Title
Effects of Two Chemosterilants, Metepa and Hempa, on the Hemolymph Proteins in the Last Instar Larvae and Pupae of the Smaller Citrus Dog, Papilio xuthus LINNE

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Citation
防虫科学 (1971), 36(3): 105-110

URL
http://hdl.handle.net/2433/158689

Type
Departmental Bulletin Paper

Textversion
publisher

Kyoto University

Botyu-Kagaku 36, 105, (1971)

Introduction

In recent years, the effect of chemosterilants on the external form of pupae in Lepidoptera has been mainly investigated on the silkworm, *Bombyx mori* L. (Hirano 1965, Sugai and Hirano, 1967). Hirano (1965) reported that degeneration of antennae, legs and wings in deformed pupae, were induced by apholate administered orally to larval stages of the silkworm. 

Similar results were also obtained in this laboratory using pupae of the smaller citrus dog, *Papilio xuthus* L., in which metepa was administered orally to insects in the last larval instar. However, no deformed pupae were observed when hempa was administered orally to the smaller citrus dog (Unpublished data). The results of this work suggest that there may be differences in the action of these two chemosterilants to some insects.

The present investigation was undertaken to investigate whether the two chemosterilants have a similar effect on the hemolymph proteins of the last instar larvae and pupae of the smaller citrus dog.

Materials and Methods

*Chemicals and Applications;* Metepa (Tris (2-methyl-1-aziridinyl)-phosphine oxide) and hempa (Hexamethylphosphoric triamide) were dissolved in distilled water at concentrations of 1,000 pg, 4,000 pg and 8,000 pg per 0.1 ml immediately prior to treatment. 

*Insect;* Female adults of the smaller citrus dog were collected from the field in Kikugawa and reared in the laboratory at a temperature of 25°C, relative humidity of 60% and all day illumination (24 hour light). Eggs of these adults were reared on leaves of the mandarine orange, *Citrus unshiu* Marcos. Chemicals were administered orally to the larvae of fifth instar at several ages at a rate of 0.1 ml per larva by means of a microsyringe. Treated larvae were reared on the orange leaves until they were used for electrophoretic studies.

*Electrophoresis;* Hemolymph and testis were
collected from the male larva of 4-day-old, fifth instar larvae treated with chemicals. Hemolymph was obtained from 7th dorsal segment of the larvae (Kitagaki et al., 1970). A pair of testes was removed as quickly as possible, was homogenized in 20 μl of veronal buffer, pH 8.6 with a glass homogenizer, and the resulting homogenate was centrifuged at 8,000 rpm for 4 minutes. The supernatant was used for the electrophoretic investigations.

The electrophoresis was carried out as described by Kitagaki et al. (1970), using the following buffer system and method.

Agar…………………………………… 400mg
P. V. P. (Polyvinylpyrrolidone, K-90)…………200mg
Veronal buffer, pH 8.6, μ=0.025…………40ml
Size of a glass plate…………………… 50 × 200mm
Thickness of gel layer…………………… 1mm
Constant current……………………….2.5mA/cm
Time of electrophoresis…………………60min.
Temperature of electrophoresis…………5°C

A sample of 2 μl was applied to a short slit of 10mm width on the agar gel plate by means of a microsyringe and subjected to electrophoretic separation. Proteins were detected by Amido Black 10B staining under conditions recommended by Yushima and Kamano (1964). Each fraction was determined by a densitometer, QUICK of Atago Optical Works Co., Ltd., using 650 μν and a slit width of 8mm. Human serum (Moni-Trol 1, Dade Reagents Inc.) was used as a standard.

**Results**

As the first step, a relationship between the deformed pupae and concentrations of two chemosterilants was studied. When three different doses of metepa and humpa were orally administered to newly molted 5th instar larvae of the smaller citrus dog, abnormal pupae were classified into 8 ranks according to the degree of deformation of the pupa as shown in Table 1, that is 1, 2, … 7 and normal pupa shown as 8.

As shown in Table 1, it was very clear that metepa treatment with doses higher than 4,000μg per larva greatly induced the formation of deformed pupa. Following treatment with 8,000μg of humpa the larvae did not grow and thereafter died. In order to elucidate the difference of action between metepa and humpa, as shown in Table 1, electrophoretic patterns of the proteins of hemolymph and testes from larvae and pupae orally treated with the two chemosterilants after the 4th moulting larva were investigated.

**The effect on hemolymph proteins of larvae:**

In a previous paper, the authors (1970) reported that main four protein fractions could be found in the hemolymph of untreated 4day-old,

<table>
<thead>
<tr>
<th>Chemicals*</th>
<th>Dosage (μg/larva)</th>
<th>Rank</th>
<th>No. of Larvae</th>
<th>No. of Kills</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Humpa</td>
<td>8,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>1,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Metepa</td>
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<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4,000</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Chemicals were orally administered at larvae.
fifth instar. These fractions were named $A_1$, $A_2$, $G_1$ and $G_2$, starting with the designation of $A_1$ for the most rapidly migrating component.

In this investigation, four main fractions were again detected in the hemolymph of untreated 4 day-old, fifth instar larvae. However, as shown in Figure 1, fraction $G_1$ decreased markedly and fraction $G_2$ decreased slightly, when 4,000 $\mu$g of hempa or metepa and 1,000 $\mu$g of metepa were orally administered to larvae immediately after the fourth moult. Following treatment with 1,000 $\mu$g of hempa the decrease of fraction $G_1$ was scarcely noticeable. Fraction $G_2$ in hempa decreased more than the metepa and untreated.

When 1 day-old, fifth instar larvae were treated with 4,000 $\mu$g or 1,000 $\mu$g of metepa per larva fractions $G_1$ and $G_2$ were more obvious than in the case of larvae treated immediately after the 4th moult. It is thought that these fractions might be the amount of protein which was accumulated in hemolymph before the treatment of metepa.

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**The effect on proteins in hemolymph of pupae:**

The electrophoretic patterns of proteins in the hemolymph of pupae following oral administration of 4,000 $\mu$g of metepa or hempa per larva immediately after the 4th larval moult are shown in Figure 2.

Fraction $G_2$ on the hemolymph of 1 day-old pupa decreased substantially were following hempa treatment than metepa treatment and was smaller than in the controls. Similar results were observed in 3 day-old pupa. In the pupal stage, only one fraction $A_1$ was found. Fractions $A_1$ and $A_2$ which were detected in 4-day-old fifth instar larvae were not observed. There was no significant difference between the hempa and metepa treatments in the amounts of fraction $A$. However, this fraction decreased slightly compared to the untreated.

**The effect on proteins in the soluble fraction of the testes:**

Electrophoretic patterns of proteins in soluble fraction of the testis obtained from 4 day-old
Discussion

The above observations revealed that metepa and hempa treatment causes changes in the protein content and A/G ratios in the hemolymph of larvae and pupae of the smaller citrus dog. Palmquist and Lachance (1966) demonstrated a sterilization effect in the parasitic wasp, *Bracon hebetor* Say (Habrobracon), treated with the alkylating agent, tepa and its nonalkylating analog, hempa. These compounds induced a high frequency of recessive lethal mutations in the sperm of this insect, although tepa was the more efficient mutagen. Sugai and Hirano (1967) reported that apholate inhibited markedly the growth of the wings in pupae of the silkworm, *Bombyx mori* Linnae, Sharma and Rai (1969) also suggested that there are similarities between the action of apholate and the synthetic juvenile hormone.

On the other hand, Kitagaki et al. (1970) reported that G1 and G2 fractions in hemolymph of the smaller citrus dog were corresponded to globulins in the rice stem borer, *Chilo suppressalis* Walker, hemolymph and human serum. Koike (1962) suggested that G1 fraction of globulins in the rice stem borer have an important physiological function.

The A/G ratios of untreated 4 day-old, fifth instar larvae and of pupae were 1.54 and 11.50 to 11.82, respectively, as shown in Table 2. However, the A/G ratio of treated larvae changed substantially due to the decrease of G1 and G2 fractions. The effect of metepa on the globulin content was more marked than that from hempa, at high dosages. The A/G ratio in hemolymph of hempa-treated pupae was changed by the decrease of the G1 fraction and the increase of A fraction, although the hempa treatment did not induce any deformed pupae.

From these results, it is suggested that metepa and hempa may block the synthesis of the G1 and G2 protein fractions in this insect. Metepa was more effective than hempa in decreasing the G1 fraction but hempa was more effective than metepa with respect to decreasing the G2 protein content.
Fig. 1. Electrophoretic patterns of the protein from the soluble fraction of larval testes of smaller citrus dogs.

* Chemicals were orally administered to newly emerged or 1-day-old, fifth instar larvae.

The testis was taken from 4-day-old fifth instar larvae.

(1) Untreated
(2) Hempa, 1,000 µg/larva
(3) Hempa, 4,000 µg/larva
(4 and 6) Metepa, 1,000 µg/larva
(5 and 7) Metepa, 4,000 µg/larva

Table 2. Protein electrophoresis of larval and pupal hemolymph of smaller citrus dogs.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Dosage (µg/larva)</th>
<th>Time of Treatment</th>
<th>Time Collected Hemolymph</th>
<th>Days from Treatment of Collected Hemolymph</th>
<th>Percent of Total Protein</th>
<th>A/G Ratio</th>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Untreatment</td>
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<td>0**</td>
<td>4***</td>
<td>4</td>
<td>22.6</td>
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<td>61.50</td>
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<td>11.82</td>
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<td>—</td>
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<td>8.26</td>
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</table>

* Chemicals were orally administered at larval stage after the 4th moult.
** Days after the 4th moult.
*** Days after the 4th moult.
**** Days after the pupation.
The present work was undertaken to investigate the effect of metepa on the hemolymph protein of the last instar larva and pupa of the smaller citrus dog, *Papilio xuthus* Linne.

In this investigation four main fraction A₁, A₂, G₁, and G₂ were detected from hemolymph of untreated larvae in 4-day-old fifth instar larvae. At 4,000 µg of metepa and hemepa, fraction G₁ decreased remarkably and fraction G₂ decreased slightly. At a dosage of 1,000 µg of metepa, fraction G₂ was also observed to decrease but the decrease in fraction G₁ was hardly noticeable with hemepa. Fraction G₂ in hemepa decreased more than the metepa and untreated.

In hemolymph of 1-day-old pupa fraction G₂ clearly decreased following hemepa treatment and this was more than that following treatment with metepa.

Only one protein fraction was found in the soluble fraction of the testis in larval and pupal stages. This fraction did not change after treatment with the chemosterilants and the nature of this protein fraction remains unknown.

The A/G ratio of untreated larvae of 4-day-old, fifth instar and pupae was 1.54 and 11.50 to 11.82 respectively. The A/G ratio of treated larvae changed substantially due to the decrease in the G₁ and G₂ fractions.

**Acknowledgements**: We wish to express their gratitude to Dr. C. F. Wilkinson, Department of Entomology, Cornell University of New York for his valuable criticisms and for reading the manuscript. We also wish to thank, Dr. H. Koike, National Institute of Agricultural Sciences Tokyo and Dr. S. Nagasawa, FAO Agricultural Officer at the Biological Institute of Sao Paulo, Brazil for their valuable suggestions.

**Summary**

The present work was undertaken to investigate the effect of metepa on the hemolymph protein of the last instar larva and pupa of the smaller citrus dog, *Papilio xuthus* Linne.

In this investigation four main fraction A₁, A₂, G₁, and G₂ were detected from hemolymph of untreated larvae in 4-day-old fifth instar larvae. At 4,000 µg of metepa and hemepa, fraction G₁ decreased remarkably and fraction G₂ decreased slightly. At a dosage of 1,000 µg of metepa, fraction G₂ was also observed to decrease but the decrease in fraction G₁ was hardly noticeable with hemepa. Fraction G₂ in hemepa decreased more than the metepa and untreated.

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**References**