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The Effects of Hormones on Development of Embryonic and Post Embryonic
Salmonids, and Hormone Metabolism During These Stages.

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirement of the

degree of

Master of Science

Completed April 12, 1993

Commencement June 1993

APPROVED:

Professor of Fisheries in charge of major

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Date of thesis is presented April 12, 1993

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ACKNOWLEDGEMENTS

I cordially thank my major professor, Dr. Carl B. Schreck for his guidance and unwavering support throughout this study. I thank all members of the Oregon Cooperative Fishery and Research Unit, especially Dr. Martin "gentleman and a scholar" Fitzpatrick, Grant "such a dear" Feist, Cameron "sir computer" Sharpe, Dan "smiley" Farnsworth, Caleb "chinook slayer" Slater, Iris "brain kid" Knoebl, and Rob "hatchery honcho" Chitwood for their assistance. I also thank Ying-Chi Lam, Sze-Wing Ngan, and Eric Green for generously contributing their time in assisting in hatcheries and laboratories. Dr. Ted Kerstetter and Dr. Dave Hankin played instrumental roles in my decision to further my studies. Carrie Cook and Elaine Almgren provided me with the "Total American Experience" which included many censored topics and items! My beloved family; Dato' Yeoh Kian Teik, Chiew Ngai Fong, Yeoh Choo-San, Yeoh Choo-Hor, and Yeoh Lam-Poh have been most supportive and encouraging.

Dr. Carl B. Schreck is co-author of all manuscripts in this thesis because he provided the opportunity, guidance, and most importantly the enthusiasm to all my endeavours in science. Dr. Martin S. Fitzpatrick is a co-author in Chapter 2 and 3 because he assisted me in the classroom, laboratory, and hatchery. Grant Feist is a co-author in Chapter 2 because he assisted in the experimental design and in Chapter 3 because the HPLC procedure was modified from his previous work. I also thank Dr. Lawrence Curtis, Dr. Frank Moore, and Dr. James Pease for critically reviewing this thesis and Gene Pennello for his advice in statistics.

Finally, I am glad I opted for a career in Fisheries.

THE ROAD NOT TAKEN

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I-
I took the one less traveled by,
And that has made all the difference.

Robert Frost

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CHAPTER 1 - INTRODUCTION

This Master's Thesis consists of three chapters and three appendices. Chapter 1 titled "Effects of hormones on early development of steelhead trout, *Oncorhynchus mykiss*, and profiles of cortisol and cortisol glucuronide during and beyond embryogenesis" elucidates the effects of hormones on early development in steelhead trout. This chapter also determines the apparent ability of young trout to metabolize exogenous cortisol. Dynamic fluctuations of endogenous cortisol and cortisol glucuronide throughout early development support the notion of the ability to metabolize and clear cortisol as cortisol glucuronide.

Chapter 2 titled "Endogenous steroid metabolism indicated by fluctuations of endogenous steroid and steroid glucuronide concentrations in early development of the steelhead trout, *Oncorhynchus mykiss*" explores the possibility of endogenous steroid metabolism in embryos and hatched steelhead trout. Whole body profiles of steroids show dynamic fluctuations in steroid concentrations which suggest the presence of endogenous steroid metabolism. I also attempted to compare whole body profiles of steroids in bisexual populations to steroid profiles in gynogenetic (all-female) populations of steelhead trout to determine if chromosomal sex of these animals could be determined prior to the onset of gonadal differentiation.

Chapter 3 titled "*In vivo* steroid metabolism in embryonic and newly hatched steelhead trout, *Oncorhynchus mykiss*" determines the ability of embryos and alevins of steelhead trout to metabolize hormone precursors and form hormone glucuronides. There are indications that young trout have the ability to excrete hormones and hormone glucuronides.

Appendix 1 consists of a preliminary experiment using chinook salmon, *O. tshawytscha* eggs. The experimental design was similar to Chapter 1. Appendix 2 consists of a preliminary experiment exploring the feasibility of microinjecting steroids into salmonid alevins and Appendix 3 is a preliminary experiment to determine if augmentation of maternally contributed hormones affect early development of chinook salmon.

In concluding, these series of experiments provide additional information on the possible importance of hormones to early development in fish.

Effects of Hormones on Early Development of Steelhead Trout (*Oncorhynchus mykiss*) and Profiles of Cortisol and Cortisol Glucuronide During and Beyond Embryogenesis¹

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ABSTRACT

The importance of hormone reservoirs in mature teleost eggs is unknown. To investigate the effects of hormones on embryonic development, steelhead trout, *Oncorhynchus mykiss*, eggs were immersed in cortisol, testosterone, or thyroxine at two different stages of development. Elevated concentrations of cortisol were detectable in the trout eggs or embryos after immersion. Cortisol accelerated hatching in eggs exposed during water hardening but had no effect when given at the eyed stage; eggs that hatched faster were also more synchronous in time of hatching. Eggs that hatched faster had elevated cortisol and cortisol glucuronide concentrations at hatch compared with those immersed at the eyed stage or controls. The decline of these elevated concentrations of cortisol and cortisol glucuronide during embryonic development indicates conversion, clearance or both. Eggs exposed to cortisol at the earlier developmental stage did not seem to clear or convert cortisol as efficiently as those exposed at a later stage. Testosterone did not accelerate hatching in steelhead trout. Thyroxine accelerated hatching in eggs immersed at the eyed stage but not when given at water hardening; eggs that hatched faster were more synchronous in hatching time than other groups. Prior to exogenous feeding (50 days postfertilization, dpf), fish (eggs) immersed in cortisol at the eyed stage had higher mean condition factor (K_N) than other experimental or control groups, but this effect disappeared by 83 dpf. However, at 130 dpf, cortisol groups that were immersed at the eyed stage were again heavier, longer, and more robust than other groups.

At 50 dpf, fish from eggs immersed in thyroxine at water hardening were significantly longer and less robust (smaller K_T) than controls. These effects disappeared by 83 dpf.

INTRODUCTION

Mature, unfertilized teleost eggs have high concentrations of hormones such as cortisol (De Jesus et al., 1991; De Jesus and Hirano, 1992; Hwang et al., 1992), testosterone (Rothbard et al., 1987; Feist et al., 1990; Schreck et al., 1991), and thyroxine (Kobuke et al., 1987; Tagawa and Hirano, 1990), which decline throughout embryogenesis. The source of these hormones is thought to be maternally contributed because hormone concentrations found in ovarian fluid and maternal plasma parallel concentrations in the eggs (Feist et al., 1990). Steroids in ovarian fluid or plasma may enter the eggs because steroids are able to passively diffuse through egg membranes (Maneckjee et al., 1991). The importance of these maternally contributed hormones to embryonic development and beyond embryogenesis is unclear (Schreck et al., 1991).

Cortisol is at elevated concentrations in plasma of mature and ripe salmonids (Pickering and Christie, 1981) and newly ovulated killifish, *Fundulus heteroclitus* (Bradford and Taylor, 1987). Researchers are concerned about the performance of progeny from adult females that have elevated cortisol because elevated concentrations of cortisol is a good indicator of stress in teleosts (Donaldson, 1981; Schreck, 1981; Schreck, 1990), . If quality of progeny from females with elevated cortisol is affected, quality of future broodstocks could be affected. In a preliminary experiment, we found eggs from one female chinook salmon, *Oncorhynchus tshawytscha*, injected with cortisol at 24 h prior to spawning hatched 50% faster than eggs from control, sham-injected females. There is little information on the effects of cortisol on different developmental parameters such as growth or ability and time taken

to hatch and we do not know if the embryo is capable of metabolizing this steroid.

Besides cortisol, mature salmonid plasma has substantial concentrations of testosterone (Scott et al., 1983; Bromage et al., 1984; Fitzpatrick et al., 1986). The possible incorporation of testosterone into eggs might assist or accelerate embryonic development due to its anabolic properties. Mature female salmon undergoing vitellogenesis also have high plasma concentrations of thyroxine (Sower and Schreck, 1982). Teleosts treated with exogenous thyroxine demonstrate various effects (Pickford and Atz, 1957; Gorbman, 1969; Leatherland, 1982; Brown et al., 1987). Eggs and larvae of striped bass, *Morone saxatilis* (Brown et al., 1987), coho salmon, *O. kisutch* (Kobuke et al., 1987), and chum salmon, *O. keta* (Tagawa and Hirano, 1990) contain endogenous thyroxine. The role that thyroxine may potentially play, especially in embryonic development of teleosts, is unclear (Reviews by Lam, 1985 and Brown and Bern, 1989).

The high concentrations of hormones in developing teleost embryos may serve as a reservoir for ontogenesis. Not only are hormones present, but also teleostean eggs have a wide complement of steroidogenic enzymes that permit hormone metabolism (Sakai et al., 1988; Petrino, et al., 1989). Teleost embryos have the ability to metabolize hormones to metabolites and glucuronides (Antila, 1984; Yeoh et al., 1993a; Yeoh et al., 1993b).

We determined if augmentation of maternally inherited hormones on steelhead trout, *O. mykiss*, affects embryonic development by evaluating hatching times and growth expressed in weight, total length and condition factor (K_N). We also determined the amount of exogenous cortisol incorporated into the eggs after immersion in cortisol by using radioimmunoassay and established the concentrations of cortisol and cortisol

glucuronide during and beyond embryonic development to determine if metabolism or clearance of incorporated cortisol was occurring. Further, we determined whole body concentrations of endogenous cortisol and its glucuronide to establish if these profiles indicate the ability of embryos and hatched fry to use maternally contributed hormones.

METHODS AND MATERIALS

Effects of hormones on early development.

Gametes. Steelhead trout eggs (n=12,000) were pooled from four ripe females. Sperm from two ripe males were also pooled at the Cedar Creek Hatchery, Oregon. Sperm and eggs were kept separated. The gametes, stored in oxygen-supplemented airtight bags, were transported to Smith Farm Fish Genetics and Performance Laboratory, Oregon State University, Oregon. Temperature was kept constant at 12 °C.

Hormone augmentation. Upon arrival at Smith Farm Fish Genetics and Performance Laboratory, the eggs were divided into treatment groups consisting of three replicates of 400 eggs each. We exposed eggs to hormones at either water hardening, which immediately followed fertilization, or the eyed stage, 20 days postfertilization (dpf). Control groups were treated similarly to the hormone-treated groups except that no hormones were present in the water with 2 ml of dimethylsulfoxide (DMSO).

The hormones tested in this experiment were cortisol, testosterone, and thyroxine. Each hormone concentration for immersion of eggs was 1 mg/ml of water. This concentration was achieved by dissolving 1 g of hormone in 2

ml of DMSO. This solution was then added to 498 ml of hatchery water. Each immersion treatment was for 1 h.

Control groups for eggs that were immersed at water hardening were fertilized normally but were water hardened in a mixture of 2 ml of DMSO and 498 ml of hatchery water. Eggs that were exposed to hormones at the eyed stage were fertilized and water hardened in hatchery water. These eggs were then incubated in incubation trays for 20 days. At 20 dpf, all eyed groups were removed from incubation and placed in individual airtight bags that contained 498 ml of hatchery water and 2 ml of DMSO with or without (controls) hormones. These bags were supplemented with oxygen, sealed, and maintained at ambient temperature of hatchery water. After 1 h, eggs were rinsed and returned to respective incubation trays.

Growth. Embryos were weighed and measured at various stages. Total length measurements were taken with a ruler or calipers. Condition factor (K_N) was calculated using $K_N = W/aL^b$ (Anderson and Gutreuter, 1985) because the overall shape of alevins varied through time. Constants a and b were recalculated at each measurement date from a compilation of data on weight and total length ($n=500$, Table 1). After yolk sac absorption (50 dpf), the fish were removed from incubation trays. Each treatment group was randomly placed in a section of a trough. Flow rate was 12 l/min and the dimensions of each section were 42 x 42 x 38 cm (depth). Temperature was 12°C. The fish were fed at a rate of 4% of their body weight daily.

Hatch. Incubating eggs were observed every day for signs of hatching. Signs of hatching in all trays were detected on the April 2, 1992, designated as $t=0$. The eggs were checked every 6 h. The amount of time taken for 50% of each

TABLE 1.

Constants (n=500/age group) used to calculate condition factor, K_N of steelhead trout at various stages of development.

Age (days postfertilization)	a	b
50	0.08	0.76
83	0.01	2.82
130	0.02	2.64

group to hatch (median time to hatching) was documented. We also measured the synchrony of hatching, that is, the mean time for the first and the last eggs to hatch in each treatment.

Sampling. Total cortisol incorporated into steelhead trout eggs was determined by radioimmunoassay. Exogenous cortisol on the surface of eggs was rinsed off by placing the samples in a glass tray with running water for 1 min. Egg samples were then stored at - 80° C. Samples of eggs immersed in cortisol were also taken at 1 h and 10, 15, 20, and 30 days after hormone or incubation media immersion to determine cortisol and cortisol glucuronide concentrations

Endogenous cortisol and cortisol glucuronide profiles

Gametes. Steelhead trout eggs (3,000) and sperm were also collected from eight females and three males from Cole Rivers Fish Hatchery, Oregon. These gametes were used to determine fluctuations of free cortisol and cortisol glucuronide concentrations during embryonic development and beyond. The eggs were fertilized normally and incubated in incubation trays. Samples were collected from incubation trays every 3-4 days during development from 2 until 61 dpf and stored at - 80° C. Samples of unfertilized eggs, sperm, and maternal plasma from dorsal blood vessels were also collected at spawning and stored at - 80° C.

Extractions

Extraction procedures were modified from Feist et al. (1990). Prior to extraction, the whole body samples were thawed, individually weighed, and placed in culture tubes (16x125 mm) with 1 ml of 0.5 M NaOH at room

temperature. One hundred microliters of fluid samples such as maternal plasma, ovarian fluid, and sperm and seminal fluid were treated similarly. After 24 h, the samples were sonicated for 20 sec. The samples were then extracted twice with 8 ml of ethyl ether to obtain free steroids. The two extracts of each sample were combined, then dried under a stream of nitrogen. The dried extract was reconstituted in 1 ml of phosphate buffer, pH 7.0 and stored at - 80° C.

The remaining aqueous residue was adjusted to pH 4.8 with 50 µl of 100% acetic acid and 100 µl of acetate buffer, pH 4.8 (modified from Kime, 1990). The residue was treated with 800 µg of beta-glucuronidase (Sigma) in 100 µl of acetate buffer, pH 4.8. These tubes were incubated for 24 h at 37 °C. After the initial 24-h incubation, 800 µg of enzyme was again added to the tubes and incubated for another 24 h. Steroid moieties were extracted from the aqueous phase as described previously. These steroid moieties were dried and reconstituted in 1 ml of phosphate buffer and stored at - 80°C for future assay of glucuronides. Extraction efficiencies for free and conjugated cortisol were $44.7 \pm 5.0\%$ and $80.1 \pm 2.0\%$, respectively (n=24 each), and data were adjusted accordingly.

Radioimmunoassay

The lowest detectable concentration of cortisol in standards was 3.9 ng. The accuracy of the assay was validated by checking for parallelism of sample serial dilutions to standard curves. One hundred microliters of whole body extract was assayed. Anti cortisol antibody (Endocrine Sciences) was diluted at 1:100 with phosphate buffer. This cortisol assay procedure was modified from Redding et al. (1984). These samples were extracted and not exposed to heat to denature binding proteins. Final volume of each assay

tube after addition of charcoal was 1 ml. Intra- and inter-assay coefficients of variations were $2.57 \pm 0.99\%$ and $7.6 \pm 0.5\%$.

Statistical Analysis

Hatching rates, weights, total lengths, and cortisol concentrations in whole body extracts at individual sampling dates were normally distributed (chi-square goodness of fit, $P > 0.05$, Zar, 1984). Other statistic tools used were ANOVA (Statview II™, Abacus Concepts, Inc.), Tukey multiple comparison test, Bartlett's test, and Student's t test (Zar, 1984). Statistical significance was determined at $P \leq 0.05$.

RESULTS

Hatch

Replicates were pooled because of homogeneity in variance among replicates (Bartlett's test, Zar, 1984), and their means were not significantly different from each other (ANOVA). Mean times taken for treatment groups to hatch completely were not equal (ANOVA, $p < 0.0001$). Using Tukey's multiple comparison test (Zar, 1984), we found significant differences in the mean times of hatching among treatment groups. Groups treated with cortisol at water hardening hatched faster than control groups (Fig. 1) while groups treated at the eyed stage took as long as controls to hatch. Eggs immersed in thyroxine at the eyed stage hatched faster than controls. Overall, groups exposed to any hormone or DMSO at water hardening hatched faster than those treated at the eyed stage. The distribution of numbers of alevins hatching with regard to time was normal for all

FIGURE 1. Hatching time of steelhead trout eggs exposed to hormones or DMSO (dimethylsulfoxide) at two stages of development. Values are mean \pm SE of replicated identical treatments (n=3), which entailed 1 h exposure to cortisol, testosterone, thyroxine, or DMSO (control) at "Fertilization" or "Eyed" stage (i.e., visible eye pigments). For each replicate, hatching time was calculated as the mean for 400 eggs that were observed from initiation to completion of hatching. Symbol * denotes significant difference of treatment group from other groups ($p < 0.05$).

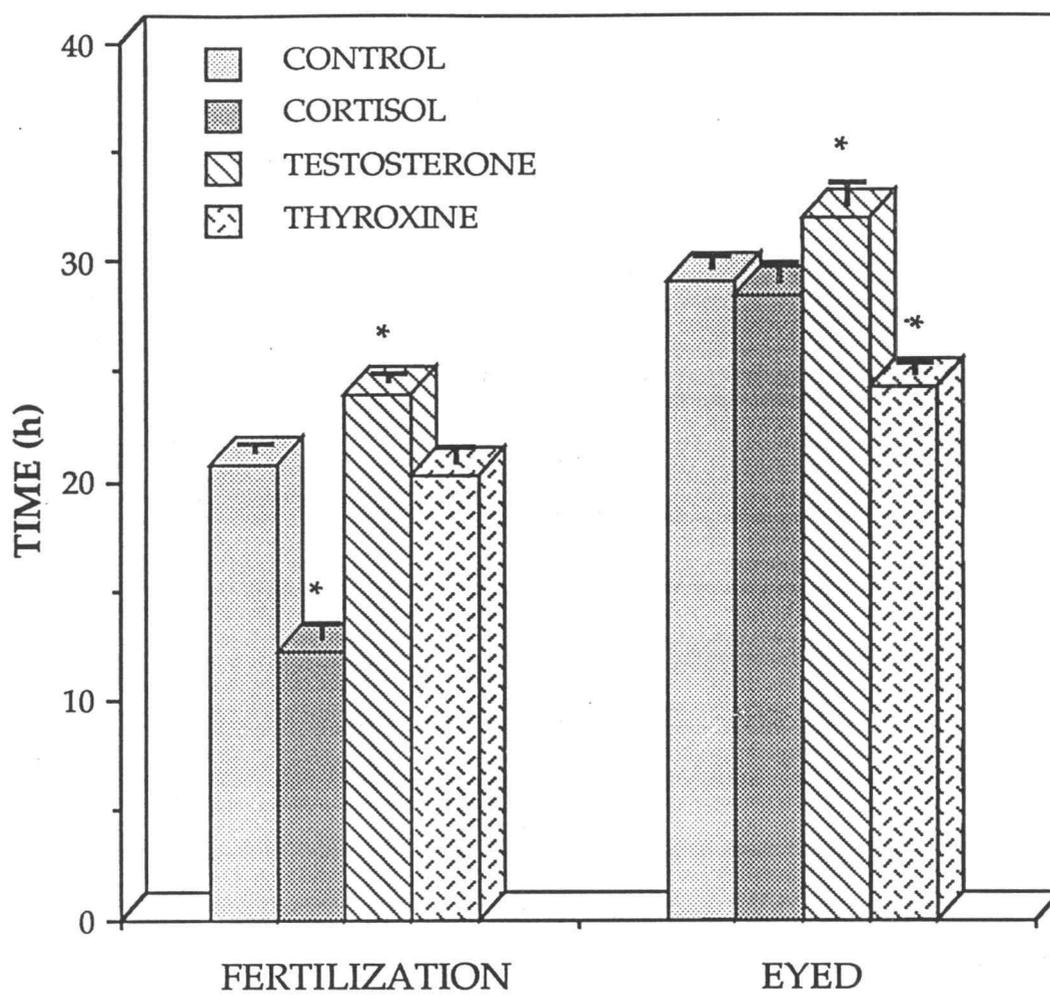


FIGURE 1.

treatment groups (chi-square goodness of fit). Mortalities of treatment groups ranged from 2% to 5% and were independent of treatment

Growth

All data from replicates of identical treatments were pooled for statistical analysis because their means were not significantly different (ANOVA) and their variances were not different (Bartlett's test). At 50 dpf, the alevins were finished absorbing their yolk sacs and were measured prior to first feeding. In groups treated at water hardening, K_n of the thyroxine group was significantly lower than those of control, testosterone, and cortisol groups (Table 2) because the total lengths of animals from this thyroxine group were significantly longer than those of other groups (ANOVA, Tukey's multiple comparison test), total length being inversely related to K_n . In treatments at the eyed stage, the cortisol groups possessed a significantly higher K_n (Table 2) than the other groups.

At 83 dpf, all groups were similar in weight, length, and K_n . Differences were again detected in K_n at 130 dpf. Condition g =factors of groups immersed in cortisol at the eyed stage (1.11 ± 0.07) were significantly larger than those of the controls (0.99 ± 0.03), testosterone (0.96 ± 0.04), and thyroxine (0.99 ± 0.03) groups (ANOVA, F test). This cortisol group was significantly heavier and longer (ANOVA, Tukey's multiple comparison test) than the other groups.

Exogenous cortisol

Cortisol and cortisol glucuronide were elevated in eggs of both treatment groups 1 h postimmersion in exogenous cortisol (ANOVA, Tukey's multiple comparison test). Ability to incorporate exogenous cortisol was not

TABLE 2.

Condition factor, weight, and length (\pm SE) of steelhead trout (N=60 for each treatment group) exposed to hormones either at water hardening (WH) or when eye pigments become visible (EYED). Fish (50 days postfertilization) had finished yolk sac absorption and were measured prior to exogenous feeding.

TREATMENT	CONDITION FACTOR (=W/aL ^b)	WEIGHT (g)	TOTAL LENGTH (cm)	
(WH)	Control	1.02 \pm 0.02	0.178 \pm 0.003	2.8 \pm 0.03
	Cortisol	1.03 \pm 0.02	0.180 \pm 0.003	2.8 \pm 0.02
	Testosterone	1.02 \pm 0.01	0.178 \pm 0.002	2.8 \pm 0.03
	Thyroxine	0.98 \pm 0.01 *	0.176 \pm 0.002	2.9 \pm 0.02 *
(EYED)	Control	0.99 \pm 0.02	0.173 \pm 0.002	2.8 \pm 0.02
	Cortisol	1.02 \pm 0.01 *	0.183 \pm 0.002 *	2.9 \pm 0.02 *
	Testosterone	0.97 \pm 0.01	0.170 \pm 0.002	2.8 \pm 0.02
	Thyroxine	0.98 \pm 0.01	0.171 \pm 0.003	2.8 \pm 0.02

Symbol * denotes significant difference of one treatment group from others exposed to hormones at the same developmental stage. ANOVA (Statview) was used to determine significant difference among treatments and Tukey's multiple comparison test ($p < 0.05$) was used to compare treatment groups.

significantly different (Student's t test) between groups exposed to cortisol at water hardening or the eyed stage. At 20, 30, and 45 days postimmersion, free cortisol concentrations of fish exposed at water hardening had declined but were still significantly higher (ANOVA, Tukey's multiple comparison test) than controls (Fig. 2). Free cortisol concentrations in eggs immersed in cortisol at the eyed stage were similar to those of controls (ANOVA) by 10 days postimmersion. Interestingly, concentrations of cortisol (Fig. 2) and cortisol glucuronide (Fig. 3) were significantly higher in groups immersed at water hardening, which hatched faster than controls and embryos immersed at the eyed stage (ANOVA, Tukey's multiple comparison test). Cortisol glucuronide concentrations of eggs immersed at both developmental stages declined to concentrations similar to those of controls (ANOVA) by 45 days postimmersion.

Endogenous Cortisol

Free cortisol and cortisol glucuronide concentrations fluctuated throughout embryonic development and beyond (Fig. 4). Concentrations of cortisol and cortisol glucuronide in maternal plasma were significantly lower (ANOVA and Tukey's multiple comparison test, $p < 0.05$) than other physiological fluids and unfertilized eggs (Fig. 5).

FIGURE 2. Mean cortisol concentrations (\pm SE) in steelhead trout embryos and alevins after eggs were immersed in either exogenous cortisol (F) or incubation media alone (control) at water hardening and the eyed stage. At 30 days postfertilization, the trout eggs hatched. Each column represents $n=30$ (10 from each replicate). Symbol * denotes significant difference between control and experimental groups at that time (ANOVA, and Tukey's multiple comparison test, $p<0.05$).

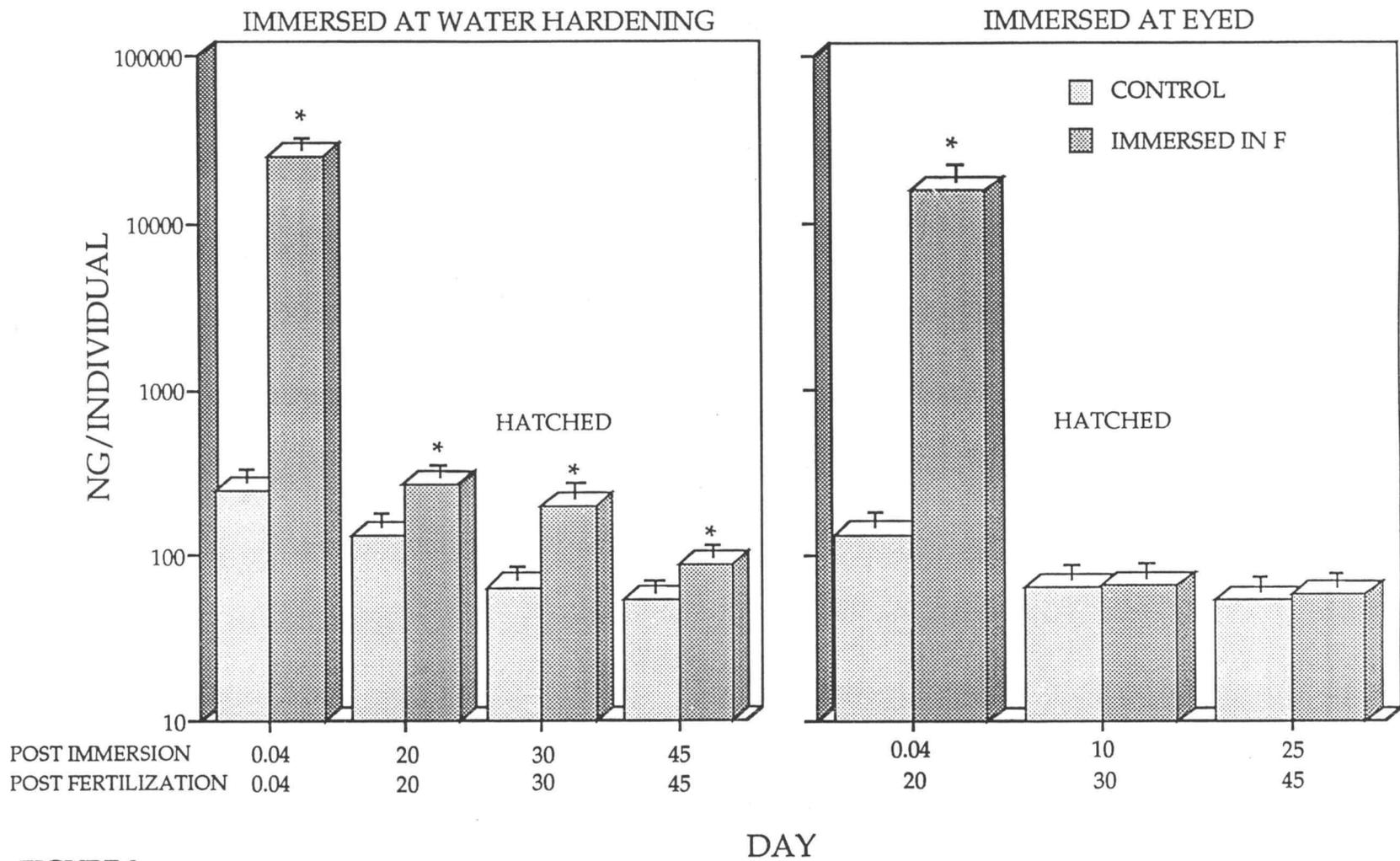


FIGURE 2.

FIGURE 3. Mean cortisol glucuronide concentrations (\pm SE) in steelhead trout embryos and alevins after eggs were immersed in either exogenous cortisol (F) or incubation media alone (control) at water hardening and the eyed stage. At 30 days postfertilization, the trout eggs hatched. Each column represents $n=30$ (10 from each replicate). Symbol * denotes significant difference between control and experimental groups at that time (ANOVA, and Tukey's multiple comparison test, $p<0.05$).

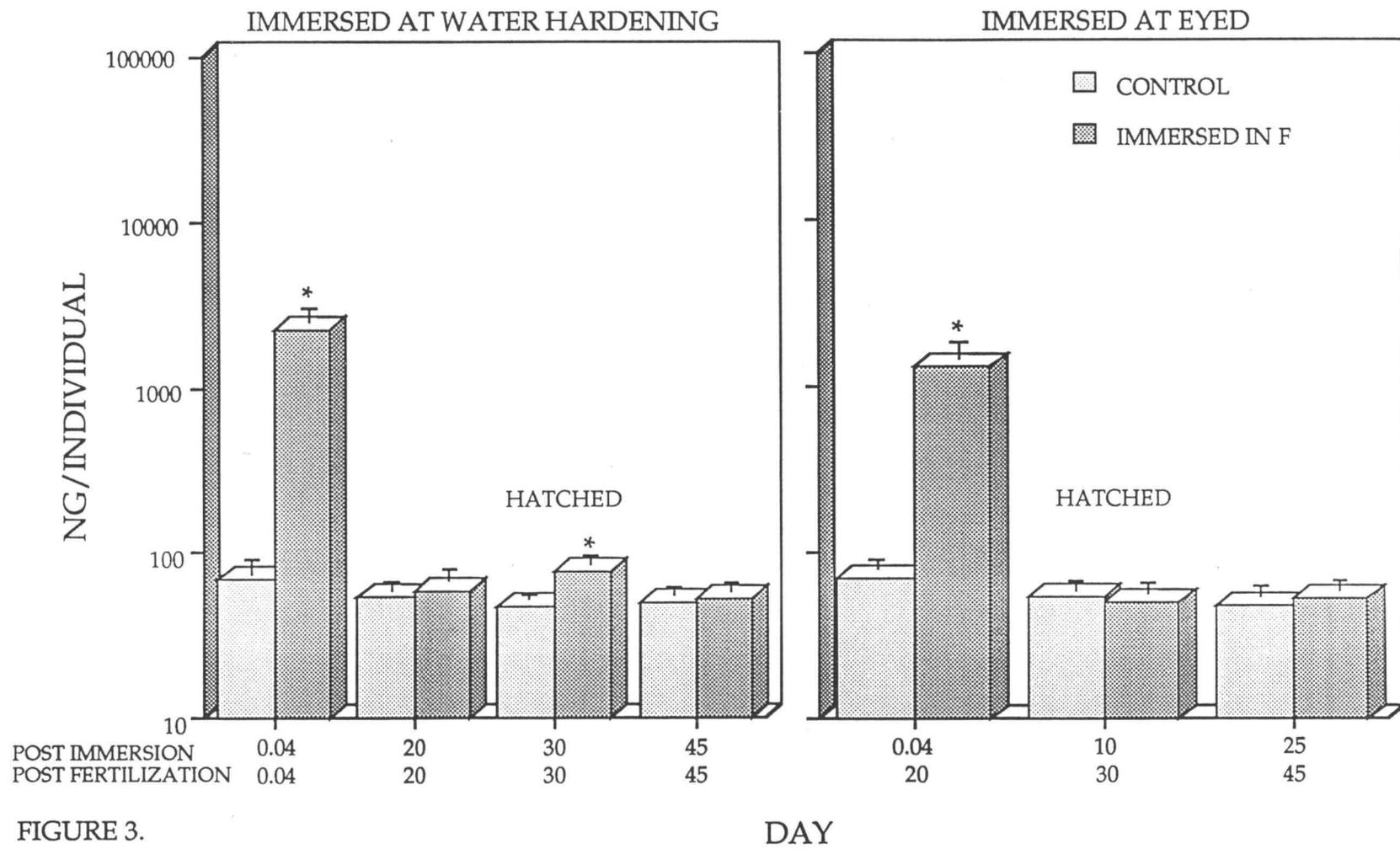


FIGURE 3.

FIGURE 4. Mean concentration (\pm SE) of whole body concentrations of free cortisol and cortisol glucuronide in steelhead trout during embryogenesis and beyond. Some error bars are not visible because of the log scale of the y-axis. Each point represents 30 individuals. Points with identical numerical or alphabetical symbols are significantly different from one another (one-tail Student's t-test, $p < 0.05$).

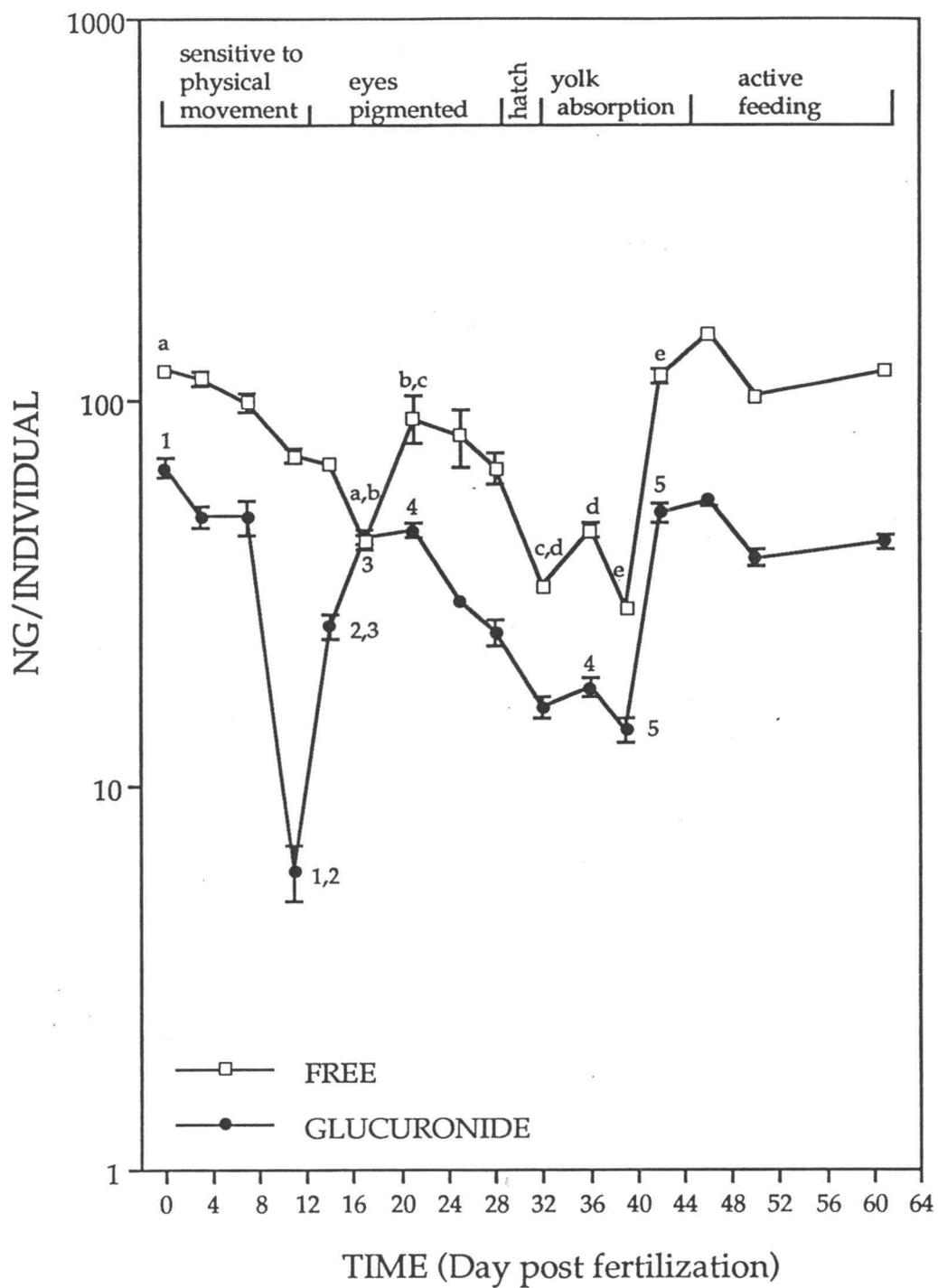


FIGURE 4.

FIGURE 5. Mean (\pm SE) concentrations of cortisol in maternal plasma, ovarian fluid, and sperm and seminal fluid of steelhead trout at spawning. Symbol * denotes significant difference of cortisol and cortisol glucuronide in maternal plasma from other fluids (ANOVA and Tukey's multiple comparison test, $p < 0.05$.)

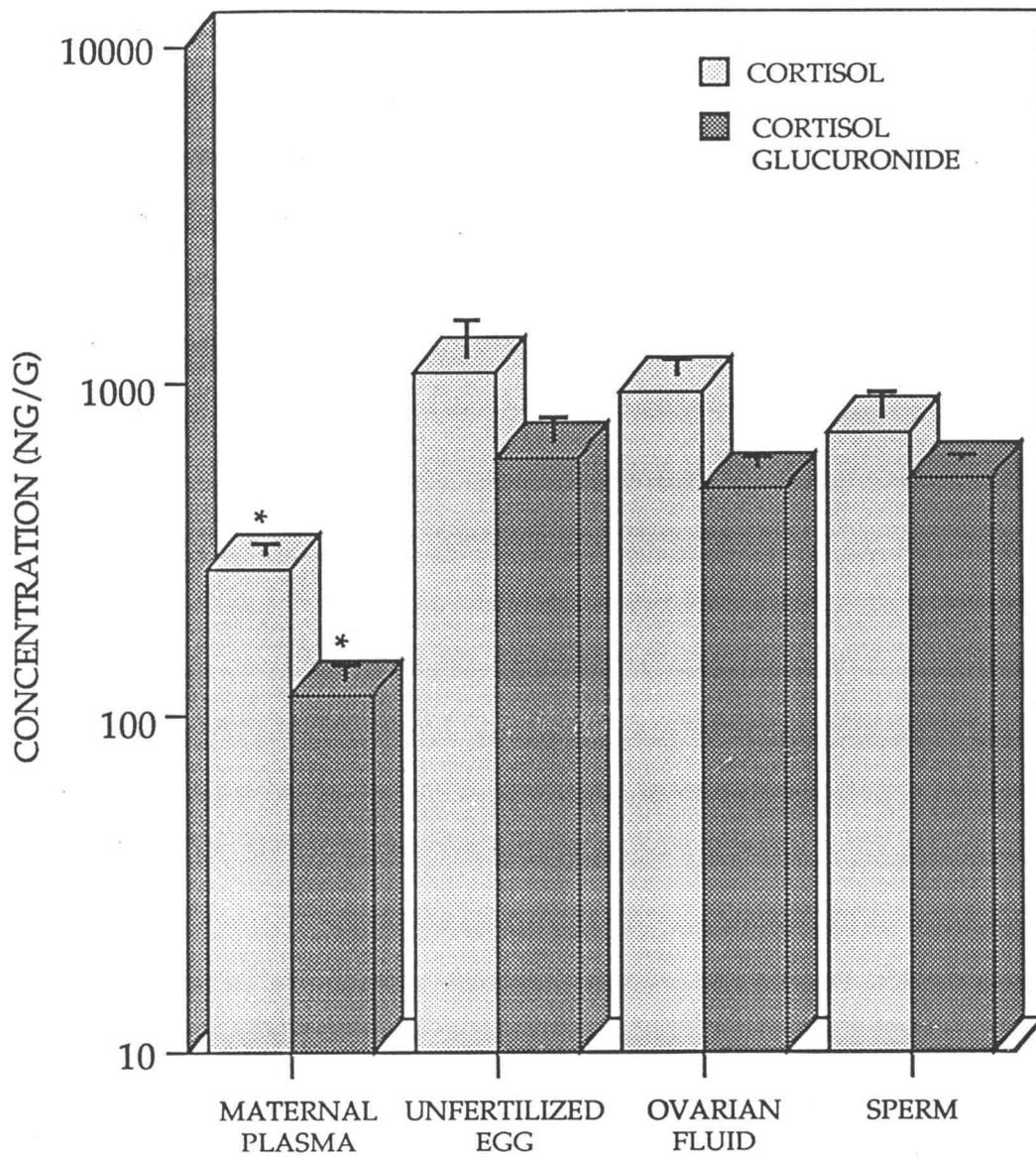


FIGURE 5.

DISCUSSION

The concept that hormones can affect embryonic processes was supported by our findings. Hormone augmentation affected early developmental processes such as hatching rates and growth.

Cortisol and thyroxine accelerated the process of hatching in steelhead trout eggs. Timing of cortisol and thyroxine exposure seems to be important in affecting the synchrony of hatching. Groups exposed to water and DMSO without hormones at water hardening hatched faster than those exposed at the eyed stage. One possible explanation is that the DMSO used as a steroid carrier may have influenced the steelhead trout eggs at the water hardening stage. However, this phenomenon was not seen in DMSO immersion of chinook salmon eggs in a preliminary experiment, while cortisol and thyroxine similarly accelerated hatching.

Steelhead trout embryos' responses to cortisol were different between the two developmental stages. We observed that eggs immersed at water hardening hatched faster and had significantly higher concentrations of cortisol at time of hatching compared with controls and embryos immersed at the eyed stage. Steelhead trout from treatment groups that hatched faster grew similarly to controls. In contrast, eggs exposed to cortisol at the eyed stage had regular hatching times but were more robust (Kn being higher), heavier, and longer than other groups when measured at 50 and 130 days after fertilization.

Testosterone did not affect hatching time in steelhead trout. Testosterone apparently was also not anabolic because it did not affect

embryonic and alevin development, growth parameters of testosterone-exposed steelhead trout were similar to those of controls.

Thyroxine accelerated hatching in steelhead trout when treated at the eyed stage. Thyroxine also accelerated hatching in chum salmon by 4-8 days (Dales and Hoar, 1954; Ali, 1961) but not in tilapia (Reddy and Lam, 1991). Thyroid hormone-deficient medaka, *Oryzias latipes*, did not have delayed hatching as one would expect, assuming thyroxine from maternal contribution was important (Tagawa and Hirano, 1991). There are many possible reasons for these observed inconsistencies and variations, such as different concentrations of thyroxine used in various studies or concentrations of sensitivity to thyroxine that may be species dependent.

Thyroxine had a transient effect on the morphology of young steelhead trout. At 50 dpf, these steelhead trout were significantly longer than other groups. This is not surprising because there are reports of thyroxine affecting teleostean morphology (Pickford and Atz, 1957; Gorbman, 1969; Leatherland, 1982; Brown et al., 1987). By 83 dpf, these animals were similar in lengths to other groups. Many studies have varied results concerning the importance and effects of thyroxine on development in teleosts (Reviewed by Higgs et al., 1982).

For hormones to play an active role in development, conversion or clearance of these hormones may occur. Fish immersed at the eyed stage were able to clear exogenous cortisol from their internal environment faster than eggs immersed in cortisol at water hardening. Elevated free cortisol and cortisol glucuronide were detected in eggs or embryos at 1 h postexposure to cortisol. Elevation of cortisol glucuronide 1 h after immersion indicates that embryos have the necessary glucuronyltransferases to form glucuronides. Glucuronides are thought of as a metabolic waste of

steroids. With elevated concentrations of cortisol glucuronide, metabolism of free cortisol may be inferred. The observed decrease of cortisol and cortisol glucuronide during development indicates clearance of free and conjugated cortisol from the embryos. The excretion routes of cortisol and its metabolites, such as glucuronides, are currently unknown. The changes in endogenous concentrations of cortisol and cortisol glucuronide also indicate that embryonic steelhead trout have the ability to convert or excrete free cortisol and cortisol glucuronide. Older (3 years) trout form glucuronides from radioactive free cortisol injected intraarterially (Truscott, 1980).

Maternal plasma has significantly lower concentrations of cortisol and cortisol glucuronide than ovarian fluid, unfertilized eggs, and sperm and seminal fluid. These cortisol concentrations were assumed to be resting concentrations in the mature female steelhead trout. However, these reported concentrations may be higher than true resting levels because the females were held in a broodstock holding facility prior spawning. Interestingly, concentrations of cortisol and cortisol glucuronide in ovarian fluid and sperm and seminal fluid were similar to concentrations found in the unfertilized egg. The source of cortisol in the unfertilized egg is unknown at present, but we speculate that it is derived from the rich source in the ovarian fluid.

ACKNOWLEDGEMENTS

We cordially thank Dr. Martin S. Fitzpatrick, Mr. Rob Chitwood, Miss Ying-Chi Lam, Miss Sze-Wing Ngan, and Mr. Eric Green for their assistance. Funding was provided by Western Regional Aquaculture Consortium and Oregon State University College Sea Grant Program (Grant R/AQ-48).

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Endogenous Steroid Metabolism is Indicated by Fluctuations of Endogenous
Steroid and Steroid Glucuronide Levels in Early Development of the
Steelhead Trout (*Oncorhynchus mykiss*)¹

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ABSTRACT

Concentrations of endogenous steroids and their glucuronide conjugates were dynamic during early development in steelhead trout, *Oncorhynchus mykiss*. Whole body content of sex steroids and steroid glucuronides of both bisexual and gynogenetic (all female) steelhead trout were quantified by radioimmunoassay. Concentrations of 17β -estradiol (E2) and cortisol (F) increased a few days before hatch. A few days after hatch, 11-ketotestosterone (11KT) increased in concentrations in both gynogenetic and bisexual populations, and 11KT glucuronide concentrations increased in the gynogenetic population. Testosterone (T) and E2 concentrations were at their lowest at 39 days post fertilization (dpf) for T and 39 and 61 dpf for E2. Levels of steroid glucuronides were not consistently parallel to free steroids through time. T-, E2-, and DHP glucuronides declined slower than their free forms. Based on fluctuating concentrations of all steroid glucuronides, both populations of fish demonstrated an ability to form glucuronide conjugates of all steroids at the embryonic stage. The dynamic fluctuations of free steroids and their glucuronide concentrations during early development of the trout indicates that steroid metabolism is active during development. This study also implicates steroid metabolism as an integral part of embryonic and postembryonic development.

INTRODUCTION

Unfertilized teleostean eggs contain a wide complement of maternally contributed endogenous hormones that decline during embryogenesis. Sex steroids were found to decrease during early development in coho salmon, *Oncorhynchus kisutch* (Feist et al., 1990), and in developing tilapia, *Tilapia sp.* (Rothbard et al., 1987). Cortisol also decreases during early embryonic development of teleosts such as chum salmon, *Oncorhynchus keta* (de Jesus and Hirano, 1992), Japanese flounder *Paralichthys olivaceus* (de Jesus et al., 1991), and steelhead trout, *O. mykiss* (Yeoh and Schreck, 1993). Thyroxine concentrations decrease during embryonic and alevin development in coho salmon (Kobuke et al., 1987), whereas triiodothyronine concentrations decrease during embryonic development in striped bass, *Morone saxatilis*, (Brown et al., 1987). Such hormone dynamics support the notion that hormones are being metabolized during early development.

The presence of steroidogenic enzymes in rainbow trout embryos (Antila, 1984) and newly hatched steelhead trout (Yeoh et al., 1993) suggests that developing teleostean embryos may metabolize hormones for development. For example, the presence of enzymes such as 5α , and 5β reduction-enzymes was implicated in development of embryonic rainbow trout (Antila, 1984). Both embryonic and newly hatched steelhead trout that had been microinjected with sex steroid precursors converted them, *in vivo*, to steroid metabolites and glucuronides (Yeoh et al., 1993). Enzymes such as 3β -hydroxysteroid dehydrogenase; 5,4-isomerase; 17α -hydroxylase; 17α , 20β -desmolase; and 11β -dehydroxysteroid dehydrogenase are found in young rainbow trout (van den Hurk, 1982; van den Hurk and Lambert, 1982).

Steroid glucuronides are steroid metabolites (Solomon and Bhavnani, 1970). There are two forms of glucuronides. The common form is thought to have minimal physiological functions and is excreted in urine (Solomon and Bhavnani, 1970). However, other studies have shown that steroid glucuronides may be sex pheromones in the male *Gobius joso* (Colombo et al., 1980; Colombo et al., 1982), the female *Brachydanio rerio* (van den Hurk and Lambert, 1983), and adult catfish (Resink et al., 1989a; Resink et al., 1989b). Scott and Canario (1992) suggested that 17α 20β dihydroxyprogesterone sulphate, which like steroid glucuronides is a steroid conjugate, detected in female teleosts reflects the maturational stage of the ovary. The second form of glucuronide is not well known in teleosts. This form undergoes further metabolic conversion (Solomon and Bhavnani, 1970). Mammals metabolize steroid glucuronides to other steroids with important physiological functions. For example, humans metabolize testosterone glucuronide, *in vivo*, to etiocholanolone 5β -androstane 3α , 17β -diol (Solomon and Bhavnani, 1970).

The biological significance of hormone metabolism and maternally contributed steroids in early development remains unknown. Teleosts have the ability to metabolize steroids during early development. In addition to observations of sex steroid metabolism, there is tentative evidence for metabolite clearance from steelhead trout embryos (Yeoh et al., 1993). In general, steroid metabolites regulate intracellular functions in the earliest stages of embryonic development (Brown et al., 1989). In addition, products of steroid metabolism direct target cells for activities such as cell division, protein synthesis, and secretion (Briggs and Brotherton, 1970). Besides steroids playing developmental roles, the genetic sex of a sexually undifferentiated fish may be determined from their whole body steroid

profiles during early development. Bimodal distributions in whole body steroids have been reported in populations of tilapia (Rothbard et al., 1987) and histologically undifferentiated coho salmon (Feist et al., 1990), which may indicate sexual differences in steroid production prior to the onset of gonadal differentiation.

Exogenous steroids affect development in teleosts. Exogenous hormones such as cortisol and thyroxine accelerated the hatching process in salmonids (Yeoh and Schreck, 1993). Exogenous steroids have various effects on teleosts, such as influencing phenotypic sex (Schreck, 1974; Hunter et al., 1986; Lewis and Sower, 1992), survival (Delabbio et al., 1988), hatching rates (Cloud, 1981; Yeoh and Schreck, 1993), and growth (Lone and Ince, 1983; Sower et al., 1983).

The first objective of this study was to determine if profiles of free steroids in developing steelhead trout were indicative of endogenous steroid metabolism. The second objective was to determine if steroid glucuronide profiles could further support the notion of endogenous metabolism. The last objective was to determine if profiles of free steroids and steroid glucuronides differ between genders. Testosterone, 17β -estradiol, 17α , 20β , dihydroxyprogesterone (DHP), 11-ketotestosterone (11KT), cortisol, and their glucuronides were quantified by using radioimmunoassay.

METHODS AND MATERIALS

Gametes. Steelhead trout (Cole River Hatchery, Oregon Department of Fish and Wildlife) eggs (n=12,000) were collected from eight ripe females. Sperm from two ripe males were also collected and pooled. About 10,000 eggs were set aside for producing gynogenetic females at Cole River Hatchery. The procedure used was modified from Thorgaard et al. (1987). Sperm extender used consisted of a 4:1 ratio of solution A (9 g/l KCl, 2.35 g/l NaCl, 0.51 g/l Na₂HPO₄, 0.29 g/l MgSO₄·7H₂O, and 0.29 g/l CaCl₂·2H₂O) to solution B (5 g/l NaHCO₃ and 5 g/l glucose). To produce bisexual populations, sperm was not exposed to ultraviolet light, and eggs were not heat shocked. After water hardening, eggs were placed in incubation trays. Twenty days postfertilization (dpf), eyed embryos spawned from the different females were transported in separate containers from Cole River Hatchery to Smith Farm Genetics and Performance Laboratory, Oregon State University, Corvallis, Oregon and reared in separate incubation trays at 12°C with flow-through well water.

Sampling. Samples were collected from incubation trays every 3-4 days during development (from 3 to 61 dpf). Thirty eggs were sampled from three trays containing gynogens or bisexuals (n=10 from each tray). These samples were stored at -80°C.

Extraction. Whole body extractions were conducted as described by Yeoh and Schreck (1993). All animals were weighed individually prior to extraction. Extraction efficiencies for free and conjugated steroids, respectively were $80.3 \pm 0.8\%$ (mean \pm SE) and $85.1 \pm 0.9\%$ for testosterone,

70.3 ± 1.0% and 82.7 ± 0.4% for estradiol, 83.9 ± 1.9% and 83.9 ± 0.8% for DHP, 75.8 ± 1.1% and 90.0 ± 0.5% for 11KT, and 39.4 ± 3.0% and 79.6 ± 0.9% for cortisol (F) (n=20 for each steroid). Reported concentrations were corrected for extraction efficiencies.

Radioimmunoassay. Radioimmunoassay procedures for whole body sex steroids were described in Feist et al. (1990) and for cortisol in Yeoh and Schreck (1993). Each animal was assayed individually. The lowest detectable concentration was 3.1 pg for testosterone, estradiol, DHP, and 11KT and 3.9 ng for F standards. Accuracy of steroid assays was validated by parallelism of serial dilutions of unknowns relative to standard curves. Intra- and inter assay variations were less than 5% and 10%, respectively. DHP antibody was a gift from Dr. A. P. Scott (United Kingdom) and 11KT was a gift from Dr. Y. Nagahama (Japan). Other steroid antibodies were purchased from Endocrine Sciences, Tarzana, California.

RESULTS

Trends for all steroids were identical, whether expressed as pg/individual or pg/g. Mean weight (\pm SE) of animals aged 3 to 28 dpf did not vary over time and was 0.136 ± 0.004 g ($n=60$ for each sampling date). Weights of developing fish at 32, 36, 39, 42, 46, 50, and 61 dpf were 0.142 ± 0.003 g, 0.154 ± 0.003 g, 0.175 ± 0.003 g, 0.185 ± 0.004 g, 0.210 ± 0.004 g, 0.222 ± 0.008 g, and 0.263 ± 0.008 g, respectively. Means and variances of steroid concentrations in animals at individual sampling dates were similar between trays in each population and were pooled for statistical comparisons (ANOVA, $p>0.05$; and Bartlett's test, $p>0.05$). All steroid concentrations in embryos and hatched fish were log transformed prior to statistical analysis. Steroid concentrations at each sampling date were normally distributed (Chi-square goodness of fit, $p>0.05$ as described in Zar, 1984). Level of statistical significance was $p<0.05$.

Free testosterone and testosterone glucuronide concentrations decreased (linear regression, $p<0.0001$) throughout embryonic development of both populations (Fig. 1). The lowest concentrations of free testosterone for both populations occurred on 39 dpf. At 42 dpf, these concentrations had increased (ANOVA and F-PLSD, Statview II, Abacus Concepts, Inc.) in both populations. An increase in free testosterone was observed at 25 dpf only in the gynogenetic population (ANOVA, F-PLSD). In the bisexual population, testosterone glucuronide concentrations increased in animals sampled at 7 and 39 dpf (ANOVA, F-PLSD). With the exception of the first week of development in the bisexual population, testosterone glucuronide

FIGURE 1. Mean whole body concentrations of testosterone (T free; open circles) and testosterone glucuronide (T gluc; closed circles) during early development of bisexual and gynogenetic steelhead trout. Values are the means (\pm SE) for N = 30 fish; "H" denotes hatching, "*" denotes significant difference of mean concentration of one sampling date from mean concentration of the previous sampling date ($p < 0.05$).

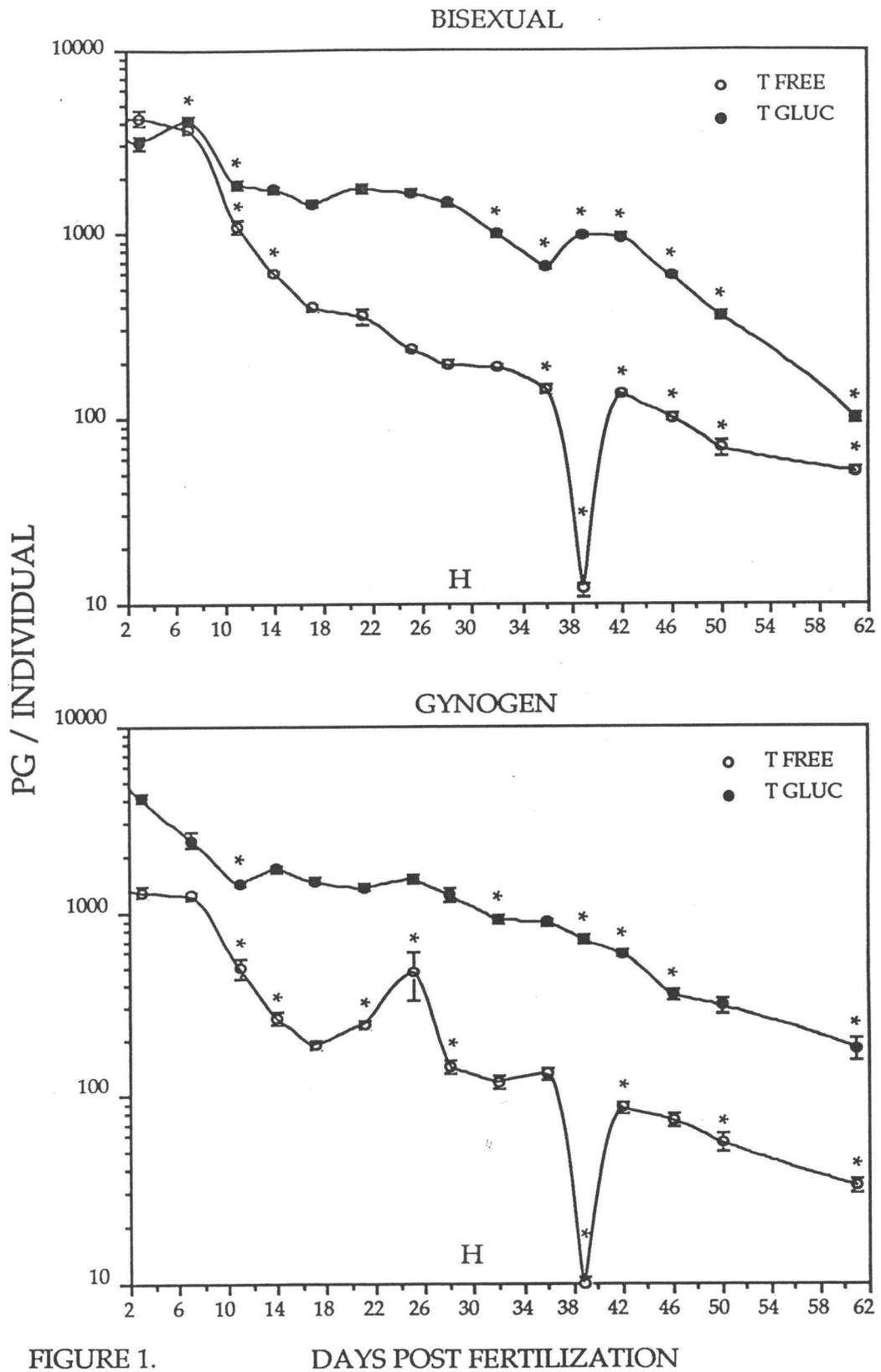


FIGURE 1.

DAYS POST FERTILIZATION

concentrations were observed to be higher than free glucuronide concentrations.

During the first 2 weeks of embryonic development, free estradiol concentrations were higher than estradiol glucuronide in both populations (Fig. 2). Thereafter, estradiol glucuronide concentrations were higher than free estradiol. Free estradiol had a more dramatic decrease in concentrations than estradiol glucuronide throughout the developmental period (linear regression of log transformed concentrations on days, Student's t-test was used in comparing slopes of regression, $p < 0.05$). Free estradiol and estradiol glucuronide concentrations increased a few days before hatch for the bisexual group and at hatch for the gynogenetic population. The peaks of free estradiol and estradiol glucuronide in the gynogenetic population occurred 3 days later than those in the bisexual population. Gynogens in this experiment hatched a day later than controls, which may indicate slower development. The lowest concentrations of free estradiol in both populations were detected at 39 dpf.

In both groups, free DHP and DHP glucuronide declined simultaneously during the first 3 weeks of development and the free form was consistently higher than DHP glucuronide (Fig. 3). Thereafter, DHP glucuronide concentrations were higher than free DHP concentrations. Overall, trends of free DHP and DHP glucuronide concentrations were similar between gynogens and controls (Fig. 3). Free DHP concentration in the gynogenetic population was lower than the bisexual population at 3 dpf (Student's t-test, $p < 0.05$). DHP glucuronide concentrations remained at stable concentrations in the bisexual population for about 2 weeks (21 to 34 dpf), with increases (ANOVA, F-PLSD) detected at 39 dpf (bisexual) and 32 dpf (gynogenetic). After hatch, DHP glucuronide concentrations in the

FIGURE 2. Mean whole body concentrations of estradiol (E2 free; open circles) and estradiol glucuronide (E2 gluc; closed circles) during early development of bisexual and gynogenetic steelhead trout. Values are the means (\pm SE) for N = 30 fish; "H" denotes hatching, "*" denotes significant difference of mean concentration of one sampling date from mean concentration of the previous sampling date ($p < 0.05$).

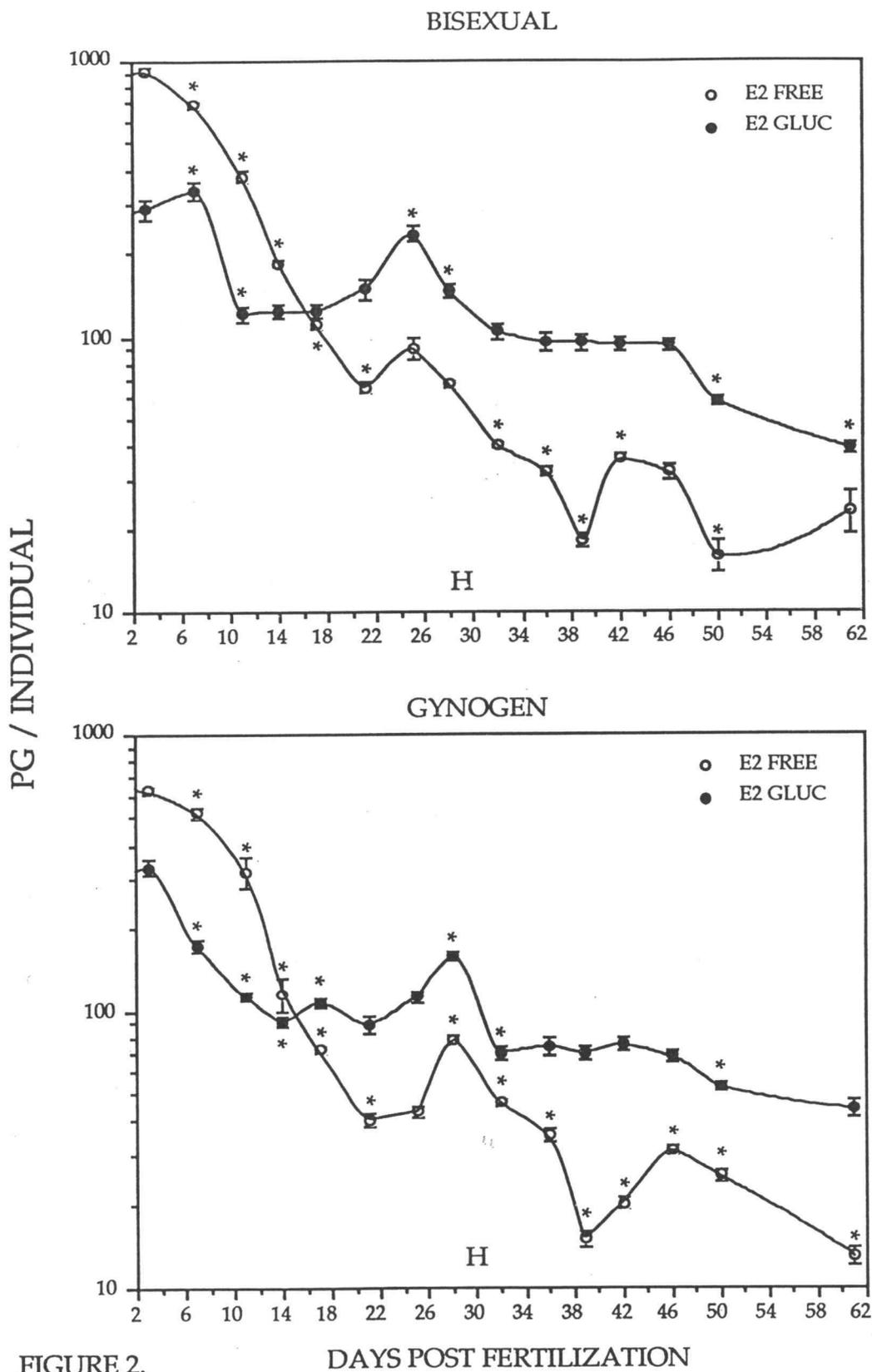


FIGURE 2.

DAYS POST FERTILIZATION

FIGURE 3. Mean whole body concentrations of 17α , 20β , dihydroxyprogesterone (DHP free; open circles) and DHP glucuronide (DHP gluc; closed circles) during early development of bisexual and gynogenetic steelhead trout. Values are the means (\pm SE) for N = 30 fish; "H" denotes hatching, "*" denotes significant difference of mean concentration of one sampling date from mean concentration of the previous sampling date ($p < 0.05$).

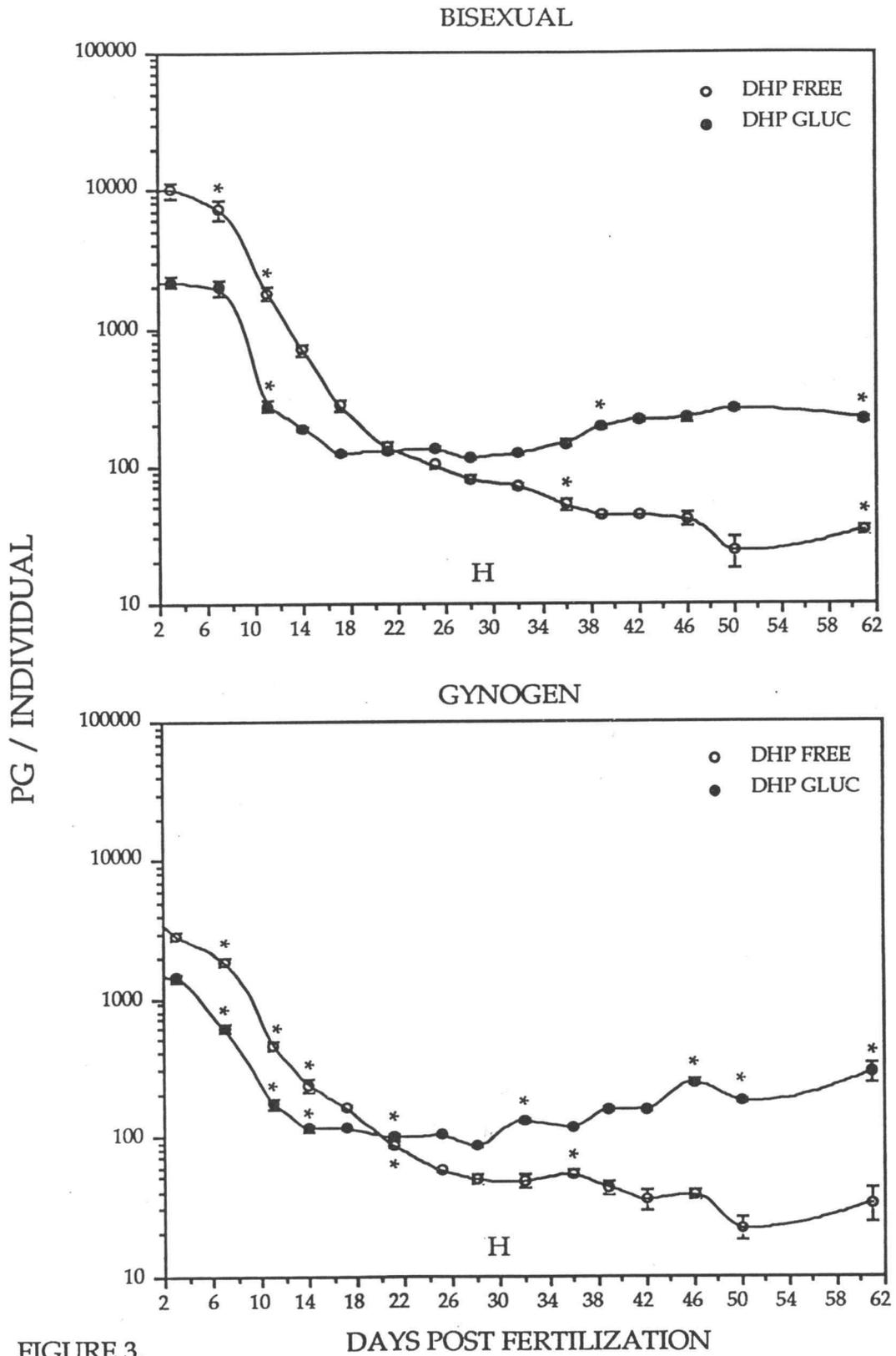


FIGURE 3.

gynogenetic population fluctuated with increases at 32, 42, and 61 dpf and with a decrease at 50 dpf (ANOVA, F-PLSD).

Unlike other sex steroids, free 11KT and 11KT glucuronide concentrations exhibited only a minor decline during early embryonic development although this decline was significant (linear regression, $p < 0.0001$) in both populations (Fig. 4). Concentrations of free 11KT and 11KT glucuronide fluctuated irregularly in both populations throughout early development (Fig. 4).

Profiles of free cortisol and cortisol glucuronide were similar between the two populations during embryonic and postembryonic development (Fig. 5). There was a gradual decrease of free cortisol during the first 2 weeks of development (linear regression, $p < 0.0001$). About 5 days (bisexual) and 11 days (gynogenetic) before hatch, free cortisol concentrations increased (ANOVA, F-PLSD). After 39 dpf, free cortisol concentrations increased and remained at stable concentrations in both populations (ANOVA, F-PLSD).

DISCUSSION

Fluctuating concentrations of free steroids strongly suggest that endogenous steroid metabolism is active during embryonic and post embryonic development. Furthermore, the presence of and fluctuations in concentrations of steroid metabolites such as steroid glucuronides is also suggestive of active steroid metabolism. The significance of hormone metabolism in early development is unknown. Rainbow trout embryos of *O. mykiss* have the ability to metabolize steroid precursors to free steroids (Antila, 1984; Yeoh et al., 1993) and steroid glucuronides (Yeoh et al., 1993).

FIGURE 4. Mean whole body concentrations of 11-ketotestosterone (11KT free; open circles) and 11-ketotestosterone glucuronide (11KT gluc; closed circles) during early development of bisexual and gynogenetic steelhead trout. Values are the means (\pm SE) for N = 30 fish; "H" denotes hatching, "*" denotes significant difference of mean concentration of one sampling date from mean concentration of the previous sampling date ($p < 0.05$).

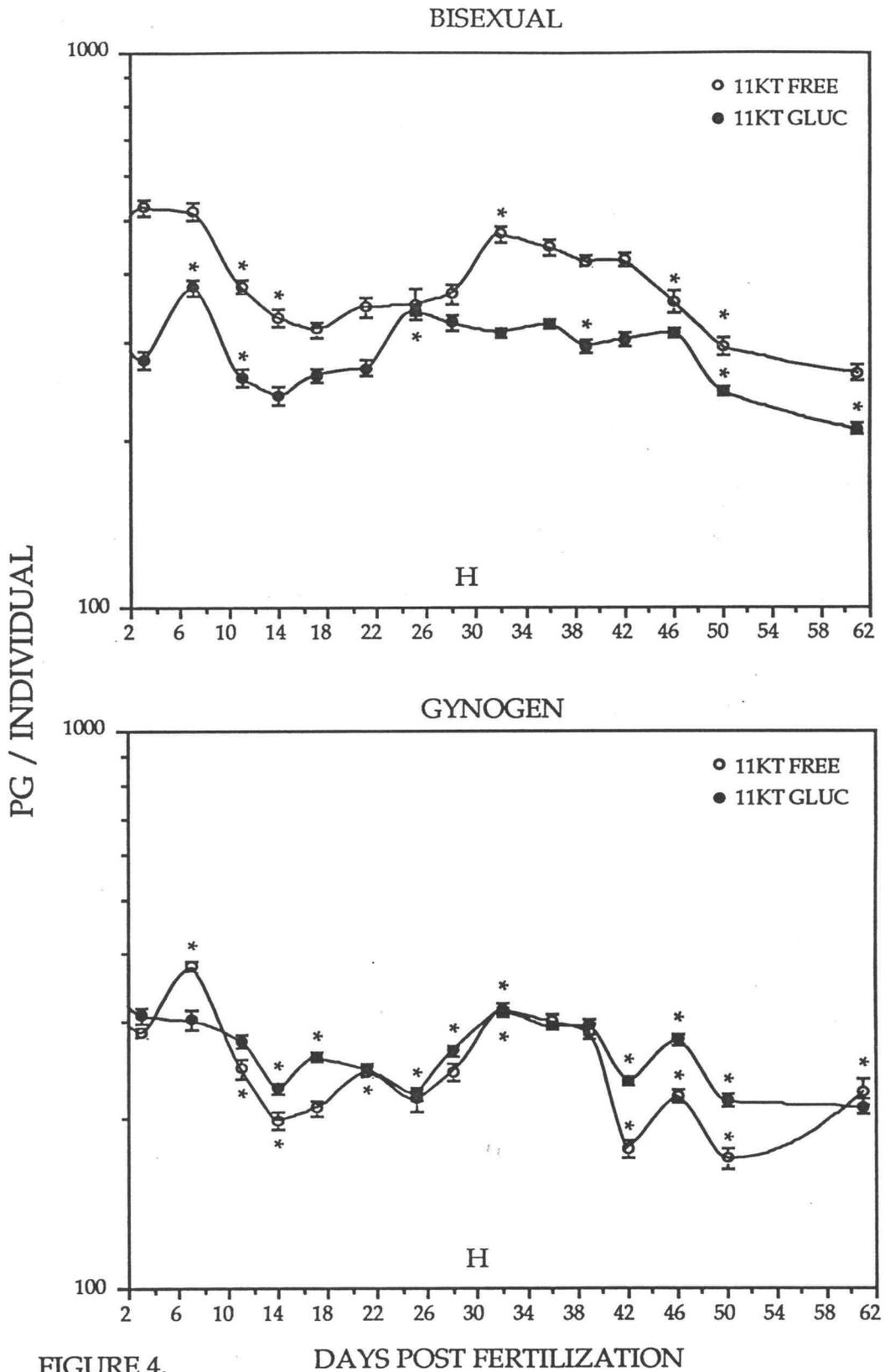


FIGURE 4.

FIGURE 5. Mean whole body concentrations of cortisol (F free; open circles) and cortisol glucuronide (F gluc; closed circles) during early development of bisexual and gynogenetic steelhead trout. Values are the means (\pm SE) for N = 30 fish; "H" denotes hatching, "*" denotes significant difference of mean concentration of one sampling date from mean concentration of the previous sampling date ($p < 0.05$).

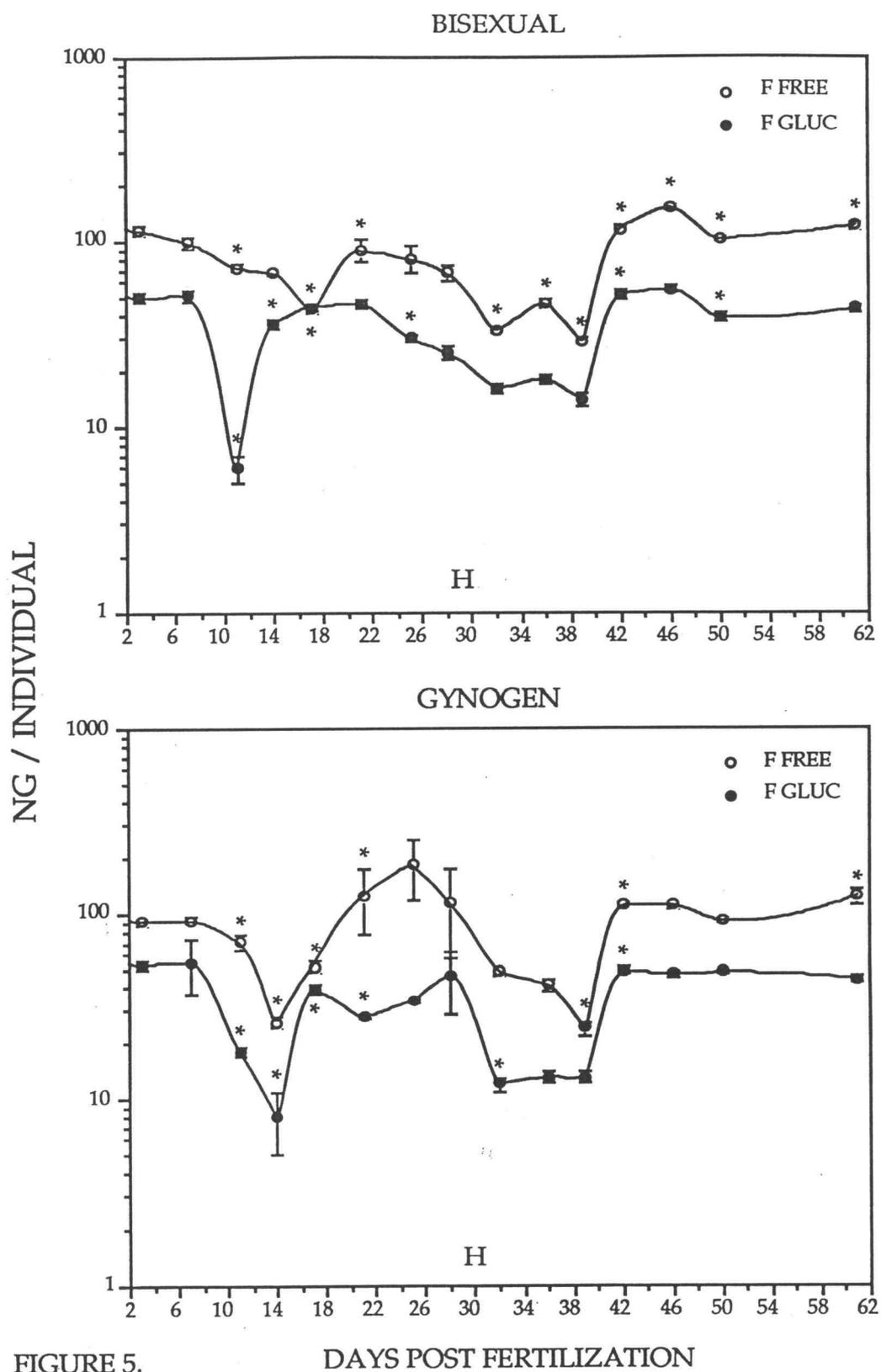


FIGURE 5.

Studies correlating steroids and their conjugates, with molecular processes of embryonic and postembryonic development are needed to determine possible roles steroids have in early development since products of steroid metabolism can direct target cells for activities such as cell division, protein synthesis, and secretion (Briggs and Brotherton, 1970) and steroid metabolites may regulate intracellular functions in the earliest stages of embryonic development in fish (Brown et al., 1989).

Mature teleostean oocytes have a large reservoir of maternally contributed hormones, which declines during embryonic development (Brown et al., 1987; Kobuke et al., 1987; Rothbard et al., 1987; Feist et al., 1990; de Jesus and Hirano, 1992; Yeoh and Schreck, 1993). These maternally contributed hormones seem to be important for early development (Schreck et al., 1991). In this study, concentrations of free testosterone, estradiol, cortisol, and DHP declined substantially during first 2 weeks of embryonic development in both populations of fish. This phenomenon implies that maternally contributed hormones are being metabolized during the earliest part of embryonic development. The importance of the maternal source of hormones may cease when the embryo can independently synthesize its own hormones.

We did not contrast steroid concentrations of the bisexual population with the gynogenetic population because the all-female population produced for this experiment developed slower at the embryonic stage. However, steroid concentrations of both populations were normally distributed at every sampling date. This inability to detect differences in frequency distributions of steroid concentrations in these two populations could be attributed to various factors, including no differences occur between males

and females in frequency distributions of hormones prior to gonadal differentiation.

In conclusion, fluctuations of endogenous steroid concentrations in this study supports the concept of endogenous metabolism of maternally contributed and possibly independently synthesized steroids during early development. In addition, steroid glucuronides, commonly thought of as by-products of steroid metabolism, fluctuated in concentrations during early development, which also supports the concept of the endogenous metabolism. More studies on the exact role of endogenous steroid metabolism in embryonic and postembryonic development are needed.

ACKNOWLEDGEMENTS

We cordially thank the Cole River Hatchery personnel for their cooperation and assistance in collection of fish gametes. We also thank Mr. Cameron Sharpe, Mr. Dan Farnsworth, Mr. Caleb Slater, Miss Ying-Chi Lam, and Miss Sze-Wing Ngan for their assistance. Funding for this research was provided by Western Regional Aquaculture Consortium and Oregon State University College Sea Grant Program (Grant R/AQ-48).

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In vivo Steroid Metabolism in Embryonic and Newly Hatched Steelhead
Trout (*Oncorhynchus mykiss*)

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ABSTRACT

Radioactive pregnenolone (P5), testosterone (T), or 17 β -estradiol (E2) were microinjected into steelhead trout, *Oncorhynchus mykiss*, embryos and newly hatched yolk sac fry (alevins) to detect *in vivo* metabolism. We also assayed the water used to incubate animals for 10 h postmicroinjection to detect possible metabolite excretion. High pressure liquid chromatography and thin layer chromatography were used to separate and tentatively identify steroid metabolites. Metabolites of P5 were androstenedione (AN), E2, T, and glucuronides of E2 and T in embryos and AN, E2, progesterone, and 17 α , 20 β -dihydroxyprogesterone, and P5 glucuronide in alevins. E2 and its glucuronide were synthesized from precursor T in the embryos and alevins; however, the amounts of E2 and E2 glucuronide synthesized in the embryos were 10 and 3 magnitudes more, than detected in alevins. Testosterone glucuronide was synthesized in similar amounts in both stages of development. Embryos did not synthesize free metabolites from E2 precursor, but E2 glucuronide was detected from E2 precursor. Estradiol in alevins was metabolized into unidentified free and glucuronide-conjugated forms of steroids. Three unknown metabolites synthesized from P5 precursors and seven unknown substances produced in animals injected with testosterone or estradiol precursors were detected. Free metabolites were detected in the incubation water that held the animals (embryos and alevins) for 10 h after microinjection with T or E2. Glucuronide metabolites were not excreted by embryos into the incubation water 10 h after microinjections with any of the steroid precursors; however, alevins excreted glucuronides into the incubation water when supplied with precursor T.

These results imply that endogenous steroid metabolism of maternally contributed steroids is active during early development.

INTRODUCTION

The unfertilized fish egg contains a reservoir of maternally contributed hormones (Kobuke et al., 1987; Rothbard et al., 1987; Feist et al., 1990; Tagawa and Hirano, 1990; De Jesus et al., 1991; Schreck et al., 1991; De Jesus and Hirano, 1992; Hwang et al., 1992; Yeoh and Schreck, 1993). The importance of sex steroids in salmonid eggs in the development of embryonic and newly hatched young is unclear. In general, the concentrations of these hormones decline during embryogenesis, for example, the observed decline of endogenous steroids and their glucuronide conjugates in embryonic steelhead trout (*Oncorhynchus mykiss*). Changes in concentrations of testosterone, 17β -estradiol, 17α , 20β -dihydroxyprogesterone (DHP), 11-ketotestosterone (KT), and cortisol in the young steelhead trout indicate that sex steroid metabolism occurs during early stages of embryonic development (Yeoh et al., 1993). Steroid profiles of other teleosts such as coho salmon, *O. kisutch* (Feist et al., 1990), and tilapia, *Tilapia* sp. (Rothbard et al., 1987), change during embryonic and postembryonic development, which may again indicate steroid metabolism.

Embryonic and postembryonic fish have the potential to metabolize endogenous steroids. Rainbow trout embryos metabolized exogenous steroids such as pregnenolone, which indicated the presence of steroidogenic enzymes (Antila, 1984). Steroidogenesis and steroid metabolism may be important in early development because steroid metabolites can regulate intracellular function in the earliest stages of fish embryonic development (Brown et al., 1989). Products of steroid metabolism can direct target cells for "growth activities" such as cell division, protein synthesis, and secretion

(Briggs and Brotherton, 1970). Exogenous steroids affect early development of young salmonids such as influencing the phenotypic sex (Schreck, 1974; Hunter et al., 1983, 1986; and Lewis and Sower, 1992) and hatching rates (Cloud, 1981; and Yeoh and Schreck, 1993). Steroids also affect growth (Schreck and Fowler, 1981; Lone and Ince, 1983; Sower et al., 1983; and Lewis and Sower, 1992) and survival (Delabbio et al., 1988) of teleosts.

Metabolites such as steroid glucuronides in fish are thought to have minimal physiological functions because they are water soluble and excreted. However, steroid glucuronides may be sex pheromones in the male black goby, *Gobius jozo* (Colombo et al., 1980, 1982), the female zebrafish, *Brachydanio rerio* (Van Den Hurk and Lambert, 1983), and adult catfish, *Clarias gariepinus* (Resink et al., 1989a; and Resink et al., 1989b). Scott and Canario (1992) detected DHP sulphate in urine and plasma of female teleosts and indicated that this conjugate reflects the maturational stage of the ovary.

There are two classes of steroid glucuronides. One class undergoes further metabolic conversion, and the other is excreted into urine (Solomon and Bhavnani, 1970). The role of glucuronides is not as simple as previously thought. Some steroid glucuronides in mammals are metabolized to free steroids that have important physiological functions, for example, human testosterone glucuronide which is metabolized, *in vivo*, to etiocholanolone 5β -androstane 3α , 17β -diol (Solomon and Bhavnani, 1970). The dynamic changes of endogenous glucuronide and free steroid concentrations during embryonic and postembryonic development indicate that steelhead trout have the ability to form steroid glucuronides at a very early age (Yeoh et al., 1993) but whether these changes are important physiologically is unclear.

Assuming the "adaptationist theory", where substances inherited from parents are important for embryonic development, the presence of and

ability to synthesize free and conjugated steroids may be important to the well being of the embryos. If embryonic and newly hatched salmonids actively metabolize steroids during early development, maternally contributed steroids probably play an important role in development. Therefore, conditions that alter maternal physiology may have cross-generational effects.

The objectives of this study were (1) to determine if developing steelhead trout are able to metabolize steroids such as pregnenolone, testosterone, or estradiol; (2) to determine if these animals have the ability to form and excrete glucuronide conjugates (3) to determine if differences in metabolite production exist between two distinct stages of early development.

METHODS AND MATERIALS

Chemicals. Tritiated pregnenolone (71 mCi/mg) was purchased from New England Nuclear Research Products, and testosterone (256 mCi/mg) and 17β -estradiol (544 mCi/mg) were purchased from Amersham Life Science. Radiochemical purity was determined by high pressure liquid chromatography (HPLC). Enzyme β -glucuronidase (bovine liver) and reference steroids were purchased from Sigma Chemical Company.

Fish. Eggs from 15 female steelhead trout were fertilized with sperm pooled from three males and incubated in egg incubation units (Heath Tray) at Cole Rivers Hatchery, Oregon Department of Fish and Wildlife, Oregon. Eighteen days after fertilization, these eggs were transferred to Oregon State University's (OSU) Fish Genetics and Performance Laboratory at Smith Farm for further incubation. At 22 (pigmented eyes) and 30 (hatch) days postfertilization (dpf), 80 eggs (embryos) and 80 newly hatched yolksac fry (alevins) were removed from egg incubation trays (Heath Trays) and stored in plastic bags filled with 100 ml hatchery water supplemented with oxygen. Temperature was kept constant at 12°C. The embryos or alevins were moved to the Food Toxicology Laboratory, OSU for microinjection with sex steroid precursors.

Microinjection. The 80 embryos and alevins were divided into four groups of 20 each. To determine actual quantities of radioactivity injected, five embryos or alevins from each group were injected with 1 μ l of either carrier (70% vegetable oil, Crisco and 30% HPLC grade acetone) alone or tritiated

pregnenolone, testosterone, or estradiol. The calculated activity of each tritiated steroid injected into each animal was 0.25 μCi . These embryos or alevins were stored at 0°C in capped vials for about 1 h before being stored at -80°C.

The remaining 15 embryos or alevins of each group were also injected with one of the steroids or carrier. Each group of embryos or alevins was incubated individually in plastic bags containing 100 ml of hatchery water supplemented with oxygen. Temperature was kept constant at 12°C by floating these bags in a water bath. Ten hours postinjection, the embryos or alevins were placed in capped vials and stored at 0°C for a few minutes before storage at -80°C. The incubation water was also collected and treated similarly to detect potential leakage of precursors and excretion of steroid metabolites and glucuronide conjugates.

The microinjection procedure consisted of placing embryos and alevins on a plexiglass platform designed to hold them in place. A 50- μl syringe with a 31- μm gauge needle controlled by an automated microsyringe pump (Hamilton) was used to dispense 1 μl of test substance into the center of yolksacs of the embryos and alevins. The only difference between injecting embryos and yolksac alevins was that the alevins were placed in a petri dish containing 5 ml of water with bubbling carbon dioxide for 3 min prior injection. After injection, the alevins were revived in a petri dish containing 5 ml of fresh water and supplied with aeration for 2 min before being placed into incubation bags. There was no mortality associated with this procedure.

Extraction. Animal samples were thawed and weighed, and each was placed in a 16 x 125 mm culture tube with 1 ml of 0.5 M NaOH. Two milliliters of incubation water from each treatment group (with two replicates) were also

treated similarly to animal samples. After 24 h, all samples were sonicated for 20 sec and extracted twice with 8 ml of ethyl ether. These extracts, referred to as free steroids, were dried under a stream of nitrogen. The aqueous residue was adjusted to pH 4.8 with 50 μ l of 100% acetic acid and 100 μ l of acetate buffer, pH 4.8 (modified from Kime, 1990). The residue was treated with another 100 μ l of acetate buffer, pH 4.8, containing 800 μ g of beta-glucuronidase. The tubes were incubated for 24 h at 37 °C. Another 800 μ g of enzyme was added for another 24 h.

Steroid moieties from the aqueous phase were extracted and dried as described above. These steroids are referred to as glucuronides. The radioactivity remaining in the aqueous phase was determined by pouring it into a scintillation vial with 5 ml of scintillation fluid (Bio-Solve) for counting in a Beckman LS 1800 liquid scintillation system. Counting efficiency of the Beckman system, calculated from the quench curve for water with the scintillation fluid, was 40%, which was used to convert cpm to dpm. Extracts from the organic or aqueous phases were reconstituted in 750 μ l of methanol, vortexed, and pipetted into 3-ml syringes fitted with 0.4- μ m Acrodisc filters. Extracts were then injected into Baker collection tubes coated with Sigmacote. The syringes were filled with another 250 μ l of methanol to rinse remaining steroids. Extracts were dried by vacuum and stored at - 80 °C until HPLC analysis.

HPLC. The HPLC system and solvent system described by Feist et al. (1991) were modified and used for eluting steroids from extracted samples. The HPLC system was an IBM LC/9533 Ternary Gradient Liquid Chromatograph and the column type used was an octadecyl C18 (Hewlett Packard). Briefly, we used an isocratic mobile phase of

water:methanol:acetonitrile:isopropanol (62:28:5:5), changing to a linear gradient of 3.3%/min of water:methanol:butanol (35:45:20). Run time was 30 min for testosterone and estradiol samples. The solvent system, for samples with pregnenolone as the precursor, was identical except instead of ending at 30 min, an extended isocratic run of the latter mobile phase for 5 min was conducted. Flow rate was 0.4 ml/min. This system allowed the separation of 17 steroid standards (Table 1). The HPLC system was linked to an on-line radioactivity counter (Flo-One beta, Radiomatic Inc.).

The radioactive samples were reconstituted in 200 μ l of mobile phase containing 16 carrier steroid standards (625 ng/steroid, Fig. 1) because endogenous steroids were present below detectable concentrations (less than 5-10 ng). Pregnenolone did not absorb at the uv wavelengths used (254 and 280 nm). The retention time for pregnenolone was determined by injecting authentic tritiated pregnenolone onto HPLC connected to the Radiomatic counter. Delay time between absorption and count peaks was 0.6 min.

A 50 μ l of each sample (n=4 animals for each treatment group) was analyzed using the HPLC procedure. The identity and quantity of presumptively identified steroids obtained after HPLC were verified by thin layer chromatography (TLC, n=2 animals for each treatment).

Thin Layer Chromatography (TLC). Samples were redried under a stream of nitrogen. The solvent system used was dichloromethane:ether (5:2). TLC plates (silica gel, Baker) were prewashed before samples, and authentic steroid standards were spotted on the plates. After 1 h, the chromatogram was allowed to dry. Cold standards were visualized under UV light. Corresponding radioactive spots were cut off and vortexed in 16 x 100 mm tubes with 3 ml of dichloromethane. The dichloromethane was decanted

TABLE 1.

Steroids standards injected onto high pressure liquid chromatography (HPLC).

ABBREVIATION	NAME	CHEMICAL NAME
Aldo	aldosterone	4-pregnene-18-al-11 β , 21-diol-3, 20-dione
E	cortisone	4-pregnene-17 α , 21-diol-3, 11, 20-trione
F	cortisol	4-pregnene-11 β , 17 α , 21-triol-3, 20-dione
KT	11-keto- testosterone	4-androstene-17-ol-3, 11-dione
OHA	11-hydroxy- androstenedione	4-androstene-11 β -ol-3, 17-dione
OHT	11-hydroxy- testosterone	4-androstene-11 β , 17 β -diol-3-one
B	corticosterone	4-pregnene-11 β , 21-diol-3, 20-dione
DOF	11-deoxy cortisol	4-pregnene-17 α , 21-diol-3, 20-dione
KP	11-keto- progesterone	4-pregnene-3, 11, 20-trione
AN	androstenedione	4-androstene-3, 17-dione
T	testosterone	4-androstene-17 β -ol-3-one
17-OHP	17-hydroxy- progesterone	4-pregnene-17 α -ol-3, 20-dione
DHP	dihydroxy- progesterone	4-pregnene-17 α , 20 β -diol-3-one
P4	progesterone	4-pregnene-3, 20-dione
20 β -OHP	20 β -hydroxy- progesterone	4-pregnene-20 β -ol-3-one
E2	17 β -estradiol	1, 3, 5 (10)-estratriene-3-17 β -diol
P5	pregnenolone	5-pregnene-3 β -ol-20-one

Figure 1: Representative high pressure liquid chromatography (HPLC) chromatogram depicting retention times of 15 steroids with uv absorbance at 254 nm. E2 was detected using uv at 280 nm wavelength (not in figure). See Table 1 for key to steroids.

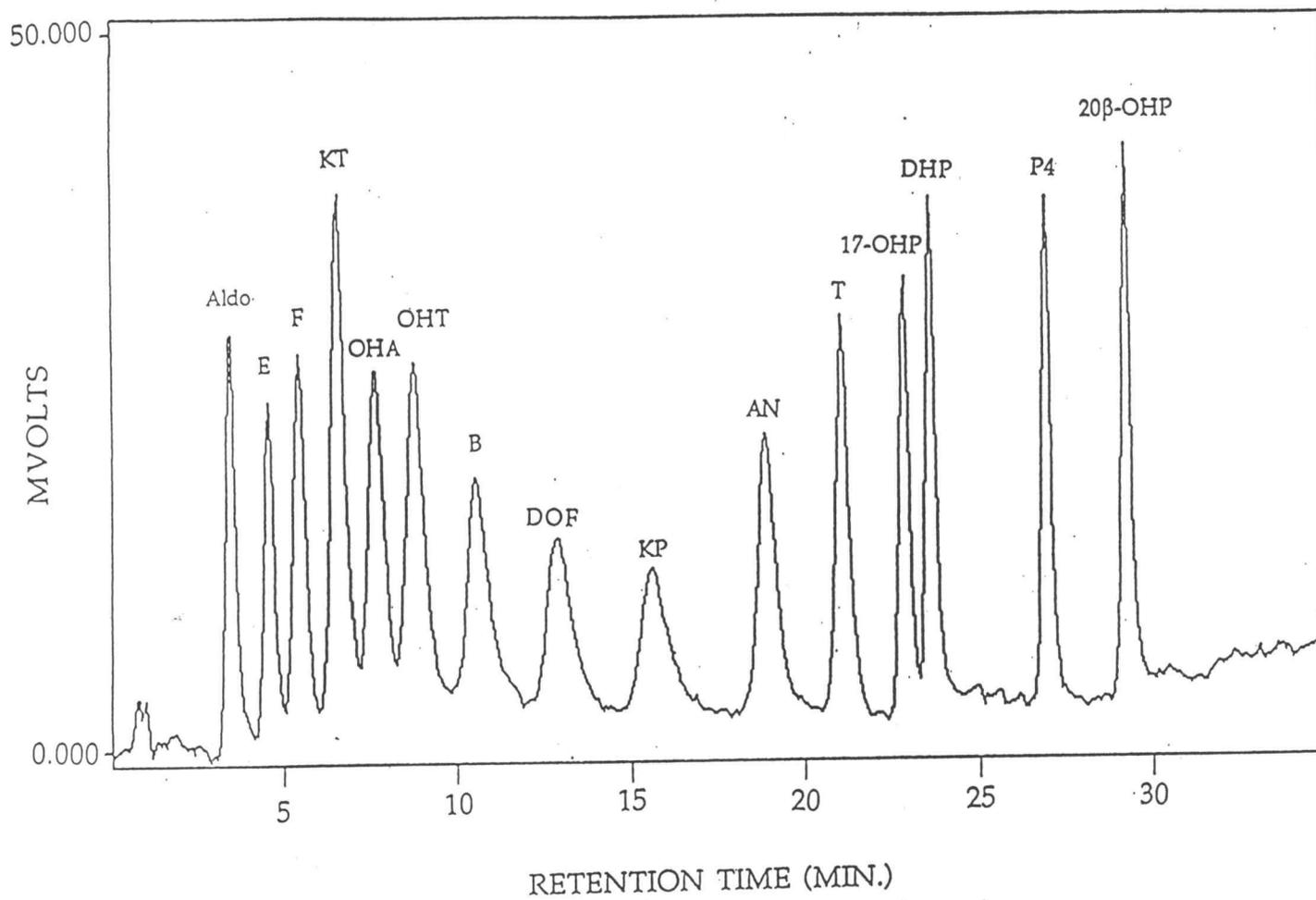


FIGURE 1.

into scintillation vials. The extraction procedure was then repeated. Combined extracts were then dried under nitrogen. Scintillation fluid (5 ml) was added and the radioactivity was determined. The R_f 's for estradiol, testosterone, pregnenolone, androstenedione, progesterone, and DHP were 0.41, 0.28, 0.34, 0.47, 0.53, and 0.16, respectively.

Based on the HPLC results, radioactive steroids that co-eluted with authentic steroids were presumptively identified and verified by TLC. Physicochemical reactions were not conducted because the objectives of this study were to determine if embryos were able to metabolize and conjugate precursors, not to rigorously identify potential metabolites.

Recovery. Non-radioactive embryos or alevins used for calculating recoveries were treated identically to actual samples. Prior to extraction, known amounts of tritiated pregnenolone, testosterone, or estradiol were added to each incubation tube. Extraction recoveries were calculated after filtration with Acrodisc filters. Total percent of recovered radioactivity of the amount injected was $75 \pm 3\%$ (pregnenolone), $85 \pm 4\%$ (testosterone), and $80 \pm 2\%$ (estradiol) for embryos and $95 \pm 5\%$ (pregnenolone), $90 \pm 4\%$ (testosterone), and 78 ± 3 (estradiol) for alevins. All data were appropriately corrected.

RESULTS

Yields of metabolites were expressed as percent of total recovered radioactivity (summation of radioactivity from free, conjugated fractions of samples, incubation water, and aqueous phase of the extraction). Unknown metabolites from animals injected with pregnenolone were named up1, up2, etc; from testosterone were named ut1, ut2, etc; and from estradiol precursors were named ue1, ue2, etc.

Pregnenolone

Embryos. Ten hours postinjection, 80.2% of the pregnenolone-derived ^3H remained in the egg (Fig. 2). Free metabolites were presumptively identified as AN (0.7%), estradiol (1.9%), and testosterone (1.3%). Three unknown free metabolites up1 (1.8%), up2 (0.4%), and up3 (1.2%) from the pregnenolone samples were detected. All free identified metabolites and free unknowns made up 8.4% of total recovered radioactivity. Free DHP and P4 were not detected. Estradiol glucuronide (0.6%) and testosterone glucuronide (0.5%) were detected but not pregnenolone glucuronide (Fig. 3). The amount of radioactivity in the incubation water was 5.8% and in the aqueous phase was 4.5%.

Alevins. An average of 85.3% of injected pregnenolone-derived ^3H was still detected in each alevin (Fig. 2). Metabolites were presumptively identified as androstenedione (0.3%), estradiol (0.5%), DHP (0.5%), and progesterone (1.3%). Unlike in embryos, testosterone and testosterone glucuronide were not found, and estradiol was detected in reduced quantity,

Figure 2. The relative distribution of radioactivity 10 h postmicroinjection of tritiated pregnenolone (P5), testosterone (T), or 17 β -estradiol (E2) into steelhead trout, *Oncorhynchus mykiss*, embryos or newly hatched alevins.

Precursor = tritiated steroid precursor remaining in the animal; free metabolite = free steroid metabolites in the animal; glucuronide conjugate = steroid glucuronides in the animal; incubation water = total radioactivity in the incubation media used for holding animals postmicroinjection; and aqueous waste = total radioactivity in the aqueous phase following extractions.

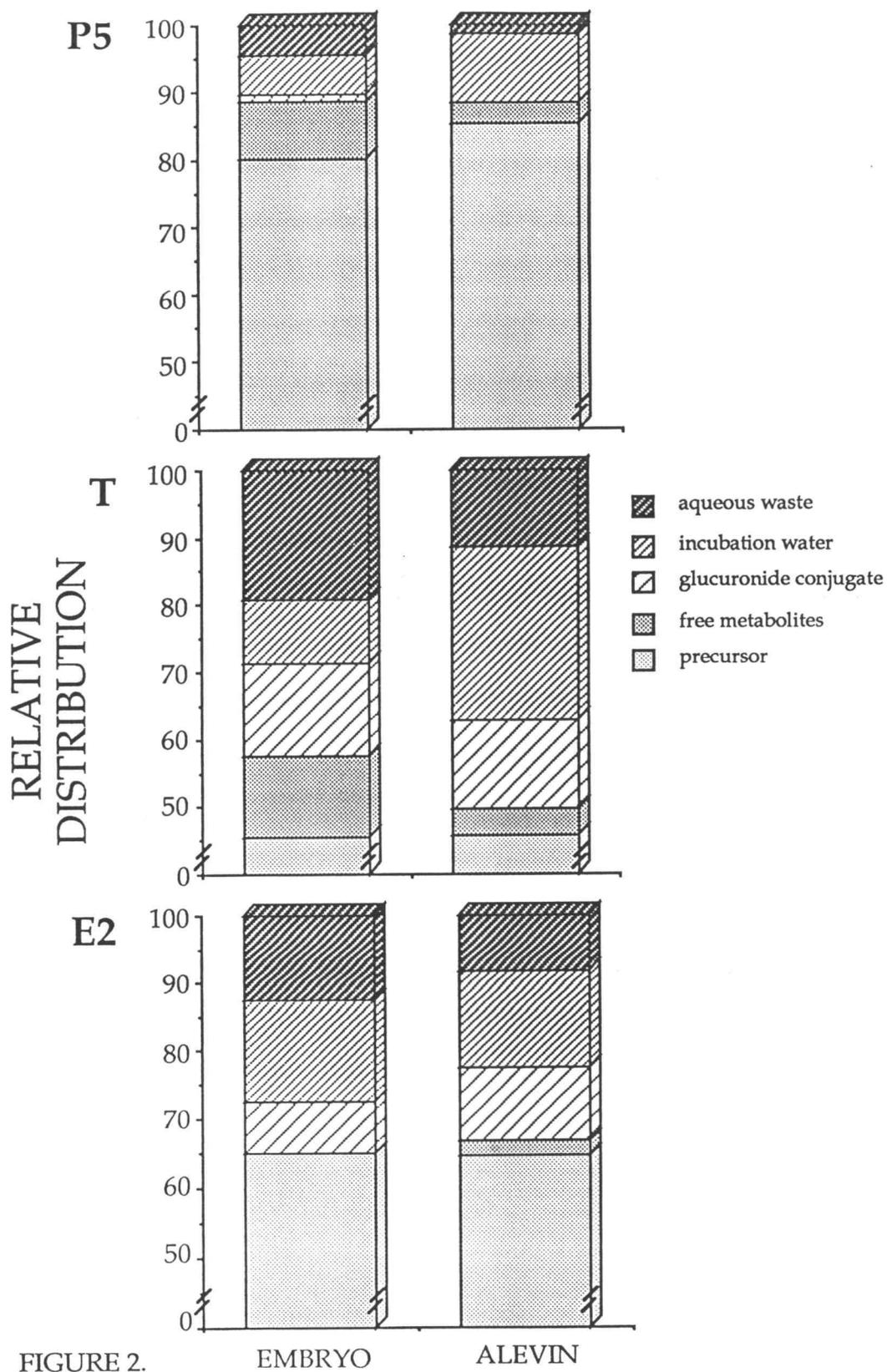


FIGURE 2.

Figure 3. A representative chromatogram of pregnenolone (P5), free and glucuronide-conjugated metabolites in steelhead trout, *Oncorhynchus mykiss*, embryos or newly hatched alevins at 10 h postinjection of P5. The abscissa is the retention time of these steroids. The mean peak height of four animals is depicted by □. The base width of each peak is similar among samples. Black dots • represent the range of samples. Black squares ■ are seen due to overlapping of the range • and mean □.

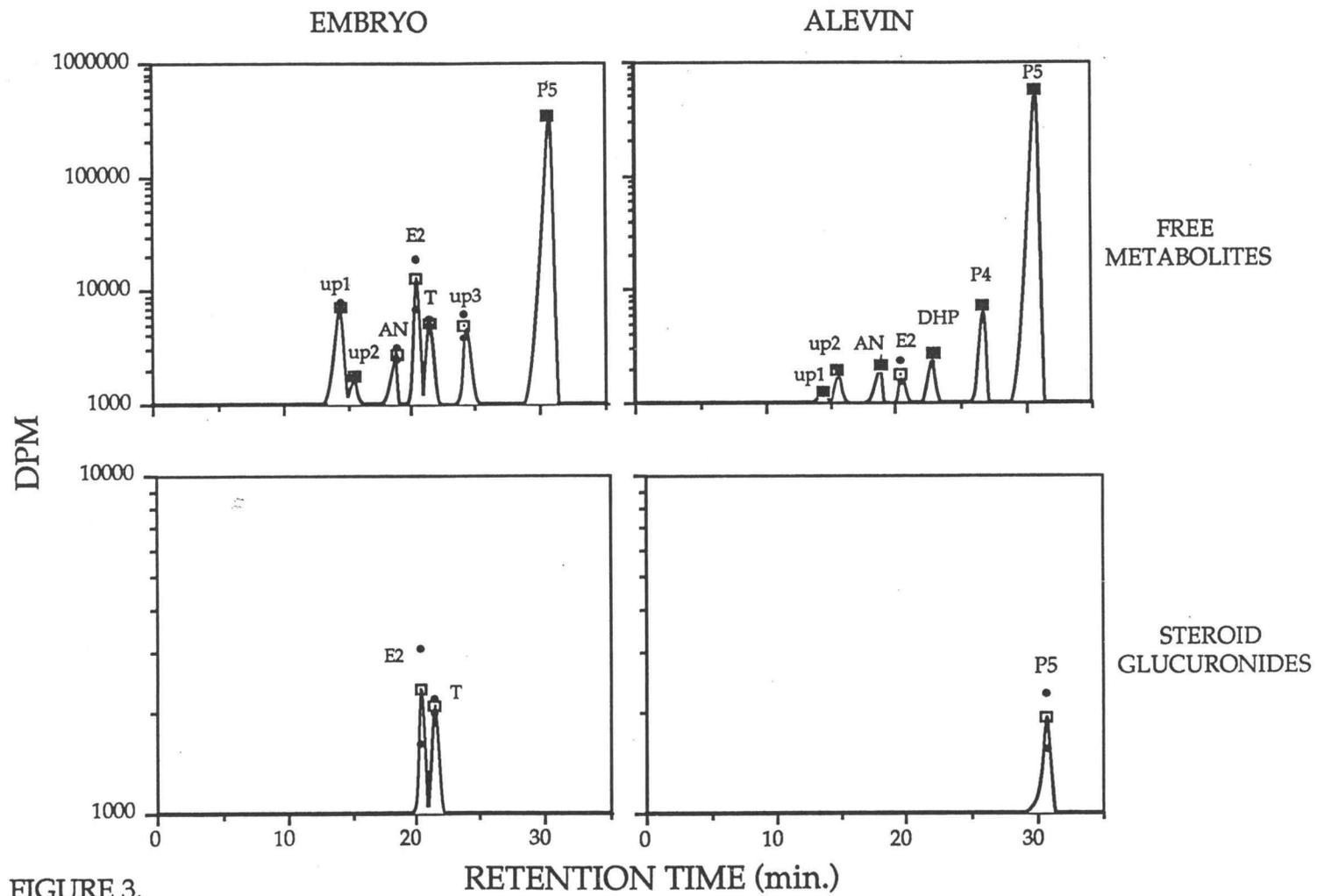


FIGURE 3.

from 1.9% (embryo) to 0.4%. Two free unknown metabolites up1 (0.2%), and up2 (0.4%) were produced. These unknown metabolites were also detected in embryos. Pregnenolone glucuronide (0.4%) was detected at this stage of development but not at the embryonic stage (Fig. 3). Radioactivity in the incubation water was 10.2% and in the aqueous phase was 1.3% (Fig. 2).

Incubation water. Radioactivity was detected in the incubation water after incubating injected alevins for 10 h. The substances and their percent yields are shown in Table 2.

Testosterone

Embryos. An average of 45.4% of administered testosterone remained free in the embryos (Fig. 2). Free estradiol (9.9%) and three unknown metabolites ut1 (0.2%), ut2 (1.2%), and ut3 (0.9%) were evident by 10 h post injection of testosterone. Glucuronides of testosterone (9.5%), estradiol (1.3%), and the three unknown metabolites ut1 (0.7%), ut2 (1.8%), and ut3 (0.4%) were also detected (Fig. 4). Unknowns ut1, ut2, and ut3 co-eluted with aldosterone, cortisone and cortisol, respectively. Steroids such as 15 α -hydroxyprogesterone and 15 β -hydroxytestosterone also may co-elute at those retention times in certain chromatography systems (Kime and Rafter, 1981). Radioactivity found in incubation water was 9.5% and in the aqueous phase was 19.1 % of total recovered radioactivity.

Alevins. An average of 45.7% of testosterone-derived ^3H remained unconverted in alevins (Fig. 2). Free estradiol (1.9%) and three free unknown elutes ut2, ut4, and ut5 with yields 0.7%, 0.4%, and 0.9%, respectively were metabolized from testosterone. Glucuronides of testosterone (8.6%), estradiol (0.4%), ut1 (0.6%), ut2 (0.8%), ut3 (0.3%), ut7 (1.0%), and ut8 (1.4%)

TABLE 2.

Yields (%) of free and glucuronide metabolites of pregnenolone (P5) microinjected into embryos or newly hatched alevins of steelhead trout, *Oncorhynchus mykiss*. The yields of tritiated steroids in the incubation water of embryos or newly hatched alevins were calculated and expressed as percent of total recovered radioactivity.

METABOLITE	EMBRYO		ALEVIN		EMBRYO INC. WATER		ALEVIN INC. WATER	
	Free	Gluc	Free	Gluc	Free	Gluc	Free	Gluc
Androstenedione	0.7	—	0.4	—	—	—	0.2	—
17 β -Estradiol	3.1	0.6	0.3	—	—	—	0.3	—
Testosterone	1.2	0.5	—	—	—	—	0.1	—
DHP	—	—	0.5	—	—	—	—	—
Progesterone	—	—	1.3	—	—	—	—	—
up1	1.3	—	0.2	—	—	—	0.2	—
up2	0.4	—	0.4	—	—	—	0.7	—
up3	1.2	—	—	—	—	—	—	—
Pregnenolone	N/A	—	N/A	0.1	N/A	—	N/A	—

— denotes that this substance was undetectable in the animals 10 h after microinjection with pregnenolone. Pregnenolone yields were not applicable (N/A) because it was used as the precursor to other metabolites.

Figure 4. A representative chromatogram of testosterone (T), free and glucuronide-conjugated metabolites in steelhead trout, *Oncorhynchus mykiss*, embryos or newly hatched alevins at 10 h postinjection of T. The abscissa is the retention time of these steroids. The mean peak height of four animals is depicted by □. The base width of each peak is similar among samples. Black dots • represent the range of samples. Black squares ■ are seen due to overlapping of the range • and mean □.

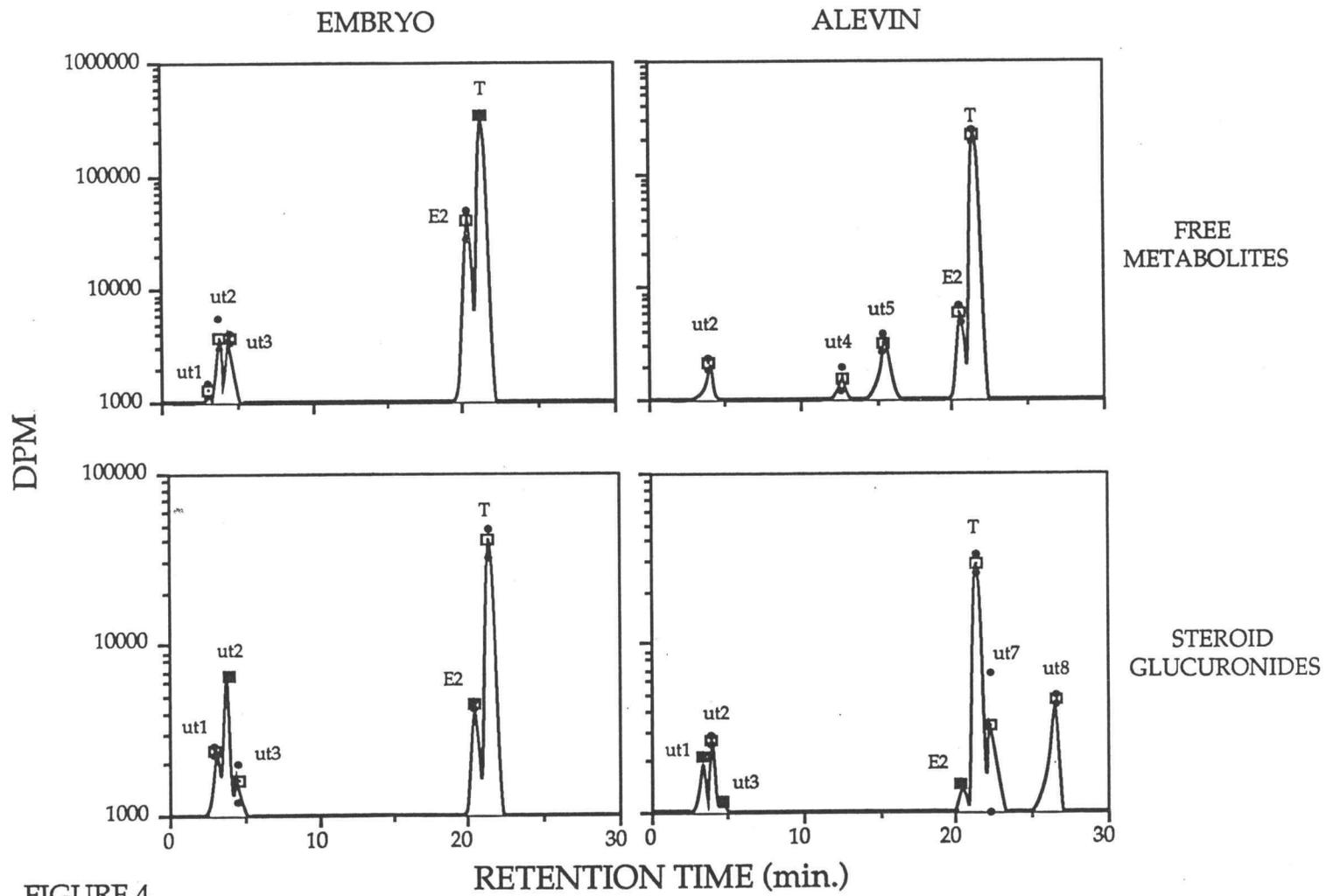


FIGURE 4.

were detected. Free ut1 and ut3 metabolites were detected in embryos but not in alevins. In alevins, ut7 glucuronide and ut8 glucuronide were detected but not in embryos. Ut7 and ut8 were less polar than testosterone (Fig. 4). Radioactivity in the incubation water was 25.9% and in the aqueous phase was 11.4% (Fig. 2).

Incubation water. Radioactivity in the incubation water of alevin samples was higher than in embryonic samples. We found glucuronides of testosterone, ut3, ut4, ut7, and ut8 (Table 3). The presence of these substances in the incubation water indicated that alevins excreted glucuronides. In addition, free metabolites detected in incubation water included unknowns ut3, ut4, ut7, and ut8.

17 β -estradiol

Embryos. An average of 64.9% of estradiol-derived ^3H remained in the animals 10 h after injection (Fig. 2). Glucuronides of estradiol (3%) and unknown metabolites called ue1 (1.6%), ue2 (1.6%), ue3 (1.4%), and ue7 (0.7%) were detected (Fig. 5). We did not detect any free metabolites. Total radioactivity in the incubation water was 15.0% and in the aqueous phase was 12.5%.

Alevins. An average of 64.6% of estradiol-derived ^3H remained in the alevins after incubation (Fig. 2). Free steroid metabolites included ue2 (0.3%), ue3 (0.8%), ue4 (0.4%), and ue5 (0.7%). Besides estradiol glucuronide (4.0%), glucuronides of ue1 (2.0%), ue2 (1.9%), ue3 (2.1%), and ue7 (0.7%) were also formed. Ue7 glucuronide was the only glucuronide recovered in alevins but not in embryos (Fig. 5). One metabolite, ue6, was detected in samples incubated for less than 1 h but not detected at 10 h postinjection.

TABLE 3.

Yields (%) of free and glucuronide metabolites of testosterone (T) microinjected into embryos or newly hatched alevins of steelhead trout, *Oncorhynchus mykiss*. The yields of tritiated steroids in the incubation water of embryos or newly hatched alevins were calculated and expressed as percent of total recovered radioactivity.

METABOLITE	EMBRYO		ALEVIN		EMBRYO INC. WATER		ALEVIN INC. WATER	
	Free	Gluc	Free	Gluc	Free	Gluc	Free	Gluc
17 β -Estradiol	9.9	1.3	1.9	0.4	0.2	—	—	—
ut1	0.2	0.7	—	0.6	—	—	—	—
ut2	1.2	1.8	0.7	0.8	—	—	—	—
ut3	0.9	0.4	—	0.3	—	—	0.3	0.3
ut4	—	—	0.4	—	—	—	0.4	0.3
ut5	—	—	0.9	—	—	—	—	—
ut7	—	—	—	1.0	—	—	0.3	0.2
ut8	—	—	—	1.4	—	—	0.2	3.8
Testosterone	N/A	9.5	N/A	8.6	N/A	—	N/A	2.1

— denotes that this substance was undetectable in the animals 10 h after microinjection with testosterone. Testosterone yields were not applicable (N/A) because it was used as the precursor to other metabolites.

Figure 5. A representative chromatogram of 17β -estradiol (E2), free and glucuronide-conjugated metabolites in steelhead trout, *Oncorhynchus mykiss*, embryos or newly hatched alevins at 10 h postinjection of E2. The abscissa is the retention time of these steroids. The mean peak height of four animals is depicted by \square . The base width of each peak is similar among samples. Black dots \bullet represent the range of samples. Black squares \blacksquare are seen due to overlapping of the range \bullet and mean \square .

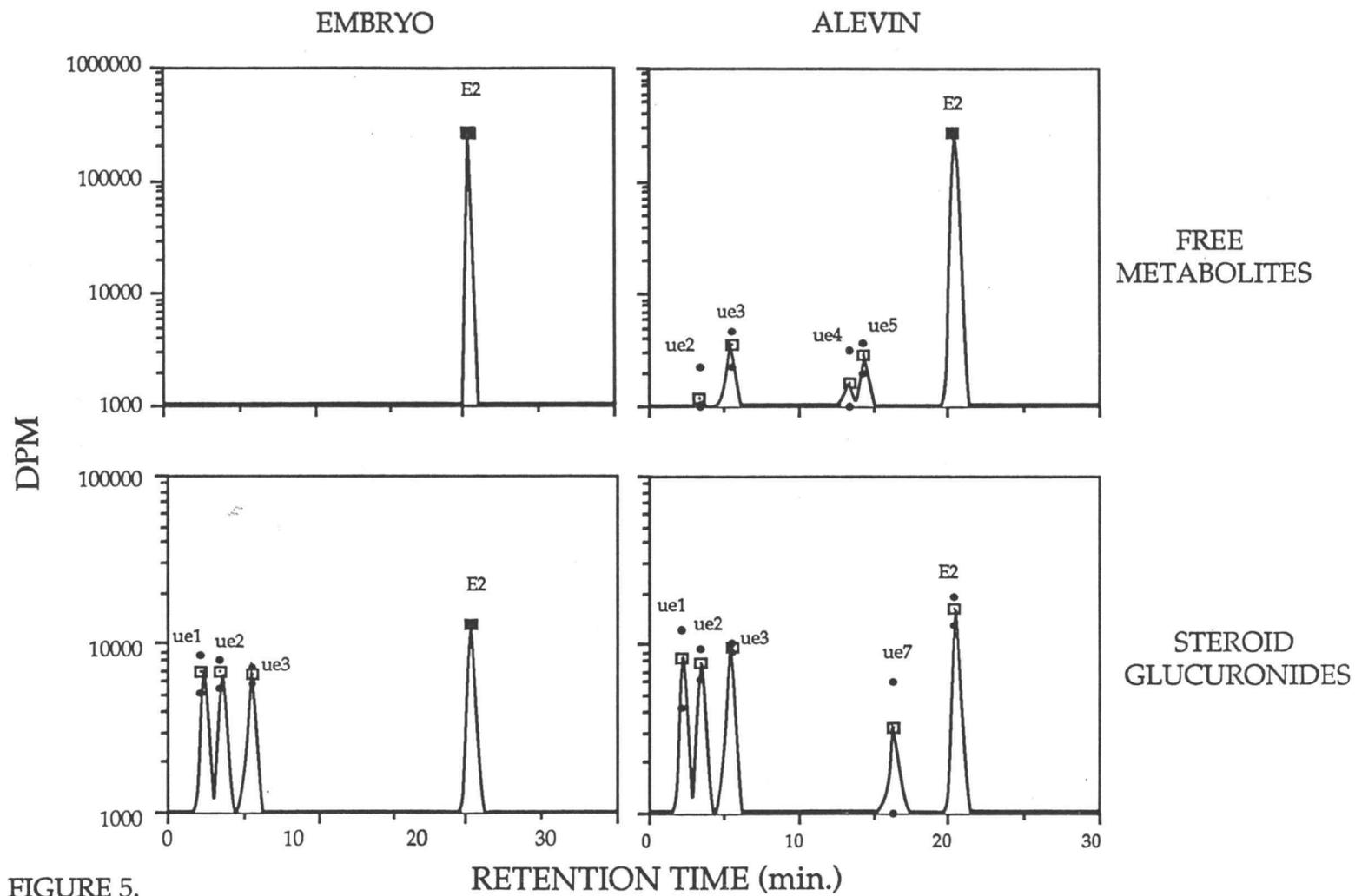


FIGURE 5.

Metabolite ue6 has a similar retention time as ut5, which was 15.5 min, indicating that they may be identical substances. Total radioactivity in the incubation water and aqueous phase were 14.4% and 8.1%, respectively.

Incubation water. Radioactivity was detected in the incubation water after incubating injected animals for 10 h. The substrates and their percent yields are shown in Table 4.

TABLE 4.

Yields (%) of free and glucuronide metabolites of 17β -estradiol (E2) microinjected into embryos or newly hatched alevins of steelhead trout, *Oncorhynchus mykiss*. The yields of tritiated steroids in the incubation water of embryos or newly hatched alevins were calculated and expressed as a percent of total recovered radioactivity.

METABOLITE	EMBRYO		ALEVIN		EMBRYO INC. WATER		ALEVIN INC. WATER	
	Free	Gluc	Free	Gluc	Free	Gluc	Free	Gluc
ue1	—	1.6	—	2.0	0.2	—	—	—
ue2	—	1.6	0.3	1.9	0.4	—	0.3	—
ue3	—	1.4	0.8	2.1	0.3	—	—	—
ue4	—	—	0.4	—	—	—	—	—
ue5	—	—	0.7	—	—	—	0.2	—
ue6	—	—	—	—	—	—	—	—
ue7	—	—	—	0.7	—	—	—	—
17β -Estradiol	N/A	3.0	N/A	4.0	N/A	—	N/A	—

— denotes that this substance was undetectable in the animals 10 h after microinjection with estradiol. Estradiol yields were not applicable (N/A) because it was used as the precursor to other metabolites.

DISCUSSION

Steelhead trout embryos and newly hatched alevins have the capacity to metabolize steroids such as pregnenolone, testosterone, and estradiol into free and glucuronide forms of steroids. The ability and capacity for steroid metabolism differed between the two developmental stages. Antila (1984) found that oocytes and early embryos of rainbow trout metabolized pregnenolone and progesterone. This difference in ability to metabolize steroids could be attributed to various factors such as presence or absence of key enzymes, concentration of steroid substrate, or the need for certain steroids for preferential development is reflected in teleosts of various ages, for example, in the adult salmon prior to oocyte maturation, plasma DHP increases during oocyte maturation (Fitzpatrick et al., 1986; So et al., 1985), and Sakai et al. (1988) inferred that there was a shift in steroidogenic activities of enzymes in medaka, *Oryzias latipes*, follicles prior oocyte maturation. In human steroid metabolism, the degree of conjugation and the preferred form of hormones vary with the physiologic state of the individual (Bongiovanni and Cohn, 1970). In developing trout, the existence of differential metabolism between two developmental stages (embryonic and alevin) may reflect two different physiological states.

This early shift in steroid metabolism may have functional significance. Exposure to exogenous steroids at these stages is potent in directing observable physiological changes such as sex ratios (Schreck, 1974; Hunter et al., 1983, 1986; Piferrer, and Donaldson 1989; Lewis and Sower, 1992) and hatching rates (Cloud, 1981; Yeoh and Schreck, 1993) while at slightly later stages growth is affected (Schreck and Fowler, 1981; Lone and Ince, 1983;

Sower et al., 1983; and Lewis and Sower, 1992). Steroid glucuronides were formed in both developmental stages, but the identity of these glucuronides was not similar between embryos and alevins. This variation in production of glucuronides is possible because glucuronyltransferases needed for glucuronidation are substrate specific (Roy, 1970).

Pregnenolone was chosen as a precursor because it is the starting point for the steroid biosynthetic cascade. Free estradiol, testosterone, and unknown metabolite up3, estradiol glucuronide, and testosterone glucuronide were synthesized from pregnenolone at the embryonic but not the alevin stage. In contrast, progesterone, DHP, and pregnenolone glucuronide, but not testosterone nor testosterone glucuronide, were synthesized in the alevin stage. Alevins also synthesized smaller amounts of free estradiol than embryos when given pregnenolone. In addition, alevins lacked the capacity to metabolize testosterone or form testosterone glucuronide compared with embryos when given pregnenolone as substrate. Endogenous concentration of testosterone in salmonid alevins is naturally low compared with newly fertilized eggs (Feist et al., 1990; Yeoh et al., 1993). We do not know the significance of the alevin's reduced ability to synthesize testosterone and estradiol at this stage of development.

A distinct change in the steroid metabolic pathway may occur in alevins. This metabolic change resulted in reduced synthesis of free estradiol, testosterone, estradiol glucuronide, and testosterone glucuronide but increased concentrations of DHP, progesterone, and pregnenolone glucuronide. Apparently, low concentrations of free estradiol in the pregnenolone-microinjected alevins was due to the absence of aromatizable androgens and not to the absence of aromatases because free estradiol was synthesized in alevins when supplied with testosterone. These low

concentrations of estradiol and estradiol glucuronide detected in alevins could be due to the absence of testosterone. Decreased estradiol secretion in post ovulatory rats was explained by the low concentrations of aromatizable androgens (Suzuki and Tamaoki, 1979). Aromatase activity appears higher in embryos than in alevins because more free estradiol was synthesized when these animals were supplied with similar concentrations of testosterone. The reduced ability of alevins to synthesize testosterone and estradiol was consistent with the presence of lower concentrations of endogenous sex steroids at that stage of development in trout (Yeoh et al., 1993).

Testosterone and estradiol glucuronides were synthesized from testosterone microinjected into alevins. The synthesis of these substances indicates that their absence in the alevins microinjected with pregnenolone was not due to inactive glucuronyltransferases but to the lack of sufficient substrate. This supports the interpretation that the steroid metabolic pathway is being diverted away from producing testosterone or estradiol and their glucuronides. Interestingly, this phenomenon of metabolic diversion was seen in protandrus *Sparus auratus* undergoing natural sex reversal. Ovarian tissue from this teleost apparently cannot convert the progestin 17 α -hydroxyprogesterone to androgens and estrogens (Colombo et al., 1972). The shift in metabolic pathway results in the synthesis of progestins such as progesterone and DHP.

Reduced synthesis of estradiol and absence of testosterone and testosterone glucuronide could be attributed to newly hatched alevins preparing an optimal internal environment. This environment is needed for sex genes, once active, to direct appropriate production of sex steroids for sex differentiation or even sex reversal. Cardwell and Liley (1991) found that

spotlight parrotfish, *Sparisoma viride*, undergoing sex reversal had decreased concentrations of estradiol. In addition, Tanaka et al. (1990) reported low serum concentrations of sex steroids in protogynous red spotted grouper, *Epinephelus akaara*, in the intersexual phase.

One common metabolite was synthesized in alevins injected with either testosterone or estradiol. This unknown substance may be an intermediate metabolite because it was only detected in alevins sampled 1 h after microinjection with estradiol but disappeared by 10 h after microinjection. The apparent metabolism of this unknown intermediate metabolite indicates active steroidogenesis.

The synthesis of progesterone and DHP from pregnenolone indicates the presence of 17α -hydroxylase and 20α -hydroxysteroid dehydrogenase in alevins. Embryos seem to lack enzymes such as 17α -hydroxylase, as proposed by Antila (1984). Conversion of microinjected pregnenolone is slower in alevins compared with embryos, possibly because alevins are metabolizing and synthesizing their own steroids, which also indicates that alevins may cease to depend on maternally contributed steroids.

Interestingly, steroid moieties of unknown glucuronides ut7 and ut8 in alevins were less polar than testosterone. The range of ut8 productions was small, but the range for ut7 productions was large among samples that were analyzed. Two samples had about 10-fold the concentrations of ut7 than the other samples. The identity and physiological roles of these two steroid glucuronides are unknown. They could be destined for excretion or substrates for further steroid metabolism.

Newly hatched steelhead trout appeared to excrete glucuronides of testosterone, ut3, ut4, ut7, and substantial amounts of ut8 into incubation water 10 h after incubation. The significance of excretion of testosterone

glucuronide by alevins but not embryos is unknown. Glucuronides were not detected in the ambient water holding embryos microinjected with pregnenolone, testosterone, or estradiol, possibly because these conjugates are stored in the perivitelline space until hatch. This is a logical assumption because nitrogenous metabolic excreta are stored in the perivitelline space during embryonic development (Smith, 1947; Blaxter, 1969; and Kamler, 1992). Newly hatched steelhead trout alevins may maintain low concentrations of testosterone compared with concentrations in newly fertilized eggs by conjugating and excreting this steroid as testosterone glucuronide.

The regulatory mechanism for converting precursors to estradiol seems to be at the higher end of the steroid cascade because embryos and alevins are able to synthesize estradiol from testosterone precursor. We found that embryos metabolized precursor estradiol to form glucuronides but not free metabolites. This result is consistent with that of Antila (1984) who did not detect any free metabolites produced from free estradiol in early rainbow trout embryos. However, estradiol glucuronide was detected in embryos and newly hatched trout injected with estradiol.

In conclusion, we have determined that the ability for steroid metabolism is present in steelhead trout during early development. This strongly implies that maternally contributed hormones are metabolized during embryonic development and that steroid metabolism is an integral part of early development. The exact roles of hormones in embryonic and alevin development in teleosts remain to be determined.

ACKNOWLEDGEMENTS

We cordially thank Dr. Jerry Hendricks and Mr. Dan Arbogast for the loan of the microinjection equipment and for assisting with the microinjections and Dr. Donald Buhler and Miss Marilyn Henderson for the generous loan and assistance with the Online Radiomatic Counter. This research was funded by Western Regional Aquaculture Consortium and Oregon State University College Sea Grant Program (Grant R/AQ-48).

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APPENDICES

APPENDIX 1

Effects of Hormones on Early Development in Chinook Salmon

INTRODUCTION

This preliminary experiment was conducted with chinook salmon. The aims of this experiment were identical to objectives of Chapter 1.

METHODS AND MATERIALS

Gametes Chinook salmon eggs (n=20,000) were pooled from eight females. Sperm was pooled from three males at the McKenzie Fish Hatchery, Oregon. The eggs and sperm were kept separated in oxygen supplemented airtight bags and transported to Smith Farm Genetics and Performance Laboratory (SF) at a constant temperature of 12°C. These gametes were used in hormone augmentation experiments.

Hormone augmentation. Upon arrival at SF, these eggs were divided into treatment groups. Each treatment group consisted of 1,500 eggs per group. We exposed eggs to hormones at three stages; at water hardening which immediately succeeds fertilization, at the eyed stage when eye pigments become visible (eyed, 20 days post fertilization) and at both these stages. The hormones tested in this experiment were cortisol, testosterone, and thyroxine. Procedure for hormone exposure is described in Yeoh and Schreck (1993, Chapter 1).

Growth. Embryos and fish were weighed and measured at various stages. Total length measurements were taken with a ruler or calipers. Condition factor was calculated (Anderson and Gutreuter, 1985) using $K_n = W/aL^b$ because the overall shape of alevins varied considerably through time. Constants a and b were recalculated at each measurement date from a compilation weight and total length data (n=500).

Upon completion of yolk sac absorption (83 dpf), the fish were removed from incubation trays, Heath Trays. Each treatment group was randomly placed in a section of a growout trough (described in Yeoh and Schreck, 1993) and were fed 4% of their body weight daily. Temperature was 13°C. To determine if timing of exposures and type of hormone used affected growth we pooled certain chinook treatment groups. Three month old chinook from different groups were marked differentially by cold branding. Fish that were treated at the same developmental stage but with different hormones were marked and pooled (n=30 from each treatment group). Fish that were treated with identical hormones but at different developmental stages were also marked and pooled. There were two other replicates for each treatment group. Amongst replicates, similar treatment groups were not branded at the same locations on the body. This was to account for possible differential growth caused by cold branding.

Hatch. Incubating eggs were observed every day for signs of hatching. The time for the first fish of each treatment group to hatch was designated as t=0. The amount of time taken by other groups to hatch completely was documented. The eggs were checked every 4 hours. Data regarding hatch

were expressed as median time to hatching (time taken for the first half of the group to hatch) for each treatment group.

Statistics. Statistics were calculated by using Statview II™ (Abacus Concepts, Inc.) and methods described in Zar (1984). Student's t test, ANOVA, and Tukey multiple comparison test were the statistical methods used, $p < 0.05$.

RESULTS

Hatch. Median time to hatching for eggs treated with cortisol at water hardening and at both stages (water hardening and eyed) were shorter than corresponding control groups (ANOVA, Tukey type multiple comparison for medians, $P < 0.05$). In contrast, eggs exposed to cortisol at the eyed stage did not hatch faster than controls ($p > 0.05$). Steelhead trout eggs, in a later study, that were immersed at eyed also did not hatch faster than controls (Yeoh and Schreck, 1993). All eggs treated with testosterone hatched faster than controls (ANOVA, Tukey type multiple comparison for medians, $P < 0.05$). Eggs immersed in testosterone at both water hardening and eyed hatched earliest, about 20% faster than corresponding controls (Fig. 1). All thyroxine groups also hatched faster than the controls (ANOVA, Tukey type multiple comparison for medians, $P < 0.05$). Animals exposed to thyroxine at both stages hatching earliest. Mortality of treatment groups ranged from 2 to 13%.

Growth. In one month old (30 dpf) embryos, the thyroxine group immersed at eyed (0.261 ± 0.009 g) weighed more than other groups immersed at eyed

FIGURE 1. Median hatch times of chinook salmon (*O. tshawytscha*) after exposure to hormones (cortisol, testosterone, or thyroxine) at various stages of development. "Fertilization" refers to the stage when salmon eggs are water hardened for 1 hour immediately post fertilization. "Eyed" refers to the stage when eye pigments become visible. "Both stages" refer to eggs that were exposed at "Fertilization" and "Eyed". Each column represents a sample size of 1,500 and temperature was at 13°C.

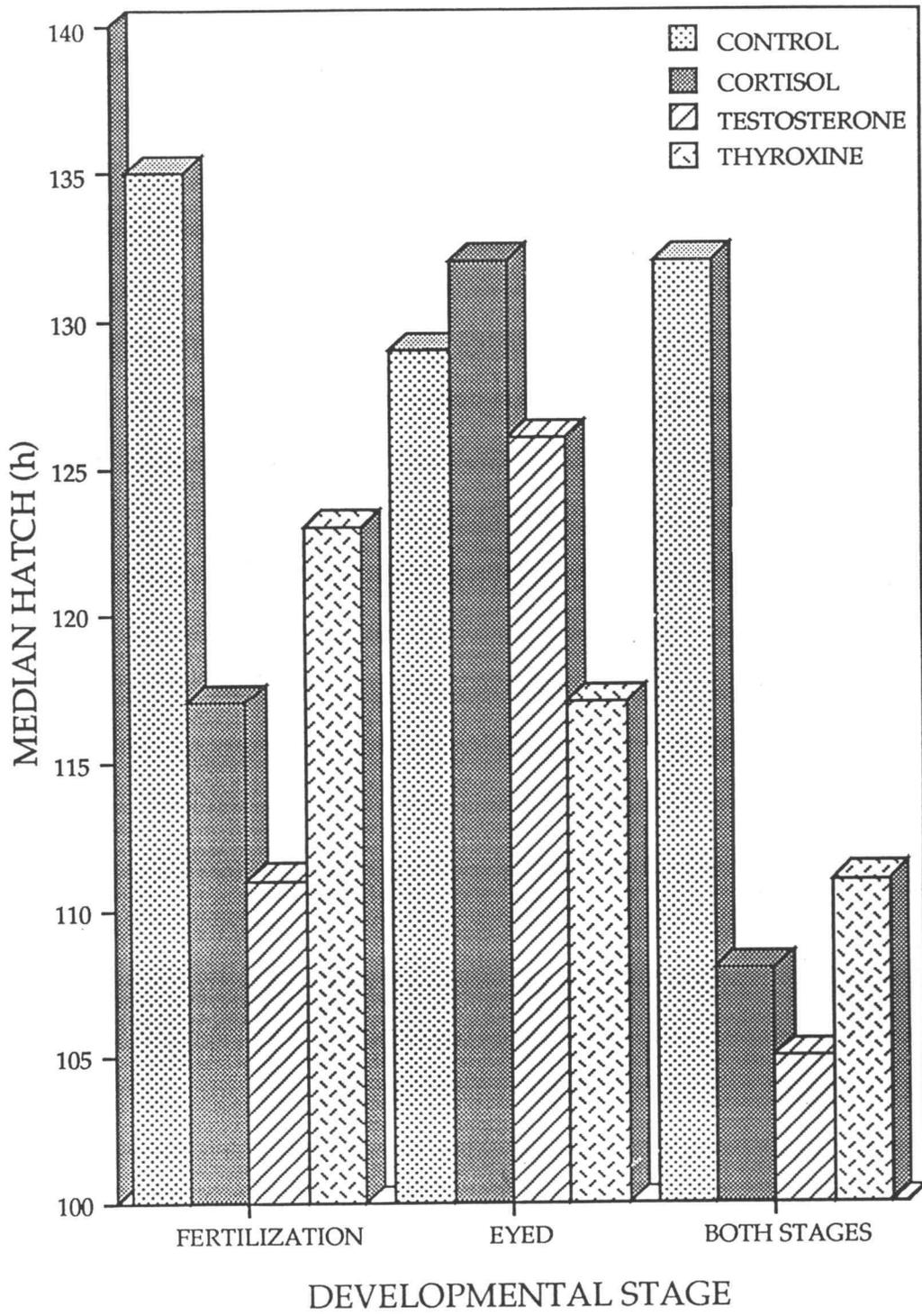


FIGURE 1.

(ANOVA, Tukey Multiple Comparison Test, $p < 0.05$). Other groups that were statistically different in weights were testosterone (water hardening, 0.267 ± 0.009 g) and cortisol (water hardening, 0.248 ± 0.003 g), control (both stages, 0.250 ± 0.005 g) and thyroxine (both stages, 0.270 ± 0.001 g). In 2.5 month old alevins, prior to first feed, a clear pattern emerged in the condition factor (Kn) of the alevins of cortisol groups, especially treated at the eyed and at both at water hardening and eyed stages. These groups were heavier than other treatment groups.

At three months post fertilization, the fry are actively feeding. These fish have been differentially marked. The cortisol groups again were similar to other groups in terms of condition. However, animals immersed at eyed and water hardening in testosterone (1.04 ± 0.01) had a higher Kn than other groups immersed at those stages too, control (1.00 ± 0.01), thyroxine (0.99 ± 0.01), and cortisol (0.99 ± 0.01). Animals immersed in testosterone at both water hardening and eyed stages had different Kn than other testosterone groups immersed at water hardening (0.98 ± 0.01) and at eyed (1.00 ± 0.01).

Kn of five month old chinook immersed in testosterone at water hardening (0.98 ± 0.01) were significantly lower than controls (1.03 ± 0.02), and cortisol (1.03 ± 0.02) but was similar to thyroxine animals also immersed at water hardening. In comparing eyed groups, the thyroxine group (1.07 ± 0.04) has a higher Kn than controls (0.98 ± 0.01) but was similar to testosterone and cortisol groups. At eight months of age, all groups were similar in weight, length and Kn ($p > 0.05$). Cold branding did not appear to adversely affect growth of experimental animals because weights, total lengths, and Kn of replicates of individual treatments that were either branded or not were similar (ANOVA, $p > 0.05$).

APPENDIX 2

Microinjection of Sex Steroids into Newly Hatched Chinook Salmon

INTRODUCTION

This experiment was designed as a preliminary experiment to Chapter 3. The objectives of this experiment were to determine if the microinjection procedure and the introduction of high doses of steroids adversely affected yolksac chinook salmon, *Oncorhynchus tshawytscha*, by inducing high mortalities, influencing sex ratio of injected populations, or inhibiting somatic growth. Further, I determined if animals at this stage of development could clear these steroids after microinjection.

METHODS AND MATERIALS

Chinook salmon gametes were collected from Fall Creek Hatchery, Oregon and were transported to Smith Farm Genetics and Performance Laboratory (SF), Oregon State University (O.S.U.) at a constant temperature of 8°C. The gametes were fertilized and incubated in complete darkness using incubation trays (Heath Tray). Two days post hatch, 400 alevins (100 each in an oxygen supplemented plastic bag) were transported to the Food Technology Laboratory (O.S.U.) at a constant temperature of 12°C. One hundred alevins were microinjected with one substance, estradiol, testosterone, or were sham injected. One hundred alevins were handled similarly injected animals but were not microinjected.

The steroid carrier used consisted of 75% vegetable oil and 25% acetone. One hundred ng of steroid (estradiol or testosterone) in 1 μ l of steroid carrier was microinjected into the alevins and 1 μ l of steroid carrier was injected into sham control alevins. The microinjection procedure consisted of placing 10 alevins at a time into MS222 (2.5 mg/l) for one minute. The alevins were then injected similarly to steelhead trout alevins in Chapter 3. After injection, the alevins were placed in plastic bags supplied with oxygen. Temperature was constant at 12° C. At SF, the alevins were placed in a flow-through system.

To determine if estradiol or testosterone injected animals could clear either of these steroids, these animals were sampled at 1.5, 8, 9, and 18 days post injection and whole body contents of estradiol and testosterone were determined by radioimmunoassay. These samples were collected live and stored at -80° C. Radioimmunoassay protocol for estradiol and testosterone was described in Chapter 2. Mortalities of all these fish were noted from the first day till 229 days post injection. At 229 days post injection, weights, total lengths, and gonadal sex of individual fish were obtained.

RESULTS

Cumulative mortality of alevins 229 days post injection was small. Five, 8, 6, and 10 fish that were control uninjected, sham, testosterone, and estradiol injected died, respectively. A decline in whole body content of estradiol (Fig. 2) or testosterone (Fig. 3) was observed in alevins that were injected with either estradiol or testosterone (n = 10, respectively for each sampling date). Weights and total lengths of different treatment groups were not significantly different (Table 1). There were 33 males and 22

females in the uninjected control group, 16 males and 36 females in the sham injected group, 23 males and 27 females in the estradiol injected group, and 23 male, 31 female survivors in the testosterone injected group.

DISCUSSION

The decrease of steroids post injection indicates that chinook salmon alevin have the ability to metabolize or clear these steroids at a rapid rate. Leaching of estradiol or testosterone is unlikely due to the lipophilic nature of steroids. Another possibility for the declines in estradiol and testosterone is that the animals have the ability to form and excrete steroid conjugates such as steroid glucuronides.

Mortalities in all groups appeared to be independent of treatments indicating that both the microinjection procedure and high concentrations of steroids did not cause differential mortalities. All treatment groups were similar in weight and total length indicating that the microinjection procedure and high concentrations of steroids did not interfere with somatic growth. The percentages of males in individual treatment groups were 60% (control), 30% (sham injected), 46% (estradiol injected), and 42% (testosterone injected).

This experiment demonstrated that microinjecting steroids such as estradiol and testosterone is a feasible technique especially high concentrations of steroids in minute volumes are needed.

Figure 2. Whole body concentrations of estradiol in chinook salmon alevins at specific times after microinjection with estradiol. * denote significant differences analyzed using Nonparametric Tukey-type multiple comparisons, $p < 0.05$.

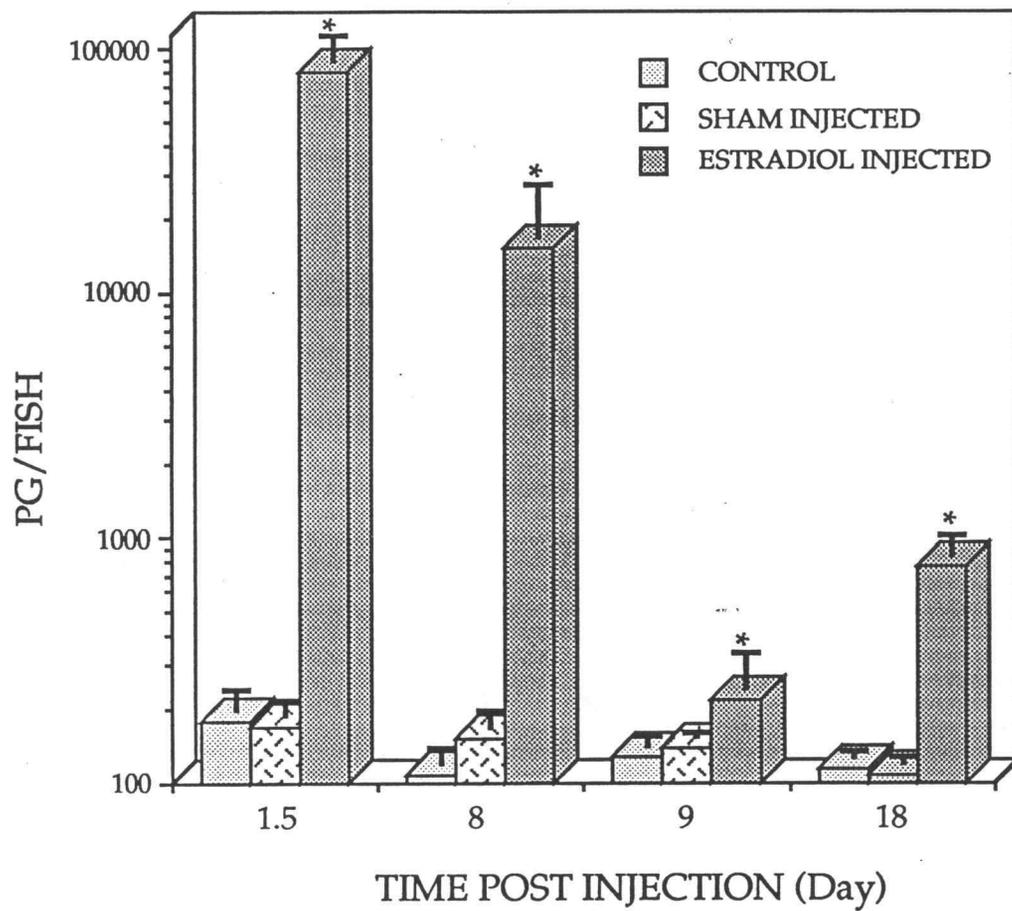


FIGURE 2.

Figure 3. Whole body concentrations of testosterone in chinook salmon alevins at specific times after microinjection with testosterone. * denote significant differences analyzed using Nonparametric Tukey-type multiple comparisons, $p < 0.05$.

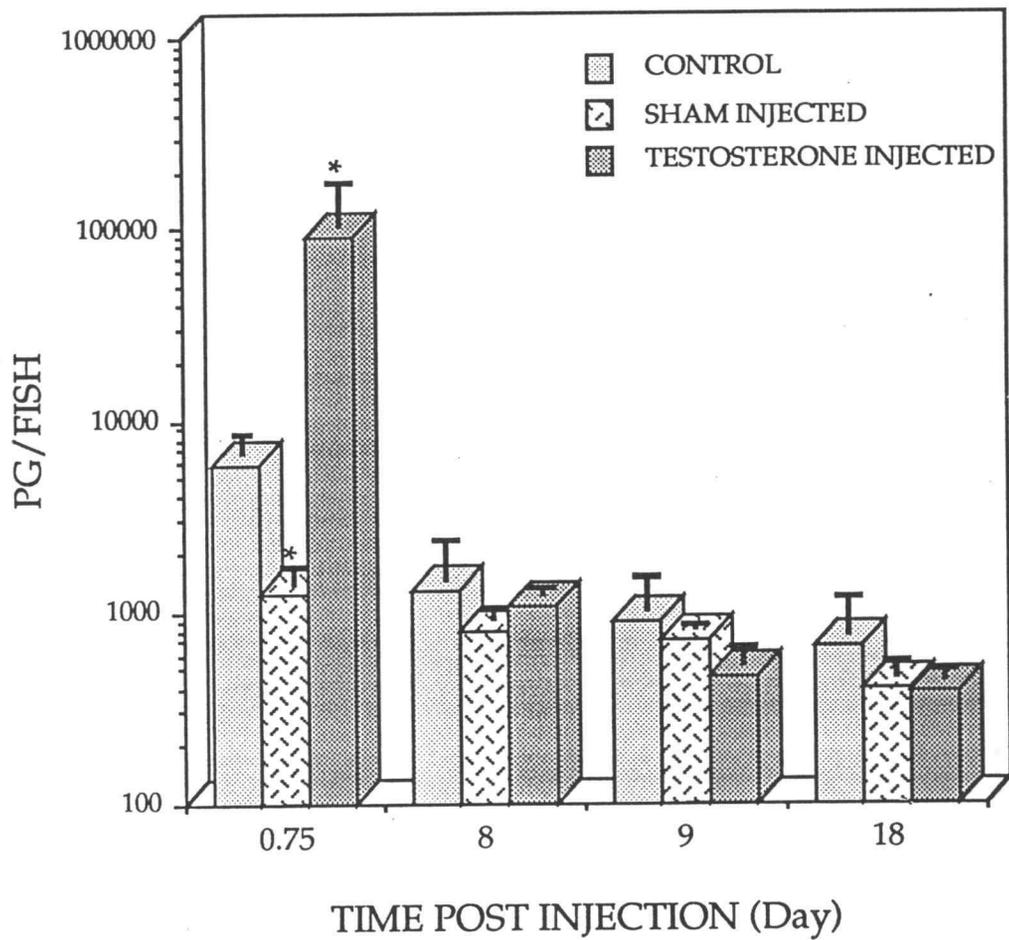


FIGURE 3.

TABLE 1.

Mean weight and total length of fish (\pm SE) 229 days post microinjection of steroid, sham, or uninjected controls.

Treatment	Weight (g)	Total Length (cm)
Uninjected	8.5 ± 0.4	9.6 ± 0.1
Sham	8.4 ± 0.3	9.7 ± 0.1
Estradiol	8.1 ± 0.3	9.6 ± 0.1
Testosterone	8.4 ± 0.3	9.6 ± 0.1

Data analyzed using ANOVA, $p=0.05$

females in the uninjected control group, 16 males and 36 females in the sham injected group, 23 males and 27 females in the estradiol injected group, and 23 male, 31 female survivors in the testosterone injected group.

DISCUSSION

The decrease of steroids post injection indicates that chinook salmon alevin have the ability to metabolize or clear these steroids at a rapid rate. Leaching of estradiol or testosterone is unlikely due to the lipophilic nature of steroids. Another possibility for the declines in estradiol and testosterone is that the animals have the ability to form and excrete steroid conjugates such as steroid glucuronides.

Mortalities in all groups appeared to be independent of treatments indicating that both the microinjection procedure and high concentrations of steroids did not cause differential mortalities. All treatment groups were similar in weight and total length indicating that the microinjection procedure and high concentrations of steroids did not interfere with somatic growth. The percentages of males in individual treatment groups were 60% (control), 30% (sham injected), 46% (estradiol injected), and 42% (testosterone injected).

This experiment demonstrated that microinjecting steroids such as estradiol and testosterone is a feasible technique especially high concentrations of steroids in minute volumes are needed.

APPENDIX 3

INTRODUCTION

The objective of this experiment was to determine if administering high concentrations of hormones into adult females (intramuscularly) 24 h prior spawning would affect early development such as hatching.

METHODS AND MATERIALS

Four females and 2 males from Willamette Hatchery, Oregon were used for this preliminary experiment. The females were anaesthetized in MS222 at 2.5 mg/ml. One female was injected with 0.5 g cortisol, testosterone, thyroxine (all respectively dissolved in 2.4 ml of DMSO), or sham (2.4 ml of DMSO). Half of the hormone or sham solution (1.2 ml) was injected into the muscles located at 3 cm above the right lateral line and 1.5 cm below the dorsal fin. The other half was injected at the same location above the left lateral line. After injection, the females were tagged with a spaghetti tag at the adipose fin for identification purposes and revived in a flow trough circular tank at Smith Farm Genetics and Performance Laboratory, Oregon State University, Corvallis, Oregon. Approximately 24 h post injection of hormones, respective females were stripped of their eggs and were fertilized with sperm pooled from two males. Eggs from individual females were kept separate from each other and incubated in incubation trays (Heath Trays).

RESULTS

Eggs from the cortisol group started hatching first (Fig. 4) A note of caution is that although sample size of each treatment consists of approximately 4000 eggs, the sample size of females injected with identical hormones is 1.

DISCUSSION

Due to the sample size of 1 female per hormone injection, I was concerned that the phenomenon of early hatching of eggs collected from the female injected with cortisol was due to possible genetic differences among females. However, hatchery personnel informed me that eggs fertilized on identical days will begin hatching on the same day regardless of possible maternal genetic differences, assuming that temperature was consistent among groups. The water supply to Smith Farm is constant at 12°C. If genetic differences among adult females do not affect hatching rates of their eggs, cortisol injected into female chinook affects hatching rates of eggs. Although I collected maternal blood and egg samples prior and post injection of respective hormones, I did not have the opportunity to determine the amount of hormones remaining in maternal blood or in the eggs 24 h post injection of these hormones.

Figure 4. Cumulative % hatch of eggs from females that were injected with either cortisol, testosterone, thyroxine, or vehicle (DMSO) through time. When the first hatched fish were detected (in cortisol group), t=0 was designated and 100% hatch represents approximately 4000 eggs.

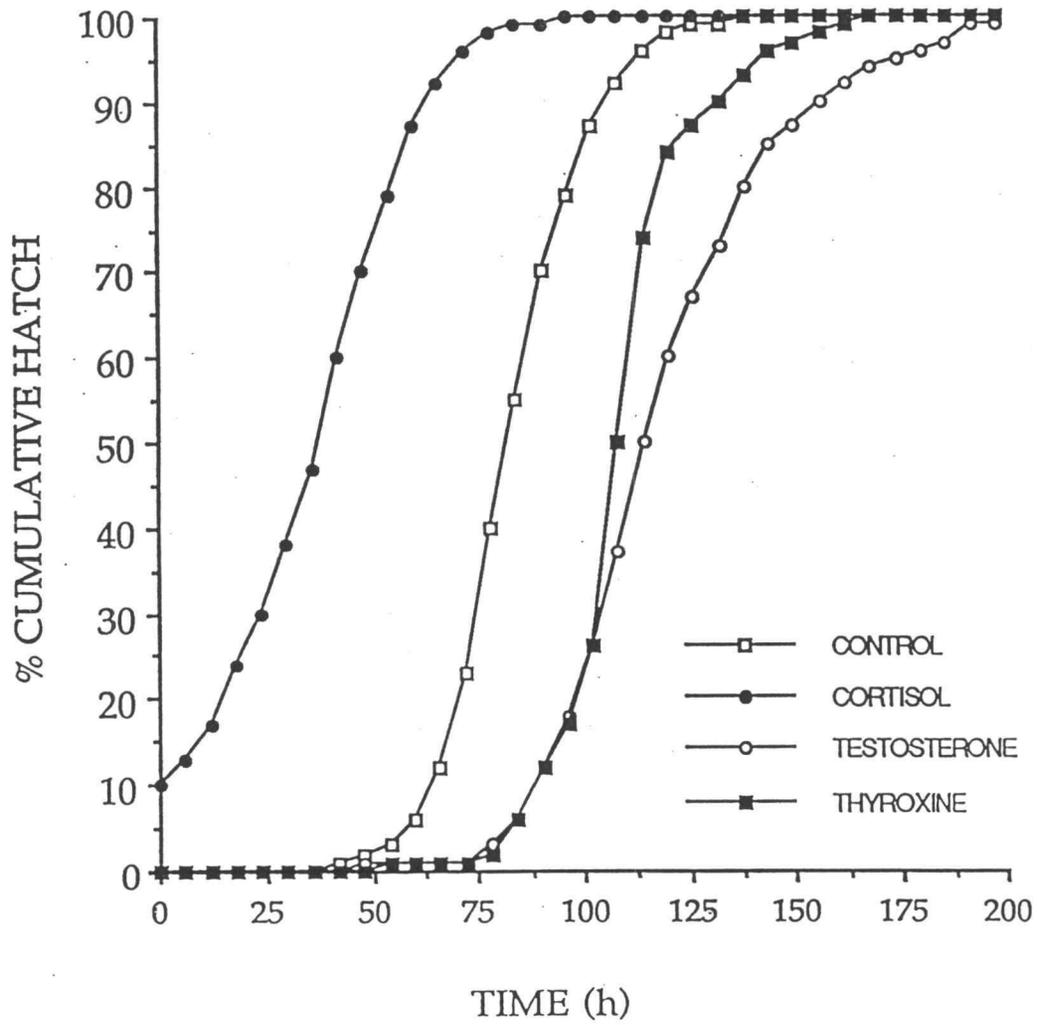


FIGURE 4.