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Statistical Models for Assessing the Influence of Hygienic Behaviour of Worker Bees on the Level of Lysozyme and Total Protein Content in Their Haemolymph

Research Article

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ABSTRACT

The hygienic behaviour of 26 bee colonies of apiaries in different regions of Bulgaria was tested. The degree of expression of hygienic behaviour was evaluated by the pin-killing method. A test field of square 5 x 5 cm was stuck onto a section of a honeycomb with a sealed worker brood. The bee colonies are divided into two groups: hygienic (cleaned over 95% of cells in the testing area on the 48th hour) and nonhygienic (cleaned less than 95% of the cells in the testing area on the 48th hour). Haemolymph has been taken from bees from each bee colony at 48th hour and lysozyme levels and total protein content has been defined. Multivariate analysis (ANOVA) was used to determine significant differences between hygienic and nonhygienic colonies. The results obtained show significant differences between both groups ($P<0.05$) according to percent cleaned cells after killing the brood at 3rd, 24th and 48th hours and insignificant differences between hygienic and nonhygienic colonies ($P<0.05$) for both parameters-lysozyme and total protein. Bivariate correlation was applied to investigate the impact of hygienic behaviour on lysozyme levels and total protein content. Low negative correlation ($r=-0.33$) between total protein and hygienic behaviour was established which means that an increase of the activities related to the cleansing of honeycomb cells leads to a decrease in the total protein content. Low correlation ($r=0.35$) between hygienic behaviour and lysozyme levels was found i.e. higher levels of lysozyme are reported with more pronounced hygiene behaviour. The calculated regression model for lysozyme ($P=0.24826$) is statistically insignificant but the regression model for total protein is statistically significant ($P=0.003153$) and can be used to predict the relation between total protein content in the haemolymph and hygienic behaviour of bee colonies.

KEY WORDS honeybees, hygienic behaviour, lysozyme level, total protein.

INTRODUCTION

Honey bees have existed for millions of years and in the process of evolution they have built up mechanisms for survival depending on the changing environmental challenges. A major mechanism that protects individual bee specimens is the bee colony. Tens of thousands of worker bees, from a few hundred to a few thousand drones and queen bee, coexist in it. As a separate biological unit, the bee colony exists due to the unity and harmony between the individual specimens. This synchronization is accomplished through conditional, unconditional reflexes and instincts (Nenchev and Zhelyazkova, 2010; Comman *et al*. 2012; Haritonov, 2015).

One of the important behavioral reactions in honeybees, which is congenital and is passed on to the offspring, is

"hygienic behaviour". Hygienic behaviour is a natural mechanism for disease control and is related to the detection and removal of infected and dead larvae and bees outside the hive. In this way, "hygienic" bee colonies clean their nests well ahead of the appearing disease's signs.

Rothenbuhler (1964) found that two recessive genes control cell production and the removal of infected pupae. Later, Taber (1982) reported that one of them determines the removal of dead larvae from the beehives and called it "removing." It is marked with the "rr" symbol. The other one determines the uncapping of the cells and is called "uncapping", marked with the "uu" symbol. More recent studies show that hygienic behaviour is a quantitative feature and its expression in bees can be affected by six (Oxley *et al*. 2010) or seven quantitative loci (Lapidge *et al*. 2002).

Natural immunity in bees largely depends on the content of antimicrobial peptides in their haemolymph (Glupov, 2004). Its protective function includes cellular and humoral barriers (Gillespie *et al*. 1997).

The amount of antimicrobial peptides increases with injury and bacteria entering the haemolymph (Aronstein and Saldivar 2005; Aronstein *et al*. 2010; Ilyasov *et al*. 2014). Glinski and Jarosz (2001) found that bacteria swallowed by bees are digested by the released hydrolytic enzymes, predominantly lysozyme (N-acetylmyramylhydrolase), alkaline phosphatase, ribonucleases, and phospholipases. A number of authors believe that lysozyme content of the haemolymph increases the protective functions of the bee organism.

According to them, the higher levels are influenced by some factors such as bee age, way of rearing the bee colonies, protein content in food, supplementary feeding of bee colonies with stimulating products, presence of pathogenic microorganisms and parasites, etc. (Zyuman *et al*. 1988; Gechev, 1995; Nagornaya *et al*. 1996; Kanchev *et al*. 1997; Zhelyazkova and Gurgulova, 1997; Zhelyazkova and Gurgulova, 2000; Gurgulova *et al*. 2001; Darkazanli, 2008; Shumkova, 2016).

The analysis of the available literature shows that correlations have been established between the hygienic behaviour and the productivity of bee families (Guler and Hakan, 2013; Mansourizalani *et al*. 2018), between the hygienic behaviour and the degree of infection with *Varroa destructor* (Nganso *et al*. 2017).

Lysozyme and total protein in bee haemolymph are considered to be factors of immunity, and hygienic behaviour is a factor for disease resistance. In this regard, it is of interest to develop statistical models to determine the relationship between hygienic behaviour, lysozyme levels, and total protein content in worker bee haemolymph.

The objective of the study is to explore the degree of influence of hygienic behaviour of worker bees on the level of lysozyme and the quantity of total protein in their haemolymph and to develop statistical models that can be used to predict the relation between investigated parameters.

MATERIALS AND METHODS

Testing bee colonies for the degree of expression of hygienic behaviour

Testing about the degree of expression of the hygienic behaviour of bee colonies and the analysis of the haemolymph obtained from them were conducted during the active beekeeping season from May to September.

Bee colonies with bees from the Local Bulgarian bee (*Apis mellifera*) were used for studying. Twenty-six bee colonies from apiaries in different regions of Bulgaria shown at Figure 1 were tested for expression of hygienic behaviour. Haemolymph for analysis was obtained from the tested bee colonies.

Testing for the degree of expression of hygienic behaviour was carried out in bee colonies equal by strength, quantity of sealed worker brood and food stock. The modified method of Gurgulova *et al*. (2003), similar to the method by Petrov (1997) has been applied. Honeycombs with the largest area of sealed worker brood have been selected.

The test field on the combs has been marked by using a square template measuring 5 × 5 cm (100 worker bee cells), which is placed in a section of the comb with continuous worker brood. The brood within the borders of the template was killed by using a thin entomological needle by piercing the caps of the sealed cells without breaking them. Empty cells within the test area have been reported in advance and excluded from the test field. The cells uncapped and cleaned by the bees were counted on the 3rd, 24th and 48th hours of the piercing (Darkazanli, 2008; Gramacho and Gonçalves, 2009).

Obtaining haemolymph and establishing the lysozyme levels and total protein content in it

From each bee colony young worker bees were taken (200-250 pieces), from which haemolymph (cumulative samples) was obtained. The haemolymph was extracted by a Pasteur pipette at the border between the IInd and IIIrd abdominal tergite.

The lysozyme content in the haemolymph was determined by the method of Motavkina *et al*. (1979), modified by Kostov and Bonovska (1983). Live culture of *Micrococcus lisodeicticus* was used in the assay, which is lysed specifically by the lysozyme. To determine the total protein quantity in the haemolymph samples the Biuret method was used.

Figure 1 Geographical location of tested apiaries in the Republic of Bulgaria

Laboratory assays to determine the lysozyme and total protein content in the studied haemolymph samples were carried out at the National Reference Laboratory "Bee Health" at the National Diagnostic Research Veterinary Medical Institute – Sofia, Bulgaria.

Statistical processing of the data

The data obtained were processed variationally and statistically with software STATISTICA 12, Copyright © Stat Soft Inc. 1984-2014 (StatSoft, 2014). To determine the presence of significant differences between bee colonies with hygienic or nonehygienic behaviour Multivariate ANOVA with Hotelling and Wilks Tests for the 3rd, 24th and 48th hours was applied. The same statistical analysis was used to define significant differences between hygienic and nonehygienic groups based on lysozyme levels and total protein content measured at 48 h.

Correlation analysis was used to determine the strength and direction of the relation between the levels of lysozyme in the haemolymph and the hygienic behaviour of worker bees and between the total protein content in the haemolymph and the hygienic behaviour of bees as well. To assess the degree of influence of hygienic behaviour of worker bees on the level of lysozyme and the quantity of total protein in the haemolymph, regression models have been developed.

RESULTS AND DISCUSSION

Table 1 presents the results from the conducted Multivariate ANOVA tests on bee colonies for the degree of expression of hygienic behaviour.

From the total of 26 bee colonies included in the study 14 of them (53.85%) have been determined as colonies with a high degree of hygienic behaviour (hygienic) and 12 of them (46.15%) as colonies with a low degree of hygienic behaviour (nonhygienic), respectively.

As can be seen from Table 1 bee colonies from the hygienic group had uncapped and cleaned on the 3rd hour an average of 2.20 ± 0.32% of the cells with killed brood within the template area. In the 3rd–24th hour range these bee colonies had cleaned over 90% (91.24%) of the total number of uncapped and cleaned cells established detected on the 48th hour (98.83±0.23%).

The analysis of the results concerning the percentage of uncapped and cleaned cells from the group of non-hygienic bee colonies shows that on the 3rd hour they had cleaned an average of 0.63 ± 0.33%, which is 1.57% lower than the hygienic group at that time. On the 24th and 48th hour, bee colonies from this group had uncapped and cleaned an average of 69.04 ± 1.99% and 86.28 ± 1.09%, which is, respectively, 23.33% and 12.55% less than the hygienic group. In the period from the 3rd to the 24th hour, bees from colonies with a low degree of hygienic behaviour had cleaned less than 70% (68.41%) of cells, which compared to the number recorded on the 48th hours is 79.29%. It was found that for the indicated period (3rd-24th hour) the non-hygienic bee colonies had cleaned 11.95% fewer cells compared to the group of hygienic colonies.

Based on the results obtained, it can be concluded that the hygienic bee colonies recognize at an earlier stage the killed brood.

Table 1 Results from testing bee colonies for the degree of expression of hygienic behaviour[1, 2, 3, 4]

Groups		Mean	SE	SD	Tests of between-subjects effects		
						F	P-value
% opened and cleaned cells at 3 h	Hygienic	2.202	0.319	2.48	Wilks	28.270	0.000
	Nonhygienic	0.625	0.332	1.27	Hotellng	28.270	0.000
% opened and cleaned cells at 24 h	Hygienic	91.780	1.911	8.85			
	Nonhygienic	69.036	1.989	14.52			
% opened and cleaned cells at 48 h	Hygienic	98.739	1.044	1.50			
	Nonhygienic	86.275	1.087	9.28			

[1] Multivariate tests of significance for levels of expression of hygienic behaviour (ANOVA).
[2] Sigma-restricted parameterization.
[3] Effective hypothesis decomposition.
[4] n= 26.
* (P<0.05).
SE: standard error and SD: standard deviation.

According to Spivak and Downey (1998), the presence of a dead brood provokes the hygienic behaviour of worker bees. Bee colonies with a high degree of expression of hygienic behaviour quickly discard the dead larvae and pupae from the beehive well before signs of disease appear, which contributes to the resistance of bees to pathogenic microorganisms and ectoparasites (Nemkova, 2004). A similar opinion is shared by other authors (Palacio *et al.* 2010).

Table 1 shows the significant differences between bee colonies with different levels of hygienic behaviour. Based on the Hotelling and Wilks tests, a statistically significant difference (P≤0.05) was found between the groups of hygienic and non-hygienic bee colonies at the 3rd, 24th and 48th hours, separately.

Table 2 shows the results of lysozyme levels in the haemolymph of worker bees with different degrees of expression of hygienic behaviour. A higher level of lysozyme in the worker bees' haemolymph has been found in the group of nonhygienic colonies (13.71±1.76 µg/mL). In this group, a wide variation in terms of the minimum and maximum value of the studied trait has been reported, 3.13 µg/ml and 38.28 µg/mL, respectively. The mean value of lysozyme content in the haemolymph of bees in the group with a high degree of expression of the hygienic group is 10.33 ± 0.93 µg/mL, which is 3.38 µg/mL less than the value of this indicator in the nonhygienic bee colonies. As can be seen from Figure 2 the differences between hygienic and nonehygienic group for lyzozyme levels in bees' haemolymph based on Hotelling and Wilks tests are statistically insignificant (P=0.11613<0.05).

A probable cause for the higher lysozyme levels in the nonhygienic bee colonies is due to the fact that they detect the presence of the dead brood at a later stage. This, in turn, leads to the activation of humoral protective immunity factors, one of which is lysozyme. According to some authors, this enzyme is not the only factor of immunity, but it is important in initiating the synthesis of peptides and low molecular proteins with bactericidal activity (Nagornaya *et al.* 1996; Wilson-Rich *et al.* 2008; Gätschenberger *et al.* 2013).

The obtained values of the studied parameters lysozyme level in the haemolymph and degree of hygienic behaviour of worker bees reflected on Figure 3 have distribution close to the normal distribution with mild left asymmetry at P < 0.05, i.e. the test for normality of distribution is statistically reliable.

This is a prerequisite for the results of the correlation and regression analyses of the relation between the lysozyme level and the hygienic behaviour of worker bees to be considered common and applicable to all bee colonies from the studied population.

The correlation analysis performed (Figure 3) has established the strength and direction of the relationship between lysozyme levels in the haemolymph and the hygienic behaviour of worker bees. The results show a weak relation between the studied parameters (r=0.351) i.e. higher levels of lysozyme are reported with more pronounced hygiene behaviour. The results from correlation analysis are evidenced by the regression analysis presented in Table 3 and Figure 4. The resulting regression model is statistically insignificant (P>0.05). The value of the determination coefficient R^2= 0.01662784 (Table 3) in turn indicates that only 1.7% of the variations in the lysozyme levels studied are accounted for by the degree of hygienic behaviour of the bees.

The insignificance of the model is a reason to continue the assay with a larger data set or to further study the nature of the dependence between the variables. So far, no similar study has been conducted in Bulgaria on the influence of worker bees' hygiene behaviour on lysozyme levels and total protein content in their haemolymph. In the studied literature the authors hadn't found a similar analysis on these parameters.

Table 4 presents the results of total protein content in the haemolymph of worker bees in colonies with high (hygienic) and low (nonhygienic) degree of expression of hygienic behaviour. In the group of nonhygienic colonies, the value of this indicator is higher (22.63±2.97 g/L), which is 3.58 g/L higher than that found in the group of hygienic bee colonies (19.05±1.95g/L).

Table 2 Amount of lysozyme in the haemolymph of worker bees from bee colonies with different level of expression of hygienic behaviour

Level of expression of hygienic behaviour	Amount of lysozyme in worker bee haemolymph (µg/mL)				
	Mean	SE	SD	min	max
Hygienic bee colonies (n=14)	10.33	0.93	6.17	3.13	25.00
Nonhygienic bee colonies (n=12)	13.71	1.76	10.85	3.13	38.28
P-value			0.11613		

* (P<0.05).
SE: standard error and SD: standard deviation.

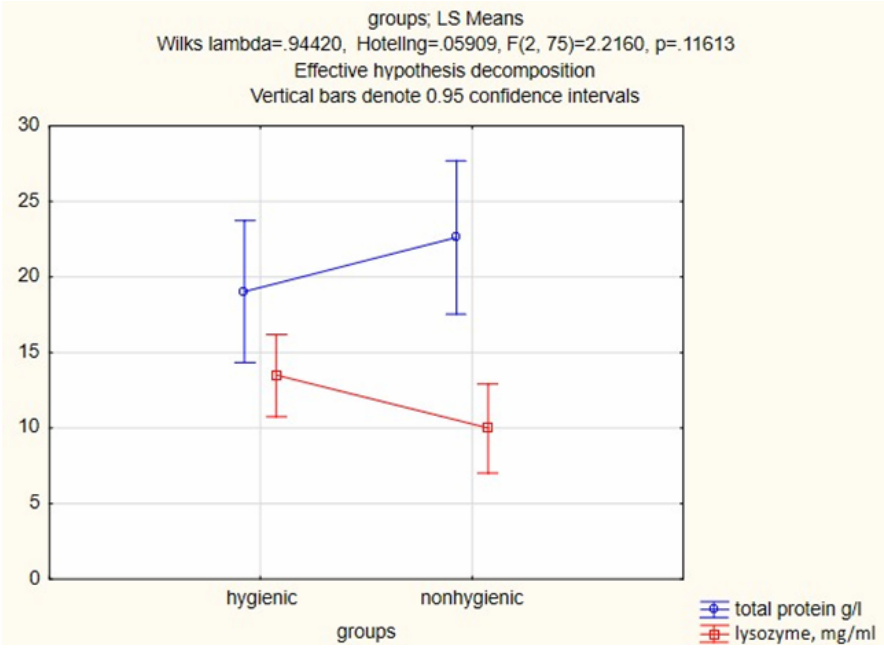

Figure 2 Multivariate ANOVA analysis for significant differences between hygienic and nonehygienic groups based on lysozyme levels and total protein content

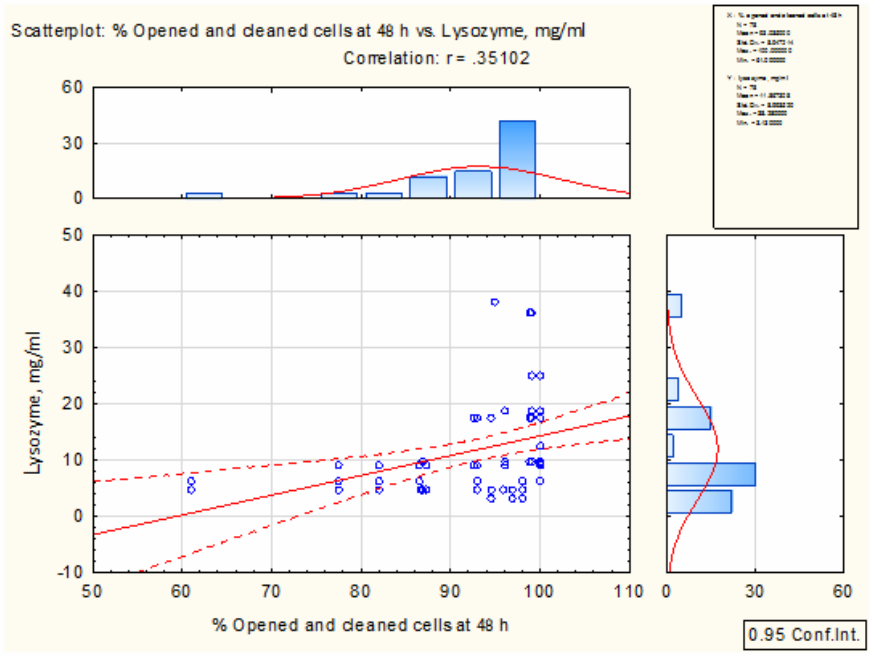

Figure 3 Histogram of distribution and correlation between lysozyme levels in haemolymph and hygienic behaviour of worker bees

Table 3 Regression model showing the dependence of lysozyme levels in haemolymph from the hygienic behaviour of worker bees[1]

No. of cases: 82	b*	SE of b*	b	SE of b	t(80)	P-value
Intercept	-	-	24.21476	10.63377	2.27716	0.025447
% cleaned cells at 48 h	-0.12895	0.110870	-0.13216	0.11363	-1.16306	0.248261

[1] Regression summary for dependent variable: lysozyme, mg/mL; R= 0.12894898, R^2= 0.01662784; Adjusted R^2= 0.00433569; F (1; 80)= 1.3527; P < 0.24826 and Standard error (SE): 8.7507.
* (P<0.05).

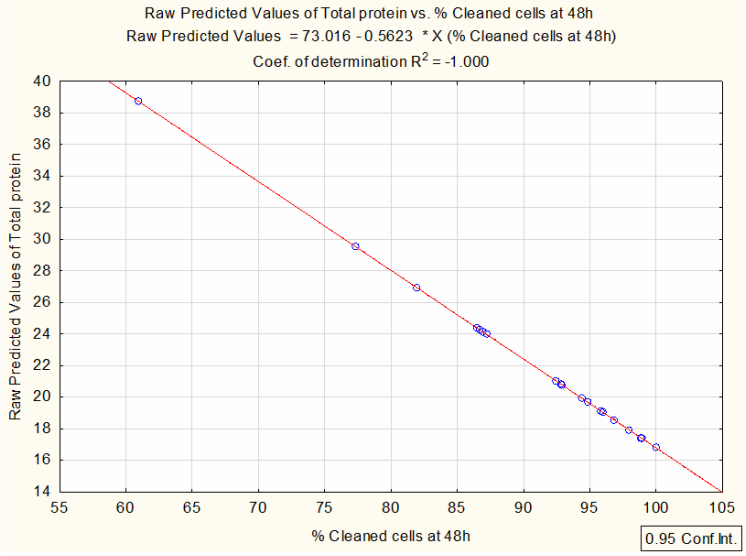

Figure 4 Regression model showing the dependence of lysozyme levels in haemolymph and hygienic behaviour of worker bees

Table 4 Amount of total protein in the haemolymph of worker bees from bee colonies with different level of expression of hygienic behaviour

Level of expression of hygienic behaviour	Total protein g/L				
	Mean	SE	SD	min	max
Hygienic bee colonies (n=14)	19.05	1.95	12.64	1.20	43.78
Nonhygienic bee colonies (n=12)	22.63	2.97	17.79	3.18	71.04
P-value			0.11613		

* (P<0.05).
SE: standard error and SD: standard deviation.

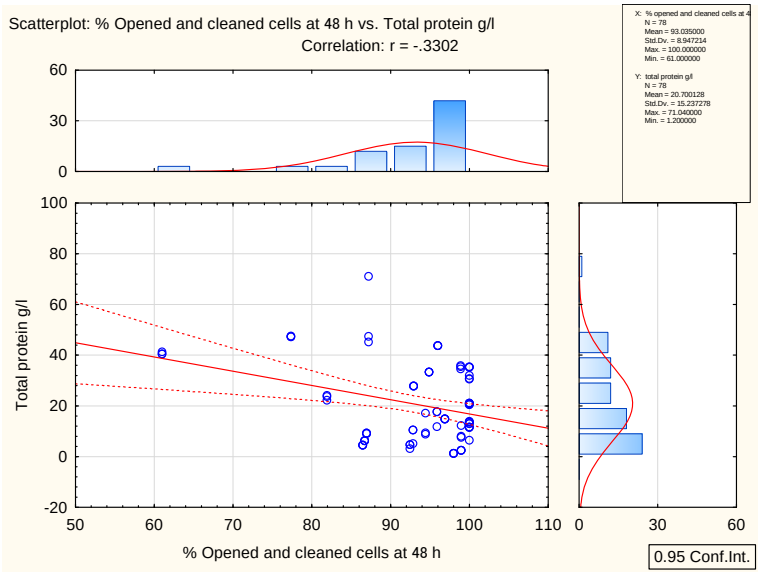

Figure 5 Histogram of distribution and correlation between a total protein in haemolymph and hygienic behaviour of worker bees

Larger variation in minimum - maximum limits are reported in colonies with a low level of expression of hygienic behaviour (min=3.18 to max=71.04 g/L), compared to variations in colonies with a high level of expression (min=1.20 to max=43.78).

The reported differences between hygienic and nonhygienic group for total protein content calculated by Hotelling and Wilks tests (Figure 2) are statistically insignificant (P<0.05).

Concerning the total protein content in the haemolymph and the degree of hygienic behaviour of the worker bees (Figure 5), the results show that the data are close to the normal distribution with a small asymmetry on the left, i.e. the distribution normality test is statistically reliable at significance level P < 0.05. This again gives reason to assume that the results of the correlation and regression analyses for the indicator total protein in the hemolymph could be applied to the entire studied population of bee colonies.

The results of the conducted correlation analysis (Figure 5) show that there is a weak negative relation (r=-0.33) between total protein content and hygienic behaviour was established which means that an increase of the activities related to the cleansing of honeycomb cells leads to a decrease in the total protein content.

As can be seen from Figure 3 and Figure 5 the are a lot of outliers for both data sets (lysozyme and total protein quantity). A possible reason for this is the larger variations in the data. This could be avoided in subsequent experiments by working with more samples or further investigating the nature of the relationship between the variables.

The parameters of the calculated linear regression model are presented in Table 5 and Figure 6 graphically shows the predicted values of the studied parameter total protein content and the overall view of the regression equation. As can be seen from the Table, the model is statistically significant (P<0.05).

Table 5 Regression model showing the dependence of total protein content on worker bee haemolymph from hygienic behaviour[1]

No. of cases: 78	b*	SE b*	b	SE of b	t(76)	P-value
Intercept			73.01644	17.23314	4.23698	0.000063
% cleaned cells at 48 h	- 0.33019	0.108274	-0.56233	0.18439	-3.04962	0.003153

[1] Regression summary for dependent variable: total protein, g/L; R= 0.33019552; R^2= 0.10902908; Adjusted R^2= 0.09730578; F (1; 76)= 9.3002; P < 0.00315 and Standard error (SE): 14.477.
* (P<0.05).

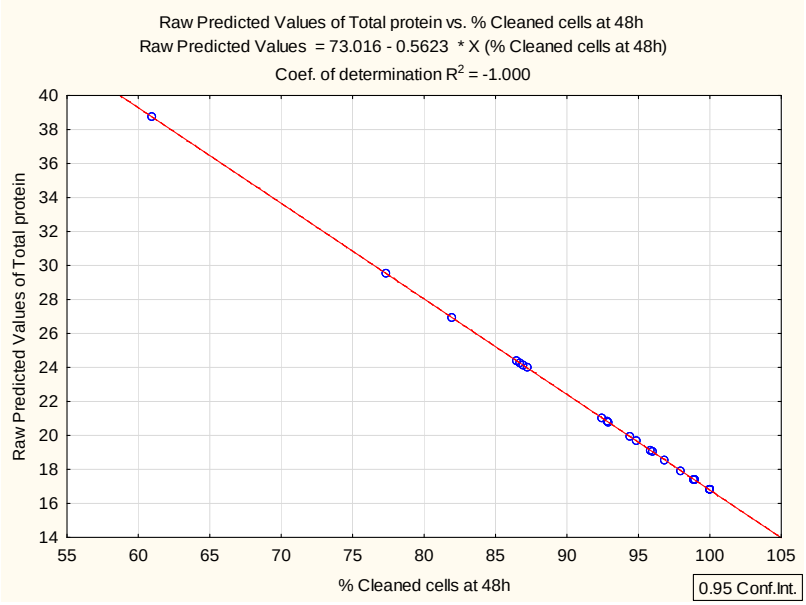

Figure 6 Regression model showing the dependence of total protein content in the haemolymph from hygienic behaviour of worker bees

The value of the determination coefficient R^2= 0.10902908 (Table 5), in turn, shows that around 11% of the variations in the studied total protein content in the haemolymph can be explained by the degree of hygienic behaviour of the bees. This percentage is much higher compared to the percentage of variations in lysozyme levels that can be explained by the bees' hygienic behaviour. As this is the first study in Bulgaria concerning the relationship between the hygienic behaviour of bees and lysozyme levels and total protein content of their haemolymph, it is not possible to answer unequivocally whether the percentage is sufficient for the relevant analysis.

CONCLUSION

The mean values obtained for both analyzed parameters (lysozyme and total protein) in the haemolymph of worker bees from non-hygienic bee colonies were higher (13.71±1.76 μg/mL and 22.63±2.97g/L) compared to those of hygienic bee colonies (10.33±0.93 μg/mL and 19.05±1.95 g/L). The differences found between the mean values of the stated parameters for hygienic and nonhygienic bee colonies are statistically insignificant at significance level (P≤0.05). The correlation analysis shows a weak relationship (r=0.35) between the lysozyme levels in the haemolymph and the hygienic behaviour of worker bees and a weak negative relationship (r=-0.33) between the total protein content and the hygienic behaviour, respectively. Based on the correlation analysis, it can be concluded that there is a tendency to increase the levels of lysozyme and decrease the total protein content in the worker bees' hemolymph with increase the degree of hygienic behaviour. The developed regression model showing the degree of dependence of lysozyme levels from hygienic behaviour of bees is statistically insignificant but the regression model for the dependence of total protein is statistically significant and can be used to predict the relation between total protein content in the haemolymph and hygienic behaviour of bee colonies. In summary, it can be concluded that the insignificancy of the model for predicting the lysozyme levels in the haemolymph confirms the need to continue the analysis with a greater number of observations, as well as further study of the nature of the dependencies among the variables.

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