The effects of 17β-estradiol injection on *in vitro* thyroid hormone deiodination in liver, brain, gill, heart, and kidney of female and male rainbow trout (*Oncorhynchus mykiss*) at different states of sexual maturity

by

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A Thesis
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THE EFFECTS OF 17ß-ESTRADIOL INJECTION ON *IN VITRO* THYROID HORMONE DEIODINATION IN LIVER, BRAIN, GILL, HEART, AND KIDNEY OF FEMALE AND MALE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AT DIFFERENT STATES OF SEXUAL MATURITY

BY

SUSANNA C. WIENS

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University

of Manitoba in partial fulfillment of the requirements of the degree

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Master of Science

Abstract

17β-estradiol (E_2) effects on *in vitro* thyroid hormone (TH) deiodination in various tissues were studied in 3 experiments (ExI, ExII, ExIII) for female and male rainbow trout at different states of sexual maturity. ExII trout were the biggest and most sexually mature (ripe ovaries), followed by ExI trout which were vitellogenic, and finally ExIII trout, which were previtellogenic and a different genetic stock from ExI and ExII. Trout were injected intraperitoneally on Days 1 and 3 with E_2 in peanut oil or peanut oil alone, and sacrificed on Day 7. In ExI and ExII, one dose was used (0.5 mg E_2/100 g body mass (bm)); in ExIII, 3 doses were used (0.05, 0.1 or 0.5 mg E_2/100 g bm). Depending on the experiment, the activities of the following pathways were measured *in vitro* in liver, brain, gill, heart and kidney microsomes: T_4-outer-ring deiodination (T_4-ORD), producing 3,5,3′-triiodothyronine (T_3, the biologically active TH) from thyroxine (T_4, the secreted prohormone); T_4-inner-ring deiodination (T_4-IRD), producing inactive 3,3′,5′-triiodothyronine (rT_3); T_3-IRD, producing inactive 3,3′-diiodothyronine (T_2); rT_3-ORD, producing inactive T_2. In ExI and ExII but not ExIII, plasma [T_3] was significantly lower in E_2-treated trout than controls except for ExII female plasma [T_3], which was not significantly lower. Plasma [T_4] was not affected. In ExI and ExII but not ExIII, liver T_4-ORD activity was significantly lower in E_2-treated trout than controls. Liver rT_3-ORD was also significantly lower in E_2-treated trout than controls. In ExI but not ExII or ExIII, liver T_3-IRD activity was slightly but significantly lower in E_2-treated trout than controls and brain T_3-IRD activity was slightly higher in E_2-treated trout than controls. Kidney T_3-IRD activity was significantly higher and female kidney T_4-IRD was higher in E_2-treated trout than controls. In ExIII, there were no significant differences in

deiodination rates or plasma [TH] between controls and E_2-treated trout at any dose. Therefore no conclusion could be drawn on whether doses lower than 0.5 mg E_2/100 g bm affect TH deiodination. The lack of responsiveness to E_2 in ExIII trout was likely due to their different genetic stock from ExI and ExII trout. This study indicates that E_2 depresses thyroidal status in female and male rainbow trout, in later stages of sexual maturity, seen as extremely depressed liver T_4-ORD and plasma [T_3]. E_2 had no effect on liver IRD pathways that could have contributed to decreased plasma [T_3]. Although E_2 substantially depressed liver rT_3-ORD activity *in vitro*, this likely has little influence on plasma [TH] *in vivo*. E_2 also depresses local thyroidal status in brain and kidney seen as increased IRD activity. In kidney, this E_2 effect to increase IRD activity could have contributed to the depressed plasma [T_3] observed. It is concluded that in vitellogenic and sexually mature rainbow trout, E_2 depresses thyroidal status mainly by decreasing systemic T_3 availability by depressing liver T_4-ORD activity, with some limited induction of IRD activities in peripheral tissues to decrease local T_3 availability. State of sexual maturity, sex and genetic stock influence response to E_2 by deiodination pathways.

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List of Abbreviations

ANOVA	analysis of variance
bm	body mass
CPM	counts per minute
DTT	dithiothreitol
ER	estrogen nuclear receptor
ExI	Experiment I
ExII	Experiment II
ExIII	Experiment III
E_2	17β-estradiol
GSI	gonadosomatic index (gonad weight as percentage body weight)
GTH	gonadotropin
HR	heart rate
HSI	hepatosomatic index (liver weight as percentage body weight)
HPLC	high performance liquid chromatography
IRD	inner ring deiodination
K_m	enzyme affinity (Michaelis-Menten Constant)
MBC	maximum binding capacity
NSB	non-specific binding
ORD	outer ring deiodination
ppm	parts per million
PST	parr-smolt transformation
RBC	red blood cells
RIA	radioimmunoassay
rT_3	reverse T_3 (3,3',5'-triiodothyronine)
$*rT_3$	^{125}I-labeled rT_3
T	testosterone
T_2	3,3'-diiodothyronine
T_3	3,5,3'-triiodothyronine
$*T_3$	^{125}I-labeled T_3
T_4	thyroxine
$*T_4$	^{125}I-labeled T_4
TG	thyroglobulin
TH	thyroid hormones
*TH	^{125}I-labeled TH
TMS	tricaine methanesulfate
V_{max}	maximal velocity of enzyme reaction
VTG	vitellogenin

1 Introduction

In poikilotherms, including fish, the major function of the thyroid system is in mediating metabolic processes such as growth, differentiation (or maturation) and reproduction by various, mainly permissive, actions of thyroid hormones (TH) (Eales, 1990). This is in contrast to homeotherms, where TH have major metabolic roles but which are primarily thermoregulatory (McNabb, 1992).

17β-estradiol (E_2) is an essential hormone for sexual maturation in female salmonid fish. Its specific reproductive function is to induce oocyte growth by stimulating hepatocytes to both synthesize the egg-yolk precursor vitellogenin (VTG) (Ng and Idler, 1983) and to synthesize zona radiata proteins (Oppen-Bernsten *et al.*, 1992).

During the seasonal reproductive cycle of salmonid fish, there are dramatic fluctuations in levels of E_2 and other sex steroids. However, some non-reproductive hormones including TH also fluctuate at this time. For example in females of several species, as the ovary matures, E_2 levels increase and TH levels decrease, and once E_2 levels drop, TH levels increase sharply again. In salmonids, TH promote E_2 synthesis and the onset of vitellogenesis, however for majority of the reproductive cycle and periods of somatic growth, thyroid hormone function (thyroidal status) generally appears to have an inverse relationship with reproductive function (Cyr and Eales, 1996).

In fish, thyroxine (3,5,3'5'-tetraiodothyronine; T_4) is the main TH secreted from the thyroid gland into the plasma. However, T_4 is relatively inactive compared with its metabolite, 3,5,3′-triiodothyronine (T_3), which is formed from T_4 by the enzymatic

removal of iodine from the outer ring of the TH molecule. This process is referred to as T_4-outer-ring deiodination (T_4-ORD). There are also deiodination pathways that remove iodine from the inner ring of the TH molecule, converting T_4 into an inactive triiodothyronine isomer (3,3′,5′-T_3 = rT_3) and degrade T_3 to an inactive diiodothyronine (3,3′-diiodothyronine = T_2). These pathways are known as T_4-inner-ring deiodination (T_4-IRD) and T_3-inner-ring deiodination (T_3-IRD), respectively. The major site of T_3 formation and the probable systemic T_3 source is the liver, which can be considered the hub of TH metabolism (Eales and Brown, 1993). Deiodinating enzymes also occur in brain, gill, heart, kidney, gut, retina and skin, but they likely regulate local TH levels and do not contribute significantly to systemic T_3 levels (Eales *et al.*, 1999). Thus by altering activities of these various deiodination pathways, T_3 availability at the tissue level can be tightly controlled.

Because of this potential for regulated control of T_3 generation and degradation by deiodination, TH deiodination is an important regulator of active TH levels in the blood and tissues and therefore of thyroidal status (Eales and Brown, 1993). Furthermore TH deiodination is an effective indicator of thyroidal status (Eales *et al.*, 1999).

TH deiodination activity is sensitive to physiological states. A general pattern that has been observed is that when salmonid fish are in anabolic states (e.g. increased food, increased growth hormone levels, increased testosterone levels) the activities of various deiodination pathways are adjusted to maximize T_3 availability (increased T_4-ORD activity, decreased IRD activity). In contrast, when salmonid fish are in catabolic states (e.g. starvation, stress factors), the activities of deiodination pathways are adjusted

to minimize T_3 availability (T_4-ORD activity decreased, IRD activity increased) (Eales and MacLatchy, 1989).

In order to establish how the thyroid system and reproductive system in salmonid fish are related, there have been several studies in which E_2 was administered to fish and responses of various indicators of thyroidal status were measured, most commonly plasma T_3 and T_4 levels (Cyr and Eales, 1996). Studies on E_2 effects on TH deiodination in rainbow trout have been limited to liver T_4-ORD activity (Cyr *et al.*, 1988a; Flett and Leatherland, 1989a) and the most conclusive studies have been limited to immature fish (Cyr *et al.*, 1988a).

Cyr *et al.* (1988a) injected immature rainbow trout of both sexes with 0.5 mg/100 g body mass (bm) E_2-3-benzoate in peanut oil on days 0 and 3, which raised plasma [E_2] to levels comparable with vitellogenic females, and depressed thyroidal status by day 7, observed as decreases in plasma [T_3] and liver microsomal T_4-ORD activity. E_2 effects on the other deiodination pathways in liver and other tissues of rainbow trout were not studied. Furthermore, sex differences in response to E_2 were not established. Also, it is unknown whether the thyroidal status of sexually mature trout would also be depressed by E_2 treatment.

The overall objective of my Master's research was to explore more completely the effects of E_2 on TH deiodination of rainbow trout. There were four specific objectives. First, I wanted to determine whether the effect of E_2 to depress T_4-ORD activity and plasma [T_3] occurred in females and males at various stages of sexual maturity and of different genetic stocks. Genetically manipulated all-female trout are commonly used in hatcheries and therefore it is of interest to determine if their response to E_2 is different

from normal trout. Second, I wanted to determine whether liver deiodination pathways other than T4-ORD are affected by E2. Based on studies where inhibition of liver T4-ORD by various treatments was accompanied by an induction of T4-IRD and T3-IRD, I would expect that E2 treatment will induce T4-IRD and T3-IRD in liver. Third, I wanted to determine whether E2 has effects on deiodination in tissues other than liver. Fourth, I wanted to determine whether intraperitoneal doses lower than 0.5mg/100 g bm affected TH deiodination in rainbow trout liver and brain. Given that plasma E2 levels lower than achieved by this dose occur over the course of the annual reproductive cycle and might be encountered due to pollution of aquatic ecosystems, it is important to determine whether lower doses alter thyroidal status.

In order to address these objectives, three *in vivo* E2-exposure experiments were performed on rainbow trout at three different stages of sexual maturation, following the general protocol of Cyr *et al.* (1988a). The fish used in each experiment were chosen to vary in size and age and state of sexual maturity. In Experiment I and II, approximately equal numbers of males and females were administered 0.5mg/100g bm E2 or E2-free vehicle by intraperitoneal injection. Experiment III was similar except that a genetically manipulated all-female stock was used and two additional lower doses of E2 were administered. Deiodination rates of several deiodination pathways in various tissues were measured and other indicators of thyroidal status were measured and compared between control and E2-injected fish. Control groups from the three experiments were also compared in order to quantify the differences and establish the nature of the differences in both state of sexual maturity and thyroidal status among the three groups.

2 Literature Review

2.1 The Thyroid System of Salmonid Fish

2.1.1 Overview of the Salmonid Thyroid System

Thyroid system function in salmonid fish comprises TH biosynthesis and secretion, TH transport, TH metabolism and excretion, and TH actions (Fig. 1) (for review see Eales and Brown, 1993). Like most fish, but unlike most other vertebrates, salmonid fish have an diffuse, non-encapsulated thyroid gland, with follicles scattered around the base of the first three branchial arches. Thyroglobulin (TG) is a glycoprotein synthesized in the thyroid follicle cells, which is then exocytosed into the colloid where it is stored. While TG is in the colloid but adjacent to the apical membrane of the follicle cells, its tyrosine residues are iodinated and coupled by iodoperoxidase in the apical membrane of the follicle cells. Under the stimulation of thyroid stimulating hormone produced by thyrotropes in the pituitary in response to signals from the hypothalamus, iodinated TG is taken up from the colloid into the follicle cells by pinocytosis. Lysosomes fuse with the TG vacuole, and TG is digested, releasing T_4 and possibly other TH to a lesser degree, which are then secreted to the blood stream. T_4 is an amino acid with an inner tyrosyl ring and an outer phenolic ring which both have iodine substituted at the 3 and 5 positions (Fig. 2). Once secreted into the blood stream, TH are reversibly bound to plasma proteins, which facilitate TH transport and entry into tissues. Once in cells, various pathways metabolize TH. T_4 may be deiodinated by specific deiodinase enzymes that remove iodine atoms to generate iodide. This either activates or inactivates

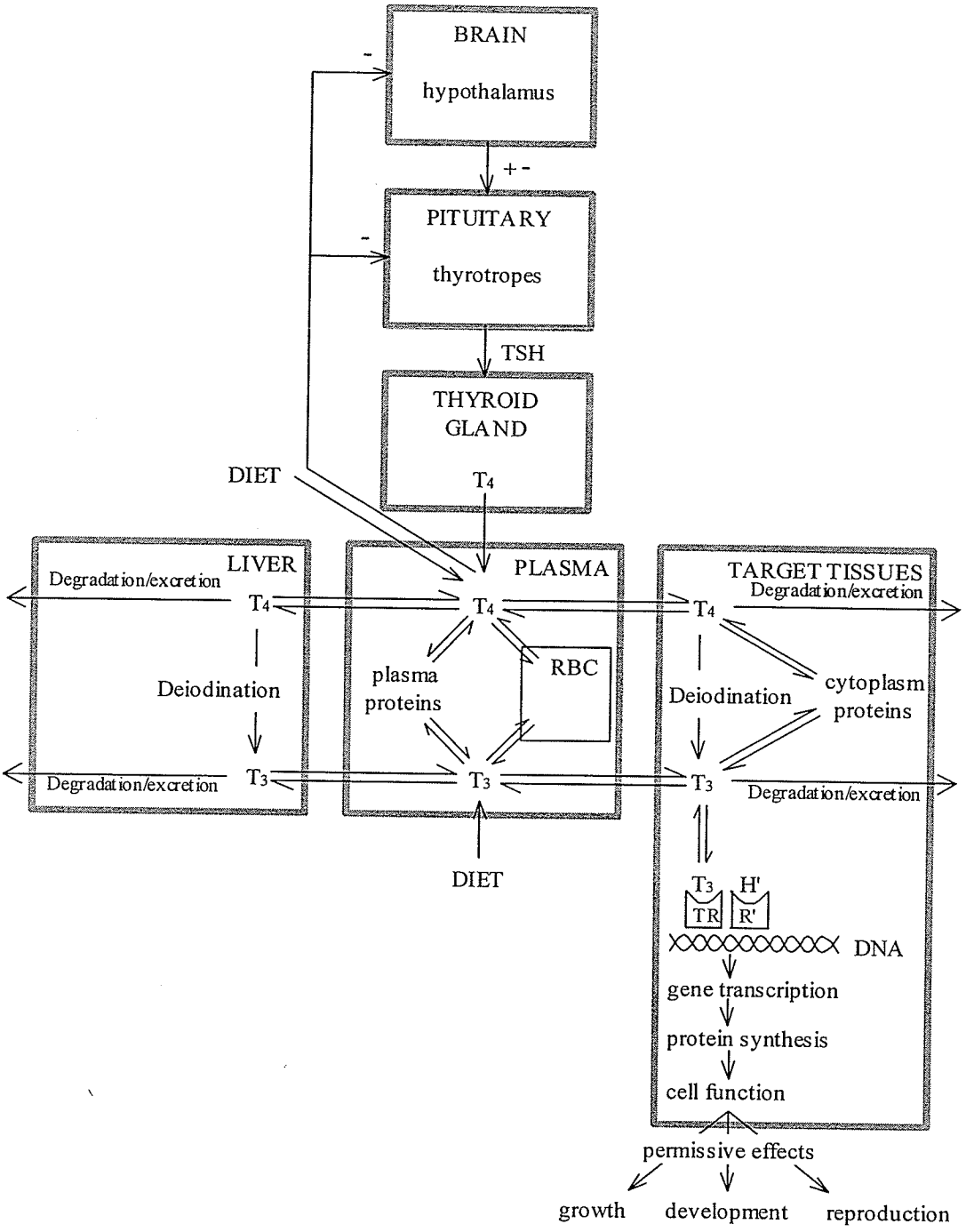

Fig. 1 Overview of thyroid system function in salmonid fish. Abbreviations used and not defined in the text: TSH = thyroid stimulating hormone; TR = TH receptor; H′/R′ = the hormone/receptor complex forming a dimer complex with TH/TR. (Adapted from: Eales and Brown, 1993; Eales *et al.*, 1999.)

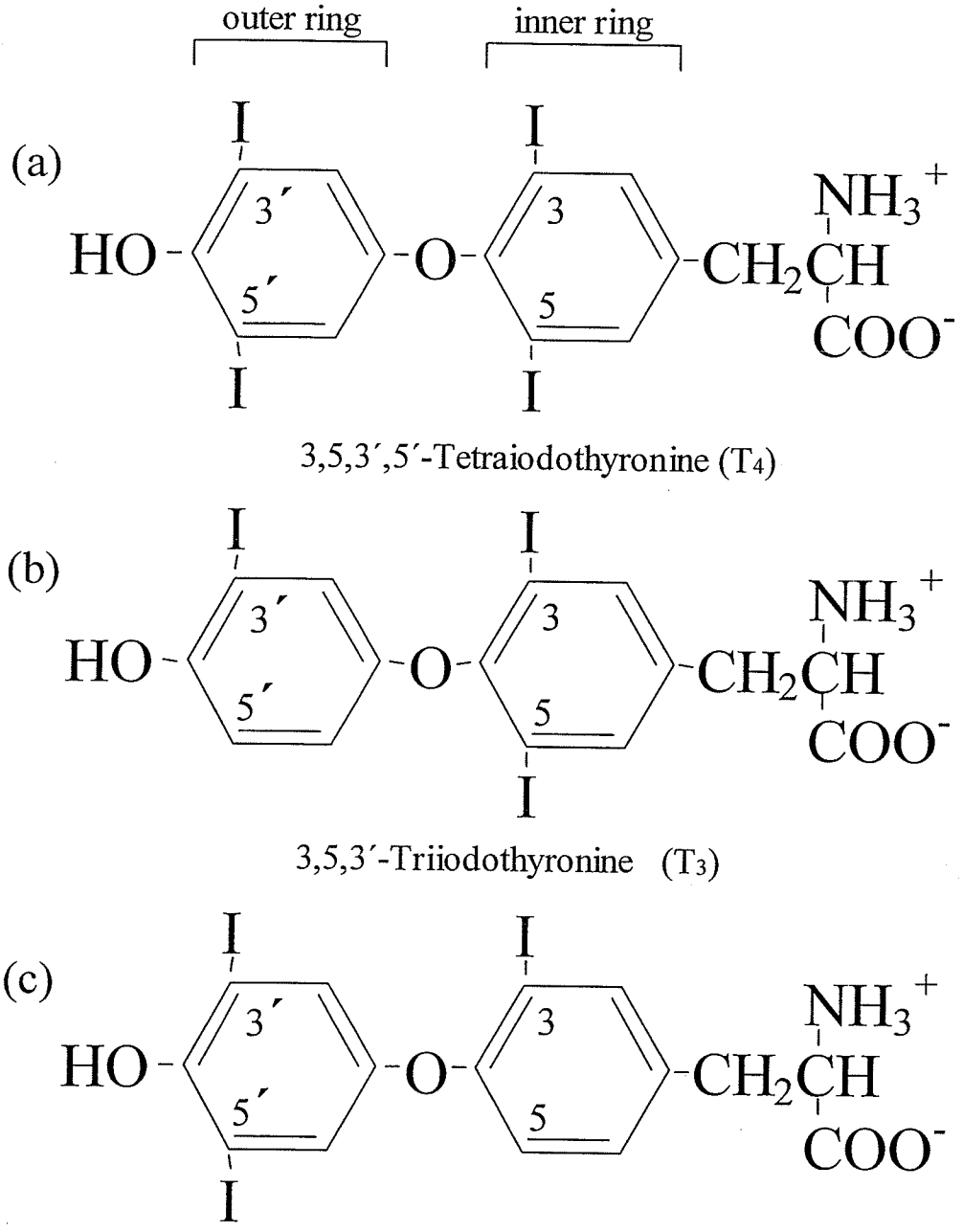

Fig. 2 Molecular structure of three thyroid hormone forms: (a) T_4, the TH secreted from the thyroid gland and relatively inactive; (b) T_3, the active form; (c) rT_3, the inactive form. The number of iodines and their positions on the inner or outer ring of the molecule affect the biological activity of the hormone.

the hormone, depending on the ring position from which it was removed. T_3 (Fig. 2) is the biologically active form of TH formed by deiodination of T_4. TH may also be conjugated forming glucuronides or sulphates, which are more water-soluble than non-conjugated TH and therefore are more readily excreted in bile and urine. T_3 may act in the tissue in which it is formed, or it may be transported in the blood to other target tissues where it exerts actions. The classical route of TH action is via nuclear receptors, to which T_3 binds forming either a homodimer with another T_3 molecule bound to a receptor, or a heterodimer with retinoic acid bound to its receptor. The dimer complex then binds to DNA acting as a transcription factor, affecting transcription and eventually protein synthesis. Several nongenomic T_3 actions have also been documented in mammals (Davis and Davis, 1996) and salmonid fish (Cyr and Eales 1989a). In salmonid fish, TH act mainly permissively on development, growth and reproduction.

2.1.2 Thyroid Hormone Transport

2.1.2.1 TH Binding in the Bloodstream

TH are transported by the bloodstream to tissues where they will be metabolized or will exert their actions. TH in the bloodstream are found both in red blood cells (RBC) and plasma.

In rainbow trout, 5-11% of T_4 and 14-23% of T_3 and 23-24% of rT_3 in whole blood is bound reversibly to sites in RBC (McLeese *et al.*, 1998). T_4 in RBC exchanges slowly with plasma and T_3 in RBC exhanges rapidly. The rapid T_3 exchange may buffer plasma T_3 levels, assist T_3 delivery to tissues, or supply RBC T_3 receptors with T_3 (McLeese *et al.*, 1998).

The majority of T_3 and T_4 in whole blood is found in the plasma, either bound to plasma proteins or free. About 99% of both plasma T_3 and T_4 is reversibly bound to plasma proteins (e.g. brook trout (*Salvelinus fontinalis*) (Falkner and Eales, 1973); rainbow trout (Eales and Shostak, 1985; Cyr and Eales, 1989b; Cyr and Eales, 1992); Arctic charr (*S. alpinus*) (Eales and Shostak, 1985)). T_3 binds with greater affinity to binding sites on plasma proteins than T_4 (Eales, 1987) and therefore the ratio of free T_3 to bound T_3 is usually lower than the ratio of free T_4 to bound T_4 (Eales and Brown, 1993). Free TH are thought to be the proportion available for uptake by tissues (Eales and Brown, 1993; Ekins, 1986).

Total TH is not always proportional to free TH because various physiological factors can affect TH-binding by proteins thus affecting the proportion of TH that are free and therefore available to tissues (Eales and Brown, 1993). For example, temperature and pH affected the proportion of free T_3 and T_4 in plasma of Arctic charr *in vitro* (Eales and Shostak, 1986) and E_2-treatment affected the proportion of free T_3 and T_4 in rainbow trout (Cyr and Eales, 1989b, 1992).

TH-binding proteins are hypothesized to have several functions. They have a passive role in TH transfer from blood to cells. They act as a TH reservoir, minimizing the effects of rapid fluctuations in TH levels due to changes in secretion or use by tissues thus protecting sensitive tissues from major changes in TH. Finally, they prevent TH from being excreted in the urine by preventing glomerular filtration (Ekins, 1986).

2.1.2.2 TH Transport into Cells

In vitro studies on TH uptake by rainbow trout hepatocytes indicate that some TH (about 15% of T_4 and less than 10% of T_3) diffuses across cell membranes, but the

majority is taken up by a more complex mechanism (Riley and Eales, 1993, 1994). The majority of T_4 and T_3 is transported into trout hepatocytes by energy-dependent, carrier-mediated endocytosis. However some features of T_3 uptake are different from that of T_4, suggesting that T_3 and T_4 have distinct iodothyronine transporters (Riley and Eales, 1994).

TH are able to cross the blood-brain barrier and enter brain cells in fish. It has been proposed for mammals that T_4 is transported from the choroid plexus epithelial cells that form the blood-brain barrier into the cerebral spinal fluid in conjunction with the TH-binding protein transthyretin that is synthesized in the choroid plexus epithelial cells (Schreiber *et al.*, 1990). Transthyretin is found in fish brain (Santos and Power, 1999), and therefore this model of TH transport to the brain for mammals may apply to fish.

T_3 and T_4 in the blood exchange with a small, rapid-equilibrating tissue compartment, which could be tissues such as kidney or liver, and also with a slow-equilibrating tissue compartment, which likely is muscle (Eales *et al.*, 1999).

The majority of TH, then, are transported by the blood, bound to plasma proteins. The unbound fraction of plasma TH is available for transport into target tissue cells, which occurs by energy-dependent carrier mediated endocytosis.

2.1.3 Peripheral Metabolism of Thyroid Hormones

2.1.3.1 Deiodination

Deiodination enzymes

Thyroid hormone deiodination is the step-wise enzymatic removal of iodine atoms from T_4 and other iodothyronines, thus activating or inactivating them (Eales and Brown, 1993). The enzymes that carry out TH deiodination are referred to as

deiodinases. Deiodinases are membrane-bound enzymes, found in the cytosolic face of the plasma membrane or endoplasmic reticulum of cells (microsomal fraction) (reviewed for mammals by Köhrle, 1996). Rainbow trout deiodinases are found in the microsomal fraction of liver homogenates and likely in the endoplasmic reticulum rather than plasma membrane (Shields and Eales, 1986). Lower levels of deiodination activity in other subcellular fractions of liver were found to be due to contamination of the fractions with microsomal material.

Deiodinases are selenoproteins, *i.e.* they contain the trace element selenium, and in at least one mammalian isoform of the enzyme and in fish deiodinases, there is a selenocysteine at the active site of the enzyme (St. Germain, 1994; Valverde *et al.*, 1997; Sanders *et al.*, 1997). In rainbow trout, as in mammals, deiodinase activity depends on a thiol cofactor that interacts with the enzyme and its substrate (Shields and Eales, 1986; Köhrle, 1996). The selenocysteine at the active site of the deiodinase is oxidized as part of the deiodination reaction and a selenium-iodine association is formed. The thiol cofactor then reduces the selenocysteine, breaking the selenium-iodine association to regenerate the deiodinase (Köhrle, 1996). The endogenous thiol cofactor has not yet been identified, but for *in vitro* studies, dithiothreitol (DTT) is effective in salmonids (Shields and Eales, 1986).

Deiodination pathways

In the mammalian literature, TH deiodination is generally discussed in reference to particular deiodinase types (distinct enzymes), characterized by several different properties (for review see St. Germain, 1994). Mammalian deiodinases are distinguished by substrate preference, substrate affinity and enzyme mechanism, determined by effects

of thiol cofactors and inhibitors (Leonard and Visser, 1986). However, there is some controversy on the existence and homology of all of the mammalian deiodinases in fish. Therefore, for the purpose of this research, it is more appropriate to consider only the rate of activity of a particular deiodination pathway, without assigning it to a particular deiodinase.

The pathways are named based on the TH deiodinated and the molecular position of the iodine removed (Fig. 2). The tyrosyl ring is referred to as the inner ring, and by convention the iodines are referred to as being at the 3 and 5 positions. The phenolic ring is referred to as the outer ring and by convention, the iodines are referred to as being at the 3′ and 5′ positions.

In salmonids, there are four important deiodination pathways (Fig. 3) (for review see Eales *et al.*, 1993). T_4-ORD is an activating pathway that converts T_4 to T_3 by removal of the 5′ iodine on the outer ring. T_4-IRD is an inactivating pathway, converting T_4 to inactive rT_3 by the removal of the 5 iodine from the inner ring. T_3-IRD is also inactivating, converting T_3 to T_2 by removal of the 5 iodine from the inner ring. rT_3-outer-ring deiodination (rT_3-ORD) is likely an iodine-salvaging step, converting rT_3 to T_2 by removal of the 5′ iodine from the outer ring. T_3-ORD and rT_3-IRD activities are negligible in salmonids, and deiodination of diiodothyronines and monoiodothyronines have not been studied in salmonids (Eales *et al.*, 1999).

Deiodination activity in various tissues

Deiodinases are found in many tissues in salmonids, but their activities vary greatly between tissues (MacLatchy and Eales, 1992a). Furthermore, the relative activities of the ORD and IRD pathways within a tissue indicate whether there is an

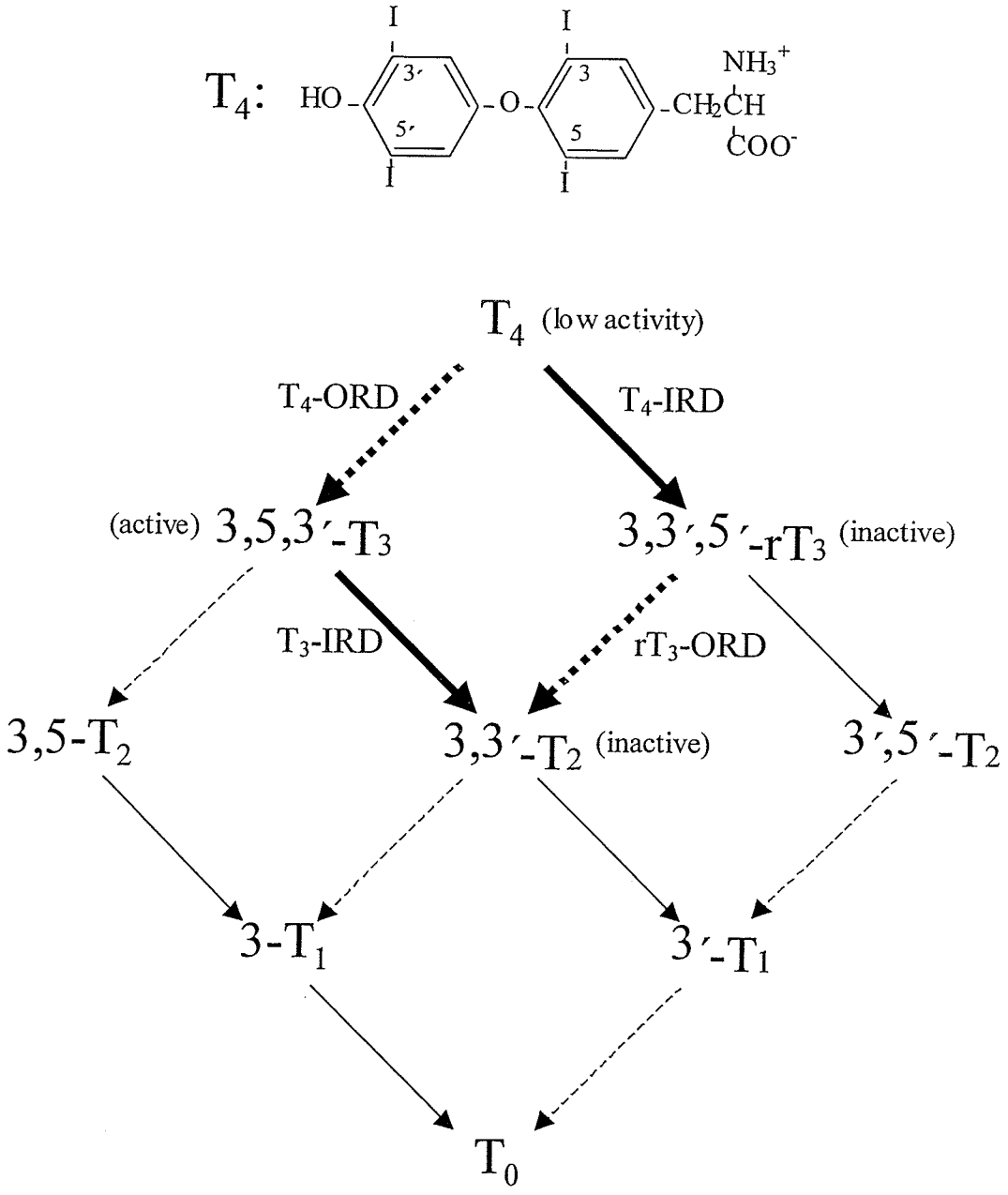

Fig. 3 Stepwise deiodination of the secreted prohormone T_4 by outer ring deiodination (ORD) pathways (dashed arrows) and inner ring deiodination (IRD) pathways (solid arrows) to more or less active TH forms. The four most active deiodination pathways in rainbow trout tissues are T_4-ORD, T_3-IRD, T_4-IRD and rT_3-ORD. These pathways are indicated by heavy arrows. (Adapted from: Eales and Brown, 1993.)

overall production or breakdown of T_3, indicating to some extent whether the tissue is a contributor to systemic T_3 levels, or whether it acts more to regulate local T_3 levels.

a) Liver

In rainbow trout, liver T_4-ORD is the predominant deiodination pathway in comparison with other liver deiodination pathways and deiodination pathways in other tissues. High performance liquid chromatography (HPLC) analysis of *in vitro* hepatic deiodination products of T_4 and T_3 indicates that rT3 formation by T_4-IRD and T_2 formation by T_3-IRD are negligible compared to T_3 formation by T_4-ORD (Sweeting and Eales, 1992a; MacLatchy and Eales, 1993; Frith and Eales, 1996). In Atlantic salmon (*Salmo salar*) throughout 5 weeks of parr-smolt transformation (PST), liver had high T_4-ORD activity compared to other tissues and T_4-ORD activity was correlated with T_3 levels, suggesting that liver was the main source of T_3 for systemic use (Morin *et al.*, 1993). Therefore in salmonids, T_4-ORD activity is a useful index of systemic potential for T_3 production, and hence of thyroidal status (Eales and Brown, 1993).

MacLatchy and Eales (1992a) determined the enzyme kinetics of liver T_4-ORD. They determined maximal velocity of enzyme reaction (V_{max}, an indicator of the number of functional units of enzyme) and enzyme affinity for substrate (K_m, Michaelis-Menten constant). Enzyme kinetics indicated that there were two distinct T_4-ORD systems in rainbow trout liver. One was a low-K_m enzyme (low K_m indicates high enzyme affinity), with K_m of 0.098 nM and V_{max} of 3.74 pmol/hr/mg protein with the relatively high V_{max}/K_m ratio (measure of enzyme efficiency) of 38.2. This K_m suggests the enzyme would function to deiodinate T_4 at physiological levels. The other was a high K_m enzyme, with K_m of 10.0 nM and V_{max} of 8.21 with a relatively low V_{max}/K_m ratio of 0.82.

MacLatchy and Eales (1992a) suggested that the high K_m T_4-ORD would only be active when plasma T_4 levels were high, like during parr-smolt transformation, or ingestion of T_4 (e.g. thyroid gland of another fish consumed).

rT_3-ORD activity is present in liver (Finnson *et al.*, 1999). rT_3 is deiodinated in liver by the low K_m T_4-ORD system (Frith and Eales, 1996) and a high K_m rT_3-ORD system (Finnson *et al.*, 1999).

b) Brain

T_4-IRD and T_3-IRD are the predominant deiodination pathways in rainbow trout brain (Frith and Eales, 1996). Thus, Frith and Eales (1996) concluded that under normal conditions, whereas liver has the potential for T_3 production (high T_4-ORD activity), brain has the potential for T_3 inactivation or degradation (T_4-IRD and T_3-IRD higher than T_4-ORD).

Fines *et al.*, (1999) also found that T_3-IRD was the most active deiodination pathway in the brain, followed by T_4-IRD, and there was low T_4-ORD activity. T_3-ORD activity was undetectable. Furthermore, T_4-IRD and T_3-IRD activities differed between brain regions (Fines *et al.*, 1999). Variations in IRD rates in the different regions could either reflect different TH requirements in different parts of the brain or different rates of TH entry into the various parts of the brain across the blood-brain barrier (Fines *et al.*, 1999). T_4-ORD activity did not vary between brain regions.

In newborn rats, Type 3 deiodinase activity, which corresponds functionally to T_4-IRD and T_3-IRD in fish (Frith and Eales 1996), was selectively expressed in brain regions relating to sexual differentiation during the first 10 days after birth, suggesting

that at least in mammals, local regulation of deiodination pathways in brain is important in determining sexual function (Escamez *et al.*, 1999).

c) Gill, kidney, muscle

MacLatchy and Eales (1992a) determined enzyme kinetics of T_4-ORD activity in gill, kidney, and white skeletal muscle of rainbow trout. Gill and muscle had a low K_m T_4-ORD resembling the low K_m liver T_4-ORD. Kidney had a high K_m T_4-ORD system resembling the high K_m liver T_4-ORD. V_{max}/K_m ratios indicated that gill and muscle had 10-fold lower enzyme efficiency than the low K_m T_4-ORD of liver, but 5 to 10 times greater enzyme efficiency than the high K_m T_4-ORD of kidney or liver.

The moderate T_4-ORD activity in muscle could contribute substantially to muscle T_3 content and even plasma T_3 levels (MacLatchy and Eales,1992a) since muscle makes up 67% of body mass, and contains 80% of carcass T_3 (Fok *et al.*, 1990).

2.1.3.2 Roles of deiodination activity in maintaining or modulating thyroidal status

In rainbow trout faced with a modest T_3 challenge, T_3 homeostasis is maintained by co-ordinated changes in deiodination activities (Eales *et al.*, 1993). Raising plasma T_3 levels by administering T_3 depresses T_3 generation by liver T_4-ORD by decreasing V_{max} (Sweeting and Eales, 1992b; MacLatchy and Eales, 1993). An experimental increase of T_3 levels also stimulates liver T_4-IRD and T_3-IRD activities. This reduces the amount of T_4 available to produce T_3 and degrades T_3 to T_2 (Sweeting and Eales, 1992b; MacLatchy and Eales, 1993; Fines *et al.*, 1999). In gill, T_3 challenge depressed T_4-ORD and induced T_4-IRD but not T_3-IRD (MacLatchy and Eales, 1993). In kidney, T_3 and T_4 challenge depressed the high K_m T_4-ORD but did not enhance T_3-IRD or T_4-IRD activities

(MacLatchy and Eales, 1993). This suggests that there is a T_3 set-point in plasma or tissue, and this set-point is autoregulated.

Eales and MacLatchy (1989) suggest that under different physiological states there are different set points for systemic T_3 levels and that transition to a new set point is achieved and then maintained by changes in deiodination activity. The general trends that have been observed are that T_3 generation (T_4-ORD activity) is enhanced when fish are in conditions favouring somatic growth (anabolic states) such as high nutrient intake, androgen administration or growth hormone administration (Eales and MacLatchy, 1989). T_3 generation is suppressed (depressed T_4-ORD and enhanced T_4-IRD and T_3-IRD) when fish are in conditions unfavourable to somatic growth (catabolic states) such as starvation, and stress. Changes in liver T_4-ORD activity are generally due to changes in V_{max} with no effect on K_m (Eales *et al.*, 1993).

Short-term treatment of immature arctic charr (*Salvelinus aplinus*) with testosterone (T), methyl T, or T propionate all increased thyroidal status by increasing plasma T_3 levels, which at least in part was due to increased hepatic T_4-ORD activity (MacLatchy and Eales, 1988). Short-term treatment of immature rainbow trout with growth hormone also increased plasma T_3 levels and liver T_4-ORD activity. IRD activities were not measured (MacLatchy, 1991). Warmer acclimation and assay temperature also increase T_4-ORD activity in rainbow trout liver microsomes. Temperature did not affect the negligible liver T_3-IRD or T_4-IRD activities (Johnston and Eales, 1995).

A decrease in nutritional state due to decrease in food quantity or quality depresses thyroidal status seen as a lowering of liver T_4-ORD activity, and consequently

decreased plasma T_3 levels (for review see Eales, 1988). Shields and Eales (1986) found that starvation of juvenile (100g) rainbow trout for 2 weeks resulted in decreased liver microsome T_4-ORD activity due to lower V_{max}. Transport and handling stress also appear to depress thyroidal status temporarily. Transport from the hatchery to the laboratory temporary depressed T_4-ORD activity and induced T_4-IRD and T_3-IRD activities in 2-year-old male and female rainbow trout from 1-3 days after transport (Johnston *et al.*, 1996). Handling and blood removal from 4-5-year-old male and female rainbow trout induced liver T_3-IRD one day after handling, but did not alter T_4-ORD, T_4-IRD, or plasma T_3 or T_4 levels (Todd and Eales, 2002).

2.1.3.3 Conjugation

Conjugation is an important alternate route of peripheral thyroid metabolism. Glucuronidation is the linking of glucuronic acid to the 4′-hydroxyl group of a TH molecule, catalyzed by glucuronyl transferases in endoplasmic reticulum (Finnson and Eales, 1997). Sulfation is the linking of sulfate to the 4′-hydroxyl group of a TH molecule by sulfotransferases (Finnson and Eales, 1998). T_4, T_3, rT_3 and T_2 are all glucuronidated and sulfated in rainbow trout liver, however rT_3 is the preferred iodothyronine substrate for these enzymes (Finnson and Eales, 1998; Finnson and Eales, 1999). Conjugated TH are biologically inactive and have higher water solubilities than non-conjugated TH and are excreted via bile (Finnson and Eales, 1996) and possibly in urine and across the gill (Eales *et al.*, 1999).

2.1.4 TH Target Tissues

2.1.4.1 TH Receptor Distribution

Bres and Eales (1988) identified putative T_3 receptors in rainbow trout liver, brain, gill, kidney, RBC, but not in spleen. These putative receptors were high-affinity, limited-capacity T_3-binding sites. T_4 also likely binds to the same sites but with 7 to 10 times lower affinity than T_3, while rT_3 has negligible affinity (Bres and Eales, 1986; Van Der Kraak and Eales, 1980). Similar binding sites have been identified in coho salmon (*O. kisutch*) liver and brain (Darling *et al.*, 1982). Maximum binding capacity (MBC, number of receptors) varied between tissues: liver had the highest MBC, followed by gill, kidney, brain and RBC (each about half the MBC of the previous tissue listed) (Bres and Eales, 1988). These differences support the binding site as a physiological receptor, since the MBC should reflect tissue responsiveness to the hormone, and in all of the tissues where T_3 receptors were found, T_3 is known to or hypothesized to have actions. T_3 and T_4 receptors were also present in arctic charr and rainbow trout pituitary, binding T_3 with 20 to 50 times greater affinity than T_4 (Bres and Eales, 1990). The binding affinity for T_3 was greater in pituitary than for liver, gill, kidney and brain, but comparable to RBC. The binding capacity in pituitary was less than for liver but greater than that for all other tissues. Binding capacity could have been under-estimated, since all pituitary cell types were included but only certain cell types (e.g. thyrotropes) may contain T_3 receptors.

2.1.4.2 Tissue Sources of Receptor-Bound T_3

MacLatchy and Eales (1992b) determined major differences in the source of receptor-bound T_3 in rainbow trout liver, gill and kidney. In gill, 76% of receptor-bound T_3 was formed intracellularly indicating that in gill, T_3 acts more as an intracellular

regulator than a hormone. They suggested that the role of T_3 in gill could be as a second messenger in that various signals could affect gill T_4-ORD, and the modified T_3 levels would result in some effect in gill. In liver, 50% of receptor-bound T_3 was formed intracellularly. At the other extreme, in kidney, 28% of receptor-bound T_3 was formed intracellularly. Therefore, in kidney, T_3 was acting as a classic hormone, in that it was transported in the blood to its target tissue. Both gill and liver have a low K_m (high affinity) T_4-ORD, which was associated with the high proportion of nuclear-bound T_3 intracellularly. Kidney, on the other hand, only has a high K_m (low affinity) T_4-ORD, which was associated with the relatively low proportion of nuclear-bound T_3 formed intracellularly (MacLatchy and Eales, 1992b).

This may be relevant to T_3 autoregulation (MacLatchy and Eales,1992b). If liver and kidney have roles in TH metabolism and excretion, and furthermore if T_3 formation in those tissues contributes to systemic T_3 levels, then by binding T_3 from extracellular sources the tissue could effectively monitor systemic T_3 levels and adjust net T_3 formation in the tissue by alterations in deiodination activities.

2.1.4.3 Thyroid Hormone Function in Liver, Brain, Gill, Heart and Kidney

TH have several permissive actions in various tissues, many of which are only hypothetical. However it can be assumed that where there are TH receptors, and where there are regulated TH deiodination pathways that control the levels of TH in the tissue, TH likely have a physiological function or action in that tissue.

Liver forms the bulk of systemic T_3. However, there are also T_3 receptors in liver (Bres and Eales, 1988; MacLatchy and Eales, 1992b). TH likely have several hepatic

functions including possible feedback control of deiodination enzyme activity (Eales *et al.*, 1993).

Brain is a target tissue for TH in mammals. Although TH actions in fish brain have not been extensively studied, TH can enter fish brain from blood. Furthermore, rainbow trout brain has putative high-capacity, limited-affinity T_3 receptors (Bres and Eales 1988). TH may have some role in olfactory imprinting during PST of Atlantic salmon (Morin *et al.*, 1993).

There are nuclear T_3 receptors in gill (Bres and Eales, 1988) and the majority of T_3 that binds to them is formed in gill by T_4-ORD (MacLatchy and Eales, 1992b). This means that there would be an intracellular T_3 supply even when plasma T_3 levels are low. It has been hypothesized that T_3 could have a role in osmoregulation because T_3 levels are correlated with gill ATPase activity during PST of salmonids (Folmar and Dickhoff, 1981). If TH have a role in osmoregulation, they could also affect the kidney (Bres and Eales, 1988).

T_3 formation by T_4-ORD in heart appears to have possible roles during parr-smolt transformation. For Atlantic salmon, heart size (cardiac somatic index) increased over PST (Morin *et al.*, 1993). Heart rate also increased during PST (Morin *et al.*, 1989). Oxygen consumption (metabolic rate) is higher in smolts than parr (Higgins, 1985). Morin *et al.* (1993) suggested that increased cardiac output facilitated by increased heart rate helps to meet the increased metabolic rate in smolts. Furthermore, TH could increase heart rate or heart excitability by increasing activity of adrenergic receptors (Morin *et al.*, 1989). Since T_4-ORD increased in heart from week 1 to 2 during PST, increased T_3 in

the heart or in the plasma (plasma T_3 did increase from week 1 to 2) could be stimulating heart rate and heart size (Morin *et al.*, 1993).

2.2 E_2 Production and Function in Salmonids

2.2.1 E_2 Production

E_2 is a steroid hormone formed from cholesterol by a series of enzymatic steps involving the P-450 mono-oxygenase system (Lehniger *et al.*, 1993). Its immediate precursor is an androgen, mainly T, which is converted to the estrogen by a series of steps by the enzyme aromatase (Nagahama *et al.*, 1982). Therefore, E_2 can be produced wherever there is androgen precursor and aromatase. Aromatase is present in brain and pituitary of male and female salmonids (Andersson *et al.*, 1988) and has been detected in several other tissues (Fostier *et al.*, 1983). However, in female salmonids, the major site of E_2 synthesis is the ovarian follicle (Lance and Callard, 1978 in Kagawa *et al.*, 1982a). E_2 is produced in the ovarian follicle in two phases: T is produced in thecal cells in response to gonadotropin (GTH) I by a cAMP-dependent mechanism, then T is transported into granulosa cells where it is aromatized to E_2 (for review see Nagahama, 1983). Metabolic and stress-induced hormones affect ovarian E_2 production, including TH (Cyr and Eales, 1988a), growth hormone (LeGac *et al.*, 1993) and cortisol (Pankhurst and Dedual, 1994).

2.2.2 Estradiol Transport and Metabolism

E_2 is transported in the blood, bound to plasma proteins (Fostier and Breton, 1975; Lazier *et al.*, 1985; Ovrevik *et al.*, 2001). E_2 is conjugated in liver into water-soluble glucuronides and sulfates, which are excreted in feces or urine (Scott and

Vermeirssen, 1994). It is also metabolized by the cytochrome oxidase P-450 system in various tissues, into active and inactive compounds (Hannson and Rafter, 1983).

2.2.3 E_2 Actions

In salmonids, E_2 likely exerts most of its actions through the estrogen nuclear receptor (ER) although it is possible that some of its actions are non-genomically mediated as has been shown for mammals (Kelly *et al.*, 1999). The ER is a ligand (E_2)-activated transcription factor, which binds to DNA sequences called estrogen response elements and regulates transcription of estrogen-regulated genes (Beato, 1989). Binding of E_2 to the hormone-binding domain of the ER activates the ER. The activated ER binds to the estrogen response elements as dimers, and transcription of specific genes is activated (Carson-Jurica *et al.*, 1990) or repressed (Beato, 1989). The major rainbow trout tissues containing ERs are liver, brain and pituitary (Salbert *et al.*, 1993). E_2 has effects on ER expression, generally enhancing it in its target tissues, thus having a positive feedback effect by increasing its ability to bind to ERs and therefore increasing the magnitude of its actions (Salbert *et al.*, 1993; Knudsen *et al.*, 1998).

In teleost fish, the main role of E_2 is in oocyte growth (for review see Ng and Idler, 1983). Oocyte growth is in great part due to the uptake of the yolk protein precursor, VTG, from the plasma into the oocyte, a process often referred to as exogenous vitellogenesis (van den Hurk and Peute, 1979). The annual reproductive cycle of female rainbow trout is often characterized as four distinct physiological periods. A previtellogenic period occurs from March to April, endogenous vitellogenesis occurs from May to June, exogenous vitellogenesis occurs from August-December, and ovulation and spawning occur from January-February (Van Bohemen and Lambert,

1981). During the period of exogenous vitellogenesis, E_2 levels rise drastically. Rainbow trout plasma [E_2] ranges from 1 to 7 ng/ml during previtellogenesis and endogenous vitellogenesis, and rises to levels as high as 60 ng/ml at the peak of exogenous vitellogenesis. Plasma [E_2] levels begin to decrease before final oocyte maturation and ovulation, and are at levels around 2 ng/ml when ovulation occurs (Van Bohemen and Lambert, 1981).

Plasma VTG levels rise as E_2 levels increase during exogenous vitellogenesis in rainbow trout and brown trout (van Bohemen and Lambert, 1981; Bromage *et al.*, 1982; Norberg *et al.*, 1989). Experimental studies have shown that in rainbow trout, E_2 directly stimulates hepatocytes to produce VTG (Anderson *et al.*, 1996; Bieberstein *et al.*, 1999) which is then transported to the oocytes and incorporated into egg yolk (Wahli *et al.*, 1981). Another direct role of E_2 on oocyte growth is its stimulation of hepatocytes to synthesize zona radiata proteins which make up the egg envelope (Oppen-Bernsten *et al.*, 1992; Knudsen *et al.*, 1998).

In addition to oocyte growth, a major role of E_2 is feedback to the hypothalamo-pituitary axis to control gonadotropin production and secretion, thus controlling its own production (Peter, 1982; Kah *et al.*, 1997). E_2 exerts both positive and negative feedback effects depending on the stage of development and phase of the reproductive cycle (Melamed *et al.*, 1998).

In salmonids, E_2 also has several actions peripheral to reproduction. For example, calcium is necessary for VTG synthesis, and E_2 may act on calcified tissues to increase available calcium levels during vitellogenesis (Persson *et al.*, 1998). E_2 also affects the function of other hormone systems that impact reproduction, for example it affects the

thyroid system (Cyr and Eales, 1996), growth hormone production (LeGac *et al.*, 1993), and melatonin production (Begay *et al.*, 1994). E_2 may play a role in sex-specific differences in the cytochrome oxidase P-450 system in liver (Förlin and Haux, 1990; Pajor *et al.*, 1990).

Thus, E_2 is an essential molecule for sexual maturation in female salmonid fish. It is responsible for egg development and has key roles in regulating ovarian steroidogenesis by feedback to the brain and pituitary. It also interacts with other hormone systems and tissues to optimize its actions during the appropriate time in the reproductive cycle.

2.3 Reproductive and Thyroid System Relationships

There is good evidence that E_2 affects the salmonid thyroid system and TH have effects on E_2 production (for review see Cyr and Eales, 1996). Temporal correlations between E_2 and TH levels over the course of the reproductive cycle as well as experimental administration of E_2 indicates generally an inverse relationship between female reproductive status and thyroidal status with the exception that TH stimulate ovarian E_2 synthesis at the onset of vitellogenesis. This is not necessarily the pattern for non-salmonid groups. For example in several Indian teleosts, it appears that female reproductive status and thyroidal status are positively correlated (Bandyopadhyay *et al.*, 1991).

2.3.1 Seasonal Correlations between TH and E_2 levels

In studies of wild and captive salmonids, the generally consistent finding is that TH levels are high before the onset of vitellogenesis when E_2 levels are low, and that they

decrease over the course of vitellogenesis as E_2 levels increase (Dickhoff *et al.*, 1989; Cyr and Eales, 1996). For example, in Atlantic salmon maintained in outdoor pens over 4 to 10 months (Dickhoff *et al.*, 1989) and rainbow trout maintained under specific photoperiods (Cyr *et al.*, 1988b), and in brown trout (Norberg *et al.*, 1989), the temporal relationship between plasma TH levels and E_2 was similar. Generally, as female E_2 levels increased as vitellogenesis progressed, T_3 and T_4 levels decreased. T_3 and T_4 levels continued to decrease while E_2 levels fell just prior to ovulation. Just after ovulation, there was a sharp rise in T_3 and T_4 levels.

In Atlantic salmon and a wild strain of brown trout, T_4 levels were generally higher than T_3 levels throughout vitellogenesis and both T_3 and T_4 levels fluctuated equally (Dickhoff *et al.*, 1989; Norberg *et al.*, 1989). In rainbow trout, T_4 levels were generally lower than T_3 levels and fluctuated less markedly than T_3 (Cyr *et al.*, 1988b). In a cultured strain of brown trout, T_4 levels were very low and did not change at all throughout vitellogenesis (T_3 levels were not measured) (Norberg *et al.*, 1989). Therefore, the degree to which T_4 levels are affected during vitellogenesis (increased E_2 levels) depends on species and degree of domestication.

During sexual maturation of salmonids, there are sex differences in TH levels (Dickhoff *et al.*, 1989). In Atlantic salmon, male and female T_3 levels were elevated and similar from December until June. With the onset of vitellogenesis in June, female E_2 levels began to rise (Dickhoff *et al.*, 1989). From June until August, female T_3 levels dropped and remained low until ovulation in November. Male T_3 levels remained elevated until August, and although they dropped from August until November, they were still significantly higher than female levels. Male T_4 levels were also generally

higher than female levels over the course of vitellogenesis. Since female TH levels were generally lower than male levels when female E_2 levels were high, it seems that E_2 could be responsible for lower TH levels in females at these times, however generalizations should not be made for all salmonid species (Dickhoff *et al.*, 1989). These correlations suggest that E_2 depresses salmonid thyroidal status, but there is also experimental evidence indicating both a TH role in E_2 production, and E_2 suppression of thyroidal status.

2.3.2 TH Effects on Steroidogenesis

TH have effects on reproductive status (Cyr and Eales, 1996), and influence ovarian follicle E_2 secretion (Cyr and Eales, 1988a, b). Cyr and Eales (1988a) determined the effects *in vitro* of T_3 and T_4 on GTH-stimulated E_2 secretion from rainbow trout ovarian follicles. They determined that T_3 had a biphasic effect on GTH-stimulated E_2 secretion: low T_3 doses (10^{-8}M) enhanced GTH-stimulated E_2 secretion, whereas high T_3 doses (10^{-7}M) had no effect or inhibited GTH-stimulated E_2 secretion. T_3 was significantly more potent in inhibiting ovarian E_2 secretion than T_4.

In a related study, rainbow trout were experimentally rendered hypothyroid or hyperthyroid (Cyr and Eales, 1988b). Hypothyroid trout had lower gonadal somatic indices (gonad mass as percentage bm; GSIs) and lower *in vitro* ovarian follicle GTH-stimulated E_2 secretion than controls. Slightly hyperthyroid trout (T_3 levels increased by ~2-fold) had greater GSIs and greater *in vitro* ovarian follicle GTH-stimulated E_2 secretion than controls. Very hyperthyroid trout (T_3 levels increased by ~10-fold), however, showed no difference in GSI and either no difference or lower *in vitro* ovarian follicle GTH-stimulated E_2 secretion compared to controls. These biphasic *in vivo* effects

of T_3 levels on gonad development and *in vitro* E_2 secretion supported the findings of the *in vitro* studies, low T_3 levels enhancing reproductive state and high T_3 levels either having no effect or depressing reproductive state.

The mechanism of action of low concentrations of T_3 to stimulate E_2 secretion was investigated (Cyr and Eales, 1988a; 1989a). Low concentrations of T_3 stimulated E_2 secretion by rainbow trout ovarian follicles by an indirect, non-genomic mechanism, possibly by inhibiting cAMP turnover in thecal cells (Cyr and Eales, 1989a). At higher concentrations, T_3 did not stimulate E_2 secretion. Cyr and Eales have discussed the potential physiological significance of this phenomenon. They proposed that stimulatory effects of low concentrations of T_3 on GTH-stimulated E_2 secretion by ovarian follicles may be physiologically important at the onset of vitellogenesis when thyroidal status, and therefore T_3 levels, are low (Cyr and Eales, 1988b). T_3 might be necessary to initiate E_2 synthesis by the ovarian follicles, when GTH levels are too low to stimulate E_2 synthesis itself. This initiation of E_2 synthesis would be necessary to start VTG production by the liver. Furthermore, it was proposed that the inhibitory effects of high levels of T_3 on E_2 secretion may not be pharmacological but may also have physiological significance during periods of somatic growth when T_3 levels are high (Cyr and Eales, 1988b). T_3 might inhibit E_2 synthesis and therefore inhibit the energetically expensive processes of ovarian growth and reproduction, thereby keeping available energy for somatic growth (Cyr and Eales, 1988b).

It is interesting to note that in *in vitro* cultures of ovarian follicles from spawning rainbow trout, T_3 appeared to stimulate maturation inducing hormone secretion and oocyte maturation by increasing GTH-sensitivity of the follicles (Sullivan *et al.*, 1989).

Like T_3 action on vitellogenic follicles to stimulate E_2 secretion, this response was biphasic, with low concentrations of T_3 stimulating maturation inducing hormone secretion and ooctye maturation, and high concentrations having an inhibitory effect or none at all (Sullivan *et al.*, 1989).

2.3.3 Effects of E_2 Administration on Thyroidal Status

2.3.3.1 Plasma Thyroid Hormone Levels

Several studies have measured TH levels in response to E_2 administration to salmonids. In rainbow trout, intraperitoneal injection or implant of E_2 depresses plasma T_3 levels and generally has no significant effect on T_4 levels (Cyr *et al.*, 1988a; Flett and Leatherland, 1989b; Holloway and Leatherland, 1997; Mercure *et al.*, 2001). A single, slow release implant of 0.1, 0.5 or 1.0 mg/100 g bm E_2 in coconut oil elevated E_2 levels when sampled at 4 weeks and 8 weeks, although levels at 8 weeks were lower than 4 weeks, and T_3 was depressed at 8 weeks (slow release because the coconut oil solidifies after injection) (Flett and Leatherland, 1989b). Intraperitoneal injection of juvenile rainbow trout with 0.5 mg/100 g bm E_2 in peanut oil on days 0 and 3 resulted in increased E_2 levels (similar to those in vitellogenic females) from day 1 to 12, peaking at day 4, and T_3 levels were depressed at day 7, but not day 12 (Cyr *et al.*, 1988a). Yearling masu salmon (*O. masou*) injected with 0.1 mg/100 g bm E_2 in ethanol on days 0 and 3 and sampled on day 7, had depressed T_3 and T_4 levels compared to vehicle-injected controls (Yamada *et al.*, 1993). Effect of E_2 on TH levels is affected by food intake (Holloway and Leatherland, 1997). Plasma T_3 levels were depressed in fed rainbow trout but not fasted rainbow trout injected with E_2, and T_4 levels of fasted trout were actually significantly elevated by E_2. Thus, in studying E_2 effects on thyroidal status, it is

important to control nutritional status and to determine the time course over which your method of E_2 administration raises E_2 levels and affects TH levels.

In rainbow trout and other salmonids, experimentally raising E_2 levels in the blood results in decreased T_3 levels, and appears to not significantly or at least consistently affect T_4 levels. This does not necessarily indicate that E_2 is itself affecting T_3 levels. Flett and Leatherland (1989b) suggested that the increased Ca levels associated with vitellogenesis could be responsible for depressing T_3 levels, although a specific mechanism was not proposed.

2.3.3.2 T_3- and T_4-Binding Proteins

Cyr and Eales (1989b, 1992) investigated the effects of intraperitoneal E_2-3-benzoate injection to juvenile rainbow trout on T_3- and T_4-binding proteins in plasma. As well as decreasing total plasma T_3 levels, E_2 also decreased the percentage of free T_3 due to increased capacity of the low-affinity, high-capacity T_3 binding site. It was concluded that E_2 effects on T_3-binding of plasma proteins might contribute to E_2's overall effect to decrease T_3 availability to tissues (Cyr and Eales, 1989b). E_2-treatment also decreased the percentage of free T_4 due to a shift in binding between high-affinity and low-affinity binding sites, and also due to a small amount of T_4 binding to presumed lipoprotein and VTG (Cyr and Eales, 1992).

2.3.3.3 Plasma TH Kinetics

E_2 could depress T_3 levels by decreasing T_4 secretion from the thyroid gland, or by suppressing deiodination of T_4 to T_3 or by increasing T_3 turnover. In order to determine how E_2 depressed rainbow trout plasma T_3 levels, Cyr and Eales (1990) investigated changes in TH kinetics in plasma in response to E_2 benzoate injection. They determined

that T_4 plasma clearance rate decreased and T_4 secretion rate decreased, but T_4 levels did not drop. Furthermore, they determined that the T_3 plasma appearance rate decreased markedly (*i.e.* lower plasma T_3 levels), which was interpreted as being due to suppression of T_4-ORD activity and possibly T_4 transport into tissues where deiodination takes place (Cyr and Eales, 1990). Cyr and Eales (1990) proposed that the decreased T_4 plasma clearance rate was due to an E_2 effect to decrease TH demand in peripheral tissues, rather than on the brain-pituitary axis to decrease T_4 secretion. Leatherland (1985) found that E_2 treatment depressed thyroid gland secretion of T_4 in response to thyroid stimulating hormone, but Cyr and Eales (1990) suggested that was likely not due to E_2 action on the brain-pituitary-thyroid axis, but rather a homeostatic response of the axis to keep T_4 levels constant despite lowered T_4 demand in tissues.

2.3.3.4 TH Deiodination in Peripheral Tissues

Cyr *et al.* (1988a) determined that in immature rainbow trout, liver T_4-ORD is substantially depressed by E_2. Not only did E_2-3-benzoate injection lower plasma T_3 levels on Day 7, but it also depressed T_4-ORD activity of liver microsomes *in vitro* on Day 7. The depressed T_4-ORD activity was due to a 10-fold decrease V_{max}. Since, in rainbow trout, the liver is the main systemic source of T_3 by conversion from T_4 by T_4-ORD, E_2 could decrease T_3 levels, and therefore thyroidal status, by shutting down liver T_4-ORD, possibly by inhibiting synthesis of T_4-ORD proteins (Cyr *et al.*, 1988a). Liver T_4-ORD was the only pathway studied in response to E_2 injection, so it is not yet known if in rainbow trout, E_2 affects thyroidal status by modulating activity of any other deiodination pathway, such as the IRD pathways.

In juvenile Masu Salmon injected with 0.1 mg/100 g bm E_2, the decreased plasma T_3 and T_4 were associated with depressed T_4-ORD in the liver, gill, and head kidney but not kidney. T_4-IRD was the only other deiodination pathway measured, and was found to be substantially and significantly enhanced in liver and gill, and less substantially in head kidney (Yamada *et al.*, 1993). This pattern of ORD depression accompanied by increased IRD parallels effects of T_3 challenge on rainbow trout and other physiological factors that depress liver T_4-ORD.

2.3.3.5 T_3 Receptors

T_3 exerts many of its actions through nuclear receptors. It can be assumed that the greater the number of receptors bound by T_3, the greater the effect of T_3 on the tissue (Bres *et al.*, 1990). Therefore, another way that E_2 could affect thyroidal status is by affecting the maximum binding capacity (MBC) and binding affinity of T_3 receptors (Bres *et al.*, 1990). Bres *et al.* (1990) found that intraperitoneally injecting rainbow trout with E_2 decreased the number of T_3 receptors in hepatocyte nuclei but did not affect the receptors' binding affinity for T_3. They determined that decreased plasma T_3 levels alone did not decrease number of T_3 receptors, therefore it is possible that E_2 had some direct effect in decreasing T_3 receptor number, rather than it being an indirect effect of E_2 through lowered T_3 levels (Bres *et al.*, 1990).

3 Materials and Methods

3.1 Experimental Animals

Three *in vivo* E_2 administration experiments were carried out using similar protocol but different groups of rainbow trout that varied in genetic strain, size, and age. These experiments were denoted Experiment I (ExI), Experiment II (ExII) and Experiment III (ExIII) based on the order in which they were performed.

Trout used for ExI and ExII were rainbow trout (Tagwerker strain) from Glacier Springs Fish Farm, Inc. (Gunton, Manitoba). For 2-5 yr prior to experimental acclimation, they were held in the Duff Roblin Animal Holdings Facility, in cylindrical, 2400-L tanks with flow-through, dechlorinated, aerated Winnipeg city tap water, at 12°C, with a 12 hr:12 hr light:dark photoperiod, and once daily fed a 1% bm ration of trout pellets (Martin Feed Mills, Tavistock, Ontario). ExI trout were 2.75 years old and weighed from 388-694 g. Ex II trout were 3.5 or 6.5 years old and weighed from 799-2122 g (Table 1). For ExI and ExII there were approximately equal numbers of females and males.

Trout used for ExIII were all female rainbow trout (Kamloops strain) from Praire Springs Fish Farm (Gunton, Manitoba, 14 hr:10 hr light:dark, 1%bm ration once daily). The all-female stock was produced at Troutlodge, Inc. (Sumner, WA) by sex reversal of females. By feeding female salmonids methyl-testosterone, they become phenotypic males with a female genotype (XX), producing all X-chromosome sperm instead of eggs. Thus when eggs (X-chromosome) are fertilized by these all X-chromosome sperm, only

TABLE 1

NON-THYROIDAL VARIABLES FOR CONTROL AND E_2-TREATED FEMALE AND MALE RAINBOW TROUT FROM EXPERIMENTS I, II, AND III.

Experiment	E_2 Treatment (mg/100g bw)	sex	n	Body Mass (g) Mean	Body Mass (g) SEM	HSI (%bw) Mean	HSI (%bw) SEM	Liver Microsomal Protein (mg/g) Mean	Liver Microsomal Protein (mg/g) SEM	GSI (%bw) Mean	GSI (%bw) SEM
I	Control	F	7	530	20	1.29	0.07	11.3	0.4	0.7	0.2
		M	11	560	20	1.24	0.05	11.4	0.7	0.6$^{\bullet}$	0.2
	0.50	F	8	580	20	1.85*	0.09	10.5	0.6	0.8	0.1
		M	10	520	30	1.87*	0.07	11.8	0.6	0.25$^{\bullet}$	0.02
II	Control	F	9	1500	200	1.8	0.2	12.5	0.8	14	2
		M	9	1200	100	1.5$^{\bullet}$	0.2	12.5	0.6	1.0$^{\bullet}$	0.2
	0.50	F	10	1600	100	2.2*	0.2	13.5	0.8	14	1
		M	8	1110$^{\bullet}$	50	2.0$^{\bullet}$*	0.2	13.2	0.7	0.8$^{\bullet}$	0.1
III	Control	F	18	310	10	1.29	0.08	10.5	0.5	0.113	0.008
	0.05	F	18	310	20	1.63*	0.08	11.4	0.5	0.115	0.007
	0.10	F	18	310	10	1.72*	0.07	10.6	0.4	0.098	0.009
	0.50	F	18	310	10	1.82*	0.08	10.8	0.3	0.118	0.008

Note: Statistical differences between females and males and control and E_2-treated trout for each variable within each experiment were determined using two-factor ANOVA or the Kruskal-Wallis test in conjuction with Dunnett's T3 test. Differences are indicated by symbol superscripts:

* indicates a significant difference between control and E_2-treated trout within a sex ($p \leq 0.05$);

$^{\bullet}$ indicates a significant difference between females and males within a treatment group ($p \leq 0.05$);

$^{\underline{\bullet}}$ indicates a marginally significant difference between females and males within a treatment group ($0.05 < p \leq 0.10$).

female embryos (XX) are formed (Donaldson and Hunter, 1982, CJFAS39). Trout were held in the Duff Roblin Animal Holding Facility under the same conditions as ExI and ExII trout for one month prior to experimental acclimation. Ex III trout were about one year old and weighed from 201- 465 g (Table 1).

3.2 Experimental Protocol

3.2.1 Holding Procedures

Five to nine days before the injection protocol began, fish were transferred to 180-L cubic fibreglass tanks, with flowing dechlorinated Winnipeg tap water at 12°C. Each tank was aerated and housed in a controlled environment room with a 12hr:12hr light:dark photperiod (light = 7am to 7pm). For ExI and ExII, fish of each sex were assigned randomly to experimental tanks, with equal numbers of females and males in each tank. For ExI and ExII there were 6 fish per tank, and for ExIII there were 8 fish per tank. Each morning fish were fed trout pellets (Martin Feed Mills, Tavistock, Ontario) at a subsistence ration of 0.6% bm.

The dates of acclimation and experimentation were May 11-May 23, 2000 (ExI), March 19-April 2, 2001 (ExII), and June 26-July 5, 2001 (ExIII).

3.2.2 Injection Procedures

Injection and sampling procedures are summarized in Fig. 4. At 30 to 60 min before injection, E_2 was weighed out into a glass injection vial, and a volume of peanut oil (Planter's) was added. The vial was sealed with a rubber sleeve stopper and the contents mixed with a vortex mixer for 4 one min intervals with 15- to 30-s breaks during which the vial was shaken and swirled by hand. E_2 did not dissolve completely, but was

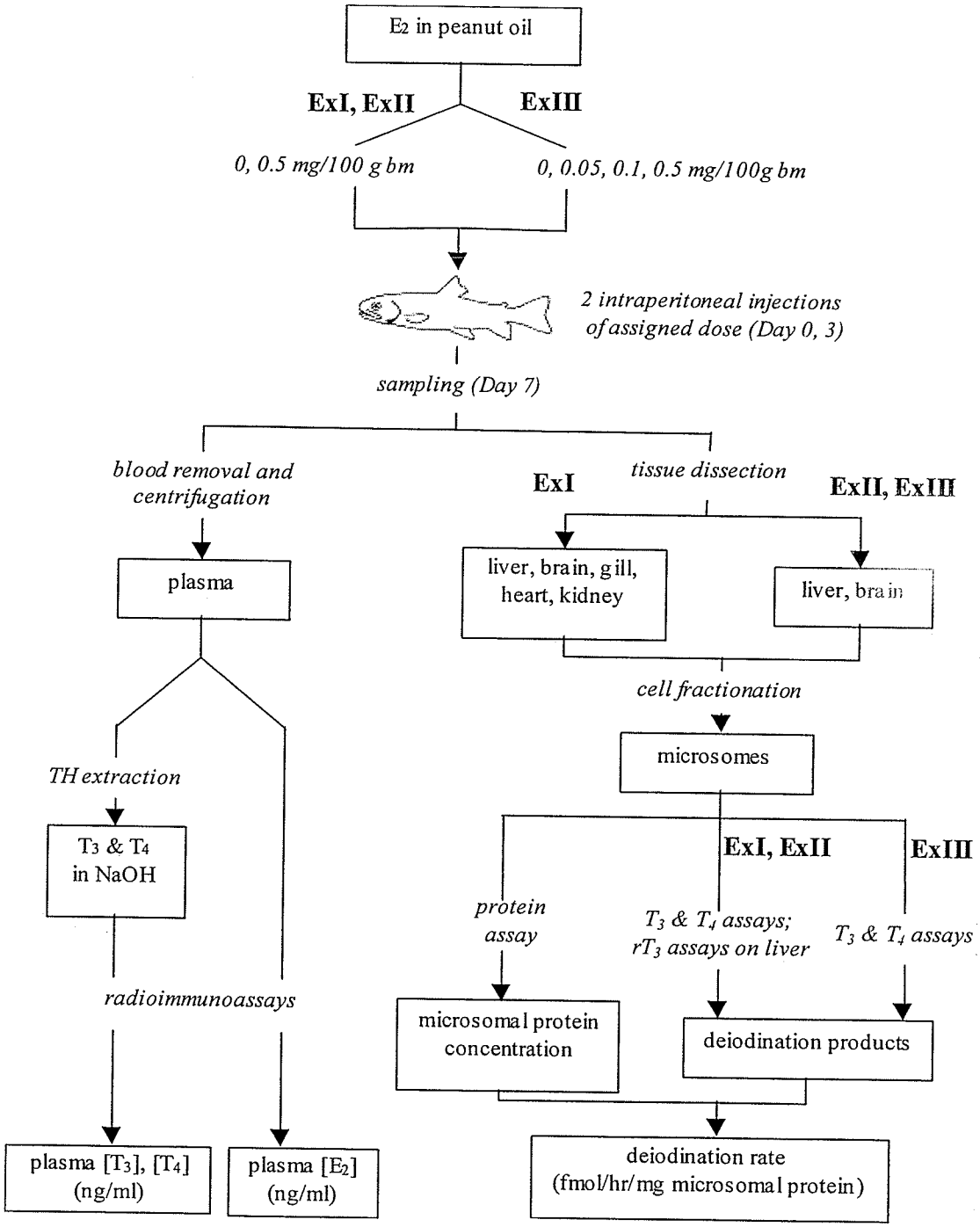

Fig. 4 Experimental protocol and analyses for Experiments I, II and III.

finely suspended in the oil. Peanut oil to be used for controls was comparably treated but contained no E_2. From 0.05-0.20 ml was injected depending on bm, to deliver 0.5 mg/100 g bm (ExI and ExII). For ExIII the doses were 0.05, 0.1 and 0.5 mg/100 g bm.

Trout were injected with E_2 on Days 0 and 3, and killed on Day 7. Injections were in late morning to mid afternoon. Day 3 injections were staggered over time in anticipation of Day 7 sampling times (ExI, 09:00-14:45; ExII, 11:45-17:00; ExIII, 11:20-15:50). Thus fish were killed between 96-98 hr after Day 3 injection for all 3 experiments.

Fish were fasted for 24 hr before injection but were fed after all injections were completed in the late afternoon. They were monitored to determine whether they were feeding post-injection, indicating recovery from handling. Generally all food was consumed within 10 minutes. Fish were fed in the morning the day after injection. Fish were also fasted 24 hr before sampling on Day 7.

Prior to injection, trout were anaesthetized one tank at a time with tricaine methanesulfonate (TMS). TMS was dissolved in a small volume of water and poured into the experimental tank immediately after the water flow had been turned off. This provided an approximate concentration of 0.07 g/L. Once all the fish had lost equilibrium, they were netted and placed into a tub with aerated water containing 0.07 g/L TMS.

After a few minutes once fish showed now response to tail pinching, one fish at a time was removed from the tub and weighed to determine the injection volume. The injectate was delivered into the intraperitoneal cavity through the body wall using a 1-ml syringe and 22-gauge needle. The needle was withdrawn slowly to ensure that oil was

not seeping out of the injection site, confirming that injectate had entered the intraperitoneal cavity and not muscle. The injected fish was then put back in the experimental tank into flowing water containing no TMS.

3.2.3 Sampling Procedures

On Day 7, two trout at a time were transferred to a tub with aerated water containing 0.07 g/L TMS. Fully anaesthetized fish were removed one at a time from the tub and blotted dry with a paper towel. Body mass was recorded. Six ml of blood were taken from the caudal vein using two 3-ml syringes with an 18-gauge needle and kept on ice in the syringes until centrifuged. Plasma was separated by spinning the blood in 1.5-ml centrifuge tubes for 3 min and then frozen at –76°C until analysis. The fish was then killed by a blow on the head and the liver and gonads were removed and weighed for calculation of hepatosomatic index (HSI, liver mass as percentage of bm) and gonadosomatic index (GSI, gonad mass as percentage bm). The sex and general condition were noted. Several tissues were removed for determination of TH deiodination rates. For all three experiments, brain and liver were removed and for Experiment I only, gill, heart and kidney were also removed. The dissected tissues were wrapped in aluminum foil, frozen in liquid nitrogen and stored at –76°C.

3.3 Plasma Hormone Analysis

3.3.1 TH Radioimmunoassays

Total plasma T_3 and total plasma T_4 were quantified by radioimmunoassay (RIA) after extracting hormones from the plasma.

3.3.1.1 TH Extraction from Plasma

Total TH were extracted from plasma samples and reconstituted in NaOH. For each sample, 1 ml plasma was mixed with 2 ml MeOH:NH_4OH (99:1 v/v) with a vortex mixer, to precipitate protein (TH would remain in MeOH:NH_4OH). The mixture was centrifuged at 1420 *g* for 20 min at 4°C, and then the supernatant fraction was pipetted off and saved. The pellet was resuspended in another 2 ml MeOH:NH_4OH, centrifuged, and the supernatant fraction saved. This was repeated a third time, then the pellet was discarded. The supernatant fractions were pooled in a 16x100 mm glass tube and the contents were dried down in a hot water bath with gentle airflow to enhance evaporation. After 30 min of drying, the walls of the sample tubes were rinsed with 250 µl of MeOH. Once completely dry (several hours) and cooled to room temperature, the residue containing the TH was reconstituted in 700 µl 0.05N NaOH (70% original volume to increase detection by assay) using a vortex mixer. The samples were then pipetted into micro-centrifuge tubes, capped, and stored at 4°C.

Extraction efficiency was quantified following exactly the same extraction procedure but with ^{125}I-labelled T_3 ($^*T_3$) added to 1 ml rainbow trout plasma (in triplicate) or ^{125}I-labelled T_4 ($^*T_4$) added to 1 ml plasma (in triplicate). The amount of radiolabeled TH in the sample (counts per minute, CPM) was recorded at the beginning of the extraction procedure and at the end, after reconstitution in NaOH. Extraction efficiency was calculated as the percentage of original radioactivity remaining at the end of the extraction procedure, correcting for radioactive decay. Mean extraction efficiencies for T_3 were 85-91%, and for T_4 were 90-92%. Extraction efficiency was taken into account for TH concentration calculations from the RIAs.

3.3.1.2 RIA Theory and Procedure

The T_3 and T_4 RIAs were performed on Sephadex columns following the established protocol of Brown and Eales (1977). The TH RIA used is a competitive binding assay utilizing a limiting amount of specific T_3 or T_4 antibody, a constant amount of $*T_3$ or $*T_4$ and the unlabelled TH-containing sample. The labelled and unlabelled TH compete for antibody binding sites. The greater the amount of unlabelled TH in the sample, the less labelled TH will bind to the antibody. Using the Eales and Brown protocol (1977), unbound TH stay on the Sephadex column, bound to Sephadex beads, whereas antibody-bound TH are eluted from the column into collecting tubes. Therefore a greater TH concentration in a sample will result in a smaller amount of labelled TH eluted (antibody-bound). The radioactivity in the collecting tube is then quantified and the TH concentration is calculated using a standard curve from a series of T_3 or T_4 standards at varying concentrations.

Sephadex columns were prepared in 5-ml Quik-Sep columns (Isolab, OH) with 0.3 g G-25 fine Sephadex per column (dry mass) and stored in 0.1 N NaOH. T_3 and T_4 RIAs were carried out separately. Samples were run in duplicate and the standard curve was run in triplicate. The following protocol was used:

1. Columns were allowed to drain.
2. Columns were rinsed with with 3 ml barbital buffer for the T_4 assay (12.15 g/L sodium barbital; pH 8.6) and phosphate buffer for the T_3 assay (14.21 g/L Na_2HPO_4 (anhydrous), 11.17 g/L Na_2EDTA; pH 7.4). Throughout the assay, when buffer was called for, barbital buffer was used for the T_4 assay and phosphate buffer was used for the T_3 assay.

3. Drains of the columns were capped.
4. 100 µl of *T_3 or *T_4 in 0.1 N NaOH containing 12,000 CPM was added to all columns (for standards, samples and non-specific binding (NSB)) and also to three reference tubes for measuring total counts added.
5. 100 µl of either standards (diluted in 0.05 N NaOH) or samples (extracted and reconstituted in 0.05 N NaOH) were added to the appropriate columns and columns were swirled to mix.
6. NSB columns were transferred to iodide collecting tubes, then caps were removed from all column drains and the sample/tracer was allowed to drain onto the column.
7. Columns were eluted with 3.5 ml of the appropriate buffer to remove iodide and drained to waste tubes except for the NSB columns, which were drained to iodide collecting tubes.
8. Columns were transferred to collecting tubes, and NSB columns to NSB tubes.
9. T_3 and T_4 antibodies were diluted with the appropriate buffer by a dilution factor determined separately for each assay. Diluted T_4 antibody (0.5 ml) was added to all T_4 columns except the T_4 NSB columns to which was added 0.5 ml barbital buffer. Diluted T_3 antibody (0.75 ml) was added to all T_3 columns except the T_3 NSB columns to which was added 0.75 ml phosphate buffer.
10. Columns were incubated overnight for 14-16 hr.
11. Columns were eluted with 3.5 ml of the appropriate buffer into collecting tubes.
12. Once eluted into test tubes, all collecting tubes and reference tubes were counted on a Packard Cobra II gamma counter for 10 min.

The standard curve comprised 9 concentrations from 0 to 20 ng/ml, and the detection limit was determined separately for each assay. The standard curve was plotted as the logit value of radioactivity (CPM) of each standard concentration (average of triplicates) as a function of the log value of standard concentration. Sample concentrations in ng/ml were then interpolated from the sample values in CPM using the standard curve, correcting for non-specific binding and iodide contamination (determined from NSB tubes and iodide collecting tubes), as well as for concentration factor and extraction efficiency for the extracted samples.

3.3.2 E2 Radioimmunoassays

E_2 RIAs were done by personnel in the lab of Dr. Mark McMaster at the National Water Research Institute, Burlington, ON using their published procedure (McMaster *et al.*, 1992).

3.4 Deiodination Rate Assays

3.4.1 Isolation of Microsomes

Three tissue samples at a time were removed from the deep-freeze and kept on ice. For liver, 1.0-1.5 g was cut from the whole liver with a razor blade and the exact mass removed was recorded. Gill filaments were removed from the gill arches. The entire brain, heart, and kidney were used. Liver pieces, brain and kidney were homogenized with a motorized pestle (Tri-R instruments Inc., New York, NY). Gill filaments and heart were homogenized with a Polytron (Brinkman Instruments, Toronto, ON). The tissues were homogenized with about 2 ml ice-cold buffer (0.1 M KH_2PO_4/Na_2HPO_4, 0.25 M sucrose, 1 mM Na_2EDTA, 20 mM DTT, pH 7.2).

Homogenate was transferred to 38.5-ml plastic Beckman ultracentrifuge tubes held on ice, topped up to the tube shoulder with buffer and kept on ice until 12 tissue samples were homogenized.

The microsomal fraction of tissue homogenates was isolated using a sequence of three centrifugations at 4°C in a Beckman Ultracentrifuge, with a 12-place Ti 50.2 Beckman rotor. The first spin was at 730 *g* for 20 min. For each sample, the pellet, containing nuclei and large particles, was discarded. The supernatant fraction was transferred to a clean centrifuge tube and topped up with ice-cold buffer. The second spin was at 25,200 *g* for 20 min. Again, the pellet, containing lysosomes and mitochondria, was discarded, and the supernatant fraction transferred to a clean tube and topped up with buffer. The third and final spin was at 110,000 *g* for 67 min. The cytosolic supernatant fraction was discarded, and the pellet, containing the isolated microsomes, was resuspended in 1.5 ml ice-cold buffer by agitation with a vortex mixer and a pipette. Microsome suspensions were transferred to cryovials and frozen at -76°C. Duplicate small aliquots of each microsome sample were used to measure preliminary microsomal protein concentration (see method below) in order to determine the necessary dilution factor for each sample to obtain equal protein concentrations among samples for the deiodination assay.

3.4.2 Deiodination Assays

3.4.2.1 Substrate Purification

One day before a deiodination assay, *T_4 or ^{125}I-labeled rT_3 (*rT_3) was purified to remove contaminant iodide using LH-20 Sephadex in HCl in a plastic Quick-Sep column (*T_3 was not purified because its contaminant iodide was much lower). *T_4 or *rT_3 was

allowed to drain onto the column, then iodide was eluted from the column with 3 ml doubly distilled water. Purified *T_4 or *rT_3 was then eluted from the column with approximately 3 ml 0.1N NH_4OH/EtOH, and the eluate dried down in a hot water bath under a gentle air stream. Once dry, the purified *T_4 or *rT_3 was dissolved in 100 µl 0.1 N NaOH, by agitation with a vortex mixer, and stored at 4°C until the assay.

3.4.2.2 Incubation

Frozen microsome samples were thawed on ice at 0-4°C, and then diluted, based on the previous preliminary determination of protein content, with ice-cold KH_2PO_4/Na_2HPO_4 buffer to approximately 0.35 mg/ml. This buffer was the same as for microsome isolation, except for all gill and rT_3 assays, where 10 mM DTT was used instead of 20 mM (MacLatchy and Eales, 1992a; Finnson *et al.*, 1999).

For each sample, duplicate 500-µl aliquots of diluted microsomes were pipetted into 16x100 mm glass culture tubes. Four blanks containing only 500 µl buffer were also run for each assay. All samples and blanks were preincubated for 30 min in a shaking water bath at 12°C (fish acclimation temperature).

For each substrate type (T_4, T_3 or rT_3), a mixture of the ^{125}I-labeled TH (*TH) and its corresponding cold TH was prepared so that a 10 µl aliquot delivered 50,000 CPM *TH and enough cold TH for each sample tube to contain the assigned substrate level. For liver, gill, heart and kidney, the T_4 level was 0.63 nM and the T_3 level was 0.75 nM. For brain, the T_4 level was 0.08 nM and the T_3 level was 0.09 nM. rT_3 was used as substrate for deiodination assays on liver from ExI and ExII only. A low and a high cold substrate level were used for the rT_3 assays (0.75 nM and 200 nM, respectively), to

determine whether the high-K_m rT_3-ORD pathway responded differently to E_2 treatment than the low-K_m rT_3-ORD pathway.

Incubation time (2 hr) began with the addition of 10 µl *TH/cold TH to the first sample tube. *TH/cold TH was added systematically to each tube every 20 s. Each tube was vortexed, and returned to the water bath. Once *TH/cold TH had been added to all sample tubes and blanks, the water bath was covered, and the tubes were incubated in darkness. Ten µl *TH/cold TH was also added to each of three reference tubes and counted on a Packard Cobra II gamma counter, to determine the average CPM corresponding to total substrate levels for deiodination rate calculations. Incubation was ended with the systematic addition of 650 µl MeOH with 10^{-3} M methyl mercaptoimidazole to each tube every 20 s followed by vortexing, exactly 2 hr after start of incubation. Samples and blanks were immediately transferred to micro-centrifuge tubes and capped to prevent evaporation, and centrifuged at 1420 *g* for 5 min to separate TH from precipitated protein. The supernatant fractions were prepared for HPLC analysis by transferring 400 µl to a 700-µl amber HPLC vial and covering with a thin layer of parafilm.

The protein content of each diluted microsome sample was determined after the assay. This exact protein concentration was used in deiodination rate calculations. The Bio-Rad Protein Assay reagent was used, following the Bradford protein-dye binding method (Bradford, 1976). In brief, 100 µl of diluted microsomes in duplicate and 100 µl of a range of bovine serum albumin standard dilutions in duplicate were mixed with 5 ml Bio-Rad reagent diluted in doubly distilled water, 1:5. Samples were incubated at room temperature for at least 5 min, and absorbance read on a Spectrophotometer (Bausch and

Lomb) at 595 nm. Absorbance of each standard concentration was plotted as a function of standard concentration, and sample protein concentrations were interpolated from this linear plot.

3.4.2.3 Identification of Deiodination Products

The proportions of the parent radiolabeled substrate and its various radiolabeled deiodination products were determined for each sample using reverse-phase HPLC as described by Sweeting and Eales (1992a). The HPLC system used was a Gilson on-line gamma detecting system in association with Gilson Unipoint software on a Compaq Presario computer system. The HPLC column was a 150mm x 4.6mm C_{18} (5 μm) Alltech Econosphere column with a C_{18} guard column. The solvent system used was a mixture of acetonitrile and water containing 0.1% trifluoroacetic acid. Solvents were degassed by bubbling with helium gas before use in the HPLC system. Once attached to the HPLC, solvents were mixed in a 36:64 ratio of acetonitrile:water, and pumped through the column at a flow rate of 1 ml/min. Sample (200 μl) was injected onto the column and eluted through the column with solvent flow, achieving separation of the various THs by reverse-phase. As solvent was eluted from the column, it passed through a Ray Test Ramona 90 gamma detector which recorded the amount of radioactivity in the solvent over time. These data were transmitted to the software, generating a chromatograph of peaks of radioactivity over time, representing relative amounts of the various radiolabeled iodothyronines in the sample. The peaks were identified as specific iodothyronines based on retention times and relative peak positions previously determined (Sweeting and Eales, 1992a). The peaks were integrated, and peak area was

calculated as the proportion of total area under all peaks. These proportions were used in the calculation of deiodination rate.

3.4.2.4 Deiodination Rate Calculations

Deiodination rates were calculated as the amount of TH substrate deiodinated per hr per mg of microsomal protein (fmol/hr/mg protein). The amount of TH substrate deiodinated was calculated as the product of total substrate in the incubation tube times the proportion of substrate deiodinated to deiodination product. Total substrate in the incubation tube was determined from the reference tubes from the deiodination assay, corrected for radioiodide contamination determined using LH-20 Sephadex filtration. Proportion of substrate deiodinated to deiodination product was determined from the HPLC chromatograph peaks, corrected for radioiodide contamination by subtraction of iodide peaks from the blanks from the deiodination assay. Specific equations for calculation of T_4-ORD, rT_3-ORD, T_4-IRD, and T_3-IRD are shown in Appendix A.

3.5 Chemicals Used

TMS was from Syndel Laboratories (Vancouver, BC). E_2, barbital buffer, DTT, TH antibodies, and cold T_4, T_3 and rT_3 were from Sigma Diagnostics (St. Louis, MO). Na_2HPO_4, Na_2EDTA, sucrose, MeOH, NaOH, HCl, and acetonitrile were from Fisher Scientific (Fair Lawn, NJ). KH_2PO_4 was from Mallinckrodt Inc. (Kentucky). NH_4OH was from J.T. Baker Chemical Co. (Phillipsburg, N.J.). EtOH was from Commercial Alcohols, Inc. (Toronto, ON). Sephadex G-25 and LH-20 were from Pharmacia Biotech (Uppsala, Sweden). Radiolabeled T_4, T_3 and rT_3 (specific activities of 1250, 3390, and

1084 μCi/μg respectively) were from NEN Life Science Products, Inc. (Boston, MA). Methyl mercaptoimidazole was from Eastern Chemical Corporation (Pequannock, NJ).

3.6 Statistical Analysis

Normality of the data for each variable within a treatment group was determined with D'Agostino's D-test (Zar, 1996) (using StatWorks™ on a Macintosh SE). Homogeneity of variance of the data for each variable within a treatment group was determined with the Levene Test (using SPSS™ software on a Pentium III computer).

For each variable within Ex I and II, if the ANOVA assumptions of normality and homogeneity of variance were met, General Linear Model univariate two factor ANOVAs using Type III Sum of Squares were used to determine the statistical significance of differences between E_2 treated trout and controls, and between males and females. This test also indicated whether the differences due to treatment and sex were independent of each other (insignificant interaction). Because there were only two levels per factor (control vs. E_2-treated for treatment and male vs. female for sex), no further analysis was needed if there was insignificant interaction. If there was significant interaction between sex and E_2 treatment for a variable, Tukey's Honestly Significant Difference Test (pairwise multiple comparison procedure based on the studentized range statistic) (Zar, 1996) was used to determine which factor-level pairs were significantly different (how each sex had been affected by E_2 treatment). For Ex III, single factor ANOVAs were used to determine the statistical significance of differences among controls and the three E_2 doses for each variable (single factor since there was only one

sex). Since there were four levels within the one factor, Tukey's Honestly Significant Difference Test was used to determine which level pairs were significantly different.

If the ANOVA assumptions of normality and homogeneity of variance were not met, then the data were transformed by various functions until the data were normal and variance was homogeneous (Zar, 1996) and ANOVAs and multiple comparison procedures were performed on the transformed data, as above.

If the ANOVA assumptions of normality and homogeneity of variance were not met and data transformations failed to normalize the data distribution and homogenize the variance, then non-parametric tests were employed. For each variable within one experiment, the Kruskal-Wallis test (analysis of variance using data ranks instead of data values) (Zar, 1996) was used to determine the statistical significance of the differences between E_2-treated trout and controls and males and females. Dunnett's T3 test (pairwise multiple comparison procedure based on the studentized maximum modulus which does not assume homogeneity of variance) (Hochberg and Tamhane, 1987) was used in conjunction with the Kruskal-Wallis test to determine which factor-level pairs were significantly different for Ex I and II, or which level pairs were significantly different for Ex III .

To compare the reproductive status of controls among the three experiments and and thyroidal status of controls among the three experiments, General Linear Model univariate two factor ANOVAs using Type IV Sum of Squares were used to compare separately the mass, plasma [E_2], HSI, liver [protein], GSI, plasma [T_3] and [T_4], and liver T_4-ORD of the controls among the three experiments, including differences between males and females. Tukey's Honestly Significant Difference Test was used to determine

which factor-level pairs were significantly different. If the ANOVA assumptions of normality and homogeneity of variance were not met and data transformations failed to normalize the data distribution and homogenize the variance, then the Kruskal-Wallis test was used to determine the statistical significance of the differences between controls among the three experiments and sexes for each variable measured. Dunnett's T3 test was used in conjunction with the Kruskal-Wallis test to determine which factor-level pairs were significantly different.

Throughout this document, for all tests, p-values less than or equal to 0.05 ($p \leq 0.05$) are considered to indicate a significant difference, and p-values greater than 0.05 but less than or equal to 0.10 ($0.05 < p \leq 0.10$) are considered to indicate a marginally significant difference. All ANOVAs, Kruskal-Wallis tests, and multiple comparison procedures were performed with SPSS™ software on a Pentium III computer. Results of all data analyses are presented in Appendix B.

4 Results

4.1 Experiment I

4.1.1 Plasma E_2 Concentrations

Plasma E_2 concentrations are shown in Fig. 5. E_2-treated trout had markedly higher plasma [E_2] than controls ($p<0.001$). E_2-treated females had plasma [E_2] of 24.5±2.9 ng/ml, 9-fold higher than female controls and E_2-treated males had plasma [E_2] of 19.6±2.4 ng/ml, 35-fold higher than male controls. Control males had significantly lower plasma [E_2] than control females ($p<0.001$).

4.1.2 Body Mass, HSI, Liver Microsomal Protein Concentration, and GSI

Non-thyroidal parameters are shown in Table 1. Control and E_2-treated trout of both sexes were of similar mass. E_2-treated trout had 47% higher HSIs than controls ($p<0.001$) and there were no sex differences. Liver microsomal [protein] did not differ due to E_2 treatment or sex. Control and E_2-treated trout had similar GSIs. The female GSI corresponded to rainbow trout ovarian growth stage IV as defined by Ruby and Eales (1999) and one female had free eggs in the intraperitoneal cavity.

4.1.3 Plasma TH Concentrations

E_2-treated trout had 32% lower plasma [T_3] than controls ($p=0.006$) (Fig. 6a). For a given treatment, males had lower plasma [T_3] than females ($p=0.019$) (Fig. 6a). Control and E_2-treated trout had similar plasma [T_4] (Fig. 6b). For a given treatment,

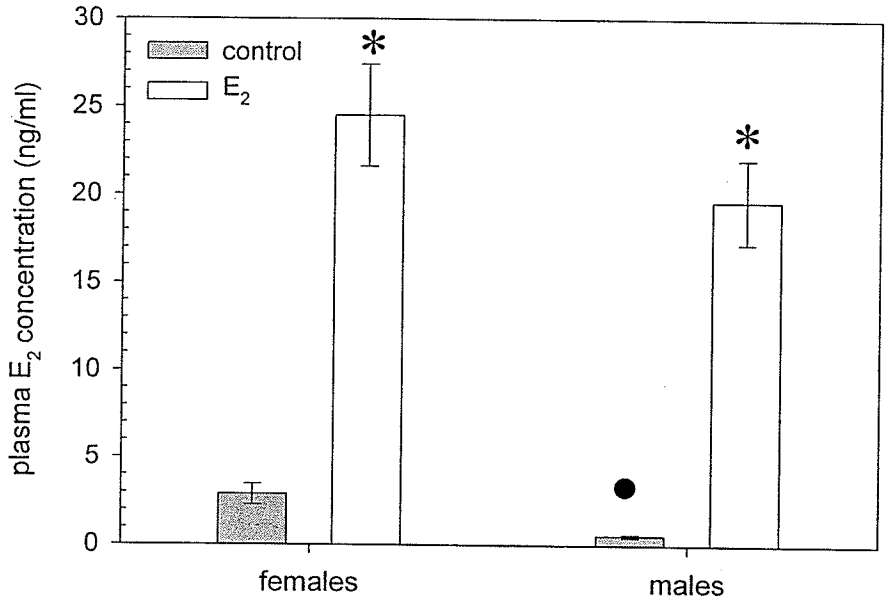

Fig. 5 Experiment I: Plasma E_2 concentrations (±SEM) for control and E_2-treated female and male rainbow trout (n = 7 for control females, n = 8 for E_2-treated females, n = 11 for control males, n = 10 for E_2-treated males). A * indicates a significant difference ($p \leq 0.05$) between control and E_2-treated trout of a given sex. A ● indicates a significant difference ($p \leq 0.05$) between females and males for a given treatment.

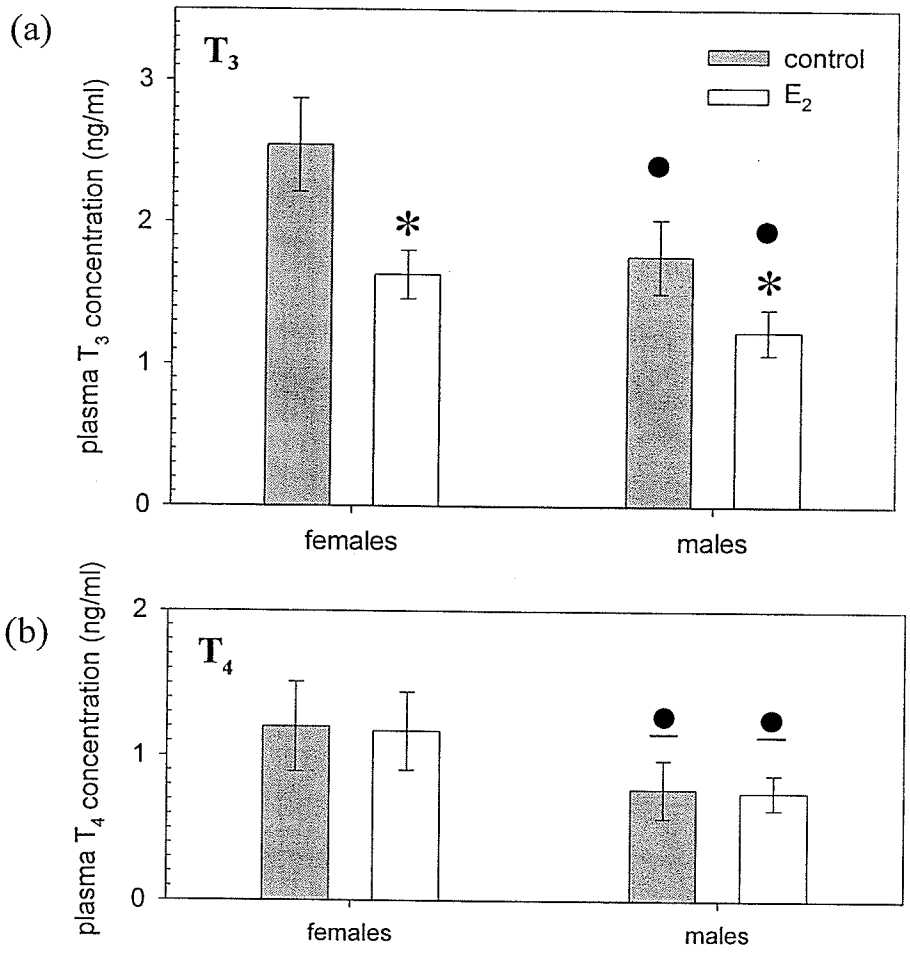

Fig. 6 Experiment I: (a) Plasma T_3 and (b) plasma T_4 concentrations (±SEM) for control and E_2-treated female and male rainbow trout (n = 7 for control females, n = 8 for E_2-treated females, n = 11 for control males, n = 10 for E_2-treated males). A * indicates a significant difference ($p \leq 0.05$) between control and E_2-treated trout for a given sex. A ● indicates a significant difference ($p \leq 0.05$) and ● a marginally significant difference ($0.05 < p \leq 0.10$) between females and males for a given treatment.

males had marginally significantly lower plasma [T_4] than females ($p=0.066$) (Fig. 6b).

4.1.4 Tissue Deiodination Rates

4.1.4.1 Liver

E_2-treated trout had an approximately 6-fold lower mean liver T_4-ORD rate than controls ($p<0.001$) (Fig. 7a). Females and males had similar T_4-ORD rates and were affected comparably. T_3-IRD rates were low and E_2-treated trout had lower liver T_3-IRD rates than controls (Fig. 7a), however only in females was this difference significant ($p=0.046$) and was lower by 86% (Fig. 8). Liver T_4-IRD rates were low and were similar in control and E_2-treated trout and males and females (Fig. 7a).

rT_3-ORD was the only rT_3 deiodination activity detected using both low and high substrate concentrations in the rT_3 assay (Fig. 9). rT_3-ORD rates assayed at the low substrate concentration (rT_3-ORD-lo) were several fold more active than the pathways assayed with T_3 or T_4 at a similar substrate concentration. rT_3-ORD-lo was 26% lower in E_2-treated trout than controls ($p=0.016$), and for a given treatment, male rT_3-ORD-lo was higher than female rT_3-ORD-lo ($p=0.002$) (Fig. 9a). rT_3-ORD rates assayed at the high substrate level (rT_3-ORD-hi) were marginally significantly lower in E_2-treated trout than controls by 29% ($p=0.074$) (Fig. 9b). For a given treatment, male rT_3-ORD-hi was higher than female rT_3-ORD-hi ($p=0.008$).

4.1.4.2 Brain

Brain T_4-ORD rates were very low and did not differ due to E_2 treatment or sex (Fig. 7b). Brain T_3-IRD was the most active of the measured deiodination pathways in

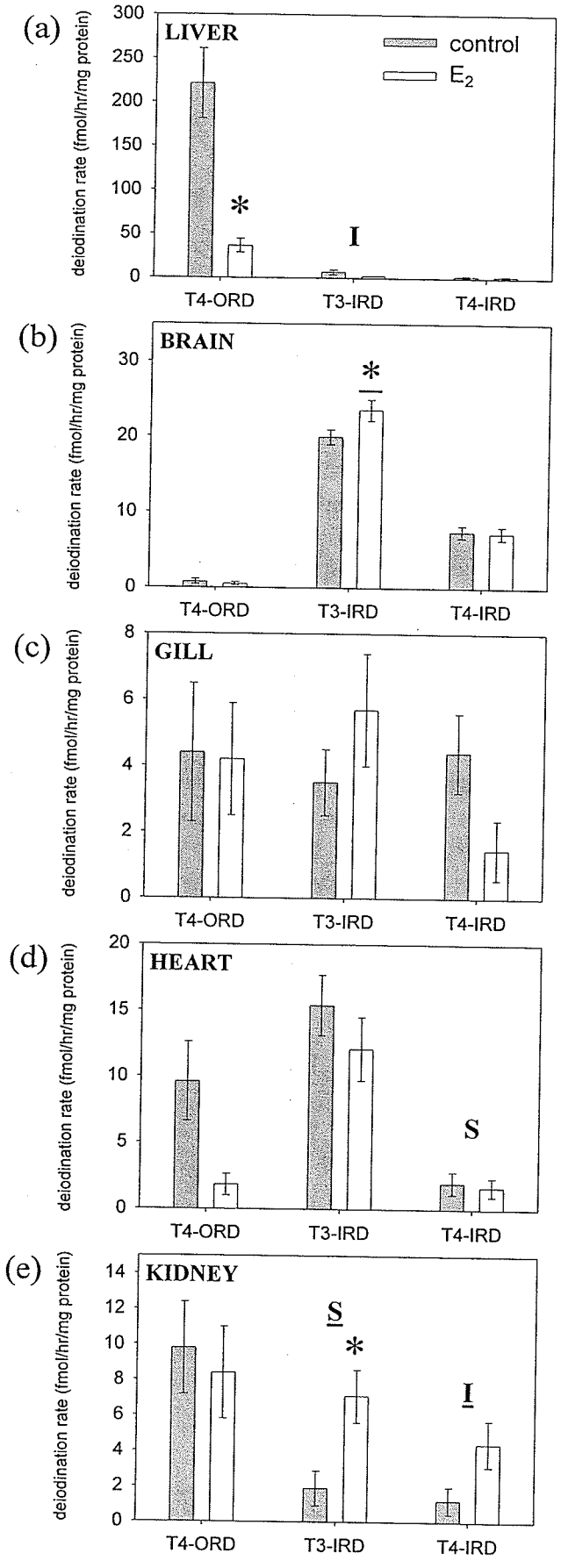

Fig. 7 Experiment I: Microsomal deiodination rates (±SEM) of T_4-ORD, T_3-IRD and T_4-IRD pathways in the five tissues analyzed: (a) liver; (b) brain; (c) gill; (d) heart; (e) kidney, for control and E_2-treated rainbow trout (sexes pooled, n=18 except for control kidney, n=17). A * indicates a significant difference ($p \leq 0.05$) between control and E_2-treated trout and $\underline{*}$ a marginally significant difference ($0.05 < p \leq 0.10$) between control and E_2-treated trout. An "I" indicates significant interaction between treatment and sex (shown in detail for liver T_3-IRD in Fig. 8). An "S" indicates a significant sex difference (shown in detail for heart T_4-IRD in Fig. 10). The "$\underline{I}$" and "$\underline{S}$" indicate marginally significant interaction and sex differences respectively ($0.05 < p \leq 0.10$).

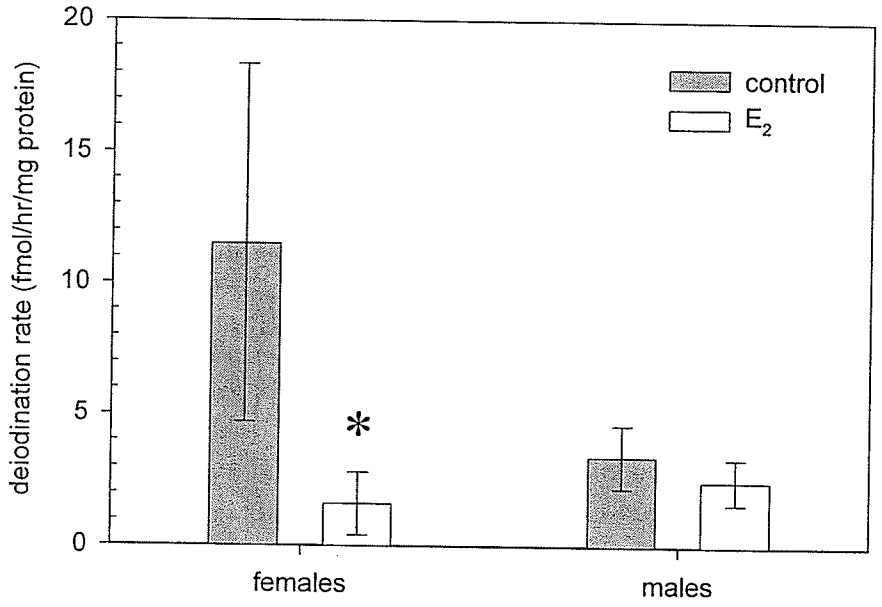

Fig. 8 Experiment I: Liver T_3-IRD rates (±SEM) for control and E_2-treated female and male rainbow trout (n = 7 for control females, n = 8 for treated females, n = 11 for control males, n = 10 for E_2-treated males). A * indicates a significant difference ($p \leq 0.05$) between control and E_2-treated trout for a given sex.

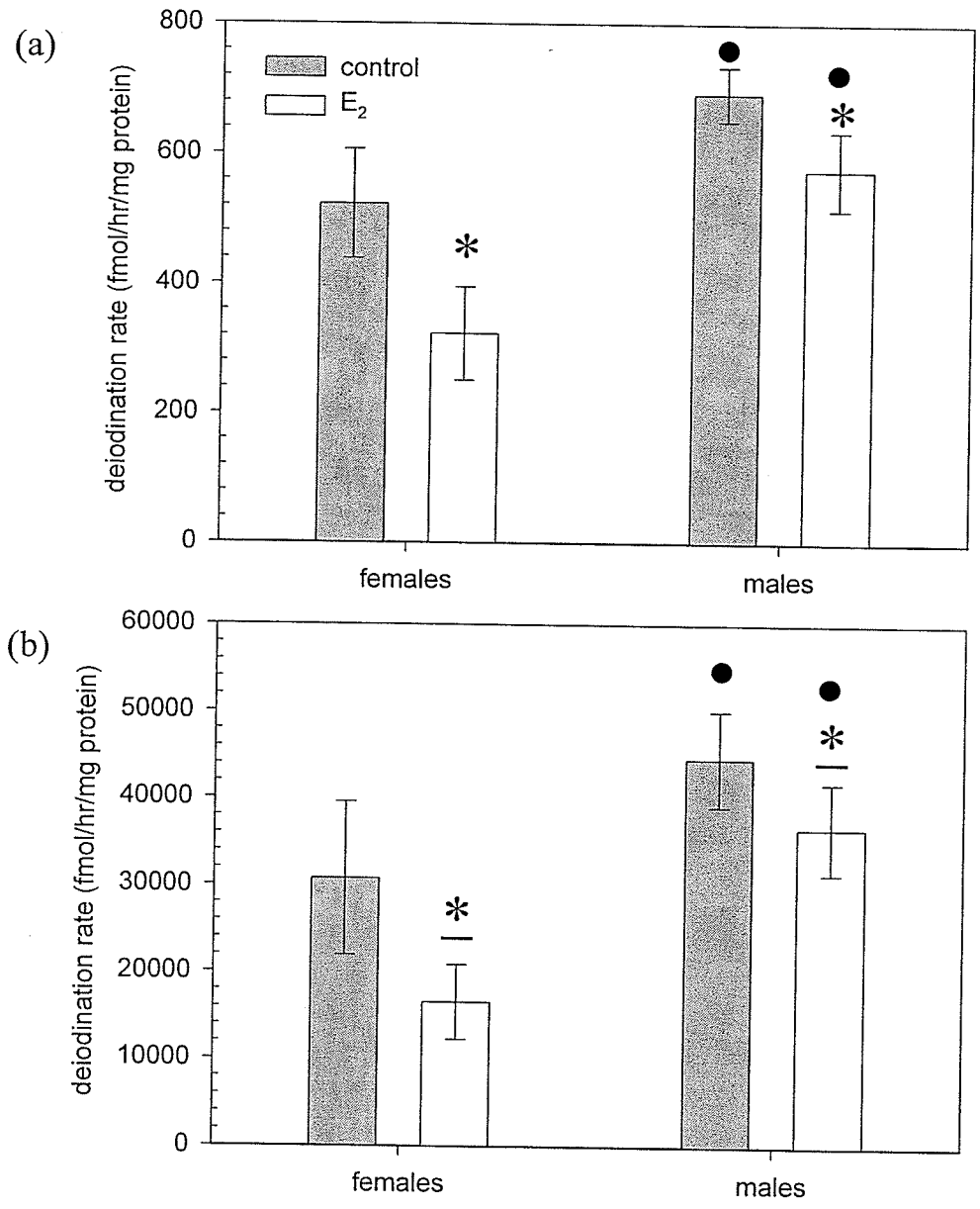

Fig. 9 Experiment I: Liver rT_3-ORD rates (±SEM) assayed at (a) low substrate concentration, and (b) high substrate concentration for control and E_2-treated rainbow trout (n = 7 for control females, n = 8 for treated females, n = 11 for control males, n = 10 for E_2-treated males). A * indicates a significant difference ($p \leq 0.05$) and $\underline{*}$ a marginally significant difference ($0.05 < p \leq 0.10$) between control and E_2-treated trout for a given sex. A ● indicates a significant difference ($p \leq 0.05$) between females and males for a given treatment.

brain. E_2-treated trout had marginally significantly greater brain T_3-IRD rates than controls by 18% ($p=0.068$) (Fig. 7b). Brain T_4-IRD rates were low and did not differ due to E_2 treatment or sex (Fig. 7b).

4.1.4.3 Gill

All gill deiodination pathways had low activity. For gill T_4-ORD, T_3-IRD and T_4-IRD rates there were no significant differences due to E_2-treatment or sex (Fig. 7c).

4.1.4.4 Heart

E_2-treated trout had lower mean heart T_4-ORD rates than controls, but there was a great amount of variance, so this difference was not significant (Fig. 7d). However, on examining the T_4-ORD rate for each individual, there were several fish in both control and E_2-treated groups with a T_4-ORD rate of zero, but in the control group, the non-zero values were consistently higher than the E_2-treated values (11-38 fmol/hr/mg protein vs. 1 –11 fmol/hr/mg protein). There were no differences due to sex. T_3-IRD was the most active deiodination pathway in heart. Heart T_3-IRD rates did not differ due to E_2 treatment or sex (Fig. 7d). Heart T_4-IRD activity was very low and was similar in controls and E_2-treated trout (Fig. 7d). For a given treatment, male T_4-IRD rates were lower than female T_4-IRD rates ($p=0.041$) (Fig. 10).

4.1.4.5 Kidney

T_4-ORD was the most active deiodination pathway measured in the kidney (Fig. 7e). Kidney T_4-ORD rates did not differ due to E_2 treatment or sex (Fig. 7e). E_2-treated trout had 73% higher kidney T_3-IRD rates than controls ($p=0.006$). For a given treatment, male kidney T_3-IRD rates were marginally significantly lower than female T_3-

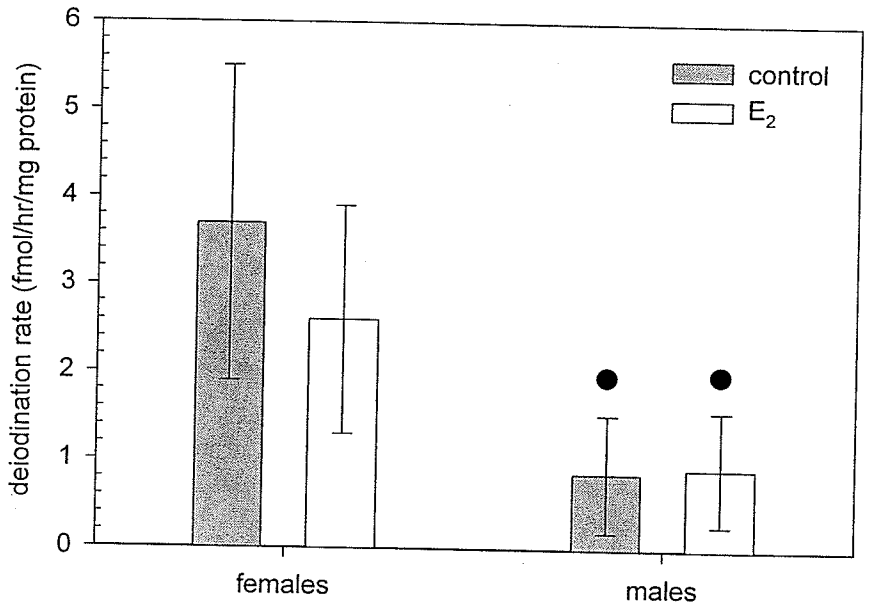

Fig. 10 Experiment I: Heart T_4-IRD rates (±SEM) for control and E_2-treated female and male rainbow trout (n = 7 for females, n = 8 for treated females, n = 11 for control males, n = 10 for E_2-treated males). A ● indicates a significant difference ($p \leq 0.05$) between females and males for a given treatment.

IRD rates (p=0.073). Overall, E_2-treated trout had higher kidney T_4-IRD rates than controls (Fig. 7e), although this was more pronounced for females and marginally significant for females only (p=0.074).

4.2 Experiment II

4.2.1 Plasma E_2 Concentrations

Plasma E_2 concentrations are shown in Fig. 11. E_2-treated females and males had plasma [E_2] of 8.4±2.6ng/ml and 8.1±3.2 ng/ml respectively, about 2.5-fold higher than controls (p=0.025). There were no differences in plasma [E_2] due to sex.

4.2.2 Body Mass, HSI, Liver Microsomal Protein Concentration, and GSI

Non-thyroidal parameters are shown in Table 1. Control and E_2-treated trout were of similar mass, but males were generally slightly smaller than females, although this was only significant for the E_2-treated group (p=0.007). E_2-treated trout had 29% higher HSIs than controls (p=0.007). For a given treatment, male HSIs were marginally significantly lower than female HSIs (p=0.093). Liver microsomal [protein] did not differ due to E_2 treatment or sex. GSIs of control and E_2-treated trout were similar. Female GSI corresponded to ovarian growth phase V as defined by Ruby and Eales (1999). The majority of females had ripe ovaries while others had ovulated eggs in the intraperitoneal cavity, some of which were being resorbed, and their gonads were small and recrudescent. For those females with many free eggs and recrudescent ovaries, their actual gonad mass was recorded as the greatest ovary mass recorded for females whose eggs were not ovulated.

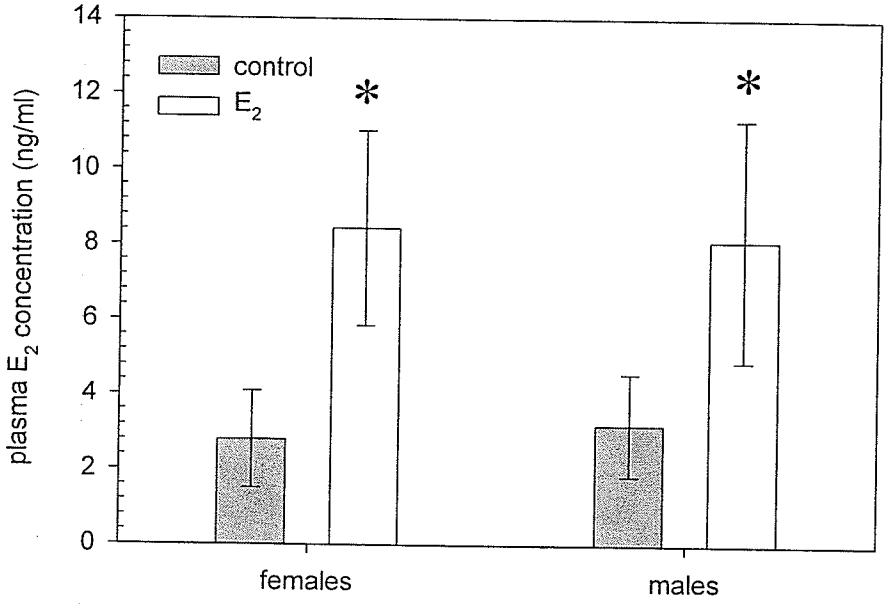

Fig. 11 Experiment II: Plasma E_2 concentrations (±SEM) for control and E_2-treated female and male rainbow trout (n = 9 for control females, n = 10 for E_2-treated females, n = 9 for control males, n = 8 for E_2-treated males). A * indicates a significant difference ($p \leq 0.05$) between control and E_2-treated trout for a given sex.

4.2.3 Plasma TH Concentrations

Overall, E_2-treated trout had lower plasma [T_3] than controls, but this difference was only significant in males where the E_2-treated mean was 64% lower than controls ($p<0.001$) (Fig. 12a). For a given treatment, there were no significant differences between female and male plasma [T_3]. One E_2-treated female had an especially high plasma [T_3] that corresponded to an extremely high liver T_4-ORD rate, and therefore was considered an outlier and excluded from statistical analysis of plasma [T_3]. Plasma [T_4] was at or below the assay detection limit (0.16 ng/ml, about 10-fold lower than plasma [T_3]) and did not differ due to E_2 treatment or sex (Fig. 12b).

4.2.4 Tissue Deiodination Rates

4.2.4.1 Liver

E_2-treated trout had almost no T_4-ORD activity (female T_4-ORD rate=0.3±0.2 fmol/hr/mg protein, male T_4-ORD rate=3.6±1.2 fmol/hr/mg protein), with 51-fold lower T_4-ORD rates than controls ($p<0.001$) (Fig.13a). For a given treatment, male T_4-ORD rates were higher than female T_4-ORD rates ($p=0.019$) (Fig. 14). One E_2-treated female liver T_4-ORD rate was 210 fmol/hr/mg protein, about 200-fold greater than the average E_2-treated T_4-ORD rate and 2-fold greater than the average control T_4-ORD rate, and was therefore considered an outlier and excluded from statistical analysis of liver T_4-ORD. For liver T_3-IRD rates and T_4-IRD rates, there were no differences due to E_2-treatment or sex (Fig. 13a).

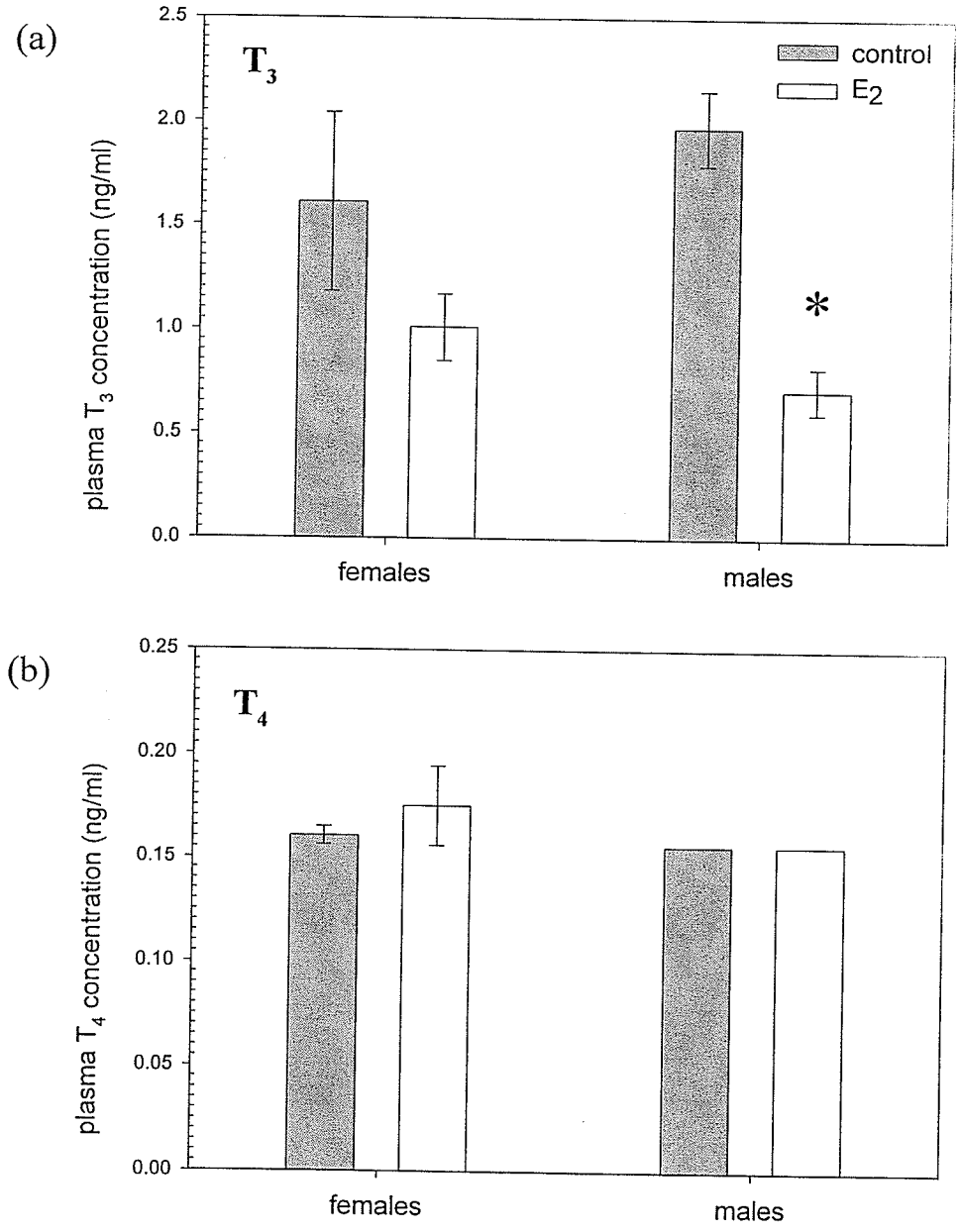

Fig. 12 Experiment II: (a) Plasma T_3 and (b) plasma T_4 concentrations (±SEM) for control and E_2-treated female and male rainbow trout (n = 9 for each group except for E_2-treated males, n = 8). A * indicates a significant difference ($p \leq 0.05$) between control and E_2-treated trout for a given sex. Male plasma [T_4] was below the detection limit (0.16 ng/ml).

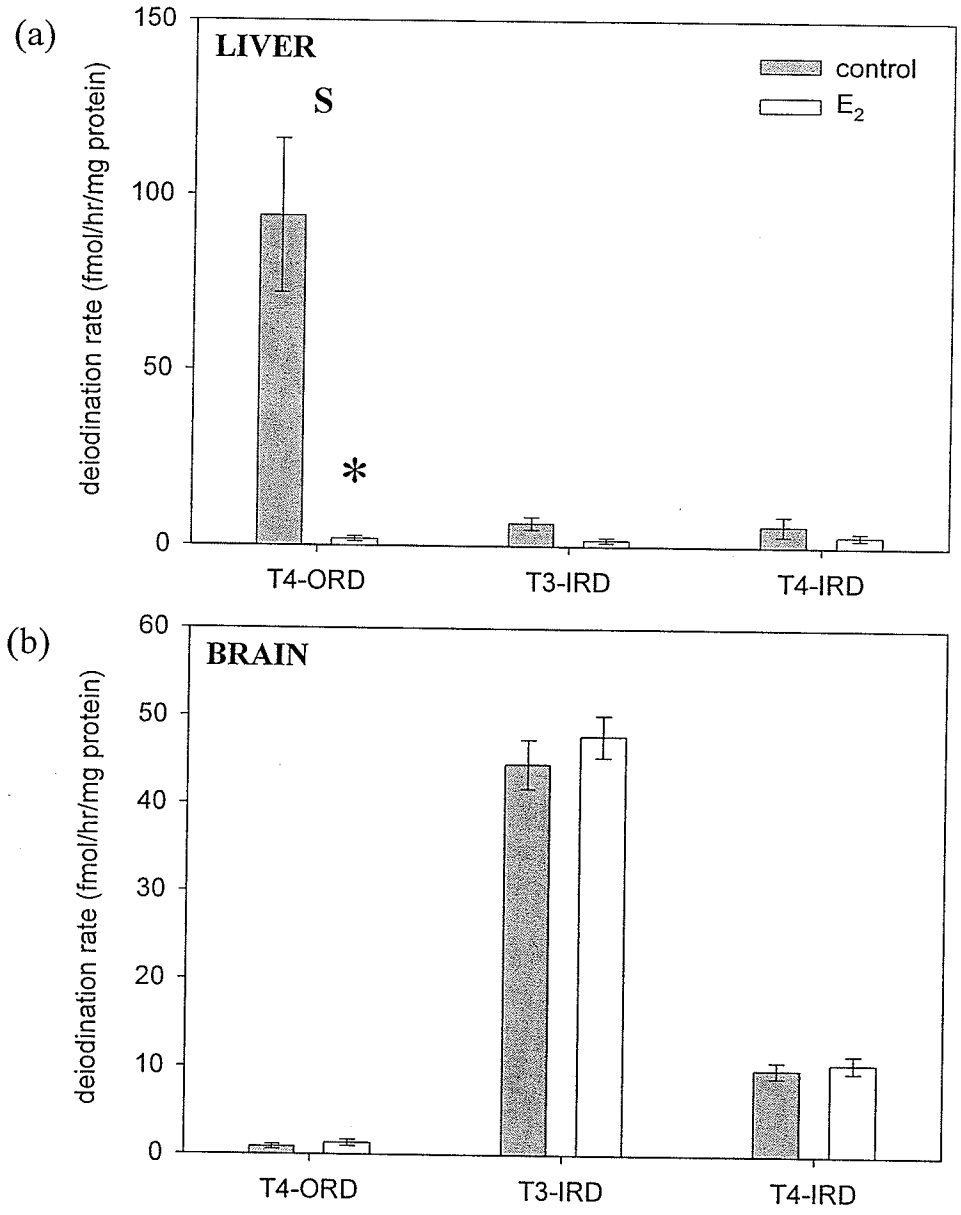

Fig. 13 Experiment II: Microsomal deiodination rates (±SEM) of T_4-ORD, T_3-IRD, and T_4-IRD pathways in (a) liver and (b) brain for control and E_2-treated rainbow trout (sexes pooled, n = 18 except for E_2-treated liver T_4-ORD, n = 17). A * indicates a significant difference ($p \leq 0.05$) between control and E_2-treated trout. A "S" indicates a significant sex difference (shown in detail for liver T_4-ORD in Fig. 14).

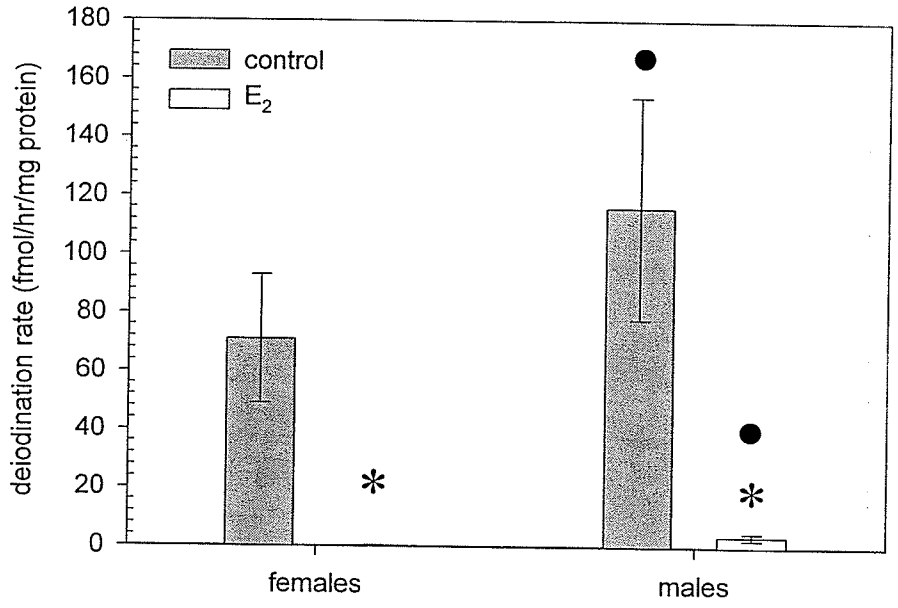

Fig. 14 Experiment II: Liver T4-ORD rates (±SEM) for control and E_2-treated female and male rainbow trout (n = 9 for all groups except for E_2-treated males, n = 8). A * indicates a significant difference ($p \leq 0.05$) between control and E_2-treated trout for a given sex. A ● indicates a significant difference ($p \leq 0.05$) between females and males for a given treatment.

As in ExI, rT_3-ORD was the only rT_3 deiodination activity detected using both low and high substrate concentrations in the rT_3 assay (Fig. 15). Also as in ExI, rT_3-ORD rates assayed at the low substrate concentration (rT_3-ORD-lo) were several fold more active than the pathways assayed with T_3 or T_4 at a similar substrate concentration. rT_3-ORD-lo was 40% lower in E_2-treated trout than controls ($p=0.024$) (Fig.15a). There were no differences due to sex (Fig. 15a). Liver rT_3-ORD rates assayed at the high substrate level did not differ significantly due to E_2-treatment or sex, although there were similar trends to rT_3-ORD-lo (Fig. 15b).

4.2.4.2 Brain

Brain T_4-ORD rates were very low, and did not differ due to E_2-treatment or sex (Fig. 13b). T_3-IRD activity was the greatest of the 3 measured pathways in the brain and did not differ due to E_2 treatment or sex (Fig. 13b). Brain T_4-IRD rates were low and did not differ due to E_2 treatment or sex (Fig. 13b).

4.3 Experiment III

4.3.1 Plasma E_2 Concentrations

Plasma E_2 concentrations are shown in Fig. 16. E_2-treated trout at all three dose levels had significantly higher plasma [E_2] than controls. Plasma [E_2] of trout administered 0.05 mg/100 g bm was 5.1±1.1 ng/ml, 9-fold higher than controls ($p=0.003$). Plasma [E_2] of trout administered 0.10 mg/100 g bm was 10.5±2.2 ng/ml, 18-fold higher than controls ($p=0.002$). Plasma [E_2] of trout administered 0.50 mg/100 g bm was 12.7±1.5 ng/ml, 21-fold higher than controls ($p<0.001$). Although this indicated a trend of dose-dependence, there were no significant differences among E_2 doses, except

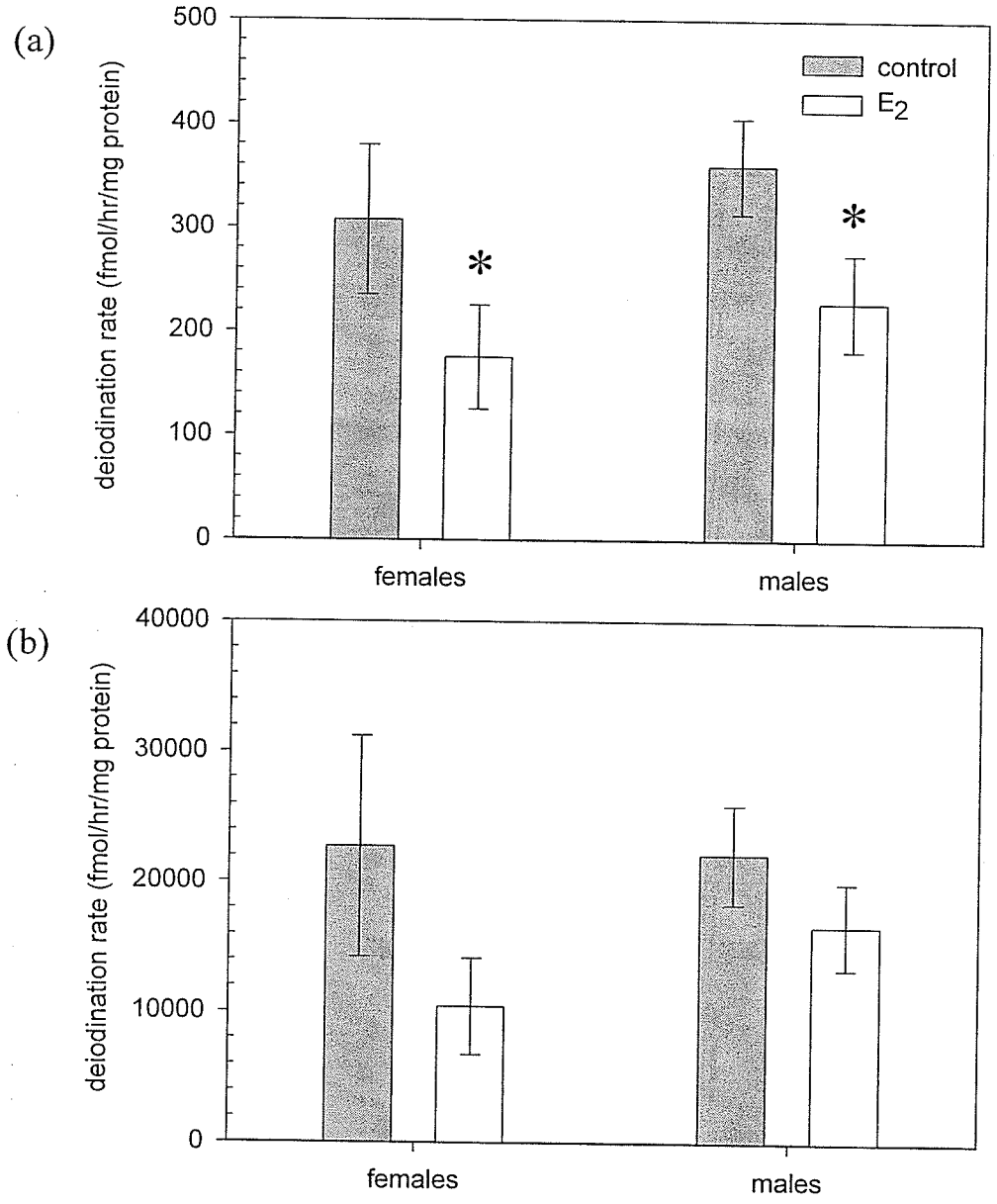

Fig. 15 Experiment II: Liver rT_3-ORD rates (±SEM) assayed at (a) low substrate and (b) high substrate concentration for control and E_2-treated rainbow trout (n = 9 for control females, n = 10 for E_2-treated females, n = 9 for control males, n = 8 for E_2-treated males). A * indicates a significant difference ($p \leq 0.05$) between control and E_2-treated trout for a given sex.

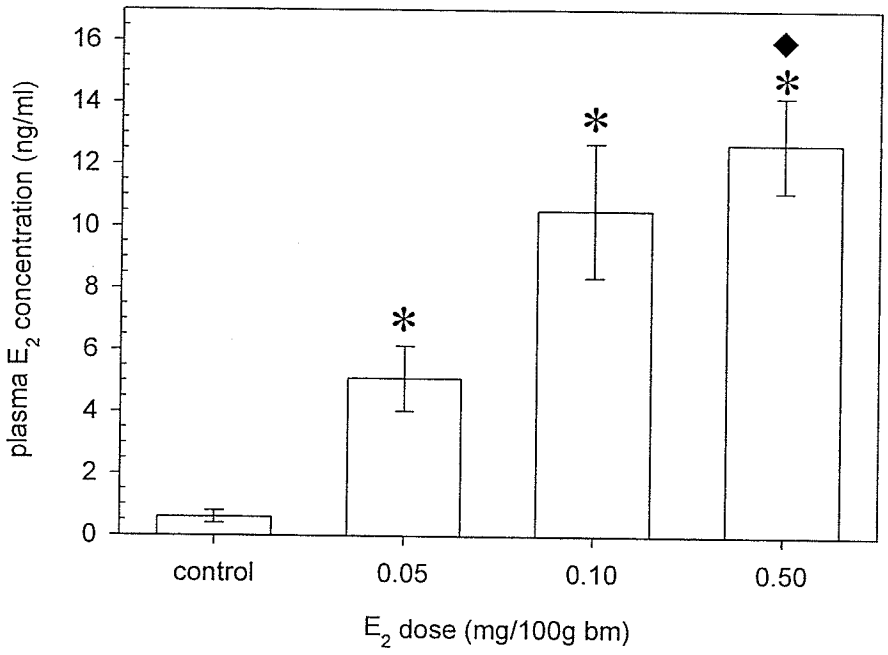

Fig. 16 Experiment III: Plasma E_2 concentrations (±SEM) for control and E_2-treated female rainbow trout given three different E_2 doses (n = 18 for each group). A * indicates a significant difference ($p \leq 0.05$) between control and E_2-treated trout of a given dose. A ◆ indicates a significant difference ($p \leq 0.05$) between the 0.05 mg/100 g bm dose group and the given dose group.

between the low and high dose groups (p=0.002).

4.3.2 Body Mass, HSI, Liver Microsomal Protein Concentration, and GSI

Non-thyroidal parameters are shown in Table 1. Control and E_2-treated trout at all three dose levels were of similar mass. E_2-treated trout at all three dose levels had significantly higher HSIs than controls. HSI of trout given 0.05 mg/100g bm was 26% greater than controls (p=0.020). HSI of trout given 0.10 mg/100g bm was 33% greater than controls (p=0.002). HSI of trout given 0.50 mg/100g bm was 41% greater than controls (p<0.001). Although this indicated a trend of dose-dependence, there were no significant differences among the HSIs of trout in the three dose groups. Liver microsomal [protein] did not differ due to E_2 treatment at any dose. GSIs also did not differ due to E_2 treatment at any dose. Ovaries were generally very small, and in several fish it was necessary to examine developing gonads with a dissecting microscope to determine that all fish were indeed female. A few had no developing oocytes at all but were assumed to be female since it was an all-female stock. GSI corresponded to ovarian growth stage I as defined by Ruby and Eales (1999).

4.3.3 Plasma TH Concentrations

There were no differences in plasma [T_3] among control and E_2-treated trout at all three doses (Fig. 17a). There were no significant differences between plasma [T_4] of controls and E_2-treated trout at all three dose levels (Fig. 12B (ExIII T_3&T_4)). Among the three doses, the only marginally significant difference in plasma [T_4] was between the lowest and highest doses (2.4-fold difference; p=0.086).

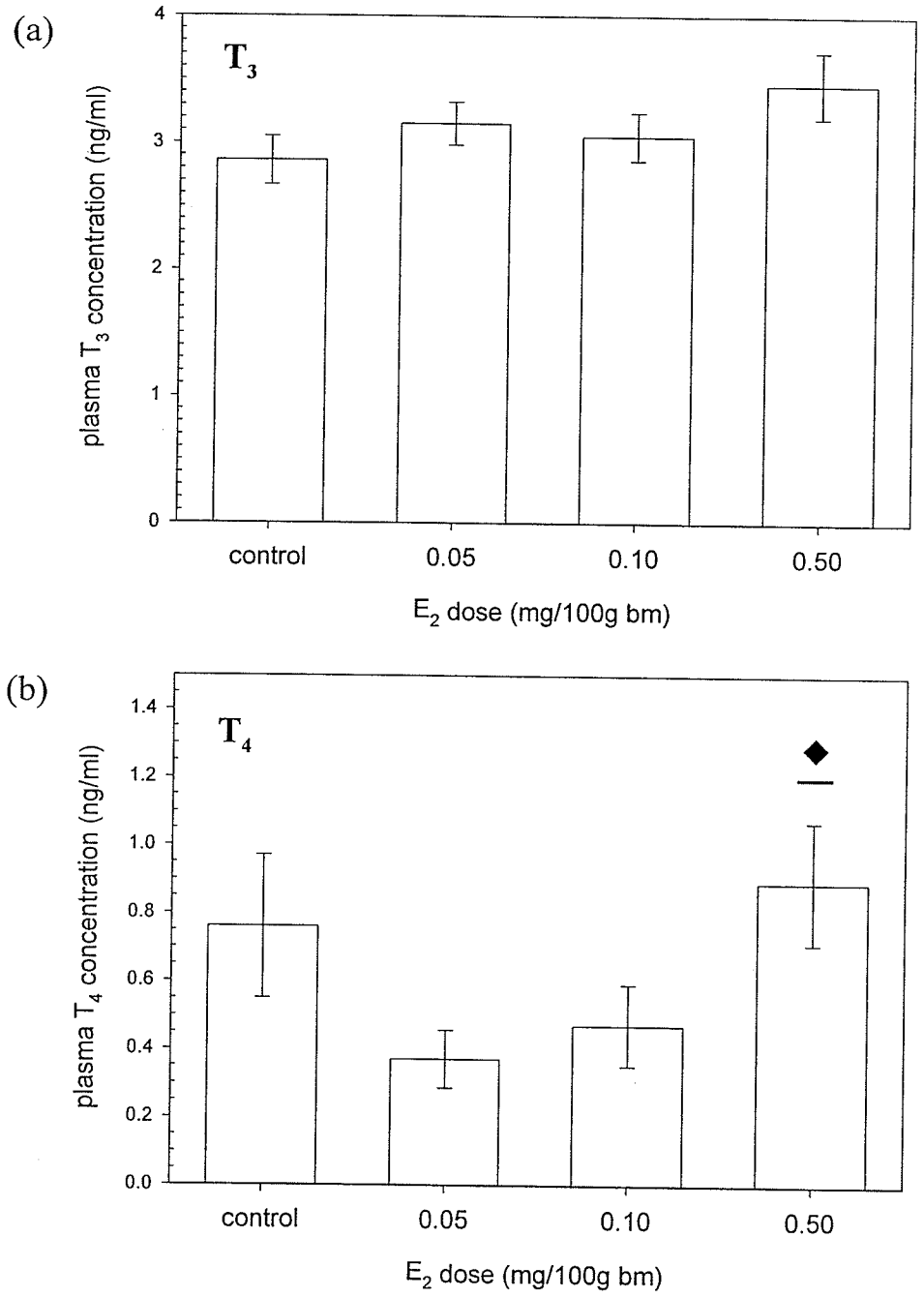

Fig. 17 Experiment III: (a) Plasma T_3 and (b) Plasma T_4 concentrations (±SEM) for control and E_2-treated female rainbow trout given three different E_2 doses (n = 18 for each group). A ◆ indicates a marginally significant difference ($0.05<p\leq0.10$) between the 0.05 mg/100 g bm dose group and the given dose group.

4.3.4 Tissue Deiodination Rates

4.3.4.1 Liver

There were no significant differences between liver deiodination rates of control and E_2-treated trout for any of the deiodination pathways measured (Fig. 18a). T_4-ORD was the most active deiodination pathway in the liver (Fig. 18a). Although not significant, mean T_3-IRD rate for all three E_2-doses was about 3-fold higher than the mean control T_3-IRD rate. On examining the liver T_3-IRD rate for each individual, control T_3-IRD rates were consistently between 0 and 14 fmol/hr/mg protein, whereas in each E_2-treated dose group, there were several T_3-IRD rates much greater than 20 fmol/hr/mg protein. This suggests that some individuals may have been affected by E_2 treatment while others were not.

4.3.4.2 Brain

There were no significant differences between brain deiodination rates of control and E_2-treated trout for any of the deiodination pathways measured (Fig. 18b). T_3-IRD was the most active deiodination pathway in the brain (Fig. 18b).

4.4 Reproductive Status and Thyroidal Status of Controls

In order to quantify the differences and establish the nature of the differences in both state of sexual maturity and thyroidal status among the three groups of fish used in the three experiments, the control means of each sex from each experiment were

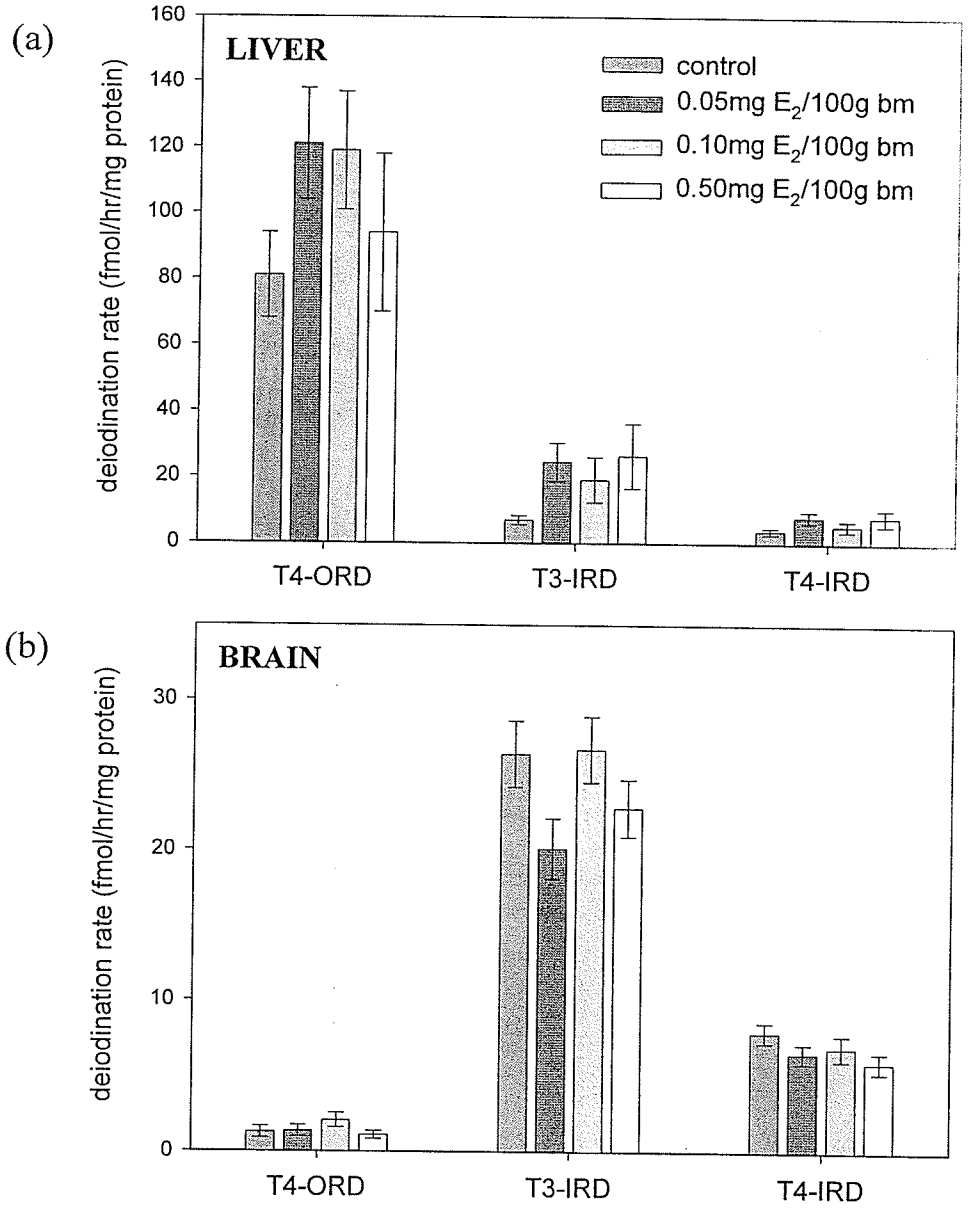

Fig. 18 Experiment III: The microsomal deiodination rates (±SEM) of T_4-ORD, T_3-IRD, and T_4-IRD pathways in (a) liver and (b) brain of female rainbow trout. There was one control group (n = 18) and three E_2 groups (each n = 18) injected with different doses.

compared for mass, plasma [E_2], HSI, liver microsomal protein concentration, GSI, plasma [TH], and liver T_4-ORD (Table 2). All data compared here have been presented in the above sections by Experiment number, but for ease of comparison and presentation of statistics for inter-experiment comparisons, the data from control fish for these variables are presented again in Table 2.

ExII control trout had significantly greater body mass than ExI and ExIII trout, and ExI trout had significantly greater body mass than ExIII trout (for all pairwise comparisons $p<0.010$). ExI females and ExII females and males had similar and the highest mean plasma [E_2], however only ExI females had marginally significantly higher plasma [E_2] than ExIII trout (females) ($p=0.056$). Mean HSI values of control trout from all three experiments were similar except for ExII females, whose HSI was significantly higher than ExI males ($p=0.043$) and marginally significantly higher than ExI females ($p=0.094$) and ExIII trout ($p=0.098$). There were no significant differences among liver microsomal protein concentrations of the three experiments, although the ExII means were the highest, followed by ExI and then ExIII. Comparing the sexes separately, ExII female control trout had a significantly greater GSI (stage V) than ExI females (stage IV) ($p<0.001$) and ExIII trout ($p<0.001$). ExI females had a significantly greater GSI than ExIII trout (stage I) ($p=0.048$). There were no significant differences between ExI and ExII male GSIs.

ExIII control trout had the highest plasma [T_3] which was significantly greater than the ExI male mean ($p=0.021$) and marginally significantly greater than ExII female mean ($p=0.056$) and ExII male mean ($p=0.057$). ExI females and males and ExIII trout had similar plasma [T_4], all of which were marginally significantly higher than ExII

TABLE 2

INTER-EXPERIMENTAL DIFFERENCES AMONG CONTROL FEMALE AND MALE RAINBOW TROUT FROM EXI, EXII, AND EXIII IN VARIABLES INDICATING REPRODUCTIVE STATUS AND THYROIDAL STATUS

Experiment Number and Date	sex	n	Body Mass (g)		$[E_2]$ (ng/ml)		HSI (%bw)		Liver Microsomal [Protein] (mg/g)		GSI (%bw)		$[T_3]$ (ng/ml)		$[T_4]$ (ng/ml)		T_4-ORD (fmol/hr/mg protein)	
			Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
I	F	7	530^{a}	20	2.9^{a}	0.6	$1.29^{\underline{a}}$	0.07	11.3^{a}	0.4	0.7^{a}	0.2	2.5^{ac}	0.3	$1.2^{\underline{a}}$	0.3	220^{ac}	90
May	M	11	560^{a}	20	0.54^{b}	0.08	1.24^{a}	0.05	11.4^{a}	0.7	0.6^{ac}	0.2	1.8^{b}	0.3	$0.8^{\underline{b}}$	0.2	220^{a}	40
II	F	9	1500^{b}	200	3^{ab}	1	1.8^{b}	0.2	12.5^{a}	0.8	14^{b}	2	$1.6^{\underline{ab}}$	0.4	0.16^{c}	0.00	70^{bd}	20
March	M	9	1200^{b}	100	3^{ab}	1	$1.5^{\underline{a}}$	0.2	12.5^{a}	0.6	1.0^{a}	0.2	$2.0^{\underline{ab}}$	0.2	0.16^{c}	0.00	120^{ac}	40
III July	F	18	310^{c}	10	$0.6^{\underline{b}}$	0.2	$1.29^{\underline{a}}$	0.08	10.5^{a}	0.5	0.113^{c}	0.008	2.9^{c}	0.2	$0.8^{\underline{ab}}$	0.2	80^{cd}	10

Note: Significant differences among the three experiments were determined with pairwise comparisons of all means for each variable, using Tukey's Honestly Significant Difference Test or Dunnett's T3 Test. Differences are indicated by letter superscripts:
For each variable, means that are not statistically different have at least one letter superscript in common. Means that are significantly different ($p \leq 0.05$) have no letter superscript in common. If means are only marginally significantly different ($0.05 < p \leq 0.10$), one of the two means will have an underlined letter superscript.

female and male plasma [T_4] by 5 to 7.5-fold (for all pairwise comparisons $p \cong 0.100$). ExI females and males had the highest liver T_4-ORD rates, the ExI female mean being significantly higher than ExII female mean ($p=0.024$) and ExI male mean being higher than ExII female mean ($p=0.002$) and ExIII mean ($p=0.027$).

5 Discussion

5.1 Differences in the Three Control Groups of Fish

The three groups of fish used in the three experiments were different ages and sizes (mass), were studied at different times of year, and the ExIII group was of a different genetic stock. Comparison of variables indicating reproductive status in controls confirmed that these groups of fish were at different states of sexual maturity, decreasing with age and body mass, with ExII trout the most sexually mature, followed by ExI trout, then ExIII trout the least mature.

Based solely on female GSIs, ExII trout were in the most advanced ovarian growth stage, stage V (Ruby and Eales, 1999), which actually represented recent ovulation for most individuals. ExII plasma [E_2] was also at the low levels expected during ovulation and spawning in rainbow trout (Bohemen and Lambert, 1981). Ovarian growth stage IV for ExI females indicates that they were in the mid stages of exogenous vitellogenesis (Ruby and Eales, 1999). However, low ExI control plasma [E_2] indicates that the plasma E_2 surge associated with exogenous vitellogenesis had not yet occurred. Ovarian growth stage I for ExIII indicates that these trout were previtellogenic (Ruby and Eales, 1999) and the low plasma [E_2] also reflected the lowest plasma [E_2] expected in rainbow trout during endogenous vitellogenesis (Bohemen and Lambert, 1981). The significantly greater HSI in ExII females compared to all other groups also indicates that ExII females were in a more advanced state of sexual maturity, since a larger HSI reflects a greater amount of VTG production.

The variables measured in this study were not ideal indicators of male reproductive status, however plasma [E_2] could reflect plasma [T] since E_2 is formed from T by the enzyme aromatase, which is present in male brain and other tissues (Andersson et al., 1988). The higher mean plasma [E_2] in ExII males than ExI males although not significant indicates that there were individuals in ExII with high plasma [E_2] and therefore suggests that some of the ExII males were in a more advanced state of sexual maturity than ExI males. Also, ExII males had significantly greater GSIs than ExIII, suggesting a generally more advanced sexual state than ExIII trout. For the purposes of this study, the male reproductive state for each experiment is assumed to parallel the female reproductive state.

Comparison of variables representative of thyroidal status for controls confirmed that there were some differences in thyroidal status among the three groups, which could have bearing on their response to E_2 and furthermore might reflect to some extent their different reproductive states. ExII trout had very low plasma [T_4] (at or below the assay detection limit) in comparison to the other two groups, suggesting that the T_4 secretion rate from the thyroid gland was lower in ExII trout. ExII females also had significantly lower liver T_4-ORD activity than the ExI trout. ExII plasma [T_3] however was not significantly lower than ExI plasma [T_3], therefore without further data on differences in thyroid hormone binding proteins, it is difficult to state equivocally whether there was a difference between ExI and ExII in T_3 availability to tissues. ExIII trout had lower or similar T_4-ORD activity to all groups yet had higher plasma [T_3] than all groups except ExI females. This high plasma [T_3] is not surprising since these fish were young and possibly in an active growth phase, thus requiring higher circulating T_3 levels. However,

some parameter other than liver T_4-ORD may have been different in this group of fish, such as TH binding proteins or TH plasma clearance rates, causing higher plasma [T_3]. Ruby and Eales (1999) found that 2-year-old female rainbow trout in ovarian growth stage I had higher T_4-ORD rates than those in stage IV, which had higher rates than those in stage V. The female T_4-ORD rates in ExI (stage IV) and ExII (stage V) agree with this finding, however the lower ExIII (stage I) T_4-ORD rate does not. Therefore, it is possible that age or genetic differences, rather than state of sexual maturity, were responsible for differences in thyroid function in ExIII trout compared to ExI and ExII.

Overall, ExII trout were the oldest and largest trout at the peak of sexual maturity, and most females had recently ovulated. There was some indication of lower thyroidal status in ExII trout in comparison with the other two groups. ExI trout were slightly younger and smaller, and were slightly less mature, the females undergoing exogenous vitellogenesis. ExIII trout were the youngest and smallest trout and were previtellogenic. Furthermore they were a different strain of rainbow trout from ExI and ExII and were an all-female stock.

Now that the physiological states of the fish have been established, the effects of E_2 administration on deiodination will be discussed. The first point to address is whether E_2-injection was effective in raising E_2 levels.

5.2 Efficacy of E_2 Injection

For all three experiments, measurements on Day 7 showed that E_2-treated trout had significantly higher plasma [E_2] than controls. For all experiments, the plasma [E_2] was raised to levels encountered during exogenous vitellogenesis over the increase to or decrease from peak plasma [E_2] levels (Van Bohemen and Lambert, 1981). Only the

mean plasma [E_2] of E_2-treated ExIII low-dose trout (5 ng/ml) was a concentration encountered during endogenous vitellogenesis in rainbow trout (Van Bohemen and Lambert, 1981). However, it is possible for all groups that E_2 levels had reached higher levels over the course of the 7-day protocol. Cyr *et al.* (1988a) found in their similar E_2-injection study on juvenile rainbow trout that E_2 levels were at approximately 40 ng/ml on Days 1 and 4, but by Day 7 had dropped to 20 ng/ml (controls were at approximately 1 ng/ml). This Day 7 level is very similar to the levels measured in E_2-treated female and male trout in ExI of the present study. In addition to higher plasma [E_2], HSI was significantly greater in E_2-treated trout than controls for all experiments, which indicates that vitellogenesis was induced or increased due to E_2 treatment in all experimental groups.

Even though E_2 was administered identically for all three experiments, E_2 levels in E_2-treated fish from the three experiments were not the same on Day 7, and they were elevated from controls to different degrees. These differences could be attributed to the different E_2 levels in the three groups before experimentation. This is however not an adequate explanation since ExI and ExII controls had similar mean plasma [E_2] and yet their E_2-treated means were substantially different (21.7±1.9 for ExI vs. 8.3±2.0 for ExII). These differences could instead be due to differences in E_2-binding to plasma proteins or E_2 metabolism in the three groups of fish. Higher E_2 turnover in ExII and ExIII fish would explain the lower E_2-treated E_2 levels in these groups. Since ExII trout were at the end of vitellogenesis when E_2 is no longer needed and ExI trout were in previtellogenesis when plasma [E_2] is low and is not required for several months, it seems

plausible that E_2 turnover could be high in these two groups of trout to keep E_2 levels low.

Despite differences in the plasma [E_2] levels in E_2-treated trout of the three experiments, experimental E_2 levels and HSIs were higher than controls for all three experiments. Therefore we could assume that we had in fact raised plasma [E_2] and stimulated vitellogenesis and therefore we could determine whether this had effects on TH deiodination.

Since microsomal [protein] is in the denominator of the equation for deiodination rate, an increase in microsomal [protein] due to E_2 treatment would result in a lower deiodination rate. Although HSIs were greater, liver microsomal protein concentrations for E_2-treated trout were not greater than controls for all experiments. Therefore the differences in liver deiodination rates between controls and E_2-treated groups were indeed due to differences in deiodination activity and not due to different protein concentrations.

5.3 E_2 Effects on Liver T_4-ORD

In this study, an E_2 dose of 0.5 mg/100 g bm in peanut oil, administered to female and male rainbow trout, resulted in significantly and substantially lower liver microsomal T_4-ORD rates and plasma [T_3] than control trout, but only in ExI and ExII. There were some differences between these two experiments in E_2 effects on liver T_4-ORD and plasma [T_3]. The T_4-ORD pathway in E_2-treated trout of ExII was almost completely inactive, operating at 0.3-3% of the control rate (0.24 – 3.6 fmol/hr/mg protein), whereas E_2-treated trout of ExI were operating at about 17% of the control rate (24.2-46 fmol/hr/mg protein). This suggests that liver in ExII trout was more sensitive to E_2

treatment than ExI trout, which could be due to higher levels of liver ERs in ExII trout. Since E_2 up-regulates ER expression in rainbow trout hepatocytes (Salbert *et al.*, 1993, Knudsen *et al.*, 1998, Mackay and Lazier, 1993) and ExII trout were at the end of exogenous vitellogenesis and therefore had recently had high plasma [E_2], it seems likely that ExII trout had higher hepatocyte ER levels than ExI trout.

In ExII, E_2-treated female plasma [T_3] was not significantly lower than control levels, despite the fact that liver T_4-ORD activity in E_2-treated females was 0.3% of control activity. In addition to this, ExII control females had significantly lower liver T_4-ORD activity than ExII males or ExI trout, and yet the control plasma [T_3] was not significantly lower in ExII females than the other groups. This lack of significance appears to likely be due to the great variance in control female plasma [T_3] in ExII, which could be due to a wider range of physiological states among individuals of this group. However, plasma [T_3] could also be higher than expected in ExII females because T_3 generation in other tissues was higher in ExII females than in the other groups, or because plasma T_3 clearance rates were lower.

Enzyme kinetics of deiodination activity were not determined in the present study, however, Cyr *et al.* (1988a) found for immature trout that lower liver T_4-ORD in E_2-treated trout was due to a 10-fold lower V_{max}. In other studies where experimental treatment (other than E_2 administration) depressed T_4-ORD, this was also generally due to major decreases in V_{max} (Eales *et al.*, 1993). Therefore, it is very likely that a lower V_{max}, or number of functional units of enzyme, was the cause of the lower T_4-ORD rate in the ExI and ExII E_2-treated trout. The mechanism by which E_2 might decrease the amount of T_4-ORD enzyme in liver has not been investigated in this study. As suggested

by Cyr *et al.* (1988a), if there is rapid turnover of deiodinase in liver, E_2 could have depressed *de novo* synthesis of the T_4-ORD enzyme in hepatocytes, thus decreasing the total level of enzyme in the liver. Alternatively, E_2 could have increased rate of degradation of the T_4-ORD enzyme.

The depression of T_4-ORD and plasma [T_3] in ExI and ExII indicates that in rainbow trout approaching the peak of sexual maturity (ExI) or having recently reached peak sexual maturity (ExII), an increase in plasma [E_2] by E_2 injection results in decreased thyroidal status at least in part by decreasing T_3 formation by liver T_4-ORD and thereby reducing T_3 availability to tissues. During vitellogenesis in rainbow trout, T_3 levels decrease as E_2 levels increase (Cyr *et al.*, 1988b). E_2 injection has been shown to decrease plasma [T_3] in mature but not reproductively active trout (Flett and Leatherland, 1989b) and to decrease both plasma [T_3] and liver T_4-ORD activity in immature rainbow trout (Cyr *et al.*, 1988a; Flett and Leatherland, 1989a). This study is the first to examine E_2 effects on liver microsomal deiodination rates in mature rainbow trout. These results are evidence of a direct effect of E_2 to lower T_3 levels by suppressing liver T_4-ORD activity in vitellogenic rainbow trout. This supports the theory proposed by Cyr *et al.* (1988a) that the increasing E_2 levels encountered during vitellogenesis act to depress T_3 levels which would suppress somatic growth thereby leaving energy resources for ovarian growth.

In ExIII, E_2 treatment did not depress liver T_4-ORD or plasma [T_3] in contrast to the results of ExI and ExII and previous studies on immature rainbow trout (Cyr *et al.*, 1988a). Sexual immaturity is therefore not an adequate explanation for the insensitivity of T_4-ORD activity to E_2 administration in ExIII trout. There have been only a few other

studies where E_2 administration did not depress thyroidal status (Cyr and Eales, 1996). It has been suggested that in these cases, there was limited scope for depression of thyroidal status, due to a pre-existing hypothyroid state (Milne and Leatherland, 1980). Given the plasma [T_3] and [T_4] and liver T_4-ORD of control ExIII trout, however, it is evident that these trout were not hypothyroid in comparison to ExI and ExII trout, so this explanation does not hold.

ExIII trout were a different genetic strain from ExI and ExII trout, and there may have been genetic selection for fast growth in this strain. It is possible that one mechanism through which fast growth is promoted is by maintenance of high plasma [T_3] by some alteration in the T_4-ORD enzyme to resist activity depression by factors such as E_2. Alternatively, the process for producing all female offspring could have had some effect on DNA of sperm or testosterone levels in sperm, which might have affected endocrine function in offspring in some way. For example, a lower rate of T_4-ORD enzyme degradation would maintain T_4-ORD enzyme levels even if E_2 decreased *de novo* synthesis of T_4-ORD enzyme. Low levels or absence of ERs in liver would also explain the insensitivity to E_2, however E_2 treatment resulted in greater HSIs in a dose-dependent manner, suggesting that E_2 was inducing vitellogenesis, which is dependent on the presence of ERs in hepatocytes (Yeo and Mugiya, 1997).

Thus, liver T_4-ORD was depressed by E_2 treatment in ExI and ExII, which corresponded to lower plasma [T_3] in E_2-treated trout. E_2 could also modulate activity of other deiodination pathways in liver, thus contributing to decreased plasma [T_3] and overall thyroidal status.

5.4 E_2 Effects on Liver T_3-IRD, T_4-IRD, and rT_3-ORD

5.4.1 Liver T_3-IRD and T_4-IRD

In various physiological states that result in depressed T_4-ORD activity, activities of IRD pathways tend to be enhanced thus contributing to an overall decrease in plasma [T_3] and therefore thyroidal status (Eales *et al.*, 1993). It was expected that in tissues where T_4-ORD was depressed by E_2 treatment, T_3-IRD and T_4-IRD activities would be enhanced. In liver of ExI and ExII fish, then, where T_4-ORD activity was substantially and significantly depressed by E_2 administration, I expected to find higher T_3-IRD and T_4-IRD activities in E_2-treated fish. Neither T_3-IRD nor T_4-IRD activities of E_2-treated fish in either ExI or ExII were higher than controls. On the contrary, in ExI, E_2-treated females had significantly lower T_3-IRD than controls. Therefore, it appears that unlike other physiological states that reduce T_4-ORD activity in rainbow trout, liver T_4-ORD activity suppression by E_2 is not accompanied by liver T_3-IRD and T_4-IRD activity induction.

It should be noted that in the previtellogenic trout of ExIII there was some evidence to suggest that liver T_3-IRD activity had been increased by E_2 treatment. Liver T_3-IRD activity was higher in some E_2-treated individuals than controls for all three doses resulting in means over 3-fold higher than controls from all three experiments (not significant). It would be useful to repeat this experiment with larger sample sizes to determine if this is a true effect of E_2 on liver T_3-IRD in this particular stock of trout.

5.4.2 Liver rT_3-ORD

Liver rT_3-ORD was the most active deiodination pathway in the liver when assayed at the low substrate concentration, which was the same as the T_3 substrate level used for assaying T_3-IRD activity, and approximately the same as the T_4 substrate level used for assaying T_4-ORD and T_4-IRD. Like liver T_4-ORD, E_2-treated trout had significantly lower rT_3-ORD than controls in both ExI and ExII. This suggests that the low substrate rT_3-ORD could be carried out by the T_4-ORD system (*i.e.* the same enzyme) as described by Finnson *et al.* (1999). One difference between T_4-ORD and rT_3-ORD activities, however, was that in ExI, male rT_3-ORD was significantly greater than female rT_3-ORD whereas there was no sex difference in ExI liver T_4-ORD. This in fact suggests that the liver low substrate rT_3-ORD and T_4-ORD systems are somewhat different. Overall, E_2 appeared to have similar effects on rT_3-ORD activity assayed at the high substrate level as rT_3-ORD activity assayed at the lower substrate level and T_4-ORD activity. However, the differences between rT_3-ORD-hi rates for E_2-treated and control fish in ExII were not statistically significant. Finnson and Eales (1999) found that T_3 challenge and fasting had different effects on high substrate rT_3-ORD activity than on T_4-ORD activity, which is in contrast to the similar effects of E_2 on these pathways determined in the present study.

Since rT_3-ORD is degrading inactive rT_3 to inactive T_2, its physiological function is likely to salvage iodide for the system (Eales and Brown, 1993). However, plasma rT_3 levels are very low in rainbow trout (Eales *et al.*, 1983). This is partly due to the high deiodinating capacity of the rT_3-ORD pathway in liver, because rT_3 formed locally by T_4-IRD would immediately be deiodinated to inactive T_2 as soon as it was formed (Finnson

et al., 1999). Even in E_2-treated trout where rT_3-ORD activity was lower than controls, the activity was still very high and likely not limiting rT_3 deiodination or iodide salvaging.

Thus in liver, the only E_2 effect on deiodination that likely contributed to decreased plasma [T_3] in ExI and ExII was a major suppression of T_4-ORD activity with no contribution by changes in IRD activities. However deiodination changes in other tissues could also affect plasma [T_3] as well as local tissue [T_3] levels.

5.5 E_2 Effects on Deiodination in Tissues Other than Liver

In all three experiments, brain T_4-ORD was very low. Thus, there was little scope for T_4-ORD depression and indeed, there were no differences between control and E_2-treated T_4-ORD rates. In ExI, both female and male E_2-treated trout had greater brain T_3-IRD activity than controls, suggesting that E_2 treatment increased brain T_3-IRD activity which would act to decrease brain T_3 levels. In ExII and ExIII there was no significant increase in brain T_3-IRD. This suggests that ExI trout brain was more sensitive to E_2 treatment than ExII and ExIII trout brain either due to state of sexual maturity and age or genetic stock. Another possibility is that since ExII trout had likely recently been exposed to high plasma [E_2] during the peak of vitellogenesis, brain T_3-IRD rates were already higher due to E_2, and therefore there was less scope for E_2 treatment to increase T_3-IRD rates.

Fines *et al.* (1999) found that a T_3 challenge that increased plasma [T_3] depressed brain T_4-ORD and T_4-IRD rates, but had no effect on brain T_3-IRD rate in immature rainbow trout. This suggests that in the present study, the higher E_2-treated brain T_3-IRD

activity in ExI was not due to altered plasma [T_3], but rather may have been due to a direct effect of E_2 on the brain to increase T_3-IRD activity.

Gill, heart and kidney all had higher T_4-ORD activities than brain, but control rates were still very low compared to liver T_4-ORD activity of ExI. This is consistent with the findings of MacLatchy and Eales (1992a) that indicated that liver T_4-ORD activity was far greater in liver than in gill, kidney or muscle. Of the four non-hepatic tissues studied, heart was the only tissue that demonstrated depression of T_4-ORD activity by E_2 although this effect was not statistically significant. Thus, overall, there was no clear-cut effect of E_2 to depress T_4-ORD in these extra-hepatic tissues.

Of gill, heart and kidney, kidney was the only tissue in which E_2 treatment appeared to increase activity of IRD pathways. Kidney T_3-IRD activity was higher in female and male E_2-treated trout compared with controls and kidney T_4-IRD activity was higher in female E_2-treated trout only compared with controls. Furthermore, in control and E_2-treated groups, female kidney T_3-IRD activity was higher than male T_3-IRD activity and was elevated to a greater extent than males. This suggests female kidney IRD activity is more sensitive to E_2 than male kidney. MacLatchy and Eales (1993) found that both T_3 and T_4 challenge to rainbow trout that increased plasma [T_3] resulted in depressed kidney T_4-ORD and enhanced kidney T_3-IRD, although in contrast to my results, they found no alteration in T_4-IRD. Since in this study, E_2 treatment increased the rate of T_3 breakdown to T_2, and increased the rate of T_4 conversion to inactive rT_3, T_3 levels in the kidney may have decreased substantially, especially in females. T_3 produced in the kidney by deiodination is not retained in kidney but instead goes into the general

circulation (MacLatchy and Eales, 1992b), therefore E_2 induction of IRD pathways in kidney could contribute to decreased plasma [T_3] observed in response to E_2 treatment.

In tissues other than liver, there is no clear-cut evidence of T_4-ORD activity suppression by E_2 treatment. IRD activity on the other hand was increased by E_2 treatment in brain and kidney. This action of E_2 to enhance IRD activity may contribute to the decreased plasma [T_3] observed in response to E_2 administration or may decrease local tissue [T_3]. These E_2 effects on IRD were specific to ExI trout for brain, and were more pronounced in females for kidney, indicating that the E_2 effect to enhance IRD is not as general as the E_2 effect to suppress liver T_4-ORD.

5.6 Effects of Lower E_2 Doses

In ExIII, all three E_2 doses raised plasma [E_2] and increased HSI above control levels, however there were no significant differences between controls and E_2-treated trout for plasma [TH] or liver and brain deiodination rates for any dose. The lowest and highest doses administered, however, elevated plasma [E_2] to significantly different levels. Furthermore, the highest dose group had higher plasma [T_4] than the lowest dose group. This suggests that the low dose of 0.05 mg/100 g bm, could affect thyroidal parameters differently than the high dose of 0.5 mg/100g bm. Overall, we were unable to determine whether lower doses than 0.5 mg/100 g bm depress liver T_4-ORD and plasma [T_3] since this high dose had no effect in ExIII trout.

5.7 Conclusions

In female and male rainbow trout in the later stages of sexual maturity, liver T_4-ORD activity is substantially depressed by an E_2 dose of 0.5 mg/100g bm administered in

two intraperitoneal injections in peanut oil over the course of 7 days. Lower plasma T_3 levels accompany this depressed liver T_4-ORD activity. In at least one genetic stock of rainbow trout, there is no effect of E_2 on thyroidal status.

Liver IRD activities are not enhanced in conjunction with liver T_4-ORD depression by E_2, and in females in earlier stages of exogenous vitellogenesis, T_3-IRD is depressed by E_2 treatment. Like liver T_4-ORD activity, liver rT_3-ORD activity is depressed by E_2. These E_2 effects on liver deiodination pathways other than T_4-ORD would not contribute to the depressed plasma [T_3] observed.

E_2 has limited effects on deiodination in brain, gill, heart and kidney. T_4-ORD activity is generally not depressed by E_2 in these tissues. Brain T_3-IRD activity is enhanced by E_2 treatment in trout in the early stages of exogenous vitellogenesis but not at the peak of sexual maturity. Kidney T_3-IRD and T_4-IRD activities are also enhanced by E_2 treatment and this effect is more pronounced in females. These E_2 effects could contribute to the depressed plasma [T_3] observed but likely have a greater role in lowering local T_3 levels and therefore tissue thyroidal status.

In the present study, it could not be conclusively determined whether E_2 doses lower than 0.5 mg/100 g bm have effects on TH deiodination.

In conclusion, in female and male rainbow trout in later stages of sexual maturity, plasma T_3 levels are depressed by E_2 administration. This effect is due at least in part to E_2 effects on TH deiodination activity, the most important of these being T_4-ORD activity depression in the liver. E_2 may also depress local T_3 levels in brain and kidney by induction of IRD pathways. Overall it appears that E_2 mainly regulates systemic rather

than local T_3 availability. State of sexual maturity, sex, and genetic stock have influences on the response to E_2 by deiodination pathways.

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Appendix A

Calculations of deiodination activity.

$$T_4\text{-ORD (fmol/hr/mg protein)} = \frac{\text{Total } T_4 \text{ substrate (fmol) x proportion of } T_4 \text{ deiodinated to } T_3 \text{ x } 2}{\text{Incubation time (hr) x microsomal protein (mg)}}$$

$$rT_3\text{-ORD (fmol/hr/mg protein)} = \frac{\text{Total } rT_3 \text{ substrate (fmol) x proportion of } rT_3 \text{ deiodinated to } T_2 \text{ x } 2}{\text{Incubation time (hr) x microsomal protein (mg)}}$$

$$T_4\text{-IRD (fmol/hr/mg protein)} = \frac{\text{Total } T_4 \text{ substrate (fmol) x proportion of } T_4 \text{ deiodinated to } rT_3}{\text{Incubation time (hr) x microsomal protein (mg)}}$$

$$T_3\text{-IRD (fmol/hr/mg protein)} = \frac{\text{Total } T_3 \text{ substrate (fmol) x proportion of } T_3 \text{ deiodinated to } T_2}{\text{Incubation time (hr) x microsomal protein (mg)}}$$

Appendix B

Summary of results of statistical analyses for each experiment and comparison of control factors among the three experiments.

- Each table represents the analysis done on one particular variable. Mean ± SEM is presented for females and males of control and E_2-treated groups and for sex means pooled for each treatment or treatment means pooled for each sex.
- Numbers in black-shaded cells are p-values from ANOVA testing. For ExI and ExII the top right corner is the treatment effect, the bottom left is the sex effect, and the bottom right is the interaction effect. Numbers in grey-shaded cells are p-values from Kruskal-Wallis testing.
- If multiple comparison procedures were performed, the p-values are presented. For ExI and ExII they appear in the bottom row and right-most column. For ExIII and ExI vs. ExII vs. ExIII they appear in the bottom 3 or 4 rows.
- If a transformation was required to homogenize variance, there is a * by the variable title, and the transformation type is specified.
- If heterogeneity of variance could not be homogenized, there is a ** by the variable title.

EXPERIMENT I

Mass (g)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	545±20	547±20	0.783
female	558±21	530±39	582±20	
male	538±18	555±22	519±29	
α: f vs. m	0.499			0.126

HSI (% body mass)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	1.260±.038	1.861±.053	0
female	1.592±.094	1.293±.069	1.854±.091	
male	1.538±.080	1.239±.045	1.867±.066	
α: f vs. m	0.76			0.622

GSI (% body mass)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	0.65±.15	0.492±.086	0.429
female	0.755±.10	0.71±.15	0.79±.13	
male	0.44±.13	0.60±0.23	0.254±.016	
α: f vs. m	0.066			0.219

[protein] in liver (mg/g)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	11.34±.42	11.25±.46	0.803
female	10.87±.37	11.29±.41	10.51±.59	
male	11.60±.45	11.37±.66	11.84±.64	
α: f vs. m	0.271			0.336

Plasma [TH] (ng/ml)

[T3]

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	2.06±.22	1.41±.12	0.006
female	2.06±.21	2.54±.33	1.63±.17	
male	1.51±.16	1.76±.26	1.23±.16	
α: f vs. m	0.019			0.438

[T4]

tmt pooled	control	E2	α: Cvs.E2
XXX	0.94±.17	0.93±.14	0.490
1.18±.20	1.20±.31	1.17±.27	
.76±.12	0.77±.20	0.75±.12	
0.066			0.997

[E2]* (Log10+1)

tmt pooled	Control	E2	α: Cvs.E2
XXX	1.45±.36	21.7±1.9	0.000
14.4±3.2	2.88±.59	24.5±2.9	0.000
9.6±2.4	.535±.83	19.6±2.4	0.000
	0.000	0.441	0.006

EXPERIMENT I: Continued

Liver Deiodination (fmol/hr/mg protein)

T4-ORD* (Log10+1)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	221±40	36.5±7.9	0
female	117±48	223±88	24.2±5.5	
male	138±28	221±38	46±13	
α: f vs. m	0.187			0.546

T3-IRD* (Log10+1)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	6.5±2.8	2.11±.71	0.030
female	6.2±3.4	11.5±6.8	1.6±1.2	0.046
male	2.95±.75	3.4±1.2	2.47±.86	0.993
α: f vs. m	0.466	0.264	0.833	0.064

T4-IRD** (unequal variance)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	1.8±1.1	1.6±1.0	0.865
female	3.2±1.6	2.6±2.6	3.7±2.1	1.000
male	0.65±0.41	1.23±.76	0±0	0.519
α: f vs. m		0.995	0.477	

rT3-ORD-[low substrate]

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	626±45	461±54	0.016
female	415±59	522±84	322±72	
male	636±37	692±42	574±60	
α: f vs. m	0.002			0.517

rT3-ORD-[high substrate]

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	39100 ±4900	27600 ±4100	0.074
female	23100±4900	30700±8800	16500 ±4300	
male	40700±3800	44500 ±5500	36500 ±5200	
α: f vs. m	0.008			0.614

Brain Deiodination (fmol/hr/mg protein)

T4-ORD

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	0.75±.36	0.48±.20	0.57
female	0.61±.31	0.69±.44	0.54±.21	
male	0.61±.23	0.78±.53	0.43±.33	
α: f vs. m	0.983			0.822

T3-IRD* (reciprocal)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	19.93±.98	23.5±1.4	0.068
female	23.4±1.7	22.2±1.7	24.5±2.8	
male	20.52±.89	18.49±.99	22.8±1.2	
α: f vs. m	0.148			0.193

T4-IRD

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	7.43±.80	7.23±.84	0.748
female	8.36±.93	8.8±1.3	7.9±1.4	
male	6.60±.69	6.54±0.94	6.7±1.1	
α: f vs. m	0.136			0.674

Gill Deiodination (fmol/hr/mg protein)

T4-ORD

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	4.4±2.1	4.2±1.7	0.95
female	4.6±1.9	4.4±2.9	4.8±2.7	
male	4.1±1.9	4.4±3.0	3.7±2.3	
α: f vs. m	0.849			0.836

T3-IRD

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	3.5±1.0	5.7±1.7	0.349
female	5.0±1.8	4.4±1.7	5.5±3.2	
male	4.4±1.1	3.0±1.3	5.8±1.8	
α: f vs. m	0.815			0.687

T4-IRD

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	4.4±1.2	1.46±.90	0.102
female	2.4±1.2	3.1±1.7	1.8±1.8	
male	3.3±1.1	5.2±1.7	1.20±.88	
α: f vs. m	0.629			0.392

Heart Deiodination (fmol/hr/mg protein)

T4-ORD** (unequal variance)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	9.6±3.0	1.83±.82	0.236
female	5.8±2.9	8.9±5.9	3.2±1.6	0.910
male	5.6±2.0	10.0±3.4	0.77±0.57	0.110
α: f vs. m		1.000	0.673	

T3-IRD

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	15.4±2.3	12.1±2.4	0.432
female	14.0±2.5	13.2±3.4	14.7±3.9	
male	13.6±2.2	16.8±3.1	10.0±3.0	
α: f vs. m	0.875			0.228

T4-IRD

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	1.98±0.84	1.67±.70	0.617
female	3.1±1.1	3.7±1.8	2.6±1.3	
male	0.89±0.46	0.86±.67	0.92±0.65	
α: f vs. m	0.041			0.584

Kidney Deiodination (fmol/hr/mg protein)

T4-ORD

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	9.8±2.6	8.4±2.6	0.486
female	11.1±3.5	15.4±5.3	9.0±3.2	
male	7.8±2.0	6.7±2.6	7.8±4.7	
α: f vs. m	0.322			0.198

T3-IRD* (trans: log10 +1)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	1.89±.97	7.1±1.5	0.006
female	7.4±2.0	3.3±2.0	10.4±2.7	
male	2.70±.87	1.1±1.0	4.4±1.3	
α: f vs. m	0.073			0.981

T4-IRD** (unequal variance)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	1.21±.77	4.4±1.3	0.057
female	3.8±1.5	0±0	6.7±2.1	0.074
male	2.17±0.88	1.9±1.2	2.5±1.4	1.000
α: f vs. m		0.529	0.487	

EXPERIMENT II

Mass** (unequal variance) (g)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	1330±100	1392±89	0.025
female	1540±100	1450±180	1620±110	0.748
male	1159±56	1203±97	1110±49	0.941
α: f vs. m		0.770	0.007	

HSI (% body mass)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	1.63±.11	2.11±0.12	0.007
female	2.02±0.11	1.78±.15	2.23±.15	
male	1.71±0.13	1.48±.17	1.97±.19	
α: f vs. m	0.093			0.921

GSI (% body mass)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	7.4±1.7	8.2±1.8	0.991
female	14.01±0.98	13.9±1.6	14.1±1.3	
male	0.908±.098	1.01±0.15	0.79±.11	
α: f vs. m	0.000			0.829

[protein] in liver (mg/g liver)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	12.48±.48	13.39±.49	0.221
female	13.02±.53	12.47±.77	13.51±.75	
male	12.84±.45	12.49±.63	13.23±.65	
α: f vs. m	0.854			0.833

Plasma [TH] (ng/ml)

[T3]** (unequal variance)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	1.79±.23	0.87±.10	0.006
female	1.31±.24	1.61±.43	1.01±.16	0.722
male	1.37±.19	1.97±.18	0.71±.11	0.000
α: f vs. m		0.964	0.565	

[T4]** (unequal variance)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	0.1584 ±.0022	0.167 ±.010	0.606
female	0.1681±.0 099	0.1606 ±.0044	0.175 ±0.019	0.966
male	0.156±0	0.156±0	0.156±0	---
α: f vs. m		0.883	0.884	

[E2]

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	2.99±.91	8.28±1.96	0.025
female	5.76±1.60	2.79±1.29	8.43±2.59	
male	5.50±1.73	3.19±1.37	8.10±3.21	
α: f vs. m	0.986			0.873

Liver Deiodination (fmol/hr/mg protein)

T4-ORD

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	94±22	1.85±.71	0.000
female	36±14	71±22	.25±16	
male	63±24	116±38	3.6±1.2	
α: f vs. m	0.019			0.651

T3-IRD** (unequal variance)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	6.5±1.8	1.69±.75	0.026
female	5.4±1.7	8.3±3.2	2.9±1.3	0.563
male	2.6±1.0	4.7±1.7	0.21±.21	0.139
α: f vs. m		0.891	0.288	

T4-IRD* (log10+1)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	5.9±2.9	3.1±1.0	0.386
female	2.86±.78	2.65±.99	3.0±1.2	
male	6.3±3.1	9.2±3.1	3.1±1.8	
α: f vs. m	0.629			0.35

rT3-ORD-[low substrate]

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	333±42	199±34	0.024
female	237±45	307±72	175±50	
male	297±36	359±46	228±46	
α: f vs. m	0.346			0.990

rT3-ORD-[high substrate]* (log10+1)

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	22400 ±4500	13200 ±2600	0.323
female	16200 ±4600	22700±85 00	10400 ±3700	
male	19500 ±2600	22100 ±3800	16600 ±3300	
α: f vs. m	0.082			0.650

Brain Deiodination (fmol/hr/mg protein)

T4-ORD

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	0.83±.27	1.29±.36	0.281
female	0.86±.23	0.73±.32	0.97±.33	
male	1.29±.40	0.93±.45	1.69±.70	
α: f vs. m	0.314			0.575

T3-IRD

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	44.5±2.8	47.7±2.4	0.422
female	45.6±2.0	42.7±2.1	48.2±3.1	
male	46.7±3.3	46.4±5.4	47.1±4.0	
α: f vs. m	0.731			0.541

T4-IRD

	tmt pooled	control	E2	α: Cvs.E2
sex pooled	XXX	9.81±.92	10.5±1.0	0.687
female	10.32±.89	9.5±1.1	11.1±1.4	
male	9.9±1.0	10.2±1.5	9.7±1.6	
α: f vs. m	0.796			0.458

EXPERIMENT III

Mass** (unequal variance) (g)

	control	high	medium	low	α: anova
mean	307±13	306±14	310±11	314±15	**0.978**
control	XXX	1.000	0.999	0.985	XXX
high	XXX	XXX	0.997	0.978	XXX
medium	XXX	XXX	XXX	0.997	XXX

HSI (% body mass)

	control	high	medium	low	α: anova
mean	1.29±0.08	1.82±.08	1.72±.07	1.63±.08	**0.000**
control	XXX	**0.000**	**0.002**	**0.020**	XXX
high	XXX	XXX	0.797	0.304	XXX
medium	XXX	XXX	XXX	0.835	XXX

GSI (% body mass)

	control	high	medium	low	α:anova
mean	0.1127 ±.0077	0.1180 ±.0076	0.0980 ±.0094	0.1148 ±.0072	**0.308**
control	XXX	0.965	0.568	0.998	XXX
high	XXX	XXX	0.298	0.992	XXX
medium	XXX	XXX	XXX	0.456	XXX

[protein] in liver (mg/g liver)

	control	high	medium	low	α: anova
mean	10.53±.46	10.76±.29	10.63±.37	11.38±.46	**0.448**
control	XXX	0.979	0.998	0.450	XXX
high	XXX	XXX	0.996	0.694	XXX
medium	XXX	XXX	XXX	0.559	XXX

Plasma [TH] (ng/ml)

[T3]

	control	high	medium	low	α: anova
mean	2.86±.19	3.46±.26	3.05±.19	3.15±.17	**0.227**
control	XXX	**0.176**	0.910	0.754	XXX
high	XXX	XXX	0.505	0.709	XXX
medium	XXX	XXX	XXX	0.988	XXX

[T4]**
(unequal variance)

	control	high	medium	low	α:KW
mean	0.76±.21	0.89±.18	0.47±.12	0.37±.09	0.257
control	XXX	0.997	0.792	0.450	XXX
high	XXX	XXX	0.307	**0.087**	XXX
medium	XXX	XXX	XXX	0.985	XXX

[E2]**
unequal variance

	control	high	medium	low	α:KW
mean	0.59±.20	12.7±1.5	10±2	5.1±1.0	**0.000**
control	XXX	**0.000**	**0.002**	**0.003**	XXX
high	XXX	XXX	0.958	**0.002**	XXX
medium	XXX	XXX	XXX	0.173	XXX

Liver Deiodination (fmol/hr/mg protein)

T4-ORD**
unequal variance

	control	high	medium	low	α: KW
mean	81±13	94±24	119±18	121±17	0.253
control	XXX	0.997	0.439	0.384	XXX
high	XXX	XXX	0.953	0.936	XXX
medium	XXX	XXX	XXX	1.000	XXX

T3-IRD*
trans: log10+1

	control	high	medium	low	α:anova
mean	7.0±1.4	26.4±9.8	19.1±6.8	24.5±5.8	**0.210**
control	XXX	0.545	0.640	0.153	XXX
high	XXX	XXX	0.999	0.855	XXX
medium	XXX	XXX	XXX	0.778	XXX

T4-IRD

	control	high	medium	low	α: anova
mean	3.8±1.0	7.9±2.4	5.3±1.6	8.0±1.7	**0.255**
control	XXX	0.358	0.933	0.331	XXX
high	XXX	XXX	0.718	1.000	XXX
medium	XXX	XXX	XXX	0.687	XXX

Brain Deiodination (fmol/hr/mg protein)

T4-ORD

	control	high	medium	low	α: anova
mean	1.27±.37	1.10±.28	2.06±.48	1.35±.38	**0.316**
control	XXX	0.989	0.478	0.999	XXX
high	XXX	XXX	0.302	0.969	XXX
medium	XXX	XXX	XXX	0.302	XXX

T3-IRD

	control	high	medium	low	α:anova
mean	26.4±2.2	22.8±1.9	26.7±2.2	20.1±2.0	**0.089**
control	XXX	0.614	1.000	0.154	XXX
high	XXX	XXX	0.554	0.802	XXX
medium	XXX	XXX	XXX	0.127	XXX

T4-IRD

	control	high	medium	low	α: anova
mean	7.86±.67	5.89±.65	6.86±.82	6.49±.62	**0.246**
control	XXX	0.195	0.738	0.510	XXX
high	XXX	XXX	0.757	0.926	XXX
medium	XXX	XXX	XXX	0.983	XXX

EXI vs. EXII vs. EXIII CONTROLS

Mass-controls** (unequal variance) (g)

	ExI-CF	ExII-CF	ExIII-CF	ExI-CM	ExII-CM	K-W
means	530±39	1450 ±180	307±13	555±22	1203±97	0.000
ExI-CF	XXX	0.006	0.008	XXX	XXX	
ExII-CF	XXX	XXX	0.002	XXX	XXX	
ExI-CM	XXX	0.009	0.000	XXX	XXX	
ExII-CM	0.001	XXX	0.000	0.001	XXX	

HSI-controls** (unequal variance) (% body mass)

	ExI-CF	ExII-CF	ExIII-CF	ExI-CM	ExII-CM	K-W
means	1.293 ±.069	1.78±.15	1.293 ±.081	1.239 ±.045	1.48±.17	0.044
ExI-CF	XXX	0.094	1.000	XXX	XXX	
ExII-CF	XXX	XXX	0.098	XXX	XXX	
ExI-CM	XXX	0.043	0.999	XXX	XXX	
ExII-CM	0.950	XXX	0.958	0.791	XXX	

GSI-controls** (unequal variance) (% body mass)

	ExI-CF	ExII-CF	ExIII-CF	ExI-CM	ExII-CM	K-W
means	0.71±.15	13.9±1.6	0.1127 ±.0077	0.60±.23	1.01±.15	0.000
ExI-CF	XXX	0.000	0.048	XXX	XXX	
ExII-CF	XXX	XXX	0.000	XXX	XXX	
ExI-CM	XXX	0.000	0.408	XXX	XXX	
ExII-CM	0.808	XXX	0.003	0.781	XXX	

[protein] in liver – controls (mg/g liver)

	ExI-CF	ExII-CF	ExIII-CF	ExI-CM	ExII-CM
means	11.3±.4	12.5±.8	10.5±.5	11.4±.7	12.5±.6
ExI-CF	XXX	0.756	0.911	XXX	XXX
ExII-CF	XXX	XXX	0.131	XXX	XXX
ExI-CM	XXX	0.732	0.797	XXX	XXX
ExII-CM	0.744	XXX	0.124	0.719	XXX

anova	
1-WAY	0.081
Ex# main	0.064
sex main	0.934
interaction	0.958

Plasma [TH] (ng/ml)

[T3]-controls* (square)

	ExI-CF	ExII-CF	ExIII-CF	ExI-CM	ExII-CM
means	2.54±.33	1.61±.43	2.86±.19	1.76±.26	1.97±.18
ExI-CF	XXX	0.612	0.882	XXX	XXX
ExII-CF	XXX	XXX	0.056	XXX	XXX
ExI-CM	XXX	1.000	0.021	XXX	XXX
ExII-CM	0.617	XXX	0.057	1.000	XXX

anova	
1-WAY	0.008
Ex# main	0.027
sex main	0.245
interaction	0.241

[T4]-controls** (unequal variance)

	ExI-CF	ExII-CF	ExIII-CF	ExI-CM	ExII-CM	K-W
means	1.20±.31	0.1606 ±.0044	0.76±.21	0.77±.20	0.156±0	0.000
ExI-CF	XXX	0.102	0.911	XXX	XXX	
ExII-CF	XXX	XXX	0.101	XXX	XXX	
ExI-CM	XXX	0.102	1.000	XXX	XXX	
ExII-CM	0.100	XXX	0.097	0.099	XXX	

[E2]-controls

	ExI-CF	ExII-CF	ExIII-CF	ExI-CM	ExII-CM	K-W
means	2.9±.6	2.8±1.3	0.6±0.2	0.54±.08	3.2±1.4	0.009
ExI-CF	XXX	1.000	0.056	XXX	XXX	
ExII-CF	XXX	XXX	0.637	XXX	XXX	
ExI-CM	XXX	0.602	1.000	XXX	XXX	
ExII-CM	1.000	XXX	0.523	0.493	XXX	

Liver Deiodination (fmol/hr/mg protein)

T4-ORD controls* (Log10 + 1)

	ExI-CF	ExII-CF	ExIII-CF	ExI-CM	ExII-CM
means	223±88	71±22	81±13	221±38	116±38
ExI-CF	XXX	0.024	0.186	XXX	XXX
ExII-CF	XXX	XXX	0.613	XXX	XXX
ExI-CM	XXX	0.002	0.027	XXX	XXX
ExII-CM	0.518	XXX	0.988	0.203	XXX

anova	
1-WAY	0.002
Ex# main	0.011
sex main	0.158
interaction	0.383