ARTIFICIALLY INDUCED SEX-REVERSAL IN GENOTYPIC MALES OF THE MEDAKA (ORYZIAS LATIPES) ¹

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THREE FIGURES

INTRODUCTION

A number of experiments have been reported on artificial sex-reversal in animals. Unfortunately, however, the materials used in most experiments have not been sufficiently analyzed genetically or embryologically. Furthermore, the sex-ratio has been employed as the evidence of sex-reversal in almost all experiments. The sex-ratio criterion, however, is unreliable unless sufficient attention is paid to the differential mortality in the two sexes. In fact, a great difference in the rate of juvenile mortality between the sexes is known in some forms, especially when feeding has been insufficient.

The fact that the secondary sexual characteristics in vertebrates are modified by sex hormones is well known. Recently a number of investigators have noticed that sex hormones also have an effect on the gonad; i.e., androgens induce the masculinization of the ovary and estrogens result in the feminization of the testis. There are excellent reviews concerning hormonal control of the differentiation of embryonic gonads (Willier, '42; Witschi, '42) in birds and lower vertebrates respectively. Therefore, only a brief review is given here on the hormonal control of gonads in fishes. Padoa

¹ Read before the 23rd annual meeting of the Genetics Society of Japan held at Hiroshima on October 12, 1951.
Toki-o Yamamoto ('37, '39) has reported that the injection of follicular hormone produces ova in the testes, while testosterone induces the process of transformation of ovaries into testes in the rainbow trout (*Salmo irideus*). Berkowitz ('38, '41) caused the production of ovo-testis in the guppy (*Lebistes reticulatus*) by the oral administration of estrogens to young males. He reported that the optimal dosage resulted in an almost complete reversal of testis to ovary. Okada ('43) showed that subcutaneous administration of estrogens (estriol and stilbestrol) causes the formation of ovo-testis in adult males of the medaka (*Oryzias latipes*). On the other hand, he found ('44) that subcutaneous implantation of crystals of androgen (methyl-dihydro-testosterone) brings about no change in the ovary of the adult medaka, while he succeeded in the production of ovo-testis in the males by estrogens and induction of male germ cells in the ovary by an androgen in the young top-minnow (*Gambusia affinis*). These experiments have induced only feminization of the testis by estrogens, and masculinization of the ovary by androgens, so that complete sex-reversal has not been attained. Further, Okada ('49) reared the young (within two weeks after hatching) of the orange-red medka in very dilute solutions of an androgen (methyl-dihydro-testosterone) and an estrogen (Ovahormon) and examined the sex-ratio of the young after fixation. Although the number of fishes used in his experiments was not great, he claimed that both androgen and estrogen retard the differentiation of the testis and that not only the transformation of testis into ovary (intersex) but complete sex-reversal, from male to female, was attained.

In the sword-tail (*Xiphophorus helleri*), which has no sex-chromosomes and is a factorial hermaphrodite, the sex is said to be determined by the environmental conditions. Essen-berg ('26) and Friess ('33) reported cases of complete sex-reversal, from female to male, in this fish.

On the other hand, geneticists in their normal breeding experiments frequently have found exceptional males and females in forms whose sex is normally determined by sex-
ARTIFICIAL SEX-REVERSAL IN FISH

chromosomes. Thus in the guppy (Lebistes reticulatus), where the male is heterogametic (XY), Winge ('30, '34) found X X-males and XY-females. In Oryzias (Aplocheilus) latipes whose sex-chromosomes are also XY type, Aida ('36) found two XY-females and XX-males. Although these findings are accidental, they clearly suggest the ambisexualuty of the two sexes.

The present report deals with complete artificial sex-reversal in the male genotype of the medaka (Oryzias latipes), the genotype of which had been analyzed genetically. The one-sided masculine sex-limited inheritance, or father-to-son inheritance, was employed as the criterion of sex-reversal, instead of sex-ratio. Thus the sex-reversed individuals in these experiments are easily distinguishable by their body color. It will be seen that complete sex-reversal from male to female has been attained under the influence of estrogens, and that sex-reversed females of the male genotype were fertile and yielded offspring.

MATERIALS AND METHODS

The materials used were the white and orange-red (called red) varieties of the medaka (Oryzias latipes). There are a number of varieties of the medaka in respect to body color. The studies of Aida ('21) on the inheritance of this fish have shown that the various color effects may be referred to the action of genes, as indicated by the following formulae (when homozygous): brown (wild) BBRR, blue BBrr, red (orange-red) bbRR, white bbrr, variegated red B'B'RR and variegated white B'B'rr. The triple allelomorphic genes B, B' and b control melanin formation and are autosomal. The genes R and r control the formation of the orange-red pigment and are heterosomal. Since both white (bbrr) and red (bbRR or bbRr) medakas contain the common recessive bb genes, they can be phenotypically expressed merely as r and R respectively. According to Aida ('21), the male is heterogametic and the red (R) and white (r) genes are located in X and Y chromosomes.
White females (X'X') and heterozygous red males (X'Y") were used. Since the dominant red gene R is located in the Y chromosome only, the progeny will be white females and red males in equal numbers. The genealogical diagram of the material used is illustrated in figure 1. In 1946 a white female (X'X') and hundreds of red medaka were bought from a dealer at Yatomi, Aichi pref., and the white female was mated with a red male. Judging from the size of the fish, it was estimated that they were hatched in 1945. Since the F_1 progeny were all red in both sexes (♀24, ♂21), the P_1 red male was judged to be homozygous regarding R genes. Its genetic constitution was assumed to be X"Y". The genotypes of the F_1 red males and F_1 red females were X'Y" and X'X" respectively. In 1947 a heterozygous red male (X'Y") was back-crossed with another white female (X'X') obtained
from Yatomi. Of the FR₁ progeny, all the females (13) were white and all the males (19) were red, as was expected. These white females and the heterozygous red males were inbred. The FR₂ progeny yielded 18 white females and 20 red males. The FR₃ progeny showed 14 white females and 21 red males. It will be seen that all the white fish were females and all the red fish were male, as would be predicted. Theoretically there should be an equal number of white females and red males. The slight preponderance of males may be due to the lower viability of white females, which are double recessive (bbrr). Neither exceptional males (white) nor exceptional females (red) were found for three generations. These breeding experiments indicate that accidental sex-reversal did not take place in our strain when they were reared under normal conditions (normal diet, indoor breeding).

In 1950 FR₃ white females (X’ X’) were mated with FR₃ heterozygous red males (X’ Y*). Newly hatched fry of the FR₄ generation were exclusively used as material. Vita-yoso (a nutrient agent) was used as the standard diet. The chief ingredients of this nutrient agent are dried fish powder and vitamins. The hatched fry (9/VII–9/VIII) were successively reared with (C) normal diet (control), (E) estrone-diet (1,250 I.U./gm standard diet) and (S) stilbestrol-diet (5,000 I.U./gm standard diet). Tablets of Ovahormon (1950) ² and Oestimon ³ were used as the source of estrone and stilbestrol (4,4’-dioxo-α,β-diethylstilben) respectively. Tablets were finely ground in a mortar and mixed with the Vita-yoso. The estrogens were administered per os from the very beginning of the food uptake. Feeding with the estrogens was continued for about 8 months. Water-fleas (Moina macrocopa) and tubificid worms (Limnodrilus, etc.) were used as live food only occasionally. The fish were reared indoors in 10-litre glass jars.

² Teikoku-zoki-selyaku-kaisha, Tokyo. It may be remarked that recently (1951) the hormone contained in Ovahormon is ethynyl estradiol instead of estrone.
³ Tanabe Gohei & Co., Osaka.
Although the two phenotypes \((R\) and \(r\)) cannot be distinguished in newly hatched fry, they are distinguishable in very young fish. Thus the genotypic females (white) and genotypic males (red) are easily recognizable in our case. After 8 months the sex was examined in the majority of the fish externally as well as internally. The fish were fixed with Bouin’s solution, decalcified with Perényi’s chromo-nitric acid, and serial sections were stained with Delafield’s hematoxylin and eosin. Some were reared to sexual maturity and were submitted to genetic analysis. The body length in this paper is the length from the tip of the snout to the end of the caudal fin of the living fish measured in an anesthetized condition. It may be remarked that fixation causes considerable reduction in size.

**Sexual differentiation of the gonads**

Before proceeding further it will be useful to give a brief outline of the differentiation of the gonad in normal medakas. Observations on the sexual differentiation of the gonad were made in post-hatching stages. The newly hatched fry of the medaka measures 4.5–5.4 mm in length. Serial sections indicate that morphologically indifferent gonads or gonad primordia are already differentiated at the time of hatching. The gonads of the newly hatched fry are suspended in the coelom between the pronephric ducts and the gut in the posterior trunk region. They are bilaterally distended lobes constricted in the median part. In the adult medakas both the ovary and the testis are median, unpaired organs. In the fry, however, the paired origin of the gonad is apparent. The gonad of the newly hatched fry consists of gonia and peritoneal epithelium. It is solid and the gonocoel is not yet differentiated. The gonia are asymmetrically distributed to the gonad primordia, the right lobe receiving the larger number in some fry while in others the right lobe receives the larger number in the anterior half and the left lobe receives the larger number in the posterior half. Although serial
sections of 14 newly hatched fry were examined, no sexual
difference between the gonads was apparent at this stage.
Therefore the gonad of the newly hatched fry may be re-
garded as indifferent. The phenotypes (R or r) can be dis-
tinguished when the young become about 5.7 mm in length.
The condition of the gonad in the 5.7 mm young is much the
same as that found in the newly hatched fry except that the
fusion of the paired lobes of the gonad is more advanced.

In 6.5 mm young, however, the morphological differentiation
of the gonad is apparent. In females of this stage some of the
gonia transform into oocytes and undergo growth. Coinci-
dent with the growth of the oocytes there is a marked in-
crease in the size of the ovary. The ovocoel is not yet differ-
entiated at this stage. In 7.5–8.0 mm young, the ovary is
suspended in the coelom by a mesovarium derived from the
peritoneum, extending between the ventral wall of the swim-
bladder and the gut. In 10 mm young, the ovocoel becomes
apparent and the ovary is suspended dorsally by the meso-
varium and ventrally by the dorsal mesentery. Some oocytes
are undergoing cytoplasmic differentiation. In 12 mm young,
the ovocoel, which is T-shaped in cross section, is greatly
enlarged. The germ cells are mainly present beneath the
ovocoel, and the dorsal roof of the ovary consists only of a
connective tunic which covers the entire ovary. An incom-
plete septum projects into the ovocoel from the median part
of the dorsal roof of the ovary. The nuclei of the growing
oocytes are losing their reticular structure and their chro-
mation collects into numerous rounded bodies (nucleoli) per-
ipherally arranged along the nuclear membrane.

The differentiation of the testis apparently proceeds at a
slower rate than does that of the ovary. In the 6.5 mm fish,
the size and the organization of the testis are not greatly
different from those of the indifferent gonad. In the 10 mm
young the testis is suspended along the ventral wall of the
swim-bladder, dorsally by the mesorchium and ventrally by
the dorsal mesentery. It has the appearance in cross section
of double lobes. In the 12 mm young, the gonocoel (testocoel)
is differentiated. The testis of the 12–15 mm young is, however, very small as compared with the ovary of the same stage.

Although in *Oryzias latipes* there is a period of gonadal development prior to sex differentiation, it is a period of indifferent gonad, such as has been reported by Wolf ('31) in the platy (*Platypoecilus maculatus*), and Johnstone ('51) in the large-mouthed black bass (*Micropterus salmoides salmoides*), and not of juvenile hermaphroditism such as that reported by Mršić ('23) in the rainbow trout, Essenberg ('23) in the sword-tail (*Xiphophorus helleri*), Goodrich et al. ('34) and Dildine ('36) in the guppy (*Lebistes reticulatus*). The gonad of the species under observation exhibits sex-differentiation directly from the indifferent state. In this respect *Oryzias latipes* may be regarded as a "sex-differentiated" species in the sense of the definition by Witschi ('14, '15). Genetically, the X Y type of sex determination has been proved by Aida ('21). The sex-ratios support the theory of the X Y mechanism of sex determination. Of 237 orange-red medakas obtained from Yatomi in 1947, 118 were females and 119 were males, so that the sex-ratio was 1:1. At the time of hatching the gonad is in the state of morphological indifference, and morphological sex-differentiation of the gonad occurs after hatching. Therefore it may be safe to conclude that both embryologically and genetically the sex-determining mechanism of the present material is relatively stable among fishes.

*Secondary sexual characteristics and their development in the medaka*

Although Oka ('31) has already described the secondary sexual characteristics in the adults of this fish, a brief outline of them and their development is included since they are used for the external discrimination of sex in this work. In reading the following description the reader is asked to consult figure 3, C18 and C6. In the female, the anal fin is smaller than that of the male and has a right-angled triangle in shape,
without a pronounced saw-toothed edge. In the male, the anal fin is larger and has the shape of a parallelogram. In typical females the anal-fin rays are bifurcated, except for the first two or three. The bifurcation of the rays, however, appears only in full-grown females whose body length is greater than 28-34 mm. There are considerable individual variations as to the age at which bifurcation begins. Females larger than 35 mm usually have dichotomous anal-fin rays. The most prominent feature of the male anal fin is the presence of papillar processes on the rays of the posterior part. The margin of the male anal fin has a markedly saw-toothed edge. The dorsal fin of the male is longer than that of the female and has a pronounced cleft between the 5th and 6th rays. The female has well-defined fleshy urinogenital papillae between the anus and the oviduct pore. In the male the corresponding structure is a single less developed protuberance between the anus and the urinogenital pore.

Sexual dimorphism first appears when the fish have reached a length of about 16-18 mm. The urinogenital papillae of the female become larger than that of the male and the anal fin of the male becomes slightly larger than that of the female. The urinogenital papillae may be used as the criterion for sex-discrimination when fish become more than 16-18 mm in length. The differences in the anal and dorsal fins become prominent when the fish reach 20 mm in length. The papillary processes in the male anal fin begin to appear in 26 mm fish, and sexual maturity is attained in both sexes when the fish reach 26-28 mm in length. Fish whose body length is more than 28 mm may be regarded as fully grown.

**Sex-differentiation in genotypic males and females**

**in control medakas (normal feeding)**

The sex-differentiation in the genotypic males and females of the control is shown diagrammatically in figure 2 (C) and in tabular form in table 1. The parents were FR\(_3\) white females (X\(X^c\)) and FR\(_3\) red males (X\(Y^r\)). The secondary
sexual characteristics were observed under anesthesia. Some of them were submitted to histological examination of the gonad in order to determine the sex while others were reared to sexual maturity and were used for breeding. Among many FR₄ offspring in the control, 12 red (X'Y") fishes and 8 white

### TABLE 1

**Sex-differentiation in genotypic males and females in control medakas** *(Normal feeding)*

<table>
<thead>
<tr>
<th>No. of Fish</th>
<th>Color of Fish</th>
<th>Genotype</th>
<th>Body Length at Obs. in mm</th>
<th>Sec. Sex Character.</th>
<th>Gonad</th>
<th>Sex</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 1</td>
<td>Red</td>
<td>X'Y&quot;</td>
<td>16</td>
<td>Testis</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>C 2</td>
<td>Red</td>
<td>X'Y&quot;</td>
<td>20</td>
<td>♂ type</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>C 3</td>
<td>Red</td>
<td>X'Y&quot;</td>
<td>23</td>
<td>♂ type</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>C 4</td>
<td>Red</td>
<td>X'Y&quot;</td>
<td>27</td>
<td>♂ type</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>C 5</td>
<td>Red</td>
<td>X'Y&quot;</td>
<td>30</td>
<td>♂ type</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>C 6</td>
<td>Red</td>
<td>X'Y&quot;</td>
<td>32</td>
<td>♂ type</td>
<td>♂</td>
<td>Breeding, fertile</td>
<td></td>
</tr>
<tr>
<td>C 7</td>
<td>Red</td>
<td>X'Y&quot;</td>
<td>33</td>
<td>♂ type</td>
<td>♂</td>
<td>Sterile, degener. testis</td>
<td></td>
</tr>
<tr>
<td>C 8</td>
<td>Red</td>
<td>X'Y&quot;</td>
<td>34</td>
<td>♂ type</td>
<td>♂</td>
<td>Breeding, fertile</td>
<td></td>
</tr>
<tr>
<td>C 9</td>
<td>Red</td>
<td>X'Y&quot;</td>
<td>34</td>
<td>♂ type</td>
<td>♂</td>
<td>Breeding, fertile</td>
<td></td>
</tr>
<tr>
<td>C 10</td>
<td>Red</td>
<td>X'Y&quot;</td>
<td>34</td>
<td>♂ type</td>
<td>♂</td>
<td>Breeding, fertile</td>
<td></td>
</tr>
<tr>
<td>C 11</td>
<td>Red</td>
<td>X'Y&quot;</td>
<td>37</td>
<td>♂ type</td>
<td>♂</td>
<td>Breeding, fertile</td>
<td></td>
</tr>
<tr>
<td>C 12</td>
<td>Red</td>
<td>X'Y&quot;</td>
<td>39</td>
<td>♂ type</td>
<td>♂</td>
<td>Breeding, fertile</td>
<td></td>
</tr>
<tr>
<td>C 13</td>
<td>White</td>
<td>X'X'</td>
<td>13</td>
<td>Ovary</td>
<td>♀</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>C 14</td>
<td>White</td>
<td>X'X'</td>
<td>16</td>
<td>Ovary</td>
<td>♀</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>C 15</td>
<td>White</td>
<td>X'X'</td>
<td>18</td>
<td>♀ type</td>
<td>♀</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>C 16</td>
<td>White</td>
<td>X'X'</td>
<td>26</td>
<td>♀ type</td>
<td>♀</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>C 17</td>
<td>White</td>
<td>X'X'</td>
<td>32</td>
<td>♀ type</td>
<td>♀</td>
<td>Breeding, fertile</td>
<td></td>
</tr>
<tr>
<td>C 18</td>
<td>White</td>
<td>X'X'</td>
<td>34</td>
<td>♀ type</td>
<td>♀</td>
<td>Breeding, fertile</td>
<td></td>
</tr>
<tr>
<td>C 19</td>
<td>White</td>
<td>X'X'</td>
<td>37</td>
<td>♀ type</td>
<td>♀</td>
<td>Breeding, fertile</td>
<td></td>
</tr>
<tr>
<td>C 20</td>
<td>White</td>
<td>X'X'</td>
<td>37</td>
<td>♀ type</td>
<td>♀</td>
<td>Breeding, fertile</td>
<td></td>
</tr>
</tbody>
</table>

(X'X') ones survived after 8 months. All the red individuals except C1 and C7 had secondary sexual characteristics of the male type. In fish C1, which was 16 mm in length at the time of observation, secondary sexual characteristics were not yet developed. The secondary sexual characteristics of C7 were of the neutral type. The anal and the dorsal fin
were of the female type and the urinogenital papillae were not developed as in the male. Relatively young red fishes (C1, C2, C3, C4 and C5) were fixed after 8 months and were examined histologically. They were found to be males. Fully grown red fishes (C6, C7, C8, C9, C10, C11 and C12) were used for breeding. They were fertile, except for C7, which was found to be sterile. The histological examination of C7 indicated that the testis was completely degenerated. It will be seen that all (12) red (X' Y") fish in the control were male, as would be expected. All the white fish except C13 and C14 had secondary sexual characteristics of the female type. In C13 and C14, which were 13 mm and 16 mm in length, respectively, the secondary sexual characteristics had not yet been developed at the time of observation. Relatively young white fish (C13, C14, C15 and C16) were fixed and examined histologically. They were found to be females. Fully grown white fish (C17, C18, C19 and C20) were used for breeding. They were found to be fertile. Therefore, all the white (X' X') fish in control (8) were found to be female as would be expected.

It will thus be seen that neither exceptional females (red female) nor exceptional males (white male) were produced in the FR4 generation in the control (normal feeding).

**Effect of estrone-feeding on sex-differentiation in genotypic males and females**

The effect of estrone-feeding on sex-differentiation in genotypic males and females is shown diagrammatically in figure 2(E) and in tabular form in table 2. The parents were FR3 white females (X' X') and FR3 red males (X' Y"), as in the controls. Estrone was administered *per os* to the FR4 offspring for a period of about 8 months from the time of hatching. Among numerous estrone-treated FR4 offspring, 10 red (X' Y") fish and 9 white (X' X') ones survived after about 8 months.

Among these 10 red fishes, the older ones (E5, E6, E7, E8, E9 and E10) showed secondary sexual characteristics of the female type, while in the younger ones (E1, E2, E3 and E4),
the secondary sexual characteristics were not yet developed at the time of observation. Fish E1, E2, E3, E4, E5, E6 and E7 were fixed and examined histologically in order to determine the sex. They were found to be female, since they had typical ovaries. Careful observation indicated that the ovaries of all these individuals contained only female germ cells. The oocytes were normal, like those of normal (X X) females. Each oocyte had a large germinal vesicle which contained numerous nucleoli peripherally arranged along the nuclear membrane. These may be associated with the vitellogenesis in the cytoplasm of the oocyte. The egg membrane is formed around the larger oocytes by the follicle cells. No male germ cells were found in all the ovaries examined. In

TABLE 2
Effect of estrone-feeding on the sex-differentiation in genotypic males and females of the medaka
Parents: FR, White (X' X') ♀ '49, FR, Red (X' Y') ♂ '49

<table>
<thead>
<tr>
<th>NO. OF FISH</th>
<th>COLOR OF FISH</th>
<th>GENO-TYPE</th>
<th>BODY LENGTH AT OBS. IN MM</th>
<th>SEC. SEX CHARACT.</th>
<th>GONAD</th>
<th>SEX</th>
<th>REMARKS</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>Red</td>
<td>X' Y*</td>
<td>11.5</td>
<td>Ovary</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>Red</td>
<td>X' Y*</td>
<td>14</td>
<td>Ovary</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>E3</td>
<td>Red</td>
<td>X' Y*</td>
<td>15.5</td>
<td>Ovary</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>E4</td>
<td>Red</td>
<td>X' Y*</td>
<td>16.5</td>
<td>Ovary</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>E5</td>
<td>Red</td>
<td>X' Y*</td>
<td>17</td>
<td>♀ type</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>E6</td>
<td>Red</td>
<td>X' Y*</td>
<td>17</td>
<td>♀ type</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>E7</td>
<td>Red</td>
<td>X' Y*</td>
<td>19</td>
<td>♀ type</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>E8</td>
<td>Red</td>
<td>X' Y*</td>
<td>30</td>
<td>♀ type</td>
<td>♂</td>
<td>Breeding, fertile</td>
<td></td>
</tr>
<tr>
<td>E9</td>
<td>Red</td>
<td>X' Y*</td>
<td>32</td>
<td>♀ type</td>
<td>♂</td>
<td>Breeding, fertile</td>
<td></td>
</tr>
<tr>
<td>E10</td>
<td>Red</td>
<td>X' Y*</td>
<td>34</td>
<td>♀ type</td>
<td>♂</td>
<td>Breeding, fertile</td>
<td></td>
</tr>
<tr>
<td>E11</td>
<td>White</td>
<td>X' X'</td>
<td>13</td>
<td>Ovary</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>E12</td>
<td>White</td>
<td>X' X'</td>
<td>15</td>
<td>Ovary</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>E13</td>
<td>White</td>
<td>X' X'</td>
<td>16</td>
<td>Ovary</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>E14</td>
<td>White</td>
<td>X' X'</td>
<td>17</td>
<td>♀ type</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>E15</td>
<td>White</td>
<td>X' X'</td>
<td>20</td>
<td>♀ type</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>E16</td>
<td>White</td>
<td>X' X'</td>
<td>20</td>
<td>♀ type</td>
<td>♂</td>
<td>Fix. micr. obs.</td>
<td></td>
</tr>
<tr>
<td>E17</td>
<td>White</td>
<td>X' X'</td>
<td>32</td>
<td>♀ type</td>
<td>♂</td>
<td>Breeding, fertile</td>
<td></td>
</tr>
<tr>
<td>E18</td>
<td>White</td>
<td>X' X'</td>
<td>34</td>
<td>♀ type</td>
<td>♂</td>
<td>Breeding, fertile</td>
<td></td>
</tr>
<tr>
<td>E19</td>
<td>White</td>
<td>X' X'</td>
<td>37</td>
<td>♀ type</td>
<td>♂</td>
<td>Breeding, fertile</td>
<td></td>
</tr>
</tbody>
</table>
ARTIFICIAL SEX-REVERSAL IN FISH

other words, no intersexes occurred. It may be said that they are sex-reversed females of the (XY) genotype.

The structure of the ovary and oviduct of these sex-reversed females of the male genotype is similar to that of the normal (XX) females. The ovary is a median, unpaired sac-like organ with a main lumen. The oviduct is a single, thick-

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**Fig. 2** Diagrams of the effect of estrogens on sex-differentiation in *Oryzias latipes.*
walled tube as contrasted to the male genital duct (*vas deferens*), which is a thin tube. The walls of the oviduct consist largely of circular muscle fibers and are continuous with the walls of the ovary. The duct opens behind the urinogenital papillae, between the anus and the urinary pore. In the female, the oviduct and the urinary duct open separately, while in the male the genital duct (*vas deferens*) and the urinary duct are fused near the end of the protuberance (corresponding to the urinogenital papillae of the female) and open as the urinogenital pore. Artificially sex-reversed females have typical urinogenital systems like those of normal females.

Fully grown sex-reversed red fish (E8, E9 and E10) have typical secondary sexual characteristics of the female type. The anal and the dorsal fin is of the female type and the anal fin has no papillary processes. The urinogenital papillae are more developed than those of normal (control) females. The secondary sexual characteristics of E9 are shown in figure 3. E8, E9 and E10 were mated with normal red (*X' Y'*) males. E10 (34 mm) died before the production of eggs. Laparotomy indicated that her ovary contained well-developed ova. E8 (30 mm) and E9 (32 mm) laid eggs during June 22–July 9, '51 and May 27–June 7, '51 respectively and then they were fixed. The eggs developed normally into embryos and fry hatched out. The offspring of the sex-reversed females are thriving quite normally. Therefore it follows that all the 10 red fish of the genotype of *X' Y'* showed completely reversed sex as the result of estrone feeding and became perfect females phenotypically. They are not only female in primary and secondary sexual characteristics but are functional females.

Relatively young white fish (E11, E12, E13, E14, E15 and E16) were fixed and examined microscopically. They had typical ovaries with growing oocytes. E14 (17 mm), E15 (20 mm), and E16 (20 mm) showed such secondary sexual characteristics of the female as the urinogenital papillae. The fully grown white fish (E17, E18 and E19) have the urino-
genital papillae more developed than those of the control females. On being mated with normal red \((X^r Y^r)\) males they were found to be fertile, and yielded eggs which developed into normal fish. Therefore, all (9) white fish with the genotype of \(X^r X^r\) differentiated into normal females, as would be expected.

Fig. 3 Secondary sexual characteristics of normal (control) \(Oryzias latipes\) and of experimentally sex-reversed \(XY\) females. a, anus; af, anal fin; d, dorsal fin; u, urinogenital papillae; v, ventral fin; p, papillar processes.
The effect of stilbestrol-feeding on sex-differentiation in genotypic males and females is shown diagrammatically in figure 2 (S) and in tabular form in table 3. The parents were FR3 white females (X' X') and FR3 red males (X' Y') as in

**TABLE 3**

Effect of stilbestrol-feeding on the sex-differentiation in the genotypic males and females of the medaka

<table>
<thead>
<tr>
<th>NO. OF FISH</th>
<th>COLOR OF FISH</th>
<th>GENOTYPE</th>
<th>BODY LENGTH AT OBS. IN MM</th>
<th>SRC. SEX CHARACT.</th>
<th>GONAD</th>
<th>SEX</th>
<th>REMARKS</th>
</tr>
</thead>
<tbody>
<tr>
<td>S 1</td>
<td>Red</td>
<td>X' Y'R</td>
<td>17.5</td>
<td>2 type</td>
<td>Ovary</td>
<td>♀</td>
<td>Fix. mier. obs.</td>
</tr>
<tr>
<td>S 2</td>
<td>Red</td>
<td>X' Y'R</td>
<td>18</td>
<td>2 type</td>
<td>Ovary</td>
<td>♀</td>
<td>Fix. mier. obs.</td>
</tr>
<tr>
<td>S 3</td>
<td>Red</td>
<td>X' Y'R</td>
<td>18</td>
<td>2 type</td>
<td>Ovary</td>
<td>♀</td>
<td>Fix. mier. obs.</td>
</tr>
<tr>
<td>S 4</td>
<td>Red</td>
<td>X' Y'R</td>
<td>20</td>
<td>2 type</td>
<td>Ovary</td>
<td>♀</td>
<td>Fix. mier. obs.</td>
</tr>
<tr>
<td>S 5</td>
<td>Red</td>
<td>X' Y'R</td>
<td>20.5</td>
<td>2 type</td>
<td>Ovary</td>
<td>♀</td>
<td>Fix. mier. obs.</td>
</tr>
<tr>
<td>S 6</td>
<td>Red</td>
<td>X' Y'R</td>
<td>24</td>
<td>2 type</td>
<td>Ovary</td>
<td>♀</td>
<td>Fix. mier. obs.</td>
</tr>
<tr>
<td>S 7</td>
<td>Red</td>
<td>X' Y'R</td>
<td>24</td>
<td>2 type</td>
<td>Ovary</td>
<td>♀</td>
<td>Fix. mier. obs.</td>
</tr>
<tr>
<td>S 8</td>
<td>Red</td>
<td>X' Y'R</td>
<td>34</td>
<td>2 type</td>
<td>Ovary</td>
<td>♀</td>
<td>Breeding, fertile</td>
</tr>
<tr>
<td>S 9</td>
<td>Red</td>
<td>X' Y'R</td>
<td>34.5</td>
<td>2 type</td>
<td>Ovary</td>
<td>♀</td>
<td>Breeding, fertile</td>
</tr>
<tr>
<td>S10</td>
<td>White</td>
<td>X' X'</td>
<td>12</td>
<td>...</td>
<td>Ovary</td>
<td>♀</td>
<td>Fix. mier. obs.</td>
</tr>
<tr>
<td>S11</td>
<td>White</td>
<td>X' X'</td>
<td>17</td>
<td>...</td>
<td>Ovary</td>
<td>♀</td>
<td>Fix. mier. obs.</td>
</tr>
<tr>
<td>S12</td>
<td>White</td>
<td>X' X'</td>
<td>21</td>
<td>2 type</td>
<td>Ovary</td>
<td>♀</td>
<td>Fix. mier. obs.</td>
</tr>
<tr>
<td>S13</td>
<td>White</td>
<td>X' X'</td>
<td>21</td>
<td>2 type</td>
<td>Ovary</td>
<td>♀</td>
<td>Fix. mier. obs.</td>
</tr>
<tr>
<td>S14</td>
<td>White</td>
<td>X' X'</td>
<td>33</td>
<td>2 type</td>
<td>Ovary</td>
<td>♀</td>
<td>Breeding, fertile</td>
</tr>
</tbody>
</table>

the controls. Among numerous stilbestrol-treated FR₄ offspring, 9 red (X'Y") fish and 5 white (X'X') ones survived after about 8 months.

All the red fish, of the male genotype (X' Y"), had sexual characteristics of the female type. At a relatively young stage, 7 of these (S1, S2, S3, S4, S5, S6 and S7) were fixed and examined histologically and all had typical ovary and oviduct. The ovary contained oocytes of various sizes. In the large oocytes, yolk formation was in progress in the cytoplasm
and the egg membrane was being formed around the oocytes by the follicle cells. Careful observation indicates that the ovaries contain only female germ cells; no male germ cells were found there. The structure of the ovary and the oviduct was quite similar to that of the normal (X X) females. The urinary duct also opened separately behind the genital duct (oviduct) as in normal females. Obviously they may be regarded as completely sex-reversed females with the male genotype (X Y). The fully grown red fish (S8 and S9) with the male genotype (X' Y") had well-developed secondary sexual characteristics of the female type. The secondary sexual characteristics of S9 are illustrated in figure 3 (S). The anal and dorsal fins are of the female type and the urino-genital papillae are more developed than those in normal females. S8 (34 mm) and S9 (34.5 mm) were mated with normal red males (X' Y"). S8 and S9 produced eggs during June 11–July 1, '51 and June 11–July 21, '51 respectively. At the final dates they were fixed. The eggs produced by the sex-reversed females developed normally and fry hatched out. They are thriving quite normally. Obviously all (9) red fish of the male genotype (X' Y") differentiated into phenotypic females under the influence of the synthetic estrogen (stilbestrol). Sex-reversal was so complete that they were fertile and yielded normal offspring.

Relatively young white fish (S10, S11, S12 and S13) of the female genotype (X' X') which had been reared with the stilbestrol-diet were fixed and examined microscopically. They had typical ovaries with growing oocytes. The fully grown white fish (S14) of the female genotype (X' X') had the urino-genital papillae more developed than those of control females. She was mated with a normal red (X' Y") male. Fertilized eggs were obtained during June 10–June 29, '51. The eggs developed normally and the fry hatched out. It is obvious that all (5) white fish of the female genotype differentiated into normal females as would be expected.
DISCUSSION

It is known that sex chromosomes in fish are weakly differentiated, since the evidence for their existence comes only from genetic data. They have not been identified cytologically. Iriki ('32), who studied the chromosomes of Oryzias (Aplocheilus) latipes could not identify heterosomes. Ralston ('34), Friedman and Gordon ('34) and Wickbom ('41), who studied the gonads of Poeciliidae such as Xiphophorus helleri and Platypoecilus maculatus, could not establish a morphological difference between the heterosomes and the autosomes with any certainty. These facts favor the view that the mechanism of sex determination in fish is only poorly established.

Sex differentiation of the gonads in fish, however, shows various gradations from hermaphroditism to typical gonochorism. Even among gonochorists there are juvenile hermaphrodites or "sex-undifferentiated" species and "sex-differentiated" species as Witschi ('14, '15) has revealed in amphibians. In discussing the problem of sex-reversal in fish it is important to know the degree of sex-differentiation of the species under consideration, both from a genetic and an embryological standpoint.

In certain races of the guppy (Lebistes reticulatus), in which the male is heterogametic (XY), Winge ('30) reported the occurrence of a few XX-males in normal breeding. Winge ('34) obtained some females of the male genotype (XY) in certain races of the fish. These facts indicate that the sex differentiation in certain races of Lebistes is very weak. This is in accord with the embryological studies on the differentiation of the gonads by Goodrich et al. ('34) and Dildine ('36), who showed juvenile hermaphroditism in this form. Obviously Lebistes may be regarded as a "sex-undifferentiated" species. According to Bellamy and Queal ('51), the sex-determining mechanism of the platy (Platypoecilus maculatus) is relatively stable among fish. Gordon ('46) reported a single case of a sex-reversed male of the female genotype and Bellamy and Queal ('51) found that such exceptional males occur with a frequency of one in about 1,800 in this
form. The embryological study of Wolf ('31) indicates that this species is "sex-differentiated" and not a case of juvenile hermaphroditism.

In *Oryzias latipes*, in which the male is heterogametic (XY), two allelomorphs, determining the body color orange-red (R) and white (r), are located in the sex chromosomes (Aida, '21). When white females (X'X') are crossed with heterozygous orange-red (called red) males (X<rY'), red females (X'rX') and white males (X'rY') will be produced in a criss-cross manner in the F₁ progeny. Using this method of crossing, Aida ('36) reported that exceptional males, or sex-reversed males of the female genotype (XX) appeared frequently in his strain in normal breeding experiments. According to him, sex-reversal from female to male in his strain may be due to decrease in the potency of the X chromosome on account of many years' close inbreeding, since an XX male in a cross with a wild (brown) female yielded only female offspring, whereas in a cross with his colored strain it yielded both females and males of the female genotype (XX) in various ratios. He stated that sex-reversed females of the male genotype (XY) rarely occur, and only two cases are reported. As mentioned before, the ovary and the testis are differentiated directly from the indifferent gonad and there is no evidence of normal juvenile hermaphroditism in this fish. Therefore, *Oryzias latipes* may be regarded as a "sex-differentiated" species.

The sex-linked characteristics were also used as the outward signs of the genotypes in the present experiments, but the method of crossing was different from that of Aida ('36). When a white female (X'X') is mated with a heterozygous red male (X<rY'), the offspring will be white females (X'rX') and red males (X'rY') in equal numbers. Since only the Y chromosome contains the dominant R gene in this case, one-sided male sex-limited inheritance, or father-to-son inheritance, is established, so that white fish will always be female and red fish always male unless sex-reversal takes place or crossing over between X and Y chromosomes occurs. In fact, no ex-
exceptional fish was found for three generations of interbreeding of white females \((X'X')\) with heterozygous red males \((X'Y^*)\) in our strain. This shows that our materials cannot be regarded as a strain which will undergo sex-reversal easily under normal conditions.

Recently a number of experiments have been reported indicating that estrogens act on the sex-differentiation of the gonad and produce feminization of genetic males. Most of the experiments, however, were concerned with the experimental production of an intersexual ovo-testis. Complete artificial sex-reversal by means of estrogens seems to be very rare. In our experiments estrone \((1,250\text{ I.U./gm dried diet})\) or stilbestrol \((5,000\text{ I.U./gm dried diet})\) was administered per os to the newly hatched fry of genetically analyzed *Oryzias latipes*, and this feeding with estrogens was continued for about 8 months. Thus genotypic females \((X'X')\) and genotypic males \((X'Y^*)\) were treated with estrogens from the stage of indifferent gonad to a young fish stage which was far beyond the period of sex-differentiation. The results show that the sex of all \((100\%)\) the genotypic males \((X'Y^*)\) was reversed and they became phenotypic females while all \((100\%)\) of the genotypic females \((X'X')\) developed into normal females. The reversal of sex was complete since no intersexes were produced. Furthermore, the sex-reversal was persistent. Fully grown sex-reversed females of the male genotype \((XY)\) were fertile and produced offspring on mating with normal males \((XY)\). Thus our sex-reversed females with the male genotype became functional females while the controls of the male genotype developed into normal males. It may be remarked that the difference between juvenile mortality in estrogen-administered fish and that in the controls is very small.

As mentioned above the sex-linked characters \(R\) and \(r\) have been used as the outward criterion of the genotype. There is another possibility instead of sex-reversal to explain the production of red females in the offspring of the cross between white females \((X'X')\) and heterozygous red males
(X*Y*). If crossing-over between X' and Y* chromosomes occurs in the male germ cells, spermatozoa having X* or Y' may be produced. Then if the eggs (X') are fertilized by X* spermatozoa, red females (X'X*) will be produced. According to Aida (’30), crossing-over between X and Y chromosomes is of very rare occurrence in *Oryzias latipes*. He estimates the frequency of such crossing-over as 1:300. Since all (100%) of the red fish developed into phenotypic females in the estrogen-treated groups and no red female occurred among the controls, even if such an X'X* female may have been produced in our case, it is certain that the majority of our red females are sex-reversed females of the male genotype. This can be further clarified by genetic analysis of these red females, on mating with normal red males (X'Y*). The data now available indicate that the sex-ratio of the offspring is not 1:1, as would be expected when the red females have the genetic constitution of X'X*, but males predominate, as would be expected when the mothers have the genetic constitution of X'Y*. It is concluded that estrogens induce sex-reversal in the genotypic males (X'Y*) of *Oryzias latipes*, and that the artificially sex-reversed females with the male genotype can develop into functional females.

**SUMMARY**

1. The sex-differentiation of the gonads in the normal medaka (*Oryzias latipes*) indicates that it is a "sex-differentiated" species. There is no evidence of juvenile hermaphroditism in normal development.

2. The effect of estrogens on sex-differentiation was studied in a genetically analyzed strain. The sex-linked characters R (orange-red, called red) and r (white) were used as indicators of the genotypes.

3. The crossing of white females (X'X') with heterozygous red males (X'Y*) for three generations (FR₁, FR₂ FR₃) with normal feeding showed that females were always white and males were always red, since the dominant R gene is located only in the Y chromosome.
4. The FR₄ offspring of FR₂ white females (XₚXₚ) and FR₄ red males (XₚYₚ) have been reared on an estrone-diet (1,250 I.U./gm diet) and stilbestrol-diet (5,000 I.U./gm diet) from the time of hatching up to the age of about 8 months. In both cases, all white (XₚXₚ) fish and all (100%) red (XₚYₚ) ones developed into complete females, no intersex being found. These red fish are sex-reversed females of the male genotype (XₚYₚ).

5. Fully grown sex-reversed females of the male genotype have reproductive capacity, yielding offspring on mating with normal males. It is concluded that estrogens induce sex-reversal in the genotypic males and transform them into functional females.

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