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Relationship between dead pupa removal and season and productivity of honey bee (*Apis mellifera*, Hymenoptera: Apidae) colonies

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Abstract: This study was conducted to evaluate the dead pupae removal behaviour of 90 honey bee (*Apis mellifera* L.) colonies during the beekeeping season and the relationships between removal and colony productivity. The liquid nitrogen technique was used in May, June, July, August, and September. The number of removed pupae was significantly ($P < 0.01$) correlated with the month and changed throughout the season. The highest cleaning efficiency of the colonies were recorded in July and September, and the lowest were in May and August. While the average dead pupae removal was 83.75% in the 90 colonies, the percentage of removed pupae varied between 56.4% and 99.3% during the 5 months. There were significant positive correlations between dead pupae removal and honey yield ($P < 0.01$; $r = 0.295$), bee wax production ($P < 0.01$; $r = 0.334$), and adult worker bee population ($P < 0.05$; $r = 0.233$). No correlation was found between dead pupae removal and brood production and average temperature. Although hygienic behaviour has positive effects on many characters relating to productivity in untreated breeding populations, it is affected by many biotic and abiotic factors. This behaviour decreases with many stressful conditions (wasps, *Merops* sp., predators, honey harvesting) and increases with colony strength during the season.

Key words: *Apis mellifera*, colony, pupae removal, season, productivity

1. Introduction

Understanding that chemical use against diseases and pests is not a solution (1), scientists have placed emphasis on the hygienic behaviour displayed by honey bees (2). Nearly 10%–12% of the colonies forming many honey bee populations demonstrate hygienic behaviour (3) and there have been differences in hygienic behaviour among colonies of the same subspecies and in the same apiary (4). Spivak and Gilliam (5) estimated that only 10% of honey bee colonies in the United States are hygienic. A general test of hygiene, the removal of freeze-killed brood by colonies (6), correlates relatively well with the removal of *Varroa*-infested brood (7,8).

Hygienic behaviour is performed by 15- to 20-day-old worker bees and prior to foraging (9). The honey bees performing hygienic behaviour are highly capable of detecting disease agents and they also uncap and remove a portion of the brood infested with the parasitic mite *Varroa destructor* (10–13). Hygienic behaviour, in which individual honey bees detect chemical stimuli from diseased larvae and subsequently remove the diseased brood from the nest, is one type of social immunity that reduces pathogen transmission (12). Therefore, it is accepted that hygienic behaviour is the basic mechanism of resistance to diseases and pests. Moreover, this behaviour trait can be improved through selective breeding (14–17). Assessed in this way, colony-level hygienic behaviour has high heritability, such as $h^2 = 0.63$ and 0.65 (13). Furthermore, *Varroa destructor*- and *Paenibacillus larvae*-resistant honey bee genotypes have been produced by at least 3 breeding programs in North America (15,18).

Honey bee colonies that remove dead pupae at rates greater than or equal to 95% in at least 2 assays are considered hygienic. However, we have inadequate information on the factors affecting hygienic behaviour during the active beekeeping season or over 6 months. This is because all activities and behaviours of a honey bee colony, such as reproduction, worker bee age, worker bee population, brood production, comb construction, pollen and nectar collection, development, and production of bee products, change with the season (19,20). Furthermore, all these activities change according to the honey bee subspecies (4). There are important effects of worker bee age (21), strength of bee colony, nectar flow, temperature, and area (22) on the level of hygienic behaviour. For instance, a queen bee lays 2500–3000 eggs per day only in

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May and June, when the season is optimal or suitable for nectar and pollen flow (19,20,23). The number of young worker bees changes with the season; as with populations of foragers, it reaches its highest level at the main nectar flow period (19,24,25). Additionally, there are no worker bees in the colony that are between 15 and 20 days old age during the late autumn and winter season for 4 to 5 months.

The aim of the present study was to evaluate the relationships between the hygienic behaviour of honey bee (*Apis mellifera* L.) colonies and beekeeping season and colony productivity.

2. Material and methods

2.1. Honey bee material

The study was conducted at the Bee Research and Application Unit of the Agricultural Faculty of Ondokuz Mayıs University. Honey bee colonies belonging to the region and having 2-year-old queen bees were used as material. At the beginning of spring (in April), the colonies were equalised in relation to the age of the queen bee, number of frames with bee and brood, nutrition and nurse, comb foundation, disinfection, control, settlement in apiary, and transportation (20,24). Each colony was numbered and a registration system was formed. Medicine (Perizin; coumaphos = Asuntol) was applied to the colonies against *Varroa destructor* in the early spring. Apart from this, no other chemicals were applied to the colonies. In the spring, the colonies were fed with sucrose syrup (1:1, water:sugar). The colonies renewing the queen bee and swarming during the course of the study were excluded from the experiment. Migratory beekeeping was applied and honey was harvested the third week of August.

2.2. Liquid nitrogen application and pupae counting method

In this study, the liquid nitrogen (–196 °C) method was used (5,6,11). The first and fifth nitrogen applications were done in Samsun (41.2°N, 36.2°E), and the other 3 applications were done in the vicinity of Gülaçar valley, near Gümüşhane (40.274°N, 39.29°E). In these 2 experimental areas, chemical usage for agricultural products is low because of low agricultural activity.

For each application, one frame with pupae was taken from each colony. Approximately 300 mL of liquid nitrogen was poured into the cylindrical metal template covering 165 pupae cells (3). Liquid nitrogen was applied 5 times at monthly intervals starting at 15–20 May, June, July, and August and terminating at 15–20 September. The hour at which the frame was placed in the hive was recorded on the colony card. This frame was taken from the hive 48 h after liquid nitrogen application. The label was fixed near the area where the liquid nitrogen was applied (165 cells) and later photographed with a digital camera. The frame was placed in the hive from which it was taken. At each period, these pictures were loaded onto a computer, and later the removed cells were counted and recorded on the colony card. Empty cells with a fixed funnel were counted at the beginning and recorded on the colony cards (3).

2.3. Productivity characteristics of colonies

Worker bee population: Total number of frames covered with adult bees (frame number/colony) of each colony was recorded every month in the period of May to November (20,24,26).

Brood rearing: Frames covered with open (egg and larvae) and closed (pupae) brood of each colony were counted (frame number/colony) and recorded in May, June, July, August, and September.

Honey yield: The first frames with honey in each colony were determined, and after leaving the required honey for the colony, the remaining was recorded as honey yield. Before the centrifuge process, frames with honey from each colony were weighed, and after the centrifugation, the same frames were weighed again and their tares were found. The honey amount produced by each colony (kg/colony) was then found by excluding the tare from the first measurement (24–26).

Wax production: Colonies were checked every 5–6 days in May, June, and July and the standard foundation combs were given when needed; this was recorded to the colony chart. The total number of foundation combs of each colony built was then counted during the honey harvest and, from this number, wax amounts produced in the each colony (g/colony) were determined. To this aim, after the honey harvest, combs with honey dishes were marked and returned to their own hives, and after a standby period of 3 days, the honey on them was cleaned by worker bees. The total wax produced by the colonies was then calculated (g/colony) by multiplying the amount of wax required for building a foundation comb with the number of the combs built by each colony (20).

Temperature and other environmental factors: Meteorological data such as air temperature, humidity, and CO_2% were measured daily in the apiary by using a data logger during the experiment (22).

2.4. Statistical evaluation

One-way variance analysis (ANOVA) was performed to determine the number of cleaned dead pupae of the colonies in the 48 h after each of the 5 months throughout the beekeeping season by using a completely randomised plot design. Tukey's multiple comparison test was used for comparison of the means (27).

3. Results

3.1. Dead pupae removal during the beekeeping season

The means, standard errors, percentages, and lowest and highest rates of removed dead pupae by the 90 colonies

during May, June, July, August, and September are presented in Table 1. There were significant differences ($P < 0.05$) in the number of dead pupae removed in the 48 h after each of the 5 months. The mean value of the number of dead pupae removed in the population was 138.18 ± 1.37 per colony (83.75%). In 48 h, the lowest and highest numbers of removed dead pupae were 38 and 165, respectively. The highest dead pupae removal rates per colony were 141.86 ± 2.73, 144.30 ± 2.32, and 146.98 ± 2.62 for treatments in June, July, and September, respectively. The lowest were 126.47 ± 3.42 and 131.28 ± 3.52 for treatments May and August, respectively.

3.2. Productivity of honey bee colonies

3.2.1. Worker bee population

The means of frames covered with adult worker bees, frame brood production, bee wax production, and honey yield values for each colony are given in Table 2. The average number of frames covered with adult worker bees of the colony was 13.12 ± 0.293. There were no significant differences between colonies ($P > 0.05$) in relation to the adult worker bee population during the experiment. However, a significant positive correlation was found between dead pupae removal and the worker bee population (Figure 1). The coefficient of correlation, regression, and regression equation were $r = 0.233$, $R = 0.135$ ($R^2 = 0.018$), and $Y = 129.506 + 0.65x$, respectively, for these 2 characters (Table 2).

3.2.2. Brood rearing activity

No difference was found ($P > 0.05$; $r = 0.035$) between the colonies in terms of brood rearing. The mean of frames covered with broods of the colony was 3.687 ± 0.09 frames per colony (Table 2). In addition, there was no significant relationship between dead pupae removal and brood rearing ($P > 0.05$).

3.2.3. Honey yield

The mean of honey yield was 21.465 ± 0.9 kg per colony. There were significant differences between colonies in terms of honey yield ($P < 0.01$). A significant positive correlation was also found between dead pupae removal and honey yield ($P < 0.01$, $r = 0.295$; Figure 2). Although some colonies had higher yield, some others had lower yields. For instance, colonies 103 and 13 had no honey yield, whereas colonies 18 and 302 had 49 kg honey yield per colony. Colony 23 was found to have the highest hygienic behaviour in the present study, giving 16.33 kg honey per colony, which was 5 kg lower than the colony average.

3.2.4. Wax production

Colonies produced significantly ($P < 0.01$) different amounts of wax from each other. The mean of wax production was 1207.43 ± 48.5 g per colony (Table 2). Significant negative correlation was found between dead pupae removal and wax production ($P < 0.05$; $r = 0.233$; Figure 3). While the highest wax (2530.85 g per colony) was produced by colonies 302 and 18, the lowest (158.18 g per colony) was produced by colonies 103 and 13. The highest wax-producing colonies (colonies 302 and 18) removed 89.9% and 91.3% of dead pupae, respectively. The lowest wax-producing colonies (colonies 103 and 13) removed 90.4% and 79.9% of dead pupae, respectively.

3.2.5. Temperature

During the experiment, average temperatures of May, June, July, August, and September were 22, 17, 27, 29, and 24 °C, respectively. No relationship was found between the dead pupae removed and average temperature ($P > 0.05$; $r = -0.031$). On the other hand, there was significant negative correlation between temperature and brood production ($P < 0.001$; $r = -0.391$).

4. Discussion

There were significant differences and relationships among the months with regard to dead pupae removal behaviour and colony productivity during beekeeping season in the untreated breeding population. While the highest dead

Table 1. The mean ($\bar{X}$), standard errors ($\pm S_{\bar{X}}$), and percentage (%) of removed dead pupae during May, June, July, August, and September in 90 colonies.

Season	n	$\bar{X} \pm S_{\bar{X}}$	%	Lowest	Highest
May	90	126.47 ± 3.42 ^{c*}	76.65	38	165
June	90	141.86 ± 2.73 ^{ab}	85.98	59	165
July	90	144.30 ± 2.32 ^{a}	87.45	82	165
August	90	131.28 ± 3.52 ^{c}	79.56	47	165
September	90	146.98 ± 2.62 ^{a}	89.08	75	165
$\bar{X} \pm S_{\bar{X}}$	90	138.18 ± 1.37	83.75	38	165

Asterisk indicates main effect of treatments, $P < 0.05$ level of significance. $^{a, b, c}$: means with different letters are significantly different ($P < 0.05$).

Table 2. The relationships between dead pupae removed (number per colony), brood production (frame number per colony), adult worker bee population (frame number per colony), honey yield (kg/colony), wax production (g/colony), and temperature (°C).

	Dead pupae removed	Brood rearing	Adult worker bee	Honey yield	Wax production
Brood rearing	0.035	1			
Adult worker bee population	0.233*	0.727**	1		
Honey yield	0.295**	0.615**	0.893**	1	
Wax production	0.334**	0.510**	0.827**	0.926**	1
Temperature	–0.031	–0.391**	0.063	–	–

*: $P < 0.05$, **: $P < 0.01$.

pupae removal rate was recorded in the second (June), third (July), and fifth (September) applications, the lowest was in the first (May) and fourth (August) applications. Many researchers reported that the level of hygienic behaviour showed variability depending on the subspecies of honey bee and colonies in the same apiary (1,3,6,11,28). For that reason, this behaviour has to be evaluated as a skill arising from genetic differences (2,14,29). On the other hand, in our study during the 5 months, the experimental colonies experienced many internal and external factors, including brood rearing, changes in adult worker bee population, building of combs, nectar and pollen collection, harvesting of honey, parasites, and predators. Differences in the treatments were attributed to these internal and external factors. For instance, colony weakness or lack of incoming nectar has been shown to reduce the removal response to mite-infested and dead-brood cells, respectively (2,6,30). Previous studies showed that colonies had higher dead pupae removing behaviour during the nectar-flowing period (3,6,11,22,30). According to us, apart from nectar flow, there might be many other reasons for differences in dead pupae removal behaviour among the treatments for both the previous studies and the present study. For example, these reasons might include the number of worker bees, pests, and all other stress factors causing danger in the hive. In our study, the lowest dead pupae removal rate occurred after the first (May) and fourth (August) treatments. While nectar flow was good in May, it was low or absent in August. The colonies even had to draw out honey comb and store some honey. We measured the weight of the hives every day during the study and over 2 t of honey was harvested from the colonies in the experiment. Therefore, we believe that nectar flow was not the main reason for the low hygienic behaviour. It was thought that the intensive bee-eater (*Merops persicus*) population in the experimental site might have been another factor causing low hygienic behaviour. The area where the experimental colonies were situated was along the main route of these birds during the time of the first treatment (May). The second factor might have been wasps (*Vespula vulgaris* and *Vespula germanica*), which had the highest population during the period of the fourth treatment (August). It was thought that the stress caused from these 2 factors and others might have led to less dead pupae being removed. Honey bees change their hygienic behaviour according to the intensity of the source of danger. They orient towards the source of the dangers creating stress for the colony. In the present study, the findings that the colonies with high hygienic behaviour (95% and above) did not display the same performances after the first (May) treatment and fourth (August) treatments support this hypothesis (data not given). The third reason might be

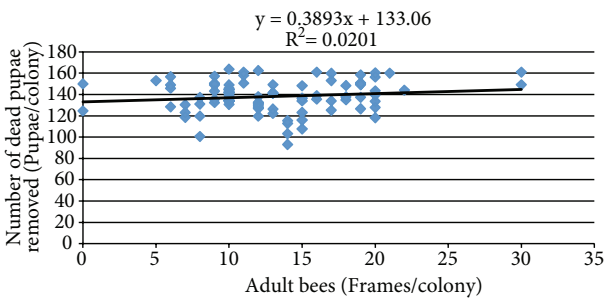

Figure 1. The relationship between dead pupae removal and the adult worker bee population.

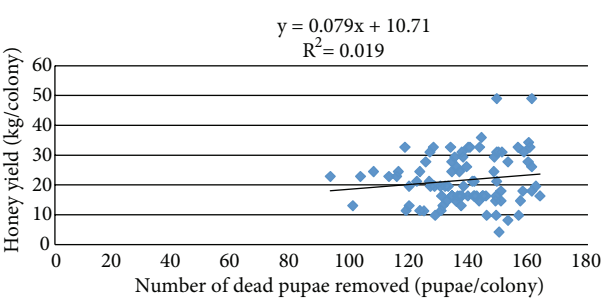

Figure 2. The relationship between dead pupae removal and honey yield.

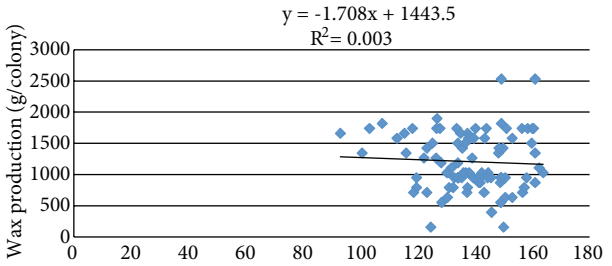

Figure 3. The relationship between dead pupae removal and wax production.

the harvest of honey during the time period of the fourth (August) treatment, which can create considerable stress. Therefore, it was considered that dead pupae removal behaviour can change and/or decrease depending on the source of danger and stress. The colonies removed the highest number of dead pupae after the fifth (September) treatment, where nectar flow was low or absent and the colony population was lower. On the other hand, colonies removed the second highest numbers of dead pupae during the third (July) treatment, where nectar flow was high and the colony population was at its highest level. In July, nectar flow was 1200 g per colony per day. Therefore, we thought that the high dead pupae removal rates during the period of rich nectar flow were not directly related to hygienic behaviour. We consider that the highest dead pupae removal rate during the rich nectar flow period was due to the importance of nectar for the colonies. Nectar storage has great importance for the future of honey bee colonies. During this period, the colony limits all its activity, especially brood rearing (19,20), and prepares the area on the comb for nectar storage. Rinderer et al. (3) reported that there was an increase in dead pupae removal in both hygienic and nonhygienic colonies during nectar flow, and Panasiuk et al. (22) stated that the nectar flow impact seems to be a more complicated factor. Therefore, we suggest that high pupae removal rates during the rich nectar flow period should be accepted not as a direct but as an indirect behaviour.

In the present study, it was found that dead pupae removal behaviour had positive effects on the honey bee colony productivity. Although the regression coefficients were low (R^2 = 0.020, R^2 = 0.003, and R^2 = 0.019, respectively), relationships between dead pupae removal and the adult bee population ($P < 0.05$, $r = 0.233$) and production of bee wax ($P < 0.01$, $r = 0.334$) and honey ($P < 0.01$, $r = 0.295$) were significant (Table 3). Namely, the colonies removing the highest numbers of dead pupae had a stronger adult bee population and produced higher amounts of honey and bee wax. These relationships were supported by many researchers (22). We think that this advantage is a result of having genetic nest-cleaning behaviour.

In conclusion, dead pupae removal behaviour of the honey bee colonies belonging to the untreated breeding population was observed to change during the season after each treatment of liquid nitrogen. This change might have resulted from colony strength, nectar flow rate, honey harvest, natural enemies, or all other the stress factors. For that reason, it was thought that honey bee colonies can perform their hygienic behaviour optimally under conditions lacking stress.

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