Studies on environmental factors associated with stress response and modulation of sex determination in pejerrey Odontesthes bonariensis

Doctoral Dissertation

STUDIES ON ENVIRONMENTAL FACTORS ASSOCIATED WITH STRESS RESPONSE AND MODULATION OF SEX DETERMINATION IN PEJERREY

Odontesthes bonariensis

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Forewords

Sex determination in fish

Fish are one of the most diverse groups in the animal kingdom and at the same time shown a high plasticity of sexual fate and development (Devlin and Nagahama, 2002). Different fish species may adopt different reproductive strategies such as gonochorism or hermaphroditism. In gonochorist teleosts, for example, individuals develop as males, with a differentiated testis, or as females, with differentiated ovaries, and keep this condition throughout their life. In contrast, hermaphrodites produce both male and female gametes simultaneously or at different points during their life cycle (e.g. sequential hermaphrodites; Devlin and Nagahama, 2002).

In addition to the variety of reproductive strategies, fish also have varied sex determination mechanisms and different pathways of gonadal sex differentiation. According to Devlin and Nagahama (2002), sex determination refers to the genetic and environmental processes and variables that influence sex differentiation whereas sex differentiation refers to the physical realization of these events in terms of testicular or ovarian development. In gonochoristic fish species, sex determination can be divided in genetic sex determination (GSD), whereby sex is determined by parental inheritance, and environmental sex determination (ESD), whereby factors like temperature, salinity, pH, density, social interactions or background color can affect the sexual fate (Baroiller and D´Cotta, 2001; Devlin and Nagahama, 2002).

Knowledge on how sex is determined, and the mechanisms involved has application in fields such as aquaculture, where monosex populations may have higher commercial value (Martínez et al., 2014). Moreover, there are also concerns about the effects of rising water

temperatures due to climate change/global warming and related events (e.g. plankton blooms, organic matter deterioration leading to hypoxia, increased rainfall implicated in drastic shifts in salinity and pH, among others) on sex determination and sex ratios of natural fish populations (Kopprio et al., 2010; Strüssmann et al., 2010).

The pejerrey as a model species for sex determination studies

The pejerrey, which belongs to the Atherinopsidae family, is an inland water fish species native from South America (Somoza et al., 2008). Pejerrey inhabits shallow water lakes (Pampean lakes with an average depth of 4 m) which are characterized by high physical and chemical variability. Most of these water bodies are eutrophic lakes with high concentrations of dissolved salts (sometimes in excess of 50 g/L; Bucher and Etchegoin, 2006) and temperatures varying from 5°C in winter to 30°C or more in summer (Gómez, et al., 2007; Kopprio et al., 2010). Although pejerrey inhabits from clear water to turbid water lakes, it is in turbid lakes with a high presence of phytoplankton that this species is most abundant (Quirós et al., 2002). Pejerrey is a visual zooplanktivore which lives in shoaling communities (Boveri and Quirós, 2002).

The pejerrey has been introduced in many countries for game fishing and for aquaculture because of its good adaptability and highly appreciated flesh (Somoza et al., 2008). In Japan, this species was introduced in 1966 and has been kept in captivity over 37 generations with successful domestication (Tsuzuki, et al., 2001; Miranda et al., 2006; Somoza et al., 2008). In natural environments, pejerrey reproduces from late winter/beginning of spring, when the water temperature reaches around 13-15°C, to early summer, and also in autumn when temperatures start to decrease again (Strüssmann et al., 1996).

Recently, pejerrey has become a good model for biological research, especially in the area of climate change and its effects on fish reproduction and population dynamics

(Strüssmann et al., 2010). This is because the process of sex determination in this species is very sensitive to temperature, resulting in all male or all female populations. Female-biased sex ratios are produced at low temperatures (female-producing temperatures or FPT, <17°C), mixed sex ratios are produced at intermediate temperatures (mixed sex-producing temperatures or MixPT, 20-28°C), and all-males are produced at high temperatures (male-producing temperatures or MPT, >29°C) (Strüssmann et al., 1996). Sex is determined in pejerrey in the first five weeks after hatching (Strüssmann et al., 1996), making this time a critical window to explore other factors that could also affect sex determination. Interestingly, pejerrey combines marked TSD with a form of genotypic sex determination. For instance, recently, a copy of the anti Müllerian hormone *amh* was identified in what appears to be the pejerrey Y chromosome; hence, this gene has been termed *amhy* (Yamamoto et al; 2014). This gene seems to be crucial for male sex determination at intermediate temperatures because it is tightly linked with phenotypic sex at intermediate temperatures and its transcription coincides with the period of sex determination period. *amhy* also shares several structural characteristics with the *amhy* of the congeneric species Patagonian pejerrey *Odonthestes hatcheri*, where *amhy* is the master gene triggering male sex differentiation (Hattori et al., 2012).

Stress response in fish and its role in ESD

Stress is any kind of variation in the external environment or the internal physiology of an organism which disturbs the normal homeostasis (Pickering, 1981). Some studies have proposed that temperature variation acts like an external stressor on the fish physiology and may affect sexual development (Martínez et al., 2009). The main response of fish when confronted by external or internal stress is the secretion of cortisol (Pickering, 1981;

Martínez et al., 2009). Cortisol is the main glucocorticoid secreted by the interrenal tissue, located in the head kidney, and constitutes the primary organismal stress response in fish.

Stress in organisms has been grouped in three categories or levels of response (Wendelaar-Bonga, 2011). The primary stress response in fish involves a key brain area (pallial area of the telencephalon that is considered the teleost homolog of the mammalian hippocampus), which integrates and produces a response through activation of adrenocorticotropic hormone (ACTH) for releasing cortisol. The secretion of cortisol involves the hypothalamic-pituitary-interrenal axis (HPI). The secondary stress response is the increase in stress hormones in the blood stream whereas the tertiary response includes the chemical and physical changes produced in the organism which could be adaptive or pathological (Tort, 2011).

Cortisol is the final messenger of the HPI axis and is involved in many actions like growth, reproduction, immune system, osmoregulation, and others. In spite of its major role in stress situations, cortisol is always present in vertebrates playing housekeeping roles. The signaling for cortisol production includes the activation of corticosteroid receptors in target tissues necessary for important functions like sex determination (Wendelaar-Bonga, 2011). In Japanese flounder, the cortisol ligand-bound glucocorticoid receptor shows a relation with the cAMP-responsive element (CRE), which is involved in the expression of *cyp19a1* gene. Further, cortisol induces down-regulation of *cyp19a1* mRNA triggering masculinization of XX fish (Yamaguchi et al., 2010). In medaka, high water temperature inhibits the proliferation of female germ cells, down-regulates follicle-stimulating receptor (*fshr*) mRNA expression, and leads to masculinization of genotypic XX fish, and all these actions are triggered by an increase in cortisol (Hayashi et al., 2010).

In pejerrey, MPT and to some extent also MixPT produce increases in cortisol levels; likewise, fish treated with high cortisol doses shown up-regulation of *amh* gene, down-regulation of *cyp19a1*a and gonadal apoptosis, which induce masculinization (Hattori et al.,

2009). Interestingly, 11-ketotestore (KT; the primary androgen in fish) and cortisol are related to the activities of the same enzymes, 11 hydroxylase (11-H) and 11β hydroxysteroid dehydrogenase (11β-HSD). For instance, the biosynthesis of 11-KT requires the conversion of testosterone into 11-hydroxytestosterone by 11-H, and conversion of 11-hydroxytestosterone into 11-KT by 11β-HSD. On the other hand, both 11-H and 11 β-HSD are also necessary for the synthesis and inactivation of cortisol (Perry and Grober, 2003). Hence, masculinization in pejerrey is thought to be a by-product of cortisol inactivation (Fernandino et al., 2013). Nevertheless, it has been shown also in pejerrey that cortisol up-regulates the synthesis of 11-KT and consequently drives masculinization (Fernandino et al., 2012, 2013).

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Effects of tank background color on sex determination of pejerrey *Odontesthes bonariensis*

Abstract

The pejerrey *Odontesthes bonariensis* is an atherinopsid species from South America that presents a combination of genotypic (GSD) and environmental (temperature-dependent; TSD) sex determination. Low and high temperatures are associated with feminization and masculinization regardless of the genotype. Masculinization involves a heat-induced stress response with high release of cortisol. In this context, we tested whether differences in background color could elicit a similar stress response as of temperature and affect the sex ratios of pejerrey. Two progenies from single crosses of pejerrey with XX and XY genotypes were exposed to different tank background colors (black, gray, dark blue, light blue, green, red and white) at 10 and 15 larvae/L (first and second progeny, respectively), during the

critical period of sex determination (1-5 weeks after hatching) and 25°C, a mixed-sex promoting temperature. Fish were sampled at the middle of the critical period (about 3 weeks) for whole-body cortisol and 11-KT titers by ELISA and after completion of gonadal sex differentiation for determination of sex reversal rates. Sex reversals were inferred from histological analysis of the gonads and detection of *amhy* gene as the genotypic sex marker by PCR. Background color did not affect the cortisol titers and the sex reversal rates in a consistent manner although some groups showed elevated cortisol.

Introduction

Pejerrey *Odontesthes bonariensis* is a brackish water fish species which in natural environments inhabits shallow lakes of the Pampa region (Argentina). These lakes produce abundant phytoplankton which in turn produce high levels of chlorophyll, giving the waters a green and turbid appearance (Quirós et al., 2002). Most of these lakes are eutrophic and have a high content of dissolved organic matter. The organic matter and phytoplankton absorb short wavelengths of light, causing longer wavelengths to be dispersed through the surrounding water (Bowmaker, 2011). However, these shallow lakes are variable environments that pass-through periods of high and low water levels, causing marked shifts in their physical and chemical variables (Quirós et al., 2002; Gómez et al., 2007). These facts suggest that pejerrey are frequently exposed to color changes in the surrounding color environment in its natural habitat, and these may be associated with stress. Interestingly, pejerrey presents strong temperature-dependent sex determination (TSD), a form of environmental sex determination (ESD), and masculinization by high temperatures in pejerrey is associated with thermal stress and elevated blood cortisol concentrations (Hattori et al., 2009).

Background color and light are important environmental conditions for the recognition of conspecifics, avoidance of predators, searching for food, as well as regulation of reproductive and migratory cycles, and therefore have pervasive effects on the physiology of fish (Wagner, 2011). Background color and light conditions are also important in aquaculture, for example, where they are used to optimize growth, control reproductive cycles or produce a desirable body pigmentation such as in the case of aquarium fish (Mclean et al., 2008; Franke et al., 2013).

Many fish species show stress responses when subjected to changes in background color (McLean et al., 2008). For example, Nile tilapia have reduced cortisol levels in light blue tanks during confinement (Volpato and Barreto, 2001). The same color also positively affects reproduction in Nile tilapia, with improved courtship behavior and male nest construction (Volpato et al., 2004). Interestingly, transfer of southern flounder *Paralichthys lethostigma* juveniles to blue tanks at 60 days after hatching causes masculinization, and this phenomenon appears to be related to increased cortisol levels after transfer (Mankiewicz et al., 2013).

Since background color and light conditions may induce a stress response and cortisol increases have been associated with environmental sex determination in fish (Hattori et al.,2009; Yamaguchi et al., 2010; Fernandino et al., 2012, 2013; Mankiewicz et al., 2013) it is possible that background color represents an environmental sex determination form for pejerrey. In this context, this study was designed to explore the effects of background color on the sex determination of this species.

Materials and Methods

Source of broodstock fish and fertilized eggs

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Experimental design

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Determination of genotypic and phenotypic sex

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EIA measurement of whole-body cortisol and 11-Ketotestosterone

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Statistical analysis

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Results

Growth, survival and skin color pigmentation of fish

以下の内容は、学術雑誌論文として出版する予定があるため公表できない（５年以内に出版予定）。

Percentage of phenotypic males among XX and XY genotypes

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Whole-body cortisol measurements

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Whole-body 11-Ketotestosterone measurements

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Discussion

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Effects of rearing density, available space and perceived proximity of conspecifics on sex determination of pejerrey *Odonteshes bonariensis*

Abstract

The pejerrey is an atherinopsid species from South America that presents a combination of genotypic and environmental (temperature-dependent) sex determination whereby low and high temperatures induce feminization and masculinization, respectively. Masculinization involves a heat-induced stress response leading to increased circulating

cortisol and androgens. We tested whether crowding would elicit a similar response as high temperature and affect the sex ratios of pejerrey. Larvae with XX and XY genotypes were reared at 15, 62 and 250 larvae/L in 0.4, 1.6, and 6.4 L containers during a period considered critical for sex determination at 25 °C, a mixed-sex promoting temperature. Fish were analyzed at 3-7 weeks for whole-body cortisol and 11-ketotestosterone (11-KT) titer and hydroxy-steroid dehydrogenase (*hsd11b2*) mRNA transcript abundance, and after completion of gonadal sex differentiation (10-14 weeks) for determination of phenotypic and genotypic sex mismatches. Crowding was associated with depressed growth, higher cortisol and 11-KT titers, increased *hsd11b2* transcription, and increased frequency of masculinization compared to intermediate and/or low rearing densities. Perceived crowding (by rearing in containers with mirror-finish, reflecting walls) also caused masculinization. These results suggest the possibility that other environmental factors besides temperature can also affect sex determination in pejerrey and that a stress response leading to increased cortisol and androgen levels, which is potentially perceived by the brain, may be a common feature among different forms of environmental sex determination in this species.

Introduction

The pejerrey *Odontesthes bonariensis* is a South American inland water species which belongs to the family Atherinopsidae. This species is characterized by a strong temperature-dependent sex determination (TSD) whereby the fate of the gonadal sex is determined in response to the temperature experienced during the first weeks after hatching, which is considered as the critical period of sex determination for this species (CPSD; Strüssmann et al., 1997). Female-biased sex ratios are produced at low temperatures (female-promoting temperatures or FPT, <19 °C), mixed sex ratios are produced at intermediate temperatures (mixed sex-promoting temperatures or MixPT, 20-28 °C), and all-male progenies are

produced at high temperatures (male-promoting temperatures or MPT, >29 °C) during this period (Strüssmann et al., 1996a, 1997). Interestingly, pejerrey combines this marked TSD with a putative form of genotypic sex determination (GSD). Thus, in this species the Y chromosome carries a copy of the anti-Müllerian hormone *amh*, hence termed *amhy*, that acts as a male sex determinant at MixPT (Yamamoto et al., 2014).

In pejerrey, exposure of larvae during the CPSD to increasing temperatures from MixPT to MPT induces a stress response with elevated cortisol levels and an increase in the proportion of males in the population (Hattori et al., 2009). Similarly, treatment with cortisol during the CPSD induces down-regulation of *cyp19a1a* expression and up-regulation of *amh* expression and gonadal apoptosis, leading to masculinization (Hattori et al., 2009). The mechanism of stress- and cortisol-induced masculinization in pejerrey at high temperatures has been examined by Fernandino et al. (2012, 2013). Those authors noted that the enzymes involved in the synthesis (e.g. 11β-hydroxylase) and inactivation (e.g. 11β-hydroxysteroid dehydrogenase) of cortisol (Perry and Grober, 2003), are also responsible for the conversion of testosterone into 11-hydroxytestosterone and then into 11-ketotestosterone (11-KT), the primary androgen in fish. Hence, masculinization by high temperature in pejerrey has been considered as an indirect consequence of cortisol synthesis and subsequent inactivation (Fernandino et al., 2012, 2013).

Some studies have shown that other environmental factors besides temperature may also act like external stressors and affect sexual development in critical life stages of fish (Strüssmann and Patiño, 1999; Baroiller and D'Cotta, 2001). In southern flounder *Paralichthys lethostigma*, for example, significantly male-biased sex ratios and increases in cortisol were observed when young were reared in blue tanks compared to black and gray backgrounds (Mankiewicz et al., 2013). Other forms of environmental modulation of sex determination also include photoperiod, as in California grunion *Leuresthes tenuis*, where male-biased ratios were reported at 12L:12D and to female-biased ratios at 15L:9D (Brown

et al., 2014), and the pez blanco *Chirostoma estor*, where continuous light induced masculinization (Corona-Herrera et al., 2018), water pH, as in the African cichlid *Pelvicachromis pulcher*, where an acidic (5.5.) pH induced male-biased sex ratios (Reddon and Hurd, 2013), rearing density and social factors as in species of *Anguilla*, where crowding generally leads to male development (Geffroy and Bardonnet, 2016), and water salinity as in European sea bass *Dicentrarchus labrax*, where abrupt changes in salinity induced a higher proportion of males possibly due to osmotic stress (Saillant et al., 2003). Thus, there is increasing evidence linking cortisol with masculinization in fishes (Fernandino et al., 2012, 2013; Goikoetxea et al., 2017).

In this context, it is plausible that non-thermal environmental factors could also affect the sex ratios in pejerrey through the stress axis, but this possibility has never been tested. Knowledge on possible synergisms and antagonisms between environmental factors in terms of stress responses, which could be cumulative, and sex differentiation effects in pejerrey may be key to accurately forecasting sex ratios of wild populations that typically inhabit a multifactorial and ever-changing environment (Hattori et al., 2018; Yamamoto et al., 2019). In this study, we begin this search by examining the effects of rearing density and container volume on the sex determination of pejerrey, including a preliminary assessment of the possibility of cortisol- and 11-KT mediation of this process and of the existence of genotypic sex dimorphism in hormonal levels.

Materials and Methods

Source of broodstock fish and fertilized eggs

All procedures and fish handling in this study were done in accordance with the Guide for the Care and Use of Laboratory Animals from Tokyo University of Marine Science and Technology (TUMSAT), Japan. Fertilized eggs of known genotype (XX and XY) were

obtained from single-pair crosses of pejerrey broodstock from two strains, the Yoshida and Yasuda strains, that are kept at the Aquatic Animal Rearing Facilities of TUMSAT. The Yoshida strain is a population bred for several generations at the Yoshida Experimental Station of TUMSAT (Shizuoka, Japan) whereas fish of the Yasuda strain were a kind gift from a local aquaculture farm (Yasuda Co. Ltd, Saitama, Japan). Both strains have been separated and bred under different thermal conditions (e.g. lower temperatures for the Yoshida strain) for at least 25 years (about 8 generations), and the former is considered to have a stronger genotypic sex determination than the latter (Zhang et al., 2018). Fertilized eggs and embryos were incubated at 17 °C until hatching.

Experimental design, rearing conditions, and sampling methods

Three trials were performed: one trial with fish from the Yoshida strain and two with fish from the Yasuda strain. Fish were reared in circular containers with volumes of 0.4 (small), 1.6 (medium), and 6.4L (large) at densities of 15 (low), 62 (moderate), and 250 (high) larvae/L. The combinations of density and volume were used to discriminate between the effects of fish density and space availability on physiological responses. Combinations of the largest volume (6.4L) with the two highest densities (62 and 250 larvae/L) could not be tested in any of the trials because of limitations in space and larvae available per progeny. Nevertheless, this volume was used in combination with the lowest density to test by Generalized Linear Model (GLM) analysis whether space availability would have any effect per se on sex ratios or modulate the sex ratio response to rearing density. The third trial had only one volume (1.6L) since the GLM analysis in the first two trials revealed only a minor contribution of volume (see details in results) and was designed to corroborate the findings of the first two trials and to provide samples for hormone and gene expression analysis which could not be taken in the first two trials. Each combination of density and volume per family was run in replicate for sex reversal analysis. Additional containers for selected densities

were set aside in the trial with Yoshida strain and in the second trial with Yasuda strain to collect samples for hormone and gene expression analysis as described below.

The rearing containers had white non-gloss bottom and walls which were fitted with regularly-spaced meshed windows, to allow exchange of water with the outside. All containers were immersed in a large communal tank provided with mild aeration and constant supply of dechlorinated tap water containing 0.1-0.2 % NaCl from the four corners to ensure water circulation through the containers and similar water quality regardless of the volume, rearing density, or location of the container inside the communal tank. Mild aeration was provided also inside each of the individual containers to ensure adequate water mixing and oxygen supply. The set of rearing containers was illuminated by a 10W white LED fixture set with a photoperiod of 14L:10D. Water temperature was maintained at the MixPT of 24.5 ± 0.5 °C (Strüssmann et al., 1997). A small number of rearing containers identical to the white containers in shape, size, and presence of meshed windows but made with mirror-finish, reflecting materials were used in paired comparisons with the standard, unreflecting containers to test the sex ratio response to visually simulated high density. Due to limitations of space, mirrored containers were run only for selected combinations of rearing density and volume.

Pejerrey larvae were stocked in the containers at the prescribed rearing densities on the day of hatching. Larvae were fed to satiation only with *Artemia nauplii* three times per day during the first 5 weeks after hatching (wah) and subsequently supplemented with TetraMin flakes. Dead fish were removed and counted daily to monitor changes in rearing density, but no attempt was made to replace dead fish. Fish were sampled individually from each container as they reached a minimum standard length of 32 mm, a size that allows easy identification of gonadal sex by histological analysis (Ito et al., 2005), but only after 10 wah to ensure that all fish had spent longer than the presumptive CPSD and the sex differentiation period (Strüssmann et al., 1997; Ito et al., 2005) under as close as possible to the target

densities. All remaining fish intended for sex ratio and sex reversal analysis were sampled at 14 wah. Fish were collected for hormone and gene expression as indicated below.

Determination of phenotypic and genotypic sex

The body trunks of fish were used for histological determination of phenotypic sex. Trunks were fixed in Bouin's solution and histological specimens were prepared by embedding in Paraplast-Plus, sectioning at 6 μm of thickness, and staining with Hematoxylin-Eosin following the methods described in previous studies (Yamamoto et al., 2014; Zhang et al., 2018). Histological criteria for identification of phenotypic sex followed Ito et al. (2005). The caudal fin of each fish was stored in 100% ethanol and used for genomic DNA extraction and genotypic sex determination following Yamamoto et al. (2014). The genotypic sex was inferred by amplification of the *amhy* promoter gene as a male marker following the methods and conditions described in Yamamoto et al. (2014) and Zhang et al. (2018). Fish without *amhy* amplification were denoted as XX and those with it as XY.

EIA measurement of whole-body cortisol and 11-ketotestosterone

Fish for hormone measurements from the second trial with the Yasuda strain were sampled from the three rearing densities at 3, 4, 5, and 6 wah to cover the CPSD of pejerrey (Strüssmann et al., 1997), when body cortisol levels seem to affect sex determination in this species (Hattori et al., 2009). A single point sampling was available for the Yoshida strain at 7 wah. Four to six larvae were sampled from each of the groups. All fish were quickly collected and immediately anesthetized by immersion in ice-water. Care was taken to standardize the time elapsed from collection to cooling in ice-water (within 30 secs and 1 min for all groups). Standard length and body weight were recorded, and the caudal fin was collected for sex genotype analysis with the fish still on ice. The body was then stored and kept at -80 °C until hormone extraction which was performed following a previous report

that validated the methodology for pejerrey larvae (Hattori et al., 2009), with minor modifications. Briefly, fish were homogenized individually in PBS on ice and suspended in 2 ml of diethyl ether for 15 min at 4°C. Homogenates were then centrifuged at 2.500 rpm for 3 min and frozen at -80 °C to recover the diethyl ether-based liquid phase. This procedure was repeated three times. The recovered phase was evaporated under N_2 and samples were immediately resuspended in EIA buffer. The analysis was performed using Cortisol Express EIA and 11-ketotestosterone EIA kits (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's instructions. The recovery factor for hormone extraction was determined by the cold spike method following the manufacturer's instructions for each batch of samples and was always higher than 90% (intra- and inter-assay variation range of 4-10%). Cortisol EIA plates were analysed in a microplate reader Bio-Rad model 550 (Hercules, CA, USA) and 11-KT EIA plates were analysed in SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA).

Transcriptional analysis of 11β-hydroxysteroid dehydrogenase

The 11β-hydroxysteroid dehydrogenase (*hsd11b2*) is a key enzyme for the conversion from cortisol to cortisone and from 11-hydroxytestosterone to 11-ketotestosterone (Fernandino et al., 2013). For the transcriptional analysis of *hsd11b2*, trunks of individual larvae from the moderate and high rearing densities in the second trial with the Yasuda strain were collected following the same schedule for hormone measurements and stored in RNAlater (Thermo Scientific, Waltham, MA) at -80 °C until use. Procedures of mRNA extraction, cDNA synthesis, qRT-PCR, and the gene-specific primers for *hsd11b2* and *b-actin* (*actb*; as a stable endogenous control) were the same as described in previous studies (Yamamoto et al., 2014; Zhang et al., 2018).

Statistical analysis

The statistical significance of the differences in the percentage of phenotypic males between experimental groups (rearing density/container volume) within each progeny and genotype (XX or XY) was compared with the Chi-Square Test with Yates' correction using GraphPad Prism (v.7.00; GraphPad software, San Diego, CA, USA). The magnitude of the effects of rearing density and container volume on the percentage of males among XX fish was analysed by means of logistic regression models and a best fit model for independent variables (density and volume) was determined using Akaike's Information Criterion (AIC) using R (version 3.3.2; R Development Core Team 2016). The statistical significance of the differences in whole-body cortisol and 11-KT levels and of hydroxy-steroid dehydrogenase gene expression among fish of the same genotype from different treatments was analysed with the Student's T test for paired comparisons and One-way ANOVA followed by the Tukey's multiple comparison test for 3 or more groups using GraphPad Prism (v.7.00).

Results

Fish survival, growth, and percentage of phenotypic males among XX and XY genotypes

The mean, minimum, and maximum survival rates per replicate at 10 wah were 78, 44, and 100%, respectively, in the trial with Yoshida strain, 53, 25, and 76%, respectively, in the first trial with the Yasuda strain, and 65, 52 and 76 % in the second trial with the Yasuda strain. The rearing densities after 10 weeks in the low-, moderate-, and high-density groups at 10 wah were 14, 47, and 152 larvae/L, respectively, in the Yoshida trial, 9.3, 38.2, and 149.8 larvae/L, respectively, in first trial with Yasuda strain, and 11, 32, and 130 larvae/L, respectively, in the second trial with Yasuda strain. Body sizes were recorded weekly only in the second trial with the Yasuda strain and revealed a marked growth depression at the highest rearing density in both XX and XY genotypes compared to the other densities that

became significant at 6 wah. The body sizes of fish in the other trials were measured only sporadically but also revealed marked size disparity in favour of the lower densities by 6-7 wah.

Fish from the two replicates of each combination of rearing density and container volume were pooled for statistical analysis of sex ratios because of the low number of XX and XY individuals in the low density/low volume containers. In most comparisons there was a significant increase in the percentage of phenotypic males among genotypic XX fish reared at 250 larvae/L compared to the low and moderate densities. In the first trial with the Yasuda strain, but not in the other trials, there was also a significant increase in the percentage of sex-reversed XX males at the moderate density (62 larvae/L) compared to the lowest density. These results of the GLM analysis using logistic regression and the Akaike´s Information Criterion indicated a major influence of rearing density and a minor influence of container volume on the percentage of sex-reversed XX males. For example, the lowest AIC values were obtained with models using both density and volume for the trial with Yoshida strain (AIC 200.55) but only density for the first trial with Yasuda strain (AIC 175.04), and yet in both progenies the values for models with only density and with both variables were very approximate.

In the case of XY genotypes, the percentage of phenotypic males was similar regardless of rearing density in the trial with the Yoshida strain (90-100%) and in the second trial with the Yasuda (70-80%). In first trial with the Yasuda strain, however, several XY fish reared at low and moderate densities in the smallest container volume were female. Nevertheless, caution is necessary in interpreting the resulting female-biased sex ratios as they may simply reflect the low number of XY fish available in these groups (particularly the low-density/low volume).

Fish stocked in the mirrored containers at a density of 62 larvae/L in the trial with the Yoshida strain and at a density of 15 larvae/L in 6.4L in the first trial with the Yasuda strain had significantly higher proportions of sex-reversed XX males than in white containers. The single comparison performed with Yasuda 2 indicated a higher percentage of males in the mirrored container but this and all other comparisons of sex ratio between mirrored and white containers yielded no significant differences.

Whole-body cortisol and 11-ketotestosterone measurements

Cortisol titers of fish in the second trial with Yasuda strain showed large individual variation and in general seemed to decrease at 5 wah and then increase at 6 wah. The cortisol titers at the highest rearing density were visibly higher at 6 wah but the differences were not statistically significant. A similar observation was made for fish collected at 7 wah in the trial with the Yoshida strain (results not shown). Fish reared at the highest density had significantly higher 11-KT titers compared to the lowest density at 6 wah whereas fish at the moderate density had intermediate values. The 11-KT titers at 7 wah in the trial with the Yoshida strain were also significantly higher in fish at the highest density compared to the lowest density (results not shown).

Expression profile of hsd11b2

Transcript abundance of *hsd11b2* did not change significantly over time in either rearing density up to 5 wah but was significantly higher in the highest rearing density compared to the moderate density at 6 wah.

Discussion

This study provides evidence that rearing density for larvae during the CPSD can affect sex determination in pejerrey, a species previously known to have another form of environmental sex determination (ESD), e.g. TSD. The experiments showed that increased rearing density, particularly in small rearing volumes, caused an increase in the occurrence of males in both XX in fish of the Yasuda and Yoshida strains. The effects of rearing density on XY fish were not as clear as a response in sex ratio was observed in only one of the two progenies of the Yasuda strain. The Yasuda and Yoshida strains have not yet been fully characterized but it has been hypothesized that the latter has stronger genotypic sex determinants compared to the former, which conversely, is more susceptible to environmental (so far thermal) influences on gonadal sex determination (Zhang et al., 2018). The results of this study lend support to this hypothesis as the magnitude of the differences in the percentage of XX males between rearing densities was greater in the Yasuda strain (e.g. 0-100%) than in the Yoshida strain (0-51.5%) under the same conditions. It is also noteworthy that all XY fish of the Yoshida strain were males whereas part of those of the Yasuda strain, particularly at the low and moderate densities of one progeny, were female. A masculinizing effect of high rearing density has been previously demonstrated in eels (Davey and Jellyman, 2005; Geffroy and Bardonnet, 2016) and in domesticated zebrafish strains (Ribas et al., 2017).

We had hypothesized that crowding would induce masculinization in pejerrey starting with a stress response, as previously demonstrated for heat-induced masculinization (Hattori et al., 2009). This was expected based also on reports that the constant interaction with conspecifics and/or low space availability under high densities are often a cause of stress in fish (Schreck, 1981). In the Japanese eel, for example, solitary-reared individuals present 2 ng/ml of cortisol in blood against 8 ng/ml in group-reared individuals (Chiba et al., 2002), which probably explains the masculinization rates observed under high densities (Davey and Jellyman, 2005; Geffroy and Bardonnet, 2016). However, in this study the cortisol titers

fluctuated widely, and we could not demonstrate a clear pattern of stress response to rearing density. Nevertheless, cortisol titers seemed to increase at the highest density compared to the other densities at around 6-7 wah. This time point may seem beyond the CPSD (3-5 wah; Strüssmann et al., 1997) of pejerrey at the experimental temperature (24.5 ± 0.5 °C) but might likely reflect the severely retarded development of fish in this condition compared to the lower densities. In fact, fish at 6 and 7 wah in trials with the Yasuda (second trial) and Yoshida strain had body sizes comparable to 4-5 wah at the other densities. Since the degree of development in pejerrey is more dependent on body size than age (Chalde et al., 2011), it is plausible that sex was determined later at the highest density in both trials. Interestingly, we noted that pejerrey larvae at higher densities had higher blood levels of 11-KT and *hsd11b2* transcription on the same weeks. All these evidences support the notion that sex was determined late at the highest density and are in concordance with previous reports on cortisol administration- and heat-induced masculinization in this species and the proposed biochemical basis for this phenomenon (see Introduction; Hattori et al., 2009; Fernandino et al., 2012, 2013). In zebrafish, Ribas et al. (2017) also showed evidence that cortisol produced by crowding stress can modulate the androgen pathway and induce male differentiation. Still with relation to the mechanism of crowding-induced masculinization, an interesting finding of this study was that fish reared in containers with mirror-finish, reflecting walls had higher rates of masculinization than those in containers with unreflecting surfaces. This observation suggests that crowding may be perceived as a visual input and this in turn would implicate the brain in the process of rearing density-dependent stress and sex determination effects in pejerrey, just as recently hypothesized for TSD in medaka (Castañeda et al., 2019).

The ecological and evolutionary bases for density-dependent sex determination in pejerrey remains undetermined, as are those for TSD (Strüssmann et al., 1996a; Strüssmann and Patiño, 1999). Pejerrey usually lives in shoaling communities (Gómez et al., 2007) but the details of these associations, such as interactions between individuals, are unknown.

Interestingly, one point in common between the two forms of environmental sex determination is the association of higher cortisol levels with masculinizing conditions (Hattori et al., 2009), and its general association with poor growth. In the case of high crowding-induced masculinization, there is an almost immediate slowdown of growth at high densities, even though every effort was made to provide enough food and prevent competition for this resource. On the other hand, at high, masculinizing temperatures, larvae initially display high growth rates but eventually become smaller than those reared at intermediate and low temperatures (see Ito et al., 2005). In other species too, increases in cortisol above a certain level become deleterious for larval and juvenile growth (Mathiyalagan et al., 1996; Lin et al., 1999; McCormick, 1999). In pejerrey, as in some other atheriniformes, growth rate does not appear to be a determinant of gonadal fate (Conover, 1984, 1992; Strüssmann et al., 1996b, 1996c) but in many of these species, fecundity increases faster with body size in females than males (Conover, 1984, 1992; Strüssmann et al., 2010). Thus, the reproductive fitness of an individual is theoretically affected by the future perspectives for growth and how accurately these perspectives are perceived at the earliest developmental stages and translated into gonadal sex determination. With these considerations in mind, and because the known implications of cortisol on sex determination in many species as discussed earlier, it would be interesting to examine critically in pejerrey and other species with ESD whether stress/cortisol are the ultimate transducers of environmental information during early development, signalling whether an individual should chose a male or female pathway to increase its lifetime reproductive fitness, as suggested by Geffroy and Bardonnet (2016) for eels and Geffroy and Douhard (2019) for other species as well.

In conclusion, this study shows that other environmental stressors besides temperature (rearing density) can also influence sex determination in pejerrey and suggests the possibility that a stress response mediated by cortisol, and eventually also 11-KT titers could be a

common feature of different ESD forms in this species. The relevance of these findings for natural populations remains to be determined and the systematic collection of data on sex ratios, year class strength (recruitment), and environmental variables in its natural habitat may prove crucial to understand this relevance and forecast future populational trends under a variety of future climate scenarios. To this aim, future studies should be directed also towards the examination of the effects on sex determination of other environmental factors such as salinity, dissolved oxygen, pH, food availability, and photoperiod, among others, and their interactions (synergisms/antagonisms).

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Effects of water salinity concentration on sex determination of pejerrey *Odontesthes bonariensis*

Abstract

The pejerrey *Odontesthes bonarsiensis* is an euryhaline fish that presents a combination of environmental (temperature-dependent) and genetic sex determination systems. This species has been used as a model in the study of the effect of environmental stressors in the modulation of sex determination. At high rearing temperatures and high rearing densities the pejerrey presents a high percentage of masculinization of XX and XY genotypic fish, associated with whole-body increases of cortisol and 11-Ketotestosterone (11-KT) titers. In this study I examined the effects of water salinity on pejerrey sex determination. Fish were reared from hatching until completion of sex determination and gonadal differentiation under six salinity concentrations (0, 0.05, 0.1, 0.3, 1 and 3 %). Sex ratios were determined at the end of the experiment by genotypic and phenotypic sex correlation. Whole-body hormone (cortisol and 11-KT) and gene expression (*hsd11b2*, *gr*s, *ar*s, *cyp19a1a* and *amha,*) analyses were conducted at 2, 4 and 2, 4, 6, 8 weeks after hatching (wah), respectively. The results indicated that the percentage of males among XX fish increased stepwise between 0.3 and 1 % salinity whereas in XY larvae the percentage of males increased gradually between 0 and 1-3 % salinity. The cortisol levels at 2 wah were lowest at 1 and 3% for both genotypes whereas at 4 wah there was no clear relation with water salinity. In case of 11-KT, the levels at 2 wah were higher at 0% for XX but the same trend was not evident in XY genotypes; at 4 wah the levels were variable and unrelated to salinity concentration. Transcription of *hsd11b2* increased transiently between 2 and 8 wah in some XX fish at 0, 1 and 3 % salinity and in XY fish at 1 and 3 %. Thus, although there was no clear association between cortisol, 11-KT titers, and sex ratios, masculinization was clearly promoted at the highest water salinities. The fact that a stress response could not be demonstrated in the male-biased groups may be related to the rapid hormone clearance by HSD11B2 action and/or a role of cortisol in osmoregulation. This finding emphasizes the need to explore alternative molecular

pathways such as those involved in osmoregulation and thyroid axis that could potentially interact with the sex determination mechanism in pejerrey.

Introduction

Environmental sex determination (ESD), whereby external variables (temperature, density, photoperiod, etc) can induce sex-reversal and unbalanced sex ratios has been reported in several fish species (Baroiller and D´Cotta, 2001). It was demonstrated in some of these species that environmental factors might cause a stress response leading to increased cortisol titers and to masculinization (Fernandino et al., 2013; Goikoetxea et al., 2017). One of these species, the pejerrey *Odontesthes bonariensis*, is a teleost species that inhabits shallow lakes from the Pampa region of South America, where it is a valuable resource for game fishing and aquaculture (Somoza et al., 2008). The species is characterized by a strong temperature-dependent sex determination (TSD) (Yamamoto et al., 2019). Sex ratios are generally 100% female or 100% male when larvae are reared at temperatures below 17 °C (female-promoting temperatures or FPT) or above 29 °C (male-promoting temperatures or MPT), respectively. At intermediate temperatures, (mixed sex-promoting temperatures or MixPT; usually around 24-26 °C), mixed-sex ratios are produced (Ito et al., 2005). The critical period of sex determination (CPSD) for TSD in pejerrey has been estimated to be between 1 and 5 weeks after hatching (Strüssmann et al., 1997). Noteworthy, pejerrey combines this marked TSD with a genetic sex determination system (GSD) based on the presence of a testis-determining duplication of the anti-müllerian hormone located in the Y chromosome, which has been accordingly termed *amhy.* (Hattori et al., 2018; Yamamoto et al., 2014)

In a recent study in which pejerrey larvae were reared during the CPSD at different rearing densities it was shown that this environmental factor can trigger masculinization of XX individuals (this thesis, 3rd Chapter). In that study, the high rearing density was shown

to cause stress in pejerrey larvae, rising cortisol titers, and leading to masculinization just as observed during exposure to MPT. The mechanism involved in stress-associated masculinization following cortisol buildup involves the deactivation of cortisol and the synthesis of 11-Ketotestosterone (11-KT), the principal androgen in fish, which are both mediated by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2; Fernandino et al., 2012; Yamamoto et al., 2019). This kind of cortisol-androgen crosstalk has been associated with other environmental cues related to ESD besides temperature, such as photoperiod, density, pH, and salinity in other species (Brown et al., 2014; Davey and Jellyman, 2005; Reddon and Hurd, 2013; Saillant et al., 2003). In some species, more than one factor might affect the sex determination. For example, in the California grunion *Leuresthes tenuis* both low temperature and long day photoperiods produced female-biased populations (Brown et al., 2014). In the European seabass *Dicentrarchus labrax*, shifting individuals 93 days after fertilization (the sexually labile period) from a salinity of 1.5% to 4% produced a male-biased population, and this was attributed to osmotic stress (Saillant et al., 2003).

The pejerrey is an euryhaline species that can survive up to salinities around 5% in natural environments (Bucher and Etchegoin, 2006). On the other hand, laboratory studies on survival, oxygen consumption, and stress response in larvae, juveniles and adults of pejerrey showed that the salinity optima for this species is around 0.5 to 2 % (Tsuzuki et al., 2000a; 2000b; 2001; 2008). It is well known that salinity has complex interactions with cortisol in fishes. Cortisol participates in ion uptake and gill remodeling during sea water and freshwater adaptation (McCormick, 2011). Moreover, the direct transfer of freshwater-acclimated fish to sea water and/or vice versa can elicit stress responses, which produces physiological imbalance during and after transfer (Eddy, 1981).

In this context, this study evaluated the possibility that water salinity concentration could affect sex determination of pejerrey. Experiments tested the effects of six different salinities

during the CPSD of pejerrey on gonadal sex, blood cortisol and 11-Ketotestosterone titers, and on the expression of important sex related genes. The genes selected for this analysis were *hsd11b2, gr1, gr2, ar1, ar2, cyp19a1a* and *amha* and the reasons are follows *hsd11b2* is a key enzyme for the conversion from cortisol to cortisone and from 11-hydroxytestosterone to 11-KT, as mentioned above. The action of cortisol is mediated by the glucocorticoid receptors (Grs) that acts like ligand-dependent transcription factors. A similar process occurs with the androgen action that is mediated by the androgen receptors (Ars) that in fish binds with more specificity to 11-KT than testosterone. Finally, gonadal aromatase (*cyp19a1a*) and anti-Mullerian hormone (*amha*) are sex related genes with crucial roles during ovary and testis differentiation, respectively (Fernandino et al., 2012).

Materials and Methods

Rearing procedures and experimental design

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General sampling procedures

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Determination of genotypic sex

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ELISA measurement of whole-body cortisol and 11-KT

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qRT-PCR quantification of hsd11b2, gr1, gr2, ar1, ar2, cyp19a1a and *amha mRNA*

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Phenotypic sex determination and sex ratios

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Statistical analysis

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Results

Survival rates and changes in condition factor K

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Sex ratios and genotypic/phenotypic sex mismatches

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Whole-body cortisol and 11-KT titers

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Transcript abundance of hsd11b2, gr1, gr2, ar1, ar2, cyp19a1a and *amha*

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Discussion

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General Conclusions and Future Perspectives

The pejerrey *Odontesthes bonariensis* has become a good model to study the biological mechanisms involved in environmental sex determination. Previous studies have shown that this species has a strong and well-characterized temperature-dependent sex-determination and that masculinization under high temperatures is correlated with a stress response via an

increase in the level of cortisol. All male or all female single-sex populations of this species can be obtained when the fish are exposed during the critical period of sex determination (CPSD) to low temperatures (at or below17ºC, the female promoting temperatures) and high temperatures (at or above 29ºC, male promoting temperatures). At intermediate temperatures (24~25ºC; mixed-sex producing temperatures), mixed-sex populations can be produced. In the present study, an analysis of three environmental factors (background color, rearing density/space availability, and water salinity) was conducted to know if these factors might act like stressors and affect sex determination in pejerrey, in a similar process as it is triggered by temperature.

Background color did not show any specific relation with cortisol levels and/or rates of sex reversal but it had influence over the physiology of the fish, affecting skin coloration, hormone levels, and survival rate. Exposure to a constant background color might not be a strong stressor since the hormonal stress respons (e.g. cortisol) was not stable. However, it is still possible that a change in color during the CPSD, such as when fish are transferred from one color environment to another, could result in significant stress and cause sex reversal.

On the other hand, rearing density and space availability showed a correlation with the rate of XX male-sex reversal with cortisol, 11-KT, and hsd11b2 expression. Moreover, it became evident that the presence of conspecifics, as revealed by using mirror wall tanks, is perceived visually and that when in excess, becomes a source of stress for pejerrey larvae.

Overall, this study indicates that crowding could be a significant stressor for pejerrey and therefore constitute a form of environmental sex determinant for this species, particularly at intermediate temperatures. It could be interesting to explore the interactions of this factor with rearing temperature, specifically at low temperatures where the molecular and physiological basis of feminization have still not been clarified.

Finally, I showed that water salinity can also affect sex determination and sex ratios in pejerrey. Salinities of 1 and 3% produced a higher incidence of sex reversed XX males and conversely a reduction in the percentage of XY sex reversed females. However, the biochemical (cortisol and 11-KT) and molecular analyses of sex-related genes did not provide a clear link between physiological responses to salinity and masculinization. Although negative, the results suggest the possibility of alternative pathways to masculinization as a result of osmotic stress and osmoregulation. For example, I suggest analyzing the thyroid hormones T3 and T4 that play a critical role during osmoregulation and that have been recently found to be correlated with androgen production and testes development in fish.

In conclusion, future studies should focus on the exploration of alternative genetic and hormonal factors to elucidate their role in stress responses and sex determination in pejerrey. The environment is always undergoing constant change and fish are exposed to a complex network of factors that deeply affect their physiology. These may include, but not be limited to, factors such as pH, food availability, photoperiod, chemical contaminants, etc and their interactions (synergisms/antagonisms).