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Influence of Metyrapone on the Morphology of Hemocytes of the Egyptian Cotton Leafworm *Spodoptera littoralis* (Boisd.)

Ivan Gelbič[1,*], Jana Strbáčková[1], and Josef Berger[2]

[1]*Institute of Entomology AS CR, Department of Morphology, Branišovská 31, 370 05 České Budějovice, Czech Republic*
E-mail:gelbic@entu.cas.cz

[2]*Faculty of Health and Social Studies, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic*

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Ivan Gelbič, Jana Strbáčková, and Josef Berger (2006) Influence of metyrapone on the morphology of hemocytes of the Egyptian cotton leafworm *Spodoptera littoralis* (Boisd.). *Zoological Studies* **45**(3): 371-377. The morphology of hemocytes in *Spodoptera littoralis* (Boisd) (Lepidoptera: Noctuidae) and the effects of metyrapone on the differential count were studied. Four types of hemocytes were found in the hemolymph of *S. littoralis*: plasmatocytes, granulocytes, spherulocytes, and oenocytoides. The proportion of hemocytes slightly changed during development, but plasmatocytes were the most numerous in all studied instars. Application of metyrapone caused developmental changes accompanied by variations in the differential count (i.e., the relative count) of granulocytes. Metyrapone induced higher metabolism in comparison with untreated specimens and resulted in precocious larval-pupal transformations. The body weight of treated larvae was higher, and the length of the last larval instar was shorter by 3 d in comparison with control animals. The significant decrease of granulocytes in the last larval instar treated with metyrapone simulated the differential count in older control animals. Observed changes following metyrapone administration were similar to the induction of a faster process of aging. http://zoolstud.sinica.edu.tw/Journals/45.3/371.pdf

Key words: Hemocyte, Morphology, *Spodoptera littoralis*, Development, Metyrapone.

Many moths of the family Noctuidae (Lepidoptera) are serious pests of agriculture and forestry. Some of them are also interesting from a theoretical point of view. Caterpillars of some migratory species vary in color dependent on their population density. Faure (1943a b) suggested that these larvae may form gregarious and solitary phases as do locusts. The parallelism with locusts indicates the hormonal control of phases in noctuids. This was confirmed by Sehnal et al. (1976), who described the effects of juvenoids on egg development, larval-pupal transformation, and body color. Juvenoids also prolong the feeding period, growth, total body metabolism (Kryspin-Sörensen et al. 1977) as well as nutrient content (Gelbič and Němec 1978) probably due to delays in larval development. Later, Gelbič and Sehnal (1986) found in the Egyptian cotton leafworm, that induction of the perfect supernumerary larval instar by application of juvenoid depended on sex, with about 90% of the supernumerary larvae being females. Induction of an extra larval stage in male larvae is rare. The majority of juvenoid-treated 12-h-old last instar larvae develop into various types of intermediate forms. The effects of the compounds mentioned above on the morphology of hemocytes and their differential count have not previously been studied.

Morphological characteristics of hemocytes of *S. littoralis* were studied by Harapaz et al. (1969) and Stettler et al. (1998) with different findings as to hemocyte types: Stettler observed spherulocytes but did not find adipocytes.

The economic significance of noctuids, especially of the Egyptian cotton leafworm, *Spodoptera littoralis*, calls for a detailed examination of the

* To whom correspondence and reprint requests should be addressed.

effects exerted by biologically active compounds on these insects. Various natural and synthetic compounds with different chemical structures show very broad spectra of biological activities in insects. In our study, we show some portions of the broad spectrum of effects of metyrapone on the development of larvae, on hemocyte morphology, and their differential counts.

MATERIALS AND METHODS

Experimental insects

The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) (Noctuidae: Lepidoptera), were reared at 25 ± 1°C, in 75% ± 10% relative humidity, with a 16L: 8D photophase, and were fed an artificial diet (PREMIX, prepared by Stonefly Industries, Bryan, TX, USA). Rearing of the moth was described in detail by Sehnal et al. (1976). Larvae, pupae, and adults were used in our experiments. In each test group, 50 larvae were used in 3 replications.

Tested chemicals

Our experiments were performed with metyrapone (purchased from Aldrich, U.S.A.). Metyrapone was diluted or emulsified by sonication in sterile distilled water and injected (2 μl/larva) into anesthetized larvae of the penultimate or last instar of the Egyptian cotton leaf worm. The applied doses were 0.2, 2, and 20 μg/specimen.

Observations

Experimental larvae of *S. littoralis* were reared in Petri dishes (in dishes with a diameter of 6 cm, 1 larva/dish was used, in those with a diameter of 10 cm, 5 larvae/dish were used). The following parameters were assessed: durations of the larval instars, changes in body weight in control and treated larvae, pigmentation, morphological changes in pupae and adults, the number of eggs laid and their hatchability, and the morphology of hemocytes and their differential counts in different developmental stages.

Hematology

Hemocytes were stained using the Pappenheim panoptic method for light microscopy. Samples for electron microscopy were fixed in 2.5% glutaraldehyde for 24 h, incubated in OsO_4 for 2 h, and then stained with uranyl acetate for 30 min and lead citrate for 20 min. The ultrastructure was studied using a Jeol 1010 Jem electron microscope (Jeol, Japan).

Statistical analysis

Results are expressed as the mean ± S.E.M. and evaluated by the two-sided Mann-Whitney U test at a significance level $2\alpha = 0.05$.

RESULTS

Normal development

Embryonic development lasted 4 days. Larval development was divided into 6 larval instars. The last larval instar could be divided into 2 stages: a period of growth and the prepupal stage. The growth or feeding period is the time of the maximum rate of growth. During this time, larvae consumed a large amount of food, and the

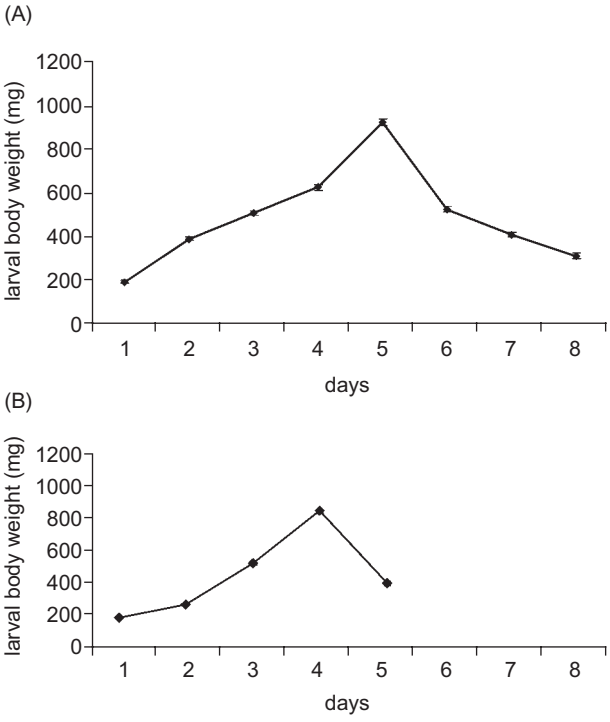

Fig. 1. Effects of metyrapone on the growth rate of the last larval instar of *Spodoptera littoralis* (Boisd.). (A) Untreated control; (B) metyrapone-treated animals (injected with 20 μg/larva). Vertical bars represent standard deviations from the mean values.

body weight rapidly increased (Fig. 1A). The duration of the feeding period was 5 day. On the last day of the feeding period, the larvae reached maximal body weight and size (Fig. 1A). This was followed by the prepupal stage (3 day). In this stage, the body weight decreased to 1/3 of the maximal larval weight. The pupal stage of females lasted 8 day. The pupal stage of males was longer by 2 day. The Egyptian cotton leafworm is a typical proterogynic species.

Effects of metyrapone

Applications of metyrapone to freshly molted larvae of the last instar caused a significant increase in body weight (Fig. 1B). In comparison with the control animals, the feeding period was shorter. The maximal body weight of treated larvae was reached 1 or 2 day earlier (depending on the dose used). Treated larvae were bigger than the control animals. After application of metyrapone, the prepupal stage was also shorter. The length of the last larval instar in treated larvae was 5 day, and in control larvae was 8 day. The length of the last larval instar and the weight of fresh pupae depended on the applied dose (Table 1).

Changes in metabolism of the experimental larvae were accompanied by changes in body pigmentation. The control larvae were black. The tested larvae were light gray or light green. The same pigmentation was observed after application of metyrapone to larvae of the penultimate instar. Application of metyrapone to the larvae of the penultimate instar induced shortening of the subsequent last larval instar.

Morphological changes were negligible. Only a few larvae molted into malformed pupae. Malformed adults were not observed in *S. littoralis*. However, it was observed that there was a decrease in the number of eggs laid and their hatchability (Table 1). Eggs laid by treated females were smaller than those of control females.

Hematology

We found hemocytes to be composed of plasmatocytes, granulocytes, spherulocytes, and oenocytoids (Fig. 3). Granulocyte (see Fig. 3-*Gr*) cytoplasm on transmission electronograms was characteristically granular, with extensive endoplasmic reticula and large cisternae. Plasmatocyte cytoplasm is abundant and rich in endoplasmic reticu-

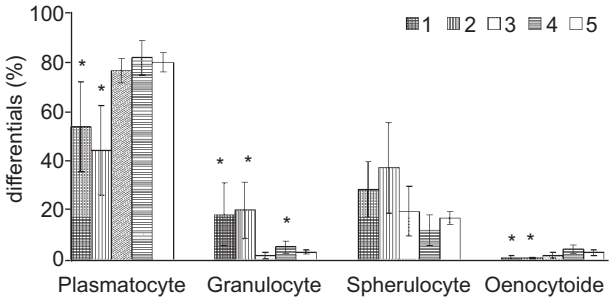

Fig. 2. Changes in the proportions of different types of hemocytes during the development of *Spodoptera littoralis* (Boisd.). (1) Third larval instar; (2) 4th larval instar; (3) 5th (penultimate) larval instar; (4) 6th or last larval instar; (5) pupae. * - indicates significance as compared with 5th penultimate instar, using the Mann-Whitney test, $p < 0.05$. Vertical bars represent standard deviations from the mean values.

Table 1. Effects of metyrapone on last larval instar and egg hatchability of *Spodoptera littoralis* (Boisd.)

Compound	Dose µg/larva	No of treated larvae	Treated instar (days)	Duration of instar	Pupal weight (mg)	No of death		No of intermediates	No of adults		Hatchability of eggs %
						L	P		♂	♀	
untreated		150	5	3.4 ± 0.5	270.5 ± 23.6	4	3		71	72	90
		150	6	8.1 ± 0.6	275.6 ± 32.1	3	9		63	75	85
water	2 µl	150	5	3.2 ± 0.4	278.6 ± 34.2	4	6		69	71	90
		150	6	8.2 ± 0.4	280.9 ± 43.7	2	5		74	71	90
metyrapone	0.2	150	5	3.1 ± 0.3	298.7 ± 33.3	7	9	1	62	71	50
		150	6	7.6 ± 0.7	321.7 ± 32.6	11	8	3	67	61	40
	2	150	5	3.2 ± 0.4	334.6 ± 25.7	9	14	5	59	63	10
		150	6	6.1 ± 0.4	356.7 ± 45.9	18	10	7	61	54	0
	20	150	5	3.1 ± 0.2	409.3 ± 46.7	12	7	2	60	69	0
		150	6	5.1 ± 0.2	453.2 ± 54.3	26	12	4	51	57	0

la, while electron-dense granules were absent or few. Spherulocyte (Fig. 3-*Sp*) cytoplasm contained large spherules, whose contents were in a circular structure. The structure of rare, unidentified cells was similar to that of plasmatocytes (Fig. 3-*Pl*), but they were larger; their morphology was generally similar to that of oenocytoids (Fig.3-*Oe*). We observed no differences in the morphology of hemocytes among larval instars.

The proportions of the various hemocyte types in untreated *S. littoralis* hemolymph changed during the course of development (Fig. 2). We found an increase in relative plasmatocyte counts simultaneously with larval growth; the augmentation of this characteristic in 5th instars (77%) was statistically significant compared with that found in 4th instars (43%). The change in the granulocyte proportion was opposite (from 20% to 2%). Spherulocyte relative counts fluctuated: decreasing from 36% in 4th instars to 19% in 5th instars, then to 11% in 6th instars, followed by a significant increase to 17% in pupae. The incidence of oenocytoids was low in all stages, at around 1%.

We also observed fluctuations in hemocyte counts during the last larval instar (Table 2). From days 4 to 7, the proportion of plasmatocytes in intact larvae in this instar did not significantly change (from 79.3% ± 6.6% to 71.2% ± 12.5%), as with granulocytes (from 6.4% ± 4.6% to 3.1% ± 1.68%) and oenocytoids (from 1.4% ± 0.8% to 2.3% ± 1.8%), but the proportion of spherulocytes slightly increased (from 12.2% ± 6.1% on day 5 to 18.2% ± 1.9% on day 6 and 23.3% on day 7). Pupation of the control animals began on day 8.

No morphological changes were observed in hemocytes after metyrapone administration. No significant differences were found when data from sham-treated and intact animals were compared. A significant reduction in granulocyte counts was found in the 6th instar 2 day after metyrapone administration (8%), as well as an increase in spherulocyte number (23%) compared to 22% granulocytes and 14% spherulocytes observed in the controls. Pupation of metyrapone-treated larvae began on day 5.

DISCUSSION

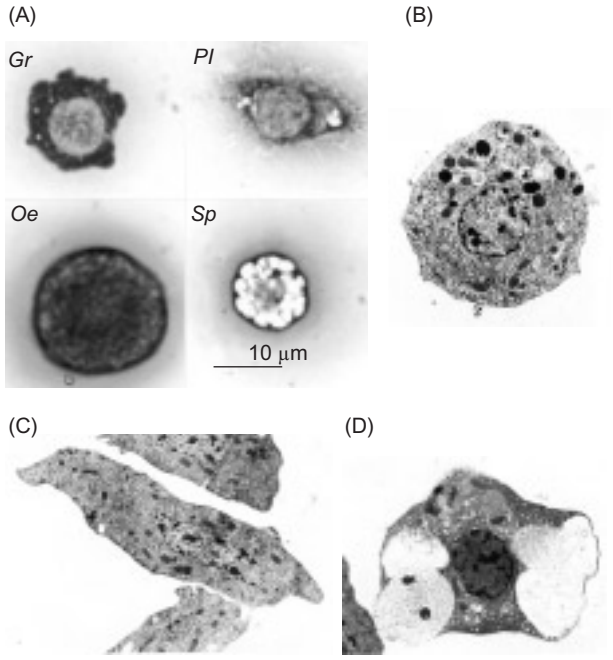

Fig. 3. Morphology of hemocytes of *Spodoptera littoralis* (Boisd.). (A) Different populations of hemocytes detected by the Pappenheim panoptic stain. (*Gr*) Granulocyte; (*Pl*) plasmatocyte; (*Oe*) oenocytoid; (*Sp*) spherulocyte. The bar indicates 10 μm. (B) Electron micrograph of a granulocyte; (C) electron micrograph of a plasmatocyte; (D) electron micrograph of a spherulocyte.

Table 2. Effect of metyrapone on the differencial count of haemocytes in the last larval instar of *Spodoptera littoralis* (Boisd.)

Group	Type of haematocytes	Day 1	Day 2	Day 3	Day 4
Controls	plasmatocyte	57.5 ± 19.9	63.1 ± 9.0	76.2 ± 8.2	83.8 ± 4.7
	granulocyte	24.8 ± 2.1	21.8 ± 4.4	12.2 ± 5.2	4.8 ± 1.5
	spherulocyte	16.2 ± 8.6	14.0 ± 6.8	9.3 ± 3.9	8.8 ± 3.8
	oenocytoide	1.5 ± 1.3	1.3 ± 0.4	2.3 ± 1.0	2.6 ± 1.4
Metyrapone	plasmatocyte	54.2 ± 11.7	67.8 ± 9.3	80.0 ± 4.7	81.0 ± 6.6
	granulocyte	27.9 ± 9.7	7.6 ± 3.6	4.8 ± 1.7	4.6 ± 3.8
	spherulocyte	16.0 ± 4.5	22.8 ± 7.3	13.0 ± 5.1	12.9 ± 8.4
	oenocytoide	2.0 ± 0.4	1.8 ± 1.1	2.3 ± 1.3	1.4 ± 1.2

We observed a distinct spectrum of hemocytes in *S. littoralis* as compared with the data published by Abdel-Rahman and co-workers (2000). We classified cells with a morphology close to the so-called spindle cells as plasmatocytes, because the spindle form was the only characteristic which differed from round or oval plasmatocytes; spindle cells may be a reactive (active) form of these cells. We observed no adipocytes. We supposed that the cystocytes described by Abdel-Rahman et al. (2000) were damaged spherulocytes; oenocytoids in their published figure are rather small spherulocytes compared with Gupta's (1979) criteria for hemocyte classification. Using Gupta's criteria, several spherulocytes in Abdel-Rahman's figures may actually be large granulocytes. We confirm the findings of Harapaz et al.'s electron microscopic studies (Harapaz et al. 1969), describing 4 hemocyte types in *S. littoralis*, although we did not observe typical oenocytoids with the morphology described by Gupta (1979).

In addition, we confirm findings of Settler et al. (1998), as we found spherulocytes and no adipocytes (Harapaz et al. 1969). We observed that hemocyte differential counts fluctuated not only as a consequence of different instars but also within a given instar. These changes may be a result of developmental processes. The fact that we observed no differences between untreated and sham-treated animals documents that comparative hemotoxicological studies performed under standardized laboratory breeding conditions are meaningful (cf. Berger 1987, Berger et al. 2003).

Our hematological data showed changes in the differential counts after metyrapone treatment, and these changes simulated an acceleration in the aging process: the hemolymph picture in 6th instars on day 2 after metyrapone treatment was close to that on day 7 in the controls, although a reverse trend toward the control values followed on days 3 and 4. This acceleration of development of the last instar was also confirmed by the findings that metyrapone-treated animals completed their last larval instar after 4 day, while control animals needed 7 day.

The results of this study demonstrate that metyrapone influenced some metabolic processes which resulted in a significant increase in body weight and precocious pupation. These effects were dose dependent. Higher doses (> 20 μg/specimen) were toxic, while lower doses influenced larval development, pigmentation, and reproduction (Table 1). In treated larvae, the feeding period was shorter. The body weight of treated larvae was evidently higher compared with control ones as a consequence of more-intensive feeding and metabolism. The same results were obtained by Gelbič and Němec (1978) and Kryspin-Sörensen et al. (1977) when applying methoprene; in those cases, however, the feeding period was longer than that of the control animals. Juvenoids also induce prolongation of the prepupal stage. In contrast, metyrapone caused a significant shortening of this stage by 1/3 of the normal length (3 d).

Comparing the effects of juvenoids, which have an action similar to that of metyrapone, revealed that metyrapone obviously acted by inhibiting the activity of juvenile hormone or its secretion (Gelbič and Němec 2001). However Darvas et al. (1991) observed in *Neobellieria bullata* that metyrapone and its analogues cause disturbances to pre-ecdysial processes, delay larval development due to inhibition of molting, and decrease the weight of tested insects. These differences are probably due to different enzymatic systems and different interactions of metyrapone with the body's ecdysteroids, which may depend on the insect species tested. This suggestion is supported by the fact that metyrapone reduced fertility. The number of eggs laid was lower, and the eggs were smaller. Metyrapone also inhibited synthesis of vitellogenins (ie., proteins which are precursors of vitelin) or their transport with a consequent decrease in the number of eggs laid and their hatchability. This fact is in congruence with the suggestion made above that metyrapone inhibits the action of juvenile hormone. Juvenile hormone is necessary for synthesis of vitellogenins. The metabolic interactions exerted by metyrapone will be analyzed in our next paper.

Although the low doses of metyrapone that were injected in the course of this study induced only slight disruptions in the larval-pupal transformation of *S. littoralis* larvae, they were sufficient to inhibit melanin synthesis; larvae were light gray or creamy-white. A similar change in pigmentation was described by Sehnal et al. (1976) in larvae of *S. littoralis* when juvenoids were applied. With regards to metyrapone, Darvas et al. (1987) discovered that this compound and its derivatives interfere with P-450-dependent monooxigenases (MFOs) which also participate in the synthesis of melanin and ecdysteroids.

Many papers have examined the antifeeding effects of hormonal antagonists (Rembold et al. 1982, Schlüter et al. 1985, Malazewska et al. 1988). We observed no antifeeding effects in lar-

vae of *S. littoralis* injected with metyrapone. For an antifeeding effect to occur, it might be necessary to apply higher doses than we used. On the other hand, when considering the fact that metyrapone significantly decreased fertility at doses below 20 μg/specimen, the use of these substances for integrated pest control seems to have good potential.

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