Production of YY Rainbow Trout Males by Self-Fertilization of Induced Hermaphrodites

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Dietary administration of various estrogens for three months from swim-up stage resulted in excess of females, the remainder of the treated groups consisting of males and hermaphrodites. Mature hermaphrodites were self-fertilized or mated with standard males and females. These hermaphrodites and some of the estrogen-treated females proved to be genetic males; the frequencies of males obtained from their ova averaged 76.8%, suggesting viability of the YY genotype. Four of nine tested males of those progenies provided all male offspring when mated with standard females.

Natural hermaphroditism is frequent in the plant kingdom where it is associated either with cross- or self-fertilization. In animals, hermaphrodite species are found less frequently, and in vertebrates they are found only in fish. In some teleosts, male and female gonads may mature simultaneously in the same animal, and internal self-fertilization has been reported in *Rivulus marmoratus*.⁵ Nevertheless, most fish are strict gonochorists. Sex chromosomes have been reported in <10% of the species examined¹⁵ and sex-linked genes have been detected only in some aquarium fish.¹² However, sex determination has been analyzed in a number of species by progeny testing hormonally sex-inverted adults.⁷

Female homogamety has been demonstrated in the rainbow trout by the production of all female progenies from some androgen-treated males.^{11,13} Two females resulting from estrogen treatment provided an excess of males (1:2 and 1:2.4) suggesting male heterogamety.¹¹ A difficulty in this type of experiment is that only some of the adults obtained after hormonal treatment are sex inverted, and therefore their genetic sex is not known before the progeny testing. This problem may be solved by uniparental reproduction. Three methods can be utilized: 1) gynogenesis, which confirmed female homogamety in rainbow trout by providing females only³; 2) androgenesis, expected to produce bisexual groups¹⁴; and 3) self-fertilization of hermaphrodites induced by incomplete sex inversion with dietary administration of estrone.⁹ These hermaphrodites are likely to be genetic males, and their self-

and cross-fertilization should confirm that male is the heterogametic sex.

In rainbow trout, functional sex inversion has been induced in both directions. Moreover, sex chromosomes have been detected cytologically;¹⁷ all females and most males have two and one subtelocentrics, respectively. Some males cannot be distinguished from females by the observation of their karyotypes after Giemsa staining. Therefore, Thorgaard^{17,18} assumed that rainbow trout represents a primitive step in sex chromosome differentiation. Data about viability of the YY genotype may permit reexamining Thorgaard's hypothesis. In addition, if YY males are fertile, they should provide all male offspring which are highly desirable in the culture of other fish-like tilapias. Viability of YY individuals has been demonstrated in the medaka¹⁹ by individual progeny testing males obtained from estrogen-treated mothers, the progenies being all male. This suggests that X and Y chromosomes do not differ markedly in genic composition in this species. We report here: 1) the effects of various estrogen treatments on sex ratio; 2) confirmation of male heterogamety by progeny testing hermaphrodites and females thus produced; 3) establishment of YY viability by the observation of all male offspring resulting from four males obtained from hermaphrodite selfing and crossbreeding.

Materials and Methods

Estrogen Treatment

Methods of dietary administration of es-

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trogens were described by Jalabert et al.⁹ We tested 3 month long periods of treatment (starting at swim-up stage) with one dose of estrone (60 mg/kg) and two doses each of three new hormones (estradiol-17 β , 12 and 24 mg/kg; ethynyl-estradiol, 3.5 and 7 mg/kg; hexestrol, 0.8 and 1.6 mg/kg). Sexual maturation was studied in each group after 2 or 3 years. The adults were divided into two classes: 1) Those that ovulated were sacrificed immediately after ova collection and autopsied; some had developed testes (hermaphrodites) and were self-fertilized, whereas the others were strictly female. 2) Those that did not ovulate were killed after the third year; some were hermaphrodites with more or less developed oocytes, whereas the remainder were strictly male but did not necessarily provide semen by stripping.

Progenies of Estrogen-Treated Individuals

Nine hermaphrodites were self-fertilized (batches HH). Sperm collection required the homogenization of the testes in diluent 532 + KCl 0.2% which reversibly arrests sperm motility.² Part of the ova collected from some of these hermaphrodites were also fertilized by sperm of three to five standard males (batches HM). Part of the sperm collected from hermaphrodite testes was also used to inseminate the ova of one standard female (batches FH). One additional control involved these standard males and females (batches FM). Seven females obtained after estrogen treatment were also fertilized by three to five standard males (batches TFM). The experimental design is summarized in Table 1. All fertilizations were performed by the dry method using diluent 532.²

A sample of 13 groups (5 obtained from hermaphrodites, self- or crossbred, 1 control FM, 7 obtained from treated females) were sexed by dissection after 1 year. Chi-square tests were used to compare observed and expected proportions of males: 1) In the case of male heterogamety, batches FH are expected to show balanced sex ratios, whereas 66% to 75% of males (depending on YY viability) are expected from HH and HM. Paradoxical effects of estrogens¹⁶ (masculinization) would lead to monosex female in HH and FH and to balanced ratios in HM. 2) In case of female heterogamety, monosex males are expected in HH and HM and balanced sex ratios in FH. Paradoxical masculinization would lead to 50% of males in HM and to 25% to 33% in batches HH and FH (depending on WW viability).

Table 1. Scheme of fertilizations and expected sex ratios

Source of gametes		Mode of reproduction	Designation	Expected sex chromosomes when female homogametic	Expected sex segregation when YY viable ^a
Female	Male				
Hermaphrodite	Hermaphrodite	Selfing	HH	XY $\times$ XY	1:3
Hermaphrodite	Standard	Crossbreeding	HM	XY $\times$ XY	1:3
Standard	Hermaphrodite	Crossbreeding	FH	XX $\times$ XY	1:1
Standard	Standard	Crossbreeding	FM	XX $\times$ XY	1:1
Sex inverted	Standard	Crossbreeding	TFM	XY $\times$ XY	1:3
Standard	HH	Progeny testing		2 (XX $\times$ XY): 1 (XX $\times$ YY)	1:1 0:1
Standard	HM	Progeny testing		2 (XX $\times$ XY): 1 (XX $\times$ YY)	1:1 0:1

^a Female : male.

Progenies of Hermaphrodite Sons

Nine males obtained from one hermaphrodite (number 4; five from batch HH and four from batch HM) were mated individually with a pool of standard females. Sex ratios were evaluated after 1 year in these progenies. The appearance of monosex male progenies would prove male heterogamety and YY viability up to the adult stage.

Results

Sex Inversion by Estrogen Treatments

All estrogen-treated individuals exhibited external signs of sexual maturation after 2 or 3 years. Out of 388 fish, 18% were males, 69% were females, and 13% were hermaphrodites. No significant differences in sex ratios were found between the six treatments tested (Table 2).

Out of 50 hermaphrodites produced in the experiment, 35 did not ovulate. Their ovaries were more or less well developed and the size of their oocytes showed considerable individual variation (Figure 1). Anatomical inspection of the testis in some males revealed abnormalities of shape and morphology of the vas deferens which may explain inability to strip semen. The 15 hermaphrodites that ovulated possessed

mature testes, but their vas deferens were abnormal or absent (Figure 2). Occasional obstructions of the genital pore are probably responsible for the frequent overripening of the collected ova. Nine ovulated hermaphrodites were self-fertilized with sperm from their homogenized testes (see Materials and Methods). Four of these batches did not provide any embryo, even when mated with standard males, but the other five resulted in progenies studied in the following sections.

Progenies of Estrogen-Treated Parents

Sex ratios of immature fish (Table 3) are in good agreement with male heterogamety and suggest a good viability of YY males. Progenies from hermaphrodite ova (HH and HM) were predominantly male. Two females (TFM) had predominantly male offsprings; presumably, these are sex-inverted females. All other sex ratios were compatible with 50%, although 66% could not be rejected in two cases. Pooling all sex ratios incompatible with 50% gave an average segregation of 1 female:3 males. Pooling results compatible with 50% gave an average sex segregation of 1:1. There is no evidence from these results for paradoxical effects of estrogen treatments or for male homogamety.

Table 2. Sex ratios following seven hormonal treatments^a

	Estrone (60 mg/kg)	Estradiol- 17- β		Ethynyl- estradiol		Hexestrol		Treat- ment not identi- fied ^b	Con- trol
		12 mg/kg	24 mg/kg	3.5 mg/kg	7 mg/kg	0.8 mg/kg	1.6 mg/kg		
Male	6	5	1	4	4	0	1	48	11
Female	22	67	12	33	7	19	15	94	11
Hermaphrodite	3	4	2	4	0	2	1	34	0
Total	31	76	15	41	11	21	17	176	22

^a Sex was determined on mature fish that had been tagged and grouped after treatment.

^b Treated individuals that had lost their tag before sexing.

Figure 1. Gonads of 10 estrogen-treated adults (animals 1 to 10); testis of male 1 has an abnormal vas deferens and differs in general shape from the testis of nontreated males. Gonads 2 to 10 present female sectors gradually increasing in size.

Individual Progeny Testing of Males Resulting from Hermaphrodite Ova

Nine males were individually progeny tested by mating with standard females (Table 4). Five of these belonged to HH and four to HM; all resulted from the same hermaphrodite. Four of these nine males

fathered predominantly male offsprings (397 of 399 individuals sexed). Sex ratios in the progenies of the five other males were close to 1:1. These results demonstrate the viability of the YY genotype, because about one of three males gave rise to all male progenies.

Figure 2. Body cavity of a hermaphrodite that ovulated; its two abnormal testes were homogenized and used for self-fertilization and cross-fertilization of standard females. A sample of its ova was also inseminated by sperm collected from standard males.

Table 3. Sex ratios determined on immature fish belonging to progenies of two hermaphrodites and seven estrogen treated females

Group	Total sexed	Male (%)	Compatibility with 50%, 66%, or 75%*
HH4	50	76	66% and 75%
HM4	44	66	66% and 75%
FH4	51	51	50%
FM4	50	52	50%
HH5	10	100	75%
HM5	116	78	75%
TFM10	110	48	50%
TFM13	50	56	50% and 66%
TFM14	48	50	50%
TFM15	52	44	50%
TFM16	44	84	75%
TFM17	61	71	66% and 75%
TFM18	50	54	50% and 66%

* Compatibility with theoretical male frequencies 50%, 66%, and 75% has been examined by χ^2 test (level 5%).

Discussion and Conclusions

The presented data show that hexestrol and ethynyl-estradiol efficiently feminize the rainbow trout. This is the first demonstration of the effectiveness of these estrogens in salmonid fish. Most estrogen treatments tested here induced deviations in sex ratios to a significant excess of females, but residual males and hermaphrodites were also present. This confirms the results of Jalabert et al⁹ obtained with estrone. In contrast, several attempts in salmonids with various estrogens do not mention the induction of hermaphroditism,^{4,8,10} probably because sexing was carried out too early. This does not permit distinguishing between females and hermaphrodites having small testicular sectors.

Deviations of sex ratio from 1:1 after

Table 4. Sex ratios in batches resulting from the fertilization of standard females by nine males obtained from hermaphrodite 4*

Male tested	Total sexed	Male	Female	Deviation from 50%
HH-4-1	100	99	1	**
HH-4-2	100	100	0	**
HH-4-3	100	55	45	NS
HH-4-4	100	62	38	*
HH-4-5	100	56	44	NS
HM-4-1	99	98	1	**
HM-4-2	97	52	45	NS
HM-4-3	102	48	54	NS
HM-4-4	100	100	0	**
Control (standard males)	102	42	60	NS

* Five belonging to HH4 and resulting from self-fertilization; 4 belonging to HM4 and resulting from cross-fertilization by standard males. Deviation from 50% is estimated at levels 5% (*) and 1% (**).

hormonal treatment do not necessarily mean that sex reversal has occurred, because mortalities caused by the hormone administration might affect one sex preferentially. Progeny testing is the most reliable method for demonstrating sex reversal. In many cases, available facilities do not enable growing a large number of separate progenies up to a size sufficient for easy sexing. In this study, the appearance of hermaphrodites was in itself a good indication of sex reversal, and this was confirmed by the excess of males in progenies obtained from their ova, and also in progenies of two females resulting from estrogen treatment. Sex ratios in several of such groups differed from 66% and not from 75%. The average sex ratio of 1:3 obtained by summing all data incompatible with a 1:1 ratio suggested a good viability of YY individuals. Similar observations have been made in coho salmon⁸ on progenies of estradiol-treated females, whereas a similar attempt in rainbow trout¹¹ raised some doubts about YY viability. In this study, YY viability was demonstrated by the fact that four of the nine males resulting from one hermaphrodite provided almost all male offspring (only two females were found in these progenies, but they may result from misclassification).

Isolation of YY individuals and propagation of YY lines have been carried out in the medaka.^{19,20} This work was greatly facilitated by using strains polymorphic at a sex-linked marker for pigmentation. The presence of recessive lethal factors closely linked to the allele born on the Y chromosome caused a very low viability of YY individuals in this particular strain. Crossing over between X and Y have been demonstrated in the medaka^{1,18} and in the guppy²⁰ by genetic analysis of sex-linked markers. This suggests a relative similarity of X and Y chromosomes permitting YY viability and X-Y meiotic pairing. In this respect, Thorgaard's assumption based on the observation of trout sex chromosomes is supported by our data.

Furthermore, the all male production from YY fathers has two potential applications. First, the basic investigations of rainbow trout male sex (females excluded) are now permitted from the onset of sex differentiation; one example may be the study of the differentiation itself and another may be the optimization of fem-

inizing treatments (any female resulting will be sex reversed).

Nevertheless, the main perspective of the all male production remains in its application to commercial breeding of fish other than salmonids. As a matter of fact, salmonid culturists find all female populations more desirable for rearing (females mature later than males and therefore do not suffer the negative consequences of sexual maturation as early as them). In the case of tilapias, monosex populations are also of great interest, because the high sexual activity causes an overcrowding in ponds; but in such a situation, all male populations are more attractive than all female populations, because an accidental contamination of males by a few females has much less consequence than the opposite. One rather extraordinary solution appeared from Hickling's work,⁶ in which interspecific crosses such as "*Sarotherodon mossambicus* female $\times$ *S. hornorum* male" provided all male offspring. This was interpreted by assuming that the *S. mossambicus* female is homogametic and the *S. hornorum* male is homogametic, the crossbred being all male XZ. Unfortunately, the all male production has not been consistent in other such crosses.

Therefore, two methods other than hybridization were expected to produce all male progenies; in the male homogametic species, there were some perspectives in the use of ZZ mothers obtained by feminization, but the all male production on a large scale by this method would require first the individual progeny testing of numerous estrogen-treated females. The situation appears more favorable in female homogametic species, such as *S. niloticus* and *S. mossambicus*, which are the most cultivated tilapias in the world; the solution would consist in mating of several YY males (produced according to the present study) with many ordinary females. Demonstration of YY viability in these species would stimulate interest in this methodology.

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