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Spatiotemporal Correlations between *amh* and *cyp19a1a* Transcript Expression and Apoptosis during Gonadal Sex Differentiation of Pejerrey, *Odontesthes bonariensis*

Munti Sarida[a, c] Ricardo S. Hattori[b] Yan Zhang[a] Yoji Yamamoto[a]
Carlos A. Strüssmann[a]

[a]Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Tokyo, Japan; [b]UPD-Campos do Jordao – Sao Paulo Fisheries Institute/APTA, Campos do Jordao, Brazil; [c]Fisheries and Marine Sciences Department, Faculty of Agriculture, University of Lampung, Bandar Lampung, Indonesia

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Apoptosis · Gene expression · Gonadal differentiation · Histological gradient · Temperature-dependent sex determination

Abstract
Sex determination in pejerrey is genetically prescribed by the Y chromosome-linked anti-müllerian hormone *amhy* but is also strongly influenced by water temperature during the critical period of sex determination. Its gonadal differentiation is characterized by a cephalocaudal and left-to-right histological gradient in both sexes that presumably helps prevent discrepant intersex development in different regions of the gonads in response to ambiguous thermal and genetic stimuli, but the relation of this gradient to molecular processes of sex differentiation is unknown. In this study, we investigated the spatiotemporal expression patterns of *amh*, gonadal aromatase (*cyp19a1a*), and apoptosis in relation to the histological gradient in ovaries and testes at an intermediate, sexually neutral temperature. The location and timing of expression of *amh*, *cyp19a1a*, and apoptosis seemed to be highly coordinated with the time of gonadal sex differentiation and the histological gradient of gonadal sex differentiation. Apoptosis occurred predominantly in the anterior region of the right gonads and is surmised to be a process to delay differentiation in this area compared to the left gonad, possibly as a means to ensure uniform development in both gonads. Aromatase expression early during development was noted even in putative XY males, supporting the notion of primacy of female development in pejerrey gonads. Thus, apoptosis may be particularly important to prevent discrepant gonadal differentiation in XY individuals where genetic pro-male (*amhy*), pro-female (*cyp19a1a*), and thermal stimuli may antagonize.

Teleost fishes exhibit 2 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 2 forms represent a continuum of mechanisms that extend from GSD on one end, whereby the gonads differentiate according to the

E-Mail karger@karger.com
www.karger.com/sxd

Yoji Yamamoto
Department of Marine Biosciences
Tokyo University of Marine Science and Technology
Tokyo 108–8477 (Japan)
E-Mail yoji@m.kaiyodai.ac.jp

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 2 sex determination systems coexist [Yamamoto et al., 2014; Zhang et al., 2018]. In this species, we have previously shown 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 leads 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 ESD and GSD and to examine the ecological implications of TSD in wild populations [Hattori et al., 2018].

An intriguing aspect of GSD 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, i.e., 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 of a mechanism for a 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 on and downregulated by the end of the sex determination period (1–4 weeks after hatching, 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 the genotype [Yamamoto et al., 2014]. Another key player, 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 high male-promoting temperatures [Strüssmann et al., 2008; Yamamoto et al., 2013].

In this study, we conducted a detailed in situ analysis of the expression of *amh* and *cyp19a1a* and apoptosis 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.

Material 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-L tanks at 25°C and reared for up to 14 wah in order to produce both

female and male individuals [Strüssmann et al., 1996a, 1997]. Larvae were fed live *Artemia nauplii* to satiation 3–4 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% paraformaldehyde/phosphate-buffered saline (PFA) overnight, dehydrated in an 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. 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. All fish used in this study were sacrificed after anesthetization by immersing in ice cold water in order to minimize animal suffering during 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 as described in a previous study [Yamamoto et al., 2014]. The forward and reverse primers for sex genotyping were 5′-AGTCAGCTCAGATGCT-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 among the embedded specimens for each time point for ISH, TUNEL, and histological analysis. 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 (online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000498997). 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 were the gonads sampled at 1 wah. These gonads were too small so they were divided only into 3 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 the 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 755–1282; GenBank accession no. EF030342.1) was amplified with forward (5′-GACCGGTGTTCAGGATTATATTTGT-3′) and reverse (5′-TGATCAGCACAGTCTGCCAT-3′) primers using cDNA synthesized from an adult ovary. The fragment was then cloned into the pGEM-T Easy Vector (Promega Corporation, Madison, WI, USA), and after confirming the insert orientation the plasmid was linearized by appropriate restriction enzymes 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 1 mg/mL RNA probe at 65°C for 16 h. 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 min at room temperature with blocking solution (Roche, Basel, Schweiz) and for 1 h at 25°C with anti-DIG-alkaline phosphatase-conjugated antibody (Roche) diluted 1:2,000 with blocking solution. Finally, sections were rinsed and signals were detected by NBT/BCIP (Roche) for 3 and 6 h 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 3 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).

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 pretreatment (1 µg/mL; Thermo Fisher Scientific) for 10 min at 37°C, slides were re-fixed in 4% PFA at room temperature for 20 min. Slides were incubated with terminal deoxyribonucleotidyl transferase (TdT) (Roche) at a dilution of 40 units/mL for 80 min in a humidified chamber at 37°C. For positive control, slides were incubated in 1 µg/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 3 categories as for the gene expression (Fig. 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

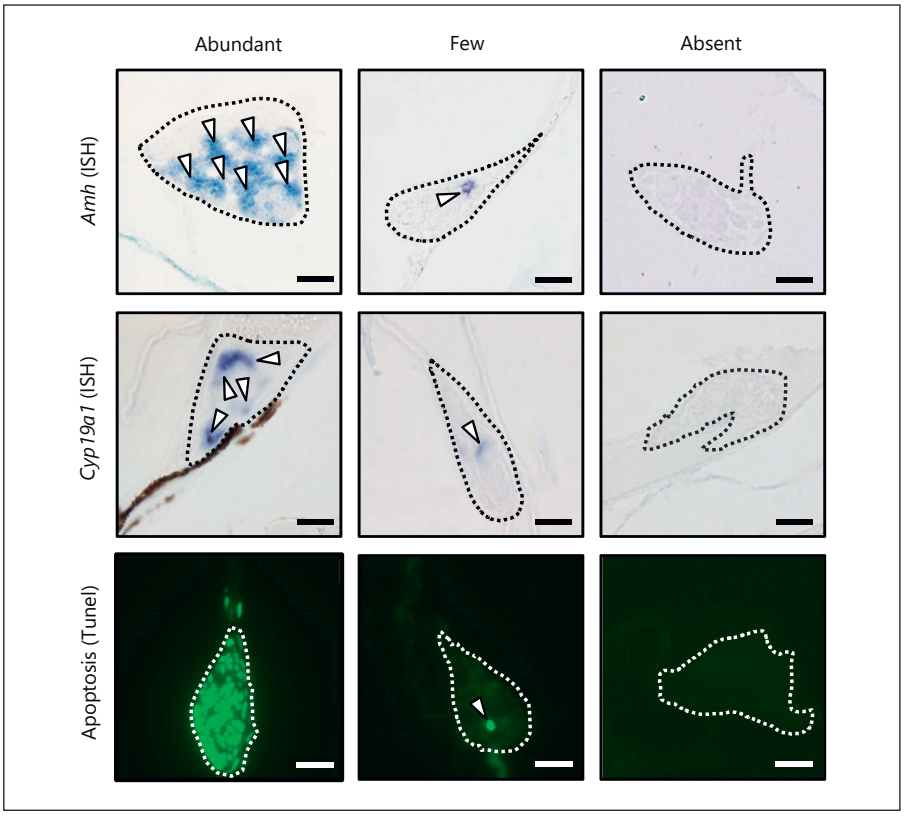

Fig. 1. Criteria and color scheme for classification of the intensity of the expression of *amh* (top) and *cyp19a1a* (middle) and apoptosis (bottom) in cross sections of larval pejerrey gonads. Scales bars, 10 μm.

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) 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.

Table 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

Expression Pattern of amh*,* cyp19a1a*, and Gonadal Apoptosis in XY and XX Genotypes*

An example of the results of gene expression and apoptosis in different segments of the left and right gonads of one individual and their graphical representation are shown in online suppl. Figure 2. Only the results for segments 6–20 were compiled because of the lack of differentiation in more proximal sections as noted above. 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 (Fig. 2A, B). The results of *amh* ISH for XY fish collected between 2–7 wah are summarized in Figure 3. 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 2 had no signal of *amh* throughout the entire left gonad (Fig. 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

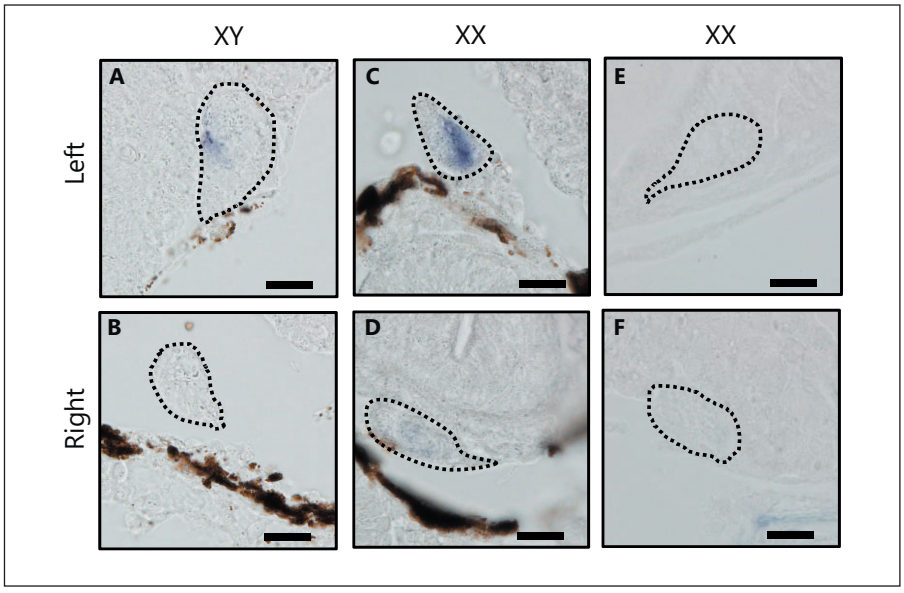

Fig. 2. Typical results of *amh* expression in anterior segments of the left and right gonads of XY (**A**, **B**) and XX (**C–F**) individuals at 1 week after hatching. The XX individuals showed 2 patterns, namely, with (**C**) or without (**E**) *amh* expression. Scale bars, 10 μm.

segments (e.g., 2–4 wah) and subsequently also in the middle segments (5–7 wah). 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 (Fig. 2C, D; 3) whereas the remaining 60% did not show any *amh* signals on both sides of the gonads regardless of the sampling time (Fig. 2E, F; 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. 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 (Fig. 3). 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. 3). In both cases, apoptosis was first observed in the anteriormost segments 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 anteroposterior (cephalocaudal) and left-to-right gradient of gonadal sex differentiation described in pejerrey by conventional histological analysis [Ito et al., 2005; Strüssmann and Ito, 2005], although with genotype-specific peculiarities as noted. Also, the

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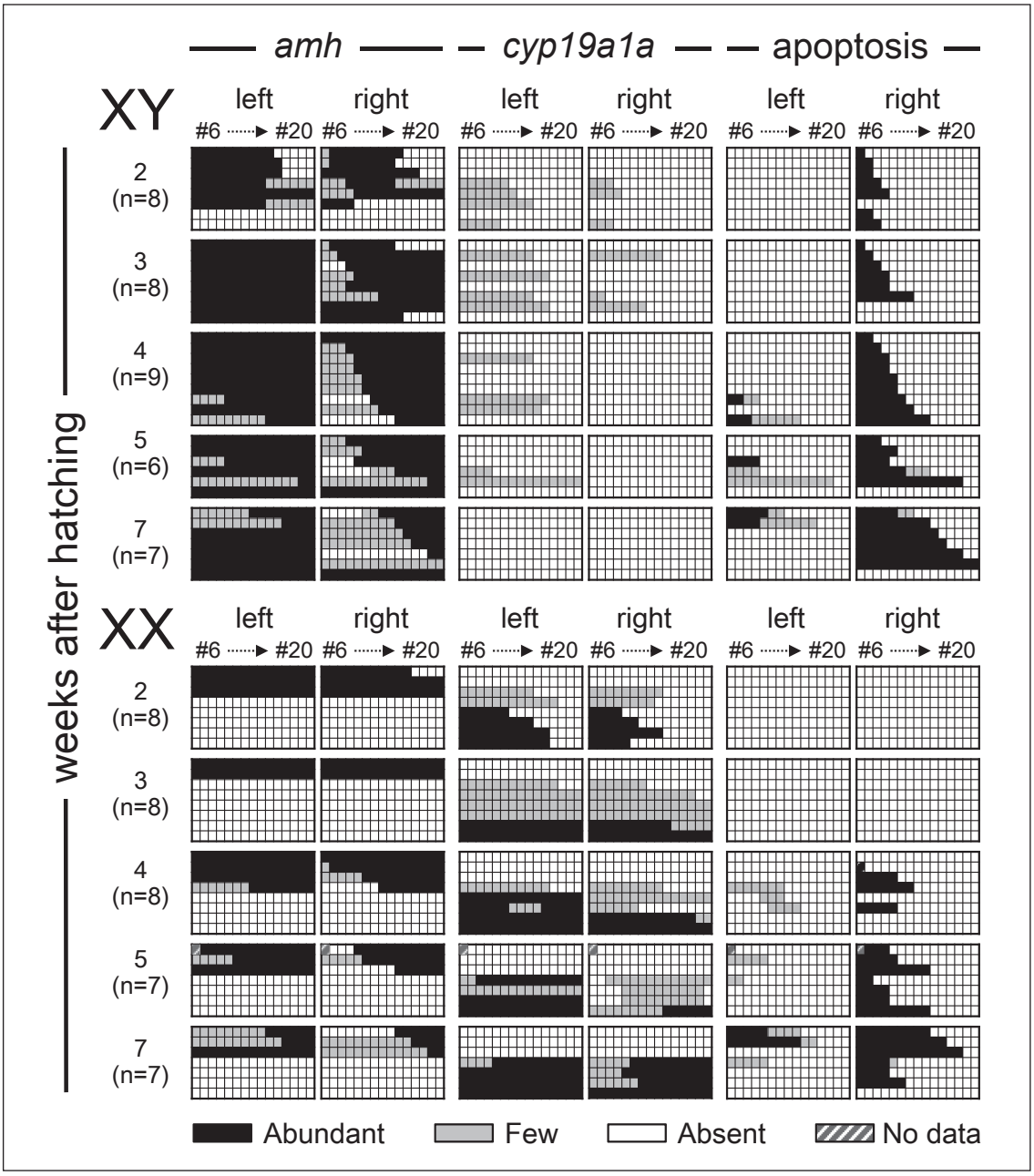

Fig. 3. Summary of the results of the expression of *amh* and *cyp19a1a* and apoptosis in the left and right gonads of XY (top) and XX (bottom) individuals at 2, 3, 4, 5, and 7 weeks after hatching. Each line represents the results of *amh*, *cyp19a1a*, and apoptosis of the same individual and each cell represents the result for a particular gonadal segment (6–20) of that individual. The color scheme follows the description in Figure 1: dark grey, light grey, and white represent abundance, few, and absence of positive cells, respectively. Cells with a slashed pattern represent missing histological sections.

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*) dur-

ing 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* 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 the 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* expression in pejerrey, studies about this gene may be needed for further understanding 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 2 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 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. 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, the lack of discrepant development throughout the gonads strongly hints at the existence of some form of developmental hierarchy. There are 2 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]. 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 one discussed above. It takes into consideration the possibility that female is the default state in pejerrey and, therefore, the existence of a conflict between the endogenous male (*amh*) and female (*cyp19a1a*) 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, a similar number of *cyp19a1a*-positive cells were found 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 supports the histological gradient of gonadal sex differentiation at the molecular level. Apoptosis in the right gonad 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 downregulation of apoptosis-related genes may contribute to understanding how a dimorphism in apoptosis expression in the left and right gonad is related with sex differentiation in this species.

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Statement of Ethics

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.

Disclosure Statement

The authors have no conflicts of interest to declare.

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