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Testicular Ecdysteroid Level in the Silkmoth, *Bombyx mori*, with Special Reference to Heat Treatment during the Wandering Stage

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ABSTRACT—To elucidate the induction mechanism of abnormal apyrene sperm by high temperature (32°C, 72 hr) treatment during the wandering stage of the silkmoth, *Bombyx mori*, ecdysteroid titres in the haemolymph and in the testes were determined. The fluctuation of ecdysteroids in the haemolymph was not disturbed by the high temperature treatment. The amount of ecdysteroids in the testes of control animals was low during the two-thirds of the last larval stage and an abrupt increase was observed on day 8. On the other hand,the elevation of ecdysteroid titre was suppressed in the testes of the treated animals during the period of treatment. After pupation, the fluctuation pattern of the ecdysteroid titre was almost the same both in control and treated animals. These results indicate that the deficiency of ecdysteroids in the testes could be the cause of the abnormal differentiation of the apyrene spermatozoa brought about by the high temperature treatment during the wandering stage of the silkmoth, *Bombyx mori*.

INTRODUCTION

Two types of sperm are produced in the testis of the silkmoth, *Bombyx mori*: one is the nonnucleate, apyrene sperm and the other is nucleated eupyrene sperm. By the effort of a number of authors who described in detail the cytological aspects of spermatogenesis in the silkmoth, *Bombyx mori*, it is well known that the differentiation of the eupyrene spermatozoa occurs mainly at the early stage of the 5th instar and the formation of apyrene spermatozoa initiates from the wandering stage and lasts until the early pupal stage [11, 14, 20–22, 28, 33]. The apyrene sperm bundles break down as they leave the testis but the eupyrene bundles remain intact up to the spermatophore stage in the bursa copulatrix [11, 25, 26]. It has been reported that apyrene spermatozoa stir the highly viscous content of the spermatophore to promote dissociation of eupyrene bundles by accelerating many reactions to result in the formation of separate eupyrene spermatozoa [25, 26].

It has been generally accepted that spermatogenesis is stimulated by ecdysteroids [3, 8–10, 22, 27, 29, 39, 40]. Testes of the tobacco budworm, *Heliothis virescens* and the gypsy moth, *Lymantria dispar*, secrete ecdystroids *in vitro* during the pharate pupal stage and in the middle to late adult development period during the pupal stage [16–19]. Also, testes of *Mamestra brassicae* and *Spodoptera littoralis* have been found to spontaneously secrete ecdysteroids *in vitro* [7, 30, 31].

When male 5th instar larvae of the silkmoth, *Bombyx mori*,are exposed to high temperature (32°C) for 72 hr at the wandering stage, the adults become completely sterilized [5, 12, 13, 33–37]. Histological and cytological studies strongly suggest the abnormal differentiation of the apyrene spermatozoa brought about by heat treatment during the wandering stage results in the induction of male sterility [12, 13].

In the present study, we attempted to determine whether the abnormal apyrene spermatozoa caused by heat treatment in the wandering stage are due to a deficiency of ecdysteroids in that period. Here we report that a low level of ecdysteroid in the testes during heat treatment may result in the abnormal differentiation of the apyrene spermatozoa in the silkmoth, *Bombyx mori*.

MATERIALS AND METHODS

Insects

Larvae of the silkmoth, *Bombyx mori* (DAIZO race), were reared with mulberry leaves in a rearing room of our laboratory at 25 ±2°C under a 16-hr light 8-hr dark photoperiod. Larvae were staged on the day of 4th ecdysis, and this day was designed as Day 0 of the 5th instar.

Heat treatment and copulation

At the onset of wandering, male larvae were maintained in an incubator, in which the temperature was kept at 32°C with high humidity, for the designed periods. After the treatment,the animals were transfered back to the rearing room. Female animals were reared and kept at 25±2°C throughout the experiment.

After eclosion, female moths were mated with normal or heat-treated male moths for 2 hr. The eggs laid by these females were counted 4 days after oviposition. The number of non-pigmented eggs was counted and the ratio of these eggs to the total number of eggs laid was calculated. In this experiment, non-pigmented eggs were considered to be unfertilized, because the pigmentation should

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occur 2 to 3 days after oviposition if fertilization had been completed [5].

Estimation of ecdysteroids in haemolymph and in testes

Ecdysteroids in 10 μl haemolymph were extracted with 300 μl of absolute methanol, and aliquots of the supernatant were assayed for ecdysteroids by radioimmunoassay (RIA) [38]. Since 20-hydroxyecdysone was used as a standard, the amount of ecdysteroids is expressed as μg of 20-hydroxyecdysone equivalent $\pm$ standard deviation. The tritiated ligand [23,24-^{3}H (N)] ecdysone (60.3 Ci/mmol) was purchased from NewEngland Nuclear.

Testes ecdysteroids were extracted with ethanol and partially purified [15]. Testes were homogenated with 80% aqueous ethanol. After centrifugation (10,000 rpm, 15 min, 4°C), the supernatant was evaporated *in vacuo*. The residue was partitioned between 5 ml of petroleum ether (b.p. 35–60°C) and 5 ml of water. The aqueous layer was subjected to a Sep-Pak C-18 cartridge column (Waters, Milford) [15]. Aqueous methanol (60%) eluted fraction from the Sep-Pak cartridge was assayed by RIA. In this step the recovery of the initially applied standard 20-hydroxyecdysone was estimated as 65–70%.

RESULTS AND DISCUSSION

In female moths mated with normal males, about 90% of the eggs were deposited during the following night, and the oviposition was completed 2 days later. The percentage of fertilized eggs was about 99% in the control group (Table 1). When the male larvae were subjected to high temperature of 32°C for 3 days in the wandering stage, the resulting moths were completely sterilized. Thus, in the case of the female moths that had mated with the treated male moths, the oviposited eggs were unfertilized (Table 1). Oviposition pattern of the females that had mated with the sterilized moths was similar to that of the virgin females [5]. As shown in Table 1, the number of eggs oviposited by the female moths which had mated with treated males decreased when the duration of the treatment was increased. When the male animals were treated for 72 hr at 32°C in the wandering stage, none of the eggs was fertilized and the number of eggs deposited was about 1/4 of the control's (Table 1).

The mechanism controlling the switch from virgin to mated behaviour in female animals is not known. The migration of sperm from the bursa copulatrix to the spermatheca was found to induce the activation of oviposition [23, 24]. In a previous study [5], we demonstrated that females copulated with sterilized males displayed an inactive oviposition behaviour. Histological and cytological studies indicate that the abnormal differentiation of the apyrene spermatozoa brought about by the heat treatment during the wandering stage results in the induction of male sterility [12, 13]. After mating drastic biochemical events occur in the spermatophore [25, 26]. Thus, the apyrene sperm in the spermatophore seem to act as "stirring bars" to promote the dissociation of the eupyrene bundles [11, 25, 26]. The liberated and activated eupyrene sperm then use a spermatophore-provided energy source to migrate to the spermatheca of the female [25, 26]. Therefore, it can be assumed that the suppression of oviposition may be due to the incomplete maturation of the sperm in the spermatophore of the females copulated with the heat-treated males. In connection with the present study, we determined the amount of prostaglandins as a candidate of the oviposition stimulating substance in the testes of the treated animals but prostaglandins do not seem to be involved in the stimulation of oviposition in the silkmoth, *Bombyx mori* (unpublished data).

The growth of the testes was recorded during the larval-pupal-adult differentiation on a daily basis (Fig. 1). The dissected testes were rinsed with distilled water, blotted on a filter paper to remove excess water and immediately weighed. The wet weight of a pair of testes of the 0-day-old 5th instar larvae was 3.0 ± 1.0 mg. Their weight gradually increased with age and reached a peak on day 0 of pupal stage in the control group. Thereafter the weight of testes remained almost constant. The weight of the testes from moths subjected to high temperature during the wandering stage was similar to that of the controls. There was no appreciable difference in the testes weight between the treated animals and the control.

It has been reported that the prothoracic gland hormone (ecdysone) accelerates the whole process of spermatogenesis of this insect [39] indicating that the high temperature during the spinning stage results in disturbance of the ecdysteroid metabolism in the prothoracic glands and/or set an obstacle to the ecdysteroid secretion into the haemolymph. We therefore focused our research on the fluctuation of ecdysteroids in the haemolymph of the heat treated animals.

The changes of ecdysteroid level in the haemolymph are shown in Fig. 2. The fluctuation of ecdysteroids in the haemolymph during the larval-pupal-adult development in

TABLE 1. Induction of the male sterility by high temperature during the wandering stage of the silkmoth

Treatment (temp. & time)	Total no. of eggs laid/moth/4 days	Fertilized eggs	% of fertility (mean $\pm$ SD)
25°C 72 hr	483.0 $\pm$ 21.0	476.7 $\pm$ 19.0	98.71 $\pm$ 0.52
32°C 24 hr	462.6 $\pm$ 29.5	456.4 $\pm$ 28.7	98.67 $\pm$ 1.18
32°C 48 hr	331.9 $\pm$ 185.7	282.9 $\pm$ 226.1	78.57 $\pm$ 28.6
32°C 72 hr	120.9 $\pm$ 50.0	0	0

Male animals were treated at 32°C for the indicated number of hours at the wandering stage. After the treatment they were kept at 25 $\pm$ 1°C until their eclosion. At least 16 animals were used in each trial. After eclosion, they mated with normal female moths for 2 hr. The eggs laid per moth were counted 4 days after oviposition.

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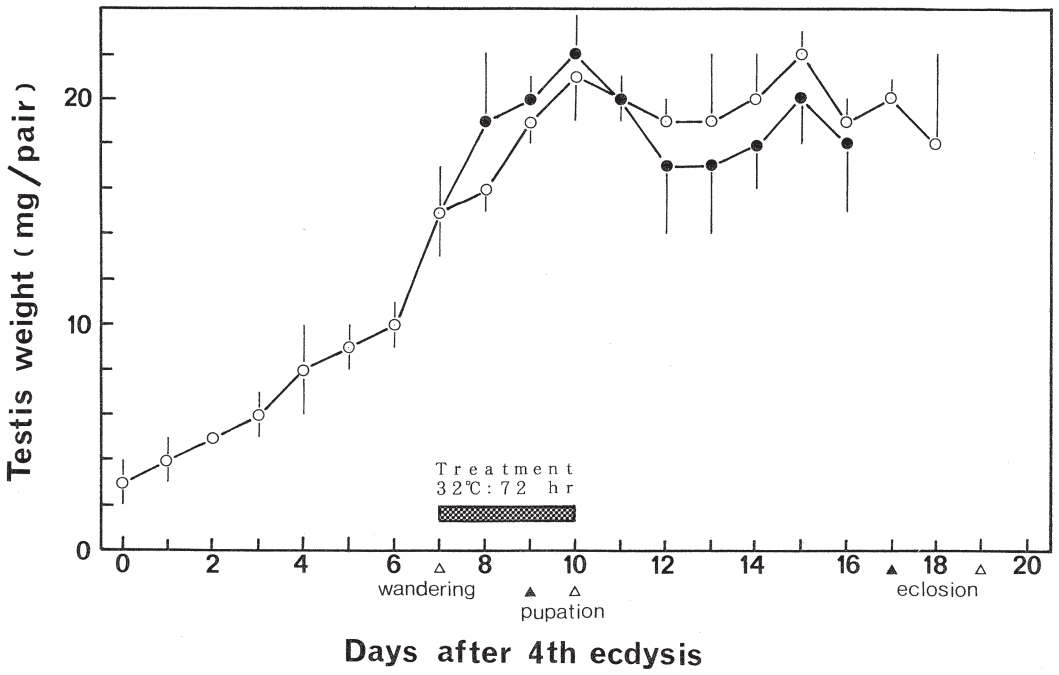

FIG. 1. Testes development in the silkmoth, *Bombyx mori*. The larvae in the wandering stage were maintained at 32°C for 72 hr. Subsequently, they were kept at 25±2°C until eclosion. Open circles indicate the control and solid circles the heat treated animals. White arrow heads indicatethe developmental events in the control and black arrow heads those of the treated animals. Each piece of data is the mean of 10 different determinations with S.D.

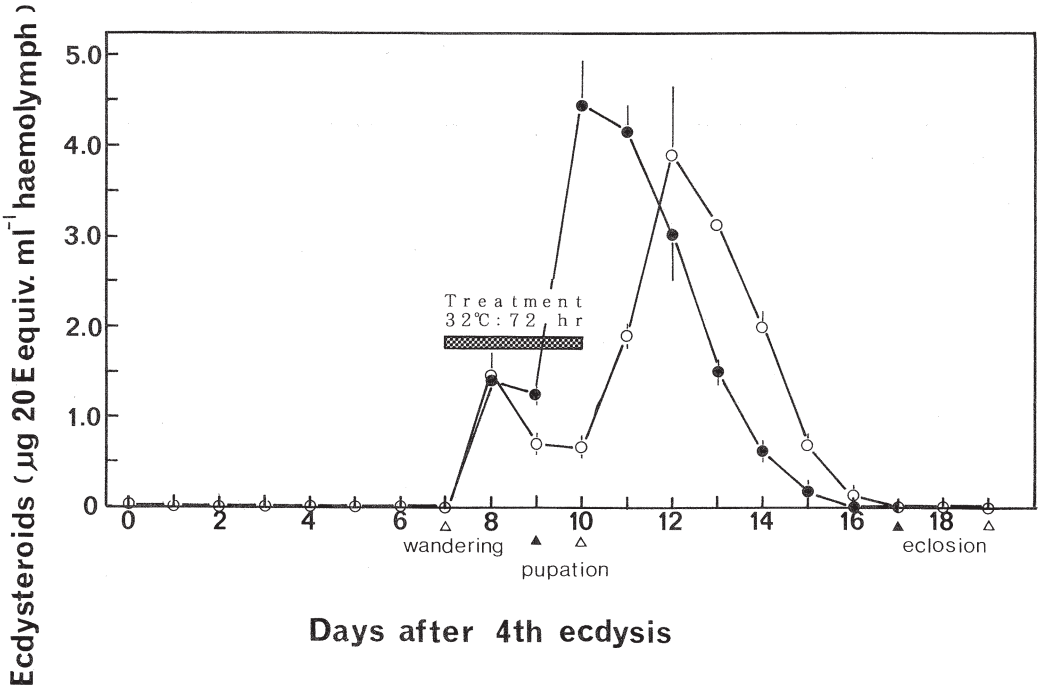

FIG. 1. Testes development in the silkmoth, *Bombyx mori*. The larvae in the wandering stage were maintained at 32°C for 72 hr. Subsequently, they were kept at 25±2°C until eclosion. Open circles indicate the control and solid circles the heat treated animals. White arrow heads indicate the developmental events in the control and black arrow heads those of the treated animals. Each piece of data is the mean of 10 different determinations with S.D.

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the control group was quite similar to that in reports previously documented [1, 2, 6]. In the heat-treated animals, the peak titres of ecdysteroid in the haemolymph was almost the same as the control's but it was found 1–2 days earlier than that of control. Since the duration of the developmental processes was 1 to 2 days less in the treated animals than in the controls, the fluctuation of ecdysteroids in the haemolymph seems to be due to the acceleration of the development brought about by the high temperature. Basically, there were no difference in the morphological characters and behavioural patterns during the pupal-adult development between the treated animals and control ones.

We next determined the amount of ecdysteroids in the testes, since ecdysteroid is involved in the initiation of the testicular maturation [3, 10, 16, 39]. In addition, testes of several insects contain and secrete ecdysteroids during the pharate pupal stage although the role of such secretions is not elucidated in detail [3, 4, 7–10, 16–19, 22, 27, 29–32, 40]. The results are shown in Fig. 3. Until the 7th day of the last larval stadium, the ecdysteroid titre in the testes was below 100 pg/pair. In control animals maintained at 25°C throughout the experiment, the titre of the ecdysteroids in testes abruptly increased to 500 pg at the onset of wandering and then declined to 250 pg at one day before pupation. After pupation, the ecdysteroid titres elevated again on day 2 of the pupal stage and its levels were maintained between 400 and 500 pg for 4 days. Thereafter, ecdysteroids in the testes decreased to an undetectable level up until eclosion.On the other hand, the ecdysteroid titre in the testes during the heat treatment (32°C for 72 hr) was significantly lower than the control's (Fig. 3). During the treatment, the amount of ecdysteroids did not change and remained steady at about 80 pg per pair of testes. On day 11, the day corresponding to day-1 control pupa, the amount of ecdysteroids began to increase and reached a maximum level of about 600 pg at day 3 of the pupal stage. Afterwards, the amount of ecdysteroids in the testes fell rapidlly (Fig. 3). In general, the pattern of the changes in the ecdysteroid titre in the testes in both groups was similar in the pupal stage, but not in the wandering stage.

The induction of male sterility is easily induced by heat treatment during the wandering and pharate pupal stage in the silkmoth, *Bombyx mori*. This phenomenon is stage specific : male sterility cannot be induced by high temperature treatment at any other develomental stages. Testicular ecdysteroid level is reduced significantly by heat treatment, indicating that the deficiency of ecdysteroids in the testes may result in male sterility, although the haemolymph ecdysteroid titre is not basically affected by such a treatment in this insect. However, the target sites of heat stress and the involvement of ecdysteroids in the differentiation of the apyrene spermatogenesis are not yet clearly understood. Furthermore, the relationship between haemolymph ecdysteroids and the endogenous ecdysteroids production in testes that was proposed for several insects [7, 16–19, 29–32] still remains to be resolved.

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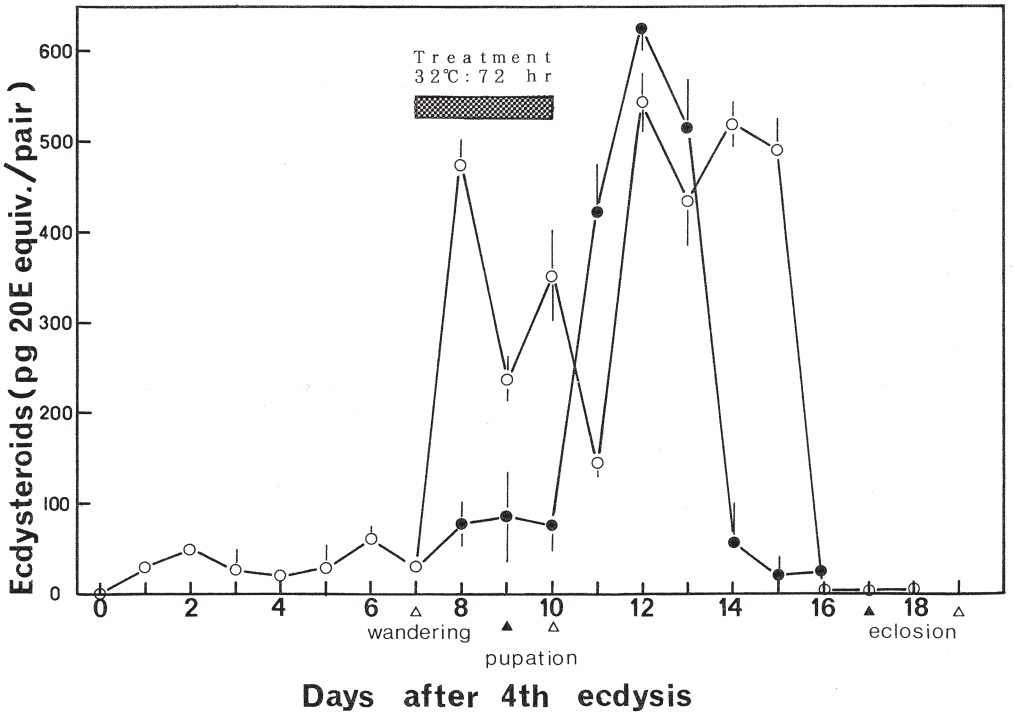

Fig. 3. Change of ecdysteroids in testes of the heat treatedanimals. Ecdysteroid titres are expressed as pg of 20-hydro-xyecdysone (20E) per pair of testes. Bars represent standarddeviation of the means. n=6–8 for each data point. Symbols are the same as in Fig. 1.

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