Induction of Female-to-Male Sex Reversal by High Temperature Treatment in Medaka, *Oryzias latipes*

Tadashi Sato*, Tomokazu Endo, Kazunori Yamahira, Satoshi Hamaguchi and Mitsuru Sakaizumi

Department of Environmental Science, Faculty of Science, Niigata University, Ikarashi, Niigata 950-2181, Japan

ABSTRACT—Medaka, *Oryzias latipes*, has a firm XX-XY sex-determining system with the sex-determining gene, *DMY*, on the Y chromosome. However, previous studies have suggested that high water temperature might affect sex determination in Medaka. In the present study, the influence of high water temperature on sex reversal was examined. Fertilized eggs of two inbred strains of Medaka were developed at high water temperature (32°C) until hatching. The hatched fry were kept at normal water temperatures (27°C) until adulthood, and the phenotypic and genotypic sex was examined. As a result, 24% (N=105) and 50% (N=36) of XX fish developed a male phenotype in the Hd-rR and HNI inbred strains, respectively. These XX sex-reversed males had a normal testis and were fully fertile. On the other hand, all XY fish were male in the both strains. These results demonstrate that high water temperatures can induce XX sex reversal and that elevated water temperatures during the embryonic stage is a simple and useful method for getting XX males in Medaka.

Key words: Medaka, sex determination, sex reversal, TSD, XX male

INTRODUCTION

Sex in many vertebrates is determined by major sex factors contained in sex chromosomes, and this sex determination system is called the genotypic sex determination (GSD) system. On the other hand, it is also known that sex determination is affected by environmental factors in some vertebrates, and this sex determination system is called the environmental sex determination (ESD) system. In vertebrates, temperature-dependent sex determination (TSD) appears to be the most common form of ESD. Although TSD is very common in reptiles, it is not known to occur in mammals, birds. It occurs in all studied crocodilians and tuataras, is prevalent in turtles, and infrequent in lizards (reviewed by Valenzuela and Lance, 2004). In some amphibians, it is also known that sex determination is affected by temperature during embryonic or larval stages (e.g. reviewed by Hayes, 1998; Wallace and Wallace, 2000). In most fishes, sex is determined by genotype under normal circumstances. In some fishes, however, sex determination is greatly influenced by temperature (e.g. Conover and Kynard, 1981; Middaugh et al., 1987; Craig et al., 1996; Yamamoto, 1999). Evidence of a thermal influence on sex ratio has now been documented in about 54 species (seven orders) of fishes, and TSD is thought to be widespread in fishes (reviewed by Conover, 2004).

In Medaka, *Oryzias latipes*, which has a firm XX/XY sex-determining system (Aida, 1921), a Y-linked gene, *DMY*, was found to be a prime candidate for the sex-determining gene (Matsuda et al., 2002). It is also well known that sex-reversed fish, XX males and XY females, can easily be obtained by the administration of sex steroids to fry (Yamamoto, 1953, 1958) or to developing embryos (Iwamatsu, 1999; Matsuda et al., 1999) in Medaka. Recently, Nanda et al. (2003) reported that spontaneous XX males occurred in several Medaka strains. These reports show that Medaka has sexual bipotentiality, and that the genetic sex determination can be influenced by environmental factors.

Aida (1936) suggested that distortion of sex ratio might be induced by water temperature during embryonic development. The study showed that temperature influences the sex ratio which may differ in the offspring of the same parents produced early in the year or during mid-summer. Gen-
eraly, in a hotter climate the number of male offspring was greater than in a colder one. Moreover, Gresik and Hamilton (1977) developed fertilized eggs at elevated temperatures (32°C) in Medaka and this preliminary study showed that 47% of XX fish were male, 12% XY fish were female. Thus, it is expected that TSD also exists in Medaka.

In the present study, to confirm whether sex reversal in Medaka is induced by high water temperature, we treated fertilized eggs of two Medaka inbred strains at 32°C during embryonic development and examined the phenotypic and genotypic sex of the treated adult fish. Consequently, we found that high water temperature induces XX sex reversal in Medaka.

MATERIALS AND METHODS

The Hd-rR and HNI inbred strains (Hyodo-Taguchi and Sakai-zumi, 1993) used in this study originated from stocks kept at the Faculty of Science, Niigata University, Japan. They were fed on a normal flake diet consisting of a mixture of one part fish and one part grain, and reared at 27±2°C, under a photoperiod cycle of 14 hr light and 10 hr dark. Fertilized eggs were incubated at 32.0±0.2°C (water temperature) and, after hatching the fry were fed under previous condition (27±2°C) until adulthood. One group of fertilized eggs was developed at 27±2°C and the hatched fry were also fed at 27±2°C until adulthood as a control group. Phenotypic sex was judged from secondary sex characters (reviewed by Egami, 1975) at about 70 days after hatching. The sexually mature female has well developed urogenital papillae and less developed anal and dorsal fins. The male anal and dorsal fins have a deep cleft between the last and preceding fins. Genotypic sex, XY or XX, was determined by the presence or absence of the DMY gene using PCR analysis from caudal fin clip DNA extracted according to Shinomiya et al. (1999). PCR was performed with the following primers for DMY: PG17.5, CCGGTGCCCAAGTGCTCCT, and PG17.6, GATGTCCTCCACAGAGAGAGAGAGAT. PCR products were analyzed by electrophoresis in a 1% agarose gel.

 Gonads of the mature fish, whose genotypic and phenotypic sex was judged, were dissected out, and fixed in Bouin’s solution, embedded in paraffin. Complete serial sections at 5 μm thickness were prepared for histological observation with a light microscope.

RESULTS AND DISCUSSION

Results of high water temperature treatment are shown in Table 1. XX males were present in the 32°C treated groups in both the HNI and Hd-rR strain. The percentages of XX sex reversal were 24% (N=105) and 50% (N=36) in the Hd-rR and HNI strains, respectively. The percentages of XX sex reversal in the 32°C treated groups was significantly higher than in the control group (27°C) for both strains (χ² test, P<0.01). On the other hand, no XY females were obtained in the 32°C treated groups in both strains. These results demonstrate that XX sex reversal can be induced by high water temperature treatment during the embryonic stage and high water temperature relates to the presence of XX males in Medaka.

In the control group (27°C) of the Hd-rR strain, phenotypic sex completely agreed with genotypic sex. On the other hand, five XX males were obtained in the control group of the HNI strain. The percentage of males in the XX fish of the HNI strain was 11% (N=44). In a previous study (Nanda et al., 2003), one male was also obtained from 13 XX fish from the HNI strain under the same conditions (at 27°C, with a light cycle of 14 hr light and 10 hr dark). These results indicate that common spontaneous sex-reversed XX males in the HNI strain were present under control conditions at 27°C. Temperature-dependent sex reversal might also be induced at 27°C in the HNI strain. Thus, in order to confirm whether temperature-dependent sex reversal can be induced at 27°C in the HNI strain, it is necessary to develop fertilized eggs at lower temperatures and examine the sex of the treated fish.

In most fishes where TSD has been reported, it is not clear whether TSD occurs only at extreme temperatures (reviewed by Conover, 2004). In Medaka, because genotypic sexing of fish from wild populations is based on the presence or absence of DMY, we can determine whether ESD, including TSD, occurs in nature. Genotypic and phenotypic sex of 2274 wild-caught Medaka from 40 locations throughout Japan was examined (Shinomiya et al., 2004). The phenotypic sex type agreed with the genotypic sex type in most fishes, while 11 males of 1185 XX fish and 12 females of 1089 XY fish were found. With the progeny test, it was shown that all the XY females (Shinomiya et al., 2004) and the four examined XX males (Shinomiya, unpublished data) were mutants of sex determination or differentiation. Phenotypic sex agreed with genotypic sex in more than 99% of fish from wild populations. Hence, the sex seems to be determined only by genetic factors under natural conditions, and the TSD appears to occur only at extreme temperatures in Medaka.

Although induction of XX sex reversal by high water

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment Temperature</th>
<th>No. of adult fish obtained</th>
<th>Percentage of XX sex reversal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>XX ♀</td>
<td>XX ♂</td>
</tr>
<tr>
<td>HNI</td>
<td>27°C</td>
<td>39</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>32°C</td>
<td>18</td>
<td>18</td>
</tr>
</tbody>
</table>

* indicates a significant difference (χ² test, P<0.01) from control group (27°C) in each strain.
temperature was confirmed in Medaka, the mechanisms affecting sex determination remain unknown. Kitano et al. (1999) reported that induction of sex reversal of genetically female larvae to phenotypic males by rearing them at a high water temperature (27°C) caused a suppression of P450 aromatase gene expression in flounder, Paralichthys olivaceus. However, information on the mechanisms affecting sex determination in fish is still poor. Since information on sex determination and differentiation has been accumulating for Medaka, detailed developmental studies on the sex reversal by temperature in Medaka may help to understand mechanisms of TSD in fish. Furthermore, as for the 32°C treatment, the percentage of XX sex reversal in the HNI strain was significantly higher than that in the Hd-rR strain ($\chi^2$ test, P<0.01). The genetic mechanism underlying the differences between the two strains may provide clues to better understand the mechanism of temperature-dependent sex reversal.

Gonads from two XX sex-reversed males and two XY males in the Hd-rR strain were histologically examined. Tissue architecture of the gonads in the XX males appeared completely normal. Seminiferous tubules radiated from a centrally located efferent duct. The type A spermatogonia occupied the most peripheral region. Cysts of type B spermatogonia, spermatocytes and spermatids were aligned in the seminiferous tubules toward the central region. Spermatogenesis seemed to be also normally occurred, and oocyte-like cells were not observed in the gonads of the XX males. Therefore, high temperature-treated XX males are thought to have a normal testis.

The XX males that were obtained by high temperature treatment were mated with normal females. We obtained fertilized eggs from all 10 observed pairs of the XX male of the Hd-rR strain and a normal Hd-rR female. The fertilized eggs developed and hatched, and the fry normally grew to female adulthood. These offspring were also fertile. Fertilized eggs were also obtained from pairs of the XX males of the HNI strain and normal females. These mating results of the XX males and normal females show that the XX males are fully fertile. The fact is consistent with the histological observation of the testis of the XX males.

Although it is known that XX sex reversal is also induced by androgenic steroids such as methylandrosterone (MT) in Medaka, XX males induced by MT treatments are frequently sterile (Yamamoto, 1958). The high temperature treatment of embryos, with no androgenic steroids, is a simple and reliable method for getting fertile sex-reversed XX males in Medaka, compared with Yamamoto’s method in which a large amount of MT is administered orally in the diet for 7–8 weeks months after hatching (Yamamoto, 1958) and Iwamatsu’s method in which MT is administered to developing embryos (Matsuda et al., 1999).

ACKNOWLEDGMENTS

This study was partially supported by the Global Environmental Research Fund from the Ministry of the Environment, Japan (FS-14: FY2003-2004).

REFERENCES

cristatus reared at extreme temperatures. Intl J Dev Biol 44: 807–810


(Received May 13, 2005 / Accepted August 18, 2005)