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The Biosynthesis of Steroid Glucuronides in the Testis of the Zebrafish, *Brachydanio rerio*, and Their Pheromonal Function as Ovulation Inducers

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In female zebrafish ovulation could be induced by male holding water, testis homogenates, and testis fractions containing steroid glucuronides. Deglucuronidation of these fractions led to a loss of ovulation-inducing potency, indicating steroid glucuronides as ovulation inducers. The chemical substances were perceived by the recipient females by means of olfaction. Incubation experiments showed the capacity of the testes to synthesize various C_{19} and C_{21} steroids and seven different steroid glucuronides, i.e., 17α,20β-dihydroxy-4-pregnen-3-one-, testosterone-, androsterone-, epiandrosterone-, 5α-androstane-3α,17β-diol-, and 5α-androstane-3β,17β-diol glucuronide. GC–MS analysis showed the presence of glucuronides of 5α-androstane-3α,17β-diol and cholesterol in male holding water, the latter probably originating from the liver. These compounds may be among the steroid glucuronides functioning as ovulation-inducing pheromones.

Pheromones involved in reproductive processes, such as attraction of the opposite sex, parental behavior, and fertility, are reported to be produced mainly in the gonads or accessory sex organs in fish (Liley 1982; Colombo *et al.*, 1982; Stacey, 1983; Liley and Stacey, 1983). In zebrafish, *Brachydanio rerio*, intersexual attraction can be caused by extracts of postovulatory zebrafish ovaries (van den Hurk and Lambert 1983; Lambert *et al.*, 1986) and ovulation can be induced by male holding water (Chen and Martinich, 1975).

Little is known about the biochemical nature of these fish pheromones. Algranati and Perlmutter (1981) suggested a cholesterol–ester-like component and van den Hurk and Lambert (1983) pointed to steroid glucuronides as sex attractants for zebrafish. In the guppy, *Poecilia reticulata*, and the black goby, *Gobius jozo*, such steroid glucuronides could evoke attraction of conspecifics (Colombo *et al.*, 1982). In the scope of these findings, it might be of biotechnological importance to identify the molecular structures of compounds that can induce ovulation. Administration of these substances might simplify common fish-farming procedures for obtaining viable eggs of fish that do not ovulate spontaneously under culturing conditions.

The present paper deals with the effects of male and female holding water, testicular homogenates, and different fractions of these homogenates on ovulation.

In addition, whether or not ovulation-inducing pheromones are perceived by olfaction was examined. Furthermore, the biosynthesis of steroid glucuronides and steroids in the testes of zebrafish was studied by incubating pieces of testes with [^{3}H]pregnenolone and [^{3}H]androstenedione, respectively. The incubation products were identified by means of thin-layer chromatography, derivatization, and recrystallization to constant specific activity. A preliminary analysis of steroid glucuronides in aquarium water containing

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male zebrafish was carried out by means of selected ion monitoring with capillary column gas chromatography–mass spectrometry (GC–MS) to determine the excretion of steroid glucuronides by male zebrafish.

MATERIALS AND METHODS

Chemicals

[7-^{3}H]Pregnenolone (8.8 Ci/mmol) and [7-^{3}H]androstenedione (9.7 Ci/mmol) were purchased from Radiochemical Centre (Amersham), and purity was checked by thin-layer chromatography. Nonlabeled steroids and steroid glucuronides were obtained from Steraloids, Makor, and Sigma. Hepes and β-glucuronidase of *Escherichia coli* (100 U/ml) were obtained from Boehringer, Leibovitz L15 medium was from Serva, and *N,O*-bis-(trimethylsilyl)acetamide (BSA) and trimethylchlorosilane (TMCS) were from Fluka. All chemicals and solvents (Baker) were of analytical grade. The solvents used for isolation of the steroid glucuronides out of holding water were distilled twice before use.

Animals

Zebrafish were reared at 25°–26° in aquaria and kept on a light regime of 14 hr light and 10 hr darkness. Under these circumstances, females show a reproductive cycle of 4 to 5 days and males show a continuous spermatogenic cycle and are willing to court females.

For the ovulation test "ovulation-ready" females are needed. To that end, a male and a female were kept together overnight in a 20-liter tank but separated from each other by a net. The next morning when the female is confronted with the male, the male will court the female, resulting in oviposition. Using this method, 50–70% of the females will ovulate. To bring them into preovulation condition, the ovulated females are stocked for about 1 week in aquaria and then used in the ovulation test. A confrontation of such ovulation-ready females with males again leads to 50–70% ovulation.

Twenty females were made anosmic by thermocauterizing the nasal epithelium with a tip of 0.1 mm; MS 222 (0.02%) was used as anesthetic. Another group of 20 fish, cauterized between the nostrils, served as controls. After a recovery period of 5 days the females were used in the ovulation test. After the test all females were histologically examined for the presence or absence of intact nasal epithelium. Heads were fixed in Bouin–Hollande, dehydrated in graded ethanol, and embedded via xylene in paraffin. Histological studies were made with 5-μm-thick sections stained with hemalum–eosin.

Ovulation Tests

From a central reservoir (20 liters), with an inflow of Cu-free tap water of 1.5 liters/hr, water was siphoned (0.3 liter/hr) to five or six aquaria, each containing one female zebrafish in preovulation condition. The water content in these aquaria was maintained constant at 15 liters. Visual contact between the fish was prevented by partitions between the aquaria. Several hours before the start of an experiment, and after termination of it, ovulation was determined by stripping the females by gently pressing the belly.

To test the influence of holding water, eight males or females were placed in the central reservoir for 18 hr. All experiments started at 1600 hr and were terminated at 1000 hr the following day. Homogenates of pooled testes and their fractions were diluted in 70 ml distilled water and tested by pumping them (4 ml/hr) from a cooled container into the central reservoir for 18 hr. The amount of tested material for one female always corresponded with testicular material of one male zebrafish.

Statistics

Comparisons were made between the numbers of ovulated fish in each group and those in the Cu-free tap water group using 2×2 chi-square tests with an individual error of $P = 0.005$, i.e., and experimental error of $P < 0.05$. Separately, the number of ovulated anosmic females after addition of male holding water was compared with that of sham-operated females ($P = 0.05$).

Testicular Fractions

After anesthesia with MS 222 the testes of several animals were removed, pooled, and homogenized on ice in a Teflon–glass homogenizer with distilled water (0.3 ml/animal). From this homogenate (Fraction F_1) the lipid material, including the free steroids, was extracted with dichloromethane ($3\times$ 10 ml). The combined dichloromethane extracts were evaporated and the residue was redissolved in a few drops of propylene glycol and then diluted in 15 ml of distilled water (Fraction F_2). The remaining water fraction (Fraction F_3) was evaporated under nitrogen at 40°, redissolved in 2 ml of sodium acetate buffer (0.1 *M*, pH 6.5), and treated with 100 μl of β-glucuronidase at 37° for 18 hr under continuous shaking in an air atmosphere. The enzyme reaction was terminated by the addition of 10 ml of dichloromethane, and the deglucuronidated steroids were extracted. The remaining water fraction (Fraction F_4) might contain steroid con-

jugates but no steroid glucuronides. A summary of the fractioning is given in Fig. 1.

In Vitro Bioconversion of Steroids

Incubation procedure. Incubations were carried out with pooled minced testes of 10 animals in Leibovitz-15 medium (1:4, w/v) with 15 m*M* Hepes containing [7-^{3}H]pregnenolone (1 μCi) or [7-^{3}H]androstenedione (1 μCi) dissolved in 44 μl propylene glycol. No cofactors were added. The incubations were carried out at 25° under continuous shaking in an atmosphere of air. The enzyme reactions were terminated after 24 hr by the addition of 1 ml dichloromethane.

Extraction and fractioning. Before extraction with dichloromethane (3× 10 ml) 25 μg of each of the following carriers were added based on pilot studies. To incubations with pregnenolone, pregnenolone, progesterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 5-pregnene-3β,17α,20β-triol, 17α,20β-dihydroxy-4-pregnen-3-one, androstenedione, testosterone, 11-ketotestosterone, 11β-hydroxytestosterone, 5α-androstane-3,17-dione, androsterone, and 5α-androstane-3α,17β-diol were added. To incubations with androstenedione, androstenedione, 11β-hydroxyandrostenedione, 11-ketoandrostenedione, testosterone, 11-ketotestosterone, 11β-hydroxytestosterone, 5α-androstane-3,17-dione, androsterone, and 5α-androstane-3α,17β-diol were added.

The free steroids and steroid conjugates were extracted as described previously. After evaporation, the fractions with the free and the deglucuronidated steroids were both redissolved in dichloromethane: methanol (9:1) and subjected to TLC in system I to separate apolar compounds (triacylglycerols) from steroids. In this system the steroids remain on the baseline, so it is possible to use the same plate for a first separation of the steroids.

Chromatography. TLC and detection of carrier and reference steroids were carried out as described by Schoonen and Lambert (1986a). The following systems were used: I, toluene–cyclohexane (1:1); II, benzene–ethylacetae (3:1); III, chloroform–ethanol (95:5); IV, diisopropylether–chloroform–hexane (7:2:1); V, benzene–ethanol (95:5); VI, cyclohexane–ethylacetate (1:1); VII, hexane–acetone (2:1); and VIII, ethylacetate–hexane–acetic acid (75:20:5).

Microchemical reactions, recrystallizations, and measurements of radioactivity were carried out according to Schoonen and Lambert (1986a).

Isolation and Identification of Steroid Glucuronides from Male Holding Water

Chromatography. Baker C_8 columns were used for isolation of the steroid glucuronides from male holding water. Ten liters of holding water, in which eight males had been swimming for 18 hr, was transferred through the column. After the column was rinsed with distilled water, the steroid glucuronides were eluted with 10 ml of methanol. The eluate was evaporated and the residue treated with β-glucuronidase, after which the free steroids were derivatized for GC–MS determination.

Derivatization. Trimethylsilyl and oxime-trimethylsilyl derivatives were prepared by reaction first with hydroxylamine hydrochloride (HAH) in pyridine and then with a mixture of BSA and TMCS. The deglucuronidated steroid fraction was transferred with ethanol into a small reaction vial and, following evaporation at 60° under nitrogen, 200 μl of a freshly prepared 2% HAH reaction mixture was added. Then the vial was closed, vortexed, and incubated for 1 hr at 100°. Under these conditions keto groups were converted to oximes. Following evaporation, 200 μl of a freshly made mixture of BSA:TMCS (9:1) was added to the residue and incubated for 1 hr at 70°.

After evaporation the residue was dissolved in 2 ml of hexane, and polar compounds (nonsteroid derivatives) were removed by extraction with acetonitrile (2 × 0.2 ml). Finally the hexane fraction was reduced

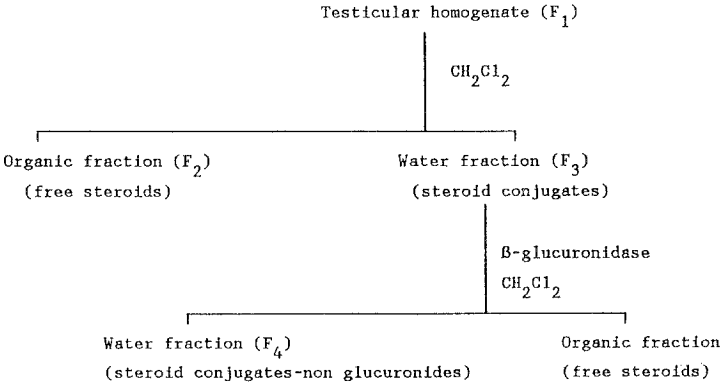

FIG. 1. Fractionation scheme of testicular homogenates of *Brachydanio rerio*.

to 50 μl and aliquots of 2 μl were subjected to GC–MS.

Capillary gas chromatography–mass spectrometry. A Hewlett–Packard 5992 B gas chromatograph–mass spectrometer with a Chrompack fused-silica wall-coated open tubular CP Sil 5 column (film thickness 0.15 μm; 25 m × 0.34 mm i.d.) was used with helium as carrier gas at a flow of 2 ml/min. The injection port temperature was 250° and the oven temperature was set at 160° and increased 1 min after injection with a rate of 5°/min to 190°, followed by a second increase with a rate of 2°/min to 235°. For total ion monitoring with a scan reach of 50–600 *m/z*, the multiplier detector was set at 1600 V, and for selected ion monitoring at 2800 V. The mass spectrometer was optimalized for the higher masses (*m/z* 414) and the obtained mass spectra were nonnormalized spectra.

RESULTS

Ovulation Tests

The results of the ovulation induction tests are summarized in Table 1. Control experiments carried out with Cu-free tap water resulted in only one ovulated female out of 18 animals. Male holding water, however, does promote ovulation significantly, whereas female holding water does not. Fifty percent of the sham-operated fish had ovulated after introduction of male holding water, whereas under the same conditions only 3 out of 20 anosmic females ovulated. Histological examination of these 3 fish revealed that the olfactory epithelium was still partly intact and the nostrils were open. In all other anosmic females the skin epithelium had proliferated and closed the nostrils completely. After administration of testes homogenates (Fraction F_1) and testicular steroid conjugate fraction (Fraction F_3), significantly more females ovulated than in the control group. Testicular Fractions F_2, with the free steroids, and F_4, containing the steroid conjugates without glucuronides, did not significantly enhance the percentage of ovulation.

Ovaries of nonovulated females contain a mixed population of follicles, most of which are stage 3 follicles (van Ree, 1976), containing oocytes in postvitellogenesis with a central germinal vesicle and a diameter up to 800 μm. Ovulated females in addition have postovulatory follicles. Oocytes with a migrating germinal vesicle, or with the germinal vesicle broken down, were absent from all ovaries examined.

In Vitro Bioconversion of Steroids

Incubation with [^3H]pregnenolone. After extraction with dichloromethane about 92% of the radioactivity was present in the

TABLE 1
EFFECTS OF ZEBRAFISH HOLDING WATER AND TESTICULAR FRACTIONS ON OVULATION IN ZEBRAFISH

Test material	Females	Number of ovulated fish	Total number of fish	Ovulation percentage
Cu-free tap water	Intact	1	18	5.6
Male holding water	Intact	11	16	68.8*
Female holding water	Intact	4	15	26.7
Male holding water	Sham operated	10	20	50.0*
Male holding water	Anosmic	3	20	15.0**
Testicular Fraction F_1[a]	Intact	9	17	52.9*
Testicular Fraction F_2[a]	Intact	1	15	6.7
Testicular Fraction F_3[a]	Intact	9	15	60.0*
Testicular Fraction F_4[a]	Intact	4	15	26.7

[a] F_1, testicular homogenate; F_2, free steroids; F_3, steroid conjugates; F_4, steroid conjugates without glucuronides.

* $P = 0.05$ compared with Cu-free tap water group.

** $P = 0.05$ compared with sham-operated females.

organic fraction. The first separation was done by TLC in system II (3×) and resulted in eight radioactive areas (A–H) corresponding to the following carriers: (A) 11-ketotestosterone, 17α,20β-dihydroxy-4-pregnen-3-one, and 5-pregnene-3β,17α,20β-triol; (B) 11β-hydroxyandrostenedione; (C) 11-ketoandrostenedione, testosterone, and 5α-androstane-3α,17β-diol; (D) 17α-hydroxypregnenolone and 17α-hydroxyprogesterone; (E) androsterone; (F) androstenedione and pregnenolone; and (G) 5α-androstane-3,17-dione. Area H is present on the front line.

After TLC in different systems, derivatization, and recrystallization to constant specific activity (Table 2), it appeared that the tritium activity in the different areas was caused by (A) 11-ketotestosterone, 17α-20α-dihydroxy-4-pregnen-3-one, 17α,20β-dihydroxy-4-pregnen-3-one, and 5-pregnene-3β,17α,20β-triol; (B) 11β-hydroxyandrostenedione; (C) 11-ketoandrostenedione and testosterone; (D) 17α-hydroxypregnenolone and 17α-hydroxyprogesterone; (E) androsterone and epiandrosterone; (F) pregnenolone and androstenedione; (G) 5α-androstane-3,17-dione; and (H) a steroid ester, which was identified as a pregnenolone ester.

The evidence of the presence of four of these steroids, i.e., 17α,20α-dihydroxy-4-pregnen-3-one, 11-ketotestosterone, androsterone, and epiandrosterone, is based only on their chromatographic behavior. 17α,20α-Dihydroxy-4-pregnen-3-one was identified indirectly by eliminating other possibilities based on derivatization and R_f values (Schoonen *et al.*, 1987b). The synthesis of 11-ketotestosterone, androsterone, and epiandrosterone was confirmed after incubations with [^{3}H]androstenedione.

The water fraction after β-glucuronidase treatment, extraction, and separation of the free steroids by TLC in system II (3×) after scanning revealed only two radioactive areas. These areas corresponded with areas A and C of the organic fraction. Rechromatography, derivatization, and recrystallization (Table 2) demonstrated that the radioactivity originated from 17α,20β-dihydroxy-4-pregnen-3-one, 5-pregnene-3β,17α,20β-triol, testosterone, and 5α-androstane-3α,17β-diol.

The quantitative data of the yields of the steroids are also given in Table 2. It appears that after an incubation of 24 hr the main products are 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 17α,20α-dihydroxy-4-pregnen-3-one, and androstenedione.

Incubation with [^{3}H]androstenedione. After extraction, the organic fraction contained 87% of the radioactivity. TLC in system II (3×) and scanning made it possible to distinguish six different radioactive areas. These areas were characterized by the following carriers: (A) 11-ketotestosterone; (B) 11β-hydroxyandrostenedione; (C) 11-ketoandrostenedione, testosterone, and 5α-androstane-3α,17β-diol; (D) androsterone; (E) androstenedione; and (F) 5α-androstane-3,17-dione.

After TLC in several systems, derivatization, and finally recrystallization to constant specific activity (Table 2), it could be demonstrated that tritium activity in areas A–F belonged to the following steroids: (A) 11-ketotestosterone; (B) 11β-hydroxyandrostenedione; (C) 11-ketoandrostenedione, testosterone, 5α-androstane-3α,17β-diol, and 5α-androstane-3β,17β-diol; (D) androsterone and epiandrosterone; (E) androstenedione; and (F) 5α-androstane-3,17-dione.

Glucuronidase treatment of the water fraction (13% of the total radioactivity), followed by extraction with dichloromethane and chromatography of the organic fraction by TLC in system II (3×), showed the presence of two radioactive areas corresponding to areas C and D of the organic fraction. After TLC in several

TABLE 2

IDENTIFICATION OF INCUBATION PRODUCTS FROM TESTES OF ZEBRAFISH, *Brachydanio rerio*, *IN VITRO*

Substrate	Area	Metabolite	Percentage yield of steroids	TLC systems				Recrystallization (dpm/mg)			
								Original	1	2	3
Pregnenolone	A	11-Ketotestosterone	1	II (3×)	III (2×)	VIII (2×)	A^+, II (3×)	—	—	—	—
		17α,20α-Dihydroxy-4-pregnen-3-one	7	II (3×)	III (2×)	VIII (2×)	C^+, II (3×)	—	—	—	—
		17α,20β-Dihydroxy-4-pregnen-3-one	4	II (3×)	III (2×)	VII (3×)	A^+, II (3×)	2476	1852	1811	1787
		5-Pregnene-3β,17α,20β-triol	2	II (3×)	III (2×)	VI (3×)	A^+, II (3×)	1751	1429	1434	1373
	B	11β-Hydroxyandrostenedione	3	II (3×)	III	A^-, II (3×)		2395	1857	1957	1850
	C	11-Ketoandrostenedione	2	II (3×)	III	VI (3×)	A^-, II (3×)	1590	1570	1593	1652
		Testosterone	1	II (3×)	III	VI (3×)	A^+, II (3×)	630	180	189	180
	D	17α-Hydroxypregnenolone	14	II (3×)	III	F^+, II (3×)		13583	13174	13238	12984
		17α-Hydroxyprogesterone	18	II (3×)	III	A^-, II (3×)		11615	11220	10604	10669
	E	Androsterone	1	II (3×)	III	A^+, II (3×)		—	—	—	—
		Epiandrosterone	1	II (3×)	III	A^+, II (3×)		—	—	—	—
	F	Pregnenolone	17	II (3×)	III	VI (2×)	A^+, II (3×)	15616	14214	14187	14289
		Androstenedione	9	II (3×)	III	A^-, II (3×)		4842	4663	4477	4490
	G	5α-Androstane-3,17-dione	2	II (3×)	IV (3×)	A^-, II (3×)		931	630	628	590
	H	Pregnenolone-ester	2	II (3×)	S^+, II (2×)			661	562	580	559
	A	17α,20β-Dihydroxy-4-pregnen-3-one–GL	3	II (3×)	III (2×)	VII (3×)	A^+, II (3×)	2148	2065	2197	2116
		5-Pregnene-3β,17α,20β-triol–GL	1	II (3×)	III (2×)	VI (3×)	A^+, II (3×)	1064	947	922	980
	B	Testosterone–GL	3	II (3×)	III	A^+, II (3×)		3220	2378	2415	2356
		5α-Androstane-3α,17β-diol–GL	1	II (3×)	III	IV (3×)	V (5×)	1064	947	922	980
Androstenedione	A	11-Ketotestosterone	2	II (3×)	III (2×)	VIII (2×)	A^+, II (3×)	1070	925	883	891
	B	11β-Hydroxyandrostenedione	5	II (3×)	III	A^-, II (3×)		3700	2973	2768	2871
	C	11-Ketoandrostenedione	2	II (3×)	III	A^-, II (3×)		1970	1988	1969	2031
		Testosterone	2	II (3×)	III	A^+, II (3×)		2491	2294	2431	2407
		5α-Androstane-3α,17β-diol	1	II (3×)	III	IV (3×)	V (5×)	2046	1847	1752	1756
		5α-Androstane-3β,17β-diol	1	II (3×)	III	IV (3×)	V (5×)	1897	480	486	448
	D	Androsterone	9	II (3×)	III	V (3×)	A^+, II (3×)	2739	1658	1583	1630
		Epiandrosterone	5	II (3×)	III	V (3×)	A^+, II (3×)	1569	1443	1437	1368
	E	Androstenedione	39	II (3×)	III	A^-, II (3×)		30981	28749	29826	29645
	F	5α-Androstane-3,17-dione	13	II (3×)	IV (3x)	A^-, II (3×)		7125	5607	5979	5524
	A	Testosterone–GL	7	II (3×)	III	A^+, II (3×)		12629	12443	12142	12294
		5α-Androstane-3α,17β-diol–GL	3	II (3×)	III	IV (3×)	V (5×)	2320	2229	2185	2137
		5α-Androstane-3β,17β-diol–GL	1	II (3×)	III	IV (3×)	V (5×)	2028	232	266	241
	B	Androsterone–GL	1	II (3×)	III	V (3×)	A^+, II (3×)	1059	657	684	626
		Epiandrosterone–GL	1	II (3×)	III	V (3×)	A^+, II (3×)	—	—	—	—

Note. For definitions of Roman numerals, see Materials and Methods. A^+, positive acetylation; A^-, negative acetylation; S^+, positive saponification; F^+, positive formylation; C^+, CrO_3 oxidation; and GL, glucuronide.

systems, derivatization, and recrystallization, the following steroids were identified: testosterone, 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, androsterone, and epiandrosterone (Table 2).

The quantitative data of these steroids, obtained from duplicate incubations, are also given in Table 2. The main products appeared to be 5α-androstane-3,17-dione, androsterone, epiandrosterone, and the glucuronide of testosterone.

Isolation and Identification of Steroid Glucuronides from Male Holding Water

The steroid glucuronides were isolated from male holding water by reversed-phase chromatography and then treated with β-glucuronidase. The deglucuronidated fraction was subjected to GC–MS analysis, first to total ion monitoring and then to selected ion monitoring (SIM). The compounds looked for were all steroids which were found as glucuronides after incubation of testes material with [^{3}H]pregnenolone or [^{3}H]androstenedione, i.e., 17α,20β-dihydroxy-4-pregnen-3-one, 5-pregnene-3β,17α,20β-triol, testosterone, 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, androsterone, and epiandrosterone. With spectral analysis none of these steroids could be identified. Cholesterol, however, was found by this procedure. A full spectrum of cholesterol–trimethylsilane (TMS) could be detected at a retention time of 35.4 min, comparable to standard cholesterol–TMS (Fig. 2). With the SIM analysis only 5α-androstane-3α,17β-diol could be demonstrated. The mass spectrum of standard 5α-androstane-3α,17β-diol-diTMS (MW 436.3) has typical fragment ions with *m/z* 436.6 (M^+), *m/z* 421.3 (M^+-CH_3), and *m/z* 346.3 (M^+-OTMS) (Fig. 3A). The retention time is 17.8 min. The theoretical abundance ratio for the ions M^+, $M^+ + 1$, $M^+ + 2$, i.e., 436.3, 437.3, and 438.3, is 100:38:14. With SIM analysis these characteristic fragment ions

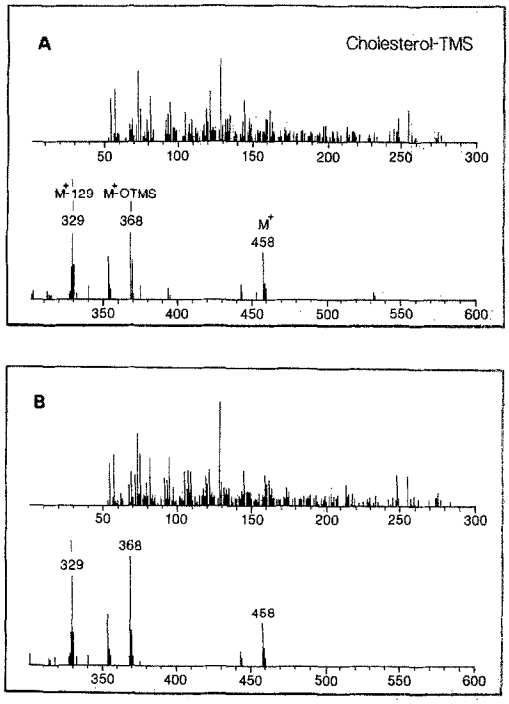

FIG. 2. (A) Mass spectrum (nonnormalized) of cholesterol–TMS. The molecular ion and particular mass fragments are *m/z* 458, *m/z* 368, and *m/z* 329. M^+, molecular ion; OTMS, *O*-trimethylsilyl. (B) Mass spectrum (nonnormalized) of a compound isolated from the steroid glucuronide fraction of male holding water of *Brachydanio rerio* after treatment with β-glucuronidase and derivatization with particular ions with *m/z* 458, *m/z* 368, and *m/z* 329.

were also monitored in the extract of male holding water at a retention time of exactly 17.8 min. (Fig. 3B). The abundance ratio for the ions M, M + 1, M + 2 was 100:36:15. This indicates that at least 5α-androstane-3α,17β-diol-glucuronide was present in the male holding water.

DISCUSSION

The present data indicate that male holding water is much more potent in inducing ovulation (about 70%) in zebrafish than female holding water (27%). The value obtained with male holding water was significantly different from the results with tap water, whereas that of female holding water was higher but not significantly different.

The percentage of ovulation due to male holding water falls within the range of ovulation values (50–70%) that occur when a male is brought together with a nonovulated female. The ovulation-inducing effect of male holding water confirms previous data of Chen and Martinich (1975). These authors, however, achieved better results, i.e., 100% ovulation, as a consequence of different experimental procedures. In their experiments, for example, female zebrafish were allowed two successive opportunities to ovulate, and fish were only counted as nonovulated females when afterward they were able to ovulate in the presence of a male.

Histological examination of the ovaries of nonovulated females demonstrates the presence of oocytes with central germinal vesicles only; thus oocytes with germinal vesicle migration and/or germinal vesicle breakdown were absent. Anosmic fish with closed nostrils do not ovulate after introduction of male holding water. The few fish of the anosmic group that ovulated after addition of this holding water had partly intact olfactory epithelia and open nostrils, and thus were apparently able to smell. The present data indicate that ovulation-inducing male pheromones of zebrafish are perceived by olfaction. This method of chemoreception corresponds to that of sex attractants in *B. rerio* (van den Hurk and Lambert, 1983) and other teleost fish, i.e., *Bathygobius soporator* (Tavolga, 1976), *Carassius auratus* (Partridge *et al.*, 1976), and *Plecoglossus altivelus* (Honda, 1979).

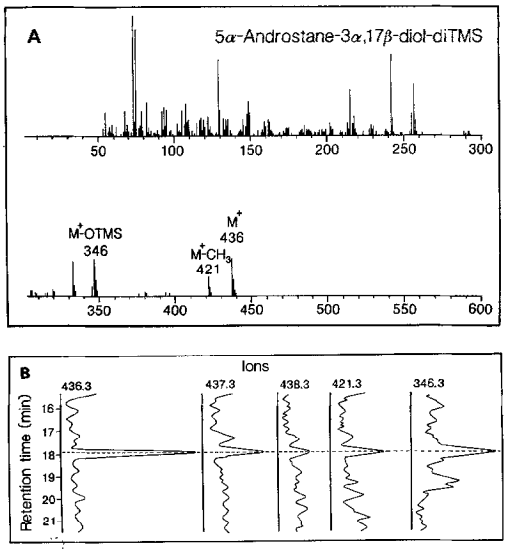

FIG. 3. (A) Mass spectrum (nonnormalized) of 5α-androstane-3α,17β-diol (diTMS). The molecular ion with *m/z* 436 and the mass ions with *m/z* 421 and *m/z* 346 are indicated. M^+, molecular ion; OTMS, *O*-trimethylsilyl. (B) SIM analysis of the characteristic ions *m/z* 436.3, *m/z* 437.3, *m/z* 438.3, *m/z* 421.3, and *m/z* 346.3 of the steroid glucuronide fraction of male holding water of *Brachydanio rerio* after treatment with β-glucuronidase and derivatization.

In view of the ovulation-inducing effect of male holding water, the testis is a good candidate for the production of sex pheromones. Indeed, experiments with testis homogenates of zebrafish show a strong ovulation response (52.9%). The steroid conjugate fraction of this homogenate also results in a high percentage of ovulated females (60%), whereas the free steroid fraction is unable to evoke such an effect. After β-glucuronidase treatment, the steroid conjugate fraction looses a great deal of its ovulation-promoting effect. This points to glucuronidated compounds, most likely steroid glucuronides, as the active chemical cues. These findings correspond with those of the few studies concerning the nature of sex attractants in fish. In zebrafish, steroid glucuronides produced by postovulatory ovaries evoked attraction of conspecific males (van den Hurk *et al.*, 1982, van den Hurk and Lambert, 1983). In *G. jozo*, etiocholanolone glucuronide formed in the mesorchial gland of the male has an attractive effect (Colombo *et al.*, 1980, 1982), and the same compound has been shown to be attractive for male *C. auratus*, and *P. reticulata* (Colombo *et al.*, 1982).

Incubation studies with tritium-labeled pregnenolone and androstenedione have demonstrated that the testis of the zebrafish is able to produce steroid glucuronides apart from the more common C_{21} and C_{19}

steroids. Pregnenolone will first of all be converted into 17α-hydroxypregnenolone and 17α-hydroxyprogesterone. From 17α-hydroxyprogesterone onward, two main steroidogenic pathways can be distinguished. The first one runs via a C_{20} oxidoreductase to 17α,20α-dihydroxy-4-pregnen-3-one and 17α,20β-dihydroxy-4-pregnen-3-one, and to a glucuronide of the latter. Apart from these products, a conversion into 5-pregnene-3β,17α,20β-triol and its glucuronide could also be demonstrated. This conversion, however, must in all probability be considered as artificial, because it may be the result of a temporary accumulation of 17α-hydroxypregnenolone.

The second route runs via a C_{17-20} lyase to the formation of androstenedione and is followed by a conversion into testosterone and its glucuronide as well as into 11-oxygenated androgens, i.e., 11β-hydroxyandrostenedione, 11-ketoandrostenedione, and 11-ketotestosterone. Also, a 5α-reduction of androstenedione may occur, resulting in the synthesis of 5α-androstane-3,17-dione, androsterone, epiandrosterone, and the glucuronide of 5α-androstane-3α,17β-diol. Incubations with tritiated androstenedione confirmed these conversions and moreover showed the synthesis of the glucuronides of 5α-androstane-3α,17β-diol, androsterone, and epiandrosterone.

These data indicate that the testis of zebrafish is able to synthesize, apart from unconjugated C_{19} and C_{21} steroids, at least six or seven steroid glucuronides. Only a few steroid glucuronides have been demonstrated in the testis of teleosts. Testosterone glucuronide is found to be produced by the testes of rainbow trout *Salmo gairdneri* (Hews and Kime, 1978) and goldfish, *C. auratus* (Kime, 1980). The 5α-reduced steroid glucuronides, however, have so far not been described as being synthesized by teleost testes, whereas 5β-reduced steroid glucuronides have been found among the products of steroidogenesis in the testes of the black goby, *G. jozo* (Colombo *et al.*, 1977) and African catfish, *Clarias gariepinus* (Schoonen *et al.*, 1987b). Concerning African catfish, it must be mentioned that the seminal vesicle is the main source of steroid glucuronides (Schoonen and Lambert, 1986b, Schoonen *et al.*, 1987a). The formation of 17α,20β-dihydroxy-4-pregnen-3-one by the testes has previously been described in some other teleosts, i.e., the rainbow trout, *S. gairdneri* (Arai and Tamaoki, 1967; Kime 1979; Depêche and Sire, 1982), and the African catfish, *C. gariepinus* (Schoonen and Lambert 1986a; Schoonen *et al.*, 1987b). According to Ueda *et al.* (1984) it is the sperm that is involved in the final synthesis of 17α,20β-dihydroxy-4-pregnen-3-one. The synthesis of the glucuronide of this steroid is a new finding.

Whether all steroid glucuronides produced by the testis of the zebrafish have a pheromonal function remains to be investigated. Until now only one of these compounds (5α-androstane-3α,17β-diol-glucuronide) could be detected in male holding water by GC–MS analysis. Cholesterol glucuronide, also demonstrated in this holding water, probably originates from the liver, although a testicular origin cannot be excluded.

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