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The seminal vesicle synthesizes steroids in the round goby *Neogobius melanostomus*

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Abstract

In this study, we examine the possible contribution of the seminal vesicles of the male round goby to the production of putative steroidal pheromones. A previous study showed that the testes of the round goby are rich in steroid-producing Leydig-like cells; and when incubated *in vitro*, convert tritiated androstenedione to at least six other steroids, including one not previously identified in fish — namely 3α-hydroxy-5β-androstane-11,17-dione (11-oxo-etiocholanolone, 11-oxo-ETIO). The seminal vesicles of reproductively mature males were examined by conventional histology, transmission electron microscopy and immunocytochemistry (utilizing an antibody against 3β-hydroxysteroid dehydrogenase — a key enzyme in vertebrate steroid synthesis). All three procedures identified Leydig cells in the proximal and medial regions of the seminal vesicles. *In vitro* incubation of seminal vesicles with tritiated androstenedione demonstrated biosynthesis of 11-oxo-androstenedione, 11-oxo-testosterone (more commonly known as 11-ketotestosterone) and 11 oxo-ETIO. These data indicate that the seminal vesicles, as well as the testes are involved in the synthesis of steroidal compounds that may function as pheromones.
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Keywords: Steroid; Testis; Round goby; Seminal vesicle; Androgen; Pheromone

1. Introduction

Previous histochemical and biochemical studies have shown that glandular accessory gonadal structures, the seminal vesicles, in male African catfish, *Clarias gariepinus* (Schoonen and Lambert, 1986; Schoonen et al., 1987, 1988) and male urohaze goby *Glossogobius olivaceus* (Asahina et al., 1989) have the capacity for steroid biosynthesis. In fish of the Gobiidae family (order Perciformes), the seminal vesicles are wing-like bilateral sperm duct outgrowths situated caudal to the testis, with ducts attached to the *vas deferens* at the inner end of the genital papilla (Fishelson, 1991; Immler et al., 2004; Miller, 1984; Moiseyeva and Ponomareva, 1973). The chamber-like cavities of the seminal vesicles store sperm in the redhead goby, *Paragobiodon echinocephalus* (Fishelson, 1991) and the black goby *Gobius niger* (=*G. jozo*) (Rasotto and Mazzoldi, 2002). In the black goby (Rasotto and Mazzoldi, 2002) and the grass goby, *Zosterisessor ophiocephalus* (Lahnsteiner et al., 1992), the seminal vesicles add a viscous sticky fluid to spermatozoa released from the testes. Histochemical studies have shown that the epithelial lining secretes sialoglycoprotein mucins in the redhead goby (Fishelson, 1991) and the black goby (Rasotto and Mazzoldi, 2002). In the urohaze goby, steroid metabolites were found following *in vitro* incubation of seminal vesicles (Asahina et al., 1989). The location of steroid biosynthesis may be interstitial Leydig cells. These have been observed in the lamina propria adjacent to the secretory epithelium, in paraffin sections stained with hematoxylin and eosin, from the round goby *Neogobius melanostomus* (Moiseyeva and Ponomareva, 1973) and the giant goby, *G. cobitis* (Fishelson, 1991).

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We have previously observed *in vitro* conversion of tritiated androstenedione (androst-4-ene-3,20-dione; Ad) into 3α-hydroxy-5β-androstane-11,17-dione (*i.e.*, 11-oxo-etiocholanolone, 11-oxo-ETIO), 11-oxo-ETIO-sulfate; 11-oxo-testosterone (11-ketotestosterone; 11-KT), 3α-hydroxy-5β-androstan-17-one (etiocholanolone, ETIO), 11β-hydroxy-androstenedione (11β-OH-Ad), ETIO-sulfate and testosterone (T) by the testes of the round goby (Arbuckle et al., 2005). Other work has shown that C19 steroids in which the carbon A ring is 5β-reduced are able to evoke strong olfactory epithelial extracellular field potentials in the round goby (Murphy et al., 2001) and bring about olfactory-mediated increases in gill ventilation in the same species (Murphy and Stacey, 2002; Belanger et al., 2006, 2007). Preliminary data show that the gonadal steroids evoke olfactory epithelial responses (Belanger, 2003) and that mixtures stimulate attraction responses from female round gobies (Moscicki et al., unpublished data). The significance of these findings lies in the potential application of reproductive pheromones to assist with population management of round gobies in the Laurentian Great Lakes, where this species is an invader threatening survival of native fish populations.

In this study, our objectives were to confirm the presence of Leydig cells in the seminal vesicles of the round goby through three techniques — conventional histology, transmission electron microscopy and immunocytochemistry. In addition, we investigated the steroidogenic capacity of this tissue by *in vitro* incubation with tritiated Ad and identified the steroids through thin-layer chromatography and microchemistry.

2. Materials and methods

2.1. Experimental animals

All animal collection, maintenance and experimental procedures were in accordance with the Canadian Council of Animal Care and the Ontario Animals for Research Act guidelines. Round gobies were obtained from the Detroit River by angling between May and October (2003–2005), then maintained at water temperatures of 18–20 °C in a flow-through system using dechlorinated tap water, in the Department of Biological Sciences at the University of Windsor. Fish were used for experiments usually within two weeks of capture. Specimens of various sizes and gonadosomatic index (GSI) values were included. Spermiating males were identified by swollen cheeks and black coloration (MacInnis and Corkum, 2000). The values for fish length and weight and for gonadal weight were obtained following euthanasia by MS222. During dissection, the seminal vesicle was identified as per Fishelson (1991) and Lahnsteiner et al. (1992). The testes and seminal vesicles were included when calculating the gonadosomatic index (GSI) values. In previous studies, mean (±standard error, S.E.) GSI values were found to be 1.54±0.19% for reproductive males and 0.24±0.03% for non-reproductive males (Belanger et al., 2007).

Gonadal tissue from 30 male round gobies was utilized for the histological and immunocytochemical studies. Tissue was fixed for hematoxylin and eosin staining and for immunocytochemistry between April and October in 2004 and 2005. For the ultrastructural studies, seminal vesicles from two male round gobies were fixed in July 2004. For the first, the total length was 16.8 cm, the total mass was 45 g and the GSI was 1.92. For the second male, these values were 18.2 cm, 53 g and 1.3. The seminal vesicle incubation experiments were conducted in early July of 2003 (year 1) and 2004 (year 2). The GSI values from two animals in year 1 were 1.74 and 0.77, and two animals for year 2 were 2.11 and 2.62, respectively. The total length and total weight values were: year 1 — 44.2 g, 14.5 cm; 61.15 g, 15.5 cm; year 2 — 35.6 g, 13.8 cm; 40.1 g, 13.8 cm, respectively.

2.2. Histology

Conventional histology was used to localize Leydig cells in the round goby seminal vesicle. The gonads were removed by dissection, fixed by immersion in Bouin's solution, then processed in paraffin and sectioned (5–7 μm thick). The paraffin was removed through a xylene series, rehydrated in an ethanol series, then stained with haematoxylin and eosin. The sections were photographed on a Zeiss Axioskop 2 microscope and the images were captured using a Northern Eclipse (Empix) acquisition system.

2.3. Transmission electron microscopy

The ultrastructure of seminal vesicle epithelial cells and of Leydig cells was observed by transmission electron microscopy. This technique enabled direct identification of abundant smooth

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Table 1
HPLC and TLC elution positions, steroid name and identification tests carried out on the four compounds (A to D; Fig. 3) formed by *in vitro* incubation of round goby seminal vesicles with tritiated Ad

Peak	HPLC (min)	R_T^a	Identified metabolite	Microchemistry performed
A	39,40,41	0.56	17β-Hydroxy-androst-4-ene-3,11-dione (11-KT)	Acetylation product comigrates on TLC
B	42,43,44	1.23	Androst-4-ene-3,11,17-trione (11-oxo-Ad)	Neither standard nor radioactive metabolite could be acetylated. Enzymatic reduction product had the same mobility as 11-KT on TLC
C	47	0.74	3α-Hydroxy-5β-androstan-11,17-dione (11-oxo-ETIO)	Enzymatic oxidation product had the same mobility as 5β-androstane-3,11,17-trione on TLC
D	54	1.38	Androst-4-ene-3,17-dione (Ad)	Enzymatic reduction product had the same mobility as testosterone on TLC

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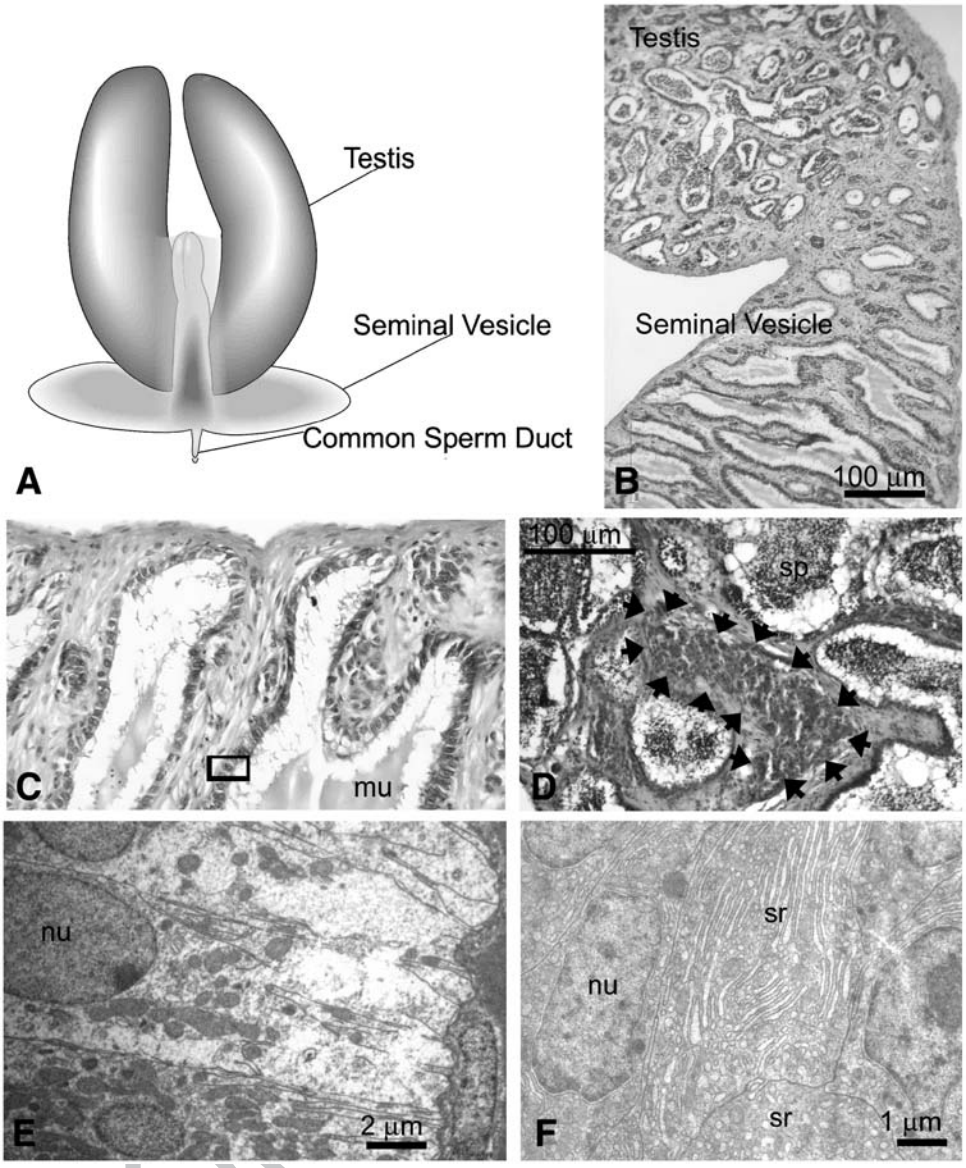

Fig. 1. The seminal vesicle in the round goby. A. The diagramatic representation of the male reproductive tract shows the seminal vesicle located at the base of the testes. B, C and D. are hematoxylin and eosin stained paraffin sections. C and D are the same magnification. The scale bar for C and D is shown in D. E and F are transmission electron micrographs. B. A low power micrograph shows the histology of the testis and the seminal vesicle. C. The chambers of the seminal vesicle are lined by simple cuboidal or low columnar epithelium. A region of epithelium, similar to that enclosed by the box, is shown by transmission electron microscopy in E. In this specimen, the lumen of the chambers contains a mucoid (mu) product. D. In this specimen, there are abundant sperm (sp) within the chambers of the seminal vesicle A cluster of Leydig cells is seen in the center of the micrograph (surrounded by arrows). E. An epithelial cell has a low columnar shape, and contains abundant mitochondria. F. The Leydig cell cytoplasm contains prominent cisternae of smooth endoplasmic reticulum (sr) nu, nucleus.

endoplasmic reticulum, which is characteristic of steroidogenic cells (Lofts and Bern, 1972). Seminal vesicles were removed from the two reproductive-phase round gobies and immersed in modified Karnovsky's fixative (0.075 M cacodylate buffer, 2.2% paraformaldehyde, 2% glutaraldehyde) for 18 h. The tissue was cut into 1 mm sliced with a razor blade, immersed in fresh fixative for 4 h, then post-fixed in 1% osmium tetroxide (in 0.075 M cacodylate buffer) and dehydrated through an ascending series of ice-cold ethanol. The tissue was then passed through propylene oxide and embedded in epoxy resin. The tissue blocks were sectioned and viewed by transmission electron microscopy at the All India Institute of Medical Sciences in New Delhi, India. Serial semi-thin sections (1 µm) were obtained on an ultra microtome, stained with 1% toluidine blue and viewed by bright field microscopy. The areas of interest were identified and the tissue blocks were further trimmed. Ultra-thin sections were obtained, then viewed and photographed under a transmission electron microscope (Philips CM-10).

2.4. Immunocytochemistry

The enzyme 3β-hydroxysteroid dehydrogenase (β-HSD) is necessary for the key conversion of Δ^5-3β-hydroxy to Δ^4-3-

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oxo steroids, making it a useful marker of steroid-producing cells in the testis (Kobayashi et al., 1998; Madekurozwa et al., 2002). In the present study, immunocytochemistry against β-HSD was used to confirm Leydig cell localization in the seminal vesicle. Wax sections were deparaffinized in xylene, rehydrated in an ethanol series, rinsed in 0.1 M PBS and incubated in 3% hydrogen peroxide to quench endogenous peroxidase activity. The sections were again rinsed in 0.0 1 M PBS three times for 10 min, incubated with normal blocking serum for 15 min, then incubated overnight with diluted (1:500, 1:1000) rabbit anti-β-HSD antibody (5062-1501, Biogenesis, MorphoSys, Kingston, NH, USA) and visualized with the anti-rabbit ABC-Vector Elite Kit (Vector laboratories, Burlington, Ont. Canada). Sections were counterstained with haematoxylin. Negative controls were included by replacing the primary antibody with normal rabbit serum.

2.5. In vitro incubation with tritiated Ad

To determine the ability of the seminal vesicles to produce steroid metabolites, two pairs of seminal vesicle were incubated with [1,2,6,7-^{3}H]androst-4-ene-3,17-dione (Amersham Biosciences). Two fish were anaesthetized with MS222, killed and the seminal vesicles dissected into small pieces and placed in ice-cold Leibowitz L15 medium (Sigma-Aldrich, USA) and transported on ice from University of Windsor to Michigan State University. The bits of tissue were placed in fresh 50 mL plastic tubes containing 10 mL Leibowitz L 15 medium and 50 μCi tritiated Ad. 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 cartridge (Waters Chromatography, Millipore, Milford, MA,USA). This was washed with 5 mL of distilled water and then eluted with 5 mL methanol. The extract was stored at −20 °C. The procedure was repeated the following year.

The methanol extracts from 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-KT, reconstituted in 1 mL acetonitrile/water/trifluroacetic acid (28/72/0.01;v/v/v) and then separated on an analytical reverse-phase HPLC column as described previously (Arbuckle et al., 2005).

2.6. Identification of steroids: thin-layer chromatography (TLC) and microchemistry

Full details of the strategy for identifying steroids by thin-layer chromatography and microchemical transformations are provided by Arbuckle et al. (2005). Briefly, *ca.* 20,000 dpm of each HPLC peak was mixed with 10 μg T, dried down, reconstituted in 30 μL ethyl acetate and spotted and run on TLC. 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 RT values in Table 1 of Arbuckle et al. (2005). By doing this, it was possible to narrow down the range of possible matching standards for each HPLC peak. A more precise match was then obtained by mixing 20,000 dpm of radioactive steroid with 10 μg each of those steroids that had the most similar RT values

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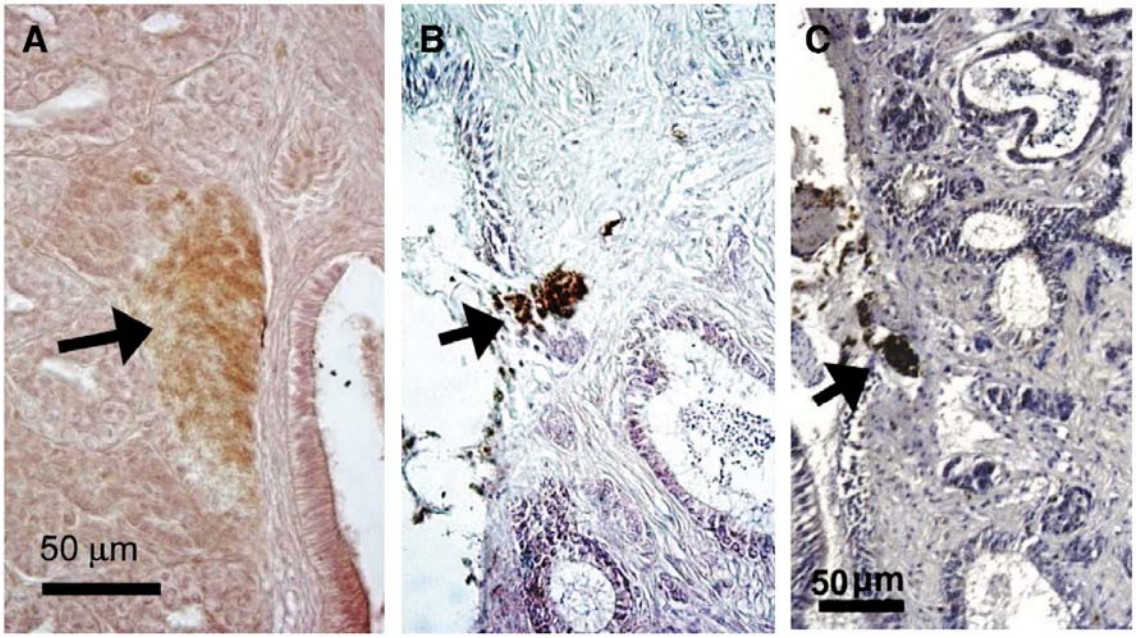

Fig. 2. β-hydroxysteroid dehydrogenase (β-HSD) immunoreactivity in the testis and the seminal vesicle. A and B are counterstained with hematoxylin. The arrows point to β-HSD-immunoreactive cells. A. In the testis, β-HSD immunoreactivity is seen in a cluster of Leydig cells adjacent to the spermatic duct. B. A small cluster of β-HSD immunoreactive cells is seen at the medial edge of the seminal vesicle, adjacent to the testis boundary. C. In this seminal vesicle, a large cluster of β-HSD-immunoreactive Leydig cells is located near the edge of the seminal vesicle, and a smaller cluster is located more centrally.

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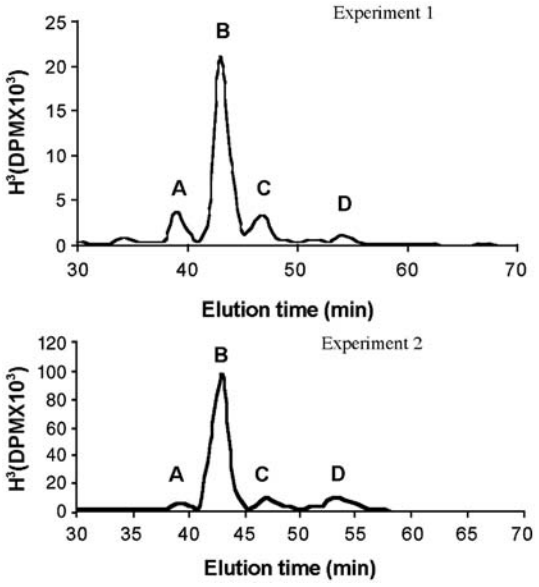

Fig. 3. HPLC separation of media from round goby seminal vesicles incubated with tritiated Ad. Each incubation used a pair of seminal vesicles from two reproductive males. Incubations I and II are from two consecutive summers. Disintegrations per minute (dpm) are along the y axis and time of elution in min along the x axis. See Table 1 for identification information on the steroids that were found in each of the labeled peaks.

and then running them together on TLC. 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.

3. Results

The seminal vesicles appeared as wings extending from the base of the testes — a morphology and arrangement seen previously in other goby species (*e.g.*, Fishelson, 1991; Lahnsteiner et al., 1992). The seminal vesicles were paired glands attached at the medial/caudal end of the testes, close to the sperm duct (Fig. 1A). The internal structure consisted of chambers interconnected with each other by wide openings (Fig. 1B), and lined by simple epithelium and underlined by a lamina propria (Fig. 1C). The lumen of the chambers contained an acellular substance (Fig. 1B, C) sometimes with embedded spermatozoa (Fig. 1D).

Clusters of interstitial Leydig-like cells were observed in the lamina propria beneath the epithelium lining the chambers (Fig. 1D), and in the proximal region of the seminal vesicle.

The low columnar epithelial cells lining the seminal vesicle chambers contained abundant mitochondria and supranuclear cytoplasm (Fig. 1E), as previously observed in the grass goby *Z. ophiocephalus* (Lahnsteiner et al., 1992). In comparison, the Leydig cell cytoplasm contained extensive smooth endoplasmic reticulum (Fig. 1F); a characteristic feature of steroid producing cells (Lofts and Bern, 1972).

Immunocytochemistry against β-HSD confirmed the steroidogenic capacity of the Leydig cells (Fig. 2). The β-HSD-immunoreactivity of Leydig cell aggregates adjacent to the spermatic duct in the testes (Fig. 2A) indicated the ability of this antibody to label Leydig cells in the round goby. Leydig cells at this testicular location have been observed previously (Arbuckle et al., 2005). Prominent groups of β-HSD-immunoreactive cells were seen in the seminal vesicle (Fig. 2B, C). These clusters were seen adjoining the testis (Fig. 2B) and in more distal locations (Fig. 2C). This combined use of histology, transmission electron microscopy and immunocytochemistry demonstrated the location of steroidogenic Leydig cells within the seminal vesicle in the round goby.

3.1. Incubation of seminal vesicle with tritiated Ad

The seminal vesicles incubated with tritiated Ad following HPLC separation (Fig. 3) yielded four prominent peaks of radioactivity — labeled A, B, C, and D. Ad was >95% converted into other steroids. All four peaks were studied by TLC and microchemistry (including acetylation, enzymatic oxidation and enzymatic reduction. It was possible to match them all to known standards (Table 1). No conjugated steroids were detected. In order of abundance, the products were: 11-oxo-Ad (peak B) 78.4%; 11-KT (peak A) 12.3%; 11-oxo-ETIO (peak C) 5.3%; Ad (peak D) 4.1%.

4. Discussion

In this study, we have demonstrated clusters of Leydig cells in the seminal vesicles of male round gobies as well as in testes. We also showed that the seminal vesicles are able to convert tritiated Ad into at least three other steroids. Overall, these results indicate that the seminal vesicle in gobiids is involved in steroid production in addition to their previously identified functions of sperm storage and 'glue' production (referenced in Introduction).

Leydig cells in the proximal and medial positions have been previously identified through 3β-HSD histochemistry in the seminal vesicles of the Indian catfish *Heteropneustes fossilis* (Nayyar and Sundararaj, 1969) and the African catfish *C. gariepinus* (Van den Hurk et al., 1987).

The most abundant steroid formed in the incubation experiments was 11-oxo-Ad. This enzymatic pathway involves a two-step reaction with the insertion of an oxygen atom at the C11 position of Ad (11-hydroxylase activity) followed by removal of two hydrogen atoms (11β-hydroxysteroid dehydrogenase activity). From this compound, 11-KT can be formed by reduction of the 17-oxo group (17β-hydroxysteroid dehydrogenase activity), while 11-oxo-ETIO can be formed by reduction of the double bond between the 4th and 5th carbon atoms (to the 5β-configuration) and subseqent reduction of the 3-oxo group (3α-hydroxysteroid dehydrogenase activity). The presence of all the necessary enzymes: 5β-reductase, 11-hydroxylase, 11β-HSD, 17β-HSD and 3α-HSD is already well-established in teleosts (Borg, 1994; Fostier et al., 1983; Kime, 1993). Our previous study of the round goby testes demonstrated 11-oxo ETIO as a final metabolite (Arbuckle et al., 2005). We now show that this novel steroid can be formed in the seminal vesicle as well as the testis.

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Interestingly, 11-oxo-Ad was not identified in the testis incubations (Arbuckle et al., 2005), but it was present in the seminal vesicles. A small amount of sulfated (but not glucuronidated) 11-oxo-ETIO was found in the goby testis incubations (Arbuckle et al., 2005). However, there was no indication of the formation of any conjugates in the seminal vesicle incubations. This is strikingly different from the African catfish, in which the seminal vesicles were shown to synthesize large amounts of steroid glucuronides (Schoonen and Lambert, 1986; Schoonen et al., 1987, 1988). In fact, in the African catfish, the seminal vesicles synthesize more and a wider variety of steroid glucuronides than the testes; some of these glucuronides also being shown to have putative pheromonal functions in that species.

A pre-requisite for any steroid (whether free or conjugated) being a pheromone is that it is released by the males into the water. At the moment, we only have preliminary (unpublished) data that shows that 11-oxo-ETIO is indeed detectable in water conditioned by male round gobies. However, evidence from many other fish species (Scott and Vermeirssen, 1994; Vermeirssen and Scott, 1996; Scott and Ellis, in press) makes it highly likely that, whatever steroids are made by the testis or seminal vesicle of the round goby, they will readily find their way into the water *via* either the gills (the main route for free steroids), urine (the main route for sulfates) or feces (the main route for glucuronides). In the reproductive male round goby, there are the further options of the seminal vesicles (*i.e.* direct from the Leydig cells into the lumen of the seminal vesicles without first entering the bloodstream) and the milt (as suggested by Locatello et al., 2002). Steroids (free and conjugated) have been detected and measured in the seminal fluid of the African catfish (Schoonen et al., 1988) and in milt of several species (Scott and Vermeirssen, 1994), although these observations do not necessarily imply that any fish actually use these fluids for pheromone dispersion. In the case of milt, for example, unless there is a mechanism for some sort of 'leakage' prior to the spawning act, it is hard to imagine how the steroids in the milt could act as an attractant for females to nests of males prior to spawning. On the other hand, milt steroids may attract subordinate males during spawning events. In some Gobiid species, these sneaker males enter the nests of dominant males during spawning (Magnhagen, 1992; Immler et al., 2004). Previous study has shown that milt steroids facilitate school spawning in the Pacific herring, *Clupea harengus pallasi* (Carolsfeld et al., 1997).

To back up the hypothesis that some, if not all, of the steroids that are made by the testis and seminal vesicle of the male round goby are also probably released into the water, it has, as already mentioned, been shown that reproductive females respond behaviorally to mixtures of synthetic steroids (Moscicki et al., unpublished data) and that the peripheral olfactory organs of females also generate extracellular field potentials (electro-olfactogram, EOG) to the same steroids (Murphy et al., 2001; Belanger, 2003). Two of the seminal vesicle steroids (11-KT; 11-oxo-Ad) were tested on round gobies by Murphy et al. (2001), who did not observe EOG responses, while Belanger (2003) observed EOG responses to these compounds. Also, in even earlier studies, we showed attraction of reproductive females to water previously occupied by reproductive males as well as strong olfactory epithelial responses to this water (Belanger et al., 2004; Gammon et al., 2005).

One possibility that we have not yet taken into account, but needs to be investigated, is that steroids might undergo further modification between the time that they are secreted by the Leydig cells and finally released into the water. Apart from a small amount of sulfation observed in two out of four testis incubations (Arbuckle et al., 2005) neither the testes nor the seminal vesicles of the round goby appear to have any capacity for steroid conjugation. Steroid conjugation is relatively common in teleost gonads (Scott and Vermeirssen, 1994) and the black goby testis in particular is able to produce an abundance of ETIO glucuronide (Colombo et al., 1977, 1979, 1980, 1982). We have previously speculated (Arbuckle et al., 2005) that the absence of conjugation in the round goby might have something to do with the 3 h delay between the sacrifice of the animals and the incubation of the tissues with radioactive Ad. However, whether this eventually proves to be true or not, conjugating enzymes will still be present in the liver. Although the main purpose of the liver conjugating enzymes is to metabolize compounds, they could still have a role in generating putative conjugated steroidal pheromones from steroids that originated in the gonads. The liver of the African catfish has been shown (Cavaco et al., 1997) to contain 11β-HSD and 17β-HSD activity that is necessary for the final transformation of 11β-OH-Ad to 11-KT in this species. Blood cells have also been shown to contain the 17β-HSD that is necessary for the final transformation of 11-oxo-Ad to 11-KT in the stickleback *Gasterosteus aculeatus* (Mayer et al., 1990). Evidence indicates that 11-KT (Miura et al., 1991; Kime, 1993; Borg, 1994) is the key androgen in teleosts (*i.e.* stimulation of spermatogenesis and male secondary characteristics) despite the fact that a specific receptor for this steroid has not yet been conclusively identified.

In conclusion, this study has shown that, like the testis, the seminal vesicle of the round goby contains Leydig cells. While six steroids were formed from *in vitro* incubation of the testis with tritiated androstenedione, only three were formed from *in vitro* incubation of the seminal vesicles. Furthermore, one of these steroids (the most abundant, 11-oxo-AD) was not one of those produced by the testis. The two steroids produced by both testis and seminal vesicle were 11-KT and 11-oxo-ETIO.

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