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**RESEARCH ARTICLE**

# Bioactive factors from male accessory glands - a potent ecofriendly approach in the control of *Spodoptera litura* Fab

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## *Abstract*

Origin of male factors that regulate calling, mating and oviposition behaviour characteristic of mated moths was investigated in *Spodoptera litura* by injecting different tissue extracts of male reproductive system into the body cavity of day 2 virgin female moths. Male accessory glands (MAG) extract injected virgins could mimic the mated moth's behaviour while rest of the extracts failed to induce the same. Fractionation of MAG extract in different buffers resulted in Bennett's buffer fraction carrying the active factors. Unravelling the nature of compound/s involved in switchover of virgin to mated behaviour may help to develop bio-control strategy for pest insects.



## Introduction

*Spodoptera litura (*Fabricius*)* or tobacco cutworm a polyphagous pest finds its host in over 120 plant species falling into 44 families including cotton, groundnuts, jute, maize, paddy, soybeans, tea, tobacco, vegetables (Qin *et al*., 2004) the commercial crops from the tropic. Development of resistance to several of chemical pesticides has forced the agriculture scientists to look for newer and safer methods to control this pest. A novel approach is to use behaviour modifying peptides/proteins indigenous to pest to deregulate the pest's own mechanisms of regulating reproductive behaviour resulting in its failure to reproduce successfully. These strategies have the advantages of the likelihood of being specific, eco-friendly and sustainable, provided an effective method is evolved to deliver them to the insects at a right time. The accessory gland secretions when transferred, together with sperm to females at the time of mating exert wide-ranging effects on female reproductive activity. Inhibiting calling behaviour, rendering her unwillingness or unable to remate for some time or permanently, increase in the number and rate of development of eggs are some of the changes attributed to accessory gland secretions. Male's chance of siring a significant proportion of that female's offspring is also influenced by these secretions.

Several studies have shown the presence of variety of bioactive molecules in the male accessory glands. Carbohydrates, lipids, and small amounts of amino acids and amines (Gillott, 1988), uric acid (Roth, 1967), prostaglandins (Gillott, 1988), juvenile hormones (JH) (Park *et al.,* 1998), and various toxic materials (Blum and Hilker, 2002) are found in MAG secretions of various insects, proteins and/or peptides being the major components.Though the influence of proteinaceous components of male accessory glands on the female has been demonstrated in number of insects, very little information is available on *S. litura* (Armes et al., 1997). Present investigation is to explore such active factors in *S. litura*.

## Materials and Methods

National Bureau of Agriculturally Important Insects (NBAII), Bangalore provided the source material to raise a colony of *S.litura* in the laboratory. A continuous culture was necessary to meet the requirement of large number of insects for isolation of peptides from MAG-duplex complex as well as to carry out bioassays. Rearing was carried out on artificial diet, with 28±2$^0$C, 70±5% relative humidity, with 12:12 :: L: D cycle. (Divakar *et al.,* 2011).

*Dissections*

Day2 and day3 males were dissected under ice-cold saline (Bindokaas andAdams, 1988), and the complete reproductive system was transferred into a clean petriplate containing saline. The male accessory glands (MAG), ejaculatory duct duplex (duplex) and seminal vesicle along with testis (SV &T) were separated and pooled from several insects in to vials placed in dry-ice. All the dissections were carried out after two hours of onset of scotophase.

*Preparation of tissue extract for bioassay*

Tissues pooled from fifty moths were homogenized in 200µl ice-cold distilled water and centrifuged at 15,000g for 20min at $4^0$ C. The fat free supernatant was lyophilized and stored at $-80^0$ C until used. The lyophilized extracts were dissolved in 75µl of saline for bioassay to study their influence on calling, receptivity, and oviposition behaviour.

*Bioassays*

Bioassays were carried out on the day2 virgin females. The insects were cold anesthetized for 5min at $4^0$ C before injection to minimize the hemolymph loss due to wriggling movements. In to the abdominal cavity of these insects 3µl of crude extract of MAG/Duplex/SV&T was injected through the inter-segmental membrane. Females were allowed to recover for 15min at $4^0$C after injection. Saline injected females served as controls.

*Fractionation*

MAG-duplex tissue extracts were prepared in 20-fold excess (w/v) of different extraction buffers viz. Bennett's buffer, 50% methanol in distilled water (DW) containing 2mM HCl, Methanol : DW : glacial acetic acid in the ratio 90:9:1 and 2% NaCl. The extract of 2% NaCl was incubated in a boiling water-bath for 5min (Suzuki *et al.*, 1982). All the extracts were centrifuged at 12,000g for 15min at $4^0$ C and supernatants were collected separately. The supernatant was defatted by passing it through a cheese cloth and later desalted by using C18 Sep - Pak Cartridges (Waters, USA). Bioassay was carried out with desalted extracts again as it was done previously.

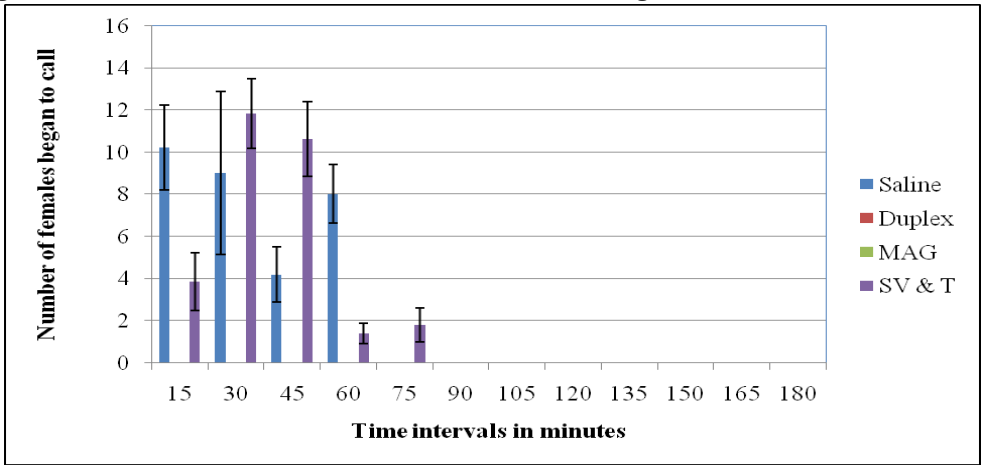
## Results

Mated moths behave differently from virgin moths in more than one way with respect to reproductive behaviour. The experiments conducted with injection of various tissue extracts of male reproductive system in to the female said it again with respect to the calling, mating and oviposition behaviour. Influence of the tissue extracts on the above mentioned behaviours in comparison with saline injected controls are detailed as follows.

*Calling Behaviour*

Following injection of saline and various crude tissue extracts, the moths were observed for calling activity on the day of treatment. All the treated moths except the MAG extract treated and the duplex extract treated groups began to call on the same day of treatment (Fig. 1).

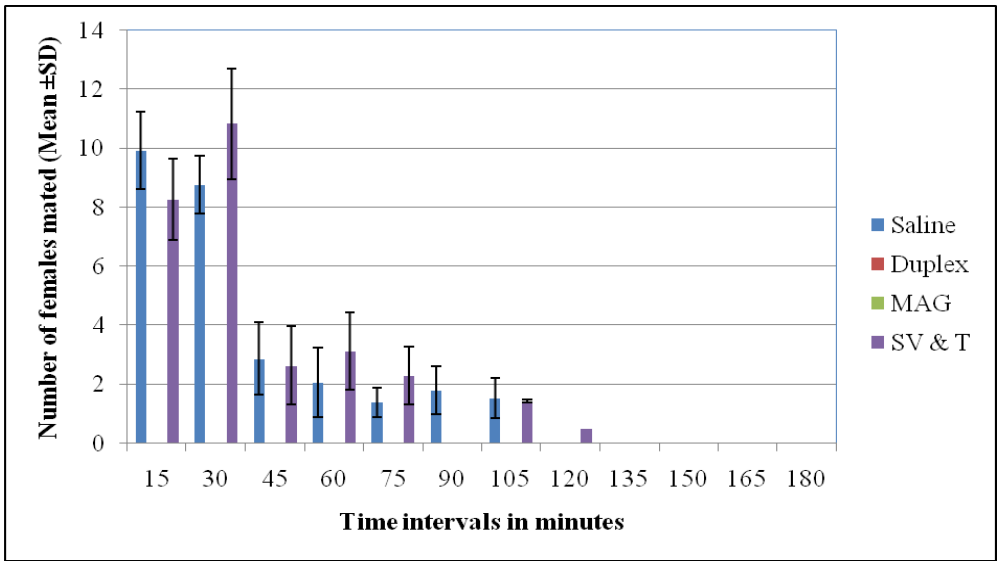
**Fig.1. Period at which *S. litura* females first called following treatments with various tissue extracts.**



*Mating Behaviour*

Both MAG and duplex treated moths remained inactive on the day of treatment like the normal mated females and none of them mated on the day of treatment. But, 96% of the saline treated and all of the SV & T treated females were observed to copulate on the day of treatment (Fig. 2).

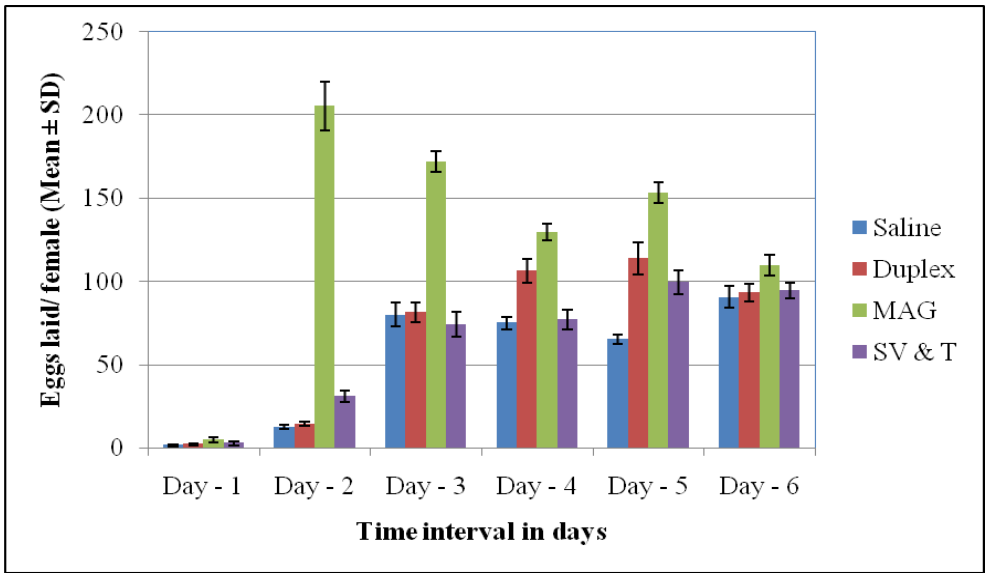
**Fig.2. Period at which *S.litura* females mated following treatments with various tissue extracts.**




*Oviposition Behaviour*

Moths belonging to all the groups began to lay eggs on the same scotophase following treatment. Virgin females injected with saline laid very few eggs (90.63±6.49) during the 6-day observation period after treatment (Fig. 3.).

**Fig.3. Number of eggs laid by *S. litura* females following treatment with various tissue extracts recorded on day basis.**



The females injected with SV & T extract also laid eggs similar to the saline injected moths on all the days. On the other hand, on all the 6 days of observation, females injected with crude accessory gland material or duplex material laid more eggs (357.6±2.15) when compared to the moths receiving either saline or SV & T. The females injected with MAG (6± 2) or duplex extract (4±2) laid only few eggs during the first 24h period, but in this group, oviposition increased significantly after the 2nd day following the treatment (MAG: 217± 34), and the trend continued during the subsequent days. There was no significant difference in the number of eggs laid by both MAG extract treated or duplex extract treated moths on all the days ($p<0.05$). These results appear to show that the substance that temporarily inhibits calling and receptivity is present in both male accessory glands and the duplex.

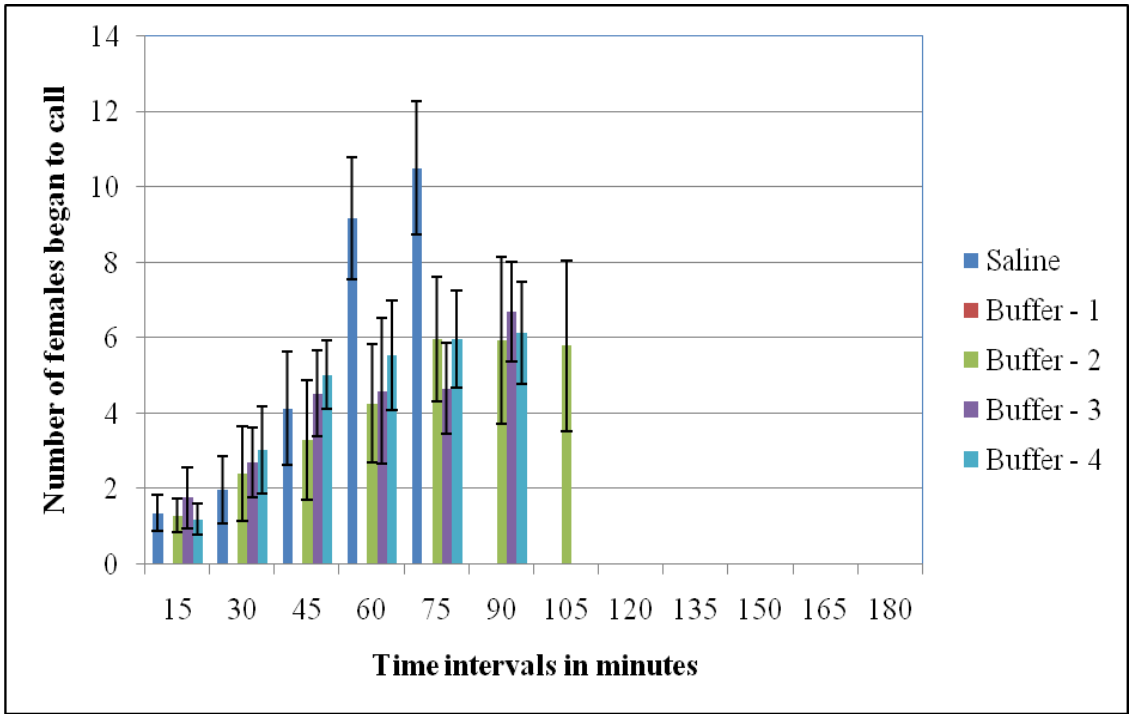
*Effect of partially purified MAG extract*

Similar studies on egg laying activity by (Bali *et al.,* 1996) in newly colonized *H.zea* moths and *H. armigera* (Shobha, 2007) moths found that protein factors eluted from MAG-duplex extracted using Bennett's buffer stimulated this activity. Hence fractionation of MAG extract was carried out in Bennett's buffer, along with three other buffers having different properties in order to prevent loss of any other probable active factors during extraction with acid based Bennett's buffer.MAG extracts in different buffers were tested for similar activities as was done with the crude extract. The results for different parameters chosen are as follows

*Calling Behaviour*

It was observed that all saline injected females began to call during the period of observation. Calling was completely suppressed in females injected with MAG-duplex extract with Bennet's buffer immediately after injection (Fig. 4.), but about 16 % females injected with extracts in rest of the buffers started calling within 15 minutes after injection and all of them resumed calling in about 105minutes after injection.

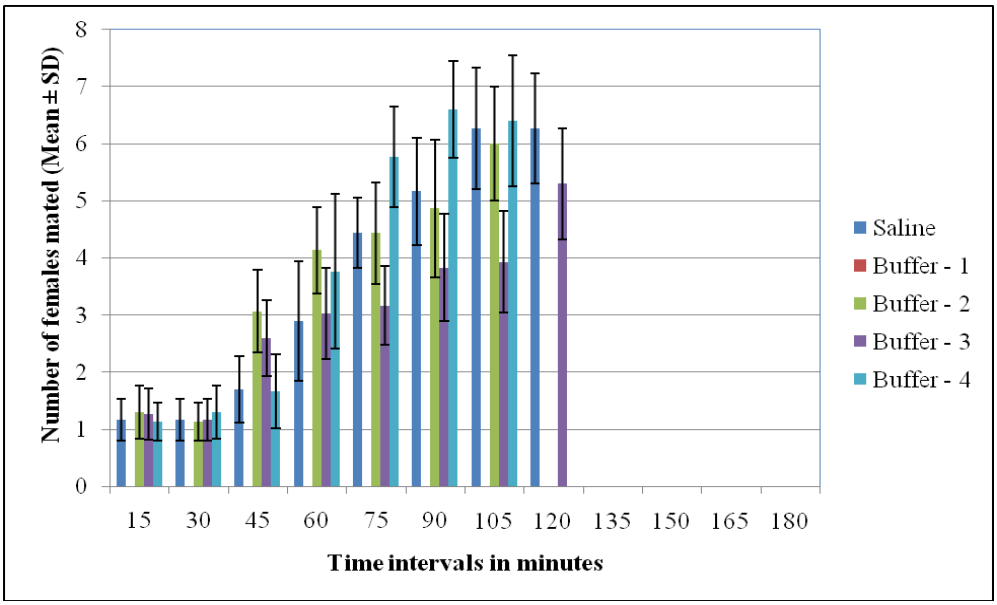
**Fig.4.Period at which *S. litura* females first called following treatment with MAG proteins extracted using different buffers.**



*Mating Behaviour*

Virgins injected with the MAG-duplex extract extracted with Bennet's buffer were unreceptive to mating attempts and behaved like mated moths. The moths in the rest of the groups were receptive to mating attempts on the day of treatment (Fig. 5.)

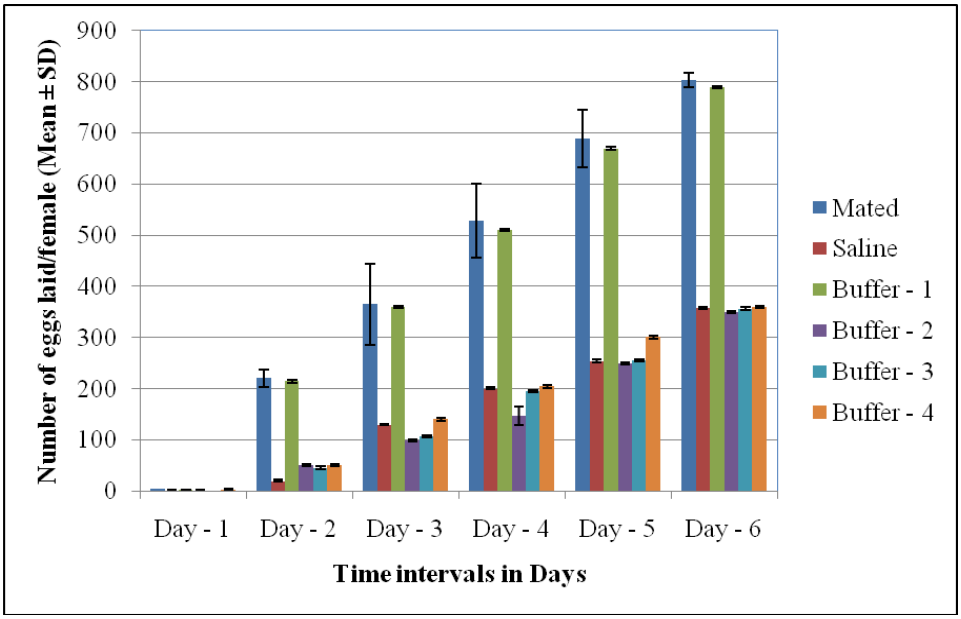
**Fig.5. Period at which *S. litura* females mated following treatment with MAG proteins extracted using different buffers.**




*Oviposition Behaviour*

Females injected with desalted accessory gland extract with Bennet's buffer laid more eggs (796 ± 27) compared with the moths receiving saline (452 ± 31). The oviposition increased significantly after the 2nd day following the treatment, and the trend continued for the rest of the period in Bennet's buffer extract received moths. Mated females laid the highest number of eggs at any given time compared with females in the treatment groups; the group treated with Bennet's buffer extract was comparable to the mated group (Fig. 6.). These results suggest that Bennett's buffer is the ideal choice for extraction of factors influencing post-mating responses in *Spodoptera litura*.

**Fig.6. Number of eggs laid by *S. litura* female following treatment with MAG proteins extracted using different buffers.**



## Discussion

Present studies found that the SV & T do not influence the post-mating changes in *Spodoptera litura*, as the moths treated with extract of these tissues were observed to behave like saline treated virgins in all respects including the number of eggs laid. They exhibited calling and were receptive to males on the day of treatment just like the virgin females.

The accessory gland or duplex crude extracts were found to influence rate of egg laying *i.e.*, temporal pattern of egg-laying. The fecundity was also found to be significantly increased when compared to control females. Also the calling and mating were temporarily inhibited by these extracts. The results from the present studies were similar to the studies in newly colonized *H.zea* moths (Bali *et al.,* 1996). Mated females were observed to lay highest number of eggs when compared to the treatment groups suggesting that along with MAG or duplex secretions, some other factors transferred during mating are necessary for deposition of their full complement of eggs. These may include a purely mechanical stimulus provided by the act of mating as in cockroach (Roth and Stay, 1962) or the presence of eupyrene sperm in the spermatheca of *Zeiraphera diniana* (Benz, 1969), *Mamestra configurata* (Gerber *et al.,* 1991) and *Manduca sexta* (Sasaki and Riddiford, 1984).

Similar to the studies in *H.zea* obtained by (Bali *et al.,* 1996) and in *H. armigera* obtained by (Shobha, 2007) in the present study also, the post mating changes could be brought about in virgins by proteinacious material of MAG-duplex complex extracted in Bennett's buffer and desalted using a C18 Sep-Pac. It was not so with other buffers, indicating that the bioactive material was not a high molecular weight protein, as it retained its activity when acidified extraction buffer was used. According to Bennett (1978), formic acid in this buffer, prevents degradation of peptides and prevents precipitation, NaCl helps in adsorption of peptides onto C18 Sep-Pac, and HCl prevents ionic bonding of peptides to other proteins in solution, while TFA prevents blocking of the extraction column. Probably buffers containing NaCl, methanol, or methanol-acetic acid precipitated the bioactive material.

## Conclusion

During the present investigations, it was observed that mated female behaviour differed in several ways from that of virgins indicating the probability of transfer of chemicals from male to female during the time of mating inducing these changes. Efforts made to localize these factors in *Spodoptera litura* by injecting crude extracts of different reproductive tissues to virgin female moths led to MAG and MAG extract with Bennett's buffer yielded the active factor. Further purification and characterization of this factor is required to develop a strategy for biocontrol of this pest insect.

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