Seminars in Cell & Developmental Biology 20 (2009) 256–263

Contents lists available at ScienceDirect

Seminars in Cell & Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

Review

Sex determination in flatfishes: Mechanisms and environmental influences

J. Adam Luckenbach*, Russell J. Borski, Harry V. Daniels, John Godwin

Department of Biology, North Carolina State University, Raleigh, NC 27695, USA

ARTICLE INFO

Article history:
Available online 11 December 2008

Keywords:
Environmental sex determination
Temperature-dependent sex determination
TSD
Sex differentiation
Aromatase

ABSTRACT

Flounder of the genus *Paralichthys* exhibit a unique mode of sex determination where both low and high temperatures induce male-skewed sex ratios, while intermediate temperatures produce a 1:1 sex ratio. Male differentiation is thus easily induced in genetic females creating a combination of genetic (GSD) and environmental sex determination (ESD). Since male flounder become reproductively fit at substantially smaller body sizes than females, temperature or other environmental variables that elicit lower growth rates may also influence sex differentiation toward male development. This review covers our current knowledge of sex determination and differentiation in flatfishes including possible adaptive significance of ESD and involvement of factors such as aromatase (*cyp19*).

Contents

1. Introduction

Teleost fishes are the most abundant vertebrates on Earth, showing a diversity of species unmatched by other classes. Not surprisingly given this extreme diversity, fish species exhibit all known forms of vertebrate sex determination. The most common mode of sex determination appears to be genetic sex determination (GSD) where sex is determined by the inherited combination of sex-determining genes or 'minor' genetic factors (i.e., polygenic systems) [1,2]. However, various forms of environmental sex determination (ESD) have also been documented. For example, environmental factors such as behavior, water temperature, and pH are known to control or influence sex determination and sex differentiation in some species [2,3]. Temperature-dependent sex determination (TSD) is the most common form of ESD in vertebrates and has been reported in over 50 fishes [4]. TSD occurs when the temperature during a critical period of development either determines the direction of sex differentiation or acts in combination with other sex determination mechanisms (e.g., genotype-by-environment interaction) to ultimately influence the phenotypic sex of the animal. Growing ecological and commercial interests in fish species, in areas such as conservation, stock enhancement and aquaculture, have fueled a recent surge in research related to ESD/TSD. Patterns of TSD in fishes also offer unique opportunities to better understand the evolution and regulation of sex determination generally.

* Corresponding author at: School of Aquatic & Fishery Sciences, University of Washington, Box 355020, Seattle, WA 98195, USA. Tel.: +1 206 860 3463; fax: +1 206 860 3467.
E-mail address: jalucken@u.washington.edu (J.A. Luckenbach).

1084-9521/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.semcdb.2008.12.002

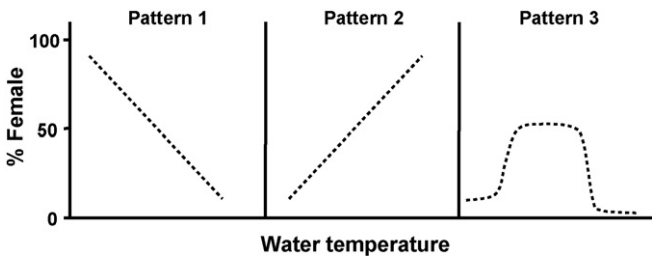

Fig. 1. Three general patterns of temperature-dependent sex determination (TSD) reported in fishes. Pattern 1 shows a decrease in the proportion of females with increasing temperature, pattern 2 shows an increase in the proportion of females with increasing temperature, and pattern 3 shows a decrease in the proportion of females at both low and high temperatures.

This review addresses our current knowledge of sex determination in fishes, focusing largely on a peculiar teleost group, the flatfishes (order Pleuronectiformes), including possible evolutionary significance of ESD in these species and critical endocrine factors involved in early sex differentiation. For a more comprehensive review of sex determination and differentiation in fishes, we recommend several recent articles [1–7].

2. TSD patterns in fishes

Three general patterns of TSD have been reported in fishes: (1) In the majority of species the proportion of males increases with exposure to high water temperatures and female differentiation is promoted by low temperatures. This pattern of TSD is seen in families such as Atherinidae, Poecilidae, Cichlidae, Pleuronectidae, and Cyprinidae [7,8] (Fig. 1). (2) Conversely, in a few species (e.g., channel catfish, *Ictalurus punctatus*), higher temperatures induce female differentiation while low temperatures induce male-skewed sex ratios. (3) Flounder of the genus *Paralichthys* are unique in that male-skewed sex ratios are generated at both low and high temperatures while an intermediate temperature yields a 1:1 sex ratio [9,10].

Although temperature influences sex determination in a variety of fishes, the occurrence of TSD in the wild has been demonstrated thus far in only two species, the silversides, *Menidia menidia* and *M. peninsulae* [4,7,11]. For other cases of TSD, it remains unclear whether this mode of sex determination may be a normal part of the species life history or only occurs in artificial/experimental conditions. For example, distorted sex ratios may occur in some species when reared at extreme temperatures or under constant thermal regimes, but such conditions may never be encountered in the wild, or at least not during the thermosensitive period, typically occurring prior to morphological differentiation of the gonads. In this scenario, extreme conditions may simply be altering the process of sex differentiation in a purely GSD species and yielding skewed sex ratios (so-called anomalous GSD or GSD + thermal effects) [4,7].

So how can true ESD/TSD be distinguished from anomalous GSD? This can be challenging because it requires knowledge of the range of conditions normally encountered in the wild by a particular species or population during the sensitive period of development. This can be a daunting task with species that spawn year round or over an extended season, and particularly for marine species that utilize large expanses of the sea. Furthermore, mimicking natural temperature fluctuations can be difficult experimentally. A recent treatment of this subject [7] proposed that several previously reported TSD species actually represent anomalous GSD because temperature effects were thought to occur at extreme temperatures outside the range of temperatures during early development in the wild (a similar situation also occurs in amphibians, see Nakamura, this issue). However, because the sensitive period of development has not been defined in many species, modes of sex determination should be examined in wild populations on a species by species basis before ESD is ruled out. It is also important to note that even if TSD does not presently occur in a species, increased water temperatures associated with global warming could rapidly change this scenario in temperature sensitive species [7].

The sections below will focus primarily on sex determination and differentiation in flatfishes, which as a group appear to exhibit either pure GSD or a combination of GSD and ESD. The possible adaptive significance of ESD in flatfishes will be briefly explored as a way to understand why ESD may have arisen in some flatfish species.

3. Sex determination in flatfishes

3.1. Genetic frameworks of sex determination

Monofactorial sex determination systems have been conceptualized for gonochoristic (non-hermaphroditic and non-sex changing) species, which includes flatfishes. However, due to the potential influence of environmental and minor genetic factors these models often provide only a rough framework to describe the mechanism of sex determination. Induction of diploid gynogenesis has proven to be a valuable technique for establishing and classifying fish into a particular GSD framework [12,13]. Diploid gynogenesis is typically induced by activating eggs with genetically inactivated spermatozoa and then blocking second polar body extrusion from the eggs or preventing the first embryonic cleavage, resulting in diploid offspring that inherit only maternal chromosome sets. Sex ratios of gynogenetic offspring can thus reveal female homogamety or heterogamety. For instance, if 100% female offspring are produced through diploid gynogenesis, the given species utilizes an XX–XY system with the female parent being the homogametic sex. Conversely, a 1:1 sex ratio in gynogens suggests a ZW–ZZ system. Perplexing results from gynogenetic studies suggest the existence of ESD or minor sex-determining genes that function independent of the major sex-determining system.

Gynogenesis studies in marbled sole (*Limanda yokohamae*), Japanese flounder (*Paralichthys olivaceus*), and Atlantic halibut (*Hippoglossus hippoglossus*) have shown that these species utilize an XX–XY sex chromosome system [9,14,15]. Furthermore, accumulating evidence in other flatfish species suggests the XX–XY system may be widespread in flatfishes (Table 1). Methods for induction of diploid gynogenesis in southern flounder (*Paralichthys lethostigma*) were recently developed by our research group [30,31], and experiments have been conducted targeting the underlying genetic mechanism of sex determination in this species. All evidence to date suggests that southern flounder possess an XX–XY system similar to its congener, the Japanese flounder. However, due to environmental influences on sex determination in southern flounder, it has been extremely difficult to unambiguously conclude upon this point. Likewise, Yamamoto [9] and other researchers struggled for years to establish the genetic mechanism of sex determination in Japanese flounder due to the confounding impact the environment exerts on genetic females as discussed below.

3.2. Effects of temperature

Japanese flounder and southern flounder of the genus *Paralichthys* exhibit a unique pattern of TSD mentioned above (Fig. 1, pattern 3), where both low and high temperatures promote male differentiation, and female differentiation occurs at an intermediate temperature. Low temperatures tested for Japanese and southern flounder were 15 and 18 °C, intermediate temperatures were 20 and 23 °C, and high temperatures were 27.5 and 28 °C,

Table 1
Summary of flatfish sex determination, including documented environmental effects.

Scientific name	Common name	Environmental factor(s)	Genetic mechanism	Reference(s)
Family Cynoglossidae				
Cynoglossus semilaevis	Tongue sole	na	WZ–ZZ	[16]
Family Paralichthyidae				
Paralichthys olivaceus	Japanese flounder	WT	XX–XY	[9,17]
Paralichthys lethostigma	Southern flounder	WT, TC, LI	XX–XY[b]	[10,18]
Paralichthys dentatus	Summer flounder	WT	Unclear	[19]
Family Pleuronectidae				
Hippoglossus hippoglossus	Atlantic halibut	nt	XX–XY	[15,20,21]
Limanda yokohamae	Marbled sole	WT	XX–XY	[14,22]
Pleuronectes platessa	Plaice	na	WZ–ZZ[b]	[23]
Pseudopleuronectes americanus	Winter flounder	na[a]	na	[24]
Verasper moseri	Barfin flounder	WT	XX–XY[b]	[25]
Family Scophthalmidae				
Scophthalmus maximus	Turbot	na	XX–XY[b]	[26]
Family Soleidae				
Solea solea	Common sole	na	Unclear	[27]

Please note that studies that analyzed experimental sex ratios were included above, whereas studies that predicted sex determining mechanisms based on karyotype analyses alone are not shown [e.g., 28, 29]. WT = water temperature; TC = tank color; LI = light intensity; nt = no significant effect of temperature; na = not addressed.
[a] Skewed sex ratio(s) observed in captivity but more work necessary.
[b] Suggested based on results.

respectively (Fig. 2). High temperature appears to be more potent than cooler temperatures in driving male differentiation in these species and regardless of rearing conditions the proportion of females in normal crosses never exceeds 50%. Hence, genetic males (XY) are unaffected by temperature and always differentiate into phenotypic males whereas genetic females (XX) are susceptible to environmental influences and may differentiate into phenotypic males (XX males).

Two other flatfishes exhibit a TSD response most similar to pattern 1 (Fig. 1). In both barfin flounder (*Verasper moseri*) [25], and marbled sole [22], exposure to high temperatures prior to and during sex differentiation significantly increases the proportion of males. Like the Paralichthid flounders, the proportion of females never exceeds 50% in these species. Barfin flounder maintained at 14 °C showed a 1:1 sex ratio, while shifting fish to 18 °C for 62 days, before morphological sex differentiation, resulted in 100% males. Similar results were obtained for marbled sole, but the high temperature (25 °C) yielded a maximum of 82% males. Only two temperatures were tested in the barfin flounder and marbled sole. Therefore male-skewed sex ratios are also possible at lower temperatures (i.e., lower than those tested), which would present a TSD pattern similar to the Paralichthid flounders.

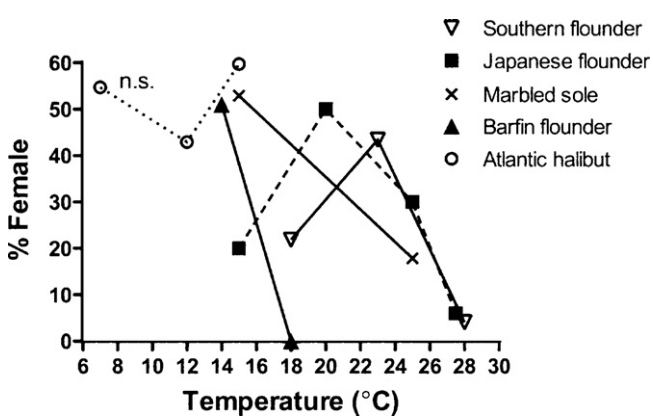

Fig. 2. Summary of results from temperature studies conducted with flatfish species. Points represent the mean female percentage at each water temperature. Data were obtained from the following publications: southern flounder [10], Japanese flounder [9], marbled sole [22], barfin flounder [25], and Atlantic halibut [21]. n.s., no significant effect in Atlantic halibut.

Unlike the flatfishes discussed above, no significant effect of temperature on sex determination was documented in two studies of Atlantic halibut targeting slightly different developmental windows and testing temperatures ranging from 7 to 15 °C. A trend toward male-biased sex ratios was found with early exposure to higher temperatures [20], while no temperature effect was discernable with slightly later exposure [21], prior to sex differentiation and a time when steroid treatments are known to affect halibut sex differentiation [32]. Taken together, these results suggest Atlantic halibut exhibit primarily GSD with possible effects of extreme high temperatures, outside the range of natural temperatures, on XX fish.

3.3. Latitudinal variation in TSD responses

Similar to some flatfishes, Atlantic silversides (*M. menidia*) show a genotype-by-environment interaction in response to temperature. Silverside populations from northerly latitudes exhibit a low frequency of TSD while southern populations exhibit a high frequency of TSD [11,33,34]. Thus, northern latitude silverside populations primarily show GSD, whereas GSD mechanisms appear to be lacking or are easily overridden by temperature cues in animals originating from southern populations.

Of the flatfishes discussed above, Atlantic halibut are a primarily GSD species and are also found at the highest latitudes. The barfin flounder, a TSD species with a slightly lower range, inhabits waters of Japan that are influenced by the cool Liman and Oyashio currents rather than the warmer Tsushima and Kuroshio currents of southern Japan [3]. Japanese flounder on the other hand mainly inhabit the warmer southern waters of Japan. The cold-shifted TSD response shown by barfin flounder relative to Japanese flounder (Fig. 2) may therefore represent an adaptation to these cooler water temperatures. In addition, the temperature response of southern flounder, the warmest water species among those mentioned, also appears to be shifted slightly toward warmer temperatures relative to the Japanese flatfishes. Based on observed latitudinal differences in the TSD response across populations of Atlantic silversides and what appears to be adaptation to local temperatures in the TSD response of different flounder species, it is possible that TSD responses among populations may vary with latitude in flounder. Also consistent with this idea, Japanese flounder of different cohorts exhibit variation in their response to the same temperature [9] suggesting a heritable component to the temperature

sensitive mechanism of sex determination. Potential latitudinal and among family variation in the TSD response has not been thoroughly addressed in any non-Atherinid fish, but this would certainly be an interesting area for future research.

3.4. Possible adaptive significance of TSD: interaction of growth and sex determination?

Though the adaptive significance of TSD is well established in Atlantic silversides [4,33], the significance of TSD in other fishes is unclear and could theoretically represent only an artifact of exposure to extreme temperatures. Our research in southern flounder, however, indicates that temperatures representative of the range encountered by fish in the wild during juvenile development [35,36], not only affect sex determination, but also greatly influence juvenile growth. Southern flounder reared at 23 °C beginning prior to sex differentiation were significantly larger after 245 days than those reared at 18 and 28 °C [3,10]. These growth results mirrored the proportion of females produced at each rearing temperature, with 23 °C yielding the greatest number of females (~50%) and the low and high temperatures yielding predominantly males (Fig. 2). Hence, water temperatures (both low and high) that produced the smallest animals also significantly skewed sex ratios toward males, while the temperature that maximized growth produced the most females.

Charnov and Bull [37] proposed that ESD is favored when the environments in which early development occurs vary in terms of growth potential such that expected individual fitness under these varying growth conditions differs between the sexes. Since female southern flounder (and flatfishes generally) reach larger adult sizes than males, faster growth observed at the temperature that produces the greatest proportion of females is consistent with this hypothesis. This is because females should benefit more from rapid growth than males and being small would less negatively impact male reproductive fitness. Importantly, studies in southern flounder also showed that the body size of males and females did not differ within each temperature [3]. This agrees with findings in Atlantic silversides where female-determining temperatures also produced faster growth, but no male-female size difference was found within a rearing temperature [33]. This suggests for these species, temperature rather than sex is the critical factor regulating growth and that growth rate may determine the direction of sex differentiation. A similar idea of 'growth-dependent sex differentiation' has been proposed in some other species [38–40]. More work is necessary to establish this possible adaptive role of TSD in southern flounder, as data in certain flatfishes are inconsistent with this hypothesis. For example, a study in barfin flounder where juveniles were shifted from 14 to 18 °C showed a significant enhancement of growth in the first 150 days post-fertilization, despite 18 °C producing 100% males [25].

In an effort to better understand growth physiology of southern flounder and its possible interaction with sex determination, we have initiated studies focusing on a key endocrine/paracrine growth factor known as insulin-like growth factor 1 (Igf1), a mitogen primarily synthesized and secreted by the liver in response to growth hormone, but also produced locally by many non-hepatic tissues [41]. Igf1 is widely considered the proximate mediator of vertebrate growth and has thus been studied as a possible indicator of growth rate and nutritional status in fish [42]. In southern flounder, we have demonstrated that circulating Igf1 levels at different temperatures [36] and under different nutritional states [43] are well correlated with changes in specific growth rate. A high rearing temperature (28 °C) during juvenile development significantly suppressed plasma Igf1, muscle *igf1* expression, and specific growth rate relative to flounder reared at 23 °C [36]. This suggests that plasma Igf1 and/or *igf1* mRNA levels may serve as markers of flounder growth status in future TSD studies, particularly for animals where growth history may be unavailable (e.g., wild-caught flounder). The potential influence of Igf1 or other growth factors on sex determination might also shed light on possible control the endocrine-growth regulatory axis plays in mediating this process.

4. Regulation of sex differentiation and ESD

Driven by the development of quantitative PCR (qPCR) and genomic technologies, molecular mechanisms of sex determination and early gametogenesis in fish have been studied extensively over the past decade [6,44–47]. A number of key gonadal factors involved in these processes have now been identified and progress is being made on linking these factors to environmental stimuli. Since estrogens are generally associated with female development and androgens or a lack of estrogens is associated with male development [1,48,49], factors involved in steroid biosynthesis and steroid receptors have been major areas of focus.

4.1. Cytochrome P450 aromatase

One steroidogenic enzyme that stands out as a key player in sex differentiation and ESD is cytochrome P450 aromatase (P450arom). This enzyme is responsible for biosynthesis of 17β-estradiol (E_2) and is not only critical to the process of sex differentiation but its expression may also be influenced by environmental factors. Fishes possess two forms of the *cyp19* gene encoding P450arom, termed *cyp19a1a* and *cyp19a1b* based on current gene nomenclature. The *cyp19a1a* isoform encodes the gonadal form of aromatase predominantly expressed in the ovary, while *cyp19a1b* encodes the brain form of aromatase predominantly expressed in the brain [46]. In Japanese and southern flounder, *cyp19a1a* mRNA levels increase dramatically during early female differentiation, whereas *cyp19a1a* remains low in differentiating males [50,35]. Beginning at ~65 mm total length, juvenile southern flounder of wild and hatchery origin segregate into two distinct groups based on *cyp19a1a* expression; those with elevated *cyp19a1a* expression (putative females) and those with low or undetectable *cyp19a1a* expression (putative males) (Fig. 3). Gonadal histology in larger juvenile southern flounder confirmed that high *cyp19a1a* expression coincided with ovarian differentiation [35]. Since the bifurcation of *cyp19a1a* levels begins prior to morphological sex differentiation in this species [10,35], the increase in *cyp19a1a* appears to be a good predictor of ovarian differentiation, while low or undetectable levels are characteristic of testicular differentiation (Fig. 3). Similarly, studies in a number of other fishes suggest increases in *cyp19a1a* correspond with female differentiation and suppression of *cyp19a1a* is necessary for both normal and temperature induced male differentiation [8,46,51].

Recent studies have focused on both ovarian and brain P450arom isoforms (*cyp19a1a* and *cyp19a1b*) in adults and early developing Atlantic halibut [20,52,53]. Due to the small size of halibut prior to and during sex differentiation, RNA for PCR analysis was isolated from whole bodies of small larvae [20,52] and head and gonad regions of larger animals [16,49]. Both *cyp19a1a* and *cyp19a1b* mRNAs were abundant in the gonad and brain regions before morphological sex differentiation and levels appeared to increase somewhat earlier (i.e., smaller body size) in the brain than the gonad region [53]. These authors posited that earlier *cyp19a1b* expression in the brain might represent the onset of sex differentiation that is later manifested in the gonads, as hypothesized in some other vertebrates (see [54]). High temperatures (10 and 13 °C) appeared to suppress gonadal *cyp19a1a* expression, whereas temperature did not affect brain *cyp19a1b* [20]. In early developing mixed sex halibut (of unknown genotype), *cyp19a1a* expression

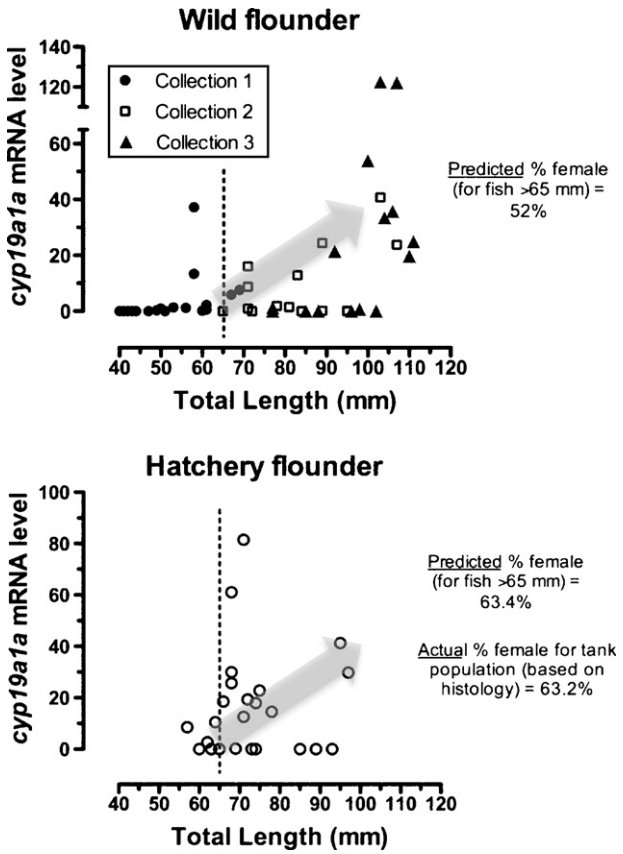

Fig. 3. P450 aromatase (*cyp19a1a*) mRNA levels in gonads of wild juvenile southern flounder (upper graph) and gonads of hatchery-reared southern flounder (lower graph) as determined by quantitative PCR. The range of bottom temperatures at capture sites for wild flounder collections 1, 2, and 3 were 23.2–24.1 °C, 24.6–26.7 °C, and 29.1–29.3 °C, respectively. The gray arrows denote the increase in aromatase expression in differentiating females beginning at ~65 mm total length (dotted line). Expression of *cyp19a1a* was normalized to elongation factor-1 alpha (*ef1a*) expression. Figure modified from Luckenbach et al. [35].

data were unclear and showed an expected on–off pattern in one study [52] and no clear sexually dimorphic pattern in another study [53]. In adults, however, *cyp19a1a* was highly expressed in ovary and not detected in testis [52] similar to the Paralichthid flounders.

To further elucidate the role of P450arom related to TSD in flounder, Kitano et al. [50] demonstrated that a high rearing temperature (27 °C) suppressed *cyp19a1a* mRNA and whole-body E_2 levels, and induced male differentiation in populations of genetically female (XX) Japanese flounder. Additionally, dietary exposure to an aromatase inhibitor, fadrozole, yielded results similar to high temperature and produced 100% males at the highest concentration tested (100 μg fadrozole/g diet) [55]. Together these data suggest endogenous E_2 biosynthesis (via P450arom) is critical for female differentiation in flounder, and in its absence, male differentiation will occur. Furthermore, high temperature may disrupt this process in XX flounder by suppressing *cyp19a1a* expression and thus E_2 biosynthesis.

4.2. Regulation of P450 aromatase

Several ideas have been proposed for environmental regulation of P450arom, including direct effects where temperature for instance may up- or down-regulate *cyp19a1a* expression and/or P450arom activity, or indirect effects mediated by factors upstream of *cyp19a1a* [5,46]. Promoter regions of *cyp19a1a* and *cyp19a1b* have been cloned in several fishes and analysis has revealed binding sites for a number of transcription factors within regulatory regions of the promoters [46]. For example, androgen response elements (ARE), cAMP response elements (CRE), estrogen response elements (ERE), progesterone response elements (PRE), glucocorticoid response elements (GRE), forkhead box (Fox), SRY/Sox, and Wilms tumor 1 (WT1-KTS) elements have been identified in some species. Analysis of the *cyp19a1a* promoter in Japanese flounder revealed two Ad4 binding protein/steroidogenic factor 1 (*nr5a1*) binding sites, an ERE-half site, a CRE-like sequence, and Fox sites [56]. These regulatory regions will likely continue to be a major focus as the search continues for factors that operate upstream of the *cyp19* genes.

The search for regulators of *cyp19a1a* transcription in Japanese flounder has revealed two candidates. Using juvenile XX flounder reared at either 18 or 27 °C, Yamaguchi et al. [56,57] first showed that the forkhead box L2 (*foxl2*) gene was expressed at low levels prior to morphological sex differentiation and then increased in fish held at 18 °C (differentiating females), whereas high temperature suppressed *foxl2* and *cyp19a1a* expression and induced male differentiation in XX fish. *In situ* hybridization showed that *foxl2* and *cyp19a1a* mRNAs were co-localized in interstitial cells of differentiating ovaries, suggesting possible interaction of these factors. Secondly, genes related to gonadotropin signaling were analyzed in the pituitary and gonad of XX flounder reared at the above temperatures. Follicle-stimulating hormone beta (*fshb*) and luteinizing hormone beta (*lhb*) subunit mRNAs were abundant in pituitaries of fish reared at both temperatures, with *fshb* appearing to be more highly expressed than *lhb*. Fsh receptor (*fshr*) and lh receptor (*lhr*) mRNAs were also quantified in the gonad and interestingly both *fshr* and *lhr* were expressed at 18 °C, but *fshr* expression was strongly suppressed by high temperature. A luciferase transfection assay using a human cell line showed that both Foxl2 and a cAMP analog, which simulates gonadotropin signaling, activated *cyp19a1a* transcription *in vitro*. Together these results demonstrate that high temperature suppresses *foxl2*, *cyp19a1a*, and *fshr* expression in the XX gonad of Japanese flounder and suggests a possible role of Foxl2 and gonadotropin signaling in regulation of *cyp19a1a* transcription. This proposed regulatory role of Foxl2 over *cyp19a1a* in flounder is supported by results in other fishes (e.g., [58]).

Other possible regulators of P450arom include heat shock factors (Hsf) and heat shock proteins (Hsp), which in theory could mediate temperature effects. It is unlikely, however, that these factors respond to such minor shifts in temperature as seen in TSD vertebrates [20]. A promising final possibility is epigenetic regulation of the *cyp19a1a* gene. Recent results in sea bass, *Dicentrarchus labrax*, suggest that high temperature exposure during early development increases DNA methylation of the *cyp19a1a* gene, leading to decreased *cyp19a1a* expression and male development [59].

4.3. Other gonadal factors potentially involved in ESD

It is important to note that in addition to P450arom, a host of other gonadal factors are known to play a role in teleost sex differentiation and possibly ESD. Few of these have been studied in flatfishes, and a comprehensive summary is not possible here, but we will touch on a few of them. Transcript levels of anti-Müllerian hormone (*amh*, or Müllerian-inhibiting substance) and the estrogen receptors, *esr1* and *esr2*, have been examined in flatfishes. In Japanese flounder, *amh* levels increase significantly during male differentiation [60] similar to other fishes [61,62], suggesting *amh* is a reliable marker and key factor for testicular differentiation. In Atlantic halibut, partial cDNAs were obtained for *esr1* and *esr2* and their expression was studied during early development [20]. Transcripts for *esr1* and *esr2* increased over early development and although temperature had no significant effect, *esr1* appeared to be somewhat down-regulated by the highest temperature tested.

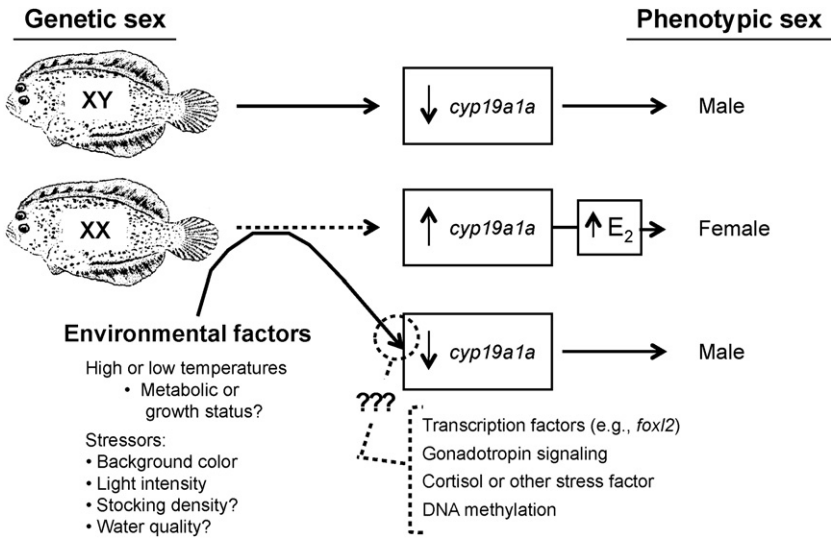

Fig. 4. A simplified model summarizing what is known concerning mechanisms of sex determination in flounder. The XY genotype is stable and not influenced by environmental factors, thus phenotypic males are produced through pure GSD. Conversely, the XX genotype is unstable and the phenotypic sex may be determined by environmental factors through suppression of *cyp19a1a* expression and endogenous E_2. The mechanism(s) by which environmental factors influence *cyp19a1a* transcription is unknown. However, several candidates have been proposed and are being further investigated.

Other intriguing early markers of sex differentiation include steroidogenic factors like P450scc, cholesterol side-chain cleavage enzyme (*cyp11a1*), 11β-hydroxylase (*cyp11b2*), and 3β-hydroxysteroid dehydrogenase (*hsd3b*), and transcription factors such as doublesex and mab-3-related transcription factor 1 (*dmrt1*), *dax1* (now termed *nr0b1*), and Ad4BP/steroidogenic factor-1 or fushi tarazu factor-1 (*nr5a1*) [8,62,63]. Other factors like R-spondin1 (*rspo1*), *wnt4*, *sox9*, follistatin (*fst*), factor-in-the-germline alpha (*figa*), and androgen receptors (*ar1* and *ar2*) are also thought to play important roles in sex differentiation [39,44,49,61,64]. Recent work in tilapia profiled transcript levels of a number of the above genes in gonads of XX and XY fish prior to and during sex differentiation [57]. Expression of *foxl2* showed a pattern very similar to *cyp19a1a* in XX gonads, thus agreeing with findings in Japanese flounder [52], while other genes examined either increased later in sex differentiation or showed no clear dimorphic pattern. In regard to XY gonads, *dmrt1* was specifically expressed in XY gonads and showed an early increase, similar to *cyp19a1a* in XX gonads. In teleosts generally, ovarian differentiation is often correlated with increases in *foxl2*, *cyp19a1a*, *fst*, *figa*, and E_2 levels, while testicular differentiation is often correlated with increases in *dmrt1*, *sox9*, *nr5a1*, and *amh*. Mechanisms regulating normal sex differentiation and environmentally controlled sex differentiation are likely to share many common features, thus studies should continue to investigate effects of the environment on these gonadal factors.

5. Conclusions and future directions

Flatfishes appear to exhibit either pure GSD or a combination of GSD and ESD with the XX genotype being prone to the influence of environmental factors (Fig. 4). In our experience, the XX genotype in southern flounder is highly unstable in the culture environment and exposure to low or high temperatures during early development can cause sex reversal to phenotypic males [10,36]. In addition, recent studies have demonstrated that other environmental factors such as tank color and light intensity can also have this effect in southern flounder [18]. This creates a scenario where various environmental factors may induce the same phenotypic result likely through a pathway involving *cyp19a1a* regulation (Fig. 4). Studies in Japanese and southern flounder [35,50,55] suggest *cyp19a1a* is a critical factor in the process of flounder sex differentiation, and importantly, suppressing expression of this gene leads to male differentiation regardless of genotype. Temperature and other environmental factors likely affect flounder sex differentiation by suppressing *cyp19a1a* expression, and other possible regulatory factors such as *foxl2* and *fshr*, in genetic females. However, the precise mechanism by which an environmental cue leads to down-regulation of P450arom and male development is unknown.

In some species such as southern flounder, the line between observing GSD and TSD is quite narrow (5 °C or less for southern flounder). As indicated by estuarine temperatures measured where juvenile southern flounder were captured, water temperatures rise dramatically to over 29 °C during summer months during sex differentiation (Fig. 3). Other studies of wild southern flounder show temperatures well in excess of 30 °C in estuaries representative of the northern range of this species [36]. Based on this information and recent evidence of other environmental factors that influence sex determination in southern flounder [18], it would appear that ESD, and specifically TSD could occur naturally in this species and potentially other flatfishes as well.

Among fishes, GSD is probably best understood in medaka fish (*Oryzias latipes*), a species in which the male-determining gene, *DMY* (i.e., the equivalent of mammalian *SRY*), has been identified [6,65]. To date, the male-determining gene(s) in flatfishes is unknown and no sex-linked DNA markers have been identified in Paralichthid flounders that could be used to address whether XX male flounder exist in the wild. There is interest, however, in developing such a tool as some flounder populations are in decline and fisheries managers are eager to better understand potential reasons for these declines. An interesting recent study modeled the genetic risk associated with the existence of XX male flounder in the wild [66]. In Japan, this is a real concern because stock enhancement of Japanese flounder has been conducted for decades and some sex reversal in captivity prior to fish release is likely to have occurred. Results showed that the presence of XX males could ultimately result in extinction of the male-determining gene. The greatest danger would apparently be created by hatcheries using hatchery-produced broodstock (potentially XX males) for reproduction of subsequent generations of flounder for stock enhancement. Obviously this possibility warrants further study

of ESD in flatfishes before stock enhancement proceeds in other species.

It remains unclear how environmental cues regulate gonadal P450arom. Indeed, this connection between environmental cues and P450arom remains an enigmatic issue in all ESD vertebrates [54] and has become a 'holy grail' of sorts in ESD research. Analysis of *cyp19* promoters for potential regulatory factors and research related to *cyp19a1a* methylation hold promise. Still, another possibility supported by data in southern flounder is that stress factors, such as cortisol, may influence sex determination [18]. In this scenario, several environmental cues could be perceived as a 'stressor' and lead to suppression of *cyp19a1a* through a shared mechanism (Fig. 4).

Clearly many questions remain with regard to genetic and environmental control of sex determination in flatfishes and teleosts generally. Emerging research related to sex determination and differentiation in several additional flatfish species, such as summer flounder (*Paralichthys dentatus*), Brazilian flounder (*Paralichthys orbignyanus*), winter flounder (*Pseudopleuronectes americanus*), and turbot (*Scopthalmus maximus*) should provide further insight into mechanisms of sex determination in flatfishes and the possible natural occurrence of ESD in this order.

Acknowledgements

Sincere thanks to all those involved in the southern flounder sex determination research covered in this review, especially Ryan Murashige, Andrew J. Morgan, and Poem M. Turner. North Carolina Sea Grant, the Salstonstall-Kennedy Program of the National Marine Fisheries Service, and USDA Cooperative State Research, Education, and Extension Service (CSREES), supported much of our research in this area. J.A.L. is currently supported by National Research Initiative Competitive Grant no. 2007-35203-18082 from the USDA CSREES.

References

[1] Devlin RH, Nagahama Y. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 2002;208:191–364.
[2] Oldfield RG. Genetic, abiotic and social influences on sex differentiation in cichlid fishes and the evolution of sequential hermaphroditism. Fish Fish 2005;6:93–110.
[3] Godwin J, Luckenbach JA, Borski RJ. Ecology meets endocrinology: environmental sex determination in fishes. Evol Dev 2003;5:40–9.
[4] Conover DO. Temperature-dependent sex determination in fishes. In: Valenzuela N, Lance V, editors. Temperature-dependent sex determination in vertebrates. WA: Smithsonian Institution Press; 2004. p. 11–20.
[5] Baroiller JF, D'Cotta H. Environment and sex determination in farmed fish. Comp Biochem Physiol 2001;130C:399–409.
[6] Matsuda M. Sex determination in the teleost medaka, *Oryzias latipes*. Annu Rev Genet 2005;39:293–307.
[7] Ospina-Álvarez N, Piferrer F. Temperature-dependent sex determination in fish revisited: prevalence, a single sex ratio response pattern, and possible effects of climate change. PLoS ONE 2008;3:e2837.
[8] Baroiller JF, Guiguen Y, Fostier A. Endocrine and environmental aspects of sex differentiation in fish. Cell Mol Life Sci 1999;55:910–31.
[9] Yamamoto E. Studies on sex-manipulation and production of cloned populations in hirame, *Paralichthys olivaceus* (Temminck et Schlegel). Aquaculture 1999;173:235–46.
[10] Luckenbach JA, Godwin J, Daniels HV, Borski RJ. Gonadal differentiation and effects of temperature on sex determination in southern flounder (*Paralichthys lethostigma*). Aquaculture 2003;216:315–27.
[11] Conover DO, Kynard BE. Environmental sex determination: interaction of temperature and genotype in a fish. Science 1981;213:577–9.
[12] Pandian TJ, Koteeswaran R. Ploidy induction and sex control in fish. Hydrobiologia 1998;384:167–243.
[13] Felip A, Zanuy S, Carrillo M, Piferrer F. Induction of triploidy and gynogenesis in teleost fish with emphasis on marine species. Genetica 2001;111: 175–95.
[14] Aida S, Arai K. Sex ratios in the progeny of gynogenetic diploid marbled sole *Limanda yokohamae* males. Fish Sci 1998;64:989–90.
[15] Tvedt HB, Benfey TJ, Martin-Robichaud DJ, McGowan C, Reith M. Gynogenesis and sex determination in Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture 2006:573–83.
[16] Chen S-L, Deng SP, Ma HY, Tian Y-S, Xu JY, Yang J-F, et al. Molecular marker-assisted sex control in half-smooth tongue sole (*Cynoglossus semilaevis*). Aquaculture 2008;283:7–12.
[17] Tabata K. Induction of gynogenetic diploid males and presumption of sex determination mechanisms in the hirame *Paralichthys olivaceus*. Nippon Suisan Gakk 1991;57:845–50.
[18] Turner PM., Effects of light intensity and tank background color on sex determination in southern flounder (*Paralichthys lethostigma*). M.Sc. Thesis. North Carolina State University, Raleigh, NC, USA, 2008.
[19] Colburn HR, Nardi GC, Borski RJ, Berlinsky DL., Induced meiotic gynogenesis and sex determination in summer flounder (*Paralichthys dentatus*). Aquaculture; accepted for publication.
[20] van Nes S, Andersen Ø. Temperature effects on sex determination and ontogenetic gene expression of the aromatases *cyp19a* and *cyp19b*, and the extrogen receptors *esr1* and *esr2* in Atlantic halibut (*Hippoglossus hippoglossus*). Mol Reprod Dev 2006;73:1481–90.
[21] Hughes V, Benfey TJ, Martin-Robichaud DJ. Effect of rearing temperature on sex ratio in juvenile Atlantic halibut, *Hippoglossus hippoglossus*. Environ Biol Fish 2008;81:415–9.
[22] Goto R, Kayaba T, Adachi S, Yamauchi K. Effects of temperature on sex determination in marbled sole *Limanda yokohamae*. Fish Sci 2000;66:400–2.
[23] Purdom CE. Induced polyploidy in plaice (*Pleuronectes platessa*) and its hybrid with the flounder (*Platichthys flesus*). Heredity 1972;29:11–24.
[24] Fairchild EA, Rennels N, Howell WH, Wells RE. Gonadal development and differentiation in cultured juvenile winter flounder, *Pseudopleuronectes americanus*. J World Aquacult Soc 2007;38:114–21.
[25] Goto R, Tatsunari M, Kawamata K, Matsubara T, Mizuno S, Adachi S, et al. Effects of temperature on gonadal sex determination in barfin flounder (*Verasper moseri*). Fish Sci 1999;65:884–7.
[26] Cal RM, Vidal S, Martínez P, Álvarez-Blázquez B, Gómez C, Piferrer F. Growth and gonadal development of gynogenetic diploid *Scophthalmus maximus*. J Fish Biol 2006;68:401–13.
[27] Howell BR, Baynes SM, Thompson D. Progress towards the identification of the sex-determining mechanism of the sole, *Solea solea* (L.), by the induction of diploid gynogenesis. Aquaculture Res 1995;26:135–40.
[28] Vitturi R, Catalano E, Colombera D. Chromosome analysis of *Bothus podas* (Pisces, Pleuronectiformes) from the Mediterranean Sea. J Fish Biol 1993;43:221–7.
[29] Azevedo MFC, Oliveira C, Pardo BG, Martínez P, Foresti F. Cytogenetic characterization of six species of flatfishes with comments to karyotype differentiation patterns in Pleuronectiformes (Teleostei). J Fish Biol 2007;70(Suppl. A):1–15.
[30] Luckenbach JA, Godwin J, Daniels HV, Beasley JM, Sullivan CV, Borski RJ. Induction of diploid gynogenesis in southern flounder (*Paralichthys lethostigma*) with homologous and heterologous sperm. Aquaculture 2004;237:499–516.
[31] Morgan AJ, Murashige R, Woolridge CA, Luckenbach JA, Watanabe WO, Borski RJ, et al. Effective UV dose and pressure shock for induction of meiotic gynogenesis in southern flounder (*Paralichthys lethostigma*) using black sea bass (*Centropristis striata*) sperm. Aquaculture 2006;259:290–9.
[32] Hendry CI, Martin-Robichaud DJ, Benfey TJ. Hormonal sex reversal of Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture 2003;219:769–81.
[33] Conover DO. Adaptive significance of temperature-dependent sex determination in a fish. Am Nat 1984;123:297–313.
[34] Lagomarsino IV, Conover DO. Variation in environmental and genotypic sex-determining mechanisms across a latitudinal gradient in the fish, *Menidia menidia*. Evolution 1993;47:487–94.
[35] Luckenbach JA, Early LW, Rowe AH, Borski RJ, Daniels HV, Godwin J. Aromatase cytochrome P450: cloning, intron variation, and ontogeny of gene expression in southern flounder (*Paralichthys lethostigma*). J Exp Zool 2005;303A:643–56.
[36] Luckenbach JA, Murashige R, Daniels HV, Godwin J, Borski RJ. Temperature affects insulin-like growth factor I and growth of juvenile southern flounder, *Paralichthys lethostigma*. Comp Biochem Physiol 2007;146A:95–104.
[37] Charnov EL, Bull JJ. When sex is environmentally determined. Nature 1977;266:828–30.
[38] Kraak SBM, De Looze EMA. A new hypothesis on the evolution of sex determination in vertebrates; big females ZW, big males XY. Neth J Zool 1993;43:260–73.
[39] Piferrer F, Blázquez M, Navarro L, González A. Genetic, endocrine, and environmental components of sex determination and differentiation in the European sea bass (*Dicentrarchus labrax* L.). Gen Comp Endocrinol 2005;142:102–10.
[40] Lawrence C, Ebersole JP, Kesseli RV. Rapid growth and out-crossing promote female development in zebrafish (*Danio rerio*). Environ Biol Fish 2008;81:239–46.
[41] Wood AW, Duan C, Bern HA. Insulin-like growth factor signaling in fish. Int Rev Cytol 2005;243:215–65.
[42] Picha ME, Turano MJ, Beckman BR, Borski RJ. Endocrine biomarkers of growth and applications to aquaculture: a minireview of growth hormone, insulin-like growth factor (IGF)-I, and IGF-binding proteins as potential growth indicators in fish. N Am J Aquacult 2008;70:196–211.
[43] Ueda A., Assessment of growth and its regulation through insulin-like growth factor-I in southern flounder, *Paralichthys lethostigma*. M.Sc. Thesis. North Carolina State University, Raleigh, NC, USA, 2004.
[44] Kanamori A. Systematic identification of genes expressed during early oogenesis in medaka. Mol Reprod Dev 2000;55:31–6.
[45] Baron D, Houlgatte R, Fostier A, Guiguen Y. Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol Reprod 2005;73:959–66.

[46] Piferrer F, Blázquez M. Aromatase distribution and regulation in fish. Fish Physiol Biochem 2005;31:215–26.
[47] Luckenbach JA, Iliev DB, Goetz FW, Swanson P. Identification of differentially expressed ovarian genes during primary and early secondary oocyte growth in coho salmon, *Oncorhynchus kisutch*. Reprod Biol Endocrinol 2008;6:2.
[48] Guiguen Y, Baroiller J-F, Ricordel M-J, Iseki K, McMeel OM, Martin SAM, et al. Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*). Mol Reprod Dev 1999;54:154–62.
[49] Vizziano D, Baron D, Randuineau G, Mahè S, Cauty C, Guiguen Y. Rainbow trout gonadal masculinization induced by inhibition of estrogen synthesis is more physiological than masculinization induced by androgen supplementation. Biol Reprod 2008;78:939–46.
[50] Kitano T, Takamune K, Kobayashi T, Nagahama Y, Abe S-I. Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). J Mol Endocrinol 1999;23:1–10.
[51] Rashid H, Kitano H, Lee KH, Nii S, Shigematsu T, Kadomura K, et al. Fugu (*Takifugu rubripes*) sexual differentiation: CYP19 regulation and aromatase inhibitor induced testicular development. Sex Dev 2007;1:311–22.
[52] van Nes S, Moe M, Andersen Ø. Molecular characterization and expression of two *cyp19* (P450 aromatase) genes in embryos, larvae, and adults of Atlantic halibut (*Hippoglossus hippoglossus*). Mol Reprod Dev 2005;72:437–49.
[53] Matsuoka MP, van Nes S, Andersen Ø, Benfey TJ, Reith M. Real-time PCR analysis of ovary- and brain-type aromatase gene expression during Atlantic halibut (*Hippoglossus hippoglossus*) development. Comp Biochem Physiol 2006;144B:128–35.
[54] Lance VA, Is regulation of aromatase expression in reptiles the key to understanding temperature-dependent sex determination?, J Exp Zool, doi:10.1002/jez.465, in press.
[55] Kitano T, Takamune K, Nagahama Y, Abe S-I. Aromatase inhibitor and 17a-Methyltestosterone cause sex-reversal from genetic females to phenotypic males and suppression of P450 aromatase gene expression in Japanese flounder (*Paralichthys olivaceus*). Mol Reprod Dev 2000;56:1–5.
[56] Yamaguchi T, Yamaguchi S, Hirai T, Kitano T. Follicle-stimulating hormone signaling and Foxl2 are involved in transcriptional regulation of aromatase gene during gonadal sex differentiation in Japanese flounder, *Paralichthys olivaceus*. Biochem Biophys Res Commun 2007;359:935–40.
[57] Yamaguchi T, Kitano T. The mechanism of transcriptional regulation of P450 aromatase gene by Foxl2 in Japanese flounder (*Paralichthys olivaceus*). Cybium 2008;32(2 suppl.):74–6.
[58] Wang D-S, Kobayashi T, Zhou L-Y, Paul-Pransanth B, Ijiri S, Sakai F, et al. Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with Ad4 binding protein/steroidogenic factor 1. Mol Endocrinol 2007;21:712–25.
[59] Piferrer F, Navarro-Martin L, Ospina-Álvarez N, Blázquez M, Viñas J. Temperature-dependent sex determination in fish, effects of temperature on gonadal aromatase gene expression, and epigenetic regulation after early exposure to high water temperature. In: Proceedings of the Sixth International Symposium on Fish Endocrinology. 2008. Abstract S8-4.
[60] Yoshinaga N, Shiraishi E, Yamamoto T, Iguchi T, Abe S-I, Kitano T. Sexually dimorphic expression of a teleost homologue of Müllerian inhibiting substance during gonadal sex differentiation in Japanese flounder, *Paralichthys olivaceus*. Biochem Biophys Res Commun 2004;322:508–13.
[61] Rodríguez-Marí A, Yan Y-L, BreMiller RA, Wilson C, Cañestro C, Postlethwait JH. Characterization and expression pattern of zebrafish *anti-Müllerian hormone* (*amh*) relative to *sox9a*, *sox9b*, and *cyp19a1a*, during gonad development. Gene Exp Patterns 2005;5:655–67.
[62] Ijiri S, Kaneko H, Kobayashi T, Wang D-S, Sakai F, Paul-Prasanth B, et al. Sexually dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia *Oreochromis niloticus*. Biol Reprod 2008;78:333–41.
[63] von Hofsten J, Olsson P-E. Zebrafish sex determination and differentiation: involvement of FTZ-F1 genes. Reprod Biol Endocrinol 2005;3:63.
[64] Smith CA, Shoemaker CM, Roeszler KN, Queen J, Crews D, Sinclair AH. Cloning and expression of *R-Spondin I* in different vertebrates suggests a conserved role in ovarian development. BMC Dev Biol 2008;8:72.
[65] Matsuda M, Shinomiya A, Kinoshita M, Suzuki A, Kobayashi T, Paul-Pransanth B, et al. DMY gene induces male development in genetically female (XX) medaka fish. Proc Natl Acad Sci USA 2007;104:3865–70.
[66] Kanaiwa M, Harada Y. Genetic risk involved in stock enhancement of fish having environmental sex determination. Popul Ecol 2002;44:7–15.