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The effect of indole – 3 – butyric acid (IBA), indole – 3 – pyruvic acid (IPA) and their synergetic effects on biochemical contents on the silkworm, *Bombyx mori*

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Topical application with indole – 3 – butyric acid (IBA) showed a significant increase in glycogen, protein and total lipids in the body fat and trehalose and protein contents in the haemolymph in all the groups of the silkworm, *Bombyx mori*. Topical application with indole – 3 – pyruvic acid (IPA) showed a significant increase in glycogen, protein and total lipids in the body fat and trehalose and protein contents in the haemolymph in all the groups except 100 and 150 μ g/ml glycogen and 100 μ g/ml protein in the body fat of the silkworm, *B. mori*. Topical application with mixture of IBA and IPA showed a significant increase in the glycogen, protein and total lipids in the body fat and trehalose and protein contents in the haemolymph in all the groups except 100 μ g/ml proteins in the body fat and trehalose and protein contents in the haemolymph in all the groups except 100 μ g/ml proteins in the body fat and trehalose and protein contents in the haemolymph in all the groups except 100 μ g/ml proteins in the haemolymph of the silkworm, *B. mori*.

Key words: Biochemical contents, indole – 3 – butyric acid (IBA), Indole – 3 – pyruvic acid (IPA), silkworm.

INTRODUCTION

Insects are unique in having morphological and physiological features manifested for adopting themselves to varied environment. One of the important adaptative responses in insects is mainly achieved by altering the metabolic process. The silkworm is entirely dependent on mulberry leaves as a food source and the protein content of the leaves play an important role in the silk production. There is evidence that the plant growth regulators may act through their effect on the insect's neuroendocrine system or perhaps directly on insect cells (Osborne et al., 1968). It has been reported that the plant growth regulators mimic the moulting hormone ecdysone and restricts the insect growth of Drosophila hydei

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Abbreviations: IBA, Indole – 3 – butyric acid; IPA, indole – 3 – pyruvic acid; DNA, deoxyribonucleic acid; PABA, paraminobenzoic acid; TCA, trichloroacetic acid; BSA, serum albumen; CRBD, complete randomized block design; LSD, least significant difference; NOA, naphthoxy acetic acid.

(Alonso, 1971; Neumann, 1980, 1982). It has been reported that the reduced reproduction effected by low concentrations of ABA and GA₃ added to host grass may mean that these compounds act on insects metabolism via its hormone pathways, they serve as biochemical signals to regulate growth of insects, Deoxyribonucleic acid (DNA) synthesis and reproduction of Aulocare ellitt, since ABA, GA₃ and JH-III are biochemically similar trepenoid compounds derived from mevalonate. DeMan et al. (1981) have suggested that dietary supplementation with plant growth regulators may stimulate the insect growth and reproduction by altering the rate of DNA synthesis and/or the rate of synthesis of the insect moulting hormone. There are a number of reports regarding the supplementation of various plant growth regulators that affect the physiological processes, growth and development in different insects (Edwards, 1966; Osborne et al., 1968; Gurra, 1970; Becker and Roussaux, 1980; Neumann, 1982; Chrominiske et al., 1982; Bur, 1985). It has been reported that supplementation with plant hormones influences on the physiological process, growth and development of silkworm Bombyx mori L (Kochi and Kaliwal, 2005;

Etebari et al., 2004). It has been reported that the dietary supplementation with paraminobenzoic acid (PABA) affects proteins profile in the haemolymph and silkgland (Pramodkumari, 1990). Rup et al. (1997) have reported that the qualitative changes in the protein, lipid and carbohydrate contents under the influence of GA₃ in Zaprinus paravittigeal. Hugar and Kaliwal (1997) have reported that the topical application with BAP and IAA increases the body fat glycogen, protein and haemolymph protein where as haemolymph trehalose decreases. Goudar and Kaliwal (2001) have reported that 2- 4 D and NOA increases the body fat glycogen, haemolymph trehalose and body fat protein. It was showed that the plant growth regulators viz., IBA, IPA and their mixtures enhances the larval weight, silk gland weight, female cocoon weight, shell weight, filament length, weight and denier, fecundity, hatching percentage and eggs per ovariole in the silkworm, B. mori. Plant hormones play a very important role in the growth of plants which is a source of food and also helps in enhancing the metabolism of silk worm which in turn helps in the production of silk. Therefore, the present study was undertaken to find out the effects of IBA, IPA and their synergetic effects on the glycogen, protein and total lipids in the body fat, trehalose and protein in the haemolymph of the silkworm, B. mori.

MATERIALS AND METHODS

Animals

In the present study the commercially exploited bivoltine crossbreed silkworm race $CSR_2 \times CSR_4$ was selected. The race was procured from Rayapur grainage center, Dharwad, Karnataka, India. Leave variety is S_{36} .

Test material

The plant growth regulators IBA and IPA were procured from the Central Drug House, New Delhi and Hi Media chemical Company Pvt. Limited., Mumbai, India.

The fifth in star larvae were selected randomly and grouped into different batches for the experiment. Each group consists of five replications each with 20 worms. The IBA and IPA was individually dissolved completely in distilled water and diluted to form 100, 150 and 200 μ g/ml. The dietary supplementations of these chemicals started from the beginning of V stadium to the maturation of silkworm larvae. The regulators are topically used on the silkworm body. Amongst the four feedings per day, feeding of treated leaves was alternated with feeding of untreated leaves (Kochi and Kaliwal, 2006).

Tissue preparation

The silkworm *B. mori* larvae were dissected in *Bombyx saline* at pH 6.5 on 6th day of V in star. The body fat was immediately collected and used for the glycogen and protein estimation. The haemolymph was collected by amputating one of the larval thoracic legs in prechilled centrifuge tube. The haemolymph collected from 2 to 3 silkworms was used almost immediately for trehalose and protein estimation (Kochi and Kaliwal, 2006).

Glycogen estimation

Anthrone method of Sciefter et al. (1950) was used to determine the body fat glycogen. A known quantity of body fat was homogenized with 2 ml of 20% potassium hydroxide. The glycogen was precipitated by adding equal volume of 80% ethanol and incubated overnight at room temperature. It was centrifuged at 3000 rpm for 15 min and the supernatant was discarded. The residue was dissolved in a known volume of distilled water. Glycogen content was estimated with known aliquots in triplicate by the Anthrone method. Glucose-D was used as the reference standard and the intensity of the colour was read on the spectrophotometer at 620 nm.

Trehalose estimation

The estimation of haemolymph trehalose was carried out according to the method of Roe (1955). Known quantity of haemolymph was collected in each test tube, and added 0.5 ml of 2% of sodium hydroxide to each test tube. After shaking, the tubes were kept in boiling water for 10 min and then the tubes were cooled in an icebox. Then 5 ml of anthrone reagent (0.05% anthrone in 70% sulphuric acid) was added to the tubes, and they were again kept in boiling water for 15 min for the development of colour. Then the tubes were cooled to room temperature. Then the colour intensity was read on spectrophotometer at 620 nm. For the reference standard the trehalose (Sigma, USA) was used. Anthrone positive carbohydrate in the haemolymph is considered as trehalose.

Protein estimation

The method of Lowry et al. (1951) was used for the total protein estimation. The tissue protein was precipitated by the addition of 1 ml of 30% trichloroacetic acid (TCA) solution followed by centrifugation at 3000 rpm for 30 min. It was repeated twice, and then the precipitate was dissolved in 1 ml of 0.1 N sodium hydroxide. A known aliquot of this solution was then mixed with 5 ml of alkaline copper reagent (20% sodium carbonate prepared in 0.1 N sodium hydroxide containing sodium potassium tartrate and 1% copper sulphate). After 10 min 0.5 ml of Folin Ciocalteu's reagent was added to the tubes and the tubes were shaken thoroughly. Then the tubes were kept for 20 min for colour development. The readings were taken on the spectrophotometer at 650 nm. The total haemolymph protein estimation was also carried out. A known quantity of haemolymph was diluted with 0.5 ml of distilled water. A known aliquates of this solution was added with 5 ml of alkaline copper reagent. After 10 min 0.5 ml of Folin Ciocalteu's reagent was added and were mixed thoroughly, then kept for 20 min until the colour develops. The readings were taken on the spectrophotometer at 650 nm. For the reference, standard Bovine Serum Albumen (BSA) (Fatty acid free) was used.

Extraction and estimation of lipids

The method of Folch et al. (1957) was used for the lipid estimation, using chloroform: methanol mixture (2:1 V/V). First, all of the body fat was homogenized with appropriate volume of chloroform: methanol mixture (1:10). The homogenate was then quantitatively transferred to a 50 ml separating funnel and then added similar volume of chloroform. The two solvents were partitioned by the addition of 0.2 volume of water. After the funnel was shaken, the mixture was allowed to stand overnight. The lower chloroform layer

containing lipids was drawn off. The lipids sample was kept in vacuum desiccators until constant weight was obtained.

Statistical analysis

The experiments were designed by complete randomized block design (CRBD) method. The date collected were fed to the computer for statistical analysis by using the software SPSS version 6, to study the significance of variance among the treatment groups (one way variance test 'F'). To determine the significant difference among the treatment groups, the least significant difference test (LSD) was carried out. The percentage values were transferred into sine angular values only (Snedecor and Cochran, 1967; Raghava, 1983).

RESULTS

The results on the effects of topical application with different concentrations that is, 100, 150 and 200 μ g/ml of plant growth hormones viz. IBA, IPA and their synergetic effects on glycogen, protein and total lipids content in the body fat and protein contents in the haemolymph of CSR₂ x CSR₄ crossbreed race of the silkworm, *B. mori* are presented in Tables 1 to 3.

Fortification of IBA on the biochemical contents of the silkworm, *B. mori*

Topical application with 100 µg/ml IBA to silkworm larvae resulted in an increase of 37% glycogen, 28% protein and 4% total lipids in the body fat and 1% protein, 25% trehalose in the haemolymph when compared with those of the corresponding parameters of the carrier control. The increase in glycogen, protein, and total lipids in the body fat and protein and trehalose contents in the haemolymph were statistically significant. Topical application with 150 µg/ml IBA to silkworm larvae resulted in an increase of 40% glycogen, 29% protein and 7% body fat total lipids in the body fat and 8% protein, 41% trehalose in the haemolymph when compared with those of the corresponding parameters of the carrier control. The increase in glycogen, protein and total lipids in the fat body and protein and trehalose contents in the haemolymph were statistically significant. Topical application with 200 µg/ml IBA to silkworm larvae resulted in an increase of 44% glycogen, 32% protein and 10% body fat total lipids and 16% protein, 52% trehalose in the haemolymph when compared with those of the corresponding parameters of the carrier control. The increase in glycogen, protein and total lipids in the body fat and protein and trehalose contents in the haemolymph were statistically significant (Table 1).

Fortification of IPA on the biochemical contents of the silkworm, *B. mori*

Topical application with 100 µg/ml IPA to silkworm larvae

resulted in an increase of 2% glycogen, 22% protein and 8% total lipids in the body fat and 11% protein, 15% trehalose in the haemolymph when compared with those of the corresponding parameters of the carrier control. The increase in total lipids in the body fat and protein and trehalose contents in the haemolymph was statistically significant. Topical application with 150 µg/ml IPA to silkworm larvae resulted in an increase of 6% glycogen, 28% protein and 20% total lipids in the body fat 13% protein, 16% trehalose contents in the haemolymph when compared with those of the corresponding parameters of the carrier control. The increase in protein and total lipids in the body fat and protein and trehalose contents in the statistically significant. haemolymph was Topical application with 200 µg/ml IPA to silkworm larvae resulted in an increase of 19% glycogen, 31% protein and 56% total lipids in the body fat and 18% protein, 26% trehalose contents in the haemolymph when compared with those of the corresponding parameters of the carrier control. The increase in glycogen, protein, total lipids in the body fat and protein and trehalose contents in the haemolymph were statistically significant (Table 2).

Synergetic effects of IBA and IPA on the biochemical contents of the silkworm, *B. mori*

Topical application with 100 µg/ml IBA and IPA mixture to silkworm larvae resulted in an increase of 39% glycogen, 16% protein and 11% body fat total lipids in the body fat and 8% protein and 23% trehalose contents in the haemolymph when compared with those of the corresponding parameters of the carrier control. The increase in glycogen, protein and total lipids in the body fat and trehalose contents in the haemolymph were statistically significant. Topical application with 150 µg/ml IBA and IPA mixture to silkworm larvae resulted in an increase of 48% glycogen, 22% protein and 11% body fat total lipids in the body fat and 11% protein and 25% trehalose contents in the haemolymph when compared with those of the corresponding parameters of the carrier control. The increase in glycogen, protein and total lipids in the body fat and protein and trehalose contents in the haemolymph were statistically significant. Topical application with 200 µg/ml IBA and IPA mixture to silkworm larvae resulted in an increase of 91% glycogen, 41% protein and 16% body fat total lipids in the body fat and 32% protein and 26% trehalose contents in the haemolymph when compared with those of the corresponding parameters of the carrier control. The increase in glycogen, protein and total lipids in the body fat and protein and trehalose contents in the haemolymph were statistically significant (Table 3).

DISCUSSION

The last penultimate in star is the most active feeding

Treatment	Dose µg/ml ──	Body fat (μg/mg)			Haemolymph (μg/ml)	
		Glycogen	Protein	Total lipid	Trehalose	Protein
IBA	100	9.866 * (137)	59.99 * (128)	313.3 * (104)	390.90 * (125)	2869* (101)
IBA	150	10.133 * (140)	60.40 * (129)	323.3* (107)	440.90 * (141)	3101* (108)
IBA	200	10.399 * (144)	62.00 * (132)	330.0* (110)	472.50 * (152)	3320* (116)
Carrier control	distilled water	7.199 (100)	46.66 (100)	300.0 (100)	310.80 (100)	2854 (100)
Normal control	-	7.133 (99)	48.99 (104)	293.3 (97)	300.30 (97)	2200 (77)
		(S)	(S)	(S)	(S)	(S)
S.Em± CD at 5%		1.166 2.438	3.06 8.76	4.172 9.304	12.20 34.87	3.494 11.785

Table 1. Effect of Indole-3-butyric acid (IBA) on biochemical contents in the body fat and haemolymph of the silkworm, B. mori.

* - Significant increase/decrease at 5%, S.Em ± - standard error mean, CD- critical difference, S - significant percentage increase/decrease over that of the carrier controls in parenthesis.

Table 2. Effect of Indole-3-pyruvic acid (IPA) on biochemical contents in the body fat and haemolymph of the silkworm, B. mori.

Treatment	Dose µg/ml ──	Body fat (µg/mg)			Haemolymph (µg/ml)	
		Glycogen	Protein	Total lipid	Trehalose	Protein
IPA	100	17.60 (102)	24.66 (122)	280* (108)	290 * (115)	3714* (111)
IPA	150	18.26 (106)	25.86 * (128)	310*(120)	294 * (116)	3774 * (113)
IPA	200	20.46* (119)	26.46 * (131)	402* (156)	318 * (126)	3939* (118)
Carrier control	distilled water	17.13(100)	20.13 (100)	257 (100)	252 (100)	3324 (100)
Normal control	-	16.59 (96)	19.06 (94)	140 (54)	264 (104)	3576 (107)
		(S)	(S)	(S)	(S)	(S)
S.Em± CD at 5%		1.12 2.27	2.639 5.571	3.334 9.976	1.401 4.327	128.90 366

* - Significant increase/decrease at 5%. S.Em ± - standard error mean, CD - critical difference, S - significant, percentage increase/decrease over that of the carrier controls in parenthesis.

stage during which the larvae accumulate large quantity of fuel reserves in various tissues and is endowed with unique biochemical adaptations to conserve nutritional resources available during active larval stage of the silkworm, *B. mori.* Chapman (1998) has suggested that insects have adapted to a range of strategies in order to derive and store adequate energy nutrients and water from the food they consumed. The carbohydrates, protein and lipids biomolecule are supplied by feeding on mulberry leaves. Although the mulberry leaves are complete diet for silkworm it is possible that some deficiencies occur for different reasons (Etebari et al., 2004). Hence, dietary supplementation of plant hormones may influence on the biochemical contents of the silkworm, *B. mori*. The biomolecules are stored in the body fat and haemolymph during the fifth in star stage. It is well demonstrated that carbohydrates are stored in the body fat as glycogen, which is converted into trehalose in the body fat before it is released into the haemolymph for its utilization. Body fat, apart from converting stored glycogen into trehalose, is a major site of protein synthesis, which is essential for the maintains of the growth and reproduction.

Hence, body fat in insects plays an important role

Treatment	Dose µg/ml —	Body fat (μg/mg)			Haemolymph (μg/ml)	
		Glycogen	Protein	Total lipid	Trehalose	Protein
IBA + IPA	100	22.90 * (139)	84 * (116)	360.0 * (111)	616.35 * (123)	3158 (108)
IBA + IPA	150	24.24 * (148)	88* (122)	360.0 * (111)	627.05 * (125)	3266 * (111)
IBA + IPA	200	31.25 * (191)	102 * (141)	376.6 * (116)	630.00 * (126)	3858 * (132)
Carrier control	distilled water	16.36 (100)	72 (100)	323.3 (100)	497.70 (100)	2922 (100)
Normal control	-	11.22 (68)	64.8 (90)	320 (99)	502.95 (101)	2900 (99)
		(S)	(S)	(S)	(S)	(S)
S.Em± CD at 5%		0.745 2.332	0.989 3.133	6.610 14.742	28.72 60.32	154.92 323.78

Table 3. Synergetic effects of Indole-3-butyric acid (IBA) and Indole-3-pyruvic acid (IPA) on biochemical contents in the body fat and haemolymph of the silkworm, B. mori.

* - Significant increase/decrease at 5%, S.Em ± - standard error mean, CD - critical difference, S – significant percentage increase/decrease over that of the carrier controls in parenthesis.

in the synthesis of proteins and trehalose and that haemolymph serves as a vehicle for the transportation of these substances for their utilization in the body. Lipids are important constituents of cuticle and help in acylation of glucose-6-phosphate during chitin synthesis (Wyatt, 1967). The lipid in the body fat is an energy resource which can be mobilized rapidly during starvation, oogenesis, embryogenesis and moulting and is used to sustain continuous muscular activity (Gilbert and Chino, 1974). It has showed that the plant growth regulators affect the economic parameters of the silkworms, it is likely that they may also affect the synthesis, storage and release of the biological molecules from the body fat to the haemolymph which will help for the growth and development of silkworm, B. mori. As we have observed in earlier reports that dietary supplementation with plant growth regulators influences the glycogen, protein and total lipids in the body fat and trehalose and protein in the haemolymph of the silkworm, B. mori (Bur, 1985; Pramadokuimari, 1990; Rup et al., 1997; Hugar and Kaliwal, 1997; Goudar and Kaliwal, 2001; Kochi and Kaliwal, 2005; Etebari et al., 2004). In the present study, therefore, the effects of topical

application with IBA, IPA and their mixture on biochemical contents were studied.

Effects of IBA, IPA and their mixture on glycogen in the body fat and trehalose in the haemolymph of the silkworm, *B. mori*

The results of this present study showed that there was a significant increase in the glycogen and the body fat in all the treated groups with IBA, IPA and their mixture. The results obtained in the present study are in agreement with the reported of the bivoltine silkworm, B. mori after the treatment with BAP and IAA (Hugar and Kaliwal, 1997). Goudar and Kaliwal (2001) have also reported an increase in glycogen in the body fat of the silkworm treated with NOA and 2, 4 - D. The increase in glycogen in the body fat might be due to the stimulatory effect of these plant growth regulators on glycogenesis reported to increase during feeding period (Pant and Morris, 1969). Wyatt (1967) reported that the quantity of these biomolecules in insect tissue depends on quantum of food intake and variation in their levels, due to the utilization during growth and

metamorphosis. Siegert and Ziegler (1982) have reported decreased metabolism in Menduca sexta at the end of last larval in star and further the sugar in the haemolymph and glycogen in the body fat decreased during moulting and increased at the resumption of feeding. It is reported that the homeostatic of trehalose in the haemolymph is maintained through hormonal regulation at the expense of glycogen in the tissue probably from the body fat (Saito, 1963). In the present results, there was a significant increase in trehalose in the haemolymph in all the groups treated with IBA, IPA and their mixture. Hugar and Kaliwal (1997) have reported that there was a significant increase in trehalose in the haemolymph in BAP and IAA treated groups might possibly be due to the conversion of glycogen into trehalose and it's subsequent release into the haemolymph by the fat body (Hugar and Kaliwal, 1997). The results are also in agreement with that reported for the silkworm, B. mori after the treatment with NOA, PABA and 2, 4 – D (Goudar and Kaliwal, 2001). There was an increase in glycogen in the body fat and trehalose in the haemolymph as it may be utilized as additional source of fuel or energy required during the pupal and adult transformation. However, the mechanism of action of these plant growth regulators on the body fat synthetic activity and trehalose in the haemolymph is not known. Hence, further investigation is essential in this regard.

Effects of IBA, IPA and their mixture on protein contents in the body fat and haemolymph of the silkworm, *B. mori*

It is well known that the body fat of an insect is regarded as liver of vertebrate in carrying out intermediary metabolism as well as protein synthesis and its storage (Wigglesworth, 1972). Therefore, body fat is an important organ in the insects, which plays an important role in anabolic as well as catabolic activities of insects. The results of the present study indicated that there was significant increase in protein in the body fat and haemolymph in all the groups treated with IBA, acid IPA and mixtures. The results obtained in the present study support the views of Hugar and Kaliwal (1997) where the body fat protein and haemolymph protein increased significantly in the groups treated with BAP and IAA. Similar results were also obtained by Goudar and Kaliwal (2001) in the silkworm, B. mori treated with PABA, 2, 4 -D and NOA. The increased of protein contents in the body fat and haemolymph protein may possibly be due to the stimulatory effects of the plant growth regulators at a given concentration on the synthetic activity of the body fat and the increased haemolymph protein might be due to the release of excess of protein by the body fat into the haemolymph and at the same time the weight of the silk gland has also increased significantly. This also coincides with the subsequent increase in the cocoon weight and its shell weight of the silkworm, B. mori.

Effects of IBA, IPA and their mixture on total lipids content in the body fat and haemolymph of the silkworm, *B. mori*

In the present study, the total lipids in the body fat are increased in all the groups treated with IBA, IPA and their mixtures. Similar results have been reported that the body fat total lipids, phospholipids and neutral lipids in the body fat were increased in all the groups treated with 2, 4- dichlorophenoxy acetic acid (2, 4 - D) and naphthoxy acetic acid (NOA) may be due to the stimulatory effect on the synthetic activity in the body fat (Goudar and Kaliwal, 2001). Increase in the total lipids in the body fat might possibly be due to stimulatory effect of IBA, IPA and their mixtures at a given concentration on the body fat synthetic activity and ovarioles might have not sequestered the lipids from the body fat in excess of its requirements, so, the accumulation of total lipids was seen. Moreover, there was significant increase in fecundity might possibly suggest that the ovariole might have sequestered the lipids from the body fat to the eggs

but the ovariole has not sequestered the lipids from the body fat in excess of its requirements, hence, there was an accumulation of total lipids in the body fat. Guerra (1970) citing the references of Harper (1963) have quoted, that the metabolic processes taking place within the living organism are nearly the reflection of the chemical composition of the body.

Since, the concentration of the most chemical substances in the body fluids varies within rather narrow limits, significant changes in the normal metabolism which are detrimental to insect development and/ or reproduction could be produced by inducing an excess or a deficiency of essential metabolites. The sensitive and efficient regulation of the rates of metabolic processes controlling life is made possible by several known mechanism. These are the nervous system, hormones, the stimulation or inhibition of enzyme activity, feedback inhibition and the induction or suppression of enzyme synthesis whether the increase/decrease in alvcogen. protein and total lipids in the body fat and trehalose and protein contents in the haemolymph after treatment with all these plant growth regulators in the present study, may be due to their influence on nervous system or hormones or the stimulation or inhibition of enzymes activity or the induction or suppression of enzyme synthesis were not known. Hence, further investigation on mechanisms of plant growth regulators on the biochemical contents of the silkworm is necessary. In the present study the plant growth hormones that are IBA and IPA and their mixtures affect the biochemical contents such as glycogen, protein and total lipids in the body fat and trehalose and protein contents in the haemolymph thus affecting the physiological process of the silkworm, B. mori. The contents were dose dependent. The mulberry leaf itself has different levels of plant growth hormones but dietary supplementation of plant hormones alters the levels of biochemical contents leading to variations in physiological activities, either improving or reducing the economic traits of the silkworm, B. mori. However, the exact effects of these hormones on the body fat and haemolymoh are essential in the silkworm, B. mori.

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