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In vitro biosynthesis of novel 5β-reduced steroids by the testis of the round goby, *Neogobius melanostomus*

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Abstract

Previous studies indicate that, in the round goby *Neogobius melanostomus*, the reproductively mature male releases a pheromone that attracts ripe females. Furthermore, studies suggest that the pheromone may be a steroid (more specifically a 5β-reduced androgen) produced by specialized glandular tissue in the testes. In the present study, it is shown that the testis of the male round goby contains such specialized glandular tissue. In vitro, the testes convert [^{3}H]androstenedione into 3α-hydroxy-5β-androstane-11,17-dione (i.e., 11-oxo-etiocholanolone, 11-oxo-ETIO); 11-oxo-ETIO sulfate (11-oxo-ETIO-s); 11-oxo-testosterone (i.e., 11-ketotestosterone), 3α-hydroxy-5β-androstan-17-one (etiocholanolone, ETIO); 11β-hydroxy-androstenedione; ETIO sulfate and testosterone. Glucuronidated steroids were not identified. Neither 11-oxo-ETIO nor 11-oxo-ETIO-s has previously been identified in teleost gonads. Both these steroids are formed in the round goby testis even when [^{3}H]17-hydroxyprogesterone is used as a precursor. The fact that, for both steroids, the carbon A ring has a 5β-configuration (already linked with olfactory sensitivity and behavior induction in two other species of gobies) makes them likely candidate pheromones in the round goby. However, their in vivo production and pheromonal activity remain to be proved.

Keywords: Sex steroid; Goby; Teleost; Pheromone; Teleost; 11-Oxo-etiocholanolone; Testis; Teleost; 11-Oxo-etiocholanolone sulfate; Testis; Teleost; Testis histology

1. Introduction

The parental male round goby (*Neogobius melanostomas*), a bottom-dwelling teleost fish, maintains, and guards a nest into which many females deposit eggs (MacInnis and Corkum, 2000; Wickett and Corkum, 1998). It has been hypothesized that male round gobies release a pheromone (or a mixture of pheromones) to attract reproductive females to their nests, and that this pheromone is a steroid originating in the testes (Murphy et al., 2001). Strong evidence to support this hypothesis comes from studies on another species from the family Gobiidae, the black goby, *Gobius jozo* (= *G. niger*). Male black gobies have been shown to emit a pheromone that attracts females to their nests (Mozzi, 1968). Colombo et al. (1977, 1982) have shown that the testes of black gobies contain prominent Leydig (steroid-secreting) cells, concentrated in the region where the testis is suspended from the body wall by lengthwise mesenteries known as mesorchia, and hence termed the 'mesorchial gland.' Colombo et al. (1970, 1977) showed that the mesorchial gland was capable of transforming

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radioactive pregnenolone into predominantly conjugated and 5β-reduced steroids. The most prevalent of these was 17-oxo-5β-androstan-3α-yl glucuronide (etiocholanolone glucuronide; ETIO-g). This steroid was also shown to act as an attractant to gravid females (Colombo et al., 1980).

Previous studies of the round goby support the view that this species also releases steroids with pheromonal properties. Murphy et al. (2001) examined olfactory epithelial (electro-olfactogram, EOG) responsiveness of the round goby to over 100 steroids and prostaglandins. The prostaglandins were all inactive, however, 19 steroids elicited responses. Cross-adaptation studies with these steroids revealed that there were four classes of olfactory receptors that Murphy et al. (2001) named E1, E2-3-g, ETIO, and DHEA-s, after the steroids that gave the highest response to each class of receptor: estrone [E1], 17β-estradiol 3-glucuronide [E2-3g], etiocholanolone [ETIO] and 17-oxo-androst-5-en-3β-yl sulfate [dehydroepiandrosterone sulfate; DHEA-s]. Murphy et al. (2001) discovered that males markedly increased their gill ventilation rate in response to steroids that acted on the E1, E2-3g, and ETIO receptors, while females only responded to steroids that acted on the ETIO receptor. In a subsequent study (Murphy and Stacey, 2002), females responded to E1 and E2-3g following treatment with methyl testosterone, suggesting that these classes of receptors were involved in female-to-male communication.

The aims of the present study were: first, to establish whether the testis of the round goby also contains a homolog of the mesorchial gland; second, to determine whether this gland was able to convert radioactive androstenedione (androst-4-ene-3,20-dione; Ad) and 17-hydroxypregn-4-ene-3,20-dione (17-P) into other steroids in vitro; third, to determine the identity of these steroids. Due to time constraints, identification studies were mainly restricted to the steroids that were formed from tritiated Ad. Underlying this decision was the fact that there are a relatively small number of other steroids into which Ad (a C_{19} steroid) can be converted, compared with when pregnenolone or 17-P (both C_{21} steroids) are used as precursors. There is a risk to this strategy in that the pheromone may actually be a C_{21} steroid or even a 5-ene steroid. However, although Murphy et al. (2001) have shown that round gobies gave an EOG response to six 5β-reduced C_{21} steroids, this response was much weaker than that to ETIO and could also be significantly reduced by cross-adaptation with ETIO. The work on the black goby (see above) also strongly implicates 5β-reduced C_{19} steroids as pheromones.

The importance of this work lies in the fact that the round goby has invaded the Great Lakes of North America and is posing a threat to native fish species (Corkum et al., 1998). If a male sex pheromone can be identified in this species, it may possibly be useful for trapping females and/or disrupting their spawning.

2. Materials and methods

2.1. Experimental animals

Round gobies were obtained from the Detroit River (Windsor, Ont., Canada) by angling and were maintained at a water temperature of 18–20 °C in dechlorinated tap water. Spermiating males were captured in June 2002 (fish I and VI) or July 2003 (fish II–V). Spermiating males were identified by swollen cheeks and black coloration (MacInnis and Corkum, 2000). The mean (SE) gonadosomatic index (GSI) of 8 reproductive males was 1.92 (0.10) compared with GSI values at 0.27 (0.06) for 10 non-reproductive males. There was a significant difference in the GSI values between these two groups of males ($t_{(16)} = 15.29$, $P < 0.001$). Furthermore, the presence of active sperm and histological examination of the testis was completed in this study to confirm reproductive maturity. The fish were anaesthetized with MS222, killed and the testes dissected and placed in ice-cold Leibowitz L15 medium (Sigma–Aldrich, USA).

In July 2003, two males (fish II and III) were injected with 10 IU/g body weight of human chorionic gonadotropin (HCG)—dissolved in saline at a concentration of 250 IU/ml. The testes were dissected out 12 h after the HCG injection. These HCG injections were performed to determine if in vivo stimulation by gonadotropin would have any noticeable effect on the types and relative amounts of steroids that were produced.

2.2. Histological preparations

Following deep anesthesia of two spermiating round gobies in MS 222, the testes were removed and immediately immersed in Karnovsky fixative (0.075 M cacodylate buffer, 2.2% paraformaldehyde, and 2% glutaraldehyde) overnight, then cut into 1 mm slices by hand, and on the following morning, immersed in fresh fixative for 2 h. The fixed tissue slices were post-fixed in 1% osmium tetroxide (in 0.075 M cacodylate buffer), dehydrated through an ascending series of ice-cold ethanol, passed through propylene oxide and embedded into epoxy resin. Serial semi-thin (1 μm) sections were taken on an ultramicrotome, stained with 1% toluidine blue (in 1% sodium tetraborate), viewed by brightfield microscopy and photographed on a Zeiss Axioscop 2 FS.

Testes from a further 10 round gobies were prepared in paraffin, sectioned serially at 3 μm, and were also examined. This procedure, that was less time consuming and less technically demanding than the plastic method, confirmed the same cellular organization in all of the animals that were examined. Here, we present the data obtained from the plastic sections because of clarity and high resolution by brightfield light microscopy.

2.3. In vitro incubation of testes with tritiated precursors

Five pairs of testes (fish I–V) were incubated with [1,2,6,7-^{3}H]androst-4-ene-3,17-dione (including those from the two fish that had been injected with HCG) and one pair of testes (fish VI) with 17-hydroxy[1,2,6,7-^{3}H]progesterone. Both radiochemicals were purchased from Amersham Biosciences. The diffuse spatial arrangement of the Leydig cells in the round goby testis, as seen at high power in the histological preparations, precluded selection of regions that were rich in Leydig cells (cf. mesorchial gland of the black goby: Colombo et al., 1970, 1977, 1982) for in vitro incubation. The testes were thus dissected from the fish, separated and then finely minced, suspended in ice-cold Leibowitz L15 medium (Sigma) and transported on ice from the University of Windsor to Michigan State University (ca. 3 h). Here, the bits of tissue were distributed to fresh 50 ml plastic tubes containing 10 ml Leibowitz L15 medium and 10–50 μCi tritiated Ad or 17-P. The tubes were laid on their side and gently agitated at 16 °C for a further 6 h (Kime and Scott, 1993). At the end of the incubation period, the medium was filtered and then passed through a Sep-Pak C-18 cartridge (Waters Chromatography, Millipore, Milford, MA, USA). This was washed with 5 ml distilled water and then eluted with 5 ml methanol. The extract was stored at −20 °C.

2.4. Separation of tritiated metabolites from reproductive male round goby by high-performance liquid chromatography

The methanol extracts from the Sep-Pak C-18 cartridges were dried down either under a stream of nitrogen at 45 °C or in a rotary evaporator, mixed with 10 μg each of Ad and 11-oxo-T, reconstituted in 1 ml acetonitrile/water/trifluoroacetic acid (28/72/0.01; v/v/v) and then loaded onto an analytical reverse-phase high-performance liquid chromatography (HPLC) column (Rainin Dynamax Microsorb; 5 μm octadecylsilane; 4.6 mm × 25 cm; fitted with a 1.5 cm guard module). Two pumps were used to deliver solvents through the column at a rate of 0.5 ml/min. Solvent A was 0.01% trifluoroacetic acid (TFA) in distilled water and solvent B was 70% acetonitrile and 0.01% TFA in distilled water: 0–10 min, 28.6% B; 10–60 min, 28.6–100% B; 60–80 min, 100% B; 80–82 min, 100–28.6% B; and 82–100 min, 28.6% B. One minute fractions were collected between 20 and 70 min. A volume of 5 μl from each HPLC fraction was then counted in a scintillation counter and fractions that formed distinct peaks on HPLC were saved for identification studies. Part of the identification criteria for metabolites involved the position that they eluted on HPLC. This was established for the synthetic standards by running 10 or 20 μg of each on the HPLC column under the same running conditions as above and monitoring the eluate with a diode-array detector. Steroids with a 4-ene configuration (e.g., Ad and 11-oxo-T) gave a strong peak of UV adsorption at ca. 248 nm. Steroids with a C=O group (or groups) gave a much weaker, but nevertheless distinct, peak of UV adsorption at ca. 200 nm.

2.5. Identification of steroids by thin-layer chromatography (TLC) of HPLC fractions

The standard synthetic steroids used in this study (Table 1) were purchased from Sigma Chemical Company or Steraloids. We tried to obtain all the steroids into which Ad might be converted via 'well-established' biosynthetic pathways. Using this criterion, we might expect there to be metabolites with either androst-4-ene, 5α-androstan or 5β-androstan configurations. In the 5α- and 5β-reduced steroids, we might expect to find either a 3-oxo, a 3α-hydroxyl or a 3β-hydroxyl group. In the androst-4-ene steroids, we would expect to find only a 3-oxo group. We would not expect to find 3-reduced androst-4-ene steroids as, to our knowledge, direct reduction of the 3-oxo group of 4-ene steroids has not been demonstrated in any vertebrate (although such steroids may be formed as intermediates when 5-ene steroids are used; Inaba et al., 1966). We might expect the 17-oxo group to be either retained or reduced to form a 17β-hydroxyl (but not 17α-hydroxyl) group. We might expect the 11-carbon to remain as it is or to be 11-oxygenated to form an 11β-hydroxyl (but not 11α-hydroxyl) group and then also further oxidized to form an 11-oxo group. All these possible combinations are shown in Table 1 (including eight steroids that we were unable to obtain).

There is always the possibility that Ad will be converted to C_{18} (estrogen-like) steroids. However, we have not included these in Table 1 as in vitro estrogen formation by testes has not been reported in teleosts (Fostier et al., 1983). No estrogen formation was found in previous studies on the testes of black goby (Colombo et al., 1977) or urohaze-goby *Glossogobius olivaceus* (Asahina et al., 1985).

We might also expect that any steroid in Table 1 that has either a 3α-, 3β-, or 17β-hydroxyl group could be glucuronidated or sulfated. Only four examples (that are referred to within the present paper) are shown in Table 1. Identification of conjugated steroids normally involves their deconjugation through either enzyme hydrolysis (which works on glucuronides and some sulfates) or acid solvolysis (which works on sulfates only) (Scott and Vermeirssen, 1994). Only the latter method needed to be applied in the present study (see below).

All steroids were dissolved in ethanol at a concentration of 500 μg/ml. At the outset of the identification work, 10 μg of each steroid was combined with 10 μg testosterone (T), spotted onto separate lanes of the TLC plates and developed with chloroform/methanol (50/2, v/v).

Table 1
Standard synthetic steroids against which the tritiated metabolites were compared

Steroid[a]	Common name	R_T[b]	HPLC elution (minutes)
Available steroids			
3α,17β-Dihydroxy-5β-androstan-11-one		0.12	
5α-Androstane-3β,11β,17β-triol		0.14	
11β,17β-Dihydroxyandrost-4-en-3-one	11β-OH testosterone	0.16	40
3β,11β-Dihydroxy-5α-androstan-11-one		0.27	
17β-Hydroxyandrost-4-ene-3,11-dione	11-Oxo-testosterone (11-oxo-T)[c]	0.4	40
3β,11β-Dihydroxy-5α-androstan-17one		0.44	
3α,11β-Dihydroxy-5β-androstan-17-one		0.45	
5β-Androstane-3α,17β-diol		0.48	
17β-Hydroxy-5β-androstane-3,11-dione		0.48	45
3α,11β-Dihydroxy-5α-androstan-17-one		0.53	
3β,11β-Dihydroxy-5β-androstan-17-one		0.57	
17β-Hydroxy-5α-androstane-3,11-dione		0.58	
3α-Hydroxy-5β-androstane-11,17-dione	11-Oxo-etiocholanolone (11-oxo-ETIO)	0.67	47
11β-Hydroxyandrost-4-ene-3,17-dione	11β-OH androstenedione (11β-OH-Ad)	0.72	43
5α-Androstane-3α,17β-diol		0.72	
3α-Hydroxy-5α-androstane-11,17-dione		0.77	
5α-Androstane-3β,17β-diol		0.78	
3β-Hydroxy-5α-androstane-11,17-dione		0.79	
5β-Androstane-3β,17β-diol		0.86	
17β-Hydroxyandrost-4-en-3-one	Testosterone (T)	1.0	52
3α-Hydroxy-5β-androstan-17-one	Etiocholanolone (ETIO)	1.0	60
11β-Hydroxy-5α-androstane-3,17-dione		1.03	
11β-Hydroxy-5β-androstane-3,17-dione		1.06	
17β-Hydroxy-5β-androstan-3-one		1.12	
3β-Hydroxy-5α-androstan-17-one		1.14	
3α-Hydroxy-5α-androstan-17-one		1.20	
17β-Hydroxy-5α-androstan-3-one		1.28	
3β-Hydroxy-5β-androstan-17-one		1.28	
5β-Androstane-3,11,17-trione		1.33	48
Androst-4-ene-3,11,17-trione	11-Oxo-androstenedione; androstenetrione	1.38	45
5α-Androstane-3,11,17-trione		1.49	47
Androst-4-ene-3,17-dione	Androstenedione (Ad)	1.70	55
5β-Androstane-3,17-dione		1.82	
5α-Androstane-3,17-dione		1.94	
Conjugated steroids			
17-Oxo-5β-androstan-3α-yl glucuronide	Etiocholanolone glucuronide (ETIOg)	0	44
17-Oxo-5β-androstan-3α-yl sulfate	Etiocholanolone sulfate (ETIOs)	0	37
11,17-Dioxo-5β-androstan-3α-yl glucuronide	11-Oxo-etiocholanolone glucuronide (11-oxo-ETIOg)	0	38
11,17-Dioxo-5β-androstan-3α-yl sulfate	11-Oxo-etiocholanolone sulfate (11-oxo-ETIOs)	0	30
Steroids (in same range as above) that were not available for testing			
5α-Androstane-3α,11β,17β-triol			
5β-Androstane-3β,11β,17β-triol			
5β-Androstane-3α,11β,17β-triol			
3α,17β-Dihydroxy-5α-androstan-3-one			
11β,17β-Dihydroxy-5α-androstan-3-one			
3β,17β-Dihydroxy-5β-androstan-11-one			
11β,17β-Dihydroxy-5β-androstan-3-one			
3β-Hydroxy-5β-androstane-11,17-dione			

R_T is the relative position of each steroid in relation to testosterone (T) when run on TLC. HPLC elution times were only determined for steroids of interest.

[a] The nomenclature of steroids in this paper follows that recommended by IUPAC (Kime, 1995; www.chem.qmul.ac.uk/iupac/steroid) and not that used by Steraloids (www.steraloids.com), Sigma–Aldrich (www.sigma-aldrich.com) or Murphy et al. (2001).

[b] The R_T values and HPLC elution times shown in Table 1 are only approximate and are expected (and were found) to differ slightly in response to temperature and exact composition of solvents; however, approximate R_T provided a useful 'first step' in the identification of products from incubation of testis with tritiated Ad.

[c] Also known incorrectly as 11-keto-testosterone.

The positions of the androst-4-ene steroids (e.g., Ad, T, and 11-oxo-T) were detected by shining a UV lamp at the plate. Steroids with reactive hydroxyl groups (e.g., 3α-hydroxy-5β-androstane-11,17-dione; 11-oxo-ETIO) were detected by spraying with a solution of phosphomolydic acid in ethanol (10% w/v) and heating the plate for 5 min at 150 °C. Steroids with no reactive hydroxyl groups (e.g., 5β-androstane-3,11,17-trione) were detected

by spraying with concentrated sulfuric acid in methanol (10% v/v) and heating the plate for 5 min at 150 °C. After the bands were revealed, their distance to the origin was measured to the nearest millimeter. The relative position of each steroid in relation to that of T was then calculated. This ratio (R_T) formed the basis of a table (Table 1) against which the relative elution positions of radioactive bands could subsequently be compared.

To determine the identity of the radioactive steroids in the various peaks, ca. 20,000 dpm was mixed with 10 μg T, dried down, reconstituted in 30 μl ethyl acetate and spotted and run on TLC as described above. The position of T was marked with a pencil and each lane then divided into 5 mm strips that were scraped off, mixed directly with 7 ml scintillation fluid, and counted. The position of the radioactivity relative to that of T was then compared with the R_T values in Table 1. By doing this, it was possible to narrow down the range of possible matching standards for each HPLC peak. A more precise match was obtained by mixing 20,000 dpm of radioactive steroid with 10 μg each of those steroids that had the most similar R_T values and then running them together on TLC. After the bands had been revealed, they were marked and scraped off, as was 2×0.5 cm of silica gel from either side of the bands. All the scrapes were mixed with scintillation fluid and counted. In any situation where the radioactivity was >90% associated with a band, microchemical studies were then carried out to further establish the co-identity of radioactive metabolite, and standard steroid.

2.6. Microchemical reactions

Prior to each microchemical reaction, the radioactive metabolite (ca. 20,000 dpm) and the cold standard (10–20 μg) were mixed together and evaporated under a stream of nitrogen at 45 °C. In some situations, in addition to the above, the radioactive steroid was treated in the absence of cold standard and then mixed with 10 μg of the 'expected' product prior to being run on TLC.

2.6.1. Enzymic oxidation

The steroid was redissolved in 1 ml 0.05 M Tris–HCl (pH 9) containing 1.5 mg NAD (Sigma N-1511) and 1.2 mg hydroxysteroid dehydrogenase (HSD; Sigma H-8879). The reaction mixture was left overnight at room temperature and then extracted twice with 4 ml ethyl acetate.

2.6.2. Enzymic reduction

The steroid was redissolved in 1 ml 0.05 M Tris–HCl (pH 7.6) containing 3.3 mg HSD (Sigma, H8879) and 2.4 mg NADH (Sigma, N-8179). The reaction mixture was left overnight at room temperature and then extracted twice with 4 ml ethyl acetate.

2.6.3. Chemical oxidation

The steroid was redissolved in 100 μl glacial acetic acid and 60 μl of chromium trioxide solution (20 mg/ml of distilled water). The reaction mixture was left overnight at room temperature and then extracted twice with 4 ml ethyl acetate, which was washed twice with 1 ml sodium bicarbonate and twice with 1 ml distilled water before being evaporated.

2.6.4. Acetylation

The steroid was redissolved in 100 μl pyridine and 100 μl acetic anhydride and left overnight. The pyridine and acetic anhydride were removed by drying down with a stream of nitrogen at 45 °C. The plates were developed with chloroform/ethanol 50/1 (v/v) to prevent potential acetylated products from migrating too far up the plate.

2.6.5. Acid solvolysis

Acid solvolysis was carried out to remove sulfate groups from sulfated steroids. The radioactive steroid was dissolved in 5 ml ethyl acetate/trifluoroacetic acid (100 ml/1.4 ml, v/v) and incubated overnight at 45 °C. The solvents were removed by drying down under a stream of nitrogen at 45 °C.

2.7. Recrystallization

Definitive evidence for the identity of several of the radioactive steroids was obtained by mixing them with 15–20 mg of standard steroid and then repeatedly crystallizing them with either acetone/water or ethanol/water. A portion of steroid was removed after each crystallization, dried, and carefully weighed and then redissolved in ethanol, and counted to determine specific radioactivity (dpm/mg).

2.8. DEAE ion exchange protocol

The column was made from two 1 ml HiTrap DEAE FF cartridges (Amersham Biosciences) linked in series. There were two buffer solutions. Solution A consisted of Tris–HCl 0.05 M (pH 7.8) made up in a mixture of 1250 ml deionised water and 250 ml ethanol. Solution B consisted of 0.5 M NaCl made up in the same buffer as solution A.

The column was equilibrated with solution A at a flow rate of 0.15 ml/min. The test compound (20,000 dpm) was mixed with 10 μg each of free, sulfated and glucuronidated 17,20β-dihydroxypregn-4-en-3-one dissolved in 1 ml of solution A. This was injected on to the column at 0.15 ml/min. After 5 ml of solution A had passed through the column, a gradient of 0% solution B to 100% solution B over the next 10 ml was then applied. The eluate was monitored at 254 nm and 500 μl fractions were collected between 0 and 5 ml and 1 ml fractions between 5 and 15 ml. These were mixed with scintillation fluid and counted.

3. Results

3.1. Histology

The structure and cellular organization of the testis was the same in all samples that were examined in this study. The testes of the spermiating round goby were, as in many other fish, paired, elongated white-colored organs. When a testis was viewed in a cross-sectional plane, the mesorchial region, located where mesenteries extend to the body wall, was recognized by a deep fold (Fig. 1A). Seminiferous tubules, filled with spermatic cells, radiated from the center of the testis (Figs. 1A and B). The mesorchium contained mesentery tissue that extended into the fold, and contained prominent blood vessels, nerve fascicles and loose aggregates of round pale-staining cells with central nuclei (Figs. 1B and C). The staining properties and shape of these cells within the mesentery resembled erythrocytes, seen within blood vessels (Fig. 1C, arrow). Three regions with putative Leydig cells were recognized in the testes of the round goby. (1) A vascularized aggregate of cells, characteristic of endocrine cells (polyhedral cells arranged in cords and clumps, with intertwining capillaries), lined one side of the fold adjacent to the mesorchial mesentery (Figs. 1B and D). (2) A second mass of putative Leydig cells was located adjacent to the spermatic (efferent) duct, located at the base of the fold (Figs. 1B and E). (3) Clusters of Leydig-like cells were present in the center of the testis (Fig. 1F). These were arranged into cords with capillaries coursing among the cells (Figs. 1 D, E, and F). The toluidine blue staining intensity of the cytoplasm ranged from moderate (Fig. 1D) to dark (Fig. 1F). Examination of serial sections revealed that these Leydig-like cell aggregates formed "islands," rather than an uninterrupted arrangement along the length of the testis. Sperm were abundant within the spermatic (efferent) duct (Fig. 1E) and filled the lumina of seminiferous tubules (Fig. 1G). The 'pockets' of Leydig cells were not visible upon dissection, unlike the large single mass of glandular tissue in the black goby testis (Colombo and Burighel, 1974), thus making it infeasible to incubate the Leydig cells separately from the seminiferous tubules. Hence, whole testes (a mixture of glandular and spermatogenic tissues) were used for incubation studies.

3.2. Incubation of testes with [H^3]androstenedione

Following HPLC separation (Fig. 2), the incubation medium from testis I yielded seven prominent peaks of radioactivity (labeled A, C, D, D′, E, F, and G) and a region of indistinct, relatively minor peaks between the more prominent peaks A and C (labeled B). The incubation media from the other four testes yielded a similar pattern of peaks. However, peak A was only present in one other testis and there was a new peak (labeled F′) that was not present in testis I. Peak D′ was evident only in testis I. Although there were only two fish in each group, the HCG-injected fish did not have an overtly different pattern from the non-HCG-injected fish (perhaps slightly higher amounts of F′). In all cases, Ad was >95% converted to other steroids. In all cases also, >90% of the radioactivity in each of the peaks (excluding region B) formed single major bands on TLC.

Identification studies were concentrated on steroids that were produced by testis I. It proved possible through HPLC, TLC and microchemistry to match all the peaks to known C_{19} steroids (Table 1), except for D′ and for one component of the B region (Table 2). The compound in the D′ peak had an R_T value of 0.12. Although this value was close to that of three of the standards (Table 1), none of them co-migrated with the radioactive steroid after they had been mixed together and run on TLC. All the steroids that were identified had either an androsten-4-ene or a 5β-androstane configuration. None of the radioactive steroids co-migrated with any of the available 5α-androstane steroids. Microchemistry implemented in the identification of the incubation products is described in Table 3.

It was established that >90% of peak A and 50% of region B remained at the origin when run on TLC. This made it likely that these steroids were conjugated to either a sulfate or a glucuronide group (making them too hydrophilic to chromatograph on TLC). In order to establish the nature of the conjugating group(s) in peak A, some of the radioactivity was run on an anion-exchange column with 10 μg each of the free, glucuronidated and sulfated forms of 17,20β-P. The bulk of the radioactivity eluted in a position (Fig. 3) that was consistent with the steroid having a sulfate group (i.e. it was too strongly charged to be a glucuronide). This was confirmed by the fact that acid solvolysis turned >90% of the radioactivity into a free steroid that was found to have the same mobility on TLC as peak E (11-oxo-ETIO). Acid solvolysis of fraction 37 (in region B) also released a steroid that had the same chromatographic properties as ETIO. Acid solvolysis would not have been expected to deconjugate glucuronide groups.

The identities of the main peaks from testis II were confirmed in the same way as for the first incubation (i.e., TLC plus microchemistry). Partial characterization (TLC with and without acetylation) was also carried on the steroid in peak F′ to show that it was most probably testosterone. The identities of the steroids in the main peaks from testes III, IV, and V were only checked by co-migration on TLC.

Finally, the identities of solvolyzed peak A (11-oxo-ETIO-s), peak C (11-oxo-T), peak E (11-oxo-ETIO), and peak G (ETIO) were confirmed by recrystallization to constant specific activity (Table 4).

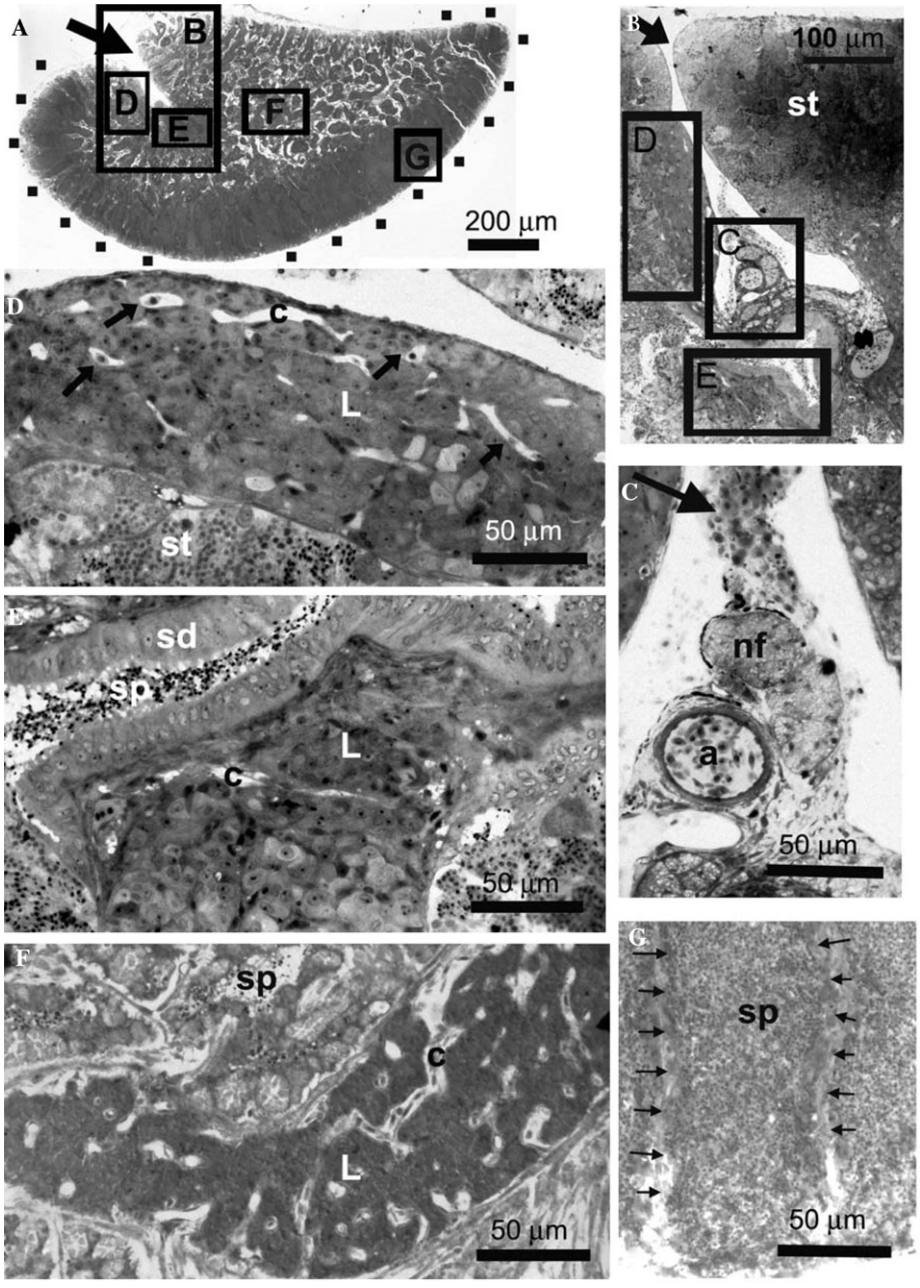

Fig. 1. The testis from a spermiating round goby. Semi-thin plastic section stained with toluidine blue. (A) A low power cross-sectional view of the testis. The black arrow points to a prominent fold located on the mesorchial side of the testis. Rectangles D, E, and F show locations with glandular cells, shown in high power in panels D, E, and F. The periphery of the testis (outlined with black squares) contains abundant seminiferous tubules filled with sperm. Rectangle G outlines a seminiferous tubule, shown at high power in panel G. A high power view of the mesorchial fold from a different specimen (outlined by a rectangle B) is shown in B. (B) The tissue surrounding the fold located on the mesorchial side of the testis. The arrow points to this fold. There are seminiferous tubules (st) located beside the fold, on one side, and a region with glandular cells on the opposite surface, enclosed by rectangle D, which is shown at high power in panel D. The tissue beneath the fold contains a spermatic duct (sd) and adjacent glandular cells, enclosed by rectangle E, enlarged in panel E. The region at the base of the fold, enclosed by the square, C, contains structures shown at high power in panel C. (C) A high power view of mesentery, with an arteriole (a), nerve fascicle (nf) and polyhedral cells which appear to form part of the mesorchium, extending into the fold (arrow). (D) Glandular cells (putative Leydig cells, L) located beside the fold at the mesorchial surface (area D in B) shown at high power. Capillaries (c) with single erythrocytes are visible (arrows). The glandular cells are polyhedral, and arranged into cords and clumps. Seminferous tubules (st) are seen adjacent to the glandular cells. (E) Glandular cells (putative Leydig cells, L) located beneath the spermatic duct (sd), which is filled with sperm (sp). These round cells are arranged in a sizeable clump, with a single capillary (c) passing through this large cellular aggregate. The staining of these cells ranges from pale to moderate. (F) "Islands" of strongly basophilic putative Leydig cells (L) located in the center of the testis (location shown by rectangle F in A). In these cells, the cytoplasm is as strongly basophilic as the nucleus, and the nucleus is not distinguishable. Capillaries (c), containing erythrocytes, are prominent. Sperm (sp) within seminiferous tubules are beside these Leydig cell islands. (G) A high power view of a seminiferous tubule shows spermatic cells (sp) filling the lumen of the tubule. The small arrows outline the follicular cells of the seminiferous tubule.

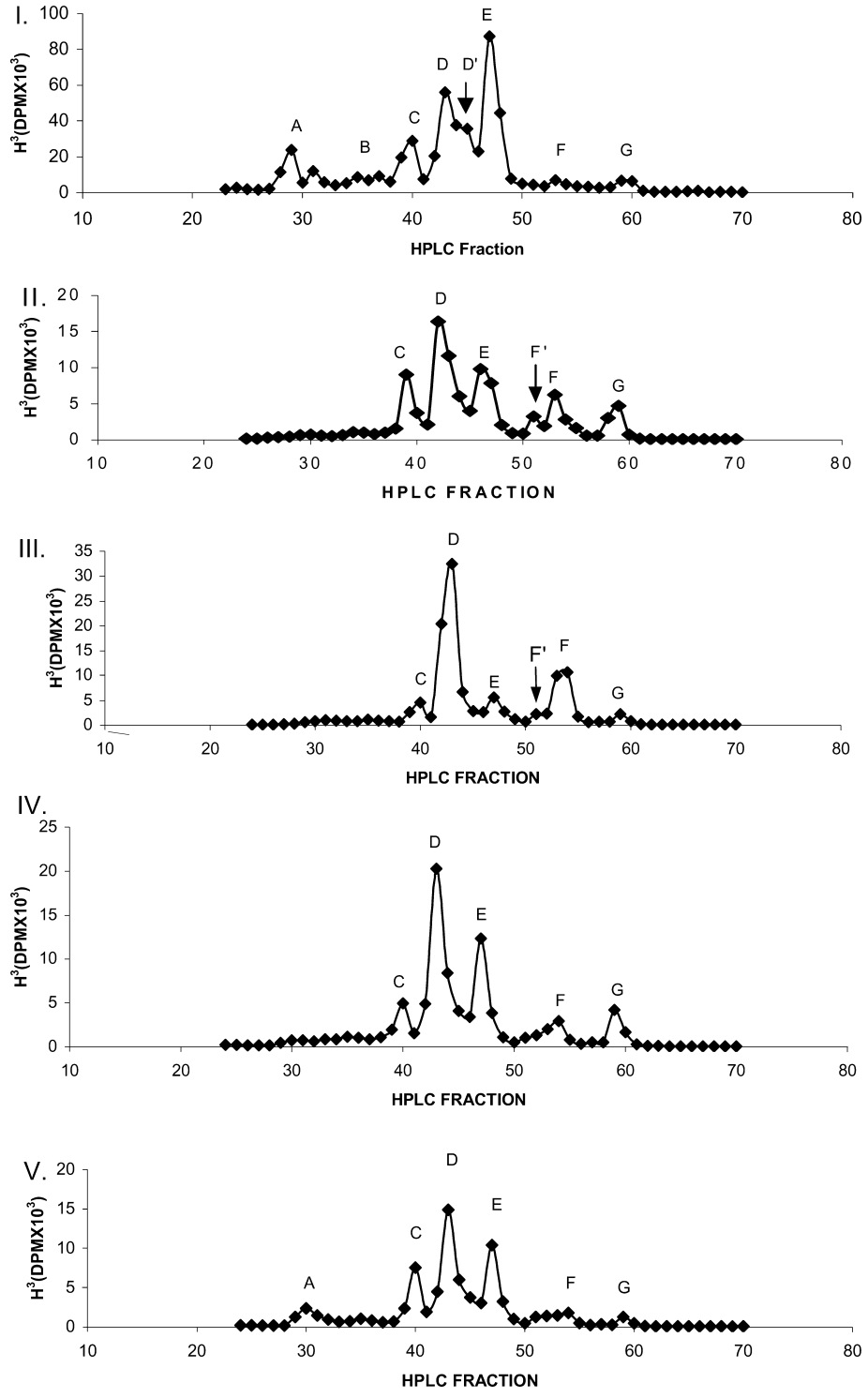

Fig. 2. HPLC separation of media from round goby testes incubated with [^{3}H]Ad. Each plot represents a single male. Fish II and III were injected with HCG the previous day. Decay per minute (dpm) is along the *y* axis and elution minutes along the *x* axis. See Table 2 for identities of the steroids that were found in each of the labeled peaks.

3.3. Incubation of a single testis (fish VI) with [H^3]-17-P

A single incubation was carried out with tritiated 17-P. HPLC separation of the incubation medium yielded at least nine peaks (Fig. 4). Only a few of these peaks could be matched to the C_{19} steroids shown in Table 1. Also two of the peaks (D and E) yielded two prominent bands on TLC. Peak A had the same properties as peak A from testis I (i.e., it could be solvolyzed to a steroid that had the same mobility as 11-oxo-ETIO). Peak F

Table 2
Main steroids produced by incubation of testes of the round goby with [^{3}H]Ad

Product	Peak	% Yield
11,17-Dioxo-5β-androstan-3α-yl sulfate (11oxo-ETIO-s)	A	13.5
17-oxo-5β-androstan-3α-yl sulfate (ETIO-s)	B	1.4 (1.7 unidentified)
17β-Hydroxyandrost-4-ene-3,11-dione (11-oxo-T)	C	16.0
11β-Hydroxyandrost-4-ene-3,17-dione (11β-OH-Ad)	D	18.3 (D′ = 12.3% unidentified)
3α-Hydroxy-5β-androstane-11,17-dione (11-oxo-ETIO)	E	28.5
Androst-4-ene-3,17-dione (Ad)	F	3.9
3α-Hydroxy-5β-androstan-17-one (ETIO)	G	4.33

Percent yields indicated in this table are expressed as a total of the radioactivity of all peaks made by testis I (Fig. 2).

Table 3
Identification evidence for peaks from testis 1

Peak	HPLC (min)	R_T[a]	Identified metabolite	Microchemistry performed
A	28–30	Origin	11,17-Dioxo-5β-androstan-3α-yl sulfate (11-oxo-ETIO-s)	Following solvolysis: acetylation products comigrate on TLC; enzyme and chemical oxidation products have same mobility as 5β-androstane-3,11,17-trione on TLC
B	37	Origin	17-oxo-5β-androstan-3α-yl sulfate (ETIO-s)	Following solvolysis: acetylation products comigrate on TLC
C	39,40	0.67	17β-Hydroxyandrost-4-ene-3,11-dione (11-oxo-T)	Acetylation products co-migrate on TLC; chemical oxidation product has same mobility as androst-4-ene-3,11,17-trione
D	43	0.66	11β-hydroxyandrost-4-ene-3,17-dione, (11β-OH-Ad)	Failure to acetylate; enzyme reduction products co-migrate on TLC; chemical oxidation product has same mobility as androst-4-ene-3,11,17-trione
D′	44	0.12	Unidentified	N/A
E	47	0.84	3α-Hydroxy-5β-androstane-11,17-dione (11-oxo-ETIO)	Acetylation products co-migrate on TLC; enzyme and chemical oxidation products have same mobility as 5β-androstane-3,11,17-trione on both TLC and HPLC
F	53,54	1.70	Androst-4-ene-3,17-dione (Ad)	Failure to acetylate
G	59,60	1.03	3α-Hydroxy-5β-androstan-17-one (ETIO)	Acetylation products comigrate on TLC; enzyme oxidation product has same mobility as 5β-androstan-3,17-dione

Microchemical reactions used were acetylation, oxidation, reduction, and solvolysis. HPLC and TLC elution positions of each peak are also given.
[a] Due to day-to-day differences in TLC running conditions (e.g., temperature), these R_T values do not necessarily exactly match the values shown in Table 1.

also had the same mobility as 11-oxo-ETIO. The identities of these two steroids were confirmed by recrystallization to constant specific activity (Table 4).

4. Discussion

Histological analysis showed that the testis of the round goby is similar to that of the black goby (Colombo and Burighel, 1974), of the rock goby *Gobius paganellus* (Stanley et al., 1965) and of the urohaze goby (Asahina et al., 1985), in possessing concentrations of Leydig cells. In these other species, the endocrine portion of the testis was found in a large glandular mass running along the mesorchium, as well as in smaller aggregates adjacent to the seminiferous tubule. The three spatially distinct zones with Leydig cells in the round goby match localizations previously observed in other gobiid species. The accumulation of Leydig cells adjacent to the fold facing the mesorchium in the round goby, parallels the large glandular mass (mesorchial gland) running along the mesorchium in the black goby (Colombo and Burighel, 1974) and the rock goby (Stanley et al., 1965) and in the central region of the urohaze goby testis (Asahina et al., 1985). Leydig cells may form a large mesorchial gland in a stage of sexual maturity of the round goby that has not been examined in this study. Leydig cells

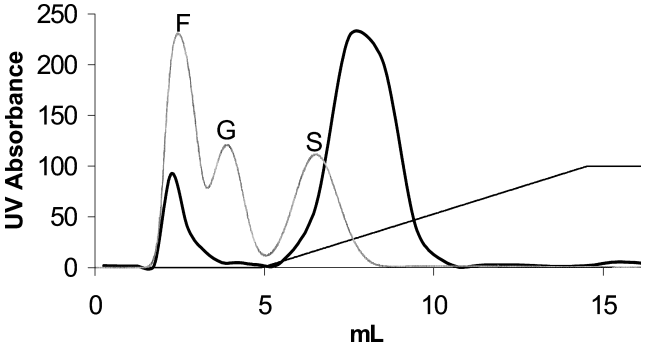

Fig. 3. DEAE–Sephadex (anion-exchange) separation of peak A from incubation of testes with [^{3}H]Ad. The continuous curved line shows the elution position of radioactivity. The hatched curved line shows the UV absorption (at 254 nm) of free (F), glucuronidated (G), and sulfated (S) 17,20β-P. The continuous straight line shows the gradient. The elution position of the radioactivity is consistent with the radioactive compound being a sulfate.

Table 4
Results of recrystallization of several standard steroids with radioactive metabolites derived from incubation of round goby testes with either tritiated Ad or 17-P

Cold standard	Radioactive steroid peak[a]	Recrystallization number			
		1	2	3	4
Testosterone (T)	E (original dpm = 10750)[b]	1628[e]	176	74	69
Testosterone	Tritiated T[c]	6337	6147	6206	5532
11-Oxo-ETIO	E	4698	4421	4315	
11-Oxo-ETIO	Solv. A	9831	8985	8960	8383
11-Oxo-ETIO	*F*[d]	9689	8733	8588	8763
11-Oxo-ETIO	Solv. *A*[d]	2401	1988	1850	1829
ETIO	G	2010	2153	1905	2331
11-Oxo-T	C	5100	4576	4533	

[a] Letters refer to labeled peaks on HPLC (Fig. 2).
[b] This was done as a negative control.
[c] This was done as a positive control; tritiated testosterone was purchased from Amersham–Pharmacia.
[d] These two steroids were derived from fish VI (17-P incubation; Fig. 4.); Solv., solvolyzed.
[e] All values are in dpm/mg of crystal.

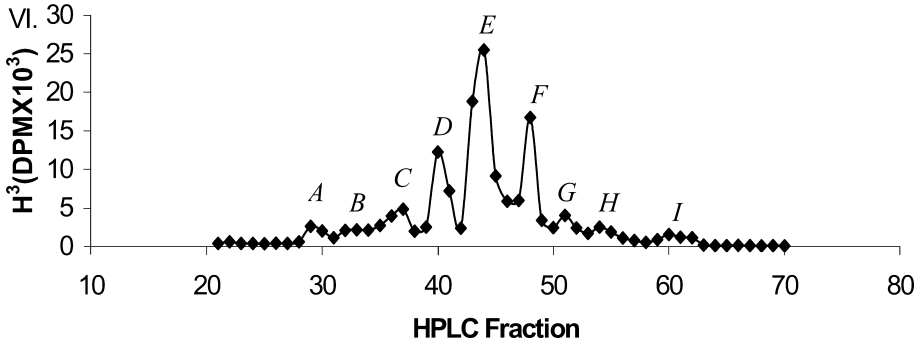

Fig. 4. HPLC separation of media from testes of a single round goby male incubated with [^{3}H]-17-P. Tentative identifications were made of 11-oxo-T in peak D and Ad in peak H. Definitive identifications were made of 11-oxo-ETIO-s in peak A and 11-oxo-ETIO in peak F. The other steroids were not identified.

concentrated beside the sperm duct have also been observed in the black goby, where the large mesorchial mass of Leydig cells extends as far as the collagenous sheath surrounding the deferent duct (Colombo and Burighel, 1974). The third location of Leydig cells in the round goby, scattered in pockets throughout the middle of the testes, is also an arrangement that has previously been observed in the rock goby (Stanley et al., 1965), and black goby (Colombo and Burighel, 1974; Rasotto and Mazzoldi, 2002). In the urohaze goby, cord-like processes with glandular cells extend between seminiferous tubules from the main central glandular mass (Asahina et al., 1985). The spatially distinct localization of Leydig cells in the testis of gobiids is different from the Leydig cell distribution in most vertebrate species, where the steroid producing endocrine Leydig cells are interstitial to the seminiferous compartment (e.g., Ross et al., 1989).

Circumstantial evidence that the glandular tissue in the testis is involved in the synthesis of pheromones in gobies comes from the work of Rasotto and Mazzoldi on the black goby (2002). These authors showed that there are two types of male—parents and 'sneakers.' Because the latter "sneak" fertilizations and do not guard or maintain nests, they do not presumably need to emit pheromones to attract females. Underlining this difference in behavior, the large glandular masses of Leydig cells are absent from the testes of the sneaker males. Furthermore, only the milt (ejaculate) of parental males has been shown to excite aggression in other parental males (Locatello et al., 2002).

The incubation of testis with [^{3}H]Ad yielded steroids that either retained their androst-4-ene configuration or were 5β-reduced. Many were also 11-oxygenated. The presence of 5β-reductase, 11-hydroxylase and 11β-HSD enzymes in teleost testes is well-established (Borg, 1994; Fostier et al., 1983; Kime, 1993). Nevertheless, despite numerous reports of the synthesis of 11-oxo-T and ETIO in the testes of a variety of species, the synthesis of 11-oxo-ETIO does not appear to have been previously reported in any teleost, apart from a study on the tilapia *Sarotherodon mossambicus* (Kime and Hyder, 1983). However, in that study, the precursor steroid was already oxygenated at the 11-position (i.e., [^{3}H]-11-oxo-Ad), making it unclear whether tilapia would normally form it if the precursor had been Ad. In the present paper, we have replicated this finding five times. We have also demonstrated the synthesis of this steroid from 17-P as well as from Ad.

There were only two other products from the 17-P incubation that we were able to match to any of the steroids in Table 1. These were 11-oxo-T and Ad (limited identification only). The other peaks were not identified (through lack of resources). Some of them probably represent C_{21} metabolites.

In common with the black goby, the testis of the round goby produces an abundance of 5β-reduced steroids. Some of the steroids in the two species are the same (e.g., ETIO and ETIO-s). However, there are two major differences between the species. One difference is that the black goby seems to lack 11β-HSD activity. Although Colombo et al. (1977) found 11β-hydroxysteroids, they found no 11-oxo-steroids in the black goby incubations. Although this difference could be due to differences in protocol (different handling procedures, incubation media and precursors), it is unlikely. In the round goby, 11-oxo-ETIO, and 11-oxo-T are not minor products. They form a large proportion of the metabolites in the incubation media—suggesting the presence of a lot of 11β-HSD activity. It is unlikely that Colombo et al. (1977) would have failed to find such activity in the black goby if it existed. The situation in vivo may be entirely different. There are several species in which it appears that it is common for C_{19} steroids to be secreted 'half-formed' from the testis and transformed to their final product by enzymes in either the blood cells (e.g., conversion of 11-oxo-Ad to 11-oxo-T in the stickleback *Gasterosteus aculeatus*; Mayer et al., 1990) or liver (e.g., conversion of 11β-OH-testosterone to 11-oxo-T in African catfish *Clarias gariepinus*; Cavaco et al., 1997).

The other difference between the round goby and the black goby is the apparent low amount of steroid conjugation in the round goby incubations. Although we found small amounts of the sulfated forms of ETIO and 11-oxo-ETIO, we were unable to conclusively demonstrate the presence of their glucuronidated forms (although they may have been present in trace amounts). In view of the results of Colombo et al. (1970, 1977), who found substantial production of both glucuronides and sulfates in both the rock goby and black goby, it seems surprising that we were unable to demonstrate the synthesis of ETIO-g. However, we caution against the interpretation that the necessary enzyme, steroid–UDP–glucuronosyl-transferase, is missing from the testis of the round goby. In one of their studies on the seminal vesicle of the African catfish, *Clarias gariepinus*, Schoonen and Lambert (1986) detected large amounts of steroid glucuronide formation. In a later study (Schoonen et al., 1987) they found hardly any at all. In this second study, they noted that there was a 2 h delay between the capture of the animals and the incubation of their seminal vesicles and speculated that the capacity for producing glucuronides was lost more rapidly than the capacity for 5β-reduction and 11β-hydroxylation. In the present study, there was a relatively large (and unavoidable) 3 h delay between sacrifice of the animals and incubation of the testes. There was also evidence that steroid sulfotransferase activity might be labile; as 11-oxo-ETIO-s appeared to be low (or absent) in three of the incubations.

In several other species, temperature has been shown to influence glucuronide formation (Kime, 1980). However, the temperatures at which we and other authors have maintained the fish and subsequently carried out the incubations have been within the normal range for the species.

Although there are similarities in steroid biosynthetic pathways between the black goby and the round goby, there appear to be very few between the urohaze-goby and the other two species. The urohaze-goby glandular tissue predominantly synthesized 5α-reduced steroids (Asahina et al., 1985). These were notably absent in the present study. However, in at least one species, the sailfin molly, *Poecilia latipinna*, Kime and Groves (1986) have demonstrated the production of both 5α-and 5β-reduced androgen within a single testis. Furthermore, both these types of androgen were present with either 3α- or 3β-hydroxyl groups.

One criticism that can be leveled at the present study is that incubations were carried out with whole testes, while Colombo et al. (1977) used just the mesorchial gland and Asahina et al. (1985) used separate 'glandular tissue' and 'seminiferous tissue.' We do not believe this is a problem. First, apart from the fact that the structure of the testis in the round goby makes the clean dissection of glandular and seminiferous tissue infeasible, Asahina et al. (1985) did in fact find no difference in the types of steroid produced by the testis of the urohaze goby—only in their yields.

One result for which we have no reasonable explanation is the presence of an unidentified metabolite ($R_T = 0.12$; peak D′) in the incubation medium from the testis of fish I, but its absence in testes from II through V. The relative mobility of the steroid suggests that it has at least two hydroxyl groups (of which there were several in Table 1 that we were unable to test). Since the incubation of testes from fish I was carried out in a different year and a different month from the others, the difference is most likely due to a difference in the reproductive status of the fish. This will need to be taken into account in future studies.

Conjugated steroids are more soluble in water than free steroids and therefore potentially more likely to be utilized as pheromones (Scott and Vermeirssen, 1994; Vermeirssen and Scott, 1996). In mammals, conjugation is usually hepatic in origin and typically leads to excretion and deactivation of the steroid hormone (Kime, 1993). Colombo et al. (1979) showed not only that the testis of the male goby produced ETIO-g, but the steroid also induced behavioral activity when it was added to the water. Although we failed to establish the production

of this particular steroid in the round goby testis, we have identified ETIO by itself and also, tentatively, ETIO-s, also identified in *Gobius jozo* by Colombo et al. (1977).

The probable route of excretion of free steroids is via the gills (Vermeirssen and Scott, 1996) and of sulfated and glucuronidated steroids via the urine and feces (Scott and Vermeirssen, 1994). The milt (Locatello et al., 2002) and seminal vesicle fluid (Schoonen and Lambert, 1986) are also potential sources of the pheromone. However, immunoassays first need to be developed for ETIO and 11-oxo-ETIO in order to investigate these various possibilities.

More evidence that would point towards a pheromonal role for either 11-oxo-ETIO-s or ETIO-s is their elution positions on HPLC. Currently, water that has been conditioned by reproductive male round gobies has been separated by HPLC and each fraction has been tested against female round gobies for physiological activity (Bélanger et al., 2004). The highest EOG activity came between fractions 30–40. This is a potentially significant finding, in that 11-oxo-ETIO-s, ETIO-s (and 11-oxo-ETIO-g) elute on HPLC between 30 and 40 min. None of these steroids was tested for EOG activity by Murphy et al. (2001). However, we have carried out preliminary studies on 11-oxo-ETIO, 11-oxo-ETIO-g, and ETIO-s and found that they evoke an EOG response at least at 10^{-10} M (own unpublished data). However, 11-oxo-ETIO-s remains to be tested for EOG activity and all four steroids remain to be tested for behavioral activity. Also, it still needs to be established that these steroids are actually produced, and released into the water, in vivo and that they also modify the behavior of the animals.

In conclusion, we have shown that the testis of the sexually mature male round goby contains islets of steroid-synthesizing glandular tissue. We have discovered that, in vitro, the testis produces at least four 5β-reduced C_{19} steroids (two of them novel). These findings lend further support to the hypotheses of Colombo et al. (1977) and Murphy et al. (2001) that sexually mature male gobies secrete pheromones that attract females and that these pheromones are derived from the testes and are probably steroids with a 5β-reduced configuration.

Acknowledgments

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