ESTRONE-INDUCED WHITE YY FEMALES AND MASS PRODUCTION OF WHITE YY MALES IN THE MEDAKA, *ORYZIAS LATIPES*

TOKIO YAMAMOTO

Biological Institute, Faculty of Science, Nagoya University, Nagoya, Japan

Received October 10, 1966

IN the medaka (*Oryzias latipes*), an oviparous cyprinodont fish, the normal sex-chromosome constitution is XX for female and XY for male (AIDA 1921). A series of our previous experiments have convincingly demonstrated that a heterotypic (atypical) sex hormone administered prior to and passing through the stage of gonadal sex differentiation induces a complete reversal in sex differentiation. In other words, a functional sex phenotype opposite to the sex genotype can be achieved at will in either direction (YAMAMOTO 1953, 1958).

In progeny tests of estrogen-induced XY females by XY males we have been confronted with an intriguing problem of viability of YY zygotes. The problem has been fully discussed on the basis of experimental results (YAMAMOTO 1964a). While viable $Y^R Y^R$ zygotes (R is a dominant gene responsible for deposition of a carotenoid) produced by mating estrogen-induced $X^r Y^R$ females with normal $X^r Y^R$ males are very rare, $Y^R Y^r$ zygotes yielded by mating estrogen-induced $X^r Y^R$ females by white $X^r Y^r$ males are, unexpectedly, all viable. At first sight, it may seem curious, but when we take into consideration the origin of the Y^r chromosome, these facts are cogently rationalized. The inference is that the regular Y^R has an inert section (—) and the ordinary X^r and X^R have a positive segment (+) essential for viability. The r of the Y^r is known to have derived from X^r by crossing over. This being so, it is quite possible that Y^r is $Y^{r,+}$ in constitution and $Y^R Y^r$ is $Y^{R,-} Y^{r,+}$, so that, the majority of $Y^R Y^R$ zygotes have double inert sections, $Y^{R,-} Y^{R,-}$, and are lethal. Presence of the inert section (—) in the regular Y^R chromosome has been inferred and experimentally proven not only in the $d-rR$ strain but in other strains, including the wild type (YAMAMOTO 1965). However, a few viable $Y^R Y^R$ zygotes are considered to be the recombinant $Y^R Y^R_c$ having the constitution $Y^{R,-} Y^{R_c,+}$, the (+) element having been transferred from $X^{r,+}$ by crossing over. (The subscript c denotes a recombinant).

In short, lethal $Y^R Y^R$ and viable $Y^R Y^R$ zygotes are radically different in genetic make-up. Experiments in conformity with this conclusion were presented earlier (YAMAMOTO 1964a, b). This being so, it is hardly surprising that practically all $Y^r Y^r$ zygotes having the (+) segment in duplicate, namely $Y^{r,+} Y^{r,+}$, are viable. In fact, we were able to detect two $Y^r Y^r$ sons among 12 F_1 offspring of estrone-induced $X^r Y^r$ females mated with $X^r Y^r$ males (YAMAMOTO 1964a). This is in strong contrast to detecting only two $Y^R Y^R$ males out of 57 F_1 offspring derived from matings of estrogen-induced $X^r Y^R$ females with normal $X^r Y^R$ males (YAMAMOTO 1959).

Estrone-induced $Y^R Y^r$ females have been reported (YAMAMOTO 1963). A further verification of the viability of $Y^r Y^r$ zygotes will now be presented. Viability of $Y^r Y^r$ males having been ascertained, the present paper deals with the production of estrone-induced $Y^r Y^r$ females. By testcrosses of both control and estrone-treated fishes, 328 $Y^r Y^r$ sons have been obtained, verifying our conclusion.

MATERIALS AND METHODS

In order to obtain YY females, estrone must be administered for two consecutive generations. In previous experiments, our $d-rR$ strain ($X^r X^r$ females, $X^r Y^R$ males), displaying father-to-son inheritance, was used. Because the present purpose is to secure estrone-induced $Y^r Y^r$ females (r = white), matings as in Table 1 were employed.

To obtain heterozygous orange-red males ($X^R Y^r$) and females ($X^R X^r$), ten homozygous orange-red females ($X^R X^R$) of our $d-RR$ strain and five white males ($X^r Y^r$) of our $d-rr$ stock were mated, producing 153 R daughters and 126 R sons. The sons ($X^R Y^r$) were used as male parents as well as testers for females, and the daughters ($X^R X^r$) were employed as testers for males.

Daily hatchings from early July to mid-July were divided each day into control and experimental groups. The initial number of each group was 220 in the first generation and 200 in the second. Control groups of both generations were fed on a standard diet composed of 60 g of dried shrimp powder, 30 g of toasted whole barley flour, 6 g of yeast preparation, and 4 g of powdered green tea. Experimental groups of both generations were treated with 100 μ g estrone per gram of the standard diet.

Hormone was administered for 7 to 8 weeks, from the time of hatching (about 4.8 mm) to the 12 mm stage or older. After termination of treatment, the young were raised on normal diet in outdoor pools until they reached maturity the next spring.

RESULTS

The results are presented in Table 1. Parents (P_1) of the first generation (Ic and Ie) were 12 white females ($X^r X^r$) and 12 heterozygous orange-red males ($X^R Y^r$). In the control (Ic), criss-cross inheritance was displayed, namely offspring were 44 orange-red daughters ($X^R X^r$) and 40 white sons ($X^r Y^r$)—a ratio of 1:1, as expected. In the estrone-treated group (Ie), all 38 orange-red ($X^R X^r$) and 24 white ($X^r Y^r$) fish developed into complete females, with only two non-reversed white males (starred in Table 1). Apparently, 92% (24/26) of white fish were estrone-induced $X^r Y^r$ females.

As parents (P_2) of the second generation (IIc and IIe), 24 estrone-induced white females, $Er(X^r Y^r)$ females, and 12 normal orange-red males ($X^R Y^r$) were used. In the control (IIc), 11 R females ($X^R X^r$), 6 R males ($X^R Y^r$) and 17 r males ($X^r Y^r + Y^r Y^r$) were produced, which approximate a ratio of 1:1:2 ($\chi^2_{(2)} = 1.5$, $P = 0.5-0.3$). Besides the control group, mass breeding of this mating type yielded 32 R females, 25 R males and 51 r males (not listed in Table 1)—a ratio of 1 R ♀ : 1 R ♂ : 2 r ♂ ($\chi^2_{(2)} = 1.3$, $P = 0.5-0.3$). Hence, the numerical relations of the classes in segregating progenies of both control and mass breeding indicate that $Y^r Y^r$ males are all viable, reconfirming the earlier conclusion (YAMAMOTO 1964a). This is verified by actual detection of $Y^r Y^r$ males in the following progeny tests.

Of 17 r males in the control group of the second generation (IIc), nine were

TABLE 1

Results of offspring produced in the first (I) and second (II) generations

P_1	$r(X^rX^r) \text{ } \varnothing \text{ } \varnothing \text{ (12)} \times R(X^RY^r) \text{ } \delta \text{ } \delta \text{ (12)}$	
	Normal diet	
I_c	$R(X^RX^r) \text{ } \varnothing \text{ } \varnothing \text{ (44)}$	$r(X^rY^r) \text{ } \delta \text{ } \delta \text{ (40)}$
P_1	$r(X^rX^r) \text{ } \varnothing \text{ } \varnothing \text{ (12)} \times R(X^RY^r) \text{ } \delta \text{ } \delta \text{ (12)}$	
	Estrone diet	
I_e	$ER(X^RX^r) \text{ } \varnothing \text{ } \varnothing \text{ (38)}$	$Er(X^rY^r) \text{ } \varnothing \text{ } \varnothing \text{ (24)}$ $Er(X^rY^r) \text{ } \delta \text{ } \delta \text{ (2)*}$
P_2	$Er(X^rX^r) \text{ } \varnothing \text{ } \varnothing \text{ (24)} \times R(X^RY^r) \text{ } \delta \text{ } \delta \text{ (12)}$	
	Normal diet	
II_c	$R(X^RX^r) \text{ } \varnothing \text{ } \varnothing \text{ (11)}$	$R(X^RY^r) \text{ } \delta \text{ } \delta \text{ (6)}$ $r(X^rY^r + Y^rY^r) \text{ } \delta \text{ } \delta \text{ (17)}$
Detected	5 + 4 (cf. Table 2)	
P_2	$Er(X^rX^r) \text{ } \varnothing \text{ } \varnothing \text{ (24)} \times R(X^RY^r) \text{ } \delta \text{ } \delta \text{ (12)}$	
	Estrone diet	
II_e	$ER(X^RX^r + X^RY^r) \text{ } \varnothing \text{ } \varnothing \text{ (28)}$	$Er(X^rY^r + Y^rY^r) \text{ } \varnothing \text{ } \varnothing \text{ (13)}$
Detected	3 + 5 (cf. Table 3)	3 + 3 (cf. Table 4)

Subscripts c and e denote control and experimental. Sex chromosome formulae in brackets following r or R are presumed sex genotypes. Figures enclosed by brackets represent numbers of parents used and offspring obtained. E denotes estronized fish. A number of animals designated with broken underline were submitted to testcrosses. Free figures are numbers of fish actually detected by testcrosses. r =white, R =orange-red.

* Nonreversals.

TABLE 2

Testcrosses of white sons of estrone-induced X^rY^r females, denoted $E(X^rY^r)$ females, mated with X^RY^r males

Parents			No. of σ^7 tested	Offspring					Genotype of tested $r\sigma^7$ parent
Females	r	Male from		$R\varphi$	$R\sigma^7$	$r\varphi$	$r\sigma^7$	Total	
X^RX^r	E(X^rY^r) $\varphi\varphi \times X^RY^r\delta\delta$	2	132	121	134	138	525	X^rY^r	
X^RX^r	E(X^rY^r) $\varphi\varphi \times X^RY^r\delta\delta$	3	23	28	26	26	103	X^rY^r	
X^RX^r	E(X^rY^r) $\varphi\varphi \times X^RY^r\delta\delta$	4	26	24	21	30	101	X^rY^r	
X^RX^r	E(X^rY^r) $\varphi\varphi \times X^RY^r\delta\delta$	5	26	35	40	28	129	X^rY^r	
X^RX^r	E(X^rY^r) $\varphi\varphi \times X^RY^r\delta\delta$	8	24	26	21	14	85	X^rY^r	
Grand total			231	234	242	236	943	$\chi^2_{(3)}=0.3$	
Ratio			1 : 1 : 1 : 1					P=0.99	
Heterogeneity			$\chi^2_{(12)}=8.4$					P=0.8-0.7	
X^RX^r	E(X^rY^r) $\varphi\varphi \times X^RY^r\delta\delta$	1	0	145	0	131	276	Y^rY^r	
X^RX^r	E(X^rY^r) $\varphi\varphi \times X^RY^r\delta\delta$	6	1*	154	1†	145	300	Y^rY^r	
X^RX^r	E(X^rY^r) $\varphi\varphi \times X^RY^r\delta\delta$	7	0	192	0	177	369	Y^rY^r	
X^RX^r	E(X^rY^r) $\varphi\varphi \times X^RY^r\delta\delta$	9	0	104	0	111	215	Y^rY^r	
Grand total			1*	594	1†	564	1,160	$\chi^2_{(1)}=0.8$	
Ratio			0 : 1 : 0 : 1					P=0.5-0.3	
Heterogeneity			$\chi^2_{(3)}=0.9$					P=0.9-0.8	

*, † See Table 5 as to testcrosses.

tested singly by $X^R X^r$ females to identify their genotype either as $X^r Y^r$ or $Y^r Y^r$. The results are shown in Table 2. They are clearly divided into two categories in respect of numerical relations of classes in their progenies. Five families comprise $R\varphi$, $R\delta$, $r\varphi$ and $r\delta$ in a 1:1:1:1 ratio. The results are in close agreement with expectation. Heterogeneity among families is also nonsignificant. From these results, the genotype of the five parental r males listed in the upper section of Table 2 is inferred to be $X^r Y^r$.

The remaining four r males, listed in the lower section of Table 2, sired all-male progenies (1,158 in number), R and r males in a 1:1 ratio, with only one exceptional R and one r females (starred and daggered in Table 2), and were identified as $Y^r Y^r$ males. The two exceptional females were submitted to progeny tests (Table 5). Although the numerical relations among the classes of progeny of the exceptional R female departed significantly from a $1R\varphi : 2R\delta : 1r\delta$ ratio ($P \approx 0.02$), we cannot label this fish other than $X^R Y^r$. The exceptional r female produced offspring conforming to a ratio of $1R\varphi : 1R\delta : 2r\delta$, and was identified as $X^r Y^r$. Hence, the two exceptions which appeared among 1,160 progenies may be regarded as XY females arising from genic imbalance between allosomal and autosomal sex-genes, i.e., from accumulation of autosomal F-genes to such an extent that the totality of sex-genes becomes $\Sigma F > \Sigma M$ in spite of their XY constitution.

For the experimental group of the second generation (IIe), the results of testcrosses of estronized R females from estrone-induced $X^r Y^r$ females mated with $X^R Y^r$ males are tabulated in Table 3. Of 28 ER females which are either estronized $X^R X^r$ females or estrone-induced $X^R Y^r$ females, 12 were submitted to testcrosses.

TABLE 3
Testcrosses of estronized orange-red daughters (ER ♀ ♀) from estrone-induced $X^r Y^r$ females mated with $X^R Y^r$ males

Parents		No. of ♀ tested	Offspring					Genotype of tested ER ♀ parent
Er female from	Male		$R\varphi$	$R\delta$	$r\varphi$	$r\delta$	Total	
$E(X^r Y^r) \varphi \varphi \times X^R Y^r \delta \delta$	$X^R Y^r$	4	67	40	0	41	148	$X^R X^r$
$E(X^r Y^r) \varphi \varphi \times X^R Y^r \delta \delta$	$X^R Y^r$	5	75	47	0	46	168	$X^R X^r$
$E(X^r Y^r) \varphi \varphi \times X^R Y^r \delta \delta$	$X^R Y^r$	6	117	54	0	55	226	$X^R X^r$
Grand total			259	141	0	142	542	$\chi^2_{(2)}=1.1$
Ratio			2 : 1 : 0 : 1					$P=0.5-0.3$
Heterogeneity							$\chi^2_{(4)}=5.0$	$P=0.3-0.2$
$E(X^r Y^r) \varphi \varphi \times X^R Y^r \delta \delta$	$X^R Y^r$	2	16	26	0	23	65	$X^R Y^r$
$E(X^r Y^r) \varphi \varphi \times X^R Y^r \delta \delta$	$X^R Y^r$	3	25	46	0	19	90	$X^R Y^r$
$E(X^r Y^r) \varphi \varphi \times X^R Y^r \delta \delta$	$X^R Y^r$	7	12	24	0	11	47	$X^R Y^r$
$E(X^r Y^r) \varphi \varphi \times X^R Y^r \delta \delta$	$X^R Y^r$	9	45	71	0	42	158	$X^R Y^r$
$E(X^r Y^r) \varphi \varphi \times X^R Y^r \delta \delta$	$X^R Y^r$	10	18	34	0	15	67	$X^R Y^r$
Grand total			116	201	0	110*	427	$\chi^2_{(2)}=1.6$
Ratio			1 : 2 : 0 : 1					$P=0.5-0.3$
Heterogeneity							$\chi^2_{(8)}=4.7$	$P=0.8-0.7$

* $Y^r Y^r$ sons.

TABLE 4

Testcrosses of estrone-induced white daughters (Er ♀ ♀) from estrone-induced X^rY^r females mated with X^RY^r males

Parents		No. of ♀ ♀ tested	Offspring				Total	Genotype of tested ER ♀ parent
Er female from	Male		R ♀	R ♂	r ♀	r ♂		
E(X ^r Y ^r) ♀ ♀ × X ^R Y ^r ♂ ♂	X ^R Y ^r	5	1	3	0	4	8	X ^r Y ^r
E(X ^r Y ^r) ♀ ♀ × X ^R Y ^r ♂ ♂	X ^R Y ^r	6	4	3	0	7	14	X ^r Y ^r
E(X ^r Y ^r) ♀ ♀ × X ^R Y ^r ♂ ♂	X ^R Y ^r	7	16	17	0	23	56	X ^r Y ^r
Grand total			21	23	0	34	78	$\chi^2_{(2)}=1.4$
Ratio			1	1	0	2		P=0.5
E(X ^r Y ^r) ♀ ♀ × X ^R Y ^r ♂ ♂	X ^R Y ^r	3	1*	27	0	24	51	Y ^r Y ^r
E(X ^r Y ^r) ♀ ♀ × X ^R Y ^r ♂ ♂	X ^R Y ^r	9	0	15	0	21	36	Y ^r Y ^r
E(X ^r Y ^r) ♀ ♀ × X ^R Y ^r ♂ ♂	X ^R Y ^r	10	0	21	0	16	37	Y ^r Y ^r
Grand total			1*	63	0	61†	124	$\chi^2_{(1)}=0.03$
Ratio			0	1	0	1		P=0.9-0.8
Heterogeneity							$\chi^2_{(2)}=1.9$	P=0.5-0.3

* See Table 5 as to testcross.

† Y^rY^r sons.

Of these, eight successfully produced offspring sufficient for identification of their genotype. The remaining four either died before reproducing or produced only a few fry which were not raised. Of eight ER females tested individually by X^RY^r males, three produced R ♀, R ♂ and r ♂ in a ratio of 2:1:1 and were identified as X^RX^r, while five did so in a ratio of 1:2:1 and were inferred as X^RY^r. None of these families deviate significantly from expectations, nor does the total.

Results of testcrosses of estrone-induced white daughters (Er ♀ ♀) among the experimental group of the second generation (IIe) are listed in Table 4. Of 13 Er females tested, three died before reproducing, two were barren owing to extreme plump abdomen, and two were lost, so that only six produced offspring. The six families are divided into distinct two categories, as expected. Although numbers of the three families listed in the upper section of Table 4 are small, segregation may be regarded as 1R ♀ : 1R ♂ : 2r ♂ indicating their maternal parents

TABLE 5

Testcrosses of exceptions appeared in offspring of estrone-induced X^rY^r females

Reference	Parents		Offspring				Total	Genotype of exceptional fish tested
	Female	Male	R ♀	R ♂	r ♀	r ♂		
Table 2	exc. R	X ^R Y ^r	163	248	0	128*	539	X ^R Y ^r
	Ratio		1	2	0	1		$\chi^2_{(2)}=8.0$, P=0.02
Table 2	exc. r	X ^R Y ^r	73	80	0	168	321	X ^r Y ^r
	Ratio		1	1	0	2		$\chi^2_{(2)}=0.9$, P=0.7-0.5
Table 4	exc. R	X ^R Y ^r	25	66	0	25†	116	X ^R Y ^r
	Ratio		1	2	0	1		$\chi^2_{(2)}=2.2$, P=0.5-0.3

*, † Y^rY^r sons.

($Er \text{ } \varnothing$) to be X^rY^r . P estimated by χ^2 test for the combined data is statistically in agreement with this. The remaining three Er females produced all-male offspring, R males and r males in a 1:1 ratio, with a single exceptional R female (starred in Table 4). Obviously, the genotype of the three Er females listed in the lower section of Table 4 must be Y^rY^r . None of the three families deviates significantly from expectation. Thus, production of estrone-induced Y^rY^r females was successfully achieved. The single exceptional R female was tested by an $X^R Y^r$ male (Table 5) and found to be an $X^R Y^r$ female arising from genic imbalance between allosomal and autosomal sex genes.

DISCUSSION

Hitherto, exceptional XY females that arose sporadically by genic imbalance between allosomal and autosomal sex-genes had been used to produce YY males in male-heterogametic fishes (WINGE 1934, and WINGE and DITLEVSEN 1947 in *Lebistes*; AIDA 1936 in *Oryzias*). However, the appearance of such exceptional XY females was too infrequent to allow them to be used to produce YY zygotes. For example, WINGE obtained seven such females, and AIDA only two.

Production of XY females through the action of estrogens (YAMAMOTO 1953, 1959) makes it possible to produce YY zygotes deliberately. For the most part of my previous studies, however, estrogen-induced X^rY^R females have been mated with normal X^rY^R males to produce $Y^R Y^R$ males. Since the genotypes X^rY^R and $Y^R Y^R$ cannot be distinguished visibly, F_1 R males were testcrossed individually. However, surviving $Y^R Y^R$ males were a great rarity: so much so that only two $Y^R Y^R$ males had been detected out of 57 R (X^rY^R and $Y^R Y^R$) males individually tested. That is to say, $Y^R Y^R / (X^rY^R + Y^R Y^R)$ is 1:28.5 instead of the expected 1:3, had all $Y^R Y^R$ been viable (cf. YAMAMOTO 1955, 1959). The reason for this is presented in the first section.

In contrast to the rarity of viable $Y^R Y^R$ zygotes, practically all $Y^R Y^r$ zygotes, produced by estrogen-induced X^rY^R females mated with X^rY^r males, were found to survive because their constitution is $Y^{R,-}Y^{r,+}$. This fact was repeatedly confirmed not only from the numerical proportions of segregating classes but by actual detection of the genotype by test crosses. Using this mating type and administering estrone again, even the production of estrone-induced $Y^R Y^r$ females was successfully accomplished (YAMAMOTO 1963). This is evidence for the presence of the (+) segment in the Y^r chromosome, and it is concluded that $Y^R Y^r$ zygotes have the constitution $Y^{R,-}Y^{r,+}$.

In the light of earlier studies (YAMAMOTO 1964a, b) pertaining to the viability of YY zygotes, it is hardly surprising that practically all $Y^r Y^r$ zygotes, which would have the (+) segment in duplicate, are viable. If a YY zygote formed at fertilization is to survive and develop into an adult fish, the viability segment (+) must be present either in single dose or in duplicate. This being so, it is rational that practically all $Y^r Y^r$ zygotes, which have the (+) section in duplicate ($Y^{r,+} Y^{r,+}$), should survive.

The present study corroborates the conclusion arrived at from the foregoing

experiments and inference. As shown in Table 2, we were able to detect four Y^rY^r males among nine white ones ($X^rY^r + Y^rY^r$), by progeny tests of the control group in the second generation. In progeny tests (Table 4) of estrone-induced white females ($X^rY^r + Y^rY^r$), three induced X^rY^r females and three induced Y^rY^r females were actually detected. Also, the 171 white (r) sons in the lower section of Table 3 (starred) and Table 4 (dagged), are Y^rY^r males. They were produced by mating either estrone-induced $X^R Y^r$ or $X^r Y^r$ females by $X^R Y^r$ males. Observed and expected segregation ratios are in fair agreement. This indicates that all Y^rY^r males are viable, as predicted in the earlier papers.

In addition to these results, 153 (128 + 25) white (Y^rY^r) males (starred and dagged in Table 5) were produced among offspring of testcrosses of exceptions. Altogether, 328 (4 + 110 + 61 + 153) Y^rY^r males were mass produced in the present study. In all these cases, there is no shortage of surviving Y^rY^r zygotes. This fact is in striking contrast to the rarity of viable $Y^R Y^R$ males among offspring derived from estrogen-induced $X^r Y^R$ females crossed by normal $X^r Y^R$ males. The present study thus provides strong evidence for the correctness of the previous thesis (YAMAMOTO 1964a) and for the reality of the genetic map of the sex chromosomes (YAMAMOTO 1964b).

Artificial production of estrone-induced white (Y^rY^r) females and mass production of Y^rY^r males not only validate the conclusion arrived at in the earlier papers, but provide an unusual opportunity to approach further researches which are otherwise difficult.

SUMMARY

Estrone-induced Y^rY^r (white) females were produced in the medaka, an oviparous cyprinodont, where the normal sex-chromosomal constitution is XX for females and XY for males. Offspring of $X^rX^r \text{♀} \times X^R Y^r \text{♂}$ (R = orange-red) displaying criss-cross inheritance, were routinely treated with estrone to obtain estrone-induced X^rY^r females, $E(X^rY^r)$, in the first generation. The offspring of $E(X^rY^r) \text{♀} \times X^R Y^r \text{♂}$ were again treated with estrone to obtain estrone-induced white females comprising $E(X^rY^r)$ and $E(Y^rY^r)$. By progeny tests, three $E(X^rY^r)$ and three $E(Y^rY^r)$ females were successfully detected. In the control group, five X^rY^r and four Y^rY^r males were identified. A total of 328 Y^rY^r males were produced. The proportion of Y^rY^r males observed among the classes segregating conforms fairly well to expectation, indicating that practically all Y^rY^r zygotes are viable in contrast to $Y^R Y^R$ zygotes, of which only rare crossovers ($Y^R Y^R_c$) can survive. The present study corroborates the earlier conclusion pertaining to viability of YY zygotes.

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