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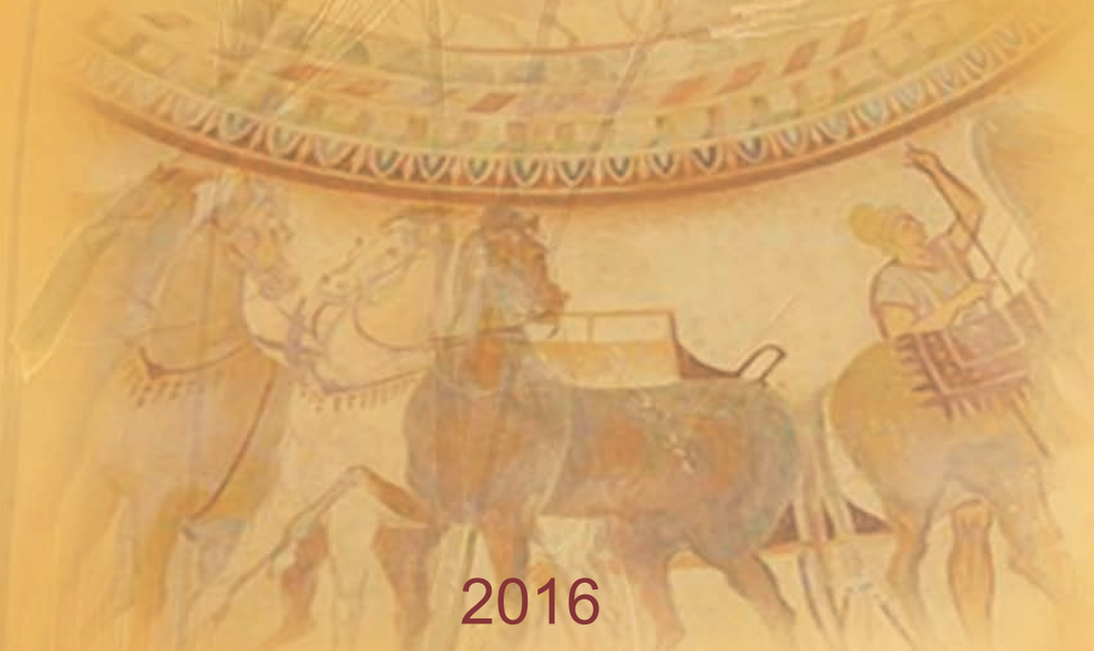

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Nutrition and Physiology

Lysozyme levels in haemolymph of worker bees (*Apis mellifera* L.) from bee colonies with different degree of expression of hygienic behaviour

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Abstract. *A total of 24 bee colonies of apiaries with different business orientation were tested for the degree of expression of hygienic behaviour by modified method, different from the traditionally used for this purpose method. To outline the test field a square sized 5 x 5 cm was used, stuck onto a section of a honey comb with sealed worker brood (the area bounded by the stencil is equal to 100 worker bee cells). The brood in the outlined square is killed by a thin entomological needle by jabbing the sealed cells, without destroying their caps. Depending on the time and extent of cleaning bee colonies are divided into 3 groups: super hygienic - colonies which of the 24th hour after the jabbing have uncovered and cleaned over 95% of the outlined area; hygienic – colonies which on the 48th hour after the jabbing have uncovered and cleaned over 95% of the outlined area; non- hygienic – colonies which have cleaned less than 95% of the cells in the area on the 48th hour. From each bee colony samples of worker bees (200-250 pcs.) have been taken and haemolymph obtained. The amount of lysozyme has been defined at the Reference Laboratory "Honeybee health" at the National Diagnostic Scientific Research Veterinary Medical Institute - Sofia by the method of Motavkina et al. (1979), modified by Kostov et al. (1983). The results obtained show different values for the amount of lysozyme in haemolymph of worker bees, depending on the degree of expression of their hygienic behaviour – 10.49 ± 1.86 μg/ml for the group of super hygienic colonies; 9.11 ± 1.37 μg/ml for the group of hygienic ones; 15.22 ± 2.37 μg/ml for the group of non-hygienic bee colonies, respectively. The established values range from 4.59 μg/ml to 38.28 μg/ml, the greatest variation being in the group of non-hygienic colonies. The data suggests that in positive direction compared to the average for the model is the deviation of LS-means of bee colonies with low level of hygiene (non-hygienic). The reported LS-estimates suggest that in the non-hygienic bee colonies there is a tendency of increase the lysozyme content in the haemolymph.*

Keywords: honeybees, bee colonies, hygienic behaviour, haemolymph, lysozyme level

Introduction

Innate instincts in the honeybee consist of complex inherited behaviour reactions that are set in their genesis. Such are feeding of larvae, building honey combs, guarding and cleaning the nest, hatching bees, the bee dance to find food, etc. One of the clearest examples of unconditional reflexes in bees' is the "hygienic behavior", which is inherited in the offspring and directly affects the health of bee colonies. The mechanisms of this behavior are related to the identification and disposal of infected and dead larvae and bees outside the hive, thereby limiting the spread of infection from various diseases within the bee colony.

The study of hygienic behaviour is crucial since it is directly related to the creation of resistant breeds of bees to a number of diseases (Rotenbuler, 1975; Milne, 1982; Taber and Gilliam, 1987, 1988; Fukae et al., 1990; Choi et al ., 1991; Spivac and Gilliam, 1993; Southwick, 1994 a,b; Hornitzky, 1995; Taber, 1996; Petrov, 1997; Zhelyazkova and Gurgulova, 2003; Darkazanli, 2008). After the European Union adopted ordinance banning the use of sulfonamides and antibiotics in beekeeping (Council Regulation, EEC, No 2377/90), attention was focused on breeding colonies with high degree of natural immunity. In this connection, the possibility of including hygienic behaviour in selection programs creates conditions for the production of ecological bee products that are safe and useful for the man.

According to Taber (1982) hygienic behavior is controlled by two recessive genes. One of them is responsible for the disposal of dead larvae out of the honeycombs and is called removing, marked by the symbol "rr", whereas the other one - uncapping and marked by the symbol "uu". In alleles of the queen bee the alternative trait for non removal of the larvae in the cells is presented in homozygous ("RR") or heterozygous ("Rr") state. The same applies to the trait uncapping, presented in homozygous ("UU") or heterozygous state ("Uu"). Drones are haploid and have only one set of chromosomes, so they could provide only one type of gametes containing "R" or "r", respectively "U" or "u". As a result of combining the gametes of the queen bee with the gametes of the drones diploid worker bees are produced that have hygienic behaviour in the colony.

Hygienic behavior in honey bees is associated mainly with resistance to diseases. In bees, as in all other insects, there are no mechanisms for building immune response, therefore natural protection plays an important role (protective and readaptation mechanisms such as external and internal structure of the bee, the protective function of haemolymph). The waterproof and rigid outer shell of the body of bees, the chemical composition of haemolymph

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as internal environment of the bee organism, the peritrophic membrane and tracheal system are the mechanical and physiological barriers that pretect the body cavity of bees from bacterial and fungal invasion.

Immunity in bees depends exclusively on the quantitative content of proteins in their haemolymph. One of the main factors of immunity in honeybees is the enzyme lysozyme (N-acetylmyramylhydrolase). The enzyme lysozyme (muramidase) in the haemolymph of bees is a factor of resistance, and its synthesis is carried out in all phases of development. Its concentration in the haemolymph could be used as a criterion for determining the degree of resistance of the bee colony (Aronstein and Saldivar, 2005; Aronstein et al., 2006; Aronstein and Murray, 2010). It was found that the content of lysozyme in the haemolymph of bees to a large extent determines the protective functions of the bee organism and depends on a number of factors: type of raising the bee colonies, age of bees, protein content in food, adding stimulating products in the supplemental feed of bee colonies, presence of pathogenic microorganisms and parasites, environmental status, etc. (Zyuman et al., 1987; Nagornaya et al., 1996; Zhelyazkova and Gurgulova, 1997, 2000; Kanchev et al., 1997; Gurgulova et al., 2001).

The information in scientific literature about the relationship between the hygienic behaviour and lysozyme levels in the haemolymph of honeybees on the one hand and resistance to diseases, on the other hand, determined the need of the present study.

The aim of the paper was to determine the lysozyme level in the haemolymph of worker bees *(Apis mellifera* L.*)* from colonies with different degree of manifestation of hygienic behaviour.

Material and methods

Study area. The study was conducted during the active bee season of 2015-2016. Bee colonies with bees from the local breed of *Apis Mellifera* L. have been used. Testing about the degree of manifestation of hygienic behaviour of 24 bee colonies from 8 apiaries in different regions of the country with different economic direction was carried out, namely: Training apiary of Trakia University, Stara Zagora; Apiary of Institute of Animal Science (IAS), Kostinbrod; Apiary at the Experimental Station in Livestock Breeding town of Smolyan; Queen bee production apiary in the village of Dimovtsi, municipality of Gurkovo; Queen bee production apiary town of Lovech; Stock apiary (honey and pollen) ,,Druzhinata", village of Voyvodinovo, municipality of Plovdiv; Stock apiary (honey) village of Okop, municipality Tundzha; Apiary village of Madzherito, municipality Stara Zagora.

Testing colonies for level of manifestation of hygienic behaviour. Testing of colonies for hygienic behaviour was carried out by modified method (Gurgulova et al., 2003), different from that of Taber and Gilliam (1988) and similar to the one applied by Petrov (1997). To outline the test field a square sized 5 x 5 cm was used, stuck onto a section of a honey comb with sealed worker brood (the area bounded by the stencil is equal to 100 worker bee cells). The method involves several stages: selection of bee colonies of equal strength; selection of honeycombs with the largest areas of sealed worker brood; outlining a square measuring 5x5 cm (100 sealed cells) over the brood area; killing the brood in the outlined square by a thin entomological needle by jabbing the sealed cells without destroying the caps of the cells; counting the uncapped and cleaned cells at the 24th and 48th hour after jabbing.

Depending on the time and extent of cleaning colonies are grouped in three groups: super hygienic – colonies that on the 24th hour after jabbing have cleaned over 95% of the cells in the outlined area; hygienic - colonies that at the 48th hour after jabbing have cleaned over 95% of the outlined area; non-hygienic - those that have cleaned less than 95% of cells in the area on the 48th hour.

Obtaining haemolymph and determining lysozyme content in it. Samples of 200 –250 worker bees were taken from the tested bee colonies and pooled samples of haemolymph were obtained from them. The haemolymph was obtained by the conventional method in beekeeping - suction with a Pasteur pipette on the border between the IInd and IIIrd typhoid tergit. The quantity of lysozyme in the haemolymph is determined by the method of Motavkina et al. (1979), modified by Kostov and Bonovska (1983). Live culture of *Micrococcus lisodeicticus* is used for the assay, which is specifically lysed by the lysozyme. Analyses were made at the National Reference Laboratory "Honeybee health" at the National Diagnostic Scientific Research Veterinary Medical Institute, Sofia.

Statistics. The survey data were processed variation-statistically by computer – Statistika software.

Results and discussion

Table 1 presents the results of testing bee colonies for level of manifestation of hygienic behaviour. The percentage of uncapped and cleaned cells with killed brood in the outlined area (5x5 cm - 100 cells) was used as a criterion. Data show that of the tested 24 colonies 6 (25%) are super hygienic (over 95% uncapped and cleaned cells on the 24th hour after killing the brood), 6 (25%) are hygienic (over 95% uncapped and cleaned cells on the 48th hour after killing the brood) and 12 (50%) are non-hygienic (less than 95% uncapped and cleaned cells on the 48th hour after killing the brood).

Table 1. Results for hygienic behaviour test of honeybee colonies

Uncapping and cleaned cells (%)									
24-th hour					48-th hour				
Mean	SE	SD	min	max	Mean	SE	SD	min	max
Super hygienic bee colonies (n = 6)									
98.29	0.57	1.39	95.92	100.00	99.50	0.34	0.84	98.00	100.00
Hygienic bee colonies (n = 6)									
85.14	3.83	9.37	69.79	94.00	97.12	0.80	1.95	95.00	100.00
Nonhygienic bee colonies (n = 12)									
70.78	3.95	13.70	47.00	89.25	87.29	2.67	9.24	61.00	94.90

The higher percentage of non-hygienic bee colonies can be explained by the fact that part of apiaries in which the testing was conducting are unisolated stock apiaries and no selection for level of hygiene has been applied in them.

Bee colonies from the group of super hygienic on the 24th hour have uncapped and cleaned an average of 98.29±0.57% of the cells, ranging between 95,92 and 100% (Table. 1). In these colonies on the 48th hour the percentage of cleaned cells increased to almost 100% (99.50±0.34%), while the variation was in a narrow range - between 98 and 100%. On the 48th hour of reading the uncapped and cleaned cells with killed brood in bee colonies from the hygienic group were 97.12±0.80% (with variation in relatively narrow range between 95 and 100%). At the same time the controlled trait in the non-hygienic colonies (87.29±2.67%) is by 12.21% less than the super hygienic and 9.83% less than the hygienic colonies, respectively. The data show variation in wide ranges (between 61 and 94.9%) for bee colonies from the non-hygienic group (Table 1).

The reliability of the reported differences between bee colonies with different level of manifestation of hygienic behaviour in terms of percentage of uncapped and cleaned cells is indicated in Table 2. On the 48th hour low level of reliability (P≤ 0.05) was determined between super hygienic/hygienic and hygienic/non-hygienic colonies. Statistically proven difference at P≤ 0.01 was established between super hygienic/non-hygienic bee colonies.

Table 2. Reliability of differences between groups of honeybee colonies with different hygienic behaviour (t-test)

	Super hygienic/hygienic			Super hygienic/nonhygienic			Hygienic/nonhygienic		
	Mean	Mean	P	Mean	Mean	P	Mean	Mean	P
24-th hour	98.29	85.14	0.007 **	98.29	70.78	0.000 ***	85.14	70.78	0.035 *
48-th hour	99.50	97.12	0.021 *	99.50	87.29	0.006 **	97.12	87.29	0.022 *

* P≤0.05 ** P ≤ 0.01 *** P ≤ 0.001

Table 3 presents the results about amount of lysozyme in the haemolymph of worker bees from families with different manifestation of hygienic behaviour. The analysis of the results shows the highest average value (15.22±2.37 µg/ml) in colonies from the non-hygienic group. For the super hygienic and hygienic bee colonies close values for content of lysozyme in the haemolymph have been determined – 10.49±1.86 µg/ml and 9.11±1.37 µg/ml, respectively. The established differences between the average values for the hygienic and non-hygienic bee colonies have low level of reliability (P≤0.05). The reported minimum value of the studied trait is 4.59 µg/ml, regardless of the level of hygiene. Maximum values are between 18.66 and 38.28 µg/ml, the variation min/max being the highest in bee colonies from the non-hygienic group. Similar results about higher values of lysozyme content in the haemolymph of worker bees were obtained in other studies on the hygienic behaviour of bees (Gurgulova et al., 2003; Darkazanli, 2008).

Table 3. Lysozyme levels in haemolymph of worker bees and LS- estimate of the impact of hygienic behaviour (n=47)

Groups according to hygienic behaviour	Lysozyme levels in haemolymph of worker bees (µg/ml)					LS - estimate
	LS - mean	SE	SD	min	max	
super hygienic	10.49	1.86	6.45	4.59	25.00	-1.71
hygienic	9.11*	1.37	5.12	4.59	18.66	-3.08
nonhygienic	15.22*	2.37	10.84	4.59	38.28	+3.03
Mean for the model	12.19	1.27	8.73	4.59	38.28	

* The reported differences between groups were significant at P≤0.05

Data suggest that in positive direction compared to the average for the model are the deviations of LS-estimates of bee colonies with low level of hygiene (non-hygienic). The reported LS-estimates suggest that in the non-hygienic bee colonies there is tendency for increase of the lysozyme content in the haemolymph. It can be assumed that the impossibility of those colonies to detect quickly and cleaned the sick larvae (in this case killed larvae) puts them in a stressful situation. Probably, as a compensation activation of the natural protective factors of the bee organism starts, including change in the protein composition of the haemolymph. The differences could be accounted for by various abiotic and biotic environmental factors in different regions - climatic conditions, bee pasture, development and physiology of bee colonies.

The results obtained in the present study indicate that the amount of lysozyme in the haemolymph of the bees is influenced except by the method of rearing the bee colonies, the age of bees, the content of protein in the diet, the addition of stimulant products in the supplemental feeding of colonies, the presence of pathogenic microorganisms and parasites, the state of the environment, but also by the degree of manifestation of hygienic behaviour of bee colonies.

Conclusion

The quantity of lysozyme in the haemolymph of worker bees from families with low manifestation of hygienic behaviour has the highest average value (15.22±2.37 µg/ml), and in super hygienic and hygienic bee colonies – 10.49±1.86 µg/ml and 9.11±1.37 µg/ml, respectively. The established differences between the average values for hygienic and non-hygienic bee colonies have low level of reliability (P≤ 0.05). The variation min/max is the highest in bee colonies from the non-hygienic group – between 4.59 µg/ml and 38.28 µg/ml. In positive direction compared to the average for the model is the deviation of the LS-estimates of bee colonies with low level of hygiene (non-hygienic), suggesting a tendency for increase of the lysozyme content in the haemolymph. The results obtained in the present study show that the amount of lysozyme in the

haemolymph of bees is affected by the level of manifestation of hygienic behaviour of bee colonies.

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Results are presented in understandable tables and figures, accompanied by the statistical parameters needed for the evaluation. Data from tables and figures should not be repeated in the text. **Tables** should be as simple and as few as possible. Each table should have its own explanatory title and to be typed on a separate page. They should be outside the main body of the text and an indication should be given where it should be inserted.

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Todorov N and Mitev J, 1995. Effect of level of feeding during dry period, and body condition score on reproductive performance in dairy cows,IXth International Conference on Production Diseases in Farm Animals, September 11–14, Berlin, Germany.

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