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SEX PHEROMONES OF THE SEA LAMPREY (*Petromyzon marinus*): Steroid Studies

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Abstract—Pheromone-containing and pheromone-devoid samples of male sea lamprey (*Petromyzon marinus*) urine were analyzed for the concentrations of nine steroids [dehydroepiandrosterone (DHEA), testosterone (T), dihydrotestosterone (DHT), progesterone (P), androstenedione (A), estrone (E_1), estradiol (E_2), corticosterone (B), and cortisol (F)] by radioimmunoassay (RIA). Samples analyzed included native urine that had been enzymatically hydrolyzed with mixed β-glucuronidase/sulfatase. Values of the analyses were used to prepare solutions of the individual steroids for bioassay at concentrations which bracketed the urinary concentrations. Results show that only testosterone elicited a preference response in spawning-run female sea lampreys, and in concentrations three to four orders of magnitude greater than those found in active, unhydrolyzed male urine. The possibility that testosterone acts as a pheromone in this species is discussed.

Key Words—Sea lamprey, *Petromyzon marinus*, pheromone, urine, steroids, radioimmunoassay, RIA, behavior.

INTRODUCTION

Intraspecific chemical signals (pheromones) have been implicated in a variety of behavioral processes in fish, including schooling, parent–young interactions, homing, pair formation, and spawning (Solomon, 1977; Liley, 1982). Preferences of spawning-run landlocked sea lampreys (*Petromyzon marinus*) for substances released by sexually mature conspecifics of the opposite sex indicate that pheromones may play a role in the reproductive behavior of this species (Teeter, 1980). Pheromone release in sea lampreys coincides with the appearance of secondary sex characteristics. At this time, male sea lampreys release in their urine a pheromone that is attractive to female conspecifics (Teeter, un-

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published observation). The possibility that this pheromone could be steroidal in nature led us to assay both pheromone-containing (behaviorally active) and pheromone-devoid (behaviorally inactive) male urine for its content of several metabolically important steroids; the values obtained from this analysis were then used to establish concentrations for examining the effects of these compounds on the preference behavior of female sea lampreys.

METHODS AND MATERIALS

Spawning-run sea lampreys (*Petromyzon marinus*) were captured in tributaries of Lake Huron and held in large raceways supplied with lake water at the Hammond Bay Biological Station, Millersburg, Michigan. Urine from both sexually immature and sexually mature male sea lampreys (approximately 400 animals per collection, four collections per day) was expressed by applying gentle abdominal pressure and allowing urine to spray into a Teflon trough. During the collection, the animals were held well away from the trough to ensure that no water, skin mucus, or other material contaminated the urine. Once in the trough, the urine was collected by pipet, pooled in daily batches, and frozen for subsequent bioassay and analysis. The results of previous experiments (Teeter, 1980) showed that the presence of a sex pheromone in male urine (obtained as previously described or by catheterization) was associated with the presence of secondary sex characteristics; milt did not, however, elicit preference responses in spawning-run females. Consequently, urine was collected from "sexually immature" males (those lacking secondary sex characteristics) and from "sexually mature" males (those which displayed secondary sex characteristics). Urine from spermiated males (that which contained a noticeable quantity of milt) was kept in a separate pool and was not used in this study. The urine was separated into pools on the basis of positive or negative preference assays, as described below.

Bioassays. Pooled male urine and steroid solutions were assayed as previously described (see Lisowski et al., 1986, for details of apparatus and procedures) for pheromone content (behavioral activity) using spawning-run female sea lampreys as test subjects in a two-choice preference test tank. As an example of a representative test, females were given a choice between a compartment containing lake water and a compartment containing lake water with 13 μl/liter male urine. Data were analyzed using the Wilcoxon matched-pairs, signed-ranks test (Brown and Hollander, 1977). Presence of a pheromone was indicated if females spent a significantly longer time in the stimulus arm of the apparatus than in the lake water arm. Gender of all animals used in the study was confirmed by autopsy.

Steroid Extraction and Separation. Ten-milliliter aliquots of male sea lamprey urine were used for the radioimmunoassays (RIAs). For samples that were

to be hydrolyzed, a mixture of 15,000 Fishman units of *Helix pomatia* β-glucuronidase/aryl sulfatase (Sigma Chemical Co., St. Louis, Missouri) in 0.8 ml water and 1.2 ml of 1.2 M acetate buffer (pH 4.8) was added; the hydrolysis was allowed to proceed at 37°C for 2 hr. At the end of the hydrolysis period, the mixture was extracted twice with 20-ml portions of acid-washed diethyl ether. Unhydrolyzed samples were also extracted twice with 20-ml portions of diethyl ether. These extracts were dried under a gentle stream of nitrogen in a warm (50°C) sand bath, redissolved in 10 ml of acid-washed diethyl ether, washed with 0.5 ml of 0.5% $NaHCO_3$, and dried again. Each extract was dissolved in 0.1 ml of a mixture of (all freshly distilled) chloroform–*n*-heptane–methanol–water, 500:500:75:3 (Zamecnik et al., 1977) and fractionated on a 2.5-g (8-ml) Sephadex LH-20 column, eluting with the same mixture. Elution patterns and recovery values of tritiated steroids run on these columns are given in Table 1. The glass columns (8 mm ID, acid-washed) and polyethylene fittings (QSH Practicolumns, Isolab Co., Akron, Ohio) were treated for 1 hr with 1% trichloromethylsilane (Aldrich Chemical Co., Milwaukee, Wisconsin) in chloroform prior to packing. Material eluted from the columns was dried and taken up in 1.0 ml steroid radioimmunoassay buffer (0.1 M sodium phosphate in saline, pH 6.9, containing 0.1% gelatin and 0.01% thimerosal).

Steroid RIA. All determinations were done on precoded samples whose identity was known only to an investigator not directly involved in the analytical procedures. Aliquots of the samples in steroid RIA buffer were analyzed in specific RIA systems (Table 2). The following antibodies and tritiated steroids were used: (1) rabbit anti-progesterone-11α-hemisuccinyl-bovine serum albu-

Table 1. Elution Patterns and Recovery Values of Standard (Prepurified) Steroids[a]

Fraction no. (and ml eluted)	Steroid eluted		Recovery (%)
	Trivial name	Systematic name	
1. (0–2.5)			
2. (2.5–5.5)	Progesterone (P)	4-Pregnen-3,20-dione	98
	Androstenedione (A)	4-Androsten-3,17-dione	96
3. (5.5–9.0)	DHEA	5-Androsten-3β,-ol-17-one	91
	Testosterone (T)	4-Androsten-17β-ol-3-one	94
	DHT	5-Androstan-17β-ol-3-one	87
4. (9.0–14.0)	Corticosterone (B)	4-Pregnen-11β,21-diol-3,20-dione	96
	Estrone (E_1)	1,3,5(10)-Estratrien-3-ol-17-one	86
5. (14.0–24.0)			
6. (24.0–33.0)	Cortisol (F)	4-Pregnen-11β,17α,21-triol-3,20-dione	72
	Estradiol (E_2)	1,3,5(10)-Estratrien-3,17β-diol	94

[a] Steroids eluted according to Zamecnik et al. (1977) from 2.5 g Sephadex LH-20 columns.

Table 2. CROSS-REACTIVITY VALUES[a] OF COELUTED STEROIDS IN RIA SYSTEMS USED DURING STUDY

Fraction	Steroid tested	Coeluted steriod's RIA system	Cross-reactivity value
2	Progesterone	Androstenedione	0.01
	Androstenedione	Progesterone	0.01
3	DHEA	Testosterone	0.01
	DHEA	DHT	1.46
	Testosterone	DHT	19.71
	Testosterone	DHEA	0.01
	DHT	Testosterone	29.33
	DHT	DHEA	0.01
4	Estrone	Corticosterone	0.01
	Corticosterone	Estrone	0.01
6	Cortisol	Estradiol	0.01
	Estradiol	Cortisol	0.01

[a] Percent cross-reactivity determined at 50% binding of tested antibody.

min (BSA) and [1,2,6,7-^{3}H]progesterone, 89 Ci/mmol; (2) rabbit anti-androstenedione-7α-carboxyethylthioether-BSA and [1,2,6,7-^{3}H]-androstenedione, 114 Ci/mmol; (3) rabbit anti-testosterone-7α-carboxymethylthioether-BSA and [1,2,6,7,16,17-^{3}H]testosterone, 150 Ci/mmol; (4) rabbit anti-5α-dihydrotestosterone-1α-carboxyethylthioether-BSA and [1,2,4,5,6,7,16,17-^{3}H]-5α-dihydrotestosterone, 190 Ci/mmol; (5) rabbit anti-estrone-6-(*O*-carboxymethyl)oxime-thyroglobulin and [2,4,6,7-^{3}H]estrone, 85 Ci/mmol; (6) rabbit anti-estradiol-17β-6-(*O*-carboxymethyl)oxime-BSA and [2,4,6,7,16,17-^{3}H]-17β-estradiol, 152 Ci/mmol; (7) rabbit anti-cortisol-21-hemisuccinyl-thyroglobulin and [1,2,6,7-^{3}H]hydrocortisone, 93 Ci/mmol; (8) rabbit anti-dehydroepiandrosterone-15α-carboxymethylthioether-BSA and [1,2,6,7-^{3}H]dehydroepiandrosterone, 79 Ci/mmol; and (9) rabbit anti-corticosterone-21-hemisuccinyl-thyroglobulin and [1,2,6,7-^{3}H]corticosterone, 89 Ci/mmol. All the antibodies were produced by Miles-Yeda, Rehovoth, Israel, except anti-dehydroepiandrosterone (anti-DHEA, Sigma Chemical Co.) and anti-estradiol, which was a gift of Dr. D. T. Armstrong, London, Ontario, Canada; the tritiated steroids were purchased either from New England Nuclear, Boston, Massachusetts, or Amersham Corp., Arlington Heights, Illinois, and were purified before use (on the Sephadex LH-20 columns). Assays were performed as previously described (Katz et al., 1982).

Separation of bound from free steroids in the RIA tubes was effected by dextran-coated charcoal methodology. Centrifuged supernatants were counted

for radioactivity in ACS II aqueous scintillation solution (Amersham) using a Packard Instruments liquid scintillation spectrometer, model 2425, and were corrected for quenching. Data reduction was performed on a Hewlett-Packard 9815A/9871A desktop computer using log–logit transformations. Extraction and recovery procedures were validated using standard steroid mixtures as previously described (Katz et al., 1982).

Behavioral Activity Assays of Urinary Steriods. As little as 0.5 ml (6.5 μl/liter of water) of pooled urine from sexually mature male sea lampreys has been shown to elicit preference responses in females in our two-choice tanks (Teeter, 1980). We arbitrarily use 1 ml of urine per test (13 μl/liter) when determining whether or not a pool of urine contains the pheromone. Consequently, samples of the steroids were tested in the preference tank at the concentrations at which they were determined to be present in 1 ml of behaviorally active male urine by RIA ("active urine–unhydrolyzed," Table 3). Solutions of the steroids were prepared in ethanol and the appropriate amount of material delivered by microliter syringe to the stimulus side of the test tank. An equivalent amount of solvent was added to the blank side of the tank as a control in each experiment.

RESULTS AND DISCUSSION

Urine of sexually immature and sexually mature male sea lampreys was analyzed by RIA for the content of nine steroid hormones (dehydroepiandrosterone, testosterone, dihydrotestosterone, progesterone, androstenedione, estrone, estradiol, corticosterone, and cortisol). To measure the degree of steroid conjugation, both active and inactive urine were hydrolyzed with a sulfatase–

TABLE 3. STERIOD CONTENT OF NATIVE AND HYDROLYZED MALE SEA LAMPREY URINE, EITHER PHEROMONE-CONTAINING (ACTIVE) OR PHEROMONE-DEVOID (INACTIVE)[a]

	Active urine		Inactive urine	
Steroid	Unhydrolyzed	Hydrolyzed	Unhydrolyzed	Hydrolyzed
DHEA	0	2.05 ± 0.43	10 ± 0.05	1.11 ± 1.41
T	1.28 ± 0.08	1.48 ± 0.04	0.42 ± 0.03	0.56 ± 0.04
DHT	0.15 ± 0.01	0.35 ± 0.01	0.18 ± 0.07	0.26 ± 0.01
P	0.38 ± 0.01	1.08 ± 0.05	0.67 ± 0.02	0.60 ± 0.02
A	1.02 ± 0.04	1.34 ± 0.19	1.13 ± 0.01	1.16 ± 0.06
E_2	0.44 ± 0.06	6.91 ± 0.86	1.64 ± 0.07	3.89 ± 0.20
E_1	0.84 ± 0.06	2.09 ± 0.09	0.60 ± 0.04	2.28 ± 0.10
B	0	19.42 ± 1.92	4.82 ± 0.27	15.30 ± 0.92
F	6.58 ± 0.61	9.65 ± 0.33	4.87 ± 0.50	5.21 ± 0.49

[a] All concentrations are ng/ml except DHEA, which are pg/ml.

TABLE 4. BEHAVIORAL PREFERENCE RESPONSE VALUES FOR SEVERAL MALE SEA LAMPREY URINARY STEROIDS, TESTED INDIVIDUALLY, AS MEASURED IN TWO-CHOICE TEST TANK (1 ml STIMULUS SOLUTION PER TEST)

Steroid	Stim. soln. concentration (ng/ml)	No. of animals	Mean % time (±SE) on stimulus side	Attractive?[a]
DHEA	100[b]	24	56.9 ± 6.2	N
	10[b]	23	53.4 ± 6.4	N
	1[b]	24	56.7 ± 6.1	N
	0.1[b]	24	52.9 ± 5.5	N
DHT	0.2	24	42.1 ± 4.9	N
P	0.4	24	57.1 ± 6.3	N
A	10	24	44.0 ± 6.5	N
	1	24	51.7 ± 5.3	N
	0.1	24	53.9 ± 5.3	N
E_1	10	24	57.9 ± 5.0	N
	1	24	52.4 ± 6.6	N
	0.1	24	55.4 ± 5.8	N
	0.01	24	53.8 ± 5.5	N
B	100	18	57.7 ± 6.0	N
	10	24	42.4 ± 5.4	N
	1	30	52.7 ± 4.4	N
F	100	24	53.0 ± 5.7	N
	10	24	49.9 ± 6.0	N
E_2	0.5	24	45.8 ± 5.8	N
Urine control (1 ml[c])		24	63.7 ± 5.0	Y

[a] $P < 0.05$, Wilcoxon matched-pairs signed-rank test, two-tailed; Y = yes, N = no.
[b] pg/ml.
[c] 1 ml of bioactive male urine used as a control.

glucuronidase enzyme mixture prior to RIA analysis. Numerical values for these analyses are presented in Table 3 and graphed in Figure 1.

Using results obtained with inactive, unhydrolyzed urine as baseline values, and comparing them with steroid concentrations found in active urine, one may note that testosterone increases threefold, and cortisol increases by 1.5 times; progesterone decreases twofold, and estradiol shows a fourfold decrease, while DHEA and corticosterone decrease to zero. DHT, androstenedione, and estrone concentrations remain roughly the same. Results for hydrolyzed and unhydrolyzed progesterone suggest the presence of a steroid capable of forming a conjugate and capable of cross-reacting with progesterone antibody. One likely candidate for this contaminant is deoxycorticosterone, which is known to cross-

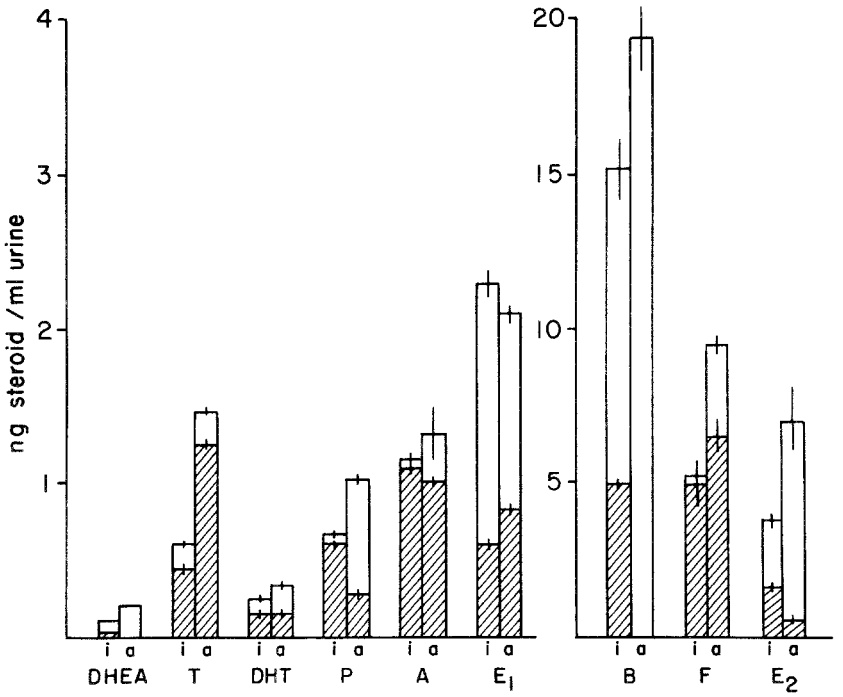

FIG. 1. Steroid content of biologically active, pheromone-containing (a) and biologically inactive, pheromone-devoid (i) male spawning-run sea lamprey urine. Shaded bars show unhydrolyzed values, clear bars represent hydrolyzed values.

react with the antibody and which coelutes with progesterone from the Sephadex LH-20 column.

Using enzyme-hydrolyzed urine as a measure of total steroid content, it can be seen that in all cases but that of estrone, active urine contains a greater quantity of the steroids measured than does inactive urine. The estrone content in hydrolyzed, active urine is not significantly lower than its unhydrolyzed counterpart.

It is known that production and/or release of male pheromone in landlocked sea lampreys corresponds with the onset of sexual maturity and concomitant development of secondary sexual characteristics (Teeter, 1980). It seemed reasonable that sea lampreys might have developed a pheromone communication system using steroid hormones whose concentrations might surge during this period of development. The possibility of chemical communication using an externalized hormone as a signal is generally recognized (Liley, 1982); however, as of the present, this has not been reliably demonstrated for any aquatic species. With the exception of testosterone (see below), the results of our bioassays with unconjugated urinary steroids were uniformly negative. In no case

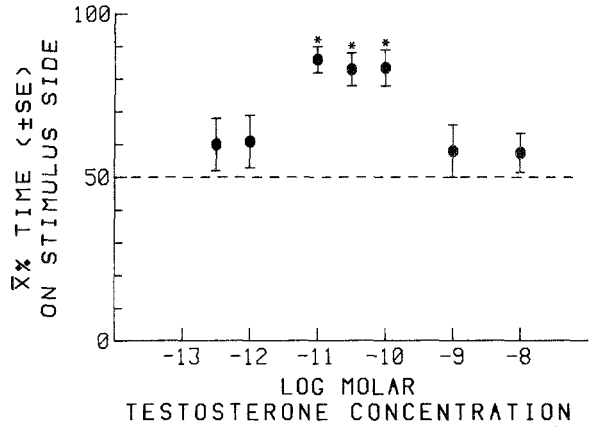

FIG. 2. Plot of log molar testosterone concentration vs. mean percent time that female spawning-run sea lampreys spend on the stimulus side of a two-choice behavioral preference tank. An asterisk denotes a significant preference for testosterone ($P < 0.05$, Wilcoxon matched-pairs, signed-tank test, two-tailed).

did any of the assayed steroids, when presented at concentrations which would be found in 1 ml of male urine (concentrations known to elicit female preference when using bioactive male urine), elicit preference in female lampreys (Table 4). Testosterone was assayed over a range of concentrations, and the results are presented in Figure 2. Bioactive male sea lamprey urine contains approximately 5×10^{-9} M unconjugated testosterone (1.5 ng/ml). When 1 ml of bioactive urine is added to the stimulus side of the behavioral test tank, the testosterone concentration that results from dilution into 78 liters of water is ca. 6.6×10^{-14} M. The behavioral response to pure testosterone occurs at concentrations between 1×10^{-11} M and 1×10^{-10} M (Figure 2). Thus, bioactive male urine may contain a substance other than testosterone which elicits a preference response in female sea lampreys. As the concentration of testosterone was increased above 1×10^{-9} M, the preference effect disappeared (Figure 2). It is clear from these results that females show a preference for water in which testosterone is present. The testosterone concentration necessary to evoke this response is approximately three to four orders of magnitude greater than the testosterone concentration of native, bioactive male lamprey urine that is observed to evoke a preference response in females.

It is possible that the preference shown by spawning-run female sea lampreys for testosterone is of no biological significance. It is also possible that testosterone, or a closely related structural derivative, functions as a sex pheromone in sea lamprey when present at the appropriate concentration. In this case, the testosterone concentrations necessary to elicit a response in females in the bioassay apparatus are representative of the amounts that the female must

sense in the open stream before preference behavior is elicited. This would argue for the release of pheromone by one or more males in close proximity to the female, perhaps taking place over the animals' spawning nest. At close range, dilution of the pheromone would be minimal, and it is possible that the critical concentration range of 10^{-11}–10^{-10} M might be reached. Even if this is the case and testosterone is functioning as a short-range attractant, then our general bioassay results indicate that there is a second substance exerting its attractive effect on females at much lower concentrations. This is evidenced by the fact that 0.5–1.0 ml of bioactive male urine, when placed in the bioassay device, will elicit a behavioral preference response in female lamprey. The concentrations that obtain in this situation are on the order of 6–13 μl of urine per liter of water. This amounts to a 1:78,000 dilution of the urine and, for testosterone in male urine, the result is that the concentration is much lower than the effective preference-producing concentration. Thus, the presence of another pheromone is implicated. Experiments directed to isolating and identifying this material are currently being undertaken in our laboratory.

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