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Original article

Infection of drone larvae (*Apis mellifera*) with American foulbrood*

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Abstract – In-vitro reared drone larvae of several sister queens from an *Apis mellifera ligustica* and a Buckfast breeding line were infected with *Paenibacillus larvae* (type strain ATCC 9545) causing American foulbrood (AFB). Although drone larvae were susceptible to AFB and could be infected under in-vitro conditions there were differences within and between lineages. Infection sensitivity was higher in the *A. m. ligustica* line compared to the Buckfast line. Different infection thresholds were found among sister queens of the *A. m. ligustica* line suggesting a considerable genetic variance for larval resistance against AFB.

Paenibacillus larvae **/ disease resistance / in-vitro rearing / mortality**

1. INTRODUCTION

American foulbrood (AFB) is caused by the spore-forming bacterium *Paenibacillus larvae* (sensu Genersch et al., 2006). The spores germinate in the larval midgut of young larvae and penetrate the midgut epithelium, and finally enter the body cavity. Infection results in death of the larva and degradation of the larval tissues to a brownish sticky mass. Dead larvae are highly contagious and may contain about 2.5×10^9 spores (Sturtevant, 1932). Worker bees become contaminated when removing dried larvae, which probably is an important infection pathway to spread the infectious spores in the colony. Infection may also be transferred horizontally between colonies by robbing of contaminated honey or drifting of spore carrying adult bees. The spores are extremely long-lived and can germinate even after more than 35 years (Haseman, 1961), probably making nesting sites and beekeeping equipment important vectors for transmission. The pathogen is often present without producing clinical disease signs visible to the beekeeper (Fries et al., 2006), but when clinical disease occur, infected colonies often succumb to the disease (Hansen and Brødsgaard, 1999). Thus, AFB is considered to be one of the most destructive brood diseases in honeybees (Burnside et al., 1949).

Although AFB was identified for more than a 100 years, it still plagues beekeeping world wide. Current strategies to fight AFB include antibiotic treatments, killing of infected colonies, large scale quarantine actions (Hansen and Brødsgaard, 1999) and breeding resistant honeybee strains.

Since the 1930's honeybee lines are known to differ in resistance to AFB at the colony level (reviewed by Rothenbuhler, 1958). Traits or conditions known to be associated with disease resistance include intensity of hygienic behaviour (reviewed by Spivak and Gilliam, 1998a, b), removal of spores from the honey by action of the honey stopper (Sturtevant and Revell, 1953) and food compounds such as fatty acids (Rose and Briggs, 1969) and pollen

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(Rinderer and Rothenbuhler, 1974) that reduce bacterial growth of *P. larvae*.

In addition, Rothenbuhler and Thompson (1956) showed that individual larvae can have an innate resistance to AFB. Palmer and Oldroyd (2003) found that workers of different patrilines within a colony differ in AFB resistance demonstrating that larval resistance has an allelic component. The finding that expression levels of antimicrobial peptides, especially abaecin, after infection with AFB differ among lineages (Evans, 2004) and that expression levels correlate with resistance at the colony level (Evans and Pettis, 2005) indicate that compounds of the immune system may play an important role in larval resistance and that larval resistance may act as a measurement for colony resistance. However, the biological mechanisms of larval resistance are not fully understood.

This study aims to detect differences in larval resistance between and within honeybee lines to test for genetic variance in larval disease resistance. We take advantage of male haploidy for testing genetic variance of AFB susceptibility among drone larvae in an exposure bioassay. Using the in-vitro rearing of larvae, we can eliminate all factors known to influence colony level AFB resistance (e.g. hygienic nurse bees, variance in infection load) and monitor larval development under highly controlled conditions.

2. MATERIALS AND METHODS

2.1. Honeybee lines and queen treatment

Seven sister queens of a Buckfast breeding line and three sister queens of an *A. m. ligustica* breeding line were used for this study. One week old virgin queens were gassed twice with CO_2 for five minutes. This procedure initiates egg-laying and since all queens were unmated they exclusively laid unfertilized eggs developing into haploid drones.

2.2. In-vitro-rearing

Larvae were reared in-vitro following a protocol according to Peng et al. (1992) modified according to Genersch et al. (2005). Drone larvae were reared in 24-well tissue culture plates with a diet consisting of 66% royal jelly (v/v) (Stakich, Inc., Bloomfield Hills, MI, USA), 3% glucose (w/v) and 3% fructose (w/v) in sterile distilled water. The wells were filled with approximately 0.3 mL of this diet using a scaled sterile syringe. Larvae younger than 24 hours were taken from the combs using a Chinese grafting tool (Graze Bienenzuchtgeräte, Germany) and carefully transferred to the surface of infectious larval diet. Larval age was estimated by size. After 24 hours the growing larvae were transferred to a new well with uninfected diet. Dead larvae were removed. On subsequent days the larvae were transferred to fresh diet every second day to reduce handling stress. Due to larval growth the number of larvae per well was ten, five, three and one at the first, second, forth, and sixth day respectively. For larger larvae a rounded sterile metal hook was used to transfer the larvae from one well to the other.

The larvae were maintained in an incubator at 35 °C with a relative humidity of 96%.

Experiments were terminated at day nine when drone larvae reached the prepupal stage because only little additional mortality after day nine was observed in a first set of five pilot experiments which were run up to 14 days.

2.3. Infection and bacterial isolates

The *P. larvae,* ERIC I genotype (Genersch et al., 2006), type strain ATCC 9545 from the American Type Culture Collection was used for all infection experiments. This strain has a comparatively low individual larval virulence in the sense of killing its host slowly (Genersch et al., 2005). Using this particular strain, we expected larger variance in mortality, thus allowing a higher resolution of detecting temporal differences in the reaction of honeybee lines to the pathogen.

The concentration of the spore stock solution was estimated by viable cell counting. A defined volume of spore solution was spread onto MYPGP-agar plates in 5 replicates. Plates were placed in an incubator (35 °C, 96% humidity, 5% CO_2) for ten days and colonies were counted (Nordström and Fries, 1995).

The concentration of the stock solution was adjusted to approximately 20.000 colony forming units (cfu) per mL. Since the number of colonies

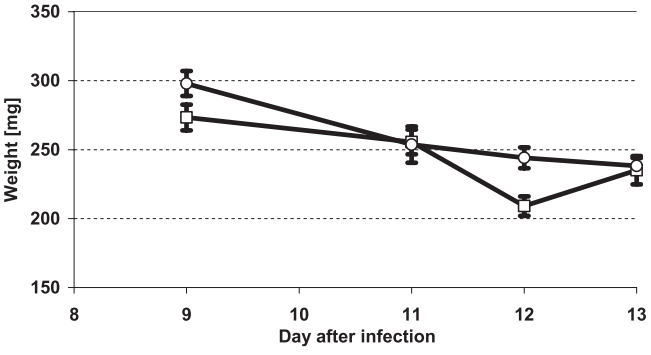


Figure 1. Average weight (mg ± s.e.) of surviving infected (open circles, n = 326) and control larvae (open squares, n = 286).

obtained in a viable count depends on a number of factors (e.g. suitability of culture medium, incubation conditions), underestimation of the actual viable cell mass within infected larvae is highly likely. Spore suspensions were stored at 4 °C.

For infection of the larvae the spore solution was thoroughly vortexed and mixed with the larval diet using a syringe to distribute the spores evenly. Larvae of the treatment group were placed on 0.3 mL of infectious diet with the defined spore concentration for 24 h. At subsequent days all larvae were fed normal diet. Control larvae were fed normal diet throughout the experiment.

2.4. Measuring mortality

Control and infected larvae were observed every 24 hours under a stereo microscope. Dead larvae were identified by ceased respiration, displayed colour change and body surface reflecting light differently due to dehydration. These combined traits made it easy to identify dead larvae. Dead larvae were removed from the experiment, weighed and frozen for future genetic analysis. At the end of the experiments all survivors were recorded, weighed and frozen. Larvae that died in the first 24 hours were excluded from the analyses because mortality due to infection of *P. larvae* never occurs before the second day post infection (Genersch et al., 2005).

Control and treatment group were checked for significant differences by making a survival analysis with Gehan's Wilcoxon test. This is a standard test to compare survival in two or more groups with censored observations (Gehan, 1969).

3. RESULTS

3.1. Larval weight

Figure 1 shows the effect of infection on larval body weight of larvae that survived the experiment. The differences in larval development between controls and treatment were not statistically significant (hierarchical nested ANOVA, days nested into treatment, $P = 0.666$). Furthermore, no obvious impact of infection on larval development was observed. 70% of the surviving larvae reached prepupal stage on day nine (100% on day 10) after infection irrespective of treatment.

3.2. Dose-mortality relationship

The mortality of drone larvae from one *A. m. ligustica* queen was tested with two different spore concentrations (Fig. 2). There were no significant difference between the control and the treatment group at the concentration of 400 cfu/mL (Gehan's Wilcoxon test, $P = 0.191$). In the treatment group with 1000 cfu/mL, however, mortality differed significantly from the control group and the group given the lower spore dose (Gehan's Wilcoxon test, $P < 0.001$).

3.3. Differences between lines

We compared the two honeybee lines, by calculating the difference between mean mortality in control and treatment group per queen

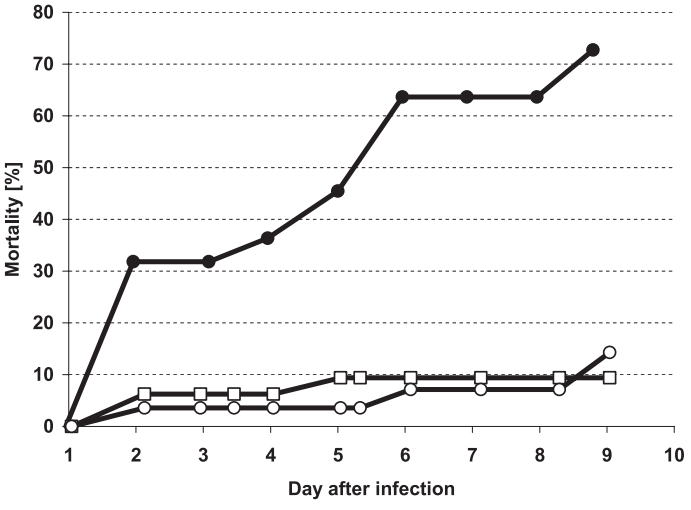


Figure 2. Dose-mortality effect. Average cumulative mortality (%) of *A. m. ligustica* drone larvae infected with 400 (open circles, n = 28) and 1000 cfu/mL (filled circles, n = 22). Untreated controls; open squares, n = 32.

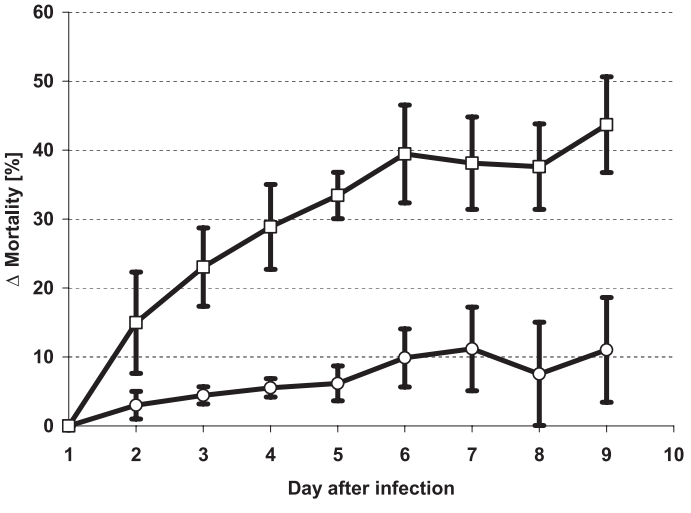


Figure 3. Average mortality (% corrected for control) of drone larvae of the Buckfast (open circles, n = 102) and the *A. m. ligustica* line, treated with 1000 cfu/mL (open squares, n = 125).

for all three *A. m. ligustica* sister queens and all seven Buckfast sister queens at a spore concentration of 1000 cfu/mL (Fig. 3). The survival analysis showed a significant difference between the treatment groups (Gehan's Wilcoxon test, $P = 0.033$, n = 102 Buckfast and 125 *A. m. ligustica* drone larvae).

In the Buckfast line infection with a concentration of 400 cfu/mL did not result in an increased mortality compared to the control group up to day nine (Gehan's Wilcoxon test, $P = 0.460$, n = 230 controls and 176 infected) but differences were highly significant at the higher concentration of 1000 cfu/mL (Gehan's Wilcoxon test, $P = 0.009$, n = 230 controls and 128 infected).

In the *A. m. ligustica* line infection resulted in an increased mortality at both concentrations, 400 cfu/mL (Gehan's Wilcoxon test, $P = 0.040$, n = 272 controls and 59 infected) and 1000 cfu/mL (Gehan's Wilcoxon test, $P < 0.001$, n = 272 controls and 140 infected). The standard errors in the treatment group were high, indicating a strong variance among queens within the *A. m. ligustica* line.

Absolute values for the mean mortality in the treatment group were higher in the *A. m. ligustica* line at both concentrations (37.8%

respectively 59.3% at day nine after infection) than in the Buckfast line (27.1% respectively 40.9%).

3.4. Differences within lines

Within the *A. m. ligustica* line differences in mortality between controls and treatment were not significant for *A. m. ligustica* queen A at the concentration of 400 cfu/mL (Gehan's Wilcoxon test, $P = 0.191$) but highly significant for the concentration of 1000 cfu/mL (Gehan's Wilcoxon test, $P < 0.001$) as well as between the two treatment groups (Gehan's Wilcoxon test, $P < 0.001$) (Fig. 4). Differences in mortality were highly significant for *A. m. ligustica* sister queen B at both concentrations 400 cfu/mL (Gehan's Wilcoxon test, $P < 0.001$) and 1000 cfu/mL (Gehan's Wilcoxon test, $P < 0.001$). Differences were highly significant in *A. m. ligustica* queen C even for the concentration of 200 cfu/mL (Gehan's Wilcoxon test, $P = 0.004$) as well as for 1000 cfu/mL (Gehan's Wilcoxon test, $P < 0.001$).

While all three progeny sets from the *A. m. ligustica* line were susceptible at a dose of 1000 cfu/mL only one of seven sets from the Buckfast line was equably susceptible (Gehan's Wilcoxon test, $P = 0.048$ at 400 cfu/mL and $P = 0.024$ at 1000 cfu/mL). The absolute values for the mortality in the treatment group at day nine after infection were 24.2% at 400 cfu/mL and 27.3% at 1000 cfu/mL.

4. DISCUSSION

4.1. Larval weight

The weight of surviving drone larvae in both treatment and controls match the values given in the literature for drone larvae reared under natural conditions (reviewed by Jay, 1963). This suggests that the in-vitro-rearing conditions per se have no negative impact on larval development.

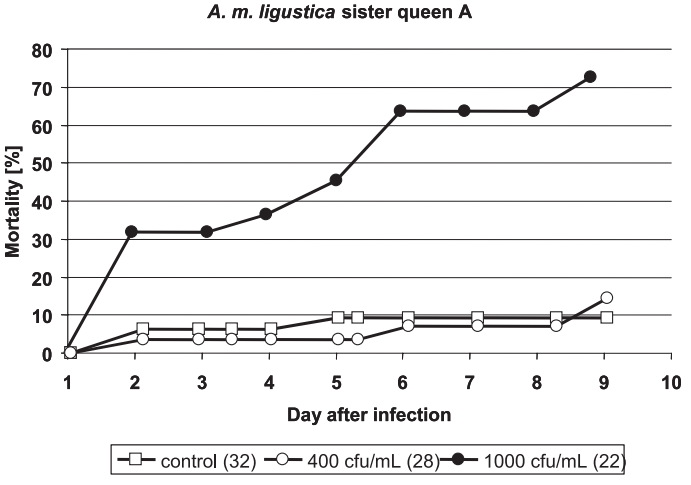
4.2. Infection of drones

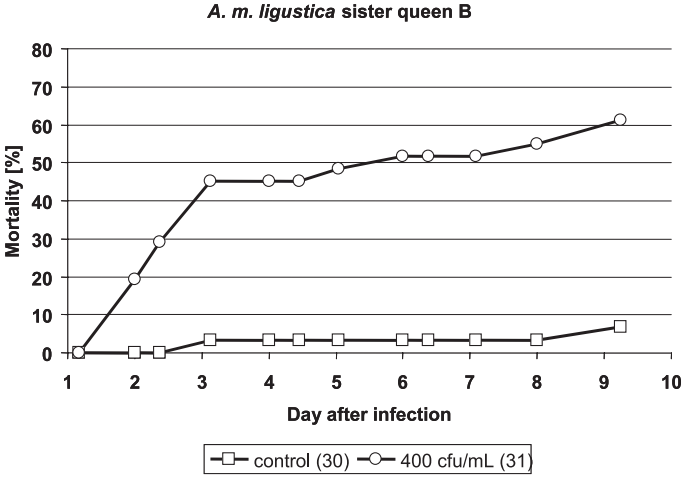
We could show that in-vitro rearing is a feasible routine method to study susceptibility of drone larvae to AFB under controlled conditions. Drone larvae were found to be clearly susceptible to AFB in this exposure bioassay confirming the observations of Woodrow and Holst (1942) that not only workers but also drone and queen larvae can become infected. Genersch et al. (2005) estimated a LC_{50} (concentration that is lethal for 50% of the infected larvae) of 200 cfu/mL for the strain ATCC 9545 with in-vitro reared worker larvae. The LC_{50} of all drone larvae used in this study would be five times higher around 1000 cfu/mL. At this concentration 128 out of 266 treated drone larvae died before day nine, which represents a mortality of 48.1% (disregarding control mortality). In our exposure bioassays the concentration of bacterial stock solution was determined by colony count on MYPGP agar and incubation with 5% CO_2, whereas Genersch et al. (2006) used Columbia sheep blood agar without carbon dioxide. Nordström and Fries (1995) demonstrated that MYPGP agar and a 5% CO_2 atmosphere enhances spore growth about five times compared to Blood agar without CO_2. Hence, the spore dose resulting in a 50% mortality of drone larvae may in fact be coherent with the lethal dose suggested by Genersch et al. (2006) for this bacterial strain in worker larvae.

Nevertheless, it is possible that drone larvae respond differently to infection with AFB than worker larvae due to developmental differences, for example a different time span between moultings. Further studies on larval resistance to AFB should include comparisons between drone and worker larvae.

4.3. Variation between and within lines

The progeny of queens from the Buckfast line was found to be less susceptible to the tested spore concentrations than the *A. m. ligustica* line. This confirms that honeybee lines do vary in their susceptibility to AFB infection at the larval level. The differences among the drone offspring of the three *A. m.*





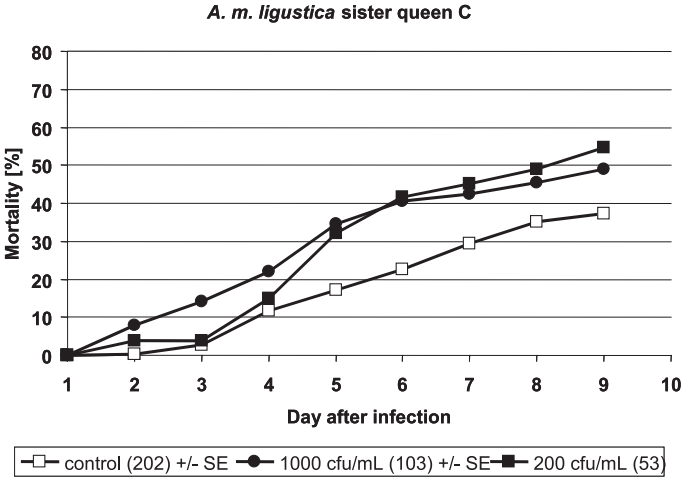


Figure 4. Average mortality (% corrected for controls) of drone larvae of the three *A. m. ligustica* sister queens (A, B and C) and treatment groups with 200 (filled squares), 400 (open circles) and 1000 cfu/mL (filled circles). Numbers in parenthesis indicate sample sizes.

ligustica queens suggest a genetic variance for a susceptibility threshold, below which infection is sublethal up to day nine after infection. This threshold seems to lie between 400 and 1000 cfu/mL for queen A, below 200 cfu/mL for queen B and probably above 1000 cfu/mL for queen C, although high control mortality makes interpretations difficult. We expect that susceptibility thresholds of the tested Buckfast queens were all above our tested concentrations.

We used only one bacterial strain for infections. Given the found differences in pathogen virulence among AFB strains (Genersch et al., 2005) it is possible that other strains would lead to different results, that certain strains are adapted to certain bee lines or that different bacterial strains differ in pathogenicity between bee lines as suggested by Fries and Camazine (2001). Considering the low number of queens we used and their close relatedness, we can not say, whether Buckfast bees in general are more resistant to AFB compared to Italian bees.

Different levels of antimicrobial peptides, as found by Evans and Pettis (2005), might have led to the observed variance. In our study larvae received food ad libitum. Thus, it is not possible to say, whether resistance had an energetic cost leading to reduced colony productivity, if larvae compensate infection by additional feeding. To understand if larval resistance to AFB has an energetic cost it would be necessary to restrict or measure the amount of diet consumed by the larvae.

Future work will aim at identifying the genes responsible for the observed variance in disease resistance and to investigate their role in the resistance mechanism.

ACKNOWLEDGEMENTS

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Infection par la loque américaine des larves de mâles de deux lignées d'abeilles domestiques (*Apis mellifera*).

***Paenibacillus larvae* / résistance à la maladie / mortalité / élevage in-vitro**

Zusammenfassung – Infektion von Drohnenlarven (*Apis mellifera*) mit Amerikanischer Faulbrut. Drohnenlarven von Schwesterköniginnen einer Buckfast und einer *Apis mellifera ligustica* Brutlinie wurden mit Sporen eines Bakterien-Typstammes von *Paenibacillus larvae*, dem Erreger der Amerikanischen Faulbrut (AFB), einer schweren Brutkrankheit der Honigbiene, infiziert. Die Sterblichkeit der Larven wurde täglich in nicht-infizierten Kontrollen und der sporenbehandelten Gruppe aufgenommen. Die Larven wurden unter kontrollierten in-vitro Bedingungen aufgezogen, um alle Faktoren auszuschließen, die bekanntermaßen AFB Resistenz auf Kolonieebene beeinflussen (z.B. hygienisches Verhalten, Futterkomponenten). Larven der untersuchten Königinnen der Buckfastlinie waren bei den untersuchten Sporendosen weniger anfällig als Nachkommen der *Apis mellifera ligustica* Linie (Abb. 1). Dies zeigt, dass in verschiedenen Honigbienenlinien unterschiedliche Resistenzniveaus existieren. Wir können allerdings nicht sagen, ob die gefundenen Resistenzniveaus repräsentativ für alle Buckfast bzw. *Apis mellifera ligustica* Linien sind und ob sie auch konsistent für andere Bakterienstämme wären. Innerhalb der *A. m. ligustica* Linie fanden wir eine Varianz für Infektionsgrenzwerte (Abb. 2). Dies zeigt, dass diese Grenzwerte auch genetisch beeinflusst sein können. Zusätzlich wurde eine Methode entwickelt, um Varianz in larvaler Resistenz gegen AFB zu bestimmen, unter Benutzung haploider Drohnenlarven.

***Paenibacillus larvae* / Krankheitsresistenz / in-vitro Aufzucht / Sterblichkeit**

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