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Sex Reversal in the Medaka *Oryzias latipes* by Brief Exposure of Early Embryos to Estradiol-17β

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ABSTRACT—To induce sex reversal of male to female, freshly-fertilized eggs of the S-rR strain medaka (*Oryzias latipes*) were immersed in saline containing estradiol-17β (E_2) in different concentrations for various durations until hatching. Results of the present experiment showed that the immersion duration in 1 μg/ml E_2 to induce 100% reversal of sex differentiation in the genotypic males was enough only for one day (24 hr) post-fertilization (dpf) and that treatment with E_2 for 1 dpf resulted in a dose-dependent manner with the maximum sex reversal of 100% at 1 μg/ml. To ascertain early developmental periods efficacious for inducing sex reversal, additional brief immersion treatments of eggs with E_2 were further performed individually for four different early developmental periods (Stages 4-9, 10-12, 13-15 and 16-18) within 1 dpf. As a result, induction of sex reversal was observed in all these short immersion periods without any restricted efficacy. Between both experimental and control groups treated with or without E_2 for 1 dpf, differences in the number of germ cells in a gonad were compared in newly-hatched fry. It was found that gonads of the genotypic males (XY) treated with E_2 revealed the female type which contained many germ cells with much dividing activity. These data suggest that a possible switch mechanism that exogenous E_2 could trigger to change the genetic cascades involved in sex determination upon fertilization exists in early developmental stages.

Key words: sex reversal, sex determination, sex differentiation, *Oryzias latipes*, estradiol

INTRODUCTION

In most gonochoristic fishes including the medaka (*Oryzias latipes*), as well as in other vertebrates, the genotypic sex of an individual is determined by the combination of sex chromosomes at the moment of fertilization, while the phenotypic sex does not become manifest until gonadal sex differentiation occurs in the later developmental stages. Newly-hatched fry of the medaka still have sexually indifferent (bipotent) gonads, which could differentiate directly into either ovaries or testes without any hermaphroditic state during the juvenile period. Using a genetically analyzed breed (d-rR strain) of the medaka, Yamamoto (1953, 1958) for the first time demonstrated in the artificial induction of complete and functional sex reversal in both sexes by oral administration of sex steroids in the period from the indifferent gonadal stage until the end of gonadal sex differentiation. Since then, on the basis of his method, a number of sex reversal experiments with exogenous sex steroids have been extensively carried out for elucidating the mechanism of sex differentiation not only in fishes (see reviews in Hunter and Donaldson, 1983; Devlin and Nagahama, 2002), but also in other vertebrate species (amphibians, review in Gallien, 1962; reptiles, review in Pieau *et al.*, 1994; birds, review in Scheib, 1983). It is an intriguing question: "In which developmental process might induction of sex reversal with exogenous sex steroids be brought about: in sex determination in the early embryonic stages after fertilization or in gonadal sex differentiation in the juvenile stages after hatching?"

Recently, Iwamatsu (1999) has devised a simple method for inducing sex reversal in the medaka, more convenient than that of Yamamoto. In this method, if developing eggs shortly after fertilization are immersed in saline containing estradiol-17β (E_2) for various durations until hatching, genotypic males permanently reverse to functional females. This method should greatly help pinpoint the switch mechanism involved in sex determination after fertilization rather than later sex differentiation after hatching, with some

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improvements in experimental strategy. Hence, the present study was carried out mainly to elucidate whether or not male to female sex reversal could be induced by immersing medaka embryos in saline containing E_2 for briefer periods in the early embryogenesis soon after fertilization. Furthermore, to assist in obtaining basic information about whether or not in genotypic males treated with E_2 in the early developmental stages, gonads differentiate into ovaries exactly via the same process as normal ovarian development in the genotypic female, sex differences in the number of germ cells and their dividing activity in gonads of newly-hatched fry (Tsuzuki *et al.*, 1966; Satoh and Egami, 1972; Hamaguchi, 1982) were also compared between control and experimental groups treated with E_2 for 1dpf.

MATERIALS AND METHODS

We used a genetically defined S-rR strain (Hagino *et al.*, 2001) of the medaka (*Oryzias latipes*) which had been maintained by mass-mating homozygous white females (X^rX^r) with heterozygous orange-red males (X^rY^R). In this strain the genotypic sex could be easily judged by body color at least by the time the fish grew to 6–7 mm in total body length 10–15 days post-hatch.

Fertilized eggs were collected within 1 hr after being spawned during mating while they were hanging as a cluster from the urogenital pores of the females. They were dissociated from each other by cutting off their attaching filaments and washed thoroughly in a sterile saline for medaka oocyte (111.2 mM NaCl, 5.4 mM KCl, 1.1 mM $CaCl_2$, 0.6 mM $MgSO_4$, pH adjusted to 7.3 with M/2 $NaHCO_3$: Iwamatsu et al., 1976). These developing eggs were usually at 2-4 cell stages at the initiation time of incubation at 26°C in the saline containing estradiol-17β in different concentrations for various durations.

Estradiol-17β (E_2, Sigma, St. Louis, MO) was dissolved in ethanol to make a stock solution with a concentration of 10 mg/ml. Aliquots of this stock solution were properly diluted with the saline to obtain final E_2 concentrations of 1, 10, 100, 500, and 1,000 ng/ml, respectively. The saline was used to obtain stable solutions containing constant concentrations of E_2. These E_2 concentrations partly referred to the results by Iwamatsu (1999). Incubation periods in E_2 saline were 1, 2, 4, 6, 8, and 10 days post-fertilization (dpf). In additional experiments for incubation, four different short periods along the early developmental process within 1 dpf were defined as follows: (I) 3–4 hrs from stage of 2-4 cell (shortly after fertilization) to stage 9 (late morula), (II) 4–5 hrs from stage 10 (early blastula) to stage 12 (late blastula), (III) 5–6 hrs from stage 13 (early gastrula) to stage 15 (midgastrula), and (IV) 5–6 hrs from stage 16 (late gastrula) to stage 18 (late neurula). Control experiments were done using both saline only and saline containing 0.02% ethanol as solvent for E_2. Developmental stages of medaka embryos were determined according to the descriptions of Iwamatsu (1994). After the end of treatment with E_2 in each experimental group, eggs were rinsed 3 times in the saline to be completely free of E_2 and followed to develop until hatching in normal saline without any exogenous sex steroids. In principle, the medium was exchanged every 2 days during incubation.

Newly-hatched fry were transferred in dechlorinated tapwater, fed on a commercially-available powdered food (TetraMin, Melle, Germany), and reared to more than 20 mm in body length for about 2 months. These fish were individually examined for sexing with a dissecting binocular microscope under anesthesia in 0.015% phenylurethane (Nacalai Tesque, Inc., Kyoto, Japan) aquatic solution. Genotypic sexes were determined by body color, and phenotypic sexes by secondary sexual characteristics (Yamamoto, 1953; Iwamatsu, 1999). In all doubtful cases, especially of phenotypic (functional) sex, gonads were removed from anesthetized fish, and sex was verified by squashing the dissected whole gonads with an ordinary light microscope (× 200).

Part of newly-hatched fry, which had been pretreated with or without E_2 (1 μg/ml) for 1 dpf, were fixed *in toto* in Bouin's solution in order to learn the sex difference by the number and dividing activity of germ cells in a gonad. Their trunk regions including gonads were excised and embedded in Paraplast (Sherwood Inc., mp, 56–57°C) by a routine procedure. Cross sections were serially made at 7 μm in thickness and stained with Mayer's haematoxylin and eosin. The total number of dividing and non-dividing germ cells in each gonad was carefully counted 3 times by a cytological examination of all the serial sections. For this experiment, both groups of XX+XY mixed fry obtained by crossing males (X^rY^R) with females (X^rX^r), and all female fry (XX) by crossing sex-reversed males (X^rX^r) with females (X^rX^r) were used.

RESULTS

With the present conditions for incubation (26°C, 14 hr light-10 hr dark), most of the fertilized eggs developed normally and hatched around 10 days in saline, despite the presence or absence of E_2 (Data not shown).

In freshly-fertilized eggs immersed in saline containing E_2 at 1 μg/ml for different durations (1–10 days) from fertilization to hatching, 100% induction of sex reversal from genotypic males (X^rY^R) to functional females was observed irrespective of the length of immersion duration (Table 1).

Table 1. Effects of various durations for treatment with estradiol-17β (E_2) (1 mg/ml) on inducing sex reversal in *Oryzias latipes*

Day from fertilization to end of E_2 treatment	No. of adult fish* obtained					Percentage of sex reversal
	Total	R ♀	R ♂	r ♀	r ♂	
0 (Control)	25	0	14	11	0	0
1	27	14	0	13	0	100
2	41	21	0	20	0	100
4	20	9	0	11	0	100
6	31	9	0	22	0	100
8	21	13	0	8	0	100
10**	24	16	0	8	0	100

*R: orange-red (X^rY^R); r: white (X^rX^r).
**around hatching time.

Moreover, there was no paradoxical sex reversal from genotypic females (X^rX^r) to functional males in any case. In addition, no intersexes with hermaphroditic gonads were observed. Consequently, in the experiment incubating freshly-fertilized eggs in saline containing 1 μg/ml E_2, the minimum duration for inducing 100% sex reversal in genotypic male was found to be for 1 dpf, the period from fertilization to just before the early neurula stage.

Under the constant immersion for 24 hr (1 dpf), effects of E_2 in various concentrations on inducing sex reversal were examined (Table 2). It was shown that sex reversal by E_2 treatment was increasingly induced in a dose-dependent

Table 2. Effects of estradiol-17β (E_2) concentrations for 24 hr treatment on inducing sex reversal in *Oryzias latipes*

E_2 (ng/m l) saline level	No. of adult fish* obtained					Percentage of sex reversal
	Total	R ♀	R ♂	r ♀	r ♂	
1000	122	66	0	56	0	100
100	110	55	2	53	0	96
10	140	57	15	68	0	79
1	137	0	69	68	0	0
0 (Control)	105	0	48	57	0	0

*R: orange-red (X^rY^R); r: white (X^rX^r).

manner in the range of 10–1,000 ng/ml. However, no sex reversal was obtained in 1 ng/ml.

When immersion treatment of freshly-fertilized eggs in saline containing E_2 at 500 ng/ml was performed for four different periods during the early embryonic stages within 1 dpf, sex reversal was induced in all of these shorter periods, but at a considerably reduced rate (Table 3). This result failed to obtain a developmental period specially sensitive for E_2 to induce sex reversal.

Table 3. Induction of sex reversal by short immersion treatment of medaka (*Oryzias latipes*) eggs in saline containing estradiol-17β at 500 ng/ml for four different periods within 24 hr after fertilization

Periods* of E_2 treatment	No. of adult fish** obtained					Percentage of sex reversal
	Total	R ♀	R ♂	r ♀	r ♂	
I	99	5	41	53	0	11
II	135	18	46	71	0	28
III	123	13	51	59	0	20
IV	131	18	39	74	0	32

*I) Stage 4 (4 cell) to Stage 9 (late morula); II) Stage 10 (early blastula) to Stage 12 (late blastula); III) Stage 13 (early gastrula) to Stage 15 (mid gastrula); IV) Stage 16 (late gastrula) to Stage 18 (late neurula).
**R: orange-red (X^rY^R); r: white (X^rX^r).

After 24 hr treatment of freshly-fertilized eggs in saline with or without E_2 at 1 μg/ml, newly-hatched fry from the control or experimental groups were examined on both the number of germ cells and their dividing activity per gonad. The result is presented in Fig. 1. In the untreated genotypic female XX group, fry had abundant numbers of germ cells in the range of 130–240 per gonad, most of which showed much dividing cell activity. A similar pattern of germ cell numbers was also seen in fry of the genotypic female XX (E_2) group treated with E_2. In fry of the untreated female and male-mixed XX+XY group, the number of germ cells consisted of two types of distribution patterns. One is the same type as that of the genotypic female pattern, while the other is probably the genotypic male which has less abundant germ cells dispersing in 30–140 with little or no dividing activity. In fry of the XX+XY (E_2) group treated with E_2, however, it was apparent that the distribution pattern of the number of germ cells, along with their dividing activity, had shifted to that of the female (XX) type irrespective of E_2 treatment.

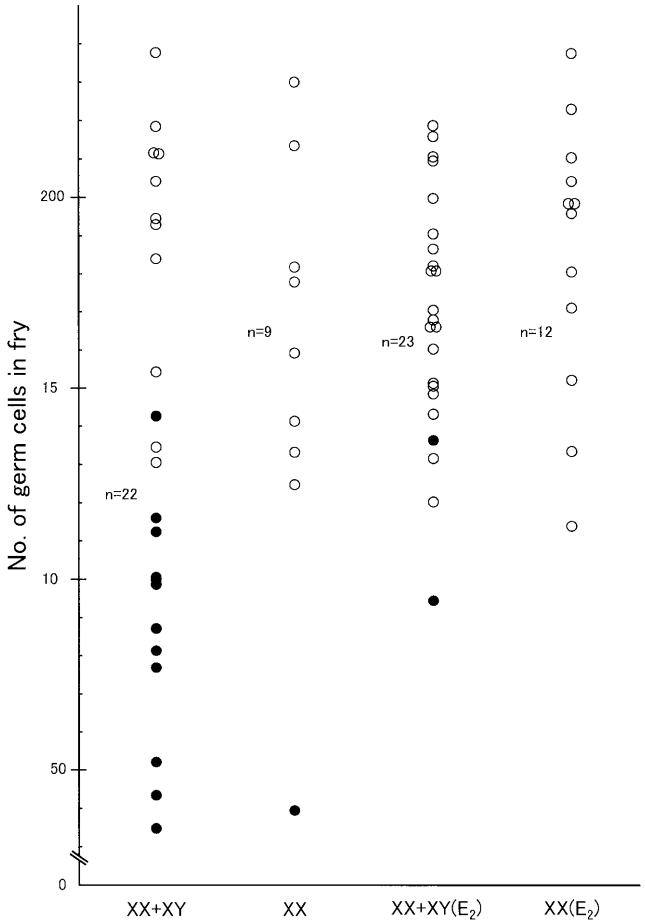

Fig. 1. Effect of treatment with E_2 for one day post-fertilization on number of germ cells in newly-hatched fry of medaka, *Oryzias latipes*. XX, genotypic female alone; XX+XY, male-female mixed. XX (E_2) and XX+XY (E_2) are of female (XX) and male-female mixed (XX+XY) treated with E_2 of 1 g/ml for one day after fertilization, respectively. Open circles show gonads containing numerous dividing germ cells; solid circles show gonads containing few if any dividing germ cells.

DISCUSSION

In the medaka (*Oryzias latipes*), gonadal blastema is formed in embryos a few days before hatching to become a sexually indifferent and developmentally undifferentiated gonad in fry at the time of hatching (Yamamoto, 1953, 1958; Gamo, 1961; Satoh, 1974; Kanamori *et al.*, 1985). Although this gonad shows no detectable morphological sex difference, it has been reported that gonadal sex differentiation is already recognizable in the number of germ cells and their dividing activity (Tsuzuki *et al.*, 1966; Satoh and Egami, 1972; Hamaguchi, 1982). However, it has still a bipotency to differentiate into either testis or ovary by the critical period, and if an adequate dosage of exogenous sex steroids is

given for an optimal period, the direction of gonadal sex differentiation could be easily changed contrary to the genotypic sex (Yamamoto, 1969).

So far, almost all experiments of sex reversal with administration of exogenous sex steroids in fishes have been carried out exclusively in the process of gonadal sex differentiation after hatching, but not in the process of sex determination shortly after fertilization. Hishida (1964) successfully induced sex reversal of *Oryzias latipes* by the microinjection of a small amount of estrogen dissolved in olive oil into the yolk just after fertilization by artificial insemination, at the time when the egg envelope (chorion) had not yet hardened. However, droplets of olive oil microinjected within a yolk sphere did not decrease their original volume during embryogenesis until hatching just as that of natural oil droplets; they usually disappear by being consumed in 7 or 10 days after hatching. Thus, it is not clear in this method when estrogen has an effect on sex differentiation through development if estrogen dissolved in oil droplets could be transferred into an egg yolk. However, Antila (1984) reported in rainbow trout, *Oncorhynchus mykiss* that egg yolk might not act as a reservoir of lipid soluble sex steroids.

Meanwhile, as a convenient method for inducing sex reversal, Iwamatsu (1999) immersed intact eggs in E_2-containing saline for different durations beginning with various days after fertilization until hatching. However, in this case, durations of hormonal immersion always included the period before hatching in which gonadal sex differentiation had commenced in the late embryonic stages.

Accordingly, in the present experiment different durations from soon after fertilization until various days before hatching were applied for the hormonal exposure so as not to include even the initiation step of gonadal sex differentiation. Thus, using Iwamatsu's method slightly modified in terms of the time of hormonal treatment, freshly-fertilized eggs were immersed in saline containing E_2 at 1 μg/ml in early developmental stages. Consequently, it was found that 100% reversal of sex differentiation from genotypic male to functional female was sufficiently achieved in shorter periods for only one day post-fertilization (dpf), well prior to the beginning of gonadal sex differentiation. Effective dosage of E_2 to induce sex reversal was more than 10 ng/ml, which was higher than the concentration (about 4 ng/ml) of endogenous E_2 for 1 dpf under natural developmental conditions (Iwamatsu *et al.*, 2005). Moreover, the effect of E_2 to induce sex reversal was dose-dependent. These results are quite consistent with the results of our previous study (Iwamatsu *et al.*, 2005). In addition, they also reveal that the number of germ cells in a genotypic male (XY) gonad shows mostly the female specific pattern including more abundant germ cells with active cell division when freshly-fertilized eggs were subjected with E_2 exposure for only 1 dpf. Moreover, sex reversal of genotypic males to functional females was induced even by shorter treatments in different developmental periods around the appearance of primordial germ cells within 1 dpf. Interestingly, there was little or no induction of intersexes with a hermaphroditic gonad in genotypic male fish treated with E_2 for only a short time in the early development, irrespective of the stages. This likely means that the action of E_2 in the early developmental steps may modify the sex determination but have no chance to form hermaphroditic gonads in the late developmental periods of gonadal differentiation, because E_2 in the egg precipitously decreases by diffusing into its surrounding medium after the initiation of development (Iwamatsu *et al.*, 2005). In contrast, in administration of E_2 just before gonadal differentiation after hatching, indifferent embryonic male gonads first differentiate towards the male direction by the stimulating effect of E_2, and ultimately result in the complete reversion into an ovary by the female inducing action of E_2 after passing through a transition stage of a hermaphroditic structure (Hishida and Kobayashi, 1985, 1990). In this case, hermaphroditic gonads are produced by chance.

Taken together, these data in the medaka may suggest that reversion of sex by treatment with exogenous estrogen could be induced not only in the process of gonadal sex differentiation after hatching, but also in that of sex determination following fertilization. Thus, the fact that very brief treatments with estrogen during the period of sex determination can result in permanent alternations in sexual phenotype regardless of genotypic sexes, suggests that a mechanism of sex determination sensitive to estrogen does exist in the early developmental period, possibly for the permanent switching of the sex determination pathway.

Like *SRY* (*Sry*) in mammals (Sinclair *et al.*, 1990; Gubbay *et al.*, 1990), the sex-determining gene dubbed *DMY* (*dmrt 1b (Y)*) has been identified on the Y chromosome in the medaka (Matsuda *et al.*, 2002; Nanda *et al.*, 2002). It is believed that this gene determines maleness by initiating development of a testis instead of an ovary, whereas the default pathway of this gene leads to femaleness. In order to clarify the multi-step mechanism of sex determination and subsequent gonadal differentiation, it will be necessary to better understand the relationship between the functions of *DMY* and the putative, estrogen-sensitive switching of sex determination that was proposed in the present study.

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