The Journal of Basic and Applied Zoology

RESEARCH Open Access

Gene cloning, recombinant expression, and bioassay of an allatotropin in *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae)

R. Mamtha, Tannavi Kiran, B. S. Chaitra, Saraswathi Saraswathi, R. Sowrabha, Karthik V. Rao and D. Manjulakumari*

Abstract

Background: Allatotropin, a neuropeptide found in several invertebrates indirectly regulates vitellogenesis by stimulating juvenile hormone biosynthesis by the corpora allata. Here, we cloned and expressed the gene encoding allatotropin of *Spodoptera litura* (tobacco cutworm), a polyphagous pest in the Asian tropics. This study is aimed at studying the effect of recombinant protein on egg-laying in females of *S. litura* as it could be used as a method to control the pests from a reproductive perspective.

Results: The protein encodes a full-length open reading frame consisting of 173 amino acids and was rich in arginine (10%) and glutamic acid (9.3%). The theoretical pI of the protein was 5.47 and a hydrophobic signal peptide of 22 residues was predicted. The recombinant allatotropin was expressed in *Escherichia coli* BL21 (DE_3) and purified by nickel exchange chromatography. The molecular weight of the recombinant protein was about 37 kDa and expression levels up to 5.3 mg/ml were achieved. Injection assay in vitro indicated that allatotropin induces egg-laying during the first scotophase after treatment in females of *Spodoptera litura*.

Conclusion: Allatotropin induces egg-laying in female moths and could be a potential molecule for the development of control strategies against *Spodoptera litura*. In this strategy, the protein if delivered to the females before mating may lead to accelerated egg deposition much before she encounters the male moths, thus the population being checked as the eggs deposited by the females are unfertilized. Thus, the present work could lead to the development of a protein based biopesticide resulting into a species-specific and an eco-friendly way of pest control.

Keywords: Allatotropin, Juvenile hormone, Prokaryotic expression, Egg-laying, *Spodoptera litura*

Background

Allatotropin, a member of the family of myoactive neuropeptides found in several invertebrate phyla stimulates juvenile hormone (JH) biosynthesis in corpora allata (CA) (Elekonich & Horodyski, 2003). Juvenile hormones are crucial in all insect developmental and reproductive events including embryogenesis, larval moulting, metamorphosis, vitellogenin synthesis, vitellogenin uptake by the ovaries and ovarian development, spermatogenesis and growth of male accessory glands (Gade et al., 1997; Koeppe et al., 1985; Nijhout, 1994; Riddiford, 1994). Allatoregulating neuropeptides are numerous and have been localized in various insect tissues. Among the identified allatotropins, Manse-AT (AT 1) was the first peptide to be isolated from the adult heads of *Manduca sexta* (Kataoka et al., 1989) and later was also known in several other insects such as *Lacanobia oleracea* (Audsley et al., 2000) and *Galleria mellonella* (Boguś & Cymborowski, 1984). In the greater wax moth *G. mellonella*, AT 1 peptide consists of 13 amino acids and stimulates JH biosynthesis in

*Correspondence: manjulakumari_doddamane@yahoo.co.in
Department of Microbiology and Biotechnology, Bangalore University, Bangalore, Karnataka 560056, India

the CA in a dose-dependent manner. The terminal steps of JH biosynthesis were reported to be affected by AT 1 in larval workers of the honey bee, *Apis mellifera* (Rachinsky et al., 2000). Furthermore, AT 1 was shown to be potent stimulators of heart rate, muscle contraction and gut peristalsis (Koladich et al., 2002). It also exerts an inhibition in the ion transport across the midgut of *Manduca sexta* (Lee & Horodyski, 2002; Lee et al., 1998). In the tomato moth, *Lacanobia oleracea*, injection of AT 1 peptide into the sixth instar larvae led to increase in mortality rate, reduction in body weight and also delay in pre-pupal development as a consequence of JH synthesis (Audsley et al., 2001). Another allatotropin reported as Spofr-AT 2 was identified and characterized by cloning the cDNA encoding Manse-AT in *Spodoptera frugiperda* (Abdellatief et al., 2004). The role of Spofr-AT 2 in controlling the synthesis and transfer of JH during mating using the RNA interference technique was reported by Hassanien et al. (2014) in *Spodoptera frugiperda*. Here, the knockdown of the AT 2 gene resulted in low levels of JH transferred from male to female as well as a lower number of eggs deposited. In many Lepidopterans, JH is crucial for egg maturation and/or production (Ramaswamy et al., 2000; Shu et al., 1998). So far, there are no reports on allatoregulating neuropeptides or their functions associated with egg production in *Spodoptera litura*.

Spodoptera litura Fabricius (Lepidoptera: Noctuidae) is considered as a serious agricultural pest attacking more than 120 host plants belonging to 44 families (Qin et al., 2004) wherein 40 species are known from India (Garad et al., 1984). The main crop species attacked by *Spodoptera litura* in the tropics include taro, tobacco, flax, cotton, jute, groundnuts, maize, alfalfa, rice, soybeans, tea, and vegetables (Chari & Patel, 1983). Due to pesticide resistance and environmental problems arising from the long-term application of synthetic chemical pesticides against the pest (Tong et al., 2013), the development of novel biopesticides has become a major research interest. At present, the target for pest control are proteins/peptides especially the behaviour modifying peptides or neuropeptides in insects (Alstein & Nässel, 2001; Lenaerts et al., 2019). Fónagy (2006) in his review grouped well-known neuropeptides such as *bombyxin* (Ishizaki et al., 1983), allatotropins/allatostatins (Kataoka et al., 1989; Schoofs et al., 2017; Stay et al., 1995), pheromone biosynthesis activating neuropeptide (Raina et al., 1989), Adipokinetic hormone (Stone et al., 1976), trypsin modulating oostatic factor (Borovsky, 2003; Borovsky et al., 1998) and so on, into four major classes such as growth and development, reproduction, metabolism and homeostasis according to function, structure, mode of action and discussed their potential roles in pest control. Thus, with this background, the present study was conceived to clone and express the allatotropin of *Spodoptera litura* in *Escherichia coli*. The *E. coli* expression system acts as a rapid and simple system for the expression of recombinant proteins in a large scale at a short time. However, the disadvantage of this system includes the formation of inclusion bodies and often requires the use of denaturants at a high concentration to solubilize these proteins. Hence, in this study a pET-32a + vector series (Novagen, Germany) was designed for expression of peptide sequences fused with the 109 aa Thioredoxin tag (Trx-tag) to avoid the formation of inclusion bodies. The effect of recombinant protein on egg-laying was further studied by an injection method. Injection method has often been used by several researchers to study the effect of purified peptides/proteins like the studies in *Drosophila melanogaster* (Monsma & Wolfner, 1988), *Spodoptera litura* (Yu et al., 2014), *Aedes aegypti* (Fuchs & Hiss, 1970); *Bombyx mori* (Ando et al., 1996), *Helicoverpa zea* and *Helicoverpa armigera* (Eliyahu et al., 2003; Kingan et al., 1993) and so on. Finally, the outcome of this study could lead to the production of a protein-based biopesticide for controlling the pests from a reproductive perspective.

Methods

Insects

The parental stock of *Spodoptera litura* (NBAII-MP-NOC-02) was obtained from National Bureau of Agricultural Insect Resources, Bengaluru. The neonates were initially reared in groups on an artificial diet (Divakara et al., 2011) and when the larvae reached 3rd instar, they were transferred into individual rearing vials, until pupation to avoid cannibalism. The insects were maintained under laboratory conditions with a temperature of 25 ± 2 °C and natural light–dark cycles. The pupae collected were disinfected with 0.02% formaldehyde, sexed and maintained separately according to their peripheral characters. Cotton dipped in 10% honey solution was provided as food to the adult female moths.

Characterization of the allatotropin gene

The nucleotide sequence of allatotropin isoform X2 (NCBI Reference Sequence: XM_022974503.1) identified from *Spodoptera litura* genome was translated into all six reading frames using ExPASy translate tool. The longest reading frame with the stop codon was selected for in-silico analysis of the theoretical pI and amino acid composition using Protparam tool provided by ExPASy (Gasteiger et al., 2005). SignalP 4.0 program was used to predict the presence of signal peptide cleavage sites in the amino acid sequence (Petersen et al., 2011).

Gene cloning

The full-length coding sequence of allatotropin was optimized using the codon frequency table of *E. coli* (Kazusa DNA Research Institute, Japan), while the amino acid sequence was kept unaltered except for the addition of a methionine at the *N*-terminal as a start codon. A pair wise alignment of the optimized sequence was performed with the allatotropin reference sequence (Accession No. XM_022974503.1) using EMBL-EBI pair wise alignment tool based on Needleman-Wunsch algorithm. The pET-32a + vector series (Novagen, Germany) fused with the 109 aa Thioredoxin tag (Trx-tag) was designed for expression of the protein to assist proper folding of the protein and to keep them from precipitating. The gene was chemically synthesized without signal peptide sequence containing cleavage site between positions 22 and 23 before ligation into pET32a$^+$ cloning vector. The signal sequence from the gene was removed in order to express a mature protein and moreover *E. coli* translocation machinery cannot recognize the signal peptide native to *Spodoptera litura*. The recombinant plasmid was supplied by Gene Universal, USA.

Transformation and expression of allatotropin gene in *E. coli*

The plasmid pET32a$^+$/allatotropin was transformed into chemically competent *E. coli,* BL21 (DE_3) cells by heat shock and colonies were grown on LB culture medium containing ampicillin (100 µg/ml) (Dagert & Ehrlich, 1979; Green et al., 2012). Positive clones were confirmed by colony PCR using universal T7 primers. PCR (30 cycles) was performed as follows: denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1.5 min. The recombinant plasmid was analyzed on 1% agarose gel and stored at − 20 °C. The clone harbouring the recombinant plasmid was inoculated into 3 ml LB broth containing ampicillin (100 µg/ml) and incubated at 37 °C. One ml of the overnight grown culture was added to 100 ml LB broth containing ampicillin (100 µg/ml) and was incubated at 37 °C on a rotary shaker until the OD_{600} reached 0.3–0.4. Protein expression was induced by the addition of Isopropyl β-d-1-thiogalactopyranoside {IPTG (Sigma)} at a final concentration of 1 mM/L directly to the cell suspension for 4 h at 37 °C. The resulting cells were harvested by centrifugation at 4000 rpm for 10 min and freeze-thawed to lyse the cells. This process of freeze-thawing was repeated thrice and the mixture was centrifuged. The expression of recombinant protein from the obtained supernatant was analyzed by SDS-PAGE (Laemmli, 1970).

Purification of the recombinant protein

The recombinant protein was purified using His-Tagged Bacterial Protein Purification kit (Himedia, India). A 100 ml induced cell pellet was re-suspended in minimum volume of distilled water and the cell lysate was prepared by freeze-thawing method. The pre-packed column was centrifuged at 1000 rpm for 1 min to devoid the preservative. The spin column was equilibrated with 0.4 ml of Equilibration buffer (10 mM imidazole) and centrifuged at 1000 rpm for 1 min. The flow through was discarded. This step was repeated once again. The sample containing the His-tagged protein was loaded onto the pre-equilibrated column and was incubated for 30 min to ensure proper binding of the protein to the resin. The column was centrifuged at 1000 rpm for 1 min to remove the unbound proteins. The column was then transferred to a new collection tube and 0.4 ml of wash buffer (25 mM imidazole) was added through the column and mixed manually be inversion. The column was centrifuged at 1000 rpm for 1 min and the flow through was discarded. This step was repeated twice for a total of three washes. The column was again transferred to a new collection tube and 0.4 ml of elution buffer (250 mM imidazole) was added to the column and mixed thoroughly for 10 min. Later the column was placed in a new collection tube and centrifuged at 1000 rpm for 1 min. The elution step was repeated twice for a total of three individual eluants. The purity was checked by SDS-PAGE and the concentration was determined using a NanoDrop 2000c Spectrophotometer (Thermo Scientific).

Western blotting

Proteins separated by electrophoresis were electroblotted onto Nitrocellulose membranes using Tris–glycine buffer at 50 V for 1.5 h at room temperature (RT) (Kurien & Scofield, 2006). Following a transfer, the membrane was blocked for 1 h at RT with 5% BSA in Tris-buffered saline (20 mM Tris pH 7.4, 150 mM NaCl and 0.01% Tween-20). The blot was further incubated for 1.5 h with anti-his tag mouse monoclonal antibody (G-Biosciences, USA) in the ratio of 1:1500 followed by incubation with Goat anti mouse IgG-HRP. The protein-antibody complex was developed using the picoLUCENTTM PLUS HRP Chemiluminescent kit (G-Biosciences, USA).

Determination of the biological activity

5, 15 and 25 pg/µl of the recombinant protein was injected using a Hamilton syringe into the abdominal cavity through the intersegmental membrane of the anesthetized 24 h old *Spodoptera litura* virgin females. Buffer injected virgin females served as control and virgin females without being injected were also observed to rule

out the effect of the injection. The number of eggs laid by each group during the first scotophase after treatment was recorded.

Statistical analysis

The experimental data from at least three different experiments are expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. A level of $p < 0.05$ was considered significant.

Results

Characterization of the allatotropin gene

The protein sequence was found to be rich in arginine (10%) and glutamic acid (9.3%). The total number of negatively charged residues (Asp + Glu) was 27 and the total number of positively charged residues (Arg + Lys) was 23. The theoretical isoelectric value of the protein was 5.47. The SignalP software predicted a hydrophobic signal peptide of 22 residues indicating the protein to be secretory in nature with the cleavage site between positions 22 and 23.

Gene cloning

The codons were optimized as per the codon frequency usage of *E. coli* and the Codon Adaptation Index (CAI) value was adjusted to 0.95. The GC content was adjusted to the optimum level of 30–70%. A pair-wise alignment performed with the allatotropin reference sequence (Accession No. XM_022974503.1) using EMBL-EBI pairwise alignment tool revealed 62.6% similarity with the reference sequence (Fig. 1). The optimized nucleotide was artificially synthesized and ligated between EcoRI and XhoI restriction sites in pET32a^{+} vector. Further, the recombinant plasmid was transformed into the cloning host BL21 (DE3).

Recombinant protein expression

After induction with IPTG, the expression of protein was checked on SDS-PAGE (Fig. 2). Purification of the expressed protein with Ni–NTA resin showed that the molecular weight of the recombinant protein was ~ 37 kDa (Fig. 3). Immunoblot analysis with an anti-his tag mouse monoclonal antibody confirmed the identity of the purified protein (Fig. 4). Moreover, the final concentration of the recombinant protein was ~ 5.3 mg/ml.

Biological activity of allatotropin

The number of eggs laid by the females upon injection of the recombinant protein at a concentration of 5, 15 and 25 pg/µl was 258 ± 49.7, 220 ± 106.5 and 248 ± 63.9 respectively on the first scotophase after treatment when compared to buffer injected virgin females and virgin females which were 17 ± 5.1 and 4 ± 3.9 respectively. The treatment group was significantly different from the control group although no significant difference was observed among the treatment groups (Fig. 5).

Discussion

This study was focused on cloning and expression of the allatotropin of *Spodoptera litura* in *E. coli*. Cloning and expression of proteins in *E. coli* has been the most common approach to study the proteins which are difficult to purify in large quantities from its source. But a major drawback associated with the use of *E. coli* is its property to form inclusion bodies of misfolded proteins whenever a protein is overexpressed. But this limitation could be overcome by the fusion of thioredoxin protein with the protein of interest which helps in the solubilization of the protein thereby avoiding precipitation of the protein to form inclusion bodies (Sachdev & Chirgwin, 1998). Heterologous expression of many insect proteins have been successfully done, for example, nitrophorins (Champagne et al., 1995), chemosensory protein (Zhang et al., 2012), vitellogenin (Wu et al., 2015), chitinase (Esther Shoba et al., 2016) and so on. However, allatotropin, though the gene sequence is known, has not been expressed in microbial system.

Here, the entire sequence of allatotropin about 1609 bp was obtained from the genome of *Spodoptera litura*. The sequence with the coding regions being 519 bp long that encodes 173 amino acids was successfully expressed in *E. coli* system along with the Trx-tag. The protein was obtained in the soluble fraction without forming inclusion bodies and purified using a Ni–NTA column as the protein contains His-tag. The protein expression levels were achieved up to 5.3 mg/ml after 4 h of induction with IPTG. The effect of allatotropin in inducing egg-laying behaviour in *Spodoptera litura* females was studied by injection assay. The experiment performed by injecting the protein into the abdominal cavity of the female moths exhibited accelerated egg-laying behaviour unlike the other two sets of moths. The number of eggs laid by the females injected with 5, 15 and 25 pg/µl of the recombinant proteins was not significantly different from each other, while they significantly varied from the buffer injected and virgin females which laid few eggs during the first scotophase of treatment. This shows that very low concentration of the protein is sufficient to stimulate egg-laying behaviour in *Spodoptera litura* females, while earlier studies by Kingan et al. (1993) have also demonstrated the activity of pheromone biosynthesis activating neuropeptide by injecting only 2 pmol of the peptide into *Helicoverpa zea* females.

```
Reference         1 ATGAACATTTCAATGCATTTGGCGGTAGCTGTGGCGGCGGCGGCCTGTCT    50

Optimized         1 --------------------------------------------------     0

Reference        51 GTGCGTGTGCGCAGCGGCACCTGAGAATCGACTCGCGCGCACCAAACAAC   100
                                  ||.|||||.|||||.||.||.||||||||||.|
Optimized         1 ----------------GCTCCTGAAAATCGCCTGGCACGCACCAAACAGC    34

Reference       101 AACGCCCCACCCGCGGCTTCAAGAACGTCGAGATGATGACCGCCAGGGGA   150
                    |.||||||.||||||||.||.||.||.||.||.||||||||||..|.||.
Optimized        35 AGCGCCCTACCCGCGGTTTTAAAAATGTTGAAATGATGACCGCACGTGGT    84

Reference       151 TTCGGCAAGCGAGACAGGCCACACACTCGGGCTGAGCACCAGGACAGCTA   200
                    ||.||.||.||.||..|.||.||.||.||.||.||.||.|||||.||.||
Optimized        85 TTTGGTAAACGCGATCGCCCGCATACCCGCGCCGAACATCAGGATAGTTA   134

Reference       201 TGACTCCCACGCTCGCAGGAAGTTTAACCCCAAGAGCAACCTCATGGTCG   250
                    |||....||.||.||..|.||.|||||.||.||.||.||.||.|||||.|
Optimized       135 TGATAGTCATGCACGTCGCAAATTTAATCCGAAAAGTAATCTGATGGTGG   184

Reference       251 CCTACGACTTTGGCAAAAGGAGTGGTAATGATGACGTTACTGATGAAGCA   300
                    |.||.||.|||||.|||.|.|||||.||||||||.|||||.||||||||.
Optimized       185 CATATGATTTTGGTAAACGTAGTGGCAATGATGATGTTACCGATGAAGCC   234

Reference       301 GTGTACGGTTTGGACAACTTCTGGGAGATGCTGGAGGCTACACCTGAGAG   350
                    |||||.|||.||||.||.||.|||||.||||||||.||.||.||.||..|
Optimized       235 GTGTATGGTCTGGATAATTTTTGGGAAATGCTGGAAGCAACCCCGGAACG   284

Reference       351 GGAAGGACAAGAGAATGACGAGAAGACTTTGGAAAGCATTCCTTTGGACT   400
                    .|||||.||.||.|||||.||.||.||..||||||||||||..||||.|
Optimized       285 TGAAGGCCAGGAAAATGATGAAAAAACCCTGGAAAGCATTCCGCTGGATT   334

Reference       401 GGTTTGTGAACGAGATGCTGAATAATCCAGATTTCGCGCGATCTGTGGTC   450
                    ||||||||||.||.||||||||||||||.|||||.||.||....||.||.
Optimized       335 GGTTTGTGAATGAAATGCTGAATAATCCGGATTTTGCACGTAGCGTTGTT   384

Reference       451 CGCAAGTTCATTGACCTCAATCAGGACGGCATGCTATCATCGGAGGAGCT   500
                    ||.||.||.|||||.||.||||||||.||.|||||.......||.||.||
Optimized       385 CGTAAATTTATTGATCTGAATCAGGATGGTATGCTGAGCAGTGAAGAACT   434

Reference       501 GTTAAGGAACGTCGTTTAAATACACATTT    529
                    |.|..|.||.||.||.
Optimized       435 GCTGCGTAATGTGGTG------------    450
```

Fig. 1 Pair-wise alignment of nucleotide sequences using Needleman-Wunsch algorithm

Allatotropins (AT) have been isolated from numerous arthropod species, identified from the sequences of cloned genes, or deduced in-silico from nucleotide sequence databases (Egekwu et al., 2014; Elekonich & Horodyski, 2003; Weaver & Audsley, 2009). Previous studies have shown that neuropeptides regulate JH biosynthesis at several steps of the synthetic pathway (Kamruzzaman et al., 2020; Wielendaele et al., 2013). *Aedes aegypti* allatotropin and farnesoic acid probably act on the terminal steps of JH biosynthesis (Li et al., 2003). Moreover, Manse-AT had stimulatory effects on JH I to JH III release through increasing the supply of acetyl- and propionyl-CoA precursors (Teal, 2002). JH production is stimulated by allatotropins and there is a direct correlation between JH titer and the number of eggs deposited. In *Spodoptera frugiperda*, silencing the allatotropin gene reduced the oviposition rates in adult females (Griebler et al., 2008). A possible mechanism in which the TOR pathway regulates vitellogenin (Vg) synthesis was proposed by Lu et al. (2016). In this pathway, the signals (AT) activates juvenile hormone acid methyltransferase (JHAMT) to methylate JH acid into JH further activating Vg synthesis. Lenaerts et al.

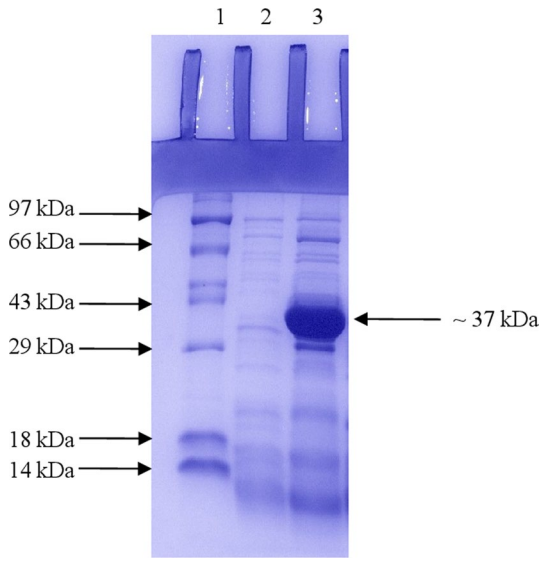

Fig. 2 Induction of pET32a + /allatotropin clone analyzed on 12% SDS PAGE. Lane 1—Protein marker, Lane 2—Uninduced sample, Lane 3—Induced sample

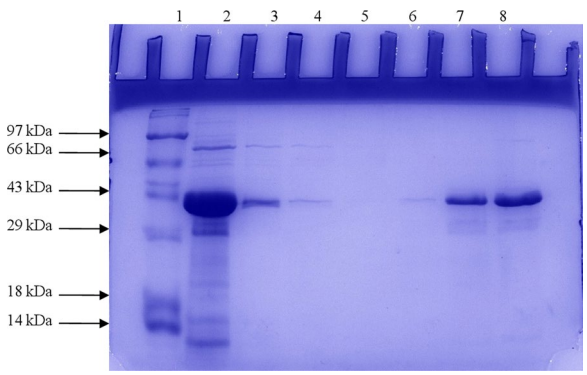

Fig. 3 Purification of recombinant protein and its analysis on 12% SDS PAGE. Lane 1—Protein marker, Lane 2—Induced sample, Lane 3—Flow through, Lane 4—Unbound fraction 1, Lane 5—Unbound fraction 2, Lane 6—Elaunt 1, Lane 7—Eluant 2, Lane 8—Eluant 3

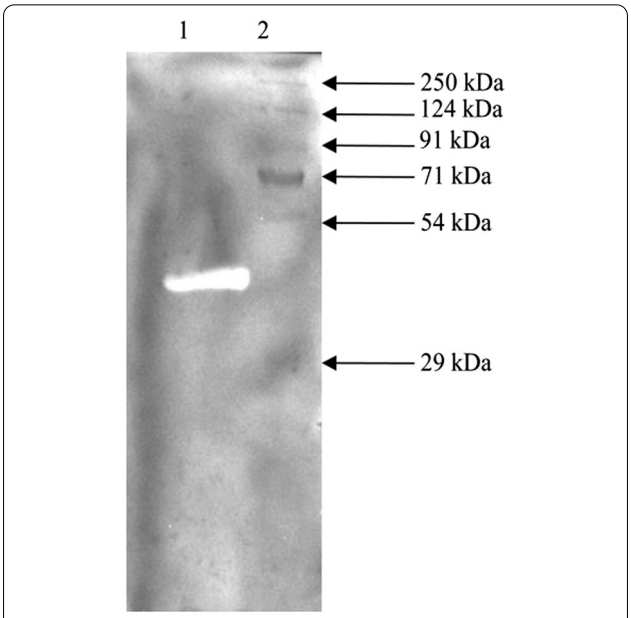

Fig. 4 Western blotting analysis for confirmation of expressed allatotropin**.** Lane 1—Recombinant protein, Lane 2—Pre-stained marker

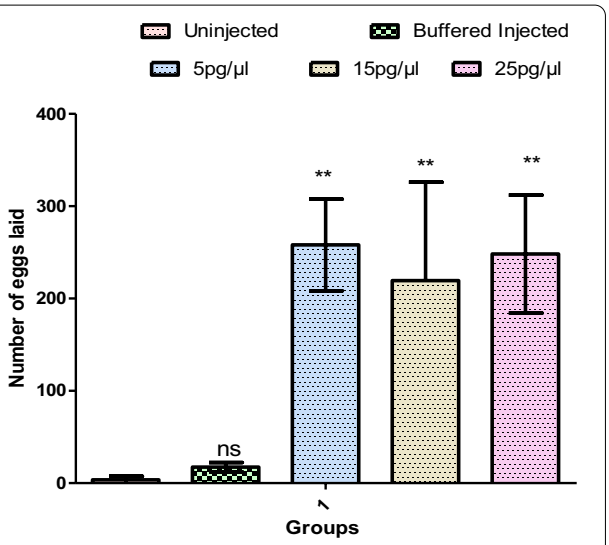

Fig. 5 Number of eggs laid by *Spodoptera litura* females followed by injection with different concentrations of recombinant protein in comparison with controls ($n = 10$)

(2019) in their review have also suggested allatotropin to be involved in insect oogenesis.

The mechanism wherein synthesis of brain factors such as the allatotropin leads to activation of JH synthesis in the CA is well established in several insects. Yet, injection of allatotropin directly into the hemolymph and its effect in female moths has not been studied so far. In our study, we have determined the role of allatotropin in inducing egg-laying behaviour when artificially injected into female moths and we speculate that the allatotropin injected into hemolymph bring about the necessary change either indirectly or directly by traversing to CA or ovary respectively (Fig. 6). The virgin females, when injected with allatotropin, behaved in a manner normally exhibited by the mated females. However, in nature, a well-known strategy developed by male insects is to transfer accessory gland proteins along with sperms to the female reproductive tract during mating, where they facilitate several processes that modify female

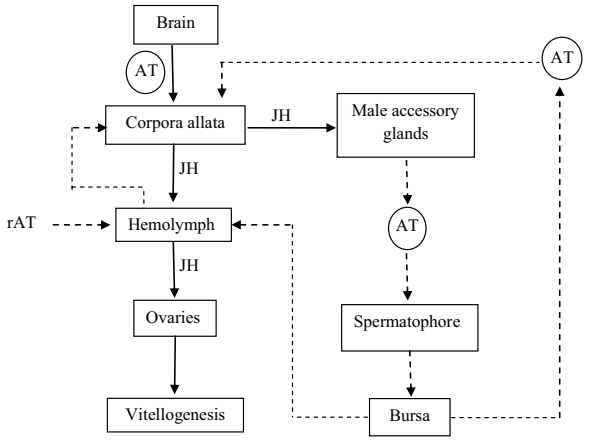

Fig. 6 Possible targets for allatotropin (AT) in *Spodoptera litura* adults, associated with vitellogenesis. Solid lines represent known pathways, while broken lines are speculative based on information from other species. The known pathway works through some of the brain factors, such as the allatotropin, to induce JH biosynthesis in the corpora allata, and JH, in turn reaching the ovaries via hemolymph, where it regulates the process of vitellogenesis in the females. While in males, the JH synthesized in CA is transferred to the accessory glands. We hypothesize the transfer of AT by means of spermatophore to the bursa of females during copulation (broken line), in turn reaching the CA either directly or via hemolymph, thus, inducing JH biosynthesis. While the recombinant allatotropin injected into hemolymph may induce JH synthesis in the hemolymph or travel to CA and bring about JH synthesis. (Juvenile Hormone: JH; Allatotropin: AT; Recombinant allatotropin: rAT)

post-mating behaviour such as sperm protection, competition, storage and activation (Harshman & Prout, 1994; Neubaum & Wolfner, 1999), as well as rendering her reluctant or unable to remate either for some time or permanently, stimulating an increase in the number and rate of development of eggs and also affecting longevity in females, thereby resulting in the males' ability to sire a significant proportion of the females' offspring.

Egg maturation and egg-laying are two functionally distinct but closely related processes. Several reports in insects demonstrate that male accessory gland (MAG) secretion alters the physiological state of virgin females and allows oviposition to occur. In one such study, Pickford et al. (1969) for the first time revealed that an egg-laying stimulant was produced by the accessory glands of the migratory grasshopper *Melanoplus sanguinipes*. In *Schistocerca gregaria*, implantation of accessory glands in the female locust invoked an increase in the number of eggs produced (Leahy, 1973). Similar observations have also been recorded for *Musca domestica* (Riemann & Thorson, 1969), *Hylemya brassicae* (Swailes, 1971) and *Drosophila funebris* (Baumann, 1974). However, in Lepidoptera studies demonstrating the effect of an accessory gland secretion on oviposition is limited. In *Helicoverpa armigera*, small peptides having a molecular weight of 6.7 kDa enhanced fecundity in treated virgin females (Shobha et al., 2009). Similarly, Jin and Gong (2001) also reported oogenesis and oviposition factors (OOSF) with an estimated molecular mass between 55 and 66 kDa in the MAGs of *Helicoverpa armigera*.

McNeil and Tobe (2001) hypothesized a sex peptide that increases JH titers in females transferred from the male accessory glands to females after mating could potentially be an allatotropin. They reported a close similarity between the AT of *Pseudaletia unipuncta* and accessory gland myotropin, a neuropeptide of *Locusta migratoria*. The accessory gland neuropeptide of *Locusta migratoria* transferred from male to female during mating stimulates oviposition (Paemen et al., 1991). Moreover, the sex peptide in *Drosophila melanogaster* is known to increase oviposition by activation of corpora allata resulting in the production of juvenile hormone (Chen, 1984; Kubli, 1992). Although the proteinaceous factors of MAGs influencing egg-laying behaviour have been widely established in several insects, very little information is available on *Spodoptera litura,* a destructive pest of many host plants across the globe. A preliminary work by Sridevi et al., (1987) reported that the MAG extracts of *Spodoptera litura* have an oviposition stimulating factor which induced oviposition in virgin female moths. Later on, Yu et al. (2014) demonstrated that MAG secretions modulate female post-mating behavior in *Spodoptera litura*, but failed to characterize these proteins.

Thus, a strategy well established in this study is that allatotropin if delivered to the females before mating may lead to accelerated egg deposition much before she encounters the male moths, consequently the population being checked as the eggs deposited by the females are unfertilized. The present work could lead to the development of a protein-based biopesticide and the use of neuropeptides could be an answer to overcome the issues related to the use of chemical pesticides, toxic proteins as well as the issues related to the use of naturally occurring viral pesticides. Hence, in the near future, insect neuropeptides such as the allatotropin could be a promising target for the generation of novel selective insecticides for successful pest management.

Conclusion

The nucleotide sequence of allatotropin isoform X2 (NCBI Reference Sequence: XM_022974503.1) identified from *Spodoptera litura* genome was artificially synthesized and ligated into pET32a + vector series fused with the 109 aa Thioredoxin tag. The plasmid pET32a^{+}/allatotropin were transformed into chemically competent *E. coli,* BL21 (DE_3) cells and expressed using 1 mM/L IPTG. The recombinant protein was analyzed by SDS-PAGE

and confirmed by western blotting. The expressed allatotropin was further purified using His-Tagged Bacterial Protein Purification kit. The effect of recombinant allatotropin in inducing egg-laying behaviour in *Spodoptera litura* females was studied by injection method. The experiment performed by injecting three different concentrations i.e. 5, 15 and 25 pg/µl of the protein into the abdominal cavity of the female moths exhibited egg-laying behaviour. Here, we propose a possible mechanism that bypasses the brain in which the allatotropin transferred from male to female flows into the hemolymph and activates JH biosynthesis thus leading to vitellogenesis. The present work could lead to the development of a protein based biopesticide resulting into a species-specific and an eco-friendly way of pest control.

Abbreviations
JH: Juvenile hormone; CA: Corpora allata; AT: Allatotropin; Trx-tag: Thioredoxin tag; LB: Luria bertani; IPTG: Isopropyl β-d-1-thiogalactopyranoside; rAT: Recombinant allatotropin; MAG: Male accessory gland; BSA: Bovine serum albumin.

Acknowledgements
We would like to thank the National Bureau of Agricultural Insect Resources (NBAIR) for supplying the insects.

Authors' contributions
MR and MD conceptualized the idea and designed the study. MR conducted the experiments, analyzed the results, and drafted the paper. TK and SR helped in cloning and expression studies, while CBS, S, and KVR provided technical guidance. All authors have read and approved the final version of the manuscript.

Funding
This research is funded by University Grants Commission (UGC), India to MR under the Award Number F1-17.1/2015-16/RGNF-2015–17-SC-KAR-23605 for the design, conduct of the study, analysis, and interpretation of data.

Availability of data and material
Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 5 June 2020 Accepted: 2 September 2021
Published online: 02 October 2021

References

Abdel-latief, M., Meyering-Vos, M., & Hoffmann, K. H. (2004). Characterization of a novel peptide with allatotropic activity in the fall armyworm *Spodoptera frugiperda*. *Regulatory Peptides, 122*(2), 69–78.

Alstein, M., & Nässel, D. R. (2001). Neuropeptides signalling in insects. *Advances in Experimental Medicine and Biology* 155–165.

Ando, T., Kasuga, K., Yajima, Y., Kataoka, H., & Suzuki, A. (1996). Termination of sex pheromone production in mated females of the silkworm moth. *Arch Insect Biochem: Published in Collaboration with the Entomological Society of America, 31*(2), 207–218.

Audsley, N., Weaver, R. J., & Edwards, J. P. (2000). Juvenile hormone biosynthesis by corpora allata of larval tomato moth, *Lacanobia oleracea*, and regulation by *Manduca sexta* allatostatin and allatotropin. *Insect Biochemistry and Molecular Biology, 30*(8–9), 681–689.

Audsley, N., Weaver, R. J., & Edwards, J. P. (2001). In vivo effects of *Manduca sexta* allatostatin and allatotropin on larvae of the tomato moth *Lacanobia Oleracea*. *Physiological Entomology, 26*(2), 181–188.

Baumann, H. (1974). The isolation, partial characterization, and biosynthesis of the paragonial substances, PS-1 and PS-2, of Drosophila funebris. *Journal of Insect Physiology, 20*(11), 2181–2194.

Boguś, M. I., & Cymborowski, B. (1984). Induction of supernumerary moults in Galleria mellonella: Evidence for an allatotropic function of the brain. *Journal of Insect Physiology, 30*(7), 557–561.

Borovsky, D. (2003). Trypsin-modulating oostatic factor: A potential new larvicide for mosquito control. *Journal of Experimental Biology, 206*(21), 3869–3875.

Borovsky, D., Powell, C. R., Dawson, W. O., Shivprasad, S., Lewandowski, D., DeBondt, H. L., De Ranter, C., & De Loof, A. (1998). Trypsin modulating oostatic factor (TMOF): a new biorational insecticide against mosquitoes. *Insects: Chemical, Physiological, and Environmental Aspects*, pp.131–40.

Champagne, D. E., Nussenzveig, R. H., & Ribeiro, J. M. (1995). Purification, partial characterization, and cloning of nitric oxide-carrying heme proteins (nitrophorins) from salivary glands of the blood-sucking insect *Rhodnius prolixus*. *Journal of Biological Chemistry, 270*(15), 8691–8695.

Chari, M. S., & Patel, S. N. (1983). Cotton leaf worm *Spodoptera litura* Fabricius, its biology and integrated control measures. *Cotton Development, 13*(1), 7–8.

Chen, P. S. (1984). The functional morphology and biochemistry of insect male accessory glands and their secretions. *Annual Review of Entomology, 29*(1), 233–255.

Dagert, M., & Ehrlich, S. D. (1979). Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene, 6*(1), 23–28.

Divakara, Y. G., Medhini, N., & Manjulakumari, D. (2011). Mass rearing of *Spodoptera litura* under laboratory conditions. In D. P. Ambrose (Eds.) *Insect pest management, a current scenario* (pp. 467–472).

Egekwu, N., Sonenshine, D. E., Bissinger, B. W., & Roe, R. M. (2014). Transcriptome of the female synganglion of the black-legged tick *Ixodes scapularis* (Acari: Ixodidae) with comparison between Illumina and 454 systems. *PLoS ONE, 9*(7), e102667.

Elekonich, M. M., & Horodyski, F. M. (2003). Insect allatotropins belong to a family of structurally-related myoactive peptides present in several invertebrate phyla. *Peptides, 24*(10), 1623–1632.

Eliyahu, D., Nagalakshmi, V., Applebaum, S. W., Kubli, E., Choffat, Y., & Rafaeli, A. (2003). Inhibition of pheromone biosynthesis in *Helicoverpa armigera* by pheromonostatic peptides. *Journal of Insect Physiology, 49*(6), 569–574.

Esther Shoba, R., Manmohan, M., Santhosh, S., Athmaram, T. N., & Manjulakumari, D. (2016). Heterologous expression of *Helicoverpa armigera* chitinase gene in *Pichia pastoris*. *IJAR, 2*(3), 738–745.

Fónagy, A. (2006). Insect neuropeptides and their potential application for pest control. *Acta Phytopathological Entomological Hungarian, 41*(1–2), 137–152.

Fuchs, M. S., & Hiss, E. A. (1970). The partial purification and separation of the protein components of matrone from *Aedes aegypti*. *Journal of Insect Physiology, 16*(5), 931–939.

Gade, G., Hoffmann, K. H., & Spring, J. H. (1997). Hormonal regulation in insects: Facts, gaps, and future directions. *Physiological Reviews, 77*(4), 963–1032.

Garad, G. P., Shivpuje, P. R., & Bilapate, G. G. (1984). Life fecundity tables of *Spodoptera litura* (Fabricius) on different hosts. *Proceedings: Animal Sciences, 93*(1), 29–33.

Gasteiger, E., Hoogland, C., Gattiker, A., Wilkins, M. R., Appel, R. D., & Bairoch, A. (2005). Protein identification and analysis tools on the ExPASy server. In *The proteomics protocols handbook* (pp. 571–607)

Green, M. R., Hughes, H., Sambrook, J., & MacCallum P. (2012). Molecular cloning: A laboratory manual. In *Molecular cloning: A laboratory manual* (pp. 1890–1890)

Griebler, M., Westerlund, S. A., Hoffmann, K. H., & Meyering-Vos, M. (2008). RNA interference with the allatoregulating neuropeptide genes from the fall armyworm *Spodoptera frugiperda* and its effects on the JH titer in the hemolymph. *Journal of Insect Physiology, 54*(6), 997–1007.

Harshman, L. G., & Prout, T. (1994). Sperm displacement without sperm transfer in Drosophila melanogaster. *Evolution, 48*(3), 758–766.
Hassanien, I. T., Grötzner, M., Meyering-Vos, M., & Hoffmann, K. H. (2014). Neuropeptides affecting the transfer of juvenile hormones from males to females during mating in *Spodoptera frugiperda*. *Journal of Insect Physiology, 66*, 45–52.
Ishizaki, H., Mizoguchi, A., Fujishita, M., Suzuki, A., Moriiya, I., O'oka, H., Kataoka, H., Isogai, A., Nagasawa, H., Tamura, S., & Suzuki, A. (1983). Species specificity of the insect prothoracicotropic hormone (PTTH): The presence of Bombyx and Samia specific PTTHs in the brain of Bombyx mori: Prothoracicotropic hormone (PTTH)/peptide hormone species specificity/ Bombyx mori/Samia cynthia ricini). *Development, Growth & Differentiation, 25*(6), 593–600.
Jin, Z. Y., & Gong, H. (2001). Male accessory gland derived factors can stimulate oogenesis and enhance oviposition in *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Arch Insect Biochem: Published in Collaboration with the Entomological Society of America, 46*(4), 175–185.
Kamruzzaman, A. S. M., Mikani, A., Mohamed, A. A., Elgendy, A. M., & Takeda, M. (2020). Crosstalk among Indoleamines, Neuropeptides and JH/20E in Regulation of Reproduction in the American Cockroach *Periplaneta Americana*. *Insects, 11*, 155.
Kataoka, H., Toschi, A., Li, J. P., Carney, R. L., Schooley, D. A., & Kramer, S. J. (1989). Identification of an allatotropin from adult *Manduca sexta*. *Science, 243*(4897), 1481–1483.
Kingan, T. G., Thomas-Laemont, P. A., & Raina, A. K. (1993). Male accessory gland factors elicit change from 'virgin'to 'mated'behaviour in the female corn earworm moth *Helicoverpa zea*. *Journal of Experimental Biology, 183*(1), 61–76.
Koeppe, J. K., Fuchs. M., Chen. T. T., Hunt, L. M., Kovalick, G. E., Briers, T. (1985). The role of juvenile hormone in reproduction. In *Endocrinology II* (pp. 165–203). Pergamon.
Koladich, P. M., Cusson, M., Bendena, W. G., Tobe, S. S., & McNeil, J. N. (2002). Cardioacceleratory effects of *Manduca sexta* allatotropin in the true armyworm moth *Pseudaletia unipuncta*. *Peptides, 23*(4), 645–651.
Kubli, E. (1992). My favorite molecule. The sex-peptide. *BioEssays, 14*(11), 779–784.
Kurien, B. T., & Scofield, R. H. (2006). Western blotting. *Methods, 38*(4), 283–293.
Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680.
Leahy, S. M. (1973). Oviposition of virgin *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae) after implant of the male accessory gland complex. *Journal of Entomology Series A, General Entomology, 48*(1), 69–78.
Lee, K. Y., & Horodyski, F. M. (2002). Restriction of nutrient intake results in the increase of a specific *Manduca sexta* allatotropin (Manse-AT) mRNA in the larval nerve cord. *Peptides, 23*(4), 653–661.
Lee, K. Y., Horodyski, F. M., & Chamberlin, M. E. (1998). Inhibition of midgut ion transport by allatotropin (Mas-AT) and Manduca FLRFamides in the tobacco hornworm *Manduca sexta*. *Journal of Experimental Biology, 201*(22), 3067–3074.
Lenaerts, C., Monjon, E., Van Lommel, J., Verbakel, L., & Broeck, J. V. (2019). Peptides in insect oogenesis. *Current Opinion in Insect Science, 31*, 58–64.
Li, Y., Unnithan, G. C., Veenstra, J. A., Feyereisen, R., & Noriega, F. G. (2003). Stimulation of JH biosynthesis by the corpora allata of adult female *Aedes aegypti* in vitro: Effect of farnesoic acid and Aedes allatotropin. *Journal of Experimental Biology, 206*(11), 1825–1832.
Lu, K., Chen, X., Liu, W. T., & Zhou, Q. (2016). TOR pathway-mediated juvenile hormone synthesis regulates nutrient-dependent female reproduction in *Nilaparvata lugens* (Stål). *International Journal of Molecular Sciences, 17*(4), 438.
McNeil, J. N., & Tobe, S. S. (2001). Flights of fancy: possible roles of allatostatin and allatotropin in migration and reproductive success of *Pseudaletia unipuncta*. *Peptides, 22*(2), 271–277.
Monsma, S. A., & Wolfner, M. F. (1988). Structure and expression of a Drosophila male accessory gland gene whose product resembles a peptide pheromone precursor. *Gene and Development, 2*(9), 1063–1073.
Neubaum, D. M., & Wolfner, M. F. (1999). Mated Drosophila melanogaster females require a seminal fluid protein, Acp36DE, to store sperm efficiently. *Genetics, 153*(2), 845–857.
Nijhout, H. F. (1994). The endocrine control of molting and metamorphosis. In H. F. Nijout (Ed.), *Insect hormones* (pp. 89–141). Princeton University Press.
Paemen, L., Tips, A., Schoofs, L., Proost, P., Van Damme, J., & De Loof, A. (1991). Lom-AG-myotropin: A novel myotropic peptide from the male accessory glands of *Locusta migratoria*. *Peptides, 12*(1), 7–10.
Petersen, T. N., Brunak, S., Von Heijne, G., & Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods, 8*(10), 785.
Pickford, R., Ewen, A. B., & Gillott, C. (1969). Male accessory gland substance: An egg-laying stimulant in *Melanoplus sanguinipes* (F) (Orthoptera: Acrididae). *Canadian Journal of Zoology, 47*(6), 1199–203.
Qin, H., Ye, Z., Huang, S., Ding, J., & Luo, R. (2004). The correlation of the different host plants with preference level, life duration and survival rate of *Spodoptera litura* Fabricius. *Chinese Journal of Eco-Agriculture, 12*(2), 40–42.
Rachinsky, A., Tobe, S. S., & Feldlaufer, M. F. (2000). Terminal steps in JH biosynthesis in the honey bee (*Apis mellifera* L.): developmental changes in sensitivity to JH precursor and allatotropin. *Insect Biochem Mol Biol, 30*(8–9), 729–37.
Raina, A. K., Jaffe, H., Kempe, T. G., Keim, P., Blacher, R. W., Fales, H. M., Riley, C. T., Klun, J. A., Ridgway, R. L., & Hayes, D. K. (1989). Identification of a neuropeptide hormone that regulates sex pheromone production in female moths. *Science, 244*(4906), 796–798.
Ramaswamy, S. B., Shu, S., Mbata, G. N., Rachinsky, A., Park, Y. I., Crigler, L., Donald, S., & Srinivasan, A. (2000). Role of juvenile hormone-esterase in mating-stimulated egg development in the moth *Heliothis virescens*. *Insect Biochemistry and Molecular Biology, 30*(8–9), 785–791.
Riddiford, L. M. (1994). Cellular and molecular actions of juvenile hormone I. General considerations and premetamorphic actions. In: *Advances in Insect physiology*. Academic Press (pp. 213–274)
Riemann, J. G., & Thorson, B. J. (1969). Effect of male accessory material on oviposition and mating by female house flies. *Annals of the Entomological Society of America, 62*(4), 828–834.
Sachdev, D., & Chirgwin, J. M. (1998). Solubility of proteins isolated from inclusion bodies is enhanced by fusion to maltose-binding protein or thioredoxin. *Protein Expres Purification, 12*(1), 122–132.
Schoofs, L., De Loof, A., & Van Hiel, M. B. (2017). Neuropeptides as regulators of behavior in insects. *Annual Review of Entomology, 62*, 35–52.
Shobha, A., Geetha, B., & Manjulakumari, D. (2009). Purification and partial characterization of a male derived peptide that enhances egg maturation and oviposition in female cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Entomon, 34*(4), 213–221.
Shu, S., Park, Y. I., Ramaswamy, S. B., & Srinivasan, A. (1998). Temporal profiles of juvenile hormone titers and egg production in virgin and mated females of *Heliothis virescens* (Noctuidae). *Journal of Insect Physiology, 44*(11), 1111–1117.
Sridevi, R., Ray, A., & Ramamurty, P. S. (1987). An oviposition stimulant in the male accessory-gland extract of Spodoptera-litura (Lepidoptera, Noctuidae). *Entomon, 12*(3), 231–233.
Stay B, Tobe SS, Bendena WG (1995) Allatostatins: identification, primary structures, functions and distribution. In: *Advances in Insect Physiology* (pp. 267–337). Academic Press
Stone, J. V., Mordue, W., Batley, K. E., & Morris, H. R. (1976). Structure of locust adipokinetic hormone, a neurohormone that regulates lipid utilisation during flight. *Nature, 263*(5574), 207.
Swailes, G. E. (1971). Reproductive behavior and effects of the male accessory gland substance in the cabbage maggot *Hylemya Brassicae*. *Annals of the Entomological Society of America, 64*(1), 176–179.
Teal, P. E. (2002). Effects of allatotropin and allatostatin on in vitro production of juvenile hormones by the corpora allata of virgin females of the moths of *Heliothis virescens* and *Manduca sexta*. *Peptides, 23*(4), 663–669.
Tong, H., Su, Q., Zhou, X., & Bai, L. (2013). Field resistance of *Spodoptera litura* (Lepidoptera: Noctuidae) to organophosphates, pyrethroids, carbamates and four newer chemistry insecticides in Hunan, China. *Journal of Pest Science, 86*(3), 599–609.
Van Wielendaele, P., Badisco, L., & Vanden Broeck, J. (2013). Neuropeptidergic regulation of reproduction in insects. *General and Comparative Endocrinology, 188*, 23–34.
Weaver, R. J., & Audsley, N. (2009). Neuropeptide regulators of juvenile hormone synthesis: Structures, functions, distribution, and unanswered questions. *Annals New York Academy of Sciences, 1163*(1), 316–329.
Wu, B., Liu, Z., Zhou, L., Ji, G., & Yang, A. (2015). Molecular cloning, expression, purification and characterization of vitellogenin in scallop *Patinopecten*

yessoensis with special emphasis on its antibacterial activity. *Developmental and Comparative Immunology, 49*(2), 249–258.
Yu, J. F., Li, C., Xu, J., Liu, J. H., & Ye, H. (2014). Male accessory gland secretions modulate female post-mating behavior in the moth *Spodoptera litura*. *Journal of Insect Behavior, 27*(1), 105–116.
Zhang, Y., Dong, X., Liu, J., Hu, M., Zhong, G., Geng, P., & Yi, X. (2012). Molecular cloning, expression and molecular modeling of chemosensory protein from *Spodoptera litura* and its binding properties with Rhodojaponin III. *PLoS ONE, 7*(10), 476.

Publisher's Note