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Egglaying behavior of silkmoths injected with the extract of male reproductive system

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Abstract
To study the egg laying behavior of silkmoths, freshly emerged female moths were injected with male reproductive system extract of whole and different tissues, the moths injected with saline served as controls. The results were compared with normal mated and virgin moths.

Keywords: Silkmoth, Oviposition, Temporal pattern of egglaying, Virgin, mated moths

Introduction
Insects occupy a unique position in the animal kingdom with respect to the diversity. This is especially true when we consider the behavioral traits. Among invertebrates, complexity in behavior seems to have attained its culmination in insects. Concomitantly the regulatory mechanisms underlying these behavioral traits are also highly complex.
Two important phases of reproduction namely mating and egg laying are very interesting in insects, especially in silkworm, as it is of commercial importance. Since the early stages of human civilization silkworm has been used as a source of silk for producing exquisite textiles. Because of its industrial importance extensive scientific studies have been carried out on silkworm to support the development of sericultural technology.
Studies are mainly aimed at improving the economic characters such as yield, and quality of silk focusing on the larvae and pupae, whereas adult moths received very little attention though reproduction involving both male and female adult moths is a critical phase for continuation of one's own progeny.

Review of literature
Studies on adult Silkmoth mainly deals with the understanding of the behavioral aspects of mating and egg laying (Yamaoka and Hirao, 1981; Manjulakumari, 1991), but not so much, the physiology of mating and egg laying, the two most important aspects of reproduction.
In insects, mating is not a mere process of inseminating the female but, has a profound influence on the reproductive behavior and physiology of female. The post-mating effect of male on females include induction of oogenesis (Pickford *et al.*, 1969), enhancement of fecundity (Danthanarayana and Gu, 1991) [14], suppression of remitting (Shirk *et al.* 1980) [52], stimulation of oviposition (Watanabe, 1988; Bali *et al.*, 1996) [60, 3], acceleration of egg laying (Herndon and Wolfner, 1995; Soller *et al.*, 1997) [26, 55], and so on.
Male is actually the source of compounds which modulate the physiology of female in several ways (Chen, 1984 [8]; Gillott, 1988 [22]; Chen *et al.*, 1988; Aigaki *et al.*, 1991 [1]; Kalb *et al.*, 1993; Herndon and Wolfner, 1995 [26]; Chapman *et al.*, 1995 [7]; Kubli, 1996 [30]; Wolfner, 1997 [61]; Shu-Xia Yi, Gillott, 1999) [53]. During insemination, male introduces not only gametes but also some of the substances secreted by different tissues of the reproductive system viz., testes (Smith, 1956), ejaculatory duct and accessory gland (Bairati, 1968; Gillott and Friedel, 1977) [2, 23]. These substances may be proteins (Rockstein, 1964; Ranganathan, 1982 [47]; Chen *et al.* 1988; Basker 1988; Wolfner, 1997 [61]), carbohydrates (Chino, 1958 [11], Blum *et al.*, 1962 [6]; Ranganathan, 1970, 1973 [45, 46]; Baumann, 1974 [4]; Pant and Sharma, 1976 [42]; Muse and Balogun, 1993) or lipids (Ranganathan and Padmanabhan, 1994) [48] entering the female and influencing its physiology. Such substances have been termed differently as fecundity enhancing substances, receptivity inhibiting substances, oviposition stimulating substances (Gillott and Friedel 1977 [23]; Shu-Xia Yi, Gillott, 1999 [53]) etc.

In addition to this males supplement zinc (Engebretson and Mason, 1980) [19] and sodium requirements of the female (Pivnik and McNeil, 1993) [44].

The male derived substances also play an important role in successful transfer of sperms (Gracia and Bellido 1964 [24]; Lefevre and Moore 1967 [34], Chen 1971 [9], Fowler 1973) [20], sperm storage (Gilbert, 1981 [21]; Neubaum and Wolfner, 1999 [59]; Uyen Tram and Wolfner, 1999) [59] and utilization (Kalb *et al.*, 1993). If female does not receive adequate quantity of these substances, it fails to store as many sperms as it could have otherwise (Kalb *et al.*, 1993). In *Drosophila melanogaster* seminal fluid molecules from the male accessory glands are responsible for initial behavioral changes like an elevation of egg laying but persistence of these changes require stored sperm (Neubaum and Wolfner, 1999) [59].

The transfer of seminal fluid molecules along with sperm during mating is of great importance to the reproductive success of male. Since seminal fluid components perform diverse functions in the female, it benefits both the male and his mate (Gillott, 1988 [22]; Eberhard, 1996 [18]; Wolfner, 1997) [61].

In most of the insects egg laying can take place irrespective of mating. Though virgins could lay eggs in some insects (Hinton 1981) [28], generally they lay fewer eggs when compared to mated females. The increased egg laying capacity can be stimulated by copulation in insects (Ridley, 1988 [49]; Danthanarayana and Gu, 1991) [14].

Oviposition in insects is an interesting phenomenon with lot of diversity - diversity in selecting the oviposition site, in the pattern of laying eggs, rate of egg laying etc. In silkworm, due to its domestication over several years and human intervention in selecting specific characters, the behavioral traits that can be observed are limited to the act of feeding and reproduction. One such interesting behavior is the laying of eggs in a monolayer. The adult female lays eggs packed close to one another and never one above the other except under a certain pathological condition. Egg laying is a multi-step process, it begins with oocyte release by the ovaries followed by egg movement down the oviducts and the deposition of eggs on to the substratum. Yael Heifetz (2000) [62] reported that the ACP 26Aa stimulates the first step in egg laying release of oocytes by the ovary. During mating ACP 26Aa begins to accumulate at the base of the ovaries, a position consistent with action on the ovarian musculature to mediate oocyte release. For successful insect reproduction there should be synchronization of copulation with a number of factors including the presence of mature or nearly mature gametes in both sexes, ability to produce the secretions necessary for sperm transfer in male and availability of nutrients for egg maturation in female. Generally mated females lay more eggs than virgins (Bali *et al.*, 1996) [3]. In virgins oviposition may be delayed or even prevented and egg maturation is slower.

In *Rhodnius*, the rate of egg laying is greatly increased by mating, but this has little effect on the rate of egg formation (Coles, 1965). Mating and producing hatch able eggs are two different, although connected events. Roth and Willis (1956) in *Blatta orientalis*, reported that mating stimulates egg laying and showed that mated females, on an average laid 160 eggs, while unmated lay only 114. Similar results were reported in *Haematosiphon inodorus* (Lee, 1954) +, *Hesperocimex sonarensis* (Ryckman, 1958) [51] and *Cimex lectularis* (Davis, 1964, 1965a, 1965b) [15-17]. A virgin moth lays fewer than 10% of the eggs on the first day and continues egg laying for 4 to 5 days without increasing the rate of oviposition. After mating, over 90% of eggs are laid at one time during the first day. The mechanism that controls acceleration of egg laying is obscure despite many studies to clarify it. Mating may be immediately followed by an outburst of egg laying, but in many species the manner of laying by mated and virgin females is very different. Thus, even when the total number of eggs laid is the same for both mated and virgin females, virgins may delay laying or lay with an irregular periodicity as compared with mated females, e.g. in the fly *Cochliomyia hominivorax* (Crystal and Meyners, 1965) and the acridids *Gamphocerous Rufus* (Loher and Huber, 1964) and *Locusta migratoria* (Mika, 1959). In the bug *Oncopeltus faciatus* a single mating with a normal male has at most a minor and temporary effect on the rate of egg production even when enough sperms are transferred to make the production of the some fertile eggs possible for several weeks. In silkworm, so far, not many attempts have been made to closely examine the role of mating on egg laying behavior. The few attempts which have been made (Omura, 1936, 1938 [41]; Yamaoka and Hirao, 1977 [66]; Thomas Punitham *et al.*, 1987) [58] have failed to demonstrate the effects quantitatively. To understand the mechanisms which control and regulate egg laying behavior, it is very essential to understand these aspects mentioned above. During the current investigations, the egg laying behavior was examined closely in virgins as well as in mated moths and the results were compared quantitatively with one another.

The stimulus to egg laying is provided by mating via chemical substances secreted by different tissues of male reproductive system. These substances may pass through the wall of the tract in a modified or unmodified form and stimulate the female tract (Baumann, 1974) to bring about the necessary changes to initiate egg laying.

Pickford *et al.*, (1969) working with the migratory grasshopper, *Melanoplus sanguinipes*, showed that an egg laying stimulant was produced by the male accessory reproductive glands. In *Schistocerca gregaria,* Leahy (1973a) implanted male accessory glands in virgin females and found their oviposition rate to be increased. Lange and Loughton (1985) showed that injection of mature male accessory gland extracts of *Locusta migratoria* stimulated an increase in oviposition rate of virgin females comparable to that of mated females.

Material and methods

Silkworm seed cocoons of bivoltine race NB_4 D_2 were collected from Government bivoltine cocoon market and maintained in the laboratory at room temperature. The males and females were segregated at the Pupal stage itself and maintained in separate cages to avoid copulation.

The newly emerged silkmoths were allowed to copulate for 6 hours. The females were then transferred on to the egg cards. By placing a plastic cellule around them, the space of egg laying was restricted. A few months were left free without placing a cellule for better observation of their movements during oviposition. The following parameters were recognized to quantify the egg laying behavior

- The pre-oviposition period: Period between termination of copulation and initiation of egg laying.

- The oviposition period: Period between initiation of egg laying and the completion of egg laying.
- The temporal pattern of egg laying: Number of eggs laid at different intervals of the oviposition period.
- Total number of eggs laid.

The egg cards were carefully changed every 24 hours after the initiation of oviposition with minimal disturbance to animal, to record the temporal pattern. Similarly the egg laying behavior of virgin females were quantified.
Egg laying behavior of silkmoths injected with the extract of whole and different tissues of reproductive system was studied by injecting the extract into haemocoel of female moths. The different organs of male reproductive system were pooled from 40 unmated freshly emerged male moths. The tissues were homogenized separately in 2 ml Silkmoth saline, (Yamaoka, 1977) [66] centrifuged at 10000_g for 10 minutes. The crude extract was injected in to the virgin females around 6 AM in the early morning soon after their emergence. The dosage injected was equivalent to that of one male moth (i.e., 50µl/moth) and the egg laying behavior was studied by considering the parameters mentioned earlier. Normal mated, virgin and the saline injected moths served as controls.

Results and discussion
To study the egg laying behavior of silkmoths injected with reproductive system extract of whole and different tissues, the moths injected with saline were used as controls. The results were compared with normal mated and virgin moths.

Complete male reproductive system extract injection
Moths that received complete male reproductive system extract, laid 426.00±16.00 eggs within 24 hours after the initiation of oviposition. The total number of eggs laid by these moths was found to be 529.00±12.00. These moths started laying eggs after a pre-oviposition period of 5.65+0.291hrs. The oviposition duration was lasted for 84.32±1.12hrs. About 80% of the eggs were laid in the first 24 hours after the initiation of oviposition. Thereafter, the egg laying rate gradually decreased.

Testes extract injection
The pre-oviposition period of the testes extract injected moths was found to be 7.52±0.130hrs which is comparable to that in virgins. The total number of eggs laid was 410.00±9.00, which is nearly similar to that of virgins injected with saline. The pre-oviposition period and the total number of eggs laid by saline injected moths were found to be 7.13±0.098hrs and 378.00±12.00 eggs respectively. The testes extract injected and saline injected virgins laid about 3.7% and 4.47% of total eggs laid in the first 24 hours of oviposition period respectively. The oviposition period of extract injected moths was similar to that of saline injected virgins.

Vas deferens extract injection
The total number of eggs laid by the moths injected with vas deferens extract was found to be 367.00±6.00 eggs. The pre-oviposition period and oviposition period were 7.45±0.131hrs and 180±1.75hrs respectively. They laid about 3.29% of the total eggs in the first 24 hours of oviposition period which is nearly comparable with that laid by moths injected with saline which laid 4.78% of the total eggs in the same time.

Seminal vesicle extract injection
The moths started laying eggs after the pre-oviposition period of 7.85±0.121hrs, the total number of eggs laid by the moths was found to be 429.00±6.00. They laid about 11.19% of the total eggs in the first 24 hours of oviposition period, which was comparable to the oviposition period of saline injected moths. The oviposition period stretches to 150.64±2.47hrs.

Ejaculatory duct extract injection
The pre-oviposition period of the moths injected with the ejaculatory duct extract was found to be 7.80±1.30hrs. In the first 24 hours after the initiation of oviposition they laid about 5.16% of total eggs which is similar to that of saline injected virgins which laid 4.78% of the total eggs in the same period. The total number of eggs laid by these moths was found to be 327.00±11.00. The oviposition duration was 172.48±0.48hrs.

Accessory reproductive gland extract injection
After the initiation of oviposition in the first 24 hours the accessory reproductive glands extract injected moths laid about 80.98% of the total eggs. This is similar to that of mated moths which laid about 80.01% of total eggs in the same period. The pre-oviposition period of the accessory reproductive glands extract injected moths and normal mated moths were 5.83±0.086 and 5.84±0.095hrs respectively. The total number of eggs laid by extract injected moths was 535.00±10.00 which is similar to that of mated moths which laid about 537.00±13.00 eggs. The oviposition duration of extract injected and mated moths was 80.8±1.8 and 92.16±5.12hrs respectively.
The egg laying behavior of moths injected with testes, vas deferens, seminal vesicle and ejaculatory duct extracts was comparable to the virgins egg laying behavior with respect to pre-oviposition period, oviposition period and total number of eggs laid. Whereas in females which received the extract of whole reproductive system and accessory reproductive gland, the egg laying behavior was similar to that of mated moths.

Egg laying data of silkmoths injected with male reproductive system extract

Table 1: Define silkmoths injected with male reproductive system

Moth Sample	Pre-oviposition period (hrs.) Mean±SE	Oviposition period (hrs.) Mean±SE	Total number of eggs laid Mean±SE
Virgin female	7.65±0.081	226.56±5.89	399.00±11.00
Mated female	5.84±0.095	92.16±5.12	537.00±13.00
Extract injected females			
Saline	7.13±0.098	196.8±2.59	378.00±12.00
Testis	7.52±0.130	189.28±1.87	410.00±9.00

Vas deferens	7.45±0.131	180±1.75	367.00±6.00
Seminal vesicle	7.85±0.121	150.64±2.47	429.00±6.00
Ejaculatory duct	7.80±0.130	172.48±0.48	327.00±11.00
Accessory reproductive gland	5.83±0.086	80.80±1.80	536.00±10.00
Complete male reproductive system	5.65±0.291	84.32±1.12	529.00±12.00

The temporal pattern of egg laying in moths injected with testes, vas deferens, seminal vesicle and ejaculatory duct extracts showed a similar pattern as in virgins. Whereas the temporal pattern of egg laying in the moths received whole reproductive system and accessory reproductive gland extract was comparable to the egg laying pattern of mated moths.

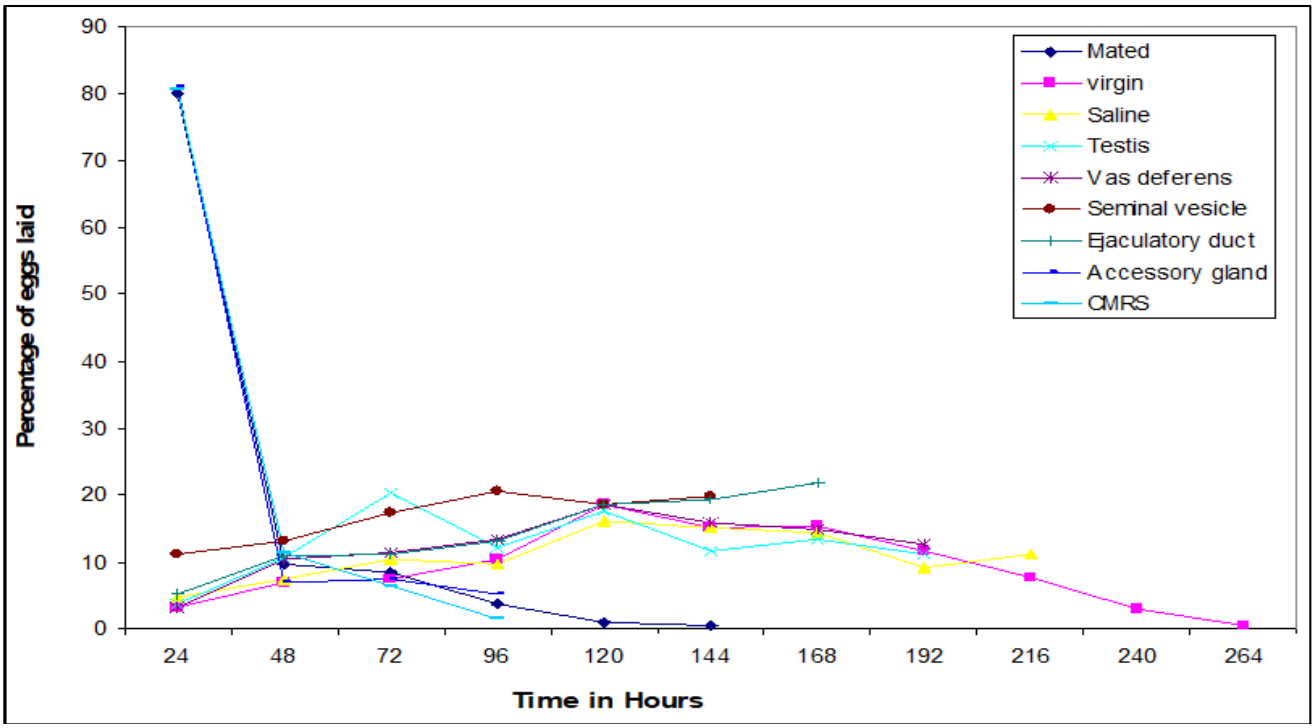

Fig 1: Temporal pattern of egg laying in females injected with different male reproductive organs extract

In *Bombyx mori* L. the results shows that even though the virgins lay egg, the egg laying behavior of the virgins is different from that of the mated moths in many respects. The pre-oviposition period is longer by 2 to 4 hours in virgins compared to that in mated moths. There are several instances where such a delay has been reported in virgins (Siddiqui, 1986). This suggests that mating stimulates oviposition in *Bombyx mori* as in many other insects.

The number of eggs laid by the mated silkmoths was more compared to that laid by the virgins. The virgins laid only about 76% of eggs laid by the mated moths. This is similar to the observations made in *Diacrisia oblique* where virgins laid less number of eggs compared to mate females (Siddiqui, 1986). In *Drosophila melanogaster*, it has been reported that mating resulted in a 2 to 3 times increase in oviposition (Chen and Buhler, 1970).

In *Bombyx mori* according to Yamaoka and Hirao (1971) [64] and Yamaoka *et al.* (1971) [63], viable sperms in the female genital tracts induce the nervous activity which inturn accelerate the oviposition activity of female. The sperms are capable of producing necessary stimulus for early active oviposition in mated female irrespective of age of male partner. Thomas Punitham *et al.* (1987) [58] observed a decrease in hatchability of eggs in subsequent mattings. The decrease in hatchability may be due to decrease in the sperm transfer. According to Lefevre and Jonsson (1962a) [35] and Davis (1965) [16] the number of sperms transferred progressively decreases when the males mate in succession with a number of females not as a result of the lack of sperms but a lack of the accessory gland secretion which is essential for the activation of sperms (Davis, 1965) [16].

Though there was a difference in the number of eggs laid by mated and virgin silkmoths, there was not much difference in the number of eggs laid by female silkmoths mated with freshly emerged males and females mated with previously mated males or the aged males. Henneberry and Clayton (1984) [27] also observed that subsequent mattings have no effect either on the number of eggs laid or on the number of eggs hatched in insect *Heliothis viruses.*

Several scientists have tried to localize the oviposition stimulating substance or the male factor which induces monogamy in several insects. These investigations have showed that the substance originates either in the testes, or the accessory reproductive glands like the opalescent glands or Patagonia or the accessory glands as it is called in some insects. According to the investigations in most of the insects the substance originates from the accessory glands. (Lange and Loghton, 1985; Sridevi *et al.*, 1987 [56[; Huignard, 1997) [29].

However, in some of the insects the substance is of testicular origin (Murtaugh and Denilinger 1985 [39]; Sugawara, 1966) [57].

Efforts made during the present investigations by injecting different parts of the male reproductive tract into the virgins and observing their egg laying behavior reveal that the oviposition stimulating substance originates from the

accessory glands and not testes. The moths injected with tissue extract other than accessory glands behaved like control moths in every aspect.

It is clear from the present work that the accessory gland material stimulates oviposition behavior because the injected virgins lay 88% of the total eggs with in two days while the untreated virgins took 3 days to lay as many eggs. Within the first 24 hours the accessory gland extract injected virgins laid 81% of the total eggs, while the untreated virgins laid only 5% over the same period. During the second day the untreated virgins laid about 8% of the total eggs.

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