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# Do Male-derived Substances Affect Female Reproductive Physiology? A Study on Oviposition, Fecundity and Longevity Aspects in Female *Helicoverpa armigera* (Hubner) Moths

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## ABSTRACT

Oviposition by the female moths of *Helicoverpa armigera* began on the third day of emergence; however, mating stimulated earlier oviposition. Hence, we carried out series of experiments to demonstrate the role of male accessory glands-ductus ejaculatoris duplex (MAG-duplex) secretions on female post-mating behaviour in *H. armigera* moth. Injection of extracts from the MAG-duplex into the abdomen of females accelerated oviposition with a marginal increase in egg production (fecundity). The secretions of MAG-duplex did not affect the longevity of the females much whereas the negative effect on females' longevity observed in mated females may be because of diversion of more resources for egg development and oviposition, leaving fewer resources for survival. On the other hand, marginal increase in fecundity but accelerated oviposition indicates that MAG-duplex secretions trigger the egg laying but for maintenance of oviposition rate, presence of sperm is necessary. Results of our study not substantiated the hypotheses, mechanical stimulation by male triggers oviposition.

***Key words*:** *Helicoverpa armigera,* Male accessory glands, Oviposition, Fecundity, Longevity

Numerous studies have demonstrated that seminal fluid proteins (SFPs) play an essential role in insect reproduction. Insect SFPs are the products of male reproductive tract, secretory tissues-accessory glands, seminal vesicles, ejaculatory duct, ejaculator bulb and testes. SFPs are transferred to females along with sperm during mating. After mating in most species of insects, male SFPs results in a profound remodelling of behavioural, physiological [1-2] and gene signalling pathways in females and typically cause transient or permanent loss of sexual receptivity and elevated egg production of the females [3-5]. Transferring SFPs that up regulate these processes can benefit males, ensuring their sperm fertilize the maximum number of eggs before the female remate and can also benefit females, in allowing increased egg production, only when sperms are present to fertilize those eggs. Of the various SFPs that may mediate the above responses, those comes from the secretions of male accessory glands, are known to play a significant role [6-8].

So far, it has been found that substances in MAG are transferred to females are hormones and/or active proteinaceous factors [9-11] influencing the physiology and behaviour of mated females, which include: alleviating attraction to males; depressing subsequent mating; enhancing oviposition; stimulating oogenesis; transferring, storing, and utilizing sperm; and shortening life span.

*Helicoverpa armigera* (Lepidoptera: Noctuidae), commonly known as cotton bollworm or American bollworm, is a serious pest of many agriculturally important crops and claims a major share in crop losses every year. The pest status of this species is derived, in part, from four characteristics of its life history (polyphagy, high mobility, high fecundity and facultative diapause) that enable it to survive in unstable habitats and adapt to seasonal changes [12]. Therefore, it is important to have the basic knowledge of the insect's biology and behaviour for the successful introduction of mating disruption technology into pest management programs. The current study was designed to test the possible involvement of the male accessory glands in influencing oviposition, fecundity, and longevity of females.

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## MATERIALS AND METHODS

### *Insect rearing*

*H. armigera* larvae (NBAII-MP-NOC-01) procured from NBAIR, Bengaluru, were reared on modified semi synthetic chickpea diet [13]. The 3rd instar larvae were

maintained individually in vials (plastic cup, size: 4.2 × 4.5 cm), at 25ºC during the photophase and at 23ºC during the scotophase, 65±5% Relative Humidity and 16 Light: 8 Dark photoperiod in a B.O.D incubator. Pupae were collected and sexed according to the characteristics of their exterior paramera. The male and female pupae were kept separately in plastic containers (25 × 15 × 8 cm) until adult emergence to ensure virginity and age. Unless stated otherwise, all female adults used in this study were 2-day-old.

*Preparation of male crude extract*

Virgin males of 2-6 day old were dissected using ice-cold lepidopteran saline [14], and the whole MAG-duplex tissue was transferred into a sterilized, pre chilled microcentrifuge tubes. The dissections were carried out after two hours of onset of scotophase.

Tissues excised from 300 moths were pooled and homogenized using a tissue homogenizer in 20-fold excess (w/v) of Bennett's extraction buffer [15], centrifuged at 15,000 g for 20 minutes at 4°C and the supernatant was collected carefully. The pellet was similarly extracted two times with buffer. All the three supernatants were pooled desalted using C18 Sep-Pak cartridge (Waters, USA) as described by [16], frozen and subsequently lyophilized and stored at -40°C until used. For bioassays the lyophilized extract was dissolved in 900 µL 1X phosphate buffered saline (PBS) of pH 7.4.

*Effect of MAG-duplex Secretions on oviposition rate, fecundity and longevity of females*:

To determine whether MAG-duplex secretions influenced female in terms of oviposition rate, fecundity i.e., total number of eggs laid in its life time and lifespan, the experiments were set up with four treatments: (1) virgin females injected with MAG-duplex extract (n=27), (2) virgin females injected with PBS (n=32), (3) virgin females mated with naive males of 2-6 day old in the ratio 1:5 (Female: Male) (n=29) and (4) untreated virgin females (n=34). Injections were given at the beginning of the second scotophase after emergence. The females which are mated for 1hour were selected for bioassay in the treatment 3.

The moths were cold anesthetized, prior to injection, by exposing them to temperature of 4ºC for 5 minutes. These insects were transferred on to ice and injected 3 µL (one male equivalent) MAG-duplex extract using Hamilton syringe into the abdominal cavity through intersegment membrane [17]. Females were allowed to recover for 15 minutes after injection. The experimental moths were kept individually in an oviposition chamber (plastic cup, size: 8.5 × 6.5 cm) covered with a black cloth serving as an oviposition substrate. 10% honey solution was provided as food for the moths. The eggs of each moth were counted daily till the death. The egg count for three days was considered to measure the effect of MAG-duplex secretions on oviposition rate whereas total number laid till death indicated the fecundity. The results from females living less than 5 days were not considered. The experiment was replicated 3 times.

*Statistical analysis*

Data on daily eggs laid, total eggs laid and longevity were not normally distributed and thus analyzed using nonparametric Kruskal-Wallis test followed by Dunn's procedure for multiple comparison [18]. The relationship between fecundity and longevity of MAG-duplex extract injected females was analyzed using a linear regression analysis. Rejection level was set when $\alpha < 0.05$ in all analyses. All analyses were made using R, version 3.0.2 software.

## RESULTS AND DISCUSSION

*Effect of MAG-duplex secretions on oviposition*

A significant difference among the treated groups ($H = 164.990$, $p < 0.05$) was observed for the data pooled for six consecutive scotophase after treatment, on analysis with nonparametric Kruskal-Wallis test. Further a pair-wise multiple comparison by Dunn's test showed $p < 0.05$ in case of MAG-duplex injected and mated females. The oviposition in virgin and PBS injected was found to be similar ($p > 0.05$). The mated females laid the highest number of eggs among the experimental groups. However, the MAG-duplex extract injected females laid more number of eggs than virgin and PBS injected females and the difference was highly significant for those six days, an indication of oviposition stimulation by MAG-duplex secretions (Fig 1).

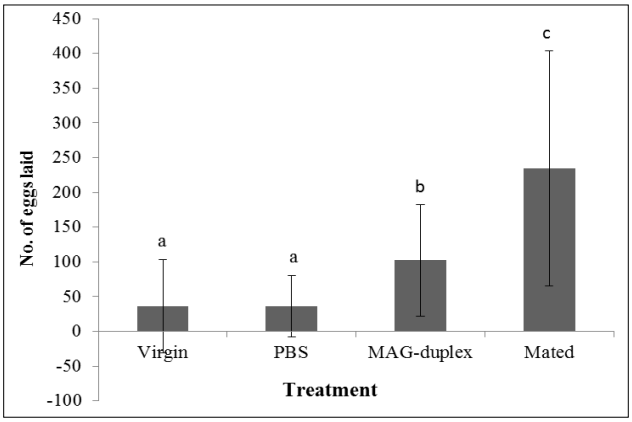


Fig 1 Effect of MAG-duplex extract on oviposition (mean±SD): Total number of eggs laid by females (six consecutive scotophase) injected with MAG-duplex extract and PBS injected (control) in comparison with mated and virgin females. The same letters on mean bar show no significant difference at p<0.05 after Kruskal-Wallis followed by Dunn's test

In *H. armigera*, mating not only increases the fecundity [19] but also triggers the oviposition just as in other insects [20-21] and generally the male accessory glands secretions are involved in it. In the present study, a bioassay was carried out for studying the physiological effects of MAG-duplex secretions on female *H. armigera* moths especially on oviposition, fecundity and longevity. The male accessory gland factors not only elicit a change in a behavioral pattern (receptivity) but also a physiological response (oviposition). The study by [22] demonstrates that the target receptors for accessory gland proteins are located in the female reproductive tract as well as in haemolymph. This may explain why male accessory gland secretions delivered by ejaculation into the female reproductive tract and injection into female body cavity have similar effect on the female physiology.

Ovulation, oviposition and egg production increased from very few eggs per day laid by virgin females to a maximum of eggs per day following mating [23-24] in several insects. This reflects that female insects carefully adjust their investments in mating and egg production depending upon

their reproductive state [25]. For elevated egg production or for somatic maintenance the materials from spermatophores are used [26-27]. However, stimulation mechanism of egg production/laying in relation to mating is species specific.

*Effect of MAG-duplex secretions on fecundity and longevity*

Nonparametric Kruskal-Wallis test showed significantly difference in the fecundity of four groups ($H = 20.665$, $df = 3$, $p < 0.05$). However, after Dunn's test, there was no significant difference in fecundity of PBS injected and virgin females ($p > 0.05$). But a significant difference was observed in the fecundity of mated females which laid more eggs compared to virgin moths as well as MAG-duplex extract and PBS injected moths ($p < 0.05$). MAG-duplex extract injected females laid less than mated females but more than PBS injected and virgin females (Fig 2).

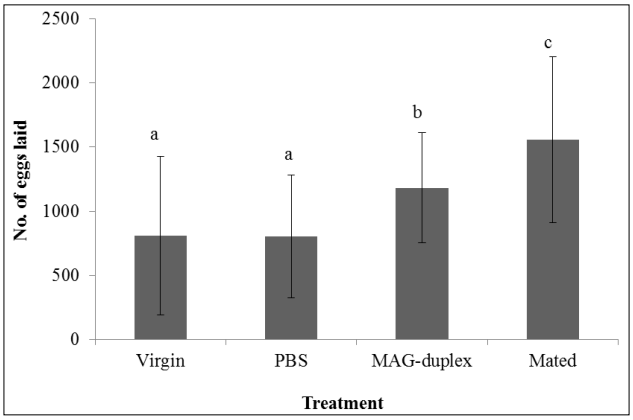


Fig 1 Effect of MAG-duplex extract on fecundity (mean±SD): Total number of eggs laid by females injected with MAG-duplex extract and PBS injected (control) in comparison with mated and virgin females. The same letters on mean bar show no significant difference at p<0.05 after Kruskal-Wallis followed by Dunn's test

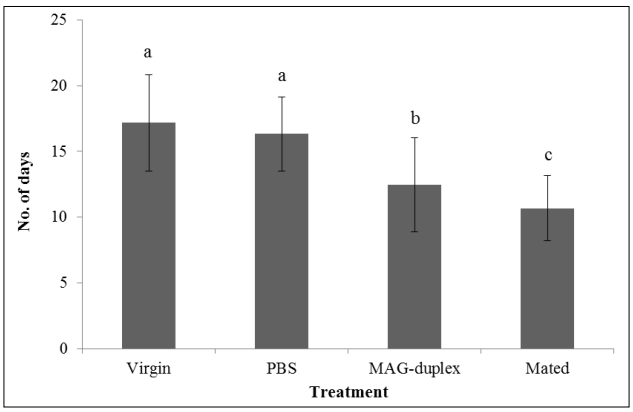


Fig 3 Effect of MAG-duplex extract on longevity (mean±SD): Life span of females injected with MAG-duplex extract in comparison with PBS injected (control), mated and virgin females. The same letters on Mean bar show no significant difference at p<0.05 after Kruskal-Wallis followed by Dunn's test

In longevity studies a significant difference was observed among the groups ($H = 53.275$, $df = 3$, $p < 0.05$). The virgin moths lived longer than the mated moths which are evident as the difference was more significant between them ($p < 0.05$) and mated moths had shortest life span than the rest of the groups. There was no significant difference in the longevity between PBS injected as well as virgin females ($p > 0.05$). The longevity of MAG-duplex extract injected moths was though slightly longer compared to mated moths, but was evidently shorter than virgin and PBS injected moths (Fig 3).

The linear regression analysis was carried out to know the relationship between fecundity and longevity. The results showed no negative relation though these two behaviours are seems to be dependent on each other. This result implies that MAG-duplex extract reduced the longevity but not to the extent of mated ones (Fig 4).

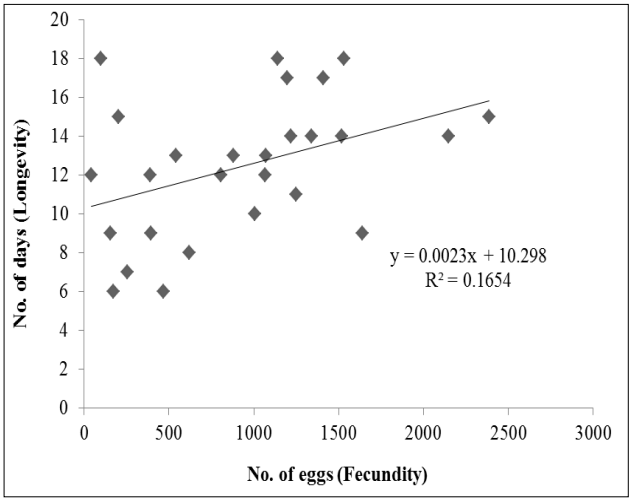


Fig 4 Relationship between fecundity and longevity in MAG-duplex extract injected females. Negative correlation is not as strong as in normal mated moths

MAG-duplex extract injected moths showed threefold increase in oviposition rate whereas mated moths showed fivefold increase compared to controls. Between MAG-duplex injected and mated it was around twofold increase in the latter. This clearly indicates that MAG-duplex secretions have contributed for acceleration of egg laying but could not match with the mated moths. Male accessory gland secretions although able to trigger oviposition [28], events such as the act of mating or contributions thereof such as the spermatophore or presence of sperm is necessary for maximum fecundity [29-31]. Several researchers have found out that stored sperm are needed to maintain the mated state: long-term inhibition of mating receptivity, and continued elevated rates of oogenesis, ovulation and egg deposition all require the presence of sperm in the female [32-35]. In some the duo, viable sperm and accessory secretions, are believed to be required for maximal oviposition response [36-37]. This could be true in case of *H. armigera* as mated females exhibited higher rate of oviposition as well as fecundity compared to virgins or MAG extract injected moths in the present study.

So far, the molecular characteristics of few proteins/peptides related to oviposition from MAG in different insects [38-43] have been determined. Except for $SP_{DS}$ in *D. suzukii* (which shares great similarity in gene sequence with SP in *D. melanogaster*), other proteins/peptides show little similarity with each other and the molecular masses widely vary from 3.99 kDa (OSS) to 60 kDa (OSP). This indicates that there may exist two possible reasons for different molecules with similar function: one is the fast evolution in the reproduction field and the other is that their targets are different. For example, Acp26Aa [44] (Heifetz et al., 2000) and OSP [11] may act on ovaries, while SP is suggested to act directly on CA [45].

A virgin female lives significantly longer than a mated female [46]. The positive correlations between oocyte degradation and female longevity suggest that longer female

lifespan is the result of recouping resources from eggs through oosorption [47-48]. A number of authors [49-50] report that mated females showed shorter longevity. Several hypotheses were put forward to explain the reason behind this physiology. According to some researchers the antagonistic co-evolution between the sexes has thus been compelled males to adapt to intra sexual competition and intersexual selection that have detrimental effects on their mates [51]. Such male effects can shorten female longevity due to physical damage from spiky male genitalia during mating [52-53], other forms of traumatic insemination [54], or altered female reproductive schedules by male toxic ejaculates [55-57].

The premature death of the mated female is due to a combination of factors. First, the energetic requirements of progeny production take their toll. Second, just the presence of males (even in the absence of mating) also decreases the female's life span [58-59]. Perhaps this is due to her inability to stop and rest and eat as she is continually being chased by males. A third component to her shortened life is independent of either of these, and requires the receipt of seminal fluid from the male [35]). To counter this female has evolved multiple-mating strategy which benefits her to increase genetic fitness as well as to harness more nutrients from the male.

According to the resource allocation model [60-61], ageing occurs because resources allocated to reproduction are unavailable for investment in somatic repair, making individuals or populations that invest more in reproduction likely to incur faster ageing and shorter lifespan. Therefore, the longevity reduction in mated *H. armigera* may not necessarily caused by male accessory gland proteins as suggested in some studies [6] as longevity was not affected in MAG injected virgins but a combination of several factors including allocation of resources affect the longevity. Resource allocation between ova and the soma before and after mating in *H. armigera* females may be a strategy for maximum reproductive success under the constraints imposed by external environment and sexual interaction. Such resource allocation between survival and reproduction may have evolved under natural and sexual selection that favours higher fecundity [62].

## CONCLUSION

Our study demonstrated the accelerated oviposition with a marginal increase in fecundity upon injecting of extracts from MAG-duplex into the abdomen of females. The secretions of MAG-duplex did not affect the longevity of females much whereas the negative effect on females' longevity in mated females was significant.

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