17,20β-P and cortisol are the main in vitro metabolites of 17-hydroxy-progesterone produced by spermiating testes of *Micropogonias furnieri* (Desmarest, 1823) (Perciformes: Sciaenidae)

Denise Vizziano Cantonnet¹, Magdalena Mateo¹, Andrés Alberro¹, Florencia Barrios² and Alexis Fostier³

The aim was to investigate the major C21 steroids produced by spermiating white croaker *Micropogonias furnieri* (Sciaenidae) in order to establish the potential mediator of gamete maturation in males of this species. The testes steroid production at the spawning season was identified incubating the ³H-17-hydroxy-4-pregnene-3,20-dione precursor through thin layer chromatography, high pressure liquid chromatography, enzymatic oxydation, acetylation and immunochemistry analyses. 17,20β-Dihydroxy-4-pregnen-3-one (17,20β-P) and 11β,17,21-Trihydroxy-4-pregnene-3,20-dione (cortisol) were the main metabolites produced. Contrary to what we expected, 17,20β,21-Trihydroxy-4-pregnen-3-one was not detected. Circulating levels of 17,20β-P were undetectable in immature testes and in those at the first spermatogenesis stages, while a clear increase was observed during the whole spermatogenesis and spermiation phases (from undetectable to 1047 pg mL⁻¹). *In vitro* studies together with plasma detection suggest that 17,20β-P is a good steroid candidate involved in *M. furnieri* testes maturation. The role of cortisol during late phases of testes development needs further studies.

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**Introduction**

The role of 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) in several teleost fish has been proven as the oocytes maturation-inducing steroid (MIS) during their final development stages. This role is based on its biological effect mediated by specific receptors and its increased level during the ovulatory surge of luteinizing hormone Lh (Fostier *et al*., 1973, 1983, 1987; Scott & Canario, 1987; Nagahama & Yamashita, 2008). However in the past the role played by 17,20β-P in males remained unclear and critical revision articles were written regarding its activity as a maturation-inducing steroid in females (Nagahama, 1987). Moreover, the involvement of 17,20β-P regulating different phases of teleost fish testes development has been highlighted (Schulz *et al*., 2010; Scott *et al*., 2010). The 17,20β-P testes synthesis and release in male blood plasma has been studied in more than 100 species as per Scott *et al*. (2010) revision article. Salmonid is the most studied male fish model. During the reproductve cycle these fish show...
two peaks of 17,20β-P. A small peak occurs during the progression of spermatogonial proliferation and a higher peak during the spermiating phase (Dépêche & Sire, 1982; Baynes & Scott, 1985; Scott & Sumpter, 1989; Vizziano et al., 1995; Khan et al., 1997; Kusakabe et al., 2006; Schulz et al., 2010). The occurrence of a small peak of 17,20β-P at early phases of testes development and the fact that trout non-flagellated germ cells were able to produce 17,20β-P in vitro when its precursor was added (Vizziano et al., 1996), suggested that early events of germ cell development could be regulated by this progesterin. Indeed, 17,20β-P has been proposed as a meiosis regulator in the Japanese eel (Miura et al., 2006) and its effect on the proliferation of early spermatogonia in zebrafish was recently demonstrated (Chen et al., 2013). Moreover, membrane progesterin receptors type β (mPRβ) were detected in zebrafish spermatogonia and spermatocytes using immunohistochemistry analyses (Hanna & Zhu, 2009), while receptors type α (mPRα) were already expressed in the Microgogonias undulatus testes in a stage when only spermatogonia and spermatocytes were present (Tubbs et al., 2010). Furthermore, in Atlantic salmon and in cod the expression of the nuclear progesterone receptor (Pgr) was restricted to Sertoli cells with a strong signal in them contacting type A/early type B spermatogonia (Chen et al., 2012, 2013). In sum, in early stages of testes development germ cells are able to convert the precursors 17-Hydroxy-4-pregnen-3,20-dione and 17P into 17,20β-P in order to regulate their proliferation and enter meiosis via progesterin receptors located at the membrane of germ cells and/or in Sertoli cells.

The high increase of 17,20β-P in salmonid during the spawning season coincides with a clear increase of Lh in plasma during spermiation (Gomez et al., 1999). Furthermore, during the complete testicular cycle, Lh or Fsh can stimulate 17,20β-P both in vivo and in vitro, but Lh stimulation is during the latest testes development phases (Le Gac & Loir, 1988; Schulz & Blüm, 1990; Schulz et al., 1992; Planas et al., 1993; Planas & Swanson, 1995; Vizziano & Le Gac, 1998). These results suggest that 17,20β-P mediates the late phases of testes development. The induction and advancement of spermiation was the clearer function suggested for this steroid (Ueda et al., 1985; Scott et al., 2010). Amplification of mit production (Baynes & Scott, 1985; Yueh & Chang, 1997) and stimulation of spermatozoa motility (Miura et al., 1992; Miura & Miura, 2003; Tubbs & Thomas, 2008) were other proposed functions.

During the spermiating season in non-salmonid male fish it was seen an increase of various progestins i.e. 17,20β,21-Trihydroxy-4-pregnen-3-one (17,20β,21-P), 17,20α-Dihydroxy-4-pregnen-3-one (17,20αP) and 17P in some cases reaching levels higher than 17,20βP (Vizziano et al., 2008). These levels suggest that different progestins could be involved in gamete maturation of other fish species as well. The same condition is seen on females, where several other candidates have been proposed as maturation-inducing steroids, the evidence favouring 17,20β,21-P as plausible alternative to 17,20βP (Scott et al., 2010).

In the Atlantic croaker, Microgogonias undulatus (Linnaeus, 1766) males the 17,20β,21-P affects sperm motility and velocity in vitro (Thomas, 1994). Furthermore 17,20β,21-P plasma levels in Atlantic croaker males have been reported only in one case by Mathews et al. (2002) being undetectable during the recrudescing phase and reaching 1.01 ng/mL at the end of the sexual cycle. Our study model, the white croaker Microgogonias furnieri (Desmarest, 1823), is an important species for fisheries in Argentina, Brazil, and Uruguay. The Río de la Plata estuary and Rocha’s coastal lagoons are their southern reproduction areas (Acha et al., 1999; Vizziano et al., 2002a, 2002b; Macchi et al., 2003). The steroid metabolism of the species female gonads have been previously studied reporting that during the oocyte maturation season female gonads produce both 17,20βP and 17,20β,21-P (García-Alonso et al., 2004). However, no data on steroid metabolism in spermiating males are available. Thus taking into account data reported about M. undulatus here we investigated if 17,20β,21-P was produced by M. furnieri spermatizing testes. However our findings showed that the major metabolites were 17,20βP and 11β,17,21-Trihydroxy-4-pregnene-3,20-dione or cortisol.

**Material and Methods**

**Chemicals.** Nonradioactive steroids were purchased from Sigma Chemical Co. (St. Louis, USA). [1,2,6,7-3H]17-hydroxy-4-pregnen-3,20-dione (3H-17-P, specific activity [sp. act.] = 77 Ci mmol-1) were from Amersham-Life Sciences (U.K.). H-17,20βP, not available commercially, was synthesised enzymatically by reduction of H-17-P with 3a, 20β-hydroxysteroid dehydrogenase Streptomycetes hydrogenans (Sigma, USA) following procedures described by Vizziano et al. (1995). The names of the steroids mentioned in this paper are shown in Table 1.

**Sampling.** Research involving animal experimentation is in accordance with the standards for the use and care of laboratory animals in compliance with Uruguayan regulations on animal welfare (Comisión Honoraria de Experimentación Animal (CHEA)).

Sampling was carried out in the white croaker Microgogonias furnieri major spawning area during the spawning season in the Río de la Plata estuary (Acha et al., 1999; Vizziano et al., 2002a; Macchi et al., 2003). Adult males (> 30 cm, Cousseau et al., 1986) were obtained from artisanal fishers of Río de la Plata coast during the spawning season. The gonad stage was determined after gentle stripping and three spermiating males were selected. The selected males were decervicated and the gonads sampled. The total weight (± g) was recorded and the wet weight of gonads was measured (± 0.1 g) prior to maintain the gonads in cold sterile RPMI Media 1640. The gonadosomatic index was calculated using body (M) and gonad (Mg) mass as: Ig = 100. Mg/M. The
GSI of males were between 2.4 to 3.6%, consistent with GSI of spermiating white croaker males (Vizziano et al., 2002b).

To verify if during the testicular cycle 17,20β-P levels changed in the bloodstream complementary samplings were taken in an oceanographic cruise in the Río de la Plata on the spawning area and season (20-30 January); the cruise was the R/V Aldebarán (DINARA, Uruguay). During the stay in the cruise, blood of 91 croakers was collected from the caudal vessels using a heparinised syringe, maintained in ice and centrifuged for 15 min at 3700 g. Plasma samples were frozen until their analysis by RIA. Fish were manipulated without anesthetic for sampling blood plasma and killed by decervication in order to sample the gonads fixed in Bouin. During the sampling the testicular stage was classified macroscopically following Vizziano et al. (2002b). The 91 males collected presented different stages of development: stage 1 (n=3), stage 2 (n=6), stage 3 (n=12), stage 4 (n=69), stage 6 (n=1).

Table 1. Meaning of common abbreviations used in text following Kime (1995).

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
<th>Other abbreviations or names</th>
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<tbody>
<tr>
<td>P</td>
<td>4-Pregnene-3,20-dione</td>
<td>Progesterone</td>
</tr>
<tr>
<td>17-P</td>
<td>17-Hydroxy-4-pregnene-3,20-dione</td>
<td>17-Hydroxyprogesterone</td>
</tr>
<tr>
<td>17,20β-P</td>
<td>17,20β-Dihydroxy-4-pregnene-3-one</td>
<td>DHP</td>
</tr>
<tr>
<td>17,20α-P</td>
<td>17,20α-Dihydroxy-4-pregnene-3-one</td>
<td></td>
</tr>
<tr>
<td>17,21-P</td>
<td>17,21-Dihydroxy-4-pregnene-3,20-dione</td>
<td>11-deoxy cortisol</td>
</tr>
<tr>
<td>17,20,21-P</td>
<td>17,20β,21-Trihydroxy-4-pregnen-3-one</td>
<td>20β-S</td>
</tr>
<tr>
<td>cortisol</td>
<td>11β,17,21-Trihydroxy-4-pregnen-3,20-dione</td>
<td>Hydrocortisone</td>
</tr>
<tr>
<td>3α,17P-5β</td>
<td>3α,17-dihydroxy-5β-pregn-20-one</td>
<td></td>
</tr>
<tr>
<td>11-deoxycorticosterone</td>
<td>21-Hydroxy-4-pregnen-3,20-dione</td>
<td>DOC</td>
</tr>
<tr>
<td>11-20βS</td>
<td>17,20β,21-Trihydroxy-4-pregnen-3,11-dione</td>
<td>20-dihydrocortisone</td>
</tr>
<tr>
<td>E</td>
<td>17,21-Dihydroxy-4-pregnen-3,11,20-trione</td>
<td>Cortisone</td>
</tr>
<tr>
<td>11β,21-P</td>
<td>11β,21-Dihydroxy-4-pregnen-3,20-dione</td>
<td>Corticosterone</td>
</tr>
<tr>
<td>A</td>
<td>4-Androstene-3,17-dione</td>
<td>Androstenedione</td>
</tr>
<tr>
<td>T</td>
<td>17β-Hydroxy-4-androstene-3-one</td>
<td>Testosterone</td>
</tr>
<tr>
<td>11KT</td>
<td>17β-hydroxy-4-androstene-3,11-dione</td>
<td>11-ketotestosterone</td>
</tr>
<tr>
<td>11βOH-A</td>
<td>11β-Hydroxy-4-androstene-3,17-dione</td>
<td>11-betahydroxyandrostenedione</td>
</tr>
<tr>
<td>Ad</td>
<td>4-Androstene-3,11,17-trione</td>
<td>Adrenosterone, androstenetriene</td>
</tr>
<tr>
<td>E2</td>
<td>1,3,5(10)-Estratriene-3,17β-diol</td>
<td>17β-estradiol</td>
</tr>
</tbody>
</table>

In vitro incubations. The testes of three males were washed twice in sterile RPMI medium 1640 (Gibco BRL, USA, 10 mM Hepes, pH 7.5) and finely chopped. Testes fragments (350 to 490 mg mL⁻¹) were incubated with 10 µCi of [3H]-17P for 6-7 h, at 22°C. The temperature corresponds to the mean temperature during spawning season in the spawning area in the Río de la Plata (Vizziano et al., 2002a). The reaction was interrupted with ethanol at a final concentration of 80% and 10 µg of unlabeled 17P was added. Incubates were frozen at -20°C until analyzed.

Hormone identification. Testicular fragments and culture medium were homogenized with an Ultra-Turrax (T25, JanKe & Kunkell, Germany) in ethanol/water at a final concentration of 80/20 v/v and steroids were extracted and analyzed as previously described (Vizziano et al., 1996). The radioactivity of the aqueous and organic phases was measured (Intertechnique, SL 4000 counter) and the organic phases were further analyzed by thin layer chromatography (TLC, 60 F²⁵⁴, Merck). The non-polar lipids were eliminated through three migrations in toluene/cyclohexane 50/50 v/v (system I). Steroid metabolites were separated in TLC (two migrations in benzene/acetone 80/20 v/v, system II) and carriers were detected under UV light. A thin layer radiochromatogram scanner (Packard, Model 7220/21) was used to measure the radioactivity of the plates. The radioactivity peaks of the different zones of the TLC were further analyzed by reverse phase HPLC (Nucleosil, C18, 5µm, 4 mm, l=250 mm) after elution with dichloromethane-methanol (90/10 v/v) and adding internal carriers. These zones were analyzed by two different solvent systems: methanol/acetonitrile/water 33/26/41 v/v/v (system III) and tetrahydrofuran/water 35/65 v/v (system IV) with a flow rate of 1 mL min⁻¹. The elution time of internal and external standards was detected at 254 nm (3000 spectromonitor detector, LDC) and the radioactivity of the sample was measured by a Radio-Chromatography Detector (Packard, Series A-500) coupled to the HPLC. The elution times of the authentic inert steroids and the authentic radiolabeled steroids were compared. The difference between online UV absorbance and radioactivity measurements was around 0.5 min. For further examination of the metabolites identified by TLC and HPLC a second replicate was extracted and purified by TLC (systems I and II) and HPLC (system III). After HPLC purification the
radioactivity peaks that co-eluted with 17,20βP and cortisol as well as the polar zone that could contain the 17,20β,21-P were extracted with dichloromethane and further analyzed as described above. The percentage of radioactivity present in each peak was calculated with the Radio-Chromatogram detector software using the radioactivity present in all peaks of the procedure as total radioactivity.

A second replicate of male 2 was extracted and analyzed without internal standards by TLC (systems I and II) and HPLC (system III) for further analysis of peaks corresponding to 17,20βP, cortisol and 17,20β,21-P by oxydation, acetylation and immunochemistry analyses.

**Enzymatic oxidation of metabolites.** After TLC and HPLC purification an enzymatic oxidation of 20β hydroxyl of the metabolite co-eluting with 17,20βP and of the putative area of 17,20β,21-P was carried out. The radiolabeled metabolites were oxidized with 10 µL of a suspension of the enzyme 3-20β-hydroxysteroid dehydrogenase (20βHSD) from Streptomyces hydrogenans (Sigma Co, St Louis, USA) in presence of 1 mM NAD+. Incubation was carried out in a final volume of 1 mL of phosphate buffer (10 mM, pH 7.6) at room temperature for 60 min. The reaction was interrupted with 20 µg of 17P and 5 mL of dichloromethane. The product obtained was extracted 3 times in 5 mL of dichloromethane and analyzed by HPLC (system III).

**Table 2.** Analysis of metabolites produced by testes fragments of three males of *Micropogonias furnieri* at spermiating time. System I = toluene/cyclohexane 50/50 v/v; System II= benzene/acetone 80/20 v/v; System III = methanol/acetonitrile/water 33/26/41 v/v/v; System IV= tetrahydrofuran/water 35/65 v/v.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>System I (TLC)</th>
<th>System II (TLC)</th>
<th>System III (HPLC)</th>
<th>System IV (HPLC)</th>
<th>Enzymatic oxidation</th>
<th>Acetylation</th>
<th>Immunochemical</th>
</tr>
</thead>
<tbody>
<tr>
<td>17,20β-P</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cortisol</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>17,20,21-P</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>17,21-P</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Hormone measurement.** 17,20βP concentration in blood plasma was measured by RIA. Plasma was extracted using ethyl-acetate: cyclohexane 1:1 v/v. Each extract was evaporated and the dry residues were dissolved with phosphate buffer (50 mM, pH 7.4, NaCl 150 mM) with 0.1% of gelatin added. Incubation was carried out for 20 h at 18°C with anti-17,20β-P serum (Fostier et al., 1981) at a final dilution of 1/9000. A cold suspension of dextran coated charcoal (500 mg of charcoal +50 mg of dextran T70 in 100 mL buffer phosphate) was added to the samples and incubated for 15 min at 0°C. Then samples were centrifuged at 3500 g for 15 min at 2°C and supernatants were counted for radioactivity. The percentages of cross reactivities (CR) of each antisera were checked and calculated in a dose which generated a 50% displacement of maximum binding of the tracer as follows: CR = 100 * (std mass / ns mass) where std stands for the antigen and ns stands for the competitive antigen against anti-17,20β-P (Table 3). Sensitivity was 6 pg/tube and the coefficient of variation of the intra assay test was 20%.

**Table 3.** Cross-reactivity of steroids with the anti-17,20β-P used for radioimmunoassay. Cross-reactivity was determined by comparing the concentrations of unlabeled cortisol and unlabeled competitors for the displacement of 50% of the antibody-bound tritiated cortisol. The full names of steroids are given shown in Table 1.

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Cross-reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17,20β-P</td>
<td>100</td>
</tr>
<tr>
<td>17,20,21-P</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>17-P</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>17,21-P</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>T</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>E2</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Results

Metabolism of tritiated 17-P by spermiating testes. The low radioactivity measured in the aqueous portion remaining after solvent extraction indicated a low conjugation capacity of the testes at this stage (0.98%, 1.6% and 8.5% of the total radioactivity for the three testes analysed, respectively). Regarding the unconjugated metabolites, three main peaks of radioactivity (Figs. 1a-c) could be observed in the TLC analyses of spermiating testes incubates, besides the precursor (Fig. 1d). The most polar peak (A) comigrated with highly polar metabolites. The second peak (B) corresponded to the distance of migration of cortisol using benzene/acetone 80/20 v/v. In this first analysis, we were not looking for cortisol therefore it was not used as standard. In subsequent studies of C21 migration using benzene/acetone 80/20 v/v it was clear that cortisol migrated with the system used at the area corresponding to peak B of radioactivity being located between the more polar 17,20,21-P (see Fig. 1, male 2, standard 2) and the less polar 17,20α-P (see Fig. 1, male 2, standard 3). The third peak (C) comigrated with di-hydroxylated progestins (Fig. 1, males 1, 2 and 3, see standard 5). Other minor radioactivity peaks occurred in the migrating zone of androgens such as 11KT, 11β-hydroxyandrostenedione, adrenosterone and androstenedione (see standards number 4, 6, 7 and 10 respectively in Fig. 1, males 1, 2 and 3). However, androgens were not further investigated.

‘A’ TLC peak or ‘17,20ß,21-P zone’. The TLC ‘A’ peak was further analyzed by HPLC showing various minor radioactivity peaks not co-eluting with standards neither with 17,20ß,21-P nor with 11-20ßS (Fig. 1). However, the polar zone that could contain 17,20ß,21-P was further investigated. A second replicate of male 2 was analyzed by TLC and HPLC and the zone that theoretically should contain 17,20ß,21-P was analyzed after specific enzymatic oxidation of the 20β-hydroxyl group by immunochemistry assays. The enzymatic treatment did not induce any change in the original radioactivity profiles observed before the treatment; 17,21-P would have been detected if 17,20ß,21-P had been present. Furthermore, the percentage of binding of TLC peak A to a high titre anti-17,20ß,21-P serum was significantly low (1.5%).

‘B’ TLC peak or ‘cortisol zone’. The ‘B’ peak which migrated with the same migration coefficient as the cortisol standard was especially evident in males 2 and 3 (Fig. 1). A further study by HPLC using different solvent systems (III and IV) has been made using cortisol as standard. HPLC analysis of this peak confirmed the occurrence of tritiated cortisol in two of the three males studied, using the solvent systems III and IV. Figure 2 represents the results obtained with system III. A similar profile was observed using system IV (data not shown). Besides, after acetylation of the peak purified by TLC and HPLC the percentage of recovery in the zone of TLC co-migrating with the acetylated true cortisol was 64% and 49% for males 2 and 3, respectively.

Chromatograms after TLC analysis

![Chromatograms](image-url)

Fig. 1. Radiochromatogram after TLC analysis of spermiating testes of *Micropogonias furnieri* males 1, 2 and 3 after toluene/cyclohexane 50/50 v/v (system I) and benzene/acetone 80/20 v/v (system II). The TLC zones further analyzed by HPLC are shown as A, B and C. The authentic standards used were: 1 = 20-dihydrocortisone; 2 = 17,20,21-P; 3 = 17,20α-P; 4 = 11KT; 5 = 17,20ß-P; 6 = 11ßOH-A; 7 = Ad; 8 = testosterone; 9 = 17-P; 10 = A.
'C' TLC peak or '17,20β-P zone'. A large radioactivity peak was detected for the three investigated males. Further investigation of the ‘C’ TLC peak by HPLC showed that the main metabolite co-eluted with 17,20β-P after analysis with two different solvent systems (systems III and IV). Figure 3 shows the results obtained with system III. A similar profile was observed using system IV (data not shown). Furthermore, specific enzymatic oxidation of the 20β-hydroxyl group of the suspected 17,20β-P gave a radioactivity peak which co-eluted with a 17-P inert standard in HPLC (system III) (data not shown). This TLC area also showed a minor radioactivity peak co-eluting with 17,21β-P, another derivative of tritiated 17-P (male 2, Fig. 3).
Fig. 3. Analysis of zone C of TLC or di-hydroxylated progestins area (see Fig. 1) by HPLC using methanol/acetonitrile/water 33/26/41 v/v/v (system III) in *Micropogonias furnieri*.

**17,20β-P circulating levels during the testicular cycle.** 17,20β-P was undetectable in the plasma of nine males caught at the immature phase (Stage 1) and first stages of spermatogenesis (Stage 2) (Fig. 4). 17,20β-P increased clearly in blood when males were at whole spermatogenesis phases (Stage 3), with variable concentrations ranging from 76 to 840 pg mL⁻¹ with a mean value of 364 ± 264 pg mL⁻¹. Males releasing sperm (Stage 4) also showed a large variability in their 17,20β-P circulating levels; 23% had undetectable levels and the other males showed levels ranging from 117 to 1047 pg mL⁻¹ with a mean ± SD value of 432 ± 204 pg mL⁻¹. One male at resting stage (stage 6) had 208 pg mL⁻¹.
to other teleost fish as salmonids (Scott et al., 1979). In this species can be considered small compared to smaller amounts of 17,20β-P. In fact, the amount of 17,20β-P in eels is interesting to note that the presence of 17,20β-P has been considered essential at the beginning of the meiosis process is coincident with the pathways proposed by Milla et al. (2009). In this revision the authors highlighted that the activity of 17α-hydroxylase is sustained by the presence of the very active 21-hydroxylase, that converts 17P into 17,21P. The presence of 17,20β-P when the precursor is present (Ueda et al., 1984). Thus, the clear increase of this progestin during spermatogenesis reflects the type of spermatogenesis of total spawners showing some degree of synchronization when germ cells mature in testes (Billard & Escaffre, 1975) and containing almost exclusively spermatozoa in spermiant testis. Contrary to total spawners, serial spawners as M. furnieri do not have a high degree of synchronisation during spermatogenesis, and the spermatogenetic wall is present even during spermatogenesis (Vizziano et al., 2002b). If M. furnieri germ cells, including spermatozoa, have the ability to convert precursors into 17,20β-P, as in salmonoids (Sakai et al., 1989; Vizziano et al., 1996), the dynamics of spermatogenesis could explain the absence of a clear peak of 17,20β-P during spermatiation in this sciaenid fish. Nevertheless, it must be reminded that not all fish have sperm with the capacity to convert 17-P into 17,20β-P (Scott et al., 2010).

Another point needing further analyses is the high production of 17,20β-P in testes in vitro during spermatiation and not reflected in high amounts in plasma during the same phase. One explanation could be that 17,20β-P is intratesticularly produced and concentrated and is not released in high amounts to blood. This could be possible if 17,20β-P play an autocrine or paracrine function in M. furnieri testes as it was demonstrated in other fish (Miura et al., 1992; Thomas et al., 2004; Milla et al., 2008; Tubbs et al., 2011). Another explanation regarding the small amounts of 17,20β-P is the possible conjugation and metabolism that may occur in peripheral tissues rapidly removing this steroid from blood (Scott et al., 2010).

The most surprising finding of this work was the cortisol production by spermiating testes since corticosteroids are primarily produced in interrenal glands (Fostier et al., 1983; Kime & Scott, 1993; Lee et al., 2000). The cortisol synthesis by spermiating testes of the white croaker under the influence of the precursor 17P is sustained by the presence of the very active 21-hydroxylase, that converts 17P into 17,21P. The latter can be converted into cortisol by the presence of an active 11β-hydroxylase. This pathway of cortisol biosynthesis in testes is coincident with the pathways proposed by Milla et al. (2009). In this revision the authors highlighted that the activity of 21-hydroxylase has not been previously shown in teleost fish testes. We are hereby providing evidence of a clear activity of 21-hydroxylase in teleost testes.

Discussion

The results demonstrate that 17,20β-P and cortisol are the main metabolites of 17P produced by Micropogonias furnieri spermiating testes. However, we expected the presence of 17,20β,21-P as a major metabolite. In a close related species, M. undulatus, 17,20β,21-P is the major progestin identified in the ovariies during final oocyte maturation after oocyte maturation (Trant et al., 1986). In males, treatments with 17,20β,21-P resulted in an increased percentage of motile sperm and in sperm velocity (Thomas et al., 2004). This effect occurred via a progestin membrane receptor α (mPRα) (Thomas et al., 2005, 2007; Thomas, 2012) highly specific for 17,20β,21-P (Thomas et al., 1997). However, there is no information validating the endogenous production of this steroid for M. undulatus. We could not identify 17,20β,21-P in M. furnieri testes during spermiation even if a high activity of 20ß-hydroxysteroid dehydrogenase (20ßHSD) and 21-hydroxylase, the two enzymes required for the synthesis of 17,20β,21-P from the precursor (17P), were detected.

Changes in plasma levels during M. furnieri testicular cycle were studied in order to clarify the participation of 17,20β-P at an endocrine level. 17,20β-P circulating levels in spermiating males were relatively low (0.1-1 ng mL⁻¹) and M. furnieri can be considered one of the species with smaller amounts of 17,20β-P. In fact, the amount of 17,20β-P in this species can be considered small compared to other teleost fish as salmonids (Scott et al., 2010). The important increase in 17,20β-P levels in blood plasma during the whole spermatogenesis and spermiation stages compared to the undetectable levels at the first development stages sustain the idea that this progestin is involved in the regulation of these two stages of testicular development. It is interesting to note that the presence of 17,20β-P has been considered essential at the beginning of the meiosis process in eels (Miura et al., 2006). This suggests that during the phase in which germ cells enter in meiosis some levels of 17,20β-P are needed to sustain the process and could explain the relative high levels observed in M. furnieri during spermatogenesis.

At the final stage of M. furnieri testicular cycle 17,20β-P plasma levels behave differently than in total spawners as salmonids. An elevated peak of 17,20β-P has been largely described in total spawners (Scott et al., 2010). This significant increase of 17,20β-P production and release to plasma can be explained by the important increase in the number of spermatozoa capable to produce large amounts of 17,20β-P when the precursor is present (Ueda et al., 1984). Thus, the clear increase of this progestin during spermiation reflects the type of spermatogenesis of total spawners showing some degree of synchronization when germ cells mature in testes and containing almost exclusively spermatozoa in spermiant testis. Contrary to total spawners, serial spawners as M. furnieri do not have a high degree of synchronisation during spermatogenesis, and the spermatogenetic wall is present even during spermatogenesis (Vizziano et al., 2002b). If M. furnieri germ cells, including spermatozoa, have the ability to convert precursors into 17,20β-P, as in salmonoids (Sakai et al., 1989; Vizziano et al., 1996), the dynamics of spermatogenesis could explain the absence of a clear peak of 17,20β-P during spermatiation in this sciaenid fish. Nevertheless, it must be reminded that not all fish have sperm with the capacity to convert 17-P into 17,20β-P (Scott et al., 2010).

Another point needing further analyses is the high production of 17,20β-P in testes in vitro during spermatiation and not reflected in high amounts in plasma during the same phase. One explanation could be that 17,20β-P is intratesticularly produced and concentrated and is not released in high amounts to blood. This could be possible if 17,20β-P play an autocrine or paracrine function in M. furnieri testes as it was demonstrated in other fish (Miura et al., 1992; Thomas et al., 2004; Milla et al., 2008; Tubbs et al., 2011). Another explanation regarding the small amounts of 17,20β-P is the possible conjugation and metabolism that may occur in peripheral tissues rapidly removing this steroid from blood (Scott et al., 2010).

The most surprising finding of this work was the cortisol production by spermiating testes since corticosteroids are primarily produced in interrenal glands (Fostier et al., 1983; Kime & Scott, 1993; Lee et al., 2000). The cortisol synthesis by spermiating testes of the white croaker under the influence of the precursor 17P is sustained by the presence of the very active 21-hydroxylase, that converts 17P into 17,21P. The latter can be converted into cortisol by the presence of an active 11β-hydroxylase. This pathway of cortisol biosynthesis in testes is coincident with the pathways proposed by Milla et al. (2009). In this revision the authors highlighted that the activity of 21-hydroxylase has not been previously shown in teleost fish testes. We are hereby providing evidence of a clear activity of 21-hydroxylase in teleost testes.

Fig. 4. Plasma level of 17,20β-P in males at different stages of development sampled at the Río de la Plata estuary. 1: Immature testis; 2: Developing testis (early spermatogenesis); 3: Fully developed testis (whole spermatogenesis); 4: Spermiating testis; 6: Resting testis. n = number of fish collected for each stage.
The physiological effect of cortisol needs a binding to receptors. In fish it has been shown that cortisol transactivate in vitro the two glucocorticoid receptors (Bury et al., 2003). Interestingly, a mineralocorticoid-like receptor initially isolated from a testes cDNA library was described to have high affinity for cortisol (Colombe et al., 2000). The receptivity for the cortisol is present in fish testes but receptors by which they exert a physiological effect are not well understood.

The role of cortisol in the reproduction of male fish shows contradictory effects at different stages of the testicular development. Cortisol at low doses can affect the early spermatogenesis by stimulating the spermatogonia mitosis but can also inhibit the effect of 11-ketotestosterone on the spermatogonia proliferation when higher doses are used. Moreover, cortisol also induces retardation on spermatogenesis development probably via inhibition of androgens (Milla et al., 2009). It has been shown that later during the spermatiation phase cortisol has a direct negative effect on the production of 17,20β-P (the MIS in male trout) testicular production (Milla et al., 2008) suggesting that cortisol affects the late phases of male reproduction. The presence of both 17,20P and cortisol at the spermatiation phase of the white croaker do not sustain the idea of a negative effect of the testicular cortisol on 17,20P in this species. Recently, it has been proposed that another corticosteroid, 11-deoxycorticosterone (DOC), and the mechanisms of action of 17,20β-P are linked during the spermatogenesis stage, in order to control milt fluidity (Milla et al., 2008). The cortisol, being the second major metabolite detected in males testes during spermatogenesis could be a regulator of testes hydration as it was proposed in trout for other corticosteroids.

The results obtained suggest that 17,20β-P is a good candidate to be considered as steroid mediator of spermatogenesis and sperm maturation and release in M. furnieri. The action of cortisol during testicular development needs further studies.

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