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Validation of the methods of hygienic behaviour evaluation in the honeybee

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Summary

The aim of this study was to determine which method of hygienic behaviour assessment is more reliable: the evaluation of the pierced brood removal rate or the evaluation of the freeze-killed brood removal rate. Additionally, we aimed to determine whether freeze-killed brood should be placed in colonies when defrosted or still frozen. Defrosted freeze-killed brood was removed faster within a 24 h period. The removal rates for pierced brood and frozen freeze-killed brood were similar in hygienic colonies. In non-hygienic colonies, pierced brood was removed at a significantly slower rate than frozen or defrosted freeze-killed brood. The mechanisms of removing frozen and defrosted freeze-killed brood were similar to each other and different from those observed in the case of pierced brood. The defrosting of brood prior to its introduction into colonies seems inadvisable, as it accelerates brood removal. Our results confirm the hypotheses of those researchers who believe that the frozen freeze-killed brood removal test is not always appropriate. A good solution is, therefore, to perform the frozen freeze-killed brood and pin-killed brood removal tests simultaneously. The time from the beginning of the tests to the moment 50% and 75% of dead-brood cells have been cleaned up should be assumed as the appropriate duration of the hygienic behavior evaluation tests.

Keywords: hygienic behaviour, pierced brood, freeze-killed brood, Buckfast

The stress laid on the quality of bee products and the rising drug resistance of apian pathogens have drawn considerable attention to the hygienic behaviour of honeybees. The correlation between the dead-brood removal rate (i.e. hygienic behaviour intensity) and the resistance of honeybee colonies to American foulbrood (AFB) (13, 19) or chalkbrood (8) have been confirmed. Hygienic behaviour is also important for the production of bees resistant to *Varroa destructor* mites (3, 7, 16). The reliability of different methods of behaviour evaluation is crucial in this context.

Hygienic behaviour is mainly assessed on the basis of the pin-killed brood (pierced brood) removal rate or the freeze-killed brood removal rate. However, results obtained by these two methods are thought to be incompatible (6, 9, 17). Assessment based on the freeze-killed brood removal rate is mostly recommended (6, 17). However, no uniform standard has been adopted so far to deal with freeze-killed brood before its introduction into the colony nest (Tab. 1). It is particularly important to determine whether brood should be defrosted prior to being introduced into colonies or not. On the

Tab. 1. Publications dealing with brood freezing conditions and the state of killed brood at its introduction into honeybee colonies in order to assess hygienic behaviour

Paper	Freezing conditions	State of the brood at introduction into colonies
Spivak and Gilliam (18)	frozen for 24 hours at –20°C	frozen
Waite et al. (20)	frozen for 24 hours, no data on temperature	completely thawed, no data on duration
Bąk et al. (1)	frozen for 6 hours at –20°C	warmed to room temperature, no data on duration
Panasiuk et al. (14)	frozen for 12 hours at –18°C	left at room temperature to unfreeze, no data on duration
Panasiuk et al. (15)	frozen for 12 hours at –18°C	left for 2 hours at room temperature to unfreeze
Present paper	frozen for 24 hours at –20°C	frozen
	frozen for 24 hours at –20°C	left for 6 hours at room temperature to unfreeze

other hand, Palacio et al. (13), as well as Békési and Szalai (2) think the rate of pierced (pin-killed) brood removal to be a better measure of hygienic behaviour expression.

The aim of this study was to determine which method of hygienic behaviour assessment, i.e. the evaluation of the pierced brood removal rate or the freeze-killed brood removal rate, is more reliable. Additionally, the study was aimed at determining whether freeze-killed brood should be placed in colonies after being defrosted (defrosted freeze-killed brood) or still in a frozen state (frozen freeze-killed brood).

Material and Methods

Two experiments were carried out. Eight colonies of Buckfast F_1 hybrids and seven colonies of Carniolan F_1 hybrid worker bees were used in experiment 1, and fifteen colonies of F_1 Buckfast hybrid worker bees in experiment 2. Their pure-bred queen mothers were naturally mated. All the colonies had a similar strength and structure.

In experiment 1, a comb with sealed brood at the eye--darkening pupal stage (14^{th}-15^{th} day of bee development) was selected from a source colony which was not one of the experimental colonies, and cut into several sections of 100 cells. The sections were refrigerated for 24 h (–20°C). Then, they were defrosted for 6 hours at room temperature to obtain the "defrosted freeze-killed brood" sections. Simultaneously, one test comb with capped brood at the eye-darkening pupal stage was taken from each of the 15 experimental colonies, and 100 pupae were put to death by being pierced with a 100-pin (diameter: 0.7 mm) "brush" to create the pierced-brood area in the comb. Immediately thereafter, a hole was cut in each test comb near the pierced--brood area, and a section of the defrosted freeze-killed brood was introduced into the hole. The test combs prepared in this way were subsequently returned to the experimental colony nests. After 24 hours, the cells in the pierced-brood areas and in the "defrosted freeze-killed brood" sections containing dead pupae that had not been completely removed were counted. The procedure was repeated four times (repetitions). The procedure in experiment 2 was similar. The only difference was that, apart from pierced brood and defrosted freeze-killed brood, an additional section (100 cells) of "frozen freeze-killed brood," i.e. freeze-killed brood taken directly from the refrigerator and therefore still in a frozen state, was inserted into each of the test combs. In this experiment, the colonies were additionally classified as hygienic and non-hygienic. Hygienic colonies were considered to be those that removed at least 60% of the pierced pupae within 24 hours.

The results were statistically analysed with both one-way and two-way ANOVAs plus Duncan multiple range tests (SAS Institute Version 9.13., 2002-2003 license 86636). Spearman's rank correlations were calculated between the removal rates evaluated for brood killed and prepared in different ways to determine whether the results for the different methods of brood killing, as well as the different states of the brood at introduction into the colonies, corresponded with one another.

Results

Both the brood killing method and the state of freeze--killed brood (frozen/defrosted) at its introduction into experimental colonies significantly influenced the efficiency of hygienic behaviour (in both experiments – 1 and 2: ANOVA $F \leq 0.01$). The genetic type of the bees (Carniolan/Buckfast; experiment 1; ANOVA $F = 0.54$) and the type of behavioural group (hygienic/non-hygienic; experiment 2; ANOVA $F = 0.07$) did not affect this trait. The effect of the repetitions was also insignificant (experiment 1; ANOVA $F = 0.86$ and experiment 2; ANOVA $F = 0.08$). These findings are confirmed by data presented in Tables 2 and 3.

Defrosted freeze-killed brood and frozen freeze--killed brood were removed faster than pierced brood by all the colonies (Tab. 2 and 3). The differences were particularly prominent in the hygienic colonies (Tab. 3). The removal rate for defrosted freeze-killed brood was higher than the rates for frozen freeze-killed brood, particularly in the non-hygienic colonies (Tab. 2 and 3). The efficiency of removing defrosted freeze--killed brood and frozen freeze-killed brood was very similar. In the non-hygienic colonies, defrosted and frozen freeze-killed brood was removed faster than pierced brood. Furthermore, the differences between the rates of removing the three types of brood (pierced/defrosted freeze-killed/frozen freeze-killed) were smaller than in the case of the non-hygienic colonies.

The pierced-brood removal rate exhibited greater variability in experiment 1. In experiment 2, this was

Tab. 2. Number of cells from which pupae were completely removed within 24 hours – Experiment 1

Group	Pierced brood		Defrosted freeze-killed brood	
	mean	CV	mean	CV
Buckfast F_1 (n = 8)	77^a	23	95^b	8
Carniolan F_1 (n = 7)	72^a	29	97^b	6
Overall mean (n = 15)	75^a	26	96^b	7

Explanations: a, b – differences are significant within rows at $p \leq 0.01$; CV – coefficient of variability; n – number of colonies

Tab. 3. Number of cells from which pupae were completely removed within 24 hours – Experiment 2

Group	Pierced brood		Defrosted freeze-killed brood		Frozen freeze-killed brood	
	mean	CV	mean	CV	mean	CV
Hygienic colonies (n = 5)	72**	19	87	18	75	26
Non-hygienic colonies (n = 10)	41^{a}**	40	91^b	13	80^c	27
Overall mean (n = 15)	51^a	41	89^b	15	78^c	26

Explanations: a, b, c – differences are significant within rows at $p \leq 0.01$; ** – difference between hygienic and non-hygienic colonies is significant at $p \leq 0{,}01$; CV – coefficient of variability; n – number of colonies

Tab. 4. Spearman's rank correlation coefficients for the removal rates evaluated for test brood prepared in different ways

Experiment 1						
Brood preparation method	Buckfast F_1		Carniolan F_1		Buckfast F_1 + Carniolan F_1	
	pierced brood		pierced brood		pierced brood	
Defrosted freeze-killed brood	0.536 p = 0.17		–0.315 p = 0.49		0.113 p = 0.68	
Experiment 2						
Brood preparation method	Hygienic colonies		Non-hygienic colonies		Hygienic + non-hygienic	
	pierced brood	frozen freeze-killed brood	pierced brood	frozen freeze-killed brood	pierced brood	frozen freeze-killed brood
Defrosted freeze-killed brood	0.100 p = 0.87	0.900* p = 0.03	0.215 p = 0.55	0.418 p = 0.22	0.018 p = 0.94	0.563* p = 0.03
Frozen freeze-killed brood	0.200 p = 0.74		–0.152 p = 0.67		–0.166 p = 0.55	

Explanations: * – correlation significant at $p \leq 0.05$

observed only in the non-hygienic colonies, whereas in the hygienic ones variability was similar (Tab. 2 and 3). Neither the genetic group nor the behavioural group types influenced this variability. Variability in the frozen freeze-killed brood removal rate was higher than that observed in the case of defrosted freeze-killed brood (Tab. 3).

There was no significant correlation, either in the hygienic or in the non-hygienic colonies, between the removal rate for pierced brood and the rate of removing both the frozen and defrosted freeze-killed brood. A significant correlation was observed between the rate of removing frozen and defrosted freeze-killed brood, particularly in the hygienic colonies.

Discussion

Pierced brood is believed to be removed faster than frozen freeze-killed brood, especially by non-hygienic and intermediate colonies, since worker bees are thought to perceive the orifices in the cell caps of pierced brood and the body fluid issuing from the pupae. This stimulates bees to remove the pupae, which distorts the results of hygienic behaviour assessment (9, 17). Surprisingly, all the colonies in our study removed defrosted freeze-killed brood faster than they removed pierced brood. The differences were particularly prominent in the hygienic colonies. The hygienic colonies in our study had similar rates of removing pierced brood and frozen freeze-killed brood. On the other hand, all the non-hygienic colonies were faster to dispose of defrosted freeze-killed brood and frozen freeze-killed brood than of pierced brood. A similar, higher rate of removing frozen freeze-killed brood, compared with the rate of removing pierced brood, was observed by Newton et al. (12). Békési and Szalai (2), however, obtained contrary results.

Spivak and Downey (17) and Waite et al. (20) regarded colonies that removed frozen freeze-killed brood out of over 95% of cells within 48 hours as hygienic. Spivak and Gilliam (18), on the other hand, defined hygienic colonies as those that cleaned out *all* the cells within 48 hours. Spivak and Downey (17) suggested that the assessment of hygienic behaviour on the basis of pierced brood removal requires the analysis to be performed already after 24 hours, in contrast to the 48-hour period assumed for frozen freeze-killed brood. They justified this by indicating that pierced brood is removed faster than frozen freeze-killed brood. The present research showed that the bee population included a high proportion of hygienic colonies. However, we used a 0.7 mm diameter pin, whereas it is more common to use an entomological pin size 2 (0.45 mm in diameter, 5), which is very important for the results (21). We evaluated the effect of brood removal after 24 hours, expecting that after 48 hours, most of the colonies would have cleaned out all types of brood (pierced, defrosted freeze-killed, and frozen freeze-killed).

Espinosa-Montano et al. (6) found that the pierced--brood and frozen-brood removal rates were correlated with each other. Our study showed that the mechanisms of dead brood removal and frozen freeze-killed/defrosted freeze-killed brood removal may be similar to each other and different from those observed in the case of pierced brood. In practice, however, researchers (13, 16) have made progress in selection, regardless of the assessment method applied (frozen freeze-killed brood/pierced-brood).

Pierced brood was the slowest to be removed. Therefore, despite the incompatibility between pierced brood removal and the removal of frozen and defrosted freeze-killed brood, the assessment of the rate of pierced brood removal appears to be useful for the purpose of identifying hygienic colonies in populations that have not yet been selected for hygienic behaviour expression. The pin-killed brood assay is preferred in most European breeding programs (4) because of its higher repeatability, correlation with the removal of *Varroa destructor*-infested brood (Hoffmann, 1996 from 4), and lower costs (6). The removal rate for frozen and defrosted freeze-killed brood might be a good selection criterion if the population has already been

subjected to selection and, therefore, includes a high proportion of hygienic colonies.

The results of our studies, as well as the results of Newton et al. (12) and Békési and Szalai (2), did not correspond to the results of Spivak and Downey (17), which are considered as the most reliable. In our experiment, the frozen freeze-killed brood removal rate was considerably higher than the pin-killed brood removal rate. Therefore, we suggest using the frozen freeze-killed brood removal and the pin-killed brood removal tests simultaneously. The duration of the hygienic behavior evaluation tests should span the time from the beginning of the tests to the moment 50% and 75% of dead-brood cells have been cleaned up (cf. 4). Our studies also suggest that the expression of hygienic behaviour is highly dependent on the environment and the bee type (11). Thus, the genetic basis of hygienic behaviour proves more complex than previously believed (10). These two factors should therefore be allowed for when choosing the optimal method of assessment.

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