International Journal of Pharmacy
Journal Homepage: http://www.pharmascholars.com

Research Article **CODEN: IJPNL6**

EXTENDED SYNTHESIS OF STORAGE PROTEINS IN SILKWORM *BOMBYX MORI* IN RESPONSE TO JUVENILE HORMONE ANALOGUE (JHA) TREATMENT

Santhy K Sivanandan

Department of Zoology, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore – 641043, India

***Corresponding author e-mail:** dr.santhyanandan@gmail.com

ABSTRACT

The female silkworm, *Bombyx mori*, rapidly accumulates two storage proteins in the haemolymph that are synthesized by the fat body during the final stage of the last larval instar. This is then sequestered from the haemolymph in to the fat body during larval – pupal transformation. Application of Methoprene (Manta) at low concentration ranging from 0.1 µg – 1 µg on the third day was found to prolong the larval period by two days in a dose dependent manner which resulted in the prolonged synthesis of storage proteins as evidenced by the SDS – PAGE and densitometric scanning of haemolymph proteins. A corresponding decline in the storage protein concentration was observed in the fat body samples in lieu of the late sequestration. The prolonged synthesis of storage protein in the study was found to have an indirect impact on the silk as observed in enhanced silk production.

Keywords: Silk, *Bombyx mori*, Methoprene, SDS-PAGE, Haemolymph, larva.

INTRODUCTION

The silkworm, *Bombyx mori*, has two varying molecular weight storage proteins which are produced more actively in the final instar larvae[1]. Studies have been advanced to explain the function of larval storage proteins[2,3] raises conflicts in understanding the processing of these proteins *in vivo*, although the resultant information gathered indicate the storage proteins in lepidopterans undergo quantitative changes during larval development.

They are found to accumulate in the hemolymph of the mid – final instar larva, reach maximum levels at spinning and then they are sequestered in to the fat body[4.] *B. mori* has been reported to possess two storage proteins, namely sp-1 and sp-2. Sp-1 corresponds to a larval female protein which is rich in methionine and found only in the haemolymph of the female larva[5]. The reduction in sp-1 at the time of moulting suggests that this storage protein may provide amino acids for the formation of tissue in internal organs, such as the midgut and silk glands of the growing larvae during the larval moulting period[6]. In the larvae of *B. mori*, the application of methoprene (Manta) did not prevent synthesis and secretion of storage proteins in to the hemolymph but completely suppressed their sequestration in to the fat body[7,8].

Therefore, methoprene could act indirectly on the fat body to suppress sequestration of storage protein, through its inhibition of ecdysone secretion from the prothoracic gland. The above reports suggests that the accumulation of haemolymph proteins like storage proteins (sp-1 & sp-2) in silkworm *B. mori* is influenced by hormone action and thereby it can serve as an important marker for the evaluation of the Methoprene. This present experiment is carried out to study the larval duration after the application of Methoprene by protein analysis.

MATERIALS AND METHODS

1. Insect rearing: The cross breed *B. mori* were used and larvae were reared on mulberry leaves in a culture room at 25^{o}C ± 12 h dark cycle. The female

larvae were collected within 4 h after ecdysis and divided in to five groups, each with 50 larvae.

2. Hormone application: Methoprene was dissolved in distilled water and different concentrations ranging from 0.1 to 1 µg was applied between 48- 72 h in the fifth instar. Distilled water treated and untreated controls were maintained.

3. Tissue preparation and SDS – PAGE: Haemolymph was collected in chilled test tubes and mixed with 9 volumes of phosphate buffered saline containing a few crystals of phenyl thio urea. The mixture was centrifuged at 16,000 g for 5 min to remove the haemocytes. Bled larvae were dissected out in ice – cold saline to collect the fat body. The tissue was homogenized with 10 times volume of phosphate buffered saline and centrifuged at 16000 g. The total protein content in haemolymph and fat body samples were estimated following the method of Lowry *et al.*[9]. Extracts of haemolymph and fat bodies were mixed with equal volume of SDS – sample buffer. The mixture was boiled for 1 min and subjected to SDS – PAGE using a 10% stacking gel and 10% separating gel. Peptides were stained with 0.025% Coomasie brilliant blue R 250. Molecular weight standards were used for blot analysis[10].

4. Immunoblotting: Haemolymph and fat body proteins separated on polyacrylamide gel was blotted onto a nitrocellulose filter which was later immersed in 100 ml of Tris buffered saline- Tween 20 (TBST with the composition of 10 mM Tris - HCl buffer, 0.9 % Nacl and 0.05 % Tween 20, pH 7.5) containing five per cent milk powder and shaken for 2 h at room temperature to block the non-specific protein binding sites. The filter was then incubated under shaking in 100 ml of TBST containing three per cent milk powder and primary antibody (sp-1 and sp-2 in combination, 1:1000 dilutions) for 3 h at room temperature. After washing three times at 10 min intervals with 100 ml TBST, the filter was transferred to 100 ml TBST containing three per cent milk powder with peroxidase conjugate goat anti rabbit IgG (1:500 dilution) for 3 h. Finally the filter was washed four times at 10 min intervals with 100 ml TBST and once without Tween - 20. The sites of IgG binding were then visualized with a substrate solution containing 4 chloro 1-naphthol plus hydrogen peroxide. Purple colour bands were visualised and photographed.

RESULTS

The protein profile of the haemolymph showed several polypeptides with molecular weights ranging from 200 to 18 k Da on both treated and control groups. The two polypeptides, sp-1 (82 k Da) and sp-2 (76 & 72 k Da) that electrophoresed to the 80 k Da region were identified as storage proteins. The coomassie blue staining intensity of haemolymph proteins during the development of the fifth instar larvae (from day 1 to 8) suggests stage specificity or developmental specificity with regard to their accumulation in the haemolymph. Their accumulation increased steadily and reached the maximum level before the onset of spinning.

Application of Manta was found to prolong the larval duration by two days in treatments as compared to the control. SDS-PAGE and densitometric scanning pattern of the protein profile of male and female larvae did not reveal any significant difference between the treated and control groups on third day which was the day of hormone application. An increase in the quantity of storage proteins in both male and female larvae was observed on day 8 compared to that of the third day. However there was no difference between the control and methoprene treated larvae on the same day. The maximum retention was detected in 0.1 µg dosage followed by 0.5 µg dosage and in control only traces of this protein was detected. This dose dependent retention continued on day 10 of both male and female larvae (Fig. 1). Western blotting performed to confirm the storage proteins using a blot from SDS-PAGE to nitrocellulose filter showed strongly reacting peptides at 82, 76 and 72 kDa regions (Fig. 2).

Throughout the feeding period of the last instar, protein concentration in the fat body remained at trace and with same fluctuation in controls as well as in methoprene treated larvae. In the control larvae, the fat body began to accumulate proteins after cocoon spinning (day 8). However there was no accumulation of proteins in treated larvae on the same day. On day 9, on 0.1 µg and 0.5 µg methoprene treated larvae, protein concentration increased steadily in a dose dependent manner. By the end of day 10, all the groups in the experiment evidenced high concentration of storage protein due to the complete sequestration and concomitant larval – pupal transformation (Fig 1 contd.). In our study, the concentration of exogenous Methoprene was so optimized that it could prolong the larval life only for two days aiming at the enhancement of silk production.

Figure 1: SDS - PAGE pattern of haemolymph and fat body proteins in *B. mori* treated with Methoprene

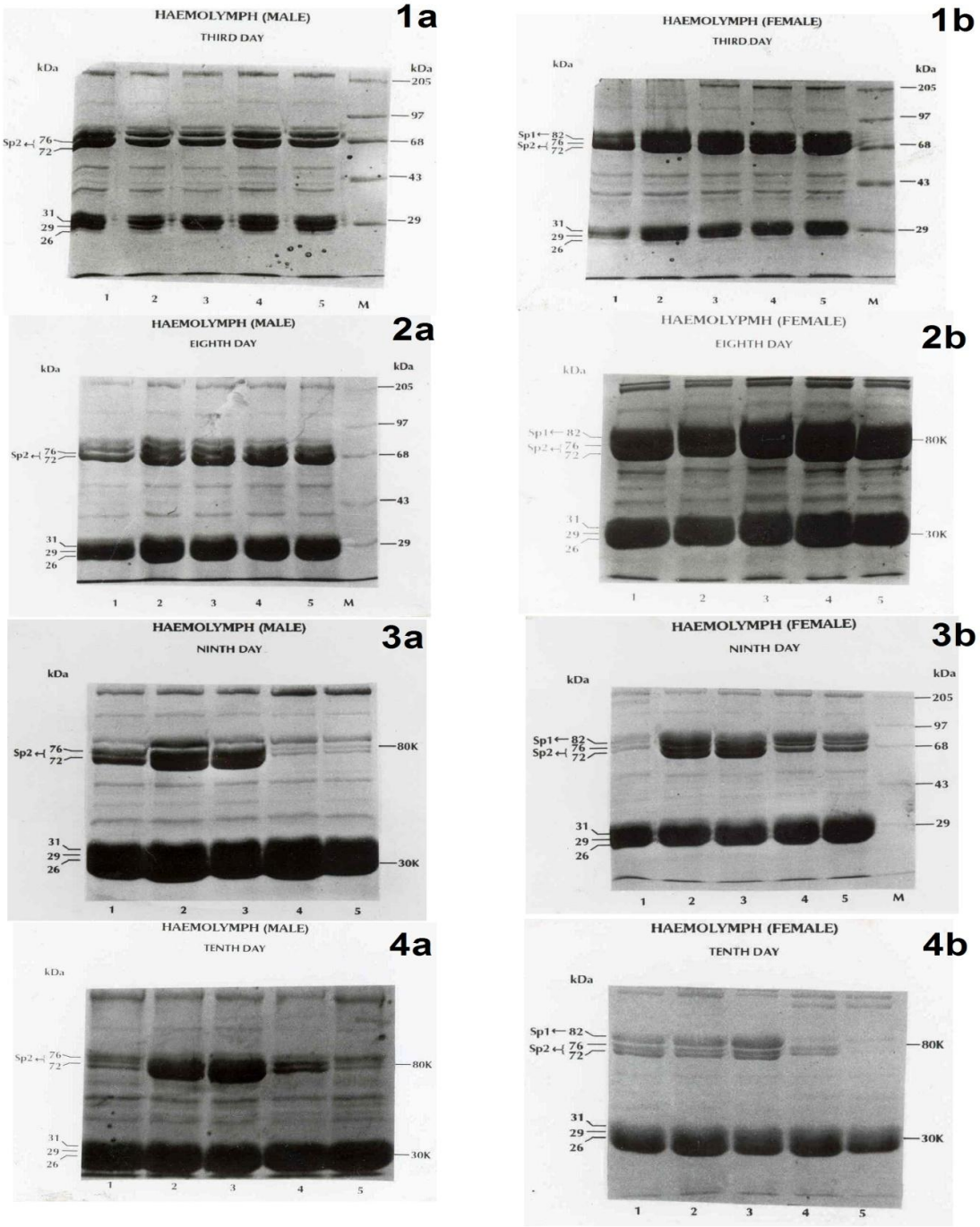

**Figure 1: Haemolymph protein profile in male and female 5th instar larvae; 1, 2, 3 and 4; a and b – Days 3, 8, 9 and 10 respectively; Lane 1-0.1 µg; Lane 2-0.5 µg; Lane 3-1.0 µg; Lane 4 -water treated control; Lane 5-Untreated control; M-Molecular weight markers

Figure 1(Contd): SDS - PAGE pattern of haemolymph proteins and fat bodies in *B. mori* treated with Manta

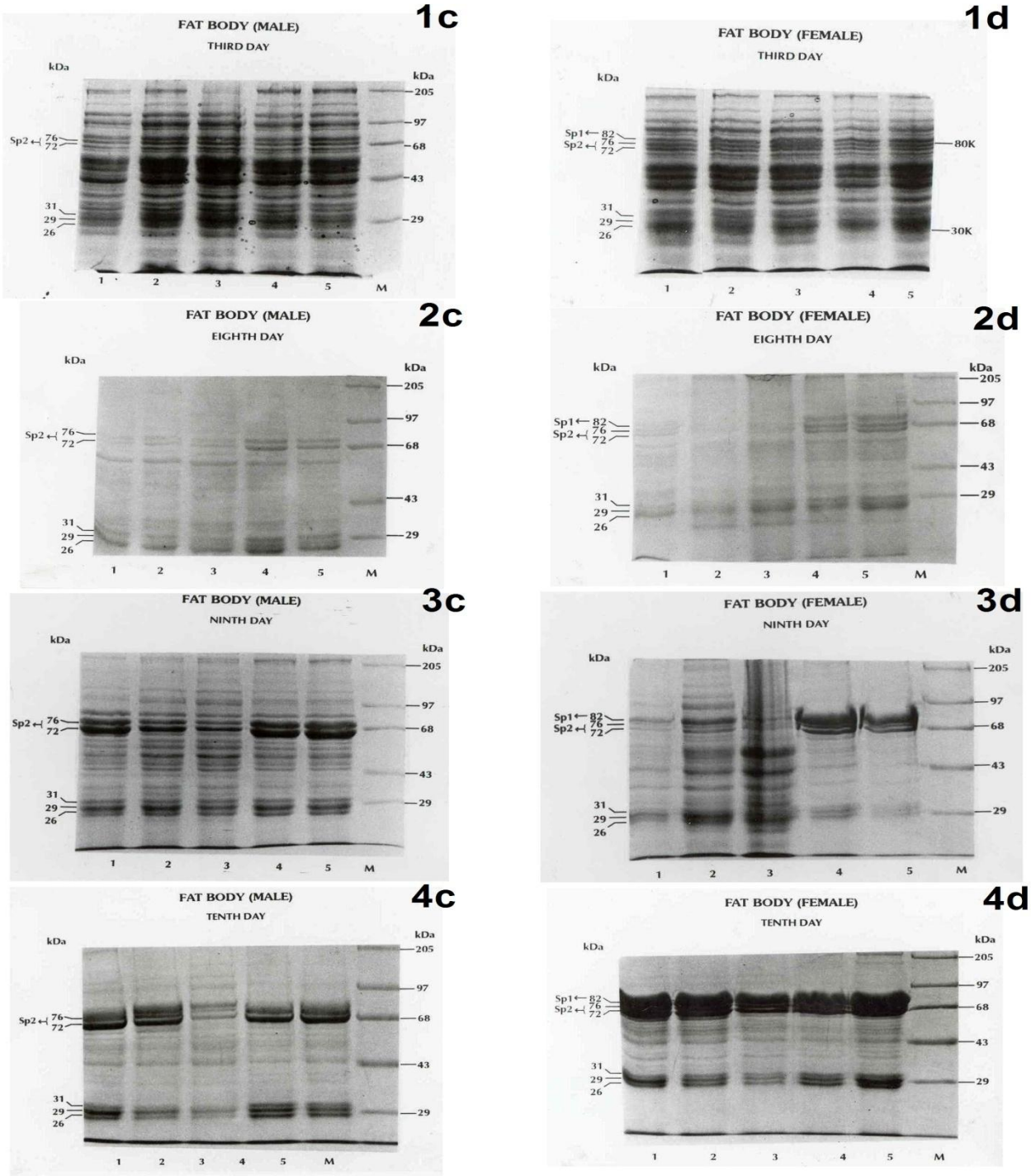

**Figure 1: Fat body protein profile in male and female 5th instar larvae; 1, 2, 3 and 4;c and d – Days 3, 8, 9 and 10 respectively; Lane 1-0.1 µg; Lane 2-0.5 µg; Lane 3-1.0 µg; Lane 4 -water treated control; Lane 5-Untreated control; M-Molecular weight markers

Figure 2: Identification of Sp-1 and Sp-2 by immunoblotting with storage protein antibodies

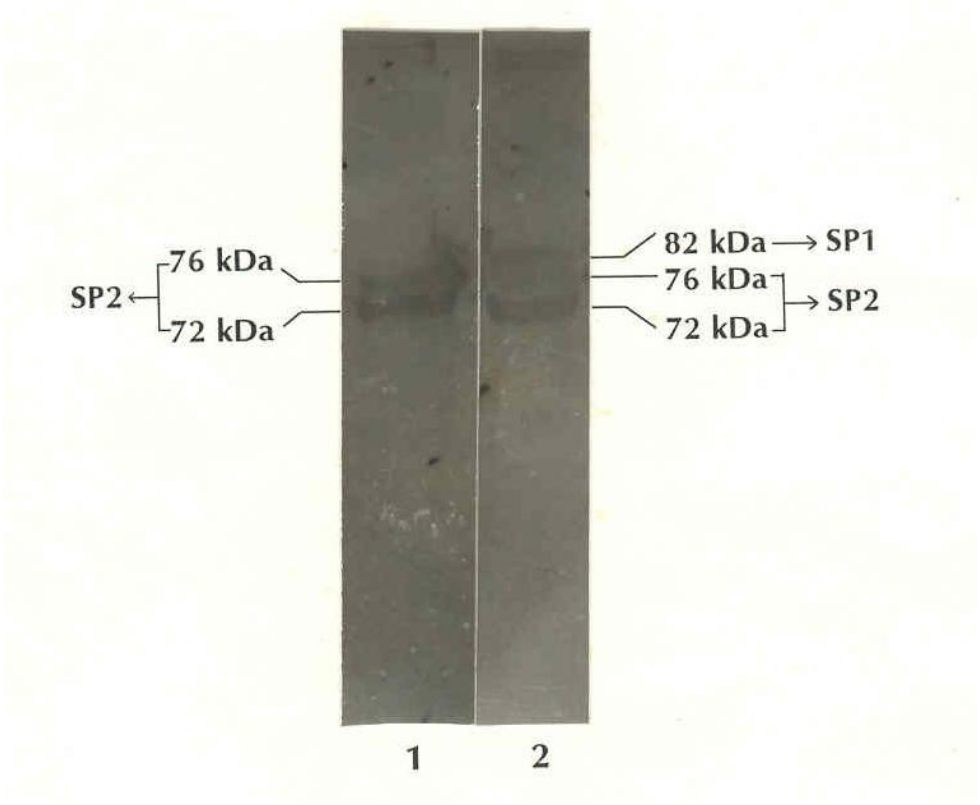

**Figure 2: Lane 1 and 2 - Male and fenale 5th instar larvae of *B. mori*

DISCUSSION

Analysis of protein profile of haemolymph showed marked difference in quantity of storage protein irrespective of sex. It was noticed that on day 9 and 10, the storage proteins concentration corresponding to 0.5 µg and 0.1 µg treatments were maximum in a dose dependent manner when compared to that of control. The exogenous application of JHA might have led to the persistence of JHA for a longer duration in the haemolymph. This existence of JHA prevented successful prothoracicotropic hormone (PTTH) transduction and there by inducing the secretion of ecdysteroid by prothoracic glands as reported by Gu *et al.*[11] in *B. mori* and in other insects[12]. This is also confirmed by the high concentration of storage proteins in the treatments for two more days. In contrast, protein concentration in the control group declined abruptly due to the high ecdysteroid. The protein uptake or sequestration and formation of intracellular storage granules under the influence of ecdysteroids as reported in *Pieris brassicae*[13], *Galleria mellonella*[14], *Sarcophaga peregrine*[15] and *Spodoptene littura*[16]. Our study suggested methoprene could act indirectly and suppressed the sequestration of storage proteins through its inhibition of ecdysone secretion. Earlier Kujiara and Yamashita[1] showed the application of methoprene did not prevent the synthesis and secretion of proteins in to the haemolymph, but suppressed completely their sequestration in to the fat body.

It is well documented that as long as the JHA titre in the haemolymph is high, the ecdysone will not be secreted. In the Manta treated worms exogenous Manta might have persisted in the haemolymph for two more days, there by resulting in the prolongation of larval duration. The high concentration of storage proteins in the haemolymph of treated larvae during extended period may be due to delayed sequestration of storage proteins by fat body. Recently, storage protein receptors identified in the silk gland of *B.mori*[17] revealed the possibility of utilization of the high concentration of storage protein from the haemolymph of Manta treated groups for the silk synthesis by the silk gland mediated by receptors.

CONCLUSION

Application of Manta at 0.75 µg and 1.0 µg dosages were found to be effective for cross breed LxNB4D2, the most exploited race in South India. The two dosages 0.75 µg and 1.0 µg were found to be optimum to prolong the larval duration by two days and further increase of the dosage will not be advantageous to the farmers from the economic point of view. Moreover, the extension of larval duration will not be profitable as it increases the cost incurred for maintenance and labor charge.

CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES

1. Kajiyura Z, Yamashita O. Arch. Insect Biochem. Physiol. 1989; 12:99-109.
2. Nagata M, Kobayashi M. J. Seric. Sci. Jpn. 1990; 56:461-468
3. Terashima J, Yasuhara N, Iwami M, Sakurai S, Sakurai S. Bombyx mori. Dev. Genes Evol. 2000; 210(11):545-558.
4. Ogawa K, Tojo S. Appl. Ent. Zool. 1981; 16:288-296.
5. Wheeler DE, Tuchinskaya I, Buck NA, Tabashnik BE. J. Insect Physiol. 2000; 46(6):951-958.
6. Tojol S, Liu Y, Zheng Y. Recent Advances in Insects and Other Arthropods. 2012; 1:32.
7. Kajiyura Z, Kodano – Okuda K, Yamashit O. J. Seric. Jpn. 1987; 56:398
8. Mamatha DM, Kanji VK, Cohly HH, Rao MR. Int. J. Environ. Res. Publ. Health. 2008; 5(2):120-124
9. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. J. Biol.Chem. 1951; 193:265-275.
10. Towbin H, Staehelin T, Gordon J. Proc. Nat. Acad. Sci. USA. 1979; 76:4350-54.
11. Gu SH, Chow YS, Yin CM. Mol. Cell. Endocrinol. 1997; 127:109-116
12. Smith WA, Rybczynski R. Prothoracicotropic hormone. In: Insect Endocrinology (L. I. Gilbert, editor), Academic Press; 2011. pp. 1-62.
13. Tojo S, Kiguchi K, Kimura S. J. Insect Physiol. 1981; 491-497.
14. Miller SG, Silhacek DL. Insect Biochem. 1982; 12:293-300
15. Ueno K, Natori S. J. Biol. Chem. 1984; 259:12107-11.
16. Tojo S, Morita M, Hiruma K. J. Insect Physiol. 1985; 31: 243-249.
17. Elmogy M, Iwami M, Sakurai S. Efflatounia. 2007; 7:1-8