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SEX DIFFERENCES IN CYTOCHROME P-450 ISOZYME COMPOSITION AND ACTIVITY IN KIDNEY MICROSOMES OF MATURE RAINBOW TROUT

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Abstract—Kidney microsomes from sexually mature male, as opposed to female, rainbow trout displayed an approximately 20-fold higher cytochrome P-450 specific content, NADPH–cytochrome *c* reductase activity, and rates of hydroxylation of lauric acid, testosterone, progesterone and aflatoxin B_1. Little or no sex difference in metabolism was observed with benzo[*a*]pyrene or benzphetamine as substrates. A similar pattern was observed in hepatic microsomes from these fish, but the difference was much less striking (approximately 2-fold higher activity in males). Juvenile trout (both sexes) possessed activities intermediate between mature males and females. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of kidney and liver microsomes of juvenile and sexually mature male and female trout suggested that the striking sex difference in kidney could be due to the high amount of trout P-450 isozyme LM_2 in sexually mature males. Immunoquantitation of LM_2, performed by Western Blotting and immunostaining with rabbit anti-trout LM_2-IgG, confirmed that mature male kidney contained much higher levels of P-450 LM_2 than juvenile or female kidney, or even of liver microsomes of all three groups. The amount of P-450 LM_2 in mature female kidney microsomes was barely detectable. The high amount of LM_2 in male trout kidney is consistent with the high activity of these microsomes towards lauric acid and aflatoxin B_1, which have been shown previously to be preferentially metabolized by trout P-450 LM_2. It is suggested that rainbow trout may serve as an alternative to the rat as an animal model for the study of sex-dependent differences in cytochromes P-450.

The liver microsomal cytochrome P-450-dependent MFO‖ system in fish is active towards numerous xenobiotics [reviewed in Refs. 1–3]. The MFO system in fish is very responsive to induction by the PAH type of inducer, such as 3-MC, but is refractive to the phenobarbital class of inducers [reviewed in Refs. 3 and 4].

The importance of the further study of the properties and regulation of fish cytochromes P-450 can be appreciated as rainbow trout are being utilized as an animal model for AFB_1-induced hepatocarcinogenesis [5]. Formation of the ultimate carcinogenic metabolite, AFB_1-2,3-epoxide, is a cytochrome P-450-dependent reaction [6]. In addition to AFB_1, rainbow trout have been demonstrated recently to develop liver tumors following dietary exposure to BP [7]. These findings could have important environmental implications as recent studies have demonstrated high incidences of hepatic neoplasms in some populations of fish which appear to be correlated to environmental exposure to PAHs [8, 9].

Recent success in the purification of cytochromes P-450 from rainbow trout [10, 11] and the marine teleost, scup [12], has demonstrated the existence of multiple forms. The major P-450 isozyme purified from BNF-induced rainbow trout, LM_{4b}¶, and the major isozyme purified from scup, $P\text{-}450_E$, are both active towards BP and display immunochemical cross-reactivity. In addition to cross-reacting with scup $P\text{-}450_E$, antibody to trout LM_{4b} inhibits liver microsomal BP-hydroxylase activity catalyzed by winter flounder and brook trout.** Immunochemical analysis by Western Blotting has demonstrated that, in liver, trout LM_{4b} is induced many-fold following exposure to BNF or PCBs [13].

A major constitutive P-450 in rainbow trout, LM_2, is active towards lauric acid [14] and is very effective at activating AFB_1 to the carcinogenic and DNA-binding metabolite, AFB_1-2,3-epoxide [15]. Trout P-450 LM_2 is not induced following exposure to BNF

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‖ Abbreviations: MFO, mixed-function oxidase; 3-MC, 3-methylcholanthrene; AFB_1, aflatoxin B_1; BP, benzo[*a*]pyrene; PAH, polycyclic aromatic hydrocarbon; BNF, β-naphthoflavone; PCBs, polychlorinated biphenyls; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; and HPLC, high performance liquid chromatography.

¶ The authors have previously [11, 13] adopted a nomenclature system patterned after that developed for rabbit cytochromes P-450. Trout P-450s are designated LM_{1-4} with the subscript reflecting relative mobility on SDS–PAGE gels. Trout LM_2 has a molecular weight of 54,000 and is a major constitutive P-450. Trout LM_{4b}, molecular weight 58,000, is the major isozyme obtained following induction with BNF. This terminology does not imply any structural or functional similarity between trout P-450 LM_2 and LM_{4b} and rabbit LM_2 and LM_4.

** Personal communication, cited with permission of John Stegeman, Woods Hole Oceanographic Institution.

or PCBs when assayed immunochemically [13]. On the contrary, its activity appears to be repressed as evidenced by lower microsomal activity towards lauric acid [14] and AFB_1 [15].

As in the case with rat [reviewed in Ref. 16], rainbow trout, brook trout and winter flounder exhibit marked sex differences in cytochrome P-450 levels and activities towards certain substrates [17, 18]. In both rat and fish, activity is higher in males. Androgens appear to be stimulatory and estrogens inhibitory [19–21]. In the rat, this sex difference is due to the synthesis of male- and female-specific isozymes of cytochrome P-450, requires an intact hypothalamus-pituitary-liver axis and is neonatally imprinted [reviewed in Ref. 22]. The studies with trout suggest a somewhat different mechanism as an intact pituitary does not appear to be required for the sex difference to be expressed [23].

We now present evidence that sexually mature rainbow trout kidney exhibits a sex difference in cytochrome P-450 specific content and activity towards lauric acid, AFB_1 and sex steroids greater than observed previously in any other animal. Furthermore, we attribute this sex difference to higher levels of a particular form of trout P-450, LM_2, in males compared to females or juveniles.

MATERIALS AND METHODS

Chemicals and equipment. [G-^{3}H]AFB_1 was from Moravek Biochemicals. [7,10-^{14}C]BP, [1-^{14}C]lauric acid, [4-^{14}C]testosterone and [4-^{14}C]progesterone were all obtained from Amersham. The unlabeled compounds, which were used to dilute the above to known specific activities, were all obtained from Sigma. Benzphetamine-HCl was provided by the Upjohn Co. [^{125}I]Protein A was purchased from ICN.

Nitrocellulose, utilized in Western Blotting and the [G-^{3}H]AFB_1–DNA binding experiments, was from Schleicher & Schuell. The apparatus and chemicals used for SDS–PAGE were from Bio-Rad and the equipment for Western Blotting was from the E-C. Apparatus Corp. Salmon sperm DNA, PMSF, cytochrome *c*, isocitrate and human serum albumin were from Sigma. Isocitrate dehydrogenase was obtained from Boehringer Mannheim and NADPH was from P-L Biochemicals.

Trout. Rainbow trout *Salmo gairdneri* were obtained from Hide Away Springs Hatchery of Kewaskum, WI. Sexually immature juvenile trout were 8 months of age and weighed an average of 200 g. Sexually mature trout were over 2 years old, weighed an average of 1200 g, and possessed ripe eggs or milt. All trout were maintained at a water temperature of 10°.

Trout were killed by a blow to the head, and the livers and kidneys were removed immediately and placed in either 0.1 M Tris-acetate, pH 7.5, 0.1 M KCl, 1 mM EDTA, 0.1 mM PMSF for liver, or 10 mM potassium phosphate, pH 7.5, 0.25 M sucrose, 1 mM EDTA, 0.1 mM PMSF for kidney. The tissues were then minced, rinsed, and homogenized with 3 vol. of the respective buffers, and microsomes were prepared by ultracentrifugation.

The microsomal pellets were washed once and resuspended in 0.1 M potassium phosphate, pH 7.25, 30% glycerol, 1 mM EDTA, 1 mM DTT and 0.1 mM PMSF and frozen in aliquots at −80°. Cytochrome P-450 content and [^{14}C]laurate hydroxylase activity were determined with fresh microsomes. All other assays were performed with microsomes that had been stored for 1–2 weeks at −80°.

Enzyme assays. Cytochrome P-450 was determined from the CO-reduced versus CO difference spectrum [24] to eliminate hemoglobin interference. NADPH–cytochrome *c* reductase was assayed at 20° [25]. The hydroxylation of [1-^{14}C]lauric acid was measured by a previously described HPLC technique [14], modified from Orton and Parker [26]. [^{14}C]BP hydroxylase was assayed by the method of DePierre *et al.* [27]. The N-demethylation of benzphetamine was assayed by the formation of formaldehyde with Nash reagent [28]. The NADPH-dependent covalent binding of [G-^{3}H]AFB to DNA was determined as previously described [6, 15]). Metabolite profiles of [^{14}C]testosterone and [^{14}C]progesterone were examined by HPLC, utilizing conditions described previously [20], or by autoradiography following TLC [29]. All assays were performed at 29°.

Other assays. SDS–PAGE was performed by the method of Laemmli [30]. The procedure for Western Blotting was from Burnette [31]. In this procedure, proteins separated by SDS–PAGE were transferred electrophoretically (20 V for 3 hr) to nitrocellulose and immunostained with rabbit anti-trout P-450 LM_2-IgG followed by [^{125}I]Protein A. Visualization and quantitation were performed by autoradiography followed by densitometry.

Rainbow trout cytochromes P-450 LM_2 and LM_{4b} were purified from hepatic microsomes of BNF-induced fish [10, 11]. Rabbits were immunized and IgG fractions prepared as described previously [11]. Protein was assayed by the method of Lowry *et al.* [32] with human serum albumin as standard.

RESULTS

Sex differences in the specific content and activity of the MFO system of rainbow trout kidney microsomes. The specific content of cytochrome P-450 and specific activity of NADPH–cytochrome *c* reductase were approximately 20-fold higher in kidney microsomes of sexually mature male trout compared to female (Table 1). This level of cytochrome P-450 of approximately 1.7 nmoles/mg is, to our knowledge, the highest reported for kidney of any animal and is higher than that reported for hepatic cytochrome P-450 levels in fish.

The specific content of kidney microsomal cytochrome P-450 in sexually immature juvenile trout was intermediate between that of sexually mature males and females. When kidney microsomal activity towards a number of endogenous substrates and xenobiotics was examined, the males displayed higher activity towards lauric acid, AFB_1, progesterone and testosterone (Table 1). The higher activity towards [^{14}C]testosterone was reflected by an increase in the formation of one of the three major polar metabolite peaks observed upon HPLC or on autoradiograms of the TLC plates (data not shown). The identity of this peak was not confirmed but is tentatively designated as 6β-hydroxytesto-

Table 1. Sex differences in trout kidney microsomal MFO components and reactions

Enzyme or reaction	Trout		
	Mature males	Mature females	Juveniles
Cytochrome P-450* (nmoles/mg)	1.74 ± 0.52	0.08 ± 0.04	0.30 ± 0.04
NADPH–cytochrome *c* reductase* (nmoles/min/mg)	172 ± 32	10 ± 4	52 ± 12
[^{14}C]Laurate hydroxylase* (nmoles/min/mg)	16.4 ± 8.5	0.88 ± 0.52	5.14 ± 1.95
[^{14}C]BP hydroxylase† (nmoles/min/mg)	0.028	0.015	0.027
Benzphetamine N-demethylation† (nmoles/min/mg)	0.90	0.90	
[^{3}H]AFB_1–DNA binding† (dpm/40 min/mg)	4522	148	368
[^{14}C]Testosterone† (nmoles/min/mg—total metabolites)	0.351	0.020	0.065
[^{14}C]Progesterone† (nmoles/min/mg—total metabolites)	0.152	ND‡	0.022

Procedures used to assay the P-450 specific content and various catalytic activities are described in Materials and Methods.

* Mean ± S.E.M. for four to eight trout.

† Mean of duplicate pooled samples.

‡ Not detectable.

sterone. Hydroxylation of [^{14}C]progesterone was 7-fold higher with male compared to juvenile trout kidney microsomes. No detectable activity was observed with kidney microsomes from females.

Sex difference in the MFO system of rainbow trout liver microsomes. Liver microsomes from sexually mature rainbow trout displayed a similar sex difference pattern as in kidney, but the difference in activity towards lauric acid, progesterone and testosterone was only about 1.5- to 2-fold (Table 2) as opposed to 20-fold in kidney (Table 1). In contrast to the 1.5- to 2-fold difference between sexually mature male and female trout hepatic microsomal P-450 levels, NADPH–cytochrome *c* reductase, and lauric acid-, testosterone- and progesterone-hydroxylases, covalent AFB_1 metabolite–DNA adduct formation was not detectable in liver microsomes from sexually mature females (Table 2). The activation of AFB_1 to covalent DNA adducts was 3-fold higher with liver microsomes of sexually mature males compared to juvenile trout.

SDS–PAGE of trout liver and kidney microsomes

Table 2. Sex differences in trout liver microsomal MFO components and reactions

Enzyme or reaction	Trout		
	Mature males	Mature females	Juveniles
Cytochrome P-450* (nmoles/mg)	0.90 ± 0.29	0.61 ± 0.19	1.22 ± 0.18
NADPH–cytochrome *c* reductase* (nmoles/min/mg)	77 ± 29	37 ± 19	73 ± 16
[^{14}C]Laurate hydroxylase* (nmoles/min/mg)	1.83 ± 1.31	1.04 ± 1.42	1.08 ± 1.32
[^{14}C]BP hydroxylase† (nmoles/min/mg)	0.020	0.044	0.092
Benzphetamine N-demethylation† (nmoles/min/mg)	0.44	0.38	0.76
[^{3}H]AFB_1–DNA binding† (dpm/40 min/mg)	2826	ND‡	943
[^{14}C]Testosterone† (nmoles/min/mg—total metabolites)	0.173	0.069	0.172
[^{14}C]Progesterone† (nmoles/min/mg—total metabolites)	0.100	0.069	0.172

Procedures used to assay the P-450 specific content and MFO activities are described in Materials and Methods.

* Mean ± S.E.M. for four to eight trout.

† Mean of duplicate pooled samples.

‡ Not detectable.

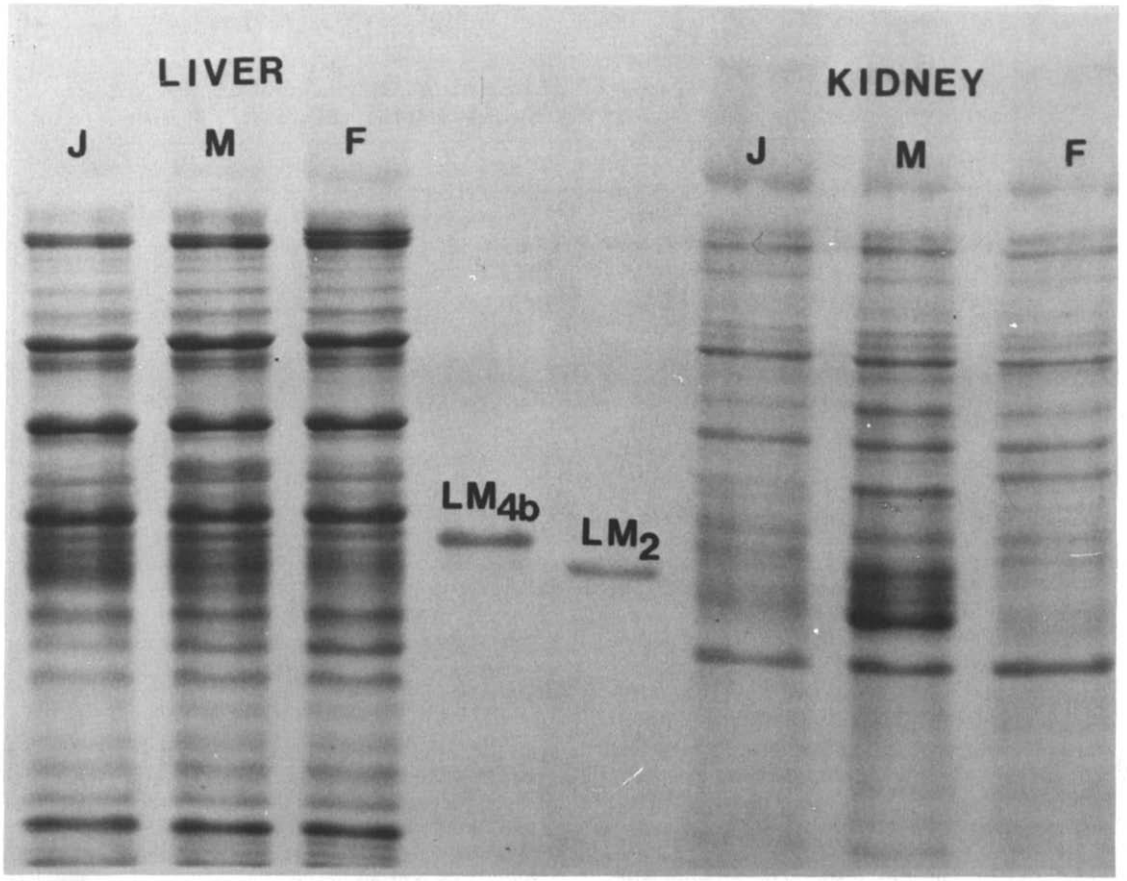

Fig. 1. SDS–PAGE of rainbow trout kidney and liver microsomes and purified trout P-450 LM_2 and LM_{4b}. From left to right, the wells contained pooled trout liver microsomes (40 μg) from juveniles (J), sexually mature males (M) and sexually mature females (F) followed by 10 pmoles of purified trout P-450s LM_{4b} and LM_2 and finally pooled trout kidney microsomes (40 μg) from juveniles (J), sexually mature males (M) and sexually mature females (F). The procedures for sample preparation, electrophoresis, and staining were from Laemmli [30].

and immunochemical quantitation of trout P-450 LM_2. It had been demonstrated previously that trout P-450 LM_2 has high activity towards the activation of AFB_1 to AFB_1-2,3-epoxide [15] which covalently binds to DNA and is the ultimate carcinogen. Trout P-450 LM_2 has little activity towards BP or benzphetamine [11] but appears to function in the (ω-1)-hydroxylation of lauric acid [14]. This substrate

Table 3. Immunoquantitation of trout P-450 LM_2 in kidney and liver microsomes.

Microsomes	Sex	(nmoles LM_2/mg*)	% Total P-450†
Kidney	Juveniles	0.20	67
	Males	0.78	45
	Females	0.03	38
Liver	Juveniles	0.30	25
	Males	0.36	40
	Females	0.22	36

* Immunoquantitation was performed by densitometry following SDS–PAGE, Western Blotting, immunostaining with rabbit IgG against trout LM_2 followed by [^{125}I]Protein A and autoradiography. The conditions for SDS–PAGE and Western Blotting were as described in Materials and Methods and as for Fig. 2. The results shown are for pooled microsomes for each group. The amount of microsomal P-450 LM_2 was calculated utilizing purified LM_2 as standard. The values shown were within the linear range of the LM_2 standard, except for the mature male kidney microsomes, which may have been overloaded. Therefore, the value shown above may be an underestimation.

† Results are given as the percentage of total P-450, determined spectrophotometrically, given in Tables 1 and 2.

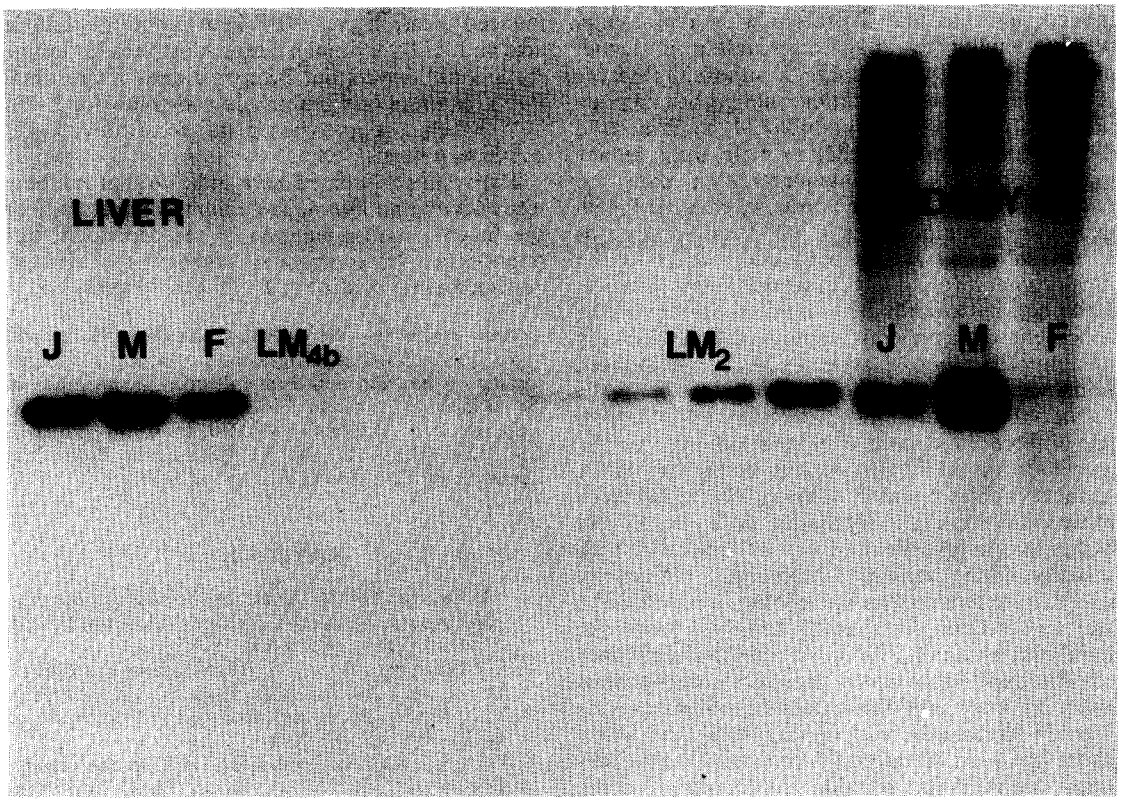

Fig. 2. Western Blotting of rainbow trout kidney and liver microsomes and purified P-450s LM_2 and LM_{4b} and immunostaining for LM_2. SDS–PAGE was performed as described above except that the microsomal protein load was 10 μg. From left to right, the first three samples were liver microsomes (10 μg) from juveniles (J), males (M) and females (F), followed by 1.8 pmoles of purified trout LM_{4b}. The next six wells were purified trout LM_2 at loads of 0, 0.18, 0.36, 0.54, 0.90 and 1.80 pmoles. The last three samples were kidney microsomes (10 μg) from juveniles (J), males (M) and females (F). The proteins were electrophoretically transferred from SDS–PAGE to nitrocellulose (20 V for 3 hr) and stained with antibody and [^{125}I]Protein A as described by Burnette [31]. Autoradiography was performed for 3 hr at −80°.

specificity is consistent with that which distinguishes sexually mature male from female trout, especially with respect to the kidney (Tables 1 and 2).

SDS–PAGE of liver and kidney microsomes from juvenile and sexually mature male and female trout with purified trout P-450 LM_2 and LM_{4b} is shown in Fig. 1. No obvious differences can be seen in liver microsomes from these trout in the molecular weight range of LM_2 or LM_{4b}. In contrast, kidney microsomes from sexually mature male trout demonstrated a band corresponding in molecular weight to P-450 LM_2, whereas this band is either faint or absent in kidney microsomes of juveniles or sexually mature females.

To more accurately assess the relative amount of trout P-450 LM_2 in these microsomes, an SDS–PAGE slab gel containing liver and kidney microsomes from all three groups of trout was analyzed by Western Blotting and immunostaining with rabbit-anti-trout LM_2 IgG (Fig. 2). Immunoquantitation was then performed by densitometry and the results are given in Table 3. These results demonstrate that P-450 LM_2 constitutes 25–40% of the total P-450 in juvenile and adult rainbow trout liver microsomes and 38–67% of the total in the kidney. Furthermore, the 25-fold higher levels of LM_2 in male kidney and 1.5-fold higher levels in male liver microsomes, compared to females, are entirely consistent with the observed differences in laurate hydroxylase activities (Tables 1 and 2). The identity of the higher molecular weight proteins observed in Fig. 2, especially with the kidney microsomes, is unknown.

DISCUSSION

Sexually mature rainbow trout displayed a marked sex difference in the level and activity of the P-450-dependent MFO system. This sex difference was much higher in kidney (20-fold) than in liver (2-fold). Furthermore, this sex difference in MFO activity was specific for the endogenous P-450 substrates lauric acid, progesterone and testosterone and the hepatocarcinogen AFB_1, but was not expressed with either BP or benzphetamine as substrates.

Lauric acid and AFB_1 have been demonstrated LM_2 [14,y to be preferentially metabolized by trout LM_2 [14, 15], a major constitutive form of trout liver microsomal P-450 [13]. SDS–PAGE results did,

indeed, indicate that a protein with a molecular weight, similar to LM_2, was present in higher amounts in kidney microsomes from sexually mature male trout. Western Blotting and quantitation by immunostaining with antibody to LM_2 confirmed the much higher levels of LM_2 in male trout kidney microsomes. The immunoquantitation data are entirely consistent with the proposal that the P-450-dependent sex differences in MFO activity towards certain substrates in liver and kidney are due to the much higher levels of LM_2 in sexually mature males. Little activity towards BP was seen with any trout, consistent with a previous immunoquantitation study demonstrating the low levels of LM_{4b} in non-induced rainbow trout [13].

The MFO activity which demonstrated the largest sex difference, in both liver and kidney, was the activation of AFB_1 to covalent metabolite–DNA adducts (Tables 1 and 2). The activity with kidney microsomes from sexually mature males was 12-fold higher than in juveniles and 30-fold higher than with sexually mature females. Interestingly, the activity with mature female liver microsomes was below the limit of detection even though LM_2 was present in significant amounts (Table 3). Such a discrepancy could be explained if female liver microsomes possess either an enzymatic (glutathione-*S*-transferases or epoxide hydrolase) or a nonenzymatic (an abundance of alternate targets) mechanism(s) that prevents the AFB_1-2,3-epoxide from binding to the exogenous DNA.

The physiological significance of the sex difference in the kidney or liver microsomal MFO system, during sexual maturation in rainbow trout, and the role of sex steroids, is unknown. The major androgen in maturing salmonids is 11-ketotestosterone, presumably derived from 11β-hydroxytestosterone [33]. Therefore, one might expect an increase in 11β-hydroxylation in male trout, yet, in mammals, 11β-hydroxylation is catalyzed by a mitochondrial P-450. Currently, little or no information has appeared describing the properties of steroid-producing cytochromes P-450 in any fish.

In this report, we confirm the previous findings on the sex difference in trout liver microsomal MFO activity [17–21] and provide evidence that this sex difference may be much more significant in kidney. Stegeman and Chevion [17] had reported previously a 1.5- to 2-fold higher P-450 specific content in kidney microsomes from sexually mature male rainbow or brook trout, compared to females, with no significant difference in BP-hydroxylase activities. The P-450 levels (0.024 to 0.086 nmole/mg) and BP-hydroxylase activities (0.001 nmole/mg) in the previous study [17] are much lower than those reported here. However, if the BP-hydroxylase results from these two studies are expressed as turnover numbers (min^{-1}), the values are almost identical (0.014 and 0.016). We have found that the buffer used in the present study, which contains 0.25 M sucrose, 1 mM EDTA and 0.1 mM PMSF, is optimal for recovery of catalytically active P-450 from trout kidney microsomes.

Furthermore, we have shown that BP is a relatively poor substrate for either liver or kidney microsomes from uninduced trout, due to the low constitutive levels of trout LM_{4b} (the major P-450 from BNF-induced trout, active in BP hydroxylation). Lauric acid and AFB_1 are much better substrates for examining constitutive trout liver or kidney microsomal P-450, as these compounds are substrates for P-450 LM_2, which appears to be a major constitutive P-450 in rainbow trout.

The results presented here indicate that rainbow trout may be an alternative animal for the study of the function and regulation of sex-dependent differences in P-450 composition and activity and in the developmental control of P-450 gene expression.

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