

## Effect of SSP - 11 on Weight and Tissue Composition of 4th Instar Larvae of Silkworm, *Bombyx mori*.<sup>1</sup>

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### ABSTRACT

The anti-juvenoid, SSP-11 ((E) 4-chloro-a, a, a-trifluoro-N [1 (1H-imidazole-1-yl)-2-propoxy ethylethene] O, toluene) was used to induce trimoulters from tetramoult larvae of the silkworm, *Bombyx mori* (race NB7). The compound was fed for two days through an artificial diet at the concentration of 200 ppm to newly ecdysed 4th stadium larvae. Body and silk gland fresh weight were recorded daily in the 4th larval stadium and protein profiles of haemolymph, fat body and silk gland were determined at the end of the stadium. In larvae treated with SSP-11, feeding period in stadium 4 was prolonged by 3-4 days and 80-85% of the larvae started to spin as trimoulters. Eight and thirty fold increases in the larval body and silk gland weights respectively were the result of SSP-11. Further, total proteins, total carbohydrates, glycogen and nucleic acid (DNA & RNA) also increased. The activity levels of alanine aminotransferase and aspartate aminotransferase increased significantly than in the control indicating increased mobilization of amino acids into transamination activities.

### Introduction

The role(s) of different juvenile hormone analogues (JHAs) viz., methoprene, triprene, and diflubenzuron in regulating growth and development in the silkworm, *Bombyx mori* have been established (Washida 1984, Gaaboub et al. 1988). They prolong the feeding period of 5th stadium larvae and increase silk protein synthesis (Washida 1984). On the other hand precocious metamorphosis could be induced by introducing chemical compounds with anti-JH bioactivity (-Akai et al. 1984, Gu et al. 1988). Kuwano et al. (1983) reported that certain biologically active terpenoid imidazole compounds when administered topically to 8 h old 3rd stadium larvae show

anti-JH activities in silkworm. SSP-11 and KK-42 were reported to be anti-juvenoids and work more effectively for the induction of trimoultization in silkworm when applied to 3rd or 4th stadium larvae (Akai et al. 1984, Kiuchi et al. 1985) where the larvae undergo moulting only three times in place of normal four moults. Several studies concerning larval development, cocoon production, quality of silk fibre and fecundity in silkworm after treatment with anti-juvenoids have been reported (Akai et al. 1984, 1986, Kiuchi et al. 1985, Kimura et al. 1986, Gu et al. 1988).

The available literature on the effect of anti-juvenoids in insects is confined to larval growth, cocoon production and fecundity. Its involvement in the weight and tissue composition remains to be explored. Therefore, the present study has been undertaken in order to assess the impact of SSP-11 on the weight and composition

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of selected tissues of silkworm, such as haemolymph, fat body and silk gland.

### Materials and methods

#### Insects

One laying (400 eggs) of silkworm (Race NB7) was brushed on artificial diet (Anonymous, 1994) and rearing was accomplished at LD 12:12 photoperiod,  $85 \pm 2\%$  RH and  $28 \pm 1^\circ\text{C}$  temperature during stadia 1st-3rd and  $70 \pm 2\%$  RH and  $26 \pm 1^\circ\text{C}$  temperature during stadia 4th and 5th.

#### Application of SSP-11

The emulsion of the anti-juvenoid SSP-11 ((E) 4-chloro-a, a, a-trifluoro-N [1 (1H-imidazole-1-yl)-2-propoxy ethylethene] O, toluidine) was used to induce trimoulters from the tetramoulter larvae. The emulsion was mixed with the diet just before feeding at a concentration of 200 ppm. This was fed to 200 newly ecdysed 4th stadium larvae for two days which were separated from the general stock. Larvae which did not respond to the treatment, underwent 4th moult to become 5th stadium.

#### Collection of tissues

Silk gland (SG), of five larvae each was dissected out from 4th stadium larvae everyday until spinning (treated) or ecdysis (control) and its weight along with the total larval body weight was recorded. The fat body (FB), haemolymph (HP) and SG were obtained from the 4th stadium larvae during spinning for biochemical analysis. The HP was drawn into clean Eppendorf tubes which contained a few crystals of phenyl thiourea and stored at  $-20^\circ\text{C}$ . The FB and SG were separated, freed from adhering connective tissue, washed in physiological saline (0.85% NaCl) and excess saline was blotted with millipore filter paper. The required amount of each tissue was weighed to the nearest 0.1 mg and used for biochemical analysis.

#### Biochemical analyses

The % silk gland over fresh body weight was determined by taking the total weight of the SG and total body weight of the larvae by using the following formula: % SG over body weight = weight of SG: weight of the body  $\times 100$

The levels of protein (Lowry et al. 1951), nucleic acids (DNA & RNA) (Munro and Fleck 1966), free amino acids (Moore and Stain 1954), total carbohydrates (Carroll et al. 1956), glycogen (Montgomery 1957) and activities of alanine aminotransferase, ALAT (EC 2.6.1.2) and aspartate aminotransferase, AAT (EC 2.6.1.1) (Reitman and Frankel 1957) were determined in the HP, FB and SG of control and treated larvae. The enzyme assays were made under conditions following zero order kinetics after preliminary standardization regarding linearity with respect to time of incubation and enzyme concentration.

### Results and discussion

#### Silk gland and body growth rate.

As seen in Figs. 1 and 2 the application of SSP-11 resulted in extension of the feeding period of 4th larval stadium from 4 to 8 days. All the larvae started to spin as trimoulters and produced smaller cocoons than the tetramoulter control larvae did. The weights of the control larvae increased in a straight line until they reached a plateau on day 4 (Figs. 1 and 2). However, the feeding period of the larvae treated with SSP-11 prolonged, and the larvae continued to increase their body weight by eight fold (2265 mg vs. 280 mg) and SG weight by thirty fold (399 mg vs. 12 mg) until day 8 during 4th stadium. But the control larvae put on only three fold increase in body weight (876 mg vs. 280 mg) and four fold increase

TABLE 1. Effect of 200 ppm SSP-11 in the larval diet on the protein, amino acid, carbohydrate and enzyme contents of the haemolymph of silkworm. Means + SD of 5 replicates.

Parameter (mg/ml)	Control	SSP-11 treated	% change & P value
Total protein	40.32 $\pm$ 4.20	48.66 $\pm$ 3.79	+20.68 (P<0.001)
Free amino acid	5.10 $\pm$ 0.31	4.08 $\pm$ 0.26	-20.00 (P<0.001)
Total carbohydrates	31.22 $\pm$ 2.78	34.35 $\pm$ 2.12	10.03 (P $\delta$ 0.05)
Glycogen	0.056 $\pm$ 0.004	0.060 $\pm$ 0.005	+7.14 (P< $\delta$ 0.05)
ALAT*	0.097 $\pm$ 0.007	0.103 $\pm$ 0.006	+5.77
AAT*	0.068 $\pm$ 0.004	0.070 $\pm$ 0.004	NS
AAT/ALAT	0.701	0.680	-3.00

\*  $\mu$  moles pyruvate formed/mg proteins/hr.

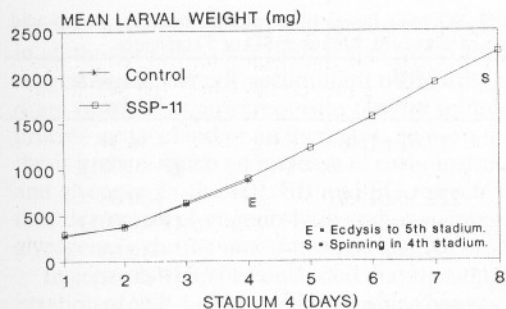


Fig. 1. Effect of SSP-11 on the larval weight of silkworm during stadium 4. Each value is mean of 5 replicates.

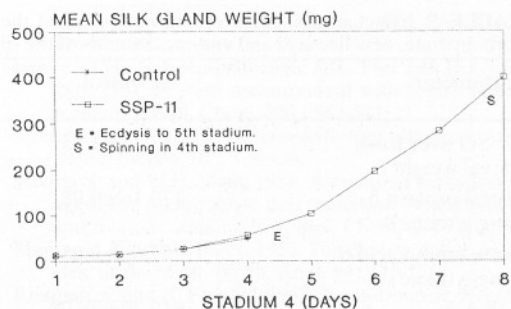


Fig. 2. Effect of SSP-11 on the silk gland weight during stadium 4. Each value is mean of 5 replicates.

in SG weight (49 mg vs. 12 mg) till day 4 of 4th stadium, the day of 4th ecdysis. The result was a significant increase in the % silk gland over the fresh body weight in the treated batches than in the control (Table 3). This allometric or heterogenic increase of larval body and SG weights and corresponding increase in the % silk gland in the treated larvae may be due to the extended feeding process during the prolonged period in the 4th stadium and the resultant higher diet consumption. Such an increase in larval weight due to prolongation of 5th larval stadium when the 4th stadium larvae were treated with methoprene has been reported by Washida (1984). Kubota et al. (1988) found that when silkworm larvae were

subjected to SSP-11 treatment once in 3rd instar or repeatedly, the trimoulters resulted had longer 4th stadium duration. This is in agreement with the present results. This may be the result of  $\delta$  metabolic and/or hormonal alterations caused by SSP-11.

**Total Proteins**

The results (Tables 1-3) suggest that major alterations occur in the composition of HP, FB & SG after the administration of SSP-11. In silkworm, FB is an active organ involved in protein synthesis while HP serves as the medium for transportation of proteins to the SG. Administration of SSP-11 resulted in a significant increase of total proteins, and nucleic acids in the FB with a corre-

TABLE 2. Effect of 200 ppm SSP-11 in the larval diet on the protein, amino acid, carbohydrate, nucleic acid and enzyme contents of the fat body of silkworm. Means  $\pm$  SD of 5 replicates.

Parameter (mg/g tissue)	Control	SSP-11 treated	% Change & P value
Total protein	156.39 $\pm$ 11.78	187.53 $\pm$ 10.05	+19.91 (P<0.001)
Free amino acid	39.76 $\pm$ 4.10	28.02 $\pm$ 2.68	-29.53 (P<0.001)
DNA*	186.20 $\pm$ 13.10	214.38 $\pm$ 13.75	+15.13 (P<0.01)
RNA	1.386 $\pm$ 0.061	1.672 $\pm$ 0.068	+20.63 (P<0.001)
Total carbohydrates	12.36 $\pm$ 0.68	13.78 $\pm$ 0.56	+11.49 (P<0.05)
Glycogen	7.03 $\pm$ 0.39	9.38 $\pm$ 0.34	+33.43 (P<0.001)
AIAT**	0.494 $\pm$ 0.02	0.618 $\pm$ 0.03	+25.10 (P<0.001)
AAT**	0.272 $\pm$ 0.01	0.305 $\pm$ 0.02	12.13 (P<0.001)
AAT/AIAT	0.551	0.494	-10.53

\*  $\mu$  moles pyruvate formed / mg proteins / hr.

\*\*  $\mu$ g/g tissue.

TABLE 3. Effect of 200 ppm SSP-11 in the larval diet on % SG over larval body weight, protein, amino acid, carbohydrate, nucleic acid and enzyme contents of the silk gland of silkworm. Means  $\pm$  SD of 5 replicates.

Parameter	Control	SSP-11 treated	% Change & P value
% SG over fresh larval weight	12.73 $\pm$ 1.13	17.61 $\pm$ 1.48	+38.34 (P<0.001)
Total protein $\delta$ (mg/g tissue)	170.16 $\pm$ 9.98	222.36 $\pm$ 13.01	+30.68 (P<0.001)
Free amino acid $\delta$ (mg/g tissue)	57.66 $\pm$ 2.05	50.38 $\pm$ 2.76	-12.63 (P<0.01)
DNA $\delta$ ( $\mu$ g/g tissue)	819.70 $\pm$ 38.08	900.96 $\pm$ 49.00	+9.91 (P<0.05)
RNA $\delta$ (mg/g tissue)	2.007 $\pm$ 0.112	2.505 $\pm$ 0.116	+24.81 (P<0.001)
Total carbohydrates $\delta$ (mg/g tissue)	16.36 $\pm$ 0.92	19.34 $\pm$ 0.87	+18.22 (P<0.001)
Glycogen $\delta$ (mg/g tissue)	9.46 $\pm$ 0.506	11.60 $\pm$ 0.489	+22.62 (P<0.001)
AIAT**	0.504 $\pm$ 0.038	0.688 $\pm$ 0.045	+36.51 (P<0.001)
AAT**	0.339 $\pm$ 0.023	0.401 $\pm$ 0.030	+18.29 (P<0.001)
AAT/AIAT	0.673	0.583	-13.37

\*\*  $\mu$  moles pyruvate formed / mg proteins / hr.

sponding increase in the HP and SG suggesting stepped-up synthetic activities or decreased degradative activities in them (Tables 1-3). The RNA or RNA/DNA ratio can be considered as an index of the capacity of an organism for protein synthesis. Total RNA is, therefore, a measure of the potential rate of protein synthesis (Ring 1973). Thus a significant increase in DNA (extra nuclear DNA) and RNA levels in FB and SG over the control indicates acceleration of protein as well as nucleic acid synthesis by the treatment. Since the synthesis of HP, FB and SG proteins and nucleic acids are controlled by ecdysteroids (Tojo et al. 1981), the increase of these substrates indicates the involvement of SSP-11 in the hormone mediated activation of protein synthesis. In contrast to this, the FAA content of HP, FB and SG markedly decreased over their respective control levels which indicates their active mobilization into silk protein synthesis and/or into oxidative activities.

Transaminase activity levels (AAT & AIAT).

The activity levels of AAT & AIAT were measured because protein synthesis requires a balanced pool of amino acids and transamination is one of the major mechanisms which functions as

regulator of this pool. The FAAs are known to play a role in meeting the energy demand by their conversion into keto acids which are fed into the citric acid cycle through the transamination process. Interestingly, in the FB and SG, the AAT and AIAT activities elevated significantly after the treatment, which indicates accelerated amino acid catabolism in the tissues. Between the enzyme activities, AIAT was higher than AAT suggesting active mobilization of amino acids for the synthesis of silk proteins. The AAT/AIAT ratios were lower in treated larvae indicating a relatively greater diversion of the transaminase pathway at the pyruvate level. The AIAT activity reflects amino acid breakdown and AAT reflects the mobilization of these into gluconeogenesis. Gluconeogenesis is the major pathway for the net synthesis of carbohydrate from non-carbohydrate substrate and occurs via reversal of the glycolytic pathway. The carbon source for gluconeogenesis are amino acids and the decreased levels of FAA in the tissues suggest that this is providing the substrate for de novo synthesis of carbohydrates. Hence, total carbohydrates and glycogen levels were also measured to establish the correlation between these and the changes in protein and FAA after the administration of SSP-11. The lev-

els of total carbohydrates and glycogen increased in all three tissues over their respective controls suggesting an overall accumulation of carbohydrate reserves as a prerequisite for the spinning process in the gland or an increased de novo synthesis in them. Such an increase in carbohydrates and glycogen in the HP, FB and SG reflects either decreased glycogenolysis and/or increased glycogenesis during maturation of the SG.

In general, it can be concluded that the administration of SSP-11 induces favourable changes in the composition of HP, FB and SG for an early larval maturation and precocious metamorphosis through the mediation of endogenous hormones. The possible cooperative and/or antagonistic interactions of SSP-11 and circulating hormones on the biochemical mechanism of silk protein synthesis is not fully known.

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KEY WORDS: *Bombyx mori*, trimoulturization, silkworm, silk gland, fat body, tissue composition, SSP-11.

## Επίδραση της SSP-11 στο Βάρος και τη Σύνθεση των Ιστών Προνυμφών Μεταξοσκώληκα 4ης Ηλικίας, *Bombyx mori*

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### ΠΕΡΙΛΗΨΗ

Η αντιορμόνη νεότητας ουσία SSP-11 ((E) 4 χλωρο-α, α-τριφθοριο-N [1(1H-ιμιδαζολ-1-ύλ)-2-προπόξυ εθυλαδεν] O, τολονθίνη) χρησιμοποιήθηκε να προκαλέσει έκδυση προνυμφών τρίτης έκδυσης από αυτές τετάρτης έκδυσης μεταξοσκώληκα, *Bombyx mori* (φυλή NB7). Η ουσία δόθηκε με τεχνητή τροφή για δυο ημέρες σε συγκέντρωση 200 ppm σε νεοεκδυθείσες προνύμφες 4ης ηλικίας. Το βάρος του σώματος και των μεταξογόνων αδένων καταγραφόταν καθημερινά στην 4η ηλικία και το προφίλ των πρωτεϊνών της αιμολέμφου, του λιπώδους σώματος και των μεταξογόνων αδένων προσδιοριζόταν στο τέλος της ηλικίας. Σε προνύμφες που δόθηκε η SSP-11 η διάρκεια διατροφής της 4ης ηλικίας επιμηκύνθηκε κατά 3-4 ημέρες και το 80-85% των προνυμφών άρχισαν να πλέκουν κουκούλι ως προνύμφες 3ης έκδυσης. Οκταπλάσια και τριακονταπλάσια αύξηση στα βάρη του σώματος της προνύμφης και των μεταξογόνων αδένων αντίστοιχα ήταν το αποτέλεσμα της χορήγησης SSP-11. Επιπλέον, οι συνολικές πρωτεΐνες, υδατάνθρακες, γλυκογόνο και νουκλεϊκά οξέα (DNA και RNA) αυξήθηκαν επίσης. Τα επίπεδα δραστηριότητας της αμινοτρανσφεράσης της αλανίνης και του ασπαρτικού αυξήθηκαν σημαντικά παρά στο μάρτυρα δείχνοντας αυξημένη κινητοποίηση αμινοξέων στη δραστητικότητα των τρανσαμινασών.