

SUSCEPTIBILITY OF BEE LARVAE TO CHALKBROOD IN RELATION TO HYGIENIC BEHAVIOUR OF WORKER BEES IN COLONIES OF CHOSEN RACES OF HONEYBEE (*APIS MELLIFERA*)

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Received 25 August 2013; accepted 03 April 2014

Abstract

The susceptibility of bee larvae to *Ascosphaera apis* infestation and the hygienic behaviour of worker bees in relation to *A. apis* infected and freeze-killed brood were evaluated in three races of bees: *Apis mellifera carnica*, *Apis mellifera caucasica*, and *Apis mellifera mellifera*. Experimental bee colonies were evaluated in field conditions during the three beekeeping seasons. The lowest percentage of infected larvae was observed in *car* GR1 and *mel* A colonies (8.5% and 15%, respectively) and the highest in *car* Mr and *cau* P colonies (21% and 24.3%, respectively). Bees in the *car* GR1 and *mel* A colonies removed mummified brood in a shorter period of time (6.5 and 7.1 days on average, respectively) than *car* Mr and *cau* P colonies (above 8 days). Bees in the *mel* A and *car* GR1 colonies cleaned significantly more cells with freeze-killed brood within 24 and 48 hours (above 70% and 80% on average, respectively) than *car* Mr and *cau* P colonies (on average 10 - 20% lower cleaning rate). A low correlation coefficient was found for the susceptibility of larvae to *A. apis* infection and hygienic behaviour.

Keywords: *Ascosphaera apis*, freeze-killed brood, hygienic behaviour, larvae susceptibility.

INTRODUCTION

Honey bee (*Apis mellifera*) diseases are a major problem in colony management. Pathogens and diseases can negatively affect colony growth and production, resulting in economic losses. Effective treatments are lacking for some diseases, and chalkbrood is a disease for which some alternative methods should be developed for control.

Chalkbrood is a common fungal disease caused by *Ascosphaera apis* (Spiltoir, 1955). The disease causes death of bee larvae of any caste and is transmitted within a colony by the nurse bees feeding larvae with food contaminated with *A. apis* spores. According to Bailey (1981), 3 to 4-day-old larvae are the most susceptible, whereas others have found that younger larvae are also susceptible (Gilliam et al., 1978). Nevertheless, Jensen et al. (2009) showed that, in 5-day-old larvae, *A. apis* spores have limited time in the gut for germination and tissue

penetration. Enzymes produced by *A. apis* enable the fungi to damage peritrophic membranes of the gut and grow in the larval body cavity (Theantana and Chantawannakul, 2008). Field recognition of the disease is based on visual identification of mummified brood. Clinical symptoms of chalkbrood appear for short periods, mostly in cold and damp weather (Aronstein and Murray, 2010). Chilling of the brood during the development period increases the number of diseased larvae (Vojvodic et al., 2011). Different methods have been used for larval inoculation with *A. apis* in studies of the disease and the selection of bees resistant to chalkbrood. In some studies, larvae were fed with sugar-honey-pollen candy inoculated with spores of *A. apis* (Milne, 1983; Taber, 1986; Taber and Gilliam, 1987; Bieńkowska and Konopacka, 1999; Panasiuk et al., 2008). Another method was spraying the comb with larvae using a suspension of mummies in sugar syrup (Gilliam et al., 1983; Koenig et al., 1987;

Chorbiński, 2004) or water (Carrera et al., 1987). However, these methods resulted in irregular larvae infection (Gliński, 1981; Carrera et al., 1987; Puerta et al., 1990; Panasiuk et al., 2008). According to Flores et al. (2004), larvae inoculation was higher after spraying water with spores over the comb or feeding colonies a spore-pollen mixture than feeding colonies sugar syrup containing spores.

Honey bees have developed colony level behaviours to control or eliminate pathogens (Cremer et al., 2007). The hygienic behaviour of honey bees is a genetically determined trait and a known mechanism for disease resistance. Hygiene involves the detection of diseased or dead brood in capped cells, and uncapping and removing them from the nest (Rothenbuhler and Thomson, 1956; Rothenbuhler, 1964; Milne, 1985). This behaviour eliminates the source of infection and limits disease transmission within the colony. The hygienic behaviour of bees was tested using different methods of brood killing, including freeze-killed and pin-killed brood (Spivak and Downey, 1998; Newton and Ostasiewski, 1986). Differences in the hygienic behaviours of bees of different races (bee subspecies) have been widely described. Hygienic bees remove a greater number of dead brood compared to non-hygienic within the same timeframe (Spivak and Gilliam, 1993; Büchler, 1996; Kefuss et al., 1996; Spivak and Downey, 1998; Arathi and Spivak, 2001; Panasiuk et al., 2008; Bąk et al., 2010). Phenotypic selection leads to populations with higher proportions of hygienic bees (Rothenbuhler, 1964; Heath, 1982; Büchler, 1996; Palacio et al., 2000). However, bees bred for hygienic behaviour against brood diseases detect and remove less *Varroa* mite-infested pupae than bees bred for specific hygienic behaviours against *V. destructor* or other mechanisms of resistance against mites (Ibrahim and Spivak, 2006; Danka et al., 2013).

We investigated the susceptibility of honey bee larvae from different races (*Apis mellifera carnica*, *Apis mellifera caucasica*, and *Apis mellifera mellifera*) to chalkbrood disease in colonies kept in field conditions. We also evaluated the hygienic behaviour of adult bees in the same colonies.

MATERIAL AND METHODS

The research was conducted at the Department of Bee Breeding in the Research Institute of Horticulture, Apiculture Division, in Puławy. The experiment was repeated in July and August during three seasons, from 2010 to 2012, after the main honey flow. In

2010, two races of bees were used: *A. m. carnica* (lines *car* GR1 and *car* Mr) and *A. m. caucasica* (line *cau* P). In the subsequent seasons, *A. m. mellifera* (line *mel* A) was introduced into the experiment. These three races of bees are currently used in Polish national breeding programs and are kept by commercial beekeepers. The race of bees taken from breeding colonies was confirmed based on automatic measurements of forewing venation using DrawWing computer software (Tofilski, 2004; Gerula et al., 2009). Each year, new colonies were generated with commercial queens and bees of the same breed. The initial bee colonies were in good condition with no symptoms of any disease.

The experimental colonies were kept in nucleus hives with six frames measuring 220 x 180 mm. The colonies were tested in the same apiary and managed in the same way. To obtain combs with brood of the same age, the queens were caged using queen excluders for 48 hours to lay eggs. Experimental hives were equipped with entrances with queen excluder nets to prevent housekeeping bees from removing chalkbrood mummies from the hive. The entire experiment was conducted during the period of moderate honey flow.

The colonies were fed with pollen loads, icing sugar, and honey candy (in 2:1:1 ratio) inoculated with spores of *A. apis* to evaluate the susceptibility of young bee larvae to chalkbrood. The mixture was prepared for inoculation using mummified brood (5 mummies per 1000 g of candy crushed and stirred in 50 mL of distillate water) (Taber, 1986; Taber and Gilliam, 1987; Panasiuk et al., 2008). Mummies originated from bee colonies naturally infected by *A. apis* that exhibited symptoms of the disease. The feeding started when larvae on an experimental comb were 2 - 3 days old. To 80 g portion of candy, 2 mL of the inoculation mixture was added, thoroughly mixed, and put on the top bars in the colonies. All uncapped brood were removed from the colonies before experimental feeding to avoid their inoculation. Bees ate the given food within 2 days. The capped brood cells on the experimental frame were counted in each colony immediately after capping.

The mummies of diseased brood were observed in entrance boxes and on hive boards. The mummies were collected from the hives each day, starting from the second day after brood capping. The observations terminated after all brood were either cleaned or emerged from the cells. The number of mummies collected from entrance boxes and hive boards was used to determine the percentage of brood infected with *A. apis*. Susceptibility was calculated as the sum

of mummies removed by bees and collected from each colony in relation to capped brood cells.

The hygienic behaviour of bees based on cleaning freeze-killed brood was also evaluated in experimental colonies. The hygienic behaviour test was performed when the experiment with *A. apis*-inoculated brood was finished in all colonies. A brood comb from a standard carniolan colony was put in the refrigerator at -18°C for 20 hours. The comb was then cut into small pieces and left at room temperature to thaw. Each experimental colony received a piece of comb with roughly 100-150 brood cells inserted in the hole cut in the centre of the middle nest comb. The number of clean cells was counted 24 and 48 hours after introducing the comb pieces into the colonies and the percentage of brood removal calculated.

ANOVA was used for the statistical analysis. The percentage of cleaned cells was transferred using arcsine transformation and compared with Tukey's HSD test. The correlation between the percentage of clean cells with freeze-killed brood and percentage of mummified brood was determined using Pearson's correlation coefficient.

RESULTS

Significant differences in the susceptibility of brood to *A. apis* were observed between bee lines in different years. In 2010, *car* GR1 and *car* Mr bees had the same percentage of infected larvae, whereas *cau* P had significantly greater rates of infection. In the successive season, although differences were still found in the percentage of infected larvae, they were not significant. *Mel* A and *cau* P colonies had moderate percentages of mummified brood. In 2012, the *mel* A, *car* GR1, and *cau* P colonies had significantly lower rates of infected larvae compared to *car* Mr (Tab.1). In 2010 and 2012, the average percentage of infected larvae was significantly lower than the average in 2011 (Tab. 1).

When comparing the tested bee populations, the lowest percentage of infected larvae, regardless of the year of research, was found in *car* GR1 bees. *Cau* P colonies had the highest percentage of mummified brood, whereas *mel* A and *car* Mr reached moderate percentages of infected larvae (Tab.1).

Table 1.

Percentage of brood infected with <i>Ascosphaera apis</i>					
Honey bee race and line	Year	No. of colonies	Average no. of sealed brood cells	Average no. of removed mummies	Average % of removed mummies
<i>A. m. carnica</i> GR1	2010	5	438.4	28.6	9.2a
<i>A. m. carnica</i> Mr		9	1016.3	106.1	9.3a
<i>A. m. caucasica</i> P		6	759.7	207.7	28.3b
<i>A. m. carnica</i> GR1	2011	5	245.6	23.2	10.4a
<i>A. m. carnica</i> Mr		5	159.2	56.2	37.7a
<i>A. m. caucasica</i> P		5	274.8	59.8	28.0a
<i>A. m. mellifera</i> A		4	220.5	53.0	25.4a
<i>A. m. carnica</i> GR1	2012	5	984.2	57.0	5.8a
<i>A. m. carnica</i> Mr		5	662.6	161.4	25.6b
<i>A. m. caucasica</i> P		5	850.6	123.6	15.8a
<i>A. m. mellifera</i> A		4	747.2	30.2	4.5a
<i>A. m. carnica</i> GR1	2010 - 2012*	15	556.1	36.3	8.5A
<i>A. m. carnica</i> Mr		19	612.7	107.9	21.1AB
<i>A. m. caucasica</i> P		16	628.4	130.3	24.3B
<i>A. m. mellifera</i> A		8	483.8	41.6	14.9AB

a, b - $p \leq 0.05$ within a year or between years

A, B - $p \leq 0.05$ between colonies of different races and lines

* *A. m. mellifera*: 2011 - 2012

Table 2.

Time required to remove mummies of brood infected with *Ascosphaera apis*

Honey bee race and line	Year	No. of colonies	Average no. of cleaning days	Range, days	Average no. of cleaning days in the year
<i>A. m. carnica</i> GR1	2010	5	5.8a	5 - 7	7.7a
<i>A. m. carnica</i> Mr		9	7.4b	5 - 10	
<i>A. m. caucasica</i> P		6	9.7b	8 - 10	
<i>A. m. carnica</i> GR1	2011	5	6.8a	6 - 8	7.5a
<i>A. m. carnica</i> Mr		5	8.0a	8	
<i>A. m. caucasica</i> P		5	7.8a	7 - 8	
<i>A. m. mellifera</i> A		4	7.2a	6 - 8	
<i>A. m. carnica</i> GR1	2012	5	7.0a	6 - 8	8.1a
<i>A. m. carnica</i> Mr		5	10.0b	8 - 11	
<i>A. m. caucasica</i> P		5	8.4b	7 - 11	
<i>A. m. mellifera</i> A		4	7.0a	7	
<i>A. m. carnica</i> GR1	2010 - 2012*	15	6.5A	5 - 8	
<i>A. m. carnica</i> Mr		19	8.3B	5 - 11	
<i>A. m. caucasica</i> P		16	8.7B	7 - 11	
<i>A. m. mellifera</i> A		8	7.1A	6 - 8	

a, b - $p \leq 0.05$ within a year or between yearsA, B - $p \leq 0.05$ between colonies of different races and lines* *A. m. mellifera*: 2011 - 2012

Bees detected diseased brood under the caps and removed dead brood from the cells. *Car* GR1 bees removed dead brood in the shortest amount of time, whereas *mel* A bees required slightly longer. *Car* Mr and *cau* P bees took significantly longer to clean infected brood. However, cleaning time did not differ significantly between tested bee lines in 2011 (Tab. 2).

The bees required 7.5 to 8.1 days on average to remove dead mummies during the course of the observed years. The shortest time period was recorded in 2011 and the longest in 2012, but the differences were not significant. Regardless of the year, *car* GR1 bees on average removed mummies from comb cells significantly faster than *car* Mr and *cau* P colonies, which took the longest (Tab. 2).

Car GR1 bees in 2010 and *car* GR1 and *mel* A bees in 2012 cleaned the highest percentage of cells at both observation time points, but the differences from *car* Mr and *cau* P were not significant. In 2011 the highest rate of removal was observed in *mel* A colonies after the first 24 hours. *Car* GR1 bees removed dead brood slightly slower, with

car Mr being significantly slower (Tab. 3). The same tendency was observed for the measurement at 48 hours but the differences were not significant. During the first day after frozen brood introduction, bees removed more than 60% of cells on average (from above 40% to >90%). During the subsequent 24 hours, bees cleaned an additional approximate 10% of the initial number of cells. The highest cleaning rate was observed in 2011, and the lowest in 2010, regardless of line being observed (Tab. 3). The *mel* A and *car* GR1 bees cleaned the most freeze-killed brood, and *car* Mr removed the fewest when all experimental years were analysed together (Tab. 3). The observation of both fast *A. apis*-infected mummy removal and fast freeze-killed brood cleaning in the *car* GR1 and *mel* A colonies indicated high hygienic behaviour among these bee populations. A significant correlation was found between removing freeze-killed brood from the colonies after 24 and 48 hours and actively removing chalkbrood mummies from colonies ($r = 0.56$ and $r = 0.66$, respectively, $p < 0.00$).

Table 3.

Percentage of freeze-killed brood removed after 24 and 48 hours						
Honey bee race and line	Year	No. of colonies	Average % of freeze-killed brood removed			
			between bee lines within a year		between years	
			24 hours	48 hours	24 hours	48 hours
<i>A. m. carnica</i> GR1	2010	5	67.1a	67.3a	49.8a	54.7a
<i>A. m. carnica</i> Mr		9	45.5a	52.3a		
<i>A. m. caucasica</i> P		6	41.9a	47.9a		
<i>A. m. carnica</i> GR1	2011	5	85.6b	96.6a	81.2b	94.6c
<i>A. m. carnica</i> Mr		4	62.3a	88.0a		
<i>A. m. caucasica</i> P		5	84.4b	95.8a		
<i>A. m. mellifera</i> A	2012	4	93.9b	100a	56.6a	74.8b
<i>A. m. carnica</i> GR1		5	64.0a	87.7a		
<i>A. m. carnica</i> Mr		5	48.2a	65.8a		
<i>A. m. caucasica</i> P		5	51.8a	71.4a		
<i>A. m. mellifera</i> A	2010 - 2012*	4	67.0a	74.3a	61.7	70.2
<i>A. m. carnica</i> GR1		15	72.2BC	83.5B		
<i>A. m. carnica</i> Mr		17	50.0A	63.9A		
<i>A. m. caucasica</i> P		16	58.3AB	70.2AB		
<i>A. m. mellifera</i> A		8	76.8C	85.3AB		

a, b - $p \leq 0.05$ within a year or between years

A, B, C - $p \leq 0.05$ between colonies of different races and lines

* *A. m. mellifera*: 2011 - 2012

DISCUSSION

Several methods of killing brood have been used to evaluate hygienic behaviour in honey bees. Panasiuk et al. (2008) reported that freeze-killed and *A. apis* spore-inoculated brood was removed more slowly, but the dynamics of removing these brood were similar, whereas pin-killed brood were removed faster and during the first 2 days after killing. Therefore, we used only *A. apis* inoculation and freeze-killed methods to address hygienic behaviour in our research.

The study was performed directly in bee colonies in field conditions. We reached an inoculation rate of 13.5 - 25% of larvae on the basis of the number of mummies removed relative to the number of capped brood cells. Carrera et al. (1987) found that 2 - 3% of larvae exhibited disease symptoms 8 days after spray inoculation using a suspension of mummies in water. Larvae mummification was >90% when spraying water with spores over comb and 86% when colonies were fed a spore-pollen mixture in

the study by Flores et al. (2004), but the authors also chilled brood to increase the effects of the disease. A much lower mummification rate (60%) was achieved after feeding colonies sugar syrup containing spores.

Some attempts at larval infection with *A. apis* failed for Carrera et al. (1987) because workers removed almost all of the treated brood from cells. In addition, Jensen et al. (2009) reported that many larvae could have died and been removed from cells before sealing them. Thus, the final number of removed mummies in our experiment may vary in accordance with the real infection level. We did not observe larvae before capping, but our research was finished when all cells on the observed comb were empty. During the first year of evaluation, bees of both *A. m. carnica* lines reached almost the same average percentage of infected larvae, but the initial numbers of capped brood cells were different. In the following 2 years, the difference in the number of capped brood cells was about 25% higher in *car* GR1, but the average percentage of infected larvae

was 3.5 times higher in *car* Mr than in *car* GR1 in 2011 and almost 4.5 times higher in 2012. The rate of brood infection could have been affected by the different number of larvae on the experimental comb. The correlation calculated between the initial number of cells with capped brood and the percentage of removed mummies was $r = -0.4$ ($p < 0.05$), which indicates a greater number of cells negatively affected the level of infection.

Car GR1 bees had the lowest percentage of mummified brood and removed mummies in the shortest amount of time. A similar level of mummification and removal time was observed in *mel* A colonies. This result was expected as *car* GR1 bees were selected for increased hygienic behaviour against *A. apis* diseased brood. Thus, *A. m. mellifera* (*mel* A) may have a higher natural resistance to disease.

Our data indicate differences in the susceptibility of larvae to *A. apis* among the analysed honey bee populations. Jensen et al. (2009) reported higher susceptibility and mortality of larvae from *A. m. caucasica* and *carnica* colonies compared to *ligustica* colonies. In the present study, *cau* P larvae were significantly more susceptible to infection than the other lines. Several factors could have influenced the results of our experiment: the tested colonies may have been affected by a lower temperature in the nest, and the regular colony status may have been interrupted when brood and food stores were removed before feeding inoculated candy. The tendency for a low rate of *A. apis* brood infection in *car* GR1 and *mel* A colonies may indicate genetic mechanisms of resistance in these subspecies.

Hygienic behaviour may be a mechanism of disease resistance in bees. In our experiment we took combs with brood of the same age from a *A. m. carnica* bee colony. This avoided a possible effect of the race of killed brood on the rate of cell cleaning. Also, Spivak and Downey (1998) found that the origin of the frozen section of brood plays no role in the hygiene behaviour of the host colony.

A negative correlation was found between colonies that quickly removed freeze-killed brood and the development of chalkbrood disease (Gilliam et al., 1988). However, we did not find a correlation between the hygienic behaviour of bees and susceptibility of larvae to *A. apis*. We observed a significant correlation between the removal of freeze-killed brood from colonies after 24 and 48 hours and the removal of chalkbrood mummies. Our findings correspond to those of Boecking and Drescher (1992), who reported a correlation between the

removal of frozen brood and brood artificially infected with *Varroa* females. These results corroborate that hygienic bees remove damaged brood regardless of the cause of damage.

The *car* GR1 colony in the first year of the experiment and *mel* A colonies in the two successive seasons had the highest percentage of freeze-killed brood removed after 24 and 48 hours. Similarly, Bąk et al. (2010) found, in small mating hives, that *A. m. mellifera* bees recognize and uncap dead brood earlier than *A. m. caucasica*, *A. m. carnica*, and *A. m. carnica* hybrid bees. However, when the authors analysed the mean percentage of cells cleaned within particular groups 24 hours after introduction into the colonies, *car* Dobra bees cleaned slightly more cells than *A. m. mellifera* bees. The same study reported 9.9% and 9.6% clean brood cells in *car* Dobra and *mel* Augustowska, respectively, 24 hours after the introduction of freeze-killed brood, which is much less than our observation. The difference may be due to our colonies being stronger with a larger population of bees of an age when they perform removal, a function identified by Krivtsova (2001); mid-Russian bees kept in strong colonies removed 87.5% of the frozen brood within 24 hours of the experiment. Koenig et al. (1987) also proposed that the ability to clean cells depends on the strength of the bee colonies.

CONCLUSIONS

Larvae of the *car* GR1 and *mel* A have lower susceptibility to *A. apis* than *car* Mr and *cau* P.

Colonies of *car* GR1 and *mel* A cleaned a higher percentage of freeze-killed brood than *cau* P and *car* Mr.

A low correlation coefficient was found for the susceptibility of larvae to *A. apis* infection and hygienic behaviour, which may indicate a lack of relationship between these traits.

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