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Toxicological and behavioral effects of Chlorfluazuron on pheromone production and perception of *Tribolium castaneum* (Coleoptera: Tenebrionidae).

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ABSTRACT

Chlorfluazuron (Atabron) is an insect growth regulator (IGR) belongs to benzoylphenyl urea for controlling the major insect pests. The present work aims to investigate the toxicological and biological effects of Chlorfluazuron on the 4th larval instar of rust red flour beetle, *Tribolium castaneum* at different concentrations (0.1, 0.5, 1, 5 and 10 ppm) under laboratory conditions. The results revealed high significant mortality in larvae, pupae and adults. The obtained results showed significant decrease in adult emergence, fecundity and fertility. Chlorfluazuron caused also a significant prolongation in larval and pupal developmental period.

When 4th larval instar treated with LC_{50} value of Chlorfluazuron (1.2ppm) female production and male's perception to pheromone affected. The results indicated that both treated and untreated sexes of the rust red flour beetle could secrete a pheromone that was able to stimulate the opposite sex as well as its own sex. Although production and responsiveness of pheromone in untreated groups were significantly higher than treated one. Females secreted a pheromone that stimulated males is sex pheromone. While the pheromone secreted by males is an aggregation pheromone.

Production and responsiveness to pheromone in untreated virgin sexes was significantly higher than treated one at photophase and scotophase. The maximum production and perception of pheromone in untreated sex were at 15.00 p.m. While a peak in treated one was at 13.00p.m.

Keywords: *Tribolium castaneum*, Chlorfluazuron, pheromone production and perception.

INTRODUCTION

The rust red flour beetle, *Tribolium castaneum* (Herbst), is one of the serious pests of flour and other cereal products in Egypt. When insecticides have been tested against this species, the degree of susceptibility often depends on the specific insecticide and formulation that is being evaluated (Arthur 2000).

IGR was introduced to describe a new class of bio-rational compounds through greater selectivity of action. Generally IGRs have very low toxicity to mammals and other non-target organisms and, usually, are rapidly degraded in the environment (Kostyukovsky *et al*., 2000). These characteristics make IGRs potential alternatives to conventional insect pest.

Chlorfluazuron (atabron) is a noval chitin synthesis inhibitor that belongs to the benzoylphenyl ureas (BPUs) and acts as an anti-molting agent, inhibit biosynthesis of chitin of an important constituent in insect cuticle, loses cuticle elasticity and causing abnormal endocuticular deposition and abortive molting (Dhadialla *et al.*, 2005).

Pheromones must be considered a major mode of intraspecific

communication in insects that acts to elicit a specific behavioral or developmental response from other organisms of the same species (karlson and Luscher, 1959) and they offer several possibilities for the manipulation of populations and behavior of such destructive insects.

The objective of this study was clarify the possibilities of using IGR (chlorfluazuron) and sex pheromone in pest control.

MATERIALS AND METHODS

Insect colony:

A laboratory colony of the red flour beetle, *T. castaneum* was maintained for many generations under constant conditions 30°C and 30-70% R.H. in the Department of Entomology, Benha University. The rearing medium was wheat flour mixed by weight with Brewer's yeast (95:5, w:w).

The chitin synthesis inhibitor:

The chitin synthesis inhibitor (5% E.C.), Chlorfluazuron (Atabron) was tested in the present study. Its chemical formula is 1-[3,5– dichloro- 4- (3-chloro -5- trifluoromethy l-2-pyridyloxy) phenyl]-3-(2,6- difluorobenzoyl)urea.

Bioassay test:

A preliminary experiment was carried out to determine the effect of different concentrations (0.1, 0.5, 1, 5 and 10 ppm) of chlorfluazuron as a chitin synthesis inhibitor against *T. castaneum*. Four replicates each containing 25 larvae/petridish was used for each concentration and for the control. The feeding technique used was according to Oberlander, (1997). Fourth larval instar were fed on treated flour till adult emerged. Mortality percentage of larvae, pupae, adults, adult emergence and the duration of larvae and pupae were estimated. The healthy and active adults produced from males and females were counted and inhibition of adult emergence was estimated according to Khazanie (1979). Ten replicates of one male and one female were run for each concentration and left for mating for 10 days and oviposition on a suitable media. The eggs laid were counted after both males and females were removed to record the number of eggs laid, hatchability percentage, sterility according to Toppozada *et al*., (1966) and oviposition deterrent index (O.D.I) according to Lundergren (1975) were estimated. The larvae obtained from hatched eggs were reared to adults.

Evidance of pheromone production on *T. Castaneum* adult treated as 4th larval instar by atabron and untreated one:

Evidence of pheromone production was carried out by bioassay treated males against treated males and treated males against treated females in compare with untreated one as a control. Also bioassay treated females against treated males and treated females against treated females in compare with untreated one as a control.

The olfactometer used in the present study was a vial type similar to that used by Burkholder (1970). It consisted of a glass vial (15x1.5cm). which had a rubber plug with a movable glass rod.The latter had a broad inner end at which a small piece of masking tape was fixed. The insect tested for pheromone production was held by the masking tape, while that tested for response was placed on the bottom of the vial. The distance between the two insects was 4 cm.

Five replicates each one contains 10 vials and in each vial two individuals were placed separately. The tested males and females were 8-10 days old.

Hexane was the solvent used for extracting pheromone in the following experiment at (0.3) female equivalents (FE) per 10µ of solvent according to Hussien (1982).

Assays were conducted at 1 p.m. and under conditions of 29-30°C and 30-70% R.H.

Effect of daytime on *T. castaneum* adult treated as 4th larval instar by atabron and untreated one:

Diurnal variations in female sex pheromone production and male response were determined by extracting treated 8-10 days old virgin female at 2 hours intervals throughout the photophase beginning at 9 a.m. This was extended throughout the scotophase for determine pheromone production by females.The treated females extracts were then tested against treated 8-10 days old males started at 9 a.m.

For determine male response, treated males were tested for sex pheromone extracted from treated female at each photophase and scotophase interval. For each test, five replicates each one contains 10 treated males placed individually in 10 vials were used.

In control untreated male and females used according to the previous manner.

Statistical analysis:

Probit analysis was determined to calculate LC_{50} (Finney, 1971), through software computer program. The results obtained were evaluated using one way analysis of variance. ANOVA and Snedecor (1971). On origin Pro. Lab (version 7.5) statistical program.

RESULTS AND DISCUSSION

To study the effect of atabron on some biological aspects of treated 4th larval instar and sub-sequent developmental stages of *T. castaneum*. Sterilized flour media which larvae feed on were treated with different concentrations of atabron.

Effect of Atabron on some toxicological and biological aspects of *T. castaneum*:

In the present study the chlorfluazuron caused appreciable toxic effect in larvae of *T. castaneum*. The data obtained in Table (1) showed that the percentages of larval mortality were 15.00 % at 0.1ppm, and increases significantly by the increase of concentration to 67.00 % at 10ppm. The present investigation is similar to the results obtained by (Ishaaya *et al.*, 1984; Gazit *et al*., 1989 on *T. castaneum* and Elek, 1998 on *Rhyzopertha dominica* and *Sitophilus oryzae*).

Table1: Effect of Atabron against *Tribolium castaneum*, treated as 4th larval instar.

Concentrations (PPM)	% larval mortality ±SE	% of larval malformation± SE	% pupal mortality ± SE	% of pupal malformation± SE	% adult mortality±SE	% of adult malformation±SE	% of emerged adult±SE	% of inhibition of adult emergence
0.1	15.00±0.25	2.00±0.28	00.00	00.00	00.00	00.00	85.00±0.25	15.00
0.5	18.00±0.74	16.00±0.41	4.00±0.47	4.00±0.47	03.00±0.47	2.00±0.28	78.00±1.22	22.00
1	31.00±0.57	24.00±0.71	6.00±0.28	5.00±0.25	12.00±0.41	12.00±0.41	63.00±0.57	37.00
5	37.00±0.90	36.00±0.47	9.00±0.47	9.00±0.47	17.00±0.47	17.00±0.47	54.00±1.37	46.00
10	67.00±0.87	49.00±1.37	12.00±0.47	9.00±0.85	18.00±0.64	18.00±0.64	21.00±0.47	79.00
Control	00.00	00.00	00.00	00.00	00.00	00.00	100.00	00.00
p-value	***	***	***	***	***	***	***	-
LC_{50}	1.2 ppm							
slop	1.2							
Chi square	25.47							

P-value of ANOVA: ***= significantly different at $P < 0.001$.

The percentages of pupal mortality were 0.0, 4.00, 6.00, 9.00 and 12.00 % at the concentrations of 0.1, 0.5, 1, 5 and 10ppm, respectively, as compared with 0.0 percent in the control. This results are in harmony with the results obtained by (Ishaaya *et al.*, 1987; Ishaaya and Yablonski, 1987 on *T. castaneum* and Haseeb *et al*., 2005 on *Diadegma semiclausum* and *Oomyzus sokolowskii*).

The explanation of this result could be due to chlorfluazuron at higher concentrations have antifeeding effect Riddiford and Truman (1978).

Also, Table (1) showed that morphogenic aberriations and abnormalities in larval, pupal, adult stages and intermediates of larval-pupal forms were increased significantly with the increasing in concentration as compared with control, these findings are in agreement with those results obtained by (Williams and Amos, 1974 on *T. castaneum*).

The morphogenic abnormalities could be grouped into four categories (malformed larvae, larval-pupal intermediates, malformed pupae and malformed adults).

All the external characteristics of deformed larvae like normal larvae but may larval exuvium was adhering to terminal abdomen as in fig. (B) or adhere to head capsule as in fig. (F), may appear restriction between head and the remaining of the body as in fig. (C), sometime fifth larval instar with wrinkled fourth instar exocuticle and inhibited to complete moulting as in fig. (D) or larva with swollen in abdomen as in fig. (E).

In fig. (G) larva with unchitinized abdomen, while in fig. (H) the head, thorax had pupal character, but abdomen still in larval form, in fig. (I) took the pupal shape with free legs and darkening colour but in fig. (J) appear C-shape larval-pupal intermediate.

In fig. (B) incomplete wings appeared in pupa, in fig. (C) head capsule which character the adult stage appeared and chitinized, while head capsule and forewing which character the adult stage appear in fig. (E), in fig. (D) pupa became dark and the terminal end was not completely formed.

In fig. (B) abnormal-looking adult with pupal exuvium adhering to its abdomen, in fig (C) adult with incomplete wing and abnormal legs but in fig. (D) adult took horse-shape.

These abnormalities could be due to changes in cuticle build up due to the decreasing level of chitin caused by an increased level of phenoloxidases. Ishaaya and Casida, (1974). Also histopathological examination of prepupal instar of *T. castaneum*, resulting from 4th larval instar fed on LC_{40} of chlorfluazuron revealed detachment of the epidermal cells from the endocuticle (Khaled, 2009) Furthermore, the abnormal adults observed were due to its resistance to metabolic detoxification during the larval and pupal stages. Ishaaya *et al.,* (1987).

In contrast in Table (1) the percentages of the adult emergence were decreased significantly with the increasing in concentrations as compared with control. Total inhibitions of adult emergence were 15.00, 22.00, 37.00, 46.00 and 79.00% at the Concentration of 0.1, 0.5, 1.0, 5 and 10 ppm, respectively, as compared with 0.0% in the control. The effect was concentration dependent. These results are in agreement with those obtained by (Ishaaya and Yablonski, 1987 and Abdel Fattah and Khaled, 2008 on *T. castaneum*).

Table (2) illustrated that the larval and pupal duration was increased significantly with the increasing of concentrations as compared with control. These observed results are in agreement with those obtained by (Kandil *et al.,* 2005 and El-Barkey, 2009) on pink bollworm *Pectinophora gossypiella*. The changes in larval and pupal durations may be induced morphogenic disruption. Prolongation in both larval and pupal durations in this study could be due to that chlorfluazuron (CSI) belongs to benzoylphenyl urea compound that interact with chitin synthase to inhibit chitin synthesis Deul *et al.,* (1978). Moreover, Ishaaya and Casida (1974) have reported that "CSI may be affecting other physiological systems, such as hormone system, that activates the chitinase and phenoloxidase". Accordingly, CSI may affect endocrine system and consequently affect the metabolic processes performed under its control Mayer *et al.,* (1990).

The fecundity and fertility were decreased significantly as a result of treatment with atabron. This decrease was negatively correlated with concentration. On the other hand, the oviposition deterrent index (O.D.I) and percentages of sterility were positively correlated with the concentrations as indicated in Table (2) for instance, (O.D.I) was 10.10, 41.57, 60.60, 100 and 100% at the concentrations of 0.1, 0.5, 1, 5 and 10 ppm, respectively.

Also the percentage of sterility was 34.89, 76.17, 94.04, 97.87 and 99.57% at the previous concentrations.

These obtained results are in agreement with Perveen, 2006 on *Spodoptera litura*.

The reduction in total number of eggs per female in this study could be due to interference of the tested IGR with oogenesis; it induced decrease in the concentration of yolk, proteins, carbohydrates, lipids (Shaurub *et al.,* 1998).

Reduction in the percentage of egg-hatch obtained in the present study could be due to sterilization of either eggs and sperms or may be due to inability of the sperms to be transferred to females during copulation (Ismail, 1980).

Table 2: Biological activity of Atabron against *Tribolium castaneum*, treated as 4th larval instar.

Concentrations (PPM)	larval duration(days) ±SE	pupal duration (days) ±SE	No. of eggs\female (fecundity)±SE	Percentage of fertility±SE	Percentage of sterility	% of oviposition deterrent index (O.D.I)
0.1	15.87±0.41	09.75±0.06	192.00±1.71	79.69±1.37	34.89	10.10
0.5	16.10±0.41	09.80±0.14	97.00±1.51	57.73±0.92	76.17	41.57
1	16.27±1.19	10.31±0.05	57.00±0.81	24.56±0.47	94.04	60.60
5	17.79±1.05	10.50±0.06	05.00±0.22	00.00	97.87	100.0
10	07.05±0.46	04.63±0.09	00.00	00.00	99.57	100.0
Control	14.07±0.22	9.10±0.04	235.00±1.74	100±0.0	00.00	00.00
p-value	***	N.S	***	***	-	-

P-value of ANOVA: ***= significantly different at P < 0.001. N.S = non significantly different

Effect of LC_{50} (1.2) of Atabron on responsiveness and production of pheromones in male and female adult beetles

Results on the response of treated virgin females and males of the rust red flour beetle, *T. castaneum*, to pheromone produced by either treated sex (8-10 days old), under constant conditions of 29-30°C and 30-40 %R .H., are given .In Table (3) and Fig. (1).

The following is an explanation of the response behavior of each sex to either male or female beetles.

Table 3: Response of virgin *Tribolium castaneum* males and females (8-10 days old) to adults of both sexes produced by treated 4th larval instar by atabron.

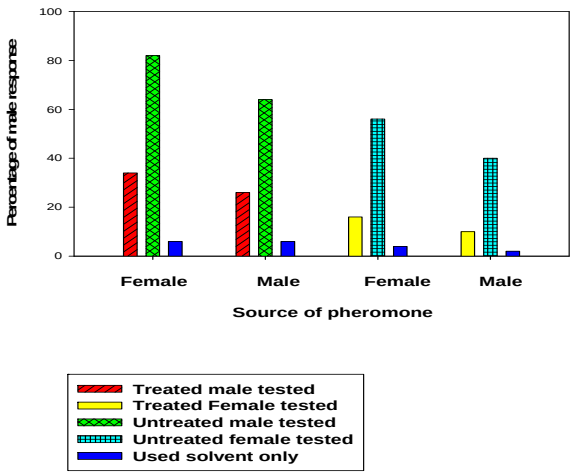
Types of Experiment	Percentage of male response				P- Value
	Untreated	With only solvent	Treated	Corrected experiment	
Male tested against female	82	6	34	29.79 ± 0.51d*	***
Male tested against male	64	6	26	21.28 ± 0.24b**	***
Female tested against female	56	4	16	12.50 ± 0.24c*	***
Female tested against male	40	2	10	08.16 ± 0.32a**	***
P- Value	***	N.S	***	-	-

Bioassays were conducted at 29-30°c and 30-70% R.H.

ANOVA P-Value:
*= Significantly different at P<0.05.
**= Significantly different at P<0.01.
***= Significantly different at P<0.001.
N.S= non Significantly different.

Student's (t) test:
a-significant difference between male tested against males and any other treated group.
b- significant difference between female tested against males and any other treated group.
c-significant difference between male tested against females and any other treated group.
d-significant difference between female tested against females and any other treated group.

Fig (1) Response of virgin *Tribolium castaneum* males and females (8-10 days old) to adults of both sexes produced by treated 4th larval instar by atabron



a- Male response behavior to female:-

The level of response 29.79 % was reached when treated males were tested against treated females . While in untreated one and used solvent only the response reach 82 and 6.00 %, respectively. The response behavior of treated male beetles to treated female consisted of a sequence of increasing levels of excitation .The treated males exhibited a sequence of events from the resting state .The first level of response included the raising of antennae, head and thorax .The second level of response included moderate activity of circular running in contrast the activity high in untreated beetles .In this level of excitation the treated male was bobbing up and down on the surface of the olfactometer vial. During the excitation, antennal and legs vibrations occurred.The duration of any level of excitation was variable. The treated males occasionally progressed from the resting state to active in a fast and continuous motion; but most commonly paused for variable lengths of time at lower levels of excitation. In other words, treated virgin female beetles were able to produce a pheromone, apparently a sex pheromone which could excite quiet a high percentage of treated males.

b-Male response behavior to male:-

Treated males also responded at a level 21.28% to treated male beetles. but response of untreated one and used solvent only were 64.00 and 6.00%, respectively, The response of treated male beetles to their own sex also consisted of a sequence of events the first level of response included the raising of antennae, head and thorax . The second level of response included low activity of circular running in other hand the activity moderate at untreated beetles. No vibration or bobbing appeared.

In other words, treated male beetles of *T. castaneum*, produced a pheromone, apparently an aggregating pheromone, that was able to excite a high percentage of treated males.

c-Female response behavior to female:-

Treated females tested against their own sex showed a level 12.50 % of response. While response of untreated one and used solvent only 56.00 and 4.00 % , respectively.

The treated females exhibited a sequence of events which also included the raising of antennae, head and thorax. Movements were generally slow. The duration of any level of excitation was short.

d-Female response behavior to male:-

The level of response 08.16 % was reached when female beetles were tested against males but response of untreated one and used solvent only were 40.00 and 2.00 %, respectively.

In this case, females also exhibited a sequence of events similar to those mentioned in male response behavior to male.

Statistical analysis of the data of the response of treated males to their own treated sex and the response of treated female beetles to the other treated sex Showed moderate significance ($P<0.01$). While the response of treated male beetles to treated females and the response of treated female beetles to their own treated sex Showed low significance ($P<0.05$).

While the response of treated and untreated (males to their own sex , the response of female beetles to the other sex, the response of male beetles to females and the response of female beetles to their own sex) Showed high significance ($P<0.001$) and in used solvent only no significance appear among previous groups.

The results indicated that both treated and untreated sexes of the rust red flour beetle could secrete a pheromone that was able to stimulate the other sex as well as its own sex. Although responsiveness and production of pheromone in untreated groups were significantly higher than treated one.The degree of response varied according to the source of pheromone . Thus, Females secreted a pheromone that stimulated and highly excited males more than females.Thus the female pheromone appeared to be a sex pheromone.

On the other hand, the pheromone secreted by males seemed to be an aggregation pheromone and both sexes were affected by this pheromone for aggregation. Results obtained in the present study are in agreement with those results obtained by (Suzuki *et al.,* 1984 in the rust red flour beetle, *T. castaneum*; Narayanan and

Nadarajan, 2005 in *Antigastra catalaunalis* and Ruther *et al*., 2007 in jewel wasps, *Nasonia vitripennis*).

Suzuki and Sugawara (1979) reported that males of the red flour beetles, *T. castaneum* secrete an aggregation pheromone attractive to both sexes.

2-Effect of daytime:-

a-On extractable female pheromone content:-

Table (4) and Fig (2) illustrated that the effect of daytime on pheromone production and showed that treated females extracted early in the morning (photophase) (9.00a.m.) stimulated 10.87% of males indicating a low pheromone titer.

Treated female extracted two hours later (11.00 a.m.) produced a higher amount of pheromone. In this case the number of males responded to the treated female extract significantly increased to 11.36%. A peak of treated male response 26.67% was reached at (13.00p.m.) Probably due to the highest pheromone titer produced by treated females at that time. Male response to treated females extracted in the afternoon (15.00p.m.) significantly decreased to 23.91%. This continued to decrease towards evening (scotophase) as it dropped to 2.22% at (21.00p.m.)

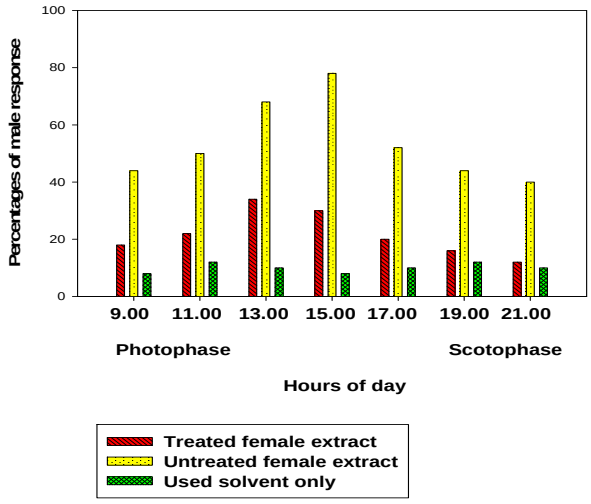
Although production of pheromone in untreated virgin females extract was significantly higher than treated one at photophase and scotophase. The maximum production of pheromone in untreated virgin females extract was 78% at (15.00 p.m.).

Table 4: Response of male *Tribolium castaneum* to virgin female extracted at 2 hour-intervals during the photophase and scotophase. Both sexes were 4-10 days old produced by treated 4th larval instar by atabron.

Daytime of female extracts (hrs)	Percentage of male response				P- Value
	Untreated	With only solvent	Treated	Corrected experiment	
9.00	44	8	18	10.87 ± 0.20	***
11.00	50	12	22	11.36 ± 0.37	***
13.00	68	10	34	26.67 ± 0.40	***
15.00	78	8	30	23.91 ± 0.32	***
17.00	52	10	20	11.11 ± 0.32	***
19.00	44	12	16	04.55 ± 0.24	***
21.00	40	10	12	02.22± 0.20	***
P- Value	***	N.S	***	-	-

Bioassays were conducted at 9 p.m. under the conditions of 29-30°c and 30-70% R.H.

Fig (2): Response of male *Tribolium castaneum* to virgin female produced by treated 4th larval instar by atabron extracted at 2 hour-intervals during the photophase and scotophase. Both sexes were 4-10 days old.



b-On male response:-

Table(5) and Fig (3) illustrated that The effect of daytime on response of treated males and showed that an increase in male response took place as the photophase progressed from early morning to afternoon. It then decreased significantly toward the late afternoon, and reached its lowest level at (21.00 p.m.). The maximum response (20.45 %) was reached at 13.00 p.m.

Although responsiveness to pheromone in untreated virgin males was significantly higher than treated one at photophase and scotophase. The maximum responsiveness to pheromone in untreated virgin males was 76% at (15.00 p.m.).

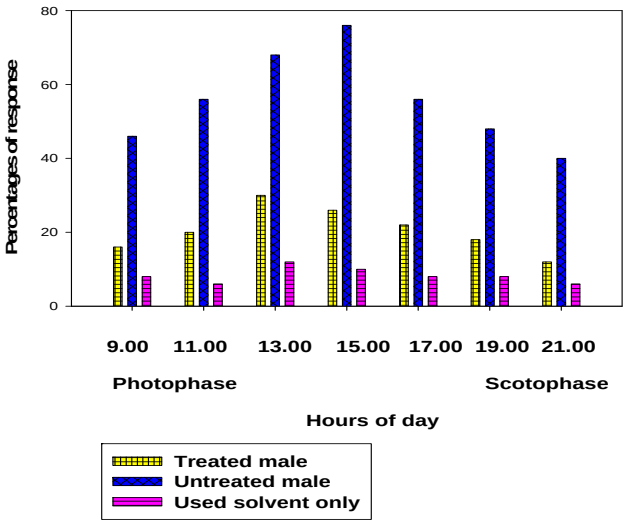
In the present study it is evident that both treated male responsiveness and female extractable pheromone titers follow diurnal and circadian rhythm. Moreover, the male response cycle of *T. castaneum* correlates with the female pheromone content during the photophase. Such finding has been observed by (Aftab, 1993 in *T. castaneum*; Wright and Morton, 1995 and Bashir *et al*., 2003 in *Rhyzopertha dominica*).

Table 5: Effect of daytime on the response of *Tribolium castaneum*(4-10 days old) males at 2 hour intervals over the photophase to a single sex pheromone extract of virgin females produced by treated 4th larval instar by atabron.

Daytime of male response (hrs)	Percentage of male response				P- Value
	Untreated	With only solvent	Treated	Corrected experiment	
9.00	46	8	16	08.70 ± 0.24	***
11.00	56	6	20	14.89 ± 0.32	***
13.00	68	12	30	20.45 ± 0.32	***
15.00	76	10	26	17.77 ± 0.24	***
17.00	56	8	22	15.22 ± 0.20	***
19.00	48	8	18	10.87 ± 0.20	***
21.00	40	6	12	06.38 ± 0.20	***
P- Value	***	N.S	***	-	-

Bioassays were conducted under the conditions of 29-30°c and 30-70% R.H.
ANOVA P-Value: ***= Significantly different at P<0.01. N.S=non Significantly different.

Fig (3): Effect of daytime on the response of *Tribolium castaneum* (4-10days old) males at 2 hour intervals over the photophase to a single sex pheromone extract of virgin females produced by treated 4th larval instar by atabron.



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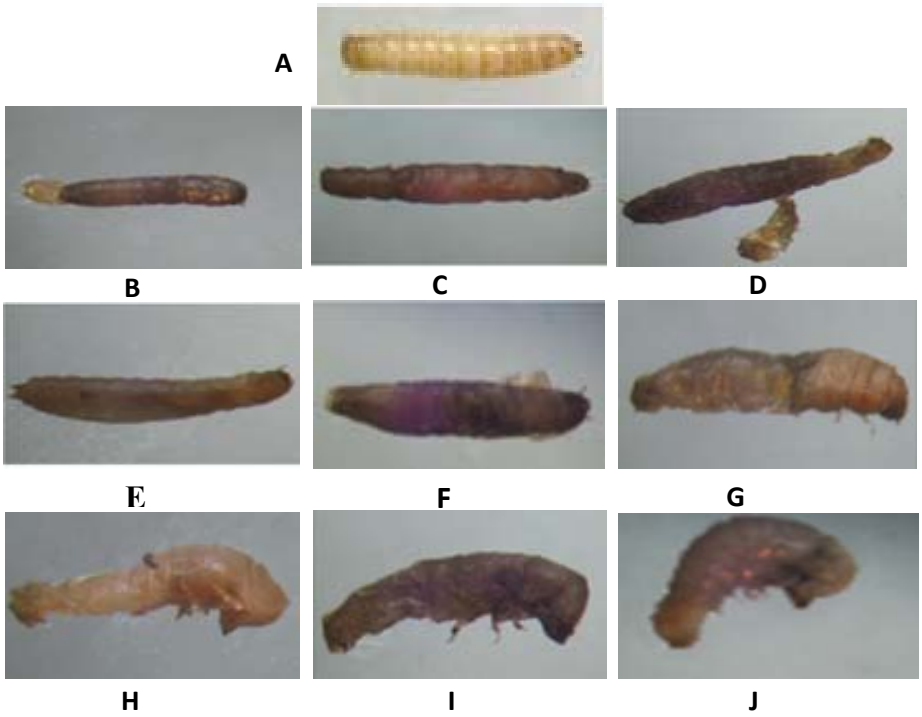

Plate 1: showed some degrees of malformed larvae and some larval- pupal intermediates.
A: Normal larvae B-J: Deformed larvae & larval-pupal intermediate

Plate 2: showed some pupal malformation.
A: Normal pupa B-E: Deformed pupae

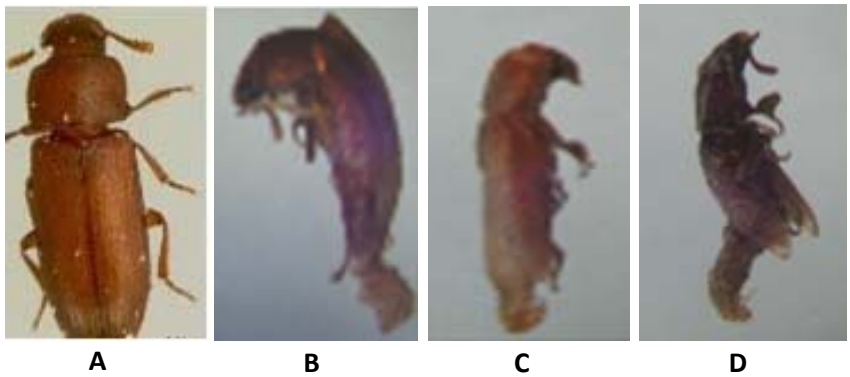

Plate 3: showed some adult malformation.
A: Normal adult B-D: Deformed adult

ARABIC SUMMARY

التاثيرات السمية والسلوكية البيولوجية لمركب الكلورفلوازرون على أنتاج وإدراك الفيرومونات لخنفساء الدقيق الصدئية.

رضا فضيل على بكر[1]- الفت محمد المنيرى[2]- نهاد محمد البرقى[2] - نانسى مجدى بيومى الشوربجى[2]
1- قسم علم الحشرات – كلية العلوم – جامعة عين شمس
2- قسم علم الحشرات – كلية العلوم – جامعة بنها

تم دراسة تاثير مركب الكلورفلوازرون الذى ينتمى لمجموعة منظمات النمو الحشرية على بعض الصفات البيولوجية لحشرة خنفساء الدقيق الصدئية و ذلك بمعاملة العمر اليرقى الرابع بالتركيزات المختلفة (0.1، 0.5، 1، 5 و10جزء فى المليون) تمت الدراسة تحت ظروف المعمل. اوضحت النتائج ان استخدام هذا التركيز تسبب فى نسب موت معنوية فى كل من اليرقات، العذارى و الطور البالغ. كما تسبب هذا التركيز فى خفض نسبة خروج الطور اليافع من العذراء، نقص عدد البيض الناتج من الانثى اليافعة و قلت النسبة المئوية للفقس كما طالت فترة عمر الطور اليرقى و طور العذراء.

وبمعاملة العمر اليرقى الرابع بالتركيز الخاص بالجرعة القاتلة للنصف وجد ان الاناث و الذكور المعاملين و الغير معاملين لهم القدرة على انتاج الفيرومونات التى تحفز الجنس الاخر او نفس الجنس للاستجابة ومع ذلك انتاج و ادراك الفيرومونات فى الافراد الغير المعاملة اعلى بصورة ملحوظة عنها فى الافراد المعاملة. كما اوضحت النتائج ان الانثى تفرز فيرومون الجنس بينما الذكر يفرز فيرومون التجمع. و بدراسة تاثير هذا التركيز على انتاج و استجابة الحشرة اليافعة الناتجة من العمر اليرقى المعامل خلال فترات النهار و الليل المختلفة، وجد ان انتاج و ادراك الفيرومونات فى الافراد الغير معاملة اعلى منها معنويا فى الافراد المعاملة، حيث وجد ان اعلى انتاج و استجابة للفيرومون فى الافراد الغير معاملة تكون الساعة الثالثة بينما فى الافراد المعاملة تكون الساعة الواحدة.