Studies on the molecular mechanisms underlying the histological gradient of gonadal sex differentiation in Pejerrey

Doctoral Dissertation

STUDIES ON THE MOLECULAR MECHANISMS UNDERLYING THE HISTOLOGICAL GRADIENT OF GONADAL SEX DIFFERENTIATION IN PEJERREY

March 2018

Graduate School of Marine Science and Technology
Tokyo University of Marine Science and Technology
Doctoral Course of Applied Marine Biosciences

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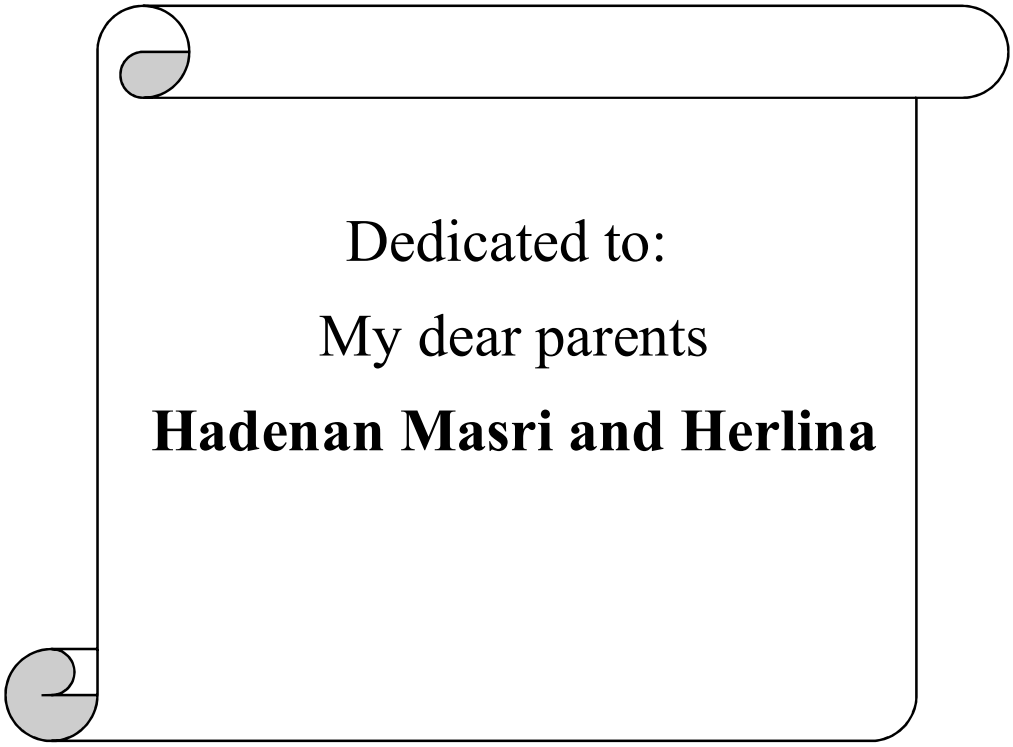
Declaration

I hereby declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research. It has neither been accepted, not submitted for any other degrees. All of the sources of information in this thesis have been duly acknowledged.

Munti Sarida

March 2018


Dedicated to:
My dear parents
Hadenan Masri and Herlina

CONTENTS

Acknowledgements

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General Abstract

The pejerrey *Odontesthes bonariensis* is considered as the most marked case of temperature-dependent sex determination (TSD) among teleosts. In this species, all-female and all-male populations can be obtained by rearing the larvae at low (17°C) and high (29°C) temperatures, respectively, whereas both sexes are formed when larvae develop at intermediate temperatures (around 24-25°C). At this intermediate range, sex is influenced by an genetic factors. The influence of temperature for sex determination in pejerrey is generally observed between 1 and 5 wah (weeks after hatching) and therefore it is conceivable that this period is critical for the sex determination in this species. In spite of its marked TSD, pejerrey belongs to the differentiated type of gonochorists (e.g. ovaries and testes develop directly from an indifferent primordia) and intersexes are extremely rare. Also, histological gonadal differentiation follows a characteristic cephalocaudal, left-to-right gradient regardless of sex, whereas intense apoptosis in the anterior region of the right gonad has been tentatively associated with testis differentiation. The sex-related genes *amha*, *amhy*, and *cyp19a1a* (gonadal aromatase) are thought to play important roles in the sex differentiation process of pejerrey, but their possible interplay and relation with the differentiation gradient are still unclear. The purpose of this study is to clarify the molecular mechanisms underlying the histological gradient of gonadal sex differentiation in pejerrey.

In the first chapter, experiments were conducted to examine the spatiotemporal correlations between amh and cyp19a1a transcript expression and apoptosis during gonadal sex differentiation of pejerrey at a sexually neutral temperature. Progeny from a single XX ($amhy^{-/-}$)

and XY (*amhy*$^{+/-}$) cross was reared for 14 weeks at 25°C and fish were analyzed weekly to examine the degree of histological gonadal differentiation and the location/intensity of gene expression in the gonads by *in situ* hybridization, and at the end of the period to estimate the sex ratio of the progeny. Ovarian and testicular differentiation began at 4 and 7 weeks, respectively and the XX and XY individuals were 36% and 96% male, respectively. *amh* expression was detected from 1 and 2 weeks in the left and right gonads, respectively, of almost all XY fish and about 1/3 of the XX fish; in both cases, *amh*-expressing cells were typically more abundant in the left gonad than in the right gonad and expression expanded from an initial site in the anterior region towards more posterior regions of the gonads. *cyp19a1a* expression was observed from 2 weeks in the anterior region of both gonads regardless of genotype but was maintained only in XX gonads without *amh* expression. Apoptosis appeared first in the anterior region of the right gonad and then expanded to the posterior regions of the same side; it was virtually absent from and when present had limited intensity in the left gonad. Gonadal apoptosis was observed from 2-4 wah and became abundant in almost all larvae reared at 25°C regardless of genotype. The expression profiles of *amhs* and *cyp19a1a* allowed to distinguish the presumptive phenotypic sex at an early stage of development in both the XX and XY genotypes. Thus, presumptive males have intense and faint or inexistent *amhs* and *cyp19a1a* expression, respectively, whereas females have the opposite pattern. Also, both genes showed a gradient of expression that was consistent with the gradient of histological differentiation, from anterior to posterior regions and from the left to the right gonad. The appearance of *cyp19a1a* expression at an early developmental stage in both genotypes and presumptive sexes suggests that the females may be the default state in pejerrey, at least at this intermediate temperature.

The second chapter examined the effects of feminizing and masculinizing temperatures on the spatiotemporal patterns of expression sex related genes and apoptosis. irst chapter. Sex ratios at 17 and 29ºC were 40% and 100% male, respectively and testicular and ovarian differentiation started at 5 and 8 wah at 29 and 17ºC, respectively. *amhs* (*amha* and *amhy*) transcripts were first detected from 1 and 2 weeks in the anterior region of the left and right gonads, respectively, in individuals reared at 29°C regardless of genotype, whereas *cyp19a1a* expression was completely suppressed at this temperature. XY fish at 17°C could be divided in two subgroups, one with faint *amhs* expression and strong *cyp19a1a* expression (72% occurrence), and another with strong *amhs* expression and faint or negligible *cyp19a1a* expression (28%) that, based on the relative concordance with the sex ratios (60% female: 40% male), were assumed to represent presumptive females and males, respectively. All XX fish at 17°C had complete suppression of *amhs* and, on the contrary, abundant *cyp19a1a* expression from 3 weeks. As 25°C, gonadal apoptosis was observed from 2-4 wah and became abundant in almost all larvae reared at 29°C regardless of genotype. At 17°C, apoptosis was observed in about half of the individuals of both sexes from 6-7 wah and was much less intense than at the other temperatures. Altogether, there was no association between the incidence of apoptosis on any particular week and the resulting phenotypic sex ratios but, interestingly, apoptosis was observed 1-2 weeks earlier in the XY than in the XX at all temperatures studied. The gradients of *amhs* and *cyp19a1a* expression observed in the first chapter were also observed and were not affected by the masculinizing and feminizing temperatures. These results indicate that the high temperature promotes expression of *amhs* and suppresses *cyp19a1a* expression, overriding the default female pathway. On the other hand, the low temperature suppresses *amhs* expression and promotes *cyp19a1a* expression,

leading to feminization in some of the XY. Moreover, at all temperatures there was a fairly high correlation between the occurrence of apoptosis in the right gonad and the disappearance or reduction of the expression of both *amhs* and *cyp19a1a* in the same area.

In summary, the spatiotemporal expression of *amhs*, *cyp19a1a*, and apoptosis supports a cephalocaudal, left-to-right gradient of gonadal sex differentiation in pejerrey at the molecular level. The location and timing of *amh*s, *cyp19a1a* and apoptosis seems highly coordinated among themselves and with the time of gonadal sex differentiation. Since all animals show *cyp19a1a* expression at an early stage regardless of the genetic or presumptive phenotypic sex, it is surmised that the female may be the default state in pejerrey. Likewise, the results also suggest that the early expression of *amhs* (conceivably related with the presence of *amhy* in XY fish and of environmentally-induced stress in the XX) antagonizes the expression and effects of *cyp19a1a*, causing to testicular formation. This study also provided a novel role for apoptosis in gonadal differentiation of pejerrey. Apoptosis was shown to occur typically in the anterior region of the right gonad in individuals of both sexes and generally coincided spatiotemporally with the reduction or disappearance of *amhs* and *cyp19a1a.* Thus, rather than a direct role of apoptosis in testicular formation, the results suggest that early apoptosis in the right gonads could be a mechanism to delay differentiation in this area until it is firmly established in the left gonad, and hence prevent gonadal ambiguity (intersexes) that could occur if different areas of the gonads responded differently to environmental signals. According to this view, in putative females there is no conflict with the default sex, so apoptosis is delayed or even unnecessary, leading to the casual observation that apoptosis is more common in putative males. In other words, the molecular data obtained in my thesis supports the notion that gonadal sex differentiation in

pejerrey starts in the anterior region of the left gonad and that the right gonad simply follows the direction set by the left side, and that the delay in the right side is probably driven by apoptosis. Further studies are needed to confirm the female-default and molecular gradient/apoptosis-based intersex prevention hypotheses put forth in this work and examine their existence in other TSD species.

General Introduction

Teleost fishes exhibit two major but not necessarily mutually-exclusive mechanisms of gonadal sex determination, genotypic (GSD) and environmental (ESD) sex determination (Strüssmann and Patiño, 1999). One view of sex determination in fishes is that the two forms represent a continuum of mechanisms that extend from GSD in one end, whereby the gonads differentiate according to the predetermined genetic sex, to ESD on the other end, whereby physical, chemical, or even social factors direct the fate of gonadal determination (Strüssmann and Patiño, 1999; Yamamoto et al., 2014, 2019). So far temperature is the most prevalent environmental factor influencing sex (temperature-dependent sex determination; TSD) whereby high temperatures generally result in masculinization and male-skewed sex ratios; low temperatures, whenever effective, can cause feminization or masculinization depending on the species (Conover and Kynard, 1981; Strüssmann and Patiño, 1995; 1999; Conover, 2004). Thus, both sex determination systems can coexist in the same individual and the ultimate direction of sexual development depends on the relative strength of the genotypic and environmental cues during the critical time of sex determination.

The first fish in which TSD was documented was the Atlantic silverside (*Menidia menidia*, family Atherinopsidae). In Atlantic silversides, exposure of larvae during early development to different water temperatures can affect sex determination; sex ratios are female-biased at colder temperatures prevalent at the beginning of the breeding season and male-biased at warmer temperatures occurring later in the breeding season. Besides, Conover (2004) and Duffy *et al*. (2015) reported that geographical distribution also influences the degree of environmental lability

of sex in Atlantic silversides. Interestingly, no populations display pure TSD or GSD, but the level of TSD is uniformly high in their southern range and then declines rapidly approaching their northern range, where GSD dominates. 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 and how sexes are determined by temperature.

The pejerrey, an atherinopsid fish species from South America, is a gonochorist teleost fish with strong temperature-dependent sex determination (TSD). It has been used as a model for the study of the molecular processes involved in the crosstalk between environment and gonadal fate. In this species, all-female and all-male populations can be obtained when larvae are reared during the critical period of sex determination (between 1-5 wah) at low (17°C, female-promoting temperature; FPT) and high (29°C, male-promoting temperature; MPT) temperatures, respectively. When larvae develop at intermediate temperatures of around 24 or 25°C (Mix sex-promoting temperature), both sexes are formed (Strüssmann et al., 1996a; 1997). This is thought to occur as a manifestation of GSD, which is driven by the *amhy* gene (Yamamoto et al., 2014).

The *amhy* gene is thought to be a duplicated copy of autosomal anti-Müllerian hormone (*amh*) and is located on the Y-chromosome (hence *amhy*). This gene is a male trigger in Patagonian pejerrey (*Odontestes hatcheri)* (Hattori et al., 2012) and pejerrey (*Odontestes bonariensis)* (Yamamoto et al., 2014). The knockdown of *amhy* in *O. hatcheri* during

embryogenesis leads to the development of ovaries in XY individuals. Moreover, *amhy* is expressed in presumptive Sertoli cells of undifferentiated gonads prior to the expression of the autosomal *amha* copy (Hattori et al., 2012; Yamamoto et al., 2014). It is considered as the first example for a vertebrate master sex determining gene that was not a transcription factor.

In order to unravel the molecular pathways underlying the TSD mechanism, a series of genes universally implicated in the sex differentiation process have been analyzed so far. Our previous studies showed that the connection between environmental temperature and sex determination was mediated by the glucocorticoid stress-related hormone cortisol, in particular during the masculinization process (Hattori et al., 2009). During the critical period of sex determination, pejerrey larvae at MPT consistently showed higher cortisol, 11-ketotestosterone (11-KT), and testosterone (T) titers than those at FPT. Similar patterns were observed in larvae at Mix-PT fed with cortisol, implying a possible link between stress and masculinization at MPT (Hattori et al, 2009).

Moreover, there are also other candidates for sex-determining gene among fishes. One is the *gsdf*^{Y} (*gonadal soma derived growth factor*) found in *Oryzias luzonensis* (congeneric of Japanese medaka *Oryzias latipes*) (Myosho et al., 2012). This gene, a TGF-β superfamily member, first found in rainbow trout as a somatic factor controlling the proliferation of primordial germ cells and spermatogonia (Sawatari et al., 2007), was identified by genetic mapping. Another is the *sdY,* which was identified by a large-scale expression profile of genes specifically expressed in differentiating testis. It encodes a transcription factor with sequence similarity to interferon regulatory factor 9 in rainbow trout and other salmonids. It is considered as one example of a duplicated gene that was newly recruited for sexual development (sexually dimorphic on the Y-

chromosome) (Yano et al., 2012, 2013). Finally, there is also *sox3Y* (*Sry* related Hmg-Box protein 3 on the Y-chromosome), which is found in *Oryzias dancena* (Takehana et al., 2014). These findings illustrate a complex scenario with unstable and variable gene hierarchy in the genotypic sex determination in fish, especially during male differentiation pathway. Thus, gonadal sex in fish is under the control of various sex determination switches or modulators and may involve not only genotypic but also environmental factors such as temperature.

Meanwhile, female sex differentiation is stimulated by the *cytochrome* P450 monoxygenase (P450aro, *Cyp19*), whic is a key factor in converting androgens into estrogens (Baroiller et al., 1999, Guiguen et al., 2010; Sandra and Norma, 2010). Teleost fish have two *cyp19a1* paralogues, *cyp19a1a* mainly expressed in the female gonads, and *cyp19a1b* predominantly expressed in the brain (Chang et al., 2005; Barney et al., 2008; Patil and Gunasekera, 2008; Piferrer and Guiguen, 2008; Siegfried, 2010). Sex steroids are primarily produced in the gonads (Schulz et al., 2010). In fish, aromatase activity and estrogen concentration are key developmental switches for ovary or testis development and the balance of the sex steroids seems to be of great importance to sexual commitment (Piferrer and Guiguen, 2008; Siegfried, 2010). Aromatase is encoded by *cyp19a1a*, and it has also been demonstrated that the *cyp19a1a* expression level is consistent with the high levels of aromatase activity and estrogen production in many fish species (Guiguen et al., 2010). As a result, *cyp19a1a* up-regulation can trigger ovarian differentiation in teleosts, while its down-regulation induces testicular differentiation (Guiguen et al., 2010; Zhang et al., 2013). Moreover, aromatase is thought to be crucial for feminization in the pejerrey (Karube et al., 2007; Fernandino et al., 2008, Zhang et al., 2018). 17β-estradiol (E2) is known to induce and maintain ovarian development and

the levels are much higher in females compared to males. In minnow, pejerrey, and zebrafish, exposure to estrogen resulted in cessation of male gonad development and sex reversal, which in turn correlated with pronounced decrease in dmrt1 expression (Schulz et al., 2007; Fernandino et al., 2008; Zhang et al., 2008). In tilapia, Dmrt1 suppresses the female pathway by repressing aromatase gene transcription and thus estrogen production (Wang et al., 2010). In this context, there seems to be a feedback loop between dmrt1, *cyp19a1a* and, by implication, the estrogen/androgen balance (Herpin and Schartl, 2011).

Another process which is involved during gonadal sex differentiation in fish is programmed cell death (apoptosis). Apoptosis is a cellular process that plays a crucial role during normal development and homeostasis of all multicellular organisms (Jacobson et al., 1997; Yamashita, 2003; Takle and Andersen, 2007). It is primarily characterized by cytoplasmic shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing and apoptotic bodies (AnvariFa et al., 2017). In recent years there has been a trend of using fish as model for studying vertebrate development and diseases in relation to apoptosis, but observations related to gonadal development are still limited. In one of such studies, apoptosis was shown to be involved in the sex reversal process of sex-changing adults, by remodeling of the gonads though a complex orchestration of cell proliferation and cell death (Liarte et al. 2007).

In other studies, apoptosis in relation to gonadal development, sex reversal, and sexual maturation has been reported in zebrafish and pejerrey. In zebrafish, apoptosis was observed both during gonadal sex differentiation and gonadal maturity. During gonadal development, apoptosis is thought to have an important role to revert “juvenile ovaries” of genotypic males into testes

(Uchida et al, 2002). Other studies also showed that oocyte survival, made possible by preventing apoptosis, is essential for ovarian development and to maintain the female phenotype (Uchida et al., 2002; Pradhan et al., 2012; Dranow et al., 2013). The pattern of occurrence of germ cell apoptosis was similar to that observed in mammals (Miranda et al., 1999; Santos et al., 2008).

In the pejerrey, apoptosis was observed in somatic cells of the anterior region of the right gonads of fish reared at high temperature (29°C) and part of the fish at intermediate temperature (24-25°C). On the other hand, it was rarely observed in gonads at low temperature (17°C) (Strüssmann et al., 2008; Yamamoto et al., 2013). In addition, treatment with exogenous estrogen reduced gonadal apoptosis at high temperature (29°C) and induced feminization whereas an aromatase inhibitor induced apoptosis in the gonads of larvae reared at low temperature (17°C) (Yamamoto et al., 2013). Thus, these results strongly suggested that apoptosis in somatic cells in the right gonads might play a key role in testicular differentiation in pejerrey and that estrogens are involved in the regulation of this process. However, what are the exact roles of apoptosis in gonadal formation is yet to be clarified, particularly in fish with pronounced TSD such as pejerrey

In spite of the marked TSD, an intriguing aspect of this species is the rarity of gonadal ambiguity or intersex in wild specimens and also in specimens from experiments with thermal or endocrine manipulation (Strüssmann et al, 2005; Fernandino et al., 2008; Hattori et al., 2009; Pérez et al., 2012). These observations indicate that environmentally labile species such as pejerrey must have a mechanism to coordinate the direction of sex differentiation in both female and male throughout the entire length of gonads. A process that could be involved in the prevention of sex discrepancies is the cephalocaudal, left-to-right gradient of gonad

differentiation (Strüssmann and Ito, 2005). This study found that sex differentiation only began in the right gonad when 10-30% of the length of the left gonad had already differentiated. Also, it found that sex differentiation started from the anterior region and then spread to the posterior region in both the left and right gonads regardless of the sexes. It is also important to emphasize that blood vessels are formed prior to sex differentiation (Ito et al., 2005), suggesting that extra-gonadal factors may be carried by the bloodstream into the left-anterior region of the gonad to trigger the sex differentiation process. However, the exact reason of such hierarchy in the differentiation of the left lobe over right and along each lobe in the gonads of pejerrey is still unknown.

Moreover, as mentioned above, apoptosis appears first in the somatic cells of the right gonads in all larvae reared at high temperature and in some larvae reared at Mix sex-promoting temperatures. On the other hand, it was rarely observed in larvae reared at low temperature (Strüssmann et al., 2008; Hattori et al., 2009; Yamamoto et al., 2013). Therefore, apoptosis is thought to have a role in testicular differentiation, but the reasons for the gradient of gonadal apoptosis and its relation to testicular differentiation remain unclear.

In this context, the main objective of this study was to clarify the molecular mechanisms underlying the histological gradient of gonadal sex differentiation in pejerrey and for that goal I used the following approaches:

1. To examine the spatiotemporal correlations between *amh* and *cyp19a1a* transcript expression and apoptosis during gonadal sex differentiation of pejerrey.
2. To analyze the effects of feminizing and masculinizing temperatures on the spatiotemporal patterns of expression of sex related genes and apoptosis.

Chapter 1

Spatiotemporal correlations between *amh* and *cyp19a1a* transcript expression and apoptosis during gonadal sex differentiation of pejerrey *Odontesthes bonariensis*

Introduction

Teleost fishes exhibit two major but not necessarily mutually-exclusive mechanisms of gonadal sex determination, genotypic (GSD) and environmental (ESD) sex determination (Strüssmann and Patiño, 1999). One view of sex determination in fishes is that the two forms represent a continuum of mechanisms that extend from GSD in one end, whereby the gonads differentiate according to the predetermined genetic sex, to ESD on the other end, whereby physical, chemical, or even social factors direct the fate of gonadal determination (Strüssmann and Patiño, 1999; Yamamoto et al., 2014, 2019). Thus, both sex determination systems can coexist in the same individual and the ultimate direction of sexual development depends on the relative strength of the genotypic and environmental cues during the critical time of sex determination. The most common form of ESD in fish is temperature-dependent sex determination (TSD) whereby high temperatures generally result in masculinization and male-skewed sex ratios; low temperatures, whenever effective, can cause feminization or masculinization depending on the species (Conover and Kynard, 1981; Strüssmann and Patiño, 1995; 1999; Conover, 2004).

The pejerrey *Odontesthes bonariensis* is a clear example where these two sex determination systems coexist (Yamamoto et al., 2014; Zhang et al., 2018). We have previously shown in this species the presence of a genotypic testis-determining factor, the Y chromosome-linked anti-Müllerian hormone *amhy* (Hattori et al., 2012), and a clear XX-XY chromosome system (Hattori et al., 2013) that lead to general compliance of phenotypic sex to genotypic sex and hence to balanced sex ratios at intermediate, "sexually neutral" temperatures (Yamamoto et al., 2014). On the other hand, monosex or highly sex-skewed populations can be easily obtained at low, feminizing and high, masculinizing temperatures during the critical period of sex determination (Strüssmann et al., 1996a; 1997; Zhang et al., 2018). Moreover, sex reversal can be monitored at the individual level by comparison of the phenotypic (gonadal) sex with

the genotypic background (presence or absence of *amhy*; Yamamoto et al., 2014; Hattori et al., 2018). With these characteristics, the pejerrey seems to be an excellent model for the study of the molecular processes involved in the interactions between environment and gonadal sex determination and to examine the ecological implications of TSD in wild populations (Hattori et al., 2018).

An intriguing aspect of gonadal sex differentiation in this species is related to the rarity of gonad ambiguities such as the co-occurrence of ovarian and testicular tissues within the same individual. The coexistence of marked TSD (but without a clear threshold for female/male determination, that is, absence of a "pivotal temperature"; Strüssmann et al., 1997) and of a genotypic determinant of sex, as described above, would suggest that intersexes may be common in pejerrey. Yet, intersexes are rarely found not only in the wild but also, and more surprisingly, in laboratory experiments where fish are subjected to complex thermal or endocrine manipulations at various developmental stages (Strüssmann and Ito, 2005; Ito et al., 2005; Fernandino et al., 2008; Hattori et al., 2009; Perez et al., 2012). These observations suggest the presence in pejerrey of a mechanism for tight coordination of histological differentiation from the rudimentary, undifferentiated gonads that prevents discrepant differentiation in different regions. One of such mechanisms could be the cephalocaudal, left-to-right gradient of gonad differentiation reported for this species (Strüssmann and Ito, 2005). Those authors demonstrated that histological sex differentiation of the testes and ovaries in pejerrey begins in the anterior region of the left gonad and proceeds caudally until 10-30% of this side has differentiated before it starts at the anterior region of the contralateral (right) gonad.

The relations of this cephalocaudal, left-to-right histological gradient to the known molecular mechanisms involved in pejerrey sex determination and gonadal differentiation are still unknown. In pejerrey the Y-linked *amhy* gene is transcribed from early embryonic stages

and down regulated by the end of sex determination period (1-4 wah) whereas the autosomal *amh*, *amha* increases significantly from the end of the same period (4 wah) at intermediate temperatures in gonads that differentiate as testes regardless of genotype (Yamamoto et al., 2014). Another key player, the gonadal aromatase *cyp19a1a*, is an enzyme that catalyzes the conversion of androgens to estrogens and is thought to be crucial for feminization in this species (Karube et al., 2007; Fernandino et al., 2008, Zhang et al., 2018). In addition, previous studies revealed a high incidence of apoptosis of somatic cells in the anterior region of the right gonad during sex differentiation, particularly at the high, male-promoting temperatures (Strüssmann et al., 2008; Yamamoto et al., 2013). In this study, we conducted a detailed *in situ* analysis of *amh*, *cyp19a1a*, and apoptosis expression in histological preparations to examine spatiotemporal associations between these molecular processes and their relation to the cephalocaudal, left-to-right gradient of ovarian and testicular histological differentiation of pejerrey.

Materials and methods

Rearing and sampling procedures

Fertilized eggs were obtained by natural spawning from an XX ($amhy^{-/-}$) female and an XY ($amhy^{+/-}$) male and incubated at 19°C until hatching as described in a previous study (Yamamoto et al., 2014). Immediately after hatching, larvae were stocked in two 60-liter tanks at 25°C and reared for up to 14 weeks after hatching (wah) in order to produce both female and male individuals (Strüssmann et al., 1996a; 1997). Larvae were fed live *Artemia* nauplii to satiation three to four times daily from the first day after hatching and gradually weaned into powdered fish food (TetraMin flakes, Melle, Germany) from the third week. Larvae were sampled at 1, 2, 3, 4, 5, and 7 wah (n=20 fish/time point). All fish were fin-clipped for genomic DNA extraction and *amhy* genotyping. The trunk portion of each larvae was fixed in 4% (w/v)

paraformaldehyde/phosphate-buffered saline (PFA) overnight, dehydrated in ascending alcohol series, and embedded in Paraplast Plus (McCormick Scientific, St. Louis, Mo., USA) for *in situ* hybridization (ISH) and TdT-mediated dUTP nick end labeling (TUNEL) assay (see details below). The remaining fish at the end of the rearing experiment (14 wah) were collected for determination of phenotypic and genotypic sex ratios by gonadal histology and *amhy* genotyping, respectively. The experiments were carried out in accordance with the guide for the care and use of laboratory animals from Tokyo University of Marine Science and Technology. All fish used in this study were sacrificed after anesthetization by immersing in ice cold water in order to minimize animal suffering prior to any sampling.

DNA extraction and sex genotyping

Genomic DNA was extracted from fin samples of all fish and used for the analysis of genotypic sex based on the presence/absence of *amhy*. Extraction procedures and subsequent amplification were conducted using the procedures described in a previous study (Yamamoto et al., 2014). The forward and reverse primers for sex genotyping were 5'-AGTCAGCTC AGATGCT-3' and 5'-AGCCGGATGCAAAACTTCCAG-3', respectively. PCR products were analyzed by 1% agarose gel electrophoresis. *amhy*-positive fish were scored as XY and *amhy*-negative as XX.

Sample preparation for ISH, TUNEL, and histological analysis of gonadal sex differentiation

After genotyping, 6-9 XX and XY individuals were chosen from among the embedded specimens for each time point for ISH, TUNEL, and histological analyzes. Blocks were serially cross-sectioned at a thickness of 5 μm and the sections containing the gonads were divided into 20 segments with approximately the same number of sections (Fig. 1.4). Representative sections from each of these segments were then picked and sequentially pasted on replicate

glass slides to be used in ISH (*amh*, *cyp19a1a*), TUNEL (apoptosis), and light histology after hematoxylin-eosin (HE) staining (analysis of the degree of gonadal sex differentiation). An exception to this protocol was the gonads sampled at 1 wah. These gonads were too small so they were divided only into three representative segments (anterior, middle, and posterior).

Histological analysis of gonadal sex differentiation

The degree of gonadal sex differentiation of larvae sampled between 1 and 7 wah and the phenotypic sex of juveniles sampled at 14 wah was judged using the histological criteria described by Strüssmann et al. (1996b) and Ito et al. (2005). Briefly, ovarian differentiation was ascertained by the appearance of an assemblage (cluster) of somatic cells in the ventral edge of the gonads, which represents the onset of ovarian cavity formation, or by the presence of the ovarian cavity and/or clearly recognizable oocytes. Testicular differentiation was evidenced by the appearance of a slit-like opening in the medullar area of the gonad, which signals the beginning of the formation of sperm duct, or by the presence of the sperm duct and/or the typical lobular structure of the pejerrey testes.

In situ hybridization of amh and cyp19a1a

The *amh* probe used in this study recognizes both *amhy* and *amha* and was prepared following our previous studies (Yamamoto et al., 2014). For the *cyp19a1a* probe, a 527-bp fragment (nucleotides +755 to +1282; GenBank accession no. EF030342.1) was amplified using forward (5'-GACCGGTGTTCAGGATTATATTTGT-3') and reverse (5'-TGATCA GCACAGTCTGCCAT-3') primers using cDNA synthesized from an adult ovary. The fragment was then cloned into the pGEM-T Easy Vector (Promega Corporation, Madison, WI) and after confirming the insert orientation, the plasmid was linearized by appropriate restriction enzyme and used for probe synthesis. Dixogenin-11-UTP-labeled riboprobe was synthesized

using T7 or SP6 RNA Polymerase to generate sense or antisense probes. ISH was carried out using previously described protocols with some modifications (Yamamoto et al., 2011, 2014). Sections were initially permeabilized with 1 mg/ml of proteinase K at 37°C for 12 min, acetylated, and incubated with 1mg/ml RNA probe at 65°C for 16 hours. After hybridization, sections were washed, and unbound probes were digested using 20 µg/ml of RNase A in order to reduce background signals. Slides were incubated for 30 minutes at room temperature with blocking solution (Roche, Basel, Schweiz) for 1 hour at 25°C with anti-DIG-alkaline phosphatase-conjugated antibody (Roche Diagnostics) diluted 1:2000 with blocking solution. Finally, sections were rinsed and signals were detected by NBT/BCIP (Roche) for 3 and 6 hours for *amh* and *cyp19a1a*, respectively. Slides were observed under a microscope (BX53 microscope, Olympus, Tokyo, Japan) and images were captured and digitalized with a CCD camera (DP73, Olympus). For interpretation of the results, the abundance of *amh*- and *cyp19a1a*-positive cells was arbitrarily classified in three visual categories as follows: abundant (positive cells occupy a significant area of the gonadal cross section in the segment), few (generally less than 10% of positive cells in the segment), and absent (absolutely no positive cells in the segment) (Fig. 1.1).

Detection of apoptosis by TUNEL assay

TUNEL assay was used for visualization of apoptotic DNA strand breaks and followed the procedure described in a previous study (Hattori et al., 2009). Briefly, after proteinase K (Thermo Fisher Scientific) pretreatment (1 µg/mL) for 10 min at 37°C, slides were re-fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline at room temperature for 20 minutes. Slides were incubated with terminal deoxyribonucleotidyl transferase (TdT) (Roche) at a dilution of 40 units/mL for 80 minutes in a humidified chamber at 37°C. For positive control, slides were incubated in 1µg/mL DNase (Thermo Fisher Scientific) for 30 min at room

temperature. Negative controls were obtained by incubating sections with only TdT buffer without transferase. The frequency of apoptosis was classified in three categories as for gene expression (Fig. 1.1).

Results

Histological sex differentiation of the gonads and sex ratios

The first morphological signs of ovarian and testicular differentiation were observed at 4 and 7 wah, respectively (data not shown). These signs were generally observed around segment 6 and more rostral segments did not show any signs of differentiation until 7 wah as reported previously by Strüssmann and Ito (2005). The analysis of the phenotypic sex at the end of rearing (14 wah) showed that 39% of the fish were female and 61% were male (total n=112). About 96% of the XY fish differentiated as males (44 out of 46) (Table 1.1) whereas the XX fish were 64% (42 out of 66) female and 36% male. No histological difference was detected between sex-reversed and non-sex-reversed testes or ovaries and no intersex gonads were found.

Expression pattern of amh, cyp19a1a, and gonadal apoptosis in XY and XX genotypes

Transcripts of *amh* were detected in the anterior region of the left gonad of most XY genotypes at 1 wah but not in the middle and posterior regions of the same side or in any region of the right gonad (Figs. 1.2A and 1.2B). The results of *amh* ISH for XY fish collected between 2-7 wah are graphically summarized in Fig. 1.3. Only the results for segments 6 to 20 were compiled because of the lack of differentiation in more proximal sections noted above. At 2 wah, *amh*-positive cells in the left gonads of XY fish were abundant in segments rostral to segments 15-16 and fewer or absent in the more posterior segments. This pattern was observed in 6 out of 8 XY individuals whereas the remaining two had no signal of *amh* throughout the

entire left gonad (Fig. 1.3). From 3 wah onward, *amh* signals were abundant throughout the left gonads in all individuals. Compared to the left, the right gonads of XY fish had fewer *amh* signals first in the rostral segments (e.g. 2-4 wah) and subsequently also in the middle segments (5-7 wah, Figs. 1.5). The same patterns of *amh* expression in the left and right gonads of XY fish were observed in about 40% (average for all weeks combined) of the XX individuals (Figs. 1.2C, 1.2D and 1.3) whereas the remaining 60% did not show any *amh* signals on both sides of the gonads regardless of the sampling time (Figs. 1.2E, 1.2F, and 1.3).

No *cyp19a1a*-positive cells were observed in the left and right gonads of both XY and XX genotypes at 1 wah (data not shown). At 2 and 3 wah, about half of the XY individuals had few *cyp19a1a*-positive cells in the rostral and middle segments of the left and right gonads whereas the other half had none (Fig. 1.3). From 4 wah onward, *cyp19a1a* expression decreased, particularly in the right gonad, until it could not be detected at all at 7 wah. In contrast to XY, about 66% of the XX individuals had *cyp19a1a* signals, first in the rostral and middle segments of both gonads from 2 wah and subsequently also in more posterior segments (Figs. 1.3 and 1.5). The remaining XX individuals, regardless of the sampling time, had absolutely no *cyp19a1a* expression.

The XY fish had abundant gonadal apoptosis in the right gonads already from 1 wah (data not shown) whereas in the XX genotypes it started only from 4 wah (Fig. 1.3). In both cases, apoptosis was first observed in the anteriormost segments (Figs. 1.5) and subsequently in the middle and posterior segments. Apoptosis in the left gonads was observed only after 4 wah in few individuals of both genotypes and, as in the right side, appeared chiefly in the anterior and middle segments of the gonads.

Discussion

Gradients of gonadal differentiation or development such as observed in pejerrey have

been reported in other teleost species including *Coptodon* (*Tilapia*) *zillii* (Yoshikawa and Oguri, 1978), *Oryzias latipes* (Yoshikawa and Oguri, 1979, 1981), and *Lates calcarifer* (Banh et al., 2017). However, the role(s) of such gradients and their molecular basis remain largely unknown. In this study, we investigated the spatiotemporal expression patterns of *amh*, *cyp19a1a*, and apoptosis in relation to the histological gradient of sex differentiation in ovaries and testis of pejerrey. The expression analysis of the male-related gene *amh* revealed that transcripts were initially found in the anterior region of the left gonad of most XY and in part of the XX larvae at 1 wah. In the following weeks, the expression of *amh* expanded from the anterior towards the posterior region of the left gonad and into the right gonad. Ovarian aromatase expression was observed first in the anterior region of the left and right gonads of both genotypes at 2 wah. It then spread to more posterior regions of the gonads in XX individuals whereas in XY it progressively disappeared. These patterns agree with the molecular findings of previous studies (Yamamoto et al., 2014; Zhang et al., 2018). More importantly, they agree relatively well with the antero-posterior (cephalocaudal) and left-to-right gradient of gonadal sex differentiation described in pejerrey by conventional histological analysis (Strüssmann and Ito, 2005; Ito et al., 2005), although with genotype-specific peculiarities as noted. Also, the fact that *amh* and *cyp19a1a* expression began before the onset of histological differentiation of testes (7 wah) and ovaries (4 wah) suggests that they are the cause rather than a consequence of the histological gradient. However, the results also point to a possible contribution from apoptosis to this gradient.

Left-right asymmetry in gene expression has also been described in rainbow trout *Oncorhynchus mykiss* during masculinization of XX fish by androgens. In this species, *amh* expression also showed left and right dimorphism whereas *cyp19a1a* did not (Guillevic and Guiguen, 2008). Differential gene expression between the left and right gonads has been extensively analyzed also in birds and the transcription factor *Pitx2* gene was shown to be a

key player in the left-right patterning (Intarapat and Stern, 2013; Guioli et al., 2014). An important target of *Pitx2* in birds is the *Bmp7* gene, a TGF-beta family member that displays higher expression in left than the right side during early gonad differentiation (Hoshino et al., 2005). Although no information is currently available about *Pitx2* and its regulation of *amh* and *cyp19a1a* expressions in pejerrey, studying about this gene may be needed for further understanding of the mechanism of the left and right gene expression gradient in pejerrey.

Apoptosis has been implicated in sex determination of zebrafish (Uchida et al., 2002). In this species, which is an undifferentiated gonochorist, all larvae first develop an ovary-like gonad but during subsequent development the oocytes in genotypic males undergo apoptosis and testes develop. A previous study also suggested the involvement of apoptosis in testicular differentiation of pejerrey, which contrarily to zebrafish is a differentiated gonochorist, because it was common in the right gonads of fish reared at male-producing temperatures and rare at feminizing conditions (Yamamoto et al., 2013). This study confirmed that apoptosis was largely restricted to the right gonads and was observed in most XY individuals, which were found to be 96% male. However, it was not clear in the previous study how apoptosis in the right gonads could be implicated in sex differentiation if, as discussed above, differentiation begins in the left gonad at both molecular and histological levels. Besides, unlike in masculinizing conditions, this study was conducted at an intermediate temperature where individuals are more likely to follow their genetically predetermined sex (although not 100%, as shown by the presence of 4% XY females and 37% XX males). Thus, we hypothesize that apoptosis in the right gonads could have two complementary roles during sex differentiation in pejerrey, namely, 1) to support a gradient of differentiation between the right and left gonads, and 2) to mitigate the conflict between male and female signals in XY individuals.

The first role takes into consideration the fact that intersexes are rare in pejerrey in spite of the coexistence of marked TSD and GSD and the absence of a pivotal temperature for

male/female transitions in the middle of the thermal range as previously mentioned (see Introduction). It is still not clear yet whether the gonads interpret the thermal cues autonomously and with equal sensitivity throughout all regions of the gonad, or through coordination from the central nervous system, which could be then imprinted upon the gonads through the blood circulation to ensure uniformity (Miranda et al., 2001; 2003). Assuming that the gonads respond to environmental stimuli locally, though, the lack of discrepant development throughout the gonads strongly hints at the existence of some form of developmental hierarchy. There are two conceivable pathways to generate such hierarchy: by selective accumulation of pro-differentiation, inducible elements in one point or by selective inactivation of putative inducible elements in the competing point(s) (DeFalco et al., 2003), and these processes may work alone or in combination. The virtual confinement of apoptosis to the anterior region of the right gonad during the initial stages of sex differentiation in pejerrey suggests that it may be involved in inactivating putative differentiation site(s) in this region, keeping it undifferentiated until a sufficient region of the left gonad has differentiated and started producing sex-inducers such as sex steroids (Strüssmann and Nakamura, 2002) that would ensure compliance throughout the gonads by paracrine signaling. The histological (Strüssmann and Ito, 2005) and molecular gradients of gene expression and apoptosis (this study) provide support for this hypothesis.

The second role complements the role discussed above. It takes into consideration the possibility that female is the default state in pejerrey and, therefore, of the existence of a conflict between the endogenous male (*amh*) and female (c*yp19a1a*) signals within XY individuals. Previous studies have suggested the primacy of female development in pejerrey based on histological and molecular evidence (Strüssmann and Ito, 2005; Yamamoto et al., 2013; Zhang et al., 2018). In this study, we noted simultaneous and sympatric expression of the pro-male (*amh*) and pro-female (*cyp19a1a*) genes in the anterior region of both gonads in XY larvae

between 2 and 3 wah, even though these fish ultimately developed as males. Equally important, this *cyp19a1a* expression had similar number of positive cells in the left and right gonads, a pattern also noted in XX fish. These observations are additional evidence that pejerrey may be predisposed to become females regardless of the genotypic sex and suggest that apoptosis could be involved in preventing *cyp19a1a*-induced feminization of the anterior region of the right gonads in the presence of a genotypic male determinant. Nevertheless, it is also possible that male signaling mediated by *amh* (*amhy* and/or *amha*) suffices to override this *cyp19a1a*-dependent, developmentally-programmed ovarian differentiation. In fact, AMH has suppressive effects on aromatase in other vertebrates (e.g., human granulosa-lutein cells, Grossman et al., 2008; Sacchi et al., 2016). This would explain the absence of *cyp19a1a* expression in some XY and XX individuals with *amh* expression but no apoptosis. In order to clarify these issues, further studies must attempt to determine which cells actually undergo apoptosis, and to compare the importance of *amhy* and *amha* expression and their timing for *cyp19a1a* suppression and induction of apoptosis. Moreover, we could not figure out the exact roles of apoptosis in the right gonads after 4 wah, although it is clear that in areas with apoptosis, the abundance of both *amh*- and *cyp19a1a*-expressing cells is greatly reduced.

In conclusion, the location and timing of expression of *amh*, *cyp19a1a*, and apoptosis seems highly coordinated with the time of gonadal sex differentiation and broadly support the histological gradient of gonadal sex differentiation at the molecular level. Apoptosis in the right gonads is surmised as a process to delay differentiation until it is firmly established in the left gonad, probably as a means to ensure uniform development throughout the gonads and prevent locally discrepant sexual differentiation. Finally, this study also provides molecular evidence supporting the primacy of female development in pejerrey gonads. Hence, apoptosis may be particularly important in XY individuals whereby genotypic male and female determinants may compete. Further analysis including up- and down-regulation of apoptosis-related genes may

contribute to understand how a dimorphism in apoptosis expression in the left and right gonads is related with sex differentiation in this species.

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Figure Captions

Figure 1.1. Criteria and color scheme for classification of the intensity of *amh* (top), *cyp19a1a* (middle), and apoptosis (bottom) expression in cross sections of larval pejerrey gonads. Scales bars indicate 10 μm.

Figure 1.2. Typical results of *amh* expression in anterior segments of the left and right gonads of XY (A, B) and XX (C, D, E, and F) individuals at 1 week after hatching. The XX individuals showed two patterns, namely, with (C) or without (E) *amh* expression. Scales bars indicate 10 μm.

Figure 1.3. Summary of the results of *amh*, *cyp19a1a*, and apoptosis expression in the left and right gonads of XY (top) and XX (bottom) individuals at 2, 3, 4, 5, and 7 weeks after hatching. Each line represents the results of *amh*, *cyp19a1a*, and apoptosis of the same individual and each cell represents the result for a particular gonadal segment (6-20) of that individual. The color scheme follows the description in Figure 1.1: dark grey, light grey, and white represent abundance, few, and absence of positive cells, respectively. Cells with a slashed pattern represent missing histological sections.

Figure 1.4. Schematic of the process of histological sectioning used for obtaining replicate slides with representative sections from 20 equally-spaced gonadal segments for the molecular and histological analyses.

Figure 1.5. Typical patterns of gene expression (*amh* and *cyp19a1a*) and apoptosis in the

anterior, middle, and posterior part of gonads during sex differentiation of pejerrey. The left and right panels show gene expression of *amh* and occurrence of gonadal apoptosis in the same individual (XY-genotype, 5 weeks after hatching), respectively. The middle panels show gene expression of *cyp19a1a* in XX-genotype, 4 weeks after hatching. Dotted boxes indicate left (L) and right (R) lobes of the gonad. Insets in panels are high magnification images of the left and right lobes of the gonad. Scale bars = 50 μm.

Table 1.1. Phenotypic (gonadal) and *amhy*-based genotypic sex ratios in fish at the end of the experiment (14 weeks after hatching).

Genotype	**Phenotype**		**Total n (%)**
	Female	Male	
XX	42	24	66 (58.9)
XY	2	44	46 (41.1)
Total n (%)	44 (39.3)	68 (60.7)	112

Figure 1.1.

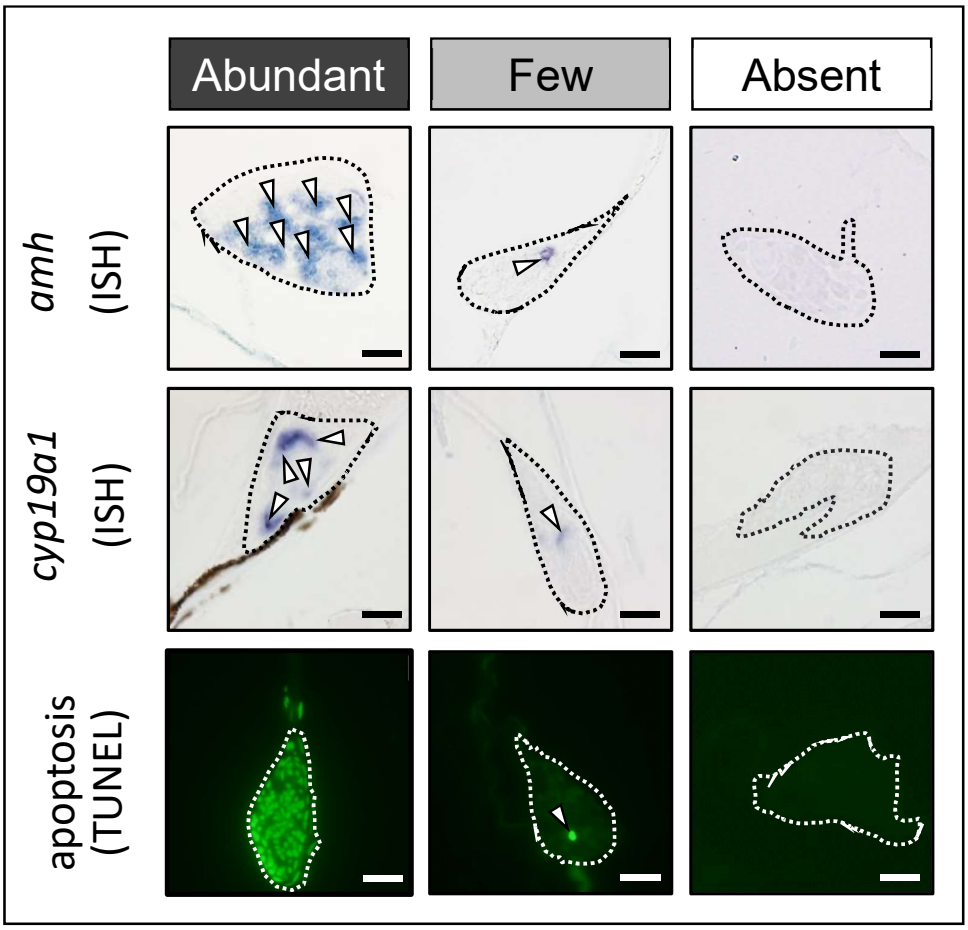


Figure 1.2.

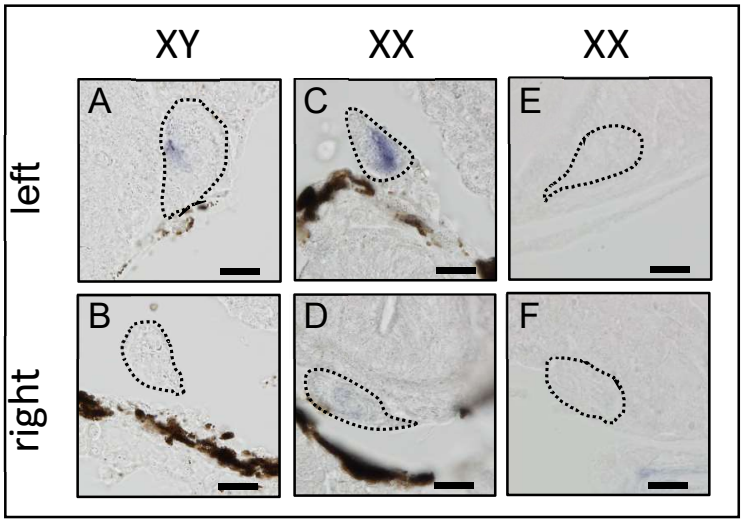
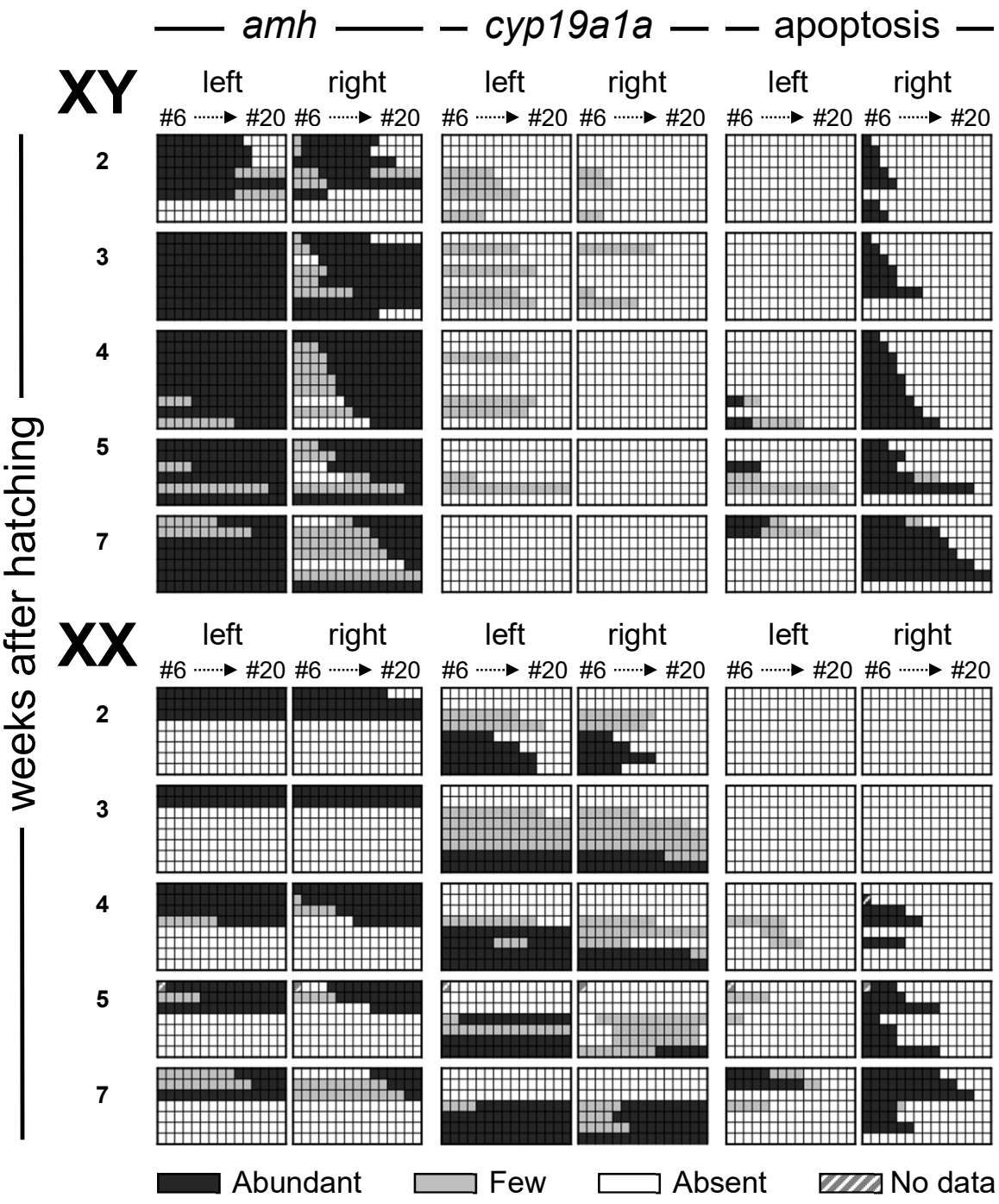


Figure 1.3.


amh
cyp19a1a
apoptosis
XY
XX
left
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#6 #20
weeks after hatching
2
3
4
5
7
Abundant
Few
Absent
No data

Figure 1.4.

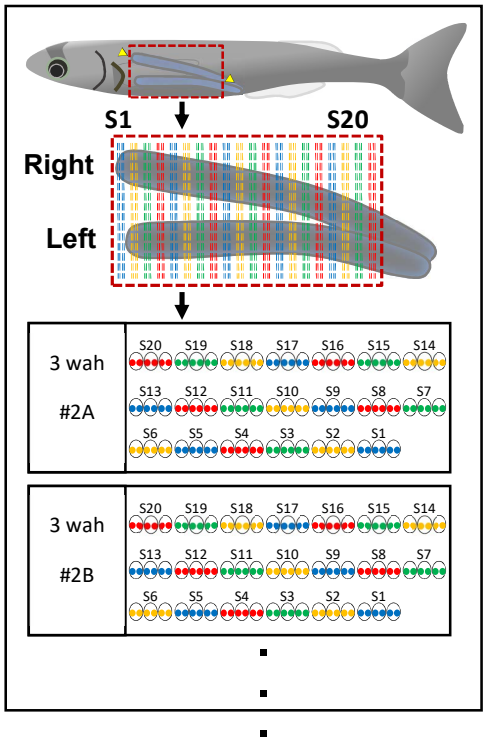
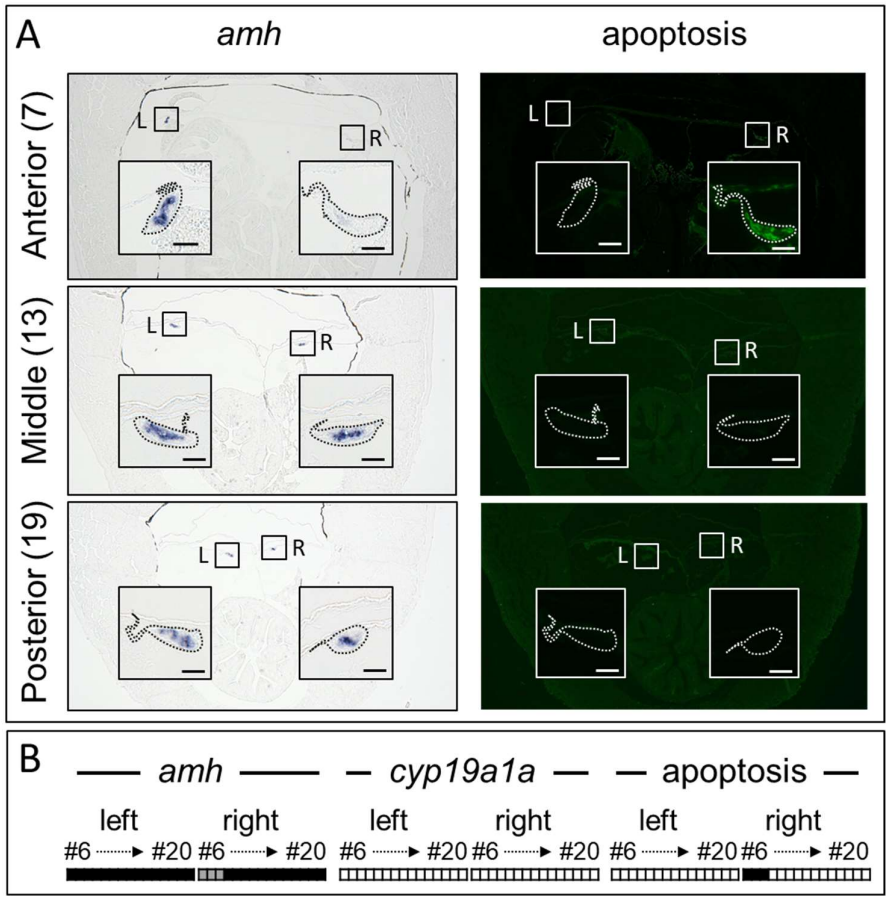

S1
S20
Right
Left
3 wah
#2A
3 wah
#2B
S20 S19 S18 S17 S16 S15 S14
S13 S12 S11 S10 S9 S8 S7
S6 S5 S4 S3 S2 S1

Figure 1.5.


A
amh
apoptosis
Anterior (7)
Middle (13)
Posterior (19)
L
R
B
amh
cyp19a1a
apoptosis
left
right
#6
#20

Chapter 2

Effects of feminizing and masculinizing temperatures on the spatiotemporal expression patterns of *amh* and *cyp19a1a* transcripts and apoptosis

Introduction

The pejerrey *Odontesthes bonariensis* is considered to possess the most marked temperature-dependent sex determination (TSD) among teleosts. In this species, the male percentages reach 0 and 100% by rearing larvae at 17°C (female-promoting temperature, FPT) and 29°C (male-promoting temperature, MPT), respectively. Whereas, when larvae develop at intermediate temperatures of around 24 or 25°C, males and females are formed (Strüssmann et al., 1996a; 1997); here sex is influenced by an interplay of genetic factors and temperature (Yamamoto et al., 2014). The influence of temperatures occurs when larvae pass through the critical period of sex determination, which has been estimated to be between 1 and 5 weeks after hatching (wah).

In spite of the marked TSD, an intriguing aspect of this species is related to the rarity of gonad ambiguity or intersex in the wild and also in experiments with thermal or endocrine manipulations (Strüssmann et al, 2002.; Fernandino et al., 2008; Hattori et al., 2009; Perez et al., 2012). These observations indicate that environmentally labile species, such as pejerrey, must have a mechanism to coordinate the direction of the sex formation in both female and male throughout the entire length of gonads. A process that could be involved in the prevention of sex discrepancies is the cephalocaudal, left-to-right gradient of gonad differentiation reported for this species (Strüssmann and Ito, 2005).

As I explained in the first chapter, the main objective of this dissertation was to explore such gradient described at histological level and to clarify its relationship with sex determination in pejerrey. In 2014, our group identified in pejerrey a homologue of the testis-determining gene Y-linked anti-Müllerian hormone (*amhy*) in pejerrey (Yamamoto et al., 2014), a testis determinant first identified in the closely related species *O. hatcheri* (Hattori et al., 2012). Particularly, Yamamoto et al., 2014 reported *amhy* is transcribed from early embryonic stages and down regulated by the end of sex determination period (1-4 wah),

whereas *amha* increases significantly from the end of the same period in gonads that differentiate as males in both genotypes (XY and XX). Therefore, *amha* and *amhy* are thought to have important roles in testis differentiation at an intermediate temperature (Yamamoto et al., 2014). Besides, gonadal aromatase *cyp19a1a,* is thought to be crucial for feminization in this species which this enzyme has function to convert from androgens to estrogens (Karube et al., 2007; Fernandino et al., 2008, Zhang et al., 2018a). Moreover, previous studies revealed a high incidence of apoptosis of somatic cells in the anterior region of the right gonad during sex differentiation, particularly at the high, male-promoting temperatures (Strüssmann et al., 2008; Yamamoto et al., 2013). Within such context, this chapter aimed to examine the effect of feminizing and masculinizing temperatures on the spatiotemporal patterns of expression of sex-related genes (*amh and cyp19a1a)* and apoptosis during gonadal sex determination/differentiation in pejerrey.

The first chapter showed the spatiotemporal profile of *amhs* and *cyp19a1a* expression and their relation with the histological gradient of gonadal differentiation at a sexually neutral temperature (25°C), showed *amhs* and *cyp19a1a* expression followed a gradient of expression from anterior to posterior regions and from the left to the right gonad. To gain better understanding of sex determination/differentiation process in pejerrey, I then continued to observe the process of gonadal differentiation involving those genes (*amhs* and *cyp19a1a* expression) and apoptosis to clarify whether they are correlated with the left-to-right and cephalocaudal gradient of both ovarian and testicular differentiation of pejerrey at low and high temperatures.

Materials and methods

Rearing and sampling procedures

Fertilized eggs were obtained by natural spawning from an XX (*amhy*$^{-/-}$) female and an XY (*amhy*$^{+/-}$) male and incubated at 19°C until hatching as described in Chapter 1. Shortly, larvae were stocked in two 60-liter tanks kept at 17°C (FPT) and 29°C (MPT) immediately after hatching and reared for up to 14 weeks after hatching (wah) in order to produce both all-female and all- male populations (Strüssmann et al., 1996a; 1997). Larvae were fed live *Artemia* nauplii to satiation three to four times daily from the first day after hatching and gradually weaned into powdered fish food (TetraMin flakes) from the third week. Larvae were sampled weekly for histological from 1 to 10 wah (n=20 fish/time point). All fish were fin-clipped for genomic DNA extraction and *amhy* genotyping, as described in Chapter 1. The trunk portion of each larvae was fixed in 4% (w/v) paraformaldehyde/phosphate-buffered saline (PFA) overnight, dehydrated in ascending alcohol series, and embedded in Paraplast Plus (McCormick Scientific, St. Louis, Mo., USA) for *in situ* hybridization (ISH) and TdT-mediated dUTP nick end labeling (TUNEL) assay (see Chapter 1). The remaining fish at the end of the rearing experiment (14 wah) were collected for determination of phenotypic and genotypic sex ratios by gonadal histology and *amhy* genotyping, respectively. The experiments were carried out in accordance with the guide for the care and use of laboratory animals from Tokyo University of Marine Science and Technology. All fish used in this study were sacrificed after anesthetization by immersing in ice cold water in order to minimize animal suffering prior to any sampling.

DNA extraction and sex genotyping

Genomic DNA was extracted from fin samples of all fish and used for the analysis of genotypic sex based on the presence/absence of *amhy*. Extraction procedures and subsequent amplification were conducted using the procedures described in a previous study (Yamamoto et al., 2014). The forward and reverse primers for sex genotyping were 5'-AGTCAGCTC

AGATGCT-3' and 5'-AGCCGGATGCAAAACTTCCAG-3', respectively. PCR products were analyzed by 1% agarose gel electrophoresis. *amhy*-positive fish were scored as XY and *amhy*-negative as XX.

Sample preparation for ISH, TUNEL, and histological analysis of gonadal sex differentiation

After genotyping, 5-8 XX and XY individuals were chosen from among the embedded specimens for each time point for ISH, TUNEL, and histological analyzes. Blocks were serially cross-sectioned at a thickness of 5 μm and the sections containing the gonads were divided into 20 segments with approximately the same number of sections and used for ISH (*amh*, *cyp19a1a*) and TUNEL (apoptosis) as described in Chapter 1.

Histological analysis of gonadal sex differentiation and sex ratios

The degree of gonadal sex differentiation of larvae sampled between 2 and 10 wah and the phenotypic sex of juveniles sampled at 14 wah was judged using the histological criteria described as described in Chapter 1.

In situ hybridization of amh and cyp19a1a

The *amh* and *cyp19a1a* probes used in this experiment and ISH conditions were same as described in Chapter 1. After the reaction of ISH for each gene, sections were rinsed and signals were detected by NBT/BCIP (Roche) for 3 and 6 hours for *amh* and *cyp19a1a*, respectively. Slides were observed under a microscope (BX53 microscope, Olympus) and images were captured and digitalized with a CCD camera (DP73, Olympus). For interpretation of the results, the abundance of *amh*- and *cyp19a1a*-positive cells was arbitrarily classified in three visual categories as follows: abundant (positive cells occupy a significant area of the

gonadal cross section in the segment), few (generally less than 10% of positive cells in the segment), and absent (absolutely no positive cells in the segment) as described in Chapter 1.

Detection of apoptosis by TUNEL assay

TUNEL assay was used for visualization of apoptotic DNA strand breaks and followed the procedure described in Chapter 1. The frequency of apoptosis was classified in three categories as described in Chapter 1.

Results

The histological sex differentiation and sex ratio

The first morphological signs of gonadal sex differentiation were observed at 8 wah for ovaries at low temperature (FPT, 17°C,) and at 5 wah for testes at high temperature (MPT, 29°C) (data not shown). The analysis of the phenotypic sex at 14 wah showed the following percentage of females: 84.3% and 0% at 17 and 29°C, respectively (Table 2.1). At 17°C XY larvae develop into either female or male (60% and 40%, respectively, n=115), whereas all XX were females (n=70). On the other hand, at 29°C all larvae were male regardless of genotypic sex (XY, n=29; XX, n=31) (Table 2.1).

Expression pattern of amh, cyp19a1a and gonadal apoptosis *in XX and XY genotypes at 29°C*

Transcripts of *amh* were detected only in the anterior region of the left gonad in XY and XX genotypes at 1 wah (Figs. 2.1A and 2.1C) but not in the middle and posterior regions of the same side or in any region of the right gonad (Figs. 2.1B and 2.1D). At 2 wah, *amh*-positive cells in the left and right gonads of XY fish were abundant in segments rostral to segments 14-17 and fewer or absent in the more posterior segments. This pattern was observed in all of XY individuals (Fig. 2.2). From 3 wah onward, *amh* signals were abundant throughout

the left gonads in most of the individuals (except 4 fish from 5 and 7 wah). Compared to the left, the right gonads of XY fish had fewer *amh* signals first in the rostral segments (e.g. 2-4 wah) and subsequently also in the middle segments (5-7 wah, Figs. 2.2). On the other hand, *amh* signals in XX individuals were were few in posterior regions of right gonads at 2 wah whereas those were abundant throughout the left gonads in all of individuals from 2 to 4 wah and right gonads from 3 and 4 wah. From 5 wah onward, the left and right gonads of XX fish had fewer *amh* signals in the rostral and the middle segments (5-7 wah, Figs. 2.2).

The XY fish had abundant gonadal apoptosis in the anteriormost segments in the right gonads from 1 wah (data not shown) whereas in the left gonad it started only from 5 wah (Figs. 2.2). In XX fish, the apoptotic signals were started to appear in the anteriormost segments in the both side of gonads from 5 wah (except one fish which showing abundant apoptotic signals in the right anterior gonad at 4wah) and more abundant in the middle segments of the right gonads in 5 and 7 wah.

No *cyp19a1a*-positive cells were observed in the left and right gonads of both XY and XX genotypes at any time point.

Expression pattern of amh, cyp19a1a and gonadal apoptosis *in XX and XY genotypes at 17°C*

Few *amh*-positive cells were observed in the anterior region of the left gonad in all of XY fish at 1 to 2 wah (Figs. 2.3). At 3 wah onward, *amh*-positive cells remained few in most of the XY fish but spread throughout the left and right gonads (Figs. 2.3). In contrast, no *amh*-positive cells were observed in the left and right gonads in XX genotypes at any time point (1-7 wah, Figs. 2.3).

The transcripts of *cyp19a1a* were first observed at 3 wah in segments rostral in left (5 out of 7) and right (3 out of 7) gonads whereas the remaining two had no signal of *cyp19a1a* throughout the entire left and right gonads (Figs. 2.3). At 4 wah, *cyp19a1a*-positive cells were

few (3 out of 8) or abundant (3 out of 8) in the left and right gonads in XY individuals whereas the remaining two had no signal of *cyp19a1a* throughout the entire gonads (Figs. 2.3). At 5 wah, few (4 out of 6) or abundant (1 out of 6) *cyp19a1a*-positive cells were observed in the entire left and right gonads in most of XY fish whereas remaining one had no signal of *cyp19a1a* throughout the entire gonads (Figs. 2.3). The similar patterns of *cyp19a1a* expression in the left and right gonads of XY fish were observed at 7 wah but *cyp19a1a*-positive cells were more abundant than 5 wah (Figs. 2.3). On the other hand, most of XX fish had abundant *cyp19a1a*-positive cells in the anteriormost segments in the left and right gonads at 3 wah and *cyp19a1a*-positive cells spread throughout entire gonads in all individuals at 4 wah onward (4-7 wah, Figs. 2.3).

At 17°C, no apoptotic cells were observed in the left and right gonads in XY and XX genotypes until 5 wah, but abundant gonadal apoptosis were observed only in the anteriormost segments in the right gonads in XY (4 out of 6) and XX (1 out of 6) at 7 wah.

Discussion

The *amh*- or *cyp19a1a*-positive cells were first observed in somatic cells in anterior-most region of left gonad in the larvae reared at high temperature or of both gonads in larvae rearing at low temperature, respectively. These genes expression occurred between 1 and 2 wah. These results suggested that anterior-most region of the gonad might differentiate at first during gonadal differentiating process. These patterns agree with the first chapter and the previous studies (Yamamoto et al., 2014; Zhang et al., 2018a). More importantly, spatiotemporal patterns of *amh*, *cyp19a1a* relatively agreed with the antero-posterior (cephalocaudal) and left-to-right gradient of gonadal sex differentiation described in pejerrey by conventional histological analysis (Strüssmann and Ito, 2005; Ito et al., 2005). The fact that *amh* and *cyp19a1a* expression began before the onset of histological differentiation of testes (5

wah) at 29°C and ovaries (8 wah) at 17°C suggests that they are the cause rather than a consequence of the histological gradient.

Exposing larvae to MPT during the critical period of sex determination elevated *amh* expression (XY; *amhy* and/or *amha*, XX; *amha*) and completely suppressed *cyp19a1a* expression, which in turn induced low aromatase activity and E_2 levels. In medaka and Japanese flounder, high water temperature induced cortisol and it directly inhibits the expression of *cyp19a1a* and induces masculinization (Hayashi et al., 2010, Yamaguchi et al., 2010). In addition, in our previous study revealed that high water temperature increased level of endogenous cortisol and induces masculinization in pejerrey (Hattori et al., 2009). Taken together, although the inhibitory mechanism of cortisol on *cyp19a1a* expression in pejerrey remains unclear, similar mechanism may also exist in pejerrey. Other possibility of the low expression of *cyp19a1a* at MPT is that *amh* could be an anti-aromatase factor as reported in mammals (Josso et al., 1998; Visser et al., 2006). Such high expression of *amh* at MPT was also observed in Japanese flounder (Yoshinaga et al., 2004, Kitano et al., 2007; Yamaguchi and Kitano, 2012), Nile tilapia (Poonlaphdecha et al., 2013), and Southern flounder (Mankiewicz et al., 2013) and sex reversed-male caused by high water temperature has also been observed in Nile tilapia and flounder.

In this study, approximately 40% of XY individuals developed as male at FPT. However, all individuals possessed *amh*-positive cells throughout both gonads during the process of gonadal differentiation. As I mentioned before, XY individuals possessed Y-chromosome-linked male sex determinant, *amhy*, in addition to autosomal *amha* in pejerrey (Yamamoto et al., 2014). Because *amha* expression did not observed in any XX fish gonads at any time point at FPT, the *amh* signals observed in XY individuals would probably originated from *amhy* gene. Except the individuals showing abundant *amh* signals at FPT, *amhy* signals as male sex determinant were not sufficient to induce masculinization at this temperature and

feminization process mediated by *cyp19a1a* may override the *amhy*-induced masculinization process.

In this study, all of XY and XX fish developed as male at MPT. However, the occurrence of apoptosis was largely restricted in the right gonads during the early gonadal differentiation process (2-4 wah) only in XY individuals and apoptosis did not observed in XX individuals during 2-4 wah at MPT. In mammalian cell line, recombinant AMH inhibited cell proliferation and induced apoptosis (Zhang et al., 2018b). In pejerrey, XY individuals have *amhy* and it strongly expresses at the beginning of sex determination period (Zhang et al., 2018a). Therefore, *amhy* may be one of the inducer of this apoptosis observed during early sexual development in XY individuals. The mechanism and reason of right side specific gonadal apoptosis remains unclear but spatial expression analysis of AMH receptor (*amhrII*) in the gonads would be interesting for the future study to solve this question. Another point to be considered is that the gonadal area where weak or absent of *amh* expressions observed in XY and XX fish were the same locations where the apoptosis were observed. The hypothesis for this occurrence of apoptosis could be related to the rarity of intersex gonads was observed either in wild population or under captivity, as in bring on the occurrence of anterior-posterior, left to-right gradient of gonadal differentiation in this species might be a mechanism to prevent intersex (Strüssmann and Ito, 2005).

In summary, the gradients sex-related gene (*amhs* and *cyp19a1a)* expressions were observed at MPT and FPT. The high temperature promotes expression of *amhs* and suppresses *cyp19a1a* expression and override the default female pathway. On the other hand, the low temperature suppresses *amh* expression and promotes *cyp19a1a* expression and induced feminization in half of the XY and all of XX individuals. Interestingly, apoptosis was observed 1-2 weeks earlier in the XY than in the XX at all temperatures studied. Moreover, there was a fairly high correlation between the occurrence of apoptosis in the right gonad and the

disappearance or reduction of the expression of both *amhs* and *cyp19a1a* in the same area. The role of this particular pattern of apoptosis during testicular/ovarian differentiation of pejerrey larvae is an intriguing topic that requires a deeper temporal and spatial approach to clarify it. Also, a study needs to be conducted to investigate the cue(s) implicated in the triggering of apoptosis and its target.

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Figure Captions

Figure 2.1. Typical results of *amh* expression in anterior segments of the left and right gonads of XY (A, B) and XX (C, D) individuals reared at 29°C at 1 week after hatching and typical results of *cyp1a1a* expression in anterior segments of the left and right gonads of XY (E, F) and XX (G, H,) individuals reared at 17°C at 3 weeks after hatching. Scales bars indicate 10 µm.

Figure 2.2. Summary of the results of *amh*, *cyp19a1a* and apoptosis expression in the left and right gonads of XY (top) and XX (bottom) individuals reared at 29°C between 2 and 7 weeks after hatching at. Each line represents the results of *amh*, *cyp19a1a* and apoptosis of the same individual and each cell represents the result for a particular gonadal segment (6-20) of that individual. The color scheme follows the description in Figure 1.1 in Chapter 1: dark grey, light grey, and white represent abundance, few, and absence of positive cells, respectively. Cells with a slashed pattern represent missing histological sections.

Figure 2.3. Summary of the results of *amh*, *cyp19a1a* and apoptosis expression in the left and right gonads of XY (top, A) and XX (bottom, B) individuals reared at 17°C between 2 and 7 weeks after hatching. Each line represents the results of *amh*, *cyp19a1a* and apoptosis of the same individual and each cell represents the result for a particular gonadal segment (6-20) of that individual. The color scheme follows the description in Figure 1.1 in Chapter 1: dark grey, light grey, and white represent abundance, few, and absence of positive cells, respectively. Cells with a slashed pattern represent missing histological sections.

Table 2.1. Phenotypic (gonadal) and *amhy*-based genotypic sex ratios at the end of the experiment (14 weeks after hatching) for fish reared at low (17°C) and high (29°C).

Genotype	**Phenotype**		
17°C	Female	Male	**Total n (%)**
XX	70	0	70(60.9)
XY	27	18	45(39.1)
Total n (%)	97 (84.4)	18(15.6)	115
Genotype	**Phenotype**		
29°C	Female	Male	**Total n (%)**
XX	0	31	31(51.7)
XY	0	29	29(48.3)
Total n (%)	0 (0)	60(100)	60

Figure 2.1.

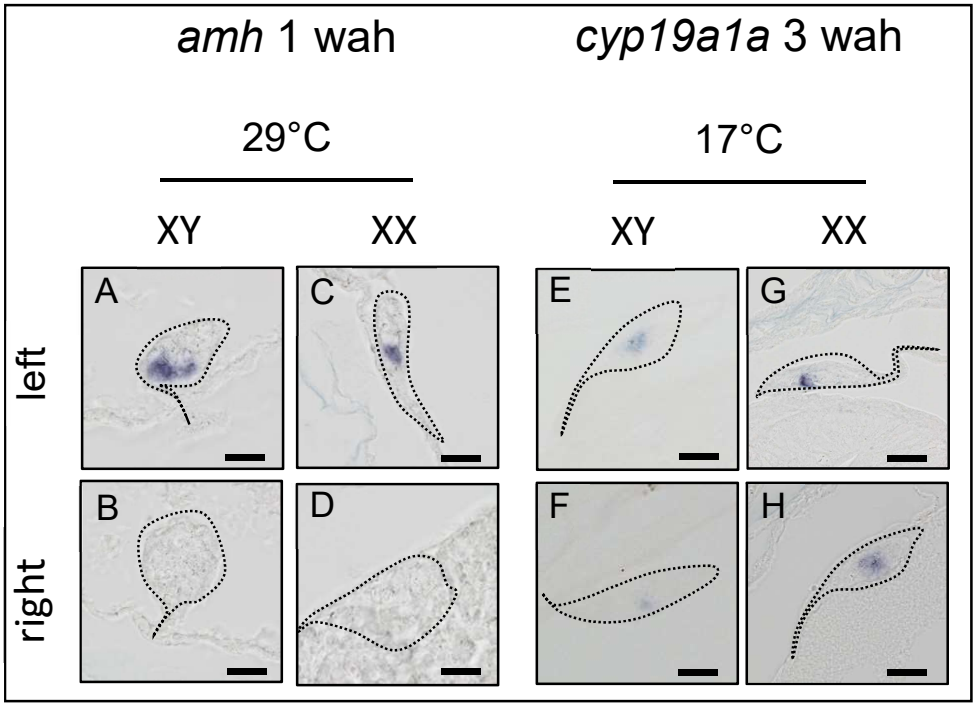

amh 1 wah
cyp19a1a 3 wah
29°C
17°C
XY
XX
XY
XX
left
right
A
B
C
D
E
F
G
H

Figure 2.2.

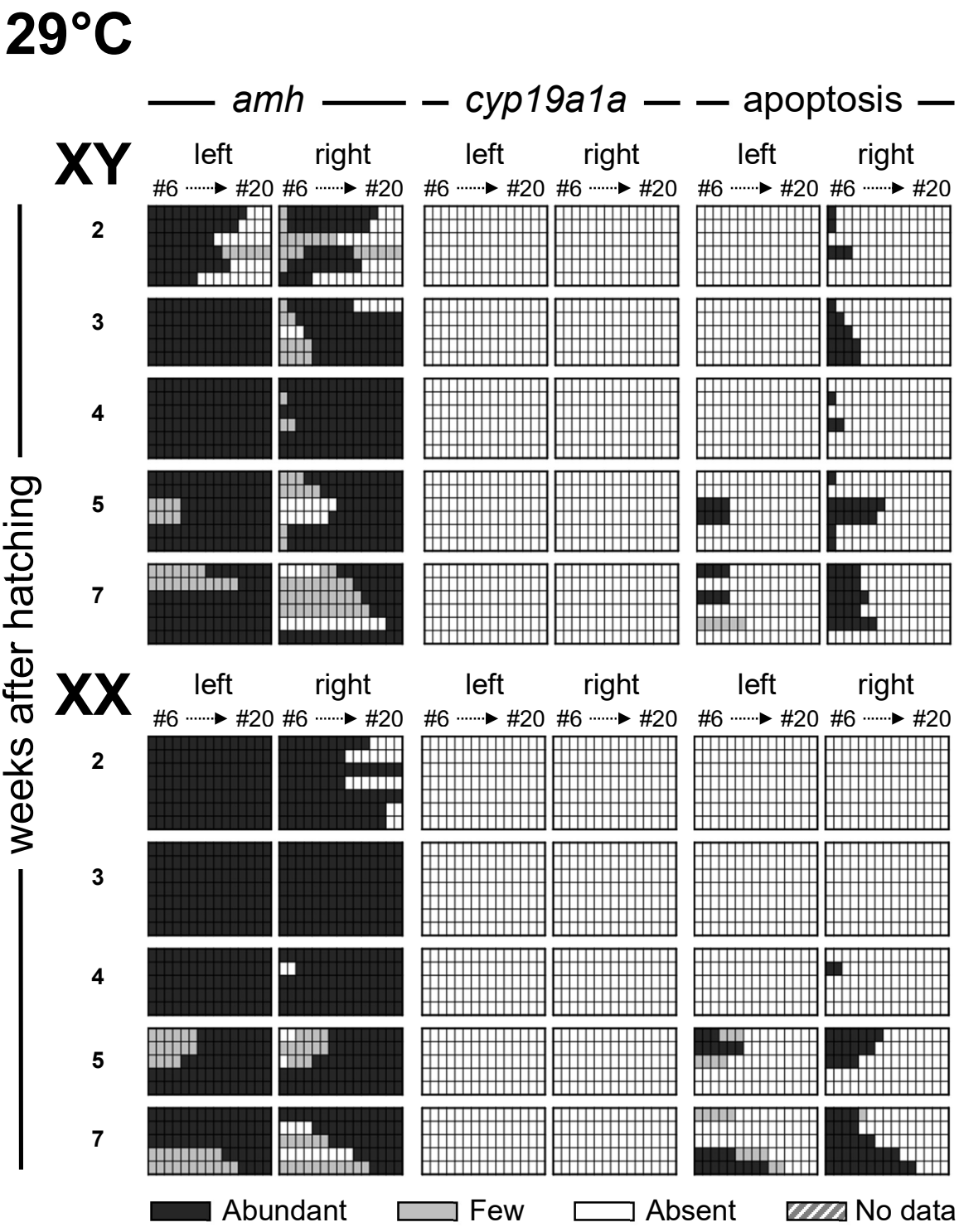
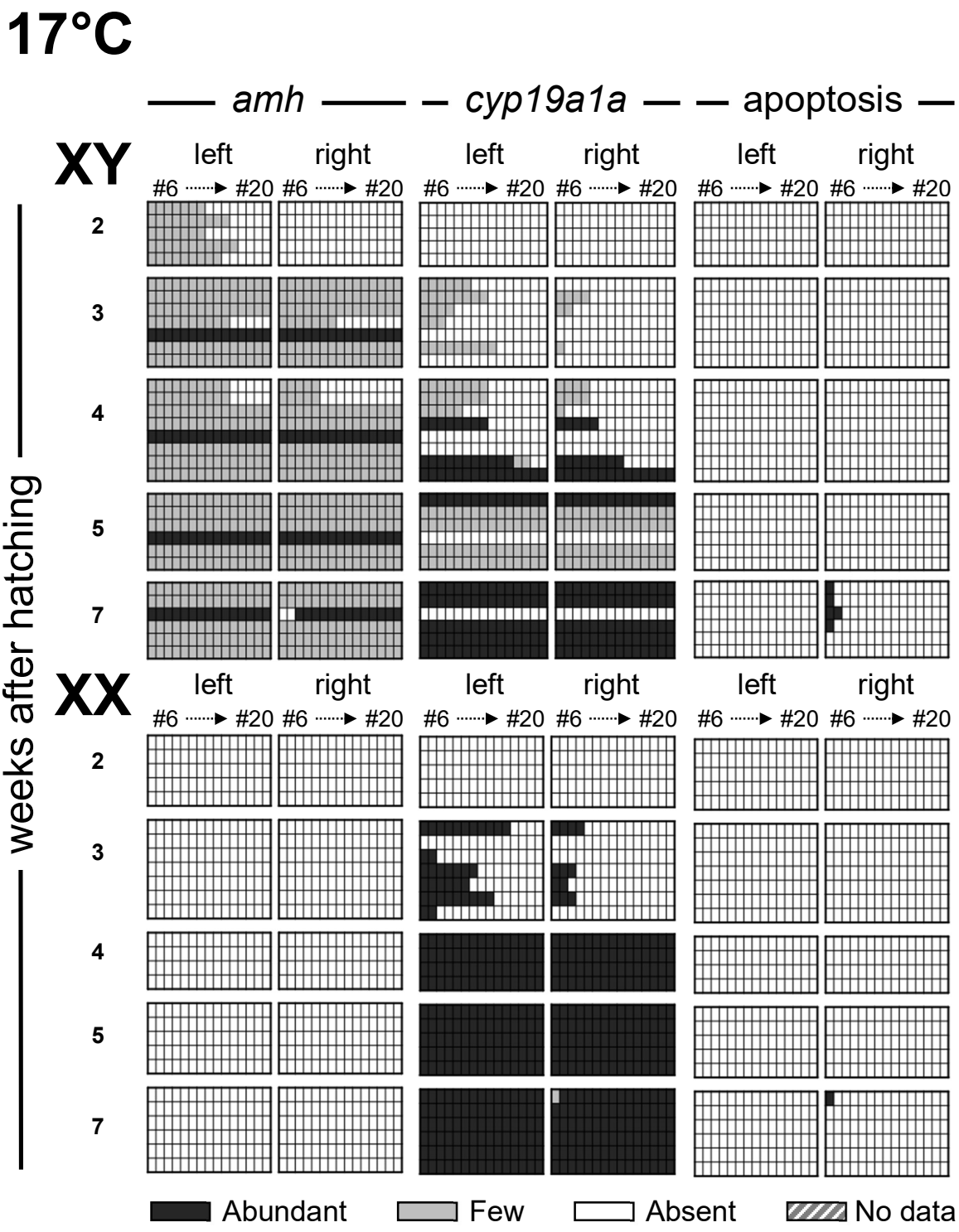

29°C
amh
cyp19a1a
apoptosis
XY
XX
left
right
#6 #20
weeks after hatching
2
3
4
5
7
Abundant
Few
Absent
No data

Figure 2.3.


17°C
amh
cyp19a1a
apoptosis
XY
XX
left
right
#6 #20
weeks after hatching
2
3
4
5
7
Abundant
Few
Absent
No data

General Discussion and Perspectives for Further Studies

Sex determination in pejerrey is temperature-dependent (TSD) at low and high temperatures and genetically-prescribed at intermediate temperatures (24-25°C). The critical time of thermolabile sex determination for these temperatures is 3 to 5 weeks after hatching (wah), 1 to 3 wah, and 2 to 4 wah, respectively (Strüssmann et al., 1996a; 1997). Pejerrey is typically gonochoristic and gonadal differentiation is characterized by a cephalocaudal/left-to-right histological gradient in both sexes and by the occurrence of apoptosis in the anterior half of the right gonad in putative males. The anti-Müllerian hormone (Amh), the ovarian Cyp19a1a, and apoptosis are conceivably implicated in these processes but their possible interplay and relation with the differentiation gradient are still unclear. To shed light on these points, I examined the spatiotemporal patterns of *amh* and *cyp19a1a* gene expression and apoptosis in the gonads during the period of gonadal sex determination/histological differentiation of pejerrey at three temperatures by *in situ* hybridization (ISH).

The expression of *amh* (note: the probe used in this study for ISH does not differentiate between *amha* and *amhy*) was first detected from 1 and 2 weeks in the anterior region of the left and right gonads, respectively, in individuals reared at 25°C and 29°C regardless of genotype. In contrast, it was first observed from 2 and 3 weeks in the anterior region of the left and right gonads, respectively, in some XY individuals reared at 17°C. *Amh*-expressing cells were typically more abundant in the left gonad than in the right gonad and expression expanded from an initial site in the anterior region towards more posterior regions of the gonads. Interestingly, *amh* expression was few or absent in the anterior region of the right gonads in presumptive male and presumptive females, respectively, at all temperatures studied.

The gonadal aromatase *cyp19a1a* expression was observed from 2 and 3 weeks in the anterior region of both gonads regardless of genotype in individuals reared at 25°C and 17°C,

respectively. However, the cyp*19a1a* expression was maintained only in XX gonads at individuals reared at 25°C and in all XX and some XY individuals without *amh* expression at 17°C, and notably absent at 29°C. Moreover, *cyp19a1a* expression was typically more abundant in the left gonad than in the right gonad and expression expanded in the following weeks from the initial site in the anterior region towards more posterior regions of the gonads. Interestingly, *cyp19a1a* expression was few or absent in the anterior region of the right gonads in presumptive females, even at low and intermediate temperatures.

Apoptosis appeared first in the anterior region of the right gonad and then expanded (cephalocaudally) to the posterior regions of the same side regardless of the rearing temperature and the genotypic sex. In most cases apoptosis was limited to the anterior segments of the right gonads, and only occasionally it was observed in both the right and the left sides of the gonad. Gonadal apoptosis was observed from 2 weeks in XY and 4 weeks in XX and became abundant in almost all larvae reared at 29 and 25°C. At 17°C, apoptosis was observed in about half of the individuals of both sexes from 6-7 wah and was much less intense than at the other temperatures. There seemed to be no association between the incidence of apoptosis on any particular week and the resulting phenotypic sex ratios but apoptosis was observed 1-2 weeks earlier in the XY than in the XX individuals at all temperatures studied. Moreover, at all temperatures there was a fairly high correlation between the occurrence of apoptosis in the right gonad and the disappearance or reduction of the expression of *amh* and *cyp19a1a* in the same area.

In summary, the spatiotemporal expression of *amh*, *cyp19a1a*, and apoptosis supports a cephalocaudal, left-to-right gradient of gonadal sex differentiation in pejerrey at the molecular level; the location and timing of *amh*, *cyp19a1a* and apoptosis expression seems highly coordinated among themselves and with the time of gonadal sex differentiation. Apoptosis in the right gonad is highly and fairly associated with suppression of *amhs* and *cyp19a1a* and these findings constitute evidence of a cellular and molecular gradient perhaps

associated with the known gradient of histological sex differentiation in this species. It also provides molecular evidence supporting the notion that that genetic and environmental sex determinants coexist in pejerrey.

Based on this study and on the scientific information available for this species, I proposed a mechanism of temperature-induced masculinization in pejerrey. First, it must be recognized that all animals showed *cyp19a1a* expression at an early stage regardless of the genetic or presumptive phenotypic sex. This observation suggests that the female may be the default state in pejerrey. In this context, I hypothesize that the early expression of *amhs* (probably *amhy* and conceivably related with the presence of *amhy* in XY fish) and/or cortisol (such as in the case of environmentally-induced stress in the XX) antagonizes the early expression and effects of *cyp19a1a*, leading to testicular formation. In other words, the alternative presence of Amhs or cortisol overrides the female default pathway and activates downstream male–related genes, leading to masculinization (Fig. 3.1).

Another important finding of this study is a possible novel role for apoptosis in gonadal differentiation of pejerrey. Apoptosis was shown to occur typically in the anterior region of the right gonad in individuals of both sexes and generally coincided spatiotemporally with the reduction or disappearance of *amhs* and *cyp19a1a.* Thus, rather than a direct role of apoptosis in testicular formation, the results suggest that early apoptosis in the right gonads could be a mechanism to delay differentiation in this area until it is firmly established in the left gonad, and hence prevent gonadal ambiguity (intersexes) that could occur if different areas of the gonads responded differently to environmental signals. According to this view, in putative females there is no conflict with the default sex, so apoptosis is delayed or even unnecessary, leading to the casual observation that apoptosis is more common in putative males (Fig. 3.2).

Further studies are needed to confirm the female-default and molecular gradient/apoptosis-based intersex prevention hypotheses put forth in this work and examine

their existence in other TSD species. In addition, it is necessary to conduct further experiments in order to elucidate more details on the process of sex determination and gonadal sex differentiation in pejerrey. For example, experiments with manipulation of gonadal sex determination by rearing larvae at 25 and 29°C and treatment with an apoptosis inhibitor, receptor blockers, or by using newly available gene inactivation technologies. In addition, it is recommended to perform more detailed analysis to discriminate whether *amhy* or *amha* is the responsible for inhibiting the expression of *cyp19a1a*. Also, the development of probe that discriminates between *amha* and *amhy* would greatly aid in the specific localization of both *amhs* genes during *in situ* hybridization. The importance of *amha* expression for sex determination in XX genotypes must also be further scrutinized.

Figure 3.1. Schematic representation of the working hypothesis of females as the default state during gonadal sex differentiation in pejerrey.

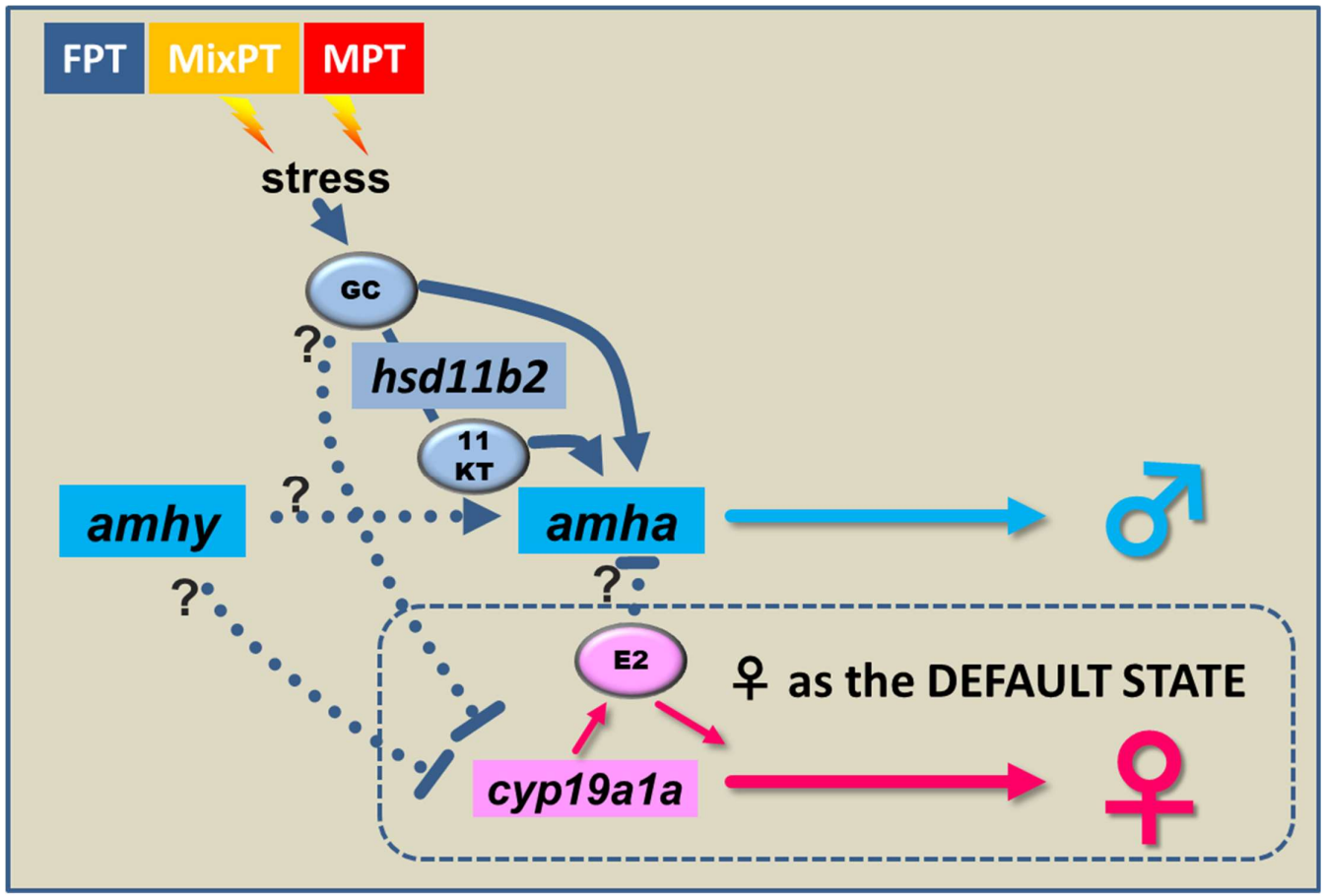
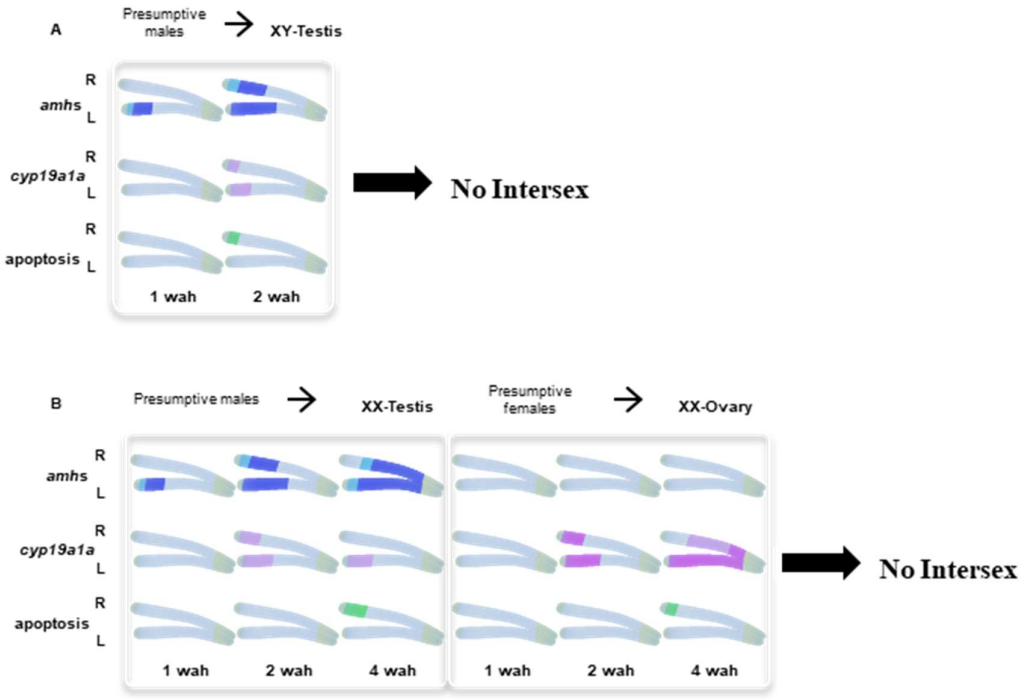


Figure 3.2. Schematic representation of the working hypothesis on the role of apoptosis in the prevention of intersex formation during gonadal sex differentiation in pejerrey.



List of Publications and Presentations

Publications

1. **Munti Sarida**, Ricardo S Hattori, Yan Zhang, Carlos Augusto Strüssmann and Yoji Yamamoto. 2019. Spatiotemporal correlations between *amh* and *cyp19a1a* transcript expression and apoptosis during gonadal sex differentiation of pejerrey Odontesthes bonariensis. Sexual Development Journal. **(accepted)**
2. Yan Zhang, Ricardo S Hattori, **Munti Sarida**, Estefany L García, Carlos Augusto Strüssmann and Yoji Yamamoto. 2018. Expression profiles of amhy and major sex-related genes during gonadal sex differentiation and their relation with genotypic and temperature-dependent sex determination in pejerrey Odontesthes bonariensis. General and Comparative Endocrinology, 265:196-201. doi: 10.1016/j.ygcen.2018.03.013
3. Yoji Yamamoto, Yan Zhang, **Munti Sarida**, Ricardo Shohei Hattori and Carlos Augusto Strüssmann. Coexistence of genotypic sex and temperature-dependent sex determination in pejerrey, *Odontesthes bonariensis.* PloS ONE 9(7): e102574. Doi:10.1371/journal.pone.0102574

Oral/Poster presentations

1. Yoji Yamamoto, Zhang Yan, **Munti Sarida**, Ricardo S Hattori and Carlos A Strüssmann. 2018**.** Genotypic and Temperature Dependent Sex Determination in Pejerrey**.** 18th International Congress of Comparative Endocrinology (ICCE18), Canada.
2. **Munti Sarida**, Yan Zhang, Ricardo S Hattori, Yoji Yamamoto, and Carlos Augusto Strüssmann. 2017 (September 22 -23). Spatiotemporal distribution of gonadal apoptosis, *amh* and *cyp19a1a* gene expression during gonadal sex determination/histological differentiation in pejerrey. International symposium fisheries science and the future generations the JSFS. Tokyo. (Oral).
3. **Munti Sarida**, Yoji Yamamoto, Yan Zhang, Ricardo Shohei Hattori, and Carlos Augusto Strüssmann. 2016 (28th June -2nd July). Spatiotemporal coordination of *amh* and *cyp19a1a* gene expression and apoptosis during testicular differentiation in pejerrey *Odontesthes bonariensis.* 8th International Symposium on Fish Endocrinology (8th ISFE), Gothenburg, Sweden. (Poster).
4. **Munti Sarida**, Yoji Yamamoto, Yan Zhang, Ricardo Shohei Hattori, and Carlos Augusto. 2016 (March 26-30). Spatiotemporal coordination of *amh* and *aromatase* gene expression and apoptosis during testicular differentiation in pejerrey *Odontesthes bonariensis.* Suisan Gakkai Spring 2016. Tokyo. (Poster).
5. **Munti Sarida**, Ricardo Shohei Hattori, Yan Zhang,Yoji Yamamoto, and Carlos Augusto Strüssmann. Male gonadal differentiation in Pejerrey *Odontesthes bonariensis*: Co-occurrence between apoptosis and anti- müllerian hormone gene expression. 2015 (April 13-17th). 7th International Symposium on Vertebrate Sex Determination. Kona, Hawaii. (Poster).