HOKKAIDO UNIVERSITY

Title	Restoration of the loach, Misgurnus anguillicaudatus, from cryopreserved diploid sperm and induced androgenesis
Author(s)	Yasui, George Shigueki; Fujimoto, Takafumi; Arai, Katsutoshi
Citation	Aquaculture, 308, S140-S144 https://doi.org/10.1016/j.aquaculture.2010.05.041
Issue Date	2010
Doc URL	https://hdl.handle.net/2115/44225
Type	journal article
File Information	arai.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Restoration of the loach, *Misgurnus anguillicaudatus*, from cryopreserved diploid sperm and induced androgenesis

George Shigueki Yasui*, Takafumi Fujimoto, Katsutoshi Arai

Hokkaido University, Graduate School of Fisheries Science, Laboratory of Aquaculture Genetics and Genomics 3-1-1 Minato, Hakodate, Japan. 041-8611.

* Corresponding author: Tel.: +81 138 40 5535; Fax: +81 138 40 5537. E-mail: george@fish.hokudai.ac.jp

Running title: Androgenesis using cryopreserved diploid sperm

ABSTRACT

In the present study, we evaluated the feasibility of using cryopreserved diploid sperm as a repository genebank for the loach, *Misgurnus anguillicaudatus*, along with a rederivation strategy utilising induced-androgenesis. Firstly, we evaluated three types of media for egg inactivation: Hank's saline solution + 0.5% bovine serum albumin (BSA), Ringer's solution + 0.5% BSA, and masu salmon seminal plasma. Haploid and diploid sperm were taken from diploid and tetraploid loaches, respectively. Fresh and cryopreserved haploid or diploid sperm were then used to fertilize intact or UV-irradiated eggs from wild diploid females. The irradiation media evaluated here successfully maintained the egg quality over 2 hours. Fertilization and hatching rates of eggs fertilized with cryopreserved diploid sperm were 11.68 ± 6.74% and 7.14 ± 6.29% respectively, compared to 63.51 ± 10.68% and 45.19 ± 16.2% for intact eggs fertilized by fresh haploid sperm. All-male inheritance was confirmed by determination of larval morphology, ploidy status and microsatellite genotypes of putative androgenetic progeny.

Keywords: Cryopreservation, sperm bank, androgenesis, loach, *Misgurnus anguillicaudatus*, teleost

1. Introduction

The cryopreservation of sperm in liquid nitrogen is a promising tool for gene banking because it permits a long-term storage of the genome. Consequently, cryopreserved fish sperm may be used as a long term gene bank for endangered strains or species. At the beginning of this century, protocols for sperm cryopreservation had been successfully established for more than 200 fish species (Billard and Zhang, 2001). Most of these protocols aimed to establish a gene bank for the maintenance of genetic diversity as well as a source of sperm for *in vitro* fertilization. In order to re-derive extinct or endangered species from cryopreserved sperm, it is necessary to use genetically-inactivated eggs to achieve successful androgenesis, providing only paternal inheritance. Although the combination of cryopreserved sperm and androgenesis protocols have already been discussed as a means to restore endangered genomes (Gwo et al., 1999; Urbányi et al., 2004), optmised protocols have only been established for a limited number of species thus far (for reviews, see Komen and Thorgaard, 2007; Pandian and Kirankumar, 2003).

Viable androgenesis includes detrimental stages such as the genetic inactivation of the egg nucleus and diploidization of paternally derived chromosomes. In recent years, research has improved the observed survival rates of androgenotes. The first stage is to develop an irradiating medium for the eggs. This type of solution must maintain the viability of unfertilized eggs during the period of irradiation. In order to do this, such a medium should be inexpensive, easy to obtain, and should present low viscosity and toxicity for the gametes. Natural solutions such as seminal plasma or ovarian fluid were successfully used as an irradiating medium in the zebrafish (*Danio rerio*, Corley-Smith et al., 1996), muskellunge (*Esox masquinongy,* Lin and Dabrowski, 1998),

common carp (*Cyprinus carpio,* Rothbard et al., 1999) and also for the loach (*Misgurnus anguillicaudatus,* Fujimoto et al., 2007). However, the content of such solutions may differ due to seasonal variation and are not promptly available around the year.

Another focus for improving induced-androgenesis was to evaluate the optimum irradiation dose for genetic inactivation of the egg nucleus, and this has successfully been achieved for several species (Komen and Thorgaard, 2007). However, when genetically-inactivated eggs are fertilized by normal haploid sperm, the resultant haploid progeny die during the early stages of embryogenesis, due to so-called haploid syndrome. Therefore, in order to achieve viable androgenesis, it is necessary to duplicate the paternal chromosomes to obtain viable diploid androgenotes. Diploidization may be achieved by preventing the first cell cleavage by temperature or pressure shocks, but this process frequently results in very low survival rates (Komen and Thorgaard, 2007). The fertilization of eggs by two sperm cells (dispermy) or fused spermatozoa was used by some authors in order to avoid the diploidization stage (Araki et al., 1995; Clifton and Pandian, 2008; Grunina et al., 2006; Kirankumar and Pandian, 2004a). For the rainbow trout, Thorgaard et al. (1990) produced diploid androgenotes by fertilizing genetically-inactivated gamma-irradiated eggs with diploid sperm of artificially produced autotetraploid males. In the loach *Misgurnus anguillicaudatus*, Arai et al. (1995) further reported the production of diploid androgenotes using fertile diploid sperm from spontaneously occurring tetraploid individuals. However, the successful production of diploid androgenotes using fresh haploid sperm followed by duplication of paternally derived chromosomes by inhibiting first mitosis is still difficult to achieve, largely due to very low survival rates (Masaoka et al., 1995). In the loach, some populations present natural clonal individuals and polyploid individuals, which

conveniently represent a source of diploid sperm (Arai, 2001, 2003; Yoshikawa et al., 2008). Nonetheless, such individuals normally represent only a small fraction of the natural population, emphasizing the need of establishing cryobanks. The application of diploid sperm for gene banking in the loach may thus be facilitated by recently developed techniques regarding sperm cryopreservation (Yasui et al., 2008, 2009), egg inactivation (Arai et al., 1992; Fujimoto et al., 2007) and the production of neo-tetraploid individuals induced by the fertilization of normal eggs from diploid wild type females with diploid sperm from spontaneously occurring natural tetraploid males followed by inhibition of second polar body release (Fujimoto et al., 2010).

Considering the aspects mentioned above, the aim of our present study was to develop the restoration of individuals by integrating the cryopreservation of rare diploid sperm with induced androgenesis.

2. Materials and methods

2.1 Gamete sampling, sperm cryopreservation and fertilization

Adult female loaches were obtained from Iwamizawa city (Hokkaido Island, Japan). Neo-tetraploid males were obtained by crossing sperm from natural-occurring tetraploid males (Arai, 2001, 2003) with eggs from normal wild type diploid females, followed by retention of the 2nd polar body release by heat shock (42°C for 2 min; 5 min after activation) (Fujimoto et al., 2010). Spawning was induced by the injection of a single dose of hCG (100 I.U. for males and 500 I.U for females). After 10-12 hours at 27°C, the fish were anesthetized in 0.1% 2-phenoxyethanol and the gametes were collected by stripping.

Sperm was collected using a capillary glass tube. For cryopreservation, we used the procedure described by Yasui et al. (2008) with some modifications. Due to the

small volume of sperm collected from neo-tetraploid individuals, the sperm content in the capillary tube was placed in a 1.5 ml microtube containing 500 µl of cryo-solution (163.5 mM NaCl, 24mM $NaHCO_3$, 10% methanol, pH 8.3) and gently vortexed. The sperm was then placed in three French straws (250 µl), adjusted to 140 µl by cutting and then sealed with straw powder. The samples were frozen at -33.3 ± 2.09°C/min from 0 to -50°C using a cooling tube introduced in powdered dry ice (Yasui et al., 2009), and then plunged into liquid nitrogen for storage. The minimum storage duration was 6 h before the fertilization trials. Thawing was performed in water bath at 25°C for 10 s. The thawed straw content was placed in a 1.5 ml microtube and gently vortexed before insemination.

Eggs were stripped into Petri dishes (85 mm diameter) covered by a polyvinylidene chloride film (SaranWrap: Asahi Kasei Co. Ltd., Tokyo, Japan). Insemination using fresh or cryopreserved samples was performed using 50 µl of sperm which was directly pipette onto the egg mass. Sperm activation was performed by a 20-fold dilution in dechlorinated tap water. After 5 min, the fertilized eggs were transferred to another Petri dish containing approximately 50 ml of water.

2.2 Evaluation of irradiation media

Eggs from three diploid wild type females (~1000 eggs each) were stripped in 2 ml microtubes, and the volume completed using Hank's saline solution (137mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1mM $MgSO_4$, 4.2 mM $NaHCO_3$, 0.5% bovine serum albumin-BSA) (Sakai et al., 1997), modified Ringer solution (128.3 mM NaCl, 23.6 mM KCl, 3.6 mM $CaCl_2$, 2.1 mM $MgCl_2$, 0.5% BSA) or masu salmon (*Oncorhynchus masou*) seminal plasma. The eggs were maintained in an incubator set at 20°C.

Groups of 150 eggs were taken from the microtubes at 10, 30, 60 and 120 minutes after storage, and fertilized with fresh haploid sperm. The inseminating dose was higher than 687.65 spermatozoa $egg^{-1}ml^{-1}$ in order to ensure fertilization (Yasui et al., 2009). One batch of "dry" eggs (without previous contact with irradiating media) was fertilized at the beginning of the experiment and served as intact control group. Hatching rates were measured after 40-50 hours post fertilization. Data were expressed as a percentage of the control group.

2.3 Androgenesis and fertilization trials

For genetic inactivation of nuclei in eggs, we used the procedure described by Fujimoto et al. (2007). Groups of ~800 eggs from three females were stripped into 2.1 ml Hank's saline + 0.5% BSA and then UV-irradiated at 150 mJ/cm^2.

Groups of ~150 irradiated and intact eggs were pipetted directly into plastic Petri dishes covered by a plastic film (saran wrap). One drop of irradiation media was added on the egg mass to prevent dehydration. Intact and inactivated eggs were then inseminated with cryopreserved haploid or diploid sperm and fresh haploid or diploid sperm. Fertilized eggs were then maintained at 20°C and fertilization (at blastula stage) and hatching rates were measured, as well as the percentage of normal and abnormal larvae.

2.4 Ploidy status and genotyping

A small piece of fin was removed and processed using the standard protocol for nuclear staining with 4´,6-diamidino-2-phenylindole (DAPI) and the relative DNA content was determined using a Partec Ploidy Analyzer PA-II (Partec GmbH, Münster, Germany).

In order to confirm paternity, PCR products from the parental fish, androgenotes (*n*=19) and triploids (*n*=10) derived using cryopreserved diploid sperm from neo-tetraploids, were amplified following the procedure described by Morishima et al. (2008), using the primers *Mac37, Mac45, Mac47, Mac229* and *Mac449.* Electrophoresis was carried out using an automated sequencer ABI3130xl (ABI) and genotyping was performed using the GeneMapper program Ver.3.7 (ABI).

2.5 Statistical analyses

Data are shown as mean ± SD. All experiments were performed in triplicates using different egg sources. A general factorial design was used in order to study the effect of sperm (fresh haploid, cryopreserved haploid, fresh diploid and cryopreserved diploid) and egg source (normal or inactivated) upon fertilization, hatching and the percentage of abnormal larvae. Data were checked for normality using the Liliefor test, and then compared using ANOVA followed by Student-Newman Keul's multiple range test ($P>0.05$). Arcsine transformation was used for the percentage data regarding abnormal larvae in order to fit a normal distribution.

3. Results

The irradiation media evaluated in this study reduced hatching rates only after 60 min of storage (Fig. 1). Differences between the solutions were observed only at 120 min of incubation. After 10 min, the relative hatching rates were 85.59 ± 7.14%, 70.78 ± 18.15% and 65.43 ± 23.28% for Hank's saline solution, Ringer and seminal plasma, respectively. After 120 min of storage, these values decreased to 32.01 ± 18.55%, 15.10 ± 9.73% and 21.19 ± 4.67%, respectively. The hatching rate of the control group was 54.29 ± 14.9%.

The fertilization rates were highly variable, especially when inactivated eggs were employed (Table 1). No significant differences were observed between normal and inactivated eggs and no interaction was observed for any of the sperm and egg sources. Fertilization decreased significantly when inactivated eggs were fertilized with cryopreserved diploid sperm. Using fresh haploid sperm, the fertilization rate was 63.51 ± 10.68%, decreasing to 28.80 ± 20.06% when irradiated eggs were employed. For fresh diploid sperm, the fertilization rate was 53.20 ± 2.71% for normal eggs and 34.92 ± 27.12% for inactivated eggs. When cryopreserved haploid sperm was used to fertilize normal eggs, a fertilization rate of 41.83 ± 17.88% was recorded. The same sperm source resulted in a fertilization of 28.35 ± 15.01% when inactivated eggs were utilized. Cryopreserved diploid sperm resulted in a fertilization rate of 21.33 ± 7.14% when normal eggs were used. Using the same sperm source, the fertilization rate of UV-irradiated eggs was only 11.68 ± 6.74%.

Hatching rate data followed similar trends as the fertilization rates. No interaction was observed when using different sperm and egg sources. Significant reductions in hatching rates were observed only in the cross between UV-inactivated eggs and cryopreserved haploid sperm (14.12 ± 9.08%) and in UV-inactivated eggs crossed with cryopreserved diploid sperm (7.14 ± 6.29%). Hatching rates following the fertilization of normal eggs with fresh haploid sperm and fresh diploid sperm were 45.19 ± 16.20% and 48.14 ± 5.76%, respectively.

As expected, the percentage of abnormal fry was significantly higher in treatments that generated haploid androgenotes (crosses between haploid sperm and inactivated eggs). In these treatments the interaction between egg and sperm sources was significant and the percentage of abnormal fry was 100%. Other treatments revealed hatching rates of abnormal fry ranging from 7.97 to 15.94%.

As shown in Table 1, flow cytometry analyses revealed that haploid progeny were created when UV-inactivated eggs were fertilized with fresh or cryopreserved haploid sperm. Diploid progeny were obtained when UV-inactivated eggs were fertilized with fresh diploid sperm, when UV-inactivated eggs were crossed with cryopreserved diploid sperm, when normal eggs were crossed with fresh haploid sperm and when normal eggs were crossed with cryopreserved haploid sperm. Triploid individuals were resulted from the crosses between normal eggs and cryopreserved diploid sperm, and between normal eggs x fresh diploid sperm.

All-male inheritance of the androgenotes was confirmed by paternally derived alleles at five independent microsatellite loci (Table 2). Female alleles were not observed among androgenotes. Male and female alleles were observed in the triploid progeny.

4. Discussion

The type of irradiating media used is a key factor when using UV-irradiation for inactivating the egg nucleus. Several types of media have been used for this purpose such as ovarian fluid (Lin and Dabrowski, 1998; Rothbard et al., 1999), synthetic ovarian fluid (Bongers et al., 1994, 1995), water (Myers et al., 1995), seminal plasma (Corley-Smith et al., 1996; Fujimoto et al., 2007) and Ringer's solution (David and Pandian, 2006b). In the present study, Hank's saline solution was effective in irradiating eggs from the loach, and also in prevent egg dehydration during fertilization trials. Although fertilization could be achieved until 120 minutes of storage, the time taken to inactivate the egg nucleus and fertilize a batch of eggs was usually less than 10 minutes.

Fertilization rates using different sources of gamete were highly variable. Irradiated eggs yielded the most variable results, as reported for other teleosts (Babiak et

al., 2002b; David and Pandian, 2006a), and our as described in our earlier studies involving the same species (Arai et al., 1992, 1995; Fujimoto et al., 2007; Masaoka et al., 1995). In most cases, irradiation reduces fertilization ability due to physical and genetic damage of the eggs (Kirankumar and Pandian, 2003; Lin and Dabrowski, 1998; Nam et al., 2002).

Both egg and sperm sources presented a significant interaction for data regarding abnormal larvae in treatments that generated haploid progenies (fresh haploid sperm x irradiated eggs and cryopreserved haploid sperm x irradiated eggs). This fact was expected due to haploid syndrome presenting in the resultant embryos, in which all the progeny possessed body malformations.

The combination of cryopreserved diploid sperm and irradiated eggs reduced the fertilization rates when compared with controls (haploid sperm and intact eggs). In fish, the usage of cryopreserved sperm to achieve androgenesis presents variable results, with reduction in survival rates (David and Pandian 2006b; Grunina et al., 2006; Kirankumar and Pandian, 2004b; Scherer et al., 1991) or no effect upon survival rates (Babiak et al., 2002b; Bercsenyi et al., 1998). In our case, such problems may have occurred due to egg irradiation combined with poor sperm quality from tetraploid males, since we observed lower sperm content in such tetraploid individuals. Sperm content may affect fertilization success if the resulting inseminating dosage is lower than 689 spermatozoa egg^{-1} ml^{-1} (Yasui et al., 2009). In our study, the survival rate of androgenotes derived from cryopreserved diploid sperm (7.14 ± 6.29%), was higher than those obtained in other teleosts described previously (Araki et al., 1995; Babiak et al., 2002a; Paschos et al., 2001) although our survival data was lower than that described previously (David and Pandian, 2006a, Kirankumar and Pandian, 2004b; Nam et al., 2002). As observed here, other studies have focused in eliminating the diploidization step in order to

improve the survival of the androgenotes. Sun et al. (2006) obtained 4.1% of diploid androgenotes using diploid sperm obtained from allotriploid hybrids in order to achieve interspecific androgenesis, similarly to Kirankumar and Pandian (2004a) using dispermic fertilization (~15%). In sturgeon *Acipenser stellatus,* Grunina et al. (2006) performed dispermic androgenesis and obtained survival rates of 17% androgenotes using fresh sperm and 7% for cryopreserved sperm. These data are similar to that observed in the present study.

Our data regarding survival rates and genotyping indicates that the cryopreservation of diploid sperm followed by induced androgenesis may be applicable for gene banking. However, it is necessary to obtain fertile diploid sperm. As demonstrated in our study, neo-tetraploid males were produced when a diploid female was crossed with a natural tetraploid male followed by inhibition of second polar body release (Fujimoto et al., 2010). This may be useful as a source of diploid sperm. Another source of diploid sperm is from an artificial tetraploid, induced by inhibition of the first cleavage. However, induction of tetraploid individuals is generally very difficult to achieve, largely due to very low viability (Arai, 2001; Sakao et al., 2006). Furthermore, a large number of artificial tetraploids were reported to produce haploid and other unexpected gametes in the loach (Nam and Kim, 2004). In the loach, however, other sources of diploid sperm may be obtained from masculinized clonal individuals (Morishima et al., 2002; Yoshikawa et al., 2007, 2009) and diploid-triploid mosaics (Morishima et al., 2004).

In the present paper, we have attempted to restore diploid individuals by induced-androgenesis using cryopreserved diploid sperm. Thus, the cryopreservation of diploid sperm is feasible to use as a repository gene bank in the loach and also for other

teleost fish, as long as diploid sperm can be readily obtained from spontaneously occurring or artificially induced tetraploids, or other sources.

Acknowledgements

This study was supported in part by Grants-in-Aid for the 21st Century COE (Center of Excellence) Program (K-2, 2004-2008 fiscal year) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), and Scientific Research (B) (no.18380108) and Scientific Research (B) (no. 21380114) from the Japanese Society for Promotion of Science (JSPS).

References

Arai, K., 2001. Genetic improvement of aquaculture finfish species by chromosome manipulation techniques in Japan. Aquaculture 197, 202-228.

Arai, K., 2003. Genetics in the loach *Misgurnus anguillicaudatus*: recent progress and perspectives. Fol. Biol. 51, 107-117.

Arai, K., Masaoka, T., Suzuki, R., 1992. Optimum conditions of UV ray irradiation for genetic inactivation of loach eggs. Nippon Suisan Gakkaishi 58, 1197–1201.

Arai, K., Ikeno, M., Suzuki, R., 1995. Production of androgeneticdiploid loach *Misgurnus anguillicaudatus* using spermatozoa of natural tetraploids. Aquaculture 137, 131–138.

Araki, K., Shinma, H., Nagoya, H., Nakayama, I., Onozato, H., 1995. Androgenetic diploids of rainbow trout (*Oncorhynchus mykiss*) produced by fused sperm. Can. J. Fish. Aquat. Sci. 52, 892–896.

Babiak, I., Dobosz, S., Kuzminski, H., Goryczko, K., Ciesielski, S., Brzuzan, P., Urbanyi, B., Horvath, A., Lahnsteiner F., Piironen, J., 2002a. Failure of interspecies androgenesis in salmonids. J. Fish Biol. 61, 432–447.

Babiak, I., Dobosz, S., Goryczko, K., Kuzminski, H., Brzuzan, P., Ciesielski, S., 2002b. Androgenesis in rainbow trout using cryopreserved spermatozoa: the effect of processing and biological factors. Theriogenology 57, 1229–1249.

Bercsenyi, M., Magyary, I., Urbanyi, B., Orban, L. and Horvath, L., 1998. Hatching out goldfish from common carp eggs: interspecific androgenesis between two cyprinid species. Genome 41, 573–579.

Billard, R., Zhang, T., 2001. Techniques of genetic resource banking in fish. In: Watson, P.F., Holt, W.V. (Eds.), Cryobanking the genetic resource: wildlife conservation for the future? Taylor and Francis, London, pp.156-170.

Bongers, A.B.J., Veld, E.P.C., Abo-Hashema, K., Bremmer, I.M., Eding, EH., Komen, J., Richter, C.J.J., 1994. Androgenesis in common carp (*Cyprinus carpio* L.) using UV irradiation in a synthetic ovarian fluid and heat shocks. Aquaculture 122, 119–132.

Bongers, A.B. J., Nguenga, D., Eding, E.H., Richter, C.J.J., 1995. Androgenesis in the African catfish, *Clarias gariepinus*. Aquat. Living Resour. 8, 329-332.

Clifton, J.D,, Pandian, T.J., 2008. Dispermic induction of interspecific androgenesis in the fish, Buenos Aires tetra using surrogate eggs of widow tetra. Curr. Sci. 95, 64-74.

Corley-Smith, G.E., Lim, C.J., Brandhorst, B.P., 1996. Production of androgenetic zebrafish (*Danio rerio*). Genetics 142, 1265-1276.

David, C.J., Pandian T.J., 2006a. GFP reporter gene confirms paternity in the androgenote Buenos Aires tetra, *Hemigrammus caudovittatus*. J Exp.Zool. 305A, 83-95.

David, C.J., Pandian T.J., 2006b. Cadaveric sperm induces intergeneric androgenesis in the fish, *Hemigrammus caudovittatus*. Theriogenology 65, 1048-1070.

Fujimoto, T., Sakao, S., Yamaha, E., Arai, K., 2007. Evaluation of different doses of UV irradiation to loach eggs for genetic inactivation of the maternal genome. J. Exp. Zool. 307A, 449 - 462.

Fujimoto, T., Yasui, G.S., Hayakawa, M., Sakao, S., Yamaha, E., Arai, K., 2010. Reproductive capacity of neo-tetraploid loaches produced using diploid spermatozoa of a natural tetraploid male. Aquaculture, this volume.

Grunina, A.S., Recoubratsky, A.V., Tsvetkova, L.I., Barmintsev, V.A., 2006. Investigation on dispermic androgenesis in sturgeon fish: the first successful production of androgenetic sturgeons with cryopreserved sperm. Int. J. Refr. 29, 379-386.

Gwo, J.C., Ohta, H, Okuzawa, K., Wu, H-C., 1999. Cryopreservation of sperm from the endangered Formosan landlocked salmon (*Oncorhynchus masou formosanus*). Theriogenology 51, 569 - 582.

Kirankumar, S., Pandian, T.J., 2003. Production of androgenetic tiger barb, *Puntius tetrazona*. Aquaculture 228, 37–51.

Kirankumar, S., Pandian, T.J., 2004a. Use of heterologous sperm for the dispermic induction of androgenesis in barbs. J. Fish Biol. 64, 1485-1497.

Kirankumar, S., Pandian, T.J., 2004b. Interspecific androgenetic restoration of rosy barb using cadaveric sperm. Genome 47, 66-73.

Komen, H., Thorgaard, G.H., 2007. Androgenesis, gynogenesis and the production of clones in fishes: a review. Aquaculture 269, 150-173.

Lin, F., Dabrowski, K., 1998. Androgenesis and homozygous gynogenesis in muskellunge (*Esox masquinongy*): evaluation using flow cytometry. Mol. Reprod. Dev. 49, 10-18.

Masaoka, T., Arai, K., Suzuki, R., 1995. Production of androgenetic diploid loach *Misgurnus anguillicaudatus* from UV irradiated eggs by suppression of the first cleavage. Fish. Sci. 61, 716-717.

Morishima, K., Horie, S., Yamaha, E., Arai, K., 2002. A cryptic clonal line of the loach *Misgurnus anguillicaudatus* (Teleostei : Cobitidae) evidenced by induced gynogenesis, interspecific hybridization, microsatellite genotyping and multilocus DNA fingerprinting. Zool. Sci. 19, 565-575.

Morishima, K., Oshima, K., Horie, S., Fujimoto, T., Yamaha, E., Arai, K., 2004. Clonal diploid sperm of the diploid-triploid mosaic loach, *Misgurnus anguillicaudatus* (Teleostei : Cobitidae). J. Exp. Zool. 301A(6), 502-511.

Morishima, K., Nakayama, I., Arai, K., 2008. Genetic linkage map of the loach *Misgurnus anguillicaudatus* (Teleostei: Cobitidae). Genetica 132(3), 227-241.

Myers, J.M., Penman, D.J., Basavaraju, Y., Powell, S.F., Baoprasertkul, P., Rana, K.J., Bromage, N., McAndrew, B.J., 1995. Induction of diploid androgenetic and mitotic gynogenetic Nile tilapia (*Oreochromis niloticus* L.). Theor. Appl. Genet. 90, 205–210.

Nam, Y.K., Kim, D.S., 2004. Ploidy status of progeny from the crosses between tetraploid males and diploid females in mud loach (*Misgurnus mizolepis*). Aquaculture 236, 575-582.

Nam, Y.K., Cho, Y.S., Cho, H.J., Kim, D.S., 2002. Accelerated growth performance and stable germ-line transmission in androgenetically derived homozygous transgenic mud loach, *Misgurnus mizolepis*. Aquaculture 209, 257-270.

Pandian, T.J., Kirankumar, S. 2003., Androgenesis and conservation of fishes. Curr. Sci. 85, 917-931.

Paschos, I., Natsis, L., Nathanailides, C., Kagalou, I., Kolettas, E., 2001. Induction of gynogenesis and androgenesis in goldfish *Carassius auratus* (var. oranda). Rep. Dom. Anim. 36, 195-198.

Rothbard, S., Rubinshtein, I., David, L., Shelton, W.L., 1999. Ploidy manipulations aimed to produce androgenetic Japanese ornamental (koi) carp, *Cyprinus carpio* L. Isr. J. Aquacult. - Bamidgeh 51, 26-39.

Sakai, N., Burgess, S., Hopkins, N., 1997. Delayed *in vitro* fertilization of zebrafish eggs in Hank's saline containing bovine serum albumin. Mol. Mar. Biol. Biotechnol. 6, 84-87.

Sakao, S., Fujimoto, T., Kimura, S., Yamaha, E., Arai, K., 2006. Drastic mortality in tetraploid induction results from the elevation of ploidy in masu salmon *Oncorhynchus masou*. Aquaculture 252(2-4), 147-169.

Scheerer, P.D., Thorgaard, G.H., Allendorf, F.W., 1991. Genetic analysis of androgenetic rainbow trout. J. Exp. Zool. 269, 382–390.

Sun, Y., Zhang, C., Liu, S., Duan, W., Liu, Y., 2006. Induced interspecific androgenesis using diploid sperm from allotetraploid hybrids of common carp×red crucian carp. Aquaculture 264, 47-53.

Thorgaard, G.H., Scheerer, P.D., Hershberger, W.K., Myers, J.M., 1990. Androgenetic rainbow trout produced using sperm from tetraploid males show improved survival, Aquaculture 85, 215–221.

Urbányi, B., Horváth, A., Kovács, B., 2004. Successful hybridization of Acipenser species using cryopreserved sperm. Aquacult. Intern. 12, 47-56.

Yasui, G.S., Arias-Rodriguez, L., Fujimoto, T., Arai, K., 2008. Simple and inexpensive method for cryopreservation of fish sperm combining straw and powdered dry ice. Cryoletters 29, 383-390.

Yasui, G.S., Arias-Rodriguez, L., Fujimoto, T., Arai, K., 2009. A sperm cryopreservation protocol the loach *Misgurnus anguillicaudatus* and its applicability for other related species. Anim. Rep. Sci. 116, 335-345.

Yoshikawa, H., Morishima, K., Kusuda, S., Yamaha, E., Arai, K., 2007. Diploid sperm produced by artificially sex-reversed clone loaches. J. Exp. Zool. 307A, 75-83.

Yoshikawa, H., Morishima, K., Fujimoto, T., Arias-Rodriguez, L., Yamaha, E., Arai, K., 2008. Ploidy manipulation using diploid sperm in the loach, *Misgurnus anguillicaudatus:* a review. J. App. Ichtyol. 24, 410-414.

Yoshikawa, H., Morishima, K., Fujimoto, T., Saito, T., Kobayashi, T., Yamaha, E., Arai, K., 2009. Chromosome doubling in early spermatogonia produces diploid spermatozoa in a natural clonal fish. Biol. Rep. 80, 973-979.

Figure legends:

Fig. 1. Hatching rates of loach eggs stored in Hank's saline solution + 0.5% BSA, modified Ringer solution + 0.5% BSA or *Oncorhynchus masou* seminal plasma. Average hatching of the control group (intact eggs) was 54.29±14.9%. Identical superscript letters above the columns denote non-significant differences as determined by the Student Newman-Keuls test ($P > 0.05$).

Table 1. Percentages of fertilization, hatching and abnormal larvae derived from combinations of gametes sources. Identical letters within columns denotes non-statistical differences as determined by the Student Newman-Keuls multiple range test ($P>0.05$)

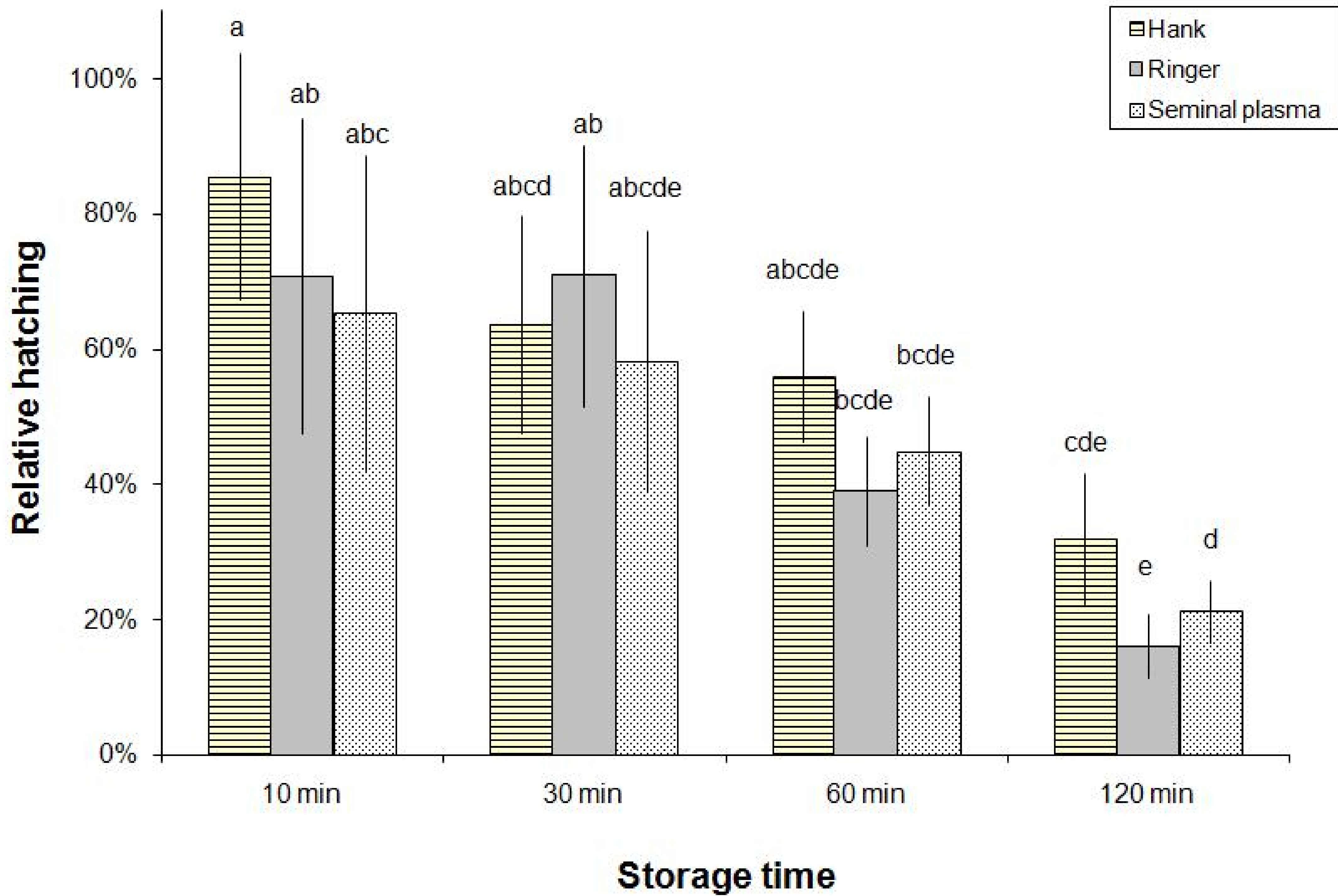
Crosses			Fertilization (%)	Hatching (%)	Abnormal (%)	Ploidy status				
Egg		Sperm				*n*	Haploid	Diploid	Triploid	Aneuploid
Normal	x	Fresh haploid	63.51 ± 10.68^{a}	45.19 ± 16.20^{ab}	7.97 ± 3.87^{b}	60	-	60	-	-
Inactivated	x	Fresh haploid	28.80 ± 20.06^{ab}	17.88 ± 12.28^{abc}	100.0 ± 0.00^{a}	60	60	-	-	-
Normal	x	Fresh diploid	53.20 ± 2.71^{ab}	48.14 ± 5.76^{a}	7.90 ± 7.47^{b}	60	-	-	60	-
Inactivated	x	Fresh diploid	34.92 ± 27.12^{ab}	23.83 ± 18.84^{ac}	15.94 ± 20.95^{b}	60		60	-	-
Normal	x	Cryop. haploid	41.83 ± 17.88^{ab}	23.82 ± 14.24^{abc}	8.14 ± 6.77^{b}	60	-	60	-	-
Inactivated	x	Cryop. haploid	28.35 ± 15.01^{ab}	14.12 ± 9.08^{bc}	100.0 ± 0.00^{a}	60	60	-	-	-
Normal	x	Cryop. diploid	21.33 ± 7.14^{ab}	19.12 ± 6.79^{abc}	09.17 ± 3.87^{b}	60	-	-	57	3*
Inactivated	x	Cryop. diploid	11.68 ± 6.74^{b}	07.14 ± 6.29^{c}	8.76 ± 10.90^{b}	60	-	60	-	-

Fertilization was measured at blastula stage.
* hyper 3n individuals.

Table 2. Microsatellite genotypes of androgenotes and triploids derived from cryopreserved diploid sperm.

Sample	Loci									
	Mac37 (10)	*n*	*Mac45 (10)*	*n*	*Mac47 (3)*	*n*	*Mac229 (3)*	*n*	*Mac449 (14)*	*n*
Male	84 / 92	-	110	-	128 / 146	-	210 / 220 / 242 / 262	-	233 / 253 / 260	-
Female	101 / 109	-	91 / 106	-	148	-	194 / 222	-	272 / 280	-
Inactivated eggs	84 / 92	19	110	19	146	13	210 / 220	1	233 / 260	13
x	-	-	-	-	128 / 146	6	210 / 242	4	253 / 260	6
Cryopreserved diploid sperm	-	-	-	-	-	-	210 / 262	2	-	-
	-	-	-	-	-	-	220 / 242	4	-	-
	-	-	-	-	-	-	220 / 262	8	-	-
Normal eggs x Cryopreserved diploid sperm	84 / 92 / 101	5	110 / 106	4	128 / 146 / 148	4	194 / 210 / 242	2	233 / 260 / 272	3
	84 / 92 / 109	5	110 / 91	6	146 / 148	6	194 / 210 / 262	1	233 / 260 / 280	2
	-	-	-	-	-	-	194 / 220 / 242	2	253 / 260 / 272	3
	-	-	-	-	-	-	222 / 220 / 242	3	253 / 260 / 280	2
	-	-	-	-	-	-	222 / 220 / 262	2		

Underlined alleles originated from the female. Number in parenthesis indicates the linkage group (Morishima et al., 2008).


Hank
Ringer
Seminal plasma
Relative hatching
100%
80%
60%
40%
20%
0%
a
ab
abc
abcd
ab
abcde
abcde
bcde
bcde
cde
e
d
10 min
30 min
60 min
120 min
Storage time