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Double copulation of a female with sterile diploid and polyploid males recovers fertility in *Bombyx mori*

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Summary

Silkworm males produce dimorphic sperm, nucleate eupyrene sperm and anucleate apyrene sperm. Apyrene sperm have been speculated to have an assisting role in fertilisation. However, the coexistence of eupyrene and apyrene sperm in the testis and female reproductive organs has made it difficult to define the role of apyrene sperm. Polyploid males are highly sterile. Microscopic observation revealed that the elimination of eupyrene nuclei by peristaltic squeezing caused the sterility of polyploids. Heat-shock applied to pupae of Daizo males (DH) also induced high sterility due to the lack of normal apyrene sperm. When eupyrene sperm of sterile DH males and apyrene sperm of sterile polyploid males were mixed by double copulation, a remarkable increase in fertility of the double-mated females was observed. This finding strongly suggests that the apyrene sperm are indispensable in fertilisation of the silkworm and that polyploid apyrene sperm function as a substitute for diploid sperm. We established an experimental system in which we can separate the two types of sperm for further studies on their functions without chemical and/or mechanical treatments.

Keywords: Apyrene sperm, Double copulation, Eupyrene sperm, Polyploid silkworm, Tubulin

Introduction

Like other Lepidoptera, males of the silkworm (*Bombyx mori*) produce dimorphic sperm, eupyrene (nucleate) sperm and apyrene (anucleate) sperm. By copulation, both types of sperm enter the female reproductive organs, but only eupyrene sperm participate in fertilisation. It is not clear how in the silkworm the different types of sperm are produced from spermatogonia bearing the same genome. In fact the eupyrene spermatocytes finish meiosis during the final (fifth) larval instar, while the apyrene ones start meiosis after the spinning stage (Tazima, 1964). Some morphological differences between eupyrene and apyrene sperm in the process of spermatogenesis are documented in axoneme elongation, the behaviour of the nucleus, basal body, acrosome as well as the amount of mitochondria and mitochondrial DNA (Yamashiki & Kawamura, 1997, 1998; Kawamura *et al*., 1998).

The function of apyrene sperm is not fully understood (Friedländer, 1997), but they definitely play a role in fertilisation (Jamieson *et al*., 1999). Several functions of apyrene sperm have been proposed: (1) the presence of apyrene sperm in the bursa copulatrix delays female remating and, hence, they seem to play an important role in sperm competition (Cook & Wedell, 1999); (2) their vigorous movement in the spermatophore promotes dissociation of eupyrene sperm bundles (Osanai *et al*., 1987); (3) they promote the ability of eupyrene sperm to cross the basement membrane of a testis (Katsuno, 1977); (4) they provide nutrients for the eupyrene sperm (Riemann & Gassner, 1973); and (5) they help in transporting eupyrene sperm into the female genital organs (Iriki, 1941).

In the silkworm, Osanai *et al*. (1989) succeeded in separating eupyrene from apyrene sperm bundles by using a Percoll density gradient in a special centrifuge. They confirmed the metabolic pathway of energy resources for motile acquisition of apyrene sperm

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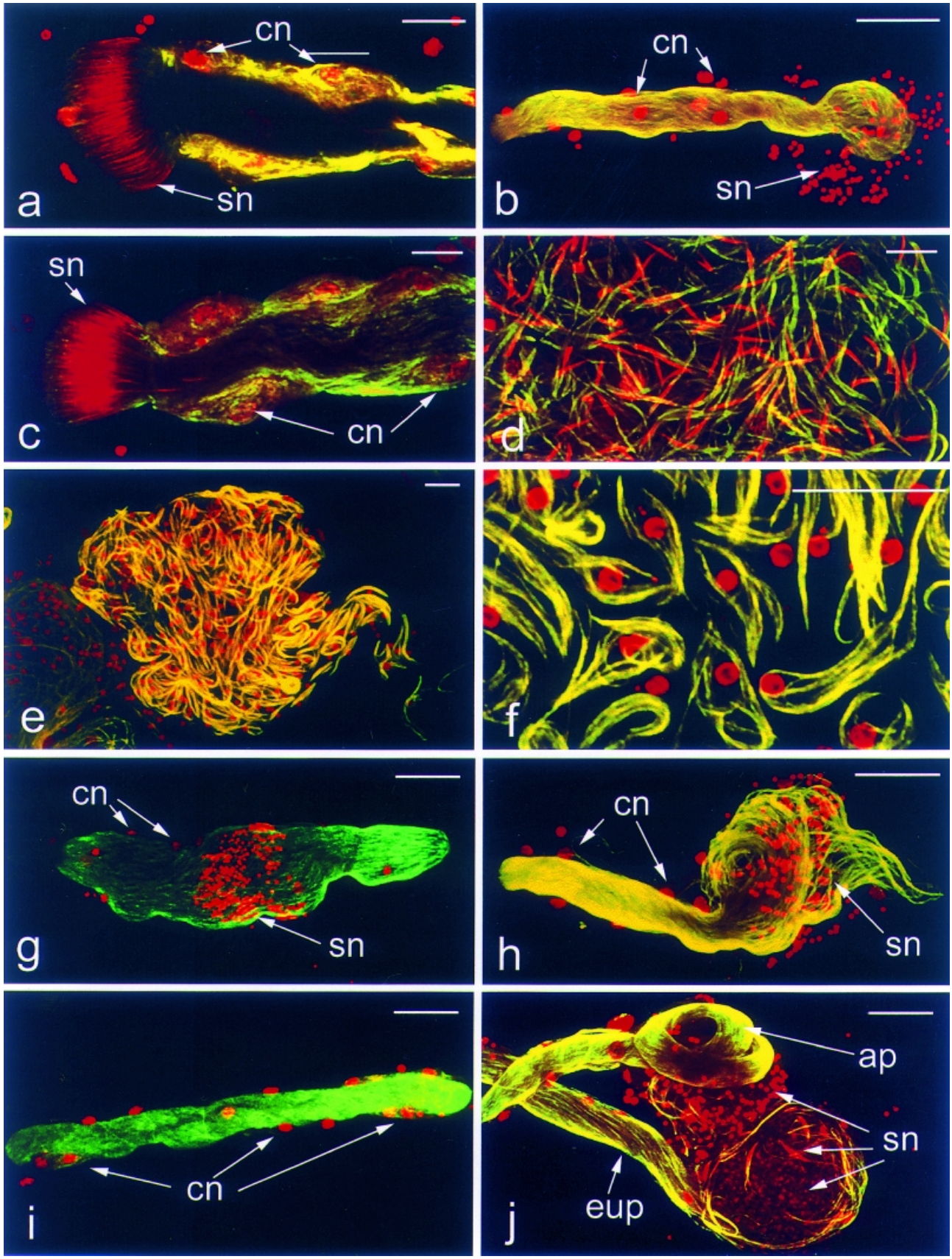
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(Osanai *et al*., 1989) and dissociation of eupyrene sperm bundles by acids and endopeptidase (Osanai & Isono, 1997). Unfortunately, there was no technique for artificial fertilisation at that time, so they could not confirm the fertilising ability of the activated sperm *in vitro*.

Sugai & Kiguchi (1967) reported that the treatment of male silkworm pupae with a high temperature produces highly sterile individuals. Later, Sugai & Takahashi (1981) speculated that morphological abnormality of apyrene sperm caused the high sterility. In the silkworm, bisexual polyploid individuals are easily produced by the low-temperature treatment of eggs at the first cleavage stage (Kawamura, 1978). The resulting tetraploid and triploid males are highly sterile in nature. Kawamura *et al*. (2001) showed that the peristaltic squeezing that occurs at the very late stage of spermatogenesis eliminates the nuclei of polyploid eupyrene sperm bundles.

We aimed to develop an experimental system to study the reproductive biology of lepidopterans by producing an individual which harbours either normal eupyrene sperm or normal apyrene sperm. We found that inducing abnormal apyrene sperm by heat-shock treatment causes the sterility of diploid males, and that sterility of polyploid males is due to the abnormal eupyrene sperm. We will discuss the crucial role of apyrene sperm at fertilisation by producing results showing that double copulation by a heat-treated male and then a polyploid male recovers fertility.

Figure 1 *(opposite)* Confocal microscopic images of the sperm bundles and spermatids stained with immunofluorescence for tubulin (green) and propidium iodide for nuclei (red). (*a*) The anterior region of a diploid eupyrene sperm bundle of a control Daizo (DC) male after peristaltic squeezing. (*b*) A diploid apyrene sperm bundle of a DC male after peristaltic squeezing. (*c*) The anterior region of a heat-treated diploid eupyrene sperm bundle of a Daizo (DH) male after peristaltic squeezing. (*d*) An abnormal eupyrene spermatid of a DH male. Spearhead nuclei and a thick axoneme have lost their longitudinal orientation. (*e*) Apyrene spermatids of a DH male. (*f*) A high-magnification image of a DH spermatid. Round nuclei and a thick axoneme display an irregular orientation. (*g*)–(*i*) Apyrene sperm bundles of a triploid-1: before peristaltic squeezing (*g*), during squeezing (*h*) and after a squeezing (*i*). (*j*) The posterior region of adjacent eupyrene and apyrene sperm bundles in a tetraploid. Round nuclei of the apyrene sperm and spearhead nuclei together with fragmented nuclei of the eupyrene sperm were eliminated from the posterior ends of the bundles. ap, apyrene sperm bundle; cn, cyst cell nucleus; eup, eupyrene sperm bundle; sn, sperm nuclei. Scale bars represent 50 μm (*a–c*, *g–j*) or 20 μm (*d–f*).

Materials and methods

The silkworms (*Bombyx mori*) used were re9 females (red egg: *re*/*re*) and Tw1 males (white egg 2: *w*-2/*w*-2) for tetraploid induction (Kawamura, 1978) and Cre males (red egg: *re*/*re*) for triploid production. For fertility examination of triploid males and Daizo males, either the females of F_1 progeny from Cre females (sex-limited sable, red egg: T(W:2) p^{Sa}, *re*/*re*) and Eq (red egg: *re*/*re*) males (CE) or Daizo (black egg (wild type) : $+^{re}/+^{re}$, $+^{w\text{-}2}/+^{w\text{-}2}$) were used. F_1 eggs of the cross between the red egg strain and the white egg 2 strain show wild-type colour (black) and the egg colour segregation ratio in the F_2 is black:red:white = 9:3:4. Room temperature was kept at 26 °C throughout a life cycle.

Induction of polyploid silkworms

F_1 eggs of the cross between an re9 female and a Tw1 male were refrigerated at –10 °C for 24 h at the first cleavage stage (120–150 min after oviposition) and then returned to room temperature. The eggs with large serosa nuclei were selected as the tetraploids (rw4n) (Kawamura, 1979). Since the meiotic chromosomes in the female perform no crossing-over, tetraploid females are as fertile as diploids. By crossing an rw4n female with a diploid male, we can obtain triploid individuals that show almost 100% hatchability and a normal growth rate (triploid-1: 4n female × 2n male). On the other hand, tetraploid males are highly sterile in nature. Starvation for 48 h at the middle stage of the last larval instar increases the fertility (Kawamura *et al*., 1995). By applying the starvation technique, we produced fertile tetraploid males and crossed them with either diploid (triploid-2: 2n female × 4n male) or tetraploid females (tetraploid: 4n female × 4n male). Ploidy of the progeny was confirmed by the egg colour segregation ratio in the backcross.

Induction of sterile Daizo males

Sterile Daizo males were induced following the method of Sugai & Hanaoka (1972) with a slight modification. The males at the spinning stage were subjected to a temperature of 33 °C for 96–120 h and then returned to room temperature.

Copulation

For a fertility test, heat-treated Daizo (DH), untreated Daizo (DC) or polyploid males were mated to a virgin CE or Daizo female for 3 h. The silkworm, in contrast to many wild lepidopteran species, can remate immediately after the first copulation. For double copulation, a polyploid male was mated with a CE female for

3 h prior to the mating of a DH male with the same female for an additional 3 h. We watched the pairing couples throughout mating, and double copulation was confirmed by the presence of two spermatophores in the bursa copulatrix.

Indirect immunofluorescence staining for tubulin

Spermatids and sperm bundles from excised testes of pupae were smeared on a coverslip coated with 3-aminopropyl-triethoxy-silane (Sigma Chemical, St Louis, MO), and fixed with 4% paraformaldehyde and then methanol. Mouse monoclonal antibody against chicken alpha-tubulin (Cederlane Laboratory, Hornby, Ont., Canada) cross-hybridises with silkworm alpha-tubulin. The antibody and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (MBL, Tokyo, Japan) with propidium iodide (Sigma Chemical) were applied on the coverslip. The stained specimens were observed with a confocal laser-scanning microscope (Fluoview, Olympus, Tokyo, Japan). Details of the immuno-staining procedure are described in Yamashiki & Kawamura (1997).

Statistical analysis

The arcsin-transformed data of fertility were analysed by one-way ANOVA and Scheffe's *post hoc* test for multiple comparisons using the statistical software StatView version 5.0 for Macintosh.

Results

Observation of sperm bundles

Control Daizo (DC)

In eupyrene spermatogenesis the spearhead-shaped nuclei proceeded to the anterior of the sperm bundles and remained in the region after the squeezing was completed (Fig. 1*a*). Round nuclei of apyrene sperm bundles that had been located in the middle region of the bundle during spermatogenesis were eliminated from the posterior end by peristaltic squeezing (Fig. 1*b*).

Heat-treated Daizo (DH)

Eupyrene spermatogenesis in DH males proceeded normally. The peristaltic squeezing occurred in the pupae that received the heat treatment. The spearhead-shaped nuclei of eupyrene sperm bundles were located in the anterior region (Fig. 1*c*). Eupyrene spermatocytes usually finish meiosis before the spinning stage and, therefore, meiotic eupyrene spermatocytes were rarely observed in the testis of pupae. Fig. 1*d* disclosed an exceptional example of one eupyrene spermatocyst in which the spearhead nuclei and the thick axoneme lost their longitudinal orientation. Meiotic figures in apyrene spermatocysts appeared morphologically normal, but elongated sperm bundles were not formed during further development (Fig. 1*e*). A thick axoneme surrounded round apyrene nuclei and their elongation assumed irregular orientation (Fig. 1*f*). Peristaltic squeezing did not occur in such abnormal apyrene spermatids. In the later spermatogenetic stage, the abnormal sperm bundles still retained the nuclei, forming a round mass. Normal apyrene sperm bundles were not observed in testes at the late pupal stage.

Polyploid

Three kinds of polyploid silkworms were prepared: triploid-1 (4n female × 2n male), triploid-2 (2n female × 4n male) and tetraploid (4n female × 4n male). In all polyploid silkworms, spermatogenesis proceeded in a similar manner. The apyrene sperm nuclei that had been located in the middle region (Fig. 1*g*) were squeezed out by peristaltic squeezing (Fig. 1*h*, *i*). These processes of apyrene spermatogenesis appeared normal just as in the untreated diploid ones (Fig. 1*b*). In eupyrene sperm bundles, however, spearhead nuclei moved towards the posterior end as the squeezing proceeded and the nuclei were finally eliminated by the squeezing action. None or only a few nuclei remained in the sperm bundle. In the debris, spearhead nuclei as well as nuclear fragments were observed just as in those of apyrene bundles (Fig. 1*j*).

Double copulation by DH and polyploid males

In three repeated mating experiments, similar results were obtained (Table 1). Males of DH, tetraploid, triploid-1 and triploid-2 were all highly sterile, whereas additional copulation with polyploid males recovered fertility of the DH males. At single copulation, tetraploid males displayed approximately 2% fertility on average, and DH and two kinds of triploid males were either completely sterile or showed less than 0.1% fertility. Double copulation by DH and polyploid males significantly increased fertility of mated females (Table 1). Analysed by ANOVA with Scheffe's *post hoc* test, the values of fertility did not differ statistically among the three groups, but were lower in the DC group.

Genetic confirmation of the progeny from double copulation

To confirm which male parent, DH or triploid-1, contributed to fertilisation, we used Tw1 females with a plain larval marker (*p*) for the mother. If sperm from DH males fertilise the eggs of Tw1 females, the progeny will bear a normal larval marking ($+^{p}$). On the

Table 1 Fertility of males in high-temperature-treated Daizo (DH), polyploids and their double copulation

	n	No. of eggs deposited	Fertility (%)	5% confidence interval[a]
DH[b]	18	110.94 ± 77.42	0	
Tetraploid (4n × 4n)	18	165.61 ± 70.09	1.81	0.00–3.39
Double copulation[c] (DH + tetraploid)	18	339.28 ± 133.51	78.47	60.83–88.25 a
DH[b]	14	141.93 ± 57.90	0.10	0–3.61
Triploid-1 (4n × 2n)	14	137.29 ± 64.09	0.05	0–2.47
Double copulation[c] (DH + triploid-1)	14	340.57 ± 97.88	74.64	62.14–88.27 a
DH[b]	15	67.13 ± 22.66	0	
Triploid-2 (2n × 4n)	15	193.27 ± 90.54	0	
Double copulation[c] (DH + triploid-2)	15	387.80 ± 139.27	78.58	64.64–89.6 a
DC[d]	4	584.5 ± 60.50	99.91	99.24–100 b

[a] The value was calculated with arcsin-root transformed data: the same letter (a or b) within a column indicates no statistical difference between groups by ANOVA with Scheffe's *post hoc* test.
[b] Heat-treated Daizo males (see Materials and Methods).
[c] A polyploid male was mated with a CE female prior to the mating of a DH male (see Materials and Methods).
[d] Untreated Daizo males.

other hand, fertilisation of Tw1 eggs with triploid-1 sperm will produce either stripe (p^S) or plain offspring. In all double crosses, the larval marking of the progeny was normal and the sex ratio was 1:1 (Table 2). The result proved that the females were fertilised with eupyrene sperm of DH males.

Table 2 Genetic analysis of progeny from the double copulation

Cross no.	No. of progeny	Egg colour	Larval marking	F/M ratio[a]
1	228	All black	Normal marking	0.982
2	89	All black	Normal marking	0.978

[a] Ratio of females to males.

Discussion

During spermiogenesis in the silkworm (*Bombyx mori*), the nuclei of eupyrene sperm change into a spearhead shape, move towards the anterior end of sperm bundles and anchor to a head cyst cell by an acrosome–basal body assembly. On the other hand, the nuclei of apyrene sperm bundles have no such assembly and, therefore, remain in the middle region without any contact with the head cyst cell (Yamashiki & Kawamura, 1997). By peristaltic squeezing at the final stage of spermatogenesis, the nuclei of the apyrene sperm are squeezed out together with the cytoplasm, while those of the eupyrene sperm stay in the anterior part of the bundle and only the cytoplasm is discarded (Kawamura *et al*., 2000). In the triploid, however, the nuclei of the eupyrene sperm are discarded due to abnormality in the acrosome–basal body assembly and incomplete anchorage to the head cyst cell (Kawamura *et al*., 2001). As shown in Fig. 1*g–i*, apyrene sperm formation appears to proceed morphologically normally in polyploid males. Therefore, the sterility in polyploid males is caused by the lack of normal eupyrene sperm.

The apyrene spermatocytes of heat-treated Daizo (DH) males appear normal until they form spermatids. Thereafter, the normal extension of the axoneme is disturbed, and a thick axoneme surrounds the nuclei, forming a spermatocyst like a thread-ball (Figs. 1*e*, *f*). One exceptional example shown in Fig. 1*d* seems to be a eupyrene spermatocyst which enters meiosis after the spinning stage. This suggests that the heat treatment has some effect on the elongation and orientation of the axoneme in both types of sperm bundles. The eupyrene sperm that complete meiosis before the heat treatment appear quite normal. DH males which produce only eupyrene sperm become as sterile as the polyploid with only apyrene sperm (Table 1).

In this study we have established an experimental system to separate eupyrene sperm from apyrene sperm. Fertility is remarkably improved when the eupyrene and apyrene sperm are mixed through double copulation. We confirm that the apyrene sperm are

indispensable in fertilisation of the silkworm and polyploid apyrene sperm function as a substitute for the diploid (Table 1). The reason why the apyrene sperm are indispensable for fertilization is unclear at present. In *B. mori*, however, there are phenomena which do not always coincide with the proposed function of the apyrene sperm in Lepidoptera (see Introduction): (1) the presence of apyrene sperm in the bursa copulatrix does not delay female remating, (2) the spermatophore is not a requisite structure for dissociation of eupyrene sperm bundles but motility of apyrene sperm may promote the dissociation of eupyrene sperm bundles without the spermatophore in the bursa copulatrix, (3) eupyrene sperm bundles crossed the basement membrane of a testis without apyrene sperm, (4) if apyrene sperm only provided nutrients for the eupyrene sperm, then a single copulation by DH males would fertilise eggs. Watanabe *et al*. (2000) suggested that apyrene sperm migrate from the spermatophore to the spermatheca earlier than eupyrene sperm in *Papilio xuthus*. If the apyrene sperm, which show vigorous movements in female reproductive organs, help in transporting eupyrene sperm as suggested by Iriki (1941), the significant improvement in fertility by double copulation can be explained.

The irregular meiosis and loss of the nuclei by peristaltic squeezing are common in apyrene spermatogenesis of any ploidy (Wolf & Bastmeyer, 1991; Kawamura *et al*., 2000, 2001). The absence of dosage compensation as confirmed in *Bombyx mori* (Suzuki *et al*., 1998, 1999) seems to be one of the reasons why polyploid apyrene sperm retain the same function as diploid sperm. In addition, the dissociation of eupyrene sperm bundles in the bursa copulatrix may play an important role for polyploid apyrene sperm. By using the experimental approach presented here, we will be able to carry out biochemical and physiological analyses to define the role of apyrene sperm in fertilisation.

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