International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2013): 4.438

Characterization of Egg Maturation Substance from *Spodoptera litura* F. Males

Divakara Y G, Mamtha R, Doddamane Manjulakumari*

Department of Microbiology and Biotechnology, Bangalore University, Bengaluru – 560056, India

Abstract: ***In Spodoptera litura, we have demonstrated the influence of male accessory gland (MAG) and ejaculatory duplex (ED) secretions on egg maturation by injecting crude extracts of these tissues and seminal vesicle with testes (SVT) into 2-day old virgin females. Proteinaceous components of MAG and ED were subjected to fractionation using weak cation exchanger and RP-UFLC. The peak fraction P-5 at 53.64min of F-3 fraction recorded highest number of matured eggs compared to other peaks. Molecular mass of this peak, analysed on MALDI-TOF, was found to be 10247.1Da.***

Keywords: Male accessory gland, egg maturation, RP-UFLC, MALDI-TOF.

1. Introduction

Male insects secrete number of soluble components when are transferred along with sperms at the time of mating, may act as chemical messenger in changing over reproductive behaviour of females. For example in house flies such components originate mainly in ejaculatory duct [29] whereas in mosquitoes, in accessory reproductive glands [10]. However, in many insects, male accessory gland (MAG) secretions are involved in such change over behaviour of females from virgin to mated conditions as seen in *Drosophila* [23]-[9]-[37], *Bombyx mori* [24], *Helicoverpa armigera* [33]. The behavioural changes include termination of calling and receptiveness, stimulation of oogenesis, acceleration of egg maturation, enhanced oviposition [7] *etc*. Such behaviour modifying substances have been identified in several insects including *Leptinotarsa decemlineata* [34], Mediterranean fruit fly [11], *Aedes aegypti* [25], field crickets [22], but has been extensively studied in Drosophila species. Baumann et al. [2] reported the isolation of 27 amino acid residue peptide from MAG of *Drosophila funebris* that was responsible for termination of receptivity in the females. Two Sex Peptide like factors (OSS and SPDS) in *Drosophila suzuki* [26]-[31] and more than 16 accessory gland proteins (Acps) in *Drosophila melanogaster* [37] have been reported. A pheromonostatic peptide (PSP) in *Helicoverpa zea* [20] and oviposition stimulating protein (OSP) in *Melanoplus sanguinipes* [39] have been purified from MAG.

Behaviour modifying molecules can probably be exploited for the control of pest population on the same lines as of pheromones that have been successfully employed in pest control [6]-[12]-[5]. Reproductive behaviour modifying molecules may prove to be species specific sparing the non-target species and environment friendly alternative over chemical pesticides to check the population of both agricultural pests and vectors of human diseases.

Spodoptera litura Fabricius (Lepidoptera: Noctuidae) commonly known as tobacco cutworm is a serious pest. According to Qin et al. [28] around120 species of plants belonging to 44 families serve as hosts to this insect. It would be of interest and economic importance to track the reproductive behaviour of this insect to bio-molecule from the perspective of pest control. Jin et al. [18] have studied the role of MAG secretions in reproduction of *Spodoptera litura*. Their studies are limited to the understanding of calling and mating behaviour, oviposition, fecundity and longevity at the level of crude extracts. Izadi and Subrahmanyam [16] have analysed the molecular mass of MAG proteins by SDS-PAGE with crude extract but no role has been attributed to these proteins. In this paper we have discussed the work which has led to identification of the peptide responsible for egg maturation in *Spodoptera litura*.

2. Methods

2.1 Insect Rearing

Spodoptera litura were reared individually in separate vials on an artificial diet [13] at 25±2 ^{o}C and 75±5% relative humidity under laboratory conditions where photoperiod of 12L:12D was maintained. Male and female moths maintained in separate chambers until use, were fed with 10% honey solution.

2.2 Effect of mating on egg maturation

In order to study the effect of MAG secretions on the rate of egg maturation two day old (Day-2) male and female moths were allowed to mate for 3 hours, after which the females were transferred to egg laying chambers for oviposition. Egg maturation was studied by counting the number of eggs laid and number of chorionated eggs present in the abdomen. Five females were dissected at an interval of every 24 hours from the time of termination of mating to remove the ovarioles to count number of chorionated eggs in the abdomen. The removed ovaries were placed in a petri plate containing 10% hot KOH and incubated for one hour at room temperature. The non-chorionated eggs/immature eggs dissolved, while the chorionated eggs/mature eggs remained intact [32]. These eggs were counted and added to the number of eggs laid till the day of dissection, to determine the total number of eggs matured. Virgin females of similar age served as controls.

2.3 Preparation of tissue extract for bioassay

The reproductive tissues i.e. male accessory glands (MAG), ejaculatory duct duplex (ED), and seminal vesicle along with testis (SVT) were dissected out from Day-2 male moths (n=200) and rinsed in ice cold saline [4], the excess saline was blotted, and the tissue was stored in separate vials at -80 ^{o}C till further use. The tissues were homogenized individually in 200µl of ice-cold distilled water and centrifuged at 15,000g for 20min at 4 ^{o}C. The supernatant was collected in a separate vial whereas the pellet was redissolved in 200 µl of ice-cold distilled water and extracted twice similarly. The supernatants were pooled and lyophilized. The lyophilized extracts were dissolved in 75µl of saline for bioassay to study their influence on egg maturation behaviour.

2.4 Bioassay

To determine the biological activity of the tissue extracts bioassay was carried out on Day-2 virgin females by injecting the tissue extracts two hours after the onset of scotophase. The insects were cold anesthetized for 5min at 4^{o}C before injection to minimize the hemolymph loss due to wriggling movements. Insects were divided into four groups with 30 each. First three groups were injected with two pair equivalent of crude tissue extracts of MAG, ED, and SVT respectively into the abdominal cavity through the inter-segmental membrane. Fourth group was injected with saline that served as control (C). Females were allowed to recover for 15min after injection. The treated moths were transferred to separate chamber for egg laying and number of eggs matured at interval of 24 hours from the time of injection was determined as explained earlier.

2.5 Extraction in different buffers

MAG and ED tissues were pooled to prepare an extract (MAG-ED) in 20 fold excess (w/v) ice cold extraction buffers with four different compositions. Buffer-1 composed of 1mol/L hydrochloride, 5% formic acid, 1% trifluroacetic acid and 1% sodium chloride [3], the Buffer-2 composed of 50% methanol in distilled water containing 2mM hydrochloric acid [30], where as Methanol: distilled water: glacial acetic acid in the ratio 90:9:1 [27] served as Buffer-3 and 2% sodium chloride as Buffer-4 extraction buffer. The latter extract was incubated in a boiling water bath for 5min as described by Suzuki et al. [35]. The extracts were centrifuged at 12,000g for 15 min at 4 ^{o}C and supernatant was collected separately in clean centrifuge tubes. Re-extraction from the pellet was done in 10 fold excess of the respective buffers and supernatant obtained were pooled and was passed through cheese cloth to remove fat content. The extracts were desalted using C18 Sep – Pak cartridges (Waters, USA) and bioassay was carried out as explained earlier.

2.6 Fractionation by Ion exchange chromatography

Further fractionation was carried out by ion-exchange chromatography with MAG-ED extract using CM Sep – Pak Cartridge (Waters, USA). The cartridge was rinsed with 10ml of 20% acetonitrile (AcN) containing 10mM ammonium acetate (AmAc) and 1M sodium chloride (pH 5.0), followed by 20ml 20%AcN, 10mM AmAc. The desalted MAG-ED tissue extract, diluted to1.5 times with distilled water were passed through the cartridge and the flow-through was collected as fraction-1 (F-1). The unbound proteins were eluted with 6ml of 20% AcN with 10mM AmAc the eluate was collected as fraction-2 (F-2). Then the weakly bound material was eluted as fraction-3 (F-3) with 6ml of 20% AcN with 10mM AmAc and 200mM sodium chloride. The remaining, strongly bound material was eluted out as fraction-4 (F-4), by increasing the concentration of sodium chloride i.e. with 20% AcN with 10mM AmAc and 1M sodium chloride. The fractions were desalted using C18 Sep – Pak Cartridges and bioassay was carried out.

2.7 Fractionation by RP-UFLC

The active fractions from the CM Sep – Pak Cartridge were subjected to reverse phase ultra fast liquid chromatography (RP-UFLC) on Phenomenex C-18 column and eluted with trifluroacetic acid (TFA) – acetonitrile (AcN) gradient solvent system [31] at a flow rate of 1ml/min. The composition of the mobile phase was 0.1% TFA in Distilled water (Buffer A) and 80% AcN (Buffer B). The gradient parameters were 0% Buffer-B for 10 seconds, 0-10% Buffer-B for 10min, 10-40% Buffer-B for 50min, 40-10% Buffer-B for 58min, and 10-0% for 60min. Fractions were collected manually and fractions showing peaks were used for bioassay.

2.8 Mass spectrometry

The active fractions of RP-UFLC were lyophilised and subjected to MALDI-TOF analysis on ultrafleXtreme (Bruker Baltonics, Germany) for mass determination. The mass spectra were acquired from Flex control (Bruker) and analysis was done using Flex Analysis 3.1 (Bruker) software, with smart beam laser (2Hz).

3. Results

3.1 Effect of Mating on egg maturation

Number of eggs matured in the mated moths was 417.1±43.3 on day2 whereas in virgin females it was only 5.5±1.95 over the same period (Fig. 1). The counts of mature eggs in mated females were significantly higher compared to virgin females on all the days.

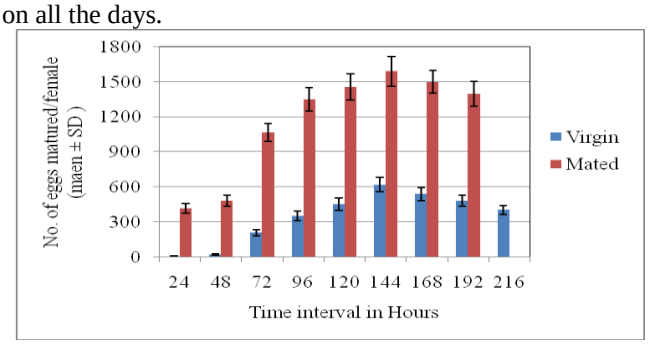

Figure 1: Number of eggs matured in virgin and mated moths recorded at the interval of 24 hours from the time of termination of mating (n=5)

3.2 Crude extract treatment

The matured eggs were significantly higher in the females injected with MAG and ED extracts as compared to the SVT extract injected moths as well as saline injected controls on all the days (Fig.2). Between MAG and ED injected groups there was no significant difference in the number of eggs matured indicating the substance that influences egg maturation is present in both male accessory glands and ejaculatory duplex.

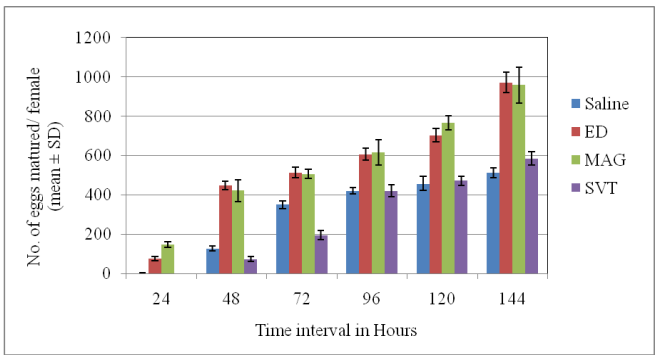

Figure 2: Number of eggs matured in moths treated with various tissue extracts recorded at the interval of 24 hours from the time of injection (n=5)

3.3 Buffer extracts treatment

Females injected with desalted crude MAG-ED extract prepared in buffer-1 (Bennett's buffer) recorded highest number of matured eggs compared to rest of the treated moths. The pattern of egg laying in buffer-1 treated moths on different days was closer to the mated moths whereas rest of the groups were similar to virgin moths (Fig. 3). These results suggest that Bennett's buffer is the ideal choice for extraction of factors influencing post-mating responses in *Spodoptera litura*.

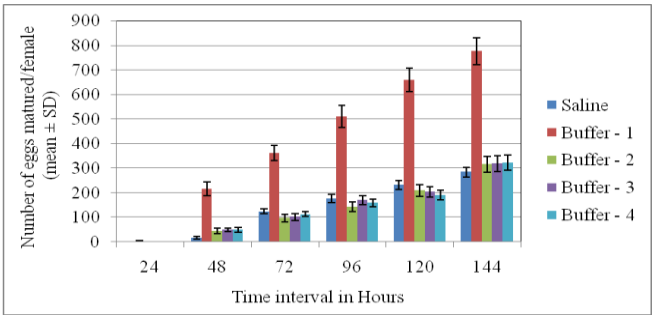

Figure 3: Number of eggs matured in moths treated with MAG-ED extracts prepared in different buffers recorded at the interval of 24 hours from the time of injection (n=5)

3.4 Cation-exchange fractions treatment

Fractions F-3 and F-4 obtained on weak cation-exchange cartridge accelerated egg maturation while fractions F-1 and F-2 failed to do so. Number of eggs matured remained higher in F-3 and F-4 throughout the observation period compared with other fractions as well as control group (Fig. 4). F-3 and F-4 fractions were eluted with buffer containing 200mM NaCl and 1M NaCl respectively.

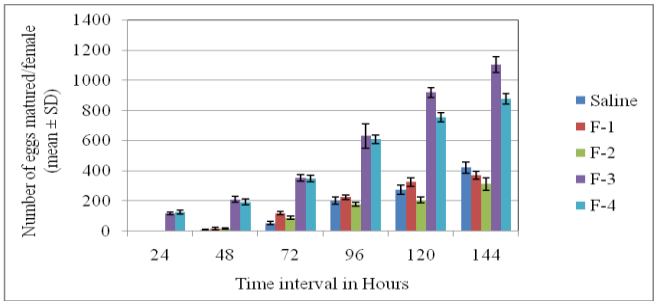

Figure 4: Number of eggs matured in moths treated with fractions of ion-exchange chromatography recorded at the interval of 24 hours from the time of injection (n=5)

3.5 RP-UFLC fractions treatment

The fractions of RP-UFLC on C-18 column showing peaks were used for bioassay to determine the egg maturation. The peak of F3-5 at 53.64min (Fig. 5) recorded highest number of matured eggs compared to other peaks of F-3 and peaks of F-4 (Fig.6) as well as saline injected females (Fig.7). The molecular mass of active peak was found to be 10247.1Da (Fig. 8).

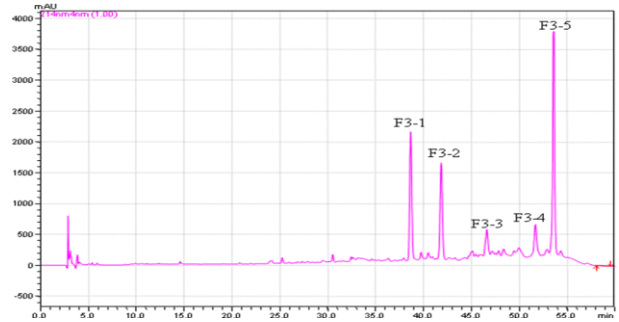

Figure 5: Chromatogram of F-3 subjected to RP-UFLC showing peaks at 38.7min, 41.9min, 46.9min, 51.9min and 53.6min.

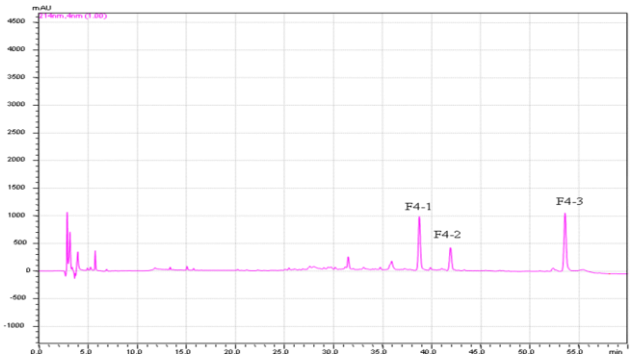

Figure 6: Chromatogram of F-4 subjected to RP-UFLC showing peaks at 38.7min, 41.9min, 53.6min.

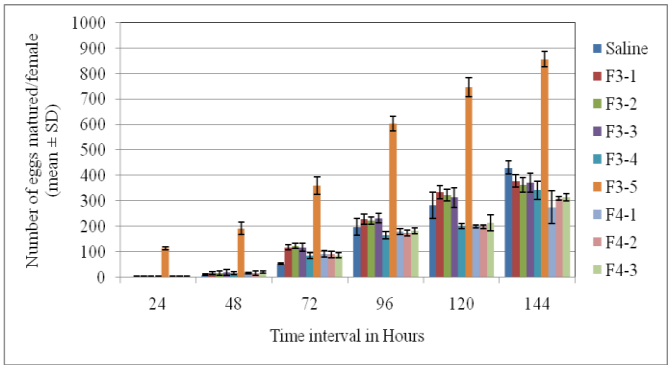

Figure 7: Number of eggs matured in moths treated with peak fractions of RP-UFLC recorded at the interval of 24 hours from the time of injection (n=5)

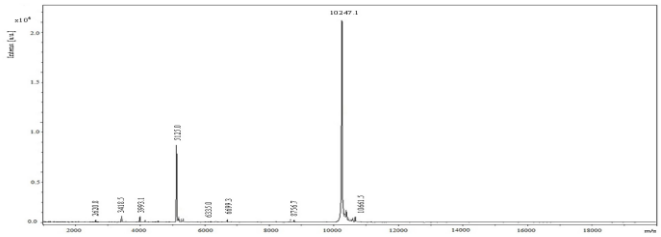

Figure 8: Peak of F3-5 on MALDI-TOF showing molecular mass of 10247.1Da

4. Discussion

Mating brings about several changes in the female moths. Non receptiveness, cessation of calling and egg production are few of the behavioural changes to be mentioned. The modulators of these changes in behaviour have been found to be present in the accessory glands of the male moths, the ejaculates of which are transferred along with sperms during mating to bring about these changes. Such proteins have been reported in several insects like SP in *Drosophila melanogaster* shown to reduce receptivity and elicit egg laying [21] whereas Ovulin (ACP26Aa) stimulates the release of oocytes from ovary [8]-[14]. Fecundity enhancing substance (FES) accelerates the rate of egg laying [33] and oogenesis and oviposition factor (OOSF) has been found to regulate egg production and egg maturation in *Helicoverpa armigera* [17].

In the present study we have tried to study the effect of mating on egg maturation and thereafter to identify the protein responsible for it in *Spodoptera litura*. The experiments to study the effect of mating on egg maturation shows that the mated moths lay eggs at much faster rate than the virgin moths. Over the same period of time the number of eggs laid by mated moths far exceeds the no of eggs laid by virgin moths. This leads to a conclusion that a factor responsible for acceleration of egg maturation is transferred during mating and the fact that virgins lay eggs affirm that the initiation of egg maturation happens even in the absence of mating.

Similar to the reports of Kingan et al. [20] in *Helicoverpa zea*, the crude extracts of MAG and duplex when injected into the abdominal cavity of the virgin females induced mated behaviour in *Spodoptera litura*. In the present study, we found that male accessory gland and duplex showed high percentage of egg maturation compared to saline and SVT injected moths indicating that the factors responsible for the accelerated rate of maturation are present in the accessory gland and duplex of male *Spodoptera litura*. These crude extracts found to promote egg maturation without mating or without presence of sperm. The process of male accessory gland secretions promoting egg maturation needs two days to reach its maximum as females, with or without mating, lay eggs only on second day after emergence. A similar situation is reported in the Mediterranean flour moth *Ephestia kuehniella* [38].

The post mating changes were induced in virgins by Bennet's buffer extract of MAG-ED tissue and further fractions of cation exchange chromatography F-3 and F-4 also brought about the similar changes in virgins. Highly acidic Bennett's buffer is known to precipitate low molecular weight factors. Whereas bioactivity was seen in F-3 and F-4 fractions eluted by increasing ionic strength with NaCl indicate that the bioactive factors were strongly adsorbed on to cation cartridge [15], thus have a net positive charge. Various researchers have used Bennet's buffer for successful extraction of small molecular weight peptides like allatostatins from *Aedes aegypti* [36], HezPSP [19], OOS from *H. zea* [1] and *Helicoverpa armigera* [17]-[33].

Further fractionation of F-3 and F-4 fractions by RP-UFLC using C18 column and bioassay with the peak fractions showed that peak F3-5 could induce acceleration of oviposition and egg maturation in virgins. Mass spectrophotometric analysis of the F3-5 fraction showed that the molecular weight of peptide in *Spodoptera litura* was 10247.1Da. Though the fraction F-4 induced mated behaviour, none of its peak fractions induced similar behaviour for unknown reason.

There are only few reports describing the purification and characterization of oviposition and egg maturation stimulating factors because of the difficulty in sequencing the pure fraction. Till date *D. melanogaster* sex-peptide [21] and 30KDa protein in *Melanoplus sanguinipes* [39] are the only well characterized male factors. Efforts are on to sequence the peptide responsible for accelerated egg maturation in *Spodoptera litura*.

5. Acknowledgments

We would like to thank Prof. R. Uma Shaankar, Department of Crop Physiology, University of Agricultural Sciences, Bangalore for providing RP-UFLC facility, Ms. Sunitha, for Mass Spectrophotometric analysis at the Indian Institute of Science, Molecular Biophysics unit, Bangalore and University Grants Commission, Govt. of India for providing financial assistance (Ref No.: F. No. 36-43/2008 (SR)).

References

[1] G. Bali, A. K. Raina, T. G. Kingan, J. D. Lopez, "Ovipositional behaviour of newly colonized corn earworm (Lepidoptera: Noctuidae) females and evidence for an oviposition stimulating factor of male

origin", Annals of Entomological Society of America 75(1), pp. 51-55. 1996.
[2] H. Baumann, K. J. Wilson, P. S. Chen, R. E. Humbel, "The amino acid sequence of a peptide (PS-1) from *Drosophila funebris*: a paragonial peptide from males which reduces the receptivity of the females", European Journal of Biochemistry, 52, pp. 521-529. 1975.
[3] H. P. J. Bennett, "Use of ion-exchange Sep-Pac cartridges in the batch fractionation of pituitary peptides", Journal of Chromatography A, 359, pp. 383-390. 1986.
[4] V. P. Bindokas, M. E. Adams, "Hemolymph composition of the tobacco budworm, *Heliothis virescens* F. (Lepidoptera: Noctuidae)" Comparative Biochemistry and Physiology, 90, pp. 151-155. 1988.
[5] R. T. Carde, A. K. Minks, "Control of moth pests by mating disruption: success and constrains", Annual Review of Entomology, 40, pp. 559-585. 1995.
[6] R. T. Carde, K. Trammel, W. L. Roelofs, "Disruption of sex attraction of the redbanded leafroller *Argyrotaenia velutinana* with microencapsulated pheromone components", Environmental Entomology, 4, pp. 448-552. 1975.
[7] T. Chapman, "Seminal fluid-mediated fitness traits in Drosophila", Heredity, 87, pp. 511-521. 2001.
[8] T. Chapman, L. A. Herndon, Y. Heifetz, L. Parteidge, M. F. Wolfner, "The Acp26Aa seminal fluid protein is a modulator of early egg-hatchability in *Drosophila melanogaster*. Proc Biol Sci 268:1647-1654. 2001
[9] P. S. Chen, "The accessory gland proteins in male *Drosophila:* structural, reproductive, and evolutionary aspects", Experientia, 52, pp. 503-510. 1996.
[10] G. B. Craig Jr, "Mosquitoes: female monogamy induced by male accessory gland substance", Science, 156, pp. 1499-1501. 1967.
[11] S. J. Davies, T. Chapman, "Identification of genes expressed in the accessory glands of male Mediterranean fruit flies (*Ceratitis capitata*)", Insect Biochemistry and Molecular Biology, 36, pp. 846-856. 2006.
[12] J. P. Deland, G. J. R. Judd, B. D. Roitberg, "Disruption of pheromone communication in three sympatric leafroller (Lepidoptera: Tortricidae) pests of apple in British Columbia", Environ Entomology, 23, pp. 1084-1090. 1996.
[13] Y. G. Divakar, N. Medhini, D. Manjulakumari, "Mass rearing of *Spodoptera litura* (fabricus.) under laboratory conditions on artificial diet and studies on their mating and egg laying behavior", in Insect Pest Management, A Current Scenario, Dunston P Ambrose (ed), India, 2011.
[14] L. A. Herndon, M. F. Wolfner, "A *Drosophila* seminal fluid protein, Acp26Aa, stimulates egg laying in females for 1 day after mating", Proceedings of National Academy of Science, USA, 92, pp. 10114-10118. 1995.
[15] Isidra Recio, Servaas Visser, "Two-ion exchange chromatographic methods for the isolation of antibacterial peptides from lactoferrin in-situ enzymatic hydrolysis on an ion-exchange membrane, J Chromatography A, 831, pp. 191-201. 1999.
[16] H. Izadi, B. Subrahmanyam, "Protein profiles of *Spodoptera litura* (F). male accessory reproductive gland and its secretions", Communications in Agricultural and Applied Biological Sciences, 70(4), pp. 857-862. 2005.
[17] Z. Y. Jin, H. Gong, "Male accessory gland derived factors can stimulate oogenesis and enhance oviposition in *Helicoverpa armigera* (Lepidoptera: Noctuidae)", Archieves of Insect Biochemistry and Physiology, 46, pp. 175-185. 2001.
[18] Jin-Feng Yu, Cong Li, Jin Xu, Jian-Hong Liu, Hui Ye, "Male accessory gland secretions modulate female post-mating behaviour in the moth *Spodoptera litura*", Journal of Insect Behaviour, 27, pp. 105-116. 2014.
[19] T. G. Kingan, W. M. Bondar, A. K. Raina, J. Shabanowitz, D. F. Hunt, "The loss of female sex pheromone after mating in the corn earworm moth *Helicoverpa zea*: identification of a male pheromonostatic peptide", Proceedings of National Academy of Sciences, USA, 92, pp. 5082-5086. 1995.
[20] T. G. Kingan, P. A. Thomas-Laemont, A. K. Raina, "Male accessory gland factors elicit change from 'virgin' to 'mated' behaviour in the female corn earworm moth *Helicoverpa zea*", Journal of Experimental Biology, 183, pp. 61-76, 1993.
[21] E. Kubli, "Sex-peptides: seminal peptides of the Drosophila male", Cellular and Molecular Life Sciences, 60, pp. 1689-1704. 2003.
[22] E. L. Larson, J. A. Andres, R. G. Harrison, "Influence of the male ejaculate on postmating prezygotic barriers in field crickets", PLoS One, 7(10):e46202, 2012.
[23] R. A. Leopold, "The role of male accessory glands in insect reproduction", Annual Review of Entomology, 21, pp. 199-221. 1976.
[24] D. Manjulakumari, Geetha Bali, "Mating: A mechanism that regulates oviposition behaviour in the silk moth *Bombyx mori* L.", in Readings in Behavior, Ramamurthy and Geetha Bali (eds.), New Age International Ltd., India, 1996.
[25] C. Naccarati, N. Audsley J. N. Keen, J. H. Kim, G. J. Howell, Y. J. Kim, R. E. Isaac, "The host-seeking inhibitory peptide, Aea-HP-1, is made in the male accessory gland transferred to the female during copulation", Peptides, 34, pp. 150-157. 2012.
[26] Y. Y. Ohashi, K. Haino-Fukushima, Y. Fuyama, "Purification and characterization of an ovulation stimulating substance from the male accessory glands of *Drosophila suzukii*", Insect Biochemistry, 21, pp. 413-419. 1991.
[27] L. Paemen, A. Tips, L. Schoofs, P. Proost, J. Van Damme, A. De Loof, "Lom-AG-myotropin: a novel myotropic peptide from the male accessory glands of *Locusta migratoria*", Peptides, 12, pp. 7-10. 1991.
[28] H. Qin, Z. Ye, S. Huang, J. Ding, R. Luo, "The correlations of the different host plants with preference level, life duration and survival rate of *Spodoptera litura* Fabricius", Chinese Journal of Eco-Agriculture, 12, pp. 40-42. 2004.
[29] J. G. Riemann, D. J. Moen, B. J. Thorson, "Female monogamy and its control in houseflies", Journal of Insect Physiology, 13, pp. 407-418, 1967.

[30] J. Rooney, S. M. Lewis, "Differential allocation of male-derived nutrients in two lampyrid beetles with contrasting life-history characteristics", Behavioral Ecology, 10, pp. 97-104. 1999.
[31] T. Schmidt, Y. Choffat, S. Klauser, E. Kubli, "The *Drosophila melanogaster* sex-peptide: A molecular analysis of structure-function relationship", Journal of Insect Physiology, 39, pp. 361-368. 1993.
[32] A. Shobha, "Characterization of peptides isolated from male accessory glands of *Helicoverpa armigera*", Ph D thesis, Bangalore University, India, 2007.
[33] A. Shobha, Geetha Bali, D. Manjulakumari, "Purification and partial characterization of a male derived peptide that enhances egg maturation and oviposition in female cotton bolloworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae)", Entomon, 34(4), pp. 213-221, 2009.
[34] H. M. Smid, A. B. Koopmanschap, C. A. D. De Kort, H. Schooneveld, "A peptide from the male accessory gland in *Leptinotarsa decemlineata*: purification, characterization and molecular cloning", Journal of Insect Physiology, 43, pp. 355-362. 1997.
[35] A. Suzuki, H. Nagasawa, H. Kataoka, Y. Hori, A. Isogai, S. Tamura, F. Guo, X. Zhong, H. Ishizaki, M. Fujishita, A. Mizoguchi, "Isolation and characterization of prothoracicotropic hormone from silkworm, *Bombyx mori*". Agricultural and Biological Chemistry, 46, pp. 1107-1109. 1982.
[36] J. A. Veenstra, F. G. Noriega, R. Graf, R. Feyereisen, "Identification of three allatostatins and their cDNA from the mosquito *Aedes aegypti*", Peptides, 18, pp. 937-942, 1997.
[37] M. F. Wolfner, "Tokens of love: functions and regulation of Drosophila male accessory gland products", Insect Biochemistry and Molecular Biology, 27, pp. 179-192. 1997.
[38] J. Xu, Q. Wang, "Seminal fluid reduces female longevity and stimulates egg production and sperm trigger oviposition in a moth", Journal of Insect Physiology, 57, pp. 385-390, 2011.
[39] S. X. Yi, C. Gillott, "Purification and characterization of an oviposition-stimulating protein of the long hyaline tubules in the male migratory grasshopper, *Melanoplus sanguinipes*", Journal of Insect Physiology, 45, pp. 143-150, 1999.