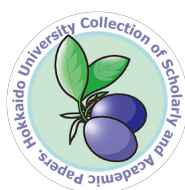




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Application of artificial insemination technique to eupyrene and/or apyrene sperm in *Bombyx mori*

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Running headline

Artificial insemination with separated eupyrene and apyrene sperm

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Footnote

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Abstract

The silkworm, *Bombyx mori*, has a dimorphic sperm system. The eupyrene sperm is the sperm to fertilize eggs and the apyrene sperm plays a crucial role for assisting fertilization. Heat-treated (33°C for 96h) Daizo (DH) males, one of the strains in the silkworm, produce only eupyrene sperm, while in triploid males only apyrene sperm are functional. Though both types of males are found to be sterile, double copulation of the two males with a single female greatly increases fertility. Here we examined the fertilizing ability of eupyrene and apyrene sperm by means of an artificial insemination technique previously established in *B. mori*. Both the eupyrene sperm collected from DH males and the apyrene sperm from triploid males do not have the ability to fertilize eggs, respectively. Artificial insemination with the mixture of eupyrene and apyrene sperm leveled up the frequency of fertilized eggs, more than 80 %. When cryopreserved DH sperm (eupyrene sperm) were subjected to the same experiment, fertilized eggs of more than 95% were obtained. These results confirmed that apyrene sperm play an important and indispensable role in fertilization in *B. mori*. Separation of functional eupyrene sperm from functional apyrene sperm and success of fertilization by means of the artificial insemination technique are applicable for further studies to elucidate the function of apyrene sperm.

Introduction

Lepidopteran males produce dimorphic sperm, eupyrene (nucleate) and apyrene (anucleate) sperm (Meves, '03). Eupyrene sperm are ordinary sperm to fertilize eggs, while the function of apyrene sperm has long been a matter of speculation (Jamieson, '87). Cook and Wedell ('99) suggested that apyrene sperm in *Pieris napi* might be instrumental in delaying female rematings. This hypothetical function is considered to be effective to prevent sperm competition. There is also a “kamikaze sperm” hypothesis (Silberglied et al., '84) that claimed apyrene function as a tool for male-to-male reproductive competition. On the other hand, three hypotheses based on vigorous movements of apyrene sperm have been proposed: 1) they help eupyrene sperm bundles to cross the basement membrane of a testis (Katsuno, '77), 2) they promote dissociation of eupyrene sperm bundles in a spermatophore (Osanai et al., '87) and 3) they transfer eupyrene from brusa copulatrix to spermatheca (Holt and North, '70; Friedländer and Gitay, '72). Because eupyrene and apyrene sperm are difficult to separate and are transferred together into the female reproductive organs by copulation, the behavior and/or crucial function of each kind of sperm have long been unclear. Sahara and Kawamura (2002), however, developed an experimental system in which separation of eupyrene and apyrene sperm becomes possible by using heat-treated diploid and triploid males. Heat-treated diploid males provide only eupyrene sperm and triploid males produce only apyrene sperm that are functional. Although both heat-treated and triploid males were almost sterile (0 and 0.10 % in average fertility), double copulation of these males with a female greatly improved the fertility (approx 75% in average). These supplied evidence that apyrene sperm is indispensable for fertilization (Sahara and Kawamura, 2002).

Artificial insemination techniques have been studied in some insects, *Aedes aegypti* (Burcham, '57), *Apis mellifera* (Watoson, '27; Harbo, '85), *Bombyx mori* (Ômura, '36), *Cimex lectularius* (Davis, '65) and *Drosophila melanogaster* (Gottschewski, '37). In *B. mori*, Takemura et al. ('96;'99) developed the most effective method of artificial insemination, by which fertilizes eggs are obtained at normal rate, almost 100%. Osanai et al. ('89) succeeded in separating eupyrene and apyrene sperm bundles by using a Percoll gradient centrifugation. However, they could not confirm the fertility of separated sperm because no artificial insemination technique for *B. mori* was applicable then. In this paper, we define that mixture of separated eupyrene and apyrene sperm can fertilize the eggs by means of artificial insemination, though eupyrene sperm without the presence of apyrene sperm failed to fertilize eggs. We will also discuss the role of

apyrene sperm in fertilization and application of this technique for maintenance of genetic stocks in the silkworm.

MATERIALS AND METHODS

Bombyx strains

The strains of the silkworm (*Bombyx mori*) used were re9 females (red egg, striped: re/re , p^S/p^S) and Tw1 males (white egg 2, plane: $w-2/w-2$, p/p) for tetraploid induction, and rs males (red egg, striped: re/re , p^S/p^S) for triploid production. For fertility examination of semen, from triploid and/or Daizo (black egg, normal marking: $+^{w-2}/+^{w-2}$, $+^{re}/+^{re}$, $+^P/+^P$) males, the females of F₁ progeny from Cre females (sex-limited sable, plane, red egg: T(W:2) p^{Sa} , p/p , re/re) and Eq (red egg, plane: p/p , re/re) males (CE) was used. Rearing temperature was kept at 26°C.

Induction of polyploid silkworms

F₁ eggs of the cross between an re9 female and a Tw1 male at the first cleavage stage (120 to 150 min after oviposition) were cooled to -10°C for 24h and then returned to room temperature. The eggs with large serosa nuclei were selected as the tetraploid (rw4n) (Kawamura, '79). Since the meiotic chromosomes in the female silkworms perform no crossing-over, the tetraploid females are as fertile as the diploid ones. By crossing an rw4n female with a diploid male, we obtained triploid individuals. Polyploidy of the progeny was confirmed by the egg color segregation ratio of black to red eggs in the backcross, 5:1 in triploid and 1:1 in diploid.

Induction of sterility in Daizo males

Sterile Daizo (DH) males were obtained following the method of Sugai & Hanaoka ('72) with a slight modification. The males at the spinning stage were subjected to the temperature of 33°C for 96 to 120h and returned to room temperature.

Cryopreservation of DH semen

Cryopreservation and thawing procedure followed the method developed by Takemura *et al.* (2000). The semen in Grace's insect cell culture medium (Invitrogen) with 5% dimethyl sulfoxide (DMSO) was placed in a 0.25 ml plastic straw. The semen straw was frozen in a deep freezer at -80°C, and then put in liquid nitrogen. The frozen semen in the straw was thawed in water at 37°C for 5 sec.

Artificial insemination of sperm

The artificial insemination was carried out according to the method reported by Takemura *et al.* ('96, '99). For collection of the semen, the whole internal reproductive organs were dissected from dozens of males. The sperm, which was collected from ruptured seminal vesicles, was kept in a sterilized ice-cold dish until use. Since the function of sperm activation in the glandula prostatica could be replaced with trypsin (Takemura *et al.*, '99), the sperm was mixed with an equal volume of 0.3 µg/ml trypsin (crystal trypsin from porcine pancreas; specific activity, 5300 USP units per mg) in Grace's medium. Five to 10µl aliquots of semen from non-treated Daizo males (DC), Daizo males sterilized by high temperature treatment (DH), triploid males and a 1:1 mixture of DH with triploid were injected into the bursa copulatrix of virgin CE female moths. The experiment was repeated twice. The same procedure was adapted to test the fertilizing ability of the semen mixture of cryopreserved DH (Cryo-DH) and triploid.

RESULTS AND DISCUSSION

Apyrene sperm bundles in DH males and eupyrene sperm bundles in triploid males had been confirmed to be morphologically abnormal (Kawamura, et al., 2001, Sahara and Kawamura, 2002). Eupyrene sperm bundles in DH males and apyrene sperm bundles in triploid males, however, accomplish maturity through peristaltic squeezing (Kawamura et al., 2000; 2001; Sahara and Kawamura, 2002). It was previously ascertained that these males produce few offspring (0 to 0.10 %), but fertility is greatly improved by copulation of both types of males with a single female (74.64 to 78.74 %) (Sahara and Kawamura, 2002).

It has been well known that apyrene sperm in Lepidoptera are produced in great numbers (Silberglied et al., 1984). In the silkworm, the ratio of apyrene sperm to eupyrene sperm in a testis is calculated at approximately 7:3 (He et al., 1996; Kawamura and Sahara, 2002). Table 1 shows the results of two independent experiments of fertilization by artificial insemination. The fertility more than 98% disclosed by the untreated Daizo control males means that the artificial insemination has been successfully carried out as in the previous report (Takemura et al., '99).

When the same technique is applied to the semen mixture of DH and triploid, the fertility is comparably high, more than 80% in two replications. Since we mixed equal volume of the semen from DH and triploid, it probably keeps the constant ratio of apyrene sperm to eupyrene sperm, 7:3. The injection of semen either from DH or from triploid males showed no fertility at all (Table 1). The results coincide well with the previous results obtained in double copulation experiment (Sahara and Kawamura, 2002). It is very hard to regulate the ratio of eupyrene and apyrene sperm in double copulation. The application of artificial insemination technique to separated eupyrene and apyrene sperm open the gate to examine whether there is a dose response of the apyrene sperm. The ratio of apyrene sperm to eupyrene sperm known in Lepidoptera other than *B. mori* is a 5:1 for *Spodopetra litura* (Etman and Hooper, '79), a 9.6:1 for *Manduca sexta* (Shepherd, unpublished data in Silberglied et al., '84), a 9:1 for *Plodia interpuncterlla* (Gage and Cook, '94) and a 9.5:1 for *Ephesia kueniella* (Koudelová and Cook, 2001). It is also interesting if the species-specific ratio of apyrene sperm to eupyrene sperm is important for successful fertilization in Lepidoptera.

The spermatophore, a pouch containing eupyrene and apyrene sperm, occurs in the female reproductive organ (bursa copulatrix) during lepidopteran mating (Ômura, '38). The number of spermatophores in the bursa copulatrix corresponds to the number of copulations (Burns, '68). On the other hand, artificial insemination produces no such

spermatophore in the bursa copulatrix (Takemura et al., '99). The absence of such obstructing structures makes it easier to observe the behavior of eupyrene and apyrene sperm in the female reproductive organs. In the double copulation experiment by Sahara and Kawamura (2002), two spermatophores existed in the bursa copulatrix of a female. Successful fertilization in both experiments implies 1) that the spermatophore itself plays no significant role in the fertilization process, and 2) that the separation of the two types of sperm by a spermatophore wall does not inhibit subsequent mixing. When DH semen was injected into a female, the eupyrene bundles stayed in the bursa copulatrix, while the injected apyrene sperm from triploids proceeded to the spermatheca. Mixture of apyrene and eupyrene sperm in the bursa copulatrix might be indispensable for eupyrene sperm to proceed into the spermatheca besides eupyrene bundle dissociation (Osanai et al., '87).

Rapid arrival of spermatozoa to the fertilization point appears to be a ubiquitous phenomenon among mammals (Blandau, '69; Bedford, '72). The vast majority of rabbit spermatozoa in this vanguard is dead and certainly plays no direct role in fertilization (Overstreet and Cooper, '78). The behavior of the vanguard spermatozoa in mammals and apyrene sperm in lepidopteran insects is parallel in the point that the sterile sperm precede the fertile sperm in female reproductive organ. The occurrence of the similar phenomenon with the vanguard spermatozoa and the apyrene sperm which belong to far distant phylogeny may be of evolutionary interest.

Karube and Kobayashi ('99) suggested that the arrival of eupyrene sperm at the vestibulum accelerates egg deposition. Since the females which received the injection of semen mixture increased the number of deposited eggs (Table 1), the advance of eupyrene sperm in the female reproductive organs may indeed be a deposition stimulus.

If triploid sperm fertilized the CE eggs, the color of deposited eggs is expected to become red or black and the larval skin to be a striped pattern (p^S). The colors of the fertilized eggs, however, were all black and the larval skin was of DH type ($+^P$). These observations disclosed that only DH eupyrene sperm participate in the fertilization.

The maintenance of genetic bioresources is one of the important goals for biological studies. In the case of the silkworm, the importance of commercial strains for effective production of silk and of mutant strains as experimental animals is recognized. The artificial insemination in *B. mori* was successfully performed using cryopreserved semen in most strains tested (Takemura and Kanda, '99; Takemura et al., 2000). This opens the gate to store the resources without rearing the strains every year. Unfortunately, cryopreserved semen had a low fertilization capacity, or none, in some strains (Takemura and Kanda, '99; unpublished data). Semen dissected from the

vesicula seminalis contained eupyrene sperm bundles and apyrene spermatozoa (Osanai et al., '88; '89). Since liquid nitrogen seems to produce some damages to the sperm during storage, failure of fertilization by artificial insemination may be caused by dysfunction of eupyrene sperm bundles and/or apyrene spermatozoa. In this experiment, we confirmed that the assistance of apyrene sperm is indispensable for fertilization by eupyrene sperm. Because fertility of the cryopreserved eupyrene sperm of DH males is increased by the addition of triploid apyrene sperm (Table 1), the damage to eupyrene sperm is probably not the limiting factor. On the other hand, injection of cryopreserved eupyrene sperm of DH into a female that had been mated with a triploid male beforehand showed only a little increase in fertility (Table 1). Therefore, the simultaneous presence of the two types of sperm in a bursa copulatrix may be important for successful fertilization. The main cause of sterility in cryopreserved semen appears to be severe damage to the apyrene spermatozoa. These results suggest that there is a possibility to use triploid apyrene sperm when apyrene sperm has become dysfunctional in cryopreservation.

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Table 1 Fertility with artificial insemination.

Semen from	n	Average no. of eggs deposited	Fertility (%)	5% confidence interval*1
DC *2	7	464	98.25	93.88 - 100
<i>Exp1-1</i>				
DH *3	9	30.2	0	
Triploid	11	74.3	0	
DH+Triploid*4	9	195.7	80.12	66.27 - 88.56
<i>Exp1-2</i>				
DH*3	8	82.8	0	
Triploid	8	117.6	0	
DH+Triploid*4	10	362.3	86.23	75.38 - 93.66
<i>Exp2</i>				
Cryo-DH+Triploid*5	3	379.3	95.08	91.20 - 97.84
Triploid+ Cryo-DH*6	3	266	1.00	0.04 - 2.96

*1 The value was calculated with arcsin-root transformed data; *2 Untreated Daizo males; *3 Daizo males placed at 33°C for 96-120h; *4 Semen mixture of DH and triploid males (see Materials and methods); *5 Semen mixture of cryopreserved DH and crude triploid males (see Materials and methods); *6 semen injection of cryopreserved DH into a female that was mated with a triploid male beforehand.