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Presence of 11-ketotestosterone in pre-differentiated male gonads of *Odontesthes bonariensis*

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Abstract The involvement of androgens during sex differentiation period was investigated in the pejerrey *Odontesthes bonariensis*, by classic biochemical studies and gonadal histology. We studied in particular whether the enzyme activities involved in 11-oxygenated androgen production were active in a gonadal/peritoneum complex (GPC) of very small larvae exposed to masculinizing temperatures previous to morphological sex differentiation (5 weeks post-hatching). The GPC was incubated with 17-hydroxyprogesterone (^{3}H-17P), and the presence of 11-KT as major metabolite in early gonads undergoing masculine pathway after temperature treatment exposure is reported. 11-KT was identified by thin-layer chromatography and high-pressure liquid chromatography. The present results show that 11-KT is produced at very early stages of testis development in pejerrey, being this androgen one of the main mediators of the masculinization induced by temperature treatment at the gonad level.

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Introduction

The involvement of steroids in sex differentiation of fish has been reported in many species (Guiguen et al. 2010; Nakamura 2010). Nevertheless, even when androgens have been recognized as inductors of fish sex differentiation since the foundational studies of Yamamoto (1969), their role as early mediators of testis differentiation is still a matter of debate (Vizziano et al. 2007, 2008; Ijiri et al. 2008; Hattori et al. 2009; Blasco et al. 2010; Nakamura 2010). The study of the gonadal androgen synthesis is generally constrained by the gonadal size during the differentiation period, limiting the possibility to establish whether there is an early production of 11-oxygenated androgens preceding testicular differentiation. It is important to note that the temperature sex determination (TSD) observed in pejerrey is of fundamental importance for the study of the processes involved in the sex differentiation. This characteristic allows assuring that a nondifferentiated gonad is going to be an ovary or a testis just by controlling rearing temperature during the first week post-hatching. In brief, those larvae at 29 °C will result in functional males after differentiation (Strüssmann et al. 1997).

In a previous work, we were unable to establish clearly whether larval trunks were able to produce

androgens previous to testis differentiation time working with gene expression of two Leydig cell markers involved in androgen production (i.e., *cyp11a* and *cyp11b*) (Blasco et al. 2010). However, the presence of a clear peak of 11-KT measured by enzyme immunoassay (EIA) in larval trunks of individual exposed to male-promoting temperatures (Hattori et al. 2009) reopened the question about the production of androgens by early gonads previous to differentiation into testis. Then, the potentiality of 11-oxygenated androgen production of the gonadal/peritoneum complex (GPC) of *O. bonariensis* in larvae exposed to masculinizing temperatures (MPT; 29 °C) previous to morphological sex differentiation was studied using a classic biochemical approach. A pool of GPCs taken form 5 weeks post-hatching (wph) larvae was incubated with radiolabeled precursors and then analyzed by thin-layer chromatography (TLC) followed by HPLC. 11-KT was determined as major androgen produced by gonads undergoing testis differentiation.

Materials and methods

As in most fish species, in pejerrey, the small size of the gonads around the period of sex differentiation precludes studies in isolated gonads. Though, in pejerrey, the isolation of the gonads together with peritoneum (gonad–peritoneum complex; GPC) has been feasible as early as 5 wph in fish reared at a male-producing temperature (MPT, 29 °C). This material allowed performing metabolic studies around the time of testicular morphological differentiation, which occur at 6 wph at MPT (Strüssmann et al. 1997). Notwithstanding the isolation of GPCs, the scarcity of the obtained material required analytical techniques displaying detection limits in the order of a few ng/mg of tissue.

The isolation of the GPCs from one hundred larvae followed by incubation with [1,2,6,7-^{3}H(*n*)]-17-hydroxyprogesterone (^{3}H-17P) in 500 ml of L-15 medium allowed us to characterize the main steroidal metabolites. The metabolites were extracted by partitioning into dichloromethane / methanol (9:1 v:v) and then resolved by silica-based TLC using benzene / acetone (4:1 v:v) as developing solvent. The radioactive zones identified by autoradiography were extracted from the silica and further analyzed by C18 reverse phase high-pressure liquid chromatography (RP-HPLC) using methanol / water 40:60 v:v as isocratic mobile phase and RP-HPLC using acetonitrile water gradient (30–100 % in 15 min, 1 ml/min flow) as mobile phase. The co-migration of the reactivity along with the authentic standard steroid was considered as a criterion of identification.

Results

At 5 wph, the gonads of the pejerrey exposed to male-promoting temperatures (29 °C) are completely undifferentiated at a morphological level (Fig. 1). These 5 wph gonads undergoing male differentiation after exposure to high temperature were able to produce 11-KT as major androgen when the precursor 17P was offered to the tissues (Fig. 2). In contrast, when 11β-OHA4 was analyzed within the same experiment, no radioactivity was observed after RP-HPLC chromatography, suggesting that this metabolite was not produced in the gonads during this period (data not shown).

Discussion

We confirmed for the first time that isolated gonads of fish larvae undergoing masculine differentiation (previous to morphological testis differentiation) were able to produce 11-oxygenated androgens. The synthesis of 11-KT when the 17P was added as precursor showed that the enzymes 17,20 hydroxylase-lyase,

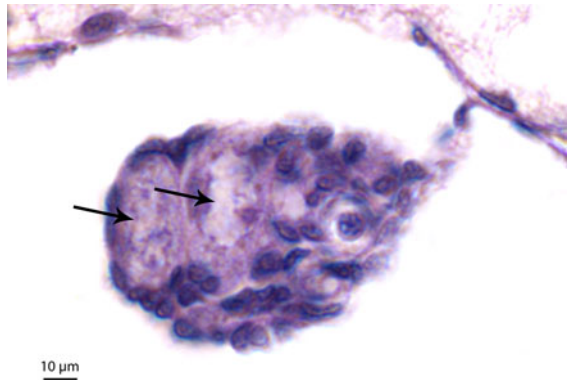

Fig. 1 Histological section of pre-differentiated 5 wph gonad from MPT (29 °C). *Arrows* indicate primordial germ cell. The *scale bar* indicates 10 µm

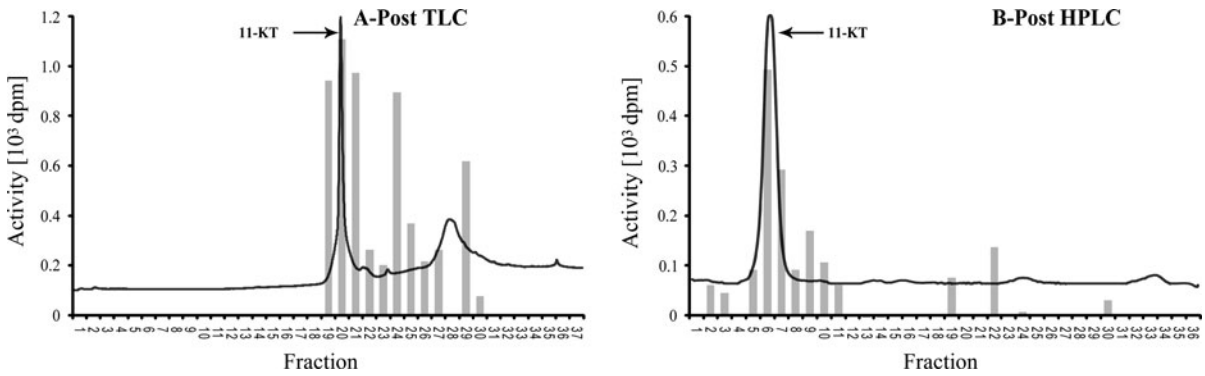

Fig. 2 11-KT identification from ^{3}H-17P-incubated undifferentiated gonads and peritoneum treated at MPT (29 °C). Chromatogram (254 nm; continuous black trace) and fraction radioactivity (*gray bars*) for the 11-KT obtained from A. 11-KT band from the TLC resolved by RP-HPLC (methanol:water 40:60 v:v). B. Fractions 19–21 from A resolved by RP-HPLC (acetonitrile–water gradient)

11β-hydroxylase, 11β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase were active very early during male gonad development, before the morphological differentiation. In previous works, Hattori et al. (2009) observed a peak of 11-KT in larval trunks coinciding with the pre-differentiation time of gonads exposed to male-promoting temperatures (29 °C). However, the gonadal origin of 11-KT has not been demonstrated by Hattori et al. (2009) as pejerrey's kidneys have also being demonstrated to produce 11-oxygenated androgens (Blasco 2010). Then, the potentiality of 11-KT production by pre-differentiated testis (present results) and the presence of 11-KT measured by EIA (Hattori et al. 2009), together with the peak overexpression of *fsh* and *fshr* in larvae at the same stage and treatment conditions (Shinoda et al. 2010), suggest the importance of 11-oxygenated androgens on the temperature sex determination system and a very early regulation of androgens by gonadotropins. This opens the question about the level of action of temperature in the brain–pituitary–gonad axis during the early gonad differentiation in the pejerrey. In previous works made in rainbow trout, *fsh* expression was observed at the pituitary (Saga et al. 1993) around 60 days post-fecundation at the time at which ovaries start to differentiate (Vizziano et al. 2007). Whether the brain–pituitary–gonad axis is already functional at these early stages of development remains to be elucidated in teleosts.

Considering that androgens are not exclusively produced in the gonads, but also in pejerrey kidneys (Blasco 2010), the involvement of androgens produced by other organs should be considered to evaluate its role in the gonadal differentiation process. Furthermore, cortisol should be used as a precursor for the androgens synthesis through the action of liver and gonads as was reported for different fish species (Kime 1978; Fostier et al. 1983). This opens the question of an involvement of the interrenal-liver-gonad axis in the androgen production at early stages of testis development.

In conclusion, we confirmed the potentiality to produce 11-KT by gonads undergoing male differentiation in the pejerrey as a consequence of the masculinization induced by temperature.

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