STEROID METABOLISM IN VITRO DURING FINAL OOCYTE MATURATION IN WHITE CROAKER *Micropogonias furnieri* (PISCES: SCIAENIDAE)

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(With 4 figures)

ABSTRACT

Final oocyte maturation (FOM) is a process involving a complex set of genetical, biochemical, and morphological mechanisms. FOM involves the shift of a post-vitellogenic follicle to a pre-ovulated oocyte, which is necessary for fertilization by spermatozoan to occur. This process is regulated by a maturation-inducing steroid (MIS) at the follicular level. In other species of scienids fish the MIS, a hydroxilated derivatives of progestagen 17, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S), was identified. Although *Micropogonias furnieri* is the second fishery resource of Uruguay, basic knowledge about its endocrine process is very scarce. The aim of this work was to investigate what steroids are synthesized in vitro by the oocyte follicle of *M. furnieri* during the maturation process. Fragments of ovary (1 g) in three stages: post-vitellogenic (PV), maturing (Mtg), and mature (M) were incubated with 1 $\mu g.g^{-1}$ of tritiated progesterone (P) at 30, 60, and 180 min. After extraction with ethanol and dichloromethane, steroid metabolites were purified by TLC and rpHPLC. Two progesterone derivatives with identical chromatographic properties of 20β-S and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) were purified. In other Teleost fish these steroids are biologically activ as MIS. The 17,20β-P was clearly detected in Mtg and M stages and confirmed by enzymatic oxidation with enzyme 20β-HSD. The 20β-S was strongly detected in all Mtg oocytes. The results do not corroborate 20β-S as a major hormone synthesized in the ovary in FOM as occurs in other scienid fish. A differential steroid synthesis in the advanced oocyte stages suggests that the 20β-S is acting as a MIS in *M. furnieri*.

Key words: steroid metabolism, oocyte maturation, reproduction, fish.

RESUMO

Metabolismo de esteróides in vitro durante a maturação ovocitária final na corvina branca *Micropogonias furnieri* (Pisces: Sciaenidae)

A maturação ovocitária final (FOM) é um processo complexo que envolve mecanismos genéticos, bioquímicos e morfológicos que conduzem à transformação de um ovócito pós-vitelogênico em um ovócito apto a ser fertilizado. Esse processo está regulado pelo hormônio esteróide indutor da maturação ovocitária final (MIS), o qual é sintetizado no folículo. Em outras espécies de Sciaenidae, o MIS foi identificado como um derivado hidroxilado da progesterona 17, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S). *Micropogonias furnieri* é um recurso superexplorado na costa uruguaia, contudo, seus processos endócrinos são pouco conhecidos. O objetivo deste trabalho foi pesquisar quais esteróides são sintetizados *in vitro* pelos folículos em maturação de *M. furnieri*. Fragmentos de ovários (1 g) foram incubados em três estágios diferentes: pós-vitelogênese (PV), em maturação (Mtg) e maduros (M) com 1 μg/g de progesterona tritriada (P) durante 30, 60 e 180 min. Depois da extração dos esteróides com etanol e

diclorometano, esses foram purificados e identificados utilizando-se TLC, rpHPLC e oxidação enzimática. Foram identificados dois derivados de progesterona com idênticas propriedades cromatográficas ao 20β-S e 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P), os quais, em outras espécies de peixes, apresentam atividade biológica como o MIS. A 17,20β-P foi observada claramente nos estágios Mtg e M e confirmada pela oxidação com a enzima 20β-HSD. A 20β-S foi claramente observada nos ovários em maturação (Mtg). Os resultados não permitiram confirmar que 20β-S é o hormônio mais sintetizado nos estágios estudados, como ocorre em outras espécies de ciaenídeos, mas a presença de uma síntese diferencial no estágio de maturação sugere que 20β-S esteja atuando como o MIS em *M. furnieri*.

Palavras-chave: metabolismo esteróideo, maturação ovocitária, reprodução, peixe.

INTRODUCTION

Teleost fish produce several reproductive steroids in distinct phases of oogenesis (Kime, 1993). During the growth phase, oocytes are arrested in prophase I of meiosis and greatly increase in size due to vitellogenin sequestration. In many fish, after oocyte growth is completed the process of final oocyte maturation (FOM) occurs: meiosis resumes, the germinal vesicle breaks down, and the ooplasm hydrates (Nagahama, 1983), which is followed by ovulation and fertilization.

Gonadotropin initiates FOM by brings on the synthesis of a maturational-inducing steroid (MIS) by the ovarian follicles. Progestagens induce oocyte maturation in Teleost fish (Goetz *et al.*, 1987; Jalabert *et al.*, 1991) and the grade of induction is related to the position of specific functional groups on the steroid nucleus (Jalabert *et al.*, 1976; Canario & Scott, 1988). It has been demonstrated that progesterone derivatives such as 17, 20β-Dihydroxy-4-pregnen-3-one (17, 20β-P) or 17, 20β 21-trihydroxy-4-pregnen-3-one (20β-S) are the MIS in several Teleost species (Nagahama, 1994). In Salmonids and Cyprinids 17, 20β-P was identified in vivo and in vitro as MIS (Suzuki *et al.,* 1981; Nagahama *et al.,* 1983; Fostier *et al.*, 1983; Scott & Canario, 1987). Kime (1993) suggested that other steroids could be implicated in FOM in other fish. For instance, 17, 20α-dihydroxy-4-pregnen-3-one (17, 20α-P) and 17, 21-dihydroxy-4-pregnen-3-one (17, 21-P) were the main metabolites in vitro in Pleuronectiforms and Siluriforms, respectively (Upadhyaya & Haider,1986; Canario & Scott, 1990). More recently 20β-Hydroxy-4-pregnen-3-one (20 -P) was identified as MIS in a *Siluriform Clarias batrachus* (Moses & Haider, 1999).

In Perciforms, like the Scienid Atlantic Croaker (*Micropogonias undulatus*), ovarian tissue incubated with tritiated pregnenolone during FOM failed to produce radioactive 17, 20β-P and 17, 21β-P. Instead, another radioactive product, identified as 20β-S, was the major steroid produced by ovaries in this stage (Trant *et al.*, 1986). In other scienid fish (*Cynoscion nebulosus*) immunoreactive 20β-S was detected in plasma at the time of FOM (Thomas *et al.,* 1987), and proven to be a potent inducer of germinal vesicle breakdown (GVBD) of croaker oocytes in vitro (Trant & Thomas, 1988). Subsequent works provided further evidence that 20β-S is a MIS in the Atlantic croaker (Trant & Thomas, 1989a,b; Patiño & Thomas, 1990).

M. furnieri is a Teleost fish widely distributed along the Atlantic coast (Isaac, 1988; Vazzoler, 1998). The Rio de la Plata estuary is one of the spawning zones of white croaker (Acha *et al.*, 1999), a population that has been overexploited in Uruguay (Pin, 1999). Not long ago, Macchi *et al.* (1992) demonstrated stress response of the white croaker to environmental degradation in Río de la Plata estuary. Consequently, basic information on reproductive physiology in *M. furnieri* of Río de la Plata is important and necessary for both aquaculture and the recovery of wild populations.

However, stages involved in the maturation process in *M. furnieri* have not been described until now, including steroid metabolism in ovaries. In vitro studies of tritiated precursor metabolism have supplied valuable information on hormonal control and enzymatic systems implicated in oocyte steroidogenesis (Baek, 1990).

Taking these into account, the aim of this work was to elucidate the dynamics of steroidogenesis in post-vitellogenic (PV), maturing (Mtg), and mature (M) ovaries, particularly the variations of this process in relation to FOM in theca-granulosa follicular cells of white croaker (*M. furnieri*). The hypothesis of

this work is that, as in other fish species of the same family (Sciaenidae), the 20β-S is the MIS in *M. furnieri*.

MATERIALS AND METHODS

a) Chemicals

[1,2,6,7^{-3}H]-progesterone was purchased from Amersham International. Steroids were obtained from Sigma and Steraloids, and solvent from Biopack. Liquid scintillator used was purchased from Packard.

b) Fish

Nine female white croakers in post-vitellogenic stages were purchased from a local supplier from December to March, held in 400 L tanks at 20°C with circulating aerated marine water. Mtg (n = 3) and M (n = 3) stages were obtained after in vitro induction of PV females with hCG at 24 h and 48 h respectively (García-Alonso & Vizziano, unpublished). Fish were anesthetized with an aqueous solution of 2-phenoxy-ethanol (3.5%) and sacrificed by decapitation. The ovaries were removed and portions (5 g) were used for incubation. The gonadosomatic index (GSI) was calculated to check the oogenesis stages in female donors.

c) Incubations and extractions (in vitro steroid production)

Pieces of chopped ovarian tissue (1 g each) were incubated in duplicate for 30, 60, and 180 min at 22°C in Dulbecco's modified Eagle medium (3 ml) and 15 mM Hepes with $NaHCO_3$ (Trant & Thomas, 1989a) containing [^{3}H]-progesterone (10 µCi). Incubations were performed at pH = 7.8, PO = 300 mOsm, and isosmotic to gonadal plasm of *M. furnieri*. In some experiments, hCG (5 IU per ml) was added for steroidogenic induction. The incubation was stopped with ethanol and excess of progesterone (10 µg). The incubation medium was extracted three times with an ethanolic solution (9 ml) of NaCl 2% (final concentration) (Baek, 1990; Guiguen, 1992). Three successive extractions were performed using 10, 8, and 5 ml of dichloromethane. Aliquots of the aqueous phase were used to evaluate steroid conjugation. The organic phase was evaporated and resuspended in ethanol (200 µl). Aliquots of all samples were counted for radioactivity. Ethanol was evaporated, and samples were resuspended in dichloromethane: methanol (9:1) and used for chromatographic analysis.

d) Chromatography

Standards containing 2 µg each of testosterone (T), 17-hydroxy-4-pregnen-3-one (17-P), 17, 20β-P, and 20β-S were added as carriers to each sample. Thin-layer chromatography (TLC), using silica Gel 60 F254, Merck, 20 x 20 cm plates with concentration zone, was performed for each fraction, using Toluene: Cyclohexane, 50:50 (system I), and Benzene: Acetone, 80:20 (system II) as mobile phases.

TLC was developed twice using system I to eliminate non-polar compounds, like vitelins. Chromatograms were afterwards developed three times using system II in order to separate the radiotracers. Radioactive metabolites were autorradiographically detected (Hyperfilm RPN 535, Amersham International, 18 x 24 cm plates). After one-week exposition, the film was developed (Processing Chemical KODAK GBX) and fixed (KODAK GBX). Identification of each metabolite was achieved by comparing its relative migration with authentic reference steroids under a UV-lamp (wavelength, 254 nm).

The peaks of radioactivity that co-migrated with 20β-S, 17, 20β-P, and 17-P or T were scraped from TLC plates and extracted with dichloromethane: methanol (9:1). The activity of aliquots of each sample was measured using PPO-bisMSB CASRN (Packard) in a Liquid Scintillation Counter LS 100 C (Beckman) and percentage of hydrophilic and hydrophobic compounds were determined by radioactive measurements.

These samples were also analyzed by reverse phase rpHPLC using a SHIMADZU (LC-10AS) Liquid Chromatograph coupled to a photodiode array detector (SPD-M10A, Shimadzu). Metabolites and standards were isocratically eluted from a C18 Nucleosil (5 µm) column, using acetonitrile: methanol: water 26:33:41 (system III) for 20β-S, and acetonitrile: water, 40:60 (system IV) for 17, 20β-P as mobile phases and a flow rate of 1 ml.min^{-1}, using a previously reported method (Mugnier, 1996). Fractions of rpHPLC eluates (0.5 ml) were collected and activity was measured on 200 µl aliquots.

Samples of the main metabolite that co-migrated and co-eluted with 17, 20β-P, was extracted in dichloromethane and analyzed by enzymatic oxidation with 3α ,20β-hydroxysteroid dehydrogenase (20β-HSD, SIGMA) in Mtg oocyte extractions. After enzymatic activity control, samples were evaporated, resuspended in buffer phosphate 10 mM, pH 7.6 (1 ml) containing NAD^+ (1 mM, 0.65 mg) and added 20β-HSD in buffer Tris 50 mM, pH 7.6 (10 µl) After

incubating at room temperature for 2 hours, metabolites were extracted with dichloromethane. The 17,20β-P and 17-P were added as standards carriers to the organic phase. The fractions were evaporated, resuspended in ethanol (25 µl), and a rpHPLC analysis, using system III was performed. Activity of each fraction was also measured.

RESULTS

a) Metabolite extraction

Table 2 shows the percentage of radioactive metabolites extracted from the incubation media. 93.5% of the activity recovered was present in the organic phase, independently of stage and incubation time.

b) Chromatography

The TLC analysis of the radioactive compounds extracted from the incubation media revealed the presence at all incubation times of several metabolites of progesterone in PV, Mtg, and M oocytes (Fig. 1). However, higher numbers of tritiated metabolites were observed with increasing incubation times.

The precursor (P) was the predominant species in all studied stages (Figs. 1 and 2). In the post-vitellogenic stage, radioactive metabolites that co-migrated with 17-P and T were found. Putative 17β-estradiol was also observed in other samples in this stage.

The metabolism pattern observed in PV changed after FOM initiation: in Mtg and M metabolites co-migrating with 17, 20β-P and 17, 21-P were observed (Figs. 1 and 2). Besides, an additional metabolite with the same migration distance of trihydroxylated progestagen, 20β-S, was found in Mtg (Figs. 1 and 2). In vitro stimulation with hCG induced the synthesis of a high number of unidentified metabolites, as shown by TLC. The effect of induction was more evident in the Mtg stage (Fig. 2).

A weak band was found in some PV samples after 180 min of incubation. This band co-eluted with 20β-S in rpHPLC in only two of three females studied (Fig. 3). However, when in vitro gonadotropin stimulation of PV oocytes with hCG was performed, it failed to produce metabolites that co-eluted with 20β-S (data not shown).

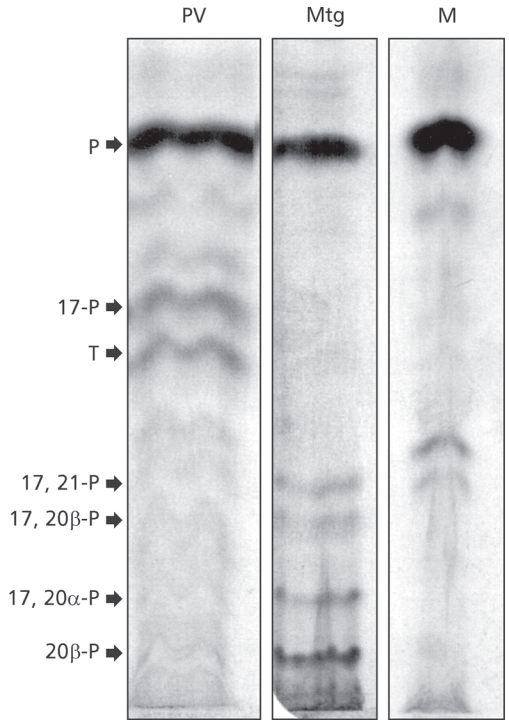

Fig. 1 — Autoradiography of TLC (system I and II) with extractions of incubated PV, Mtg, and M oocytes at 180 min.

TABLE 1
Percentage of total recovery radioactivity, after extraction.

Stage & Incubation time	Organic phase (% ± SD)	Aqueous phase (% ± SD)
PV 30 min	94.20 ± 2.99	5.80 ± 2.99
PV 60 min	95.90 ± 3.15	4.10 ± 3.15
PV 180 min	94.38 ± 3.66	5.62 ± 3.66
Mtg 30 min	92.25 ± 3.68	7.75 ± 3.68
Mtg 60 min	94.78 ± 1.41	5.22 ± 1.41
Mtg 180 min	93.40 ± 2.50	6.60 ± 2.50
M 30 min	92.68 ± 2.15	7.32 ± 2.15
M 60 min	92.40 ± 4.86	7.60 ± 4.86
M 180 min	91.20 ± 2.06	8.80 ± 2.06
Total % ± SD	93.47 ± 3.18	6.53 ± 3.18

Phases (organic and aqueous) of oocytes incubated at different stages of FOM, post-vitellogenic (PV), maturing (Mtg), and mature (M) oocyte at 30, 60, and 180 min of incubation.

TABLE 2
Migrations distance of standard steroids in TLC.

Steroids	Migrations to origin (cm)	Migration relative to P (cm)
P	11.8	1.00
E_2	8.8	0.74
17-P	8.6	0.72
20β-P	8.2	0.69
T	7.3	0.61
17, 21-P	6	0.50
17, 20β-P	4.4	0.37
17, 20α-P	3.2	0.27
20β-S	0.9	0.07

Steroid migrations after system I and II (two and three times, respectively). The distances are in relation to origin of TLC and relative to P.

Samples from Mtg oocytes showed peaks that co-eluted with 20 -S only at 180 min (Fig. 3) and the increase in metabolism of P was clear in relation to 30 and 60 min. The qualitative concentration of this metabolite was absent or unclear at 30 and 60 min.

The presence of 17, 20β-P was investigated by rpHPLC using system IV in Mtg oocytes at 30, 60, and 180 min. In this case, a tritiated metabolite with the same chromatographic profile as 17, 20β-P was found at all incubation times (Fig. 4), but showing more activity at 180 min. These results agreed with those of TLC. The presence of [^{3}H]-17-P after enzymatic oxidation confirmed the identity of putative 17, 20β-P in Mtg incubations at 180 min, but not all metabolites were transformed in 17-P. Solvent system IV, used in rpHPLC, clearly separates 17, 20β-P from other dihydroxylated steroids such as 17, 20α-P or 17, 21-P. However, rpHPLC of the TLC fraction that co-migrated with 17, 20β -P showed another peak with the same retention time as 17, 20α-P. This result may be attributable to a tailing effect in the TLC analysis. On the other hand, 17, 20α-P was detected in the MIS of other Perciforms (Canario & Scott, 1989; Mugnier, 1996).

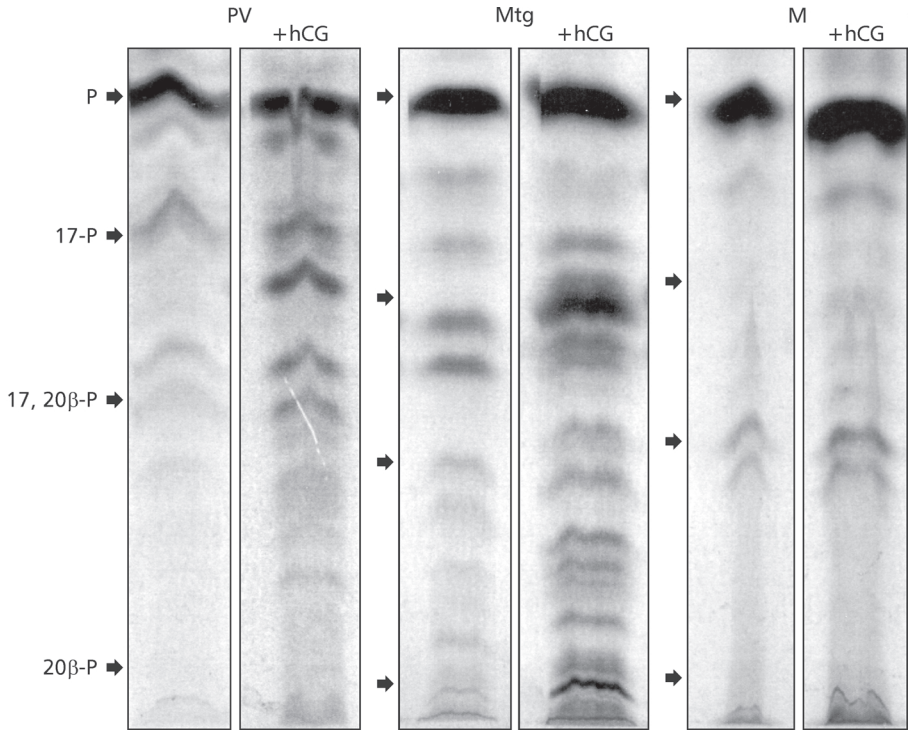

Fig. 2 — Autoradiography of TLC (system I and II) with extractions of incubated PV, Mtg, and M oocytes with and without hCG at 180 min.

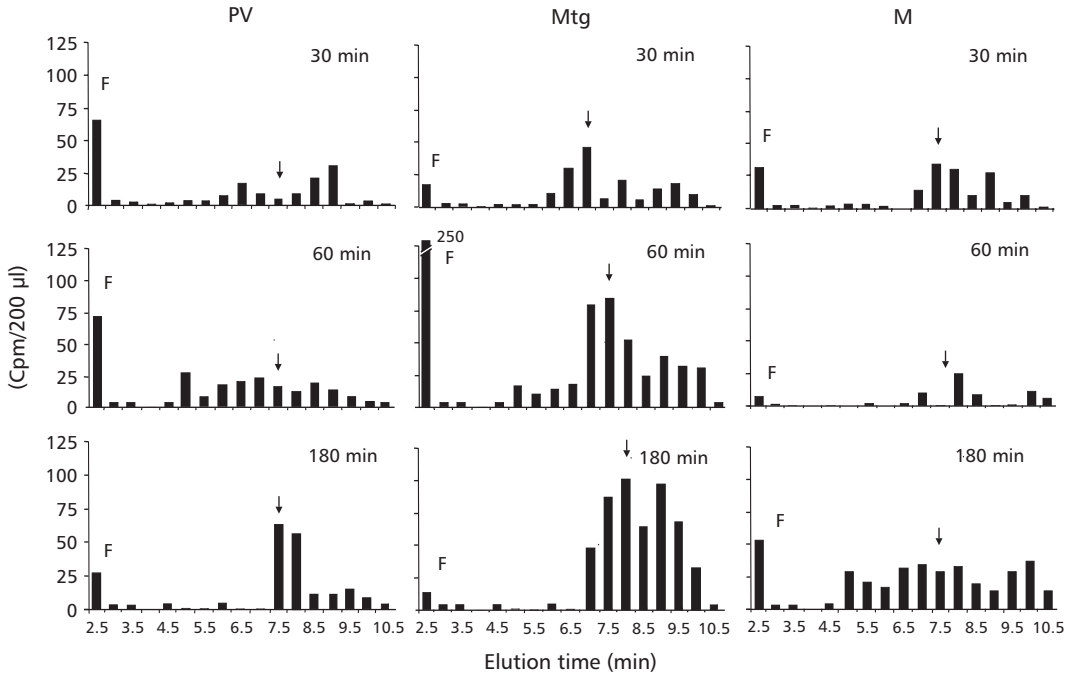

Fig. 3 — Elution times in rpHPLC (system III) of co-migrated tritiated metabolite with 20b-S in TLC of PV, Mtg, and M oocytes at 30, 60, and 180 min. Arrows indicated the elution time of carriers steroids standard; F solvent front.

Other steroids that were synthesized in all incubations co-migrated with T and 17-P. In addition, a polar metabolite, which could not be identified by rpHPLC, was observed in some samples.

To summerize: the metabolite identified by TLC and rpHPLC as 20β-S appeared specifically in Mtg oocytes at 180 min, and its synthesis was not evident in all PV, M samples, and Mtg oocytes at low incubation times (30 and 60 min). In PV, 20β-S was not produced after in vitro induction with hCG. However, 17, 20β-P appears in TLC and rpHPLC as qualitatively more synthesized in ovaries at all stages, as well as in PV after induction with hCG.

DISCUSSION AND CONCLUSIONS

The extraction of steroids from the incubation medium was efficient; radioactive products recovered were mainly lipophilic, as demonstrated by high extraction of organic solvents. This result indicates a low grade of conjugation of the steroids to hydrophilic sulphate or glucuronide derivatives. The synthesis of sulphates and glucuronides steroids has been demonstrated in Teleost gonads (Scott & Turner, 1991; Kime, 1993) even with a physiological role as a pheromone (Waring *et al.*, 1996; Scott *et al.*, 1994).

In fish like Salmoniforms, all with synchronic ovaries and one spawn per season, all the PV oocytes mature at the same time and, consequently, it is easier to identify which steroid is metabolized in each oogenesis stage. In animals with partial spawn, vitellogenic, PV, Mtg, M, and/or ovulated oocytes may coexist in the ovary. The white croaker is a partial spawn fish (Vazzoler, 1970) and, for this reason, low proportions of PV oocytes mature at the same time, and several follicular stages, including Mtg, coexist. Steroid synthesis results from the overall enzymatic activity in each stage, and in vitro progesterone metabolism reflects this situation. Our results confirm this hypothesis, since several metabolites of progesterone in PV, Mtg, and M oocytes, were observed by TLC at all incubation times (Fig. 1).

The present study suggests that PV, Mtg, and M follicles of *M. furnieri* are able to synthesize a variety of steroids, including 17, 20β-P and 20β-S, both identified as MIS in several Teleost fish with or without gonadotropic stimulation in vitro (Scott & Canario, 1987; Thomas, 1994). The presence of 17, 20β-P in females, has also been observed in testis of *M. furnieri* (Vizziano & Fostier, 1999).

Small amounts of 17-P were also detected by TLC and rpHPLC, and the low 17-P levels detected in all incubations are probably due to its extremely rapid metabolism (Kime, 1992). Synthesis of 17-hydroxylated steroids, such as 17-P, 17, 20β-P, and 20β-S evidence the activity of 17-hydroxilase and 20β-HSD enzymes, which generates key metabolites in the biosynthesis of MIS in Teleost (Nagahama, 1994). In vitro hCG induction produced an amplification of P metabolism and the activation of new biosynthetic pathways of steroidogenesis. The endogenous gonadotropin GtH II, heterologous hCG, induces maturational competence in Teleost oocytes (Redding & Patiño, 1993; Kagawa *et al.*, 1998), activating and deactivating cofactors and enzymes of ovary steroidogenesis, including the induction of dehydrogenases for MIS and synthesis of steroids receptors in oocytes (Patiño & Thomas, 1990b; Patiño, 2000). However, no metabolite was observed with chromatographic characteristics of 20β-S in PV oocytes after hCG stimulation.

In Perciforms, different steroid metabolites, including MIS, have been identified in vitro. In Tilapia (Oreochromis mossambicus), 17, 20β-P and 20β-S were observed at FOM (Rocha & Reis-Henriques, 1998). The same metabolites were identified in ovarian incubations of *Morone saxatilis* (King *et al.*, 1994) and 20β-S was demonstrated to be the MIS in the hermaphrodite, *Acanthopagrus schlegell* (Yuehl & Chang, 2000). In scienids, Thomas & Trant (1989) and Trant & Thomas (1989a) identified 20β-S as MIS in Cynoscion nebulosus and Micropogonias undulates, respectively.

Trant *et al.* (1986) and Thomas (1994) demonstrated that 20β-S is the major steroid produced in vitro by follicles during FOM in scienids. However, 17, 20β-P was the principal steroid detected in maturing oocytes of *M. furnieri*. The use of progesterone as a precursor instead of Pregnenolone may account for this difference. Progesterone was selected for our study because it is the direct precursor of a variety of ovarian steroid hormones (Fostier *et al.*, 1983).

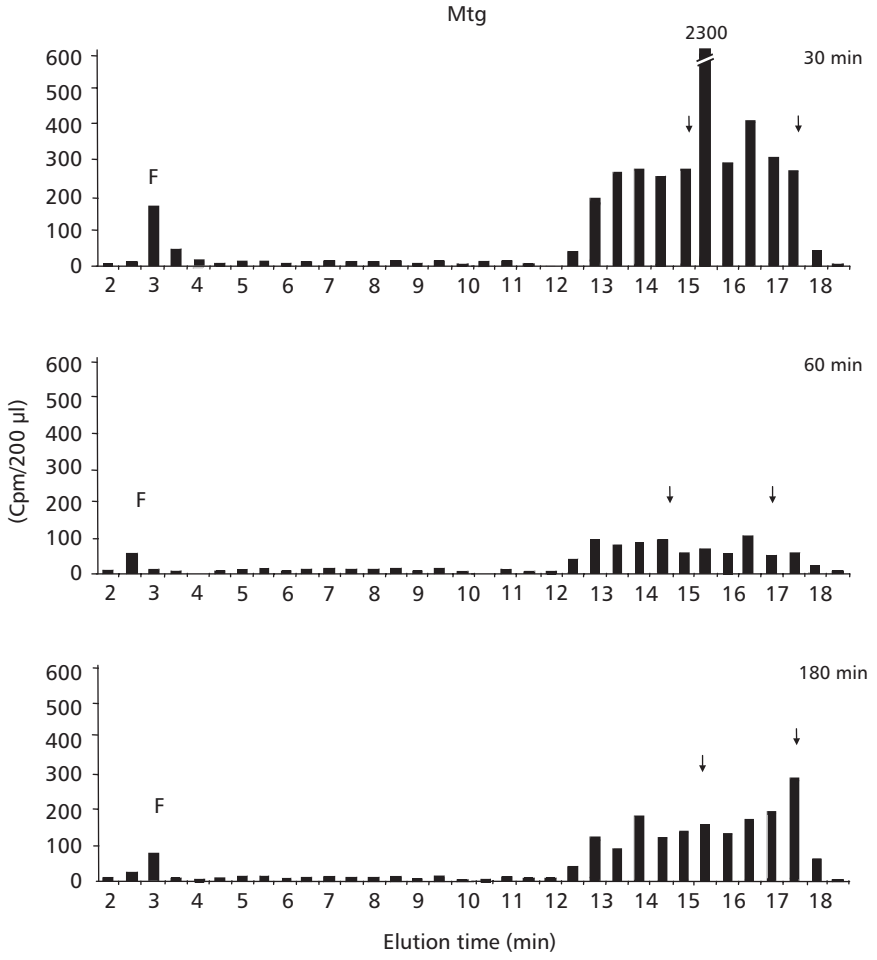

Fig. 4 — rpHPLC (system IV) of radioactive bands co-migrated in TLC with 17,20b-P. Black and grey arrows indicated the elution time of steroid standard, 17,20a-P, and 17,20b-P, respectively. F: solvent front.

Additional studies should be performed with blood or gonadal plasma using specific antibodies (ELISA or RIA) in order to demonstrate the presence of those steroids.

In conclusion, our results show that during FOM exist a steroidogenic shift and qualitative change of the metabolite pattern, and the precursor metabolization was time-dependent and determined by the exact oogenesis stage of the female donor.

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