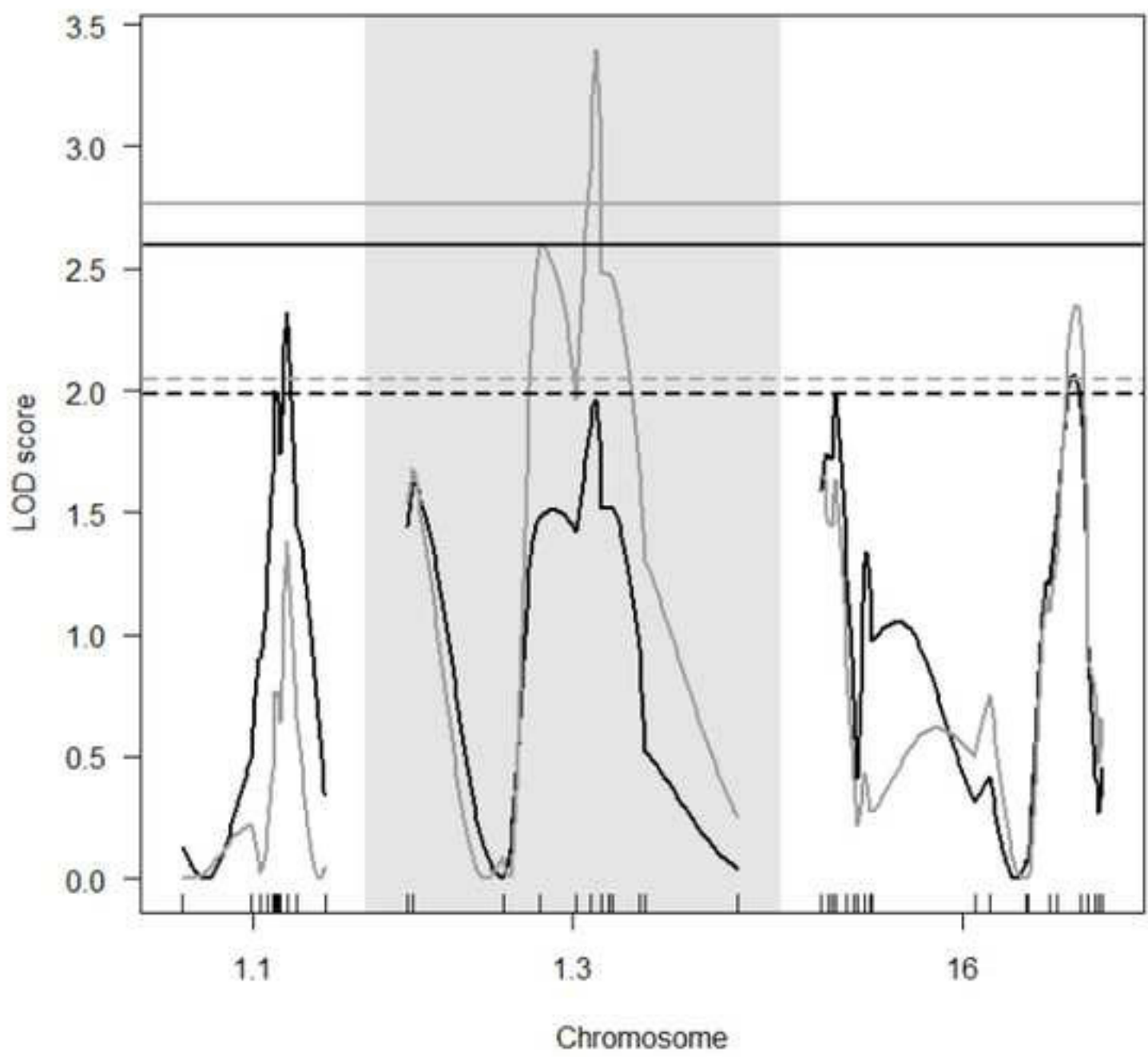| QTL	Chromosome	Start [Mb]	Length [Kbp]	Candidate genes	Marker	LOD score	Variance explained [%]
1	1	25.61	233	20	BI319	3.11	7.1
2	1	2.49	439	23	SV042	2.56	4.1
3	16	2.37	190	8	K1614	2.08	3.9
4	16	6.23	271	18	K1626	2.50	4.9
5	11	8.36			SV285	5.82	6.8

Figure 1

Figure 2

$$D = \left| \frac{R_1}{R_1 + R_2} - \frac{S_1}{S_1 + S_2} \right| + \left| \frac{R_2}{R_1 + R_2} - \frac{S_2}{S_1 + S_2} \right|$$