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Effects of 5α-dihydrotestosterone on expression of genes related to steroidogenesis and spermatogenesis during the sex determination and differentiation periods of the pejerrey, *Odontesthes bonariensis*

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ABSTRACT

Sex steroid hormones are important players in the control of sex differentiation by regulating gonadal development in teleosts. Although estrogens are clearly associated with the ovarian differentiation in teleosts, the effects of androgens on early gonadal development are still a matter of debate. Traditionally, 11-ketotestosterone (11-KT) is considered the major androgen in fish; however, 5α-dihydrotestosterone (5α-DHT), the most potent androgen in tetrapods, was recently found in fish testis and plasma, but its physiological role is still unknown. In this context, the expression of genes associated with steroidogenesis and spermatogenesis, body growth and sex differentiation were assessed in *Odontesthes bonariensis* larvae fed with food supplemented with two doses of 5α-DHT (0.1 and 10 μg/g of food) from hatching to 6 weeks of age. At the lowest dose, 5α-DHT treated larvae showed an estrogenic gene expression pattern, with low *hsd11b2* and high *cyp19a1a* and *er2* expression levels with no differences in sex ratio. At the highest dose, 5α-DHT produced a male-shifted sex ratio and the larvae exhibited a gene expression profile characteristic of an advancement of spermatogenesis, with inhibition of *amh* and stimulation of *ndrg3*. No differences were observed in somatic growth. These results suggest that in this species, 5α-DHT could have a role on sex differentiation and its effects can differ according to the dose.

1. Introduction

The manifestation of sex in vertebrates is the result of two closely and interrelated processes: sex determination and subsequent gonadal differentiation. Sex determination refers to the proximate factors that determine gonadal fate whereas gonadal differentiation is defined as the process by which a morphologically undifferentiated gonad is then transformed into an ovary or a testis through a series of molecular, cellular, and histological benchmarks (Devlin and Nagahama, 2002; Penman and Piferrer, 2008). In teleost fishes, it is well known that exposure to estrogens or androgens during a critical period of development can strongly influence the gonadal fate towards the formation of an ovary or a testis respectively (Baron et al., 2007; Devlin and Nagahama, 2002; Fernandino et al., 2008a, 2008b, 2013a; Guiguen et al., 2010; Nakamura, 2010; Vizziano et al., 2008; Yamamoto, 1969). The major products of synthesis of the gonads are the androgens (mainly testosterone, 11β-hydroxyandrostenedione and 11-ketotestosterone), and estrogens (mainly 17β-estradiol). Although androgens and estrogens are implicated in male and female sex differentiation respectively, estrogens are required for the differentiation and maintenance of the female phenotype, while androgens, in contrast, are currently viewed as the consequence of male differentiation. However, although androgens have also been shown to be involved in sex determination in fish (Fernandino et al., 2012, 2013a, 2013b; Hattori et al., 2009), most of the literature is related to estrogens and less is known about the androgen effects on early gonadal development.

Although 5α-dihydrotestosterone (5α-DHT) is the most important androgen in tetrapod males (Avila et al., 1998), in teleost fish the 11-oxygenated androgens are considered as the more potent androgenic steroids (Borg, 1994; Kime, 1993; Lokman et al., 2002), and the general assumption has been that 5α-DHT had no major physiological role in this vertebrate group (Borg, 1994; Cavaco et al., 1998) until Margiotta-Casaluci et al. (2013a) reported high plasma levels in adult fathead minnow, *Pimephales promelas*. In the same species, exposure to 200 ng/L of 5α-DHT showed an in vivo androgenic potency comparable to 11-ketotestosterone (11-KT). Both androgens stimulated the development of secondary sexual characteristics and spermatogenesis in juvenile males and, in addition altered normal ovarian physiology and morphology leading to development of intersex gonads in juvenile females (Margiotta-Casaluci and Sumpter, 2011). The inhibition of 5α-DHT synthesis, using dutasteride, affected the reproductive performance adult male and female fathead minnows (Margiotta-Casaluci et al., 2013b). However, the effects of 5α-DHT are controversial. Exposure to

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10 μg/L of 5α-DHT plasma inhibits vitellogenin levels (Marlatt et al., 2013) in adult female fathead minnows, yet there are indications that 5α-DHT treatment may also be estrogenic (Martyniuk et al., 2012, 2013; Ornostay et al., 2013). The mRNAs coding for 5α-reductase enzyme variants (with 3 molecular variants in fish: *srd5a1*, *srd5a2*, and *srd5a3*) are expressed early in development in fathead minnow (Martyniuk et al., 2013); however, their potential roles and/or relevance in fish development are not known.

The actions of sex steroids can be observed during the gonadal differentiation period through the subsequent development of ovaries or testes. Besides, these changes can be studied by the expression of key gene involved in this process, sometimes even before the appearance of morphological evidences. For example *amh* which is known to play a key role in the inhibition of the development of the Müllerian and genital tracts in male mammals (Yoshinaga et al., 2004). The expression of this gene is involved in the masculinization process in different vertebrates (Baron et al., 2005; Fernandino et al., 2008b; Klattig and Englert, 2007; Rodriguez-Mari et al., 2005; Skaar et al., 2011; Yoshinaga et al., 2004) and, although teleost fish lack Müllerian ducts, *amh* orthologues have been described in many fish species (Skaar et al., 2011). This gene has also been associated with the inhibition of spermatogenesis and it is decreased by androgens (Halm et al., 2007; Miura et al., 2002; Nobrega et al., 2010; Schulz et al., 2010; Skaar et al., 2011).

The pejerrey, *Odontesthes bonariensis*, has emerged as an interesting teleost model to study the influence of external factors on the sex determination and the gonadal differentiation process (Fernandino et al., 2013b). This species exhibits a clear temperature-dependent sex determination (TSD). The fate of the differentiating gonads is driven by the environmental temperature, experienced by the fish between 1 and 7 weeks post-hatching (11 to 18 mm body length; depending on the temperature) and the proportion of females formed changes gradually from 100% at 19 °C and below to 0% at 29 °C (Strüssmann et al., 1997). In this context, the aim of the present work was to determine the effects of 5α-DHT administration during larval development on sex ratio, growth and expression of steroidogenesis and spermatogenesis related genes in pejerrey larvae.

2. Materials and methods

2.1. Source of fish and rearing conditions

Recently hatched larvae were obtained from the Estación Hidrobiológica de Chascomús (Ministerio de Asuntos Agrarios, Provincia de Buenos Aires), and transported the next day in plastic bags at ambient temperature to the IIB-INTECH aquatic facilities. Approximately 400 larvae (6–7 mm length) were housed in 200 L tanks and maintained under a constant photoperiod (16 L:8D) in a closed circulation water system at a controlled temperature (25 ± 0.5 °C) for 11 weeks. In pejerrey, this temperature produces a sex ratio close to 1:1 female–male (Strüssmann et al., 1997). During the experimental period, the larvae were fed ad libitum three times a day with commercial food (Shullet S.A., Argentina) and once a day with live food (*Artemia spp. nauplii*). Larvae were handled in accordance with the UFAW Handbook on the Care and Management of Laboratory Animals (http://www.ufaw.org.uk) and the IIB-INTECH internal institutional regulations.

2.2. Hormonal treatments

Three treatments were conducted in duplicate tanks by feeding larvae with commercial fish food supplemented with 5α-DHT: 0 (control), 0.1 and 10 μg 5α-DHT/g of fish food. The androgen was dissolved in ethanol, added to the commercial fish food, then homogenized in a ceramic mortar and dried at 4 °C. The hormonal treatments were applied during a 6 week period from 0 days post-hatching (dph), covering the period of sex determination and gonadal differentiation in this species (Strüssmann et al., 1997). From week 7 all groups were fed with commercial fish food with no hormone added, until week 11, when the experiment ended. Dosage and mode of administration were selected according to previous studies in this species (Fernandino et al., 2008b; Hattori et al., 2009; Karube et al., 2007).

2.3. Sampling and sexing

Fifteen individuals were sampled from each treatment at 2, 4, 6 and 11 weeks post-hatching (wph). The larvae were anesthetized on ice, weighted (W) and measured with a digital caliper (total length: TL). The condition factor (K) was then calculated as follows: $K = W \times 1000 \times L^{-3}$. Ten individuals from each treatment were sampled at 6 wph and anesthetized, and whole body was submerged in RNAlater (Sigma-Aldrich) and stored at −80 °C for the gene expression studies. At the end of the experimental period (11 wph), approximately 20 individuals per treatment (10 per tank) were sampled for sexing by histology following the criteria established by Ito et al. (2005). Briefly, the fish were anesthetized, immersed in Bouin's solution, and stored overnight at 4 °C; they were then dehydrated and embedded in paraffin before being transversally cut at 7 μm with a Leica RM2125RT microtome, then stained with hematoxylin and eosin, and examined under a microscope (Nikon Eclipse E200).

2.4. RNA extraction

The larval trunks (with no head and caudal peduncle), stored in RNAlater, were individually processed to extract total RNA using Trizol reagent (InvitrogenTM, Life Technologies). The RNA concentration and the quality of each sample were established by spectrophotometry and the integrity of the samples analyzed by electrophoresis with a denaturing 1.2% agarose gel.

2.5. Quantification by real time quantitative PCR (RT-qPCR)

One microgram RNA from each larval trunk was first treated with deoxyribonuclease I (Amplification Grade; InvitrogenTM, Life Technologies) and then reverse transcribed using Superscript II (InvitrogenTM, Life Technologies) and oligo (dT) following the manufacturer's instructions. The expression profile of different genes related to pejerrey gonadal development was studied at 6 wph, characterized as the onset of the morphological sex differentiation period (Fernandino et al., 2008a, 2008b). The expression of the following genes was quantified by RT-qPCR: *amh* (anti mullerian hormone, GenBank Accession no. **AY763406**), *cyp19a1a* (gonadal aromatase, **EF030342**), *hsd11b2* (11 p hydroxysteroid dehydrogenase, **HM755972**), *ndrg3* (N-myc downstream regulated 3, **GQ381269**), *ar1* (androgen receptor 1, **HM755973**), *ar2* (androgen receptor 2, **HM755974**), *er1* (estrogen receptor 1, **EU284021**), *er2a* (estrogen receptor 2, **EU284022**), and *p-actin* (**EF044319**) as a reference gene. All primers used are given in Table 1.

Each RT-qPCR reaction was performed in 15 μL, containing 7.5 μL of FastStart Universal Master SYBR Green (Roche Applied Science), 1 pL of cDNA and 600 nM of each oligonucleotide. Samples were analyzed with MX3005P equipment (Stratagene). The amplification protocol consisted of an initial cycle of 1 m at 95 °C, followed by 10 s at 95 °C and 30 s at 60 °C for a total of 45 cycles. The subsequent quantification method was performed using the AACt method (threshold cycle, www.appliedbiosystems.com/support/apptech).

2.6. Statistical analysis

Data for wet weight (W) and total length (TL) were analyzed using one-way ANOVA followed by the Bonferroni's Multiple Comparison Test in order to compare all treatment means. The condition factor was analyzed using one-way ANOVA followed by Dunnett's post-hoc test in order to compare each treatment with the control group. Normality of

Table 1
Oligonucleotide primers used in the study.

Gene	Name	Sequence	Size	ACC #
amh	RQamhFw	CCTGTCTCCCGCACTGTTAGA	94 pb	AY763406
	RQamhRv	GGATCCACG111GCCTCACTTA		
cyp19a1a	RQcyp19a1Fw	GCGAGCTGTCTGCTGAGAA	100 pb	EF030342
	RQcyp19a1Rv	AGGAGCAGCAGCATGAAGAAGA		
hsd11b2	RQhsd11b2Fw	CGAGCTGTCTCTGATGTCCAAC	64 pb	HM755972
	RQhsd11b2Rv	TGCTCAGAGTGCCGAAGAAGT		
ndrg3	RQndrg3Fw	TCCCCAGCGGGTATCGTT	61 pb	GQ381269
	RQndrg3Rv	CCAACCATGGACGAGATGGCTGA		
β-actin	RQactinFw	CTCTGGTCGTACCACTGGTATCG	83 pb	EF044319
	RQactinRv	GCAGAGCGTAGCCTTCATAGATG		
ar1	RQarFw	CCAGGCGTGTTCTTGTCAGA	72 pb	HM755973
	RQarRv	TGACAACCCGAGGCATCAT		
ar2	RQar2Fw	GCTCGTGCTACCTCCTACCCTTA	64 pb	HM755974
	RQar2Rv	CATCCGTATGGCCGAAGTGT		
er1	RQer1Fw	GACCAGTCCTCTCGTGTTTGTG	170 pb	EU284021
	RQer1 Rv	CGGCTCCAACTCCCATCTCC		
er2	RQer2Fw	CCCGACCCCAGCATGACCAAC	110 pb	EU284022
	RQer2Rv	CCGATGACACGACACCCTCCTC		

data and homoscedasticity were tested using the Kolmogorov–Smirnov test (SPSS software). In those cases where assumptions of normality and homogeneity were not met, data were analyzed using a non-parametric test (Kruskal–Wallis) followed by Dunn's post-hoc test. Data of sex ratio were analyzed by Binomial test (SPSS sofware). In the case of gene expression fgStatistics software was used (Di Rienzo et al., 2010). In all cases statistical differences were considered to be significant if $p < 0.05$.

3. Results

3.1. Somatic growth

At the end of the experimental period individuals treated with 5α-DHT showed no differences in growth (TL, W and K) compared to the control group. However, larvae treated with the highest dose showed a lower growth rate compared to those treated with 0.1 μg 5α-DHT/g. At 2 wph larvae treated with 0.1 μg 5α-DHT/g presented an increased TL and W compared to both, the control and the 10 μg 5α-DHT/g treated groups; also, at 4 wph a significant decrease in TL and W was observed when the larvae were treated with 10 μg 5α-DHT/g (Fig. 1A and B).

3.2. Sex ratio

At 11 wph, the sex ratio was as follows: the control group presented 60% males, 30% females and 10% undifferentiated individuals; the 0.1 μg 5α-DHT/g treated group presented 59% males and 41% females; and the 10 μg 5α-DHT/g treated group presented 82% males and 18% females (Fig. 2). The treatment with both 5α-DHT doses showed no presence of undifferentiated fish, and the percentage of males in the 10 μg 5α-DHT/g was significantly different from that of the control group (Fig. 2).

3.3. Expression of steroidogenesis and spermatogenesis related genes

The expression profile of genes related to pejerrey gonadal development was studied at 6 wph. This is the time at which first morphological signs of gonadal differentiation can be observed. Most of the analyzed genes were shown to be modulated by 5α-DHT; however non-monotonic dose–response patterns were observed. The expression of *hsd11b2* was only inhibited by 0.1 μg 5α-DHT/g; meanwhile gonadal aromatase, *cyp19a1a*, was up-regulated in the same experimental group (Fig. 3A and B).

Both androgen receptors showed no statistically significant differences between the treatment (Fig. 4A and B). Only *er2* was observed to be up-regulated by 0.1 μg 5α-DHT/g whereas *er1* did not show differences (Fig. 4C and D). The expression of some genes related to testicular development and spermatogenesis was also quantified at 6 wph. While *amh* was inhibited by 10 μg 5α-DHT/g, *ndrg3* was up-regulated in both 5α-DHT treated groups (Fig. 5A and B).

4. Discussion

The present results showed that sex ratio and the expression of genes related to steroidogenesis and spermatogenesis were modified by 5α-DHT treatment during fish development. These data suggest that 5α-DHT may have a role in sex determination and/or gonadal differentiation. It is well known that sex steroids can direct gonadal morphogenesis in teleost fish (Nakamura, 1998, 2010; Piferrer et al., 1993; Piferrer and Donaldson, 1989; Yamamoto, 1969); and also the biochemical and molecular pathways related to steroid synthesis are well characterized in these vertebrates (Borg, 1994; Kime, 1993; Lokman et al., 2002; Nagahama and Yamashita, 2008; Tokarz et al., 2013). Although 5α-DHT was thought to be absent in teleost fish (Borg, 1994; Kime, 1993), this steroid was recently found at relatively high levels in fish plasma (Margiotta-Casaluci et al., 2013a). Information on its role on reproductive physiology and early fish embryogenesis is still lacking. Importantly, the 5α-reductases have recently been reported in early development and exhibit unique expression profiles in the fathead minnow (Martyniuk et al., 2013).

In pejerrey larvae, 0.1 μg 5α-DHT/g treatment induced an inhibition of *hsd11b2* with a concomitant stimulation of *cyp19a1a* and *er2* expression. The inhibition of *hsd11b2* was previously reported in rainbow trout after androgen treatment, probably reflecting the down-regulation of 11-oxygenated androgens synthesis (Vizziano et al., 2008). At the same time, *cyp19a1a* was stimulated by 5α-DHT treatment. Genes coding for enzymes related to steroids synthesis and the androgen and estrogen receptors are known to play a central role in sex determination/differentiation, not only in pejerrey fish, but also in other fish species (Fernandino et al., 2013a, 2013b; Strobl-Mazzulla et al., 2008; Vizziano et al., 2008). Among them, 11β-hydroxysteroiddehydrogenase (encoded by *hsd11b2*) and aromatase *(cyp19a1a)* play key roles in the synthesis of 11-oxygenated androgens (Borg, 1994; Fernandino et al., 2013a; Kime, 1993; Lokman et al., 2002) and 17β-estradiol (Guiguen et al., 2010) respectively. In this regard Mouriec et al. (2009) observed that 5α-DHT also caused an increment of the brain type aromatase (*cyp19a1b*), the teleost paralog of gonadal aromatase, *cyp19a1a* (Kishida and Callard, 2001) in zebrafish. Mouriec et al. (2009) proposed that aromatase expression was increased by 5α-androstane-3β-17β-diol (β-diol), a 5α-DHT metabolite that stimulates transcriptional activity of mammalian estrogen receptors (Kuiper et al., 1998) and binds to *er*β in rat cells (Handa et al., 2011; Oliveira et al., 2007). Thus, the production of β-diol

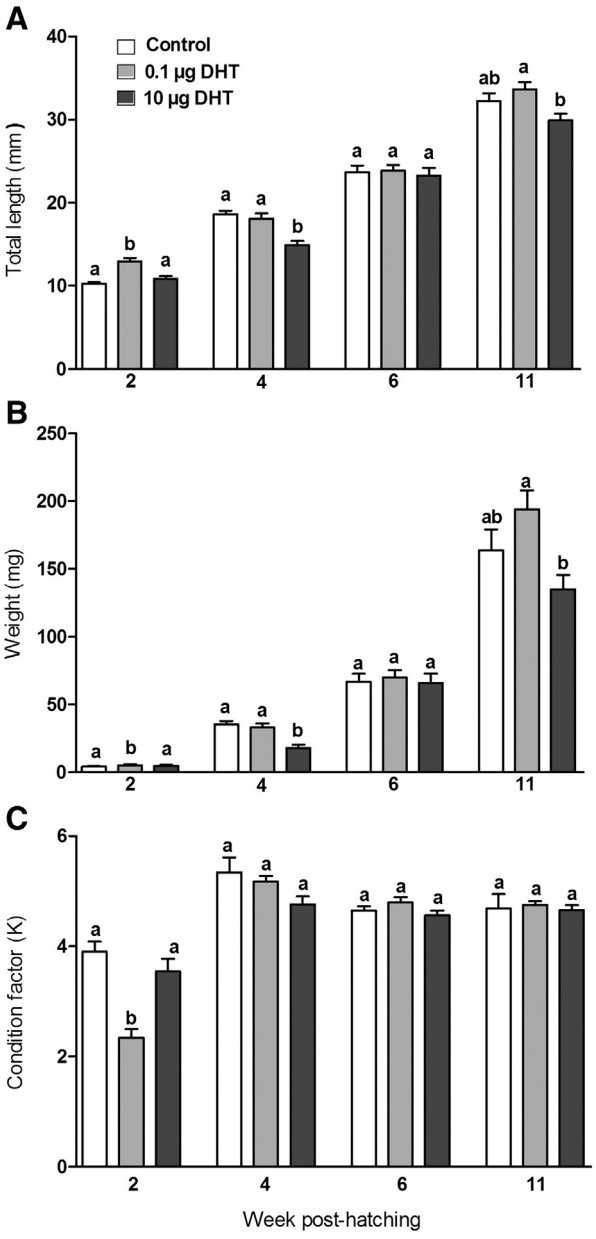

Fig. 1. Effects of 5α-DHT on somatic growth. Total length in mm. (A), total weight in mg (B) and condition factor: K (C) of pejerrey larvae sampled at 2, 4, 6 and 11 wph (n:15). Different letters within the same week means significant differences between treatments (total length and weight were analyzed using a non-parametric test Kruskal–Wallis followed by Dunn's post hoc test and the condition factor using one-way ANOVA followed by Dunnett post hoc test, $p < 0.05$).

from 5α-DHT could stimulate gonadal aromatase, *cyp19a1a*, and *er2* expression in pejerrey, as observed in some fish and mammals. Such a scenario, may explain the female related gene expression pattern at 6 wph with no male biased sex ratio at 0.1 µg 5α-DHT/g dose (Fernandino et al., 2008a, 2008b; Karube et al., 2007; Perez et al., 2012). This line of reasoning was also suggested by Marlatt et al. (2013) and Martyniuk et al. (2013) in order to explain the in vitro vitellogenin stimulation by 5α-DHT in the fathead minnow.

At the highest 5α-DHT dose, the expression profile of the selected genes was not statistically different to those of the control group, but resembled the male expression pattern observed in this species, as characterized by a high *hsd11b2*/*cyp19a1a* ratio (Fernandino et al., 2008a, 2012, 2013b; Karube et al., 2007). Although the mechanism of action is still unclear, high doses of 5α-DHT can raise the proportion of males, indicating that 5α-DHT can promote masculinization, either

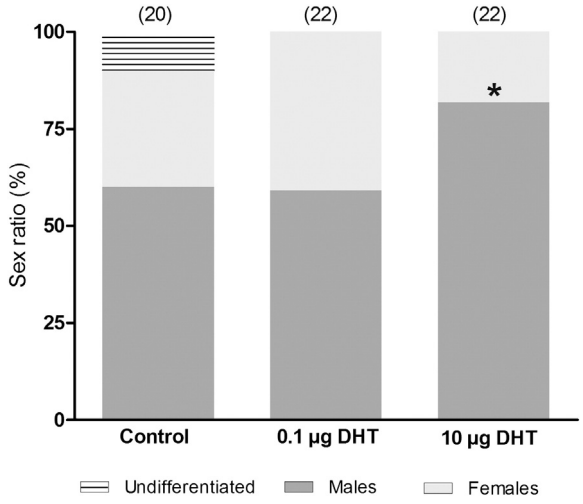

Fig. 2. Effects of 5α-DHT treatment on sex ratio. Percentage of males, females and sexually undifferentiated pejerrey larvae at 11 wph. The numbers over the bars indicate the sample number. Asterisks denote significant difference compared to control, (analyzed using Binomial test: SPSS 152 software, $p < 0.05$).

directly or through the induction of a high *hsd11b2*/*cyp19a1a* expression ratio. These data, together with data reported in fathead minnow (Martyniuk et al., 2013) suggest that 5α-DHT and/or its metabolites interfere at different steroidogenic pathways levels depending on the

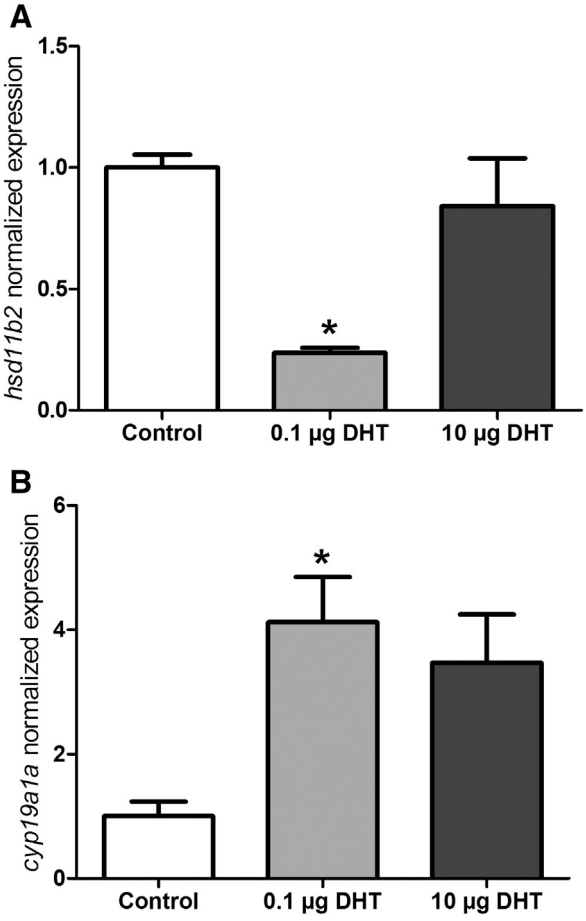

Fig. 3. Effects of 5α-DHT treatment on *hsd11b2* and *cyp19a1a* expression levels. Relative quantification of *hsd11b2* (A) and *cyp19a1a* (B) transcript abundance in pejerrey larval trunks at 6 wph (n:10) by RT-qPCR. Data were normalized against *β-actin* as a reference gene. Asterisks represent significant differences between treatments (analyzed using one-way ANOVA followed by the Bonferroni's Multiple Comparison Test, $p < 0.05$).

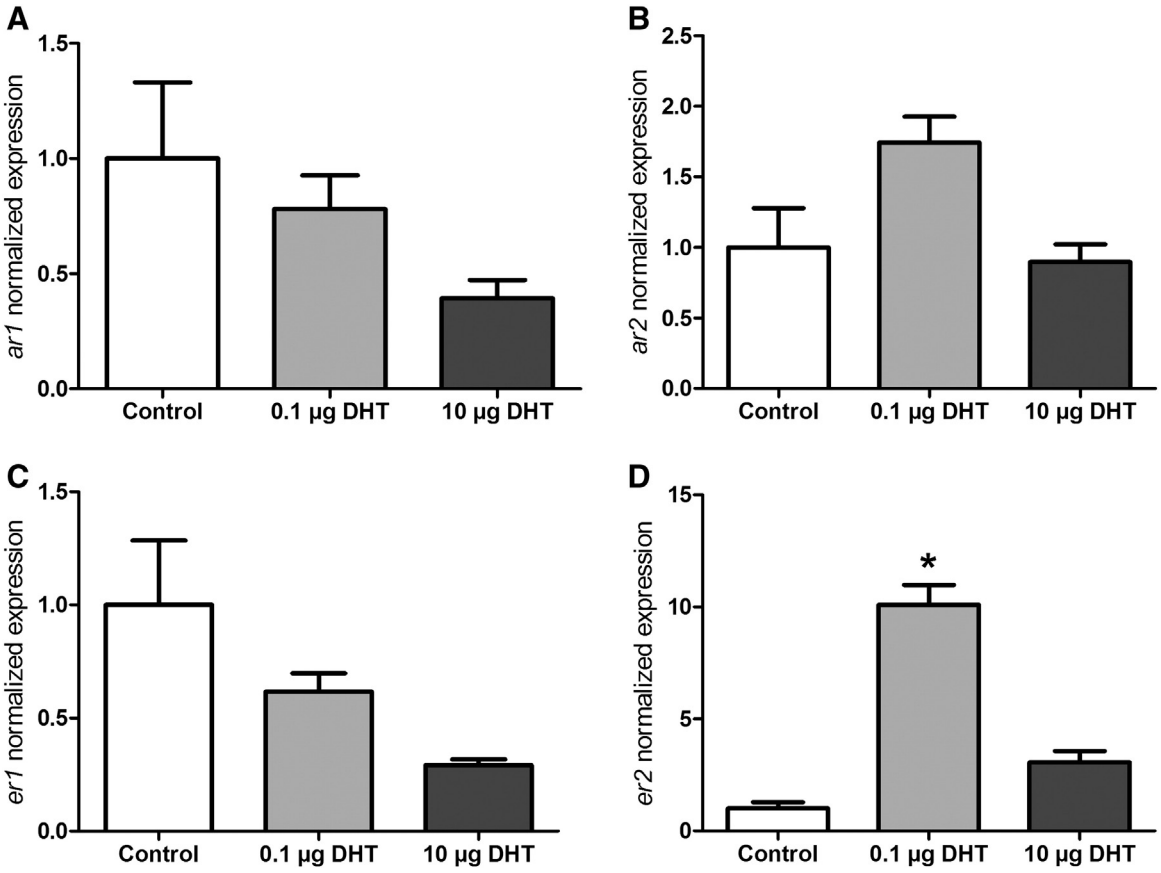

Fig. 4. Effects of 5α-DHT treatment on *ar1, ar2, er1* and *er2* expression levels. Relative quantification of ar1 (A), ar2 (B), er1 (C) and er2 (D) transcript abundance in pejerrey larval trunks at 6 wph (n:10) by RT-qPCR. Data were normalized against *β-actin* as a reference gene. Asterisks represent significant differences between treatments, (analyzed using one-way ANOVA followed by the Bonferroni's Multiple Comparison Test, $p < 0.05$).

dosage, suggesting non-monotonic dose-responses. This kind of response has also been observed for some endocrine disrupting chemicals in multiple vertebrate taxa (Vandenberg et al., 2012).

Since the 5α-DHT treatment affects male differentiation, two genes related to the spermatogenetic pathway, *ndrg3* and *amh*, were analyzed. The former plays an important role in spermatogenesis (Zhao et al., 2001), being strongly regulated by androgens (Wang et al., 2009). In pejerrey, *ndrg3* was shown to be up-regulated during testicular morphogenesis (Fernandino et al., 2011). In this work, *ndrg3* was up-regulated by 5α-DHT treatment, indicating that it is responsive to androgens. On the other hand, we followed the expression of *amh*. In adult fish the rise of *amh* is related to the inhibition of spermatogenesis and it is down regulated by androgens (Halm et al., 2007; Miura et al., 2002; Nobrega et al., 2010; Schulz et al., 2010; Skaar et al., 2011). In pejerrey, *amh* was shown to be associated with the masculinization process and found to be highly expressed in the somatic cells of the primordial testes (Fernandino et al., 2008b); with similar results reported in other teleost species (Baron et al., 2005; Skaar et al., 2011; Vizziano et al., 2008; Yoshinaga et al., 2004). Nevertheless the exact role during gonadal differentiation and spermatogenesis of both genes is still not

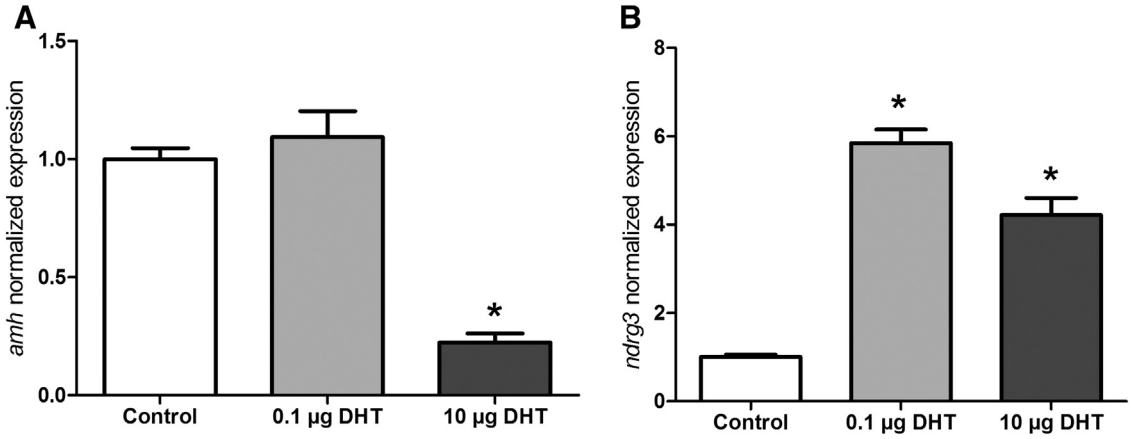

Fig. 5. Effects of 5α-DHT treatment on *amh* and *ndrg3* expression levels. Relative quantification of *amh* (A) and *ndrg3* (B) transcript abundance in pejerrey larval trunks at 6 wph (n:10) by RT-qPCR. Data were normalized against *β-actin* as a reference gene. Asterisks represent significant differences between treatments, (analyzed using one-way ANOVA followed by the Bonferroni's Multiple Comparison Test, $p < 0.05$).

fully understood in pejerrey. It has also been suggested that the inhibition of *amh* and the stimulation of *ndrg3* are both needed at the beginning of spermatogenesis in different fish species (Baron et al., 2005; Fernandino et al., 2008b, 2011; Skaar et al., 2011; Vizziano et al., 2008; Yoshinaga et al., 2004). In the present study those individuals treated with the highest dose of 5α-DHT showed an inhibition of *amh* together with a stimulation of *ndrg3*, with respect to the control group at 6 wph. As the onset of spermatogenesis in pejerrey was characterized between 14 and 17 wph (Strüssmann and Nakamura, 2002), the present results suggest that the spermatogenic process is advanced in 5α-DHT treated individuals. Similar results on the advancement of the spermatogenic processes were observed by Margiotta-Casaluci and Sumpter (2011) in the fathead minnows exposed to waterborne 5α-DHT. However similar effects were not demonstrated in the African catfish (Cavaco, 2005; Cavaco et al., 1998, 2001), suggesting either a species specific differential sensitivity or differences depending on the gonadal stages. Although it is known that androgens are required for spermatogenesis (Cavaco et al., 1997; Schulz and Nobrega, 2011), the role of 5α-DHT on this process is still under discussion (Martyniuk et al., 2013).

Finally, 5α-DHT, as other androgens, has anabolic effects on somatic growth (Margiotta-Casaluci and Sumpter, 2011; Herrera et al., 2008; Davis et al., 2010; Chakraborty et al., 2011). The anabolic effects of androgens are generally explained by the high affinity for their receptors which, in fish, are not only present in the gonads but also in muscle fibers and bones (Hofbauer and Khosla, 1999; Hossain et al, 2008). However Stanko and Angus (2007) observed that 5α-DHT reduced TL and W in mosquito fish. In the present study we could not observed clear differences on this respect.

Taken together, these results indicate that although the role of 5α-DHT was not completely elucidated in pejerrey, evidence of masculinization effects during gonadal differentiation and molecular evidences of an advancement of the beginning of spermatogenesis were observed.

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