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FACTORS RESPONSIBLE FOR SEX DIFFERENCES IN HEPATIC MIXED-FUNCTION OXYGENASE ENZYMES IN BROOK TROUT, SALVELINUS FONTINALIS

by

Ana Marie Pajor

A thesis
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ABSTRACT

The purpose of the experiments described in this thesis was to determine the factors responsible for the sex differences seen in mature fish in components of microsomal electron transfer and mixed function oxygenase (MFO) activities.

The first hypothesis tested was that the gonadal sex steroids, estradiol or testosterone, were responsible for the sex differences in microsomal electron transfer components and mixed function oxygenase activities. Immature male and female brook trout (Salvelinus fontinalis) dosed with estradiol for 10 or 19 days showed a general feminization of components of microsomal electron transport. There were no apparent effects on benzo(a)pyrene or ethoxyresorufin monooxygenase activity, nor any particular change in the influence of 7,8-benzoflavone on benzo(a)pyrene monooxygenase activity.

Dosing of juvenile trout with testosterone for 10 days resulted in masculinization of microsomal electron transport system components and a trend towards an increased activity of benzo(a)pyrene monooxygenase. In contrast, administration of testosterone for 19 days to juvenile trout resulted in feminization of microsomal electron transport components similar to that seen in estradiol-treated juveniles. Pre-

liminary studies showed elevated plasma estrogen levels in those fish. It was concluded that changes in estradiol levels could account for feminization, and testosterone for masculinization, of the MFO system at maturity.

Whereas in rats the action of steroids on the hepatic MFO enzymes is mediated by the pituitary, studies in trout suggest that the action of steroids on hepatic MFO enzymes is independent of the pituitary. To test whether the pituitary of adult fish has any effect on the MFO system, immature fish were injected with extracts from adult salmonid pituitaries. Pituitary extracts from adult salmonids had a feminizing effect on cytochromes P-450 and b5 of immature fish. In contrast, the activities of NADH- and NADPH-cytochrome c reductase were affected by steroids and not by pituitary extracts.

To test whether the pituitary gonadotropic hormone(s) are the feminizing fraction of the pituitary, immature fish were injected with partially purified adult salmon GTH in greater concentration than seen in the pituitary extract of the previous experiment. Partially purified salmon GTH had no effects on hepatic components of microsomal electron transport in the absence of the gonads. Therefore, the pituitary gonadotropic hormone(s) alone are probably not the feminizing factor of the pituitary extract.

It was concluded that both estradiol and testosterone may play a role in the production of sex differences in compo-

nents of microsomal electron transfer. The effects of steroids on cytochrome P-450 may be mediated by the pituitary. However, the other components of microsomal electron transfer are probably regulated by steroids independently of the pituitary. Finally, the feminizing fraction of the pituitary extract is probably not GTH.

RESUME

Le but des expériences décrites dans cette thèse fut de déterminer les facteurs responsables des différences sexuelles des composantes du système microsomal de transfert d'électrons et de l'activité des oxygénases à fonction multiple (MFO) chez les poissons adultes.

L'hypothèse que les stéroides sexuels des gonades, l'estradiol et le testostérone, sont responsables des différences sexuelles des composantes du système microsomal de transfert d'électrons et de l'activité des oxygénases à fonction multiple, fut la première testée. Une féminisation générale des composantes du système microsomal de transfert d'électrons fut observée chez les individus juvéniles mâles et femelles de l'omble de fontaine (Salvelinus fontinalis) dosés avec de l'estradiol pendant 10 et 19 jours. Il n'y eut pas d'effet visible sur l'activité du benzo(a)pyrène ou de l'éthoxyrésorufin monooxygénase, ni sur l'influence du 7,8-benzoflavone sur l'activité du benzo(a)pyrène monooxygénase.

Le dosage des ombles juvéniles avec le testostérone pendant 10 jours aboutit à la masculinisation des composantes du système microsomal de transport d'électrons et à une tendance vers l'accroissement de l'activité du benzo(a)pyrène

monooxygénase. Par contre, le dosage au testostérone des ombles juvéniles pendant 19 jours aboutit à la féminisation des composantes du système microsomal de transfert d'électrons et à des niveaux accrus des estrogènes dans le plasma. On peut conclure que les changements des niveaux d'estradiol peut expliquer la féminisation, et le testostérone la masculinisation, du système MFO à maturité.

Tandis que chez le rat l'effet des stéroides sur les enzymes MFO hépatiques est régi par la glande pituitaire, des études sur la truite indiquent que l'effet des stéroides sur les enzymes MFO hépatiques est indépendant de la glande pituitaire. Pour tester si la glande pituitaire de poissons adultes a un effet sur le système MFO, des poissons juvéniles furent injectées d'extraits de glande pituitaire provenant de salmonides adultes. Ces extraits eurent un effet féminisant sur les cytochromes P-450 et b5 des poissons juvéniles. En contraste, l'activité des réductases NADH- et NADPH-cytochrome c fut affectée par les stéroides mais pas par les extraits de glande pituitaire.

Pour tester si les hormones gonadotropiques pituitaires (GTH) sont la fraction féminisante de la glande pituitaire, des poissons juvéniles furent injectés de GTH partiellement purifiées provenant de saumons, à des doses plus élevées que celles présentes dans les extraits de glande pituitaire de l'expérience précédente. Le GTH de saumon, partiellement purifié, n'eut aucun effet sur les composantes hépatiques du

système microsomal de transport d'électrons dans l'absence des gonades. Par conséquent, les hormones gonadotropiques pituitaires par elles-mêmes ne sont probablement pas le facteur féminisant de l'extrait de glande pituitaire.

On conclut que l'estradiol et le testostérone peuvent jouer un rôle dans la production de différences sexuelles des composantes du système microsomal de transfert d'électrons. Les effets des stéroides sur le cytochrome P-450 peuvent être régis par la glande pituitaire. Toutefois, les autres composantes du système microsomal de transfert d'électrons sont probablement controlées par les stéroides indépendamment de la glande pituitaire. Finalement, la fraction féminisante de l'extrait de glande pituitaire n'est probablement pas le GTH.

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ABBREVIATIONS

APD	Aminopyrine N-demethylase
ANF	α-naphthoflavone or 7,8-benzoflavone (7,8-BF)
7,8-BF	7,8-benzoflavone or ANF
BNF	β-naphthoflavone or 5,6-benzoflavone (5,6-BF)
BP	Benzo(a)pyrene
CO	carbon monoxide
DMSO	dimethyl sulfoxide
ECOD	Ethoxycoumarin O-deethylase
EDTA	ethylene diaminetetraacetic acid, disodium salt
EROD	Ethoxyresorufin O-deethylase
GSI	gonadosomatic index, ratio of gonad to body weight
GTH	gonadotropic hormone
Gx	gonadectomized
HSI	hepatosomatic index, ratio of liver to body weight
3-MC	3-methylcholanthrene
MFO	mixed-function oxygenase
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form

$Na_2S_2O_4$	sodium dithionite
P-448	form of P-450 induced by PAH with absorbance (reduced-CO) at 448 nm
PAH	polycyclic aromatic hydrocarbons
PB	phenobarbital
PCB	polychlorinated biphenyl
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate
SKF-525A	β-diethyl aminoethyl diphenyl valerate
TRIS	tris(hydroxymethyl) aminomethane

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Chapter I

INTRODUCTION

1.1 INTRODUCTION

It was thought until recently that rats and mice were unique in exhibiting sex-dependent metabolism of drugs and steroid hormones. It has been shown, however, that this phenomenon is more widespread than previously thought and other animals, such as humans (Pfaffenberger and Horning, 1977), tree shrews (Kramer et al., 1979), frogs (Harri, 1980), and fish (Stegeman and Chevion, 1980; Förlin, 1980), can now be included in this group.

The hepatic sex-dependent metabolism of drugs and steroids in rats is determined by changes at maturity in levels and types of circulating hormones (Skett and Gustafsson, 1979; Gustafsson et al., 1980). These may represent a novel hormone system involving the pituitary hormone, feminotropin, the hypothalamic inhibiting factor, feminostatin, and gonadal sex steroids (ibid.). Sex differences in drug and steroid metabolism in mice are also dependent on sex steroids (Noordhoek, 1972) although the possible involvement of other factors has not been investigated. In contrast, the

sex differences in drug and steroid metabolism in fish have only recently been described and the factors responsible for their development have not yet been studied. Since the enzymes in question, the hepatic mixed-funcion oxygenases, are different in mammals and fish, it is possible that the regulation of the sex differences in these enzymes are also quite different.

The primary focus of this thesis, therefore, is the determination of factor(s) involved in the sex differences in hepatic mixed function oxygenases seen in fish at maturity.

1.2 MAMMALIAN MIXED-FUNCTION OXYGENASES

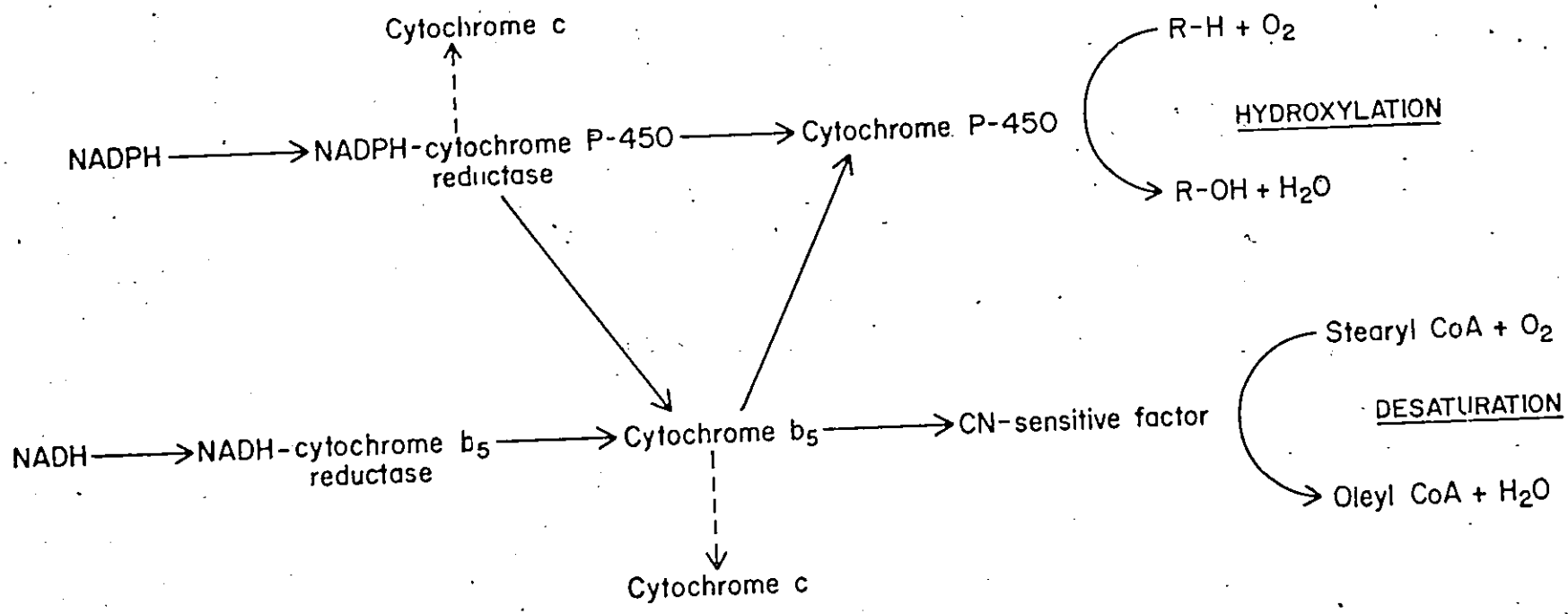
The endoplasmic reticulum of liver cells possesses two interacting electron transport systems, each of which contains a flavoprotein and a cytochrome (outlined in Fig. 1). One of these performs an NADH-dependent desaturation of fatty acids involving cytochrome b5 and cyanide-sensitive desaturase; the other is NADPH-linked and involves cytochrome P-450 in the hydroxylation of steroids and drugs.

The electron transport system associated with cytochrome P-450 is referred to as the mixed-function oxygenase system (MFO; or polysubstrate monooxygenase system, PSM). This system is found in most organisms and in most tissues of the body. One of the more striking properties of the MFO system is the great range of substrates which can be metabolized; from xenobiotics such as drugs and pesticides (Gillette, 1966) to endogenous substrates such as steroid hormones (Gustafsson, 1978). The reactions catalyzed by the MFO system involve insertion of activated oxygen into a favorably positioned bond (for example, C-H, N-H, S-H, or C-X, where X = halogen)(Cooper et al., 1979).

(from Lu, 1976)

Figure 1: Hepatic microsomal electron transport pathways in mammals

Liver microsomal electron transport



The general hydroxylation reaction catalyzed by the MFO is a monooxygenation where one atom of oxygen is used to hydroxylate the substrate and the other is converted to water, as follows:

$$R\text{-}H + 2(NADPH) + O_2 \longrightarrow R\text{-}OH + NADP^{+} + H_2O$$

This reaction is seen in the P-450 mediated metabolism of steroids (Gustafsson and Ingelman-Sundberg, 1976; Hall, 1980) and in oxidations of aliphatic or aromatic hydrocarbons (Cooper et al., 1979). Other reactions catalyzed by the MFO include oxidative N-, S-, and O- dealkylations (Cooper et al., 1979).

The general monooxygenation reaction sequence catalyzed by the MFO system involves the formation of an enzyme-substrate complex between the terminal oxidase, cytochrome P-450 (with its iron in ferric (Fe^{3+}) form), and the substrate. Then an electron is transferred from NADPH via a flavoprotein, NADPH-cytochrome P-450-reductase, to produce a reduced enzyme-substrate complex ($P\text{-}450(Fe^{2+})\text{-}S$), with the transfer of the first electron as the rate-limiting step (Holtzman et al., 1968). A molecule of oxygen is then added to cytochrome P-450 and this is followed by a second electron, which is transferred first to P-450 and then to oxygen, to produce an activated oxygen complex. This activated oxygen is probably in the form of superoxide ion (O_2^{-}) (Paine, 1978). The source of the second electron can be either NADPH (through NADPH-cytochrome P-450-reductase) or

NADH (through a second electron transfer system involving NADH-cytochrome b5-reductase and cytochrome b5). One atom of active oxygen is transferred to the substrate by inserting into the C-H bond and the other forms water, thus regenerating free ferric cytochrome P-450 (reviewed in Ullrich, 1976; Powis and Jansson, 1979). The product of monooxygenation is usually more hydrophilic and can be readily excreted or conjugated and then excreted (Ullrich and Kremers, 1977). In some cases, however, monooxygenation is required for the formation of a metabolically active form of substrate or produces a more toxic form of substrate (Gelboin, 1980).

Studies of purified, reconstituted systems have shown that the components essential for MFO activity are cytochrome P-450, NADPH-cytochrome P-450-reductase, and phospholipid. The lipid does not function directly in monooxygenation, but facilitates the transfer of electrons from the reductase to cytochrome P-450 (Lu and Levin, 1974).

Cytochrome P-450 is a collective name for a distinct group of hemeproteins. There is considerable evidence that cytochrome P-450 exists in multiple forms (for reviews see Ullrich and Kremers, 1977; Lu, 1979; Lu and West, 1979) with different, although overlapping, substrate specificities. The amounts and types of cytochromes P-450 seen can be changed by exposure to chemical inducers such as polycyclic aromatic hydrocarbons (PAH) and phenobarbital (Conney, 1967; Gillette, 1971). Cytochrome P-450 is a b-type cytochrome

containing a single glycoprotein attached to one molecule of protoheme, of minimal molecular weight between 45,000 and 60,000 daltons (Haugen and Coon, 1976). Work with reconstituted systems has shown that the substrate specificity of the monooxygenases centers on cytochrome P-450 (Lu et al., 1972). Cytochrome P-450 has unusual spectral properties; for example, the difference spectrum of reduced CO-bound P-450 has a characteristic absorption maximum at 450 nm (hence the name P-450) unlike those of other heme proteins which show absorption maxima closer to 420 nm (Omura and Sato, 1964; Omura et al., 1965).

NADPH-cytochrome P-450-reductase (NADPH: ferricytochrome oxidoreductase, E.C. 1.6.2.4) is a flavoprotein of minimal molecular weight 68,000 to 80,000 daltons and contains one molecule each of FAD and FMN (Iyanagi and Mason, 1973; Vermilion and Coon, 1974; Digman and Strobel, 1975). It exists in a ratio of 10 to 30 molecules of P-450 to each reductase (Estabrook et al., 1971). It probably does not exist in multiple forms, as P-450 does (Welton and Aust, 1975). The role of NADPH-cytochrome P-450-reductase is in the transfer of electrons from NADPH to cytochrome P-450 (Iyanagi and Mason, 1973). NADPH-cytochrome P-450 reductase also donates electrons to cytochrome b5 or to an unknown carrier to participate in fatty acid desaturation or elongation (Ilan et al., 1981). It may also donate electrons to artificial electron acceptors such as ferricyanide or cytochrome c --

the latter being the basis of a simple assay of NADPH-cytochrome P-450-reductase activity and for this reason it is often known as NADPH-cytochrome c-reductase (Phillips and Langdon, 1962).

The second electron transport chain in liver microsomes consists of a flavoprotein, NADH-cytochrome b5-reductase, and cytochrome b5 . Cytochrome b5, like cytochrome P-450, is a b-type cytochrome. It has a molecular weight of approximately 11,500 (Mathews and Czerwinski, 1976). Cytochrome b5 does not bind CO and its difference spectrum between NADH-reduced and oxidized cytochrome b5 shows an absorption maximum at 424 nm (Omura and Sato, 1964). The main physiological role of cytochrome b5 is to supply reducing equivalents from NADH to various reactions catalyzed by microsome-bound enzymes (Hrycay and Prough, 1974). For example, b5 donates electrons to a terminal oxidase known as CSF or cyanide sensitive factor to participate in fatty acid desaturation (Sasame et al., 1974; Lu et al., 1974; Lu, 1976; Schenkman et al., 1976). Cytochrome b5 can directly reduce cytochrome P-450 (Fujita and Peisach, 1977). Several P-450-dependent enzyme activities require cytochrome b5 and it has been shown that the involvement of cytochrome b5 depends on the substrate and the type of P-450 catalyzing the reaction (Lu et al., 1974b; West and Lu, 1977).

NADH-cytochrome b5-reductase (NADH: ferricytochrome oxidoreductase E.C. 1.6.2.2) is a flavoprotein of molecular

weight approximately 44,000 daltons which, unlike NADPH-cytochrome P-450-reductase, contains only one molecule of FAD per protein (Williams Jr., 1976). The reductase is arranged with cytochrome b5 in assemblies with approximately 5 molecules reductase to 50 molecules b5 in each (Ito, 1974). Its function is to transfer electrons from NADH to cytochrome b5. It can also facilitate the transfer of the first electron to P-450 from NADPH (Penglis et al., 1980). Unlike NADPH-cytochrome c-reductase, NADH-cytochrome c-reductase activity involves two enzymes, NADH-cytochrome b5-reductase and cytochrome b5 (DePierre and Dallner, 1975). Under most conditions, however, the levels of cytochrome b5 are not limiting and the activity of NADH-cytochrome c-reductase reflects the activity of NADH-cytochrome b5-reductase. The levels of cytochrome b5 and activity of NADH-cytochrome b5-reductase can be increased by inducers such as PB (Duvaldestin and Berthelot, 1978).

1.3 MIXED-FUNCTION OXYGENASES OF FISH

The major differences in mixed function oxygenase enzymes between fish and mammals are generally quantitative rather than qualitative. Most activities of MFO enzymes are approximately one tenth the activity seen in rats (Adamson, 1967; Creaven et al., 1967; Dewaide and Henderson, 1968; Walker, 1978) with the possible exception of benzo(a)pyrene hydroxylase activity in trout (Pedersen et al., 1974; Ahokas et al., 1975), scup (Stegeman and Binder,1979), and eels (Nava and Engelhardt, 1982), which is at least ten to fifteen times higher than that of rats. In addition, the temperature optima of rat MFO enzymes are generally between 35 and 40°C while those of fish are between 25 and 30°C (Adamson, 1967; Creaven et al., 1967; Pohl et al., 1974; Elcombe and Lech, 1979; Bend et al., 1977; James et al., 1979; Stegeman et al., 1979; Balk et al., 1980; Lindstrom-Seppa et al., 1981a). Studies by Pohl et al. (1980) with purified, reconstituted little skate and rat systems have identified NADPH-cytochrome P-450 reductase as the heat sensitive factor in the little skate MFO system.

There are, however, some striking qualitative differences in mixed-function oxygenases between fish and mammals. Several studies have shown that fish respond to 3-MC type (polyaromatic hydrocarbon) or mixed-type inducers (for example,

polychlorinated biphenyls) but are refractive to phenobarbital-type (PB) inducers (Buhler and Rasmusson, 1968; Elcombe and Lech, 1979; Balk et al., 1980). Mixed-type inducers in fish generally produce only the characteristics of 3-MC type inducers(Lidman et al., 1976; Melancon et al., 1981) , while in rats they produce changes similar to those seen after exposure to both PB- and 3-MC-type inducers (Snyder and Remmer, 1979).

In mammals, modifiers of MFO activity in vitro such as metyrapone, SKF 525A, and 7,8-benzoflavone (7,8-BF or ANF) are commonly used to distinguish various forms of cytochrome P-450. ANF inhibits MFO activity in 3-MC induced microsomes but activates MFO activity in control or PB-induced microsomes (Wiebel et al., 1971). Metyrapone or SKF 525A inhibit MFO activity in PB-induced microsomes but have no effect on control or 3-MC induced microsomes (Ullrich et al., 1975; Lu and West, 1979). In contrast, several fish species have control microsomes whose BP-hydroxylase activity is strongly inhibited by ANF. These include rainbow trout (Statham et al., 1978 (using 5,6-BF); Elcombe and Lech, 1979), carp (Melancon et al., 1981), and scup (Stegeman and Binder, 1979; Stegeman et al., 1979, 1981). The scup may show inhibition of BP-hydroxylase with ANF because of environmental induction of MFO; this is currently under investigation. However, this character seems to represent control cytochrome P-450 in trout and carp. High levels of control BP-hydroxy-

lase activity in trout (Ahokas et al., 1976) may be a further indication that control cytochromes P-450 in these fish are similar to the 3-MC induced form of P-450 in rats. Some species of marine fish such as croaker (Stegeman et al., 1981), little skate (Bend et al., 1979; Ball et al., 1980), sheepshead (James et al., 1979; Little et al., 1980) and southern flounder (Little et al., 1980) show 7,8-BF stimulation of BP-hydroxylase in control microsomes and inhibition of BP-hydroxylase in microsomes from polycyclic aromatic hydrocarbon treated fish, similar to the situation seen in mammals.

1.4 SEX DIFFERENCES IN MFO IN MAMMALS

In rats, sex dependent differences exist in the metabolism of drugs and steroids with activities of MFO enzymes in males generally being several fold higher than in females. Examples of drug-metabolizing MFO enzymes in this group include aminopyrine N-demethylase (Henderson, 1971; Gielen et al., 1976), aryl hydrocarbon hydroxylase or benzo(a)pyrene hydroxylase (Gurtoo and Parker, 1976), benzphetamine demethylase (Chhabra and Fouts, 1974), biphenyl-4-hydroxylase (Burke et al., 1978), p-chloro-N-methylaniline demethylase (Chhabra and Fouts, 1974), diazepam 3-hydroxylase (Nau and Liddiard, 1980), ethylmorphine N-demethylase (Castro and Gillette, 1967; Davies et al., 1969; El Defrawy el Masry et al., 1974; Nerland and Mannering, 1978), hexobarbital hydroxylase (Kuntzman et al., 1964), and p-nitroanisole demethylase (Gielen et al., 1976). In addition to increased activity, males may show a decrease in the apparent Michaelis constant (Km) for substrates, for example, hexobarbital (Kato and Onoda, 1970) and ethylmorphine (El Defrawy el Masry et al., 1974). One exception to this is diazepam-3-hydroxylase; male rats show increases in both Vmax and apparent Km with maturation (Nau and Liddiard, 1980). Other enzymes show no sex differences in activity, or sex differences only in certain strains of rats. Aniline is metabo-

lized at the same rate by adult male and female rats (El Defrawy el Masry et al., 1974) except in rats of the Wistar strain where males show higher aniline hydroxylase activity (Spence and Schnell, 1979). Some drug-metabolizing enzymes, such as ethoxyresorufin O-deethylase (EROD), are more active in females than males (Burke et al., 1978). Finally, some sex differences seen are qualitative, as in the case of DME (1,2,3,4,9,9-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-6,7- dimethyl-6,7-epoxy-1,4-methanonaphthalene) where immature rats and adult females produce only one metabolite while adult males produce two (Finnen and Hassall, 1980a).

As in the metabolism of drugs, the activities of steroid-metabolizing enzymes are generally higher in males than females (Berg and Gustafsson, 1973; Einarsson et al., 1973) with the exception of 5 α-steroid reductase (Yates et al., 1958; Einarsson et al., 1973) and a 15β-hydroxylase active on steroid sulfates (Gustafsson and Ingelman-Sundberg, 1975) which are higher in females.

The levels of cytochrome P-450 in rats (Davies et al., 1969; El Defrawy el Masry et al., 1974; Gielen et al., 1976) and activities of NADH-cytochrome c reductase (McLeod et al., 1972; Litterst et al., 1977) are higher in males than females. There are no sex differences in the levels of cytochrome b5 (Schenkman et al., 1967). Some studies have reported no sex differences in NADPH-cytochrome c reductase activity (Davies et al., 1969; Litterst et al., 1977), while

others, using the same strains of rats, have shown that NADPH-cytochrome c reductase activities in males are higher than in females (Vodicnik et al., 1981).

It has been suggested that the sex differences in hepatic mixed function oxygenases in rats may also be due to the existence of different populations of cytochromes P-450 in males and females. The extinction coefficients of total cytochromes P-450 in rats are lower in males than females (Stripp et al., 1971). In some cases, blue shifts (to 448 nm; this form is called P-448) were seen in the absorption spectra of reduced CO-bound cytochrome P-450 of females (Kahl et al., 1976). Also, MFO activities of females were greatly inhibited by ANF and slightly inhibited by metyrapone while those of males were stimulated by ANF and greatly inhibited by metyrapone (Wiebel and Gelboin, 1975; Kahl et al., 1976). This suggests that females contain both "P-450" and "P-448" and males contain only "P-450". The studies of Kamataki et al. (1980, 1981) further supported the hypothesis of sex differences in cytochromes P-450 by showing that females contained approximately equal amounts of two purified P-450 forms eluting from Sepharose columns and that males contained predominantly one form. In addition, Gielen et al. (1980) and Pasleau et al. (1981) showed that two distinct forms of steroid 16 -hydroxylase exist in rat liver. Immature rats of both sexes contained both in equal amounts, adult females had predominantly type II which was

most active in the metabolism of testosterone and adult males had type I which was most active in the metabolism of pregnenolone.

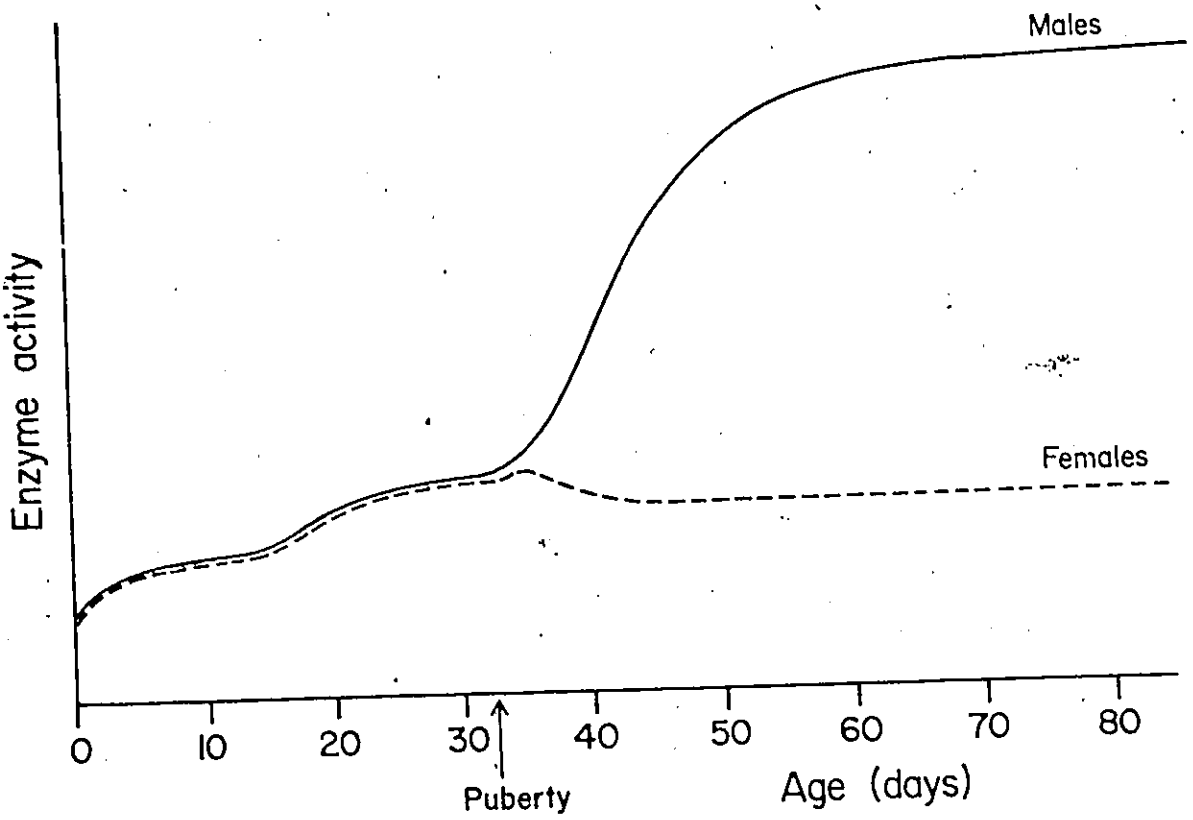
For most of these sex-dependent enzymes, the development of activities and sex-differences follows a general pattern (see Fig. 2). The activities of the enzymes are very low at birth and increase rapidly during the postnatal period but exhibit no sex differences. At puberty (around 30 to 40 days in rats) sex differences begin to appear; female activities decrease or remain at prepubertal levels and male activities continue to increase, finally levelling off at around fifty days of age (Henderson, 1971; Berg and Gustafsson, 1973; Einarsson et al., 1973; El Defrawy el Masry et al., 1974; Nerland and Mannering, 1978; Nau and Liddiard, 1980).

The activities of sex-dependent enzymes require the presence of androgen in males, but not in females. Castration of adult male rats results in a feminization of activities of sex-dependent enzymes (Yates et al., 1958; Kato et al., 1962, 1969; Kato and Onoda, 1970; El Defrawy el Masry and Mannering, 1974; Gurtoo and Parker, 1976; Dieringer et al., 1979; Spence and Schnell, 1979; Finnen and Hassall, 1980).

Ontogenic development of sex dependent MFO enzyme activities in male and female rats.

(from Henderson, 1971; Berg and Gustafsson, 1973; Einarsson et al. 1973; El Defrawy el Masry et al., 1974; Nerland and Mannering, 1978; Nau and Liddiard, 1980)

Figure 2: Activities of sex dependent enzymes in rats


Males
Females
Enzyme activity
0
10
20
30
40
50
60
70
80
Puberty
Age (days)

The subsequent administration of testosterone to these rats restores the original masculine type of metabolism (Kato et al., 1962, 1969; Kato and Onoda, 1970; El Defrawy el Masry and Mannering, 1974; Tabei et al., 1975). The administration of estradiol to intact males feminizes the sex-dependent enzymes in the same way that castration does (Quinn et al, 1958; Yates et al., 1958; Kato et al., 1962, 1969; El Defrawy el Masry and Mannering, 1974; Gustafsson and Ingelman-Sundberg, 1974; Kramer and Colby, 1976; Spence and Schnell, 1979). In contrast, ovariectomy of adult females has no effect on the activity of these enzymes (Kato et al., 1962; El Defrawy el Masry and Mannering, 1974; Gurtoo and Parker, 1976). The sex-dependent enzymes in adult females are relatively insensitive to the administration of steroids (Yates et al., 1958; Dieringer et al., 1979; Finnen and Hassall, 1980) although some have reported masculinization of female drug-metabolizing activities with testosterone (Quinn et al., 1958; El Defrawy el Masry and Mannering, 1974).

Extensive work by several groups has shown that post-pubertal activities of drug and steroid-metabolizing enzymes and responsiveness to androgens in rats are "imprinted" within the first two weeks after birth (Einarsson et al., 1973; Gustafsson and Stenberg, 1974a; Chung, 1977). Rats not exposed neonatally to androgens [i.e. normal females (De Moor and Denef, 1968; Einarsson et al., 1973), neonatally castrated males (Kraulis and Clayton, 1968; Gustafsson and

Stenberg, 1974b) or male pseudohermaphrodites (Einarsson et al., 1972)] develop a feminine pattern of metabolism at puberty and are unresponsive to androgens administered in the adult period. In contrast, rats exposed to androgens within the first twenty-four hours after birth [i.e. females and castrated males dosed with testosterone (Denef and De Moor, 1972; Gustafsson and Stenberg, 1974b; Chung et al., 1975) or normal males (Einarsson et al., 1973)] show a typically masculine pattern of metabolism and have androgen responsive MFO enzymes after puberty. Imprinting of sex-dependent enzymes, although occurring in a similar fashion to imprinting of gonadotropin secretion and sexual behavior, is thought to involve a separate center in the brain since different groups of compounds are active at imprinting each of these. For example, dihydrotestosterone is ineffective at imprinting sex-dependent gonadotropin secretion but will imprint the sex differences in steroid metabolism (reviewed by Gustafsson et al., 1980a).

Mice also show sex-dependent differences in drug and steroid metabolism but the direction of these are opposite to those seen in rats. For example, female mice show higher levels of cytochrome P-450, and NADPH-cytochrome c reductase (Davies et al., 1969), higher ethylmorphine N-demethylase (Castro and Gillette, 1967), hexobarbital hydroxylase (Noordhoek, 1972) and testosterone metabolism (Kuntzman et al., 1964), and lower aniline hydroxylase activity (Noordhoek,

1972) than male mice. While the activities of most sex-dependent enzymes do not differ much between immature mice of both sexes and adult males, those of adult females show much higher enzyme activity, opposite to the situation in rats. However, as in rats, the activities of these enzymes seem to be under androgenic control. Castration of adult mice feminizes male activities but has no effect on females and testosterone treatment of adult females decreases their enzyme activities (Noordhoek, 1972).

1.5 SEX DIFFERENCES IN MFO IN FISH

Sex differences in hepatic mixed-function oxygenase activities have also been described in fish. In some cases, these sex differences have been found to be strain dependent. In two of six strains of rainbow trout studied, females showed higher aniline hydroxylase activity than did males while there were no observed sex differences in any of the other strains (Pedersen et al., 1976). As in rats, most reports of sex differences in fish show that males have higher MFO activities than females. For example, aryl hydrocarbon hydroxylase activities in male cunner are higher than those in females at spawning (Walton et al., 1978); male vendace (Coregonus abula) showed significantly higher activities of ethoxycoumarin O-deethylase than females but no sex differences in benzo(a)pyrene hydroxylase (BP-hydroxylase) (Lindstrom-Seppa et al., 1981a).

Detailed studies of sex differences of MFO in fish were made by Stegeman (1977) and Stegeman and Chevion (1980) with rainbow and brook trout. Male trout showed significantly greater activity than females towards the substrate aminopyrine. The cytochrome P-450 levels in males were also higher than in females. Female trout had significantly higher BP-hydroxylase activities (measured by fluorescence assay) than males. There is evidence that there are different types of

cytochromes P-450 in male and female trout since microsomes from males and females could be distinguished on the basis of the influence of ANF on the activity of benzo(a)pyrene hydroxylase. In rainbow trout, ANF (at a concentration of 5 x 10^{-4} M) inhibited BP-hydroxylase activity in males but in females it activated BP-hydroxylase activity. In brook trout, ANF inhibited BP-hydroxylase activity in both sexes but microsomes of females required greater concentrations of ANF than those of males before inhibition was seen. There were no significant sex differences in NADPH-cytochrome c reductase activity, nor in cytochrome b5 levels. The activities of NADH-cytochrome c reductase were higher in males than in females but it is not known whether the metabolism of aminopyrine and benzo(a)pyrine utilizes NADH, and thus, how this sex difference affects the activity of the MFO system toward these substrates.

Studies by Forlin (1980) on rainbow trout revealed sex differences in cytochrome P-450 levels, and benzo(a)pyrene hydroxylase (measured by radiometric assay) and paranitroanisole O-demethylase activities. In these three characters, immature fish and adult females showed consistently low activity while adult males showed higher activities. Hansson and Gustafsson (1981a) have described sex differences in androstenedione metabolism in rainbow trout. As in Forlin's study, the activities of enzymes in males were higher.

There have been very few reports of developmental changes in hepatic monooxygenase activity in fish. It seems, however, that fish are similar to mammals in that they do not show sex differences until maturation. For the enzymes ethoxycoumarin O-deethylase (Koivusaari et al., 1981) and 6β-steroid hydroxylase (Hansson and Gustafsson, 1981a) the activities in males stayed relatively constant with age while they dropped considerably in females undergoing maturation. In the case of BP-hydroxylase (measured by fluorimetric assay)(Koivusaari et al., 1981), the activities in males decreased at spawning time but those in females remained constant. Finally, the activities of steroid 17-reductase (Hansson and Gustafsson, 1981a) seemed to follow the typical rat pattern (see Fig. 2, p. 17) where the activities in males rose considerably at maturation while those in females remained similar to those in immature fish. It is not known whether the sex differences seen at spawning time remain or disappear after the spawning season since these studies only reported activities to the end of the first spawning season. However, these studies suggest that the sex differences in trout only appear seasonally, unlike the situation in mammals.

1.6 EXPERIMENTAL APPROACH

The primary aim of this study was to determine the factor or factors involved in the appearance of sex differences in the MFO system seen at maturity in fish. Since changes in circulating sex steroid and pituitary hormone levels and/or types also appear at maturity in fish, it was hypothesized that these were the factors involved.

Brook trout (Salvelinus fontinalis) were chosen as experimental animals because of their availability and well-characterized MFO systems. In addition, most of the studies of sex differences in fish have been done with salmonids, including brook trout. Immature trout were used in this study since the MFO system in these fish had not yet developed sex differences. The approach used was to change the types and levels of circulating hormones in the fish by the administration of exogenous hormones and/or surgery. If the sex differences in these fish were controlled by changes in hormone levels at maturity, it was predicted that immature fish dosed with hormones would show precocious development of MFO enzyme activities, the direction of change depending on the hormone used.

To monitor changes in the hepatic MFO system in response to hormone administration, the quantities or activities of components of microsomal electron transport were measured,

possible changes in types of cytochromes P-450 were studied, and the activity of the entire system toward various substrates such as benzo(a)pyrene and ethoxyresorufin was examined.

Chapter II

EFFECTS OF ESTRADIOL AND TESTOSTERONE ON MFO IN IMMATURE TROUT

2.1 INTRODUCTION

Sex differences in hepatic monooxygenases in trout are absent until gonadal maturation (Stegeman and Chevion, 1980). At this time, differences appear between males and females in types and in relative proportions of circulating sex steroids (Campbell et al., 1980). At spawning time these levels may be increased ten-fold (Whitehead et al., 1978). In rats, the sex differences in hepatic monooxygenases appear at puberty and are regulated by the levels and types of circulating steroids and also by the pituitary which mediates the influence of steroids on the liver (Kramer et al., 1979b; Skett and Gustafsson, 1979). It is thus possible that circulating sex steroids may in some way contribute to the sex differences in hepatic P-450 in fish.

This chapter describes the results of an experiment in which immature brook trout, Salvelinus fontinalis, were

treated with one of two sex steroids of teleosts, estradiol-17β or testosterone, and characteristics of hepatic microsomal electron transport components and monooxygenase activity were monitored.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

Estradiol-17β (1,3,5,(10)-estratriene-3,17β-diol) and testosterone were obtained from Research Plus Steroid Laboratories, Denville, N.J.. Polyethylene glycol was purchased from Sigma Chemical Co., St. Louis, MO. Ethoxyresorufin was synthesized by A.V. Klotz (W.H.O.I./M.I.T.) according to the methods of Prough et al. (1977). Resorufin was purified from commercial material obtained from MCB Chemical Co.. Both compounds were judged pure by thin layer chromatography and NMR spectroscopy (by A.V. Klotz).

2.2.2 Methods

Immature brook trout were obtained from the Sandwich Fish Hatchery, Massachussetts 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 x 0.3 x 0.15 m.) located in an adjacent hatchhouse. The troughs were supplied with flowing water at 10°C 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 0700 and 1800 hours. Fish were fed 1-2% body weight once a day with Rangin's Production pellets (Zeigler Bros, Gardners, PA). All dosing and sampling of fish was conducted at the hatchery.

External sex determination of the fish in this chapter was made by L. Raymond (manager of the hatchery). Immature male and female trout can be distinguished on the basis of differences in the shape of the head and body. The sex of the fish was verified at the end of the experiment by visual examination of the gonads. The guidelines used in the identification of gonads were Vladykov, 1956 and Henderson, 1963. The ovaries in the immature fish used in this thesis had a yellow-orange colour, were opaque, and appeared granular. When crushed under a cover slip and viewed under a microscope, tiny ova could be observed. The testes in the immature fish here had a pale yellowish colour, were more transparent than the ovaries, and did not appear granular. When crushed under a cover slip and viewed under a microscope, the lobules of the testes were observed. For the rest of the experiments outlined in this thesis, fish were operated on before the start of dosing and the sexes were determined then, as well as after the end of the experiment in sham-operated fish, by visual examination of the gonads as outlined above.

Fish were injected intramuscularly once every three days with either estradiol-17β (3 mg/kg fish) or testosterone (3

mg/kg fish) in 0.1 ml polyethylene glycol (PEG). Control animals received only PEG. The three groups were dosed on consecutive days so that all the fish could be injected, and later sampled, within thirty minutes at the same time of day. On the tenth day of experimentation (three days after the third injection), blood was sampled from the caudal vein with a heparinized syringe, placed on ice, and the fish killed by cervical section. Livers were immediately excised, placed in ice-cold 0.1 M phosphate buffer pH 7.3 with 1.15% KCl and 3 mM $MgCl_2$. Samples were then transferred on ice from the hatchery to the laboratory for analysis (transit time approximately one hour). Blood was centrifuged at 1500 x g for ten minutes and plasma was frozen at -20°C. Livers were blotted dry, weighed, and then homogenized in 5 vol buffer using a Potter-Elvehjem tissue grinder. Microsomes were prepared by differential centrifugation as described by Stegeman and Binder (1979). The homogenate was first centrifuged at 800 x g for 10 minutes, followed by 10 minutes at 10,000 x g to sediment cell debris and mitochondria. The resulting supernatant was transferred to another tube and spun at 40,000 x g for 90 minutes to sediment the microsomes. The supernatant from this spin was removed and the microsomal pellet

diluted to approximately 3-8 mg protein/ml in a resuspension buffer composed of 50 mM Tris pH 7.5 10 mM β-mercaptoethanol and 20% glycerol. Aliquots were immediately frozen and stored in liquid nitrogen.

Cytochromes P-450 and b5 were analyzed as outlined by Stegeman and Binder (1979) with a Cary 118-C recording spectrophotometer. Cuvettes contained approximately 1 mg/ml microsomal protein in resuspension buffer. Amounts of cytochrome b5 were measured by reducing the contents of the sample cuvette with 0.034 mg/ml NADH and recording the difference spectrum. To measure amounts of cytochrome P-450 both cuvettes contained 0.034 mg/ml NADH (to balance cytochrome b5 and eliminate interference at 424 nm), both were bubbled with CO for one minute (to balance hemoglobin (Johannesen and DePierre, 1975), since reduced CO-bound hemoglobin has absorption maximum at 420 nm) and a few grains of $Na_2S_2O_4$ (sodium dithionite) were added to the sample cuvette to reduce cytochrome P-450. This method also allows for observation of possible mitochondrial contamination since cytochrome a_3 of cytochrome oxidase, when reduced and bound with CO, has an absorbance maximum at 430 nm and a minimum at 445 nm (Vanneste, 1966). The extinction coefficients used to calculate cytochrome concentration were taken from Omura and Sato (1964) and were 91 $mM^{-1}cm^{-1}$ for P-450 and 185 $mM^{-1}cm^{-1}$ for b5.

NADPH-cytochrome c reductase activity was assayed at 25°C by a modification (Binder and Stegeman, 1979) of the method of Phillips and Langdon (1962) with a reaction mixture containing 0.175mM NADPH and 80μM horse heart cytochrome c in 0.2M potassium phosphate buffer pH 7.7. Microsomal NADH-cy-

tochrome c reductase activity was assayed using the conditions for NADPH-cytochrome c reductase, with 0.25mM NADH replacing 0.175mM NADPH. Reduction of cytochrome c was followed at 550 nm.

Benzo(a)pyrene (BP) monooxygenase activity was assayed by a modification (Binder and Stegeman, 1979) of the radiometric procedure of Van Cantfort et al. (1977). The reaction mixture consisted of 0.05 M Tris-HCl with 1.4 mg/ml bovine serum albumin, pH 7.7, 0.40 mM NADPH (not in blanks), 69.8 μM ^{3}H-BP (approximately 150 μCi/umole) and about 60 μg of microsomes in a volume of 50 μl. Assays were done in triplicate and under red light to prevent photooxidation of the ^{3}H-BP. The reaction was initiated with 2 μl ^{3}H-BP in methanol, after which the samples were incubated 15 minutes at 29°C. The reaction was stopped with 200 μl 0.15 M KOH in 85% DMSO. Remaining substrate was extracted three times with 0.5 ml hexane. A 100 μl sample of the aqueous layer containing the metabolites was added to 3 ml Aquasol (New England Nuclear), acidified with 20 μl 0.6 N HCl and counted in a Beckman LS-100C Liquid Scintillation Counter. Counting efficiency was determined with internal standards. 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 the addition of ^{3}H-BP.

Ethoxyresorufin-O-deethylase (EROD) was measured with an assay developed by Klotz and Stegeman (pers. comm.), using a reaction mixture containing 2 µM 7-ethoxyresorufin, 0.1 M Tris pH 8.0 with 0.1 M NaCl, and 20 µl microsomes (3-8 mg protein/ml). The reaction was initiated with NADPH at a final concentration of 0.5 mM and the appearance of resorufin was monitored using a Cary 118-C recording spectrophotometer. The extinction coefficient of resorufin was determined by A.V. Klotz (pers. comm.) to be 73 $mM^{-1}cm^{-1}$.

Plasma concentrations of total estrogens, testosterone, and 11-ketotestosterone were measured by radioimmunoassay by P. Thomas (Texas A & M), as described in Stegeman et al., 1982.

Protein was determined according to the method of Lowry et al. (1958) with bovine serum albumin as a standard.

The gonado- and hepatosomatic indices (GSI and HSI, respectively) were calculated by dividing gonad or liver weight by body weight and expressing this as a percentage, as follows:

GSI = (gonad weight/ body weight) x 100

HSI = (liver weight/ body weight) x 100

The data were evaluated using a fixed-effect two way analysis of variance for unequal sample sizes (BMD program) by W. Smith (Woods Hole Oceanographic Inst.) to test for significant differences among treatment groups and between sexes. Arcsine transformations were made of the results for hepato-

somatic and gonadosomatic indices prior to statistical analysis as these were percentages (Sokal and Rohlf, 1969). Data testing 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. Differences between group means were considered to be statistically significant if $p \leq 0.05$ and were considered to show a trend if $0.05 \leq p \leq 0.10$.

2.3 RESULTS

Treatment with estradiol produced marked increases in liver to body weight ratios (hepatosomatic indices, HSI) in both males and females (Table 1, p. 39) but HSI values in testosterone-treated fish of either sex did not differ significantly from those of control animals. Conversely, the influence of hormone treatment was sex specific on the ratio of gonad to body weight (gonadosomatic index, GSI) (Table 1, p. 39). In both estradiol and testosterone-treated males the GSI values were lower than in control males. The GSI of females did not change significantly with hormone treatment. There were no significant changes in body weight (Table 1) as a result of hormone treatment, and the body colouring that is characteristic at spawning in brook trout was not seen in steroid-dosed fish.

Treatment with either steroid resulted in a significant decrease in hepatic microsomal protein content in both sexes, the reasons for which are unknown. Reduced, CO-bound hepatic microsomal P-450 had an absorption maximum at 450 nm in all fish regardless of treatment or sex. The specific content (expressed on the basis of protein concentration) of hepatic P-450 (Table 1, p. 39) was increased by testosterone in both males and females. Treatment with estradiol, on the other hand, produced a significant decrease in the specific

content of hepatic P-450 in both sexes. Although the magnitude and direction of response to treatment with either steroid were the same in both males and females, overall the specific content of P-450 in females was significantly lower than that in males (Table 1, p. 39). The difference in specific content of P-450 between males treated with testosterone and females treated with estradiol is about the same (nearly 60 percent) as the difference in this character between gonadally mature male and female brook trout (Stegeman and Chevion, 1980). P-450 levels normalized to liver weight were significantly lower in the estradiol-treated fish than in other groups, a function of the liver hypertrophy in these animals.

Hepatic microsomal cytochrome b5 levels were in all cases considerably lower than those previously seen in brook trout (Stegeman and Chevion, 1980). Nevertheless, the levels were significantly lower in hepatic microsomes of treated than in those of control fish. In many of the experimental animals the levels were so low as to be undetectable. In contrast, the activities of NADPH- and NADH-cytochrome c reductase (Table 1, p. 39) were very much like those previously seen (Stegeman and Chevion, 1980). Both NADPH- and NADH-cytochrome c reductase activities were elevated by treatment with testosterone, and there were no significant differences between the sexes within any treatment group.

The activities of hepatic microsomal BP-monooxygenase in the various groups ranged from about 50 to more than 100 pmoles/min/mg. These values, while still quite low, are substantially higher than those obtained previously with the same genetic stock (Stegeman and Chevion, 1980) but the earlier data were obtained by measurement of fluorescence of phenolic derivatives, which typically represent only thirty to forty percent of the total BP-metabolites produced by fish liver microsomes (Stegeman, 1981). There was no clear pattern of effect of steroid treatment on hepatic BP-monooxygenase activity expressed per mg microsomal protein, although there was a clear suggestion that this activity was higher in testosterone-treated males than in the estradiol-treated females. There were no significant effects of treatment on the sensitivity of BP-monooxygenase to inhibiton by 7,8-BF (Table 2, p. 40), nor were there any consistent differences between the sexes within the various treatment groups. 7,8-BF inhibited BP metabolism significantly only at the highest concentration; otherwise, it had no effect. BP-monooxygenase activity normalized to P-450, or to liver weight or body weight (not shown) was very similar in all groups. The levels of ER-O-deethylase activity were below the limits of detection (0.02 nmoles/min/mg) in all animals.

Plasma concentrations of steroid hormones are shown in Table 3 (p. 41). Both testosterone and total estrogen lev-

els were elevated in fish dosed with estradiol. Plasma steroid levels in testosterone-dosed fish were similar to those of controls. Preliminary results showed that there were probably no significant differences between dosing groups in levels of 11-ketotestosterone.

TABLE 1

MFO activity in immature trout dosed with steroids

Levels and activities of hepatic microsomal electron transport components, mixed-function oxidase enzymes, and other characters in control (C), estradiol (E) and testosterone-dosed (T) immature trout of both sexes.

Sample size is shown in parentheses.
Values shown are means ± standard deviation.

* significantly different from controls ($p \leq .05$)

Character	Control		Estradiol		Testosterone	
	Female (7)	Male (5)	Female (5)	Male (7)	Female (6)	Male (5)
Body wt. (g)	77.1 ± 14.5	76.3 ± 12.7	76.4 ± 13.4	80.3 ± 17.7	73.8 ± 16.2	91.7 ± 21.0
HSI (%)	1.4 ± 0.2	1.2 ± 0.1	2.3 ± 0.3*	1.9 ± 0.2*	1.2 ± 0.2	1.2 ± 0.3
GSI (%)	0.56 ± 0.09	0.28 ± 0.15	0.61 ± 0.21	0.10 ± 0.04*	0.73 ± 0.12	0.12 ± 0.04*
Microsomal protein (mg/g liver)	19.4 ± 2.5	19.2 ± 7.0	13.8 ± 4.2*	12.7 ± 3.6*	11.1 ± 2.2*	12.0 ± 1.5*
NADPH-cytochrome c red. (nmoles/min/mg prot)	43 ± 17	55 ± 24	46 ± 15	43 ± 14	66 ± 19*	73 ± 38*
NADH-cytochrome c red. (nmoles/min/mg prot.)	142 ± 48	145 ± 39	96 ± 29	132 ± 50	215 ± 80*	232 ± 74*
Cytochrome b_5 (pmoles/mg protein)	13 ± 7	12 ± 12	5 ± 4*	3 ± 5*	5 ± 9*	2 ± 5*
Cytochrome P-450 (nmoles/mg protein)	0.15 ± 0.04	0.18 ± 0.06	0.11 ± 0.02*	0.15 ± 0.05*	0.21 ± 0.03*	0.25 ± 0.10*
Cytochrome P-450 (nmoles/g liver)	2.9 ± 0.9	3.3 ± 0.8	1.5 ± 0.4*	1.8 ± 0.4*	2.3 ± 0.4	3.0 ± 1.2
Cytochrome P-450 (nmoles/g body)	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
BP-monooxygenase (pmoles/min/mg prot.)	63 ± 20	56 ± 18	49 ± 24	61 ± 26	60 ± 21	125 ± 79*

TABLE 2

Influence of 7,8-BF on BP-monooxygenase activity

The percent activity of benzo(a)pyrene monoxygenase remaining after in vitro addition of 7,8-benzoflavone to hepatic microsomal preparations from control (C), estradiol (E) and testosterone-dosed (T) immature trout.
Sample size is shown in parentheses.
Values shown are means ± standard deviation.

No significant differences in effects of 7,8-BF on the activity of BP-hydroxylase after dosing with estradiol or testosterone.

7,8-BF (M)	Percent activity remaining with:					
	Control		Estradiol		Testosterone	
	Female (7)	Male (5)	Female (5)	Male (7)	Female (6)	Male (5)
0	100	100	100	100	100	100
1.25×10^{-7}	94 ± 10	87 ± 9	100 ± 18	80 ± 7	92 ± 8	87 ± 13
5.0×10^{-6}	95 ± 18	125 ± 24	116 ± 36	94 ± 17	140 ± 41	94 ± 25
1.0×10^{-4}	49 ± 9	49 ± 25	66 ± 24	53 ± 20	52 ± 14	43 ± 14

TABLE 3

Plasma steroid hormone levels in steroid dosed trout

Plasma levels of total estrogens, testosterone, and 11-keto-testosterone in control, estradiol and testosterone-dosed immature trout.
Values shown are means ± standard deviation.
11-ketotestosterone values are preliminary results and represent single determinations of pooled samples.

* significantly different from controls ($p \leq .05$)

Plasma Steroid Levels (ng/ml)	TREATMENT GROUP					
	CONTROL		ESTRADIOL		TESTOSTERONE	
	Female (7)	Male (5)	Female (5)	Male (7)	Female (6)	Male (5)
Total Estrogens	0.8 ± 0.4	0.5 ± 0.2	33.9 ± 17.8*	12.8 ± 5.0*	0.4 ± 0.2	0.3 ± 0.2
Testosterone	4.5 ± 2.9	3.0 ± 1.4	20.3 ± 27.5*	25.6 ± 32.2*	3.8 ± 1.4	3.0 ± 1.1
11-keto-Testosterone	1.6		1.0		0.06	

2.4 DISCUSSION

The data presented here establish that sex steroids can modify the levels of hepatic P-450 in brook trout and that in juveniles the response to a given steroid is similar in males and females. The depression of P-450 levels by estradiol here was less marked than was the increase produced by testosterone. Hansson et al. (1980b) recently demonstrated that estradiol-17β also depressed the levels of hepatic P-450 in juvenile rainbow trout. The data thus indicate that estrogens as well as androgens could elicit the sex differences observed in P-450 in the liver of naturally maturing salmonids (appendix; Stegeman and Chevion, 1980). It should be noted that while testosterone in mammals is the major androgen and is generally regarded as being more active in producing sex differences in hepatic P-450 (Kato, 1974), in mature males of salmonids the major androgen is 11-ketotestosterone (Campbell et al., 1980). Thus the effects of androgen seen here might be the result of metabolites of testosterone rather than the administered hormone per se, although this remains to be tested. Hansson (1982) found 11-ketotestosterone to be more effective than testosterone in producing changes in cytochrome P-450 levels and in androstenedione metabolism in immature rainbow trout.

It is noteworthy that the influence of estradiol on hepatic microsomal proteins was quite explicit and revealed an SDS-PAGE profile very similar to that seen in naturally maturing females (data reported in Stegeman et al., 1982). The estimated molecular weight (56,000) of the band nearly eliminated by estradiol treatment is within the range previously observed for P-450 and the band shows heme-associated peroxidase activity. The loss of some protein in the 56,000 M.W. band could be associated with the decline in P-450 levels in estradiol-treated animals, but this remains to be confirmed.

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 (Stegeman and Chevion, 1980). The reasons for this discrepancy are not apparent. It is interesting, however, that the sex difference in naturally maturing trout parallels that in rats, in which males posses higher levels of NADH- but not NADPH-cytochrome c reductase activity (McLeod et al., 1972; Litterst et al., 1977).

Cytochrome b5 levels were markedly decreased by all steroid treatments in this study yet mature trout show no sex differences in this character (appendix; Stegeman and Chevion, 1980). Kato et al. (1968) reported no sex differences in cytochrome b5 in adult rats. Furthermore, this enzyme

in rats seems to be independent of steroid regulation since adrenalectomy and castration of adult male rats decreased cytochrome P-450 levels and activities of hepatic monooxygenases but did not affect cytochrome b5 (Ernster and Orrenius, 1973).

A consideration of dosages is quite pertinent to interpretation of studies of steroid effects in fish. The doses employed here were selected on the basis of studies of the effectiveness of similar doses of steroids in eliciting changes in thyroid function (Hunt and Eales, 1979), vitellogenin synthesis (Olivereau and Olivereau, 1979; Idler and Campbell, 1980), gonadotropic cell differentiation (Olivereau and Olivereau, 1979), and hypercalcemia (Olivereau and Olivereau, 1979; Pang and Balbotin, 1978), in various fish species. It is well established that estradiol mediates the hepatic synthesis of vitellogenin, which is reflected in increased liver weight. The increase in liver weight in estradiol-treated fish in the present study clearly indicates that an effective dose was achieved. The effects of steroids on gonadal status in fish has been found in previous studies to vary with doses and duration of treatment (Lofts et al., 1966). Thus, the apparent regression of testes seen here upon treatment with either estradiol or testosterone need not be surprising and has in fact been seen before in salmonid fish given modest doses of steroids (Yu et al., 1979).

The plasma samples from the fish in this section were taken three days after the final dose. Therefore, the low steroid hormone levels in testosterone-treated fish were probably a result of metabolism of the administered dose over the three days. In contrast, estrogen and testosterone levels were elevated in estradiol-dosed fish. This is probably due to the decreased rate of steroid metabolism seen in these fish (results reported in Stegeman et al., 1982). The levels of total estrogens in estradiol-dosed fish are in the range seen at spawning in rainbow trout (Lambert et al., 1978).

The lack of a steroid effect on estimated turnover number of BP in the fish here was somewhat unexpected in the light of the sex difference in this character in mature adults (Stegeman and Chevion, 1980). The distinction could be related to the differences in number and type of metabolites detected in the two studies. However, Forlin (1980) found sex differences in BP-monooxygenase activity in adult rainbow trout using the same radiometric method used here. Further, the direction of the sex difference found in Forlin's (1980) was opposite to that seen in the study of Stegeman and Chevion (1980). Thus, the trend seen here in male trout dosed with testosterone to show an increased activity of BP-monooxygenase may indicate that steroids can modify monooxygenase activities although the length of dosing period or type of androgen used may have been inadequate. The lack of

a clear steroid effect on the sensitivity of BP-monooxygenase to 7,8-BF was also unexpected. In trout species (Stegeman and Chevion, 1980) and in mammals (Wiebel and Gelboin, 1979) there are sex differences in sensitivity of BP metabolism to 7,8-BF, although in opposite directions. In any event, the 40 to 50 percent inhibition of BP monooxygenase activity in these fish by 10^{-4} M 7,8-BF is like that seen in some other trout (Statham et al., 1979; Stegeman and Chevion, 1980). The extent of this inhibition should not necessarily be interpreted as indicating that the animals had been inadvertently exposed to and partially induced by 3-methylcholanthrene (3-MC) type inducers since this inhibition is characteristic of uninduced P-450 in some fish, including trout (Introduction; Stegeman, 1981; Stegeman and Chevion, 1980). Moreover, the levels of ER-O-deethylase, which is catalyzed preferentially by P-450 induced by 3-MC-type inducers (Burke and Mayer, 1974), were comparable to those seen in untreated or control rainbow trout (Elcombe and Lech, 1979; Stegeman et al., 1981) or rats (Burke et al., 1978).

It is quite likely that the effect of steroids on hepatic P-450 in fish involves the regulation of forms that function 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 (Thomas et

al., 1981), including one that has a high specificity for 7α-hydroxylation of testosterone. While much less is known about the multiplicity and functions of P-450 in fish, Hansson et al. (1980) did find that hepatic 6β-hydroxylation of androstenedione was depressed in estradiol treated rainbow trout, but that 16α-hydroxylation was not, suggesting a differential regulation of steroid hydroxylating P-450s in fish liver. Further, Stegeman et al., 1982, using the samples of this experiment, found that 6β-hydroxylase activity and turnover number with testosterone as substrate was depressed by 50% in estradiol-treated brook trout, while 16α- and 16β-hydroxylation was not affected by steroid treatment. It is tempting to speculate that a distinction in catalytic activity like that seen in trout may be associated with the loss of the 56,000 molecular weight protein (putative P-450) in brook trout (data shown in Stegeman et al., 1982).

There are apparently endogenous factors that modify not only the influence of steroids on hepatic P-450, but also the response to foreign compound inducers. P-450a (testosterone 7α-hydroxylase) is induced by 3-MC and PB in immature male and female and adult female rats, but not in adult males (Thomas et al., 1981) and the induction of P-450b (benzphetamine demethylase) was mitigated by maturation in females, but not in males (Thomas et al., 1981). It has been shown that sexual and maturational differences can markedly influence the induction of P-450 in fish (Hansson

et al., 1980a; Forlin, 1980). 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.

Chapter III

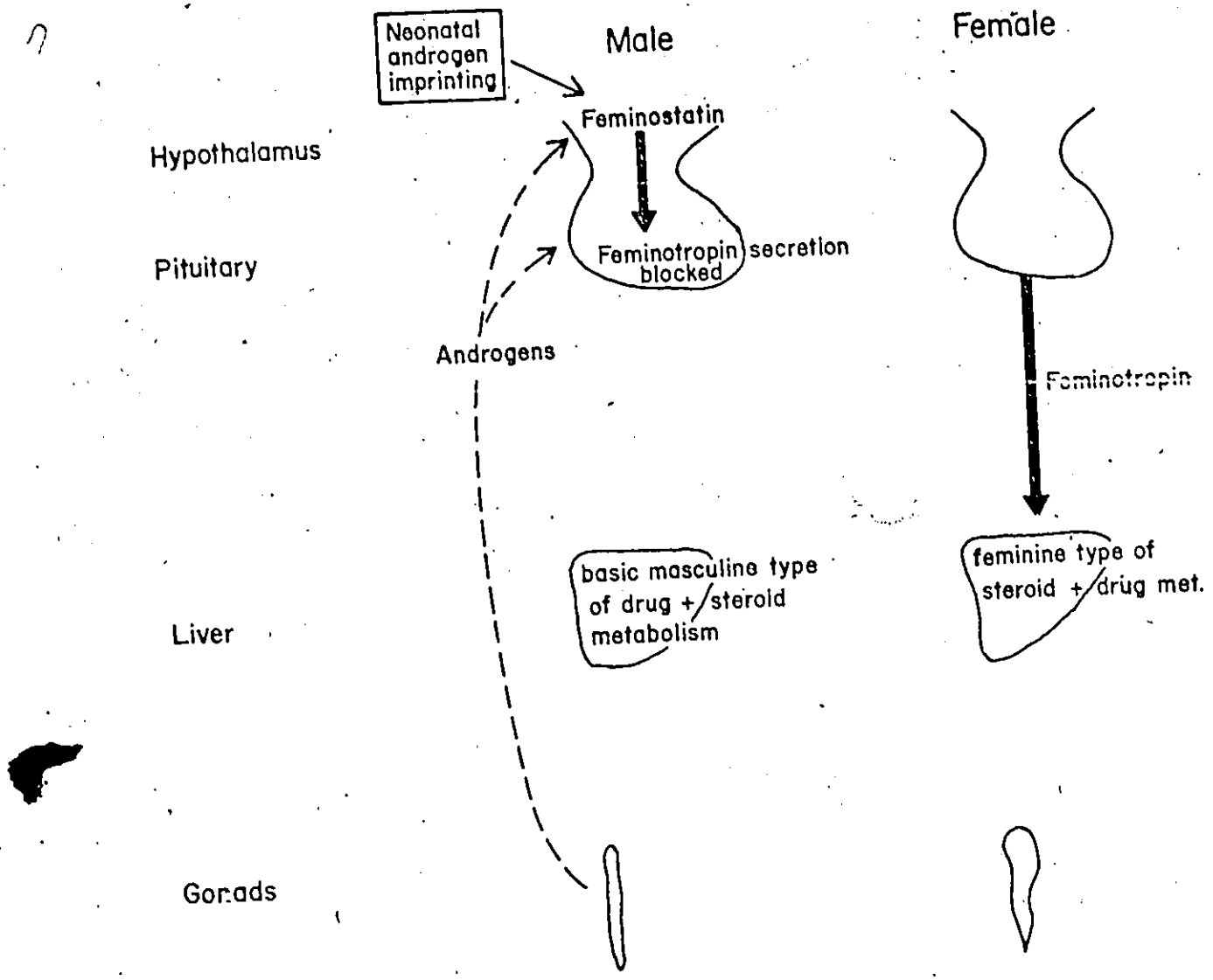
THE EFFECTS OF PITUITARY HORMONES AND STEROIDS

3.1 INTRODUCTION

The present theory of regulation of sex differences in hepatic drug and steroid metabolism in rats has been advanced by Gustafsson and co-workers (for reviews see Skett and Gustafsson, 1979; Gustafsson et al., 1980a)(summarized in Fig. 6). It centers around a pituitary feminizing factor or hormone, which they call feminotropin. In normal females, feminotropin is released from the pituitary after puberty and maintains a feminine pattern of MFO enzyme activities. In normal males, however, a hypothalamic inhibiting factor, called feminostatin, is released by the action of androgens on the brain. Feminostatin inhibits the release of feminotropin from the pituitary and thus a typically masculine pattern of MFO activities is seen. The steroid sensitive center in males also responds to estrogens which somehow prevent the release of feminotropin from the hypothalamus. The production of feminotropin is neonatally imprinted in male rats by exposure to androgens (see Introduction),

The hypothalamo-pituitary-liver axis in normal adult male and female rats (from Skett and Gustafsson, 1979; Gustafsson et al., 1980).

Figure 3: Pituitary and hypothalamic control of MFO sex differences


Neonatal androgen imprinting
Male
Female
Hypothalamus
Feminostatin
Pituitary
Feminotropin secretion blocked
Androgens
Feminotropin
basic masculine type of drug + steroid metabolism
feminine type of steroid + drug met.
Liver
Gonads

Support for this theory comes from several sources. In the absence of the pituitary, a masculine type of steroid and drug metabolism and cytochrome P-450 turnover and levels develops (Denef, 1974; Gustafsson and Stenberg, 1974c; Gustafsson and Ingelmann-Sundberg, 1975; Chung, 1977; Gillham et al., 1977; Finnen and Hassall, 1980; Skett et al., 1980). The effects of steroids are not seen in intact adult females or in hypophysectomized males (Colby et al., 1973; Gustafsson and Ingelman-Sundberg, 1975; Kramer et al., 1975, 1979b; Dieringer et al., 1979; Finnen and Hassall, 1980). The destruction of the hypothalamus or of connections between the hypothalamus and pituitary feminizes the MFO activities in adult males but does not affect those of females (Gustafsson et al., 1976; Mode et al., 1980). The implantation of an adult pituitary gland, regardless of the sex of the source, feminizes MFO activities in both adult and juvenile male rats but has no effect on those of females (Denef, 1974; Gustafsson and Stenberg, 1976; Eneroth et al., 1977; Gillham et al., 1977; Burke et al., 1978; Gustafsson and Skett, 1978; Skett and Young, 1980). The secretion of a feminizing factor begins at puberty since implantation of pituitaries from donor rats younger than day 28 had no effect on steroid metabolism in hypophysectomized male rats while pituitaries from donors aged 35 days and older resulted in a feminization of steroid metabolism (Skett et al., 1978).

The exact nature of the feminizing factor is as yet unknown. Gustafsson et al. (1977, 1981) have proposed that it is a new pituitary hormone, feminotropin. Extensive studies in rats have shown that none of the known pituitary hormones, alone or in combination, were as effective as pituitary extract in feminizing steroid metabolism in isolated hepatocytes and hepatoma cell line in culture (Gustafsson et al., 1977). Partially purified feminotropin was isolated from rat pituitary extracts and found to be as effective as whole pituitary extract in feminizing steroid metabolism. Further, it was found to be a single polypeptide of minimal molecular weight 20,000 daltons, stored in pituitary granules of similar density to those which contain prolactin (Gustafsson et al., 1977). The studies of others, such as Rumbaugh and Colby (1980), have suggested that growth hormone (GH) is the feminizing factor. When administered to gonadectomized rats, GH had effects similar to those of estradiol on hepatic MFO activity. Growth hormone also had a feminizing effect in hypophysectomized rats in the presence of thyroxin or ACTH. Other groups have suggested that other pituitary hormones, such as FSH, LH, or prolactin, are the feminizing factor (reviewed by Colby, 1980). It is clear that much work remains to be done to determine the identity of the pituitary feminizing factor.

It is unknown yet whether the same "feminizing" system operates in fish. In a recent study by Hansson et al.,

(1980b) hypophysectomy of juvenile rainbow trout altered but did not eliminate the effect of estradiol on hepatic cytochrome P-450. Hypophysectomy also failed to alter the effects of estradiol on 6B-steroid hydroxylase in rainbow trout, suggesting further that the effects of estradiol on the liver are mainly independent of the pituitary (Hansson and Gustafsson, 1981b). This suggests that sex differences in hepatic monooxygenase activity in fish may not be mediated by the pituitary. However, the possibility of pituitary involvement in this regulation in fish cannot yet be eliminated.

This chapter describes the results of an experiment to determine the extent, if any, of involvement of the pituitary in the sex differences in MFO of brook trout. Immature trout of both sexes were dosed with pituitary extracts from adult salmonids. In order to separate the effects of the pituitary extract on gonadal steroid production and subsequent steroid effects from the effects of the pituitary extract alone, half the fish used in this study were gonadectomized (Gx). In addition, possible interactions between pituitary hormones and sex steroids were examined. Finally, the experiments of the previous chapter were repeated using a longer dosing period to determine possible limits of induction or repression by sex steroids.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Estradiol-17β (1,3,5(10)-estratriene-3,17β-diol) and testosterone were obtained from Sigma Chemical Co., St. Louis, MO. Acetone dried pituitaries from adult Chum salmon (Oncorhynchus keta), containing 10-15 mg GTH/g, were purchased from Syndel Laboratories Ltd., Vancouver, B.C.. Propylene glycol was obtained from Sigma.

3.2.2 Fish

Immature brook trout of approximately 1.5 years of age were obtained from Goossen's Trout Hatchery, Otterville, Ontario in April, 1981, and shipped by air to holding facilities at the University of Ottawa. They were maintained in Living Stream Tanks (Frigid Units Inc., Toledo, Ohio), equipped with flowing dechlorinated city tap water at 10°C at densities of 36 fish per 270 l tank. The photoperiod was maintained 12L:12D with overhead fluorescent lights. Fish were fed daily with Purina Trout Chow pellets at approximately 1-2% body weight. They were acclimated to laboratory conditions at least one month before dosing.

3.2.3 Surgery

Fish were operated upon two weeks after acclimation to lab conditions. Half the fish were gonadectomized and half were sham-operated. The fish were initially anesthetized with buffered MS-222 500 mg/l pH 7.3 until the righting reflex was lost (35-40 seconds). Then the fish were maintained under anaesthesia with cooled, aerated, buffered MS-222 100 mg/l pH 7.3 continuously pumped over the gills with a recirculating pump and cooling system. For gonadectomies, a midventral incision was made and the gonads and associated ducts were removed. For sham operations the incision was made but gonads were not removed. The area of anterior insertion of the gonads was packed with a 2 x 2 mm cube of Gelfoam to prevent bleeding. The broad-spectrum disinfectant, Germiphene (Germiphene Co. Ltd., Brantford, Ont.), was used to disinfect instruments, hands and operating area (diluted 1:100), and as a pre- and post-operative swab (1:200). The incision was closed by continuous suture of the muscular layer with 4-0 chromic gut and discontinuous (mattress type) suture of the skin with 3-0 silk. The total time involved was approximately ten minutes and the fish recovered normal swimming behavior in a further 15 to 20 minutes. After one week the wound had started to close and was completely healed by the time dosing began three weeks later. There were no cases of infected wounds or opening of the sutures. Out of one hundred seventy fish operated on, three died.

3.2.4 Experimental

A preliminary group of thirty sham-operated fish was dosed i.m. in incremental doses with pituitary extract (acetone dried pituitaries) in propylene glycol. The dosing regime was as follows: day (1), 1.5 mg/kg; days (4,7,10), 3.0 mg/kg; days (13,16), 6mg/kg. They were sampled on every dosing day after day 7. This regime is similar to that suggested by Syndel Ltd for spawning adult salmonids although the dose was somewhat higher and given for a longer time (i.e. 19 days vs. 3 days). The group sampled on day 19 had slightly larger gonads than controls so this timing was used for the experiment.

Fish were divided into two sets of six groups; one set sham operated and one set gonadectomized. They were dosed three weeks after surgery with propylene glycol (controls), estradiol (3 mg/kg), testosterone (3 mg/kg), pituitary extract (Pit) (as outlined above) or a combination of pituitary extract and estradiol or pituitary extract and testosterone. The total volume injected into each fish was 0.1 ml. They were injected i.m. once every three days at the same time of day. On the nineteenth day (three days after the last dose) blood was sampled from the caudal vein with a heparinized syringe, placed on ice, and the fish killed by cervical section. Livers were immediately excised, placed in ice-cold 0.1M phosphate buffer pH 7.3 with 1.15% KCl and 3mM $MgCl_2$. Gonads were removed from sham operated fish.

Blood was centrfuged at 1500 x g for ten minutes and plasma was frozen at -30°C for later steroid analysis. The tissues were blotted dry, weighed, and then homogenized in 5 vol buffer with a Potter-Elvehjem tissue grinder. Microsomes were prepared as described by Stegeman and Binder (1979), diluted to approximately 6-10 mg protein/ml with a resuspension buffer (as in Ch. 2, Methods), immediately frozen in several aliquots in liquid nitrogen and stored at -70°C. After 1.5 months, Dewars became available so the samples were transferred to liquid nitrogen.

Ethoxyresorufin-O-deethylase, aniline hydroxylase, and aminopyrine demethylase activities were assayed on an autoanalyzer coupled with a spectrophotometer (Yagminas and Villeneuve, 1981) by A. Yagminas (Health and Welfare Canada).

All other assays have been described in Chapter 2, Methods.

Data were tested for significant differences between dosing groups using a fixed-effect three way analysis of variance (surgical treatment x sex x hormone treatment) for unequal cell sizes and Student Newman Keul's multiple range test (Sokal and Rohlf, 1969). Arcsine transformations were made of the results for hepatosomatic and gonadosomatic indices prior to statistical analysis since they were percentages (Sokal and Rohlf, 1969). Differences between group means were considered to be statistically significant if $p \leq .05$ and were considered to show trends if $.05 \leq p \leq .10$.

3.3 RESULTS

As shown in Fig. 4 (p. 63), female gonadosomatic indices (GSI) increased significantly in those fish dosed with a combination of pituitary extract + estradiol or pituitary extract + testosterone. With the same dosage of pituitary extract or steroids alone in female trout there were no signifant increases in gonad weight. In males the ratio of gonad to body weight increased in groups dosed with pituitary extract alone or in combination with testosterone. Other treatments did not result in a significant change in GSI.

Hepatosomatic indices (HSI) showed a marked increase in fish dosed with estradiol alone or in combination with pituitary extract (Fig. 5, p. 64), in some cases going above 5%. There were no significant differences in HSI between sham operated and gonadectomized fish. There were no apparent sex differences except in the case of sham operated fish dosed with pituitary extract where males had lower HSI than females. The reasons for this sex difference are not known.

Body weights of the fish used in this study are shown in Table 4 (p. 65). As shown in Table 5 (p. 66), microsomal protein levels were elevated in sham-operated fish dosed with estradiol but not in gonadectomized fish dosed with estradiol. Increases in hepatic microsomal protein levels have been reported after dosing with estradiol (Schjeide et

al., 1974) but it is interesting that the increases here were only seen in sham-operated and not in gonadectomized fish. Evidence of mitochondrial contamination of the microsomal preparation was not seen since cytochrome oxidase was not detected.

Reduced CO-bound cytochrome P-450 showed an absorption maximum at 450 nm regardless of treatment. Treatment with estradiol produced a marked decrease in cytochrome P-450 specific content (Fig. 6, p. 67), as seen in the previous chapter. The extremely low P-450 levels in sham-operated estradiol-dosed fish may possibly be a result of high protein levels in those microsomes. Other hormone treatments also resulted in decreases in cytochrome P-450 levels although not as great as those produced by estradiol. However, the administration of pituitary extract together with testosterone to gonadectomized fish had no significant effect on cytochrome P-450 levels. There were no significant differences between sham-operated and gonadectomized fish, with the exception of the estradiol dosed groups, nor between male and female fish. When P-450 levels were normalized to liver weight, the levels in pituitary extract + estradiol and estradiol dosed groups were still significantly lower than the others, while the differences between the controls and the rest largely disappeared (Fig. 7, p. 68). There were no significant differences in P-450 content when normalized to body weight (Fig. 8, p. 69).

Cytochrome b5 reduced by NADH had an absorption maximum at 424 nm and minimum at 411 nm in all cases. Cytochrome b5 levels (Fig. 9, p. 70) showed a pattern similar to that of cytochrome P-450 in that control levels were significantly higher and estradiol and pituitary extract + estradiol dosed levels were significantly lower than those of the other groups. The levels of b5 were undetectable in many fish dosed with estradiol alone (as in the previous experiment) or estradiol in combination with pituitary extract. There were no significant differences between sham-operated and gonadectomized fish and no sex differences in response to a particular hormone.

NADH-cytochrome c reductase activities (Fig. 10, p. 71) were significantly different between several sham-operated and gonadectomized groups. For example, sham-operated controls showed higher activities than gonadectomized controls. This difference was unexpected, particularly when it is considered that the gonads are thought to be inactive in immature fish. Sham-operated estradiol-dosed fish showed lower activities than gonadectomized fish dosed with estradiol. The overall effect of hormone treatment in sham-operated fish was a decrease in activity of NADH-cytochrome c reductase. There seemed to be no effect of hormone treatment in gonadectomized fish compared with gonadectomized controls, except for the groups dosed with testosterone or pituitary extract + estradiol where the activities were somewhat lower

than those of controls. There were no significant sex differences in any of the groups.

There were significant differences in NADPH-cytochrome c reductase (Fig. 11, p. 72) activity between sham-operated and gonadectomized groups: sham-operated control males had higher activity than gonadectomized control males; sham-operated fish dosed with either pituitary extract or estradiol had lower reductase activities than the corresponding gonadectomized fish. Sham-operated fish dosed with pituitary extract + estradiol had higher reductase activity than gonadectomized groups. The effect of hormone treatment on sham-operated fish was a decrease in activity, particularly in the groups dosed with estradiol or pituitary extract. However, the combination of pituitary extract and estradiol produced no change in activity compared to controls. In the gonadectomized groups there were generally no significant differences in NADPH-cytochrome c reductase activity with hormone administration except that testosterone-dosed females had lower activity than controls and both males and females dosed with testosterone had lower activity than fish dosed with estradiol. There were no significant sex differences in any of the groups.

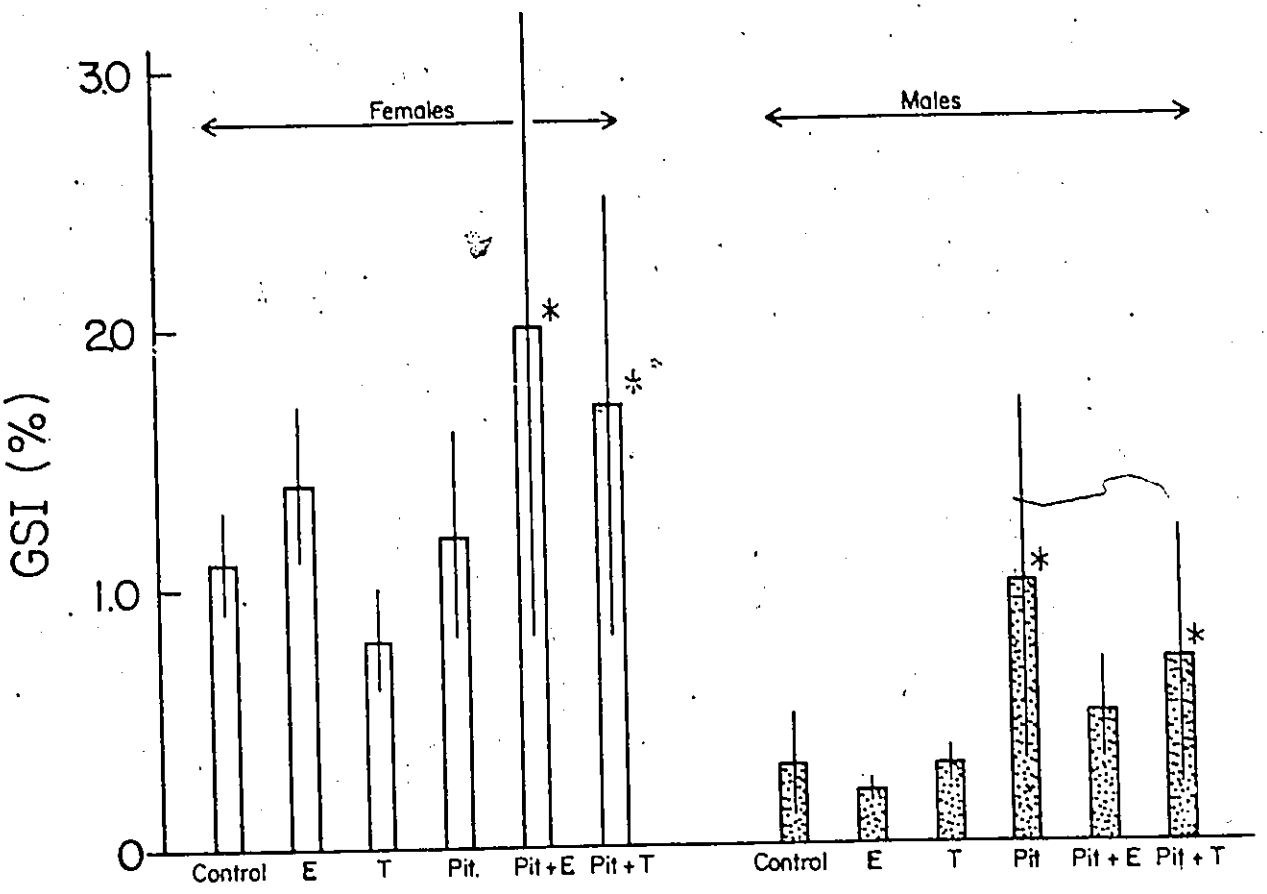
The enzyme activities measured by autoanalyzer were ethoxyresorufin-O-deethylase (EROD), aminopyrine N-demethylase (AP-demethylase), and aniline hydroxylase. There were no detectable levels of EROD (all below 0.03 nmoles/min/mg

protein) and barely detectable levels of aniline hydroxylase (all below 0.029 nmoles/min/mg protein) in these fish. The AP-demethylase activities were all approximately 0.20 nmoles formaldehyde formed/min/mg protein with no apparent differences between sexes or groups (Fig. 12, p. 73). These levels were similar to those reported by Stegeman and Chevion (1980) for adult female trout but only about one third the activity of immature and male trout. It is difficult to evaluate the AP-demethylase data since the assays were done on individual pooled samples instead of replicates. In addition, these values were near the limits of detection of the system used as it was set up for more active samples.

Gonadosomatic indices (percent) of juvenile male and female fish dosed with estradiol (E), testosterone (T) and/or pituitary extract (Pit) for 19 days.
Sample size is the same as in Table 4, p. 65.
Values shown are means ± standard deviation.

* significantly different from controls ($p \leq .05$)

Figure 4: GSI


GSI (%)
3.0
2.0
1.0
0
Females
Males
Control
E
T
Pit.
Pit + E
Pit + T
Control
E
T
Pit
Pit + E
Pit + T

Hepatosomatic indices (percent) of juvenile male and female fish dosed with estradiol (E), testosterone (T) and/or pituitary extract (Pit) for 19 days.
Sample size is the same as in Table 4, p. 65.
Values shown are means ± standard deviation.

* significantly different from controls ($p \leq .05$)

Figure 5: HSI

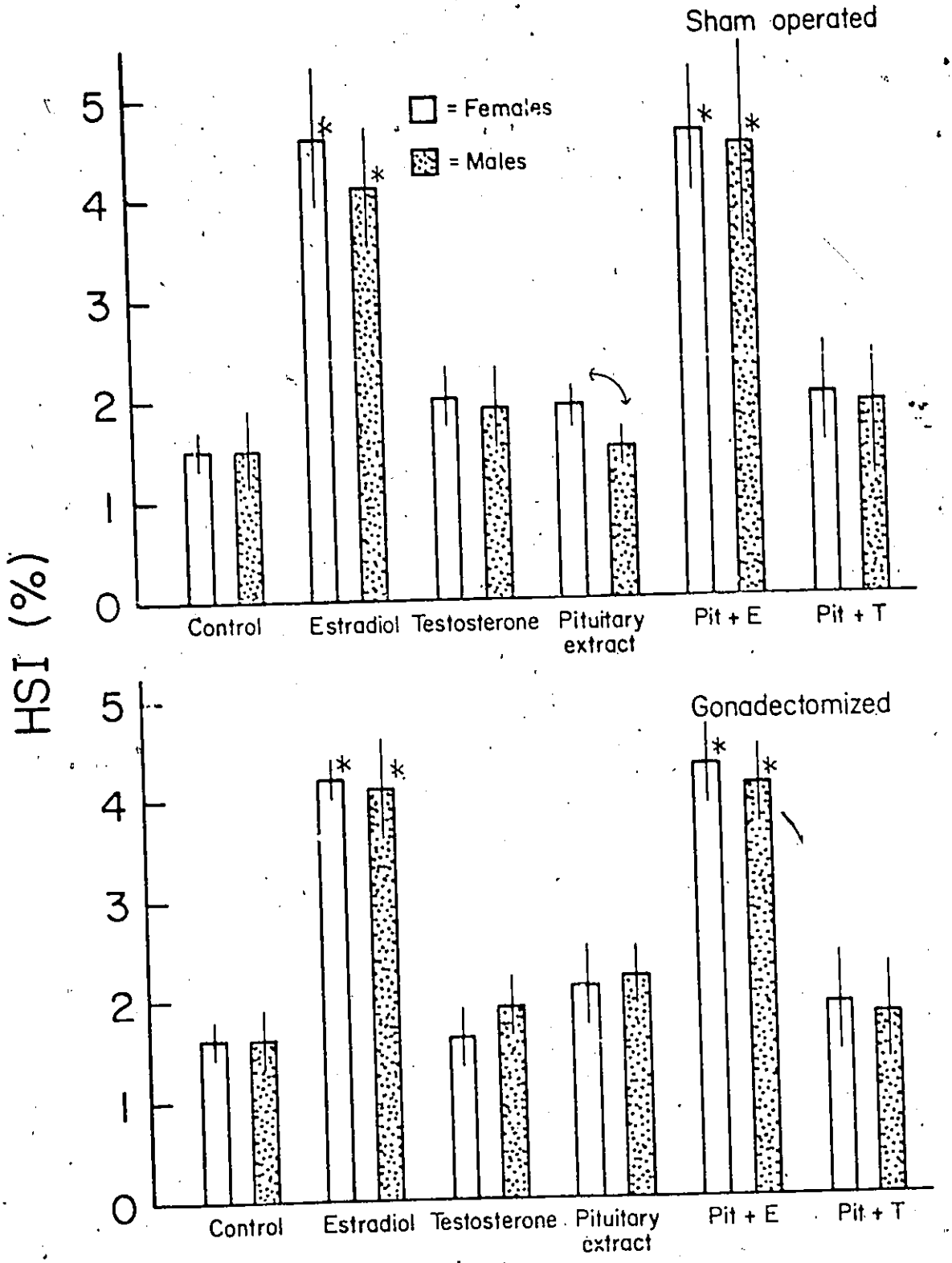

Sham operated
= Females
= Males
HSI (%)
5
4
3
2
1
0
Control
Estradiol
Testosterone
Pituitary extract
Pit + E
Pit + T
Gonadectomized
Control
Estradiol
Testosterone
Pituitary extract
Pit + E
Pit + T

TABLE 4

Body weight

Body weights (grams) of juvenile male and female fish dosed with estradiol (E), testosterone (T) and/or pituitary extract (Pit) for 19 days.
Sample size is shown in parentheses.
Values shown are means ± standard deviation.

Treatment	Sham operated		Gonadectomized	
	♀	♂	♀	♂
Control	154.3 ± 28.2(6)	187.4 ± 27.7 (6)	170.2 ± 15.8 (6)	167.8 ± 45.3 (4)
Estradiol	159.3 ± 23.7 (6)	157.7 ± 21.4 (6)	177.4 ± 12.7 (5)	169.4 ± 32.4 (5)
Testosterone	157.4 ± 65.6 (6)	184.4 ± 29.8 (6)	180.3 ± 22.1 (6)	137.3 ± 23.5 (5)
Pituitary ext.	168.2 ± 33.3 (6)	180.0 ± 11.1 (6)	168.5 ± 32.2 (6)	161.3 ± 27.8 (5)
Pit. + E'	166.9 ± 22.9 (6)	162.9 ± 31.0 (5)	169.0 ± 15.9 (6)	143.3 ± 24.0 (4)
Pit. + T	155.1 ± 34.4 (5)	165.7 ± 43.4 (5)	164.9 ± 32.2 (6)	165.2 ± 29.5 (5)

TABLE 5

Microsomal protein concentration

Microsomal protein concentrations (mg/g) in livers of juvenile male and female fish dosed with estradiol (E), testosterone (T) and/or pituitary extract (Pit) for 19 days. Sample size is shown in parentheses. Values shown are means ± standard deviation.

* significantly different from controls ($p \leq .05$)

Treatment	Sham operated		Gonadectomized	
	♀	♂	♀	♂
Control	23.3 ± 0.5 (6)	22.2 ± 2.5 (6)	23.8 ± 2.9 (6)	20.0 ± 3.1 (4)
Estradiol	34.7 ± 5.5* (6)	36.6 ± 2.7* (6)	21.1 ± 2.5 (5)	21.4 ± 4.0 (5)
Testosterone	29.3 ± 6.0 (6)	27.6 ± 3.3 (6)	25.5 ± 6.4 (6)	21.7 ± 2.5 (5)
Pituitary ext.	26.1 ± 3.3 (6)	23.2 ± 2.8 (6)	28.4 ± 4.4 (6)	26.5 ± 2.8 (5)
Pit. + E	24.5 ± 2.5 (6)	22.7 ± 2.9 (5)	22.5 ± 4.0 (6)	22.2 ± 4.9 (4)
Pit. + T	22.5 ± 2.9 (5)	21.2 ± 5.8 (5)	21.1 ± 2.8 (6)	22.2 ± 3.8 (5)

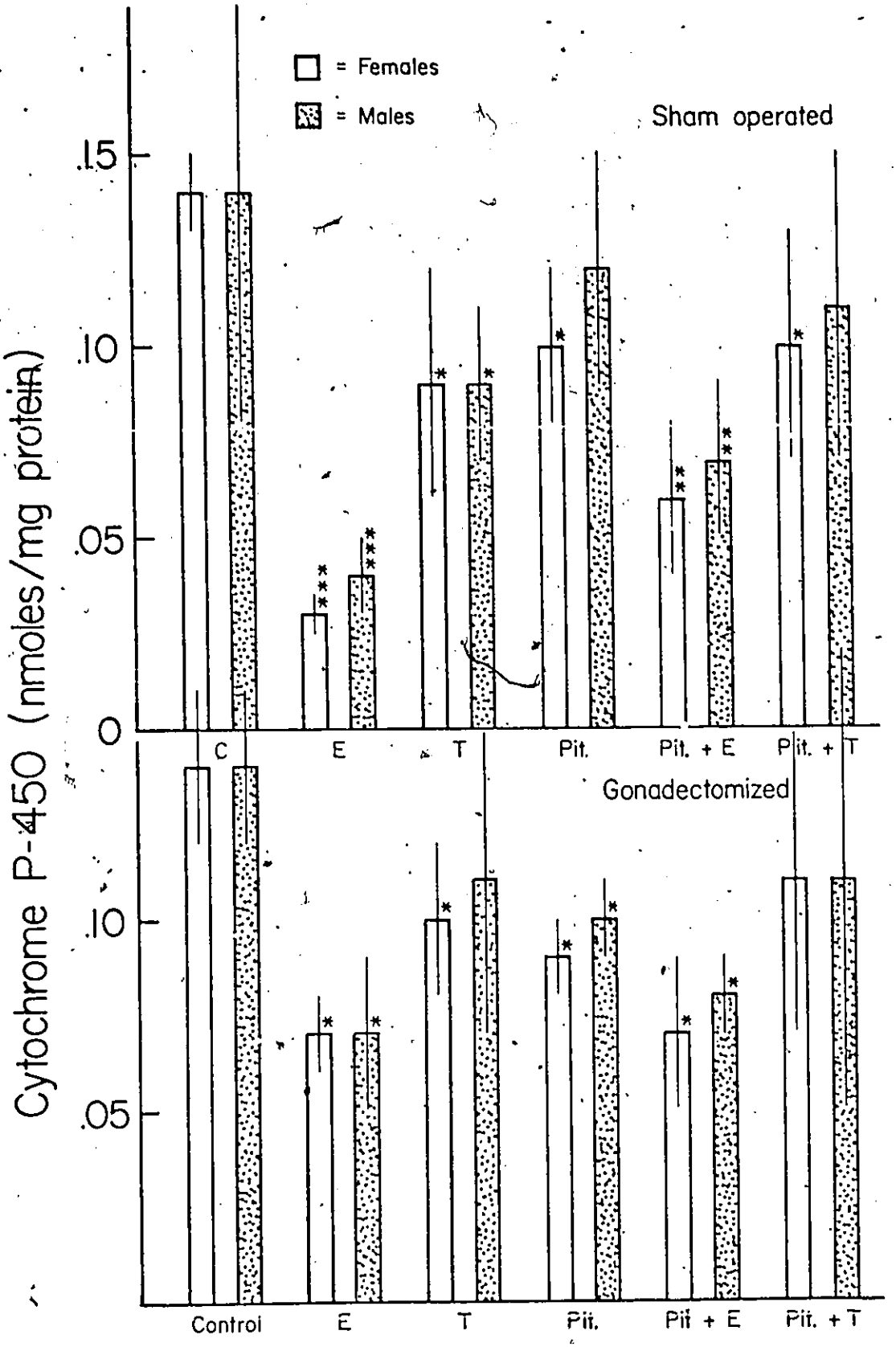
Cytochrome P-450 levels (nmoles/mg protein) of juvenile male and female fish dosed with estradiol (E), testosterone (T) and/or pituitary extract (Pit) for 19 days.
Sample size is the same as in Table 4.
Values shown are means ± standard deviation.

Statistically significant differences ($p \leq .05$):

1. Sham-operated vs. gonadectomized (Gx)
   Sham E < Gx E

2. Hormone dosed vs. control
   * significantly different from control
   ** sig. different from control and from *
   *** sig. different from all other groups

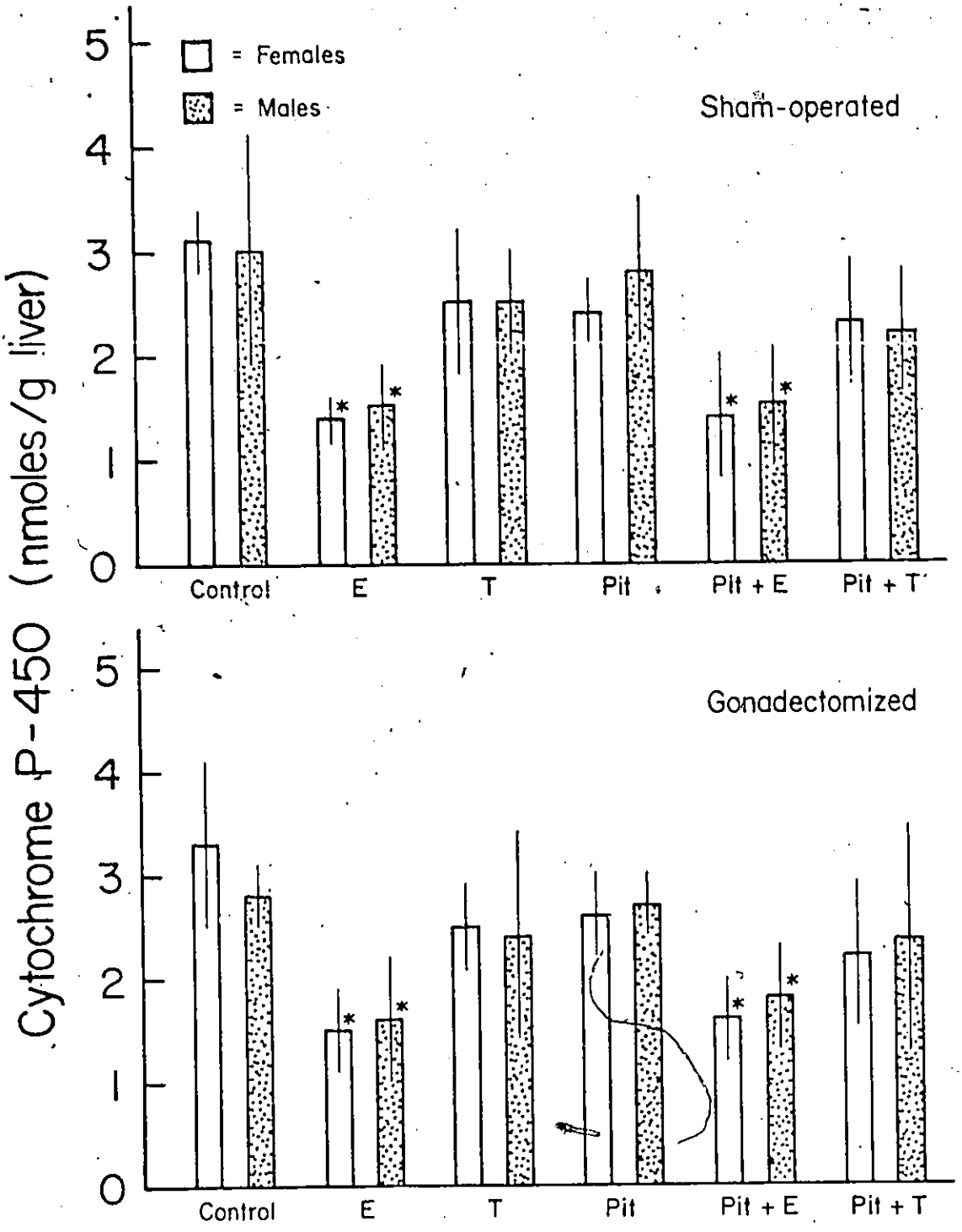
Figure 6: Cytochrome P-450 (nmoles/mg protein)


= Females
= Males
Sham operated
Gonadectomized
Cytochrome P-450 (nmoles/mg protein)
.15
.10
.05
0
C
E
T
Pit.
Pit. + E
Pit. + T
Control
E
T
Pit.
Pit + E
Pit. + T

Cytochrome P-450 content (nmoles/g liver) of juvenile male and female fish dosed with estradiol (E), testosterone (T) and/or pituitary extract (Pit) for 19 days.
Sample size is the same as in Table 4.
Values shown are means ± standard deviation.

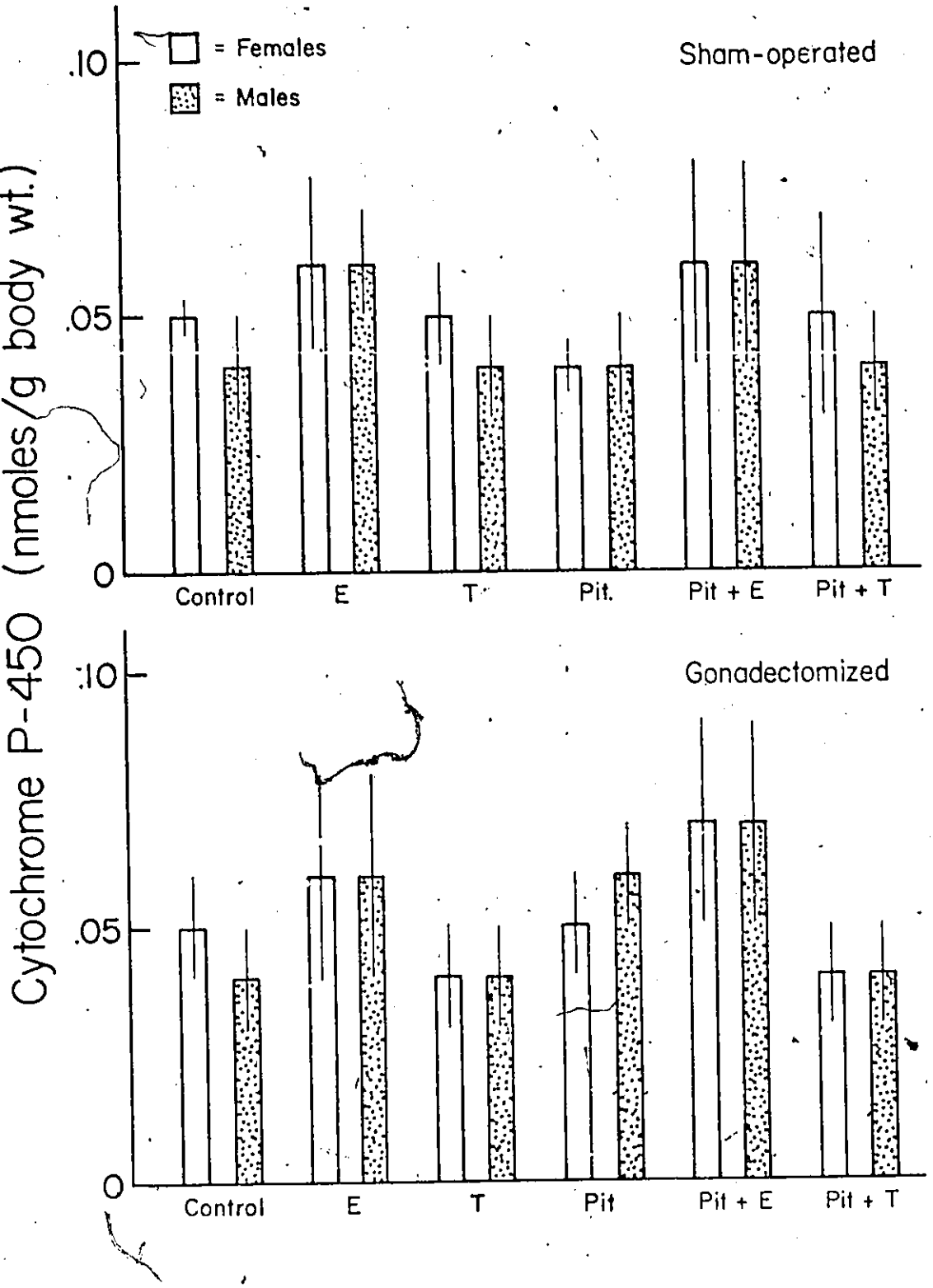
* significantly different from control ($p \leq .05$)

Figure 7: Cytochrome P-450 (nmoles/g liver)


= Females
= Males
Sham-operated
Gonadectomized
Cytochrome P-450 (nmoles/g liver)
0
1
2
3
4
5
Control
E
T
Pit
Pit + E
Pit + T
*

Cytochrome P-450 levels (nmoles/g body weight) of juvenile male and female fish dosed with estradiol (E), testosterone (T) and/or pituitary extract (Pit) for 19 days.
Sample size is the same as in Table 4.
Values shown are means ± standard deviation.
No significant differences between groups ($p \geq .05$)

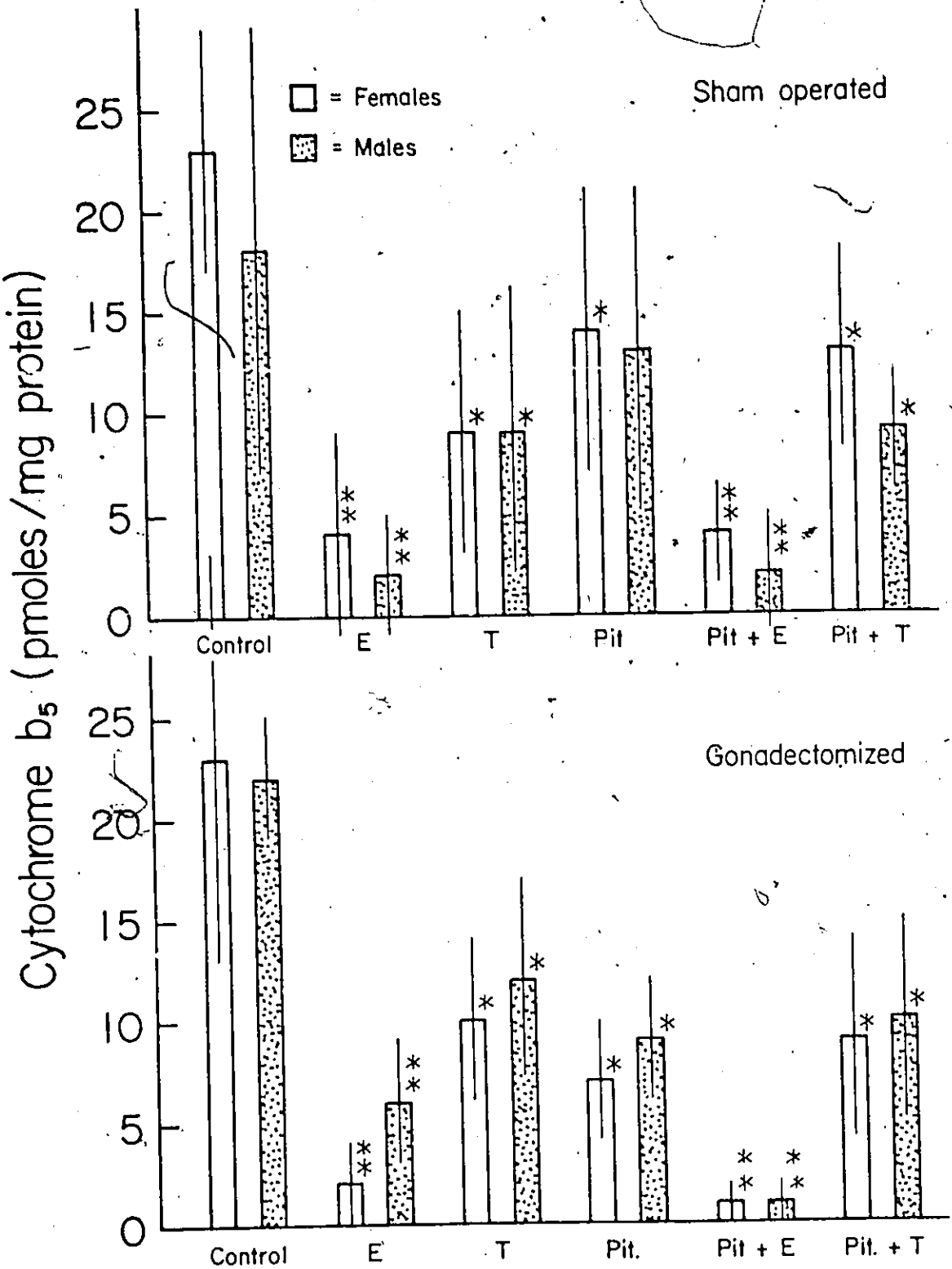
Figure 8: Cytochrome P-450 (nmoles/g body wt)

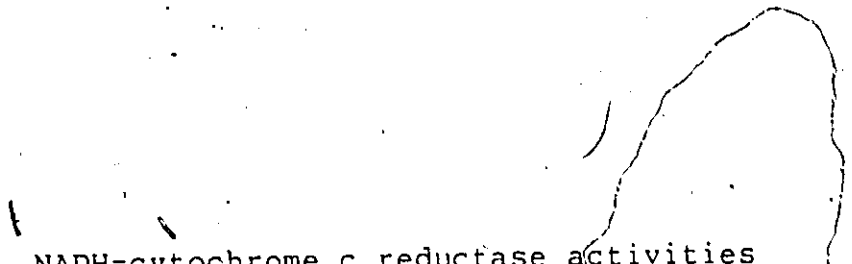

= Females
= Males
Sham-operated
Gonadectomized
Cytochrome P-450 (nmoles/g body wt.)
.10
.05
0
Control
E
T
Pit.
Pit + E
Pit + T
Pit

Cytochrome b5 levels (nmoles/mg protein) of juvenile male and female fish dosed with estradiol (E), testosterone (T) and/or pituitary extract (Pit) for 19 days.
Sample size is the same as in Table 4.
Values shown are means ± standard deviation.

* significantly different from control ($p \leq .05$)
** sig. different from control and from * ($p \leq .05$)

Figure 9: Cytochrome b5


Cytochrome b_5 (pmoles/mg protein)
= Females
= Males
Sham operated
Gonadectomized
0
5
10
15
20
25
Control
E
T
Pit
Pit + E
Pit + T
Pit.



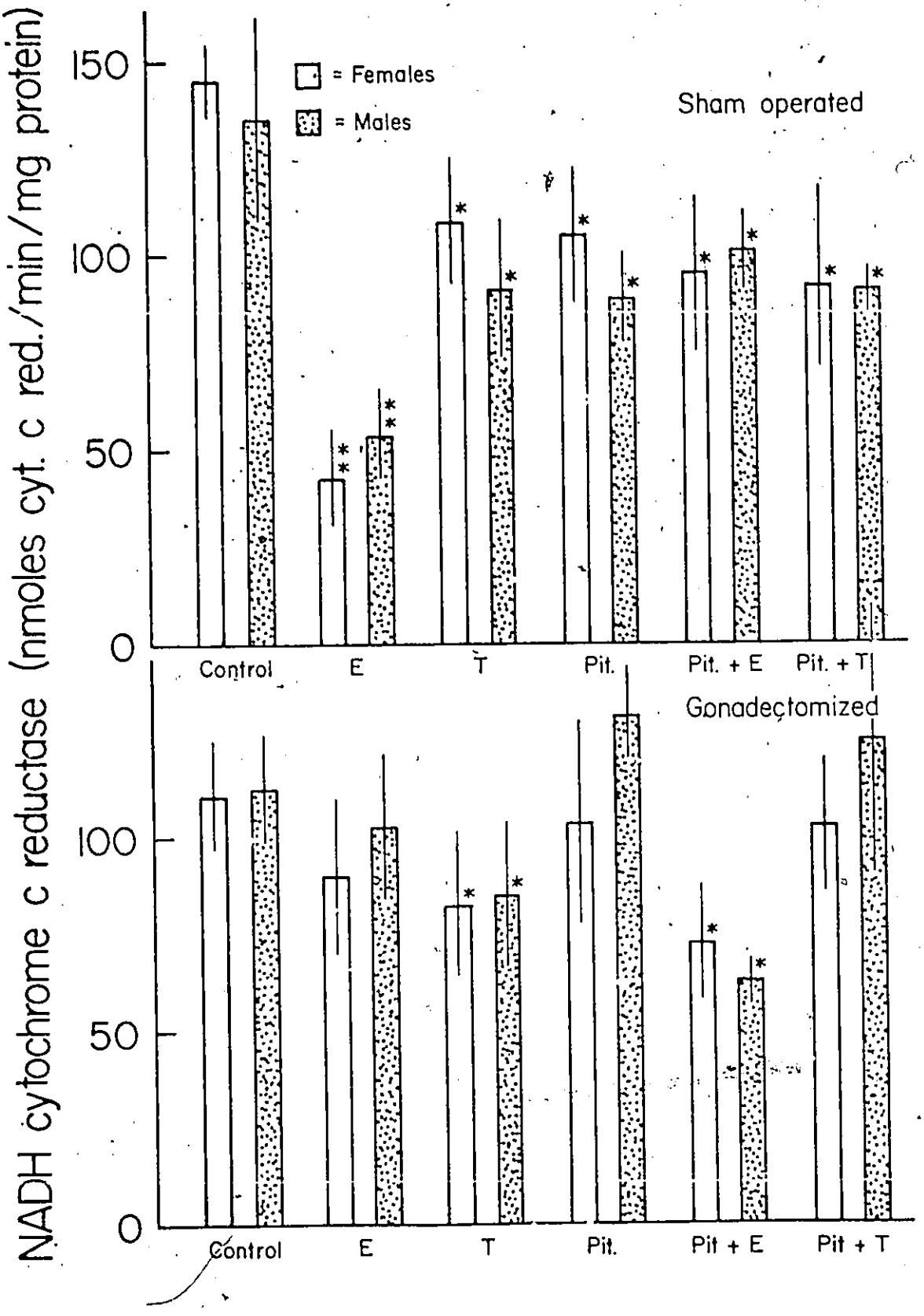
NADH-cytochrome c reductase activities
(nmoles cyt. c reduced/min/mg protein) of juvenile male and female fish dosed with estradiol (E), testosterone (T) and/or pituitary extract (Pit) for 19 days.
Sample size is the same as in Table 4.
Values shown are means ± standard deviation.

Statistically significant differences ($p \leq .05$):

1. Sham-operated vs. gonadectomized (Gx)
   Sham E < Gx E
   Sham Pit + E > Gx Pit + E

2. Hormone-dosed vs. control
   * significantly different from control
   ** sig. different from control and from *

Figure 10: NADH-cytochrome c reductase


NADH cytochrome c reductase (nmoles cyt. c red./min/mg protein)
150
100
50
0
□ = Females
▨ = Males
Sham operated
Control
E
T
Pit.
Pit. + E
Pit. + T
Gonadectomized
100
50
0
Control
E
T
Pit.
Pit + E
Pit + T

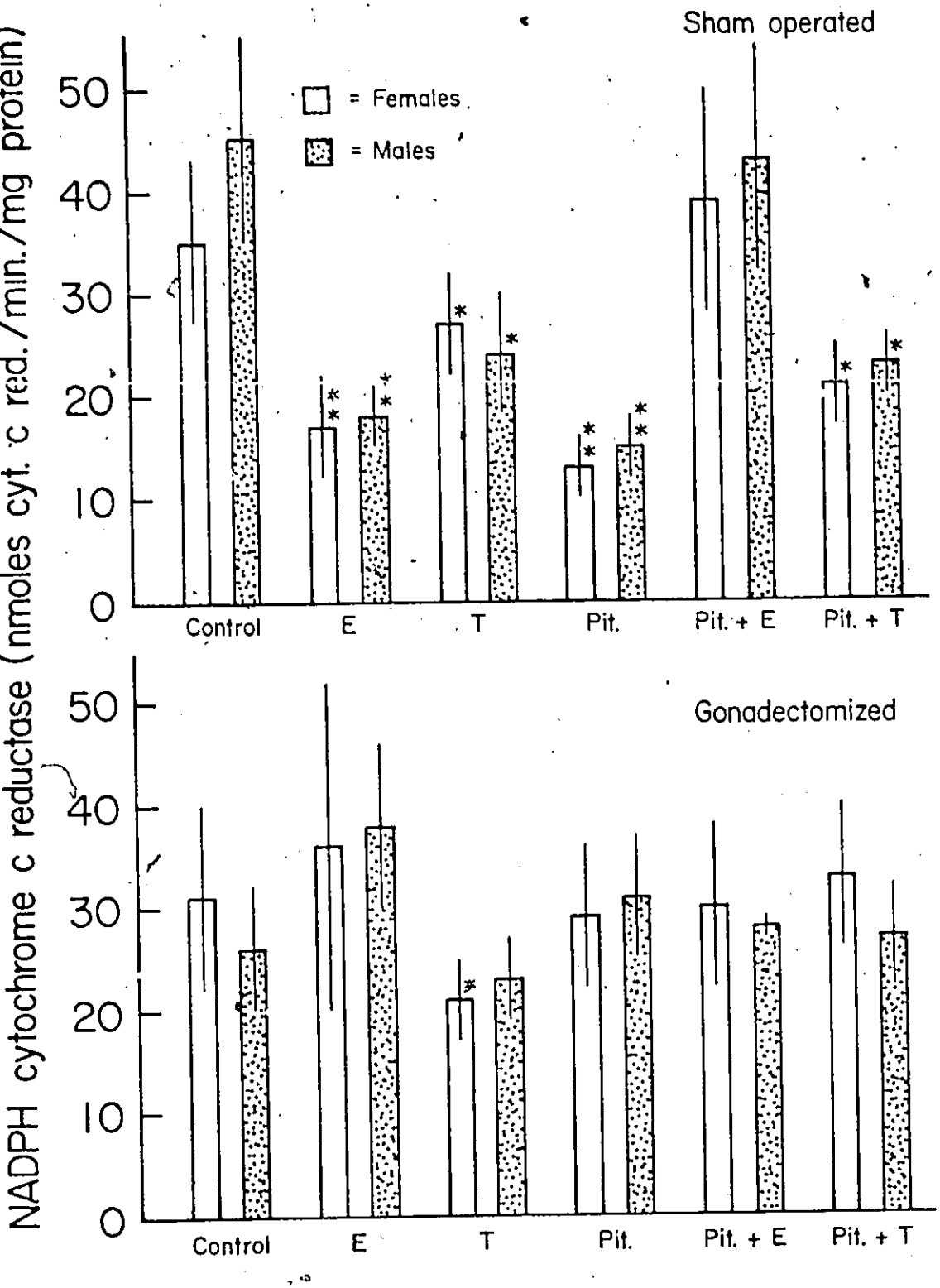
NADPH-cytochrome c reductase activities
(nmoles cyt. c red./min/mg protein) of juvenile male and female fish dosed with estradiol (E), testosterone (T) and/or pituitary extract (Pit) for 19 days.
Sample size is the same as in Table 4.
Values shown are means ± standard deviation.

Statistically significant differences ($p \leq .05$):

1. Sham-operated vs. gonadectomized (Gx)
   - Sham control o > Gx control o
   - Sham E < Gx E
   - Sham Pit < Gx Pit
   - Sham Pit + E > Gx Pit + E

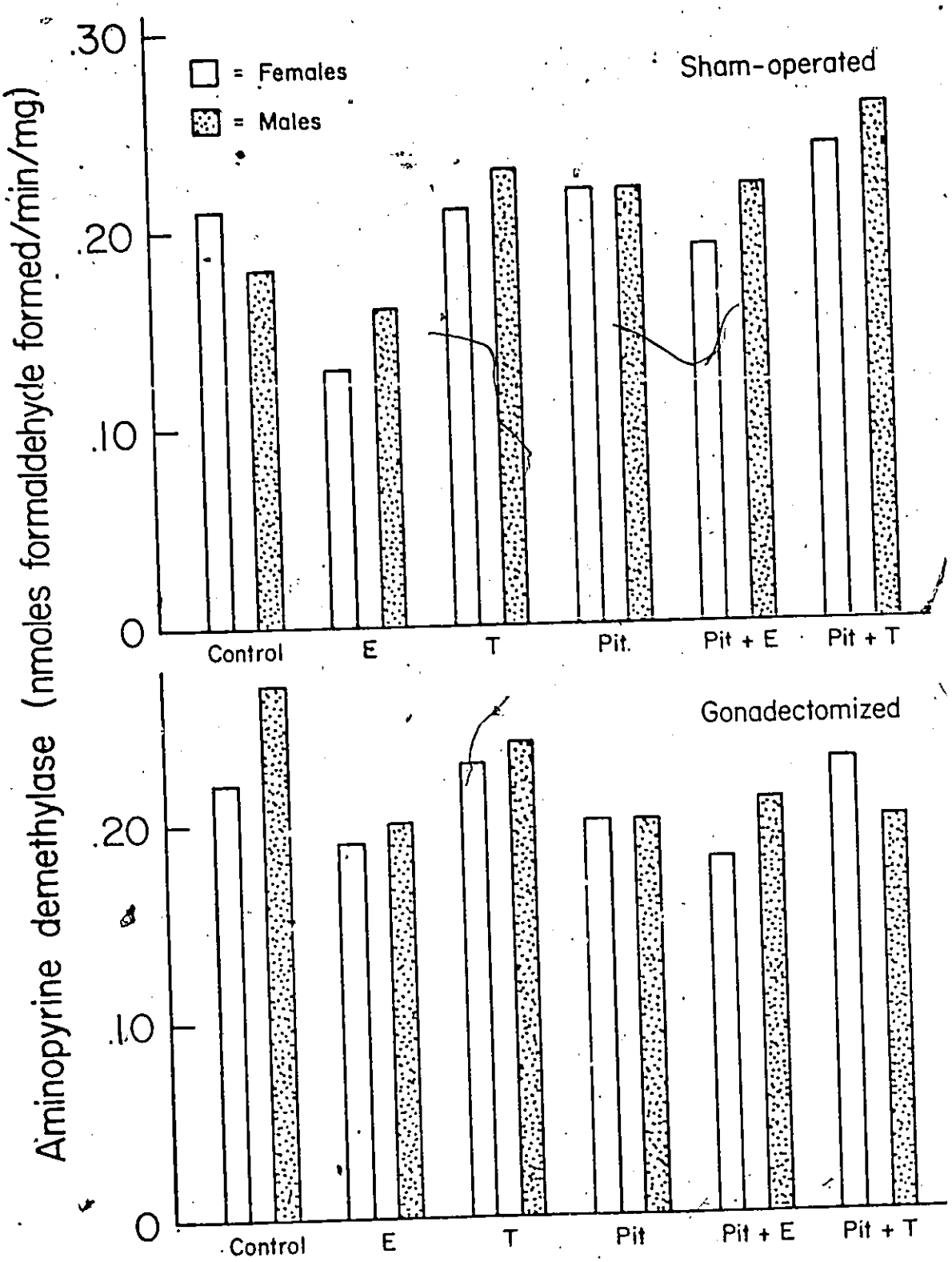
2. Hormone-dosed vs. control
   - * significantly different from control
   - ** sig. different from control and from *

Figure 11: NADPH-cytochrome c reductase


Sham operated
= Females
= Males
NADPH cytochrome c reductase (nmoles cyt. c red./min./mg protein)
0
10
20
30
40
50
Control
E
T
Pit.
Pit. + E
Pit. + T
Gonadectomized
0
10
20
30
40
50
Control
E
T
Pit.
Pit. + E
Pit. + T

Aminopyrine demethylase activities (nmoles formaldehyde formed/min/mg protein) of juvenile male and female fish dosed with estradiol (E), testosterone (T) and/or pituitary extract (Pit) for 19 days.
Values shown are results of single determination of pooled sample.

Figure 12: Aminopyrine demethylase


Aminopyrine demethylase (nmoles formaldehyde formed/min/mg)
.30
.20
.10
0
= Females
= Males
Sham-operated
Control
E
T
Pit.
Pit + E
Pit + T
Gonadectomized
.20
.10
0
Control
E
T
Pit
Pit + E
Pit + T

3.4 DISCUSSION

The data presented here verify that estradiol dosing does result in depression of cytochrome P-450 levels in juvenile fish and, when combined with the results of Chapter 2, that this effect may depend on the length of dosing period. The effects of estradiol were identical in gonadectomized and sham operated fish and, as before, in immature males and females. There was a pronounced decrease in levels of hepatic cytochrome P-450 in trout dosed with estradiol. While the direction of the change in P-450 levels was like that previously observed with estradiol dosing (Ch. 2; Hansson and Gustafsson, 1981b), the magnitude was much greater. The increased hepatosomatic index seen with estradiol dosing here was also much greater than previously seen and, therefore, the decreased P-450 levels may simply reflect this. The samples from this section also showed a decrease in 6β-hydroxylation of testosterone, but not of 16β-hydroxylation, suggesting that there was a change in a specific form of P-450 (Pajor and Stegeman, unpublished results). This is also similar to the results of previous studies of estradiol administration to trout (Hansson and Gustafsson, 1981b; Stegeman et al., 1982).

The results of this experiment showed a somewhat paradoxical depression of cytochrome P-450 levels and NADPH- and

NADH-cytochrome c reductase activities in fish dosed with estradiol and testosterone. The depression of cytochrome P-450 by both estradiol and high doses of testosterone was also observed in preliminary studies of Vodicnik et al. (1980). Recently, Hori et al. (1979) found that excessive doses of methyltestosterone and testosterone, when administered to goldfish, had estrogenic rather than androgenic effects on certain hepatic characteristics including vitellogenin synthesis; hepatosomatic index, and proliferation of hepatocyte endoplasmic reticulum, Golgi apparatus, and secretory granules. Le Menn (1979) also found that pharmacological levels of testosterone (20 mg/kg daily for 8 days) or dihydrotestosterone, a non-aromatizable androgen (DHT; 40 mg/kg daily for 8 days), had estrogenic effects on the hepatic synthesis of vitellogenin in both sexes of Gobius niger L. . Further studies by Le Menn et al. (1980) have shown that the estrogenic action of testosterone and DHT is mediated by hepatic estrogen receptors although their affinity for these androgens is approximately one hundred times lower than their affinity for estrogens. In addition, the effects of testosterone were thought to be due to a combination of aromatization to estrogens and a direct stimulation of estrogen receptors by testosterone itself (Le Menn et al., 1980).

In the previous chapter the selection of steroid dosing level was discussed. However, in addition to dosing level,

the length of dosing period must be considered. The differences in effects seen after 10 days dosing (Ch. 2) compared with 19 days of dosing (this experiment) indicate that the longer dosing period represents a higher level of steroid in the body. The injected dose was probably not totally eliminated from the fish in the interval between doses and after 19 days accumulated in the body to a greater extent than after 10 days. It is, of course, entirely possible that the differences between the two experiments are due to the different strains of brook trout used. These strains could have differences in ability to metabolize or respond to administered steroids. The levels of androgens used in other studies (Hori et al., 1979; Le Menn et al., 1979) to elicit estrogenic responses may have been excessive, in the light of the results of this section.

The results presented here indicate that pituitary extract from adult Chum salmon has a feminizing effect on cytochrome P-450, as shown by the decreased P-450 levels in both sham-operated and gonadectomized trout. This suggests the existence of a pituitary "feminizing" factor as seen in rats. In studies of hypophysectomized adult rats, the implantation of a pituitary from adult rats of either sex results in a feminine pattern of drug and steroid metabolism (Gustafsson and Stenberg, 1976; Denef, 1974; Gustafsson and Skett, 1978; Eneroth et al., 1977) and cytochrome P-450 levels (Gillham et al., 1977). In this study, the fish were

not hypophysectomized since they were immature and did not show sex differences in MFO activity or steroid levels (indirect evidence of GTH secretion from the pituitary). The results shown here are in direct contrast to those of Hansson and Gustafsson (1981b) which suggest that estradiol acts on the liver MFO enzymes and cytochrome P-450 levels independently of the pituitary. It is possible that the differences between these studies may be due to the fact that Hansson's experiments involved hypophysectomy of immature fish and the experiments of this thesis involved dosing of immature fish with adult pituitary extract. As mentioned previously, immature rats do not secrete the feminizing factor until puberty, and, while both sexes are responsive to steroids as juveniles, females lose that response after puberty. It would be interesting to repeat Hansson's experiments using adult trout.

One very consistent response to all hormone treatments in this and previous studies (Ch. 2) was a pronounced decrease in levels of cytochrome b5. These levels were in some cases so low as to be undetectable, and were still significantly decreased when expressed on the basis of body weight. Considering the possible interaction of cytochrome b5 with the P-450 dependent monooxygenase system, it would be interesting to determine the importance of these decreases. In mammalian systems, the levels of b5 do not seem to be affected by steroids since there were no changes in cytochrome b5 af-

ter gonadectomy and adrenalectomy (Ernster and Orrenius, 1974). In fish there are no sex difference in this character (Stegeman and Chevion, 1980), suggesting an insensitivity to hormonal changes associated with reproduction. The function of cytochrome b5 in mammalian systems is in the desaturation of fatty acids and it is possible that the effects of hormone administration on b5 here are mediated through changes in lipid metabolism. However, the function of b5 in fish, as well as the effects of a hormonally-influenced decrease in b5 levels, remain to be determined.

As seen in the previous chapter, the activities of both NADH- and NADPH-cytochrome c reductase responded to steroid dosing. Pituitary extract had no effect on these reductases in the absence of the gonads. Therefore, based on the effects of steroids, it is possible that the effects of pituitary extract in sham-operated fish were mediated through gonadal steroids. The importance of changes in reductase activity on the activity of the hepatic monooxygenases has not yet been determined. However, in mammalian systems, the activity of NADPH-cytochrome c reductase is thought to be the rate-limiting step of monooxygenation. If this is also true in fish, then it is possible that the changes seen in monooxygenase activity with steroid dosing could be related in part to changes in reductase activity.

As expected, the levels of EROD and aniline hydroxylase were below the limits of detection; elevated levels of these

enzymes may be used as indicators of induction by 3-MC type inducers (see Introduction). Unfortunately, it was not possible to examine BP-hydroxylase and the effect of 7,8-BF on BP-hydroxylase activity, ethoxycoumarin O-deethylase or aminopyrine N-demethylase activity (shown to be sex-dependent in trout (Stegeman and Chevion, 1980; Forlin, 1980)) in these samples. It would be interesting to study the effects of pituitary extract on these enzymes in trout.

Dosing with two compounds at once can sometimes provide insight into the location of action of these compounds. In the experiments here, the effects of pituitary extract together with steroids on cytochrome P-450 seemed to be the same as those of the steroids alone. Since all three had feminizing effects, it is difficult to conclude much from this section. However, it would be interesting to see the effects of a combination of pituitary extract and a lower dose of testosterone. If the "feminotropin" system is in effect in fish, then this combination should have a feminizing effect.

Chapter IV

EFFECTS OF GTH IN SHAM-OPERATED AND GX FISH

4.1 INTRODUCTION

The results of the previous experiment showed that the administration of adult pituitary extract to immature brook trout resulted in decreased cytochrome P-450 levels (i.e. feminization) and in decreased cytochrome b5-levels. This suggests that there may be a feminizing component of the adult pituitary.

In fish, unlike mammals, it has been suggested that sex differences in MFO are associated with seasonal changes in reproductive capacity (Hansson and Gustafsson, 1981a; Koivusaari et al., 1981). The pituitary hormones most directly associated with seasonal reproductive changes and which are regulated by sex steroids are the gonadotropins (reviews: Donaldson, 1972; Peter and Crim, 1979). These are virtually undetectable in juvenile and nonspawning fish and increase considerably during the spawning season (Crim et al., 1975). Thus it is possible that these hormones are implicated in the seasonal development of sex differences in hepatic MFO activity and may be the feminizing fraction of the pituitary extracts.

It should be mentioned at this point that the teleost gonadotropin fractions isolated thus far represent two fractions with respect to their adsorption or lack of adsorption on Con A sepharose (Ng and Idler, 1980; Ng et al., 1980a,b). One fraction, corresponding roughly in activity to mammalian LH, has vitellogenic properties. The other, corresponding to mammalian FSH, has maturational properties. However, it is unknown yet whether these are in fact two separate hormones.

The purpose of the experiment outlined in this chapter was to determine whether GTH was the feminizing fraction of the pituitary extract used in the previous chapter. To isolate the effects of GTH independently of its effects on the gonads and subsequent sex steroid production, half of the fish used in this experiment were gonadectomized before GTH administration.

4.2 MATERIALS AND METHODS

4.2.1 Hormones

The gonadotropic hormones (GTH) used in this study were purchased from Syndel Labs Ltd., Vancouver, B.C.. These consisted of the SGA-GTH fraction purified from adult Chum salmon (Oncorhynchus keta) pituitaries, and contained both the vitellogenic and gonadotropic GTH as well as some contamination by TSH (thyroid stimulating hormone). The gonadotropin activity of 1 mg of the SGA-GTH preparation was determined by Syndel Ltd. to be equivalent to 2.15 mg of the SG-G100 preparation of E.M. Donaldson as measured in the salmonid cAMP assay (Idler et al., 1975) and to 0.086 mg of NIH-LH-S19 when compared to Donaldson's, standardized in the chick bioassay (Donaldson, 1973).

4.2.2 Fish

Immature yearling brook trout were obtained from Goossen's Trout Hatchery, Otterville, Ontario in December, 1980, and shipped by air to holding facilities at the University of Ottawa. They were maintained in Living Stream Tanks (Frigid Units Inc., Toledo, Ohio), equipped with flowing dechlorinated city tap water at 8-10°C at densities of 18 fish per 270 l tank. The photoperiod was maintained 12L:12D with

overhead fluorescent lights. Fish were fed daily with Purina Trout Chow pellets at approximately 1-2% body weight.

4.2.3 Surgery

Fish were operated upon two weeks after acclimation to lab conditions. The method used was as outlined in Chapter 3 except that the abdomen was closed by continuous suture with 3-0 silk. This proved to be inadequate and improvements in procedure were made in the following experiment (outlined in Ch. 3). In five fish the sutures had come partially undone in the first week and one stitch had to be added to reinforce. In addition several fish showed signs of inflammation or infection of the wound (unfortunately numbers were not recorded). Fish were maintained three weeks after surgery before dosing.

4.2.4 Experimental

Four experimental groups, each consisting of six female and six male fish, were set up. Two of these groups were gonadectomized and the other two were sham operated. Fish were injected i.m. daily with 0.1 ml of 50 µg salmon GTH dissolved in teleost Ringer's with .01% TWEEN20 (Y.P. So, pers. comm.) or the Ringer's and TWEEN20 only (controls) for a total of ten injections.

The dosage was chosen on the basis of three studies on juvenile rainbow trout. Dosing of immature rainbow trout

via i.p. injection at levels of 20 or 200 µg GTH/100 g fish resulted in physiological levels of GTH in plasma (Crim and Evans, 1976). Furthermore, the plasma half-life of GTH at 6 or 15°C was approximately 1.5 days at both doses used. Thus, it was decided to use daily injections in order to maintain high plasma GTH levels and to use i.m. injections in order to slow the rate of absorption into the body as well as not to disturb stitches on the ventral side. Ng and Idler (1980) found that two doses (days 1 and 5) of purified gonadotropin (Con AI or maturational fraction) at a dose of 50 ug per fish (age and weight of fish not reported) resulted in an increase in plasma levels of 11-ketotestosterone and testosterone in male and testosterone in female immature rainbow trout. Idler and Campbell (1980) showed that salmon pituitary extract at a dose of 1.26 mg/kg (containing 8% GTH - con AII or vitellogenic fraction) given once every three days for twelve days stimulated estradiol and vitellogenin synthesis in juvenile female trout. The dose employed in this study was 50 µg GTH per fish (approximately 100 g body weight) daily for ten days and it contained both vitellogenic and maturational fractions. On the eleventh day of dosing fish were sampled and blood was taken as described in previous chapters. All methods used have been described previously.

4.3 RESULTS

As shown in Table 6, gonadosomatic indices increased in females ($p \leq .02$) and males ($p \leq .07$) after dosing with GTH. Hepatosomatic indices were not significantly changed with hormone treatment although sham-operated females dosed with GTH showed a trend toward a higher HSI when compared with other female groups. There were no significant differences in microsomal protein levels with treatment. The activities of NADH-cytochrome c reductase (Table 6) were very low (one third to one quarter of normal activity) and showed no significant differences between groups.

The activities of NADPH-cytochrome c reductase were similar to those previously seen (Chapter 2; Stegeman and Chevion, 1980). The only sex differences were in the sham-operated fish dosed with GTH, where the males showed higher activity than females. There were no significant differences between GTH-dosed and control females in the sham-operated fish; sham-operated males dosed with GTH, however, showed higher NADPH-cytochrome c reductase activity than control males. In the gonadectomized groups, both males and females dosed with GTH had higher NADPH-cytochrome c reductase activity than controls. There were no significant differences between sham-operated and gonadectomized controls on reductase activities. Within the GTH-dosed groups, there seemed to be a trend towards lower NADPH-cytochrome c reductase activity in sham-operated females and higher activity in sham-operated males when compared with gonadectomized fish.

The data showing levels of cytochrome P-450 are also in Table 6. The absorption maximum of reduced, CO-bound cytochrome P-450 was at 450 nm in all cases, regardless of treatment or sex. Again, as in NADPH-cytochrome c reductase activities, the only significant differences seen were in the sham-operated fish dosed with GTH; the males dosed with GTH had higher levels of P-450 than females, although neither group had levels different from their controls. There were no significant differences in P-450 levels among any other groups. When normalized to liver weight, P-450 levels still showed sex differences in the sham-operated fish dosed with GTH and in gonadectomized controls. The reasons for this latter difference are not known. There were no significant differences between groups when P-450 levels were expressed on the basis of body weight.

TABLE 6

MFO components in GTH dosed trout

Activities and levels of hepatic electron transfer components and other characters in immature trout dosed with partially purified salmon gonadotropic hormones.
Sample size is shown in parentheses.
Values shown are mean ± standard deviation.

* significantly different from controls ($p \leq .05$)
↙↘ significant sex difference ($p \leq .05$)

Character	Sham-operated				Gonadectomized			
	Saline-dosed		GTH-dosed		Saline-dosed		GTH-dosed	
	♀ (7)	♂ (5)	♀ (7)	♂ (5)	♀ (6)	♂ (6)	♀ (6)	♂ (6)
Body weight (g)	75.8 ± 17.1	100.0 ± 28.8	95.5 ± 22.9	99.6 ± 25.3	110.2 ± 15.4	106.3 ± 15.4	100.1 ± 27.7	92.2 ± 19.0
HSI (%)	1.8 ± 0.2	1.6 ± 0.1	2.0 ± 0.2	1.7 ± 0.3	1.7 ± 0.1	1.6 ± 0.2	1.8 ± 0.2	1.6 ± 0.2
GSI (%)	0.30 ± 0.12	0.06 ± 0.03	0.48 ± 0.12*	0.16 ± 0.12*	—	—	—	—
Microsomal protein (mg/g liver)	17.7 ± 5.13	18.8 ± 5.1	22.2 ± 2.8	20.3 ± 1.6	21.0 ± 4.8	24.4 ± 3.1	21.1 ± 3.2	18.4 ± 2.4
NADPH-cytochrome c red. (nmoles/min/mg prot)	45.8 ± 6.7	42.3 ± 14.4	41.1 ± 10.7	58.4 ± 7.2	35.3 ± 5.9	37.6 ± 9.9	51.1 ± 5.5*	50.6 ± 8.0*
NADH-cytochrome c red. (nmoles/min/mg prot)	56.7 ± 25.6	60.9 ± 13.7	40.3 ± 16.5	54.4 ± 14.7	34.0 ± 9.1	53.9 ± 8.0	51.8 ± 10.8	43.8 ± 7.4
Cytochrome P-450 (nmoles/mg protein)	.16 ± .04	.17 ± .03	.14 ± .02	.20 ± .03	.16 ± .03	.17 ± .03	.18 ± .04	.19 ± .02
Cytochrome P-450 (nmoles/g liver)	2.8 ± 0.8	3.1 ± 0.5	3.2 ± 0.7	4.0 ± 0.4	3.1 ± 0.5	4.2 ± 0.8	3.7 ± 0.7	3.4 ± 0.5
Cytochrome P-450 (nmoles/g body wt.)	.05 ± .02	.05 ± .01	.06 ± .01	.07 ± .02	.05 ± .01	.07 ± .01	.07 ± .02	.05 ± .01

4.4 DISCUSSION

The purpose of this experiment was to determine whether GTH was the feminizing fraction of the pituitary extract. The results here show that administration of GTH in greater concentration than in the pituitary extract used in the previous experiment (Chapter 4) did not produce similar decreases in cytochrome P-450 levels. Therefore, GTH is probably not the feminizing fraction of the pituitary. However, it is possible that a combination of hormones produces the feminizing effects of whole pituitary extract; this remains to be tested.

One assumption of this experiment was that GTH dosing would result in increased plasma steroid levels in the sham-operated group. Several studies have shown a correlation between increased plasma steroid levels and increased gonad weight (GSI) (Lambert et al., 1978) -- probably since the major sources of plasma sex steroids are the gonads (Hoar and Nagahama, 1978). The results here showed an increased GSI in both sham-operated males and females dosed with GTH. Based on the studies of Crim and Evans (1976), Ng and Idler (1980), and Idler and Campbell (1980) (see Methods) it is likely that the plasma steroid levels were increased by GTH treatment although the actual levels have not been determined. The trends seen in Chapter 2 after steroid dosing were evident in sham-operated fish dosed with GTH although it is possible that a longer dosing period is needed in order to see the steroid effects more clearly.

The results of this experiment indicate that GTH probably has no effects on MFO beyond possible stimulation of gonadal sex steroid synthesis. The only effects seen were in the sham-operated fish dosed with GTH and the direction of response was similar to that seen in the steroid-dosed fish of Chapter 2. It is interesting that the activities of NADPH-cytochrome c reductase were higher in sham-operated males dosed with GTH than in females. If it is assumed that the increased GSI in these fish represents increased synthesis of steroids, then this result is similar to the situation seen after steroid dosing but not in mature fish (Stegeman and Chevion, 1980). There may be effects of GTH on the liver independent of the gonads since there were higher NADPH-cytochrome c reductase activities in gonadectomized fish dosed with GTH than in controls. It must be noted that the GTH preparation used may have been contaminated with some TSH (according to Syndel Ltd.) and that this could be partially responsible for the effects seen.

The activities of NADH-cytochrome c reductase were unusually low in all groups, including controls, when compared with undosed and saline-dosed fish of the same stock (results not shown) and also when compared with other stocks of brook trout (Chapter 2; Stegeman and Chevion, 1980). The reasons for this are unknown particularly in view of the fact that NADPH-cytochrome c reductase activities in these samples were normal. It may be possible that the reductases show different labilities.

Several studies in rats have shown effects of gonadotropins, FSH and LH, on hepatic drug and steroid metabolism. Gustafsson and Stenberg (1975) found only FSH to be effective in increasing the activity of androgen-dependent steroid hydroxylating enzymes. Kramer et al. (1977) found that both LH and FSH, when given to gonadectomized adult male and female rats, increased (i.e. masculinized) ethylmorphine demethylase activity in the liver. However, as mentioned previously, sex differences in rat hepatic MFO are independent of reproductive cycle while those of fish seem to appear only seasonally. In this study, the parameters examined did not show any significant changes with GTH dosing although it is unknown what effects, if any, there were on drug and steroid metabolism in these fish since these were not measured.

Chapter V

SUMMARY AND CONCLUSIONS

The major findings of this thesis are summarized as follows:

1. Dosing of juvenile trout of both sexes for 10 or 19 days with estradiol had a general feminizing effect on components of hepatic microsomal electron transport. There were no apparent effects on benzo(a)pyrene or ethoxyresorufin monooxygenase activity, nor any particular change in the influence of ANF on BP-OH activity.
2. Dosing for 10 days with testosterone masculinized components of hepatic electron transport and there was a trend toward an increase (i.e. masculinization) in BP-OH activities.
3. Dosing for 19 days with testosterone resulted in a feminization of components of microsomal electron transport in juvenile fish of both sexes. Preliminary data showed that plasma estrogen levels in these fish were elevated.
4. Administration of pituitary extracts from adult salmonids to immature fish had a general feminizing ef-

fect on cytochromes P-450 and b5. Activities of NADPH- and NADH-cytochrome c reductases seemed to be influenced only by steroids and not by the pituitary.

5. The pituitary gonadotropic hormones are probably not the feminizing fraction of the pituitary. Partially purified salmon GTH had no effects on hepatic components of microsomal electron transport in the absence of the gonads.

The studies here suggest that both estradiol and testosterone are active in producing sex differences in juvenile fish. In addition, the liver of juvenile fish is responsive to the effects of pituitary extracts from adult salmonids. Therefore, as in rats, it is probably changes in other systems (ex. gonads, pituitary-hypothalamus) at maturity and not in the responsiveness of the liver that determines the sex-dependent activities.

The work of Hansson et al. (1980b) and Hansson and Gustafsson (1981b) with immature hypophysectomized fish showed that steroids act on the liver independently of the pituitary in these fish to produce changes in enzyme activity. This is similar to the situation in immature rats (as outlined in Ch. 3). The possible changes in regulation of this system at maturity in fish, however, are not known. The feminizing effect of pituitary extract from adult fish in this study suggests that the pituitary does influence this system at maturity although the feminizing factor has not

been identified. If sex differences in MFO activity in fish are regulated in the same way as those of mammals then at maturity females should develop an insensitivity to steroids and the action of steroids in males should be indirect via the pituitary. In addition, a confirmation of the seasonality of sex differences in fish should be made. This is a striking difference between fish and mammalian systems and may provide insight into the possible regulation of the sex differences in fish.

While the experiments of this thesis suggest that steroids and pituitary hormones may be involved in the regulation of sex differences in components of microsomal electron transport systems and SDS-PAGE profiles of hemeproteins (which could possibly be P-450) (data reported in Stegeman et al., 1982), it is clear that this work should be expanded to include a more extensive study of enzymes of drug and steroid metabolism. A dose-response study of both estradiol and testosterone should be made. The effects of other steroids, that are important in fish, for example 11-ketotestosterone, should be investigated. Experiments by Mode et al. (1981) have suggested that the timing between peaks of plasma hormone levels may be different in males and females and thus differences in dosing regime may determine response to a particular hormone. This should be investigated in fish.

It is interesting that the normal condition in rats with respect to hepatic MFO activity is that of the male -- in the absence of pituitary feminizing factors, the metabolism of steroids and drugs follows a masculine pattern. The MFO activities in males increase continually until well after puberty. The factors regulating this increase should be studied, both in mammals and fish.

The biological significance of sex differences in MFO activity is not yet known. It is probably related to differences in steroid profiles between males and females at maturity. It is generally thought that the original function of the MFO system was in oxygenation of endogenous substrates, and, in fact, the affinity of the uninduced system for steroids is approximately ten times greater than that for xenobiotics (Kuntzman et al., 1965). Thus, any sex differences in xenobiotic metabolism are most likely a reflection of the sex differences in steroid metabolism.

According to Skett and Gustafsson (1979), sex differences in steroid metabolism in rats serve to protect the animals from unwanted steroids. This may explain the high rate of metabolism of testosterone in female rats but not the high rate of metabolism of progesterone in male rats since this character may be important in the production of androgens. In addition, most mammals have sex differences in steroid levels and types after maturity yet do not show evidence of sex differences in hepatic MFO activity. It is possible

that, unlike rats, the hepatic metabolism of steroids in those mammals shows sex differences according to stage of reproductive cycle and therefore sex differences in MFO have been overlooked by researchers. It is also possible that those mammals use different strategies than rats to change steroid profiles. For example, while rats may primarily utilize different rates of steroid catabolism to maintain sex differences in steroids, other species may change rates of steroid synthesis or amount of plasma protein binding of steroids to achieve the same result. However, this remains to be tested.

In fish, sex differences in hepatic steroid metabolism have been characterized by Hansson and Gustafsson (1981a). During spawning season, hepatic activities of 17-hydroxysteroid oxidoreductase in male rainbow trout increase considerably together with plasma levels of 11-ketotestosterone, the major androgen in fish. It has been suggested that 17-hydroxysteroid oxidoreductase is instrumental in the synthesis of 11-oxygenated steroids (Hansson and Gustafsson, 1981a). Female trout, which have very low plasma levels of 11-ketotestosterone also show very low activities of 17-hydroxysteroid oxidoreductase. In addition, female fish show a decreased capacity for the hydroxylation of androgens such as testosterone (Stegeman et al., 1982) and androstenedione (Hansson and Gustafsson, 1981a) while male fish show a more rapid in vivo metabolism of es-

tradiol (Myers and Avila, 1980). The increased plasma levels of estrogens in vitellogenic female fish are thought to be due to decreased estrogen catabolism (Hansson, 1981). Hansson (1981) has suggested that the decreased steroid metabolizing capacity in female fish probably functions to maintain high levels of plasma estrogens during vitellogenesis.

As mentioned previously, several studies (Hansson et al., 1980; Forlin, 1980) have shown that male and female fish respond differently to inducers. For example, female trout dosed with polycyclic aromatic hydrocarbons have a higher rate of metabolism of steroids than males (Hansson et al., 1980). The overall steroid balance in these fish, however, is not known. Perhaps the fish can adapt to the "induced" situation by increasing steroid synthesis to match catabolism. But it is also possible that these changes in steroid catabolism could prove to be detrimental to the fish by decreasing circulating steroid levels. The importance of this for the reproduction of fish in contaminated areas remains to be evaluated.

Appendix A

MATURE FISH

Stegeman and Chevion (1980) have identified sex differences in brook and rainbow trout similar to those seen in rats in cytochrome P-450 content and NADH-cytochrome c reductase activity. They have also demonstrated possible differences in types of cytochromes P-450 between males and female trout according to the effect of 7,8-benzoflavone on MFO activity. In this section, various parameters measured by Stegeman and Chevion (1980) were verified in adult trout from the same sources as the fish used in this thesis.

Materials and Methods

Eight brook trout, Salvelinus fontinalis, in spawning condition were collected from the Sandwich Fish Hatchery, East Sandwich, Massachussetts Division of Fisheries and Game in November, 1981. These had been kept in outdoor raceways and fed Rangin's Production Pellets (Zeigler Bros., Gardners, PA) twice daily at about 2% body weight. The water supplying the raceways is from a small nearby pond and was at 8°C at the time of sampling. The fish were transported to the lab (transit time approximately one hour) in their own water and sampled there.

While performing gonadectomies on trout for the experiments of chapter 4 in December, 1980, four males in spawning condition were found. These were sampled and are included here for comparison. They are identified as Ontario trout since they were from Goossen's Trout Hatchery, Otterville, Ontario (other details about holding facilities etc. are found in Ch. 4, Methods).

Blood was sampled from the caudal vein with a heparinized syringe and the fish killed by cervical section. Livers were immediately excised and placed in ice-cold 50mM Tris buffer pH 7.3 with 1.15% KCl. Blood was centrifuged at 1500 x g for ten minutes and the plasma was frozen at -20°C. Livers were blotted dry, weighed, and then homogenized in 5

vol buffer using a Potter-Elvehjem tissue grinder. Microsomes were prepared by differential centrifugation as described by Stegeman and Binder (1979), and resuspended to approximately 3-8 mg protein/ml in 50mM Tris buffer pH 7.5 with 20% glycerol and 1.0 mM Dithioerythretol-EDTA. Aliquots were immediately frozen and stored in liquid nitrogen.

All other methods have been described previously.

The data were evaluated for significant differences between males and females by means of a student's t-test (SPSS program). Arcsine transformations were made of the results for hepatosomatic and gonadosomatic indices prior to statistical analysis since these were percentages (Sokal and Rohlf, 1969). Differences between group means were considered to be statistically significant if $p \leq .05$, and were considered to show trends if $.05 \leq p \leq .10$.

Results and Discussion

The data presented in Table 7 (p. 103) show a pattern of monooxygenase and other activities very similar to those previously seen by Stegeman and Chevion (1980). For example, NADPH- and NADH-cytochrome c reductase activities and cytochrome P-450 levels in the Ontario trout were similar to those reported by Stegeman and Chevion (1980). The P-450 levels in Massachussetts trout, however, were consistently lower than those seen in other trout from the same genetic stock (ibid.).

The fish were all in spawning condition and could be easily stripped. The GSI values (Table 7, p. 103) are similar to those reported by others (Stegeman and Chevion, 1980) for spawning trout. As expected, there were no significant differences in HSI since the females were past the stage of vitellogenesis (Koivusaari et al., 1981). There were no significant differences between males and females in cytochrome b5 or in microsomal protein concentrations. Cytochrome P-450 levels, in contrast, were significantly higher in males than females when expressed on the basis of protein concentration or liver weight, and showed a trend towards an increase when expressed on the basis of body weight.

The results here show that adult trout have sex differences in cytochrome P-450 content similar to those reported

by Stegeman and Chevion (1980). Therefore, the primary assumption of this thesis, that the fish used in these studies show sex differences in P-450 levels at maturity, has been verified.

TABLE 7

Mature brook trout

Activity or quantity of components of microsomal electron transport systems and other characters in spawning trout from two sources.
Values shown are means ± standard deviation.
Sample size is shown in parentheses.

↙↘ significant sex difference ($p \leq .05$)

Character	Group I (Mass. Nov. 81)		Group II (Ont., Dec. 80)
	Females (4)	Males (4)	Males (4)
Body wt. (g)	131.1 ± 13.6	158.4 ± 15.0	89.5 ± 11.0
HSI (%)	1.2 ± 0.2	1.3 ± 0.2	1.2 ± 0.4
GSI (%)	15.1 ± 4.0	2.0 ± 0.3	1.1 ± 0.4
Microsomal protein (mg/g liver)	24.8 ± 3.1	22.1 ± 0.9	14.5 ± 1.4
NADPH-cytochrome c red. (nmoles/min/mg prot.)	—	—	68.7 ± 7.1
NADH-cytochrome c red. (nmoles/min/mg prot.)	—	—	160.5 ± 30.4
Cytochrome b_5 (pmoles/mg protein)	26.0 ± 12.9	24.3 ± 6.3	—
Cytochrome P-450 (nmoles/mg protein)	0.12 ± 0.01	0.20 ± 0.02	0.36 ± 0.05
Cytochrome P-450 (nmoles/g liver)	3.0 ± 0.6	4.4 ± 0.5	5.3 ± 1.3
Cytochrome P-450 (nmoles/g body)	0.04 ± 0.02	0.06 ± 0.01	0.06 ± 0.01

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