

INFLUENCE OF GENOTYPE AND METHOD OF BROOD KILLING ON BROOD REMOVAL RATE IN HONEY BEE

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S u m m a r y

The study was done in the Department of Bee Breeding in the Institute of Pomology and Floriculture, the Apiculture Division in Puławy in 2004 and 2005. Bee colonies of two populations presenting different hygienic behavior were used. The colonies were tested using assays of bee brood killed by freezing, pinning and inoculating with spores of *Ascosphaera apis*.

Significant differences were found in the rate of removing dead brood. Bees performing high hygienic behaviour removed all dead brood within 204 hours. Bees with low hygienic behaviour removed dead brood within 250 hours. Both bee populations, recognized and removed significantly faster pin-killed brood (120 hours), then inoculated with *A. apis* spores and freeze-killed brood (respectively 204 and 250 hours).

Keywords: Hygienic behaviour, genetic conditionings, freeze-killing, *Ascosphaera apis* inoculating, pin-killing, cleaning rate.

INTRODUCTION

Hygienic behaviour of the honey bee (*Apis mellifera*) is one of the mechanisms for disease resistance. It involves the detection of diseased or dead brood in sealed cells, uncapping, and removing it from the nest (Rothenbuhler and Thompson 1956, Rothenbuhler 1964a, Milne 1985). Hygienic behaviour of bees eliminates the source of infection and limits disease transmission within the colony. Hygienic behaviour sometimes leads to the colony self-healing.

Hygienic behaviour is a genetically determined trait. Bee colonies that do not show disease symptoms and are able to remove dead brood in a short time are considered hygienic. Colonies that are susceptible to diseases and leave dead brood in the nest are considered non-hygienic. It is possible to obtain highly hygienic bees with the proper selection

(Rothenbuhler 1964a, Heath 1982, Büchler 1996, Spivak and Reuter 1998b, Palacio et al. 2000, Lapidge et al. 2002). Hygienic bees remove a higher number of dead brood compared to non-hygienic within the same given time span (Spivak and Gilliam 1993, Büchler 1996, Kefuss et al. 1996, Spivak and Downey 1998).

Many authors have demonstrated various signals provoking dead brood removal in worker bees. There are olfactory cues (Masterman et al. 1998, Arathi et al. 2006) or differences in the temperature of healthy and infected or physically damaged brood (Gramacho et al. 1997 and 1999). Worker bees uncap and remove brood from the cell when there is a *Varroa destructor* mite (Aumeier and Rosenkranz 2001) *Tropilaelaps clareae* (Woyke 2001), or *Aethina tumida* egg or larvae (Ellis et al. 2004).

Honey bees were screened for hygienic behaviour by recording the removal rate of damaged or killed brood over a certain period of time (Spivak and Gilliam 1998). Studies on hygienic behaviour were carried out on colonies that had been selectively bred into American foulbrood resistance. This was done on the basis of bee response to brood treated with *Paenibacillus larvae* bacterium that causes the disease (Woodrow 1941, Rothenbuhler 1964b, Spivak and Reuter 2001). The colony selection for chalkbrood resistance was done by feeding bees pollen cake with *Ascosphaera apis* spores (Milne 1983, Taber 1986, Taber and Gilliam 1987, Bieńkowska and Konopacka 1999). In other tests, brood combs were sprayed with syrup containing *A. apis* spores (Gilliam et al. 1983, Koenig et al. 1987b, Chorbiński 2004b) or contaminated water (Carrera et al. 1987). These tests were not appropriate due to inherent problems in inoculating colonies with bacterium or spores that resulted in irregular larvae infection (Woodrow 1941, Gliński 1981, Carrera et al. 1987, Puerta et al. 1990). In other experiments, cyanide-killed brood (Jones and Rothenbuhler 1964, Momot and Rothenbuhler 1971) or pin-killed brood (Taber 1982, Milne 1983, Newton and Ostasiewski 1986, Kefuss 1995, Spivak and Reuter 1998b and 2001, Palacio et al. 2000, Békési and Szalai 2003, Chorbiński 2004a) were used. However pin-killed brood exposes haemolymph from the pierced larvae or pupae. The exposed haemolymph may increase the rate of removal of the brood by the bees. Also the haemolymph injected under the cell capping or on top of it causes the removal of the cell contents (Gramacho et al. 1999). Bees removed pinned brood when it was killed through the bottom of the cell without damaging the capping (Spivak

and Downey 1998) or when sealed brood was injected with water or chemicals or parasite extracts (Titera and Kokkoris 1994, Romaniuk and Witkiewicz 1998).

Freeze-killed brood assays were frequently used to survey bee colonies for hygienic behaviour. The use of liquid nitrogen enables a section of comb to be frozen directly within the frame without cutting the comb sections (Spivak and Downey 1998, Spivak and Reuter 1998a).

The rate of brood removal varies with the method of killing used (Bieńkowska et al. 2004). The estimation of hygienic behaviour should be done at different periods of time after inserting killed brood into the tested colony (Büchler 1996, Békési and Szalai 2003). The age of the killed brood influences the test result. Freeze-killed brood, which has recently been sealed with a wax capping, is removed in a shorter period of time than brood sealed for a few days (Spivak and Downey 1998, Bieńkowska et al. 2004).

The aim of the research was to study bee response to dead brood inserted into the colony and to define the factors influencing the behaviour. Examined factors were: genotype of the bees and the method of brood killing.

MATERIAL AND METHODS

The observations were carried out in the Department of Bee Breeding in the Institute of Pomology and Floriculture, Apiculture Division in Puławy in 2004 and 2005. Bee colonies of two populations were tested. The first population was the *car* GR1, selected for chalkbrood resistance (hygienic). The second population was the *car* Zosia selected for high honey production and observed as

susceptible for the brood disease (non-hygienic).

Each population consisted of 5 bee colonies. Hygienic behaviour was estimated on the basis of the freeze-killed and *Ascosphaera apis* inoculated brood cleaning rate in the first year of the experiment, and freeze-killed, *A. apis* inoculated and pin-killed brood in the second year. The experiment was repeated three times every year (Table 1).

Five randomly chosen honey bee queens

was prepared with pollen loads, icing sugar and liquid honey in a ratio of 2:1:1. Then 10 chalkbrood mummies were added to the portion of 4 kilograms of prepared cake (Taber 1986, Taber and Gilliam 1987). Experimental combs with larvae stayed in the nucleuses until capping. The remaining combs were left in the bee colonies until brood reached the prepupa stage (12 to 13 days old). At that point half of them were moved to a refrigerator for 12 hours. The temperature of the refrigerator was -18°C.

Table 1.

Plan of experiment.

Year	Date of experiment	Bee population	Number of colonies	Method of brood killing	Number of experimental combs
2004	25.05–04.06	Hygienic	5	FR	5
		Non-hygienic	5	FR	5
	06.07–14.07	Hygienic	4	FR, IN	8
		Non-hygienic	3	FR, IN	6
	13.08–19.08	Hygienic	3	FR, IN	6
		Non-hygienic	4	FR, IN	8
2005	05.06–15.06	Hygienic	5	FR, IN, PIN	15
		Non-hygienic	5	FR, IN, PIN	15
	12.07–20.07	Hygienic	5	FR, IN, PIN	15
		Non-hygienic	5	FR, IN, PIN	15
	23.08–31.08	Hygienic	2	FR, IN, PIN	6
		Non-hygienic	2	FR, IN, PIN	6

Abbreviations used in the table:

FR– freeze-killed

IN – inoculated with *Ascosphaera apis* spores

PIN– pin-killed

were kept in a single frame queen excluder insulator for 48 hours with experimental comb pieces to fill them with eggs. The comb pieces in a wooden frame contained about 500 cells on each side.

When larvae were 3 to 4 days old, the third part of the combs was moved for larvae inoculation. This part was moved into the nuclei, which had been fed with pollen cake with *A. apis* spores. The cake

The other half of the brood was pinned through the wax capping using an entomological needle, with tip diameter of 0.75 mm.

The pieces of combs with experimental brood killed with three methods were moved into tested bee colonies. They were placed in the centre of bee nest, between two brood combs.

The effect of the honey bee genotype, and of the method of brood killing, was estimated after 12, 24, 48, 72 hours and then once a day up to all cells with dead brood were cleaned. The different number of experimental combs with dead brood used in the research was due to an insufficient number of experimental brood combs. This was because of unequal egg laying by the queens, natural loss of the larvae, and also the irregular *A. apis* larvae inoculation.

The average rate of cleaned dead brood was calculated and given as percentages. Data were calculated according to the Bliss' transformation. Two-way ANOVA was used for statistical calculation, and Duncan's multiple range test was applied to determine significant differences between the means.

RESULTS

In 2004 and 2005, regardless of the method of brood killing and the date of the experiment, the tendency for faster brood removing was observed in the hygienic bee colonies. In these colonies the process of total cells cleaning was completed in 204 hours while in non-hygienic colonies it

took 250 hours. Hygienic colonies cleaned cells with dead brood faster throughout the whole experiment. During the first 24 hours, hygienic colonies removed more than half of the dead brood while non-hygienic removed slightly less than 40% (Table 2). Hygienic bees had an approximately 10% higher rate of cleaning than non-hygienic bees for the whole experiment. Differences between hygienic and non-hygienic colonies were significant for all comparisons of removal with the exception of the measurement at the 48th-hour.

Regardless of the tested colony, pin-killed brood was removed the most quickly by bees (Fig. 1). Bees cleaned an average of 73% of the cells with pin-killed brood within 12 hours. Only a few unclean cells were left after 3 days of observations.

Bees cleaned cells with brood inoculated with *A. apis* spores at a considerably slower pace. The slowest were cleaned the cells with freeze-killed brood. During the first 12 hours bees removed as much as 30% of *A. apis* inoculated brood and only about 10% of the freeze-killed brood. The cell-cleaning rate increased about 15-20% for each of the compared brood killing methods.

Table 2.

Honey bee genotype influence on the rate of dead brood cleaning regardless of the applied method of brood killing.

Bee population	Number of combs	Average percent of cleaned brood cells							
		12 h		24 h		48 h		72 h	
		average (min-max)	SD	average (min-max)	SD	average (min-max)	SD	average (min-max)	SD
Hygienic	55	38.06 b (0-100)	31.93	52.34 b (1.3-100)	33.09	70.57 a (4.7-100)	29.33	85.37 b (10.9-100)	29.33
Non-hygienic	55	24.0 a (0-98.0)	27.42	39.65 a (0.9-100)	34.56	58.45 a (2.4-100)	34.47	71.59 a (5.4-100)	29.33

Different letters indicate significant differences in columns $p \leq 0.05$ (using the Bliss' transformation)
SD- standard deviation.

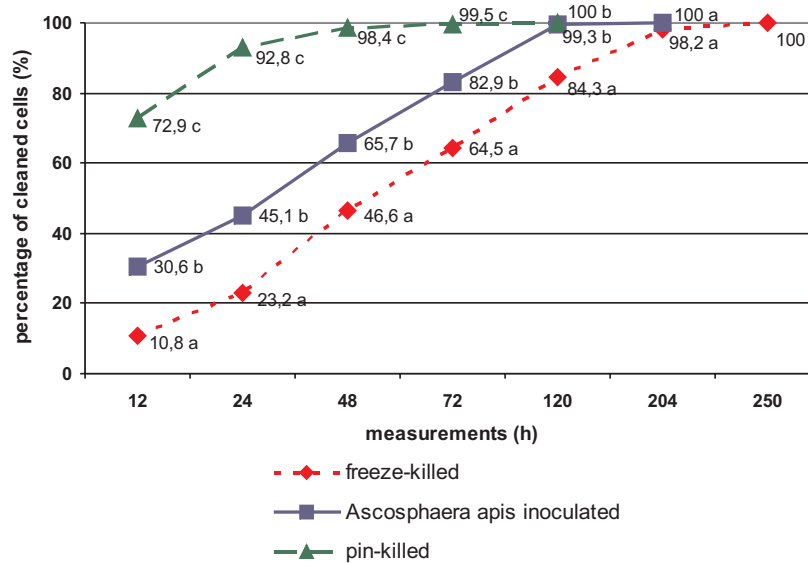


Fig. 1. The influence of different methods of brood killing on the rate of cell cleaning, regardless of the tested bee population.

Significant differences were found in the cleaning rate of cells with brood killed in different ways.

The highest differences in the cleaning rate were observed during the first 12 hours. In every successive measurement, the percentage of cell cleaning was similar for every compared group. Both bee populations removed nearly all (99.5%) pin-killed brood in the first 72 hours. Brood inoculated with *A. apis* spores were removed within 204 hours and freeze-killed within 250 hours of observation.

Only in 2005 the hygienic behavior of bee colonies from two populations was estimated on base of cleaning rate of brood killed with three methods. Analysis was done separately for measurements in particular periods of time (Table 3). Both analyzed factors: the genotype of bees and the method of brood killing differently influenced the rate of cells cleaning in particular time intervals. There was no interaction between these two factors.

Differences were found in hygienic behaviour of bees belonging to two populations, regardless of the applied method of brood killing, after 24 and 72 hours of experiment. In the other measurements (after 12 and 48 hours) no differences were found. Differences in the rate of removing brood killed in various manners were stated only in the measurements after 12 and 24 hours of experiment. In the next two measurements pin-killed brood was significantly faster removed while there were no significant differences in the rate of removing frozen and inoculated brood. The rate of cleaning pin killed brood did not vary between both bee populations at any lap of time. The rate of removing brood inoculated with *A. Apis* varied only 24 hours after inserting the combs into bee colonies. Differences between two populations in the rate of cleaning freeze-killed brood varied during all observation with the exception of the measurement after 48 hours.

Table 3.

Average percent of brood killed with three methods and removed by two populations of bees, 2005.

Measurement after	Bee population	Frozen 12+12*	Inoculated 12+12	Pinned 12+12	Average 36 + 36
12 h	Hygienic	16.3 e	35.9 ab	83.6 c	45.3 A
	Non-hygienic	5.9 d	22.9 a	62.3 bc	30.4 A
24 h	Hygienic	37.6 bc	51.4 c	96.0 d	61.7 B
	Non-hygienic	17.4 a	31.9 ab	89.7 d	46.3 A
48 h	Hygienic	58.9 ab	67.9 b	100 c	75.6 A
	Non-hygienic	43.3 a	57.2 ab	96.9 c	65.8 A
72 h	Hygienic	74.8 b	85.6 bc	100 c	86.8 A
	Non-hygienic	56.3 a	80.7 bc	99.0 c	78.7 B

* No of combs in hygienic + non-hygienic bee populations.

Different small letters indicate significant differences ($p \leq 0.05$) between means of methods of brood killing and bee populations in particular time intervals. Different capitals indicate significant differences ($p \leq 0.05$) between means of hygienic and non-hygienic populations (column).

Cell cleaning results and applied method of brood killing were characterized by different variations. The standard deviation of pin-killed removing rate varied from 2.5 to 23.3. It was the lowest in this whole experiment. Variation of freeze-killed and *A. apis* inoculated brood cleaning rate was higher, respectively from 8.2 to 33.3 and from 10.5 to 27.6.

DISCUSSION

Honey bees from the line selected for chalkbrood resistance identified and removed sealed dead brood from the cells faster than bees from the susceptible line. This occurred regardless of the method of brood killing. These results confirm numerous other reports (Rothenbuhler 1964a, 1964b, Jones and Rothenbuhler 1964, Momot and Rothenbuhler 1971, Büchler 1996, Spivak and Downey 1998).

According to Büchler (1996) bees removed 50% of pin-killed brood within 17.3 hours. In our experiment, regardless of

the tested population, bees removed more than 80% of pinned brood within only 12 hours. Significant differences in a rate of cleaning cells were stated between the bee colonies of different populations. Hygienic bees removed total dead brood within 204 hours. Non-hygienic took 250 hours to remove brood.

In our experiments, bees removed pin-killed brood significantly faster than *A. apis* inoculated or frozen brood regardless of the tested bee population. Woyke et al. (2004) found that *A. dorsata* bees removed pin-killed brood, but did not remove frozen brood. According to Gramacho et al. (1999) and Woyke et al. (2004) bees recognize pinned brood due to a drop of haemolymph extracting on a wax capping after killing the brood with a pin. This may have affected the results.

In the literature the pin-killed brood is recommended for hygienic behaviour estimation, as easy and less labour consuming (Taber 1982, Milne 1983, Newton and Ostasiewski 1986, Kefuss 1995, Büchler 1996, Spivak

and Reuter 1998b and 2001, Békési and Szalai 2003, Chorbiński 2004a). However Spivak and Reuter (1998b) did not state differences between hygienic and non-hygienic bee colonies in the rate of cleaning cells, when they tested them using pin-killed brood. For the reason of avoiding an inappropriate interpretation of a result they recommend the estimation of hygienic behaviour of bees be done within 24 hours when pin-killed brood applied, and within 48 hours when frozen brood applied.

We observed that bees removed all brood inoculated with *A. apis* spores faster than they removed freeze-killed brood. It is assumed to be the result of a smaller initial number of cells with inoculated brood that accounted for only about 50 cells, while freeze-killed brood accounted for even more than 300 cells. In the subsequent measurements the increase of cleaned cells with brood killed with these methods was similar. The dynamics of cleaning cells with pin-killed brood progressed differently as compared to other killing methods from the beginning of the observation. On the contrary, the dynamics of removing freeze-killed and *A. apis* inoculated brood was similar. It may suggest that bees behave in a more similar manner toward freeze-killed and naturally infected brood (absence of haemolymph on a cell capping).

On a basis of the results of the discussed research and results of experiments of other authors it appears that freezing is the most reliable method of brood killing for testing bee hygienic behaviour (Spivak and Downey 1998, Gramacho et al. 1999). According to Spivak and Gilliam (1998) and Spivak and Reuter (1998a) the use of liquid nitrogen for freezing brood simplifies assays and enables the killing of brood directly within the comb. Olszewski et al. (2007 and 2008)

observed that bees cleaned freeze-killed brood in a shorter time than pin-killed when it was insert into colonies when defrosted. They suggest that it accelerates the cleaning but makes the objective test evaluation impossible. In the present work the time needed for counting the sealed cells in the experimental combs before inserting them into tested colonies caused that brood was partially defrost.

Results of our experiment indicate that screening hygienic behaviour of populations that do not show high differences in this behavior may be unreliable when tested with pin-killed or *A. apis* inoculated brood. It is easier to distinguish these populations when freeze-killed brood applied. Measurements should be done during the first 24 hours of estimation.

Screening the hygienic behaviour of bees using brood inoculated with spores was inconvenient. Irregular larvae inoculation was observed in nuclei that were fed with *A. apis* spores in pollen and sugar cake. We stated that only a small part of larvae moved in experimental combs into nuclei, fed with contaminated cake, became infected. It could be an effect of various larvae susceptibility to chalkbrood (Woodrow 1941, Rothenbuhler and Thompson 1956, Gliński 1981, Spivak and Gilliam 1998) and also faster filtering of *A. apis* spores by the proventriculus of adult bees (Milne 1983, Spivak and Gilliam 1993, 1998).

Inoculating brood in bee colonies in commercial apiaries with *A. apis* spores is not recommended due to the risk of spreading the disease (Chorbiński 2004b, Heath 1982, Koenig et al. 1987a). The method can be applied only in research or experimental apiaries.

CONCLUSIONS

- Honey bees in colonies selected for chalkbrood resistance clean dead brood from the cells faster in comparison to susceptible colonies.
- Pin-killed brood is removed from the cells in a shorter time than freeze-killed or *Ascosphaera apis* inoculated.
- The dynamics of removing freeze-killed and *Ascosphaera apis* spores inoculated brood are similar.
- Irregular larvae inoculation when fed with *Ascosphaera apis* spores in pollen and sugar cake causes the samples to be unreliable.
- Freeze-killed brood should be used for hygienic behaviour screening, because it is easier to indicate differences between populations of bees with various hygienic behavior and also because of the simplicity of the method in selection of disease resistant bees.

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WPLYW GENOTYPU RODZIN PSZCZELICH I SPOSOBU ZABIJANIA CZERWIU NA SZYBKOŚĆ OCZYSZCZANIA KOMÓREK

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S t r e s z c z e n i e

Badania prowadzono w rodzinach pszczelich rasy kraińskiej należących do dwu populacji o odmiennej wrodzonej zdolności wykrywania chorego lub martwego czerwiu: *car* GR1 o silnie rozwiniętym zachowaniu higienicznym oraz *car* Zosia charakteryzującej się słabiej rozwiniętym zachowaniem higienicznym. W latach 2004 i 2005 oceniano w rodzinach pszczelich obu grup szybkość usuwania z komórek przedpoczwarek zabitych przez mrożenie, sztuczne zakażenie sporami *Ascospaera apis* i przekłuwanie igłą entomologiczną.

Stwierdzono istotne różnice w szybkości usuwania martwego czerwiu w obu grupach badanych rodzin pszczelich. Pszczoły o silnie rozwiniętym zachowaniu higienicznym wyczyściły w ciągu pierwszej doby ponad połowę komórek z czerwiem uszkodzonym różnymi sposobami, podczas gdy niehigieniczne niecałe 40%, a do końca drugiej doby odpowiednio 70,6% oraz 58,5% komórek. Cały poddany czerw, pszczoły usunęły odpowiednio w ciągu 204 oraz 250 godzin. Pszczoły obydwu populacji najszybciej wykrywały i usuwały czerw zabijany przez przekłuwanie igłą, a wolniej i w podobnym tempie czerw zabijany przez mrożenie i sztuczne zakażenie sporami grzyba. Największe różnice w dynamice usuwania martwego czerwiu zaobserwowano w ciągu pierwszej doby po wstawieniu plasterków doświadczalnych z martwym czerwiem do rodzin doświadczalnych. W ciągu pierwszych 12 godzin pszczoły wyczyściły prawie 73% komórek z czerwiem zabitym przez przekłuwanie igłą, 30,6% z czerwiem zakażonym sporami *A. apis* i niecałe 11% z czerwiem mrożonym, a do końca pierwszej doby odpowiednio 92,8%, 45,1% oraz 23,2%. Wszystkie komórki z czerwiem zabitym igłą entomologiczną pszczoły obu badanych populacji oczyściły w ciągu 72 godzin, sztucznie zakażany sporami *A. apis* po upływie 204 godzin, a zabijany przez mrożenie po 250 godzinach.

Metoda zabijania czerwiu przez mrożenie, powinna być wykorzystywana w pracach selekcyjnych gdyż lepiej obrazuje różnice między populacjami o różnym zachowaniu higienicznym oraz ze względu na łatwość wykonania pomiaru.

Słowa kluczowe: zachowania higieniczne, uwarunkowania genetyczne, mrożenie, sztuczne zakażenie, przekłuwanie czerwiu, szybkość oczyszczania.