Chapter 15
Endocrine and Environmental Control of Sex Differentiation in Gonochoristic Fish

Takeshi Kitano

Abstract Sex in vertebrates, including fish, is usually determined by genotype. In medaka (*Oryzias latipes*), a gonochoristic fish with the XX/XY sex determination system, a gene that encodes the DM domain on the Y chromosome is identified as the master sex-determining gene. However, the sex-determining genes in many non-mammalian vertebrates remain unclarified. In contrast, sex determination in some reptiles, amphibians, and fish is influenced greatly by environmental factors. For example, although the genotypic sex determination mechanism in Japanese flounder (*Paralichthys olivaceus*) is basically the XX/XY type, genotypic females can be sex reversed to phenotypic males by rearing the larvae at high or low water temperatures during gonadal sex differentiation. In addition, the phenotypic sex of many teleost fish, including flounder, can be experimentally altered by treatment with sex steroid hormones, suggesting an important role for sex steroid hormones in gonadal sex differentiation in fish. In this chapter, we review general information and recent knowledge on the basic mechanisms of sex determination and gonadal sex differentiation, and present the effects of sex steroid hormones and water temperature on gonadal sex differentiation in gonochoristic fish.

Keywords Gonadal sex differentiation · Temperature-dependent sex determination · Estrogen · Androgen · Medaka · Japanese flounder · Gonochoristic fish

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K. Kobayashi et al. (eds.), *Reproductive and Developmental Strategies*, Diversity and Commonality in Animals,
https://doi.org/10.1007/978-4-431-56609-0_15

15.1 Introduction

Sex in vertebrates, including fish, is usually determined by genotype. However, various environmental factors such as temperature also greatly influence sex determination in some nonmammalian species such as reptiles, amphibians, and fish (Adkins-Regan 1987). Pioneering medaka (*Oryzias latipes*) experiments by Yamamoto (1969) demonstrated that androgens and estrogens induce complete masculinization and feminization, respectively, leading to the hypothesis that estrogens and androgens are endogenous female and male inducers, respectively. Since then, many studies have investigated the effects of steroids on gonadal sex differentiation in gonochoristic fish.

Here, we focus primarily on several good fish models such as medaka, Nile tilapia (*Oreochromis niloticus*), and Japanese flounder (*Paralichthys olivaceus*) to study the mechanisms of sex determination and differentiation in gonochoristic fish. In medaka, which has the XX/XY sex determination system (Aida 1921), the DM domain gene on the Y chromosome (*dmy*) is identified as the master sex-determining gene localized on the Y chromosome (Matsuda et al. 2002, 2007). This species has several advantages such as small body size, short generation time, small genome size, and several useful strains (Ishikawa 2000). Therefore, the medaka is an excellent molecular genetic model of vertebrates for analyzing various biological phenomena, including embryonic development and sex determination. Nile tilapia, rainbow trout (*Oncorhynchus mykiss*), and common carp (*Cyprinus carpio*) are also useful models for studying sex differentiation because genetically all-male (XY) and all-female (XX) populations can be produced by fertilizing eggs from genotypic females (XX) with sperm from homogametic males (YY) and sex-reversed males (XX), respectively (Gimeno et al. 1996; Guiguen et al. 1999). Moreover, sex in Japanese flounder is genetically determined by the male heterogametic (XX/XY) system (Tabata 1991), and all-phenotypic female and male populations can be produced by rearing XX broods at 18 °C and 27 °C, respectively (Kitano et al. 1999), suggesting that this species is an excellent model to investigate temperature-dependent sex determination (TSD) mechanisms in fish.

In this chapter, we review general information and recent findings on the basic mechanisms of sex determination and differentiation and discuss the effects of sex steroid hormones and water temperature on gonadal sex differentiation in gonochoristic fish.

15.2 Gonadal Sex Differentiation in Gonochoristic Fish

The gonadal sex differentiation process in gonochoristic fish varies according to species, but in many species, undifferentiated gonads appear to change gradually to testes or ovaries after germ cell numbers becomes different between the sexes (three-spined stickleback—*Gasterosteus aculeatus* (Shimizu and Takahashi 1980;

Lewis et al. 2008); pejerrey—*Odontesthes bonariensis* (Strüssmann et al. 1996); chum salmon—*Oncorhynchus keta* (Robertson 1953); rainbow trout (Lebrun et al. 1982); medaka (Satoh and Egami 1972; Kobayashi et al. 2004); rosy barb—*Puntius conchonius* (Çek 2006); brown trout—*Salmo trutta* (Ashby 1957); black swordtail—*Xiphophorus helleri* (Essenberg 1923; Vallowe 1957); southern platyfish—*Xiphophorus maculatus* (Wolf 1931); and tilapia (Kobayashi et al. 2008)). Sex is first determined in gonadal somatic cells where the sex-determining genes are expressed and the sex difference in germ cell numbers occurs; subsequently, a sex difference in stromal tissue that represents formation of the ovarian cavity or efferent duct occurs.

Genotypic female (XX) medaka have more germ cells than genotypic males (XY) before hatching, and germ cells in XY gonads enter mitotic arrest, whereas they initiate meiosis in XX gonads (Fig. 15.1) (Kobayashi et al. 2004). *dmy* is expressed in somatic cells surrounding the germ cells during gonadal sex differentiation and inhibits germ cell proliferation in a male-specific manner because knockdown of *dmy* function increases germ cell numbers in XY fry at hatching (Paul-Prasanth et al. 2006). The gonadal soma-derived growth factor (*gsdf*), a member of the transforming growth factor-β (TGF-β) superfamily, is a candidate downstream *dmy* gene because its messenger RNA (mRNA) is expressed specifically by males beginning at the same stage as *dmy* in somatic cells and it appears to be involved in suppressing germ cell proliferation (Shibata et al. 2010). Actually, loss of *gsdf* function in medaka increases germ cell numbers in XY fry at hatching and causes male-to-female sex reversal in some cases (Imai et al. 2015). After hatching, the germ cells in XY gonads stop proliferating about 10 days after hatching (dah). Proliferation restarts and spermatogenesis progresses thereafter. Then, the efferent duct—which carries mature sperm, forms, and functional testis—develops. When germ cell proliferation restarts in XY medaka, DM-related transcription factor 1 (*dmrt1*) is expressed male specifically in the gonadal somatic cells (Sertoli cells) and appears to play essential roles in testicular differentiation in medaka (Masuyama et al. 2012).

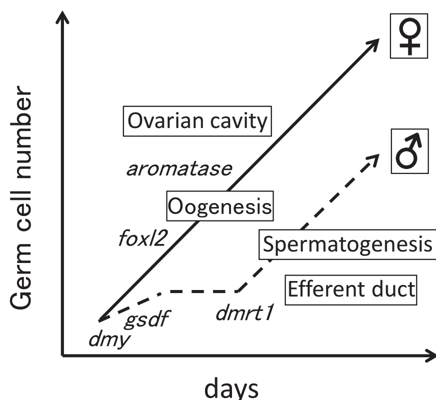

Fig. 15.1 Sexually dimorphic proliferation of germ cells and timing of transcription initiation of sex-related genes in medaka. XX medaka (solid lines) have more germ cells than XY males (dashed lines) before hatching, and subsequently germ cells in XY gonads enter mitotic arrest, whereas they initiate meiosis in XX gonads

In contrast, the molecular mechanism of germ cell proliferation during differentiation in the XX medaka ovary remains unclear. Forkhead box L2 (FOXL2) is a transcription factor that induces expression of *ovary-type aromatase (aromatase)*, which converts androgens to estrogens, in teleost fish (Wang et al. 2007; Yamaguchi et al. 2007), and *foxl2* mRNA is expressed female specifically in gonadal somatic cells of XX medaka beginning at hatching (Nakamoto et al. 2006), suggesting an important role for FOXL2 during ovarian differentiation in teleosts. After hatching, *aromatase* is expressed specifically in the somatic cells of XX gonads, the ovarian cavity forms, and the gonad changes into a functional ovary.

Follicle-stimulating hormone-β (*fsh*β) expression is detected equally in the pituitaries of both sexes of medaka (Hayashi et al. 2010), tilapia (Yan et al. 2012), and flounder (Yamaguchi et al. 2007) during gonadal sex differentiation, but follicle-stimulating hormone receptor (*fshr*) expression is much higher in the differentiating ovary than in the differentiating testis, suggesting that FSH signaling may induce gonadal *aromatase* expression in teleosts. Actually, loss of FSHR function inhibits ovarian development and causes female-to-male sex reversal in XX medaka by suppressing *aromatase* expression and the resultant estrogen biosynthesis (Murozumi et al. 2014). These findings strongly suggest that FSH regulates ovarian development and maintenance primarily through increased estrogen levels.

The germ cells of Nile tilapia enter into gonadal anlagen at 3 dah and do not change in either sex at 5–8 dah (Kobayashi et al. 2008). *gsdf* and *dmrt1* are expressed at higher levels in XY gonads beginning at 5 and 6 dah, respectively (Kaneko et al. 2015), whereas *foxl2* and *aromatase* are expressed at higher levels in XX gonads beginning at 5 dah (Ijiri et al. 2008). XX germ cells continue to proliferate at 8 dah, whereas XY germ cells do not change in number at 9–14 dah and subsequently restart proliferating, but spermatogenesis is not observed until 70 dah. The ovarian cavity and intratesticular efferent duct form at about 25 dah in XX and XY gonads, respectively, and the gonads change into a functional ovary or testis (Nakamura et al. 1998). Thus, although the process and expression of sex-specific genes during gonadal sex differentiation in tilapia and medaka are similar, the difference is that the ovarian cavity forms before and after initiating oogenesis in tilapia and medaka, respectively.

15.3 Involvement of Sex Steroid Hormones in Gonadal Sex Differentiation

15.3.1 Involvement of Estrogens

Exogenous estrogens feminize genotypic males of many species (reviewed by Hunter and Donaldson 1987). Estrogens such as estrone, 17β-estradiol (E2), and 17α-ethinyl estradiol (EE2) induce complete male-to-female sex reversal in medaka (Yamamoto 1969). Moreover, E2 downregulates *gsdf* expression and upregulates

aromatase expression during sex differentiation (Shibata et al. 2010; Kitano et al. 2012), indicating regulation of sex-specific genes by E2 in medaka. Exposure of an all-male common carp population to E2 for 90 days results in regular phenotypic female gonads containing oviducts and oocytes at various developmental stages, but no testicular tissue (Gimeno et al. 1996). Gonadal *dmrt1* expression during sex differentiation decreases during short-term E2 treatment of an all-male rainbow trout population obtained by fertilizing normal eggs (XX) with sperm from phenotypic males (YY), suggesting that the E2 feminizing treatment downregulates *dmrt1* expression in trout (Marchand et al. 2000). EE2 causes complete male-to-female sex reversal, upregulates *aromatase* expression, and downregulates *dmrt1* expression in tilapia (Kobayashi et al. 2003, 2008). Therefore, exogenous estrogens induce male-to-female sex reversal in teleosts by regulating the expression of sex-specific genes such as *gsdf*, *dmrt1*, and *aromatase*.

Brief treatment of Chinook salmon (*Oncorhynchus tshawytscha*) with the aromatase inhibitor fadrozole during sex differentiation causes genotypic females to develop into normal phenotypic males (Piferrer et al. 1994). Guiguen et al. (1999) reported that treatment of broods of rainbow trout and tilapia with an aromatase inhibitor (1,4,6-androstatriene-3-17-dione) results in a high percentage of masculinization of an all-female (XX) population. Moreover, fadrozole and the antiestrogen tamoxifen induce female-to-male sex reversal in XX flounder (Kitano et al. 2000, 2007), suggesting essential roles for endogenous estrogens during ovarian differentiation in many fish species. In contrast, treatment of medaka with fadrozole after hatching inhibits formation of the ovarian cavity and does not induce female-to-male sex reversal in XX fish (Suzuki et al. 2004). Moreover, loss-of-function of 17α-hydroxylase/17,20 lyase (P450c17), which helps synthesize androgens and estrogens, results in many diplotene-stage oocytes and spermatozoa in XX gonads (Sato et al. 2008). Therefore, endogenous estrogens may be indispensable for ovarian differentiation in many teleost fish, but not necessary in others such as medaka.

15.3.2 Involvement of Androgens

Exogenous androgens masculinize genotypic females in many species (reviewed by Hunter and Donaldson 1987). Androgens such as 17α-methyltestosterone (MT), 17α-ethinyl testosterone, and androstenedione induce complete female-to-male sex reversal in medaka (Yamamoto 1969). Treatment of tilapia with MT masculinizes genotypic females and increases gonadal expression of *dmrt1* (Kobayashi et al. 2008). MT and 11-ketotestosterone are fish-specific androgens that induce complete masculinization of Japanese flounder genotypic females, upregulate expression of anti-Müllerian hormone (*amh*)—which is a member of the TGF-β superfamily—and downregulate *aromatase* expression (Kitano et al. 2000; Yoshinaga et al. 2004). Hence, exogenous androgens likely induce female-to-male sex reversal in teleosts by regulating the expression of sex-specific genes such as *amh*, *dmrt1*, and *aromatase*.

In contrast, treatment of XY tilapia with the antiandrogen flutamide does not induce male-to-female sex reversal. Moreover, loss of P450c17 function in XY medaka results in a lack of secondary sex characteristics, but spermatogenesis proceeds as in wild-type XY fish (Sato et al. 2008). Therefore, endogenous androgens may not be required for testicular differentiation in teleosts. A functional analysis of androgen-related genes involved with testicular differentiation in gonochoristic fish is needed.

15.4 Effect of Temperature on Gonadal Sex Differentiation

Sex determination in some reptiles, amphibians, and fish is markedly influenced by environmental factors (Adkins-Regan 1987). Japanese flounder have the XX/XY sex determination system (Tabata 1991) and exhibit TSD. The details of temperature sensitivity and gonadal differentiation have been documented in this species, making it useful for studies investigating the physiology and molecular biology of TSD (Yamamoto 1995; Kitano et al. 1999). The percentages of females in normal (XX + XY) and genotypic female (all XX) larvae reared at various water temperatures from days 30 to 100 after hatching—a critical period of gonadal sex differentiation in flounder—are shown in Fig. 15.2. The percentages of females in normal and genotypic female broods reared at 18 °C were 53% and 100%, respectively. However, they decreased rapidly in both broods when the fish were reared at higher (25 °C) or lower (15 °C) water temperatures. In particular, the genotypic female broods reared at 27 °C were completely masculinized. Thus, 18 °C and 27 °C are

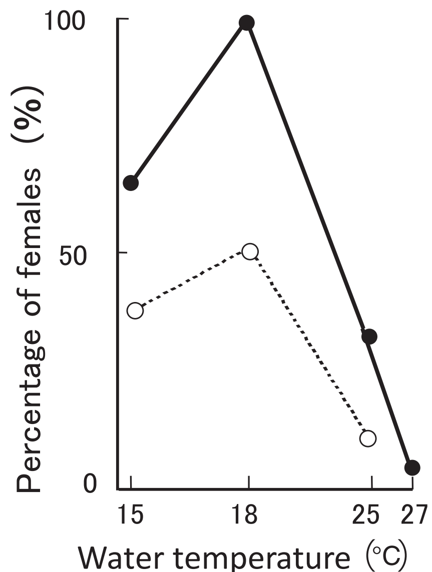

Fig. 15.2 Percentage of females in normal flounder (XX + XY, open circles) and genotypic female flounder (all XX, solid circles) reared at various water temperatures during gonadal sex differentiation. Phenotypic sex was determined at 10 months of age by histological observation of the gonad

the proper temperature conditions to change genotypic female broods to all-phenotypic females or males, respectively, from 30 to 100 days after hatching (Kitano et al. 1999). A similar thermal influence on fish has been demonstrated in studies on atherinids (Conover and Kynard 1981; Strüssmann et al. 1996, 1997), cichlids (Baroiller et al. 1995; Romer and Beisensherz 1996; Desprez and Mélard 1998), and channel catfish, *Ictalurus punctatus* (Patiño et al. 1996). Low-temperature treatment of atherinids during sex determination results in a higher percentage of females, and high-temperature treatment results in a higher percentage of males. In the cichlid *O. niloticus*, the percentage of males in all genotypic female progeny increases during rearing at high temperatures (32–37 °C) during the thermosensitive period (Baroiller et al. 1995). In contrast, the sex ratio of channel catfish is skewed toward females at 34 °C, but no effects occur at 20 °C or 27 °C (Patiño et al. 1996). These results suggest that both genotypic and TSD mechanisms are functional in some fish and that this phenomenon is more widespread in fish than previously believed.

According to current evolutionary theory, this TSD mechanism is adaptive to environments with moderate to large fluctuations in environmental conditions (Bulmer 1987). Environmental sex determination is favorable when environmental effects have different consequences for the two sexes. Conover and Heins (1987) showed that natural *Menidia menidia* populations at different latitudes, whose sex is determined by interactions between genotype and temperature during a specific critical larval development period (Conover and Kynard 1981), compensate for differences in the thermal environment and seasonality by adjusting the sex ratio response to temperature. Therefore, TSD may be a maintenance strategy for natural populations of some species.

15.5 TSD Molecular Mechanism

Phenotypic male and female Japanese flounder are produced by rearing XX larvae at 18 °C and 27 °C, respectively, during sex differentiation. The ovarian cavity and intratesticular efferent duct form at about 60 dah in the gonads of females reared at 18 °C and in the gonads of males reared at 27 °C, respectively, and the gonad changes into a functional ovary or testis (Fig. 15.3) (Yamamoto 1995; Kitano et al. 1999). *foxl2* and *aromatase* are expressed strongly in gonads of females reared at 18 °C at 50 dah when the gonad is sexually undifferentiated, whereas they are barely detected in gonads of males reared at 27 °C. In contrast, *amh* is expressed strongly in male gonads at 50 dah, but not in female gonads. Moreover, E2 treatment at a masculinizing temperature induces complete feminization of XX flounder, induces *foxl2* and *aromatase* expression, and suppresses *amh* expression (Kitano et al. 2007), suggesting that estrogens counteract masculinization in XX flounder at high temperature by regulating the expression of sex-specific genes such as *foxl2*, *aromatase*, and *amh*. To investigate whether XX gonads are temperature sensitive during sex differentiation, a gonadal organ culture experiment was performed using 55 dah

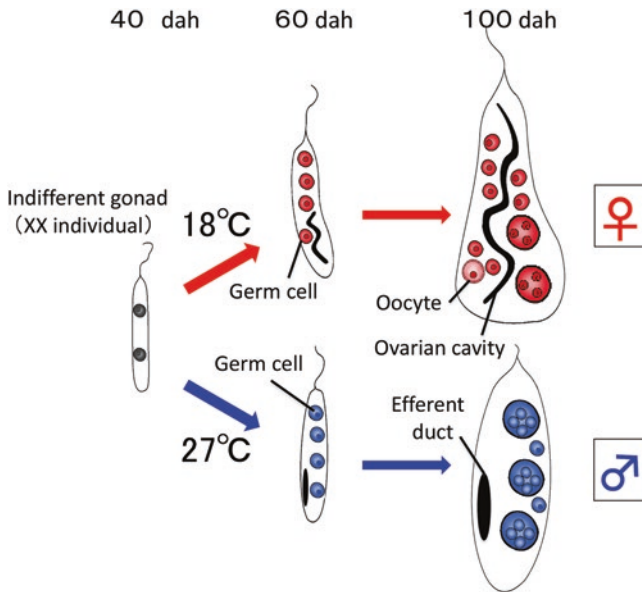

Fig. 15.3 Gonadal sex differentiation in XX flounder. The ovarian cavity and efferent duct form at about 60 days after hatching in the gonads of females reared at 18 °C and in the gonads of males reared at 27 °C, respectively

XX gonads. XX gonads cultured at 18 °C or 27 °C for 2 weeks expressed *foxl2* and *aromatase* but not *amh*, suggesting that the gonads are not temperature sensitive during sex differentiation.

Cortisol is the major glucocorticoid produced by inter-renal cells in teleosts, and production increases in response to various stressors such as heat shock. The hypothalamus–pituitary–inter-renal axis, which controls circulating cortisol levels, is highly conserved across vertebrates (Wendelaar Bonga 1997). The effects of cortisol on reproductive performance have been reported in many fish species (Wendelaar Bonga 1997). For example, cortisol suppresses E2 and testosterone secretion in rainbow trout ovarian follicles (Carragher and Sumpter 1990). Furthermore, cortisol inhibits FSH-induced estrogen production in cultured rat granulosa cells (Hsueh and Erickson 1978). Thus, a high concentration of cortisol appears to inhibit production of gonadal hormones essential for vertebrate ovarian development.

Another gonadal organ culture was performed to investigate whether cortisol directly induces masculinization of XX gonads. All XX gonads cultured with cortisol at 18 °C expressed *amh* but not *foxl2* or *aromatase*, demonstrating the masculinizing action of cortisol during sex differentiation (Yamaguchi et al. 2010). However, treatment of XX flounder with cortisol induces female-to-male sex reversal, and metyrapone (cortisol synthesis inhibitor) inhibits 27 °C–induced masculinization of XX flounder. Moreover, cortisol levels in flounder juveniles reared at 27 °C are significantly higher than in those reared at 18 °C during sexual differentiation.

Therefore, masculinization of flounder in response to high water temperature increases cortisol levels during gonadal sex differentiation.

XX medaka fry can be sex reversed to phenotypic males by high temperature (30–34 °C) during sex differentiation (Sato et al. 2005; Hattori et al. 2007). XX medaka fry were treated with cortisol and E2 at 26 °C or 33 °C during sex differentiation to investigate whether cortisol induces masculinization of XX fish. Cortisol treatment at 26 °C causes female-to-male sex reversal, and metyrapone inhibits masculinization of XX medaka at 33 °C (Hayashi et al. 2010). Moreover, the 33 °C treatment increased cortisol levels, whereas metyrapone suppressed the increased cortisol levels in the 33 °C treatment during sexual differentiation. However, high temperature or cortisol can inhibit proliferation of female-type germ cells and suppress *aromatase* expression while increasing *gsdf* expression in XX gonads during sexual differentiation. Exposure to E2 combined with either cortisol or high temperature prevents these effects (Kitano et al. 2012). Moreover, E2 completely rescues 33 °C–induced and cortisol-induced masculinization of XX medaka. These results strongly suggest that high temperature and cortisol induce female-to-male sex reversal in medaka by enhancing *gsdf* expression and suppressing *aromatase* expression, followed by estrogen biosynthesis.

Taken together, these results suggest that high temperature elevates cortisol levels, which causes female-to-male sex reversal by regulating the expression of the sex-specific genes such as *amh*, *gsdf*, and *aromatase*, and inhibiting estrogen biosynthesis during gonadal sex differentiation in teleosts with TSD. This mechanism may be common among animals with TSD.

15.6 Conclusions

This chapter has demonstrated that endogenous estrogens are indispensable for female sexual differentiation in many fish species such as tilapia, rainbow trout, and Japanese flounder, but may not be necessary in other fish such as medaka. This may be due to whether the ovarian cavity forms before or after oogenesis is initiated. Estrogens may impact early oogenesis and be involved in ovarian differentiation if the ovarian cavity forms before oogenesis is initiated. Estrogens may not affect early oogenesis and may only be involved in forming the ovarian cavity after oogenesis is initiated. The involvement of endogenous androgens in testicular differentiation remains unclear in teleosts. Therefore, a functional analysis of estrogen- and androgen-related genes needs to be performed using new technology such as genome editing.

This chapter has presented novel evidence of the involvement of cortisol in TSD of Japanese flounder and medaka. Cortisol induces female-to-male sex reversal, and metyrapone inhibits high-temperature-induced masculinization of XX fish. Moreover, high temperature increases cortisol levels, whereas metyrapone suppresses this increase during sexual differentiation, strongly suggesting that masculinization of XX fish induced by high temperature is mediated by increased cortisol.

Cortisol reportedly induces a sex change from ovary to testis through a decrease of E2 levels in the protogynous wrasse (*Halichoeres trimaculatus*) (Nozu and Nakamura 2015). Therefore, cortisol appears to have the capacity to induce masculinization not only in gonochoristic fish but also in hermaphrodites. Future studies should focus on the molecular mechanisms of masculinization and how cortisol causes female-to-male sex reversal in teleosts with TSD.

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