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Effects of Juvenile Hormone Mimic on Growth, Morphogenesis and Morphology of Hemocytes of the black cutworm, *Agrotis ipsilon* Larvae (Lepidoptera: Noctuidae)

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ABSTRACT

Effects of juvenile hormone mimic (JHM) "Ro 13-5223" on growth and morphogenesis of the black cutworm, *Agrotis ipsilon* (Lepidoptera: Noctuidae) were studied Also, the morphological changes in different haemocyte counts (DHCs) of treated last instar larvae with the high doses of JHM were investigated.. JHM dissolved in acetone was topically applied on the dorsum of the tested last instar larvae at doses of 1, 10, 50 and 100 µg/ larva. Results at high doses (50, 100 µg/ larva), induced up to 2 supernumerary larval molting expressed in (80 and 90 % of treated larvae, respectively), while the lowest JHM doses (10, 1 µg/ larva), proved a successful in supernumerary molts with less extent (75 and 42 % of treated larvae, respectively). Generally, all doses interfered with the normal development of *A. ipsilon* and resulted in the imperfect and perfect supernumerary larval molts. The use of the solvent (acetone) also led to mortality of (30%) but did not induce any significant gain in live body weight and developmental period. Eight hemocyte types: prohaemocytes, phagocytic cells (palsmatocytes, spindle cells and granular cells), adipohaemocytes, oenohaemocytes, spherulocytes and cystocytes were identified in the haemolymph of *A. ipsilon* larvae based on morphological characters. However, the DHCs in hormonal treated larvae showed some destructive cytopathological effects on haemocyte types as irregularity in cell shape, movement in nucleus towards the cell wall with lose in its central position and observation of cellular vacuolization. Acetone as control showed quite morphological changes as compared to hormone effects.

Key words: *Agrotis ipsilon*, Juvenile hormone mimic (JHM), Differential haemocyte types (DHCs).

Introduction

The black cutworm, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae) is a major agricultural pest in Egypt, which has a wide range of hosts, nearly includes all vegetables and many important grains. *A. ipsilon* larvae feed aboveground until about the 4th instar where they can do considerable damage by severing young plants in a night (Abdel-Gawaad and El-Shazil, 1971).

Juvenile hormones (JHs) are sesquiterpenoids and regulate a number of physiological processes in insect development. Larvae require JH to maintain the larval state, but it must be absent in the last larval instar for starting the metamorphosis (Riddiford, 1994 and Truman and Riddiford, 1999). More recently, several highly active synthesized compounds with less apparent similarity to JH (aromatic non-terpenoidal JH analogs) like fenoxycarb, pyriproxyfen and diofenolan (Dhadialla *et al*., 2005).). Insect growth regulators (IGRs) or third generation insecticides (Williams, 1967) are differed widely from insecticides where they affected on development, metamorphosis and reproduction of the target insects by disruption the activities of endocrine system (Oberlande *et al*., 1997). IGRs effects depend upon dose and timing of application, which resulted in production of supernumerary or permanent larvae or larval-pupal intermediates (Gadenne *et al*., 1990). Also, JH is involved in other biological activities like immune responses (Rantala *et al*., 2003). In spite of these facts, very little work has been carried out on the influence of insect hormones and their synthetic analogues on the hematology and thereby immunological properties of insects.

Haematological studies in field of insect physiology explain the vital activities performed by haemocytes (Abd El-Aziz *et al*., 2010). Haemocytes perform phagocytosis, encapsulation of foreign bodies in the insect body cavity coagulation to prevent loss of blood, nodule formation and transport

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of food materials, which may be hormones, detoxification of metabolites and biological active materials (Gupta, 1985). The morphometric changes of differential haemocyte count (DHCs) after different pests' treatment were studied by (El-Mandarawy, 1997&2005, Abd El-Aziz *et al*., 2010 and Pandey *et al*., 2017).

The present work was intended to evaluate the effect of the juvenile hormone mimic (JHM) "Ro 13-5223" on growth of *A. ipsilon*. Also, the differential haemocyte counts (DHCs) of last instar larvae with hormonal treatment were investigated.

Materials and Methods

Rearing insect:

A sample of *A. ipsilon* was obtained from the susceptible culture strain maintained for several generations in Plant Protection Research Institute, Agriculture Research center, Dokki, Giza, Egypt. Larvae reared on castor bean leaves, *Ricinus communis,* L. at conditions of 22 ± 2 °C and 65 ± 5% R.H.. Rearing technique was carried out as the same adopted by (Abdin, 1979).

Juvenile hormone mimics (JHM):

A juvenile hormone mimic (JHM) "Ro 13-5223" were supplied as a gift from Syngenta Agro, Egypt, fenoxycarb (ethyl 2-(4-phenoxy-phenoxy) ethyl carbamate.

Tests

JHM was dissolved in distilled acetone and then topically applied on the dorsum of the tested last instar larvae at doses of 1, 10, 50 and 100 µg/ larva. In the control treatments, each larva received 1µl of pure acetone. The larval cast exuviae were daily noted and removed to record the number of moults. Also, the live larval body weights and the durations of different developmental stages of treated and untreated *A. ipsilon* were observed.

Determination of differential haemocyte counts DHCs

DHCs of treated last instar *A. ipsilon* larvae were examined with JHM (at the highest the high dose of 100 µg/ larva as compared to those of acetone and control treatments. A proleg of the sixth abdominal segment was snipped off using a pair of fine scissors, and the haemolymph was allowed to ooze on a clean and grease-free microscopic slide.

For determining DHCs, blood films were made by applying one end of a slide to a drop of blood and the slide placed on a leveled surface, holding it with the thumb and index fingers of the left hand. The narrow edge of another slide was placed in the drop and held there till the blood has spread across it. It was then drawn slowly over the whole length of the first slide. The inclination of the second to the first should be 45˚, and there should be no pressure what so-ever between the two surfaces. The more slowly one slide is drawn over the other, the thinner is resulting film. Smooth spreading of the film was aided by warming the first slide on the flame of spirit-lamp before applying it to the drop of blood. After the blood was spread, it was dried by being waved rapidly in air to prevent undue shrinkage of the cells (Hunter and Bomford, 1959).

For staining films, they was fixed in absolute methyl alcohol for 2 minutes and dried in air. Then the dry film was well covered with Giemsa stain, flushed off with distilled water then the film was rinsed in distilled water and gently blotted dry with clean blot-ting paper.

The different pictures of haemocytes were performed by phase contrast microscope at 100×10x.

Statistical analysis:

Paired Student's *t*-tests ($p < 0.05$) can be used to compare between the means of treated and untreated larvae.

Results and Discussion

Juvenile hormone mimics (JHM) treatment:

When the newly ecdysed last instar larvae of *A. ipsilon* (0-24 h) treated with JH mimic at the two high doses (100, 50 µg/ larva), they had induced to undergo up to 2 supernumerary larval molting expressed in (80 and 90 % of treated larvae, respectively) as shown in Table (1). However, in low JH mimic doses of (10, 1 µg/ larva), larvae had proved to be still successful in inducing supernumerary molts but to a lesser extent (75 and 42 % respectively).

Table1: Influence of juvenile hormone mimic on growth and development of the last larval instar of *Agrotis ipsilon* when topically applied with different doses.

Treatment	Dose µg/larva	No. of tested larvae	Maximum Body Weight (mg) Mean±SD	Additional moultings					Period (d) Mean±SD	Larval Mortality %	Pupal wt.(mg) Mean±SD	Intermediate Mortality %
				Frequency			%	type				
				0	1	2						
JH Mimic	1	12	492.3±120.9 NS	7	5		42	super.	17.4±3.70*	70		30
	10	12	557.5±202.7 NS	3	9		75	super.	17.7±4.00*	50	411.3±134.9 NS	50
	50	10	727.3±134.7*	1	7	2	90	super.	19.3±1.80*	50	639.3±37.6*	50
	100	10	804.5±65.5*	2	6	2	80	super.	20.5±2.10*	40	707.5±10.6*	60
Acetone	1µl	10	453.8±112.4 *	10					11.7±1.70 NS	30	435.6±93.7*	
Untreated	0	10	486.9±80.9	10					11.3±1.70		346.1±89.7	

Period (in days) elapsed between moulting into the last (6th) larval instar and adult eclosion.
Super. : supernumerary larvae.
**Significance differences between treated and untreated (control) (taking by the comparison between means using Student's t-test) at $p < 0.05$.*
NS: non-significant

All doses interfered with the normal development of *A. ipsilon* and resulted in the imperfect and perfect supernumerary larval molts. Imperfect supernumerary larvae had a new cuticle below the old one but the larvae failed to ecdyse (Fig. 1), while the perfect supernumerary larvae had normally molted into larvae of 7th and 8th instars. As expressed in the mortality percentages, they were found to be mainly due to the formation of pupal-adult intermediates (Fig. 2).

Table 1 shows that the treated larvae with JH mimic caused a significant increase in live body weight and larval duration compared with untreated controls. The resulting pupae from treated larvae had increased in body weight with compared to Control. The use of the solvent (acetone) also led to mortality of (30%) but did not induce any significant gain in live body weight and developmental period.

(A) Normal larva **(B)** Imperfect supernumerary larvae failed to ecdyse.

Fig. 1: Showing supernumerary larvae resulting from treatment of the last instar of *Agrotis ipsilon* larvae

(A) Normal pupa **(B)** Malformed pupa

Fig. 2: Showing pupal-adult intermediate resulting from larva treated with JH mimic

Results are in agreement with the previous morphological and endocrinological study by (Abdel-Hakim, 1996 and 2005). Furthermore, in more recent studies (Abdou and Abdel-Hakim, 2017) showed that the application of methoprene to 4th instar *A. ipsilon* larvae was resulted in significantly increasing the mortality, body weight and the prolonged development of larvae as compared to control. Also, finally the pupal-adult intermediates were formed. (Sendi and Salehi, 2010) have been concluded that the juvenile hormone analogue (JHA) methoprene at high dose (100µl/ µl acetone) produced imperfect and perfect supernumerary larval instars. Kamimura and Kauchi (2002) mentioned that (JHA) fenoxycarb, has been shown to motivate additional molt when applied the 3rd and 4th larval instars of silk worm *Bombyx mori*. The 5th instar period was shortened but the 6th instar period increased from eight to twenty days depending on the amount of fenoxycarb (JH) applied.

Differential haemocyte counts (DHCs) of healthy and treated *A. ipsilon* larvae

A. DHCs of healthy A. ipsilon larvae

On the basis of light microscopy inspections, *A. ipsilon* in last instar larvae were found to consist of eight types of haemocytes:
prohaemocytes, phagocytic cells (palsmatocytes, spindle cells and granular cells), adipohaemocytes, spherulocytes, oenocytoids and cystocytes (Fig.3).

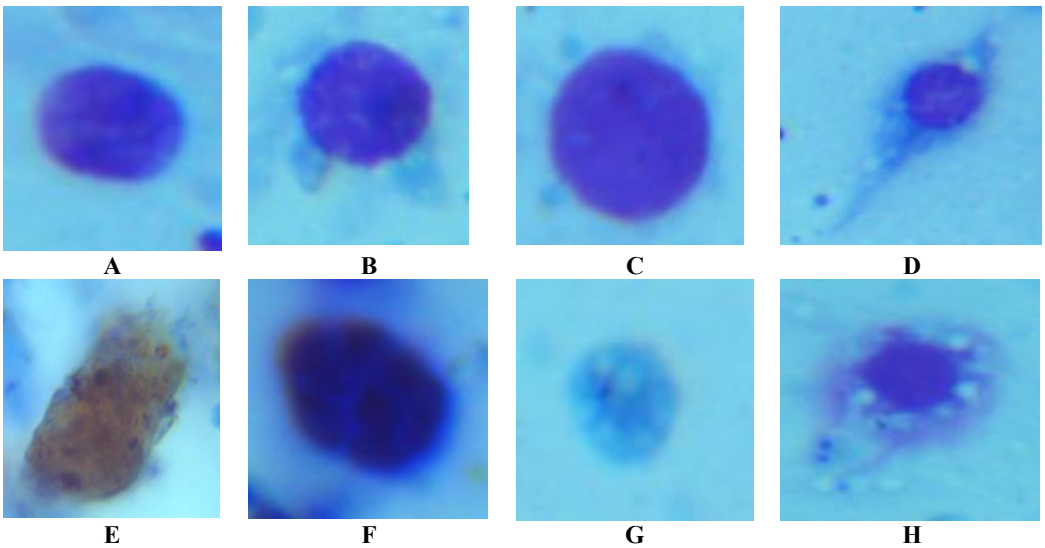

Fig. 3: Different haemocyte types of untreated healthy last instar *Agrotis ipsilon* larvae. A. Prohaemocytes, B. Plasmatocytes, C. Granular cells D. Spindle cells E. Adipohaemocytes F. Spherule cells G. Oenocytoids H. Cystocytes.

1. Prohaemocytes (PRs):

They are relatively small, round to slightly ovoid cells, distinguished by a high nuclear-cytoplasmic ratio and intensely basophilic cytoplasm. Round single nucleus with dense homogeneous chromatin is usually central although frequently seen slightly eccentric. Homogeneous cytoplasm is free of any inclusions or vacuoles, mitotic division was observed.

2. Phagocytic cells:

a.Palsmatocytes (PLs):

They have a nuclear cytoplasmic ratio which is less than that of prohaemocytes. The outline is most commonly ovoid or round in shape. Nucleus stains reddish purple, round or ovoid with punctuate or granular, chromatin and usually central in position. The cytoplasm is quite heterogeneous. Distinct granular inclusions of various dimensions and shapes together with round and/or irregular vacuoles are distributed through a matrix.

b.Granular cells (GRs):

They are larger and thicker than palsmatocytes. Granules generally tend to obscure the relatively small-round centrally located nucleus in fresh material.

c.Spindle cells (Sps):

Spindle shaped cells distinguished by the presence of very large, distinct and usually spindle nucleus, mostly appearing as solid purple structure. The cytoplasm stained dark blue violet with Giemsa.

3. Adipohaemocytes (ADs):

They are ovoid or oblong, or irregularly shaped cells and vary in size. They contain variable amounts of refringent fat droplets and several other non-lipid inclusions.

4. Spherule cells (Sphs):

They vary from oval to sepherical in shape, with slightly eccentric nuclei. The cytoplasm contains many small and large slightly translucent to phase dark spherules.

5. Oenocytoids (Oe):

Typically conspicuous, large and thick cells with eccentric nuclei. The cytoplasm contains may be filled with intricated canaliculi. After staining the cytoplasm becomes usually acidophilic.

6. Cystocytes (Cy):

Exteremely fragile cells with single, small round, cartwheel like nucleus in a basophilic or chromophilic cytoplasmic envelope containing distinct, round acidophilic inclusions. Our morphological description for these cellular types was similar to Abd El-Aziz *et al*. (2010) who found five types of haemocytes in the 4th larval instar of *A. ipsilon*: prohaemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), spherule cells (SPs) and adipohaemocytes (ADs).

B. DHCs for treated *A. ipsilon* larvae by JHM "Ro 13-5223"

Figs (4 and 5) show the effects of JHM and acetone on haemocytes of last instar *A. ipsilon* larvae. Treatments with the highest dose (100 µg/ larva) of JHM "Ro 13-5223" caused abnormalities and deformity in surface morphology in all types of blood cells. Haemocytes which generally act for defense i.e. plasmatocytes and granular haemocytes, mainly bear the effect because most of them change their contour or become fragile with irregularity and elongation of cell wall, also, twisting at the ends of spindle cells. Also, nucleus moved towards cell wall, losing its central position and cellular vacuolization was observed, particularly, in granulocytes. The cytoplasm of all cells was

badly and faintly stained. The fat droplets of adipohaemocytes shrink. Also, plasma membrane of all the cells becomes fragile leading to a gradual loss of cytoplasm and ultimately only a few interconnected cytoplasmic strands are left. Due to the damages caused to haemocytes, it can be inferred that cellular defense reactions of insect are reduced after treatments. Also, acetone showed quite morphological changes as compared to hormone effects.

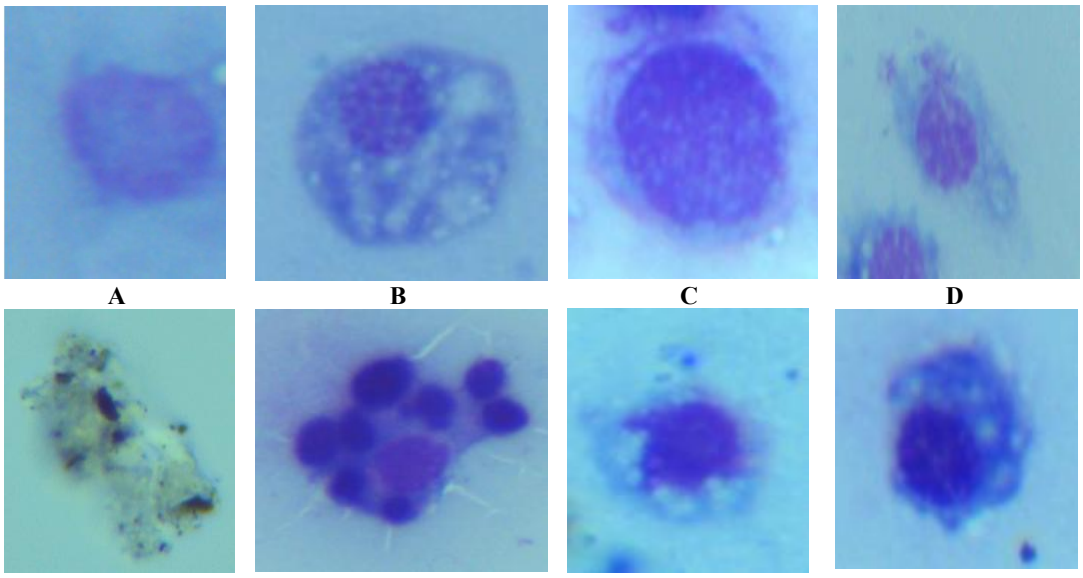

Fig. 4: Alteration in haemocyte morphology of last instar *Agrotis ipsilon* larvae exposed to acetone. A. Prohaemocytes, B. Plasmatocytes, C. Granular cells D. Spindle cells E. Adipohaemocytes F. Spherule cells G. Oenocytoids H. Cystocytes

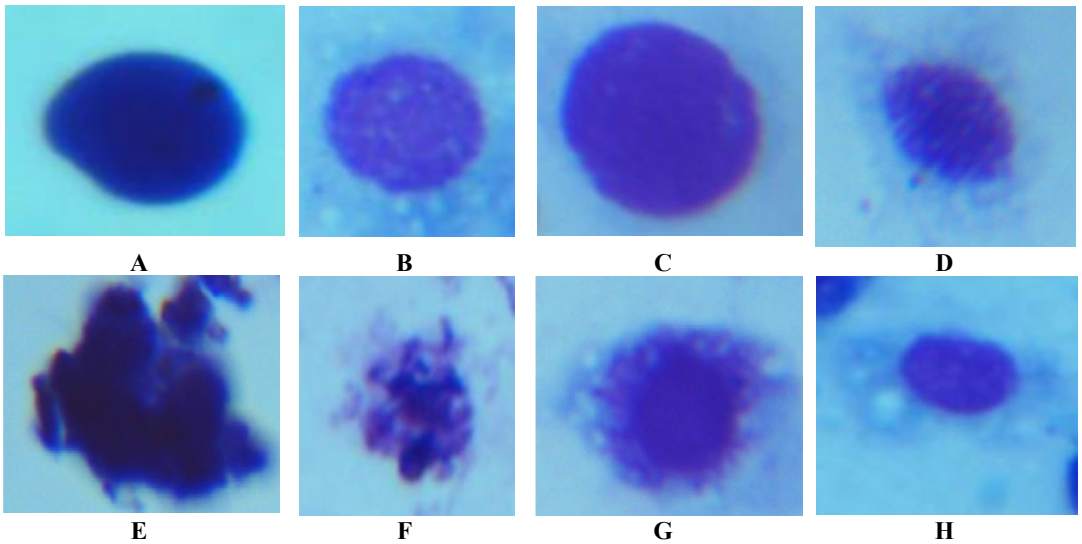

Fig. 5: Alteration in haemocyte morphology of last instar *Agrotis ipsilon* larvae exposed to 100 µg/ larva of juvenile hormone mimic "Ro 13-5223". A. Prohaemocytes, B. Plasmatocytes, C. Granular cells D. Spindle cells E. Adipohaemocytes F. Spherule cells G. Oenocytoids H. Cystocytes

Our results demonstrated that insect hormones induce certain abnormalities in the haemocyte cell types. These are in agreement with the morphological alternations and malformations of haemocyte *A. ipsilon* types observed by Abd El-Aziz *et al*. (2010). Sezer and Ozalp (2015) found that the juvenile hormone analogue pyripoxyfen caused reduction in cytoplasm and vacuolization of cells in *Galleria mellonella* haemocytes. Also, Sendi and Salehi (2010) described the pathological

symptoms influenced the haemocyte cell membrane, cytoplasm and nucleus of *Papilio demoleuse* after methoprene treatments. In addition, our foundations were similar with results obtained with *Sesamia cretica* (El-Mandarawy, 1997), *Palpita unionalis* (El-Mandarawy, 2005) and *Dysdercus koenigii* (Haq *et al*., 2005).

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