Prostaglandins Do not Release Egg-Laying Behaviour in the Silkmoth, Bombyx mori

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Prostaglandins Do not Release Egg-Laying Behaviour in the Silkmoth, *Bombyx mori*

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ABSTRACT—A re-evaluation of the role of prostaglandins (PGs) in releasing egg-laying behaviour in the silkmoth, *Bombyx*, was performed. The results revealed that PGs do not play a crucial role as oviposition behaviour stimulants. Injection of prostaglandin E_2 (PGE_2) had no effect on egg-laying behaviour of virgin females. The oviposition behaviour of females which were injected with PG biosynthesis inhibitors such as indomethacin, quinacrine and NDGA was not interrupted or suppressed. Sterilized males of *Bombyx* can be induced by heat treatment (32°C) for 72 hr during the wandering stage. The rate of oviposition of female moths mated with sterilized males was suppressed at a great deal, compared to the control group, in the ensuing mating period of 24 hr. PGE_2 injection into the females mated with the sterilized males had no effect on the restoration of oviposition behaviour. There were no significant difference in the PGE_2 content in the testes of normal and sterilized males. After mating, the amount of PGE_2 increased with age in the bursa copulatrix of females which had mated with either normal or sterilized males. These results indicate that PGs do not seem to be directly involved in the egg-laying behaviour of *Bombyx*.

INTRODUCTION

Polyunsaturated fatty acids, and in particular arachidonic acid, are the precursors of a group of extremely potent oxygenated compounds known as eicosanoids (Coreys *et al.*, 1981). These compounds have profound metabolic effects on biological processes in vertebrates, including blood flow, aggregation of blood platelets, tissue reaction to stimulants, host defence mechanisms, stimulation of smooth muscle contraction, and antibody production in vertebrates (Morris, 1991).

Eicosanoids are also biologically important in invertebrate animals (Stanley-Samuelson, 1987, 1993, 1994a, b). Eicosanoids have been detected in many insect species (Brady, 1983; Stanley-Samuelson and Loher, 1986). In regard to insect reproduction in particular, the only role of eicosanoids that has been articulated so far is the release of egg-laying behaviour in two crickets (*Acheta domesticus*: Destephano *et al.*, 1974; *Teleogryllus commodus*: Loher, 1979; Loher *et al.*, 1981; Stanley-Samuelson and Loher, 1986) and the silkmoth, *Bombyx mori* (Yamaja-Setty and Ramaiah, 1980).

Yamaja-Setty and Ramaiah (1980) demonstrated that prostaglandins (PGs) may release egg-laying behaviour in females of the silkmoth, *Bombyx mori*, by injections of PGs and PG biosynthesis inhibitors. They suggested that prostaglandin E_2 (PGE_2) is more effective than other PGs in stimulating oviposition, and they speculated that PGs are

transferred from males to females, resulting in increased oviposition behaviour (Yamaja-Setty and Ramaiah, 1980). Since their work, there has been no information about the involvement of PGs in egg-laying behaviour in the silkmoth, *Bombyx mori*, and there has also been no documentation to support the hypothesis proposed by Yamaja-Setty and Ramaiah (1980).

The mechanism that controls the shift from virgin to mated behaviour in the *Bombyx* female is not known, although various post-copulatory events are affected by substances derived from male reproductive organs (Yamaoka and Hirao, 1977; Yamaja-Setty and Ramaiah, 1980; Osanai *et al.*, 1987, 1990; Fugo and Arisawa, 1992). In female *Bombyx* moths that mated with normal males, about 90% of the eggs that had developed during the pupal stage are deposited during a night following the mating. However, in the case of female moths that had mated with sterilized male moths, these female moths display very little oviposition behaviour, similar to that of the virgin females (Fugo and Arisawa, 1992).

In order to examine the possibility that one of the oviposition stimulating substances is PGE_2, we quantified the PGE_2 contents in testes and found that the level of PGE_2 was low in the testes of sterilized male moths. This led us to determine whether the failure to release oviposition behaviour in females copulated with sterilized males is due to a deficiency of PGs in the testes of these male insects. In the present paper, we demonstrate that PGs do not release egg-laying behaviour in the silkmoth, *Bombyx mori*.

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MATERIALS AND METHODS

Insects

The Daizo race of the silkmoth, *Bombyx mori*, was used throughout these experiments, because this race has a high percentage of incidence of male sterility by heat treatment during the wandering stage as compared with the other races of *Bombyx* (Sugai and Takahashi, 1981; Fugo, unpublished data). Larvae were reared with mulberry leaves twice a day in a rearing room at $25 \pm 2°C$ under a 16 hr light: 8 hr dark (16L-8D) photoperiod. Larvae were staged on the day of 4th ecdysis, and the day was designed as Day 0 of the 5th instar.

Heat treatment and copulation

Male larvae at the onset of wandering (Day 7 in the 5th instar) were maintained in an incubator at 32°C with high humidity for 72 hr. After treatment, the animals were transferred back to the rearing conditions. Females were reared and kept at $25 \pm 2°C$ throughout the experimental procedure. The photoperiod during the pupal-adult development was 16L-8D. Adult eclosion occurs within 1.5 hr after lights-on at 9 to 10 days after pupation (Fugo *et al.*, 1984). Moths eclosed within 2 hr after lights-on were used. Animals were allowed to copulate for 2 hr. Egg-laying conditions were counted on a daily basis.

Injection of PG biosynthesis inhibitors

We used three different inhibitors which affect the biosynthesis of PGs in vertebrates: quinacrine, phospholipase A2 inhibitor; nordihydroguaiaretic acid (NDGA), lipoxygenase inhibitor; and indomethacin, cycloxygenase inhibitor (see Stanley-Samuelson, 1994a, b). Quinacrine was dissolved in distilled water. NDGA and indomethacin were dissolved in dimethylsulfoxide (DMSO) and diluted with distilled water to give the final concentration of 5% DMSO. The inhibitors and PGE_2 were purchased from Sigma Chemicals.

Extraction and determination of PGE_2

Reproductive organs (testes and bursa copulatrix) were dissected out in a cold saline solution (0.8% NaCl) and were immediately frozen at -80°C. Extraction procedure for PGE_2 was according to Kubo and Komatsu (1986). Testes or bursa copulatrix were homoginized with 80% aqueous ethanol. After centrifugation ($7,000 \times g$, 15 min 4°C), the residue was reextracted with 80% ethanol. The combined supernatants were evaporated *in vaccuo*. The residue was partitioned between 5 ml of petroleum ether (b.p. 35-60°C) and 5 ml of 0.2% potassium phosphate buffer (pH 7.0). The aqueous layer was applied to a Sep-Pak C_{18} cartridge (Waters, Milford, USA). After washing the cartridge with 5 ml of water and 2 ml of 20% aqueous methanol, the PGs were eluted with 2 ml of 80% aqueous methanol. The PG fraction was evaporated into dryness and stored at -80°C until use. The amount of PGE_2 was determined by an enzyme-immunoassay (EIA). The PGE_2 EIA kit was purchased from Cayman Chemical (USA). The buffer preparation and determination procedures were followed by protocol provided with the kit. The amounts of PGE_2 in the testes and bursa copulatrix were determined by absorbance at 415 nm using an EIA Plate Reader (Corona Elect. Japan, MTP22).

RESULTS

Effect of PGE_2 injection on egg-laying behaviour

We first tried to confirm the possibility of the involvement of PGs in egg-laying behaviour. In normal females that were mated with normal males, about 90-95% of the eggs that developed in their ovarioles were deposited within a 24 hr after mating (Table 1). On the other hand, virgin females or females that were mated with sterilized males deposited only

Table 1. Effect of the injection of prostaglandin E_2 on the egg laying behaviour of the silkmoth, *Bombyx mori*

Experiment[1] Female x Male	N	No. of eggs laid/24 hr (Mean ± SD)
Normal x Normal	10	381.3 ± 10.8
Normal x DMSO	7	344.4 ± 59.4
DMSO x Normal	8	329.5 ± 37.1
Normal x PGE_2	7	358.4 ± 45.1
PGE_2 x Normal	8	315.1 ± 45.4
Normal x Heat treated[2]	10	26.5 ± 9.7[3]
Normal x PGE_2 Heat treated[2]	10	24.6 ± 10.8[3]
DMSO : Virgin	10	26.9 ± 9.4[3]
PGE_2 : Virgin	9	31.3 ± 17.7[3]

[1] Prostaglandin E_2 (10 μg/moth/20 μl of 5% DMSO) or DMSO(20 μl 5% DMSO/moth) was injected into male or female moths 2-3 hr after eclosion. Subsequently, these moths copulated for 2 hr. After mating, female moths were kept in a rearing room ($25 \pm 2°C$, 16L-8D).

[2] A heat treatment (32°C, for 72 hr) was done at the wandering stage in males, and these animals were transferred back to a rearing room ($25 \pm 2°C$, 16L-8D) until their adult eclosion.

[3] All of the eggs were unfertilized.

about 27-31 eggs (Table 1). DMSO (20 μl of 5% DMSO/moth) had no effect on the oviposition and the number of eggs laid when it was injected into either male or female moths.

PGE_2 injection also had no influence upon the oviposition. Virgin females received DMSO or 10 μg of PGE_2 oviposited about 1/10 of the eggs in the control group and PGE_2 injection did not stimulate egg-laying behaviour. When the females mated with the sterilized males which were obtained by high temperature treatment during the wandering stage, egg-laying activity was very low, almost similar to that of virgin females. In this case, acceleration of egg-laying was not also observed, even if PGE_2 was injected into the sterilized males (Table 1). In these experiments, we could not confirm any possible involvement of PGE_2 on egg-laying behaviour in *Bombyx*.

Effect of PG biosynthesis inhibitors on egg-laying behaviour

PG biosynthesis inhibitors such as indomethacin and aspirin are potent enough to interfere with or inhibit egg-laying behaviour in *Bombyx* (Yamaja-Setty and Ramaiah, 1980). As mentioned above, we could not find any stimulatory activity of PGE_2 on the oviposition in *Bombyx*. Therefore, we next investigated the effects of the PG biosynthesis inhibitors on male and female animals to determine whether the inhibitors are potent to suppress egg-laying behaviour.

Various doses of indomethacin or quinacrine were injected into newly eclosed male moths. About 3 hr after injection these males were allowed to copulate with normal females for 2 hr. After mating, female moths were kept in a rearing room so as to deposit eggs. As shown in Fig. 1, PG biosynthesis inhibitors did not interfere with oviposition at any dosage examined.

Next, the same injection experiment was carried out in female moths. The numbers of eggs laid by drug-injected female moths were counted. The results are shown in Fig. 2.
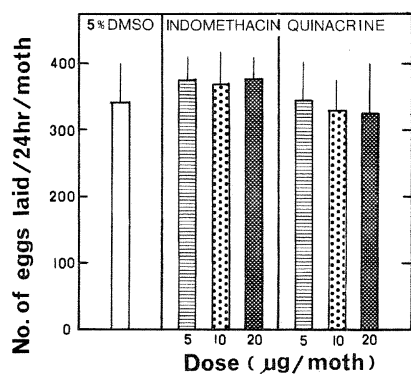
Fig. 1.   Effect of the injection of prostaglandin biosynthesis inhibitors on the egg-laying behaviour of the silkmoth, *Bombyx mori*. Male moths received various doses of inhibitor 2 to 3 hr after eclosion. Afterwards, they mated with normal female moths for 2 hr. Each datum point represents mean ± SD (n=7 to 10).
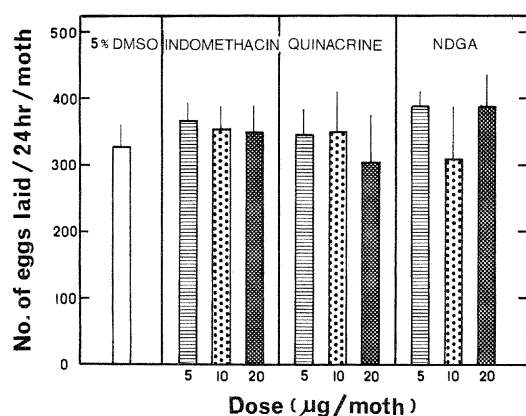

Fig. 2.   Effect of the injection of prostaglandin biosynthesis inhibitors on egg-laying behaviour of the silkmoth, *Bombyx mori*. Female moths received various doses of the test compounds 2 to 3 hr after eclosion. Afterwards, they mated with normal male moths for 2 hr. Each datum point represents mean ± SD (n=8 to 10).

Control animals that received 20 μl of 5% DMSO deposited about 330 eggs. These numbers of eggs are normal for the Daizo race and therefore DMSO did not affect oviposition. Any tested inhibitors did not suppress the egg-laying behaviour in *Bombyx* (Fig. 2).

In the former experiments, we used newly eclosed moths. The drug-injected insects were mated with male or female moths several hours after treatment. It was possible that the injected drugs did not act pharmacologically upon the several enzymatic steps of the PG biosynthetic cascade. To eliminate the possibility that the animals needed to be exposed for longer periods to the inhibitors, we used pharate adults. Two days before eclosion, female pharate adults received 5 to 20 μg of inhibitors. After eclosion, the treated moths were allowed to copulate with normal males for 2 hr. Indomethacin and quinacrine neither interfered with eclosion and oviposition behaviours (Table 2), nor suppressed the mating behaviour of males and females' reproductive behaviour when the same

injections were performed in male pharate adults (data not shown).

PGE₂ level in the testes of normal and heat-treated insects

PGE₂ levels in the testes of normal and heat-treated animals were measured daily from the beginning of final instar to 1 day before eclosion (Fig. 3). In the control animals which were reared at 25°C throughout their larval development, the amount of PGE₂ immediately after 4th ecdysis was 1.2 ± 0.4 ng/pair testes. PGE₂ levels increased from the day of the 4 th ecdysis and attained 2.6 ± 0.4 ng of PGE₂ on Day 2 of the last instar. The amount of PGE₂ then decreased gradually along

Table 2.   Effect of the application of prostaglandin biosynthesis inhibitors on the egg-laying behaviour of *Bombyx*

Inhibitor[1]	N	No. of eggs[2] laid/24 hr (Mean ± SD)
Control (5% DMSO)	8	284.4 ± 33.4
Indomethacin		
5 μg	6	282.3 ± 34.2
10 μg	6	295.3 ± 44.3
20 μg	9	305.3 ± 34.0
Quinacrine		
5 μg	6	263.8 ± 90.6
10 μg	8	312.9 ± 75.4
20 μg	6	250.5 ± 87.4

[1] Two days before eclosion, various doses of prostaglandin biosynthesis inhibitors were injected into female pharate adults.
[2] After eclosion, female moths copulated with normal male moths for 2 hr. After mating, female moths were allowed to oviposit their eggs onto an egg card in a rearing room (25 ± 2°C, 16L-8D).
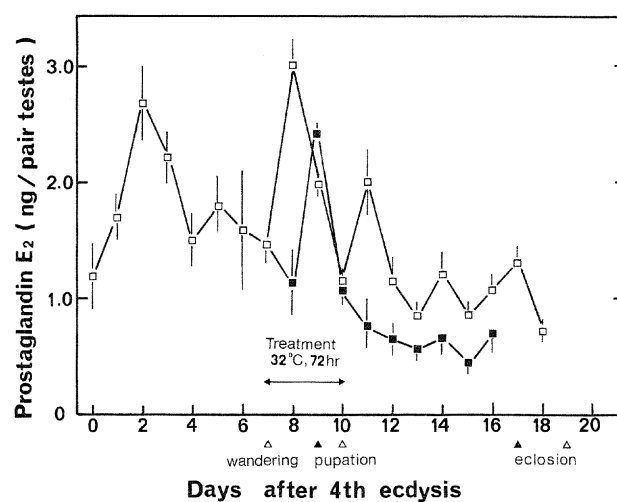

Fig. 3.   Daily changes of prostaglandin E₂ content in developing testes of the silkmoth, *Bombyx mori*. Symbols indicate normal insects (–□–) and heat-treated insects (32°C for 72 hr during the wandering stage, –■–). Each datum point represents mean ± SD (n=6).

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with larval growth. Second peak of PGE$_2$ levels was observed on Day 8 (day-1 spinning stage). After pupation, the levels of PGE$_2$ decreased with fluctuations as the pupal-adult development proceeded.

The level of PGE$_2$ in testes of the heat-treated animals was little bit lower than that of the control group, though the fluctuation of PGE$_2$ levels recorded in control animals was not so clearly observed (Fig. 3). One day before eclosion,the amount of PGE$_2$ in both testes of control and heat-treated animals was the same (Fig. 3).

PGE$_2$ level in bursa copulatrix

Female moths copulated with normal males or sterilized males for 2 hr. After mating, the bursa copulatrix was dissected to determine the amount of PGE$_2$. Female moths initiated to oviposit eggs approximately 6 to 7 hr after mating when females mated with normal males. In bursa copulatrix just after eclosion the PGE$_2$ level was negligible (less than 0.1 ng: unpublished data). Immediately after mating, the amount of PGE$_2$ elevated to about 1.6 ng in the bursa copulatrix of females which had mated with either normal or sterilized males. In control animals, PGE$_2$ levels gradually increased by 3 hr after mating and decreased dramatically at 5 hr after mating (Fig. 4). Afterwards, PGE$_2$ levels increased again with age. PGE$_2$ levels in the bursa copulatrix of females which had mated with the sterilized males fluctuated with time period after mating but the fluctuation was roughly similar to that of the control group (Fig. 4). It can be concluded that PGE$_2$ seems unlikely to be potent enough to elicit egg-laying behaviour in the silkmoth, *Bombyx mori*, despite the fact that it is present in testes and bursa copulatrix (Figs. 3 and 4).

DISCUSSION

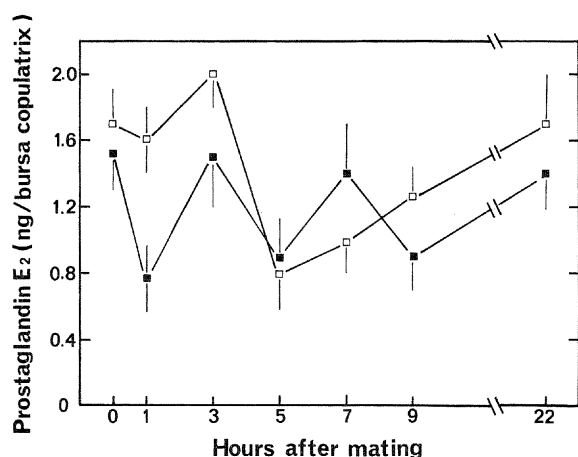The physiological roles of PGE$_2$ in the reproductive behaviour of insects have been demonstrated in only two species: crickets and silkmoth. The egg-laying behaviour is released by an injection of PGE$_2$ in *Acheta domestica*, *Telegryllus commodus* and *Bombyx mori* (Destephano *et al.*, 1974; Stanley-Samuelson, 1987, 1993, 1994 a, b; Yamaja-Setty and Ramaiah, 1980).

Yamaja-Setty and Ramaiah (1980) demonstrated that the injection of PGs caused the egg-laying behaviour in the virgin females of *Bombyx*. They showed that the number of eggs laid by virgin females was 30 while that of virgins treated with 1, 10 or 100 µg of PGs laid 32, 42 and 63 eggs per individual, respectively. Compared to virgin control group, these are stastically significant increases in oviposition. There are, however, several questions to be raised their conclusion. They reported that normal mated female moths laid about 400 to 500 eggs, but an injection of 100 µg of PGs resulted in about 12% of the number of eggs expected from a mated female moth. The aspect of pharmacological side effects by 100 µg of PGE$_2$ was not addressed at all. In addition, there was no specificity of PGs upon egg-laying behaviour: PGE$_1$ and PGF$_{2\alpha}$ equally stimulated the egg-laying behaviour in *Bombyx*, whereas there is specificity of PGs in stimulating the egg-laying behaviour of the cricket, *Acheta domestica* (Stanley-Sammuelson, 1994a).

In spite of the argument by Yamaja-Setty and Ramaiah (1980), the role of prostaglandins in releasing egg-laying behaviour in *Bombyx* has remained to be obsure. We therefore re-evaluated the role of PGs on the release of egg-laying behaviour and concluded that PGE$_2$ possesses no stimulatory effect on egg-laying behaviour in *Bombyx*.

In our experiments, females mated with normal males laid about 400 eggs during the following 24 hr but virgin females oviposited only about 1/20 as many as eggs as the control group did. Virgin females treated with 10 µg of PGE$_2$ laid 31 ± 18 eggs during the first 24 hr period. In addition, females that copulated with sterilized males and that were with sterilized males treated with PGE$_2$ oviposited 27 ± 10 eggs and 25 ± 11 eggs, respectively (Table 1). There is no statistical difference in the number of eggs laid by the females between any two of these three. In addition, we found that PGE$_2$ injections could not enhance egg-laying behaviour in this insect. Rather a high dose of PGE$_2$ (>50 µg/moth) had the reduction of the oviposition (unpublished data).

It is believed that inhibitors such as indomethacin, quinacrine, aspirine and NDGA all of which act on the enzymes involved in the biosynthetic pathway of PGs in mammals, have the ability to suppress the biosynthesis of PGs in insects as well (Stanley-Samuelson, 1987, 1993, 1994a, b). Yamaja-Setty and Ramaiah (1980) argued that PGs stimulate the egg-laying behaviour of *Bombyx*, since treatment of males and females with inhibitors resulted in a substantial reduction in oviposition by 88% with indomethacin and by 66% with aspirine. As shown in Figs. 1 and 2 and in Table 2, we could not observe any reduced oviposition after injections of inhibitors into male or female moths and into pharate adults at any dose examined. No interference with oviposition was observed by the injection of these drugs. In addition, we have examined the effect of 4-

Fig. 4. Fluctuation of prostaglandin E$_2$ levels in a bursa copulatrix of the silkmoth, *Bombyx mori*. Three to four hours after eclosion, female moths mated with normal males (–□–) or sterilized males (–■–) for 2 hr. After mating, females were allowed to oviposit in a rearing room. Each datum point represents mean ± SD (n=6).

bromo-phenacyl bromide (4BPB), (one of the inhibitors of phospholipase A2 and diacylglycerol lipase), on the oviposition in this insect, and observed that this drug exhibited no effect on egg-laying behaviour (Fugo, unpublished data). The results presented here conclusively show that PGs have no effect on the release of egg-laying behaviour in the silkmoth, *Bombyx mori*. This conclusion is good agreement with those obtained in *Manduca sexta* (Sasaki and Riddiford, 1984) and in the cabbage looper, *Trichoplusia ni* (Hagen and Brady, 1982).

Prostaglandins and/or PG biosynthetic activity is sexually transferred from male to females in a number of insects (*Teleogryllus commodus*: Ai et al., 1986; *Trichoplusia ni*: Hagen and Brady, 1982; *Locusta migratoria*: Lange, 1984; *Musca domestica*: Wakayama et al., 1986). Yamaja-Setty and Ramaiah (1980) have demonstrated that PGs are transferred from males to females, resulting in increased oviposition behaviour in *Bombyx*. Results in Figs. 3 and 4 clearly showed that the PGE$_2$ level in bursa copulatrix was elevated by about two-fold in mated females, an indication that PGs or PG biosynthetic activity are transferred from males to females during mating. However, increased oviposition did not follow the transfer of PGs or PG biosynthetic activity. We have therefore concluded that PGE$_2$ is not a candidate for oviposition stimulation in opposition to the argument by Yamaja-Setty and Ramaiah (1980), although the amount of PGE$_2$ increases after mating in the bursa copulatrix of *Bombyx*.

Various post-copulatory events are affected by substances derived from the male reproductive organs in *Bombyx* (Yamaoka and Hirao, 1977; Osanai et al., 1987, 1990; Fugo and Arisawa, 1992). One of the substances potent to stimulate egg-laying behaviour has been proposed by Yamaoka and Hirao (1977). This substance, called oviposition stimulating substance OSS, is considered to be a small peptide. OSS is present in the accessory glands of normal males and is also in sterilized male moths (Fugo and Arisawa, 1992). It can stimulate egg-laying behaviour in virgin females in a dose dependent manner but its activity was very weak and not enough to satisfy our expectations.

Male sterility is easily caused by heat treatment (32°C) for 72 hr during the wandering stage in *Bombyx* (Sugai and Kiguchi, 1968; Sugai and Takahashi, 1981; Katsuno, 1977a, b; Fugo and Arisawa, 1992; Fugo et al., 1995). Cytological observations have shown that there is a significant difference in the number and shape of spermatozoa in the testes of normal and sterilized moths. Many abnormal apyrene spermatozoa have been counted in the testes of sterilized moths (Katsuno, 1977a, b; Sugai and Takahashi, 1981). Recently, the role of apyrene spermatozoa has been postulated as an assistant function in promoting the dissociation of eupyrene sperm bundles in the spermatophore (Osanai et al., 1987, 1990). In the spermatophores of females that were mated with sterilized males, eupyrene sperm bundles remained intact, but separate eupyrene spermatozoa were not observed (Sugai and Takahashi, 1981). Oviposition activity in females which copulate with triploid male moths is very low and the oviposited eggs are unfertilized (Takizawa et al., 1976).

This was also the fact for the female moths mated with heat-treated male moths. In addition when triploid males mate with females, a larger number of apyrene spermatozoa are observed in the bursa copulatrix and in the spermatheca, while only a few eupyrene spermatozoa are observed in those organs (Takizawa et al., 1976). This appears that the simultaneous presence of normal eupyrene and apyrene spermatozoa is necessary in the bursa copulatrix for exhibiting egg-laying behaviour.

The mechanism that controls the shift from the virgin behaviour to the mated behaviour in female *Bombyx* is not known. Females that mated with normal males oviposited 400 to 500 fertilized eggs in 2 to 3 days follwing the copulation, but the number of eggs deposited by females that copulated with sterilized males or triploid males reduced to about 1/3 to 1/2 of the control group, indicating that normal spermatozoa are necessary to cause the shift from virgin to mated behaviour. Furthermore, testectomized males and sterilized males remain able to produce spermatophores and to mate with females (Fugo and Arisawa, 1992). However, these matings do not release egg-laying behaviour in *Bombyx* (Fugo and Arisawa, 1992). Therefore, the fluids that are derived from male accessory glands may play no essential role in eliciting oviposition behaviour in this insect. Therefore the presence of normal eupyrene and apyrene spermatozoa may be a stimular for the occurrence of unknown biochemical events in the spermatophore or somewhere in the reproductive organs of females. In addition, the migration of eupyrene spermatozoa from bursa copulatrix to spermatheca, as proposed by Omura (1938, 1939) and Thibout (1979), might also be a contributing factor.

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