The Cortisol and Androgen Pathways Cross Talk in High Temperature-Induced Masculinization: The 11β-Hydroxysteroid Dehydrogenase as a Key Enzyme

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In many ectotherm species the gonadal fate is modulated by temperature early in life [temperature-dependent sex determination (TSD)] but the transducer mechanism between temperature and gonadal differentiation is still elusive. We have recently shown that cortisol, the glucocorticoid stress-related hormone in vertebrates, is involved in the TSD process of pejerrey, *Odontesthes bonariensis*. Particularly, all larvae exposed to a male-producing temperature (MPT, 29 C) after hatching showed increased whole-body cortisol and 11-ketotestosterone (11-KT; the main bioactive androgen in fish) levels and developed as males. Moreover, cortisol administration at an intermediate, mixed sex-producing temperature (MixPT, 24 C) caused increases in 11-KT and in the frequency of males, suggesting a relation between this glucocorticoid and androgens during the masculinization process. In order to clarify the link between stress and masculinization, the expression of hydroxysteroid dehydrogenase *(hsd)11b2*, glucocorticoid receptors *gr1* and *gr2*, and androgen receptors *ar1* and *ar2* was analyzed by quantitative real time PCR and *in situ* hybridization in larvae reared at MPT, MixPT, and female-producing temperature (FPT, 17 C) during the sex determination period. We also analyzed the effects of cortisol treatment in larvae reared at MixPT and in adult testicular explants incubated *in vitro*. MPT and cortisol treatment produced significant increases in *hsd11b2* mRNA expression. Also, gonadal explants incubated in the presence of cortisol showed increases of 11-KT levels in the medium. Taken together these results suggest that cortisol promotes 11-KT production during high temperature-induced masculinization by modulation of *hsd11b2* expression and thus drives the morphogenesis of the testes. **(*Endocrinology* 153: 6003–6011, 2012)**

Sex steroids have been considered to play critical roles during sex determination in nonmammalian vertebrates (1). Although they are not considered as initiators of gonadal sex differentiation, their timely appearance and maintenance are fundamental for the subsequent development of ovaries and testes. In teleost fishes, for example, larvae are highly sensitive to sex steroids, and the administration of estrogens or androgens during the critical period of gonadal sex differentiation often leads to a functional sex inversion (2–4). It is currently believed that whereas estrogens are essential for female sex differentiation, androgens, on the other hand, are the product or a consequence of differentiation along the male pathway (2, 5–8). In contrast, high levels of 11-oxygenated androgens or the expression of enzymes involved in their synthesis during the critical period of sex determination/differentiation have been detected in some teleosts (9–11). These differences make the involvement of 11-oxygenated an-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2012-1517 Received May 9, 2012. Accepted September 14, 2012.
First Published Online October 5, 2012

Abbreviations: cyp11β, 11β-Hydroxylase; EIA, enzyme immunoassay; FPT, female-producing temperature; GC, glucocorticoid; GR, GC receptor; HSD, hydroxysteroid dehydrogenase; 11-KT, 11-ketotestosterone; MixPT, mixed-sex producing temperature; MPT, male-producing temperature; 11β-OHT, 11β-hydroxytestosterone; RT-qPCR, quantitative real time PCR; T, testosterone; TSD, temperature-dependent sex determination; wah, weeks after hatching.

drogens in sex determination/differentiation a fact that remains unsolved.

The enzymes involved in steroid synthesis and metabolism are important for regulating tissue-specific steroid action (12). In particular, the 11β-hydroxysteroid dehydrogenase (11β-HSD) plays a key role in the synthesis of 11-oxygenated androgens, and it is also known to be involved in the metabolism of glucocorticoids (GCs) (13, 14). In mammals, two types of 11β-HSD have been found: 11β-HSD1, which acts as an 11-oxo-reductase reduced nicotinamide adenine dinucleotide phosphate-dependent enzyme converting cortisone to cortisol, and 11β-HSD2, which acts as an oxidase nicotinamide adenine dinucleotide-dependent enzyme that converts the active ligand cortisol to cortisone, an inactive form unable to bind to GC receptors (GRs) (15). In fish, only one 11β-HSD has been described so far, and it is considered to be the homolog of the mammalian 11β-HSD2 (12). The importance of 11β-hsd2 for glucocorticoid metabolism in fish is still not clear, but it seems to be important for the final step of the major fish androgen biosynthesis, the 11-ketotestosterone (11-KT). Based on this pathway, testosterone (T) is hydroxylated by 11β-hydroxylase (cyp11β) to 11β-hydroxytestosterone (11β-OHT), which comprises a substrate for 11β-hsd2 to produce 11-KT (16). Although the biosynthesis and bioactivity of 11-KT was reported to be conserved also in mammals, the physiological role(s) of this androgen might not, as suggested by studies in mice (17, 18).

It is known that the activity or synthesis of many steroidogenic enzymes, including those related to androgens, can be affected by the stress axis during the process of gamete maturation (19). The stress axis is activated quite early during development in teleost fish (20), and recent studies have shown that GCs can also play important roles on gonadal fate, particularly during high temperature-induced testicular differentiation, a form of environmental sex determination (10, 21, 22). Elevated cortisol levels were observed during the critical period of sex determination at male-producing temperatures (MPT), and the administration of cortisol to larvae during this period produced a significant increase in the percentage of males in the progeny (10, 21). During the cortisol-induced masculinization, the expression of gonadal aromatase *cyp19a1a*, the inhibition of which is known to be tightly associated with masculinization in fish (2), was repressed. Interestingly, T and 11-KT levels began to rise before the inhibition of gonadal aromatase in pejerrey, and they were positively correlated with cortisol body levels (10, 21). This suggests an interaction between androgens and the stress axis on the masculinization process before the inhibition of the aromatase expression. However, the details of this cross talk and the possible involvement of 11β-hsd2 are still not known.

In vertebrates, cortisol actions are mediated by the glucocorticoid receptors (Grs) and, as with other steroid receptors, they act as ligand-dependent transcription factors on transactivation or repression of glucocorticoid-responsive genes (23). With the exception of zebrafish (24), two paralog *grs* (*gr1* and *gr2*) have been described in teleosts (14, 25), and their expression was shown to be modulated by cortisol in some species (26–29). Androgen actions are also mediated by specific receptors, known as androgen receptors (Ars). Once more, with the exception of zebrafish (30), two androgen receptors [*ar1* (also referred to as *ara*) and *ar2* (or *arb*)] have been described in teleosts (31–34). Teleost Ars preferentially bind 11-KT over T (30, 35, 36) and, therefore, the regulation of Ar expression is important to address androgen actions during the sex determination and differentiation periods.

In this conceptual framework, we investigated the expression patterns of *hsd11b2*, both *grs*, and *ars* during sex determination of pejerrey fish *Odontesthes bonariensis*, a species in which temperature alone can produce all-female or all-male populations (37, 38), to clarify the interactions and effects of temperature, cortisol, and androgens in the mechanism of high temperature-induced masculinization of this species. Given the dual function of 11β-hsd2 in the inactivation of glucocorticoids and the biosynthesis of 11-oxygenated androgens previously mentioned, we also examined the *in vitro* androgen production under cortisol administration.

Materials and Methods

Source of animals and experimental conditions

Fertilized eggs were obtained by artificial insemination and incubated in flowing water at 18–19 C until hatching. Newly hatched larvae were then transferred to 60-liter tanks and reared at 17 C [female-producing temperature (FPT)], 24 C [mixed-sex producing temperature (MixPT)], or 29 C (MPT) for up to 12 wk under a constant light cycle (16 h light, 8-h dark) and salinity of 0.2–0.4%. Fish were fed powdered fish food (TetraMin flakes, Melle, Germany) and live *Artemia sp. nauplii* to satiation four to five times daily. Pharmacological treatments with a cortisol-treated diet (Steraloids, Inc., Newport, RI; 0.8 mg/g of food; for more details see Ref. 8) were conducted only at the MixPT. Sampling was performed every 2 wk between 0 and 6 wk after hatching (wah) for gene expression analysis. Samples for histological determination of sex ratios (n = 30–40 per group) were taken at 12 wah and analyzed following the criteria of Ito *et al.* (39). Fish were handled in accordance with the Univesities Federation for Animal Welfare Handbook on the Care and Management of Laboratory Animals (http://www.ufaw.org.uk) and internal institutional regulations.

Quantification of *hsd11b2, gr1, gr2, ar1,* and *ar2* during gonadal sex differentiation by quantitative real time PCR (RT-qPCR)

Larvae (n = 10 for each sampling point) for RT-qPCR were stored in RNAlater (Invitrogen, Carlsbad, CA) at −80 C until use. Total RNA was extracted from larval trunks for each treatment using 1 ml of TRIzol Reagent (Invitrogen) following the manufacturer's instructions. RNA samples (1 μg) were treated with Deoxyribonuclease I Amplification Grade (Invitrogen) and reverse transcribed using SuperScript III RNase H− (Invitrogen) with oligo(dT)12–18 following the manufacturer's instructions. Primer sets for *hsd11b2*, *gr1*, *gr2*, *ar1*, and *ar2* for RT-qPCR were designed as described previously (40) using the Primer Express program (Applied Biosystems, Foster City, CA) based on sequences available in GenBank (see accession numbers in Table 1). First, the identity of the products was confirmed by sequencing. Then, melting curves were systematically monitored (temperature gradient at 1.1 C/10 sec from 65–94 C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each RT-qPCR was performed in 15-μl reaction volumes containing 2 × FastStart Universal SYBR Green Master (Roche Applied Science, Indianapolis, IN), 1 μl of first-strand cDNA (~25 ng) and each primer at a final concentration between 300 and 600 nM using the MX3000P system (Stratagene, La Jolla, CA). Transcript abundance was quantified using the Standard Curve Method and normalized against reference gene values for β-actin. The relative expression of β-actin did not significantly change over time (data not shown). Tentative threshold gene expression values for female determination/differentiation were calculated as mean values plus 2 SDs of the respective *hsd11b2*, *gr1*, *gr2*, *ar1*, and *ar2* relative expression values at FPT as described by Fernandino *et al.* (41).

In situ hydridization localization of *hsd11b2, gr1, gr2, ar1,* and *ar2* mRNAs in larval gonads

In situ hybridization was performed using previously described protocols for pejerrey tissues (40). Briefly, larvae reared for 4 wk at FPT and MPT were fixed in 4% paraformaldehyde. *In situ* hybridizations were performed using probes based on the primer amplicons listed in Table 1. The different probes were labeled with a mix of ribonucleotide tri-phosphates including biotin-16-uridine-5′-triphosphate (Roche Applied Science). Sections were pretreated with proteinase K (5 μg/ml) for 7.5 min at room temperature, hybridized with biotin-labeled sense or antisense RNA probe solution (1 mg/ml), and incubated overnight in a moist chamber at 55 C. Sections were then washed in 50% formamide/2 × saline-sodium citrate and 0.1 × saline-sodium citrate at 60 C, and incubated 30 min with Streptavidin-alkaline phosphatase-conjugate (1:2000 dilution; Roche Applied Science). These steps and the final detection with nitro-blue tetrazolium/5-bromo-4-chloro-3′-indolyphosphate followed the manufacturer's protocols. Five fish per group were analyzed, and the most representative images were used in the panels for each group.

In vitro exposure of testicular explants to cortisol

Due to the impossibility of conducting experiments with gonads isolated from larvae at these developmental stages, testes from adult males were used to examine the effects of cortisol exposure on androgen metabolism *in vitro*. Testes at maturing stage (42) were excised from the animal and immediately immersed for 2 min in PBS containing 1 mM $CaCl_2$ and $MgCl_2$. After a 2-min buffer wash, testes were cut in 2-mm slices and weighed. Basal culture medium consisted of Leibovitz-15 (Invitrogen) supplemented with 10 mM HEPES (Merck Millipore), 0.5% wt/vol BSA fraction V (Roche Applied Science), 0.4 mg/liter Amphotericine B (Sigma-Aldrich, St. Louis, MO) and 200,000 U/liter Ampicillin (Laboratorios Bagó S.A. Argentina); pH was adjusted to 7.4. Culture medium was supplemented with cortisol (Steraloids, Inc.) at 0, 0.01, 0.1, 1, 10, and 100 ng/ml. To block Grs, a Gr antagonist, RU-486 (Sigma-Aldrich), was used. RU-486 was diluted at 50 mg/ml in L15 medium following the manufacturer's protocol and added at 1 μg/ml to treatments with 0 and 0.1 ng/ml of cortisol. All treatments were performed with four technical replicates. The *in vitro* assay was repeated using testes from other four fishes.

Incubations were performed for 3 h for RT-qPCR or 12 h for enzyme immunoassay (EIA) analysis of androgen levels in a humidified air atmosphere at 22 C. For gene expression quantification, the explants were transferred to TRIzol Reagent and treated as above.

EIA measurement of 11-KT

Testes explants were removed from culture and immediately frozen at −80 C for extraction of total protein, used to normalize 11-KT values. Culture media were diluted 1:5 in EIA buffer before EIA analysis with 11-KT Express EIA Kit (Cayman Chemical, Ann Arbor, MI). Samples were analyzed following the kit manufacturer's instructions in a microplate reader (Bio-Rad model 550; Bio-Rad Laboratories, Hercules, CA). The cross-reactivity of the 11-KT antiserum was: 11-KT, 100%; 4-andro-

TABLE 1. Oligonucleotide primers used in the study

Oligo name	Method	Forward primer or sense	Reverse primer or antisense	Amplicon	Accession no.
hsd11b2	RT-qPCR	cgagctgtctctgatgtccaac	coltgctcagagtgccgaagaagt	64 bp	HM755972
	ISH	gcaattaaccctcactaaaggggcaacaacgccggtgtgtgcg	gtaatacgactcactatagggcgtgtcgggcggcgtagtaacgggcctgcg	571 bp	
gr1	RT-qPCR	cagcacttcgggaggacagag	atggtcgttattgggaggtacagg	114 bp	HQ843506
	ISH	gcaattaaccctcactaaaggggtgtcctccgcagacgtgcc	gtaatacgactcactatagggcgtgtcgggcagggtgctgtc	785 bp	
gr2	RT-qPCR	ccagaacgggcgagaccag	tgtcgtgctctcccatccttcg	178 bp	HM755976
	ISH	gtaatacgactcactatagggtgggtgtcggtgcaggacca	gcaattaaccctcactaaagggttcctgaagcggcaggctgg	534 bp	
ar1	RT-qPCR	ccaggcgtgttcttgtcaga	tgacaacccgaggcatcat	64 bp	HM755973
	ISH	gcaattaaccctcactaaaggggggccgactggagccacacag	gtaatacgactcactatagggcgtgtcgggcgacgtggggctgtcgggttg	857 bp	
ar2	RT-qPCR	gctcgtgctacctcctacccctta	catccgtatggccgaagtgt	72 bp	HM755974
	ISH	gcaattaaccctcactaaaggggccgctcccgcaccgaatgaa	gtaatacgactcactatagggcgtgtcgggcgcgccgt	886 bp	
β-actin	RT-qPCR	gctgtccctgtacgcctctgg	gctcggctgtggtggtgaagc	200 bp	EF044319

All primer sequences are shown as 5′- to 3′-, *left to right*. ISH, *in situ* hybridization.

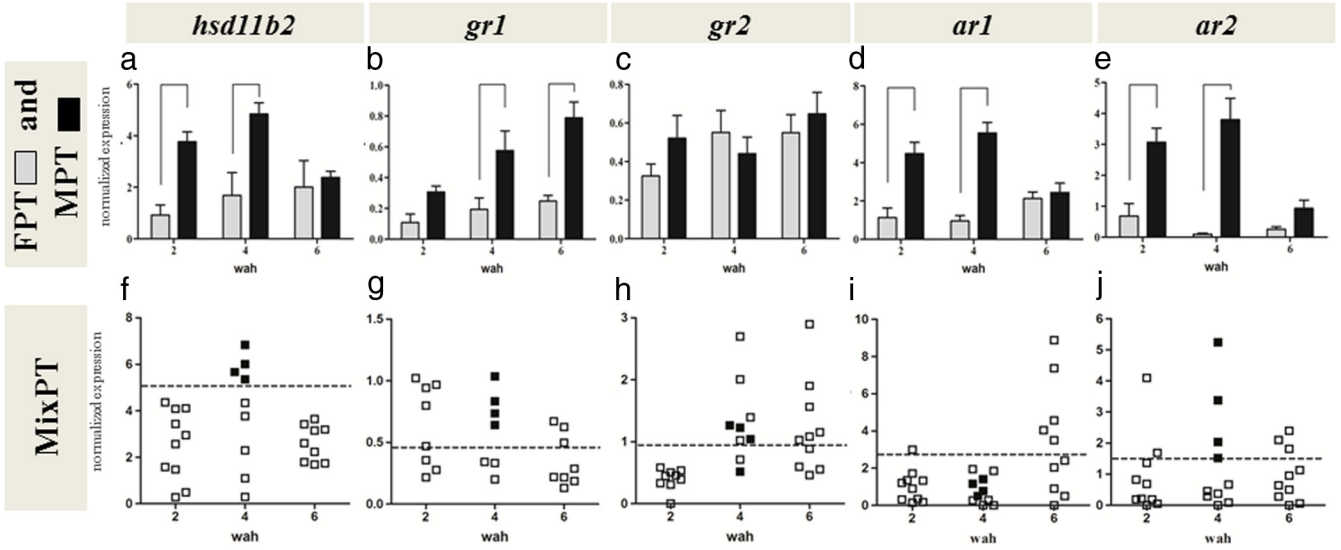

FIG. 1. Relative expression of *hsd11b2, gr1, gr2, ar1,* and *ar2* during sex determination period quantified by RT-qPCR in larvae reared at FPT (17 C, *gray bars*, a, b, c, d, and e), MPT (29 C, *black bars*, a, b, c, d, and e), and MixPT (24 C; 42.86% male, f, g, h, i, and j). Data at MixPT are shown as individual values between 2 and 6 wah. *Dotted line* shows the threshold value (mean + 2 SD) for *hsd11b2, gr1, gr2, ar1,* and *ar2* (5.04, 0.45, 0.96, 3.31, and 1.45, respectively) which were calculated using values from the FPT group. At MixPT, *solid squares* in the 4 wah values indicate the four larvae with high *hsd11b2* expression. *Connecting lines* represent statistically significant differences between temperatures for the same week (Bonferroni's multiple comparison test, $P < 0.05$).

sten-11β,17β-diol-3-one, 0.01%; and T, <0.01%. This assay has been previously validated in pejerrey (43).

Statistical analysis

Data are presented as mean and SE of the mean (SEM) for FPT, MPT, cortisol-treated groups, and *in vitro* testes culture, and as scattered plots for the MixPT experiment to highlight individual differences. Normality of data and homoscedasticity were tested using the Kolmogorov-Smirnov test (SPSS software). Then, one way ANOVA followed by the Bonferroni's multiple comparison test was used to compare the temperature treatments using GraphPad Software (version 4.00; San Diego, CA). A Binomial test (SPSS software) was used for the analysis of the expression values in larvae from cortisol treatment at MixPT. Statistical significant differences were considered at $P < 0.05$.

Results

Sex ratios at FPT, MixPT, and MPT and in cortisol-treated group

To assess whether thermal treatments were effective or not on directing sexual development, gonadal sex was determined by histology at the end of the experimental period (12 wah). In the first experiment, larvae reared at the FPT, MixPT, considered as a neutral temperature, and MPT resulted in 0% (n = 10), 42.86% (n = 21), and 100% (n = 10) males, respectively. In the second experiment, larvae treated with a diet supplemented with cortisol at the MixPT had 100% (n = 15) whereas the respective control had 69.23% (n = 13) males.

RT-qPCR analysis of *hsd11b2, gr1, gr2, ar1,* and *ar2* during gonadal sex determination and cortisol treatment

The results of relative quantification of *hsd11b2*, *gr1*, *gr2*, *ar1*, and *ar2* transcript abundance are shown in Fig. 1. The expression of *hsd11b2* in larval trunks was significantly higher at the MPT compared with the FPT at 2 and 4 wah but not at 6 wah (Fig. 1A). The expression of *gr1* increased steadily between 2 and 6 wah at the MPT but not at the FPT and was significantly higher in the former at 4 and 6 wah (Fig. 1B). On the other hand, *gr2* neither showed any significant difference between both temperatures nor did it show any clear trend during the analyzed period (Fig. 1C). Both *ars* showed similar expression patterns with significantly higher values at the MPT compared with the FPT at 2 and 4 wah followed by decreases in the former at 6 wah (Fig. 1, D and E).

At the MixPT, *hsd11b2* expression was low in all individuals examined at 2 and 6 wah. At 4 wah, however, four of nine individuals (44.4%) showed *hsd11b2* expression above the threshold value for the FPT (5.04; Fig. 1F). Thus, the expression values at 4 wah for MixPT resembled either the values observed at the MPT or FPT. The expression of *gr1* increased in four of seven individuals at the MixPT between 2 and 4 wah. As for *hsd11b2* at 4 wah, *gr1* expression values resembled either values observed at the FPT or the MPT (Fig. 1G). It is important to note that the same four larvae with high *hsd11b2* at 4 wah also presented high *gr1* expression (*solid squares* in Fig. 1, F and G). The expression of *gr2* at the

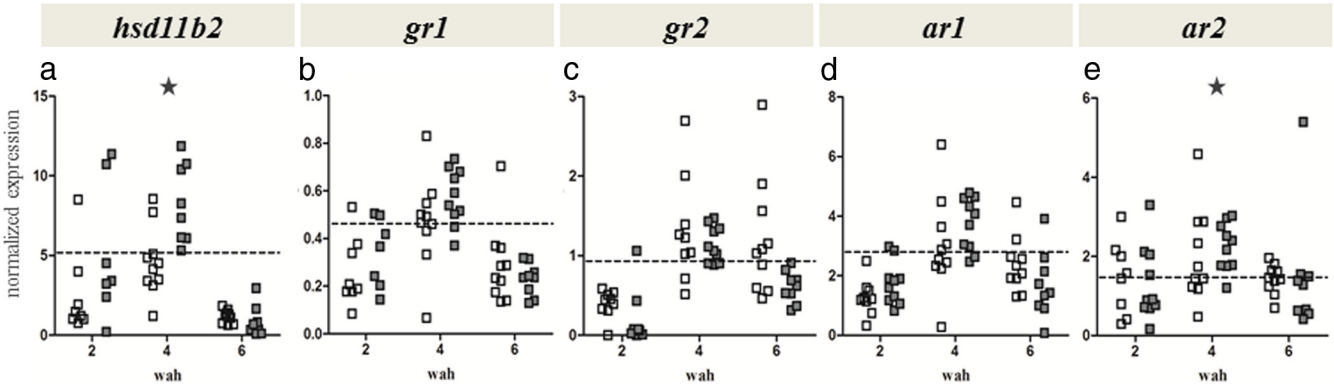

FIG. 2. mRNA abundance of *hsd11b2, gr1, gr2, ar1,* and *ar2* (a to e, respectively) in cortisol-treated (0.8 mg/g of diet) during the sex determination period. Data are shown as individual values for seven to 10 larvae each in both control and cortisol-treated groups. *Horizontal dotted lines* represent threshold values (mean + 2 SD) for *hsd11b2* (5.04), *gr1* (0.45), *gr2* (0.96), *ar1* (3.31), and *ar2* (1.45) that were calculated using values from the FPT group. *Asterisks* indicate statistically significant differences between groups (binomial test, $P < 0.05$).

MixPT showed no clear trend and no evident correlation with *hsd11b2* or *gr1* (Fig. 1H). The expression of *ar1* was low in all individuals at the MixPT between 2 and 4 wah but surpassed the threshold value for the FPT (3.31) in five of 10 individuals at 6 wah (Fig. 1I). On the other hand, *ar2* showed higher expression values in two, four, and three individuals (of 10) at 2, 4, and 6 wah, respectively (Fig. 1J). It is interesting to note that the *ar2* expression values at the MixPT resembled either the values at the FPT or MPT and that the four individuals with high expression at 4 wah also showed high *gr1* on the same week (compare with Fig. 1G).

The expression of both *hsd11b2* and *ar2* in larvae was up-regulated by cortisol treatment at 4 wah, because all larvae at this week had values above the threshold value for the FPT (Fig. 2, A and E). However, this effect was not evident at 2 and 6 wah. No clear changes were observed in cortisol-treated larvae for *gr1*, *gr2*, and *ar1* (Fig. 2, C and D).

In situ hybridization localization of *hsd11b2, gr1, gr2, ar1,* and *ar2* mRNAs in larval gonads

Signals for *hsd11b2* were detected in the gonads of 4 wah larvae at MPT but not at the FPT (Fig. 3, A and B). The expression was restricted to somatic cells that presumably

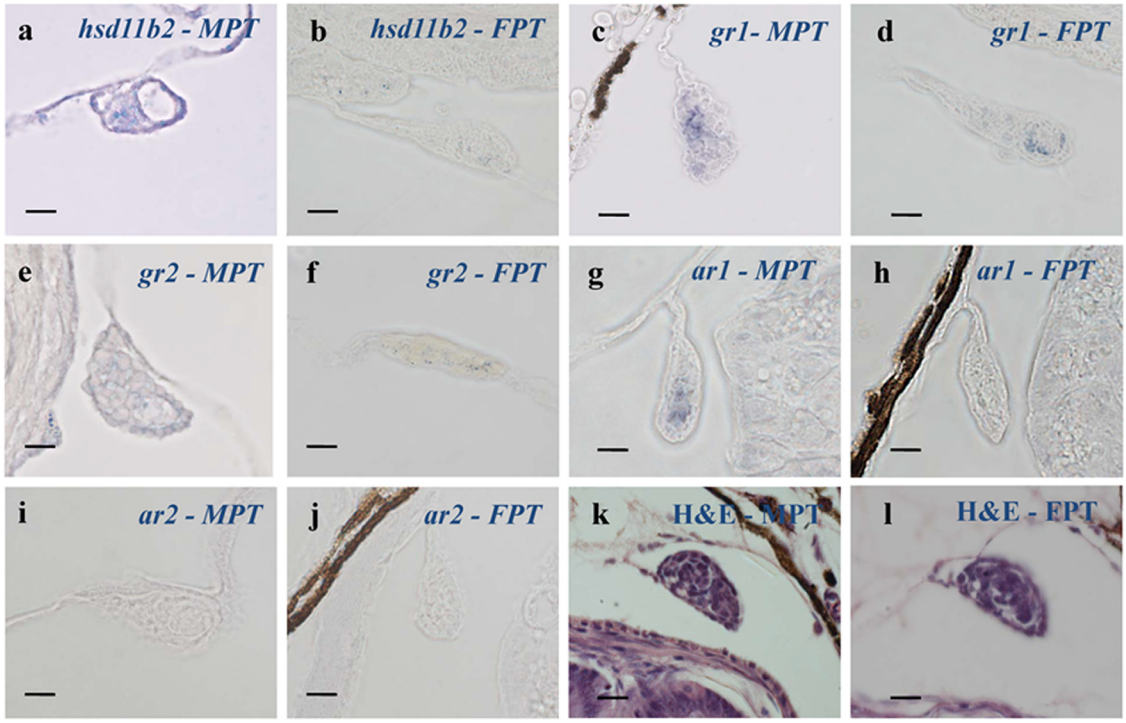

FIG. 3. Localization of *hsd11b2, gr1, gr2, ar1,* and *ar2* expression by *in situ* hybridization in larvae reared at MPT (29 C; a, c, e, g and i) and FPT (17 C; b, d, f, h and j) for 4 wah. Hematoxylin and eosin (H&E)-stained histological sections of 4 wah larvae gonad reared at MPT and FPT were included (k and l). *Scale bars*, 10 μm.

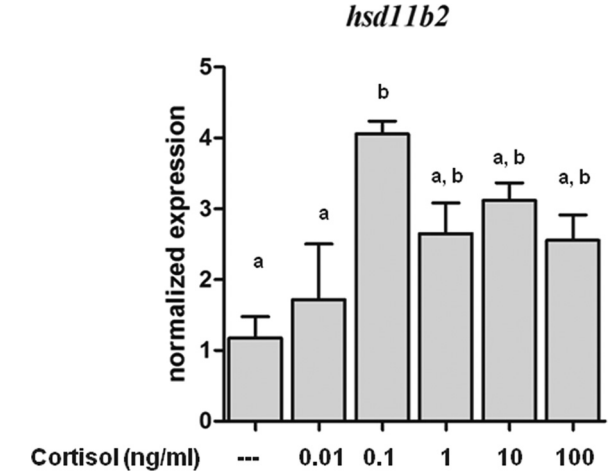

FIG. 4. Expression of *hsd11b2* in an *in vitro* testis culture incubated at increasing dosages of cortisol (0.01, 0.1, 1, 10, and 100 ng/ml). *Different letters* represent statistically significant differences between treated and control groups (Bonferroni's multiple comparison test, $P < 0.05$).

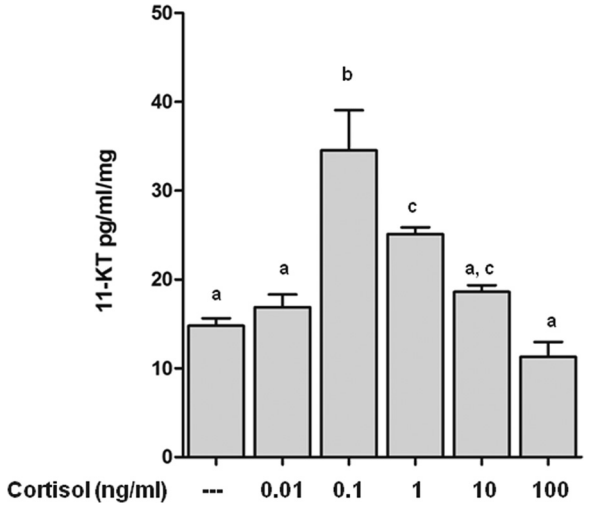

FIG. 5. Quantification of 11-KT in an *in vitro* testis culture incubated at increasing doses of cortisol (0.01, 0.1, 1, 10, and 100 ng/ml). Values were normalized by milligrams of tissue. *Different letters* represent statistically significant differences between treatments (Bonferroni's multiple comparison test, $P < 0.01$).

correspond to Leydig cells (Fig. 3A). Signals for *gr1* in larvae at the MPT were detected in somatic cells in the area of the gonad where blood vessels and sperm ducts eventually form (Fig. 3C). In contrast, signals at the FPT were found in somatic cells surrounding germ cells at a more ventral location in the gonads (Fig. 3D). Feeble signals for *gr2* were observed in gonads from both rearing temperatures with no clear differences among them (Fig. 3, E and F). Finally, *ar1* was detected in medullar somatic cells at the MPT (Figs. 3 G and H) whereas *ar2* signals could not be detected in the gonads at any of the temperatures (Fig. 3, I and J). The signal was not observed with sense probes (Supplemental Fig. 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Gene expression and androgen production in cortisol-treated testicular explants

The incubation of testicular fragments in the presence of cortisol showed an *hsd11b2* overexpression compared with the control group at 0.1 ng/ml but not at other cortisol doses (Fig. 4). The expression of *hsd11b2* was also followed after incubation with 11-KT, but no statistically differences were found (Supplemental Fig. 2).

In the same *in vitro* assay, the synthesis of 11-KT was significantly higher at 0.1 ng/ml, but it decreased with further increases in cortisol concentrations (Fig. 5). The increase in 11-KT induced by 0.1 ng/ml cortisol was blocked by the coadministration of RU486 (Fig. 6).

Discussion

Among the various forms of environmental sex determination, TSD (temperature-dependent sex determination) has been the most studied. Recent studies in fish have implicated cortisol, a stress-related hormone, in masculinization at warm temperature in pejerrey, medaka, and Japanese flounder (10, 21, 22) and also in masculinization of female morphology and behavior of adult mosquito fish (44). However, how cortisol directs the cascade of gonadal differentiation toward masculinization is still not fully understood. It is known that in adult fish the stress and reproductive axes are tightly connected. There are two enzymes that are shared by both axes: cyp11β and 11β-hsd2, responsible for the synthesis and metabolism of glucocorticoids and for the synthesis of 11-oxigenated androgens, respectively (45). These pathways, the glucocorticoids

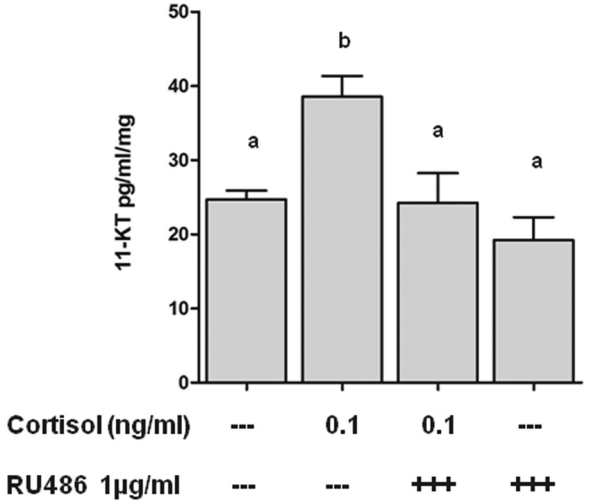

FIG. 6. Quantification of 11-KT in an *in vitro* testis culture incubated with cortisol (0.1 ng/ml) and an antagonist of GR (RU486; 1 μg/ml). Values were normalized by milligrams of tissue. *Different letters* represent statistically significant differences between treatments (Bonferroni's multiple comparison test, $P < 0.05$).

(stress) and androgens (masculinization), could be interconnected during the period of sex determination as well. Within this context, we hypothesized that the increased levels of 11-KT reported in pejerrey larvae during the sex determination period at masculinizing temperature (10) could involve the action of cortisol on the synthesis or activity of 11β-hsd2.

The expression profile of the gene codifying for 11β-hsd2, *hsd11b2*, was analyzed during the period of sex determination and showed increased expression from 2 to 4 wah at the MPT, returning to low levels that resemble those of the FPT at 6 wah. Interestingly, the peak and decline in *hsd11b2* expression coincided with the beginning and the end, respectively, of the critical period of sex determination for this species at the MPT (37–39). The *in situ* hybridization images showed that, as in other fish species (46), the expression of *hsd11b2* was restricted to somatic gonadal cells, presumably Leydig cells. These data suggest that the enzymatic machinery necessary for the local production of 11-oxygenated steroids is already active in the undifferentiated gonads during this critical period. Thus, these findings support the hypothesis that 11β-hsd2 might be responsible for the high 11-KT levels observed in larvae raised at the MPT and therefore for triggering the masculinization cascade. However, the fact that this enzyme occurs also in blood vessels of some fish species (47) should be taken into account, and the real contribution of the gonads to the production of 11-KT should be further studied.

Although in some bony fish species, the synthesis of 11-KT is preceded by the 11β-hydroxylation of T to 11β-OHT by the cyp11b (P45011β) enzyme (16, 48, 49), in pejerrey the expression of *cyp11b* transcripts showed no sexual dimorphism during the sex determination period (11). Intriguingly, those levels increased in males only at later stages of morphological gonadal differentiation. These observations could imply a posttranscriptional regulation of the activity of the cyp11b, during the sex determination period. Another alternative mechanism is based on an optional pathway for synthesizing 11-KT from androstenedione [Δ4 (48, 49)] in which *hsd11b2* would be selectively expressed at the MPT in the presence of 11β-OHT or 11-βOHΔ4. In fish, 11β-OHΔ4 has been considered as the principal steroid produced by the gonads whereas 11-KT is the predominant plasma androgen (50, 51). It is also known that the most abundant 11-oxygenated androgen in adult pejerrey testis is 11β-OHΔ4 (52). However whether 11-oxygenated androgen synthesis occurs from the T or Δ4 pathways remains undetermined.

Ozaki *et al.* (53) observed in an *in vitro* assay with Japanese eel (*Anguilla japonica*) testes explants that cortisol could induce spermatogenesis. They also found that cortisol at low doses enhanced the spermatogonial proliferation induced by 11-KT whereas higher doses inhibited it. They also demonstrated that cortisol was able to induce 11-KT production, suggesting that at optimal levels cortisol could induce spermatogenesis through the synthesis of this androgen. Because experimental procedures with larval gonads are really difficult at these stages, we used adult testes to verify whether such a mechanism would exist in pejerrey testes. Indeed, we observed that cortisol was able to enhance *in vitro* 11-KT synthesis at low doses similarly to those obtained in Japanese eel whereas higher dosages seemed to suppress this effect. The cortisol effects on 11-KT production were abolished under cotreatment with the antagonist of the glucocorticoid receptor RU486; however its action as a progesterone receptor antagonist (54) must be taken into consideration. These results suggest that a certain level of cortisol is necessary to induce 11-KT synthesis in pejerrey.

As previously described, the effects of GCs are mediated through specific intracellular receptors that act as ligand-dependent transcription factors (20, 23). Two *gr* paralogous were detected in pejerrey; however only *gr1* showed overexpression at the MPT compared with the FPT in larval trunks. The same was not observed by *in situ* hybridization, but this might be related to the lower sensitivity of this assay. Differential expression between *grs* was also observed in the Indian medaka *Oryzias dancena*, in which *gr2* was shown to be more sensitive to cortisol and dexamethasone when COS-7-transfected cells with both *gr* forms were compared (27). Glucocorticoid receptors have also been described in fish gonads as can be exemplified by the only characterized *gr* in fathead minnows (*Pimephales promelas*), which was preferentially expressed in male gonads during ontogeny (55). Although *gr2* mRNAs were not clearly observed in the gonads of pejerrey larvae by *in situ* hybridization, the high expression of *gr1* at both temperatures by RT-qPCR in larval trunks may be due to extra gonadal expression, as already observed in other teleost species (24).

In pejerrey, both *ars* were overexpressed during the critical period of sex determination in larvae reared at MPT in relation to FPT. However, in contrast to the bimodal expression pattern detected for *ar2* at MixPT during this period, which resembled those of *hsd11b2* and *gr1*, *ar1* was up-regulated in half of the samples only 2 wk later, at 6 wah, after the critical period of sex determination. Furthermore in the *in vivo* cortisol treatment only *ar2* showed an increase of expression in relation to the respective control. Nevertheless, and possibly because of differences in the sensitivity of the techniques, we could not observe any signal of this androgen receptor in larval gonads reared at MPT by *in situ* hybridization. Those results suggest that different pathways may be triggered during testis differentiation at MPT and MixPT and that their control might not be explained simply by cortisol, at least in gonads. The

association of *ars* expression and the sex determination period has been also reported in other teleost species (31–34, 56), but their role during the masculinization process remains largely unclarified. Nevertheless, both *ars* appeared to be up-regulated by high, masculinizing temperature during the sex determination period in pejerrey. Additional studies should be performed to determine the potential regulators of *ars* expression (cortisol, androgens, or other genes) as well as their possible targets.

The expression of gonadal aromatase (*cyp19a1a*, involved in the conversion of testosterone to estradiol) is inhibited by cortisol during sex differentiation (10, 21) and, as a result, blocks the female differentiation pathway. In case of pejerrey, the high 11-KT levels at MPT (7) and at very early larval stages, before the appearance of any molecular and morphological signatures of gonadal differentiation (39–41, 57), suggest that the *cyp19a1a* inhibition is a result of male differentiation rather than its triggering factor.

In conclusion, pejerrey larvae reared at warm temperature presented an overexpression of *hsd11b2*, *gr1*, and both androgen receptors (*ar1* and *ar2*) at very early stages of sex determination, much earlier than those of other sex-related genes such as *dmrt1*, *cyp19a1a*, and *amh* (40, 57). They, in turn, appear to be correlated with increased synthesis/levels of cortisol and 11-KT during the critical period of sex determination, mainly in the one induced by high temperature. The cross talk of cortisol and 11-KT via 11β-hsd2 might be crucial in TSD and also in other forms of environmental sex determination.

Acknowledgments

We thank Gabriela C. López [Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús (CONICET-UNSAM), Chascomús, Argentina] for helping with histological preparations.

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This work was supported by grants from: Consejo Nacional de Investigaciones Científicas y Técnicas, Fellowship Program for partial financing for a short training period (to J.I.F.), Agencia Nacional de Promoción Científica y Tecnológica Grants 00519 and 01383 (to G.M.S.), and Grant 22380109 from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to C.A.S).

Disclosure Summary: The authors have nothing to disclose.

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