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INFLUENCE OF ESTRADIOL AND TESTOSTERONE ON CYTOCHROME P-450 AND MONOOXYGENASE ACTIVITY IN IMMATURE BROOK TROUT, *SALVELINUS FONTINALIS*

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Abstract—Levels of hepatic microsomal cytochrome P-450 were depressed by administration of estradiol-17β and were elevated by administration of testosterone in both male and female juvenile brook trout (*Salvelinus fontinalis*). Treatment-associated changes in the levels of other microsomal electron transfer components in liver did not reflect the changes in cytochrome P-450 content and were also distinct from the changes in these components in kidney. Electrophoretic analysis of hepatic microsomes revealed that estradiol treatment reduced the amounts of several proteins including some heme-staining protein at 56,000 daltons, possibly containing cytochrome P-450. Hepatic microsomal benzo[*a*]pyrene hydroxylase and the response to 7,8-benzoflavone *in vitro* were affected little by steroid treatment, and ethoxyresorufin *O*-deethylase activity could not be detected in any of the samples. Hepatic microsomes metabolized testosterone to a suite of products including 6β-hydroxytestosterone (the major metabolite) and 16β-hydroxytestosterone, plus as many as eleven unknown metabolites. Estradiol-17β treatment depressed the rates of testosterone metabolism and particularly the rates of 6β-hydroxylase activity but did not affect 16β-hydroxylase activity. Both activities were largely unaffected by testosterone. The results are consistent with the idea that both androgens and estrogens regulate the levels of hepatic cytochrome P-450 in brook trout and that the effect, at least of estradiol-17β, involves regulation of forms that function in specific hydroxylation of testosterone. The significance of these effects and whether factors additional to steroids are involved in this regulation of hepatic cytochromes P-450 in fish remain to be established.

Hepatic microsomal cytochrome P-450-dependent monooxygenase systems in fish are, like those in mammals, involved in the biotransformation of xenobiotics such as polynuclear aromatic hydrocarbons [1, 2] and of endogenous substrates such as steroid hormones [3, 4]. Variation in microsomal monooxygenase activity in mammalian liver [5] is known to be associated with regulation of certain cytochrome P-450s by different biological and environmental factors [6]. There is much less known concerning the variation or regulation of these enzymes in lower vertebrates, although studies have demonstrated that differences in monooxygenase activity in fish can occur with differences in strain [7], chemical treatment [1, 8], and temperature [9]. Recent studies have also revealed marked sex differences in hepatic cytochrome P-450 in gonadally mature rainbow and brook trout, but not in immature fish [10, 11]. The nature and significance of these differences in brook trout and the factors responsible for them have not been clearly established, but it is logical to expect that circulating steroids contribute to the sex differences in hepatic cytochrome P-450 in these fish. It is known that the sex differences in hepatic xenobiotic and steroid monooxygenase that appear at puberty in some mammals are regulated not only by the levels and types of circulating steroids but also by the pituitary, which modulates the influence of steroids on the liver [12, 13].

Gonadal maturation is seasonal in many fish and is accompanied by changes in the levels of circulating steroid hormones, which at spawning time may be increased as much as 10-fold [14]. There are changes as well in the types and relative proportions of steroids present, depending on the sex of the animal. In serum of maturing female salmonids, for example, there is a marked increase in levels of estradiol-17β that does not appear in males [15], whereas in males the levels of any of several androgens may be greatly elevated [16]. This report describes the results of an experiment to determine whether androgens, estrogens, or both, might be responsible for the sex differences in hepatic P-450 that appear upon gonadal maturation in brook trout. Immature brook trout (*Salvelinus fontinalis*) were treated with one of two teleost sex steroids, estradiol-17β or testosterone, and characteristics of hepatic and renal microsomal electron transport components, and hepatic monooxygenase activity with xenobiotics and testosterone, were examined.

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METHODS

Chemicals. Estradiol-17β (1,3,5(10)-estratriene-3,17β-diol; abbreviated estradiol) and testosterone were obtained from Research Plus Steroid Laboratories, Denville, NJ. [^{3}H]-Benzo[*a*]pyrene (generally labeled) was from Amersham-Searle, Skokie, IL. [^{3}H]-Testosterone and [^{3}H]-toluene were purchased from the New England Nuclear Corp. Boston, MA. Authentic testosterone metabolite standards were a gift from Dr. David Waxman, M.I.T., Cambridge, MA. Polyethylene glycol was purchased from the Sigma Chemical Co., St. Louis, MO. Ethoxyresorufin and resorufin were prepared and characterized as previously described [17]. Other chemicals and supplies were obtained from sources previously indicated [10].

Animals and tissue preparation. Immature brook trout (*S. fontinalis*) were obtained from the Sandwich Fish Hatchery, Massachusetts Division of Fisheries and Game, in May 1980. One week prior to experimentation, twelve fish (at least five of each sex) were transferred from outdoor raceways at the hatchery to each of three indoor troughs (3.7 × 0.3 × 0.15 m) located in an adjacent hatchhouse. The troughs were supplied with flowing water at 10° from the same artesian source as that supplying the raceways. Natural photoperiod was maintained by means of large windows situated by the troughs, supplemented by incandescent lights between 7:00 a.m. and 6:00 p.m. Fish were fed *ad lib.* (1–2% body wt) once a day with Rangin's Production pellets (Zeigler Brothers, Gardners, PA).

All treatment and sampling of fish were conducted at the hatchery. The three groups were treated on consecutive days so that all the fish could be injected, and later sampled, within 30 min at the same time of day (10:00 a.m.). Fish were injected intramuscularly on days 1, 3 and 7 with either estradiol-17β (3 mg/kg fish) or testosterone (3 mg/kg fish) in 0.1 ml polyethylene glycol (PEG). The treatment regimen used in this study was selected based on reports of the effectiveness of varied doses of steroids in eliciting changes in thyroid function [18], vitellogenin synthesis and associated liver hypertrophy [19–21], gonadotropic cell differentiation [19] and hypercalcemia [19, 22] in various species of fish. Control animals received only PEG. On day 10 of experimentation, blood was sampled from the caudal vein with a heparinized syringe and placed on ice, and the fish were killed by cervical section.

Liver and kidneys were immediately excised and placed in ice-cold 0.1 M phosphate buffer (pH 7.3) with 1.5% KCl and 3 mM $MgCl_2$. Samples were then transferred on ice from the hatchery to the laboratory for analysis (transit time approximately 1 hr). Blood was centrifuged at 1500 *g* for 10 min and plasma was frozen in glass tubes at −20°. Livers and kidneys were blotted dry, weighed, and then homogenized in 5 vol. buffer using a Potter–Elvehjem tissue grinder. Microsomes were prepared as described by Stegeman and Binder [23], resuspended to approximately 3–8 mg protein/ml in a buffer composed of 50 mM Tris (pH 7.5), 10 mM β-mercaptoethanol, and 20% glycerol, and aliquots were immediately frozen in liquid nitrogen.

Adult brook trout in spawning condition were collected from the Sandwich Fish Hatchery in November 1981. These fish were of the same genetic stock as the juveniles and had been treated in the same way as the adults in a previous study [10]. The water supplying the raceways where they were kept came from a small nearby pond and was at 8° at the time of sampling. Fish were transported live to the laboratory in their own water (transit time—approximately 1 hr) and sampled in the same manner as the experimental groups.

Steroid assays. Radioimmunoassays (RIA) for testosterone and estradiol-17β were performed on the same plasma extract and for 11-ketotestosterone on a separate plasma extract. Plasma samples ranged from 20 to 170 μl, and [^{3}H]-testosterone (New England Nuclear) and [^{125}I]-estradiol (Radioassay Systems Laboratories) or [^{3}H]-11-ketotestosterone (Amersham) were added to each sample for determination of extraction efficiency. Samples were extracted with 5 ml methylene chloride, and the extracts were evaporated with a gentle stream of air. Samples were redissolved in 750 μl methanol, and aliquots of 50, 100 and 200 μl of the methanol extracts were transferred to two sets of tubes, dried with air, and redissolved in 0.1 M phosphate buffer (pH 7.6) for the estradiol and testosterone assays. For 11-ketotestoterone assays the dried methylene chloride extracts were redissolved directly in 150–250 μl of 0.05 M phosphate buffer (pH 7.6) containing 1 mg/ml gelatin (Type IV, Sigma Chemical Co.), and 25, 50 and 100 μl aliquots were assayed.

The estradiol antiserum, generated in rabbits against estradiol-17β-3-carboxymethylether-BSA (Radioassay Systems Laboratories), cross-reacted 6.5% with estriol and 5.2% with 17α-estradiol at 50% binding of estradiol-17β. There was no significant cross-reaction with other steroids. The protocol supplied by Radioassay Systems Laboratories for the assay for estradiol was followed using iodinated estradiol tracer. Bound estradiol-17β was precipitated with a second antibody.

The testosterone antiserum, prepared against testosterone-21-succinyl BSA (New England Nuclear), cross-reacted 56% with dihydrotestosterone but did not cross-react significantly with any other steroids including 11-ketotestosterone (< 1% cross-reaction at 50% binding). The procedure supplied by New England Nuclear using tritiated testosterone tracer was modified to assay 50 μl plasma samples. Free testosterone was precipitated with dextran-coated charcoal.

11-Ketotestosterone antiserum (lot RCB8), generated in rabbits against 11-ketotestosterone-3-carboxymethyloxime, was a gift from Dr. J. P. Sumpter, Brunel University, United Kingdom. We determined that this antiserum cross-reacted 1% with testosterone at 50% binding of 11-ketotestosterone. Aliquots (50 μl) of antiserum diluted 1/250 with buffer, and 50 μl of [^{3}H]-11-ketotestosterone tracer (approximately 1000 cpm) were added to the assay tubes and incubated overnight at 4°. Free 11-ketotestosterone was separated from bound hormone with dextran-coated charcoal.

Recovery of labeled steroids carried through the extraction procedures ranged between 60 and 80%,

and the data were corrected for this. Limits of detection under the conditions of assay here were 0.06 ng/ml plasma (estradiol), 0.08 ng/ml (testosterone), and 0.025 ng/ml (11-ketotestosterone).

Enzyme assays. NADPH–cytochrome *c* reductase activity and NADH–cytochrome *c* reductase activity were assayed at 25° by methods previously described [10]. Benzo[*a*]pyrene (BP) hydroxylase activity was assayed by a microscale radiometric procedure also previously described [24]. The reactions, done in triplicate and under red light, were initiated with 2 μl [^{3}H]-BP in methanol, incubated for 15 min at 29°, then stopped and extracted, and ^{3}H cpm was determined as before [17]. Counting efficiency was determined with internal standards. The limit of detection was about 0.002 nmole per min per mg protein. The influence of 7,8-benzoflavone (7,8-BF) on BP metabolism was determined by adding 2 μl 7,8-BF in methanol to the BP-monooxygenase reaction mixture just prior to addition of [^{3}H]-BP.

Ethoxyresorufin (ER) *O*-deethylase activity was measured using a reaction mixture containing 2 μM 7-ER, 0.1 M Tris (pH 8.0) with 0.1 M NaCl, and 22 μl microsomes (3–8 mg/ml protein). The reaction was initiated with NADPH at a final concentration of 0.5 mM, and the appearance of resorufin was monitored at 572 nm using a Cary 118C recording spectrophotometer. The extinction coefficient of resorufin was determined to be 73 mM^{-1} cm^{-1}. The limit of detection was about 0.02 nmole per min per mg protein.

Testosterone metabolites were obtained by *in vitro* incubation of reaction mixtures (1.0 ml final volume) containing 3.0 mg of microsomal protein, 50 mM Tris (pH 7.4) and 0.20 mM [^{3}H]-testosterone added in 30 μl methanol. Reactions were initiated with NADPH at a concentration of 0.5 mg/ml, incubated for 30 min at 25°, stopped by adding 1.0 ml cold acetone, and extracted twice with 2.0 ml ethyl acetate. Extracts were taken to dryness under high purity nitrogen and resuspended in methanol. Metabolites were analyzed by high pressure liquid chromatography (HPLC) using a DuPont 850 LC fitted with a 25 cm × 4.6 mm Zorbax ODS column. Elution of metabolites was achieved by running a gradient from 100% solvent A (45 parts MeOH to 55 parts water) to 100% solvent B (55 parts MeOH to 35 parts water to 10 parts acetonitrile). The procedure resulted in baseline resolution of ten authentic hydroxytestosterone standards, including 2β-hydroxy, 6α-hydroxy, 6β-hydroxy, 7α-hydroxy, 11α-hydroxy, 11β-hydroxy, 14α-hydroxy, 15α-hydroxy, 16α-hydroxy, and 16β-hydroxy derivatives of testosterone. Peaks were collected, dried, and added to neutralized aquasol, and ^{3}H cpm were measured to ±1% accuracy, using a Beckman LS-100C scintillation counter. Efficiency was determined with internal standards. Tentative identification of some metabolites was possible based on coelution with authentic standards. Recovery of authentic standards carried through the procedure was greater than 95%. Blank reactions were carried out with boiled protein or were poisoned with CO.

Other analyses. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the methods of Laemmli [25] using 8% polyacrylamide disc gels. Thirty micrograms of microsomal protein was applied to each gel, and a current of 2 milliamps/gel was maintained for the duration of the run. Gels were stained for protein as before [26]. Electrophoresis was carried out on some samples according to the methods of Thomas *et al.* [27], with subsequent staining for peroxidase activity using tetramethylbenzidine (TMBD). Standards for molecular weight determination were bovine serum albumin (66,000), glutamate dehydrogenase (53,000), ovalbumin (45,000), aldolase (39,000) and trypsinogen (24,000), all from the Sigma Chemical Co. Molecular weights were estimated by comparsion with standards run both externally and internally with samples. Densitometric analysis of gels was performed at 650 nm with a Gilford 252 spectrophotometer equipped with a linear scanner.

Cytochromes P-450 and b_5 were analyzed as outlined by Stegeman and Binder [23], with a Cary 118-C recording spectrophotometer (calibrated to within 0.4 nm). Protein was determined according to the method of Lowry *et al.* [28] with bovine serum albumin as a standard.

The data were evaluated using a fixed effect two-way analysis of variance with unequal cell sizes [29] to test for significant differences among treatment groups and between sexes. Data for liver weight/body weight or gonad weight/body weight ratios were arcsine transformed prior to statistical analysis, since these values were percentages [29]. Data on the influence of 7,8-BF on BP monooxygenase activity were analyzed using a three-way analysis of variance and then converted to percentage form for presentation.

RESULTS

The levels of plasma estradiol were greatly elevated in both female and male juvenile brook trout treated with this steroid (Table 1), although the levels achieved were lower in males than in females. The levels of plasma testosterone were also elevated in the estradiol-treated animals of both sexes. In contrast, neither plasma testosterone levels nor the levels of 11-ketotestosterone were elevated in the animals treated with testosterone. The levels of 11-ketotestosterone were significantly higher in each male group than in their female counterparts, except for those males treated with estradiol. The levels in this group, and in the testosterone-treated males, were significantly lower than in control males.

Liver to body weight ratios were increased in both males and females given estradiol (Table 1) but these values in testosterone-treated fish of either sex did not differ from those of control animals. Unlike the effect on liver weight, the influence of hormone treatment on the ratio of gonad to body weight (Table 1) was sex specific. The latter ratio was lower in both estradiol-treated and testosterone-treated males than in control males, whereas in females gonad weight did not change significantly upon treatment with either hormone. There were no significant changes in body weight (Table 1) as a result of hormone treatment. However, treatment with either steroid resulted in a significant decrease in hepatic

Table 1. Body characteristics and circulating steroids in steroid-treated juvenile and naturally maturing brook trout*

	Females				Males			
Character	Control (7)	Estradiol (5)	Testosterone (6)	Mature (4)	Control (5)	Estradiol (7)	Testosterone (5)	Mature (4)
Body wt (g)	77 ± 14	76 ± 13	74 ± 16	132.0 ± 14	76 ± 13	80 ± 18	92 ± 21	158.0 ± 15
Gonad/body wt (%)	0.56 ± 0.09	0.61 ± 0.210	0.73 ± 0.12	15.1 ± 4.0	0.28 ± 0.15	0.10 ± 0.04	0.12 ± 0.04	2.0 ± 0.3
Liver/body wt (%)	1.4 ± 0.2	2.3 ± 0.3†	1.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.1	1.9 ± 0.2†	1.2 ± 0.3	1.3 ± 0.2
Plasma Estradiol (ng/ml)	0.8 ± 0.4	33.9 ± 17.8†	0.4 ± 0.2		0.5 ± 0.2	12.8 ± 5.0†	0.3 ± 0.2	
Testosterone (ng/ml)	4.5 ± 2.9	20.3 ± 27.5‡	3.8 ± 1.4		3.0 ± 1.4	25.6 ± 32.2‡	3.0 ± 1.1	
11-Ketotestosterone§ (ng/ml)	0.48 ± 0.34	0.18 ± 0.07	0.34 ± 0.20	4.35 ± 5.33	2.40 ± 0.66	0.41 ± 0.22‖	0.70 ± 0.20‖	77.8 ± 31.6

* Values are means ± S.D. (N) = number of individuals.
† Levels in estradiol-treated animals were significantly greater than in control or testosterone-treated animals at $P \leq 0.01$.
‡ In each case, one very high value is included. Without this value, the levels are still significantly greater than in controls at $P \leq 0.01$.
§ Values for control, testosterone-treated and mature males were significantly greater than their female counterparts at $P \leq 0.05$ to $P \leq 0.01$.
‖ Values were significantly lower than in control males at $P \leq 0.01$.

Table 2. Hepatic microsomal electron transfer components and monooxygenase activities in immature brook trout treated with steroids*

Character	Females			Males		
	Control (7)	Estradiol (5)	Testosterone (6)	Control (5)	Estradiol (7)	Testosterone (5)
Microsomal protein (mg/g)	19.4 ± 2.5	13.8 ± 4.2	11.1 ± 2.2	19.2 ± 7.0	1.27 ± 3.6	12.0 ± 1.5
Cytochrome P-450† (nmoles/mg protein)	0.15 ± 0.04	0.11 ± 0.02	0.21 ± 0.03	0.18 ± 0.06	0.15 ± 0.05	0.25 ± 0.10
Cytochrome b_5 (pmoles/mg protein)	13 ± 7	5 ± 4	5 ± 9	12 ± 12	3 ± 5	2 ± 5
NADPH–cytochrome *c* red.‡ (nmoles/min/mg protein)	43 ± 17	46 ± 15	66 ± 19	55 ± 24	43 ± 14	73 ± 38
NADH–cytochrome *c* red.§ (nmoles/min/mg protein)	142 ± 48	96 ± 29	215 ± 80	145 ± 39	132 ± 50	232 ± 74
BP-hydroxylase (pmoles/min/mg protein)	63 ± 20	49 ± 24	60 ± 21	56 ± 18	61 ± 26	125 ± 79
Percent activity with 7,8-BF at:						
1.25×10^{-7} M	94 ± 10	100 ± 18	92 ± 8	87 ± 9	80 ± 7	87 ± 13
5.0×10^{-6} M	95 ± 18	116 ± 36	140 ± 41	125 ± 24	94 ± 17	94 ± 25
1.0×10^{-4} M	49 ± 9	66 ± 24	52 ± 14	49 ± 25	53 ± 20	43 ± 14

* Values are means ± S.D. (N) = number of individuals.

† Testosterone-treated fish differed significantly from controls and estradiol-treated fish at $P < 0.001$. Estradiol-treated males and females differed from the control groups at $P < 0.07$. The sex effect, males vs females, was significant at $P < 0.05$.

‡ Activity in testosterone-treated fish was significantly greater than in other groups at $P < 0.01$.

§ Activity in testosterone-treated fish was significantly greater at $P < 0.001$.

microsomal protein content in both sexes (Table 2), the reasons for which are unknown.

Reduced, CO-bound hepatic or renal microsomal P-450 had an absorption maximum close to 450 nm in all fish regardless of treatment or sex. The specific content of hepatic P-450 was increased by treatment with testosterone and depressed by treatment with estradiol in both males and females (Table 2). The direction and also the magnitude of response to treatment with either steroid were the same in both sexes. There was, however, a trend to lower specific content of P-450 in females in each control and treated group. The difference in specific content of P-450 between males treated with testosterone and females treated with estradiol was about the same (nearly 60%) as the difference in specific content between gonadally mature male and female brook trout [10]. P-450 levels normalized to liver weight were significantly lower in the estradiol-treated fish than in other groups, 1.5 to 1.8 nmoles/g liver as compared to 2.3 to 3.3 nmoles/g liver, a function of the liver hypertrophy induced by estradiol. When normalized to body weight, P-450 levels were quite similar in all groups, about 0.04 nmole/g body wt.

The levels of cytochrome b_5 in hepatic microsomes of treated fish appeared to be lower than in those of control fish. In many of the experimental animals, especially those treated with estradiol, the levels were so low as to be undetectable. Levels of both NADPH- and NADH-cytochrome *c* reductase activities, which were very much like those previously seen in adult fish [10], were elevated by treatment with testosterone but did not differ between the sexes within any treatment group (Table 2).

There was not a distinct effect of steroid treatment on the specific content of either cytochrome P-450 or cytochrome b_5 in kidney microsomes (Table 3). This may have been due partly to the fact that kidney tissue in each group was pooled for preparation of microsomes, thereby precluding statistical analyses, and partly to what may have been an inordinately high yield of "microsomal" protein in the control females. However, in contrast to these cytochromes, the specific activity of NADPH–cytochrome *c* reductase in kidney microsomes was reduced substantially by treatment with either steroid in both males and females. NADH–cytochrome *c* reductase activity (Table 3) did not vary consistently when the data were expressed as units per mg protein, but when normalized to tissue weight there was a marked reduction in this activity evident in those animals treated with estradiol.

Analysis of hepatic microsomes by SDS-PAGE revealed a pronounced reduction in the relative intensity of a band with a minimum molecular weight of about 56,000 daltons (band E in Fig. 1) in both male and female fish treated with estradiol. This same band was present with similar intensity both in control and testosterone-treated animals (Fig. 2). Estradiol also reduced the intensity of bands in region F (near 50,000 daltons), of a band near 67,000 daltons (band C), and of two doublets of even higher molecular weight (regions A and B). That the 56,000 dalton band diminished in estradiol-treated animals contained a heme protein was indicated by staining of gels for peroxidase activity. Testosterone-treated and control animals, male or female, possessed a TMBD-staining band at 56,000 daltons, but microsomes from estradiol-treated animals did not have detectable peroxidase activity migrating in that region of the gel. Analysis of hepatic microsomes from a group of untreated but gonadally mature fish revealed electrophoretic distinctions between females and males like those that appeared between estradiol-treated and control or testosterone-treated juvenile fish. The relative intensity of several bands, including a 56,000 dalton band, was reduced in mature females, as compared to the pattern in mature males.

Hepatic microsomal BP hydroxylase activity in the variously treated juvenile fish ranged from about 50 to more than 100 pmoles per min per mg. These values are higher than those seen previously in this same genetic stock [10] but the earlier data were obtained by measuring fluorescence of phenolic derivatives, which typically constitute only 30–40% of the total BP metabolites produced by fish liver

Table 3. Electron transport components in kidney microsomes of steroid-treated brook trout*

Character	Females			Males		
	Control (7)	Estradiol (5)	Testosterone (6)	Control (5)	Estradiol (7)	Testosterone (5)
Microsomal protein (mg/g kidney)	17.0	9.0	8.5	11.1	9.8	10.4
Cytochrome P-450 (nmoles/mg protein)	0.05	0.12	0.08	0.05	0.08	0.07
Cytochrome b_5 (pmoles/mg protein)	3.3	15.2	8.9	10.3	7.7	ND†
NADPH–cytochrome *c* red. (units/mg protein)‡	42	26	21	51	15	22
NADH–cytochrome *c* red. (units/mg protein)‡	79	102	142	148	65	126

* Data shown are means of replicate assays. (N) = number of fish from which tissue was pooled.
† Not determined.
‡ Units are nmoles cytochrome *c* reduced/min.

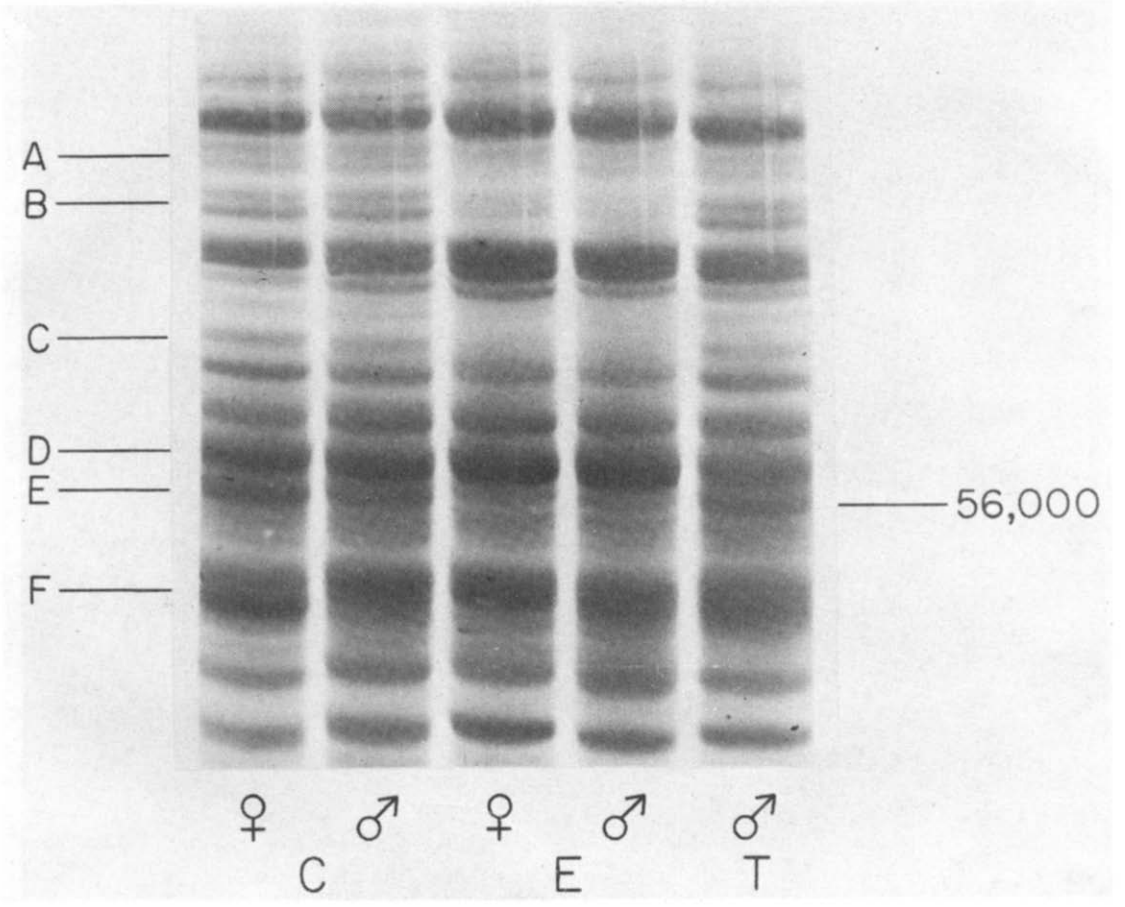

Fig. 1. SDS-gel electrophoresis of brook trout hepatic microsomes. Samples from left to right are control female, control male, estradiol female, estradiol male and testosterone male. Molecular weight regions of selected bands are (C) 66,000; (D) 58,000; (E) 56,000; and (F) 50,000.

microsomes [2]. There was no clear pattern of steroid effect on hepatic BP hydroxylase activity expressed per mg microsomal protein (Table 2), although this activity appeared to be higher in the testosterone-treated males than in the estradiol-treated females. BP hydroxylase activity that was normalized to P-450, to liver weight, or to body weight (not shown) did not differ between groups. There were no significant effects of treatment on the sensitivity of BP metabolism to inhibition by 7,8-BF (Table 2), nor were there any consistent sex differences in this character. BP metabolism was inhibited significantly only at the highest concentration of 7,8-BF. The levels of ER *O*-deethylase activity were below the limits of detection (0.02 nmole per min per mg) in all animals.

Hepatic microsomes from fish in each treated and control group formed a similar suite of testosterone metabolites. Of those whose formation was inhibited by CO (indicating the involvement of P-450) and which could be identified by coelution with authentic standards, 6β-hydroxytestosterone was the most prominent metabolite, accounting for more than 50% of the total metabolism in each case. The other major metabolite that could be identified by coelution with standards was 16β-hydroxytestosterone, but there were as many as eleven additional metabolites whose formation was not inhibited by CO or that did not coelute with our standards and, thus, have yet to be identified.

Estradiol treatment resulted in lower rates of microsomal testosterone metabolism and affected metabolism at specific positions (Table 4). The levels of 6β-hydroxylase activity per mg microsomal protein in estradiol-treated females were only a third of those in control or testosterone-treated animals, and the apparent turnover number (activity per nmole P-450) for 6β-hydroxylation was decreased more than 50% by estradiol treatment. There were some treatment-associated changes in the relative amounts of unidentified products but, unlike the effect on 6β-hydroxylase activity, estradiol produced little or no change in the specific activity or the turnover number for 16β-hydroxylase activity. In contrast to estradiol, there were no specific identifiable effects of testosterone on patterns of testosterone metabolism. Turnover numbers for the activities in Table 4 were about the same in both testosterone-treated and control females. Reliable data could not be obtained for all groups of males, due to a lack of

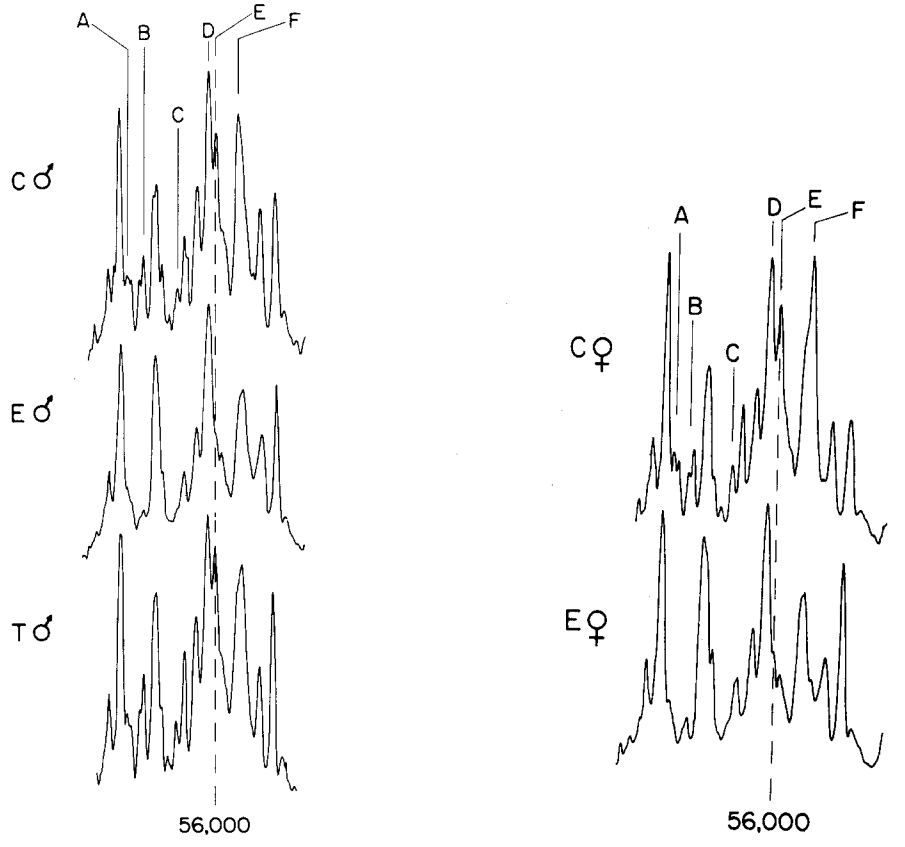

Fig. 2. Densitometric tracings of SDS-polyacrylamide gels. Left panel: male samples; and right panel: female samples. A–F are as in Fig. 1. Vertical dashed line is at 56,000 daltons.

representative microsomal samples in some groups at the time these assays were performed. However, the overall rates of testosterone metabolism and the levels of 6β-hydroxylase activity in estradiol-treated males were much lower than those in testosterone-treated males (Table 4), while 16β-hydroxylase activity was about the same in both groups. The estimated turnover numbers in each case were quite like those seen in their female counterparts.

DISCUSSION

The data presented here demonstrate that both estradiol and testosterone can regulate the levels of

Table 4. Hepatic microsomal testosterone hydroxylase activities in juvenile brook trout treated with steroids*

	Females			Males	
Activity	Control (3)	Estradiol (4)	Testosterone (3)	Estradiol (3)	Testosterone (4)
Total metabolism (units/mg)†	0.276	0.101	0.262	0.184	0.289
6β-Hydroxylase (units/mg)‡	0.152	0.051	0.145	0.090	0.158
(units/nmole P-450)	0.987	0.485	0.852	0.584	0.840
16β-Hydroxylase (units/mg)‡	0.016	0.010	0.013	0.017	0.015
(units/nmole P-450)	0.103	0.095	0.074	0.110	0.080

* (N) = number of individuals from which liver microsomes were pooled. P-450 specific contents of pooled samples were like the respective samples in Table 2. Values are means of replicate assays, and in each case replicate determinations of 6β-hydroxylase activity differed from one another by less than 5%, but replicates for 16β-hydroxylase activity differed by as much as 30%.

† Units are nmoles of testosterone metabolized/min.

‡ Units are nmoles of hydroxytestosterone produced/min.

hepatic P-450 in brook trout and that in juveniles the response to a given steroid is quite similar in both males and females. The data support a hypothesis that estrogens as well as androgens act to elicit the sex differences in P-450 in the liver of naturally maturing brook trout [10] and other salmonids.

That the estradiol treatment regimen here (chosen for reasons outlined in Methods) was effective and appropriate is indicated by the gross changes in liver weight produced by estradiol and by the measured titers of circulating steroids. Plasma estradiol levels in animals receiving this steroid were similar to those seen in naturally maturing females [15, 30]. The higher plasma testosterone levels in those animals are also consistent with observations that high levels of testosterone occur in spawning female salmonids [30, 31]. The fact that estradiol depressed the rates of testosterone metabolism could partly explain the elevation of plasma testosterone in estrogen-treated fish. But the source of these high testosterone levels and the interactive effects of estradiol and testosterone on the liver clearly deserve further exploration.

Neither testosterone nor 11-ketotestosterone levels were elevated in plasma of animals treated with testosterone, but this should not be taken to mean that insufficient doses were employed. Testosterone at similar doses was found to elicit appropriate changes in thyroid function in other species of fish [18]. Moreover, the same dose of testosterone administered to brook trout for a period of time about twice that used here produced an estrogenic response (A. M. Pajor and J. J. Stegeman, unpublished results), like that previously documented for too large doses of androgens [21]. It is quite possible that by the time blood was collected, 3 days after the last administration, the clearance of androgens by the liver had effectively reduced the circulating levels to those observed, but further study of such clearance rates is required to confirm this. As seen here, and noted by others [31], 11-ketotestosterone is a major androgen in mature salmonids, and administered testosterone can be converted to the 11-keto form [32]. Thus, 11-ketotestosterone might well be responsible for the effects of testosterone on hepatic P-450 in brook trout here and perhaps for the high P-450 levels in naturally maturing male [10] fish. By contrast, in mammals testosterone is generally regarded as being most active in producing sex differences in hepatic P-450 [33].

The depression of hepatic P-450 levels by estradiol in the brook trout here was less marked than that recently seen by Hansson and Gustafsson [34] in estradiol-treated juvenile rainbow trout. We have, however, seen a very pronounced reduction (*ca.* 80%) of hepatic P-450 content in brook trout treated for longer periods with estradiol (A. M. Pajor and J. J. Stegeman, unpublished results). In the present study, an influence of estradiol, revealed in SDS-PAGE profiles of hepatic microsomal proteins, was quite explicit, and identical, in both sexes. The estimated molecular weight (56,000) of the band nearly eliminated by estradiol treatment is within the range commonly observed for various P-450s. Because of the evidence for heme protein in this band we might speculate that loss of some protein therein is associated with the decline in hepatic P-450 levels in estradiol-treated animals and, by comparison, in mature females.

Both NADH- and NADPH–cytochrome *c* reductase activities were stimulated by testosterone administered to juvenile trout, but in naturally maturing trout only NADH–cytochrome *c* reductase activity is higher in males [10]. The reasons for this discrepancy are not apparent. It is interesting, though, that the sex difference in naturally maturing trout [10] parallels that in some strains of rat, in which males possess higher levels of NADH-, but not of NADPH–cytochrome *c* reductase activity [35, 36], whereas in other strains the reverse situation has been seen [37]. Although the mechanisms are not certain, it is possible that different modes of regulation underlie the distinctions between different strains or species. The different effects of steroid treatment on levels of NADH- and NADPH–cytochrome *c* reductase activities in liver as compared to kidney suggest that different modes of regulation of microsomal enzymes may also operate in hepatic and some extrahepatic tissues of fish. The significance of changes in the levels of these reductase activities and of cytochrome b_5 are not apparent, but they could effect changes in lipid metabolism.

The lack of a steroid effect on estimated turnover number of BP in the fish here suggests that the sex difference in this characteristic seen in mature adults [10] may have been due to factors in addition to the steroid environment, possibly including differences in the metabolites detected in the two studies. The lack of steroid effect on the sensitivity to 7,8-BF was not unexpected, since a sex difference was not seen in this characteristic in brook trout [10], although in other trout species [10], and in some mammals [38], sex differences have been seen in sensitivity of BP metabolism to 7,8-BF. The 40–50% inhibition by 10^{-4} M 7,8-BF could be interpreted as indicating that the animals had been partially induced by environmental chemicals [2, 10]. However, the undetectable levels of ER *O*-deethylase activity, which is catalyzed preferentially by P-450 induced by 3-methylcholanthrene-type inducers [39], argue against any significant effect of foreign compounds on hepatic systems in the present study. If present, such effects could alter the pattern of testosterone metabolism.

Although both testosterone and estradiol influenced the levels of cytochrome P-450, only the latter had a marked effect on testosterone metabolism. The depression of the rate of testosterone 6β-hydroxylase activity by estradiol seen here is quite similar to the results of Hansson and Gustafsson [34], who found that hepatic 6β-hydroxylation of androstenedione was depressed upon estradiol treatment of another salmonid species, the rainbow trout (*Salmo gairdneri*). Furthermore, in both studies estradiol did not appear to affect metabolism at the 16-position of the steroid nucleus. Whereas Hansson and Gustafsson [34] observed this for unspecified androstenedione 16-hydroxylase activity, in the present study we found that the primary activity at this position was 16β-hydroxylase activity and that it was largely unaffected by estradiol. Such altered patterns of testosterone metabolism are consistent with a steroid regulation of the form(s) of hepatic P-450

that functions in specific hydroxylation of steroids. It is now quite clear that some age and sex specific differences in hepatic monooxygenases in rats stem from differential regulation of three known, and some unknown, forms of P-450 [6], including one that has a high specifity for 7α-hydroxylation of testosterone. Much less is known about the multiplicity and functions of forms of P-450 in fish. Yet, the regulation of a limited number of P-450s could well be responsible for determining hepatic steroid metabolism in fish and it is quite tempting to speculate that specific effects of estradiol on testosterone metabolism in brook trout may be associated with changes in microsomal proteins observed electrophoretically. A most likely candidate for further study might be the 56,000 dalton heme-staining protein(s).

While the effects of steroids on fish liver are becoming known, the mechanisms involved are not clear. In mammals, the presence or absence of testosterone before birth determines the post-pubertal activities of some hepatic drug and steroid-metabolising enzymes and the responses to hormones, apparently by imprinting in the pituitary and hypothalamus [13]. It is possible that such imprinting by steroids during development does not exist in the same way in some fish. In spite of their juvenile status, the trout in the present study possessed some sex differences in P-450, which together with 11-ketotestosterone values suggests the onset of the maturation process, yet both males and females responded very similarly to steroid treatment. In the recent study by Hansson and Gustafsson [34], hypophysectomy of juvenile rainbow trout also failed to alter the effect of estradiol on androstenedione 6β-hydroxylase activity in rainbow trout, suggesting further that the effects on the liver were possibly direct. Sexual differentiation is very plastic in many species of fish. Hormone treatment and even subtle environmental cues [40] can elicit sex reversal that is in some cases complete and functional, even in adults. Investigating the regulation and role of P-450s and of steroid receptors in such fish might well reveal the determining factors in the action of steroids on fish liver.

Such studies may be pertinent to foreign compound effects as well. Endogenous factors related to hormonal condition can modify the response to known foreign compound inducers. P-450a (testosterone 7α-hydroxylase) is induced by 3-methylcholanthrene and phenobarbital in immature and female rats, but not in adult males [6], and induction of P-450b (benzphetamine demethylase) is mitigated by maturation in females, but not in males [6]. It has also been shown that sexual and maturation differences can influence the induction of P-450 in fish [11, 41]. However, the general significance of steroid regulation of P-450 in liver, and the interaction between steroids and foreign compounds in fish, as well as in mammals, remains to be determined.

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