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Glucose and ecdysteroid increase apyrene sperm production in *in vitro* cultivation of spermatocysts of *Bombyx mori*

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Abstract

Two types of sperm, nucleate eupyrene and anucleate apyrene, occur in the silkworm as in other lepidopteran species. Hormones and other substances have been assumed to play important roles in sperm dimorphism. We established an *in vitro* cultivation system for silkworm spermatocytes, and found that apyrene sperm are not produced when spermatocytes from larval testes are cultivated, though eupyrene spermatocytes develop normally into mature sperm. Based on the fact that ecdysteroid titers increase rapidly and peak 1 day after spinning, and that the amount of glycogen reaches its peak 1 day before the spinning stage, we studied the effects of adding glucose and/or 20-hydroxyecdysone to the culture medium. The experiments disclosed a significant additive effect of both substances on apyrene sperm production.

Key words: silkworm, *in vitro* culture, eupyrene and apyrene sperm,
20-hydroxyecdysone, glucose

1. Introduction

Males of thhe silkworm, *Bombyx mori*, produce two types of sperm, nucleate eupyrene and anucleate apyrene sperm from bipotential spermatogonia (Machida, 1929). The silkworm eupyrene and apyrene sperm differ significantly in many respects such as cytological appearance (acrosome, behavior of meiotic chromosomes and mitochondrial DNA amount), spermatogenesis, including spermiogenesis and peristaltic squeezing, and especially the time of which meiosis begins (Tazima, 1964; Yamashiki and Kawamura, 1997; Kawamura et al., 1998; 2000; 2001). These observations suggest a mechanism that causes a switch from one to the other type of spermatogenesis.

Eupyrene sperm are ordinary sperm that fertilize the eggs, while apyrene sperm are considered to have assisting roles (Osanai et al., 1987; Friedländer, 1997). Without the cooperation of apyrene sperm, eupyrene sperm lose their fertility (Sahara and Kawamura, 2002). Some speculations on the functions of apyrene sperm were proposed (Friedländer, 1997). The two kinds of sperm differentiate in distinctly different phases of development after the fourth molting: eupyrene meiosis starts on the first day of the fifth instar and finishes before the spinning stage, whereas apyrene sperm are produced after the spinning stage (Kawamura and Sahara, 2002). An influence of hormones has been assumed (Friedländer, 1982; 1989) in dimorphic sperm production. However, the factors causing the switchover from eupyrene to apyrene spermatogenesis are as yet unclear. From transplantation experiments, Jans et al. (1984) inferred the existence of an apyrene-spermatogenesis-inducing factor (ASIF), though they did not indicate what the ASIF might be.

In vitro spermatocyst cultures are a powerful tool to define the substances that contribute to spermatogenesis of the two types. *In vitro* culture of lepidopteran spermatocytes was first done by Goldschmidt (1917), using the wild silkworm, *Hyalophora cecropia*, in hanging drops of pupal hemolymph. *In vitro* cultivation experiments with lepidopteran germ cells were reported to identify the hormones and other factors that are active in spermatogenesis (Schmidt and Williams, 1953; Yagi et al., 1969). Kambysellis and Williams (1971) proposed that a macromolecule factor (MF), which induces spermatogenesis, occurs in the hemolymph. However, those researchers were unaware of apyrene spermatogenesis as well as sperm maturation.

We previously reported the establishment of an *in vitro* cultivation system of silkworm spermatocytes (Kawamura and Sahara, 2002). The cultivated spermatocysts formed elongated sperm bundles, and peristaltic squeezing occurred in eupyrene sperm bundles, forming mature sperm (Kawamura et al., 2000; 2001). In that experiment, it

became evident that (1) the addition of *Bombyx* hemolymph (BH) is indispensable for *in vitro* cultivation of spermatocytes, (2) BH of various stages in the fifth instar larvae and pupae contain different factors necessary for normal spermatogenesis, and (3) apyrene sperm are not induced when spermatocysts from larval testes are subjected to this cultivation system. The last fact may be explained by lack of ASIF activity (Jans et al., 1984). Our goal is to identify the activating molecules by using this *in vitro* system and by adding target molecules into the culture medium. Our target substances in this study are ecdysteroid and glucose, the amounts of which increase with the start of apyrene sperm production in *B. mori*; the spermatocysts used are from testes after the spinning stage which may have been under the ASIF effect.

2. Materials and methods

2.1. Experimental insect

The strains of the silkworm (*Bombyx mori*) used for *in vitro* cultivation were the F_1 generation of a cross between an re9 female and a Tw1 male maintained in the Sahara laboratory (Kawamura and Sahara, 2002). The stages are shown by hours after the fourth molt. In this material, the spinning stage is at 120h and pupation occurs some time between 168h and 192h. For estimation of the amount of glycogen and ecdysteroids, the F_1 generation of the cross between J 106 and Daizo kept in the Fugo laboratory was reared at 25°C under 16h light-8h dark photoperiod condition. Stages are disclosed by days after the fourth molting.

2.2. In vitro cultivation of spermatocysts

Excised testes of 144h-males were passed through three changes of sterilized saline solution, and the testes walls were torn open in 1.5 ml standard culture medium (TC100 medium for insect cell culture (Invitrogen Corp., Carlsbad, CA, USA) containing 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO, USA)). The cyst suspension was then collected in an Eppendorf tube, hold for 10 min, and the supernatant was transferred culture wells, 50µl per well of a 24 well-Corning culture box (Corning Glass Works, Corning, NY, USA). Fifty microliters of 144h-*Bombyx* hemolymph (BH) were added to the 400µl standard medium in the culture wells and 50µl cyst suspension was distributed to each well. The preparation of BH followed the method described by Kawamura and Sahara (2002): 375µl hemolymph from male silkworms was added to 125µl of 10% reduced glutathione (Wako Pure Chemical Industries Inc., Japan) to avoid darkening by melanization, kept at 50 °C for 25 min and then centrifuged for 15 min at 7,000 g. The culture was kept at 26°C under constant shaking at 45 rpm. In the experiments on the effect of glucose and/or ecdysone, TC100 medium with either 0 g/l, 1.1g/l (original amount), and 11g/l solution of glucose were prepared according to the formula of Invitrogen Corp. In this experiment, TC100 culture medium without glucose was used to make cyst suspensions. For ecdysteroid experiments, the prepared TC100 medium with the original concentration of glucose was used.

2.3. Indirect immunostaining for tubulin and nucleus staining

When the cultured cysts reached the age of 240h after the fourth molting, cysts and sperm bundles were collected by light centrifugation, smeared on three pieces of cover glass, and double-fixed with 4% paraformaldehyde and cold methanol. Mouse monoclonal antibody against human tubulin (Cederlane Laboratory, Hornby, Ont. Canada), and then fluorescein isothiocyanate-conjugated goat antimouse IgG solution (MBL Co. Tokyo, Japan) containing Propidium iodide was applied to the specimens. Eupyrene and apyrene sperm bundles were counted under a fluorescence microscope (Olympus, Tokyo, Japan). The detailed process of staining was described by Yamashiki and Kawamura (1997).

2.4. Estimation of ecdysteroid and glycogen amounts in Bombyx testes

Ecdysteroids: Testicular and hemolymph ecdysteroids were extracted with methanol. The radioimmunoassay (RIA) was according Fugo et al. (1995). The testis was homogenized with 100µl methanol. After centrifugation (10,000 rpm, 5min, 4^{o}C), the supernatant was evaporated in vacuum, and then subjected to RIA. Ecdysteroids in 10µl hemolymph were extracted with 300µl of absolute methanol, and aliquots of the supernatant were assayed for ecdysteroids by RIA. Since 20-hydroxyecdysone (20-E) was used as a standard, the average values of the amount of ecdysteroid were expressed as nanogram of 20-E equivalent. The antiserum for 20-E was kindly supplied by Dr. Satoshi Takeda, National Institute of Agrobiological Science, Tsukuba, Japan. The ratio of the cross-reactions of this antiserum between ecdysone and 20-E was 1:2.5 (Takeda et al., 1986). Radiolabeled ecdysone (23, 24-^{3}H) (Sp. Act. 53 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA), and unlabeled ecdysone and 20-E from Sigma-Aldrich (Tokyo, Japan).

Glycogen: The testes on various days after the fourth molting were homogenized in cold water. The homogenates were then digested in 30% KOH at 100^{o}C for 30 min, and 4 volumes of ethanol were added to precipitate glycogen. Glycogen content was determined by the phenol-sulphuric acid method (Dubois et al., 1956) with glucose as a standard.

2.5. Statistical analysis

The arcsin-transformed data of the frequency of apyrene sperm bundle were analyzed

by one-way ANOVA and Scheffe's *post hoc* test for multiple comparisons using the statistical software, StatView version 5.0 for Machintosh.

3. Results

3.1. Ecdysteroid content of in Bombyx testes and hemolymph

The ecdysteroid titer of the testes was below 500 pg/pair until the 6th day of the fifth instar. The titer abruptly increased to 6,000 pg at 1 day after spinning, remained at a high level until the 4th day of the pupal stage, and then rapidly decreased to 500 pg level until adult eclosion. Though the fluctuation pattern of ecdysteroid in hemolymph closely paralleled that of the testes, the amount was a thousand times higher (Fig. 1).

3.2. Glycogen content of Bombyx testes

In 0h-larvae, the glycogen content in each pair of testes was approximately 25μg/testis. It increased rapidly and reached a peak 1 day before spinning. Thereafter, the amount showed a drastic decrease until a half day after pupation. A slight peak appeared in 4th day pupa and then decreased to a low level again. Glycogen content expressed per milligram protein showed the same pattern (Fig. 2).

3.3. In vitro cultivation of spermatocysts with 20-hydroxyecdysone (20-E)

The spermatocysts from 144h-testes were cultivated in a medium containing 144h-BH. As shown in Table 1, the frequency of apyrene sperm bundles increased in the presence of 20-E with either 0.1 or 1μg/m1 (26.24% and 27.81%, respectively). When the values are compared with that of the control group without 20-E (17.32%), the increase in the frequency of apyrene sperm bundles is statistically significant (Table 1). The cysts cultured in medium containing 20-E at a concentration of 10μg/ml could not survive until 240h.

3.4 In vitro cultivation of spermatocysts with glucose

As previously described, the glycogen amount peaked 1 day before spinning, and then decreased rapidly. This fluctuation suggests that glycogen is consumed to produce glucose. The spermatocysts from 144h-testes were cultivated in three different glucose concentrations: standard TC100 medium (x1 glucose), TC100 medium without glucose (-glucose), and TC100 medium containing 10 times the amount of glucose (x10 glucose). When glucose was absent in the medium, the apyrene sperm bundle production was the lowest (3.74%) of the three. The frequency of apyrene sperm

bundles in standard medium was 8.84% and the value significantly increased (23.07%) as the glucose concentration increased ten fold (Table 2).

3.5 The combined effect of 20-E and glucose on apyrene sperm production

In this experiment, the effects of 20-E, glucose, and 20-E plus glucose were examined. As shown in Table 3, addition of either 20-E or glucose (x10) significantly increased the frequency of apyrene sperm (32.79% and 27.71% respectively), when compared to the value of the control (17.39%). This confirmed the results shown in Table 1 and 2. The values obtained by addition of 20-E or glucose was not significantly different from each other. Cultivation in the medium containing both 20-E and 10 times the amount of glucose induced additive effects to apyrene sperm production (42.21%). However, the value was still lower than observed in the *in vivo* control.

4. Discussion

Kawamura and Sahara (2002) reported the establishment of a long term *in vitro* cultivation system of silkworm spermatocysts, in which eupyrene spermatogenesis proceeds normally from spermatocytes to mature sperm. In this culture system, however, apyrene sperm were not produced from the spermatocysts of the fifth instar (final instar) larvae. Using this culture system, we attempted to find what substances contribute to apyrene production. Our results show that 20-hydroxyecdysone reached a peak 1 day after spinning, remained at a high level for 6 days, and then abruptly declined. The fluctuation of the glycogen content of the testis shows that the peak appeared in the larval stage shortly before spinning, i.e., 2 days earlier than that of 20-E. Fugo (unpublished data) estimated the activity of phosphorylase that digests glycogen to glucose, and found that it peaked shortly before pupation. The decline in glycogen amount strongly implies an increase in glucose availability.

High temperature exposure (32^{o}C, 72h) to *B. mori* during the spinning stage produces sterile male adults (Sugai and Hanaoka, 1972; Fugo and Arisawa, 1992). The sterility of the males is caused by dysfunction of apyrene sperm with morphological abnormality (Sahara and Kawamura, 2002). The ecdysteroid titer in the testes during the high temperature treatment was significantly lower than those in the control (Fugo et al., 1995). We show that the addition of 20-E to the culture medium significantly increased apyrene sperm production in the *in vitro* condition. Because spermatocysts used for *in vitro* cultivation were dissected from the testes at 1 day after the spinning stage (144h), the increase of ecdysteroid in individuals at the same stage may play an important role in maintaining normal apyrene spermatogenesis.

A significant increase of apyrene sperm production was also observed by the addition of glucose to the culture medium. Accumulation of glycogen was cytologically displayed in the testis walls in lepidoperan species, *B. mori* (Sugai, 1965), *Anagasta* (*Ephestia*) *kuehniella* (Szöllösi et al., 1980) and *Cydia pomonella* (Friedländer, 1989). Chippendale (1978) described that, during metamorphic changes in insects, glycogen and trehalose supply glucose, which provides an energy source and a substrate for the synthesis of pupal and adult tissues. The additive effect of 20-E and glucose in promoting an increase in apyrene sperm production implies that both molecules play important roles. Ecdysteroid is known to be a type of metamorphic hormone, which alters gene expression in the cells. Tsuzuki et al. (2001) have shown that programmed cell death in the anterior silk gland of *B. mori* is triggered by 20-E *in vitro*. Activation of genes by ecdysone and supply of energy by glucose is considered necessary for events

of metamorphosis during pupation. At the same time, production of apyrene sperm may be promoted by the utilization of glucose and ecdysone.

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Figure legends

Fig. 1. Ecdysteroid content in *Bombyx* testis and hemolymph after fourth molting. Testes were dissected every day from the 5th larvae and pupae. Each value is the mean of 4 to 5 individuals with standard deviation (SD). -●-, ecdysteroid (20-hydroxyecdyson equivalent pg/a pair of testes); -▲-, ecdysteroid in hemolymph (20-hydroxyecdysone equivalent ng/ml hemolymph); E, adult emergence; P, pupation; S, spinning.

Fig. 2. Glycogen content in *Bombyx* testis after fourth molting. Testes and the hemolymph were collected every day after the fourth molting until adult eclosion. Each value is the mean of 3 to 4 individuals with standard deviation (SD). -Δ-, glycogen content (μg/testis) -○-, glycogen content (μg/mg protein); P, pupation; S, spinning.

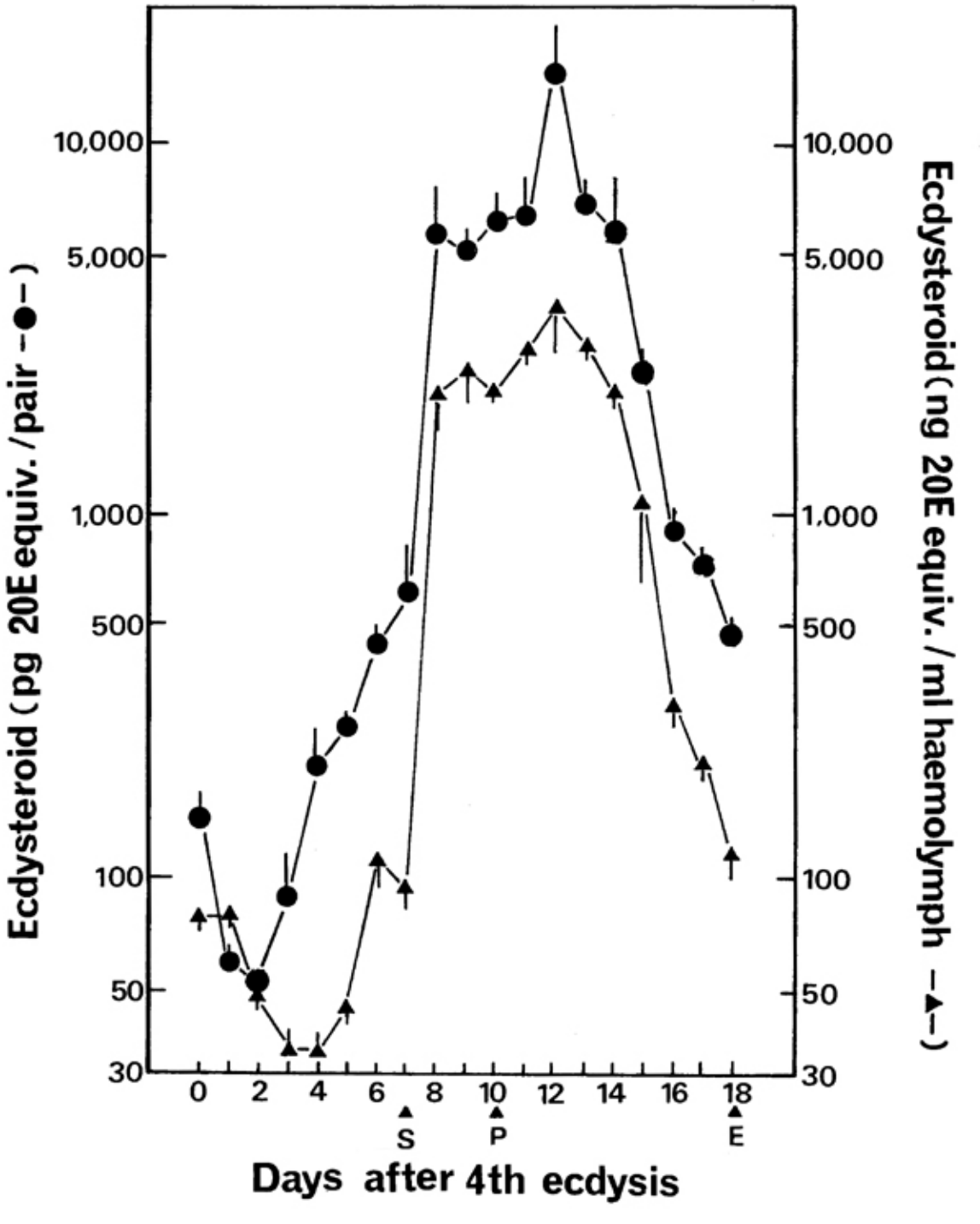

Fig. 1.

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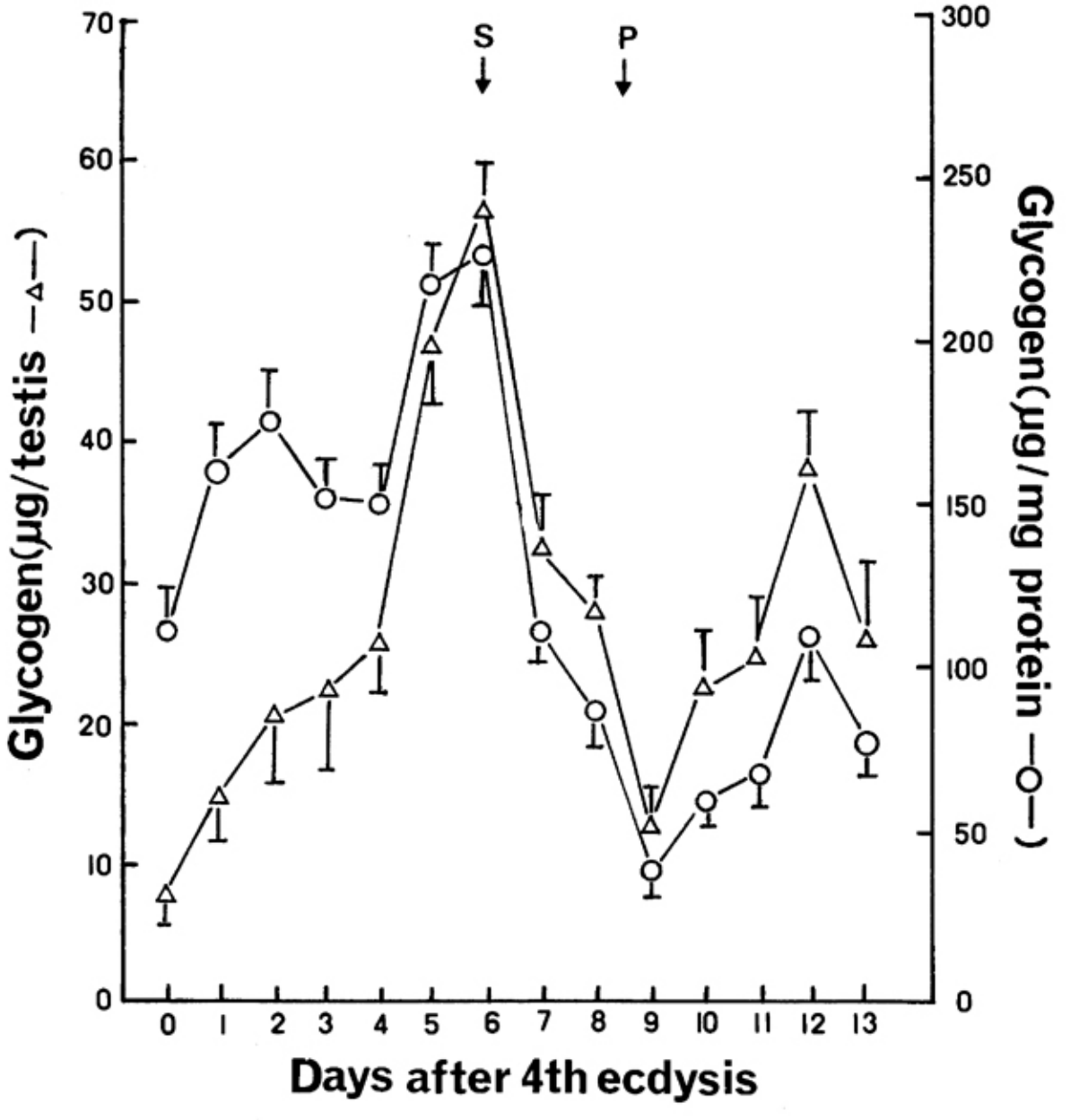

Fig. 2.

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Table 1. Frequency of apyrene sperm bundles in cultivated *Bombyx* spermatocysts for 5 days with or without 20-E.

Experimental group	n	Number of ES	Number of AS	% of AS*
Control	2	957	159	17.32 a
20-E				
0.1μg/ml	2	461	164	26.24 b
1.0μg/ml	3	1,241	478	27.81 b
in vivo control	3	804	1,720	66.72

ES: eupyrene sperm bundle, AS: apyrene sperm bundle; *: The value was calculated with arcsin-root transformed data: the identical letter (a or b) within the column indicates no statistical difference between groups by ANOVA with Scheffe's *post hoc* test.

Table 2. Frequency of apyrene sperm bundles in *Bombyx* spermatocysts cultivation for 4 days with or without glucose.

Experimental group	n	Number of ES	Number of AS	% of AS*
Standard	3	1,764	171	8.84 a
-glucose	3	2,237	87	3.74 b
x10 glucose	3	697	209	23.07 c
in vivo control	3	1,159	922	44.31

ES: eupyrene sperm bundle, AS: apyrene sperm bundle; *: The value was calculated with arcsin-root transformed data: the identical letter (a, b or c) within the column indicates no statistical difference between groups by ANOVA with Scheffe's *post hoc* test.

Table 3. Effect of glucose and/or 20-E (0.1μg/ml) on maturation of apyrene sperm bundles in *Bombyx* spermatocysts cultivation for 4days.

Experimental group	n	Number of ES	Number of AS	% of AS*
Standard	3	1,387	292	17.39 a
20-E	3	662	323	32.79 b
x10 glucose	3	741	284	27.71 b
20-E + x10 glucose	3	835	610	42.21 c
in vivo control	3	629	944	58.97

ES: eupyrene sperm bundle, AS: apyrene sperm bundle; *: The value was calculated with arcsin-root transformed data: the identical letter (a, b or c) within the column indicates no statistical difference between groups by ANOVA with Scheffe's *post hoc* test.