General and Comparative Endocrinology 166 (2010) 470–477

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Fish with thermolabile sex determination (TSD) as models to study brain sex differentiation

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ARTICLE INFO

Article history:
Received 27 July 2009
Accepted 20 October 2009
Available online 23 October 2009

Keywords:
Aromatase
Sex differentiation
Sex determination
Brain
Neurogenesis

ABSTRACT

As fish are ectothermic animals, water temperature can affect their basic biological processes such as larval development, growth and reproduction. Similar to reptiles, the incubation temperature during early phases of development is capable to modify sex ratios in a large number of fish species. This phenomenon, known as thermolabile sex determination (TSD) was first reported in *Menidia menidia*, a species belonging to the family Atherinopsidae. Since then, an increasing number of fish have also been found to exhibit TSD. Traditionally, likewise in reptiles, several TSD patterns have been described in fish, however it has been recently postulated that only one, females at low temperatures and males at high temperatures, may represent the "real" or "true" TSD. Many studies regarding the influence of temperature on the final sex ratios have been focused on the expression and activity of gonadal aromatase, the enzyme involved in the conversion of androgens into estrogens and encoded by the *cyp19a1a* gene. In this regard, teleost fish, may be due to a whole genome duplication event, produce another aromatase enzyme, commonly named brain aromatase, encoded by the *cyp19a1b* gene. Contrary to what has been described in other vertebrates, fish exhibit very high levels of aromatase activity in the brain and therefore they synthesize high amounts of neuroestrogens. However, its biological significance is still not understood. In addition, the mechanism whereby temperature can induce the development of a testis or an ovary still remains elusive. In this context the present review is aimed to discuss several theories about the possible role of brain aromatase using fish as models. The relevance of brain aromatase and therefore of neuroestrogens as the possible cue for gonadal differentiation is raised. In addition, the possible role of brain aromatase as the way to keep the high levels of neurogenesis in fish is also considered. Several key examples of how teleosts and aromatase regulation can offer more insight into basic mechanisms of TSD are also reviewed.

1. Introduction

The influence of sex steroids on the development of the central nervous system (CNS), organization, physiology and behaviour in mammals has been known since the early sixties (Barraclough and Gorksi, 1962; Whalen and Nadler, 1963; Levine and Mullins, 1964; Feder and Whalen, 1965). However the effect of 17β-estradiol (E_2) mimicking testosterone treatment was not understood until the "aromatization hypothesis" was proposed (Naftolin et al., 1971a,b; Naftolin et al., 1975). Since then, the effects of androgens on development and differentiation of the CNS were considered as the local, aromatase-mediated, conversion of androgens to estrogens. Sex-related differences in the CNS of rodents, specifically the medial basal hypothalamus and the preoptic region, appear to be driven by the hormonal milieu experienced by the individuals during early development after the conversion of androgens to estrogens (Raisman and Field, 1973; MacLusky and Naftolin, 1981; Arnold and Gorski, 1984; Toran-Allerand, 1984; Lorenzo et al., 1992; Lephart, 1996). Moreover, E_2 receptors are involved in sexual differentiation of the anterior preoptic area of mouse (Simerly et al., 1997) and estrogens modulate the sexually dimorphic dendritic branches in the hypothalamus (Simerly, 2002, 2005).

In birds, estrogens also have an important role in the organization of male neural circuits (Balthazart et al., 2004, 2009). For example, brain sex differences, are also established early in development (Morris et al., 2004), and some brain nuclei and even some neurons show sexual differences in size (Panzica et al., 1996). Sex differences have also been demonstrated in aromatase gene expression which is usually higher in males (Voigt et al., 2007).

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doi:10.1016/j.ygcen.2009.10.004

In reptiles, the brain has been proposed as a sensor of temperature and long considered as a possible driver of TSD since aromatase was differentially expressed in the brains of embryo turtles at male- and female-producing temperatures (Jeyasuriya and Place, 1997, 1998; Place et al., 2001). However, recent views point at the idea that gonadal aromatase and estrogens production are the key players responsible for TSD (Lance, 2008).

Fish are extremely versatile regarding their strategies of sex differentiation patterns including gonochorism, sequential hermafroditism, bi-directional hermaphroditism and unisexuality (Devlin and Nagahama, 2002). Particularly during the past decade, abundant information has been published on the role of aromatase and E_2 on sex differentiation in fish. The majority of these studies have been focussed on the gonads (for reviews see Devlin and Nagahama, 2002; Strüssmann and Nakamura, 2002; Piferrer and Guiguen, 2008; Guiguen et al., 2009). However the involvement of aromatase on brain sex differentiation and therefore the possible role of the brain on gonadal sex differentiation have not been studied in detail. In this context, the aim of this paper is to provide an updated view of the possible role of the brain on sex differentiation. The review is focused on species for which sex differentiation can be modulated and even overridden by the main environmental factor that is temperature.

2. Sex determination and sex differentiation in fish

As already stated by Devlin and Nagahama (2002), the distinction between sex determination and sex differentiation is often difficult and sometimes they are not very well used in the literature. The term sex determination describes "the genetic and environmental processes and variables that influence sex differentiation" whereas sex differentiation can be defined as "the physiological processes leading to the development of a testis and an ovary from an undifferentiated gonad". It is now known that both processes can be regulated by genetic factors, environmental influences or by their interaction. The mechanisms involved in sex determination are quite variable along the animal kingdom, and therefore differentially regulated in different species. However, the genes controlling sex differentiation appear to be more conserved along animal evolution (Brennan and Capel, 2004; Yao and Capel, 2005; Ferguson-Smith, 2007; Shoemaker et al., 2007; Piferrer and Guiguen, 2008; Wallis et al., 2008). Gonochoristic vertebrates exhibit two general mechanisms of sex determination namely genetic sex determination (GSD) and environmental sex determination (ESD). In GSD species, typically birds and mammals, sex is determined at conception and driven by the expression of different genes. Conversely, in ESD species, typically fish and reptiles, sex is determined during early developmental stages in response to different environmental factor(s) (Crews and Bull, 2008). Among these factors it is worth mentioning temperature (Conover and Kynard, 1981; Conover and Heins, 1987), pH (Rubin, 1985; Roemer and Beisenherz, 1996), social interactions (Francis and Barlow, 1993; Francis, 1984; Hobbs et al., 2004) and hypoxia (Shang et al., 2006; P. Thomas, personal communication). However, the most prevalent (or documented) form of ESD in teleost fish is temperature-dependent sex determination (TSD) (Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002; Strüssmann and Nakamura, 2002; Conover, 2004; Luckenbach et al., 2009). The first evidence of TSD in fish emerged from the studies in the Atlantic silverside, *Menidia menidia*, for which the influence of temperature on sex determination has been shown to change according to the latitude, ranging from high TSD in southern fish to pure GSD in northern fish (Conover and Kynard, 1981; Conover and Heins, 1987; Lagomarsino and Conover, 1993). Since then, an increasing number of fish have been found to exhibit TSD. Likewise in reptiles, several patterns of TSD have been described in fish although a recent study suggests that only one of them (producing females at low and males at high temperatures) is valid (Ospina-Álvarez and Piferrer, 2008). In this regard, although TSD was postulated to be quite widespread within fish, it now seems that "pure" TSD species are not as common as previously thought and many species initially considered to exhibit TSD are in fact species with GSD with a strong influence of temperature (GSD + TE) (Ospina-Álvarez and Piferrer, 2008). Whether pure TSD or GSD + TE, it is clear that fish offer an excellent model to study the plasticity in the mechanisms of sex determination and sex differentiation.

3. Molecular players involved in sex differentiation: effects of temperature in TSD species

For the last decade, extensive research has been focussed on the elucidation of the key players potentially involved in vertebrate sex differentiation. The development of genomic tools in several fish model species has resulted in the cloning and characterization of some genes that could be regulating the process (Baron et al., 2005, 2008; Vizziano et al., 2007, 2008; Ijiri et al., 2008). Some recent reviews have already devoted their content to some of these factors, particularly to those involved in gonadal sex differentiation (Piferrer and Guiguen, 2008; Guiguen et al., 2009). In this review we will update current knowledge on some of them, specifically those expressed in brain and possibly operating in species with TSD.

3.1. Cyp19a1 (aromatase)

Teleost fish are unique models to study the effects of neuroestrogens due to the strikingly high activity of the enzyme aromatase found in their brains, reaching values up to 100–1000 times higher than those found in mammals (Callard et al., 1990). The biosynthesis of estrogens is catalyzed by the cytochrome P450 aromatase, encoded by the *cyp19a1* gene. Aromatase, the rate-limiting enzyme for estrogens synthesis, converts C_{19} androgens into C_{18} estrogens and it has been implicated in vertebrate sex differentiation and maturation (Simpson et al., 2002). Only one copy of the *cyp19a1* gene is present in mammals and higher vertebrates, except pigs where multiple copies have been reported (Corbin et al., 1995; Choi et al., 1997). Therefore, tissue-specific expression is achieved by alternative splicing and/or different promoter usage (i.e. humans: Harada et al., 1993; Simpson et al., 1997, and mouse: Golovine et al., 2003). However, teleost fish, as result of an ancient duplication in the teleosts genome (Taylor et al., 2003), have two genes, named as *cyp19a1a* and *cyp19a1b* mainly expressed in the gonads and brain, respectively, coding for two structurally different aromatase proteins (Tchoudakova and Callard, 1998; Chiang et al., 2001; Kazeto et al., 2001).

Gonadal aromatase, *cyp19a1a*, has been strongly associated with gonadal sex differentiation (Devlin and Nagahama, 2002; Guiguen et al., 2009). However, the function of brain aromatase, *cyp19a1b*, is not well established, and the latest studies relate it to neurogenesis (Callard et al., 2001; Forlano et al., 2001, 2009; Menuet et al., 2003, 2005; Blázquez and Piferrer, 2004; Mouriec et al., 2008). Nevertheless, the possibility that *cyp19a1b* could also be dictating gonadal differentiation cannot be ruled out (Trant et al., 2001; Blázquez and Piferrer, 2004). In this regard, *cyp19a1b* has been primarily found in the neuroendocrine brain, including the telencephalon and the hypothalamus (Pasmanik and Callard, 1988; Forlano et al., 2001; Menuet et al., 2003, 2005; Strobl-Mazzulla et al., 2005; Marsh et al., 2006; Pellegrini et al., 2007), and recent results suggested that it could also be involved in the establishment of the neuroendocrine circuits during the gonadal differentiation period (Strobl-Mazzulla et al., 2008). Moreover, *cyp19a1b* is extremely sensitive to the

action of exogenous estrogens and is currently used as a biomarker for the presence of estrogenic substances in the environment (Lyssimachou et al., 2006; Cheshenko et al., 2008). The main reason why *cyp19a1b* expression can be modulated by the presence of estrogens is due to the fact that it possesses an estrogen responsive element (ERE) in its promoter (Kazeto et al., 2001, 2003; Piferrer and Blázquez, 2005). An interesting paradox comes from the fact that *cyp19a1a*, does not have binding sites for EREs in its promoter and this feature could thus be used as a way to control aromatase transcription.

In fish with TSD, temperature has been shown to regulate *cyp19a1a* gene expression (Kitano et al., 1999; D'Cotta et al., 2001; Karube et al., 2007; Fernandino et al., 2008a,b). Moreover, the temperature-dependent expression of *cyp19a1a* has also been studied in species for which this environmental factor is able to modify sex ratios as it is the case of zebrafish, *Danio rerio*, (Uchida et al., 2004) and European sea bass, *Dicentrarchus labrax*, (Blázquez et al., 2009). However, in the European sea bass, no apparent effects of temperature could be found during the first 120 days post hatching (Blázquez et al., 2009). This was probably due to the fact that rapid proliferation of primordial germ cells and thus active gonadal formation in this species does not occur until 80–140 dph depending on growth rates (Roblin and Bruslé, 1983). It is therefore, very interesting that the influence of temperature on gonadal differentiation occurs during a thermosensitive period (TSP) at which the gonadal primordium is at its earliest stages of development. Likewise, the teleost brain also acts as a sensor for temperature and therefore *cyp19a1b* gene expression can be subjected to thermal regulation (see Section 5).

High temperatures resulted in decreased *cyp19a1a* expression and increased titres of cortisol, 11-ketotestosterone and testosterone in pejerrey, suggesting a role for cortisol and thus of thermal-induced stress in testicular differentiation in a species with TSD (Hattori et al., 2009). It has also been suggested that the epigenetic regulation of gene expression could be a way to regulate aromatase transcription. In this regard, sex-related differences in the degree of DNA methylation of *cyp19a1a* promoter has been reported as a way to regulate gene expression in medaka, *Oryzias latipes* (Contractor et al., 2004), with males exhibiting high methylation levels whereas lower levels were found in females. Other possibilities suggested for the transcriptional regulation of aromatase have been reviewed in Piferrer and Blázquez (2005).

3.2. Foxl2 (forkhead box protein L2)

This gene encodes a putative winged helix/forkhead transcription factor. In several fish species *foxl2* is involved in the transcriptional regulation of aromatase including medaka (Nakamoto et al., 2006), tilapia, *Oreochromis niloticus*, (Wang et al., 2007) and rainbow trout, *Oncorhynchus mykiss*, (Vizziano et al., 2008). In the European sea bass it has also been postulated that the estrogenic effect exerted by exogenous E_2 does not involve direct up-regulation of *cyp19a1a* transcription but occurs indirectly through the activation of *foxl2* binding to regulatory sites in *cyp19a1a* promoter (Navarro-Martín et al., 2009) further evidencing its role in ovarian differentiation. In the Japanese flounder, *Paralichthys olivaceus*, a species with TSD, high water temperatures inhibited *foxl2* gene expression during early sex differentiation and results in ovarian development reinforcing its involvement in *cyp19a1a* transcriptional regulation (Yamaguchi et al., 2007; Yamaguchi and Kitano, 2008). It is also important to note that *foxl2* has also been found in the brain and pituitary gland in different fish species such as Nile tilapia (Wang et al., 2004), Southern catfish, *Silurus meridionalis*, (Liu et al., 2007), pejerrey, *Odontesthes bonariensis*, and Patagonian pejerrey, *Odontesthes hatcheri* (Hattori et al., personal communication). The involvement of *foxl2* in ovarian differentiation has been documented from fish (Wang et al., 2004; Nakamoto et al., 2006) to mammals (Loffler et al., 2003) but the brain ontogenic expression of *foxl2* in relation with sex differentiation has not been studied in detail.

3.3. Amh (anti-müllerian hormone)

Is a member of the superfamily of transforming growth factors beta and has been implicated in mammalian testicular differentiation (Brennan and Capel, 2004). In fish, sex-related differences in *amh* expression levels have been reported in Japanese eel, *Anguilla japonica*, (Miura et al., 2002), Japanese flounder (Yoshinaga et al., 2004), zebrafish (Rodríguez-Mari et al., 2005; Wang and Orban, 2007), rainbow trout (Baron et al., 2005), European sea bass (Halm et al., 2007), Nile tilapia (Ijiri et al., 2008) and pejerrey (Fernandino et al., 2008b). Although traditionally considered to be specifically expressed in testis, high *amh* levels were found in juvenile male sea bass brain and pituitary suggesting an autocrine role of this gene in the regulation of gonadotropin and steroid synthesis in the brain (Halm et al., 2007). In species with TSD, *amh* showed a temperature dimorphic expression pattern before the first signs of histological sex differentiation with markedly higher values at male-producing temperatures than at female-producing temperature (Fernandino et al., 2008b). Furthermore, this dimorphic expression was observed at a sex-neutral temperature; however, estrogens were capable to modulate *amh* expression inducing upregulation after E_2 supplementation and down-regulation in absence of E_2 and also after treatment with an aromatase inhibitor, suggesting that *amh* is more a consequence than the cause of gonadal sex differentiation at least in the pejerrey (Fernandino et al., 2008b).

4. Aromatase and estrogen receptors

Since the pioneer studies of Yamamoto (1969) it is accepted that estrogens are the "ovarian inductors" because it has been shown in a number of fish species that either early treatment with E_2 or with aromatase inhibitors could induce feminization or masculinization, respectively (Guiguen et al., 2009). Recent results also suggest that estrogens are needed to maintain the structure of differentiated ovaries (Bhandari et al., 2006; Ogawa et al., 2008), strengthening their importance in the maintenance of ovarian morphology and function.

If aromatase expression and activity and hence E_2 are related to gonadal differentiation then, as the classical mechanism of estrogens actions includes specific binding to their receptors (Bain et al., 2007), the involvement of estrogen receptors (ERs) should be taken into consideration. Much of the work on ERs in fish showed the existence of three different forms, ERα, ERβ1 and ERβ2 (also known as *esr1*, *esr2b* and *esr2a*, respectively, Hawkins et al., 2000; Menuet et al., 2002; Hawkins and Thomas, 2004; Halm et al., 2004; Filby and Tyler, 2005; Greytak and Callard, 2007), but recent studies in the trout reported the expression of two types (ERα and ERβ) each consisting of two subtypes (α1/α2 and β1/β2, Nagler et al., 2007; Boyce-Derricott et al., 2009).

In the European sea bass *cyp19a1a* expression is associated with ovarian morphogenesis. It starts to increase with the earliest signs of sex differentiation reaching a maximum by the time sex differentiation is completed (Blázquez et al., 2008). In addition, clear sex-related differences were detected for both receptors in the gonads, with higher levels in males than in females, and maximal levels coinciding with the onset of spermatogenesis. This study together with that of Blázquez et al. (2009) further support the role *cyp19a1a* in ovarian differentiation but failed to confirm an association between phenotypic sex, *cyp19a1b* and ERs. Nevertheless, a related study showed higher GnRH gene expression in sea bass females

at the start of histological sex differentiation, suggesting that the brain could be triggering gonadal development via GnRH stimulation of FSH synthesis and release (Moles et al., 2007). This direct stimulation of *cyp19a1a* by FSH has also been reported in trout (Montserrat et al., 2004). Similar observations were also made in pejerrey where both, GnRH-I and gonadotropins were suggested to be involved in the process of sex differentiation (Miranda et al., 2001, 2003). Moreover, studies in several teleosts showed that, although found in different tissues, *cyp19a1b* is highly expressed in the brain prior to the onset of *cyp19a1a* in the undifferentiated primordium (Sudhakumari et al., 2005; Wang and Tsai, 2006; van Nes et al., 2006; Blázquez et al., 2008; Strobl-Mazzulla et al., 2008) suggesting a role in early neural development and function. For example in pejerrey, the expression profile of ERs in the brain also showed different ontogenetic and temperature/sex-related differences (Strobl-Mazzulla et al., 2008). In tilapia, Tsai et al. (2003) also reported that the expression of *cyp19a1b* and ERs, varied in relation to rearing temperature and developmental period suggesting the possibility that the ontogenic expression of these genes could be involved in TSD in this species (see also Section 5).

5. Is brain aromatase related to sex differentiation in teleosts?

Although the expression of brain aromatase is related with the process of brain sex differentiation in mammals, the role of the brain and *cyp19a1b* in fish gonadal sex differentiation is unclear since contradictory results can be found in different studies. The first evidence on the involvement of *cyp19a1b* on sex differentiation was reported by Trant et al. (2001) who showed that early *cyp19a1b* expression – before morphological gonadal sex differentiation – in zebrafish brain presented a dimorphic trend. In this regard, the expression pattern could be segregated into two populations suggesting that the expression of *cyp19a1b* could be dictating gonadal sex differentiation. However, a more recent study on the same species (Kallivretaki et al., 2007), although confirmed a bimodal expression of *cyp19a1b*, showed that at that developmental stage (20 days post fertilization) the gonads were not sexually differentiated. Since there are no known genetic sex markers in zebrafish, the authors could not prove if the expression of *cyp19a1b* in brain was in fact reflecting an early sexual differentiation of the zebrafish brain that could then influence or even direct gonadal sex differentiation. After that gonadal undifferentiated stage (20 days post fertilization) sex-related changes on the expression or even distribution of brain aromatase expressing cells in the brain during gonadal sex differentiation could not be evidenced (Kallivretaki et al., 2007). Conversely, in Nile tilapia, no statistical differences could be detected in *cyp19a1b* expression levels during the period encompassing sex differentiation (Kwon et al., 2001; Sudhakumari et al., 2005).

Differential expression of *cyp19a1b* during sex differentiation has been shown in the European sea bass brain being higher in females than in males (Blázquez and Piferrer, 2004) leading to the hypothesis that the brain in this species could be involved in sex differentiation. However, a later study could not support this idea (Blázquez et al., 2008) and although *cyp19a1b* gene expression and activity in brain were detectable during early ontogenesis, no clear differences between males and females were found, in agreement to what has been reported in roach, *Rutilus rutilus*, (Lange et al., 2008). However it is worth to note that *cyp19a1b* expression in the brain during early ontogenesis, before histological sex differentiation, was much higher than that of *cyp19a1a* in the gonads during the same period, pointing at the relevance of the brain in early development. Likewise, in pejerrey, the onset expression of *cyp19a1b* and ERs in larval brains preceded the morphological differentiation of the gonads. As pejerrey sex differentiation is strongly influenced by temperature – with all-male populations a high rearing temperature and all-female populations at low rearing temperatures – (Strüssmann et al., 1996, 1997), the expression of *cyp19a1b* was measured during the temperature-sensitive window and was found to be significantly higher at a male-promoting temperature (Strobl-Mazzulla et al., 2008). Also regarding the effects of temperature on *cyp19a1b* expression, it has been suggested in tilapia mossambica, *Oreochromis mossambicus*, that the brain could be involved in TSD. In this species, low temperature before 10 days post hatching results in ovarian differentiation whereas high temperature after 10 days post hatching results in testicular differentiation in this regard, low temperature before day 10 post hatching, and thus within the thermosensitive period (TSP), induced a down-regulation of *cyp19a1b* and ERalpha that could be related to brain feminization (Tsai et al., 2003). The TSP essentially coincides with the period of highest sensitivity to exogenous estrogens and similar to the effects of temperature, estrogen administration down-regulated *cyp19a1b* expression. Conversely, upregulation of brain ER beta between days 10 and 20 post hatching may be involved in brain masculinization.

Although not conclusive, the information reported in the studies cited in the last two sections suggests the involvement of the estrogen system on brain development and possibly on brain sexual differentiation. If this influence has an effect on establishing the neuroendocrine circuits that direct subsequent gonadal differentiation is still a matter of debate.

6. Radial glial cells, neuroestrogens and neurogenesis

In contrast to previous views, the role of brain aromatase is not restricted to the regulation of reproduction. In this regard, recent findings demonstrated the participation of this enzyme in the regulation of synaptic activity, plasticity and neurogenesis in several vertebrates (reviewed by García-Segura, 2008). Additionally, brain aromatase has been shown to modulate mood and affective status in humans and mice (Kravitz et al., 2006; Dalla et al., 2004). Moreover, E_2 supplementation to adult female aromatase KO mice did not reverse their "depressive profile" induced by the chronic deficiency of estrogen, further suggesting the importance of aromatase and therefore of neural estrogens during early development (Dalla et al., 2004).

In fish, high aromatase activity levels have been found in goldfish brain Callard et al. (1990), specifically in the forebrain, known to control reproduction and sex behaviour, including the telencephalon, preoptic area and hypothalamus in different species (Lephart et al., 2001). These high levels of aromatase activity were correlated with high neural estrogen levels (reviewed by Mouriec et al., 2008). Studies in midshipman fish, *Porichthys notatus*, and zebrafish suggested that this abundance of aromatase in the teleost brain could represent an adaptation linked to the neurogenesis known to occur throughout a fish lifespan (Forlano et al., 2001; Menuet et al., 2003). Moreover, the studies also reported that the anatomical distribution of *cyp19a1b* was restricted to glial cells (Forlano et al., 2001; Menuet et al., 2003; Strobl-Mazzulla et al., 2005; Pellegrini et al., 2007), contrasting with the situation in birds and mammals for which brain aromatase gene expression is restricted to neurons (Balthazart and Ball, 1998; Zhao et al., 2007). In mammals, it was initially believed that radial glial cells could serve as a scaffold providing support to newly generated neurons for migration (Rakic, 1978). Recent studies in zebrafish have shown that glial cells can differentiate into newly born neurons and therefore can be considered as progenitor cells (Pellegrini et al., 2007; Mouriec et al., 2008). This is of particular interest in fish since they retain a high neurogenic activity in the brain during life. However, surprisingly in rainbow trout, *cyp19a1b* and ERs were not found to be expressed in the same cells, i.e., whereas aromatase is found in

radial glial cells, ERs are expressed in neurons (Menuet et al., 2003). Conversely, co-expression of *cyp19a1b* and ER alpha was recently reported in pejerrey (Strobl-Mazzulla et al., 2008). Whether this was due to methodological limitations to detect low ER levels deserves further investigations. In zebrafish larvae, *cyp19a1b* expression is under a strong estrogenic regulation with E_2 up-regulating *cyp19a1b* gene expression via interaction with an ER (Menuet et al., 2005). The complex E_2-ER subsequently binds to an estrogen responsive element (ERE) present in the promoter of *cyp19a1b* and activates gene transcription. Nevertheless, as demonstrated *in vitro* using different cell lines, complete E_2 regulation of *cyp19a1b* is not possible without the presence of a still unknown neuroglial specific factor (Menuet et al., 2005). This opens new lines of investigation as whether to explore the nature of this factor needed for the expression of *cyp19a1b* in the brain of teleost fish. Research on the possible involvement of *cyp19a1b* and estrogens in neurogenesis has been very active for the past years and is constantly updating our current knowledge. However the initial hypothesis regarding the implication of the brain in sex differentiation, particularly in gonochoristic fish, still remains unresolved.

7. Concluding remarks and future prospects

Some controversy has recently arisen about the number of fish species exhibiting TSD and also regarding their sex-ratio response pattern to the effects of temperature (Ospina-Álvarez and Piferrer, 2008). Most of the studies reporting the influence of temperature on sex ratios, apart from those of *Menidia*, have been performed in the laboratory and therefore, the possible effect of temperature on sex ratios of wild populations needs to be evaluated. There is a great deal of speculation about the possible influence of climate change and global warming on the biology of different species and also in fish. Effects on sex ratios have been predicted for some marine turtles with TSD such as the loggerhead, *Caretta caretta* (Hawkes et al., 2007) and also the painted turtle, *Chrysemys picta*, (Janzen, 1994). In the *Menidia*, however, it has been recently demonstrated that the effects caused by the presence of environmental estrogens on sex differentiation are of a much higher magnitude than those of temperature (Duffy et al., 2009) concluding the existence of a heightened sensitivity to E_2 in fish exhibiting TSD. In this regard and due to the strong estrogen-dependent regulation of *cyp19a1b* expression it can be used as a reliable biomarker for the presence of estrogenic substances in the environment.

TSD species are characterized by the absence of sex chromosomes (Valenzuela et al., 2003; Ospina-Álvarez and Piferrer, 2008) and therefore, it is impossible *a priori* to know their genotypic sex. Future studies should be intended to find early molecular markers that would aid in the identification of the genotypic sex and thus relate changes in their expression with the future phenotypic sex. Among them, *cyp19a1a* seems to be a good candidate but clearly more markers are needed. Although it seems now clear that *cyp19a1a* has a central role in sex differentiation, the mechanisms whereby temperature can regulate aromatase gene expression are still a matter of debate. These mechanisms result in *cyp19a1a* transcriptional regulation with several other possible genes and transcription factors involved. In addition, it would be very interesting to relate how the brain is involved in this process since some evidences about the importance of neural circuits in sex differentiation have already been reported.

Acknowledgments

We would like to apologize with those whose studies are not cited in this manuscript due to space limitations. We would also like to thank the editors for the opportunity to contribute to this issue of G.C.E. The work was supported by a project from the Spanish Government "TERMOSEX" PET-2007-0390 to MB and from the Argentine Scientific Agency PICT 16-38206 to G.M.S.

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