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Release of Free and Conjugated Forms of the Putative Pheromonal Steroid 11-Oxo-etiocholanolone by Reproductively Mature Male Round Goby (*Neogobius melanostomus* Pallas, 1814)¹

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ABSTRACT

Previous studies of the round goby (*Neogobius melanostomus* Pallas, 1814), an invasive fish species in the Laurentian Great Lakes of North America, have shown that this species has the ability to both synthesize and smell steroids that have a 5 β -reduced and 3 α -hydroxyl (5 β ,3 α) configuration. An enzyme-linked immunoassay (EIA) for 3 α -hydroxy-5 β -androstane-11,17-dione (11-O-ETIO) has been used to show a substantial rise in the rate of release of immunoreactive compounds into the water when males are injected with salmon gonadotropin releasing hormone analogue. Similar increases were noted for 11-ketotestosterone and 17,20 β -dihydroxy-pregn-4-en-3-one. Partitioning of the extracts between diethyl ether and water showed the presence of both free and conjugated immunoreactive 11-O-ETIO. Only conjugated immunoreactivity was found in urine (implying that free steroid is released via the gills). The identity of the conjugates was probed by using HPLC, EIA, and mass spectrometry and removal of sulfate and glucosiduronate groups. Immunoreactivity in the conjugated fraction was found to be due mainly to 3 α ,17- β -dihydroxy-5 β -androstane-11-one 17-sulfate. However, the evidence was also strong for the presence in water extracts of substantial amounts of 3 α -hydroxy-5 β -androstane-11,17-dione 3-glucosiduronate (which could be detected only by EIA after removal of the glucosiduronate group with β -glucuronidase). There were also small amounts of 3 α -hydroxy-5 β -androstane-11,17-dione 3-sulfate and 3 α ,17- β -dihydroxy-5 β -androstane-11-one 17-glucosiduronate. These studies give some idea of the types, amounts, and ratios of 11-O-ETIO derivatives that are released by reproductive *N. melanostomus* and will aid further research into the putative pheromonal roles of 5 β ,3 α -reduced androgens in this species.

androgen, fish steroids, pheromone, testis

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INTRODUCTION

The round goby (*Neogobius melanostomus* Pallas, 1814), a small teleost native to the Black and Caspian Seas of Eurasia, has invaded all five Laurentian Great Lakes of North America [1]. Multiple factors related to diet, physiology, and behavior have led to rapid expansion of *N. melanostomus* populations in the Great Lakes [2, 3], where they pose a threat to the native ecology by feeding on the eggs of native fish species [4] and by displacing other benthic fishes [5]. Currently, techniques available for controlling *N. melanostomus* populations do not target this species exclusively, and we expect the development of a biological control strategy for specifically reducing *N. melanostomus* populations will be hugely beneficial. The use of reproductive pheromones for assisting with population management holds some promise. Various studies have demonstrated the attractive properties of steroids and their conjugates in fish [6–9]. In one case (the sea lamprey), a synthetic pheromone has successfully lured females in the wild and is under development for application at the population level [10].

Thirty years ago, Colombo et al. [9] reported that 3 α -hydroxy-5 β -androstane-17-one 3-glucosiduronate (etiocholanolone glucosiduronate [ETIO-g]) was produced by the gonads of reproductive male (RM) black goby (*Gobius joso* L.) and that reproductively mature females (RF) were attracted to this conjugated steroid. During reproduction, RM *N. melanostomus* build and maintain nests on the lake bottom, within which many females deposit eggs [3, 11]. These findings have led us and others to inquire if RM *N. melanostomus* might also use a steroidal pheromone to attract females. Initial evidence was provided by Murphy et al. [8], who demonstrated strong olfactory epithelial field potential and gill ventilation responses when several synthetic steroids were added to the water. Among the stimulatory steroids were androgens (C₁₉ steroids) with a 5 β -reduced and 3 α -hydroxyl (5 β ,3 α) configuration, including 3 α -hydroxy-5 β -androstane-17-one (ETIO), ETIO-g, and 3 α ,11 β -dihydroxy-5 β -androstane-17-one 3-glucosiduronate. It was then shown that in *N. melanostomus*, RM have unusually high concentrations of steroid-producing cells in and around their testes [12, 13] and that both the testes and the seminal vesicles have a strong capacity to convert androstenedione to 5 β ,3 α -reduced steroids in vitro [12, 13], with the dominant product being 3 α -hydroxy-5 β -androstane-11,17-dione (11-oxo-etiocholanolone [11-O-ETIO]), which was later shown to evoke field potential recordings from olfactory epithelium, indicating that 11-O-ETIO is detected as an odor by *N. melanostomus* [14, 15]. Extracts of water that had been conditioned by RM evoked stronger field potential responses

from the olfactory epithelium of RF than extracts of water from nonreproductive males [16]; RF were attracted to water that has previously held RM [17]. Nonreproductive females (NRF) were attracted to a blend of synthetic, free steroids (which included 11-O-ETIO) but avoided a blend of synthetic conjugated steroids (which included 3-sulfated and glucosiduronated 11-O-ETIO), whereas RF showed a tendency (not statistically significant) to prefer conjugated steroids and avoid free steroids [18]. In relation to the last of these studies, unconjugated steroids may be suitable for attraction as the round goby olfactory system responds to many of these steroids [8, 19], and unconjugated steroids are excreted continuously from the gills [20], thus creating an uninterrupted plume. Additionally, ligand competition studies using field potential recordings from olfactory epithelium (cross-adaption) have shown that ETIO, ETIO-g, and $3\alpha,11\beta$ -dihydroxy-5 β -androstane-17-one 3-glucosiduronate all interact via the same receptor [8]. However, there is circumstantial evidence that the major component of a pheromonal mixture that attracts RF is likely to be a conjugated rather than a free steroid. This is because when RM water extracts were separated using reverse-phase HPLC, RF showed significantly higher field potential responses from their olfactory epithelium to fractions that corresponded to the elution positions of conjugated rather than free steroids [16]. It has also been shown that nesting RM release more urine in the presence of females [21], and it is well known that urine is the main route of excretion for conjugated steroids in fish [22].

Concerning the nature of any putative conjugated forms of 11-O-ETIO, a previous study [13] showed only trace production of 3α -hydroxy-5 β -androstane-11,17-dione 3-sulfate (11-O-ETIO-3-s) from radioactive precursors in vitro by *N. melanostomus* testes but not of 3α -hydroxy-5 β -androstane-11,17-dione 3-glucosiduronate (11-O-ETIO-3-g). However, in that study, it was pointed out that due to technical difficulties, there was a long time lapse (>4 h) between testis collection and subsequent in vitro incubation with radioactive steroid precursors and that such delays had already been associated in one other species with a loss of glucuronide formation [23]. Also, the possibility could be ruled out that, in vivo, 11-O-ETIO might be further modified (by reduction of the oxygen at the 17 β position and then by conjugation at either the 3α or the 17 β position) in peripheral organs (e.g., blood cells and liver) before being released into the water. This raises the question: if such conjugates are indeed made by RM *N. melanostomus*, what are the conjugates' mostly likely chemical structures? Referring to Fig. 1, 11-O-ETIO (Fig. 1A) contains only one hydroxyl group (3α) available for conjugation; this can be either sulfated (11-O-ETIO-3-s) (Fig. 1B) or glucuronidated (11-O-ETIO-3-g) (Fig. 1C). However, if the 17-oxo group is reduced to form $3\alpha,17\beta$ -dihydroxy-5 β -androstane-11-one (Fig. 1D), there are now two possible sites for conjugation (3α and 17 β), leading to sulfate or glucosiduronate conjugation via either the 17 β hydroxyl group (Fig. 1E and F) or the 3α hydroxyl group (Fig. 1G and H). Thus, structures shown in Fig. 1 constitute the "most likely" structures for a pheromone. While the possible existence of diconjugates (i.e., conjugated at both 3α and 17 β) cannot be dismissed, the formation of steroids conjugated via the 11 position is unlikely. The 11 β -hydroxyl group is notoriously unreactive and highly unlikely to accept a conjugating group.

In the present study, we developed an enzyme immunoassay (EIA) for 11-O-ETIO by using an antibody generated against the A ring of 11-O-ETIO (i.e., using a hapten in which the steroid was immobilized via the 17-oxo group). As a consequence, we expected the antibody to cross-react with

free 11-O-ETIO and conjugates of $3\alpha,17\beta$ -dihydroxy-5 β -androstane-11-one (Fig. 1D) that are coupled via the 17 β -hydroxyl group but not with any conjugates that are coupled via the 3α -hydroxyl group, an expectation that was confirmed by testing the cross-reactivity of synthetic compounds. This property of the antibody was used in conjunction with solid phase and solvent extraction, acid solvolysis (which removes sulfate groups from steroids), β -glucuronidase hydrolysis (which selectively removes glucosiduronate groups from steroids), reverse-phase HPLC, electrospray ionization mass spectrometry (ESI-MS), and thin layer chromatography (TLC) to identify which, if any, of the conjugates shown in Fig. 1 and Table 1 might be produced by male *N. melanostomus*.

MATERIALS AND METHODS

Experimental Animals

Adult *N. melanostomus* (mean weight, 36.6 ± 2.6 g SEM) were collected by angling from Lake Erie at Leamington and Colchester, Ontario, and from the Detroit River at Windsor, Ontario, during May through October in the years 2006, 2007, and 2008. Fish were held in accordance with University of Windsor animal care guidelines, and experimental procedures conformed to the guidelines of the Canadian Council of Animal Care (CCAC). After overnight acclimation in coolers containing lake water mixed with dechlorinated tap water from the University of Windsor water supply, fish were transferred into either 205-L or 50-L, gravel-lined, aerated tanks held at approximately 18°C. Tanks were set either to flow-through or recirculate (filtered, sponge, and activated charcoal) dechlorinated water. Fish were held at a constant photoperiod (16L:18D) and provided with approximately 15-cm-long segments of polyvinyl chloride piping for shelter. Fish were fed several times per week with Nutrafin fish flakes (Tetramin, Inc.).

Male fish were differentiated from females based on the appearance of urogenital papilla, as males have an elongated, triangular papilla and females have a broad, rounded papilla with the appearance of two lobes [16]. Reproductive status of males was determined by the presence of secondary sexual characteristics. A dark body, swollen cheeks, swollen urogenital papilla, larger fins, and the presence of a thick slime coat were characteristics used to identify RM. Only RM were used for the collection of urine and conditioned water. After they were collected, fish were euthanized, and their reproductive status was confirmed on the basis of gonadosomatic index (I_g), defined as gonad weight (testes, seminal vesicles, and mesorchial gland)/total body weight $\times 100$. Only males with an $I_g > 1.3$ were classified as RM [16, 17].

Collection of Urine and Conditioned Water from Reproductive Males

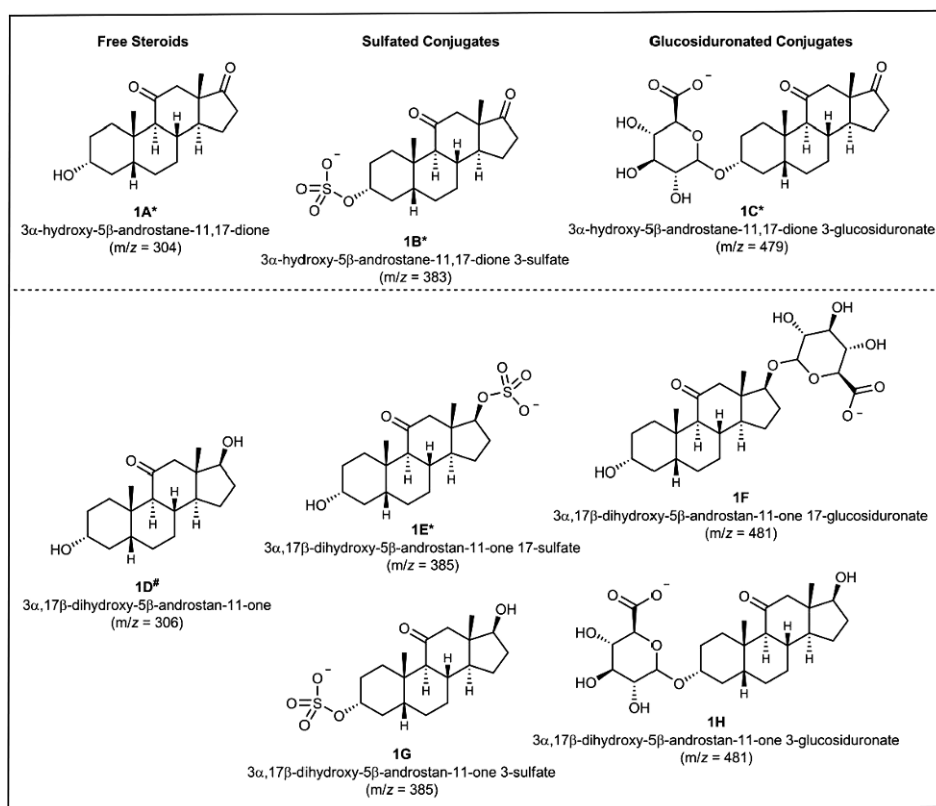
As described in previous papers [16, 17], RM were isolated in glass jars containing 1 L of dechlorinated, aerated water and left for 4 h. They were then moved to fresh jars and given 0.5% body weight of salmon gonadotropin-releasing hormone analogue (sGnRH α) (OvaRH; Syndell Labs, Vancouver, BC, Canada) dissolved at $4 \mu\text{g ml}^{-1}$ in 0.9% NaCl. Sham-treated RM were injected with 0.9% NaCl only. RM were held in 1 L of dechlorinated water for 16 h. After this period, males were injected with the same amount of sGnRH α and placed in a fresh jar for another 4 h. A vacuum assembly was used to pass all water quantities through activated Seppak C18 cartridges (Waters, Milford, MA), washed with 5 ml of water, and eluted with 5 ml of methanol.

Urine was collected from a different set of RM. When urine was collected, RM were allowed to reacclimatize to their containers for 2 h after receiving injections of sGnRH α . They were then anesthetized with 0.6% 2-phenoxyethanol (catalog no. P1126; Sigma-Aldrich, Oakville, ON), the urogenital papilla was tied shut with fishing line (0.25-mm-diameter Trilene; Berkley Fishing), and the water in the containers was changed. After 4 h, the fish was euthanized, and urine was collected directly from the distended abdomen by using a syringe and (26-gauge) needle. Methanol extracts of urine were prepared by passing the urine (diluted with distilled water) through activated Seppak C18 cartridges (Waters, Milford, MA), washing with water, and eluting with 5 ml of methanol.

All RM fish were euthanized using an overdose of MS-222 (5% Tricaine methanesulfonate; Finquel, Argent Chemical Lab), and I_g measurements were recorded.

Some RM were given intramuscular injections of androstenedione, cortisol, or 11-O-ETIO ($1 \text{ mg } 100 \text{ g}^{-1}$ of body weight). For these injections, steroids were first dissolved in a minimum amount of ethanol, and the resulting solution

FIG. 1. Steroid molecules relevant to this study listed by IUPAC name and letter designation for this article. Free steroids (**A** and **D**) are listed at the left with their corresponding sulfate and glucosiduronate conjugates to the right. Note, the structure shown in **A** is often given the trivial name 11-oxo-etiocholanolone (11-OETIO). *, Available as a standard sample from a commercial source. #, Generated from a commercial sample.



was dispersed in peanut oil. The suspension was vortexed vigorously before it was injected. Conditioned water samples were collected, and methanol extracts were prepared as described above.

EIA for Measurement of 11-O-ETIO, 11-Ketotestosterone, or 17,20 β -Dihydroxypregn-4-en-3-one

The enzyme immunoassay procedure was based closely on the methodology described previously for measuring cortisol in teleosts [24]. Essentially, the steroid to be measured is covalently bound to bovine serum albumin (BSA) that is, in turn, covalently bound to the wells of microtiter plates (CovaLink, NH). Unknown and standard amounts of steroid plus primary (i.e., antisteroid) antibody are then added to the wells. The primary antibody becomes partitioned between the immobile steroid and the steroid in solution. After equilibrium has been reached, the plate is washed and an enzyme-labeled secondary antibody is added, which is directed at the gamma-globulin of the primary antibody. After the plates are washed again, a reagent is added that changes color in the presence of the enzyme, and the color intensity is measured by a plate reader. The only substantial differences between our procedure and that described previously [24] were that we used a secondary antibody labeled with alkaline phosphatase (anti-rabbit immunoglobulin G [IgG]), together with Sigmafast nitrophenylphosphate (NPP) tablets (catalog no. N2770; Sigma-Aldrich; www.sigmaaldrich.com), rather than peroxidase. Carboxymethyloxime (CMO) derivatives of steroids were synthesized as described by Bryan et al. [25] and were conjugated to BSA as described by Yun et al. [26, 27]. The 11-O-ETIO-CMO-BSA conjugate was used not just for EIA development but also to raise an antibody to 11-O-ETIO (Fig. 1A) by injection into rabbits. This was carried out at Michigan State University.

Dried methanol extracts (1.5 ml) were dissolved in 0.5 ml of EIA assay buffer (sodium phosphate buffered saline, 0.1 M, pH 7.4, containing 0.1% BSA and 0.01% sodium azide). CovaLink NH F8 module CS-30 microtiter plates (VWR International; www.vwr.com) were activated by addition of 100 μ l of 0.125 mg ml⁻¹ disuccinimidyl suberate to each well (catalog no. S1885; Sigma-Aldrich) in a 50:50 mixture of dimethyl sulfoxide and carbonate-bicarbonate buffer, 0.05 M, pH 9.6. After plates were incubated overnight at 4°C, they were washed with distilled water and tapped dry. Then, 100 μ l of a solution of steroid-CMO-BSA conjugate in carbonate-bicarbonate buffer (25 ng ml⁻¹ for 11-O-ETIO and 50 ng ml⁻¹ for 11-ketotestosterone [11-KT] and 17,20 β -dihydroxypregn-4-en-3-one [17,20 β -P]) was added to each well. After plates were incubated for 3 h at room temperature, 100 μ l of 1% (w:v) BSA solution

in 0.1 M phosphate-buffered saline, pH 7.4, containing 0.05% Tween-20 was added to block any excess activated sites, and plates were incubated again for 3 h at room temperature, followed by thorough washing. While these plates were incubating, dilutions of standard steroids and unknowns were prepared in assay buffer in a separate set of round-bottomed 96-well polypropylene microtiter plates (Corning Life Sciences; www.corning.com), using a volume of 135 μ l in every well, to which was added 65 μ l of primary antibody (code 295/II; final dilution, 1/500 000; Michigan State University rabbit antibody). After the solution was mixed, 150 μ l was transferred to CovaLink NH plates (matching well-to-well). After plates were incubated at 4°C overnight, they were washed, and a 150- μ l solution of goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase (catalog no. A3937; Sigma-Aldrich) was added to every well. After further incubation for 3 h at room temperature, the plates were washed, and a 150 μ l NPP solution was added to each well. The plates were incubated at room temperature for up to 12 h, and absorbance was measured at 405 nm, using a microplate reader (Victor 3 1420 model Multilabel plate reader; Perkin-Elmer, Waltham, MA). In order to avoid any edge effect in the assays, all plates were sealed and wrapped in damp paper towels and placed in sealed plastic bags when not being handled directly.

Regarding our choice of EIA, initially a radioimmunoassay was developed, but preparation of sufficient radiolabeled 11-O-ETIO (Fig. 1A) proved problematic. Also, the laboratory in Windsor, Ontario, where the fish studies were carried out, was not optimally equipped to deal with radioactivity. We contemplated developing an EIA based on labeling 11-O-ETIO (Fig. 1A) with acetylcholinesterase. One of the present authors has experience in the development of such an assay, which was used to measure pheromones of the sea lamprey [26, 27]. However, despite the good correlation between EIA and MS measurements shown in that paper, subsequent studies (unpublished data) showed that when the acetylcholinesterase based EIA was applied to analyzing water extracts, it was prone to generate false-positive results. The cause of the problem was never established, but it was hypothesized that with this type of EIA, primary antibody, water extract, secondary antibody, and enzyme labels are all present contemporaneously within each well, giving ample opportunity for "matrix effects" (i.e., nonspecific interference from compounds other than cross-reacting steroids). Thus, we looked for an EIA in which neither the enzyme nor the secondary antibody was actually present during the initial incubation stage. Although the present EIA meets those criteria, admittedly, it is not (at least in our hands) nearly as sensitive as radioimmunoassay or many other reported EIA procedures. However, a lack of sensitivity was not a major problem for the present study. There are several

TABLE 1. Cross-reactivity of various steroids in the EIAs for 11-O-ETIO.

Steroids	Percentage of cross-reaction
3 α -Hydroxy-5 β -androstane-11,17-dione (11-O-ETIO)	100
3 α ,17 β -Dihydroxy-5 β -androstan-11-one	68.4
3 α ,17 β -Dihydroxy-5 β -androstan-11-one 17-sulfate	56.9
3 α ,11 β -Dihydroxy-5 β -androstan-17-one	16.6
3 α ,17,21-Trihydroxy-5 β -pregnane-11,20-dione (tetrahydrocortisone)	2.6
3 α ,17,20 β ,21-Tetrahydroxy-5 β -pregnan-11-one (β -cortolone)	0.5
3 α -Hydroxy-5 β -androstan-17-one (ETIO)	0.4
11-Ketotestosterone	0.04
Cortisone	0.01
Cortisol	0.003
11 β -Hydroxytestosterone	0.002
Testosterone	<0.001
Androstenedione	<0.001
3 α -Hydroxy-5 β -androstane-11,17-dione 3-sulfate (11-O-ETIO-3-s)	<0.001
3 α -Hydroxy-5 β -androstane-11,17-dione 3-glucosiduronate (11-O-ETIO-3-g)	<0.001

advantages: first, there appears to be no problem with nonspecific matrix effects (as there were no immunoreactive peaks with HPLC other than those due to steroids that cross-reacted with the antibody; cf. reference 28). Second, the procedure does not require radiation facilities. Third, as has been shown, EIA can be rapidly adapted to measure any steroid merely by changing the composition of the plate coating reagent and primary antibody.

Separation of Free and Conjugated Steroids

Conditioned water samples collected from RM before and after fish were injected with sGnRHa, cortisol, androstenedione, or 11-O-ETIO were passed through Seppak C18 cartridges, washed, and eluted with 5 ml of methanol, as described above. The methanol solution was then evaporated in a vacuum centrifuge. Free steroids and conjugated steroids were separated by partitioning twice between 200 μ l of water, which retained the conjugated steroids, and 5 ml of diethyl ether, which extracted free steroids. Samples from both phases were dried and reconstituted in either assay buffer (for EIA) or 10% acetonitrile (ACN) in distilled water and 0.1% trifluoroacetic acid (TFA) for subsequent HPLC.

HPLC

HPLC of methanol extracts was carried out using a J'sphere ODS-M80 (4- μ m) column (Waters, Milford, MA) and an ACN-water gradient containing 0.1% TFA as the mobile phase (Waters, Milford, MA). A linear gradient from 10% ACN-0.1% TFA:90% water-0.1% TFA to 90% ACN-0.1% TFA:10% water-0.1% TFA over 40 min was used. From the 5 ml of methanol extract prepared from a single fish, 1 ml was pooled with 1-ml extracts from seven to eight other fish and then dried. These dried extracts were dissolved in 1 ml of 10% ACN in a distilled water-0.1% TFA solution and filtered through a 0.2- μ m syringe filter into an HPLC-certified vial (Waters, Milford, MA). The injection volume was 100 μ l, and the flow rate was 0.5 ml min⁻¹; the column temperature was maintained at 20°C. Fractions were collected at 1-min intervals and dried in a vacuum centrifuge at 35°C, and the dried residues were dissolved in assay buffer and subjected to EIA to determine 11-O-ETIO immunoreactivity.

Other Tests To Ascertain the Nature of Conjugates

Dried methanol extracts were subjected to acid solvolysis to remove sulfate groups from steroids and/or to enzyme hydrolysis using a recombinant, sulfatase-free β -glucuronidase to remove glucosiduronate groups from steroids. For acid solvolysis, 1-ml samples of methanol extract, each from seven to eight males, was pooled in a glass tube and dried under vacuum at 40°C and dissolved in 200 μ l of distilled water. Free steroids were removed by extraction with diethyl ether, and the resulting aqueous phase was divided into two equal portions. One portion was kept for EIA or HPLC, and the other portion was dried and dissolved in 20 μ l of distilled water and 4 ml of TFA in ethyl acetate (1.4:100, v:v). The tube was capped, vortexed, and incubated at 45°C for about 18 h. The solvent was then evaporated under a stream of nitrogen gas, the residue was dissolved in 200 μ l of distilled water, and the solution was subjected to HPLC.

For enzyme hydrolysis, after the free steroids were removed by diethyl ether extraction, water was added to the extracts to a volume of 900 μ l, then 600 μ l of 75 mM potassium phosphate buffer, pH 6.8, containing 1% w:v BSA and 10 μ l (equivalent to approximately 400 modified Fishman units) of

recombinant *Escherichia coli* glucuronidase (catalog no. G8162; Sigma-Aldrich) was added. After addition of the enzyme solution to the tube, the contents were gently mixed by shaking, and then incubated at 37°C overnight. Steroids were then extracted using a C18 cartridge as described above. All experiments described above were carried out at least three times.

All synthetic steroids used in this study were purchased from Steraloids Inc. Although 3 α ,17 β dihydroxy-5 β -androstan-11-one 3-sulfate (Fig. 1G) was unavailable, it was prepared by enzyme reduction of 11-O-ETIO-s (Fig. 1B). To do this, 5 mg of 11-oxo-ETIO-3-sulfate (Fig. 1B) was placed in a glass vial and treated with 5 ml of 0.05 M Tris-HCl (pH 7.6) containing 0.8 mg (10 units) of β -hydroxysteroid dehydrogenase (β -HSD) (Nacalai USA, Inc.) and 10 mg of NADH (catalog no. N8179; Sigma-Aldrich). Contents were gently mixed and then incubated overnight at room temperature. The next morning, the steroid mixture was extracted using a C18 cartridge as described above.

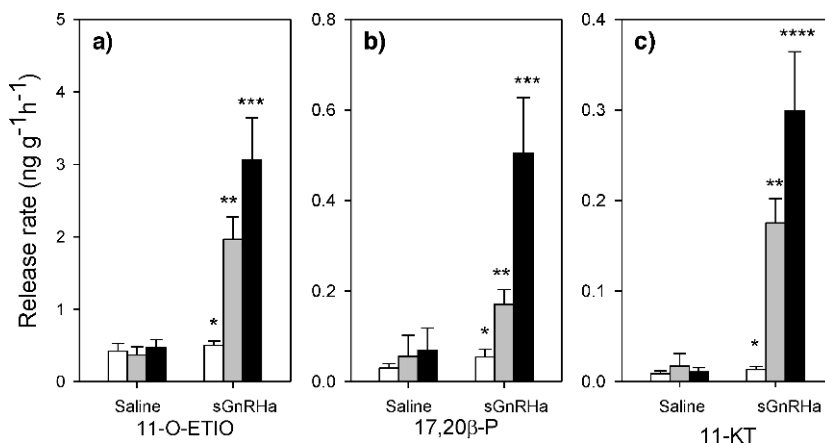
Electrospray Ionization Mass Spectrometry

Mass spectrometry of urine extracts was carried out using a Micromass LCT (Waters) instrument equipped with an atmospheric pressure ion source and a pneumatically assisted ESI interface. Nitrogen was used as the desolvation and nebulizing gas. A portion of eluting mobile phase from the HPLC column was introduced into the mass spectrometer by using a splitter. To obtain mass spectra of the synthetic compounds, solutions of the steroids were prepared in the range of 10 to 100 μ g ml⁻¹ and injected directly using a Harvard apparatus syringe pump at a flow rate of 5 μ l min⁻¹. The source temperature was maintained at 80°C, and a capillary voltage of 3 kV was used. The cone voltage was set at 50 V, and the desolvation temperature was 100°C. Spectra in the negative ion mode over a *m/z* range of 100–800 were obtained at the rate of one scan every 0.1 sec. HPLC ESI-MS spectra of solutions prepared from synthetic steroids were also obtained, and retention times for the different steroids were normalized with respect to an internal standard, ETIO 3-s (eluting at *m/z* of 369.1) and then compared with molecular peaks occurring in extracts of RM urine.

Thin Layer Chromatography

TLC test samples and synthetic standard steroids were obtained by using a development solution that allowed sulfated and glucuronidated steroids not only to run on silica gels but also to separate into distinct bands [29]. In conventional solvent solutions, conjugated steroids do not move from the origin. The extracts (in 20 μ l of methanol) were loaded onto separate lanes of precoated silica gel plates (catalog no. LK6DF; Whatman Labsales). Plates were developed for 45 min in an ethylacetate-ethanol-ammonia solution (45:45:15, v:v:v) and then dried under vacuum at 40°C for 1 h to remove all traces of the solvents. TLC lanes were marked and divided into 1- or 0.5-cm-long intervals, and silica gel from each interval was scraped off the plate and collected in glass tubes. Assay buffer (500 μ l) was added, and steroids were extracted from the silica gel into assay buffer by vigorous vortexing. The tubes were allowed to stand for 30 min to allow the silica gel to settle, the supernatant was separated, and 11-O-ETIO immunoreactivity was determined by EIA. The expected elution positions of glucuronidated, sulfated, and free steroids were established in a separate experiment by running 10 μ g each of free, sulphated, and glucuronidated 17,20 β -P and detecting the elution position of the bands with a UV lamp.

FIG. 2. Release rates ($\text{ng g}^{-1} \text{h}^{-1} \pm \text{SEM}$) of 11-oxo-etiocholanolone (11-O-ETIO) (a); 11-oxo-testosterone (11-KT) (c), and 17,20 β -dihydroxypregn-4-en-3-one (17,20 β -P) (b) by reproductive male *N. melanostomus* before and after sGnRH α (n = 24) or saline injections (n = 14). Preinjection release rates are shown by white bars; results after first injection rates are shown by gray bars; and results after the second injection release rates are shown by black bars. **, $P < 0.05$, and ***, $P < 0.005$ signify statistically significant differences between preinjection and postinjection release rates (two-tailed paired Student *t*-test).



Statistics

A Student two-tailed *t*-test was used to compare postinjection to preinjection release rates for total, conjugated, and free steroids. Release rates were expressed as either $\text{ng g}^{-1} \text{h}^{-1}$ or $\text{ng fish}^{-1} \text{h}^{-1}$.

RESULTS

Characteristics of the Enzyme Immunoassay

The standard curve for 11-O-ETIO (Fig. 1A) was roughly linear from 100 ng to 50 pg per well. The coefficient of variation within plates was 6.1% and 9.3% between plates. Of the steroids that were tested, only 3 α ,17 β -dihydroxy-5 β -androstane-11-one (Fig. 1D) and its 17-sulfate (Fig. 1E) showed substantial cross-reaction (Table 1). Most of the commonly expected teleost steroid hormones were relatively non-cross-reactive.

Release Rates of 11-O-ETIO and Its Conjugates

Injection of sGnRH α , but not of saline, into RM fish led to a significant increase in the release rates of total immunoreactive 11-O-ETIO (Fig. 1A), 11-KT, and 17,20 β -P ($P < 0.005$) (Fig. 2a, b, and c). The release rate of 11-O-ETIO (Fig. 1A) was about ten times higher than that of either of the other two steroids. Partitioning of the extracts between water and diethyl ether showed that 11-O-ETIO (Fig. 1A) immunoreactivity was present in both phases (Fig. 3a) and that prior to injection, most of the immunoreactivity was found in the water phase (i.e., in a conjugated form). After two injections of sGnRH α , there was a ninefold increase in the amount of free steroid and a twofold increase in the amount of conjugated steroid (with both increases being statistically significant [Fig. 3a]). Saline injections had no effect.

Six RF that were injected once with sGnRH α attained the total 11-O-ETIO (Fig. 1A) release rate of only $0.11 \pm 0.4 \text{ ng fish}^{-1} \text{h}^{-1}$ (mean \pm SEM; n = 6) in comparison to $67.4 \pm 28.0 \text{ ng fish}^{-1} \text{h}^{-1}$ (n = 24) in RM after the same treatment. Eight RM that were sampled directly after capture in the field had a total release rate of $67.4 \pm 8.0 \text{ ng fish}^{-1} \text{h}^{-1}$ (n = 8) in comparison to a preinjection RM release rate in the laboratory of $37.4 \pm 5.9 \text{ ng fish}^{-1} \text{h}^{-1}$ (n = 24).

HPLC Fractionation of Synthetic Steroids

With HPLC, immunoreactivity due to synthetic 11-O-ETIO (Fig. 1A) eluted at 30 min (Fig. 4a) and immunoreactivity due to synthetic 3 α ,17 β dihydroxy-5 β -androstane-11-one 17-sulfate (Fig. 1E) eluted at 24 min (Fig. 4b). When the latter compound

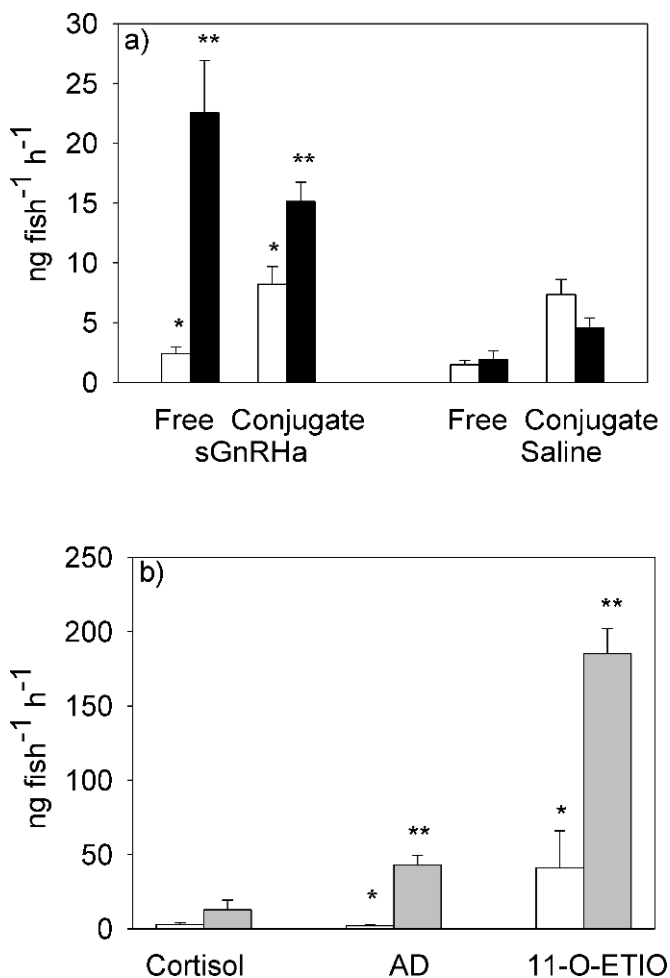


FIG. 3. a) Release rates ($\text{ng fish}^{-1} \text{h}^{-1} \pm \text{SEM}$) of free and conjugated 11-O-ETIO immunoreactivity before and after sGnRH α or saline injections. The preinjection release rates are shown by white bars and post-second injection release rates are shown by black bars. Release rates following sGnRH α injection (**) are significantly higher than preinjection release rates (*) at $P < 0.05$ (Student paired two-tailed *t*-test) for both conjugated and free 11-O-ETIO. b) Release rates ($\text{ng fish}^{-1} \text{h}^{-1} \pm \text{SEM}$) of conjugated 11-O-ETIO following injection of either cortisol, androstenedione (AD), or free 11-O-ETIO. Preinjection release rates are shown by white bars and post-first injection release rates are shown by black bars. A significant increase was shown following administration of AD or 11-O-ETIO ($P < 0.05$, Student paired two-tailed *t*-test).

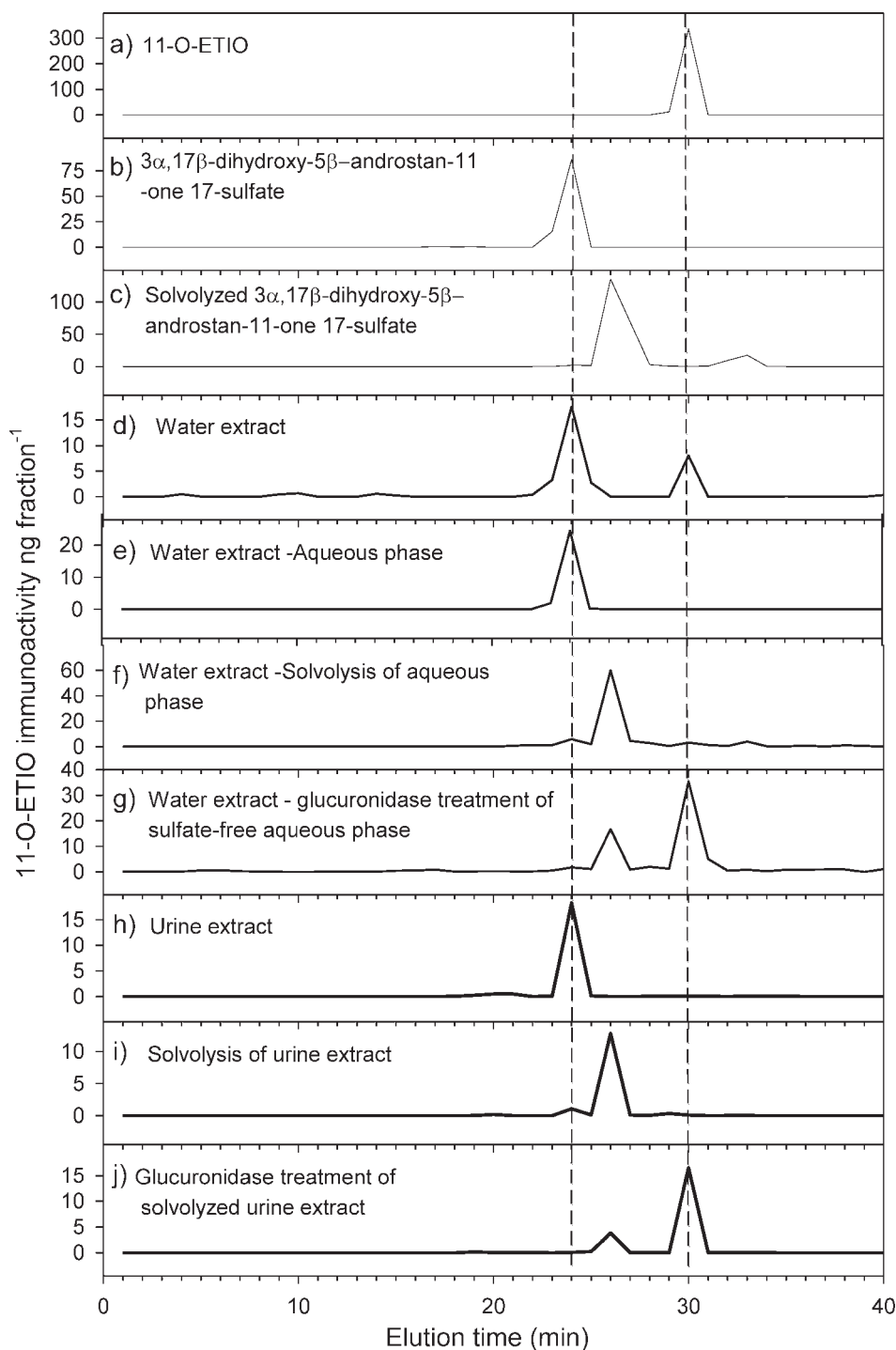


FIG. 4. HPLC results of 11-O-ETIO immunoreactivity of synthetic steroids, extracts of sGnRHa-injected reproductive male (RM)-conditioned water or urine, and acid-solvolysed and/or β -glucuronidase-treated extracts of sGnRHa-injected RM-conditioned water or urine. Extracts were prepared by solid-phase extraction (octadecylsilane), followed by elution with methanol. The 11-O-ETIO immunoreactivity (y-axis) is shown as ng fraction⁻¹ and elution time (x-axis) as min. **a)** Synthetic 11-O-ETIO; **(b)** synthetic $3\alpha,17\beta$ -dihydroxy- 5β -androstan-11-one 17-sulfate; **(c)** Acid-solvolysed $3\alpha,17\beta$ -dihydroxy- 5β -androstan-11-one 17-sulfate; **(d)** extract of RM-conditioned water; **(e)** same extract as in **d** after removal of free steroids by diethyl ether; **(f)** same extract as in **e** after acid solvolysis; **(g)** extract similar to those shown in **f** after treatment with β -glucuronidase. **h)** Extract of RM urine; **(i)** same extract as those shown in **h** after acid solvolysis; **(j)** same extract as those shown in **i** after treatment with β -glucuronidase.

was treated by acid solvolysis, a third immunoreactive peak (assumed to be free $3\alpha,17\beta$ -diol- 5β -androstan-11-one [Fig. 1D]) appeared at 26 min (Fig. 4c).

Evidence for the Identity of $3\alpha,17\beta$ Dihydroxy- 5β -Androstan-11-one 17-Sulfate in Conditioned Water and Urine Extracts

Two immunoreactive peaks were found in RM-conditioned water extracts (Fig. 4d). One peak had the same HPLC retention time as synthetic 11-O-ETIO (Fig. 1A) (30 min [Fig. 4a]), and the other peak had a retention time of 24 min (the same as synthetic $3\alpha,17\beta$ dihydroxy- 5β -androstan-11-one 17-

sulfate [Figs. 1E and 4b]). Prior extraction with diethyl ether removed the free steroid peak at 30 min but not the peak at 24 min, confirming that the immunoreactivity in the water was due to a steroid conjugate (or conjugates). The fact that the compound cross-reacted was an indication that it was not conjugated via the 3α -hydroxyl position (Table 1). When the extract was treated by acid solvolysis, it generated an immunoreactive peak at 26 min (Fig. 4f), identical to that generated by solvolysis of synthetic $3\alpha,17\beta$ dihydroxy- 5β -androstan-11-one 17-sulfate (Figs. 1E and 4c) and a residual peak at min 24.

Exactly the same results were obtained with immunoreactivity extracted from RM urine. In this case, the peak at 24 min

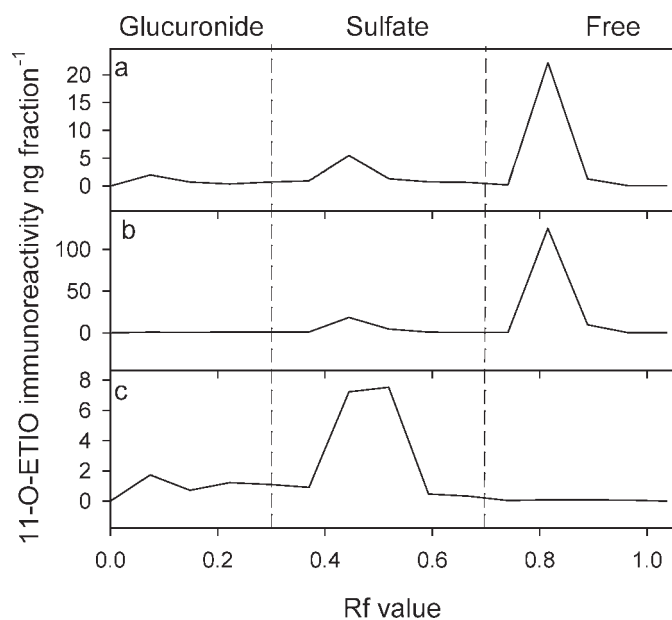


FIG. 5. Distribution of 11-O-ETIO immunoreactivity following TLC of pooled HPLC fractions, 23–25 min and 30 min from an extract of male-conditioned water (a); a mixture of synthetic $3\alpha,17\beta$ -dihydroxy- 5β -androstane-11-one 17-sulfate and synthetic 11-O-ETIO (b); and HPLC fraction 24 of an extract prepared from water conditioned by 11-O-ETIO-injected males (c).

was the only peak on HPLC (i.e., there was no free 11-O-ETIO [Fig. 1A] in urine [Fig. 4h]), and after being subjected to acid solvolysis, it generated a large peak of immunoreactivity at 26 min (Fig. 4i). When RM-conditioned water extract was run on TLC, three peaks were found (Fig. 5a): the two fastest running samples (retention factor [Rf], 0.45 and 0.82) were identical to the elution positions of synthetic 11-O-ETIO (Fig. 1A) and $3\alpha,17\beta$ dihydroxy- 5β -androstane-11-one 17-sulfate (Figs. 1E and 5b). In water that had been extracted from males injected with 11-O-ETIO (Fig. 1A), there were three peaks of activity on HPLC at 24, 26, and 30 min in the ratio 1:1.5:10. After free steroids were removed by treatment with diethyl ether, the remaining immunoreactive material ran in the position of $3\alpha,17\beta$ dihydroxy- 5β -androstane-11-one 17-sulfate (Fig. 1E) on TLC (24 min [Fig. 5c]). ESI-MS of urine established that the peak at 24 min on HPLC contained a compound with a m/z (385) and an elution position (Fig. 6c) identical to those of synthetic $3\alpha,17\beta$ dihydroxy- 5β -androstane-11-one 17-sulfate (Figs. 1E and 6g).

Evidence for the Identity of $3\alpha,17\beta$ -Dihydroxy- 5β -Androstane-11-one 17-Glucosiduronate in Conditioned Water and Urine Extracts

As described above, acid solvolysis of water and urine extracts did not entirely remove the peak of immunoreactivity at 24 min (Fig. 4f and i). This led us to suspect that some of the immunoreactivity at 24 min was due to the presence of $3\alpha,17\beta$ -dihydroxy- 5β -androstane-11-one 17-glucosiduronate (Fig. 1F) (for which we did not, unfortunately, possess a synthetic standard). This was tentatively confirmed by treatment of the residue from the peak at 24 min with β -glucuronidase. With water and urine extracts, this procedure generated peaks at 26 min and 30 min (Fig. 4g and j). The fact that the enzyme treatment generated a peak at 26 min that corresponded to the elution position of $3\alpha,17\beta$ -dihydroxy- 5β -androstane-11-one

(Fig. 1D) is an indicator that some $3\alpha,17\beta$ -dihydroxy- 5β -androstane-11-one 17-glucosiduronate (Fig. 1F) was present in the peak at 24 min. This is supported by the presence of an immunoreactive peak at Rf 0.08 on TLC (Fig. 5a and c) and the observation of a compound in urine with the correct m/z of 481 at about 24 min (Fig. 6h).

Evidence for the Identity of 11-O-ETIO-g in Conditioned Water and Urine Extracts

11-O-ETIO-3-g (Fig. 1C) does not cross-react with the antibody (Table 1) because the antibody binding site is blocked by the glucosiduronate group at position 3, and therefore, it does not show up as a peak of immunoreactivity on HPLC or TLC. However, if it is treated with β -glucuronidase, it generates 11-O-ETIO (Fig. 1A). This is indeed what happened when the peak at 24 min was treated with β -glucuronidase. For both water and urine extracts, a large peak at 30 min (Fig. 4g and j) was generated as well as a small peak at 26 min (as discussed above). Synthetic 11-O-ETIO-3-g (Fig. 1C) eluted between 23.5 and 25.5 min (Fig. 6b). RM urine extract exhibited a m/z value that was the same as that of the synthetic standard (481) in the same elution position on HPLC (Fig. 6f).

Evidence for the Identity of 11-O-ETIO-s in Conditioned Water and Urine Extracts

Acid solvolysis of RM-conditioned water and urine extracts generated only tiny amounts of immunoreactivity that eluted in the position of 11-O-ETIO (Fig. 1A) (i.e., 30 min), in contrast to that of acid solvolysis of synthetic 11-O-ETIO-3-s (Fig. 1B). In RM urine extract, there was also a weak signal at m/z of 383 (Fig. 6e) in the same elution position as 11-O-ETIO-3-s (Fig. 1B) (Fig. 6a).

Possible Presence of Other $5\beta,3\alpha$ -Reduced Compounds

In view of the fact that the antibody cross-reacted slightly with tetrahydrocortisone (Table 1), which is a potential metabolite of the stress hormone cortisol, some males were injected with cortisol. However, the release rate of 11-O-ETIO-immunoreactive conjugates did not increase (Fig. 3b), in contrast to that from males that had been injected with androstenedione or 11-O-ETIO (1A).

HPLC-MS analysis of the $3\alpha,17\beta$ -dihydroxy- 5β -androstane-11-one 3-sulfate (Fig. 1G) that had been made by enzyme reduction of 11-O-ETIO-s (Fig. 1B) with 17β -HSD showed two peaks, one peak corresponding to $3\alpha,17\beta$ -dihydroxy- 5β -androstane-11-one 3-sulfate (Fig. 1G) and the other peak, presumably, to untransformed 11-O-ETIO-s (Fig. 1B); based on the fact that the first peak had an m/z of 385, exactly 2 m/z values higher than the precursor steroid, and eluted 1.7 min earlier from the column (Fig. 6c). There was no peak of m/z of 385 at 22.1 min in the RM urine extract (Fig. 6g), strongly implying that $3\alpha,17\beta$ -dihydroxy- 5β -androstane-11-one 3-sulfate (Fig. 1G) is not made or at least not released by *N. melanostomus* males.

The m/z values predicted for disulfated, diglucosiduronated, and sulfated/glucosiduronated $3\alpha,17\beta$ -dihydroxy- 5β -androstane-11-one (Fig. 1D) were not observed in the ESI-MS spectra of RM urine extract, suggesting that such compounds are not produced or at least not released by *N. melanostomus* males.

On two occasions, when water (but not urine) extracts had been treated first by acid solvolysis followed by β -glucuronidase, an "extra" immunoreactive peak at 28 min was

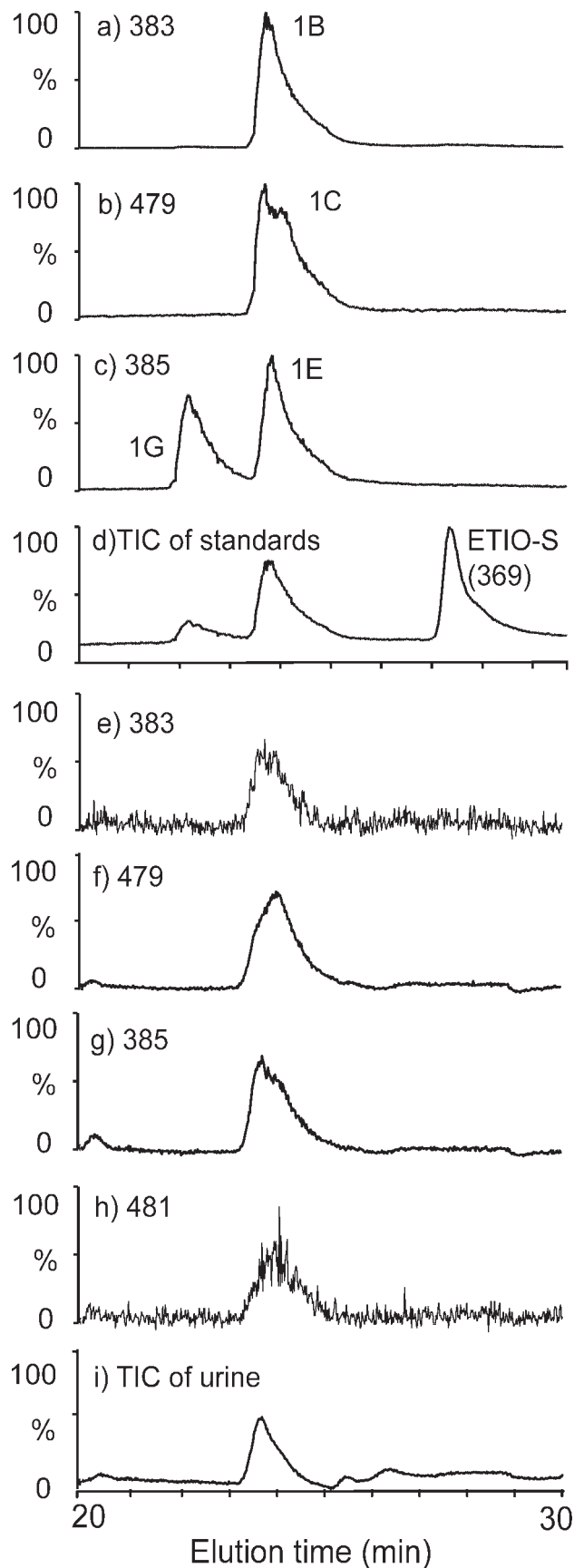


FIG. 6. ESI-MS of either a mixture of synthetic conjugated steroids (a–d) or an extract of male *N. melanostomus* urine (e–i). Plots shown in d and i show total ion chromatograms (TIC) of synthetic steroids and urine extract, respectively. All other plots show single ion chromatograms

observed in addition to the peaks at 26 min and 30 min. The possibility was explored that this extra peak might have been due to the presence of $3\alpha,11\beta$ -dihydroxy- 5β -androstan-17-one (a steroid that showed appreciable cross-reaction with the antibody [Table 1]). However, the synthetic version of this steroid eluted at 29 min on HPLC (data not shown). We are unable at present to explain the genesis of this extra peak in some but not all water extracts.

DISCUSSION

An EIA has been developed for quantifying 11-O-ETIO (Fig. 1A) and has been applied in conjunction with separation and analytical techniques, toward understanding the release of free and conjugated 11-O-ETIO (Fig. 1A) by reproductive phase *N. melanostomus* males. Results establish that males release not only 11-O-ETIO (Fig. 1A) but at least four conjugated forms. The two most abundant conjugates (as evidenced by both HPLC/EIA and ESI-MS) are $3\alpha,17\beta$ -dihydroxy- 5β -androstan-11-one 17-sulfate (Fig. 1E) and 11-O-ETIO-3-g (Fig. 1C). There are also small amounts of $3\alpha,17\beta$ -dihydroxy- 5β -androstan-11-one 17-glucosiduronate (Fig. 1F) and traces of 11-O-ETIO-3-s (Fig. 1B).

Of these four conjugates, the only one that was tentatively identified in the original study of steroid biosynthesis in *N. melanostomus* testis was the one for which there was the least evidence in the present study (i.e., 11-O-ETIO-3-s [Fig. 1B] [13]). No biosynthesis of $3\alpha,17\beta$ -dihydroxy- 5β -androstan-11-one (Fig. 1D) was demonstrated in that study, and intriguingly, no free $3\alpha,17\beta$ -dihydroxy- 5β -androstan-11-one (1D) was found in water extracts in the present study, except in the one experiment where males were injected directly with 11-O-ETIO (Fig. 1A). This suggests that although the pathway to $3\alpha,17\beta$ -dihydroxy- 5β -androstan-11-one 17-sulfate (Fig. 1E) formation probably starts with 11-O-ETIO (Fig. 1A), 17β -hydroxyl formation is followed immediately by 17β -sulfation. However, there are other possibilities. The pathway might actually start with 11-KT and follow the sequence 11-KT \rightarrow 17-sulfated 11-KT \rightarrow 5β -reduced 17-sulfated 11-KT \rightarrow $3\alpha,17\beta$ -dihydroxy- 5β -androstan-11-one 17-sulfate (Fig. 1E). Certainly, 11-KT is present in the water and produced in vitro by the testes and seminal vesicles [12, 13] and has been detected in blood plasma of nesting males [30].

Results in the present paper show clearly that sGnRH α stimulates the production and release of not only free 11-O-ETIO (1A) but also of $3\alpha,17\beta$ -dihydroxy- 5β -androstan-11-one 17-sulfate (Fig. 1E). It is likely that this also applies to the other major conjugate 11-O-ETIO-3-g (Fig. 1C). However, no specific measurements were made of levels of this compound in the sGnRH α injection studies in the present study. Even though previous behavioral studies suggest that this compound is not likely to be the only attractant pheromone [18], nevertheless, it might contribute to the efficacy of a putative pheromonal mixture. Thus, it will be important in future studies to establish how production of 11-O-ETIO-3-g (Fig. 1C) is affected by sGnRH α in comparison to other compounds.

In relation to the site of production of 17β -hydroxyl formation and conjugation, one obvious candidate is the liver,

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 corresponding to expected molecular masses of 3α and 17β sulfates and glucuronides of 3α -hydroxy- 5β -androstan-17-one and $3\alpha,17\beta$ -dihydroxy- 5β -androstan-11-one. The synthetic steroid mixture contained 3α -hydroxy- 5β -androstan-17-one 3-sulfate (ETIO-s); 11-O-ETIO-3-s (Fig. 1B); 11-O-ETIO-3-g (Fig. 1C); $3\alpha,17\beta$ -dihydroxy- 5β -androstan-11-one 17-sulfate (Fig. 1E); and $3\alpha,17\beta$ -dihydroxy- 5β -androstan-11-one 3-sulfate (Fig. 1G).

a well known site of steroid conjugation in vertebrates [22]. Other sites include blood cells, which have been shown in several fish species to contain 17 β -HSD activity [31]; the seminal vesicles, which have been shown in the African catfish (*Clarias gariepinus*) to be able to carry out steroid conjugation [23]; and the testes themselves, which produced few if any conjugated steroids in vitro [32] but might do so in vivo.

One question that arises from our studies is why we have not attempted to measure the free and conjugated forms of ETIO. As previously stated, ETIO-g appears to be able to attract *G. joso* [9], and both ETIO and ETIO-g are able to interact with a receptor in the olfactory epithelium of *N. melanostomus* [8]. One reason for concentrating our resources on 11-O-ETIO was its novelty. In addition, early studies showed it was a more abundant product of the testis than ETIO [13], and in most if not all of the HPLC-EIS runs reported in the present study, there was no evidence for the presence of compounds with masses corresponding to those expected for ETIO-g or 3 α -hydroxy-5 β -androstan-17-one 3-sulfate. However, in some more recent runs, weak but nevertheless distinct signals for these compounds have been observed in their expected elution positions. Thus, it seems very likely that compounds derived from ETIO, as well as from 11-O-ETIO, form part of the array of 5 β ,3 α -reduced steroids released by RM *N. melanostomus*.

Although the RM *N. melanostomus* used in this study displayed the physical attributes of reproductive males (black coloration, swollen cheeks), sGnRH α injections were used to make sure that the hypothalamic-pituitary-gonadal (HPG) axis of the fish was stimulated. It is well known in teleost fish that captivity and handling can adversely affect reproductive activity [33], especially sex steroid production [34]. It is also well known that in both male and female goldfish, stimulation of the HPG axis strongly enhances the release of pheromonally active steroids [35–37]. The main reason for measuring the “maturation-inducing steroid” 17,20 β -P and the “teleost androgen” 11-KT is that they are well-established male reproductive hormones [38, 39], and their release into the water has also been demonstrated in males of several other species (reviewed in references [40] and [41]). Admittedly, 17,20 β -P is not produced by males of all species [39]. Thus, there was no initial expectation that we would be able to detect its release in *N. melanostomus*. It is a key pheromone (with the ability to prime sperm production in males) in the goldfish (*Carassius auratus*) [42]. However, it is unlikely that it has a similar role in *N. melanostomus*, as its rate of release is considerably lower than that recorded in female *C. auratus* [37], and it has a much lower olfactory potency than the 5 β ,3 α steroids [8]. It was initially unknown whether this steroid would be found in water extracts. Its presence suggests that it probably has a hormonal role in stimulating sperm motility or milt hydration within the testes and that its presence (and that of 11-KT) in water is due to passive “leakage” across the gills [20, 41, 43, 44]. The release rate of 11-O-ETIO (Fig. 1A) immunoreactivity, which is greater than that of 11-KT or 17,20 β -P, is perhaps an indication of its use as a pheromone. On the other hand, in goldfish, although all steroids known to have olfactory/pheromonal activity have been shown to be released in relatively large amounts, there are other steroids without such activity that are released in equally large amounts [32, 37].

As mentioned in the previous paragraph, it is now well established that free steroids are released mainly from the gills and that conjugated steroids are released mainly via urine and/or feces. The same appears to be the case in the present study. Although we have no proof that free 11-O-ETIO (Fig. 1A)

derives from the gills, we have at least shown that it is not being excreted in urine. Also, although we have evidence that urine is a major source of 11-O-ETIO conjugates, we cannot exclude the possibility that the conjugates are also emitted from other parts of the body. Accumulation of some conjugated steroids in the bile, followed by release into the gut and then release into the water by defecation is one likely scenario [22], as feces production was evident when fish were kept in containers for water collection.

The different release routes of the free and conjugated steroids may be significant with respect to the reproductive strategy of *N. melanostomus*. It would be reasonable to hypothesize that the release of free 11-O-ETIO (Fig. 1A) via the gills is a continuous process with a rate of release that is proportional to the amount of steroid in the bloodstream, whereas the rate of release of conjugated steroids via urine is episodic. Indeed, Meunier et al. [45] recently observed that nesting males urinated more frequently in the presence of females than when held in isolation; the males also appeared to aid the dispersal of their urine by flipping their tails repeatedly.

Circumstantial evidence that 11-O-ETIO (1A) and one or more of its conjugates function as pheromones is provided by recent behavioral studies [46] that have shown that HPLC fractions from urine that contain the conjugated steroids (23 to 25 min) attracted both RF and NRF (although RF were more strongly attracted than NRF), while HPLC fractions from water that contained mainly free 11-O-ETIO (Fig. 1A) attracted NRF but repelled RF. These results are very much in line with those of the earlier study [18] that used blends of synthetic steroids (including those shown in Fig. 1A, B, and C, but not E or F). Possibly, RM release a pheromone in urine to lure RF for imminent spawning as well as to attract NRF to the vicinity of the nests. The NRF attracted to the nests may remain close to the nesting sites and avoid expending energy in the search for mating partners once they had ovulated. We expect that the NRF would soon ovulate in this scenario, because *N. melanostomus* is a multiple spawner [3], and the ovulation cycle takes about 3 weeks [47].

In conclusion, in the present study, we show that RM *N. melanostomus* injected with sGnRH α release the 5 β -reduced and 3 α -hydroxyl steroid 11-O-ETIO (Fig. 1A) into the water, as well as conjugated derivatives containing sulfate or glucosiduronate groups. These products were observed by utilizing an EIA that we developed. Much of the conjugated immunoreactivity was shown to be derived from urine, and a major contributor to this conjugated fraction was found to be 3 α ,17 β -dihydroxy-5 β -androstan-11-one 17-sulfate (Fig. 1E), with a small proportion of 3 α ,17 β -dihydroxy-5 β -androstan-11-one 17-glucosiduronate (Fig. 1F). Evidence was also strong for the release of 11-O-ETIO-3-g (Fig. 1C) (which could be detected only by EIA after removal of the glucosiduronate group with β -glucuronidase) and for only very small amounts of 11-O-ETIO-3-s (Fig. 1B). Presently, there is no direct evidence that any of the compounds we have so far identified are actually pheromonal attractants. However, what has been learned in relation to the routes of release, chromatographic properties, and amounts and ratios of free and conjugated 11-O-ETIO (Fig. 1A) derivatives is being used to design behavioral studies that will hopefully reveal such properties, if they exist.

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