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FULL-SIB MATING CAN REDUCE DELETERIOUS EFFECTS ASSOCIATED WITH RESIDUAL SPERM INHERITANCE IN GYNOGENOTES

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Abstract

Fertilization of *Oreochromis aureus* eggs with UV-irradiated sperm from the closely related species *O. niloticus*, followed by diploidy restoration, produced offspring with lower embryo viability and higher skeletal deformation rates than siblings generated with sperm from a genetically distant species *(Tilapia zillii)*. Results showed that: (a) deleterious effects due to *O. niloticus* sperm accumulate in gynogenetic fish over generations; (b) such effects are eliminated when using *T. zillii* sperm to fertilize eggs from gynogenetic mothers produced by full-sib matings. These results suggest that: (a) deleterious effects are associated with residual male DNA fragments which may be passed on to descendent offspring; (b) such fragments are significantly purged following full-sib mating. These findings suggest that biparental reproduction may play an important role in the control of genome integrality by purging supernumerary chromosome fragments.

Introduction

Tilapia, which belong to the cichlid family, are divided taxonomically on the basis of brooding behavior into three genera (Trewavas, 1983): *Tilapia (*substrate spawner), *Sarotherodon* (biparental mouthbrooder) and *Oreochromis* (maternal mouthbrooder). Due to differences in reproduction, larval development and fish morphology, crosses between mouthbrooders and substrate spawners produce nonviable larvae or very low yields of runt progeny (Fishelson, 1962; Balon, 1977; Trewavas, 1978; Chourrout and Itchkowich, 1983; Shirak, 1996). In contrast, high viability of tilapia hybrids is currently obtained in interspecific crosses of mouthbrooder tilapia (Fishelson, 1962; Trewavas, 1978). Such phenomena were also found among other fishes (e.g., Stanley, 1976; Chourrout and Quillet, 1982; Rothbard and Shelton, 1993). However, fertilization with irradiated sperm of genetically distant donors was found to be more advantageous than homologous sperm for the induc-

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tion of diploid gynogenetic individuals. For example, common carp or goldfish males were suitable sperm donors for gynogenetic induction in black carp and grass carp (Stanley et al., 1975; Stanley, 1976; Rothbard and Shelton, 1993) and even tilapia (Vardaraj, 1990; Peruzzi et al., 1993).

Inadequate irradiation of sperm used as a trigger for gynogenetic development may lead to genetic contamination by male DNA fragments. Such contamination may be one of the causes of the low yield of gynogenetic offspring (Chourrout, 1984; Thorgaard et al., 1985). In earlier studies carried out in this laboratory, successful production of stable gynogenetic *O. aureus* clones was obtained by using irradiated sperm of a closely related mouthbrooder species (*O. niloticus*), followed by full-sib mating (Don and Avtalion, 1988; Avtalion and Don, 1990; Shirak et al., 1998). In the present study, the ability of irradiated *O. niloticus* sperm to induce gynogenesis in *O. aureus* eggs was compared with sperm of the genetically distant substrate-brooder *T. zillii* over two gynogenetic generations. In addition, the effect of full-sib mating on the deleterious effects often present in gynogenotes was examined.

Materials and Methods

Fish. Males of *O. niloticus* (laboratory stock) and *T. zillii* (local Israeli stock) were used as sperm donors and ten *O. aureus* females as egg donors to produce the first gynogenetic generation.

Gamete collection and insemination. Eggs and milt were gently stripped from the fish immediately before spawning and fertilized in Petri dishes, as described by Yeheskel and Avtalion (1988). The embryos were incubated in 750-ml Zuger bottles until yolk sac absorption at a constant temperature of 28.2±0.1°C. The viability rate was determined at two developmental stages (morula and sac absorption), according to Galman and Avtalion (1989).

Fish rearing. Larvae (10-12 days) of the various groups were reared in separate cells in a closed water circulating system to the age of 6 months. They were then sexed, tagged and divided into families of 4-7 females and 1-2 males for use in the following experiments.

Gynogenetic progeny of *O. aureus* includes 10-30% males (Don and Avtalion, 1988).

UV irradiation of milt. Milt was suspended 1:10 (0.9-2x10^8 sperm/ml) in the fertilization solution [95% Dulbecco's modified Eagle medium (DMEM) and 5% pooled carp serum (PCS) pH=6.8] and examined for sperm concentration as reported by Yeheskel and Avtalion (1988). Samples of 1.5 ml of this suspension were placed into 39-mm tissue culture dishes, and then immediately irradiated with a UV dose of 276-304W/m2 (Don and Avtalion, 1988; Shirak et al., 1998). During sperm irradiation, the samples were stirred gently with a magnetic stirrer (3.5 turns/sec). Each sample was then used to fertilize 100-200 eggs. The irradiation dose which produced the highest yield of haploids at the embryo age of 168 hours (which corresponds to the swimming ability stage of a normal embryo kept at 28±0.2°C) was considered optimal and used for the subsequent experiments.

Production of first and second gynogenetic generations. Sixteen spawns of the ten *O. aureus* females were used to produce the first meiogynogenetic generation and for diploid and haploid controls. Each spawning was divided into six groups. The first and second groups of 100-200 eggs were fertilized with intact *O. niloticus* or *T. zillii* sperm, respectively, and served as diploid controls. The third and fourth groups (100-200 eggs) were fertilized with irradiated *O. niloticus* or *T. zillii* sperm, respectively, and served as haploid controls. The fifth and sixth groups (100-1000 eggs) were fertilized with irradiated *O. niloticus* or *T. zillii* sperm, respectively. After three minutes, they were heat-shocked for diploidy restoration, as described by Don and Avtalion (1988). The second gynogenetic generation was created from females of the first gynogenetic generation (Fig. 1, scheme A) and from females of full-sibs derived from the cross between females and males of the first generations (Fig. 1, scheme B).

Experimental groups. Gynogenetic lines of *O. aureus* were produced by using irradiated sperm of *T. zillii* (line *O. au-z*) or *O. niloticus* (line *O. au-n*). *O. au-n* and *O. au-z* eggs were used to produce the second gynogenetic gen-

A

O. aureus

O. niloticus
irradiated sperm

T. zillii
irradiated sperm

♂ & ♀

Heat shock

♂ & ♀

1st generation
gynogenotes

O. au-n

O. au-z

♀

♀

O. niloticus
irradiated sperm

T. zillii
irradiated sperm

O. niloticus
irradiated sperm

T. zillii
irradiated sperm

Heat shock

Heat shock

2nd generation
gynogenotes
(direct production)

♂ & ♀

O. au-nn$_1$

O. au-nz$_1$

O. au-zn$_1$

O. au-zz$_1$

B

O. aureus

O. niloticus
irradiated sperm

T. zillii
irradiated sperm

♂ & ♀

Heat shock

♂ & ♀

O. au-n

O. au-z

1st generation
gynogenotes

♂

Full-sib
mating

♀

♂

Full-sib
mating

♀

O. niloticus
irradiated sperm

T. zillii
irradiated sperm

O. niloticus
irradiated sperm

T. zillii
irradiated sperm

♀

♀

Heat shock

Heat shock

2nd generation
gynogenotes
(indirect production)

♂ & ♀

O. au-nn$_2$

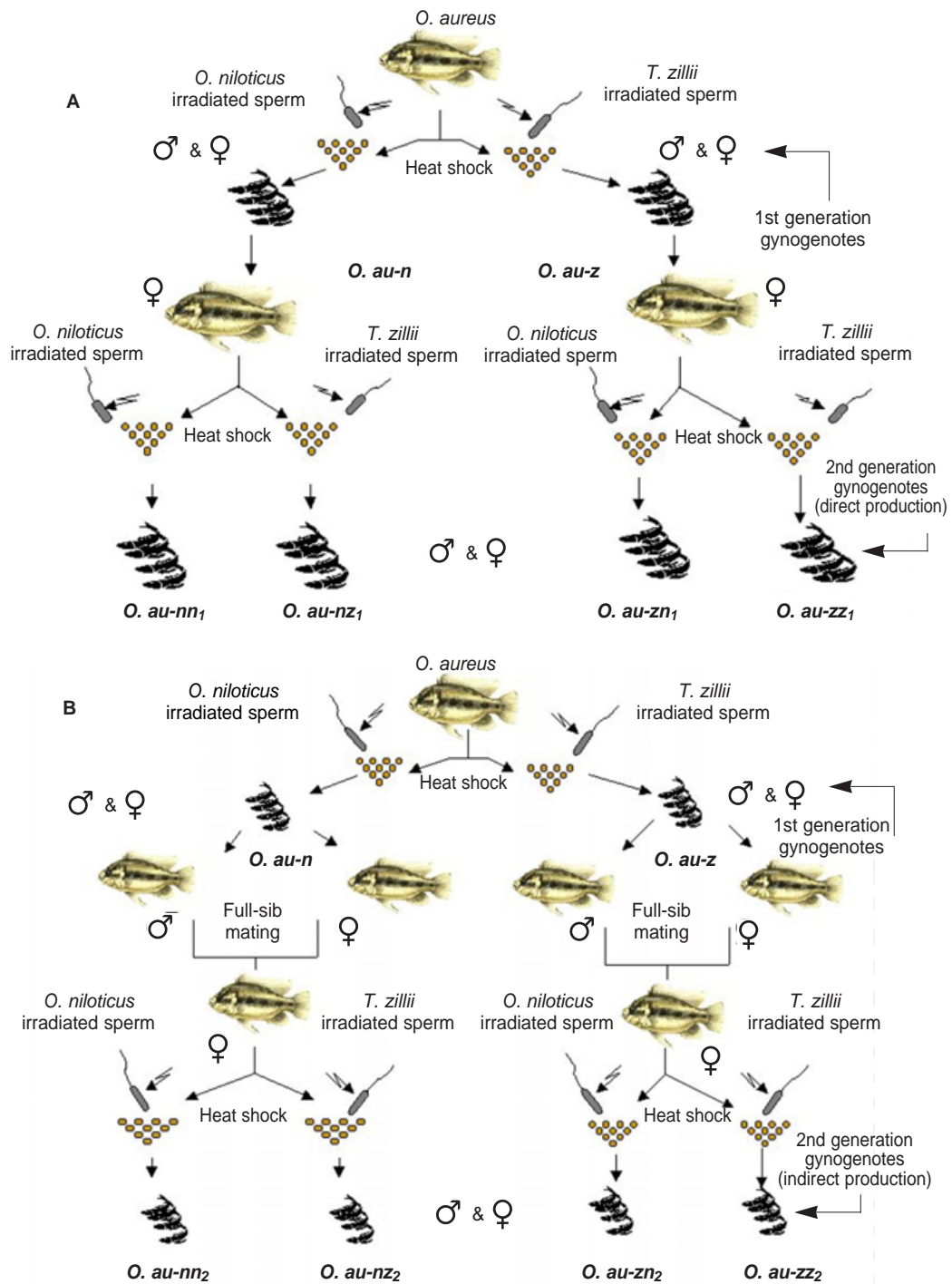**O. au-nz$_2$**

O. au-zn$_2$

O. au-zz$_2$

Fig. 1. Schematic representation of the techniques used to generate various *Oreochromis aureus* lines of (A) direct gynogenotes and (B) full-sib (indirect) gynogenotes.

eration (scheme A) and full-sibs (scheme B). Both first-gynogen generations were fertilized with irradiated sperm of, either, *O. niloticus* or *T. zillii* to produce four sublines of direct gynogenotes (*O. au-nn$_1$*, *O. au-nz$_1$*, *O. au-zz$_1$*, *O. au-zn$_1$*; scheme A) and four of indirect gynogenotes (*O. au-nn$_2$*, *O. au-nz$_2$*, *O. au-zz$_2$* and *O. au-zn$_2$*; scheme B).

Statistical analysis and calculations. Larvae viability at the stage of yolk sac resorption was calculated using the morula number as a reference of 100% (Shirak et al., 1998). The Student's *t* test (paired or unpaired) was used to compare larvae viability and abnormality rates in the different gynogenetic lines.

Results

Production of the first gynogenetic generation. UV-inactivated sperm of *O. niloticus* and *T. zillii* were compared for ability to activate *O. aureus* eggs for diploid gynogenesis. *T. zillii* sperm produced statistically higher (p=0.019; Table 1) levels of viable gynogenetic embryos (0.87±0.27%, *O. au-z*) than *O. niloticus* sperm (0.74±0.27%, *O. au-n*). However, no differences in longevity were found in their haploid sibs, which all died within eight days. The viability of the closely related hybrid controls (*O. aureus* x *O. niloticus*) varied from 42.1% to 83.7%, compared to 0% in the distant hybrid controls (*O. aureus* x *T. zillii*).

A total of 13 *O. au-n* (4 males and 9 females) and 17 *O. au-z* (5 males and 12 females) gynogenetic offspring survived until they became adults (6 months). Some of the surviving females were malformed (two *O. au-n* and one *O. au-z*) and removed from the experiment. Four full-sib pairs of *O. au-n* and *O. au-z* were tagged and used to produce the second gynogenetic generation and full-sib progeny.

Production of second gynogenetic generation. The spawns of gynogenetic females (tagged *O. au-n* and *O. au-z*) were used for direct production of a second gynogenetic generation and for the production of full-sibs (Table 1). In general, embryo viability was significantly higher in the progeny of females fertilized with irradiated *T. zillii* sperm than in progeny of those fertilized with irradiated *O. niloticus* sperm. Further, embryo viability was significantly higher (p= 0.011) in *O. au-zz$_1$* than in *O. au-nz$_1$*, and in *O. au-zn$_1$* than in *O. au-nn$_1$* (p=0.001), despite fertilization with the same type of sperm in the second generation. There were no significant differences in viability between progeny of full-sib females in the parallel experiments (e.g., between *O. au-zz$_2$* and *O. au-nz$_2$* (p=0.163), or *O. au-zn$_2$* and *O. au-nn$_2$* (p=0.133).

Malformations in gynogenotes of the second generation. No malformations were encountered among *O. au-zz$_2$* (Table 2). By contrast, 22.2% of the *O. au-nn$_1$* individuals were malformed. Malformation levels were significantly lower (p=0.003) in indirect gynogenotes of the different groups than in the comparable direct gynogenotes. Similarly, the additive effects of *O. niloticus* versus *T. zillii* sperm in the second generation of both direct and indirect gynogenotes showed a significant decrease (p=0.008) of malformations with *T. zillii* sperm.

Discussion

In vitro fertilization of *O. aureus* eggs with homogeneric *O. niloticus* sperm resulted in similar viability as previously reported for homologous sperm (Shirak, 1996). In contrast, none of the embryos of sibling eggs fertilized with the heterogeneric *T. zillii* sperm were viable. In the present study, both UV-irradiated sperm types were equally efficient in initiating haploid embryo development, but irradiated heterogeneric sperm resulted in higher gynogen viability after diploidy was restored by heat shock.

The low viability in the first gynogenetic generation produced with homologous sperm (Jaylet, 1972; Chourrout and Quillet, 1982; Don and Avtalion, 1988) has been attributed to the occurrence of paternal chromosomal residues and the toxic effect of thymidine dimers (Ijiri, 1983). Chromatin fragments derived from irradiated sperm have been shown to reside in a stable manner in first-generation gynogenotes (Chourrout and Quillet, 1982; Thorgaard et al., 1985; Thorgaard, 1986; Disney et al., 1987). Such residual fragments may integrate into the chromosomes of gynogenetic embryos by a

Table 1. The ability of genetically inactivated homogeneric (*Oreochromis niloticus*) and heterogeneric (*Tilapia zilli*) sperm to produce viable gynogenotes with *O. aureus* eggs.

Females (no.)	*Oreochromis niloticus* sperm			*Tilapia zilli* sperm			Viability (paired t test) p=
	Morula (mean no.±SD)	Embryo (mean no.±SD)	Viability (mean no.±SD)	Morula (mean no.±SD)	Embryo (mean no.±SD)	Viability (mean no.±SD)	
1st generation gynogenotes (16)	659±142	4.88±2.80	0.74±0.27 *O. au-n*	672±164	5.82±2.77	0.87±0.27 *O. au-z*	0.019
2nd generation gynogenotes							
O. au-n (7)	210±61	6.57±5.38	3.12±1.94 *O. au-nn$_1$*	210±64	10.00±6.48	4.75±2.27 *O. au-nz$_1$*	0.019
O. au-z (7)	224±78	15.71±5.56	7.02±1.20 *O. au-zn$_1$*	223±74	18.57±6.95	8.32±2.14 *O. au-zz$_1$*	0.068
Viability (paired t test) p=			0.001			0.011	
2nd generation gynogenotes from full-sibs							
O. au-n (7)	272±81	14.29±5.28	5.25±1.08 *O. au-nn$_2$*	271.85	20.14±7.99	7.42±1.77 *O. au-nz$_2$*	0.002
O. au-z (7)	182±30	12.14±3.76	6.68±2.07 *O. au-zn$_2$*	174.36	15.71±4.31	9.01±2.20 *O. au-zz$_2$*	0.005
Viability (paired t test) p=			0.133			0.163	

Table 2. Malformation rates in gynogenetic *Oreochromis aureus* in the first generation following fertilization with homogeneric *O. niloticus (n)* or heterogeneric *Tilapia zillii (z)* sperm and in their gynogenetic offspring generated directly by fertilization with *n* or *z* sperm, or indirectly from full sibs.

	Line	Malformed fish % (no. malformed No. gynogenotes)	Line	Malformed fish % (no. malformed no. gynogenotes)	O. niloticus/T. zillii sperm effect (paired t test) p=
1st gynogenetic generation	n	15.4 (2/13)	z	5.9 (1/17)	
2nd gynogenetic generation (direct)	nn_1	22.2 (2/9)	zn_1	16.7 (2/12)	
	nz_1	9.5 (2/21)	zz_1	4.3 (1/23)	
2nd gynogenetic generation of full-sibs (indirect)	nn_2	15.8 (3/19)	zn_2	12.5 (2/16)	
	nz_2	3.6 (1/28)	zz_2	0 (0/29)	0.008
Direct/indirect gynogenetic lines (paired *t* test) p=				0.003	

process analogous to recombination, as proposed by Carter et al. (1991).

The present data show lower viability with homogeneric than heterogeneric sperm. Further, the frequency of morphological abnormalities, such as scoliosis (Don and Avtalion, 1988), was significantly lower (p=0.008) with heterogeneric sperm. The optimal irradiation dose was similar for both sperm (Shirak, 1996). These results suggest that heterogeneric sperm chromatin degrades more quickly in the cytoplasm of the zygote and embryonic cells than does homogeneric sperm chromatin, significantly reducing the negative effects of residual chromosome fragments. Deleterious effects (low viability and high malformation rates) due to fertilization with irradiated homologous sperm in the first generation (*O. au-n*) persisted in the second generation (*O. au-nz$_1$*), even when it was produced with heterogeneric sperm (p=0.011). Further, results showed the cumulative deleterious effect of fertilization with irradiated homogeneric sperm on the survival of *O. au-nn$_1$* compared to *O. au-zn$_1$* (p=0.001). Clearly, the deleterious effects are inherited over at least two gynogenetic generations. This inheritance pattern is easily explained on the basis of the findings of Thorgaard et al. (1985), Thorgard (1986) and Disney et al. (1987) that male chromatin fragments reside in a stable manner in gynogenotes. However, this does not agree with the suggestion that abnormalities in gynogenotes are due to decreased heterozygosity (Lerner, 1954; Leary et al., 1985).

Of greater importance is the finding that breeding protocols which include full-sib mating result in the purging of deleterious factors

while maintaining a high level of homozygocity. This suggests that biparental reproduction (full-sib mating, in this case) plays an important role in the control of genome integrity by purging supernumerary chromosome fragments associated with deleterious effects.

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