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In-vitro cultivation of spermatocysts to matured sperm in the silkworm, *Bombyx mori*

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Abstract

In vitro cultivation of spermatocysts from the testes of *Bombyx mori* was attempted. *Bombyx* spermatocytes are bipotential, producing nucleate eupyrene sperm and anucleate apyrene sperm. In the absence of *Bombyx* haemolymph (BH), spermatogenesis proceeds normally for two or three days, and thereafter, spermatocytes and sperm bundles begin to degenerate. The present experiment has made clear that (1) BH is essential for normal eupyrene spermatogenesis in long term cultivation, (2) BH from larvae and/or pupae of different ages appears to contain different components necessary for normal eupyrene sperm production, and (3) The present culture system failed to induce apyrene spermatogenesis when spermatocysts from the larvae, especially before the middle of the fifth instar, were subjected to *in vitro* culture, probably due to the lack of contributing factors for apyrene induction in BH. With this culture system, we will be able to detect what substances contribute to apyrene sperm production.

Key words:
Silkworm, *in vitro* cultivation, eupyrene sperm, apyrene sperm, *Bombyx* haemolymph

Introduction

The silkworm males produce dimorphic sperm, nucleate eupyrene and anucleate apyrene, from bipotential spermatogonia. Since spermatogenesis of both types occurs at distinctively different stages, that is, the eupyrene spermatocytes enter meiosis during the final larval instar (the fifth), while the apyrene ones shortly before pupation, the effects of hormones are suspected for these spermatocytes to follow different paths of development (Fugo *et al*., 1996; Friedländer, 1997). To confirm this assumption, we consider it essential to establish an artificial culture system. Many *in vitro* culture experiments of male germ cells have been attempted in vertebrates such as human (Cremades *et al*., 1999; Tesarik, *et al*., 2000), fish (Saiki *et al*., 1997), and newt (Abe, 1988). Saiki *et al*. (1997) successfully cultured the premeiotic spermatocytes of the fish, *Oryzias latipes,* to fertilizable sperm. In insects, *in vitro* cultivation of spermatocytes has been reported in *Drosophila* (Fowller1973; Liebrich, 1981) cricket, *Acta domesticus* (Levin, 1972) and in Lepidoptera (Goldschmidt, 1917; Schmidt & Williams, 1953; Yagi *et al*., 1969; Kambysellis & Williams, 1971). Because of the short term cultivation, the former researchers did not pay much attentions to dimorphic sperm system in the moths. In this paper we will report a new method by which *Bombyx* spermatocytes differentiate to matured sperm in long-term *in vitro* culture conditions.

Materials and Methods

The strains of the silkworm (*Bombyx mori*) used in this study were the F_1 generation of the cross between an re9 (red egg: *re*/*re*, $+^{w\text{-}2}/+^{w\text{-}2}$) female and a Tw1 (white egg: $+^{re}/+^{re}$, *w*-2/ *w*-2) male (Kawamura, 1978). Testes were excised from the fifth instar (the final instar) larvae and pupae, and the stages were shown by hours after the fourth molting.

In vitro cultivation of spermatocysts

The excised testes were passed through three changes of sterilized saline solution and the contained cysts were isolated by tearing open the testes walls in the standard culture medium (TC100 solution for insect cell culture (Invitrogen Corp., Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO, USA)). In this experiment, spermatocysts were cultured in the medium with or without the presence of *Bombyx* haemolymph (BH). To avoid darkening by melanization, BH was prepared as follows: 375µl haemolymph was added to 125µl of 10% reduced glutathion (Sigma Chemical Co.), kept at 50 ^{o}C for 25 min, centrifuged for 15 min at 9,000 rpm, and the supernatant was filtered by a 0.20µm Minisart-plus (Sartorius AG, Göttingen, Germany). Four hundred microliters of standard culture medium and 50µl BH were put in each of 24 well-Corning culture box chambers (Corning Glass Works, Corning, NY, USA), and 50µl cyst suspension in the culture medium was added to each well. Cysts were cultured with 45 rpm shaking at 26 ^{o}C and the medium was exchanged every 72h. Living spermatocysts and sperm bundles were observed by an inverted microscope (Diaphoto TMD, Nikon, Tokyo, Japan) equipped with a Nomarski differential interference lens.

Indirect immunofluorescence staining for tubulin

The cultured cysts of an experimental group were smeared on three pieces of cover glass previously coated with 3-aminopropyl-triethoxy-silane (Sigma Chemical Co.) and fixed in 4% paraformaldehyde for 1h and in cold methanol for an additional 10 min. Mouse monoclonal antibody against human alpha-tubulin (Cederlane Laboratory, Hornby, Ont., Canada), and then fluorescein isothiocyanate-conjugated goat antimouse IgG solution (MBL Co., Tokyo, Japan) containing propidium iodide were applied to the specimens. Eupyrene and apyrene sperm bundles were counted with use of an Olympus fluorescence microscope (Tokyo, Japan). The images of sperm bundles were captured by a laser scanning confocal microscope (Fluoview; Olympus, Tokyo, Japan). This process is previously described in greater detail (Yamashiki & Kawamura, 1997).

Results

In vivo spermatogenesis in B. mori

In the strain used in this study, spinning begins at 120h and pupation occurs sometime between 168h and 192h after the fourth molting. A layer of cyst cells envelopes 64 primary spermatocytes which enter two successive meiotic divisions to form a cyst containing 256 spermatids. All eupyrene nuclei transfer to the anterior of the elongated cyst and the axonema extend toward the posterior. When the nuclei assume a spearhead shape, 256 sperm form a fully elongated sperm bundle. On the other hand, apyrene sperm nuclei stay in the middle of the bundle and the length is about a half of the eupyrene. At the last stage of spermatogenesis, peristaltic squeezing occurs in both eupyrene and apyrene sperm bundles. Some of the cytoplasmic contents of the eupyrene and the nuclei of the apyrene sperm are discarded from the posterior of the bundle by the squeezing action. Matured sperm bundles of both types appear quite slim. Figure 1 shows the *in vivo* control of living spermatocysts and sperm bundles isolated from the testes from 0h to 264h males. Immediately after the fourth molting (0h), majority of spermatocysts are in Meiosis I and II, though a few elongated spermatids already exist (Fig 1a). At 24h (Fig 1b) and 48h (Fig 1c), elongated spermatids form eupyrene sperm bundles. As seen in Figure 1d (72h), 1e (120h) and 1f (144h), small spermatocysts mixed among large spermatocysts and eupyrene sperm bundles seem to be apyrene spermatocysts. At 192h (Fig. 1g), apyrene sperm bundles appear and increase the number by 264h (Fig. 1h). As disclosed in Table 1, only eupyrene spermatogenesis proceeds up to the spinning stage, 120h. Apyrene sperm bundles first appear at one day after spinning (144h), rapidly increasing in number. After 240h (corresponding 3-day pupa), the matured apyrene sperm bundles begin dissociation to a single spermatozoon.

In vitro cultivation of spermatocysts

Cultivation of intact testes is not successful: After two days of cultivation, the testes do not disclose any sign of growth and the nuclei of spermatocytes display a heteropycnotic appearance. Therefore, spermatocysts are isolated from torn testes in the medium. Shaking is another requisite for cultivation.

Effect of *Bombyx* haemolymph (BH) is examined. In this experiment, the spermatocysts are cultured up to 264h with or without BHs that are extracted from the fifth instar larvae and pupae of various ages: 3h, 72h, 144h, and 216h. Three kinds of groups are tested according to the age the cultivation starts; 0h, 48h, and 72h. In 0h to 264h group, 5 kinds of medium are tested, ie, the standard medium (TC 100 containing

10% FBS) without BH (No BH), that with 3h BH, with 72h BH, and with 144h BH throughout the cultivation. In the fifth case, each of four kinds of BH (3h, 72h, 144h and 216h) is added to the medium respectively in every exchange of the medium. In 48h to 264h and 72h to 264h groups, the medium was prepared in the same manner. Figure 2 shows the naked cysts from 0h-testes cultured up to 264h. The first row discloses spermatocysts at 48h after cultivation started, the second row after 72h, the third row after 120h and the bottom row shows spermatocysts after 264h when they are collected, smeared on cover glass and subjected to immunostaining as described in the previous section. Figures 2a, 2d, 2g, and 2j disclose the spermatocysts cultured in NoBH medium. Until 48 hours, they develop quite normally (Fig. 2a), and eupyrene sperm bundles are formed just as seen in those of the *in vivo* case (Fig.1c). After 72h of cultivation, however, degenerating figures appeared (Fig. 2d) and the degeneration continues even when the medium is changed every 72h (Figs. 2g, 2j). This phenomenon occurs in all the groups regardless of the age the cultivation starts; after 72h of cultivation without BH, degeneration of cysts begins, and spermatocysts and sperm bundles degenerate by 264h. The middle column (Figs 2b, 2e, 2h, 2k) shows the spermatocysts cultured in the medium added by different BH of corresponding ages in every exchange of the medium. Spermatogenesis proceeds normally and large spermatocysts decrease with the increase of eupyrene sperm bundles (Figs. 2e, 2h). At 264h, many eupyrene sperm bundles are formed and squeezed sperm bundles appear to be normal, while small spermatocytes as well as apyrene sperm bundles are not observed. In the third column, 72h-BH group (Figs. 2b, 2e, 2h, 2k) is given as a representative, in which delay in the development is recognized till 264h and the delay is not so significant in 3h-BH and 144h-Bh groups.

A eupyrene sperm bundle is characterized by its elongated nuclei and the location of the nuclei in the anterior end. Figure 3a discloses a normal eupyrene sperm bundle at the peristaltic squeezing stage, in which elongated nuclei stay in the anterior end of the bundle. In some culture groups, especially cultured by the medium with 3h-BH or cultured from 0h, many abnormal eupyrene sperm bundles appear: the elongated nuclei transfer to both ends (Fig. 3b, 3c) or stay in the middle of the bundle just like in the apyrene sperm bundle (Fig. 3c, 3d). The length of these abnormal eupyrene bundles is much shorter than the normal ones. Immunostaining images of the squeezed eupyrene sperm bundles in higher magnification disclose that the nuclei of the normal eupyrene sperm bundle stay in the anterior (Fig. 3e), while the recession of the nuclei toward the posterior (Fig 3f) occur in the abnormal eupyrene sperm bundles. Figures 3g and 3h disclose the anterior and posterior end of a bundle in which the nuclei are elongated but the distribution is irregular. As shown in Table 2, the occurrence of abnormal eupyrene

sperm bundles is quite frequent in the group cultured from 0h to 264h. The frequency of abnormality is highest when cultured in the standard medium without BH (No BH). By considering the culture experiments of three groups together, the best result is obtained when the BH of the corresponding age is added in every exchange of the medium (3h-72h-144h-216h or 72h-144h-216h).

Table 3 discloses the relation between the frequency of apyrene sperm bundles in total sperm bundles and the age of the testes used at the start of the cultivation. The BH of corresponding age is added at every exchange of the medium in this experiment. The frequency of normal squeezed eupyrene sperm bundles (ESQ) increases as the age of testes grow older: In the cultured spermatocysts from 0h testes, the frequency of normal ESQ is lowest (16.52%) and it is greatly improved when testes older than 48h are used. The start of culture at the age of 120h show a peculiar result that the cultured cysts are aggregated and the chromosomes within the cell become thread-like. Only 97 eupyrene sperm bundles are recognized in three slides. We have obtained the same result in repeated experiments. The *in vivo* testes of 264h contain apyrene sperm bundles at about 70% of the total. In the artificial cultivation up to 264h, none or a few apyrene sperm bundles are produced when larval testes are used. In the cultivation of the spermatocysts and sperm bundles from the testes after the spinning stage (120h), some number of apyrene sperm bundles are produced, although the frequency is much lower than that of the *in vivo* control.

Discussion

In vitro cultivation of spermatocytes and spermatogonia in Lepidoptera was initiated by Goldschmidt (1917) who observed spermatogenesis of wild silkworms, *Hyalophora ceclopia*, in hanging drops of pupal haemolymph. Schmidt and Williams (1953) found that the culture medium must contain a certain substance that is later proved to be a heat-labile, tripsin-sensitive, non-dialysable, non-species-specific macromolecule. This substance has been termed the 'macromolecular factor' (MF). Kambysellis and Williams (1971) performed detailed experiments mainly in *Samia cynthia* on the role of MF. They found that the development of spermatocytes to sperm bundles depends, not on ecdysone, but on MF. MF-like activities were demonstrated in calf serum, fetal calf serum and newborn calf serum. Yagi *et al*. (1969) cultivated testes of the rice stem borer, *Chio suppressalis*, as a whole, or isolated spermatocytes in the medium with or without the presence of ecdysone. In their experiments, elongated spermatocysts disclosed normal shape only when they were released from the torn testis, while ecdysone caused remarkable growth and development.

The present experiments in which the naked spermatocysts of *Bombyx mori* are cultivated with or without *Bombyx* haemolymph (BH) show that BH is indispensable. The best results are obtained when the culture medium containing BH of the corresponding stage after the fourth molting is used in every exchange of the medium in 72h interval. During the cultivation, spermatocysts elongate, eupyrene sperm bundles are formed, and the peristaltic squeezing (Kawamura *et al*., 2000) occurs to form matured sperm bundles as seen in *in vivo* controls. Recently, Takemura *et al*. (1999) established a new technique for artificial insemination in *Bombyx mori*. The technique will be applicable to the matured eupyrene sperm produced by *in vitro* cultivation in this study.

In some culture groups, abnormal eupyrene sperm bundles of two types appear: In one type, short sperm bundles as long as apyrene sperm bundles are formed with elongated nuclei arranged in middle regions and/or both ends (Figs. 3b, 3c, 3d). These sperm bundles seem to be derived from apyrene spermatocytes that are probably affected by some 'eupyrene inducing factors' or by a shortage of 'apyrene-spermatogenesis-inducing-factors' proposed by Jans *et al*. (1984). In the other type, sperm bundles show a normal appearance but the nuclei recess toward the posterior and are finally discarded at the squeezing stage (Fig. 3f). The frequency of normal squeezed eupyrene sperm bundles (ESQ) reaches a high plateau when cultivation starts with spermatocysts from 48h testes or thereafter (Table 3). Many

spermatogonia exist in 0h-testis, and the number rapidly decreases up to 48h. Abnormal eupyrene sperm bundles may be derived from spermatogonia that fail normal development to become primary spermatocytes in this culture condition.

Notable results in this experiment are (1) BH is essential in *in vitro* cultivation of spermatocysts and (2) BHs from different ages appear to contain different factors that effect spermatogenesis. MF activity in the blood of *Cynthia* silkworm pupae discloses a U-shape curve during spermatogenesis (Kambysellis & Williams, 1971), while a rapid increase in testicular ecdysteroid titers at the last stage of the fifth instar is shown with a peak at 2-day pupa (Fugo *et al*., 1996). We have not yet found what molecules needed for spermatogenesis are contained in BH. In cultivation with 72h-BH, the delay in early spermatogenesis occurs. A suppressing factor in BH for early spermatogenesis is considered, or a match between the condition of sperm cells and some factors contained in BH may be needed. (3) An unexpected result is that none or only a small number of apyrene sperm bundles are produced by this culture system when the spermatocysts from larval testes are used, though eupyrene spermatogenesis proceeds normally to form matured, or squeezed, sperm bundles. The abnormal sperm bundles as long as apyrene ones suggest that BH is insufficient for the production of normal apyrene sperm bundles. In the testes after the spinning stage, some of the spermatocytes are destined to become normal apyrene sperm. Probably, they already receive the effects of the 'apyrene-spermatogenesis-inducing factor' around the spinning stage, though the number is far smaller than that of *in vivo* control. By using this culture system, we are continuing experiments on what substances contribute to the induction of apyrene sperm bundles.

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References

Abe, S. 1988. Cell culture of spermatogenic cells from Amphibians. Develop. Growth Differ., **30**, 209-218.

Cremades, N., Bernabeu, R., Barros, A. & Sousa, M. 1999. *In vitro* maturation of round spermatids using co-culture on Vero cells. Human Reprod., **14**, 1287 – 1293.

Friedländer, M. 1997. Control of the eupyrene-apyrene sperm dimorphism in Lepidoptera. J. Insect Physiol., **43**, 1085-1092.

Fowler, G. L. 1973. *In vitro* cell differentiation in the testes of *Drosophila hydei*., Cell Differ., **2**, 33-47.

Fugo, H., Yamauchi, M. & Georgiou Demos, S. 1996. Testicular ecdysteroids in the silkworm, *Bombyx mori*. Proc. Japan Acad., **72**, Ser. B, 34-37.

Levine, L. 1972. Preparation and maintenance of living house cricket (*Acheta domesticaus*) spermatocytes. Biol. Reprod., **7**, 211 –222.

Liebrich, W. 1981. *In vitro* spermatogenesis in *Drosophila*. Cell Tissue Res., **220**, 251-262.

Goldschmidt, R., 1917. Versuche zur Spermatogenese *in vitro*. Arch. f. Zellforsh., **14**, 421-450.

Kambysellis, M. P. & Williams, C. M. 1971. *In vitro* development of insect tissues. I. A macromolecular factor prerequisite for silkworm spermatogenesis. Biol. Bull., **141**, 537-540.

Kawamura, N. 1978. The early embryonic mitosis in normal and cooled eggs of the silkworm *Bombyx mori*. J. Morph., **158**, 57-72.

Kawamura, N., Yamashiki, N., Saitoh, H. & Sahara, K. 2000. Peristaltic squeezing of sperm bundles at the late stage of spermatogenesis in the silkworm, *Bombyx mori*. J. Morph., **246**, 53-58.

Jans, P., Benz G. & Friedländer, M. 1984. Apyrene-sperm-inducing factor is present in the haemolymph of male and female pupae of the codling moth. J. Insect Physiol., **30**, 495-497.

Saiki, A., Tamura, M., Matsumoto, M., Katowgi, J., Watanabe, A. & Onitake, K. 1997. Establishment of *in vitro* spermatogenesis from spermatocytes in the medaka, *Oryzias latipes*. Develop. Growth Differ., **39**, 337-344.

Schmidt, E. L. & Williams, C. M. 1953. Physiology of insect diapause. V. Assay of the growth and differentiation hormone of Lepidoptera by the method of tissue culture. Biol. Bull., **105**, 174-187.

Takemura, Y., Kanda. T. & Horie, Y. 1999. Artificial insemination using trypsin-treated sperm in the silkworm, *Bombyx mori*. J. Insect Physiol., **45**, 471-477.

Tesarik, J., Mendorza, C. & Greco, E. 2000. *In-vitro* maturation of immature human male germ cells. Molec. Cell. Endocri., **166**, 45 – 50.

Yagi, S., Kondo, E. & Fukaya, M. 1969. Hormonal effect on cultivated insect tissues. I. Effect of ecdysterone on cultivated testes of diapausing rice stem borer larvae. Appl. Entomol. Zool., **4**, 70-78.

Yamashiki, N., Kawamura, N., 1997. Behaviors of nucleus, basal bodies and microtubules during eupyrene and apyrene spermiogenesis in the silkworm, *Bombyx mori* (Lepidoptera). Develop.Growth Differ., **39**, 715-722.

Figure legends

Fig. 1. Normal spermatogenesis from 0h to 264h after the fourth molting disclosed by living spermatocysts isolated from testes. (a) 0h, immediately after molting. (b) 24h. (c) 48h. (d) 72h. (e) 120h (spinning stage). (f) 144h. (g) 192h (1 day-pupa). (h) 264h (4-day pupa). apy, apyrene sperm bundle; eup, eupyrene sperm bundle; ESQ, squeezed eupyrene sperm bundle; Lsc, large spermatocyst; Ssc, small spermatocyst. Bar, 100µm.

Fig. 2. Cultivation of spermatocysts from 0h old testes. The medium was exchanged every 72h. (a, d, g and h) Spermatocysts cultured in the standard medium without *Bombyx* haemolymph; (a) 48h after cultivation started. (d) 72h after cultivation. Degenerating spermatocysts appear as dark spermatocysts (arrow). (g and j) 120h and 264h after cultivation. Degeneration proceeds as cultivation prolonged. (b, e, h and k) Spermatocysts cultured with the medium in which 3h-, 72h-, 144h-, and 216h-BHs were added in every exchange of the medium; (b) 48h after cultivation. (e) 72h after cultivation. (h) 120h after cultivation. (k) 264h after cultivation. Squeezed eupyrene sperm bundles (ESQ) occur. (c, f, i and l) Spermatocysts cultured in 72h-BH throughout cultivation. When compared to the middle column, eupyrene spermatogenesis delays and large spermatocysts remain even at 264h of cultivation; (c) 48h after cultivation. (f) 72h after cultivation. (i) 120h after cultivation. (l) 264h after cultivation. A few matured sperm bundles (squeezed sperm bundles) appear. eup, eupyrene sperm bundle; ESQ, squeezed eupyrene sperm bundle. Bar, 100µm.

Fig. 3. Normal and abnormal eupyrene sperm bundles displayed by confocal images with immunofluorescence staining for tubulin (green) and nucleus (red). (a) Normal sperm bundle after peristaltic squeezing. Elongated nuclei are located in the anterior of the bundle. (b) Abnormal eupyrene bundle with the nuclei being transferred to both ends of the bundle. (c and d) These bundles are as short as an apyrene sperm bundle though the elongated sperm nuclei are transferred to both ends (c), or to the anterior of the bundle (d). (e) The anterior end of a normal eupyrene sperm bundle in high magnification. (f) The anterior end of an abnormal eupyrene sperm bundle. Sperm nuclei recess toward the posterior as the squeezing proceeds. (g and h) The anterior and posterior end of an abnormal eupyrene sperm bundle in high magnification. The head cyst cell is not located in the apex of the anterior in (g), and sperm nuclei are transferred to the posterior end in (f). ccn, cyst cell nucleus; hcn, head cyst cell nucleus; sn, sperm nucleus. Bars, 20µm.

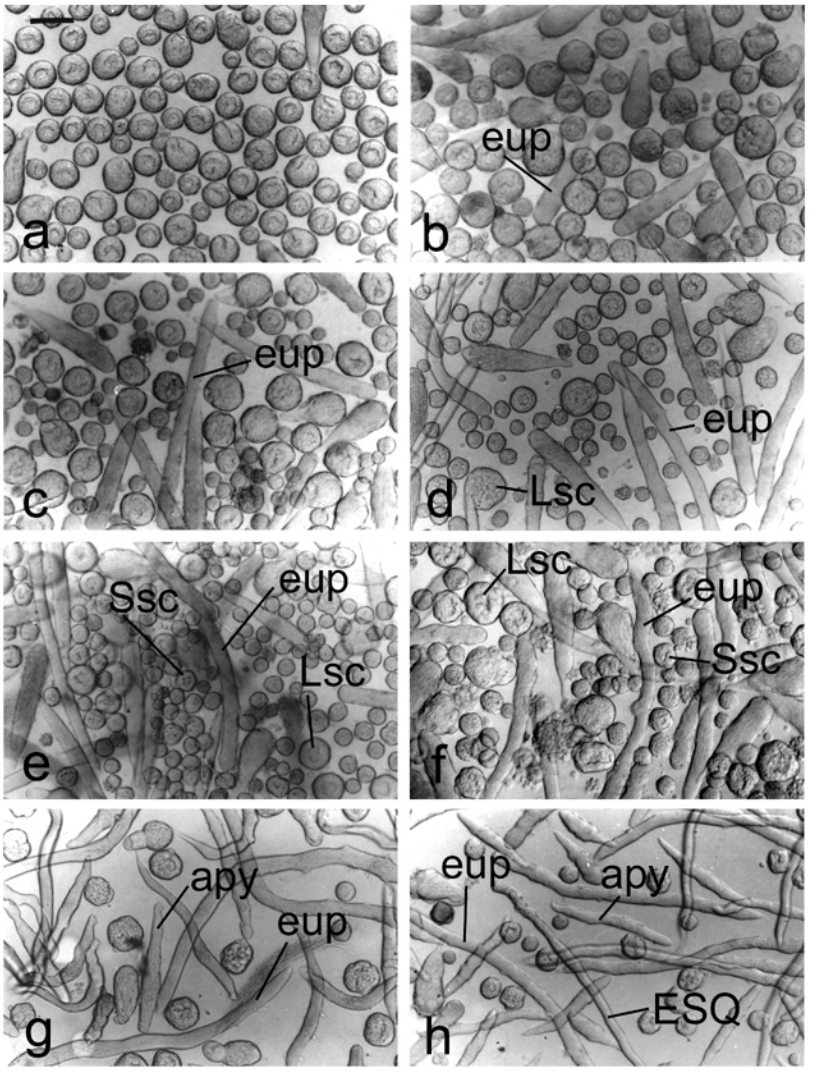

Fig. 1.

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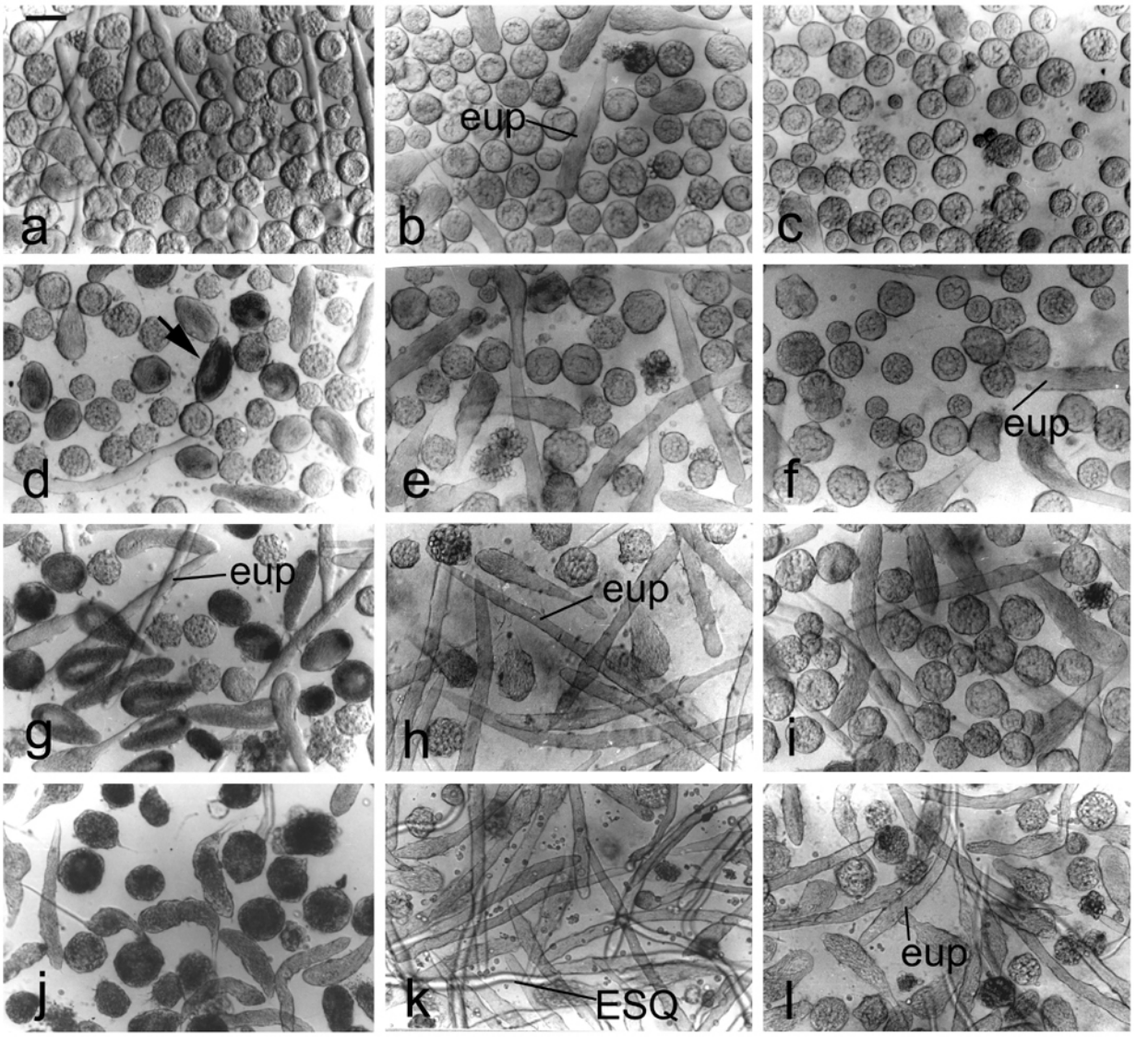

Fig. 2. Kawamura & Sahara

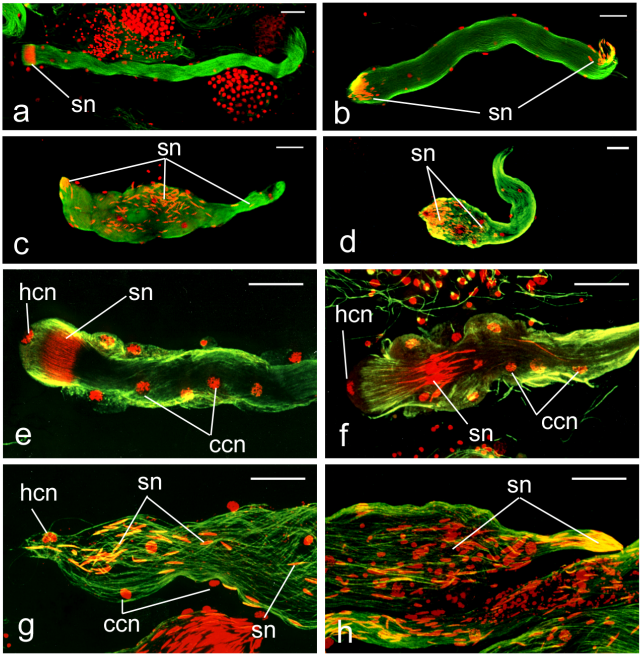

Fig. 3

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Table 1. Eupyrene and apyrene sperm bundles in the testes of the living silkworms

Testis excised at	Eupyrene bundles*	Apyrene bundles*	% of Apyrene/ total sperm bundles
0h	3	0	0
24h	81	0	0
48h	258	0	0
72h	503	0	0
96h	1,179	0	0
120h (spinning)	1,645	0	0
144h	840	4	0.53
168h	971	1,257	56.15
192h (pupation)	1,243	1,611	56.45
216h	1,614	3,225	66.65
240h	1,258	3,003	70.48
264h	938	2,197	70.08

*The number of sperm bundles given in this table is the sum of three slides.

Table 2. Effect of *Bombyx* haemolymph to cultured spermatocysts.

Cultured from	*Bombyx* haemo-lymph (BH)	Eupyrene bundles	ESQ*	Normal ESQ	Apyrene bundles	% of Normal ESQ/ESQ
0h to 264h	No BH	25	3	0	0	0
	72h BH	270	62	0	0	0
	144h BH	515	224	7	0	3.13
	3-72-144-216h BH	72	22	6	0	27.27
48h to 264h	No BH	42	31	0	0	0
	3h BH	88	11	0	0	0
	72h BH	323	103	97	0	94.17
	144h BH	209	61	59	0	96.72
	72-144-216h BH	305	111	107	0	96.40
72h to 264h	No BH	39	15	8	0	55.33
	72h BH	126	54	24	0	44.44
	144h BH	167	17	8	0	47.06
	72-144-216h BH	617	233	226	0	97.00

*Squeezed eupyrene sperm bundle

Table 3. Frequency of the normal squeezed eupyrene sperm (ESQ) and apyrene sperm bundles cultivated up to 264h.

Start of culture	BH* added	Eupyrene bundles	ESQ	Normal ESQ	% of Normal SQE/total ESQ	Apyrene bundles	% of Apyrene/ total sperm bundles
0h	A	1,505	523	72	16.52	0	0
24h	A	1,447	400	272	68.00	0	0
48h	A	716	187	174	93.05	0	0
72h	B	455	103	99	96.12	14	2.99
96h	B	1,009	225	207	92.00	4	0.39
120h	B	97	29	26	89.66	42	30.22
144h	C	1,254	465	445	95.70	319	20.28
168h	C	624	268	263	98.13	495	44.24

*<u>*Bombyx*</u> haemolymph of the corresponding ages was added in every exchange of the medium (see details in materials and methods) A, 3h-72h-144h-216h; B, 72h-144h-216h; C, 144h-216h.