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ROLE OF POLLEN AND/OR BEE BREAD AVAILABILITY IN BROOD REARING AND CELLULAR IMMUNE SYSTEM OF HONEYBEES

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ABSTRACT

The role of pollen and/or bee bread availability was evaluated on brood rearing, protein content and haemolymph of 6-days-old larvae worker honeybees. The highest area of stored pollen and/or bee bread and worker unsealed brood was recorded during August with an average 157.7±24.701 and 347.7±51.348 (sq. inch / Colony), respectively. Highly significant positive correlation was detected between stored pollen and/or bee bread and worker unsealed brood (r = 0.902**). Physiological studies on larvae and their haemolymph indicated that the, lowest protein content of both larvae and their haemolymph 22.03±6.325 % and 4.04±0.48 (µg/µl), respectively was found during April. Significant positive correlation was noticed between stored pollen and/or bee bread from one side and both larval protein content and haemolymph protein content on the other hand, (r = 0.393* and 0.345*), respectively. The highest of both total soluble solids (TSS %) and total haemocyte count (THC) was found during May and January. On the other hand, the lowest average of both (TSS %) and (THC) was noticed during April. Differential haemocytes counts (DHC) were counted in smears of their haemolymph. There were significant differences in different haemocyte types among various months. So, it turns out previously, it can be recommended for special attention protein nutrition artificially by providing nutrition supplements or pollen substitutes for bee colonies and that in periods of scarcity or lack of pollen as well as in the periods of disease spreads. In this process the chances of incidence of disease can be reduced and improve the immune system of bees. This is in addition to reducing the loss incident in bee colonies as a result of starvation.

Keywords: Honeybee, pollen, bee bread, brood rearing, protein, haemolymph, haemocytes, immunity.

INTRODUCTION

Sufficient nutrition in honeybee colony is an essential for growth and development. The development and the survival of honeybee colonies are therefore intimately associated with the availability of those environmental nutrients (Brodschneider and Crailisheim, 2010; Keller *et al*., 2005; Haydak, 1970), which suggests that the alteration of bee foraging area due to the current intensification of agriculture and landscape changes might provide a deficient nutrition and therefore affect honeybee populations (Decourty *et al*., 2010; Naug, 2009). This is further supported by beekeepers, which are ranking poor nutrition or starvation as one of the main reasons for colony

losses (Abdel-Rahman and Moustafa, 2012). Therefore, studying the connexion between nutrient availability and honeybee colony health might help to better understand the current bee losses observed throughout the world (Nneumann and Carreck, 2010; Van-Engelsdarp and Meixner, 2010). Colonies with limited protein intake decline from the combination of reduced brood rearing and a shorter lifespan for adult water. If parasitic mites and pathogens are present, the population decline can be even more severe so that the colony perishes.

Honeybee repose on pollen as their source of protein, lipids, sterols, amino acids, starch, vitamins and minerals (Roulston and Buchmann, 2000; Stanly and Linskens, 1974), is a major factor influencing the longevity of individuals (Haydak, 1970). Also, pollen is important at the colony level, since it enables the production of royal jelly by workers that is fed to larvae of all castes and to the queen (Crailsheim, 1994).

In addition to need for pollen in brood rearing and to optimize worker longevity, nutrition (particularly protein availability) is a key factor in resistance to pathogens (Ford *et al*., 2001; Kaminogawa and Nanno, 2004; Riz and Gardner, 2006; Rowley and Powell, 2007). As long as, a direct consequence of protein nutritional deficiency is a decrease in the colony population (keller *et al*., 2005) and likely a deficient health of individuals, which could also affect the resistance threshold of bees to other stress (pathogens or pesticides) (Naug, 2009). Acually, pollen intake is known for influencing the physiological metabolism (Alaux *et al.*, 2011), immunity (Alaux *et al.*, 2010), the tolerance to pathogens like bacteria (Rinderer *et al.*, 1974), virus (Degrandi-Hoffman, *et al.*, 2010) and microsporidia (Rinderer and Elliotl, 1977) reducing the sensitivity to pesticides (Wahl and Ulm , 1983).

Whilst, honeybee rarely face a total lack of pollen in their environment, but are rather challenged with variability in time and space of pollen resource nultitude, type and diversity. In addition, pollen can differ between floral species regarding their nutritional contents (Herbert and Shimanuki, 1978; Roulston and Cane, 2000; Odoux *et al*., 2012) insufflate that some are of better quality for bees than others. Thus, studying the action of pollen assimilation on bee health requires also taking into account the quality and diversity of pollen diets. Pay in addition, some studies showed that pollen quality can affect the longevity of bees and the hypopharyngeal glands development (Standifer, 1967). More recently, sugested that pollen diversity might improve some immune functions (Alaux *et al.,* 2010).

Insects' haemolymph play a very important role in transport and storage of nutrients and is crucial for the recognition and defense against microorganisms (Bogaerts *et al.,* 2009). The haemocytes can engulf and destroy smaller foreign objects such as bacteria or fungal spores, but larger parasites, bacterial clumps or fungal hyphae are encapsulated by several haemocytes and then removed from circulation (Gliński and Jarosz, 2001).

Thus, the objective of the current study aimed to study the role of pollen and/or bee bread on brood rearing and cellular immune system of honeybee, *Apis mellifera* L., colonies. Such information will be useful for improving honeybee colony status and development in Egypt especially during the pollen shortage periods.

MATERIALS AND METHODS

The present investigation was carried out in the apiary yard at Mousha village, Assiut district, Assiut Governorate, Upper Egypt, during the period extended from February, 2013 to January, 2014.

Experimental honeybee colonies:

Ten honeybee colonies of the Carniolan hybrid, *Apis mellifera carnica* Pollmann (Hymenoptera: Apidae), nearly in equal strength, contained sealed and unsealed brood, and stored food (honey and pollen and/or bee bread) in equal areas nearly. All colonies headed with sister mated queens.

Workers unsealed brood areas and stored pollen and/or bee bread:

These estimates were conducted every twelve days, using a standard frame divided into square inches. Monthly average of brood rearing was calculated (sq. inch /colony).

Total protein content:

To determine total protein content (%) of larvae and its haemolymph total protein (µg/µl haemolymph). Ten 6-days-old worker honeybee larvae were used per each colony. The biuret reagent method was used for protein determination according to Gornall *et al*, (1949). This process was repeated twice a month, and then monthly average of total protein was calculated.

Haemolymph collection:

Haemolymph samples were obtained from a small incision at the level of the 3rd dorsal tergite, using a fine hypodermic needle. Ten larvae of worker bees at 6-days-old were used. The haemolymph was put in fertilized microcapillary tubes at -20 C^{o} for later determination of protein content.

Haemolymph total soluble solids:

Total soluble solids % (TSS %) in haemolymph of 6-days-old larvae of worker bees, were determined by a hand refractometer, as described by Hussein (1978). Two readings of TSS% were carried out monthly, and then monthly average of TSS % was calculated.

Total haemocytes count:

Total haemocytes count (THC) cell/µl of haemolymph was determined in 6-days- old larvae of worker bees using Nauber's haemocytometer, as described by Amro (2009). THC was repeated twice a month, and then monthly average of THC (cell/µl) was calculated. To calculate THC (cell/µl haemolymph), formula of Predtetshensky *et al*, (1950) was used as follows:

$$\mathbf{THC} = \frac{\mathbf{a \times 4000 \times b}}{\mathbf{c}}$$

where:
THC = number of haemocytes in µl haemolymph;
a = number of haemocytes in 100 large squares;
b = haemolymph dilution;
c = number of small squares in 100 large squares.

Differential haemocytes count:

Differential haemocytes count (DHC), were determined for each larva and calculated as mean 20 randomly spots/slide (Zakaria, 2007).

Statistical analysis:

Data were analyzed and compared according to the method of Waller and Duncan (Waller and Duncan, 1969). Least significant difference (LSD) values at 0.05 probabilities were calculated using MSTAT-C software program (MSTAT-C, Michigan University, Version. 2. 10), and presented as mean ± SE (slandered error). Also, the relationships between stored pollen and/or bee bread from one side, and different studied criteria from the another side, were analyzed statistically using simple correlation.

RESULTS AND DISCUSSION

Data illustrated in figure (1) showed the monthly mean areas in, square inch/ colony, of stored pollen and/or bee bread. Statistically, data revealed that there are significant differences between pollen and/or bee bread areas for the various months.

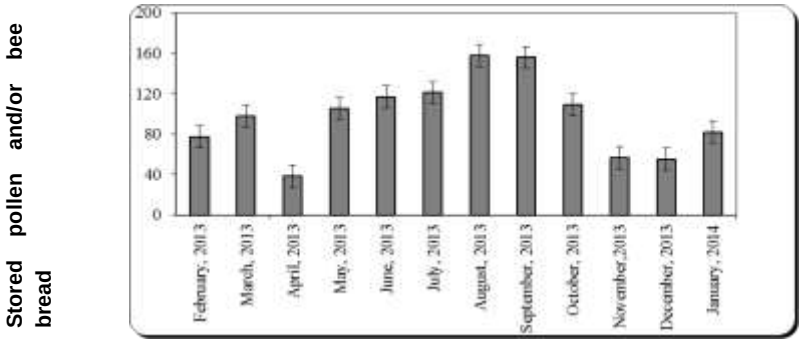

Figure 1: Monthly mean areas of stored pollen and/or bee bread (sq. inch/colony) during 2013/2014.

The maximum mean area of pollen and/or bee bread was recorded during August and September with an average 157.7±24.701 and 156.01±18.551 (sq. inch/colony), respectively. The minimum monthly mean area, 38.33±8.014 (sq. inch/colony), was noticed during April. The difference in the amount of stored pollen and/or bee bread can be attributable to the available pollen in the field, as well as, weather factors, colony requirements and strength. In a similar study in Assiut region, (Hussein *et al.*, 2005) recorded the peak of pollen stored during August, while, they found the lowest area during April.

Figure (2) exhibited the monthly mean area of worker unsealed brood in (sq. inch/colony). Variations between the monthly average areas of worker unsealed brood were significant. The obtained data showed that the highest

mean area was noticed during August with an average of 347.7±51.348 (sq. inch/colony). The lowest mean area was recorded during November and December with an average of 125.3±15.0 and 133.0±28.111 (sq. inch/colony), respectively.

In the approach, in Assiut region, (Hussein *et al.*, 2005) observed the highest and lowest mean brood areas, during May and November, respectively. Conversely, in Siwa Oasis, the results obtained by (Hagag, 2006) were distinctly different from those observed in Assiut. He recorded that the highest brood rearing activity was noticed during April and June, whereas, the lowest brood rearing was found during August, September and October.

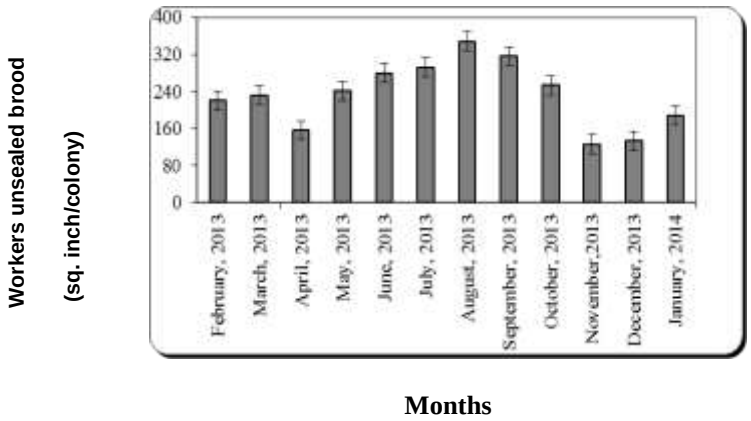

Figure 2: Monthly mean areas of workers unsealed brood (sq. inch/colony) during 2013/2014.

As shown in figure (4, a) highly significant and positive correlation was detected between stored pollen and/or bee bread and workers unsealed brood (correlation coefficient, r = 0.902**). The present results agree with the results of Campana and Moeller (1977) that reported that colonies that consume more bee bread are able to rear more bees. Horr, (1999) stated that food stored in the hive are the most critical part of early spring development. If there isn't sufficient amount of honey and pollen, development of brood will be very limited.

Data illustrated in figure (3) exhibited the monthly mean percentage of protein content in the 6-days-old larvae of workers. Variations between the monthly mean percentages of protein content were highly significant. The highest protein content percentage was recorded during May and January with an average of 43.2±9.12 % and 42.86±7.596 %, respectively. However, the lowest percentage, 22.03±6.325 % was noticed during April. Significant and positive correlation was observed between stored pollen and/or bee bread and total protein content of 6-days-old worker larvae (r = 0.393*) (figure 4, b).

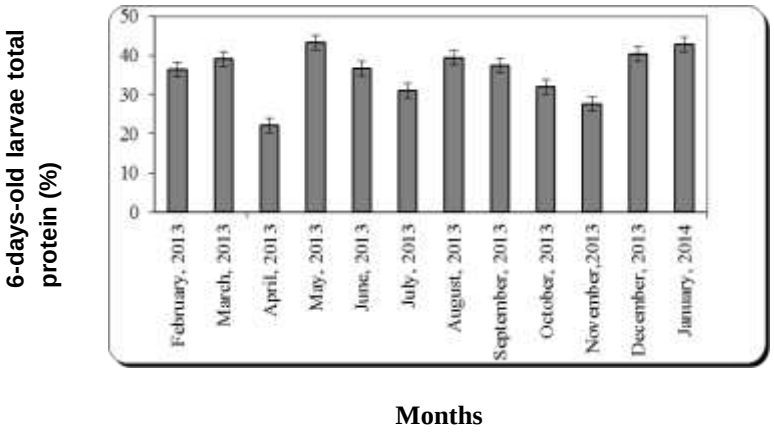

Figure 3: Monthly mean percentages of 6-days-old larval total protein during 2013/2014.

Obtained results can explain that the differences in honeybee protein content due to different amount of protein food that fed upon by those larvae or to different amount of pollen and/or bee bread available in the colony. Kunert and Crailsheim, (1988) found that, newly emerged bees deviations in weight and protein content were dependent on season and availability of food outside the hive. In general determination of food reserves enables to have better understanding about the responsibility of each constituent and its role in honeybee life.

Data in Table (1) revealed that, there were significant differences between the monthly mean of 6-days-old worker larvae haemolymph total protein (µg/µl). The highest total protein was noticed during January and May with an average of 7.5±1.264 and 7.31±1.37 µg/µl, respectively. On the other hand, the lowest total protein was observed during April with a mean of 4.04±0.48 µg/µl. The natural protein is an important factor in the functioning of the haemolymph cellular system of bees (Szymaś and Jędruszuk, 2003).

The correlation between stored pollen and/or bee bread and total protein content of 6-days-old worker larvae haemolymph (r = 0.345*) (figure 4, c). Variations in both larvae total protein and its haemolymph total protein can be attributable to variations in quantity and quality of pollen and/or bee bread.

Table 1: Monthly means of some physiological characters of 6-days-old larvae during 2013/2014.

Months	Six – days - old larvae Haemolymph							
				Differential of haemocyte count (cell/smear)				
	Protein (µg / µl)	TSS (%)	TH C (cell /µl)	Gr.	Pl.	Spl.	Mac.	Mic.
February, 2013	5.88 e ±1.16	11.45 e ±2.445	15240.4 e ± 2177.142	16.7 cde ±3.52	19.7 ab ±4.0	14.0 ef ±2.452	9.4 f ±1.89	6.3 d ±0.958
March, 2013	6.43 bcd ±1.089	12.34 b ±2.059	16070.1 b ±2365.082	17.7 cd ±4.211	19.0 ab ±3.145	15.3 cde ±2.696	13.0 de ±2.008	8.5 bcd ± 2.503
April, 2013	4.04 h ±0.48	9.98 g ±1.861	10890.0 h ±1965.658	32.0 a ±7.636	6.2 e ±1.56	5.0 h ±0.337	22.3 a ±4.112	14.3 a ±3.113
May, 2013	7.31 a ±1.37	13.32 a ±2.426	16660.5 a ±3245.636	11.6 f ±2.858	18.1 b ±3.112	16.3 bcde ±3.32	11.0 ef ±1.098	7.0 d ±1.685
June, 2013	6.36 cd ±0.891	11.95 cd ±1.09	15680.0 cd ±2681.07	16.5 cde ±4.108	20.0 ab ±2.048	17.3 bcd ±3.658	11.7 ef ±2.045	6.6 d ±1.119
July, 2013	5.73 e ±1.003	11.49 e ±2.045	15470.2 de ±2489.326	14.3 ef ±3.417	12.3 d ±1.235	11.3 g ±2.008	10.0 ef ±1.118	7.3 d ±1.684
August, 2013	6.53 bc ±1.922	12.2 bc ±1.964	15770.3 c ±3211.0	19.1 c ±5.006	20.0 ab ±4.368	18.3 ab ±3.666	12.0 ef ±2.15	7.3 d ±1.673
September, 2013	6.02 de ±0.764	11.74 d ±1.119	15370.3 e ±2225.52	16.3 e ±3.852	18.0 b ±4.71	12.0 fg ±1.568	16.0 c ±2.782	10.0 b ±2.408
October, 2013	5.27 f ±0.758	11.11 f ±1.832	14950.6 f ±2324.125	14.3 e ±3.114	17.0 bc ±4.362	15.0 de ±3.41	12.6 de ±2.406	8.7 bcd ±1.692
November, 2013	4.54 g ±0.755	10.9 f ±0.687	12990.7 g ±1968.826	25.0 b ±8.111	14.3 cd ±2.689	11.0 g ±2.504	19.3 b ±3.486	12.8 a ±1.985
December, 2013	6.8 b ±1.0	12.2 bc ±1.252	16070.1 b ±3245.0	19.2 c ±5.0	22.3 a ±5.923	17.7 abc ±5.601	15.3 cd ±2.489	9.6 bc ±1.635
January, 2014	7.5 a ±1.264	13.33 a ±2.651	16570.3 a ±3268.234	16.0 de ±4.771	19.1 ab ±3.667	20.0 a ±4.608	13.1 de ±2.777	7.7 cd ±1.069

Means followed by the same letters at the same column are not significant differences at 5 % level of probability.

Data presented in (Table 1) exhibited monthly mean percentages of total soluble solids (TSS %) in haemolymph of 6–days–old larvae. Statistical analysis showed that there were significant differences between TSS % values for various months. The highest TSS % values was recorded during January and May with an average 13.33±2.651 and 13.32±2.246 %, respectively. However, the lowest value, 9.98±1.861 %, was noticed during April. As shown as in figure (4, d) no significant correlation was found between stored pollen and/or bee bread and 6–days–old larvae haemolymph TSS % (r = 0.322).

Regarding the total haemocytes count (THC), cell/µl, significant differences were found between the various months (Table 1). The maximum monthly mean, 16660.5±3245.636 and 16570.3±3268.234 (cell/µl), was determined during May and January, respectively. While the minimum value, 10890.0±1965.658 (cell/µl). As shown as in figure (4, e) statistical analysis of the obtained data noticed that there was highly positive correlation between stored pollen and/or bee bread and THC (r = 0.496**).

Various pollen types have different nutritional value and don't have the same physiological effects according to floral sources (Lauveaux, 1963; Khodairy and Moustafa, 2008).

Data presented in (Table 1) showed clearly significant differences between mean numbers of some haemocytes in different months of 6–days–old larvae haemolymph. It is interested to note that most of haemocytes as granulocytes, macrocytes and microcytes were significantly decreased with pollen or stored bee bread increase. The highest number of granulocytes, 32.0±7.636, was found in larvae during April and the lowest number, 11.6±2.858, was found in that during May. The highest number of macrocyte, 22.3±4.112, was recorded in larvae during April and the lowest number, 9.4±1.89, was recorded in that during February. The highest number of microcytes, 14.3±3.113 and 12.8±1.692 was noticed during April and November, respectively and the lowest value, 6.3±0.958 was noticed during February.

The correlation between stored pollen and/or bee bread from one side and the previous haemocyte types on the outer side was highly negative correlation (r = -0.545**, - 0.435** and - 0.448**), respectively (figure 4, f-h).

Plasmatocytes and spindle shaped cells were statistically increased with stored pollen and/or bee bread increase. The highest number of plasmatocytes, 22.3±5.923, was observed in the larvae during December and the lowest number, 6.2±1.56, was observed in that during April. The highest number of spindle shape cells, 20.0±4.608, was found in larvae during January and the lowest value 5.0±1.56, was found in that during April.

The correlation between stored pollen and/or bee bread and plasmatocytes wasn't significant (r = 0.295), whereas this relation with spindle shape cells was significant and positive (r = 0.354*) (figure 4, i & j).

Zakaria, (2007) stated that, granulocyte and micronucleocyte cells play an important role in the immunity system and consider one of the main sign of the disease recognizer.

In conclusion, stored protein (pollen and/or bee bread) in honeybee colonies has affected the biology, brood rearing and the physiology, total protein content of larvae, total soluble solids of the haemolymph, total haemocytes counts and the differential haemocytes. In Assiut region, American foulbrood, a bacterial disease and chalkbrood, a fungal disease, were occurred during April in many apiaries. This observation are agreement with the obtained data where it was found that during April the minimum amount of stored pollen and/or bee bread and different counts of certain haemocytes according to its immunity function.

It is well known that protein level is always associated with health status of the bees and haemocytes count related to defense activity (Gliński and Jarosz, 2001). Protein deficiency also affects the ability of honeybees to resist disease (Matilla and Otis, 2006).

The fore mentioned results improved the important role of pollen and/or bee bread on the physiological status of honeybee. So, it turns out previously, it can be recommended for special attention protein nutrition artificially by providing nutrition supplements or pollen substitutes for bee colonies and that in periods of scarcity or lack of pollen as well as in the periods that are the disease spreads. In this process can reduce the chances of disease and prevention else to improve the immune system of bees device. This is in addition to reducing the loss incident in bee colonies as a result of starvation.

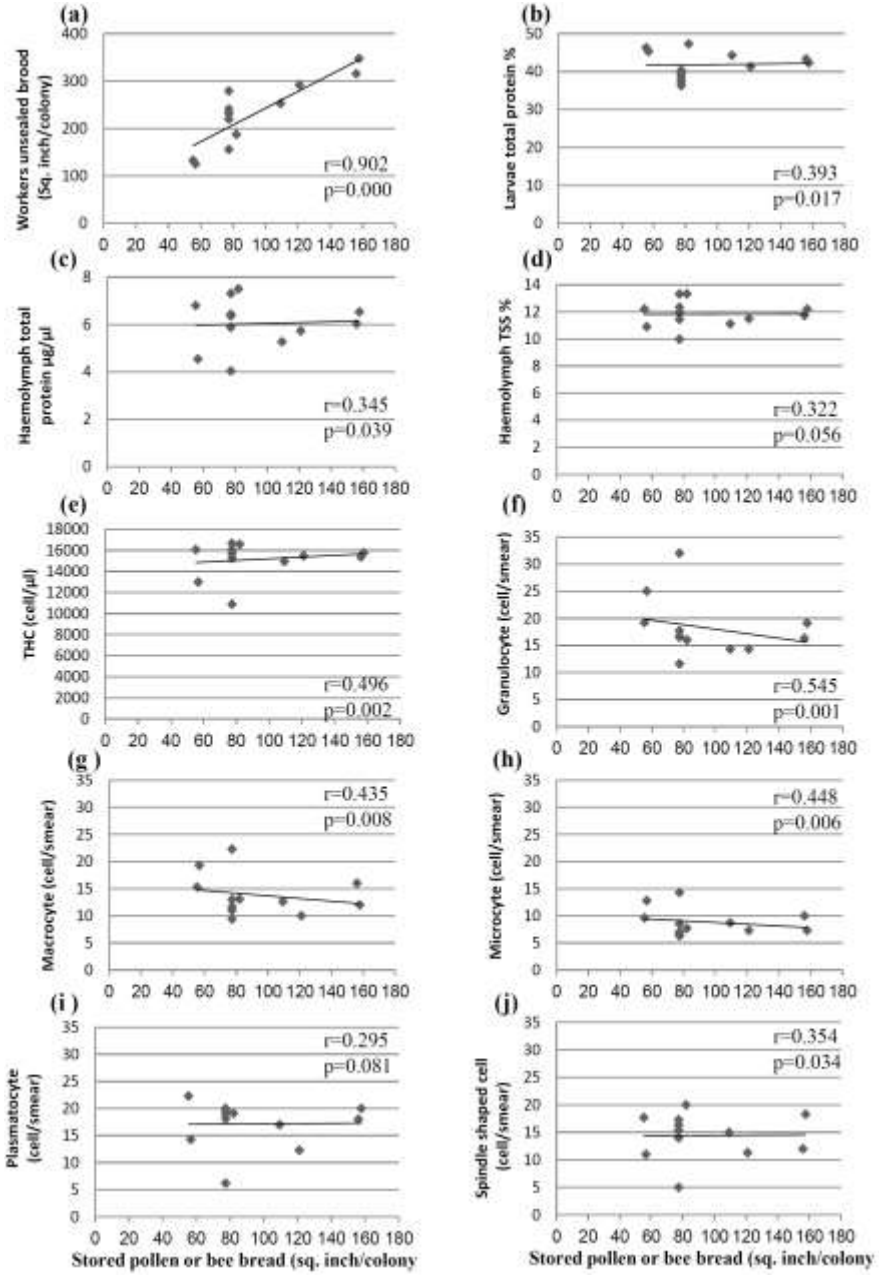

Figure 4.Correlations between stored pollen and/or bee bread and various studied characters; including both workers unsealed brood area and some physiological characters of 6-days-old larvae (r and p-values are shown).

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دور تـوافر حبـوب اللقـاح و/أو خبـز النحـل فـي تربيـة الحضـنة والجهـاز المنـاعي الخلوي لنحل العسل

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تم تقييم الدور الذي يلعبه توافر حبوب اللقاح و/أو خبز النحل في تربية الحضنة ومحتوى البروتين ودم يرقات الشغالات عمر ستة أيام. سجلت أكبر مساحة لحبوب اللقاح أو خبز النحل المخزنة ومساحة حضنة الشغالات المفتوحة خلال شهر أغسطس وذلك بمتوسط مساحات 157,7 ± 24,701 و 347,7 ± 51,348 بوصة مربعة / طائفة وذلك على التوالي. كان هناك إرتباط معنوي جدا وموجب بين مساحة حبوب اللقاح أو خبز النحل المخزنة وبين مساحة الحضنة المفتوحة للشغالات (معامل الإرتباط = 0,902**). أشارت الدراسات الفسيولوجية على يرقات الشغالات ودمها أن أدنى مستوى لمحتوى البروتين في كلا من اليرقات ودمها (22,03 ± 6,325 ٪ و 4,04 ± 0,48 ميكروجرام / ميكروليتر) على التوالي قد وجد خلال شهر أبريل. لوحظ أن هناك إرتباط معنوي وموجب بين مساحة حبوب اللقاح أو خبز النحل المخزنة من جانب وبين محتوى البروتين لكلا من اليرقات والدم من جانب آخر (معامل الإرتباط = 0,393 * و 0,345 *) على الترتيب. وجد أن أعلى نسبة مئوية للمواد الصلبة الذائبة الكلية وأكبر عدد لخلايا الدم الكلية كان خلال شهري مايو ويناير. من ناحية أخرى كان أقل متوسط لكلا من النسبة المئوية للماد الصلبة الذائبة الكلية والعدد الكلي لخلايا الدم قد لوحظ خلال شهر أبريل. تم العد التفريق لبعض أنواع خلايا الدم في سحبات الدم. وكانت هناك إختلافات معنوية في خلايا الدم المختلفة فيما بين الشهور المختلفة. لذا ومما سبق يمكن التوصية بزيادة الإهتمام بالتغذية الصناعية وخاصة البروتينية منها بتقديم مكملات أو بدائل حبوب اللقاح لطوائف نحل العسل وذلك في فترات ندرة أو عدم وجود حبوب اللقاح وكذلك في الفترات التي تنتشر فيها الأمراض.وبهذا يمكن أن نقلل من فرص حدوث الأمراض والوقاية منها وأيضا تحسين الجهاز المناعي للنحل. هذا بالإضافة إلى الحد من الفقد الحادث في طوائف النحل نتيجة للجوع.

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