THE EFFECT OF METHOPRENE ON TOTAL HEMOCYTE COUNTS AND HISTOPATHOLOGY OF HEMOCYTES IN *PAPILIO DEMOLEUS* L. (LEPIDOPTERA)

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ABSTRACT: The Juvenile hormone analogue (JHA) methoprene (ZR-515) was employed for its effects on total hemocyte counts (THC) and pathological symptoms in *Papilio demoleus* L. The results show that the low dose (1µg/µL acetone) effects cause THC reduction in general and of PLs, ADs and SPs in Particular. The hitspathological symptoms were observed as changes in PLs form. However, the high dose (100µg/µL acetone) produced imperfect and perfect supernumerary larval instars whose THC declined considerably. Extreme pathological symptoms in cell membrane, cytoplasm and nucleus were observed. The possible significance of these changes are discussed.

KEY WORDS: methoprene, hemocytes, histopathology, *Papilio demoleus*

Very little work has been carried out on the role of endocrine organs and hormones on the hematology of insects. Injection of β-ecdysone into the posterior hormone–deficient–half of the mid–ligatured larvae of *Spodoptera litura* showed that THC, which was drastically reduced after ligature, sharply rose after injection of the hormone (Prasada Rao et al., 1984). THC count was reduced following treatment of fifth instar nymphs of *Dysdercus cingulatus* with β-ecdysone and makisterone A (a phytoecdysone) (Ahmad, 1995). Injection of triol (an analogue of molting hormone) and makisterone A in the fifth instar hopper *Hieroglymphus nigrorepletrus* produced pathological symptoms (Ahmad and Khan, 1988). Similar effects have also been observed earlier by synthethic insecticides (Arvy et al., 1950; Roy and Bagchi, 1975; Zaidi and Khan, 1977; Azam and Ilyas, 1986; Younes et al., 1999, Sabri and Tariq, 2004).

In contradiction to these results is the increase in THC by some insecticides (Khalid et al., 2001; Haq et al., 2005). Phytochemicals such as plumbagin produced surface deformities in all cell types and loss of filopods in some (Saxena and Tikku, 1990). Neem gold used on larval *Spodoptera litura* brought about histopathological changes and decrease in THC of some cell types (Sharma et al., 2003). It has been shown that treating the hemocytes of *Galleria mellonella in vitro* by 20-hydroxydysone (20-E) led to a dose-dependent decrease in total cell and granulocyte number (Izzetoglu and Karacali, 2003). One aspect of hemocyte function is encapsulation of invading foreign bodies where the number of hemocytes is a key factor in combating the organism. It has been shown that the process of encapsulation has been reduced on injection of JH to *Tenebrio molitor* L..

In the view of the above studies we tried to find out if there is any relationship between hemocyte number and increasing JH in hemolymph or there is any malfunction after JH incorporation in a holometabolous insect.

MATERIAL AND METHODS

Insect *Papilio demoleus* L. eggs were collected from lemon nurseries and bred in a controlled condition (28±2 °C, 16: 8 LD and % 65±5 RH), the hatched larvae were provided daily with fresh lemon leaves. Second day old Vth instar larvae were used for the experiment.

To study total hemocyte counts (THC) the hemolymph was drawn into a Thoma white blood cell pipette up to 0.5 mark and diluted up to the 11 mark with tauber–yeager fluid (Tauber & Yeager, 1934). The pipette was then shaken for several minutes and the first three drops were discarded. A double line with improved Neubauer ruling Hemocytometer was filed with diluted hemolymph and the hemocytes counted in its four corner and one central (1mm^2) squares under a microscope (Olympus, Japan). If the distribution of cells in all the squares were not even, the sample was discarded. The number of circulating hemocytes per cubic millimeter (mm^3) was calculated using the following formula of Jones (1962).

$$\frac{\text{Hemocytes in five } 1\text{mm}^2 \times \text{Dilution} \times \text{Depth factor of chamber}}{\text{No. of squares counted}}$$

Where dilution = 20 times, Depth factor of the chamber = 10 (constant) and No. of squares counted = 5.

For blood smear slide preparation, a small drop of heat–fixed hemolymph was obtained by clipping of the proleg present on the 7th abdominal segment of the larva or piercing the cuticle of the pupa. The drop was then drawn into a thin film by the edge of another slide and the film air–dried before staining. For staining, the stock solution of Giemsa stain prepared by the method of Yeager (1945) was diluted 10 times with distilled water. The air dried smear was stained with the diluted stain for 20 minutes and subsequently differentiated in dilute lithium carbonate solution for red staining structures and then in Hcl acidified distilled water for blue staining structures. The slide was rinsed in distilled water and mounted in DPX. To determine the DHC, cell categories were counted in 200 cells chosen from random areas of the stained blood smear by a laboratory blood cell counter.

The juvenile hormone analogue (JHA) Methoprene, (ZR-515), generously supplied by Dr. F. Sehnal of Institute of Entomology, Academy of sciences Czeck Republic, were employed in this study. It was diluted in acetone and applied by a micro-applicator on the dorsum of the posterior abdominal segment in doses 1 and 100 µg/µl of acetone. Controls received 1µl of acetone alone.

RESULTS

Low dose effects

Doses of 1µg/L did not interrupt normal metamorphosis of the insect but affected the THC and morphology of hemocytes. The THC was drastically reduced (Table1) with the decline in population mainly of the PLs, ADs and SPs. Besides, the PLs also lost their typical spindle shape to acquire a some what rounded form with an irregular boundary (Figs 1-4).

High dose effects

High doses interfered with the normal development of the insect and resulted in the production of imperfect and perfect supernumerary larval molts. Imperfect supernumerary larvae had a new larval cuticle below the old one but the insect failed to ecdyse, while the perfect supernumerary larvae had normally molted into larval 6th instars. Due to the tissue breakdown and the resulting turbidity in the hemolymph, the THC could not be determined in the imperfect supernumerary larvae. In the perfect supernumerary larva, the THC was determined in 1,2 and 3 day old larvae and it was found to be much lower not only to the corresponding stages but also to older stages of the normal 5th instar larva (Table 2).

Effect on hemocyte morphology

The juvenoid employed in this study seem to affect every part of the hemocyte, i.e., cell membrane, cytoplasm and nucleus. The most sensitive cells were found to be the PLs and GRs and the most resistant ones to be the oenocytes (OEs).

Some cells loose their smooth cell boundary to become irregular (Fig. 5). Surface projections are exhibited by the PLs where cell membrane shows distinct lobes due to parts of the cytoplasm projecting into it (Fig. 2).

Thinning of the cytoplasm, is mostly observed in the PLs (Fig. 5). Vacuolisation of cytoplasm : In the GRs, SPs and ADs (but not PLs and OEs), the cytoplasm get vacuolised. The vacuoles may fill the entire cytoplasmic area (Fig. 5).

Changes affecting nucleus are seen as eccentrically pushed nuclei, under this effect, the nucleus of the cells is pushed towards the periphery, sometimes accompanied by reduction in the nuclear size (Fig. 5). In nuclear expulsion, the nucleus is pushed almost beyond the broken cell boundary as if to be thrown out, is observed in the GRs (Fig. 7).

In the PLs and PRs a furrow seems to cleave the nuclei into two halves (Fig. 8). Cellular Clumping is another feature where large patches of cytoplasm are seen to include several nuclei. This seems to have resulted from the fusion of several cells and subsequent loss of their cell boundaries (Fig. 9).

DISCUSSION

Our previous study showed six types of hemocytes in *Papilio demoleus*. They are the prohemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), spherulocytes (SPs),oenocytoids (OEs), adipohemocytes (ADs) and two subtypes, the vermicytes (VEs) and podocytes (POs) (Jalali and Salehi, 2008). However in the present investigation only two major hemocyte types were considered based on their role in immunity i.e., plasmatocytes and granulocytes. They showed sensitivity to the presence of excess JH and their THC changed drastically with various morphological symptoms.

Rizki (1957, 1962) was perhaps the first to provide direct evidence of hormonal regulation of hemocytes activity in *Drosophila* subsequently, a number of studies have, by extirpation and implantation experiments or by application of hormones, shown that the endocrine organs indeed regulate hemocyte populations and differentiation (Hoffman, 1970; Judy and Marks, 1971; Prasada Rao, et al., 1984; Ahmad and Khan, 1988). However, there are very few papers on the effects of hormones or their analogues on hemocytes . One with ecdyson (Judy and Marks, 1971, Prasada Rao et al., 1984; Ahmad and Khan, 1988) and one with juvenoid

(Gupta, 1985). Of the papers with ecdyson, (Judy & Marks, 1971) shows an increase in the migratory activity of the hemoytes *in vitro*, the other (Prasada Rao *et al*., 1984), an in crease in the THC and the third one (Ahmad and Khan, 1985), production of certain pathological conditions in the hemocytes. About the work with juvenoid, Gupta (1985) injected a juvenoid into the last nymphal instar of cockroach and found a 50% reduction of hemocytes in the adult. Since the adult hemocyte count tallied with that of the nymphal count, he postulated that the analogue had a juvenilizing effect on these cells.

In the present study with the juvenoid, methoprene, there was found, significant reduction in the THC of the treated insects and since a number of pathological symptoms were also observed. Hence, this reduction could be due to the death of pathological cells by degeneration. The pathological symptoms produced by the analogue treated every component of the cells: cell membrane, cytoplasm and nucleus. These changes, interestingly , are similar to those produced by some of the insecticides (Yeager and Manson, 1942; Gupta and Sutherland, 1968, Zaidi and Khan, 1977; Azam and Ilyas, 1986 and Younes et al., 1999; Khalid et al., 2001; Haq et al., 2005) and exotoxines of some microorganisms (Venkova, 1972).

Phytochemicals like plumbagin and neem produced somewhat similar effects (Saxena and Tikku, 1990; Sharma et al., 2003). It would thus appear that the hormone analogues – both juvenoids and ecdysteroids affect hemocytes as toxins rather than as hormones, i.e., not the way, they affect (inhibit) development of tissues like epidermis and germ cells. The possible explanation to this differential action could be that in other tissues, the hormones may be acting at the genetic level (in the nucleus), in the hemocytes they seem to affect only the cellular contents, strikingly, in almost all cases studied, only the PLs and GRs have been found to be the most sensitive cells and OEs, the most resistant ones. They remain unaffected even when all other cell types show one or the other kind of pathological symptoms. Interestingly the PLs and GRs are also the main phagocytic hemocytes in most of the insects studied (Crossley, 1964; Akai and Sato, 1973). The reason for the greater hormone sensitivity of these cells could be that, being phagocytic they are prone to be attracted to any foreign substance including synthetic analogues, and these are likely to suffer greater exposure to hormones than other cell types . OEs being thick (Zaidi and Khan, 1977; Gupta, 1979) may resist penetration of the hormone and so remaining unaffected.

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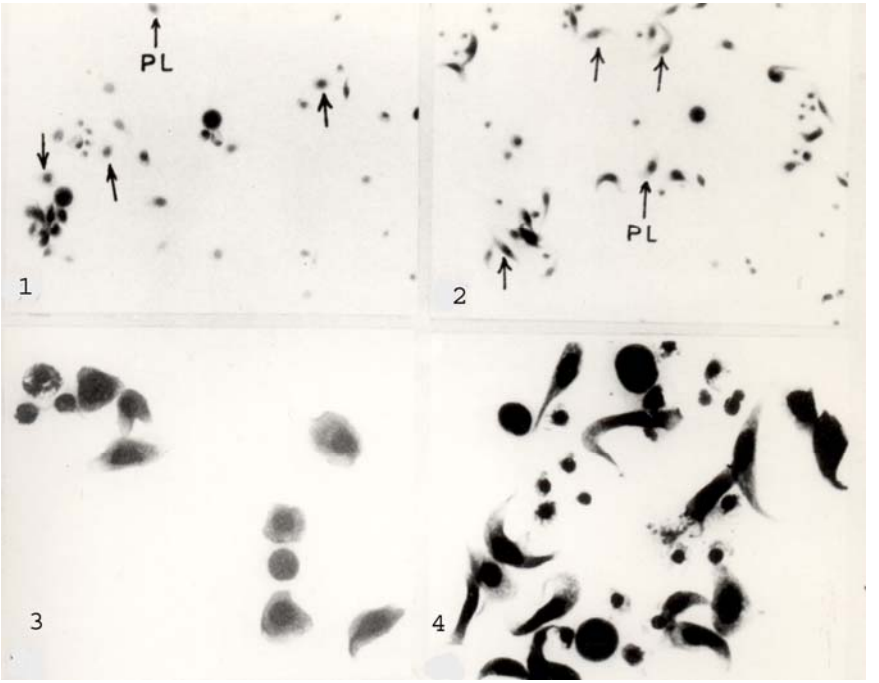
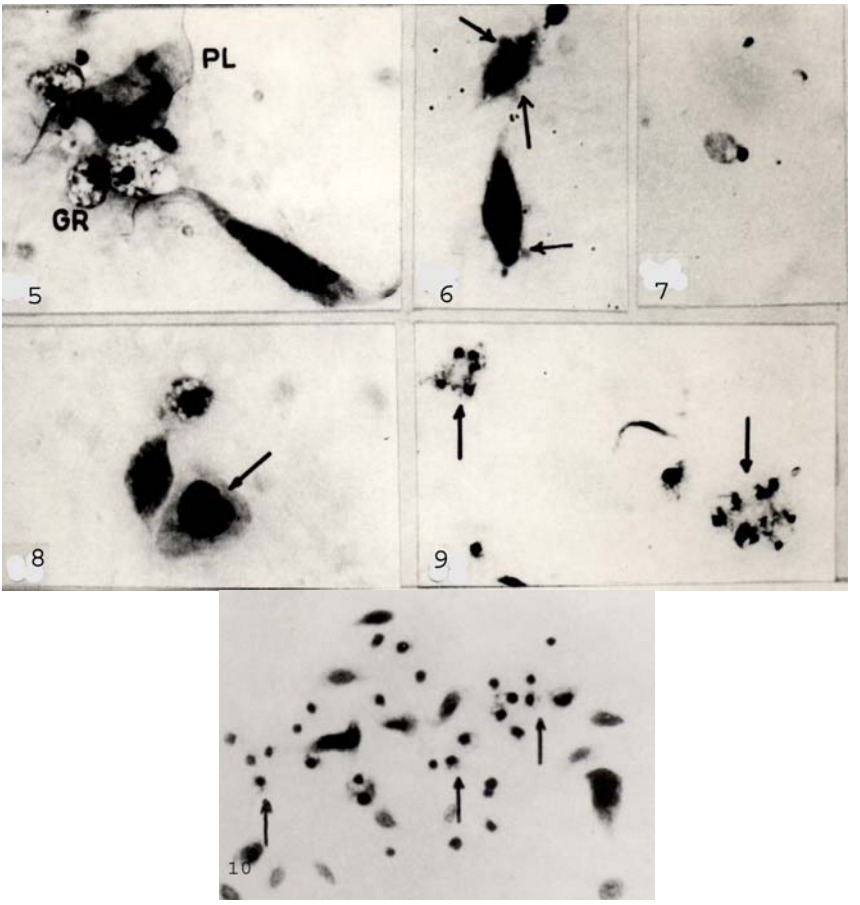


Figures 1-4. 1) Low dose JHA effects showing rounding up of PLs, X600. 2) Control of fig. 1. showing normal spindle-shaped PLs X600. 3) Low dose JHA effect in a magnified view to show irregularity in the cell boundary of rounded PLs. (oil), X1500. 4) Low dose JHA effect in a magnified view to show smooth cell boundary of spindle-shaped PLs. (oil) X1500.

Table 1. Low dose JHA effect on the THC.

Hours after Treatment	Insect No.	THC/mm³±SE		*P* values
		JHA- treated	Control	
24	10	7800.0 ± 406.4	9500 ± 423.0	<0.01
48 (prepupa)	10	13130.0 ± 1026.0	17864 ± 1264.6	<0.01
72 $(Pupa)_1$	10	3330.2 ± 312.3	5261 ± 316.7	<0.001
96 $(Pupa)_2$	10	2733.3 ± 394.5	4328 ± 763.5	Ns
120 $(Pupa)_3$	10	1472.8 ± 196.1	2255 ± 422.8	Ns

Figures subscripted to instars indicate age(Days) Ns. = Not significant



Figures 5-10. 5) High dose JHA effect showing irregular cell boundary and thinning of cytoplasm of PL, vacuolization of cytoplasm and eccentrically pushed nuclei in GRs (oil), X1500. 6) High dose JHA effect showing cytoplasmic bulges in the PLs (arrows) (oil), X1500. 7) High dose JHA effect showing nuclear expulsion possibly from a GR. X600. 8) High dose JHA effect showing lobed (cleaved) nuleus in a PL. (oil), X1500. 9) High dose JHA effect showing cell-clumping. X900. 10) Control showing normal GRs (arrows), X600.

Table 2. High dose JHA effect on the THC.

Days after Treatment	Insect No.	THC/mm^3±SE		P values
		JHA- treated (VI instar)	V instar* Control	
1	10	2223 ± 392.0	6505.4 ± 661.7423.0	<0.001
2	10	1983 ± 245.3	9440.0 ± 822.7	<0.001
3	10	1245 ± 198.5	8616.0 ± 539.8	<0.001

* Since there could not be a control for the supernumerary (VI) instar, the V instar data are included for comparison.