AN ABSTRACT OF THE THESIS OF

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Title: GROWTH AND PRECOCIOUS DEVELOPMENT OF STEELHEAD TROUT: EFFECTS OF HORMONAL COMPOUNDS

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Steroids incorporated into Oregon Moist Pellets were fed to steelhead trout (Salmo gairdneri) to determine if growth could be enhanced. An antiandrogen was administered per os in an attempt to prevent early male sexual development. The 1976 brood of Rogue River steelhead trout was treated with 17α-methyltestosterone (MT), diethylstilbesterol (DES), diethylstilbesterol plus clomiphene citrate (CC), 17α-methyltestosterone plus flutamide (FL, the antiandrogen), or flutamide (4'-Nitro-3' Trifluoromethylisobutyranilide) at the onset of feeding. The 1977 brood was started on the treated diets one month after the onset of exogenous feeding and received testosterone proprionate (TP), testolactone (TL), methylandrostenolone (MA), 17α-methyltestosterone, flutamide, estradiol (E_2), or estradiol plus progesterone (Prog).

Fish treated with FL or DES plus CC from the onset of feeding showed an increase in weight compared to controls, although lack of replication makes this conclusion very tentative. MT (1, 5, 15, or 35 μg/g feed), E_2, E_2 plus Prog, or FL (1977) decreased the growth rate compared to controls. MT (2 μg/g), MT plus FL, TP, TL, or MA failed

to induce any change of weight.

All compounds (except flutamide) fed to the fish from the onset of feeding caused abnormal sex ratios. Androgens induced more males while estrogens induced more females.

MT, MT plus FL, TL, MA, or FL (1977) advanced spermatogenesis. MT also caused a significant increase in epidermis thickness. MT (2 or 35 µg/g), TL, MA, or FL caused hypertrophied granulosa cells or atretic oocytes in some ovaries. Intersexes containing both testicular and ovarian tissue in the gonads were found in some fish treated with MT (5 µg/g), MT plus DES, MT plus FL, or DES plus CC.

Comparisons of RNA/DNA ratios failed to indicate the usefulness of this technique for determining which test compounds were anabolic or which fish had enhanced growth.

MT (1 µg/g)-treated fish that had matured precociously had an increased amount of androgen (1.92 ng/ml) as compared to the immature control fish (0.10 ng/ml). Fish treated with MT (5 µg/g) and MT plus FL had mean androgen levels of 0.26 ng/ml and 0.04 ng/ml, respectively. Androgens of fish treated with MT (15 or 35 µg/g), MT plus DES, DES plus CC, or FL averaged between 0.04-0.05 ng/ml in immature individuals.

Growth and Precocious Development of Steelhead Trout: Effects of Hormonal Compounds

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INTRODUCTION

Culture of anadromous salmonids could be facilitated if the time needed for rearing up to size at release in cold water hatcheries could be shortened. Hatchery efficiency could also be increased by preventing early maturity, thus reducing the number of males which mature precociously and hence may not contribute to the fishery. Rogue River steelhead trout (Salmo gairdneri) stocks at Cole Rivers Hatchery, Oregon, have both of the above undesirable characteristics.

Genetic and environmental factors, nutrition, and hormones all influence the growth process. Growth in terms of linear change in body weight is enhanced by the pituitary hormones, somatotropin and thyrotropin, and by the steriod hormones, androgens, estrogens, and corticosteroids. In salmonids growth has been enhanced in rainbow trout (S. gairdneri) by treatment with 4-chlorotestosterone acetate (Hirose and Hibiya 1968), norethandrolone and dimethazine (Matty 1975, Cheema and Matty 1977), and ethylestrenol (Simpson 1976); in coho salmon (Oncorhynchus kisutch) by treatment with 17α-methyltestosterone, 11-ketotestosterone (Fagerlund and McBride 1975, McBride and Fagerlund 1976), bovine growth hormone and L-thyroxine (Higgs et al. 1976) and insulin (Ludwig et al. 1977); and in pink salmon (O. gorbuscha) by treatment with 17α-methyltestosterone (Fagerlund and McBride 1977). Treatment of steelhead trout has resulted in variable growth-responses, but there is some indication that 17α-methyltestosterone may promote growth at least during certain stages of development (Fagerlund and McBride 1977).

Exact effects of the hormones on growth are not fully known. It has been assumed that the anabolic action of androgenic and to a lesser extent the estrogenic hormones increases nitrogen retention in the form of proteinaceous tissue. Nitrogen retention is indicative of an increase in the rate of protein biosynthesis (concomitant with an increase in RNA concentration) and decrease in the rate of protein catabolism which results in a progressive weight gain (Kochakian 1976). This increase in protein synthesis may be reflected by the RNA-P/DNA-P ratio. Basically the RNA-DNA ratio is an index of the growth per cell (Sable 1974). This ratio could be correlated with the anabolic effect of the steriods and also could be indicative of short term growth.

Anabolic steroids may have limited usefulness if androgenic properties are expressed. Both high doses and prolonged administration of methyltestosterone in salmon have induced structural changes in the testes (McBride and Fagerlund 1973, 1976) and discoloration and thickening of the epidermis (Yamazaki 1972). Administration of a synthetic antiandrogen with an anabolic steroid may block the androgenic effects without affecting growth. Schreck (1973) showed that the antiandrogen cyproterone acetate may inhibit androgen uptake in testes of rainbow trout. Precocious male sexual development may be a consequence of early stimulation by sex hormones. Antiandrogens could potentially be used to block this male sexual development.

The main objectives of this study are to test the possibilities that anabolic steroids can promote growth of steelhead trout, that androgens cause precocious male sexual development, and that early sexual development can be prevented by the use of antihormones. Tests

were conducted on two brood years of steelhead trout to screen potentially useful compounds and concentrations.

METHODS AND MATERIALS

1976 Brood

Summer steelhead trout from the Oregon Department of Fish and Wildlife's Cole Rivers Hatchery were transferred as eyed eggs to Oregon State University's Smith Farm facility, Corvallis, Oregon, in April 1976. Three hundred fry were randomly placed in each of 12 340 liter circular fiberglass tanks in May 1976, and maintained at 11°C well water under natural photoperiod. The fish were fed Oregon Moist Pellets (OMP) (Bioproducts, Inc., Warrenton, Oregon) several times daily at 3.3% to 6.8% of body weight, depending on weight of fish according to recommended feeding level. The treated diets were prepared by adding the test compounds to the oil fraction of the OMP before mixing with the other ingredients; the pellets were made at Oregon State University Seafoods Laboratory in Astoria. The steelhead trout were started on the treated diets immediately after buttoning-up (yolk sac completely absorbed). Only the control group was replicated. A separate group of fish fed the control diet was used for determination of androgen levels over time. Treatments were as follows:

GROUP	TREATMENT	CODE Treatment (µg/g of feed)
1	Control	—
2	Control	—
3	17α-methyltestosterone	MT (1)
4	17α-methyltestosterone	MT (5)
5	17α-methyltestosterone	MT (15)
6	17α-methyltestosterone	MT (35)
7	17α-methyltestosterone:Diethyltestosterone	MT:DES (5:5)
8	Diethylstilbesterol	DES (10)
9	Diethylstilbesterol:Clomiphene citrate	DES:CC (5:15)

cont.

10	17α-methyltestosterone:Flutamide	MT:FL (5:20)
11	Flutamide (4'-Nitro-3'Trifluoromethyl-isobutyranilide), Schering Corporation (SCH 13521), Bloomfield, N.J.	FL (20)

The fish were fed treated diets for 220 days, after which they were fed untreated control feed for 71 days. Lengths and weights of 50 individual fish from each treatment were recorded at 34, 62, 105, 156, 203, and 291 days after start of treatment. Condition factors $f = \frac{\text{Weight (g)}}{\text{Length (cm)}^{3.25}} \times 1000$ were calculated.

Gonads, liver, and skin of five fish from each treatment were preserved in Bouin's solution and imbedded in parablast for histological examination every other month. Sections (10 μ) were stained with Harris's hematoxylin and eosin. The thickness of the epidermis was measured, stages of gametogenesis were evaluated, and liver structure was noted.

Six fish from each treatment were frozen at 156 and 220 days after onset of treatment for determination of RNA-P/DNA-P ratios. Muscle and bone tissue of these fish were dehydrated and defatted in a chloroform-methanol solution (2:1 for four hours) followed by ether (100% for four hours). Both extractions were done in a Goldfisch Extraction Apparatus. The tissues were pulverized with a morter and pestal and stored in capped bottles. DNA and RNA were extracted together from the ground tissue with hot trichloroacetic acid as described by Sable (1974). RNA-P content was determined using the orcinol method (Schneider 1957) and DNA-P content was determined using the Burton (1956) and Bulow (1970) modification of the diphenylamine reaction. Analysis of nucleic acid content (μg phosphate per 100 μg dry fat-free tissue) of samples

was done colormetrically using a Beckman DB-G grating spectrophotometer.

In order to evaluate the effects of the hormones on sex determination, 80 fish from each treatment were sexed at the end of the test period by using an aceto-carmine stained gonadal squash.

To determine possible differences in plasma androgens in fish that were immature or precocious, blood from ten fish from each treatment at day 206 was obtained by severing the caudal peduncle. Plasma was stored frozen until analysis. Only male plasma samples were assayed.

The radioimmunoassay (RIA) for androgens was characterized for steelhead trout plasma. The androgen assay was slightly modified from a protocol from personal communication of Vernon Gay (Department of Physiology, University of Pittsburgh, Pennsylvania). Fifty to 100 µl plasma were extracted twice with hexane:benzene (2:1) after 1000 dpm of ^{3}H-testosterone in phosphate buffered saline-gelatin (PG) (used to estimate extraction recovery) had been incubated with the plasma. The aqueous phase was frozen in liquid N_2 and the extracts decanted and combined. Extracts were dried under air and then dissolved in 0.6 ml of PG. One-tenth ml PG-extract solution was counted for recovery. One-tenth ml of antiserum-Niswender's antitestosterone-11-BSA (S-250) of a 1:40,000 dilution in PG, 0.1 ml of ^{3}H-testosterone (10,000 dpm in PG) were added to each tube, and to the standards which ranged from 7.8 to 500 pg of testosterone. Samples were incubated for 90 minutes, placed in an ice bath for 15 minutes with 1.0 ml of charcoal-dextran mixture added to each tube. The samples were centrifuged, decanted into Insta-gel (Packard) scintillation fluid and counted in a Packard liquid scintillation spectrophotometer. Extraction efficiencies ranged between

76.5% and 100.0%. The lower limit of detection of the assay is about 4 pg. The antibody employed binds both 11-ketotestosterone (an important fish androgen) and testosterone. 11-Ketotestosterone binds with a higher affinity than testosterone. 17α-Methyltestosterone has a cross reactivity to the antibody of 12.0% compared to testosterone.

To determine the sex hormone content of control OMP, androgens and estrogens were assayed. Twelve samples each of 5 g OMP were extracted with ether (100% for four hours) in a Goldfisch Extraction Apparatus. One sample contained 10,000 dpm ^{3}H-testosterone (to determine extraction efficiency) and one sample contained 1.0 ng of unlabeled testosterone (to evaluate effectiveness of RIA). The extraction efficiencies for androgens averaged 91.3%. Androgens (average 0.8 ng/g of feed) were detected using the above RIA procedure in the OMP, but this value is probably inaccurate because the unlabeled testosterone was not reflected in the values obtained, indicating possible interference from the other oils. Due to the possible interference of the oils, androgens were extracted from the ether extracts with chloroform (100%), hexane:benzene (1:1), and separated from the estrogens by NaOH (0.4 N), with water washes between extractions. Internal standards of ^{3}H-testosterone and unlabeled testosterone were not detected after being extracted and assayed.

To determine the estrogen content of OMP, the estrogen RIA was characterized. The RIA for estrogen was modified from Korenman's (1974) procedure. Twelve samples each of 5 g OMP were extracted with ether (100% for four hours) in the Goldfisch Extraction Apparatus. One sample contained 10,000 dpm ^{3}H-estradiol (to determine extraction

efficiency) and one sample contained 2.0 ng of unlabeled estradiol (to evaluate the RIA). Ten µl of extracted feed samples in ETOH were dried and 0.2 ml of PG buffer (NaH_2PO_4, Na_2HPO_4, NaCl, thimerosal) were added to each sample and mixed, 0.1 ml of δ-globulin; 0.025 ml of diluted Niswender antiserum (S-244) of a 1:8500 dilution in PG, and 0.1 ml of ^3H-estradiol (10,000 dpm in PG) were added to each tube and to the standards which ranged from 12.5 to 200 pg of estradiol-17β. Samples were incubated for 60 minutes, placed in an ice bath for 15 minutes with 0.5 ml of charcoal suspension added to each tube. The samples were centrifuged, decanted into Insta-gel scintillation fluid and counted in a Packard liquid scintillation spectrophotometer. This assay is highly specific for 17β-E_2. No cross reactivity was found with 16-keto- or 17β-estradiol. The extraction efficiencies averaged 86.6%. Estrogens and the internal standard (unlabeled E_2) were not detected after being assayed. The estrogens separated from the androgens after the ether, chloroform, hexane:benzene, and NaOH extractions were then assayed. The internal standard of estrogen added to the OMP showed a 8.35% recovery after being assayed. Samples without an internal standard did not reflect any estrogens; this could be due to the low recovery from the extractions.

One way analysis of variance (ANOVA) was used to analyze treated fish compared to control fish of the weight data, condition factors, RNA-DNA ratios, and skin thickness samples. Least Significant Difference (LSD) was used in cases found to be significant. Chi-square analysis was used to analyze sex ratio data of the treatment groups (observed) and the control group (expected).

1977 Brood

Summer steelhead trout (Salmo gairdneri) from Cole Rivers Hatchery were transferred as one month old fry to Smith Farm facility on 3 July 1977. Two hundred and fifty fry were randomly placed in each of 12 340 liter fiberglass tanks and started on the treated diets on 18 July 1977, one month after exogenous feeding. On 2 December 1977, numbers of fish in each tank were reduced to 150 fish each to standardize densities to account for mortality that had taken place. The fish were fed OMP several times daily for the first two months of the test period and then fed once a day to satiation for the duration of the test period. The method of adding compounds to OMP and the conditions of well water and photoperiod were the same as for the 1976 brood. Treatments were as follows:

GROUP	TREATMENT	CODE Treatment (µg/g of feed)
1	Control	—
2	Control	—
3	Testosterone proprionate	TP (5)
4	Testosterone proprionate	TP (5)
5	Testolactone	TL (10)
6	Methylandrostenolone	MA (10)
7	17α-methyltestosterone	MT (2)
8	Flutamide	FL (20)
9	Flutamide	FL (20)
10	Estradiol	E_2 (5)
11	Estradiol:Progesterone	E_2:Prog (5:5)
12	Estradiol:Progesterone	E_2:Prog (5:5)

The fish were fed the treated diets for 175 days, after which they were fed control feed for 78 days. Group weights were recorded at 25 and 67 days after onset of treatment, and lengths and weights of 50

individual fish from each treatment were recorded at 137, 175, and 253 days.

Gonads and skin of five fish from each treatment were preserved in Bouin's solution on days 175 and 253 and inbedded in parablast for histological examination.

ANOVA was used to analyze the weight data and skin thickness samples. LSD test was used in cases found to be significant. The fish from this study are still being raised to check sex ratios and sexual precocious development.

RESULTS

1976 Brood

Steelhead trout treated with FL (20) weighed 23.3% more than control fish ($\alpha = 0.01$) at the end of 203 days and maintained a significant difference until the end of the test period (Figure 1). Since the mean weights of the two control groups did not differ, data for the two control groups were pooled for the comparisons with treated fish. All of the MT-treated fish (1, 5, 15, or 35) had considerably lower growth rates ($\alpha = 0.01$) compared to the weights of the control fish at the end of the study (Figures 2, 3). Fish treated with DES:CC (5:15) weighed 15.0% more than the controls ($\alpha = 0.01$) at day 291 (Figure 4). However, there were 50% fewer fish in the DES:CC group during the last 120 days of the treatment period because of a disease outbreak in this tank. Fish treated with DES (10) were deleted from the study because they had severe mortality due to disease. Mortality in all other treatments did not exceed five fish per group during the test period. MT:FL and MT:DES groups did not have any significant differences in weight compared to the control. Condition factors of the fish were not affected ($\alpha = 0.01$) by the treated diets (Table 1).

RNA-P/DNA-P ratios from treated fish sampled on days 156 and 220 were not different ($\alpha = 0.05$) from the control RNA-P/DNA-P ratio on each of those days (Figure 5). Mean RNA-P and mean DNA-P content are shown in Table 2.

The testes of the control fish sampled on day 203 consisted of small, defined tubules containing spermatogonia, and ovaries had oogonia

Figure 1. Mean weights of steelhead trout treated with methyltestosterone plus flutamide (MT plus FL) or flutamide (FL) at 5:20 or 20 µg/g of feed, respectively. FL (20 µg/g)-treated fish showed a significant difference in weight compared to the control at $\alpha = 0.01$ in March.

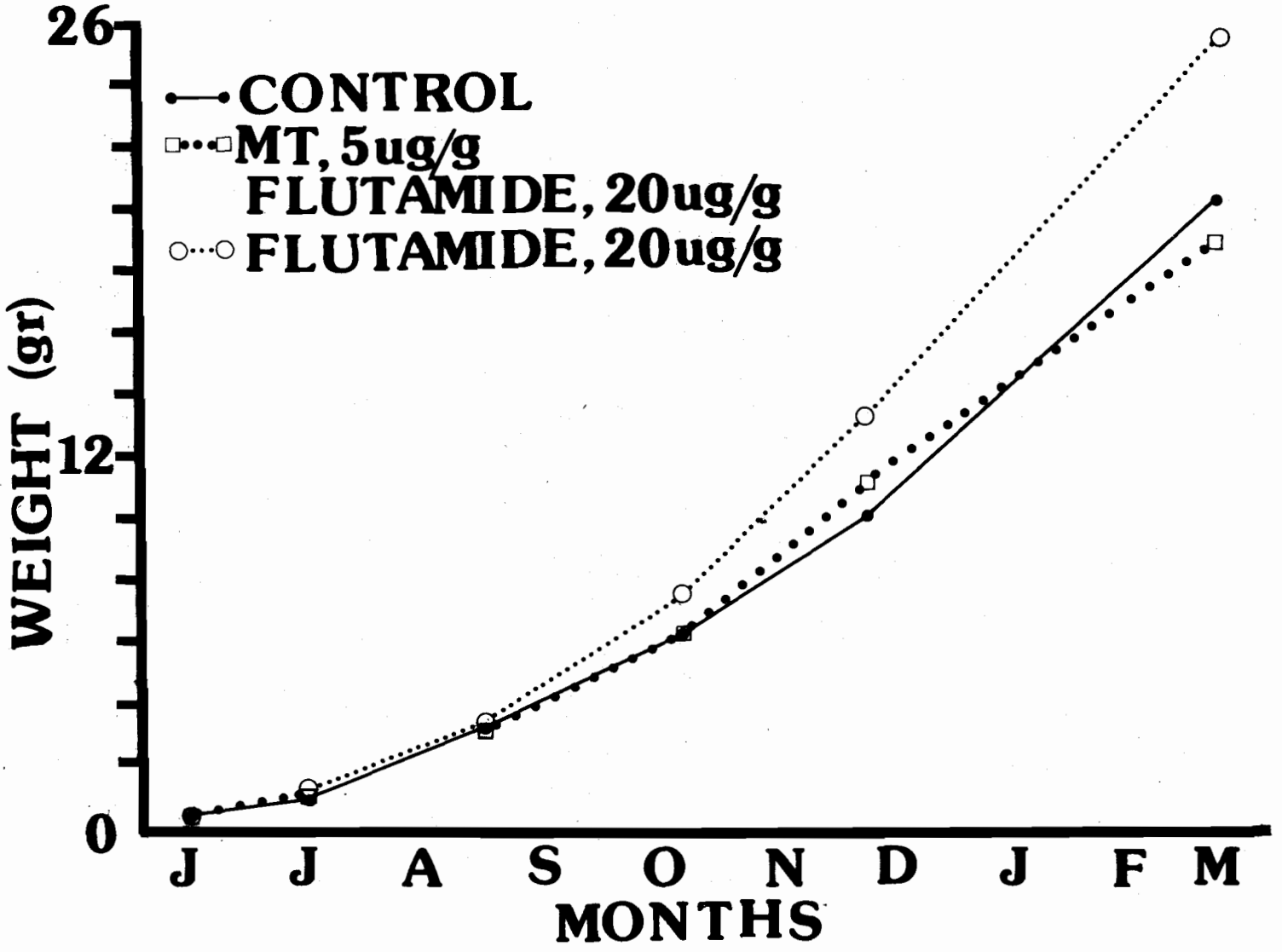
26
12
0
WEIGHT (gr)
CONTROL
MT, 5ug/g
FLUTAMIDE, 20ug/g
FLUTAMIDE, 20ug/g
J
J
A
S
O
N
D
J
F
M
MONTHS

Figure 2. Mean weights of steelhead trout treated with methyltestosterone (MT) at 5 or 15 µg/g of feed. MT (5 or 15 µg/g)-treated fish showed significant differences in weight compared to the control at $\alpha = 0.01$ in March.

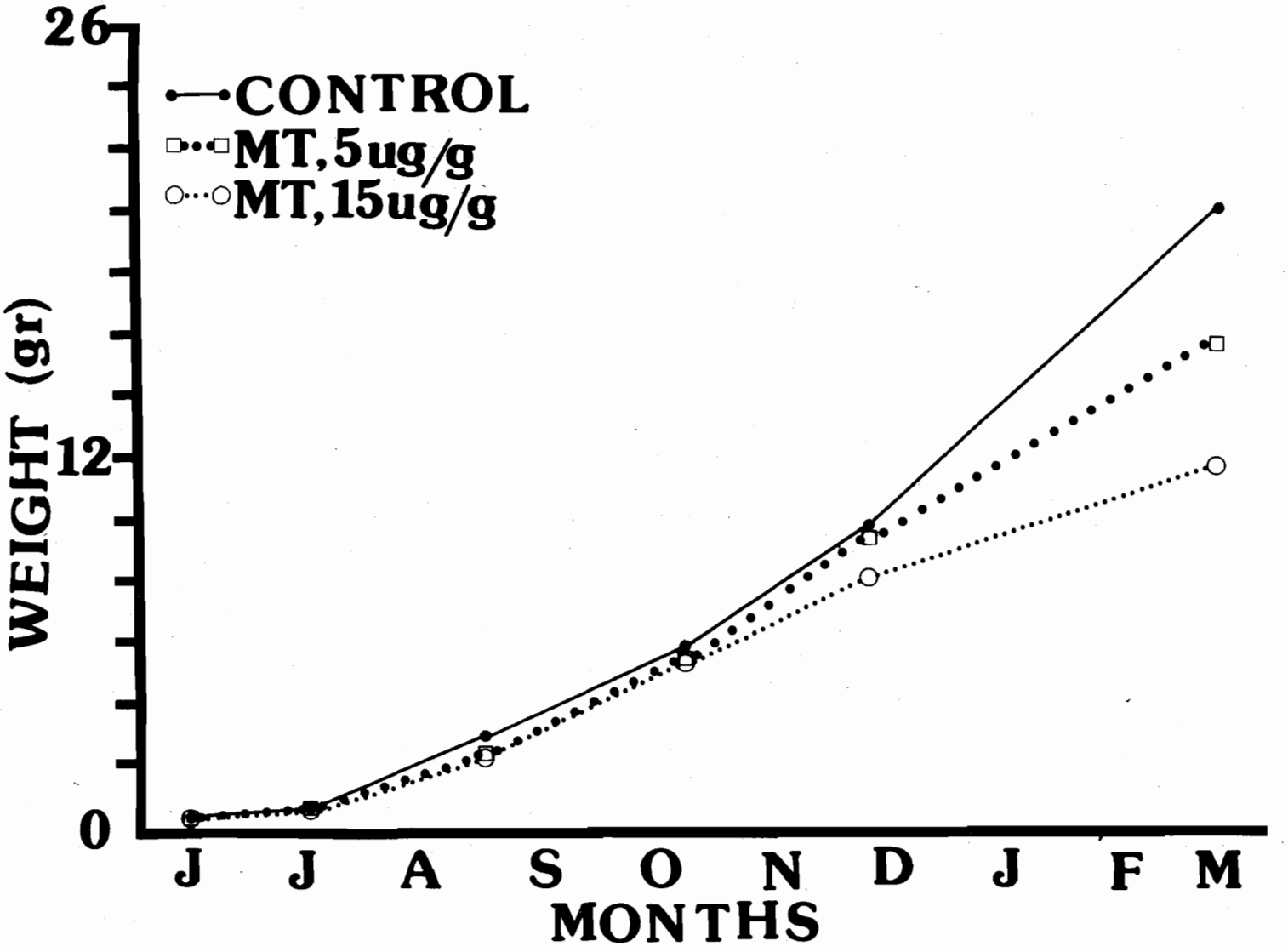
26
12
0
CONTROL
MT, 5ug/g
MT, 15ug/g
WEIGHT (gr)
J J A S O N D J F M
MONTHS

Figure 3. Mean weights of steelhead trout treated with methyltestosterone (MT) at 1 or 35 µg/g of feed. MT (1 or 35 µg/g)-treated fish showed a significant difference in weight at $\alpha = 0.01$ in March.

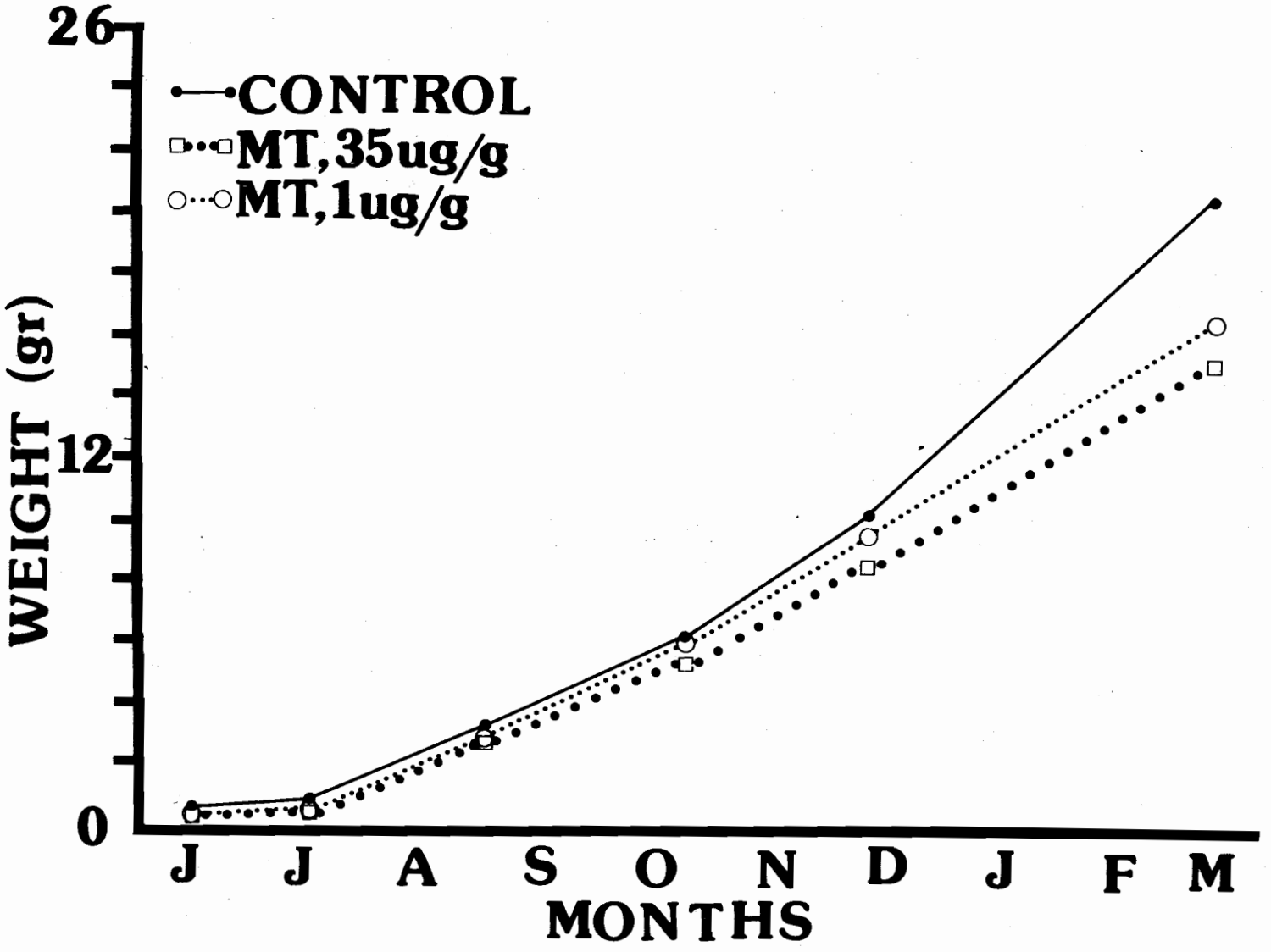
26
12
0
CONTROL
MT,35ug/g
MT,1ug/g
WEIGHT (gr)
J J A S O N D J F M
MONTHS

Figure 4. Mean weights of steelhead trout treated with methyltestosterone plus diethylstilbesterol (MT plus DES) or diethylstilbesterol plus clomiphene citrate (DES:CC). DES plus CC-treated fish showed a significant difference in weight at $\alpha = 0.01$ in March.

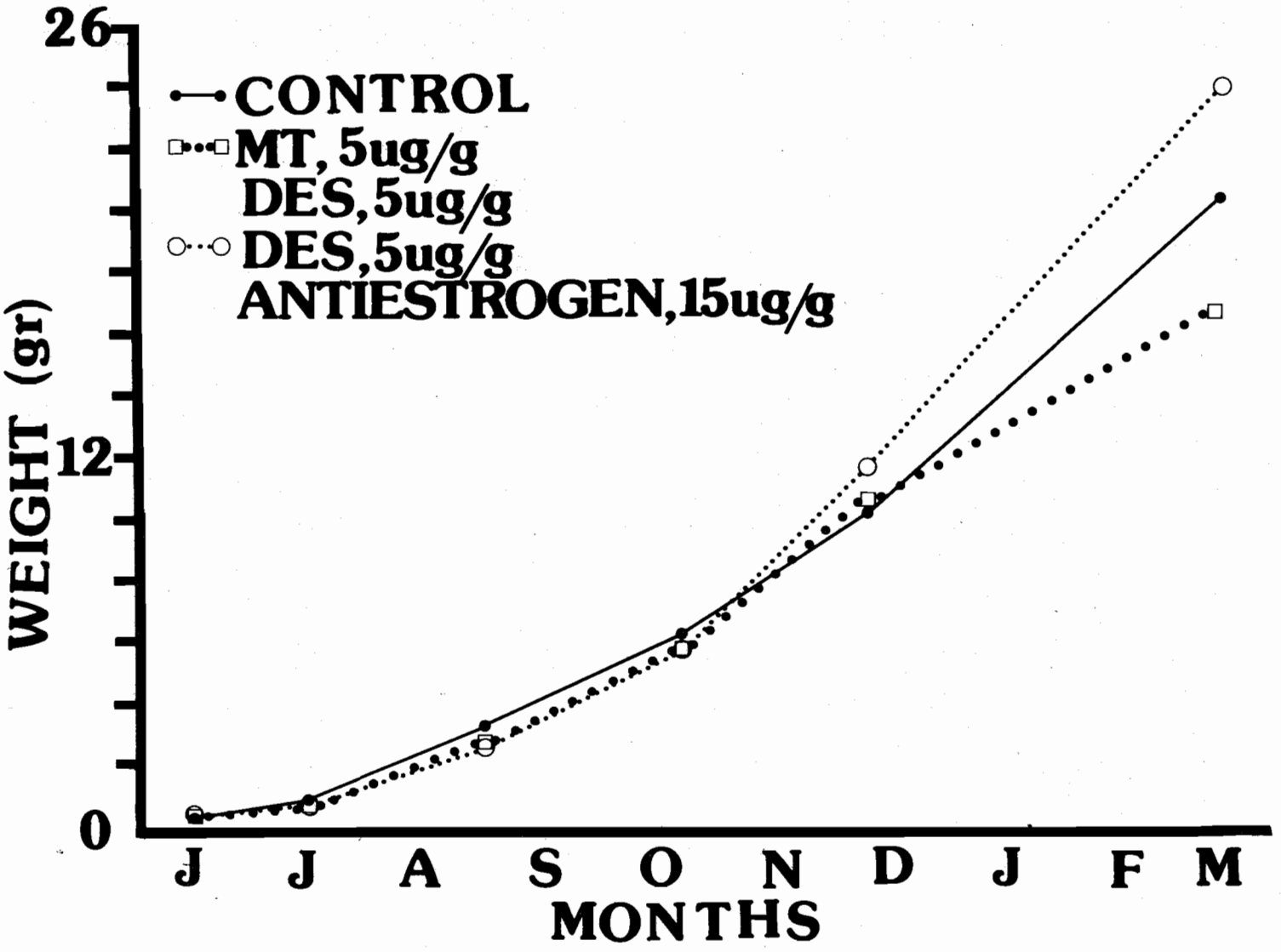
26
12
0
WEIGHT (gr)
CONTROL
MT, 5ug/g
DES, 5ug/g
DES, 5ug/g
ANTIESTROGEN, 15ug/g
J J A S O N D J F M
MONTHS

Table 1. Mean condition factors (± S.E.) of steelhead trout treated with methyltestosterone (MT), methyltestosterone plus diethylstilbesterol (MT plus DES), diethylstilbesterol (DES), diethylstilbesterol plus clomiphene citrate (DES plus CC), methyltestosterone plus flutamide (MT plus FL), or flutamide (FL). No significant differences ($\alpha = 0.01$) were noted among treated fish compared to control fish.

Treatment (µg/g of feed)	Condition factor: Day after onset of treatment					
	34	62	106	156	203	291
Control	0.84 ± 0.02	0.93 ± 0.02	1.07 ± 0.01	1.10 ± 0.02	1.11 ± 0.01	1.06 ± 0.01
MT (1)	0.86 ± 0.01	0.91 ± 0.05	1.06 ± 0.01	1.13 ± 0.03	1.07 ± 0.01	1.07 ± 0.02
MT (5)	0.89 ± 0.02	1.00 ± 0.02	1.09 ± 0.01	1.12 ± 0.01	1.10 ± 0.01	1.06 ± 0.01
MT (15)	0.93 ± 0.01	0.99 ± 0.01	1.10 ± 0.01	1.13 ± 0.02	1.14 ± 0.01	1.09 ± 0.01
MT (35)	0.89 ± 0.04	0.99 ± 0.03	1.04 ± 0.01	1.08 ± 0.03	1.13 ± 0.04	1.05 ± 0.02
MT:DES (5:5)	1.04 ± 0.06	1.04 ± 0.04	1.08 ± 0.01	1.13 ± 0.01	1.14 ± 0.01	1.08 ± 0.01
DES (10)	0.94 ± 0.01	1.01 ± 0.03	1.09 ± 0.01	-	-	-
DES:CC (5:15)	0.94 ± 0.03	0.96 ± 0.01	1.05 ± 0.01	1.10 ± 0.01	1.07 ± 0.01	1.10 ± 0.02
MT:FL (5:20)	0.96 ± 0.02	1.02 ± 0.02	1.05 ± 0.01	1.05 ± 0.02	1.13 ± 0.01	1.08 ± 0.01
FL (20)	0.89 ± 0.02	1.02 ± 0.07	1.03 ± 0.01	1.06 ± 0.01	1.07 ± 0.01	1.03 ± 0.01

Figure 5. Mean RNA/DNA ratios (µg phosphate per 100 µg dry fat-free tissue) and 95% confidence limits of muscle and bone tissue of steelhead trout treated with methyltestosterone (MT), methyltestosterone plus diethylstilbesterol (MT plus DES), diethylstilbesterol plus clomiphene citrate (DES plus CC), methyltestosterone plus flutamide (MT plus FL), or flutamide (FL). Dosage (µg/g feed) given under each compound. No significant differences noted among treated fish compared to controls in October or January at α = 0.05.

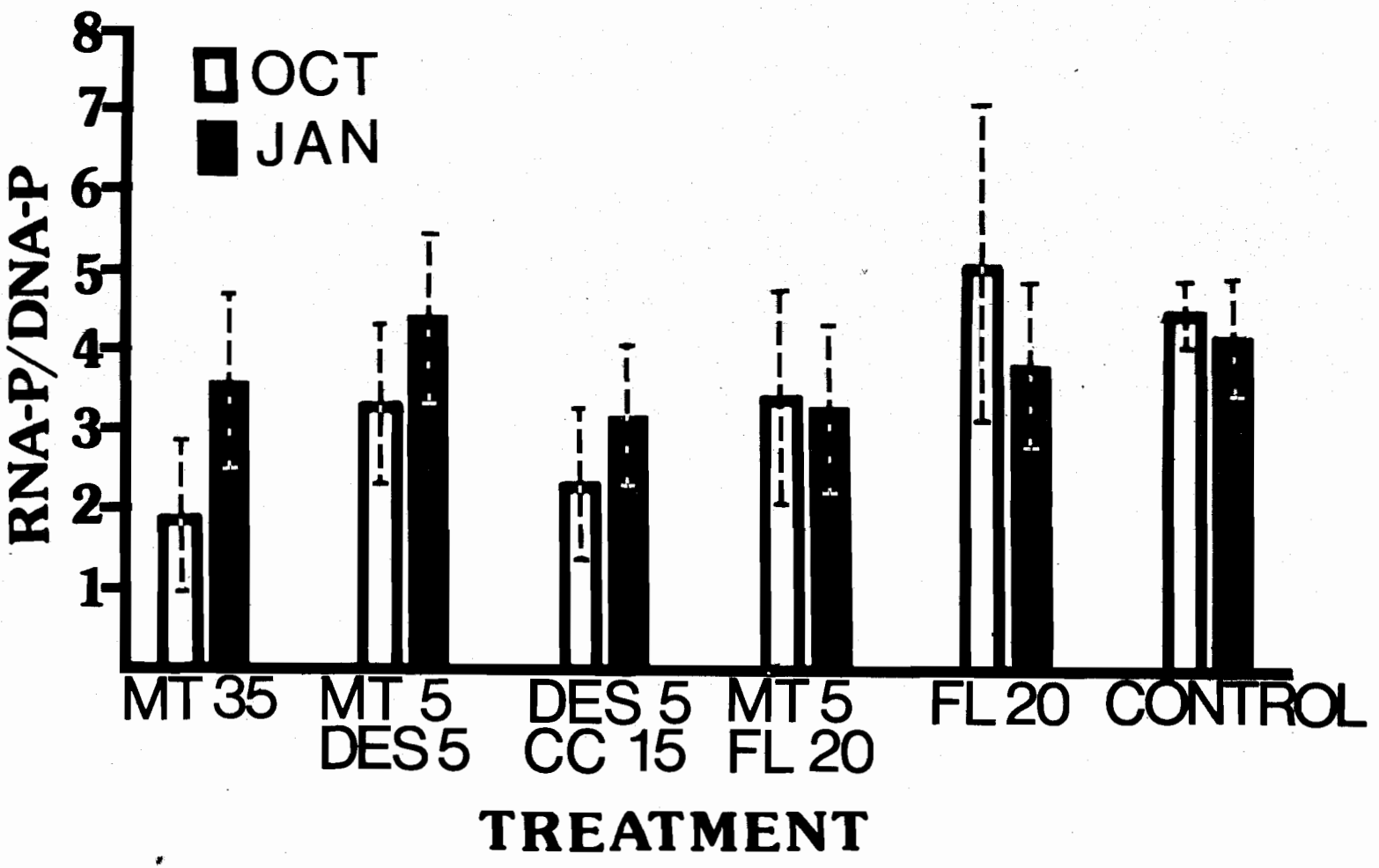
OCT
JAN
RNA-P/DNA-P
8
7
6
5
4
3
2
1
MT 35
MT 5
DES 5
DES 5
CC 15
MT 5
FL 20
FL 20
CONTROL
TREATMENT

Table 2. Mean RNA-P and mean DNA-P content (µg phosphate per 100 µg dry fat-free tissue) (± S.E.) of muscle and bone tissue from steelhead trout treated with methyltestosterone (MT), methyltestosterone plus diethylstilbesterol (MT plus DES), diethylstilbesterol plus clomiphene (DES plus CC), methyltestosterone plus flutamide (MT plus FL), or flutamide (FL). No significant differences noted among treated fish compared to control fish at day 156 or 220 at $\alpha = 0.05$.

Treatment (µg/g of feed)	RNA-P		DNA-P	
	Day after onset of treatment			
	156	220	156	220
Control	107.90 ± 07.02	128.30 ± 05.03	24.20 ± 01.05	31.10 ± 1.13
MT (1)	96.00 ± 07.02		40.80 ± 04.98	
MT (5)	133.00 ± 14.44		31.80 ± 02.30	
MT (15)	166.50 ± 20.61		93.25 ± 20.08	
MT (35)	89.83 ± 03.28	116.10 ± 26.66	33.58 ± 03.31	40.40 ± 3.18
MT:DES (5:5)	97.62 ± 03.65	155.50 ± 10.74	30.12 ± 02.21	35.40 ± 1.69
DES:CC (5:15)	124.20 ± 08.59	118.00 ± 05.00	60.00 ± 08.77	38.70 ± 3.65
MT:FL (5:20)	103.91 ± 05.65	122.30 ± 07.07	36.00 ± 06.27	37.68 ± 2.67
FL (20)	132.41 ± 14.59	121.10 ± 07.97	27.00 ± 01.55	32.44 ± 2.81

and early stages of oocyte I according to classification of Beams and Kessel (1973). All of the steroids except FL affected gonadal histology. Intersexes containing both testicular and ovarian tissue in the gonads were found in some fish treated with MT (5), MT:DES (5:5), MT:DES (5:20), and DES:CC (5:15). Advanced stages of spermatogenesis were present in fish treated with MT (1), MT (5), MT (15), MT (35), and MT:FL (5:20). MT (5), MT (15), and MT (35) induced degeneration of some gonads (no germ cells present or hypertrophy of the germinal epithilium without differentiation). The few affected ovaries of fish treated with MT (35) were degenerate. Fish treated with the highest level (35 μg/g) of MT showed extreme hyperplasia or poorly developed gonads. These alterations in the gonads apparently did not change back to normal after the fish had been on control feed for 71 days.

Male fish treated with MT (1) that had matured precociously had an increased amount of androgen (1.92 ng/ml) as compared to the immature control fish (0.10 ng/ml) (Table 3). Fish treated with MT (5) and MT:FL (5:20) had mean androgen levels of 0.26 ng/ml and 0.04 ng/ml, respectively. Androgens of the other treated groups, which were from immature fish, averaged between 0.04 and 0.05 ng/ml. Plasma androgens were not detected in the blood of control fish at days 218 and 320.

There were 61% males and 39% females in the control group (Table 4). This ratio was not significantly different from a 1:1 expected ratio. All of the compounds except FL caused a significant deviation from the control ratio ($\alpha = 0.01$, not including intersexes). FL-treated fish, however, had sex ratios similar to that of the controls. MT-treated fish had significantly higher proportions of males whereas all

Table 3. Plasma androgen concentrations (ng androgen/ml of plasma) (± S.E.) of steelhead trout treated with methyltestosterone (MT), methyltestosterone plus diethylstilbesterol (MT plus DES), diethylstilbesterol (DES), diethylstilbesterol plus clomiphene citrate (DES plus CC), methyltestosterone plus flutamide (MT plus FL) or flutamide (FL). Fish sampled were sexually immature or precociously mature.

Treatment (µg/g of feed)	Androgen (ng/ml)	
	Immature	Precociously mature
Control	0.10 ± .03	
MT (1)		1.92 ± .31
MT (5)	0.04*	0.26*
MT (15)	0.04*	
MT (35)	0.04*	
MT:DES (5:15)	0.04*	
DES:CC (5:15)	0.04*	
MT:FL (5:20)	0.05*	0.04*
FL (20)	0.04*	

*Only one sample assayed.

Table 4. Percent of male, female and intersex of steelhead trout treated with methyltestosterone (MT), methyltestosterone plus diethylstilbesterol (MT plus DES), diethylstilbesterol plus clomiphene citrate (DES plus CC), methyltestosterone plus flutamide (MT plus FL), or flutamide (FL). All treatments (except FL) are significantly different from control at $\alpha = 0.01$.

Treatment (µg/g of feed)	Male	Female	Intersex
Control	61.0	39.0	0.0
MT (5)	100.0	0.0	0.0
MT (15)	93.0	0.0	7.0
MT (35)	77.1	22.9	0.0
MT:DES (5:15)	15.3	44.0	40.7
DES:CC (5:15)	12.1	72.7	15.2
MT:FL (5:20)	89.1	0.0	10.9
FL (20)	58.3	41.7	0.0

had a higher proportion of females. DES:CC-treated fish sex ratios may have been changed due to the mortality at day 110. More females may have survived than males, therefore skewing the sex ratio. MT:FL-treated fish had 89% males and 11% intersex with no females.

All MT-treated fish, including those treated with the antiandrogen (MT:FL) as well, had a highly significant increase ($\alpha = 0.01$) in the thickness of the epidermis (Figure 6). As the dosage of MT increased (1, 5, 15 to 35), the epidermis thickness increased showing a direct, linear dose-dependent response ($r^2 = 0.998$, $t_{calc} = 32.26$). The stratum germinativum cells appeared hypertrophied in the epidermis of MT-treated fish. The DES:CC and FL-treated fish did not have an increase in epidermis thickness. None of the treatments appeared to affect the liver.

1977 Brood

No significant differences ($\alpha = 0.05$) in weight were noted between treated and control fish at day 175. Steelhead trout treated with FL (20), E_2 (5), and E_2:Prog (5:5) had significantly ($\alpha = 0.05$) smaller weight gains than control fish at the end of 253 days (Table 5). Mortality was high during the last month of the test period due to nitrogen saturation of the water supply. Total mortalities from day 0 to day 253 are shown for all treatment groups in Table 5.

Gonads of the control fish on days 175 and 253 were at the same stages as found in control fish of the 1976 brood. Some fish treated with TL (10), MT (10), MT (2), and FL (20) had altered gonads. MA (10), MT (2), and FL (20)-treated fish had some testes that showed advanced stages of spermatogenesis. The few affected ovaries of the treated fish

Figure 6. Mean epidermis thickness (mm) of steelhead trout treated with methyltestosterone (MT), diethylstilbesterol plus methyltestosterone (DES plus MT), diethylstilbesterol plus clomiphene citrate (DES plus CC), diethylstilbesterol (DES), methyltestosterone plus flutamide (MT plus FL), or flutamide (FL). Dosages (μg/g of feed) given under each compound.
**Significantly different from control at $\alpha = 0.01$.

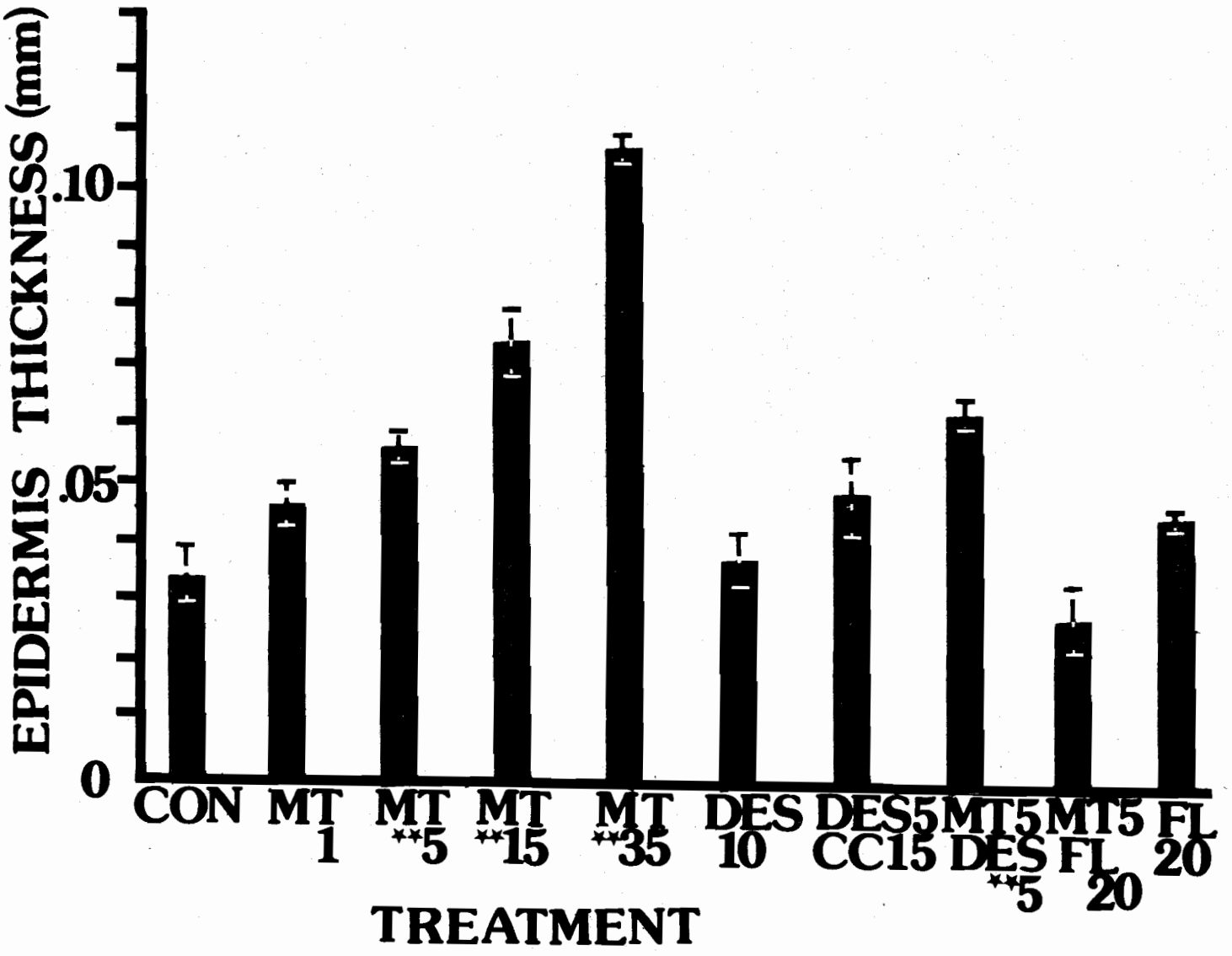
EPIDERMIS THICKNESS (mm)
.10
.05
0
CON
MT 1
MT **5
MT **15
MT **35
DES 10
DES CC15
MT5 DES **5
MT5 FL 20
FL 20
TREATMENT

Table 5. Mean weights (± S.E.) of steelhead trout treated with testosterone proprionate (TP), testolactone (TL), methylandrostenolone (MA), methyltestosterone (MT), flutamide (FL), estradiol (E_2), or estradiol plus progestrone (E_2 plus Prog). Total mortalities from day 0 to day 253 are shown for all treatment groups.

Treatment (µg/g of feed)	Mean weight (g) Day** after onset of treatment					Total mortality
	25	67	137	175	253	0-253
Control	0.812	1.83	3.10 ± 0.24	4.40 ± 0.31	12.66 ± 1.26	22
Control	0.883	1.68	3.22 ± 0.27	5.30 ± 0.41	11.21 ± 1.16	6
TP (5)	0.849	1.37	2.74 ± 0.17	3.98 ± 0.31	11.44 ± 1.42	26
TP (5)	0.829	1.48	2.68 ± 0.17	4.11 ± 0.28	8.99 ± 0.88	59
TL (10)	0.893	1.87	3.03 ± 0.24	4.32 ± 0.32	13.56 ± 1.36	9
MA (10)	0.897	1.92	2.69 ± 0.15	4.47 ± 0.29	11.56 ± 0.89	30
FL (20)	0.846	1.80	2.66 ± 0.19	3.95 ± 0.28	7.61*± 0.89	5
FL (20)	0.872	1.59	2.86 ± 0.20	4.44 ± 0.29	8.56 ± 0.63	3
E_2 (5)	0.818	1.60	2.80 ± 0.26	4.00 ± 0.24	7.86*± 1.02	8
E_2:Prog (5:5)	0.865	1.82	2.62 ± 0.19	3.76 ± 0.31	8.20*± 0.84	42
E_2:Prog (5:5)	0.909	1.64	2.71 ± 0.17	3.58 ± 0.25	9.39 ± 1.10	13
MT (2)	1.010	2.24	3.36 ± 0.26	5.84 ± 0.39	11.59 ± 0.89	3

*Significantly different from control at $\alpha = 0.05$.

**Mean weights on days 25 and 67 based on group weights for the entire group divided by the number of individuals in that group. Mean weights on subsequent days represent the average of weights of 50 individual fish.

TL (10), MA (10), MT (2), and FL (20) showed oocytes surrounded by what appeared to be hypertrophied granulosa cells. Gonads from fish treated with TP (5), E_2 (5), and E_2:Prog (5:5) did not differ from those of the controls. No intersexes were observed in this study.

No significant differences ($\alpha = 0.05$) were observed in the epidermis thickness of treated fish compared to the control fish (Table 6).

Table 6. Mean epidermis thickness (μ) (± S.E.) of steelhead trout treated with testosterone proprionate (TP), testolactone (TL), methylandrostenolone (MA), methyltestosterone (MT), flutamide (FL), estradiol (E_2), or estradiol plus progesterone (E_2 plus Prog). No significant differences ($\alpha = 0.05$) were observed in the epidermis thickness of treated fish compared to controls.

Treatment (μg/g of feed)	Epidermis thickness (μ) (± S.E.)
Control	36.50 ± 3.22
Control	37.70 ± 4.89
TP (5)	36.93 ± 3.38
TP (5)	34.16 ± 2.20
TL (10)	51.52 ± 17.45
MA (10)	51.36 ± 6.91
MT (2)	49.04 ± 4.82
FL (20)	41.80 ± 3.30
FL (20)	31.63 ± 6.95
E_2 (5)	35.66 ± 3.99
E_2:Prog (5:5)	37.70 ± 4.25
E_2:Prog (5:5)	38.30 ± 5.13

DISCUSSION

FL, the antiandrogen, was one of the only compounds that appeared to enhance growth in steelhead trout, and this only occurred when the fish were started on treatments at the onset of feeding. Unfortunately, there was no replication of this treatment in 1976. When FL treatment was initiated one month after the onset of exogenous feeding (1977), the growth rate tended to be less than that of the control fish. Feeding regimes differed between the two experiments. In 1976, fish were fed several times daily, while in 1977 they were only fed once a day during the last seven months of the test period; so in actuality the total amount of test compound the fish received may have also differed between the experiments. The effect of the fish being fed once a day was evidenced by the lower overall growth of the fish in 1977 compared to those in 1976. The nitrogen saturation in the water caused a high mortality in 1977 and may have affected the growth rates of all fish, including control fish. FL given from one month after onset of feeding advanced spermatogenesis while no signs of advanced spermatogenesis were evident in fish treated immediately after buttoning up. I cannot conclude from these data whether or not FL inhibits the sexual development by blocking the action of the endogenous hormones at the gonad. The underlying cause of enhanced growth by FL (1976) is unknown; other external factors may have affected the growth. Without a replicate it is difficult to say whether or not it was actually the FL that affected the growth rate.

Fish treated with MT at the onset of feeding weighed less than the control fish. Spermatogenesis was advanced and the epidermis was thicker than in the controls. However, in fish where treatment was started one month after feeding, there was an increased growth rate during the first 175 days, followed by a decreased growth rate so that by the termination date (day 253), MT-treated fish weighed the same as the controls. Fagerlund and McBride (1977) noted a similar trend in steelhead trout treated with MT (1 µg/g feed) from one month after buttoning up. MT (1 µg/g feed) has been shown to be an effective growth promotor for coho and chinook salmon (Fagerlund and McBride 1977). It appears that to be an effective growth promotor, the dosage of MT required varies among the Salmonidae.

The lack of growth enhancement by the other androgenic hormones (TP, TL, MA) may be due to the level of hormone used. The possibility that the androgens may be more effective at different stages of maturity cannot be excluded. Treatment of hormones at the onset of feeding may be more effective in promoting growth. The nitrogen supersaturation may also have affected the growth of the fish. Since MA and TL induced changes in the gonads, lower dosages may be more effective for growth enhancement.

DES:CC appeared to promote growth in steelhead trout in this trial; however, this increase in growth may be due to the lower density of fish or other unrecognized causes rather than the compound itself. However, DES has been shown to have a strong anabolic action in livestock. DES at a low dosage (0.6 µg/g feed) enhanced growth in plaice (Pleuronectes platessa), but the highest dosage (2.6 µg/g feed) failed

to enhance growth in these fish (Cowey et al. 1973). Bulkley (1972) found that DES (0.62 µg/g feed) decreased the growth rate in channel catfish, Ictalurus punctatus. DES may be an effective growth promotor for steelhead trout, but effective dosages need to be determined. E_2 and E_2:Prog-treated fish did not grow as rapidly as the controls. The gonads and skin thickness of these fish did not differ histologically from the controls. These differences in growth were not apparent until 253 days, previous to which time all the groups had high mortality due to nitrogen saturation. E_2 is considered to have limited anabolic action while progesterone is thought to be indirectly involved in catabolic action in mammals (Kochakian 1976). The reason E_2 failed to affect weight gain in the steelhead trout is unclear.

There were no differences in the RNA-DNA ratio or RNA-P content between treatments. In young fish, growth is occurring more by cell division (excluding muscle), and Sable (1974) suggested that RNA may be a better measurement of short-term growth. Since the samples tested were mostly muscle tissue, the RNA content may not reflect growth in these young fish. The procedures for the RNA-DNA ratio may also not be accurate enough. The orcinol and diphenylamine procedures are actually reactions of the sugars and not the phosphates, but the phosphates are the compounds that are measured for the determination of nucleic acid content. Also, the extraction efficiencies have never been determined. Other studies which showed positive results of the RNA-DNA ratio reflecting short term growth (Bulow 1970, Salbe 1974) or long term growth (Haines 1973) did not mention any validation of extraction efficiencies of the nucleic acids. I do not recommend the RNA-DNA ratio

as an indicator of short-term growth.

The changes in the sex ratio in the 1976 brood indicated that the sex determination process was affected by feeding the hormones from the onset of feeding. Sexual differentiation of the gonads in rainbow trout (S. gairdneri) occurs at six weeks after hatching (Lebrun 1977). Other sexual alterations that were observed in MT (15), MT:DES, DES:CC, and MT:FL-treated fish were the intersexes, which may or may not be functional. Jalabert et al. (1975) produced functional hermaphrodites (self-fertilizable by artificial insemination) by feeding estrone or methyltestosterone. DES induced higher percentages of intersexes than MT. The same trend was noted in Jalabert's (1975) study where estrone induced more hermaphrodites than methyltestosterone. Curiously, in the MT:DES group, there was a higher percentage of females. Apparently, the estrogenic hormones exert a stronger influence on sexual differentiation when fed to fish during the sex determination process than do the androgens at the concentrations tested.

Plasma androgen concentrations in sexually precocious fish treated with MT (1) were substantially higher than the control concentrations. The other two groups, MT (5) and MT:FL (5:20), that had precocious fish had extremely low levels of plasma androgens, which could be indicative of the exogenous hormone acting directly on the gonad and stimulating spermatogenesis. Billiard (1977) showed that administration of MT and E_2 to intact male rainbow trout inhibited gonadal development but did not change the level of circulating gonadotropin. He suggested that there might be a direct effect of the steroids on the testes.

I cannot conclude for certain that endogenously produced androgens caused precocious male sexual development. No androgen concentrations were detected in the control fish samples, and whether any of these fish became mature early is not known due to a loss of fish or tags. Exogenous androgens do cause an increase in precocious development, so it does appear than androgens could be involved naturally. FL-treated fish were not held long enough to see if FL would inhibit precocious sexual development, but the testes of FL-treated fish did not have advanced stages of spermatogenesis, and the sex ratios were close to 1:1. Carl Schreck (personal communication, 1977, Oregon Cooperative Fishery Research Unit, Oregon State University, Corvallis) in his study of winter steelhead trout found that FL at 3 μg/g appeared to inhibit precocious male sexual development at least up to 16 months if treated from the onset of exogenous feeding.

I was not able to demonstrate that androgens caused precocious male sexual development. The difficulty of serially bleeding juvenile fish prevented any conclusions on the role of endogenous androgens in precocious development. Further studies demonstrating endogenous hormone patterns of juvenile salmonids concomitant with early male sexual development should be conducted. The use of hormonal blocking agents to prevent this development would depend on whether or not endogenous hormones affected early sexual maturity. If it is found that endogenous hormones affect early sexual maturity, then administration of hormonal blocking agents could demonstrate their effectiveness in inhibiting early male sexual development.

In conclusion, the androgenic hormones did not enhance the growth of steelhead trout. The MT-treated fish actually showed decreased growth rates compared to the controls. The androgens (except TP) advanced spermatogenesis and thickened the epidermis of the fish. DES may have enhanced the growth of the fish, while the other estrogenic hormones failed to do so. FL increased the growth rate of fish fed the treated diet at the start of exogenous feeding, but further studies are needed to clearly demonstrate if FL has anabolic properties. Lack of replication of treatments failed to provide any strong conclusive evidence of the anabolic properties of these compounds. All compounds except FL fed to the fish from the onset of feeding caused abnormal sex ratios. Androgens induced more males while estrogens induced more females.

LITERATURE CITED

Beams, H. W., and R. G. Kessel. 1973. Oocyte structure and early vitellogenesis in the trout, Salmo gairdneri. Am. J. Anat. 136:105-122.

Billiard, R. 1977. Testicular feed-back on the hypothalamo-hypophyseal system in rainbow trout (Salmo gairdneri). Symp. Reprod. Physiol. Fish. Paimpont, France. 19-21 Sept. Abst. 1.

Bulkley, R. V. 1972. Diethystilbesterol in catfish feed. Trans. Amer. Fish. Soc. 101:537-539.

Bulow, F. J. 1970. RNA-DNA ratios as indicators of recent growth rates of a fish. J. Fish. Res. Board Can. 27:2343-2349.

Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62:315-323.

Cheema, I. R., and A. J. Matty. 1977. Effect of the anabolic steroids norethandrolone and dimethazine on muscle protein synthesis and growth of rainbow trout, Salmo gairdneri. J. Endocrinol. 72:11P-12P.

Cowey, C. B., J. A. Pope, J. W. Adron, and A. Blair. 1973. Studies on the nutrition of marine flatfish. The effect of oral administration of diethylstilbesterol and cyproheptadine on the growth of Pleuronectes platessa. Marine Biology 19:1-6.

Fagerlund, U. H. M., and J. R. McBride. 1975. Growth increments and some flesh and gonad characteristics of juvenile coho salmon receiving diets supplemented with 17α-methyltestosterone. J. Fish. Biol. 7:305-314.

Fagerlund, U. H. M., and J. R. McBride. 1977. Effect of 17α-methyltestosterone on growth, gonad development, external features, and proximate composition of muscle of steelhead trout, coho and pink salmon. Fish. Marine Serv. Can. Tech. Rep. No. 716. 37 p.

Haines, T. A. 1973. An evaluation of RNA-DNA ratio as a measure of long-term growth in fish populations. J. Fish. Res. Board Can. 30:195-199.

Higgs, D. A., E. M. Donaldson, H. M. Dye and J. R. McBride. 1976. Influence of bovine growth hormone and L-thyroxine on growth, muscle composition, and histological structure of the gonads, thyroid, pancreas, and pituitary of coho salmon (Oncorhynchus kisutch). J. Fish. Res. Board Can. 33:1585-1603.

Hirose, K., and T. Hibiya. 1968. Physiological studies on growth-promoting effect of protein-anabolic steroids on fish. I. Effects on goldfish. Bull. Jap. Soc. Sci. Fish. 34:473-481.

Jalabert, B., R. Billiard, and B. Chevassus. 1975. Preliminary experiments on sex control in trout: production of sterile fishes and simultaneous self-fertilizable hermaphrodites. Ann. Biol. Anim. Bioch. Biophys. 15(I):19-28.

Kochakian, C. D., ed. 1976. Anabolic-androgenic steroids. Handbook Exp. Pharm. 43. Springer-Verlag, Berlin. 725 p.

Korenman, S. G., R. H. Stevens, L. A. Carpenter, M. Robb, G. D. Niswender, and B. M. Sherman. 1974. Estradiol radioimmunoassay without chromatography: procedure, validation and normal values. J. Clin. Endocrinol. Metab. 38:718-720.

Lebrun, C. 1977. Sexual differentiation of gonads in the rainbow trout (Salmo gairdneri). Symp. Reprod. Physiol. Fish. Paimpont, France. 19-21 Sept. Abst. 7.

Ludwig, B., D. A. Higgs, U. H. M. Fagerlund, and J. R. McBride. 1977. A preliminary study of insulin participation in the growth regulation of coho salmon (Oncorhynchus kisutch). Can. J. Zool. 55:1756-1758.

Matty, A. J. 1975. Endocrine control of growth and protein metabolism in aquaculture. Proc. Pac. Sc. Cong. 1:58.

McBride, J. R., and U. H. M. Fagerlund. 1973. The use of 17α-methyltestosterone for promoting weight increases in juvenile Pacific salmon. J. Fish. Res. Board Can. 30:1099-1104.

McBride, J. R., and U. H. M. Fagerlund. 1976. Sex steroids as growth promoters in the cultivation of juvenile coho salmon (Oncorhynchus kisutch). Proc. 7th Ann. Meet. World Mariculture Soc., San Diego, Calif. Publ. by Lousiana State Univ. Div. Continuing Education. P. 145-161.

Sable, D. W. 1974. RNA-DNA ratios as indicators of short-term growth in channel catfish. Ph.D. thesis. Iowa State University. 87 p.

Schneider, W. C. 1957. Determination of nucleic acid in tissue by pentose analysis. In Methods in enzymology. S. P. Colowick and N. O. Kaplan, eds. Vol. 3. Academic Press, Inc., N.Y. P. 680-684.

Schreck, C. B. 1973. Uptake of ^{3}H-testosterone and influence of an antiandrogen in tissues of rainbow trout (Salmo gairdneri). Gen. Comp. Endocrinol. 21:60-68.

Simpson, T. H. 1976. In Day of the farmed Scotch salmon. T. Loftas, auth. New Scientist 70:20-21.

Yamazaki, F. 1972. Effects of methyltestosterone on the skin and the gonad of salmonids. Gen. Comp. Endocrinol. Suppl. 3:741-750.