TOLERANCE MECHANISMS AGAINST AMERICAN FOULBROOD IN HONEYBEE LARVAE AND COLONIES

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Abstract

Recently, increasing incidences of AFB and problems controlling the disease – based on resistance build-up in the bacteria, increased residue problems, and loss of natural resistance in bees – have renewed the interest for AFB tolerant honey bee strains world-wide. The honey bee possesses different tolerance mechanisms against AFB – e.g. larval resistance, hygienic behaviour and food inhibition of bacterial growth. Up till now, in Denmark, the only method used by queen breeders in testing strains for AFB tolerance has been testing for degree of hygienic behaviour mainly by inserting killed capped brood and examining removal.

Therefore, the aim of our study was to examine general hygienic behaviour regarding removal of both non-capped as well as capped larvae and larval tolerance against AFB in three different strains of honey bees. The ability to remove capped brood killed by freezing and removal of infected non-capped and capped brood was tested and correlated. Furthermore, the dose-response relationship of P.l. larvae *was examined in 24-28 h old honey bee larvae of the same strains reared and inoculated in the laboratory.*

The results show that there were significant differences in removal behaviour and larval tolerance among the different strains. However, none of the strains removed all killed brood and there were no seasonal variations in the removal behaviour within the different strains.

Some Danish Buckfast strains seem to have lost most of their tolerance mechanisms except hygienic behaviour. Our findings suggest that the hygienic behaviour expressed through removal of frozen capped brood alone or in combination with larval tolerance may not be sufficient to model whether a honey bee strain will be able to overcome an AFB infection.

Key words: *American foulbrood /* Paenibacillus larvae larvae */ tolerance / honey bees /* Apis mellifera

Introduction to tolerance

Paenibacillus larvae larvae (WHITE) is the causative agent of American foulbrood (AFB) in honey bees (*Apis mellifera* L.). This disease is lethal to honey bee colonies if curative control actions are not taken[15].

The honey bee possesses different tolerance mechanisms against AFB. *P.l. larvae* spores can infect the larvae of workers, queens and drones[39]. Woodrow[38, 39] studied the susceptibility of honey bee worker larvae tended by nurse bees by individual inoculation of spores in larval food. He demonstrated an effect of age on susceptibility showing that larvae younger than 24 hours are the most susceptible. The susceptibility of honey bee larvae has been further studied and shown to be associated with their genetic constitution[2, 16, 17, 31]. HOAGE and ROTHENBUHLER[16] found that 18-24-hours-old larvae from a resistant honey bee strain had an LD_{50} 2,500 spores and a slope of 0.496 compared with an LD_{50} of 1,300 spores and a slope of 0.663 for a less resistant strain. Contrary to the previous studies the larvae in the study of BRØDSGAARD et al.[4] were laboratory reared. In this way it was possible to exclude the influence of nurse bees thereby getting a more precise picture of the dose-mortality relationship in individual larvae. Larvae 24-28-hours old were found to be susceptible to infection with *P.l. larvae* with a clear dose-response relationship. The used bee strain was a common Danish *Apis mellifera ligustica* strain which had an LD_{50} of 8.49 spores. Older larvae become more and more resistant to infection so that no significant dose-mortality relationship existed when the larvae were older than 48 hours.

Several studies report that colonies without clinical symptoms of AFB may contain honey contaminated with *P.l. larvae* spores[e. g. 13]. Over a number of years (1978-1981 and 1985-1990) 9.7% of examined Danish colonies contained honey contaminated with *P.l. larvae* spores while only 3.7% of the colonies showed clinical symptoms of AFB[11]. Colonies can contain honey with a large number of spores for several years and still not show clinical symptoms of AFB[13]. Furthermore, field experiments with induced infection by *P.l. larvae* have shown that infected colonies may eliminate the infections and that no simple correlation exists between the number of spores in the honey and the first visible signs of AFB in capped brood cells[14].

No strain of honey bees is immune to AFB, but different degrees of resistance to the disease have been reviewed by ROTHENBUHLER[28]. The resistance in the various bee castes is associated with the food. Queen larvae which are fed the least amount of pollen are most susceptible, worker larvae fed moderate amounts of pollen are intermediate and drone larvae fed mostly pollen are least susceptible[25]. Pollen contains micro-organisms which act as antagonists against *P.l. larvae*[23]. These antagonists, isolated from the midgut of larvae as well as from adult bees and pollen collected by the bees were able to inhibit spore germination and colony growth of *P.l. larvae* under *in vitro* conditions. This inhibition is also likely to occur in the intestine of the larvae as feeding pollen to larvae 6-18 h old significantly reduced the mortality of experimentally infected larvae[26].

Other compounds in the larval food beside pollen play a role in resistance to AFB. In resistant honey bee strains the larval food is more effectively inhibiting the germination of *P.l. larvae* spores than larval food

from susceptible strains. Furthermore, the larval food from reistant strains is also more effectively reducing the number of vegetative cells of the bacterium than larval food from susceptible strains. One of the inhibitory components is thought to be fatty acids in the royal jelly[27].

The honey stomach in the adult bee plays a role in the resistance to the disease. Spores are removed from the food suspension in the honey stomach by action of the honey stopper. To some extent this ability prevents spore contamination of the food fed to the larvae[34]. Resistant bee strains filter the spores more efficiently than susceptible strains do[22, 34].

Adult bees are resistant to AFB when fed spores of *P.l. larvae*[37]. This resistance of adult bees may be due to substances with inhibitory activity in the midgut[6].

When honey bee larvae of different strains were infected at an age less than 24 h different levels of innate resistance to AFB has been revealed[31], making the gut environment less favourable for the bacteria in larvae from resistant strains[30], but the mechanism of this early resistance is not known. In some honey bee strains the majority of the experimentally infected larvae became completely resistant to infection with *P.l. larvae* at an age of approximately 1.5 days[2, 16]. At that age the peritrophic membrane is visible by light microscope[1], therefore, it was assumed that complete resistance was associated with the presence of this membrane. However, electron microscopy has shown the membrane to be present at a larval age of only 8 h[7] so the presence of the membrane is not the source of resistance. As the larva develops, the membrane changes in composition and increases in thickness and may, therefore, restrict the bacterium to the lumen of the intestine[7, 8]. Furthermore, the midgut epithelium also play a role in resistance to *P.l. larvae* as it act as a physical and chemical barrier.

Another very important factor in the resistance mechanism is the colony's ability to detect and remove diseased brood before *P.l. larvae* sporulates. Normally the first visible signs of AFB appear in the capped brood cells. Therefore, it has been the general opinion that bees with hygienic behaviour have to remove the cell capping and the diseased brood. According to ROTHENBUHLER this behaviour was throught to be dependent upon two recessive genes – one for uncapping and one for removal behaviour[29]. MORITZ has re-evaluated the model and concluded that uncapping was controlled by one gene but that removal behaviour might be controlled by two genes[18]. He also suggested that there could be some kind of interaction between them.

When individual larvae are inoculated with foulbrood spores many larvae die before the time of cell capping. In the bee colonies the first signs of AFB are not visible to human eyes until day 4 after infection. But at day 3 nurse bees remove about 50% of the larvae each inoculated with a high dose of spores[5]. Therefore, early removal behaviour is a very important trait to focus on when breeding for resistance against AFB.

PALACIO et al.[19] found that the total hygienic behaviour increased in a population after four years selection of queens without mating control. The hygienic colonies had a lower frequency of naturally occurring brood disease than non-hygienic colonies. Furthermore, it has been demonstrated that diseased non-hygienic colonies produce less honey than hygienic colonies[33].

Recently, increasing problems with AFB in Europe[9, 12, 20, 21] have renewed the interest and need for selection for AFB tolerant honey bee strains. In Denmark, the experience with control of AFB indicates that the problems have especially increased in some Buckfast strains[12].

Aim

The aim of the present study was to examine general hygienic behaviour and larval tolerance to AFB in selected strains of honey bees. The ability to remove capped brood killed by freezing and the dose-response relationship of *P.l. larvae* was examined in three different strains. The dose-response relationship was investigated on honey bee larvae reared and inoculated in the laboratory.

Material and methods

Three different honey bee strains were used. Two *ligustica*: L-1015, L-0603 and one Buckfast: B-0107. The queens of the colonies were sister queens of Danish strains. The queens were mated on small islands with known lines of drones. Prior to and during the experiments the colonies were checked for clinical symptoms of *P.l. larvae* according to WHITE[36] and honey samples were analysed according to HANSEN[10]. Furthermore, during a week every month the natural varroa drop-down on inserts was counted and the mite population in the colonies was estimated according to BRØDSGAARD and BRØDSGAARD[3].

Removing capped brood

Five colonies of each of the three strains were established and used for the freeze treatment. Furthermore, the natural removal behaviour of the colonies was registered in three control colonies of each strain three times during the experiment. The position of 100 newly capped brood cells were marked on a sheet of transparent plastic[16]. After 48 hours the empty cells were counted and the control removal percents were calculated. The removal test of capped frozen brood was carried out according to TABER and

GILLIAM[35] every second week from end May to end August. Totally, seven repetitions were carried out. A piece of comb was cut from an area with newly sealed brood cells using a sharp knife and a metal template of 5 cm x 6 cm covering 105 cells. The piece of comb was placed in a freezer at 18 °C for 24 hours. Thereafter, the piece of comb was returned to the colony and the number of cells from which the brood was removed was counted.

Dose-response relationship

Larval material

The test larvae originated from colonies of the three strains. The larvae were obtained by caging queens for 4-hour periods on brood less combs in the colonies. When the larvae reached the age of 24-28 hours, they were thereafter reared *in vitro*.

In vitro *rearing of larvae*

The larvae were grafted in a moist (RH>95%) glass chamber accessible from two sides at 35 °C as described by BRØDSGAARD et al.[4]. Thereafter, the larvae were reared by the technique described by REMBOLD and LACKNER[24] in an incubator with precise regulation of humidity and temperature (Biomed CO_2 incubator, ASSAB Classic T305 GF) with the modification described by BRØDSGAARD et al.[4]. The total number of dead larvae and pupae was recorded once a day.

Inoculation with P.l. larvae *spores*

Larvae were inoculated with different dosages of *P.l. larvae* spores through feeding directly to the mouthparts with 2 µl spore solution at a larval age of 24-28 hours. The spores originated from foulbrood scales containing spores of a Danish *P.l. larvae* strain, JT-79 and were prepared and counted as described in BRØDSGAARD et al.[4].

Results

Figures 1-3 show the average removal percent of killed brood of the three honey bee strains on the different sampling dates during the experiment. There were no significant seasonal differences in removal behaviour in the different strains. The average removal of the three strains varied from approximately 49% to 61%. The L-0603 and L-1015 strains were significantly different from each other while there were no significant differences between B-0107 and L-0603 or L-1015 (Table I). The bees of the control colonies removed significantly fewer larvae than in the treated colonies (X^2, $p<0.05$). In the control groups the mean removal percent were: 4.42 (±2.87 s.e.) in L-1015, 2.32 (±2.17 s.e.) in L-0603, and 4.67 (±0.91 s.e.) in B-0107. There were no significant differences in removal among the strains in the control group.

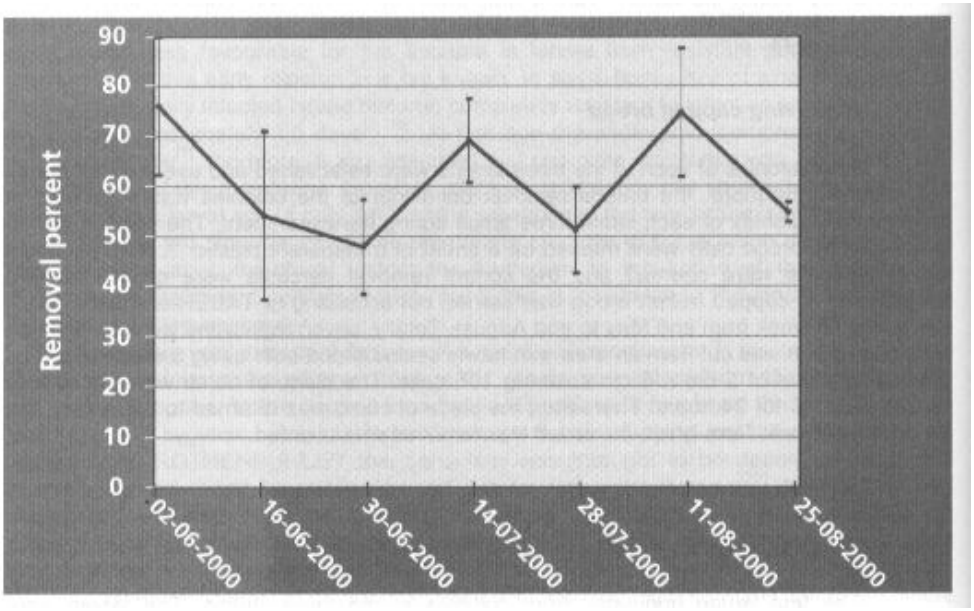

Figure 1 – The average removal (± s.e.) of capped frozen brood in the L-1015 honey bee strain on different sampling dates.

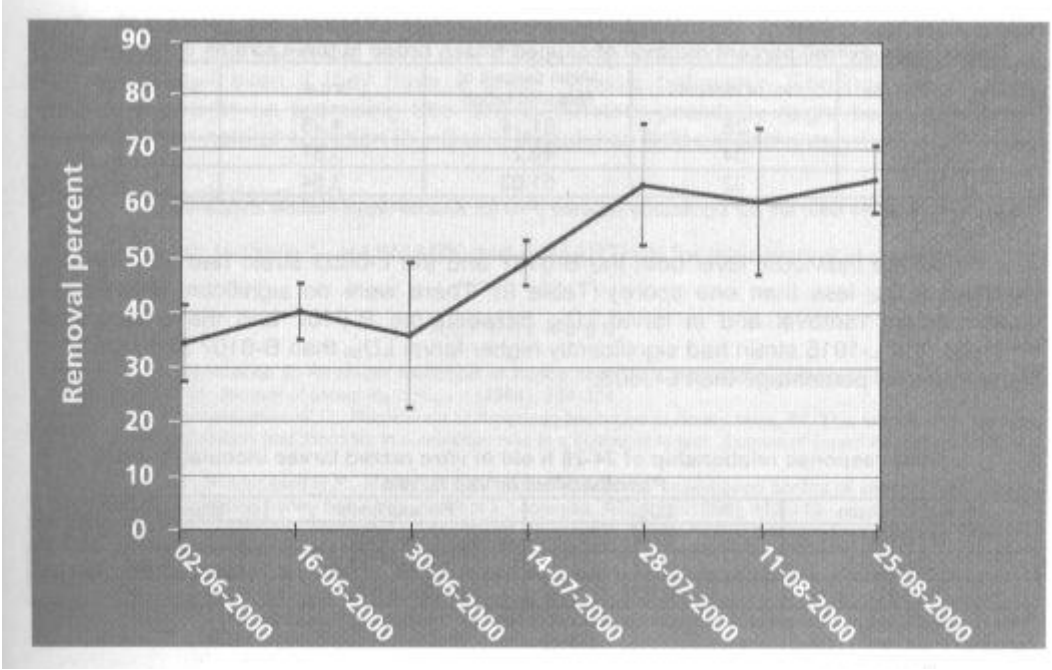

Figure 2 – The average removal (± s.e.) of capped frozen brood in the L-0603 honey bee strain on different sampling dates.

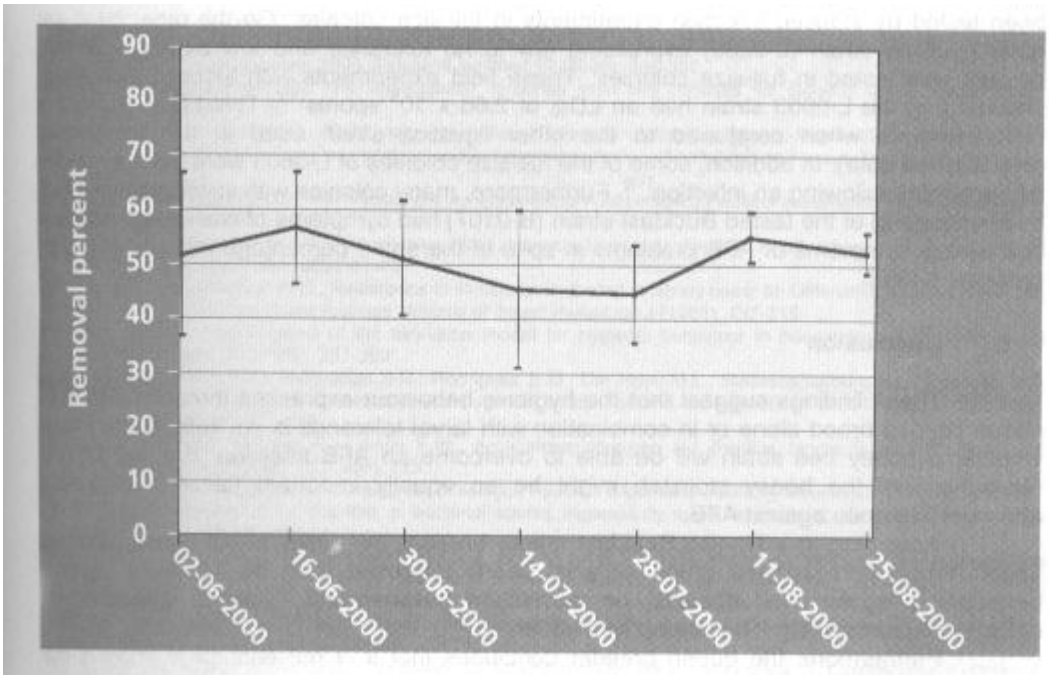

Figure 3 – The average removal (± s.e.) of capped frozen brood in the B-0107 honey bee strain on different sampling dates.

Table I

The average overall percent removal of capped frozen brood in three strains of honey bees

Honey bee strain	No. of samples	Mean percent of removed brood	± s.e.	Statistical grouping[1]
L-1015	28	61.14	4.28	A
L-0603	34	49.27	3.87	B
B-0107	32	51.03	3.54	AB

[1] *Means with the same letter are not significantly different (P<0.05, Kruskal-Wallis multiple comparisons)*

At the individual level both the B-0107 and the L-0603 strain had very low larval tolerance (LD_{50} less than one spore) (Table II). There were no significant differences in mean percent removal and in larval LD_{50} between the B-0107 and the L-0603 strain. Whereas, the L-1015 strain had significantly higher larval LD_{50} than B-0107 and significantly higher removal percentage than L-0603.

Table II

Dose-response relationship of 24-28 h old *in vitro* reared larvae inoculated with *Paenibacillus larvae larvae*

Honey bee strain	LD_{50}	Fiducial limits	Statistical grouping[1]
L-1015	8.42	7.00-9.97	A
L-0603	0.97	$1.15x10^{-5}$-13.42	AB
B-0107	0.07	$3.06x10^{-8}$-2.00	B

[1] *Means with the same letter are not significantly different (based on overlapping fiducial limits)*

Discussion

One of the tested honey bee strains (L-1015) clearly differed from the two others in having both a better removal behaviour and a higher larval tolerance. This strain might be suitable for beekeeping in areas with high incidences of AFB. However, this strain has never been tested by induced infection experiments in full-size colonies. On the other hand the other *ligustica* strain (L-0603) expressing low larval tolerance and low average removal percent was tested in full-size colonies. These field experiments with induced inoculation showed that the L-0603 strain had an LD_{50} of 2.66×10^9 spores[15]. This LD_{50} indicated a high tolerance when compared to the other ligustica strain used in this experiment (unpublished data). In addition, some of the full-size colonies of L-0603 were able to remove all the spores following an infection[15]. Furthermore, many colonies with sister queens (used by beekeepers) of the tested Buckfast strain (B-0107) had symptoms of chalkbrood infection and clinical symptoms of AFB problems in spite of the same percentage removal of frozen brood as L-0603.

Conclusion

These findings suggest that the hygienic behaviour expressed through removal of frozen capped brood alone or in combination with larval tolerance is not sufficient to model whether a honey bee strain will be able to overcome an AFB infection. E.g. the filtering mechanism of the honey stomach might be an equally important factor in explaining apparent tolerance against AFB.

According to a Danish Buckfast queen breeder the ability of his bees to remove frozen brood from capped brood cells is clearly improved after an 11 years hygienic behaviour program. Nevertheless, no correlation between the hygienic behaviour and clinical symptoms of e.g. chalkbrood was observed[32].

Furthermore, the queen breeder concludes that it is not enough to improve the hygienic behaviour of the Buckfast bees to improve the tolerance to AFB[32]. This indicates that some Buckfast strains have lost most of their tolerance mechanisms except hygienic behaviour.

Therefore, the present recommendations to Danish beekeepers and queen breeders are as follows: Avoid using bee strains with indications of poor tolerance against brood diseases – even if they have a good hygienic behaviour. Continue the hygienic behaviour programs on promising bee strains. Another possibility might be to select bee strains based on natural selection in induced inoculation field experiments.

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