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Effect of Sublethal Concentrations of Some Pesticides on Physiological Characters of Honeybee Workers Under Laboratory Conditions

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ABSTRACT

Several pesticides may be directly responsible for reducing the performance and losses of honeybee colonies. The action of feeding a nursing honeybee worker sugar solution with a sublethal concentration of seven pesticide groups was evaluated under laboratory conditions. Various physiological characteristics of nursing honeybees including food consumption, development of food glands, body water content, and food reserves were considered as indications for the functional status of nursing honeybee workers. Food consumption rate was significantly reduced in all treatments except two treatments, chlorpyrifos, and thiophanate-methyl. Also, the development of hypopharyngeal glands was significantly reduced in all treatments the reduction ranged between 31 to 55.62%. At the end of the nursing period, the state of metabolism as a food reservoir was determined. A maximum decrease in adult water content was recorded under the effect of lambada-cyhalothrine pesticide treatment without any significant difference in all treatments. Otherwise, the protein content of adults significantly decreased in all treatments except with lambada-cyhalothrine pesticide treatment. Fat content significantly increased in all treatments. The present data reflects the negative effects of sublethal concentration after dietary oral application of certain pesticide groups during the nursing period of the honeybee.

INTRODUCTION

Honeybees are the most economically valuable pollinator worldwide (Klein *et al*., 2007; Petts *et al*., 2010). Honeybees also produce several products which are used in the food and cosmetics and pharmaceutical industries. In certain areas, the honeybee populations decline has significant negative implications for plant pollination and there for limitation of crop yield (Aizen *et al*., 2009; Neumann and Carreck, 2010). The physiological condition of worker honeybees varies according to their life span, temporal differentiation, and seasonality (Amdam & Omholt, 2002; Amdam & Seehuu, 2006). Various physiological impacts on honeybee workers such as effects on survival, food consumption change in body weight and development of food glands would offer a general idea of the physiological status of the bee. Nursing adults require protein and energy for their growth and development that are contained in nectar and pollen (Babendreier *et al*., 2004).

Hypopharyngeal glands (HPGs) are the most important organ of honeybees which play a critical role in honeybee physiology. The size of HPGs reflects their functional status (Deseyn & Billen, 2005). With antiseptic and antimicrobial properties which are added to honey (Hrassnigg & Crailsheim, 1998). Development of HPGs varies depending on the age of the bees (Snodgrass, 1956; Dade, 1962; Škerl & Gregorc, 2010). Also, their development is adversely affected by multiple biotic and abiotic stress factors (Al Ghamdi *et al*., 2011; Wang *et al*., 2018; Ali *et al*., 2019; Gorby-Harris *et al*., 2019). Also, the development of HPGs was significantly affected by the application of pesticides (Hatjna *et al*., 2013 and Faita *et al*., 2018). The action of pesticides that are used in crops and in the hive reduces the size of the glandular acini awing to cell death in these structures (Alaux *et al*., 2010 and Hatjina *et al*., 2013). Several pesticides may be directly responsible for colony losses, and they can potentially reduce the performance of honeybees (Pettis *et al*., 2013).

Several pesticides negatively affected the morphology, histology, and physiology of honeybee workers. Genna *et al*. (2020) reported that the use of pesticides is a factor in reducing bee health. Nurse bees that remain in a hive for 2-3 weeks prior to two onsets for foraging perform a number of important tasks during their development including feeding young larvae and queens (Winston, 1987). Pesticides use in nearby agricultural areas has a significant impact on pollen and nectar production (Ruiz *et al*., 2020). Residues of systematic pesticides can be present at a range of sublethal concentrations in honeybee products such as pollen, nectar, and other section plants (David *et al*., 2016).

The aim of this investigation is to study the effects of sublethal concentration from certain pesticides on the physiological status of nursing honeybee workers under laboratory conditions.

MATERIALS AND METHODS

The laboratory experimental works were conducted at the Plant Protection Department, Faculty of Agriculture, Assiut University, Egypt.

Honeybee Used: A local Carniolan hybrid of honeybee *Apis mellifera* L. colonies was selected for use in the experiment. Newly emerged honeybee workers (0-24 hours age) were obtained by incubation of sealed brood frames from one colony inside a cage at 34±2°C and 65% RH as a standard condition for honeybee colony.

Pesticides: Commercial products of seven pesticides representing different groups were selected for this study (Table 1). The bioassay test was carried out under laboratory conditions to determine the sublethal concentration for every pesticide (Saad ***et al*., 2023**). The stock of solutions with sublethal concentration was freshly prepared and diluted with distilled water at room temperature.

Table 1. List of pesticide groups and their sublethal concentration used in the experiments.

Common Name	Chemical Group	Trade Name, active ingredient percent, and type of formulation	Recommend Field Rate	Sublethal concentration used
Emamectin benzoate	Avermectin	BASHA 1.9% EC	1.25ml /1L	0.002 ppm
Chlorpyrifos	Aryl organothiophosphate	Delta Fos 48% EC	5 mL/1L	0.4 ppm
Imidacloprid	Nitroguanidine	Midacleed 70% WG	0.116 g /1L	0.12 ppm
Indoxacarb	Oxadiazine	Easo plus 30% WG	0.3 g /1L	0.045 ppm
Lambda-cyhalothrin	Pyrethroid	LEBRA 10% EC	0.5 ml /1L	2.930 ppm
Glyphosate	Glycine derivative	ROUNDUP STAR44.1% SL	12.5 ml /1L	468.060 ppm
Thiophanate-methyl	Thiophanate	Ambition 70% WP	0.8 g /1L	12.726 ppm

* The sublethal concentration of all pesticides for honeybee worker was determined by (Saad *et al*., 2023).

Experimental Cages: Plastic cages **(**Fig.1**)** according to Williams *et al*. (2013) were used in the experiment each cage consists of a clear plastic cup with 2 opened holes, one in the base to introduce the sugar syrup solution using a 5 ml syringe and the second one in the side of the cup to introduce pollen dough using 2 ml. Eppendorf tube that was opened to form a dish. A piece of the new organic wax comb was placed inside the cup to enhance the honeybees' natural state (Williams *et al*., 2013).

Fig. 1. Plastic cages used for preliminary tests under laboratory conditions.

Experimental Design:

Seven treatments from pesticide groups and a negative control treatment with three cages per treatment and 30 bees per cage were set up. All groups received fresh mix pollen dough which was made by adding 10% (w/w) water as a protein food (Williams *et al*., 2013). The dough was frozen at (-20°C) and thawed before use. Feeding sugar solutions with sublethal concentration were given to the bees adlibitum at the start of the experiment and were replenished every 24±2h. Cages were placed in dark incubators.

Measurements:

The effects on physiological functions were assessed by the following characters:

1. Food Consumption:

Food consumption was recorded daily for 18 days. Dead bees were counted and removed from the cage daily, assuming that had not consumed any solutions or pollen, then the average pollen dough and sugar syrup intake was recalculated for exactly 24 h, then divided by the number of living workers in each cage to get the average consumption per bee.

2. Hypopharyngeal Gland (HPGs) Development:

To determine the effect of sublethal concentration of the tested pesticides on HPG development, the size of HPGs acini was studied at the age of 6- days when the glands were at their most developed (Omar, 2017). Subsample (five bees per cage) was collected after the 6- days of pesticide exposure for HPGs measurements when the glands were at most development. The heads of bees were dissected in insect saline solution and part of HPGs were removed. Sixteen acini volumes per gland per bee (n= 15) were performed.

The length and width of each acinus were measured using an ocular micrometer (SPI 10/0.25) microscope Reichard Daivari at 100-fold magnification to calculate the acinus volume using the same equation as described by Omar *et al*. (2017) (Eq. 1).

$$\text{The volume of an acinus} = \frac{4}{3}\ x\ \pi\ x\ \left(\frac{a+b}{4}\right)^3 \quad (1)$$

Where (a) is the maximum length and (b) is the maximum width of the acini and π= 3.14.

3. The Wet and Dry Weight of Honeybee Workers:

At the end of the experiment (18 days of pesticide exposure), 10 bees in each cage were frozen until death and then weighed to determine the fresh weight following that the bees were dried in an incubator at 55°C ±2 for 3 days to calculate the dry weight. Human *et al*. (2013) water content of different treatments was calculated by sub subtracting the dry weight from the fresh weight.

4. Protein Determination:

Protein was determined in the central laboratories of the Faculty of Agriculture, Assiut University, according to the Kjeldahl method adapted by Rabie *et al.* (1983).

5. Fat Content Determination:

The dried individuals were soaked in petroleum ether. The solvent was changed once every day for three days. The insects were dried by using filter paper and then by heating in an oven at 70°C for a few minutes in order to evaporate all of the petroleum ether from them then reweighted. Fat contents were calculated by subtracting the day weight after extraction with petroleum ether from that before (Omar, 1979).

Statistical Analysis:

The CoStat software (Version 6.303, CoHort, USA, 1998-2004**)** was used to perform an analysis of variance (ANOVA) procedure on all gathered data. A 5% threshold of significance was used to compare mean differences using the revised least significant difference (RLSD) method **(**Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Effect of Exposure to Pesticides on Food Consumption Rate:

Food consumption from sugar solution which was treated and untreated with a sublethal concentration of seven groups of pesticides was recorded daily as ml/bee. Natural mixed pollen dough was consumed mainly by honeybee workers during the first week of their nursing period (Fig. 2A). However, honeybees tended to consume larger quantities of sugar solution after the first week of adult life when they stopped taking pollen (Fig. 2B). Our results agreed with Hrassingg and Crailsheine (1998), who reported that pollen consumption was high during the period of HPGs development, and it completely stopped later contrary to pollen, honeybees only consumed small quantities of sugar solution. In general, at the end of the exposure period (18 days), it appeared that the amount of sugar solution consumption was higher in untreated solution (control) than in all bees exposed to sublethal doses from certain pesticides (Table 3). At the same time, also pollen consumption was reduced in most treatments exposed to sublethal concentrations. The decreasing percentages ranged between 9.53 to 18.79%.

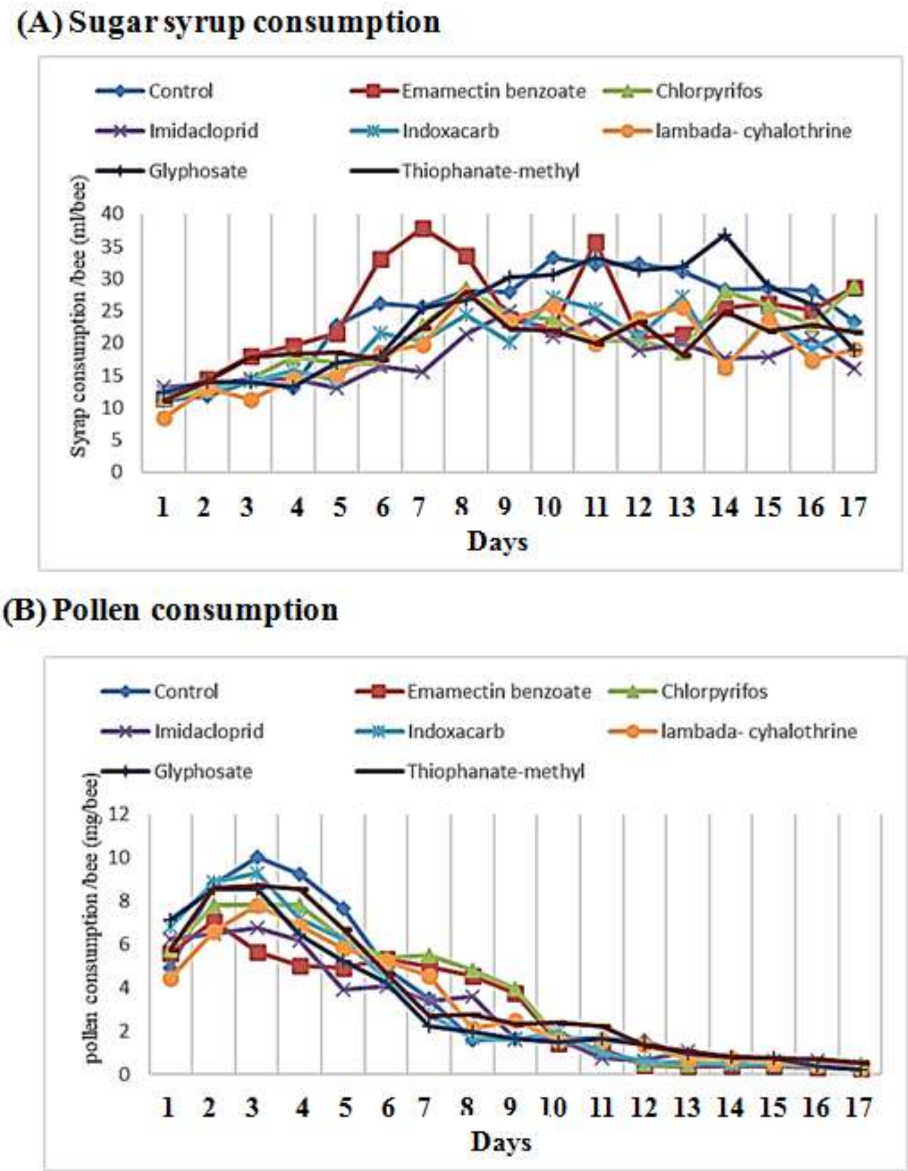

Fig. 2. Variation of sugar syrup consumption **(A)** and pollen consumption **(B)** treated with sublethal concentration tested pesticide during the nursing period of honeybee workers.

Table 2. The total amount of untreated pollen consumed by honeybee workers during the nursing period (18 days).

Treatments	Consumed pollen/bee (mg/bee)	
	Mean ± SE	Percentage of change
Emamectine benzoate	3.04±0.36 bc	-13.91
Chlorpyrifos	3.57±0.42 a	0.96
Imidacloprid	2.87±0.34 c	-18.79
Indoxacarb	3.20±0.45 b	-9.53
Lambada cyhalothrine	3.14±0.36 bb	-11.23
Glyphosate	3.16±0.40 bc	-10.54
Thiophanate-methyl	3.57±0.43 a	-0.94
Control	3.53±0.37 a	0.00
L.S.D. (0.05)	0.31	

*On the same % change: (treatment-control)/control*100

Table 3. The total amount of treated sugar solution consumed by honeybee workers during the nursing period (18 days).

Treatments	Consumed syrup/bee (ml/bee)	
	Mean ± SE	Percentage of change
Emamectine benzoate	24.64±1.19 a	+0.21
Chlorpyrifos	20.86±0.91 b	-15.15
Imidacloprid	17.82±0.68 c	-27.53
Indoxacarb	19.94±0.92 b	-18.90
Lambada cyhalothrine	19.03±0.85 bc	-22.61
Glyphosate	24.01±1.20 a	-2.34
Thiophanate-methyl	20.31±0.97 b	-17.38
Control	24.58±1.17 a	0.00
L.S.D. (0.05)	1.93	

A significant decrease in hoarding behavior for treated sugar solution was recorded in only five pesticides. The percentages of decreasing ranged between 15.15 to 27.15%. An insignificant amount from the treated sugar solution was recorded in two pesticides. Different authors have debated whether pesticides affect honeybee food consumption. Some pesticides cause honeybees to become anorexic and thus reduce food consumption (Helmer *et al*., 2015; Gonalons and Farina, 2018). The present study showed that the toxicity of sublethal concentration is not linked directly to the mortality of honeybee workers during the nursing period.

Effect of Exposure of Honeybee Workers on Oral Sublethal Concentrations on HPGs Development:

Morphological evaluations of HPGs development were recorded in Table (4) and Figure (3) as a volume of HPGs acini. Data showed that when nursing bees were exposed orally for 7 days to sublethal concentrations of certain pesticides had a significant decrease in the volume of HPGs acini in all treatments. The reduction percentages ranged between 31.28 to 55.62%. The highest reduction of acinus volume was recorded when nursing bees were orally treated with chlorpyrifos (55.62%) and imidacloprid (54.34%) pesticides. Based on the percentage of change in HPG size (mm^3×100/acini**)**, results showed that, the most to least effect pesticides were arranged as followed: chlorpyrifos > imidacloprid > glyphosate > indoxacarb > thiophanate–methyl > lambada cyhalothrine > emamectin benzoate. The size of the HPG lobes is an indirect indication of HPG activity (Harssnigg and Cralilsheins, 1998; Deseyn and Bilen, 2005). HPGs development corresponds with pollen consumption. We can infer that smaller sizes of acini of HPGs in honeybees exposed to pesticides could influence the protein synthesis of the glands and therefore lead to nurse honeybees producing royal jelly of lesser quality and/or quantity and could be reduced the production of brood

Table 4. Development of hypopharyngeal glands of nurse bees treated with sublethal concentrations of certain pesticides.

Treatments	HPG size (mm^3×100/acini) ± SE	Percentage of change
Emamectine benzoate	1.338±0.046 b	-31.28
Chlorpyrifos	0.864±0.059 e	-55.62
Imidacloprid	0.889±0.136 c	-54.34
Indoxacarb	1.063±0.020 cde	-45.40
Lambada cyhalothrine	1.142±0.021 bcd	-41.35
Glyphosate	1.019±0.110 de	-47.66
Thiophanate-methyl	1.246±0.030 bc	-36.00
Control	1.947±0.066 a	--
L.S.D. (0.05)	0.218	

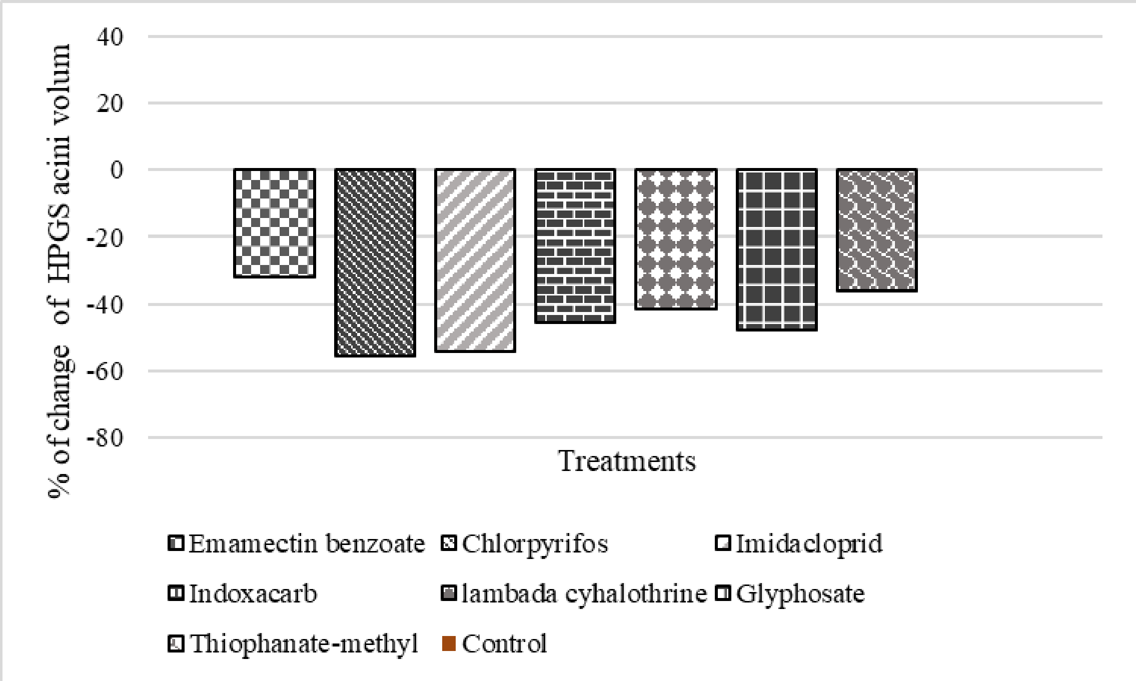

Fig. 3. Reduction effect of sublethal concentration of pesticides on HBGs acini volume.

Our results showed that continuous oral exposure to individual insecticides during the first 7 days of adult life in honeybees negatively affected HPG development. Also, in the present date under experimental conditions, the decreased size of HPGs acini can be attributed to insufficient consumption of pollen by honeybees exposed to treated sugar solution as distributed in Tables (2 and 3). The primary function of HPG in nursing bees is the production of royal jelly (RJ) which is the primary source of nutrition for future queens and larvae (Klose *et al*., 2017). Pesticides cause apoptotic and necrotic cell death in the HPGs (Keri & Gregore, 2010) and thus caused a decrease in RJ secretion by nurse bees (Zaluski *et al*., 2017). According to Faita *et al*. (2018) sublethal concentrations of herbicide (glyphosate) induced cytotoxicity in HPG and disrupted their bio-functions.

Effect of Sublethal Concentration Exposure on Water Content and Food Reserves During the Nursing Period of Honeybee Workers:

1. **Effect on Water Content:** The change in honeybee adults' weight and their bodies' water content after exposure to sublethal concentration during the nursing period of honeybees until 18 days old are considered criteria for studying honeybee nutrition. As shown in Table 5 and Figure 4 the water content of honeybee workers fed on untreated and treated sugar solution was calculated as a difference between the wet and dry matter of honeybee adults (18 days old). The water content of adults (18 days) was slightly increased in untreated against the treated bees. This result reflects that there is less mobilization in untreated bees than in other treated bees water content in adult bodies in all treatments was lesser than in the control (78.04) which reflects a maximum decrease in water content was observed with an adult of honeybee worker fed on Lambada sublethal concept of insecticide. No significant differences were detected among control other treatments.

Table 5. Effect of exposure to tested pesticides sublethal concentration on water content at the end of the nursing period of honeybee workers.

Treatments	Water content ± SE (%)	Percentage of change
Emamectine benzoate	71.45±4.27 a	-8.44
Chlorpyrifos	75.23±7.08 a	-3.60
Imidacloprid	72.59±3.70 a	-6.98
Indoxacarb	78.57±3.14 a	+0.67
Lambada cyhalothrine	70.11±3.32 a	-10.16
Glyphosate	71.40±1.98 a	-8.50
Thiophanate-methyl	72.59±4.47 a	-6.98
Control	78.04±2.58 a	-
L.S.D. (0.05)	12.24	

No significant differences were observed.

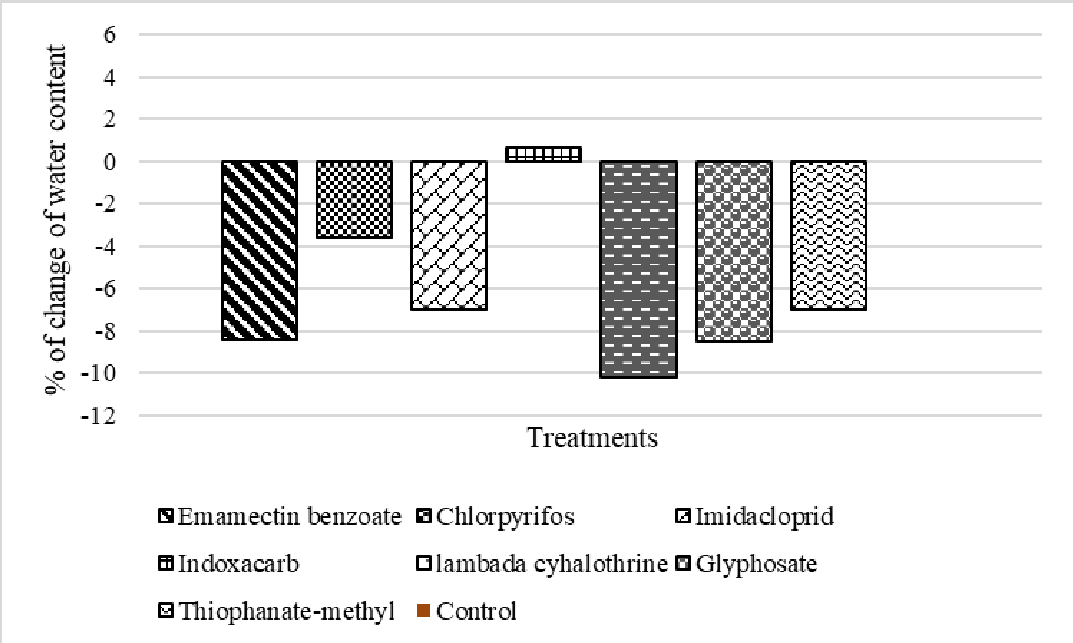

Fig. 4. Reduction effect of sublethal concentration on water content at the end of the nursing period of honeybee worker

2. Effect on Protein Content:

Data in Table (6) and Figure (5) shows the effect of the addition of sublethal concentrations of certain pesticides to sugar solution on the protein content of honeybee adults during the nursing period. Protein content decreased in all treatments in the adult after 18 days from emergence. The negative percentage ranged between -1.26 -12.03%. Significant differences were recorded with the indoxacarb, lambada-cyhalothrin, glyphosate and thiophanate methyl pesticides and the control. Derecka *et al*. (2013) reported that sublethal neonicotinoid exposure can affect honeybee metabolic physiology. The exposure of imidacloprid showed down-regulation of genes modulating the rate of glycolysis and protein synthesis.

Table 6. Effect of exposure of sublethal concentrations of pesticides on protein content during the nursing period of honeybee workers.

Treatments	Total protein (mg/gm) Mean ± SE	Percentage of change
Emamectine benzoate	167.60±1.10 bc	-2.94
Chlorpyrifos	170.50±1.10 ab	-1.26
Imidacloprid	172.63±1.18 a	-0.04
Indoxacarb	166.40±1.10 cd	-5.95
Lambada cyhalothrine	155.57±1.07 e	-11.84
Glyphosate	155.90±0.98 e	-12.03
Thiophanate-methyl	163.30±1.10 d	-7.57
Control	172.67±1.68 a	-
L.S.D. (0.05)	1.7678	

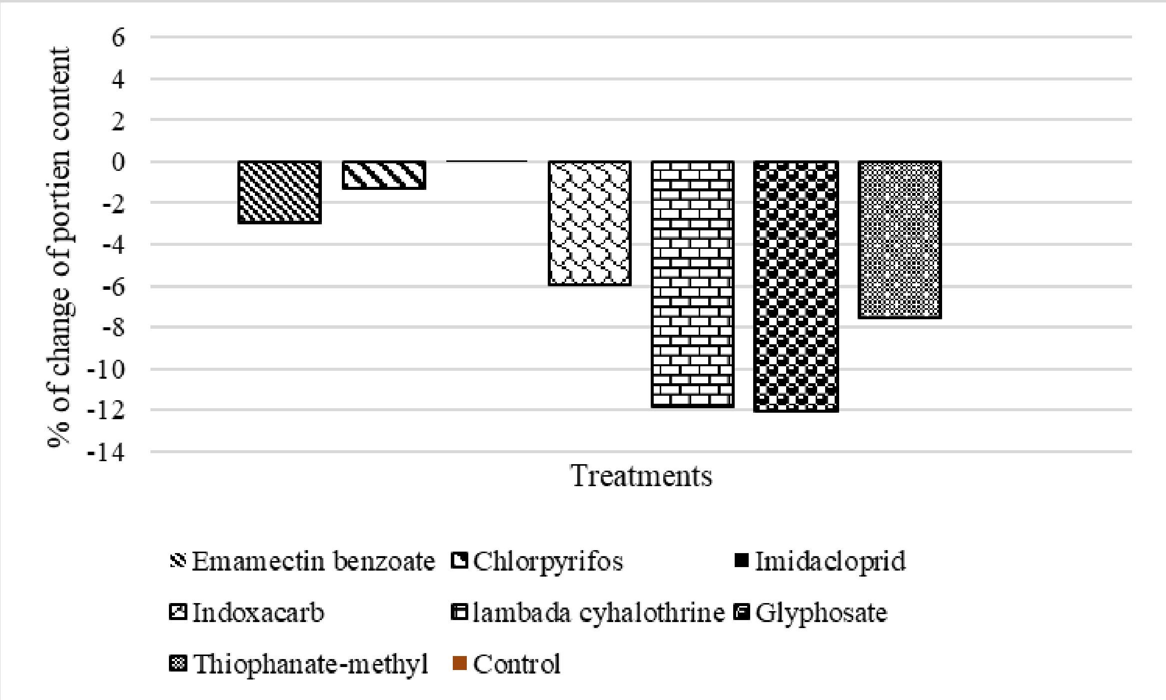

Fig. 5. Reduction effect of sublethal concentration of pesticides on protein content during the nursing period of honeybee workers.

3. Effect of Fate Content:

The state of metabolism as food reservoir results at the end of the nursing period in the body of an adult (18 days old) was determined. Continuously, oral feeding with sugar solution contains sublethal concentrations from certain pesticides to adult emergency until 18 day-olds. As shown in Table (7) and Figure (6) fat content significantly increased after exposure to sublethal concentrations from certain pesticides. The fat bodies in honeybee adult abdomen are vitally important for bees by providing energy, antimicrobial peptides, and lysozyme (Gillesple, Kanost and Tranczek, 1997). Under normal conditions, nurse bees have hypertrophied fat bodies (Amdam and Omholt, 2002). Honeybees respond to pesticide stress through immune detoxification and various antioxidant metabolic pathways which require a great deal of energy from the rich nutrients in their food (Castaireda *et al*., 2009). Dereka *et al*. (2013) recorded that larvae honeybees transiently exposed to two doses of pesticide imidacloprid showed down-regulation of genes modulating the rate of lipid metabolism and protein synthesis.

Table 7. Effect of exposure of sublethal concentration on fat content at the end of the nursing period of honeybee workers.

Treatments	Fat content (%) Mean ± SE	Percentage of change
Emamectine benzoate	15.58±2.06 c	+33.85
Chlorpyrifos	15.25±1.55 c	+31.01
Imidacloprid	17.33±0.25 bc	+48.88
Indoxacarb	19.68±0.37 ab	+69.07
Lambada cyhalothrine	20.85±0.63 a	+79.12
Glyphosate	19.46±0.77 ab	+67.18
Thiophanate-methyl	17.81±0.93 abc	+53.01
Control	11.64±1.06 d	--
L.S.D. (0.05)	3.33	

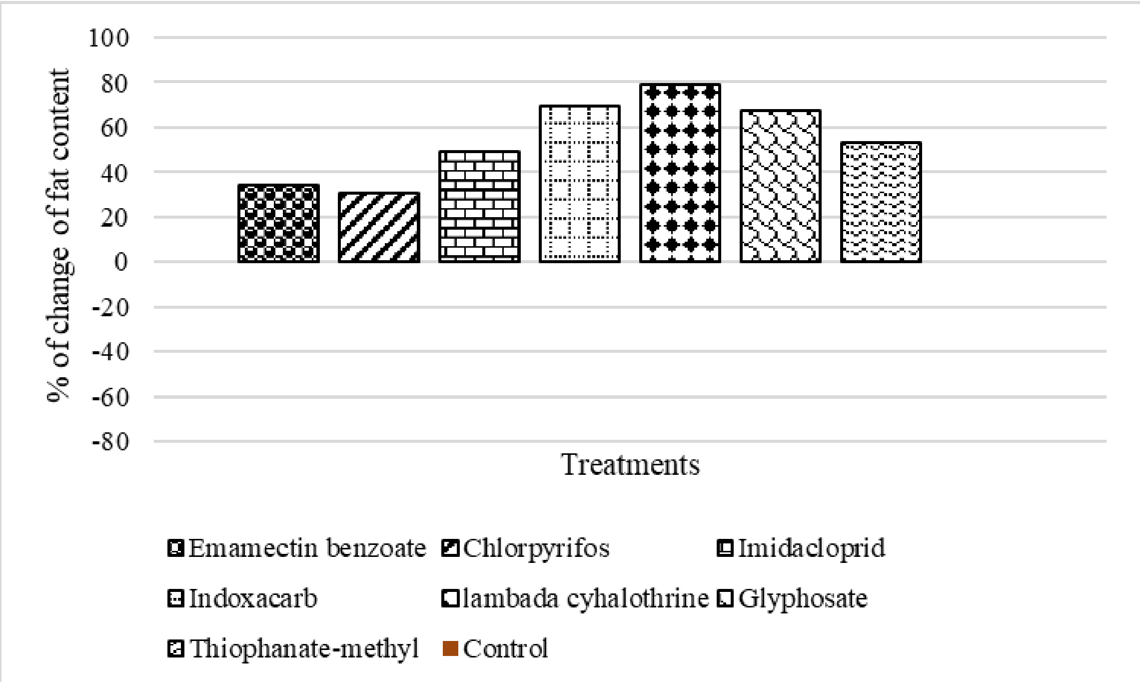

Fig. 6. The positive effect of sublethal concentration on fat content at the end of the nursing period of honeybee workers**.**

In conclusion, the sublethal concentration of agro-pesticides (chlorpyrifos, imidacloprid, indoxacarb, lambada cyhalothrine, emamectin benzoate, glyphosate and thiophanate–methyl) may be an effect on various physiological characteristics of nursing honeybee workers such as food consumption, development of food gland, body water content and food reserves. Food consumption rate was significantly reduced in all treatments except two treatments, chlorpyrifos and thiophanate-methyl. Also, the development of HPGs was significantly reduced in all treatments. A maximum decrease in adult water content was recorded under the effect of lambada-cyhalothrine pesticide treatment without any significant difference in all treatments. Furthermore, the protein content of adults significantly decreased in all treatments except with lambada-cyhalothrine pesticide treatment. Fat content significantly increased in all treatments. The present data reflects the negative effects of sublethal concentration after dietary oral application of certain pesticide groups during the nursing period of the honeybee. These results indicated that the application of pesticides has significant negative effects on the physiological characteristics of honeybee workers and honeybee health under laboratory conditions.

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ARABIC SUMMARY

تأثير التركيزات تحت المميتة لبعض المبيدات على الخصائص الفسيولوجية لشغالات نحل العسل تحت الظروف المعملية

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يعتبر العديد من مبيدات الآفات مسئولة عن خفض الأداء وفقد أعداد كبيرة من طوائف النحل وقد تم تقييم تأثير إضافة تركيزات تحت المميته من سبعة مجاميع من المبيدات إلي المحاليل السكرية لتغذية النحل حديث العمر. وقد تم تقييم تأثير بعض الخصائص الفسيولوجية علي النحل الحاضن مثل معدل استهلاك الغذاء ودرجات نمو غدد الغذائي الملكي والمحتوي المائي للشغالات الكاملة ومحتواها من المخزون الغذائي كدلالة تعكس الحالة الصحية لشغالات نحل العسل صغيرة السن. ومن خلال النتائج تم تسجيل انخفاض معنوي في معدل استهلاك الغذاء في كل المعاملات باستثناء معاملتي مبيدات الكلوربيروفوس – والثيوفينات مثيل. كما سجلت النتائج انخفاضاً معنوياً في حجم فصوص غدة الغذاء املكي في جميع المعاملات بنسبه ما بين ٣١-٥٥% مقارنة بالكنترول. وعند تقدير المحتوي المائي والمخزون الغذائي في نهاية فترة النحل الحاضن (عمر ١٨ يوم) كدلالة للحالة الميتابولزمية للشغالات فقد سجلت النتائج انخفاضاً غير معنوي في المحتوي المائي لجسم الحشرة الكاملة وقد بلغ الانخفاض أقصاه تحت تأثير مبيد اللامبادا – ثيهالوثرين. علي العكس انخفض المحتوي البروتين بشكل معنوي في جميع المعاملات ما عدا مبيد اللامبادا. بينما ارتفع محتوي الدهون بشكل معنوي تحت تأثير المعاملة بالجرعات تحت القاتلة في جميع المعاملات . وقد عكست النتائج بالتأثيرات السلبية العالية علي شغالات نحل العسل صغير السن خلال فترة النحل الحاضن عقب تطبيق التركيزات تحت المميتة للمبيدات المختبره.