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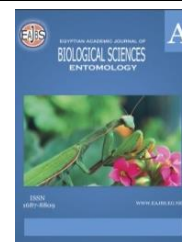
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**The Effect of An Alternative Diet Fermented by Bee Bread Microorganisms on Hypopharyngeal Glands Development and Acini Size of Honey Bee Workers, (*Apis mellifera* L.)**

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**ABSTRACT**

The hypopharyngeal glands of worker bees located in the head; consist of thousands of two-cell units that are composed of a secretory cell and a duct cell and that are arranged in sets of about 12 around a long collecting duct. The glands contribute to the production of the royal jelly fed to queens and larvae. They are highly sensitive to the quantity and quality of the food as pollen and pollen substitutes that the nurse bee consumes. The role of the worker honey bee *Apis mellifera* L. changes depending on age after eclosion (age polyethism): young workers (nurse bees) take care of their brood by synthesizing and secreting brood food (royal jelly), while older workers (foragers) forage for nectar and process it into honey.

In our experiment, we tested how diets impact hypopharyngeal gland development and their acini size, where our diets compared were (bee bread diet; unfermented diet; fermented diet in a simulation method for nature; and sucrose syrup). Also, we mentioned understanding the role of these glands in hive health. For this study, we have examined the morphogenesis of the hypopharyngeal gland during different ages of workers honeybee *Apis mellifera* L. that fed on the different diets; we measured the size of glandular acini in a robust measure. These results obtained indicated that the hypopharyngeal gland development has flexibility and can depending on the condition of the colony as the pollen substitute diet we prepared in the periods of food shortage in nature. This described the hypothesis that feeding plays an important role in the development of HG according to diet nutritional values, ensuring the importance of the fermentation process for the better health of honey bees.

**INTRODUCTION**

Special attention must be given to the role that food plays in the development of the hypopharyngeal glands (HG) (Wcislo and Cane 1996). HG of workers of *Apis mellifera* L. (Hymenoptera: Apidae) have been morphologically and physiologically studied. The anatomy and the cellular and subcellular organization have been studied extensively by use of various techniques, i.e., histology, electron microscopy, and fluorescence microscopy

(Heylen, *et al.*, 2011; Kheyri, *et al.*, 2013; & Richter, *et al.*, 2016). In particular, several studies have addressed the structural changes that occur in the hypopharyngeal gland as worker bees age and/or adopt other tasks (Knecht and Kaatz, 1990 & Richter, *et al.*, 2016). Studied due to their importance on the production of royal jelly (Gatehouse *et al.* 2004; Seehuus *et al.* 2007; & Cruz-Landim 2009). However, few studies have evaluated the development of glands in honeybees as a function of the diet offered to them.

The HG, located inside the worker's head, produces a protein-based substance that is responsible for the differentiation among castes and is also used to feed young larvae, drones and the queen (Feng *et al.*, 2009 & Kamakura 2011). Age polytheism play an important role in HG development, in particular, the hypopharyngeal gland in worker bees has a developmental cycle closely related to the division of labor, young honeybees that function as nurses have active HG with large acini, while older honeybees that present foraging activity (Winston, 1987; Knecht and Kaatz, 1990; & Deseyn and Billen, 2005) Protein sources are extremely important to physiological development, especially to the young workers, as pollen is the main source of protein for their development (Zahra and Talal 2008). Apparently, the activation of the HG will occur with the presence of a protein-based food, (Al-Ghamdi *et al.*, 2011b). During periods of the year characterized by pollen scarcity, other food sources rich in protein such as soybean extract and milk powder might be used to feed the hives (Zahra and Talal 2008; DeGrandi-Hoffman *et al.*, 2010; & Al-Ghamdi *et al.*, 2011a). However, despite those diets' high protein contents, their protein types may not be digested or absorbed by the bees, resulting in a negative impact on the overall development of the hive (Pernaland Currie 2000).

Several factors should be considered regarding the types of food offered to the honeybees, being that food characteristics are fundamental to acceptance, palatability, consumption and ingestion of the diet. In order to develop artificial protein diets that are nutritious and attractive to bees, it would make sense to make them as similar as possible to their natural proteinaceous food in the hive, beebread. Protein levels in the diet affect the resulting protein levels in honey bee hemolymph as vitellogenin (Basualdo *et al.*, 2013). Honey bees also ferment pollen to preserve it from harmful microorganisms (Herbert and Shimanuki, 1978; Vasquez and Olofsson, 2009; & Almeida-Dias *et al.*, 2018).

The size of HG is correlated with glandular production and generally increases with age from 6 to 18 days in nurse bees (Deseyn and Billen, 2005). Gland acinus size (Hrassnigg and Crailsheim, 1998; Ohashi, *et al.*, 2000; Deseyn and Billen, 2005; & Feng and Fang, 2009), or protein content (Knecht and Kaatz, 1990 & Heylen *et al.*, 2017) of the entire head where they are located. Each method has its own pros and cons. We prefer the resolution obtained from measuring the gland acini, though this method can be obtaining an accurate measure of each acinus. Under a dissecting light microscope, the glands appear clear and the micrometer we used to offer researchers an easy, accurate, and replicable method for achieving multiple glands.

## MATERIALS AND METHODS

The experiments were carried out in Assiut, Insect Research Laboratory, Plant Protection Research Institute. The experiment was conducted with the first hybrid of Carniolan honey bee, *Apis mellifera carnica* Pollmann workers in October 2019, after we collected all the available types of bee bread from hives that were placed in different regions in Assiut governorate at the successive seasons.

### **Inoculum Preparation for Fermentation:**

The inoculum was developed and prepared in our lab from a mixture of all the available types of bee bread that we collected (Clover, *Trifolium alexandrinum*; Maize, *Zea*

mays; Bean, *Vicia faba*; Fennel, *Foeniculum vulgare*; Anise, *Pimpinella Anisum*).

All the glassware and mixing implements were sterilized with 70% ethanol prior to inoculum preparation. After pooling and mixing, 10 g of the mixture of the collected bee bread was added to 300 ml of previously boiled sucrose syrup (50% w/v). This mixture was manually homogenized, was put in 250 ml - colored bottles, and placed in an incubator at 35 °C and controlled relative humidity (70%) for 25 days.

In order to release the CO<sub>2</sub> produced during fermentation, the bottles were briefly opened every 48 h. After the end of this fermentation period, the bottles were sealed and stored at 6–8 °C for up to 20 days. A new inoculum was prepared from freshly collected beebread every 20 days to help reduce contamination with opportunistic fungi and other microorganisms. (Almeida-Dias, JM. *et al.*, 2018).

#### **Diets Preparation:**

**We Firstly Prepared the Unfermented Diet:** 4.5-part powdered sugar, 3-part powdered soy meal, 1-part powdered yeast, 0.5-part powdered milk; sufficient previously boiled water to make a paste; (Mostafa, 2000) and linen oil as an attractive smell.

**Then We Prepared the Fermented Diets:** The same ingredients as the unfermented diet, with 40 ml fermented inoculum for each kilogram of the paste added and mixed well. Then we stored them in an incubator in a loosely covered plastic food-grade container at 35°C, for 28-days.

#### **Preparation of Bee Cages and Bioassay Protocol:**

Experimental wooden cages were prepared for the experiment, every cage (15 × 15 × 5) Cm dimensions with a glass side and other was covered with black muslin, was provided with a vial of tap water and another vial of sugar solution 1:1 (w:v), food source and pieces of the wax foundation were attached to the cage side. We prepared four groups of cages. Newly emerged workers aged 0 - 12 hours were confined in the cages; each cage containing 100 newly emerged bees; five replicates were used for each group. The cages were held also in a dark incubator at 32°C ±1 and 70% RH. (Fig-1).

The cages were continuously supplied with water, sucrose solution and food source, they were divided into four groups depending on the food source they were introduced as follows:

- Group < 1 >, cages contained bee bread diet as a control: (A).
- Group < 2 >, cages contained fermented diet (mixture 28-days): (B).
- Group < 3 >, cages contained unfermented diet: (C).
- Group < 4 >, cages contained only sucrose solution 1:1 (w: v): (D).



**Fig. 1:** Experimental wooden cages

To study the hypopharyngeal glands (HG) development, five bee workers were removed from each cage every three days. This procedure was repeated six times at three-day intervals. The head of each worker was dissected under the stereo-microscope at 40 x

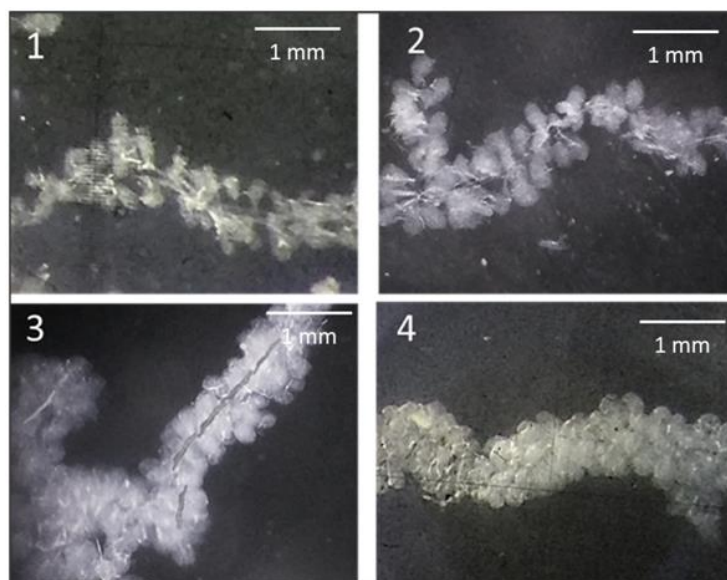
magnification force and their hypopharyngeal glands were pulled and removed outside the head using modified blades and then transferred into insect saline solution on a glass slide to determine the degree of (HG) development. The degree of (HG) development was determined according to (Maurizio, 1954). An arbitrary scale (1 to 4) was used to determine the degree of development; grade 1, represented undeveloped gland and grade 4, represented complete development. (Fig. 2 & 3). Measurements of acini size were made using a micrometer, gland diameters were measured (width and length) under light microscopy. The maximum length (L) and width (W) of ten acini per slide were taken for each individual bee (Al-Ghamdi *et al.* 2011a& 2011b); only acini with clear borders were measured. The acinal surface area (SA) was calculated using the following formula (Maurizio, 1954):

$$\text{Acinal surface area} = \pi \times ((a \times b) / 2)$$

where a = maximum length, b = maximum width, and  $\pi = 3.14$ .



**Fig. 2:** determination of the development degree of (HG)



**Fig. 3:** Hypopharyngeal glands development in worker bees is categorized according to Maurizio's (1954), where (1) grade 1, (2) grade 2, (3) grade 3 and (4) grade 4.

#### **Statistical Analysis:**

Statistical differences between hypopharyngeal mean gland size were compared using ANOVA and Duncan's multiple range test (DMRT). Hypopharyngeal gland sizes (mean  $\pm$  S.E.) were calculated by SPSS Test (version 17.0.2).

## RESULTS AND DISCUSSION

Since bee bread, which is pollen fermented by the bees, is the main food of the worker-nurse bees that feed and care for the bee larvae, pollen substitutes should have similar attributes. In an attempt to simulate this natural food source, an inoculum prepared from bee bread was used to ferment a pollen-substitute diet. After preparing inoculum for fermentation and diets Preparation (unfermented & fermented); we tested the effect of these diets on the development of the hypopharyngeal glands and measuring their acini size at the different ages of the workers that fed on the different diets.

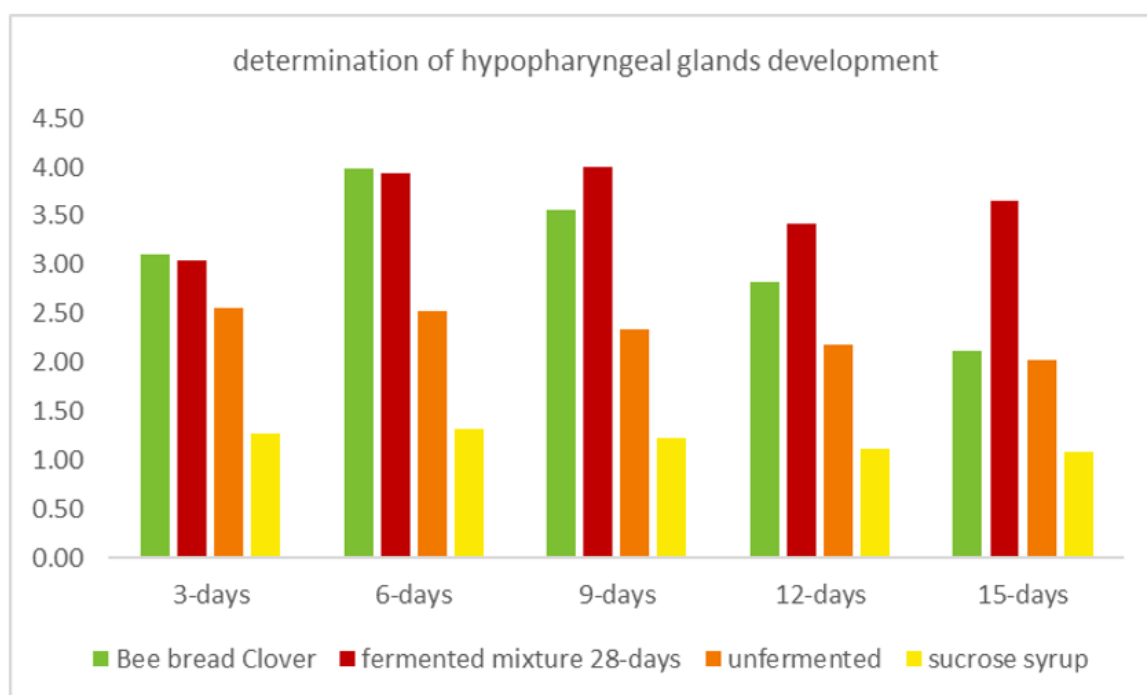
The development of hypopharyngeal glands (HG) of worker bees fed on different diets is shown in (Table.1 and Figs.-4–A, B & C). The results clearly showed significant differences in the development of HG grand means between the workers in the cages fed on sucrose syrup only, (1.20) and those fed on the other diets, (3.12; 3.61 & 2.32) for the workers fed on the bee bread diet; fermented diet and unfermented diet, respectively. It was noticed that HG development decreased at 15- days old for all diets, except for the fermented diet, whereas HG developmental degree was the lowest at 3- days old and increased at 6- days and the development continue insignificant increase till the end of measurement at 15- days old of the worker's age.

The full degree of HG development appeared in the bee bread diet at (6- & 9- days old) of the workers, they were (3.98 & 3.56), respectively; and in the fermented diet at (6-; 9- 15- days old), they were (3.94; 4 & 3.66), respectively.

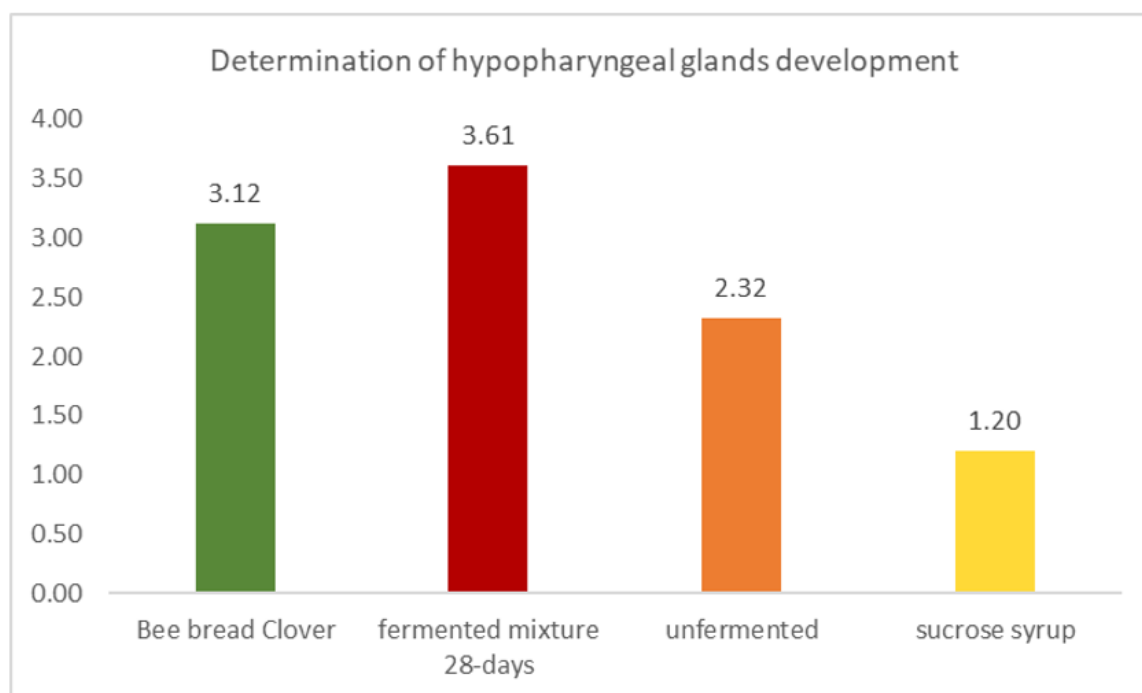
**Table-1:** determination of hypopharyngeal glands development.

Age	3 days	6 days	9 days	12 days	15 days	Grand mean
Control Bee bread Clover	3.30	4.00	3.50	2.70	2.20	
	3.10	4.00	3.70	3.00	2.00	
	3.10	4.00	3.50	2.70	2.30	
	3.00	3.90	3.50	2.80	2.00	
	3.00	4.00	3.60	2.90	2.10	
	<b>3.10</b> <b>±0.1225 AB</b>	<b>3.98</b> <b>±0.0447A</b>	<b>3.56</b> <b>±0.0894A</b>	<b>2.82</b> <b>±0.1304AB</b>	<b>2.12</b> <b>±0.1304B</b>	<b>3.12 A</b>
Fermented Mixture 28- days	3.20	3.90	4.00	2.60	3.70	
	3.00	3.80	4.00	3.90	3.60	
	3.10	4.00	4.00	3.50	3.90	
	2.80	4.00	4.00	3.50	3.50	
	3.10	4.00	4.00	3.60	3.60	
	<b>3.04</b> <b>±0.1517 AB</b>	<b>3.94</b> <b>±0.0894 A</b>	<b>4.00</b> <b>±0.0000 A</b>	<b>3.42</b> <b>±0.4868 AB</b>	<b>3.66</b> <b>±0.1517 A</b>	<b>3.61 A</b>
Unfermented	2.70	2.80	2.10	2.00	2.00	
	2.70	2.50	2.70	2.20	2.10	
	2.50	2.30	2.60	2.20	2.00	
	2.70	2.50	2.20	2.30	2.00	
	2.20	2.50	2.10	2.20	2.00	
	<b>2.56</b> <b>±0.2191 B</b>	<b>2.52</b> <b>±0.1789B</b>	<b>2.34</b> <b>±0.2881B</b>	<b>2.18</b> <b>±0.1095 B</b>	<b>2.02</b> <b>±0.0447B</b>	<b>2.32 B</b>
Sucrose syrup	1.22	1.32	1.24	1.00	1.10	
	1.32	1.33	1.20	1.18	1.09	
	1.20	1.30	1.22	1.15	1.09	
	1.40	1.28	1.20	1.15	1.07	
	1.23	1.35	1.25	1.08	1.05	
	<b>1.27</b> <b>±0.0841 C</b>	<b>1.32</b> <b>±0.0270 C</b>	<b>1.22</b> <b>±0.0228 C</b>	<b>1.11</b> <b>±0.0726 C</b>	<b>1.08</b> <b>±0.0200 C</b>	<b>1.20 C</b>

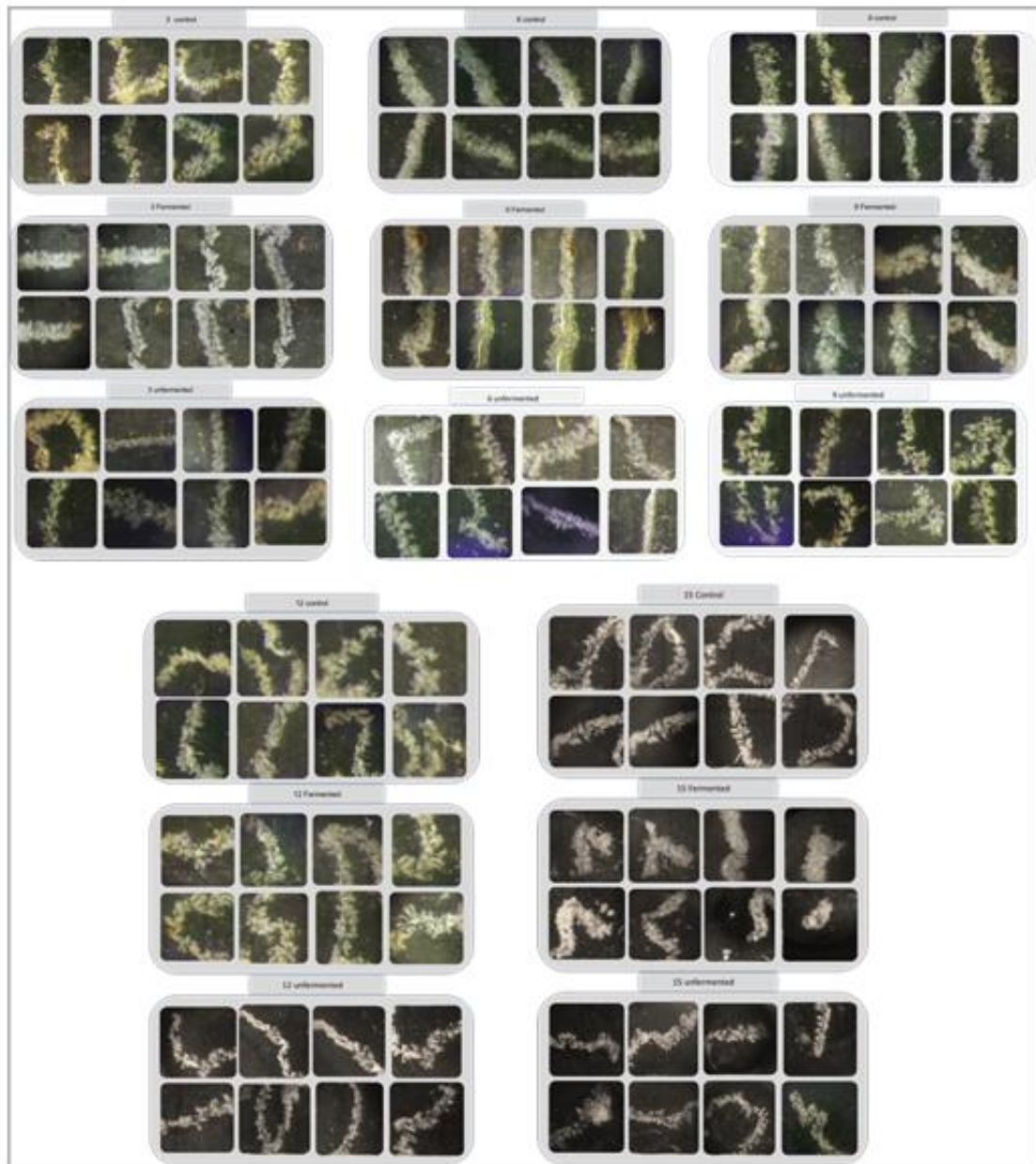
(Each number is an average of multiple replicates)



**Fig.-4-A:** determination of hypopharyngeal glands development



**Fig.-4-B:** determination of hypopharyngeal glands development



**Fig.-4-C:** determination of hypopharyngeal glands development

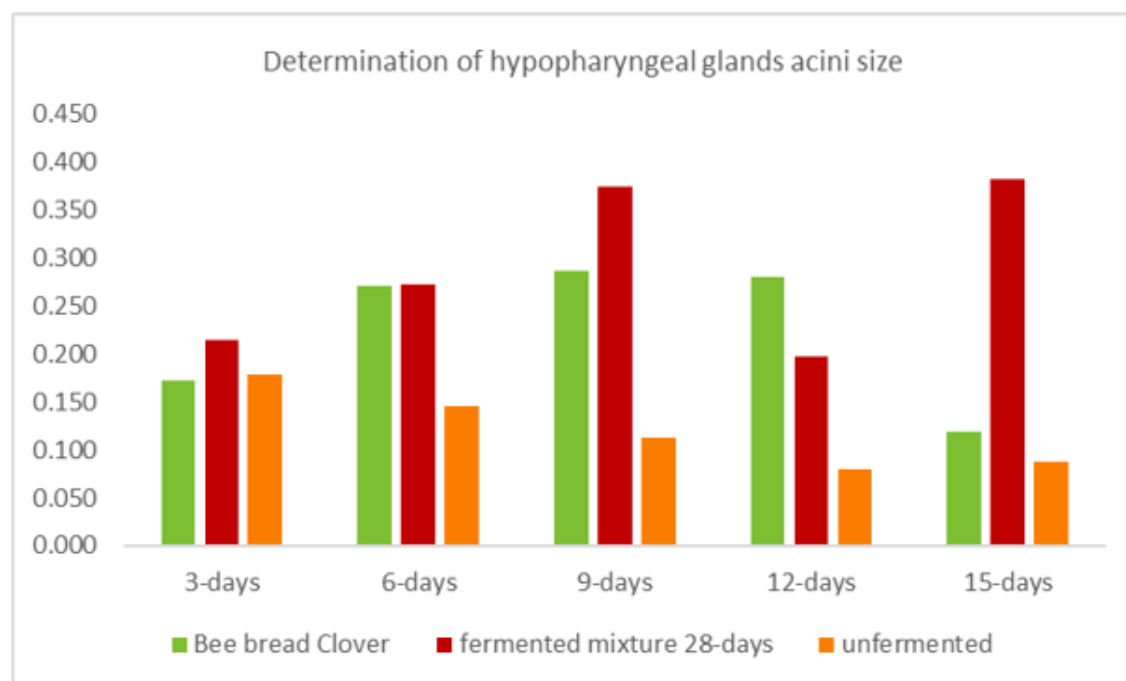
The type of diet offered to the workers affected on mean acini area values. The Smallest acini were observed for honey bees fed with a sucrose syrup, they were the same tiny acini size for all worker's ages, followed by honey bees fed with an unfermented diet ( $0.080$  &  $0.087 \mu\text{m}^2$ ). Mean acini areas of workers fed with bee bread and fermented diets aren't significantly different they are high area ( $0.226$  &  $0.288 \mu\text{m}^2$ ), respectively. Whereas the acini area was significantly lower in workers fed an unfermented diet ( $0.121 \mu\text{m}^2$ ). (Table-2 and Fig.- 5-A; B & C).

The age of the workers also had a significant effect on acini areas. After 15-days of exposure, acini were not statistically different. However, it was observed that the general morphology of the acini for all diet treatments presented a poor development when compared to acini extracted from HG of nursing bees aging from 6 to 9 days. Where the largest area of acini appeared at (6-; 9-; 12- days old) in the case of the bee bread diet ( $0.271$ ;  $0.287$   $0.280 \mu\text{m}^2$ ), respectively; and appeared at (6-; 9-; 15- days old) in the fermented diet ( $0.273$ ;  $0.374$  &  $0.383 \mu\text{m}^2$ ), respectively.



**Table-2:** determination of hypopharyngeal glands acini size

Age	3 days			6 days			9 days			12 days			15 days			Grand mean
	L	W	area	L	W	Area	L	W	area	L	W	area	L	W	area	
control	0.328	0.264	0.136	0.448	0.408	0.287	0.576	0.424	0.383	0.488	0.344	0.264	0.440	0.184	0.127	
	0.424	0.296	0.197	0.432	0.384	0.260	0.472	0.336	0.249	0.496	0.352	0.274	0.464	0.200	0.146	
	0.360	0.264	0.149	0.464	0.408	0.297	0.584	0.392	0.359	0.520	0.336	0.274	0.464	0.176	0.128	
	0.352	0.328	0.181	0.456	0.384	0.275	0.488	0.352	0.270	0.512	0.352	0.283	0.424	0.136	0.091	
	0.432	0.296	0.201	0.392	0.384	0.236	0.464	0.240	0.175	0.536	0.360	0.303	0.432	0.152	0.103	
	0.173 ±0.0290 AB			0.271 ±0.0239 A			0.287 ±0.0847 A			0.280 ±0.0147 A			0.119 ± 0.0219 B			0.226 A
fermented	0.368	0.352	0.203	0.440	0.352	0.243	0.592	0.464	0.431	0.464	0.264	0.192	0.544	0.440	0.376	
	0.408	0.376	0.241	0.448	0.384	0.270	0.592	0.472	0.439	0.472	0.264	0.196	0.592	0.448	0.416	
	0.408	0.368	0.236	0.464	0.416	0.303	0.568	0.488	0.435	0.456	0.224	0.160	0.528	0.440	0.365	
	0.336	0.320	0.169	0.408	0.392	0.251	0.496	0.360	0.280	0.472	0.288	0.213	0.568	0.432	0.385	
	0.392	0.368	0.226	0.456	0.416	0.298	0.472	0.384	0.285	0.48	0.296	0.223	0.520	0.456	0.372	
	0.215 ±0.0296 AB			0.273 ±0.0270 A			0.374 ±0.0836 A			0.197 ±0.0241 AB			0.383 ±0.0199 A			0.288 A
Unfermented	0.344	0.320	0.173	0.464	0.248	0.181	0.464	0.128	0.093	0.376	0.136	0.080	0.344	0.160	0.086	
	0.368	0.312	0.180	0.424	0.224	0.149	0.456	0.144	0.103	0.368	0.120	0.069	0.344	0.184	0.099	
	0.392	0.304	0.187	0.448	0.208	0.146	0.488	0.184	0.141	0.400	0.152	0.095	0.336	0.144	0.076	
	0.352	0.296	0.164	0.424	0.192	0.128	0.464	0.160	0.117	0.352	0.144	0.080	0.360	0.128	0.072	
	0.368	0.320	0.185	0.432	0.176	0.119	0.496	0.144	0.112	0.352	0.136	0.075	0.368	0.176	0.102	
	0.178 ±0.0094 AB			0.145 ±0.0238 B			0.113 ±0.0180 B			0.080 ±0.0096 B			0.087 ±0.0134 B			0.121 B

**Fig. 5-A:** determination of hypopharyngeal glands acini size

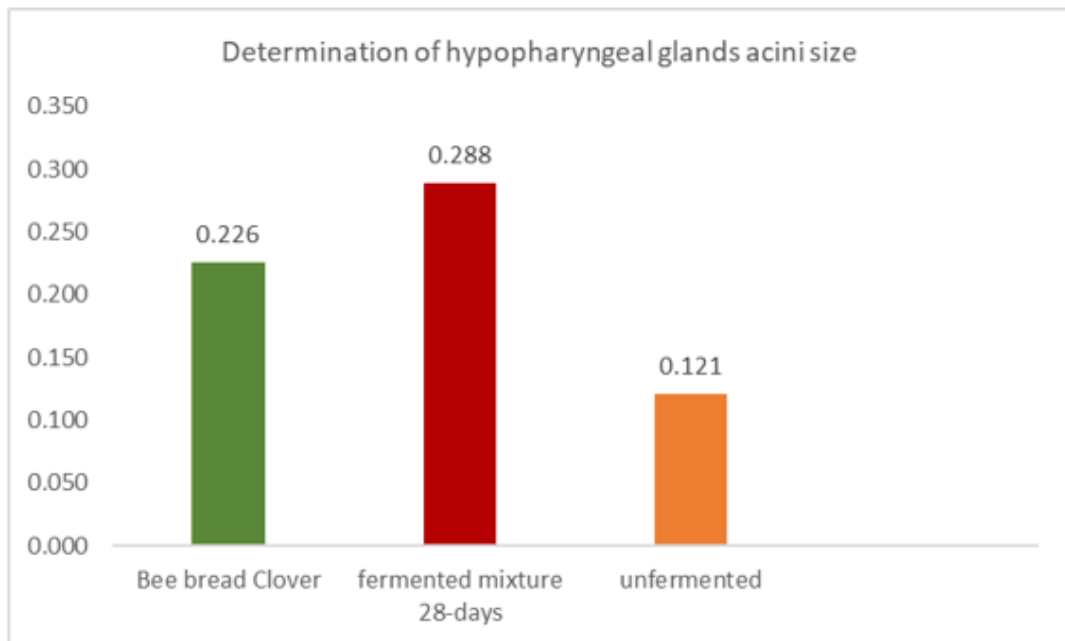


Fig. 5-B: determination of hypopharyngeal glands acini size.



Fig.5-C: determination of hypopharyngeal glands acini size

The results obtained from this study showed significant differences among treatments, where the fermented diets show the highest development of the HG and the largest acini compared to the unfermented diets.

The secretion produced by the HG is the main constituent of the royal jelly, a substance rich in protein and other nutrients that feed young larvae, helps in caste differentiation due to its morphogenetic properties and is also the exclusive nutrient source for the queen (Michener 2007&Kamakura 2011). However, the development or activation of this structure is linked to some factors such as protein availability and quantitative or qualitative variations of this resource, food palatability and absorption are factors that should be taken into account when providing honeybees with supplemental diets. (Al-Ghamdi *et al.* 2011a).

The results obtained from this study demonstrated that the type of diet does affect the development of the workers'glands, as the best results were observed for the group fed on the fermented diets. Protein availability affected the size of acini, as the fermentation process increased the protein content. groups of honeybees fed with protein supplements

presented acini with greater areas as compared to groups that were fed exclusively with pollen and/or honey (DeGrandi-Hoffman *et al.* 2010).

Fermentation affected the stability and the digestibility of bee-collected pollen. (Raffaella, *et al.*, 2019) where they mentioned that these changes in nutritional composition are a result of the metabolic activity of the microflora that is present in stored pollen. Although a large microbial diversity characterized flowers and fresh pollen, most lactic acid bacteria species disappeared throughout the bee bread maturation, giving way to *Lactobacillus* to dominate long-stored bee bread and honeybee crop. Bee bread preservation seemed related to bacteria metabolites, produced especially by some *Lactobacillus* strains, which likely gave lactic acid bacteria the capacity to outcompete other microbial groups. A protocol to fermentation, the fermentation process increased the digestibility and bioavailability of nutrients and bioactive compounds naturally occurring. These beneficial enzymes and bacteria create a protein-rich substance from the mixture. Bee Bread has a higher vitamin content, is less acidic, and has lower amounts of complex polysaccharides (which are hard to eat) than stored Pollen.

Colony microbiotas differed substantially between sampling environments and were dominated by several anaerobic bacterial genera never before associated with honey bees, but renowned for their use by humans to ferment food. (Heather *et al.*, 2012).

This variation, just like beer and other fermented foods humans eat, can have a huge variety in recipe, flavor and change in nutrition over time because the bees ferment it in order to preserve it. As we understand fermentation, it adds significant nutrition to the food we eat. Bees process and increase the nutritional value of the flower pollen through a fermentation process of their own. In addition to preservation, the fermentation process of the pollen also renders its nutrients more available. Some proteins are broken down into amino acids, starches are metabolized into simple sugars, and vitamins become more bio-available. In this sense, bee bread is even more health-giving than the more commonly available fresh bee pollen, (Evans, 2015).

Zahra and Talal (2008), observed that the effect of supplemental feeding in hives of *Apis mellifera* promoted an increase in mean acinus size and HG duct length.

The workers that were fed with a sucrose solution only presented the smallest acini area values, this is due to the lack of protein resources for the maintenance of the gland activity. This outcome is in agreement with what was presented by (Pernal and Currie, 2000), who did not observe differences in the amount of protein secretion from glands of honeybees fed on sources of inferior nutritional values.

Hypopharyngeal gland development is also directly related to the age of the workers, and this fact is due to physiological changes triggered by hormones mediated by biogenic amines as the honey bees get older (Huang *et al.*, 1989). These physiological changes cause the young workers of *A. mellifera* not to present active or developed HG right after the bee's emergence so that they will perform tasks as cleaning the hive; afterward, when they reach 6 to 12 days of age, the glands reach their full development, and the bees will then act as nurses (Otto 1955&Huang *et al.*, 1989).

A change in age-dependent roles (Age polyethism) is one of the most characteristic features of the honey bee *Apis mellifera* L. society. Young workers (nurse bees, usually younger than 13 days after eclosion) take care of their brood by synthesizing and secreting brood food (royal jelly), while older workers (foragers, usually older than 18 days) forage for nectar and process it into honey by converting sucrose to glucose and fructose (Lindauer, 1952; Sakagami, 1953; &Winston, 1987).

In parallel with this age-dependent shift in the roles, physiological changes occur in certain organs of the worker. For instance, the hypopharyngeal gland, which synthesizes

brood food (Halberstadt, 1980 & Knecht and Kaatz, 1990), is well developed in the nurse bee, whereas it shrinks in the forager and develops the enzymatic activity to hydrolyze sucrose (Simpson *et al.*, 1968 & Sasagawa *et al.*, 1989).

Previously, identification of three major proteins from the hypopharyngeal gland of nurse bees, that are synthesized as brood food proteins (50-, 56- and 64-kDa proteins), and one major protein,  $\alpha$ -glucosidase, from the forager-bee gland as an  $\alpha$ -glucosidase (Kubo *et al.*, 1996; Ohashi *et al.*, 1996; & Ohashi *et al.*, 1997).

Additionally, glandular sizes were the greatest in nurse bees. Hypopharyngeal glandular size is known to be positively correlated with gland activity and is influenced by larval feeding (Free, 1961; Crailsheim and Stolberg, 1989; Hrassnigg and Crailsheim, 1998; & Ohashi, *et al.*, 2000). These glands gradually decrease in size when honeybees become guards, cease feeding, and begin defending the colony (Deseyn and Billen, 2005).

Hypopharyngeal gland size is sensitive to the amount of protein in the diet and is a critical marker of nourishment in young adult bees.

Vitellogenin that is one of the most important proteins in honey bee hemolymph, increased when the workers fed on fermented diets, (Almeida-Dias, JM. *et al.*, 2018). They mentioned the data show considerable variability in protein content in the hemolymph of bees fed on different diets even though consumption was similar, the density of vitellogenin bands of hemolymph from bees fed the fermented diet was significantly greater compared to that from bees fed on an unfermented diet. The levels were similar to and not significantly different when compared to bee bread-fed bees. The comparison of Vitellogenin protein band densities for the most common bands demonstrated that the protein concentrations differ significantly from each other according to the different diets and the honey bee age.

vitellogenin has several functions, it serves as a precursor to brood food proteins secreted by the hypopharyngeal glands of worker bees. Amdam and Omholt (2003), theorized that vitellogenin does, in fact, serve a specific biological function in worker bees, and they adopted the hypothesis abandoned by Rutz and Lüscher that vitellogenin acts as an amino acid donor to royal jelly. They showed that much of the contemporary empirical data regarding correlations between worker behavioral ontogeny and changing vitellogenin titers and synthesis rates could be explained if vitellogenin was indeed an amino acid donor to jelly.

The import of vitellogenin into cells and tissues has previously been described as a receptor-mediated process (Dhadialla *et al.*, 1998 & Sappington *et al.*, 1995). As a first step toward understanding worker vitellogenin function, therefore, Amdam *et al.* (2003a) tested for the presence of a vitellogenin receptor protein in the membranes of the workers' hypopharyngeal glands. The result confirmed that the hypopharyngeal glands contained a single vitellogenin receptor protein with an apparent molecular weight of ~205 kDa.

Vitellogenin governs a variety of physiological aspects including development, behavior, life span and immunity (Amdam *et al.*, 2004; Corona *et al.*, 2007; Nelson *et al.*, 2007; Münch *et al.*, 2008; & Peso *et al.*, 2016), and is considered to be a general marker for honey bee health (Amdam *et al.*, 2003 & Dainat *et al.*, 2012).

This outcome may be valuable in the development of supplemental diets for hives during periods of natural food scarcity. In order to reduce the costs, diets that are fermented with bee bread microorganisms showed a great effect on the development of the hypopharyngeal glands and

should be tested in future studies to evaluate the development of the HG in the hive and other structures such as the mandibular glands. Providing the hives with an adequate amount of fermented diet might also act as a means to prevent the occurrence of pathogens

and epizootics such as CCD, which has been the cause of millions in losses to the apicultural worldwide. Further, to demonstrate the functional significance of the protocol, it was subsequently to identify vitellogenin protein in the hypopharyngeal glands of nursing worker bees and the role of vitellogenin in these tissues supports previously hypothesized roles of vitellogenin in social behavior. This protocol thus provides deeper insights into the functions of vitellogenin in the development of the hypopharyngeal glands of the honeybee.

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