HOKKAIDO UNIVERSITY

Title	Apyrene sperm from the triploid donors restore fecundity of cryopreserved semen in Bombyx mori
Author(s)	Takemura, Yoko; Sahara, Ken; Mochida, Yuji; Ohnuma, Akio
Citation	Journal of Insect Physiology, 52(10), 1021-1026 https://doi.org/10.1016/j.jinsphys.2006.06.010
Issue Date	2006-10
Doc URL	http://hdl.handle.net/2115/16978
Type	article (author version)
File Information	JIP52-10.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Apyrene sperm from the triploid donors restore fecundity of cryopreserved semen in *Bombyx mori*

Yoko Takemura[a], Ken Sahara[b*], Yuji Mochida[a] and Akio Ohnuma[a]

[a] Institute of Sericulture, 1053 Iikura, Ami-machi, Ibaraki 300-0324, Japan

[b] Laboratory of Applied Molecular Entomology, Graduate School of Agriculture,

Hokkaido University, Sapporo 060-8589, Japan

*To whom Correspondence should be addressed,
Dr. Ken Sahara

Fax: +81-11-706-2402. Tel: +81-11-706-2402
E-mail: sahara@abs.agr.hokudai.ac.jp

Phone & Fax: 011-706-2402

Running title: Cryopreserved sperm in *Bombyx mori*

Abstract

Female moths of *Bombyx mori* were artificially inseminated with cryopreserved semen. The fertility of inseminated females varied from 0% to 76.9% depending on the strain. Addition of fresh semen from triploid males, which are infertile but whose semen includes intact apyrene sperm, greatly improved fecundity of cryopreserved semen from normal males. Frozen apyrene sperm from the triploid donors also improved the fecundity of females, inseminated with cryopreserved normal semen, but less than fresh semen from triploid males. Fertilization success in *B. mori* requires the presence of both, intact eupyrene and apyrene sperm. Our results show that eupyrene sperm tolerate the cryopreservation process better than apyrene sperm. Hence, we recommend to add apyrene sperm from the triploid donors as helper sperm routinely to cryopreserved semen in artificial insemination. This may advance the application of cryopreservation as a routine technique to maintain silkworm resources. The technique may also be applicable to other moth and butterfly species which, like *Bombyx mori*, possess eupyrene and apyrene sperm.

Key words: apyrene sperm; artificial insemination; cryopreservation; eupyrene sperm; silkworm

1. Introduction

Cryopreservation of sperm and artificial fertilization and/or artificial insemination serve practical and research purposes in animals (Polge and Rowson, 1952; Graham et al., 1978; Polge, 1978). In insects, methodological progress in this field may reduce work cost and laboratory space in genetically important species such as *Drosophila melanogaster, Apis mellifera* and *Bombyx mori*.

B. mori is not only genetically but also economically important. More than 3,000 strains (Yamamoto, 2000) and 400 mutations are known which have to be maintained by rearing year after year. Recently, a feasible artificial insemination technique has been developed in this species (Takemura et al., 1996; 1999), and successfully applied to some races and hybrids using cryopreserved semen (Takemura and Kanda, 1999; Takemura et al., 2000). This would open the gate to the frozen storage of silkworm genetic resources without rearing the strains every year. The fertility of cryopreserved semen and the number of fertilized eggs, however, varies depending on the race used (Takemura and Kanda, 1999). Unfortunately, there are even races that do not tolerate routine cryopreservation at all (this study).

Semen dissected from the vesicula seminalis contains eupyrene sperm bundles

(fertilizing) and apyrene spermatozoa (non-fertilizing but indispensable for fertilization; Osanai et al., 1988; Sahara and Kawamura, 2002, 2004; Sahara and Takemura, 2003; also see review: Friedländer et al., 2005). Here we investigate the question of which type of sperm is affected by the freezing and thawing process. We set out to develop a more reliable method of artificial insemination using cryopreserved semen. This study, therefore, may contribute to the establishment of a widely applicable, long-term, silkworm semen cryopreservation and artificial insemination protocol.

2. Materials and methods

2.1. Bombyx mori *strains*

The *B. mori* L. strains used were Pure Mysole, Uryu, re9, Tw1, p50 and hybrids of Hitachi × Nishiki and W2. re9 is a red egg (*re*/*re*) and stripe larval skin (p^S/p^S) mutant. Tw1 and W2 are white egg (*w*-2/*w*-2) and plane (*p*/*p*) larval skin mutants. The other strains all yield fertile eggs with a wild-type (black) color ($+^{w\text{-}2}/+^{w\text{-}2}$, $+^{re}/+^{re}$). p50 is a regional Chinese strain, which was used in whole genome shotgun sequencing (Mita et al 2004). Pure Mysole and Uryu are regional Indian and Chinese strains respectively, and Hitachi × Nishiki is a practical hybrid for silk production. Since wild-type (black)

egg color is genetically dominant over the white egg mutant, eggs obtained by cross-mating of W2 and wild-type egg color races become pigmented after fertilization, whereas eggs remain whitish if they are not fertilized. This is a phenotypic marker for assessing fertilization. Fertility of cryopreserved Tw1 semen was determined by monitoring embryogenesis since the sperm carried the white egg mutation.

2.2. Induction of triploids

Type-A triploid

F_1 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 ^{o}C for 24h and then returned to room temperature. Eggs with large serosa nuclei were selected as tetraploids (rw4n) (see Kawamura, 1979). Since meiotic chromosomes in female silkworms perform no crossing-over, tetraploid females are as fertile as diploid ones. By crossing an rw4n female with a diploid male (rs), 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 for triploids and 1:1 for diploids.

Type-B triploid

Type-B triploid eggs were obtained using CO_2 gas treatment (Tazima and Onuma, 1967). F_1 females (black egg; $+^{pe}$ *re/pe* $+^{re}$) were obtained from mating between an re9 (red egg; *re*/*re*) female and a pe male (pink egg; *pe/pe*). Ten minute old eggs from the F_1 females crossed with pere (white egg; *pe re/pe re*) males were treated with CO_2 gas for 2h at room temperature. Because crossing-over does not occur in female silkworms, diploid eggs from the cross are either red ($+^{pe}$ *re/pe re*) or white (*pe* $+^{re}$*/pe re*). We selected exceptional black color eggs as polyploids, a mixture of triploids and tetraploids (see Tazima and Onuma, 1967). The type-B triploid males used in our experiments resulted from crosses of the tetraploid females with diploid males.

2.3. Cryopreservation

For cryopreservation and thawing we followed the method developed by Takemura et al. (2000). A 10% dimethylsulfoxide (Me_2SO) solution in Grace's medium was added to the same volume of semen as a cryoprotectant. For the freeze-and-thaw damage experiment using Hitachi × Nishiki, we exceptionally used 5% Me_2SO in Grace's medium. The semen was placed in a 0.25ml straw and frozen in a mechanical freezer (-80 ^{o}C) for 10 min. Then the straw was transferred to liquid nitrogen. After being kept

at -196°C for 30 min, the straw was thawed in water at 37 °C for 10 seconds. At the time of insemination, the thawed semen was mixed with the trypsin solution as described below or with trypsin solution plus sperm from the triploid individuals.

2.4. Artificial insemination

Artificial insemination was carried out according to the method reported by Takemura et al. (1996, 1999). For collection of semen, whole internal reproductive organs were dissected from dozens of males. The semen collected from ruptured seminal vesicles was kept in a sterilized ice-cold dish until we started the freezing process. Because the function of sperm activation in the glandula prostatica can be mimicked with trypsin (Takemura et al., 1999), the thawed semen was mixed with 1/4 volume of 0.75μg/ml trypsin (crystalline trypsin from porcine pancreas; specific activity, 5300 USP units per mg, Wako Pure Chemical Industries Ltd., Osaka, Japan, cat. No. 207-09891) in Grace's medium. A 10-12μl aliquot of a 1:1 mixture of semen and Grace's medium was injected into the bursa copulatrix of virgin moths.

In the experiments with helper sperm, the apyrene sperm from the triploid donors was mixed with frozen-and-thawed semen before trypsin treatment. The final mixture contained sperm from the triploid individuals, semen and Grace's medium in a

1 : 1 : 1 ratio.

3. Results

When female moths are artificially inseminated with cryopreserved semen from hybrid males, they lay almost as many eggs as control moths which have mated normally (Takemura and Kanda, 1999; Mochida et al., 2003). Semen of some pure races, however, is adversely affected by cryopreservation (Takemura and Kanda, 1999). This was confirmed in the present study. We even found one strain, Tw1, whose semen completely lost fertility after cryopreservation. Previously published and new fertilization data have been compiled in Table 1.

These results prompted us to investigate improvements of the cryopreservation and artificial insemination technique. Since Sahara and Takemura (2003) had successfully used a mixture of apyrene sperm from triploid males with cryopreserved eupyrene sperm, we investigated the benefits of this method. As a test strain, Tw1 could have been the best if the white egg 2 mutant had not prevented easy discrimination of fertilized from unfertilized eggs by egg color (see Materials and Methods). Instead, we selected for the experiments two races, Uryu and p50, with low fertility and one race,

Pure Mysole, with moderately high fertility after insemination with cryopreserved semen (see Table 1).

Fecundity in these strains was tested after (1) natural mating, (2) artificial insemination with cryopreserved semen and (3) artificial insemination with cryopreserved semen plus fresh, unfrozen semen from type-A triploids. Fertility after artificial insemination of cryopreserved semen was about the same as that in the previous experiments and noticeably lower than after natural mating (Table 2). The addition of fresh semen from type-A triploid individuals substantially increased the fecundity of the inseminated females even though the concentration of eupyrene sperm in the inseminating mixture was less (2/3, see materials and methods). It is therefore evident that the damage done to semen by the freeze-and-thaw procedure can be remedied by the addition of fresh but infertile semen from triploid donors. The fertilized eggs of all three races hatched with similar percentage success rates (Table 2).

Cryopreserved semen of the hybrid Hitachi × Nishiki retained the ability to fertilize eggs even after one year of storage (Takemura et al., 2000). In order to investigate freeze-and-thaw damage to apyrene sperm we subjected semen of this cryotolerant hybrid to harsher conditions, namely a reduced concentration of the cryoprotectant (5% Me_2SO). Under these conditions, Hitachi × Nishiki semen

completely lost its fertilization ability (Table 3, compare lines 1 and 2). Nevertheless, addition of fresh semen from the type-B triploid donors restored fecundity (Table 3, line 3). Addition of semen from the same triploid donors, which had been cryopreserved using 10% Me_2SO restored fertility to a similar extent as fresh semen from triploid donors (Table 3, line 4). Presumably, therefore, it is the apyrene sperm of Hitachi × Nishiki that is damaged by the 5% Me_2SO freeze-and-thaw procedure.

The generation of triploid males as donors for fresh apyrene sperm is a tedious procedure which would inhibit the application of our results as a routine technique. In a first step to reduce the expenditure of time and work, we tested the performance of the two types of hybrid semen (required for the generation of both type-A and type-B triploids) after cryopreservation. The fertility of both types of cryopreserved semen after artificial insemination (without addition of helper sperm from the triploid donors) was only slightly reduced when compared with that of fresh semen after natural mating (Table 4). This shows that cryopreserved semen can be used for the induction of both types of triploids, thus saving time and money for a routine application. Type-B triploid apyrene sperm cryopreserved with standard conditions (10% Me_2SO) was able to restore the fertility of Hitachi × Nishiki semen that had been kept under harsher conditions (5% Me_2SO) (Table 3, lines 2 and 4). Although not quite as good as fresh

apyrene sperm (Table 3, compare lines 3 and 4), the use of cryopreserved helper sperm from the triploid donors would make the technique much more convenient. Therefore we tested the performance of cryopreserved helper sperm from triploid donors systematically in combination with cryopreserved Uryu semen. In this experiment, frozen Uryu semen completely lost fertility when used without the helper sperm (Table 5, compare lines 1 and 2). The restoration of fecundity by addition of fresh apyrene sperm from triploid donors was good (Table 5, lines 3 and 4). In experiments with cryopreserved helper sperm from triploid donors, the performance of type-B sperm was noticeably better than that of type-A sperm (Table 5, lines 5 and 6).

4. Discussion

In this paper we have shown that cryopreservation of semen from some silkworm genotypes results in a loss of fertility, but that this loss of fertilizing ability can be rescued by mixing the cryopreserved sperm with fresh semen from triploid donors. We interpret these results as indicating that apyrene sperm being preferentially disabled by the freeze-and-thaw procedure, but that these apyrene sperm can be replaced by the addition of fresh apyrene sperm from the triploid donors. Our results show furthermore

that the infertile nucleated (“eupyrene”) sperm of the triploid males do not interfere to a noticeable extent with the ability of fertile eupyrene sperm from the diploid donors. Our results also confirm that apyrene sperm do not distinguish “self” from “non-self” eupyrene sperm, as previously shown by Sahara and Takemura (2003). This fits well to the hypothesis that Lepidoptera avoid sperm competition by delay of re-mating (Cook and Wedell, 1999). On the other hand, the “kamikaze sperm” hypothesis (Silberglied et al., 1984), in which apyrene function is claimed to be a tool for male-to-male reproductive competition, does not fit the results.

The greater sensitivity of apyrene sperm to freeze-thaw cycles is illustrated by the experiment (Table 3) in which Hitachi × Nishiki semen was cryopreserved using either 5% or 10% Me_2SO. When high Me_2SO conditions were used, both types of sperm were successfully preserved, and the fertility of inseminated females was high. But when low Me_2SO was used, damage to apyrene sperm led to seriously compromised fertility. The lost fertility was, however, completely rescued when semen from triploid donors was added. This must mean that the eupyrene sperm from this diploid strain are able to survive freezing in 5% Me_2SO, while apyrene sperm from this strain are not.

In the vesicula seminalis (from which we collected the semen samples) eupyrene sperm are maintained in bundles, whereas the corresponding bundles of apyrene sperm

are already fully dissociated, so that these sperm are found singly (Katsuno, 1977). The preferential disabling of apyrene sperm by freezing and thawing may result from this difference. A similar loss of fertility has been found in frozen semen pretreated with trypsin solution before freezing (Takemura et al., 2000).

We systematically showed that cryopreserved apyrene sperm from both types of triploid donor restored the fecundity of frozen Uryu semen which had completely lost its fertilization ability (Table 5). While both types of triploid semen proved adequate for most practical purposes, we recommend to use semen from type-B triploids when high fertilization efficiency is required. Our results extend the possibilities to maintain silkworm resources by cryopreservation in a routine procedure by either fresh or frozen helper sperm from the triploid donors.

The different performance of cryopreserved type-A and type-B helper sperm (Table 5), like that of different strains (see Table 1), suggests that genetic factors may affect the cryotolerance of apyrene sperm. A search for alternative strain combinations in triploid induction may be useful for further improvements of cryotolerance of apyrene sperm from the triploid males.

The experiments reported here clearly demonstrate that cryopreservation of semen together with artificial insemination is applicable for storage of genetic silkworm

resources. Since individuals of both sexes of *B. mori* can be obtained from the combination of cryopreserved ovary and semen (Mochida et al., 2003), our improvements may help to establish a system for the maintenance of whole genomes from widely different races. The techniques may also prove applicable to the preservation of rare species in Lepidoptera.

Acknowledgements

We thank Mr. Yasuhiro Yamada (Field Science Center for Northern Biosphere, Hokkaido University, Japan) for rearing the silkworms. Our thanks also go to Prof. Walther Traut (Medizinische Universtät zu Lübeck, Germany) for critical discussion and Dr. Hajime Inoue (Institute of Sericulture, Japan) for valuable advice. This study was partially supported by a Grant-in-Aid for Scientific Research no. 15380044 from JSPS (K.S).

References

Cook, P.A., Wedell, N. 1999. Non-fertile sperm delay female remating. Nature 397, 486.

Friedländer, M., Seth, R.K., Reynolds, S.E. 2005. Eupyrene and apyrene sperm:Dichotomous spermatogenesis in Lepidoptera. In: S.J. Simpson, S.J. (ed.)

Advances in insect physiology. Academic press, London, vol. 32, pp. 206-308.

Graham, E.F., Schmehl, M.K.L., Evenson, B.K., Neson, D.S. 1978. Semen preservation in non-domestic animals. In: Watson P.F. (Ed.), Artificial breeding of non-domestic animals. Academic press, London, Symposia of the zoological society of London, No. 43 pp. 153-173.

Katsuno, S. 1977. Studies on eupyrene and apyrene spermatozoa in the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) I. The intratesticular behaviour of the spermatozoa at various stage from the 5th-instar to the adult. Applied Entomology and Zoology 12, 142-153.

Kawamura, N. 1979. Polyploidy and size of serosa nuclei and cells in eggs of the silkworm, *Bombyx mori*. The Journal of Sericultural Science of Japan 48, 77-85.

Mita, K., Kasahara, M., Sasaki, S., Nagayasu, Y., Yamada, T., Kanamori, H., Namiki, N., Kitagawa, M., Yamashita, H., Yasukochi, Y., Kadono-Okuda, K., Yamamoto, K., Ajimura, M., Ravikumar, G., Shimomura, M., Nagamura, Y., Shin-i, T., Abe, H., Shimada, T., Morishita, S., Sasaki, S. 2004. The genome sequence of silkworm, *Bombyx mori*. DNA Research. 11, 27-35.

Mochida, Y., Takemura, Y., Kanda, T., Horie, Y. 2003. Fertilized eggs obtained from transplantation of frozen ovaries and parthenogenesis in combination with artificial insemination of frozen semen of the silkworm, *Bombyx mori*. Cryobiology 46, 153-160.

Osanai, M., Kasuga, H., Aigaki, T. 1988. Functional morphology of the glandula prostatica, ejaculatory valve, and ductus ejaculatorius of the silkworm *Bombyx mori*. Journal of Morphology 198, 231-241.

Polge, C. 1978. Fertilization in pig and horse. Journal of Reproduction and Fertility 54, 461-470.

Polge, C., Rowson, L.E.A. 1952. Fertilizing capacity of bull spermatozoa after freezing at –79^{o}C. Nature 169, 626-627.

Sahara, K., Kawamura, N. 2002. Double copulation of a female with sterile diploid and polyploid males recovers fertility in *Bombyx mori*. Zygote 10, 23-29.

Sahara, K., Kawamura, N. 2004. Roles of actin networks in peristaltic squeezing of sperm bundles in *Bombyx mori*. Journal of Morphol. 259, 1-6.

Sahara, K., Takemura, Y. 2003. Application of artificial insemination technique to eupyrene and/or apyrene sperm in *Bombyx mori*. Journal of Experimental Zoology Part A 297A, 196-200.

Silberglied, R.E., Shepherd, J.G., Dickinson, J.L. 1984. Eunuchs: the role of apyrene sperms in Lepidoptera? The American Naturalist 123, 255-265.

Takemura, Y., Kanda, T., Tamura, T., Shinbo, H., Horie, Y. 1996. Development of new method for artificial insemination of the silkworm, *Bombyx mori.* The Journal of Sericultural Science of Japan 65, 456-463.

Takemura, Y., Kanda, T. 1999. Artificial insemination and oviposition using cryopreserved sperm in several strains of the silkworm, *Bombyx mori*. The Journal of Sericultural Science of Japan 68, 327-332.

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

Takemura, Y., Kanda, T., Horie, Y. 2000. Artificial insemination using cryopreserved sperm in the silkworm, *Bombyx mori*. Journal of Insect Physiology 46, 491-497.

Tazima, Y., Onuma, A. 1967. Experimental induction of androgenesis, gynogenesis, and polyploidy in *Bombyx mori* by treatment with CO_2 gas. The Journal of Sericultural Science of Japan 36, 286-292.

Yamamoto, T. 2000. Silkworm strains. In: Nakatsuji, N. (Ed.), Strain maintenance and databank for life science. Kyoritsu Shuppan, Tokyo, pp.45-49.

Table 1
Fertility of semen after cryopreservation and artificial insemination

name of strain	season*	N	recipient female	eggs laid	fertilized eggs				fertility (%)			hatching larvae (%)**	references
					total	MAX	MIN	Median	MAX	MIN	Median		
Hybrid													
Fu Yo × Tsukuba Ne	summer	10	sister	6,238	6,048	706	511	619	98.4	95.5	96.9	95.6	1
Hitachi × Nishiki	winter	10	W2	5,520	5,352	609	472	544	97.9	94.6	97.3	96.1	2
Kai Ryo × Ake Bono	winter	10	sister	5,007	4,847	572	449	476	99.6	94.9	95.8	91.0	1
Hitachi × Nishiki	-***	15	W2	7,211	6,804	595	175	474	98.1	95.1	95.1	95.5	3
Asa Hi × To Kai	summer	10	sister	5,347	4,872	630	370	471	98.3	75.6	95.8	97.5	1
Race													
Tsukuba	winter	15	sister	5,923	5,454	545	232	363	96.8	85.8	92.2	94.5	1
re9	summer	10	sister	3,718	2,787	394	169	275	83.9	55.1	76.9	78.5	4
To	winter	15	sister	3,418	3,088	275	154	199	93.9	83. 4	91.0	76.8	4
re9	winter	9	W2	1,809	1,316	492	26	102	93.4	20.6	67.9	ND	1
Asa	winter	15	sister	3,996	1,568	193	18	89	60.1	12.9	35.7	95.2	1
Koishimaru	spring	15	sister	3,157	2,275	373	28	86	98.7	22.8	68.3	83.3	1
Hi	summer	15	sister	2,481	1,035	204	1	65	75.0	2.0	37.0	90.7	1
Uryu	autumn	15	sister	3,501	985	251	1	65	59.3	1.52	25.0	86.4	1
Pure Mysole	winter	15	sister	851	540	47	39	37	87	62.2	68.3	90.3	1
P50	winter	9	W2	1,867	328	92	2	27	71.8	0.4	21.1	ND	4
Uryu	winter	10	W2	1,834	272	198	0	2.5	58.9	0	1.4	ND	4
Tw1	summer	8	W2	620	0	0	0	0	0	0	0	-	4

*reared with mulberry leaves during spring-autumn and artificial diet in winter
** % values relative to fertilized eggs
***semen cryopreserved for one year
ND: not determined

1 Takemura and Kanda (1999); 2 Mochida et al. (2003); 3 Takemura et al. (2000); 4 This study

Table 2
Fecundity of p50, Uryu and Pure Mysole in natural mating and artificial insemination using cryopreserved semen with and without fresh semen from the type-A triploids

Race	semen	insemination	N	recipient female	eggs laid	fertilized eggs				fertility (%)			hatching larvae (%)*
						total	MAX	MIN	Median	MAX	MIN	Median	
p50	fresh	by mating	8	p50	3,349	3,319	492	364	417	99.8	98.1	99.3	91.2
	frozen	artificial	6	p50	206	54	39	0	44	76.5	0	12.1	82.9
	frozen + fresh triploid	artificial	5	p50	928	795	339	68	129	93.9	61.8	87.2	88.6
Uryu	fresh	by mating	8	Uryu	3,116	3,002	405	349	377	99.0	93.8	96.4	90.9
	frozen	artificial	7	Uryu	420	162	53	5	21	48.2	28.6	38.2	87.2
	frozen + fresh triploid	artificial	15	Uryu	4,816	4,050	369	138	260	98.1	47.6	85.0	90.8
Pure Mysole	fresh	by mating	15	Pure Mysole	5,302	5,091	386	280	348	98.7	89.2	96.6	85.7
	frozen	artificial	10	Pure Mysole	1,318	988	138	11	104	67.0	31.4	72.0	82.4
	frozen + fresh triploid	artificial	12	Pure Mysole	2,782	2,328	325	19	209	93.9	35.8	83.3	83.8

* % values relative to fertilized eggs

Table 3
Addition of apyrene sperm from the triploid donors restores fecundity of Hitachi × Nishiki semen cryopreserved in 5% Me_2SO

Hitachi × Nishiki semen	semen from triploids added	insemination	N	recipent female	eggs laid	fertilized eggs				fertility (%)			
						total	MAX	MIN	Median	total	MAX	MIN	Median
fresh	-	by mating	9	W2	5,810	5,709	485	704	661	98.1	93.6	99.9	99.1
cryopreserved	-	artificial	9	W2	201	0	0	0	0	0	0	0	0
cryopreserved	fresh	artificial	9	W2	5,754	5,446	658	525	618	94.7	97.1	87.7	95.4
cryopreserved	cryopreserved in 10% Me_2SO	artificial	9	W2	3,997	3,436	552	151	412	84.3	94.2	59.6	87.5

Table 4
Cryotorelant hybrid sperm for the induction of triploids

hybrid	for triploid	semen	insemination	N	recipient female	eggs laid	fertilized eggs				fertility (%)		
							total	MAX	MIN	Median	MAX	MIN	Median
[re9×Tw1] × rs	type-A	fresh	by mating	5	W2	2,341	2,070	493	341	397	92.7	75.0	90.4
		crypopreserved	artificial	9	W2	4,320	3,264	396	334	358	78.7	73.6	74.1
[re9×pe] × zepere	type-B	fresh	by mating	10	W2	4,628	4,499	487	391	461	99.6	89.0	98.4
		crypopreserved	artificial	9	W2	4,093	3,804	426	380	403	94.1	86.2	94.1

Table 5
Cryotolerance of helper sperm from the triploid donors tested in combination with Uryu semen

Uryu semen	triploid male	apyrene sperm from triploids	insemination	N	recipient female	eggs laid	fertilized eggs				fertility (%)			hatching larvae (%)*
							total	MAX	MIN	Median	MAX	MIN	Median	
fresh	-	-	by mating	10	Uryu	3,435	3,137	509	124	308	98.8	81.3	92.1	86.0
cryopreserved	-	-	artificial	8	Uryu	1,529	0	0	0	0	0	0	0	-
cryopreserved	type-A	fresh	artificial	15	Uryu	4,816	4,050	369	138	260	98.1	47.6	85.0	90.8
cryopreserved	type-B	fresh	artificial	12	Uryu	4,162	3,418	410	215	268	95.6	58.9	87.0	88.0
cryopreserved	type-A	cryopreserved	artificial	7	Uryu	2,123	936	223	59	122	71.5	24.4	40.4	88.8
cryopreserved	type-B	cryopreserved	artificial	7	Uryu	2,266	1,534	326	49	235	86.1	11.0	82.3	91.0

* % values relative to fertilized eggs