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Title: SEX STEROID PROFILES OF COHO SALMON, ONCORHYNCHUS KISUTCH, DURING EARLY DEVELOPMENT AND SEXUAL DIFFERENTIATION

Abstract approved: Redacted for Privacy
Carl B. Schreck

Whole body sex steroid levels of coho salmon, Oncorhynchus kisutch, were measured via radioimmunoassay during early development and sexual differentiation. Steroid profiles of testosterone (T), 11-ketotestosterone (KT), androstenedione (A), progesterone (P4), 17α-hydroxy-20β-dihydroprogesterone (DHP), and 17β-estradiol (E2) were generated beginning with unfertilized eggs and ending at 87 days post-fertilization (DPF). Ovarian fluid was also examined for steroid content. At hatching (42 DPF) an androstenedione assay was substituted for the P4 assay. Onset of exogenous feeding occurred at 60 DPF. Steroid profiles of unfertilized eggs essentially paralleled that found for ovarian fluid. Following fertilization, steroid levels in developing embryos declined precipitously until 30 DPF. At hatch, levels of all steroids increased slightly and then declined during yolk sac absorption. Levels of the androgens (T, KT, and A) followed similar patterns during this time while DHP and E2 content also showed

similar trends to one another. During yolk sac absorption yolk and embryos were separated from each other and assayed individually. Steroid content of yolk declined while it generally increased or remained constant in the embryos. Varying degrees of bimodality appeared in the data regarding steroid levels within samples of fish collected between 42 and 56 DPF. The bimodal distributions of steroids disappeared at the onset of feeding and did not return again until 87 DPF. The hormone levels generally decreased or remained constant following the onset of exogenous feeding, except for E2, whose levels increased during this time. Histological analyses showed the presence of undifferentiated gonads between hatching and 70 DPF. By 77 DPF ovarian development could be easily discerned. Fish born the following brood year were presumptively sexed at 101 DPF and steroid levels were determined; a sexual dimorphism was apparent in levels of T, KT, and A, but not for DHP or E2. The increase in steroid content of developing coho at hatch, coupled with their bimodal distributions during yolk sac absorption and the increase in E2 levels during ovarian development may suggest a possible role of sex steroids in the process of sexual differentiation apparent later in development. Changes in whole body steroid levels at hatch may also be indicative of the onset of sexual differentiation even though no signs of gonadal differentiation were histologically discernable at that time.

SEX STEROID PROFILES OF COHO SALMON, ONCORHYNCHUS KISUTCH, DURING EARLY DEVELOPMENT AND SEXUAL DIFFERENTIATION

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INTRODUCTION

Administration of sex steroids during early development results in varying degrees of sex reversal in a variety of teleostean fishes (see reviews by Yamamoto, 1969; Schreck, 1974; Donaldson and Hunter, 1982; Hunter and Donaldson, 1983). Androgens typically act as andro-inducers and estrogens as gyno-inducers. By virtue of this ability to cause sex inversion in the medaka, *Oryzias latipes*, Yamamoto (1962, 1969) advanced the theory that sex steroids are the natural inducers of sexual differentiation in fishes.

Surprisingly few studies have examined the onset of steroidegenesis in the differentiating teleostean gonad. Administration of radioactive steroid precursors has demonstrated the presence of steroid synthesizing enzymes during early life stages for the guppy (*Poecilia reticulata*) (Takahashi and Iwasaki, 1973) and the rainbow trout (*Salmo gairdneri*) (van den Hurk *et al.*, 1982; Antila, 1984). Only Rothbard *et al.* (1987) has examined endogenous steroid levels during sexual differentiation in teleosts. Levels of three sex steroids were measured during early life stages in three species of tilapia. Testosterone and 17β-estradiol were present in ng/individual concentrations just after fertilization and declined to undetectable levels in 3-4

week old fry. The concentrations of both steroids began to increase in 4-6 week old fry. The increase of testosterone was 10 fold higher than that of estradiol. Estradiol and 11-ketotestosterone concentrations in differentiated fry at 6-8 weeks were very low. They concluded that among the steroids examined, only testosterone reflected the process of sexual differentiation.

Our objective was to elucidate possible endocrine control mechanisms of sexual differentiation in fish by evaluating development of sex steroid profiles of coho salmon (Oncorhynchus kisutch) through the period of sexual differentiation and by correlating hormonal events with gonadal development. Steroid profiles of whole body extracts were generated via radioimmunoassay (RIA) specific for testosterone (T), 11-ketotestosterone (KT), androstenedione (A), 17α-hydroxy-20β-dihydroprogesterone (17α,20β-dihydroxy-4-pregnen-3-one) (DHP), progesterone (P4), and 17β-estradiol (E2).

MATERIALS AND METHODS

Fish Rearing and Sampling. Coho salmon gametes were obtained from Oregon Aqua-Foods hatchery in Springfield, Oregon. After fertilization in November, 1985, embryos were raised at Oregon State University's Smith Farm facility in Corvallis at a constant water temperature of 12^{o} C. During gamete collection, ovarian fluid, sperm, and unfertilized eggs were sampled and stored at -20^{o} C until analyzed for T,

KT, DHP, P4 and E2. A random sample of 8000 eggs from 72 females was fertilized with sperm from 18 males.

Eggs at 1 hour postfertilization and developing embryos at 10, 20, 30, 42, 49, 56, 63, 70, 77, and 87 days post-fertilization (DPF) were sampled. A sample size of 50 embryos was taken at each date. Steroid levels were determined for 30 fish, 10 fish were fixed in Bouin's solution for histological analysis, and 10 animals reserved for high pressure liquid chromatography (HPLC) analysis. All values reported for steroid levels are for sample sizes between 27 and 30 unless stated otherwise. An androstenedione assay was substituted for the P4 assay 40 days into the study.

All fish sampled for steroid determinations were killed by freezing with dry ice. A lethal dose of anesthetic was not used because it was found that compounds like tricaine methanesulfonate (MS-222) produced interfering peaks in HPLC. Biomoist^{TM} diet was fed from the onset of exogenous feeding (60 DPF) 3-4 times daily to satiation.

Extractions. Developing embryos to be analyzed for whole-body sex steroid content were sonicated for 30 seconds in 1.0 ml of 0.1 N NaOH and extracted twice with 8 volumes of diethyl ether. Tubes were vortexed vigorously for 30 seconds after addition of ether. Tubes were spun at 2000 g for 20 minutes after each ether extraction. Aqueous phase was removed by snap freezing in liquid nitrogen. Combined extracts were dried in a Speed Vac^{TM} centrifuge. Extraction

efficiencies for all steroids examined were determined at each sampling date (n=5) by addition of tritiated steroids to tubes containing whole fish that were then sonicated and extracted as described above. Spiked extracts were resuspended in 1.0 ml of assay media-phosphate buffered saline with gelatin (PBSG) and 0.5 ml was counted in a scintillation spectrophotometer. Extracts to be analyzed for steroid content were resuspended in 1.0 ml of PBSG and 0.1 ml aliquots were removed for each RIA. Sperm and ovarian fluid samples (0.5 ml each) were homogenized, extracted, and resuspended in an identical manner. Average extraction efficiencies for developing embryos for T, KT, A, P4, DHP, and E2 were 59.8, 56.1, 53.9, 51.8, 67.1, and 65.4%, respectively. All steroid assays were corrected for recovery.

Assays. Whole body sex steroid content for T, KT, P4, DHP, and E2 were measured by RIA as described by Sower and Schreck (1982) and modified by Fitzpatrick (1986). An androstenedione assay was also used. Anti-androstenedione-antiserum (AN6-22) was obtained from Endocrine Sciences (Tarzana, CA.) and diluted 1:30. A slightly more concentrated charcoal solution (6.25 g charcoal and 4.0 g dextran/liter PBSG) was used for all assays to reduce non-specific binding. One ml of dextran coated charcoal was added to each tube for the T, KT, A, DHP, and P4 assays, and 0.5 ml for the E2 assay. The lower limit of detection was 3.1 pg/tube for all assays except E2 which was 1.25 pg/tube.

The intra-assay coefficient of variation for all assays was less than 5%.

To determine if differences existed between males and females with regard to whole body sex steroid content, coho salmon fertilized in November of 1986 were presumptively sexed at 101 DPF and steroid content determined via RIA. Fish were sexed presumptively based on gross gonadal morphology with females having an enlarged gonad anteriorly. Five presumptive females and five males, were also sectioned for histological confirmation of sex.

Steroid levels determined via RIA were validated by verifying that serial dilutions were parallel to standard curves. Sex steroid RIAs were also validated by assaying extracts of eggs collected at one hour post-fertilization which were subjected to separation by thin layer chromatography. After correcting for recovery, thin layer chromatographed and non-chromatographed extracts showed similar levels of authentic steroids for T, KT, A, E2, P4, and DHP. Assays were further validated by accounting for addition of cold steroids (5% or 25% of endogenous steroid present) to samples. Steroids present in quantities sufficient for detection by HPLC (T and DHP) were also validated by correlating levels with those found by RIA. Validation by quantitative HPLC was only possible during early life stages (unfertilized eggs and embryos at 10 DPF) when steroid levels were relatively high.

High Pressure Liquid Chromatography. A method modified from that of Huang et al. (1983) was used for HPLC analysis. Samples for HPLC were prepared in the same way as that for RIA followed by an additional extraction with Baker-10 SPE^{TM} octadecyl (C_{18}) cartridges (see Zief et al., 1982). Steroids were eluted from the C_{18} cartridge with two 0.5 ml aliquots of methanol, filtered through an Acro^{TM} 0.45 μm mesh (PVC/nylon and P.T.F.E.), dried down, resuspended in mobile phase and injected onto HPLC. The HPLC system consisted of an IBM LC/9533 Ternary Gradient Liquid Chromatograph, an IBM 9000 computer, two fixed wavelength UV detectors at 254 and 280 nm and a Hewlett-Packard 100 x 2.1 mm reverse phase (C_{18}) column with a pore size of 5 μm. Steroids were eluted starting with an isocratic mobile phase (0.4 ml/min) of water:methanol:acetonitrile:isopropanol 62:28:5:5 immediately followed by a linear gradient of 3.3%/min of water:methanol:butanol 35:45:20 for 30 minutes. This solvent system allowed for separation of 16 steroids used as standards and gave sensitivities of 5-10 ng per steroid.

Histology. Fish sampled for histological analysis were fixed in Bouin's solution and embedded in paraffin. Serial sections (8-10 μm) were taken either transversely or sagitally and stained with hemotoxylin and eosin. Descriptions of gonadal differentiation of coho salmon outlined by Goetz et al. (1979) and of other salmonids outlined by Robertson (1953), Ashby (1957), Takashima et al.

(1980) and van den Hurk and Slof (1981) were used to identify stages of germ cell and gonadal differentiation.

Statistics. Data for sex steroid content were analyzed by the nonparametric Mann-Whitney rank sum statistic. This statistic computes the significances of differences between medians of two populations after a preliminary analysis of variance. The level of significance used for all tests was $P< 0.05$.

Since bimodal distributions with regard to sex steroid content of developing embryos is a possible indication of a sexual dimorphism, thus pointing to a potential involvement of sex steroids in the process of sexual differentiation, data for sex steroid distributions for each sampling date were analyzed by measuring skewness (g1) and kurtosis (g2). Formulas for these tests can be found in Sokal and Rohlf (1969). The tests for g1 and g2 were undertaken to determine if levels of steroids in developing coho were normally distributed. The ratio of g1 or g2 to their standard errors is a test of normality. Formulas for standard errors of g1 and g2 are from Cramer (1946). The expected value for a normal distribution is zero. Large positive values for the g1 test are indicative of an asymmetric distribution which is skewed to the right. Negative values indicate a skewing to the left. Large positive values for the g2 test are indicative of a leptokurtic distribution with more items near the mean and at the tails when compared to a normal distribution.

Negative values are indicative of a platykurtic distribution with more items in intermediate regions. A bimodal distribution is extremely platykurtic.

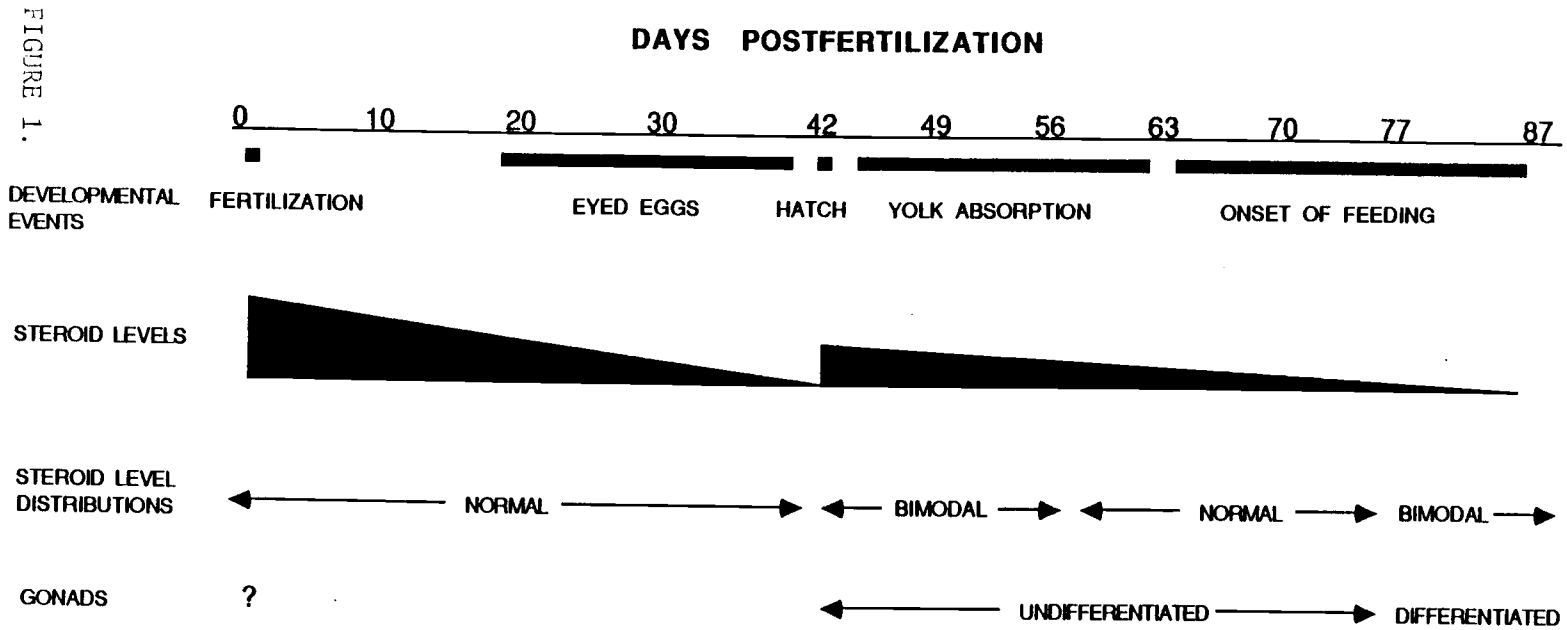
Tests for g1 and g2 are extremely sensitive to outliers, especially for sample sizes less than 75. For this reason, all values greater than three standard deviations from the mean for each distribution were deleted before applying tests. Deletions of data involved only one or two data points, if any, from each sample size of 30. Histograms of steroid distributions were also examined for this reason since visualization of histograms can also reveal trends in bimodality.

RESULTS

A generalized developmental scheme which includes hormonal events is given in figure 1. Eggs became eyed from 20 to 42 DPF. Hatching occurred at 42 DPF. Yolk sac absorption progressed from 42 to 60 DPF, and exogenous feeding began at 60 DPF. Steroid levels were relatively high in developing embryos following fertilization which then declined during the first 42 days of development. At hatch steroid levels increased slightly and then declined during the next 45 days. Steroid level distributions appeared to be normal up to hatch and then became bimodal for approximately 2 weeks. Bimodal distributions disappeared at the onset of feeding and did not return until 87 DPF. Gonads remained in a histologically

Figure 1. Developmental events, steroid levels, steroid level distributions, and gonadal development in a population of coho salmon. Steroid levels conceptually depicted by the height of the bars are not drawn to scale.

FIGURE 1.


DAYS POSTFERTILIZATION
0
10
20
30
42
49
56
63
70
77
87
DEVELOPMENTAL EVENTS
FERTILIZATION
EYED EGGS
HATCH
YOLK ABSORPTION
ONSET OF FEEDING
STEROID LEVELS
STEROID LEVEL DISTRIBUTIONS
NORMAL
BIMODAL
NORMAL
BIMODAL
GONADS
?
UNDIFFERENTIATED
DIFFERENTIATED

undifferentiated state from hatch to 70 DPF. At 77 DPF ovarian development could be discerned.

Steroid content of sperm, ovarian fluid and unfertilized eggs. Sperm and seminal fluid contained relatively high levels of all steroids investigated. Sperm and seminal fluid contained mean concentrations of 5.3, 9.6, 6.8, 0.2, and 0.1 ng/ml were found for T, KT, DHP, P4, and E2 respectively. Sex steroid content of unfertilized eggs showed a pattern very similar to that found in ovarian fluid (Fig. 2). Relatively large amounts of T and DHP and smaller amounts of KT, P4, and E2 were present in both unfertilized eggs and ovarian fluid.

Post-fertilized eggs to 30 DPF. Relatively high levels of steroids were also found in eggs at one hour post-fertilization, this was followed by a precipitous decline in steroid content by 30 DPF (Figs. 1, 3). Non-eyed eggs at 30 DPF were also analyzed for sex steroid content but are not included in Figure 3. Levels of T, KT, and P4 were similar for both eyed and non-eyed eggs (mean values of 473 $\pm$ 43, 106 $\pm$ 10 and 325 $\pm$ 38 pg/egg respectively for non-eyed eggs and 611 $\pm$ 46, 223 $\pm$ 36, and 312 $\pm$ 19 for eyed eggs). Levels of DHP and E2, however, were much higher in non-eyed eggs (mean values of 4092 $\pm$ 554, and 406 $\pm$ 52 pg/egg respectively for non-eyed eggs and 500 $\pm$ 24, and 48 $\pm$ 4 for eyed eggs).

Hatch and yolk sac absorption. Steroid profiles of developing coho during the three weeks following hatching at 42 DPF are given in figure 4 (see Fig. 1 also). The

Figure 2. Concentration (ng/gm) of sex steroids in ovarian fluid and unfertilized eggs from coho salmon for testosterone (T), 17α-hydroxy-20β-dihydroprogesterone (DHP), 11-ketotestosterone (KT), progesterone (P4), and 17β-estradiol (E2). Each value represents the mean $\pm$ SE for a sample size of 9 for ovarian fluid and 28 for unfertilized eggs.

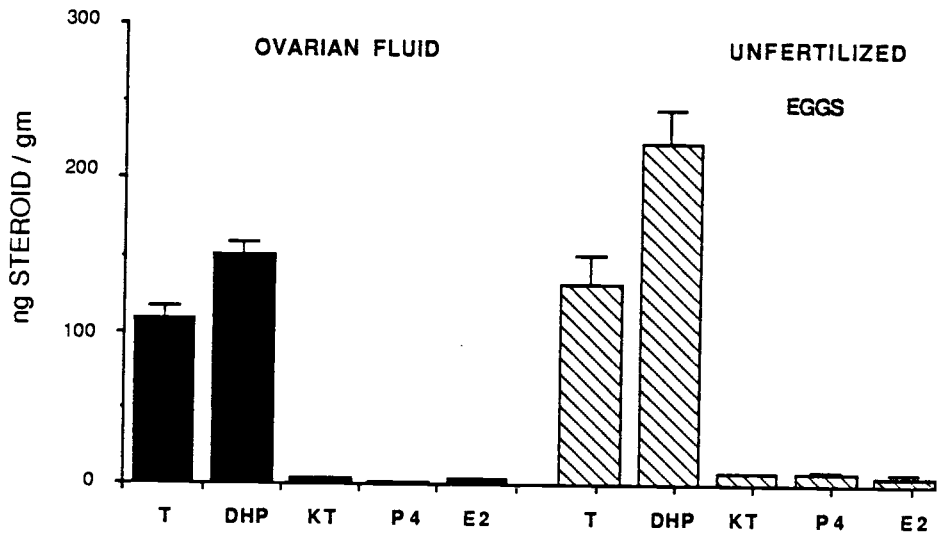

300
200
100
0
ng STEROID / gm
OVARIAN FLUID
UNFERTILIZED
EGGS
T
DHP
KT
P4
E2
T
DHP
KT
P4
E2

FIGURE 2.

Figure 3. Concentrations (pg/fish of sex steroids in coho salmon Figure 3. unfertilized eggs (U), eggs at 1 hour postfertilization (0), and developing embryos at 10, 20, and 30 days postfertilization (DPF). Steroids include testosterone (T), 11-ketotestosterone (KT), 17α -hydroxy-20β -dihydroprogesterone (DHP), progesterone (P4), and 17β -estradiol (E2). Each value represents the mean $\pm$ SE for a sample size of 27-30 for all dates except 30 DPF where n=22.

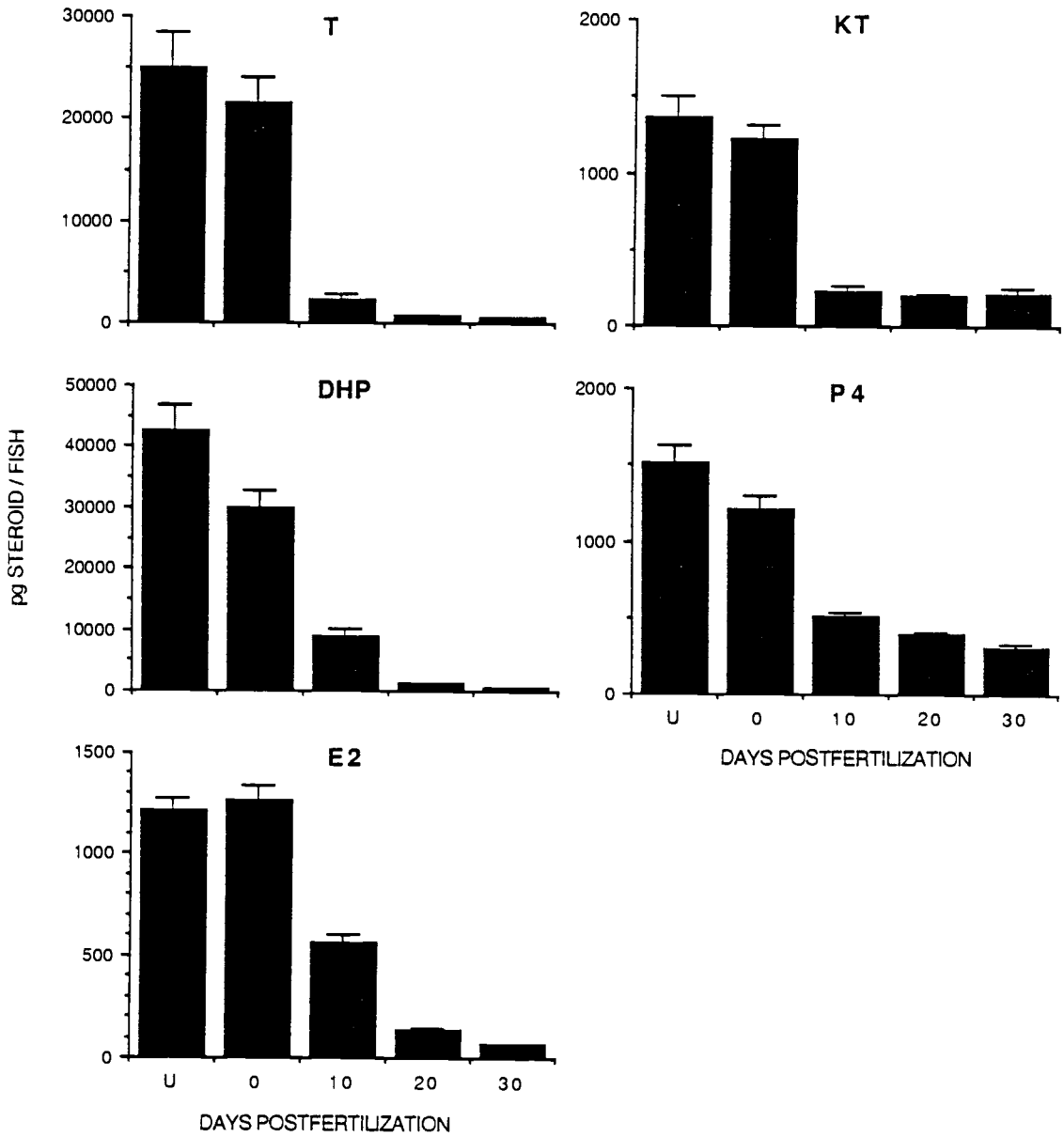

T
KT
DHP
P4
E2
pg STEROID / FISH
30000
20000
10000
0
2000
1000
50000
40000
30000
1500
500
U
0
10
20
30
DAYS POSTFERTILIZATION

FIGURE 3.

Figure 4. Concentrations (pg/fish) of sex steroids in yolks, embryos, and totals (yolk + embryo) of coho salmon from 30 to 87 days postfertilization (DPF). Steroids include testosterone (T), 11-ketotestosterone (KT), androstenedione (A), 17α-hydroxy-20β-dihydroprogesterone (DHP), and 17β-estradiol (E2). Each value represents the mean $\pm$ SE for a sample size of 27-30 for all dates except for 30 DPF where n=22, 87 DPF where n=16 and for androstenedione at 42, 49, and 56 DPF where n=17, 13, and 10 respectively. Hatching occurred at 42 DPF and onset of feeding at 60 DPF.

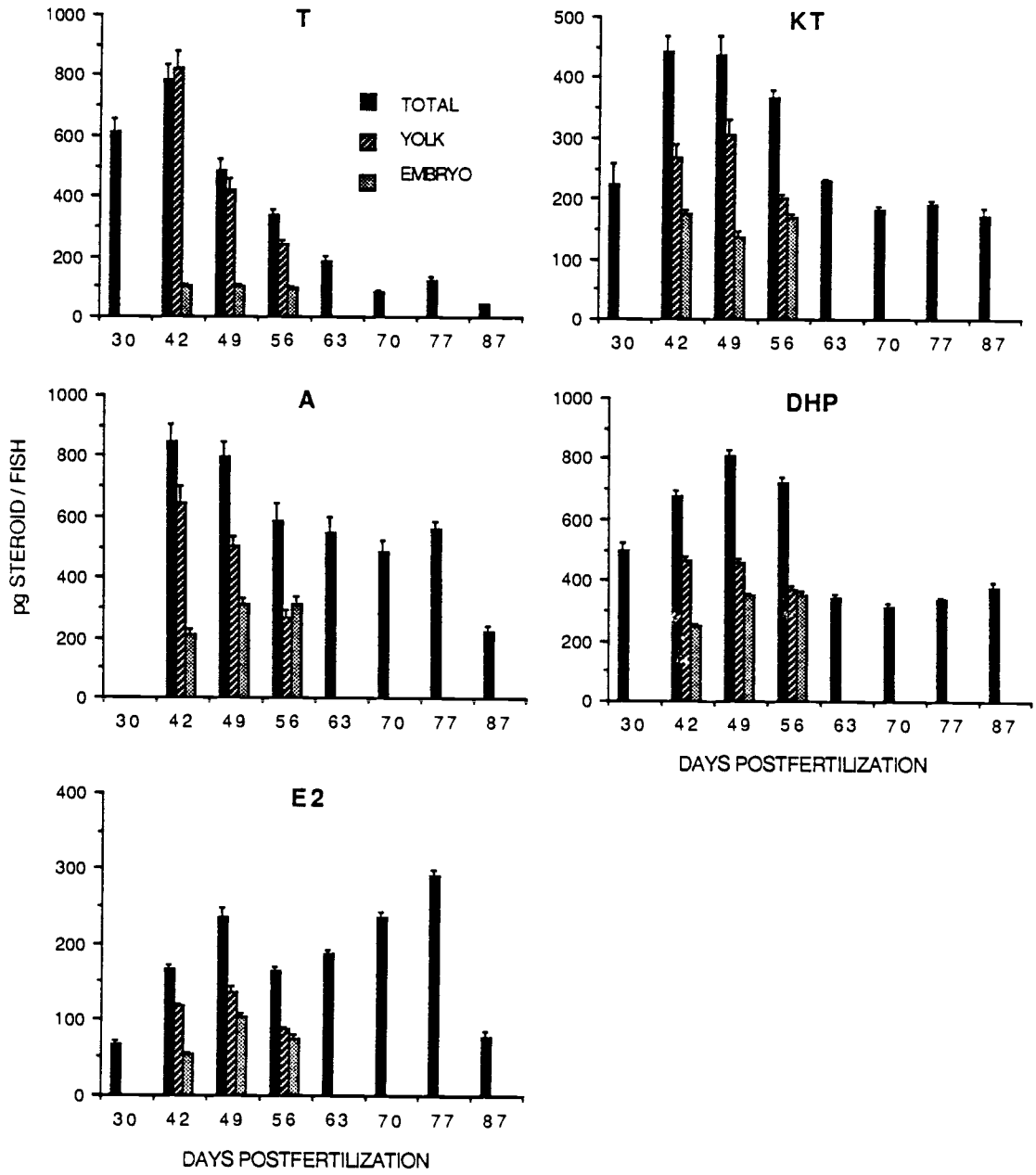

T
KT
A
DHP
E2
TOTAL
YOLK
EMBRYO
pg STEROID / FISH
DAYS POSTFERTILIZATION
DAYS POSTFERTILIZATION
30 42 49 56 63 70 77 87

FIGURE 4.

androgens T, KT, and A all showed similar patterns, increasing in total (yolk + embryo) steroid content at 42 DPF (assuming that androstenedione followed the same trend as the other five steroids during the first 30 days of development - androstenedione was found at a mean level of 7.35 ± 0.75 ng/egg for a sample of 13 eggs at 1 hour post-fertilization). This increase was followed by a slight decrease during the next two weeks.

Total steroid content for E2 and DHP showed a different pattern than that for the androgens but were similar to one another. Both steroids increased in total concentration between days 30 and 49 DPF and then decreased during the next week.

In some cases, steroid content of yolk and embryo when added together are larger than that given for total steroid content. This is because an average extraction efficiency for yolk and embryo was used to calculate total steroid present. This was done to ensure a conservative estimate for total steroid concentration. In all cases, the increases in steroid content seen at hatch do not seem to be an artifact of adding steroid levels of yolk and embryo. Steroid levels of unseparated yolk and embryo were also determined and further substantiate this contention.

Steroid content of embryos after hatch. Embryonic steroid content of both T and KT remained relatively constant during the two weeks following hatching. DHP and A

increased in embryos at 49 DPF and remained relatively constant for the next two weeks. E2 levels in embryos showed an increase up to 49 DPF and decreased during the next week.

Steroid content of yolks after hatch. Steroid content in yolks showed a similar trend for all five hormones investigated. Generally, steroid levels in yolk decreased as yolk sac absorption progressed.

Onset of feeding. Levels of T, KT, A, and DHP remained relatively constant during the four weeks following onset of feeding at 60 DPF. A decrease in the amount of A, however, was seen at 87 DPF. Estradiol gradually increased during this period followed by a dramatic decrease at 87 DPF.

Presumptively sexed coho. Steroid levels for presumptively sexed animals at 101 DPF are given in Figure 5. A sexual dimorphism with regard to androgen levels was found with males having significantly higher levels than females (confirmed by the Mann-Whitney test statistic). Although DHP levels tended to be higher in males, it was not significantly different from that of females. Surprisingly, no difference in E2 content was found between males and females.

It should also be noted that the patterns and relationships among steroids throughout this study do not change when steroid concentrations are calculated as pg steroid/ gm tissue as well as on a per fish basis as

Figure 5. Concentrations (pg/gm tissue) of sex steroids in presumptively sexed coho salmon at 101 days postfertilization. Steroids include testosterone (T), 11-ketotestosterone (KT), androstenedione (A), 17α-hydroxy-20β-dihydroprogesterone (DHP), and 17β-estradiol (E2). Each value represents the mean $\pm$ SE for a sample size of 16. Stars indicate statistical significance for the Mann-Whitney test for $p < 0.05$.

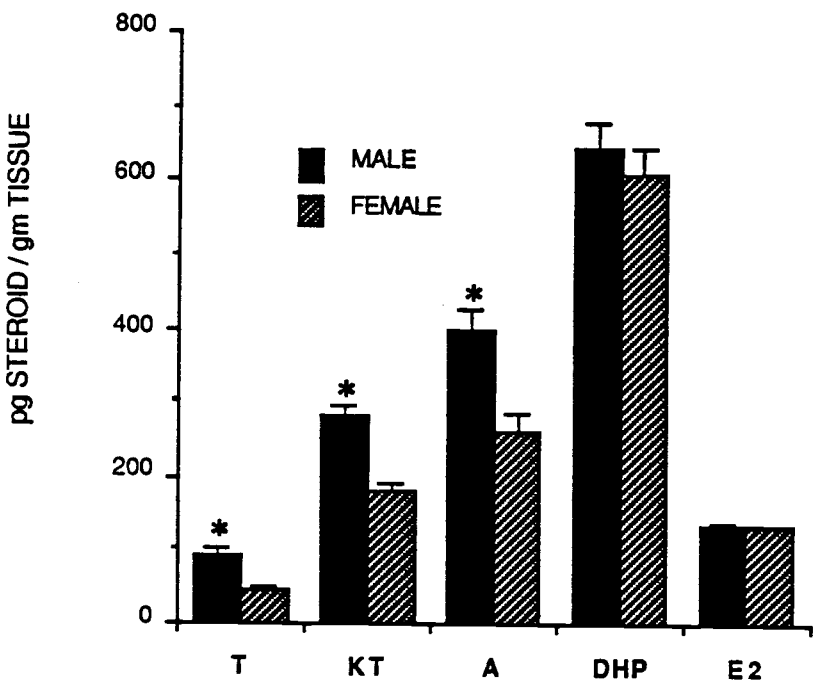


FIGURE 5.

previously presented. All trends and dynamics remain intact.

Histograms and g2 analysis. Values for g1 and g2 from unfertilized eggs to 87 DPF indicated that steroid levels were not normally distributed throughout some of this early development (Fig. 1). Although no g2 values were statistically different from each other through time, some trends in steroid level distributions became apparent. Generally, g2 values became negative during the latter stages of yolk sac absorption (49 and 56 DPF), one to two weeks after hatching. This trend towards bimodality was seen one week earlier for levels of T than for KT, A, and E2. The possibility of bimodality was particularly pronounced for KT and A levels one to two weeks post-hatch. E2 also showed a tended towards a bimodal distribution during this period. The degree of bimodality was least pronounced for DHP levels. These trends held when steroid levels in yolks and embryos were examined separately or when total steroid content was considered. Steroid levels, when expressed either as per fish or as per gram tissue, yielded similar results.

Bimodalities for the androgens can clearly be seen in histograms and g2 analyses of the presumptively sexed fish (Fig. 6). Individuals with relatively low levels of T, KT, and A are generally female. Those with relatively high levels are generally male. Values for g2, however, can be misleading. Although E2 levels usually appear rather

Figure 6. Histograms and g2 (kurtosis) tests (g2/standard error) of sex steroids (pg/gm tissue) in presumptively sexed coho salmon at 101 days postfertilization. Steroids include testosterone (T), 11-ketotestosterone (KT), androstenedione (A), and 17β-estradiol (E2).

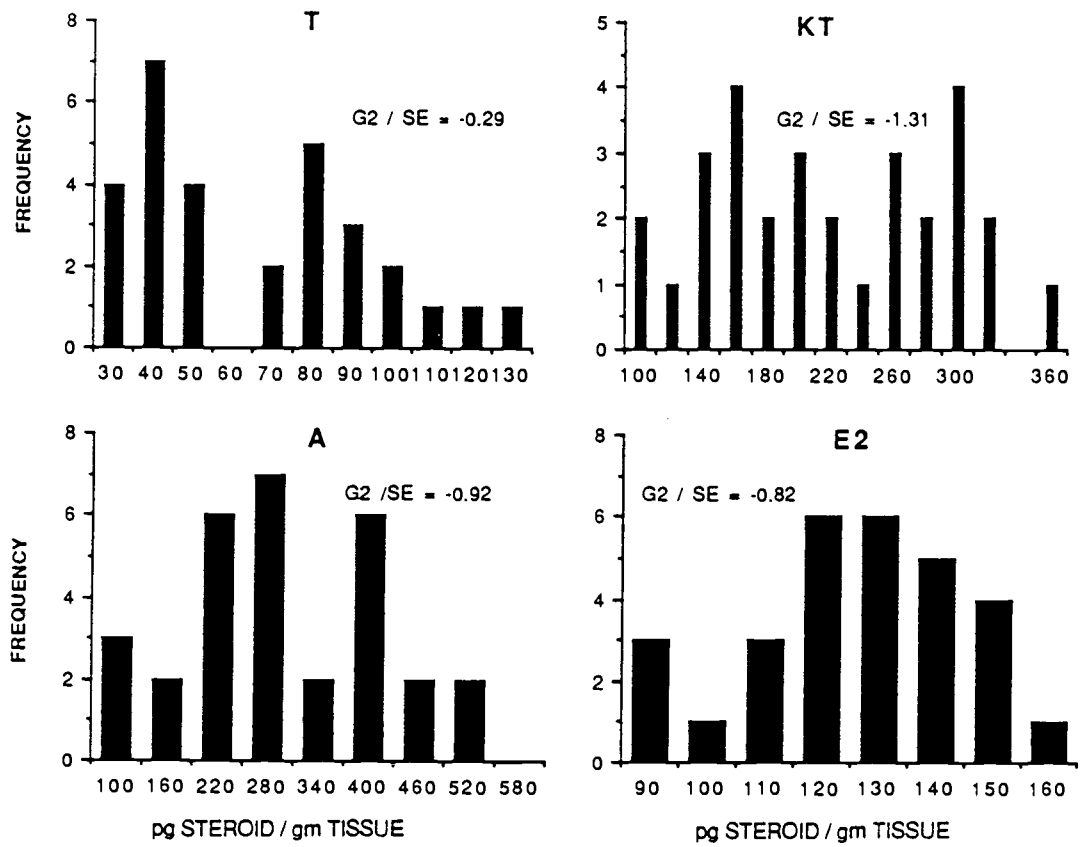

T
G2 / SE = -0.29
KT
G2 / SE = -1.31
A
G2 /SE = -0.92
E2
G2 / SE = -0.82
FREQUENCY
FREQUENCY
30 40 50 60 70 80 90 100 110 120 130
100 140 180 220 260 300 360
100 160 220 280 340 400 460 520 580
90 100 110 120 130 140 150 160
pg STEROID / gm TISSUE
pg STEROID / gm TISSUE

FIGURE 6.

normally distributed, the value for g2 indicates a trend towards bimodality.

Histograms and g2 values of select steroid levels during certain periods of development are given in Figure 7. The best examples of normal and bimodal distributions are given out of the 30 histograms generated for each steroid. Histograms for DHP are not shown because it tended to be the least dynamic and histograms for A levels are not depicted because the sample size was smaller than that for the other steroids. A trend towards bimodality was seen first for levels of T at 42 DPF (hatch); this trend was also evident at 30 DPF but not at 20 DPF. Trends in bimodality for KT, A, and E2 were seen at 56 DPF, two weeks later than for T; the distributions for these three steroids were relatively normal at hatch.

This apparent trend in bimodality appearing after hatch disappeared at the onset of feeding (60 DPF) for T and KT and did not return until 87 DPF. This was also true for E2 and A, except that possible bimodal distributions returned by 70 DPF for E2 and 77 DPF for A.

Histology. Gonads were evident at hatch (42 DPF) but appeared to be undifferentiated (Figs. 1, 8). The gonads consisted of an aggregation of primordial germ cells on the germinal ridge. At 49 DPF the gonad had developed into a cyst like structure that was still undifferentiated. The gonads of developing coho remained in an undifferentiated state until 70 DPF. At this time slight differences could

Figure 7. Histograms and g2 (kurtosis) tests (g2/standard error) of sex steroids (pg/gm tissue) for developing coho salmon at 20, 42, and 56 days postfertilization (DPF). Steroids include testosterone (T), 11-ketotestosterone (KT), and 17β-estradiol (E2). Hatching occured at 42 DPF.

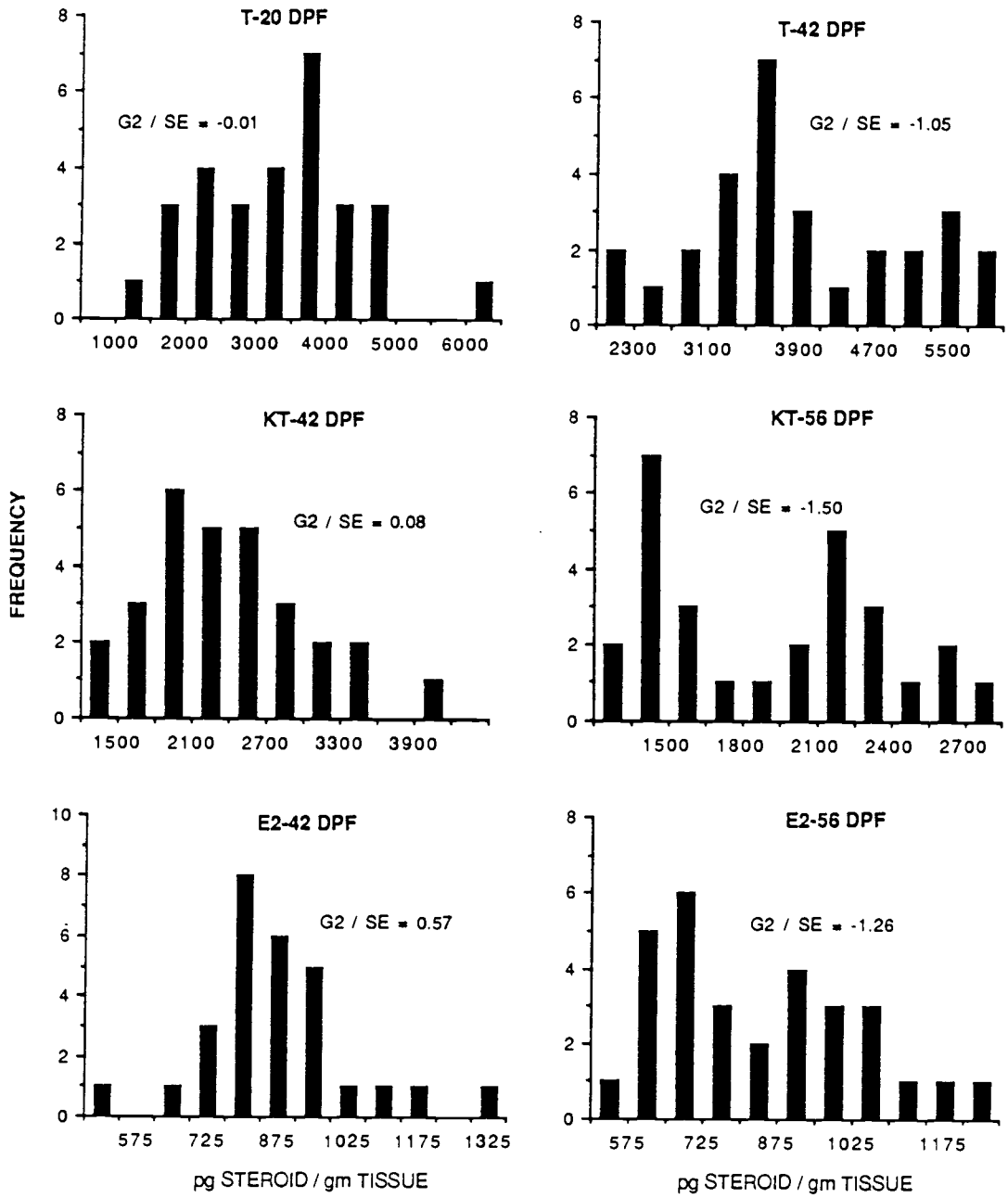

T-20 DPF
G2 / SE = -0.01
1000 2000 3000 4000 5000 6000
T-42 DPF
G2 / SE = -1.05
2300 3100 3900 4700 5500
KT-42 DPF
G2 / SE = 0.08
1500 2100 2700 3300 3900
KT-56 DPF
G2 / SE = -1.50
1500 1800 2100 2400 2700
E2-42 DPF
G2 / SE = 0.57
575 725 875 1025 1175 1325
E2-56 DPF
G2 / SE = -1.26
575 725 875 1025 1175
FREQUENCY
pg STEROID / gm TISSUE
pg STEROID / gm TISSUE

FIGURE 7.

Figure 8. Transverse section of a coho salmon undifferentiated gonad at hatch (42 days postfertilization) showing a nest of primordial germ cells. Bars represent 25 μm.

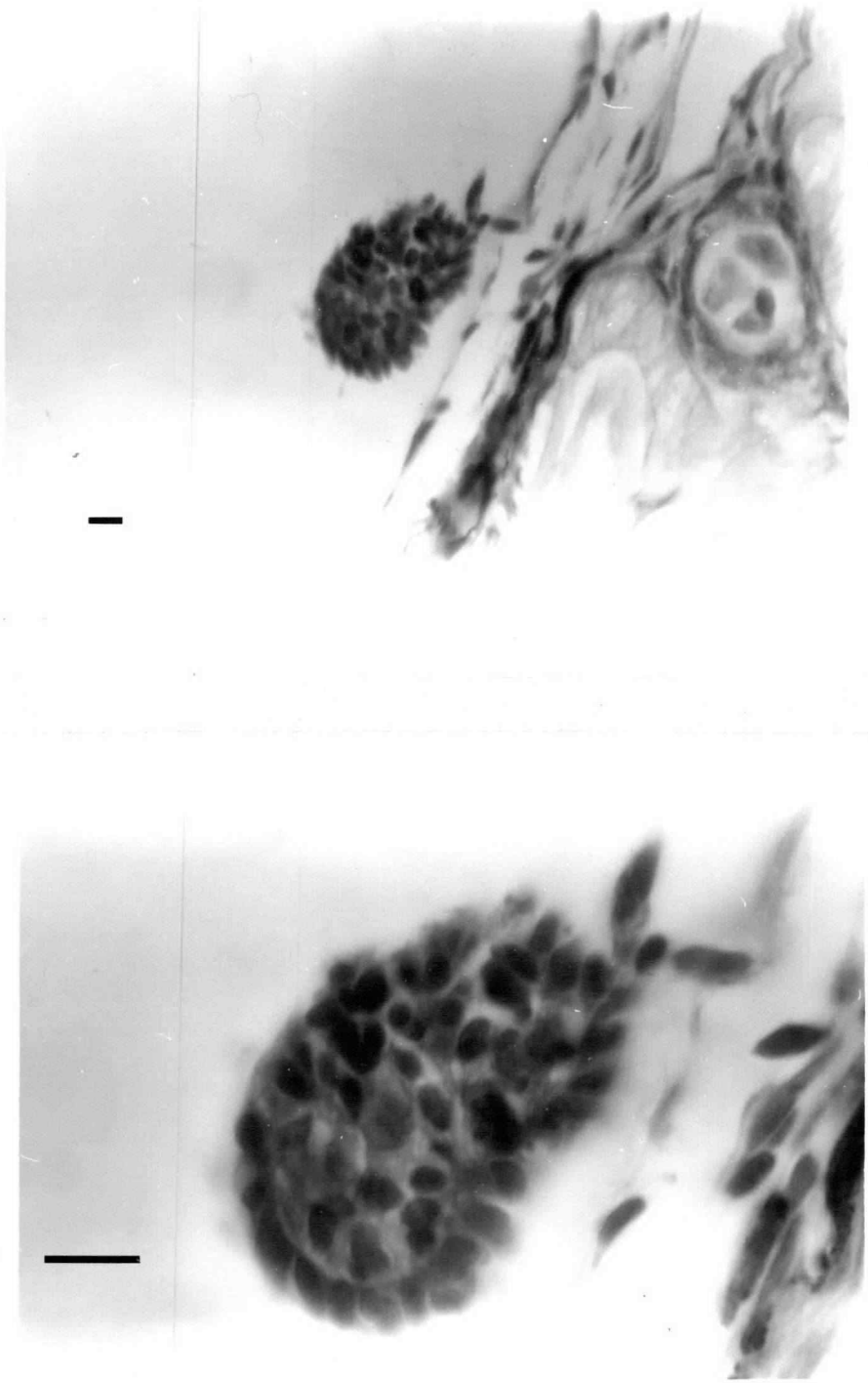


FIGURE 8.

be seen with some gonads containing larger and lighter staining gonocytes than others. We suspect that these gonads were destined to become ovaries. Differences, however, were very subtle. At 77 DPF a clear difference between males and females could be seen. Ovaries showed the typical lamellar structure and contained oogonia and perinucleolar oocytes (Fig. 9). In some cases oocytes were surrounded by follicular elements. The development of oocytes within this one week period was remarkable. Ovaries at 87 DPF appeared similar to those at 77 DPF (Fig. 10).

Testes at 77 DPF appeared granular (Fig. 11). In some cases testes contained blood vessels and a sperm duct. At 87 DPF testes contained sertoli-like cells and lobule and sperm duct formation was more complete (Fig. 12). It appeared that gonadal differentiation, at this level of resolution, occurred four to five weeks after hatch.

Histological analysis of sexed fish confirmed the presence of oocytes and a gonad with a lamellar structure for "females". "Males" typically had a granular gonad with smaller and darker staining germ cells present.

DISCUSSION

We have monitored hormonal events during early development of coho salmon as a means of elucidating possible endocrine control mechanisms of sexual differentiation. Results from this study indicate that developing coho are able to synthesize sex steroids either

Figure 9. Transverse section of a coho salmon ovary at 77 days postfertilization showing development of oogonia and follicular elements. Bars represent 100 μm.

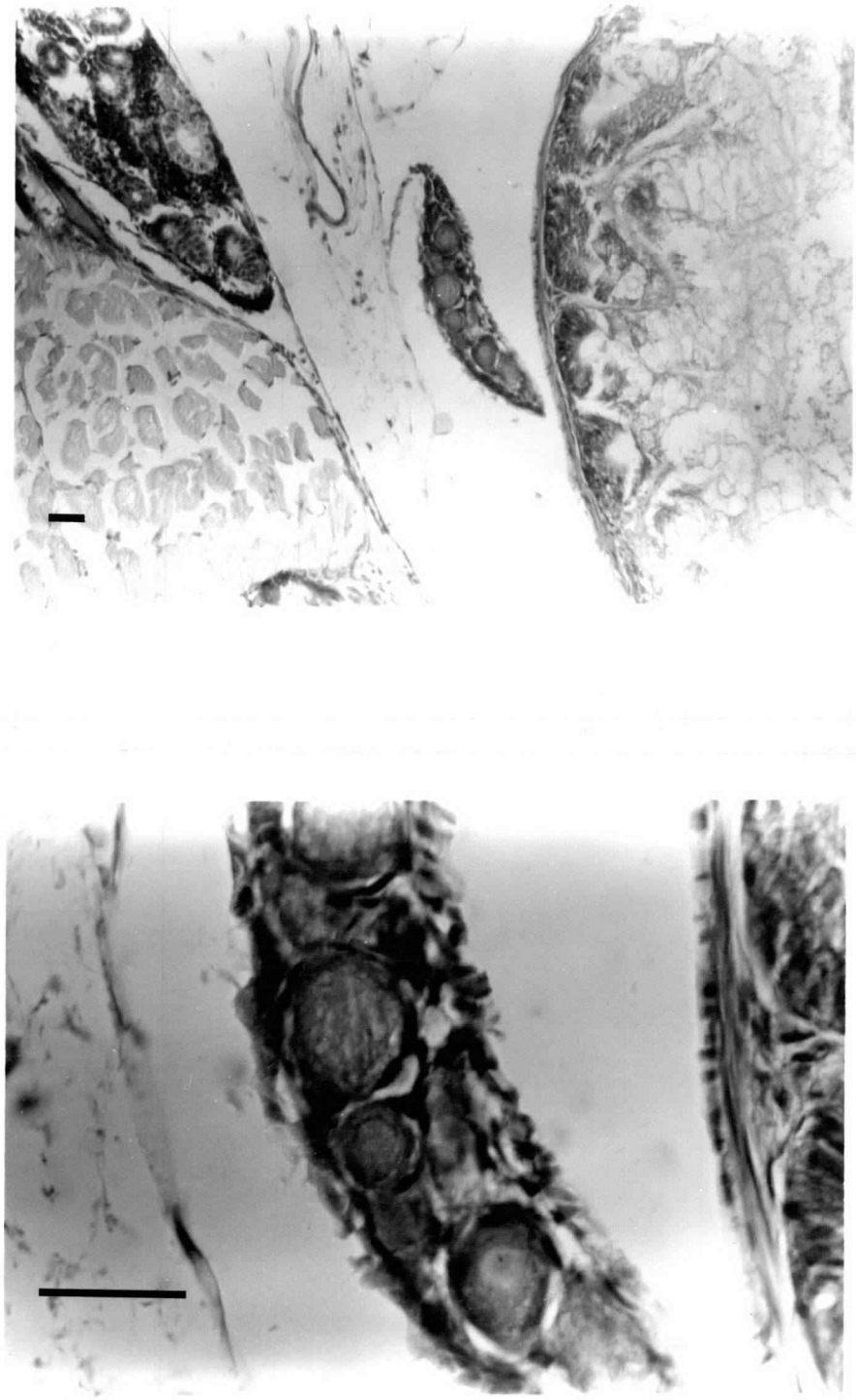


FIGURE 9.

Figure 10. Sagittal section of a coho salmon ovary at 87 days postfertilization showing lamellar structure, oogonia, and oocytes in the perinucleolus stage. Bar represents 200 μm.

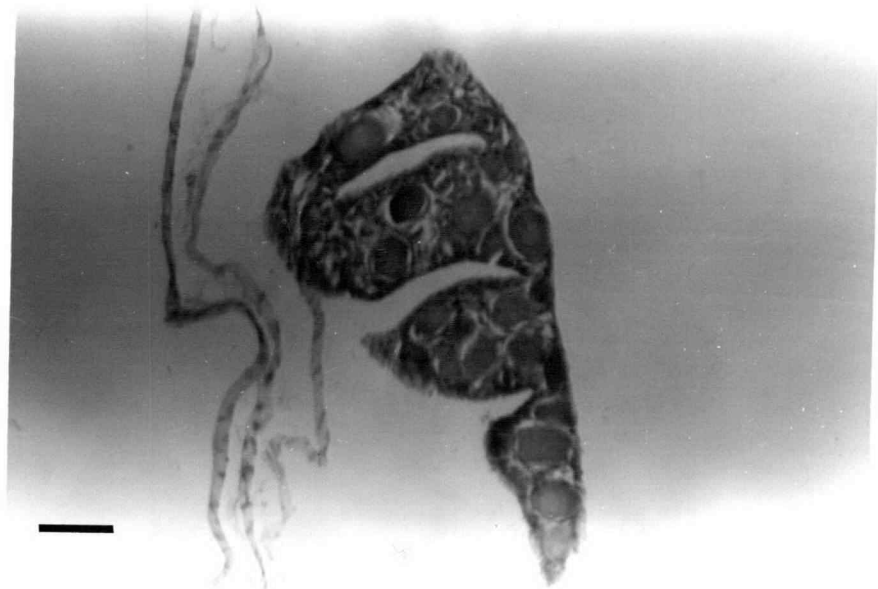


FIGURE 10,

Figure 11. Transverse section of a coho salmon testis at 77 days postfertilization. Bars represent 100 μm.

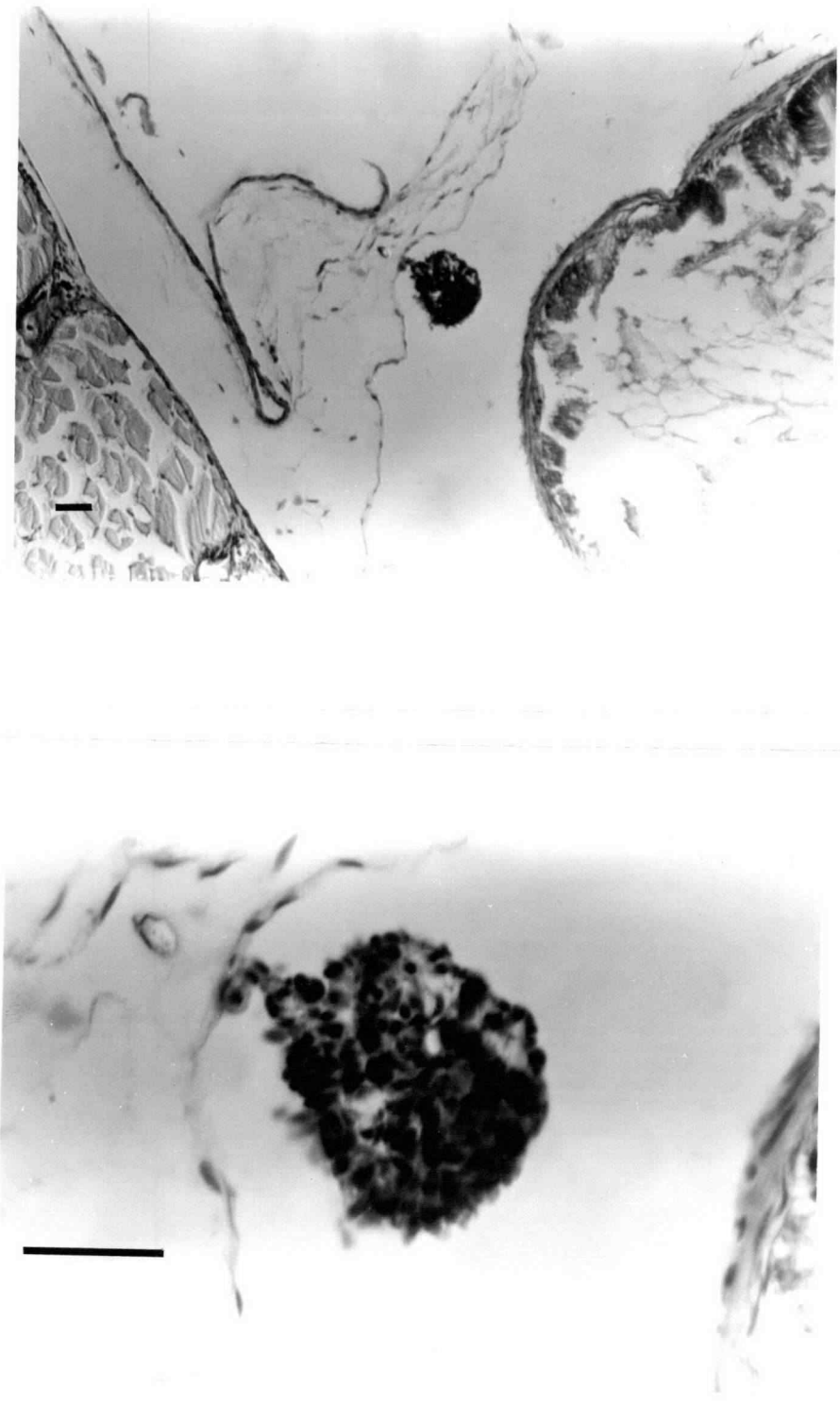


FIGURE 11.

Figure 12. Sagittal section of a coho salmon testis at 87 days postfertilization. Bar represents 200 μm.

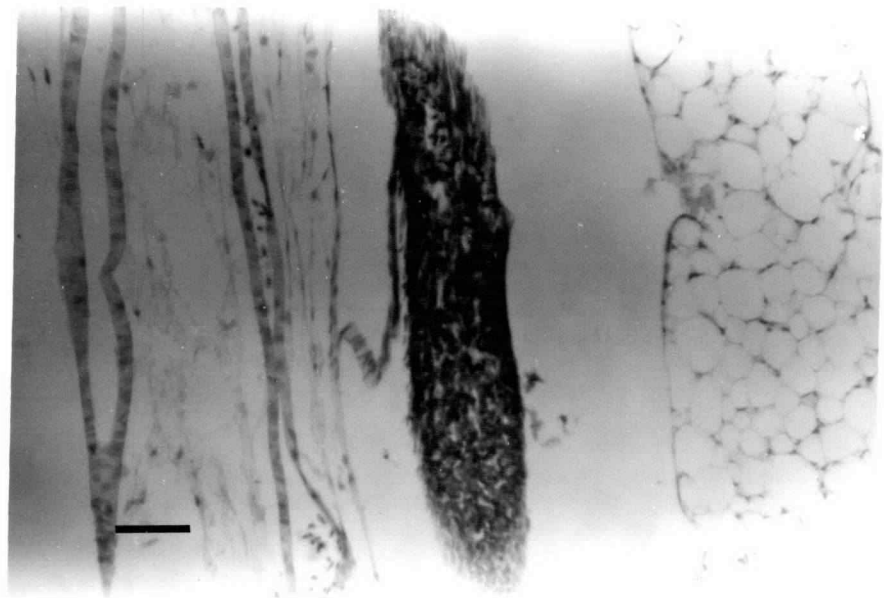


FIGURE 12.

before or concomitantly with the onset of gonadal differentiation and that patterns of hormonal concentrations distinguish themselves dimorphically before gonadal differentiation can be identified with light microscopy.

Steroid levels were found to be relatively high in unfertilized eggs and paralleled the profile seen in ovarian fluid. Steroid levels in embryonic fish then decreased dramatically during the first 30 days of development and next increased slightly at the time of hatching. It was also shortly after hatch that bimodal distributions of steroids became apparent. Hormone levels remained relatively constant during the month following yolk sac absorption for all steroids examined except for E2 which increased slightly during this period. Androgen, but not E2 or DHP, levels in presumptively sexed coho showed sexual dimorphisms. Histological analyses showed the presence of an undifferentiated gonad up to 4 weeks post hatch (70 DPF), followed by remarkable ovarian and testicular development one week later. This development occurred approximately one month after the slight increase and appearance of bimodal distributions of steroids at hatching.

If the hypothesis that sex steroids are the natural inducers of sex differentiation in fishes is true (Yamamoto, 1969), then one may expect to see changes in steroid levels in developing fish either before or during gonadal differentiation. We found this to be the case in coho salmon. The increase in whole body content of all steroids

examined at hatch, coupled with their bimodal distributions shortly after hatching, and the increase in E2 levels seen during ovarian development, may indicate a possible role of sex steroids in the process of gonadal differentiation seen later in development. Changes in steroid levels at hatching and bimodal distributions may also be indicative of the onset of sexual differentiation even though no signs of gonadal differentiation were histologically discernable at this time.

Although few studies have examined the onset of steroidegenesis in the differentiating teleostean gonad, most have provided evidence which supports our finding of steroidegenic capabilities of coho during very early developmental stages. Early steroidegenic capabilities have been documented for the guppy (Takahashi and Iwasaki, 1973), rainbow trout (van den Hurk et al., 1982; Antila, 1984) and tilapia (Rothbard et al., 1987).

We found that developing coho are able to synthesize sex steroids either before or concomitantly with the onset of gonadal differentiation. The authors cited above concluded that steroid production in developing teleosts is possible only after the onset of gonadal differentiation. Experiments in this lab (Fitzpatrick, Redding, and Schreck, unpublished data), have indicated that species differences between coho salmon and rainbow trout may exist with regard to steroid synthesizing capability of the developing gonad. In vitro gonadal incubations suggest that steroidegenesis or

steroid secretion is initiated much earlier in the coho than in the rainbow trout. Coho salmon also appear to differ from mammals, where gonadal differentiation precedes steroid synthesis (Wilson et al., 1981).

Observations that exogenous steroids can influence gonadal development support the thought of sex steroids as possible inducers of sexual differentiation in fish. The ability of exogenous androgens to influence gonadal development in a male direction in salmonids has been documented for coho salmon (Goetz et al., 1979; Redding et al., 1987), and rainbow trout (Jalabert et al., 1975; Simpson et al., 1976; Yamazaki, 1976; Johnstone et al., 1978; Okada et al. 1979; van den Hurk and Slof 1981; Sower et al., 1983; van den Hurk and van Oordt, 1985). Feminization by administration of estrogenic compounds has been seen in salmonids for coho salmon (Goetz et al., 1979; Hunter et al., 1986; Redding et al., 1987), chinook salmon (O. tshawytscha) (Hunter et al., 1986), chum salmon (O. keta) (Redding et al., 1987) rainbow trout (Okada, 1973; Jalabert et al., 1975; Simpson et al., 1976; Sower et al., 1983), and Atlantic salmon (S. salar) (Simpson, 1975-76; Johnstone et al., 1978).

Experiments concerned with the administration of sex steroids to coho salmon during early development in our laboratory (Redding, Schreck, Fitzpatrick, Feist, unpublished data), have shown that feeding of both testosterone and 17α-methyltestosterone (MT) have failed to

affect sexual differentiation. Immersion of newly hatched embryos in MT, however, produced all-male populations while T induced ovarian regression. The increase in androgens seen at hatch may be playing a role similar to that of exogenously administered steroids. Salmonid embryos may only be sensitive to androgens during a very early period of development (before the onset of feeding). Administration of E2 from first feeding, however, does affect gonadal differentiation. The findings that whole body E2 content not only rose at hatch, but also increased concomitantly with the rapid development of ovaries between 70 and 77 DPF also substantiate this finding. E2, unlike the androgens, may have an effect on sex differentiation at a later period in development. Speculatively, increases in androgen and estrogen levels at hatch may be required to induce gonadal differentiation and a further increase in E2 later in development is required for female differentiation.

Although no difference in E2 content between presumptively sexed male and female coho was found, we do not feel comfortable that we have proven that differences in E2 do not exist. E2 levels were relatively low and close to detection limits on the RIA standard curve. Non-specific binding was also higher than that for the other steroid assays. These problems may have obscured slight differences if they existed.

The finding that the steroid profile seen in ovarian fluid is paralleled by unfertilized eggs is not surprising.

Steroids are lipophilic and easily cross cell membranes. Sex steroid content of ovarian fluid found by us in this study is also similar to that seen in plasma for ovulating coho salmon (Fitzpatrick, 1986). Similarly, sex steroid content of sperm and seminal fluid parallels levels found in plasma of mature males (Fitzpatrick, 1986). E2 has also been measured in ovarian fluid from the killifish (Fundulus heteroclitus) (Bradford and Taylor, 1987), and was found to be similar to circulating levels at that time. We wonder about a possible role for these steroids in either gametes or the early developing embryo, but at this point any thoughts would be pure speculation.

The precipitous decline in T, KT, and E2 content of developing embryos from unfertilized eggs to 30 DPF has also been documented in tilapia (Rothbard et al., 1987). Antila (1984) has also shown the ability of rainbow trout embryos to metabolize steroids between the ages of 0 and 8 DPF. In our study levels of E2 and DHP were higher in non-eyed eggs when compared to eyed eggs. It is difficult to believe that the decline in steroids can be explained by their leaching out of eggs, particularly when considering the lipophilic nature of these molecules. The decline in steroid content during the first 42 days of development is most likely due to metabolic processing of maternal steroids by embryos. It is also intriguing that a relatively small increase in steroids seen at hatch may affect or be involved in sexual

differentiation after embryos have been exposed to extremely high levels of maternal hormones during early development.

The increase in T and E2 content of embryonic coho at hatch has also been documented for tilapia (Rothbard et al., 1987), although the increase for this species was seen in fry at 4-6 weeks post fertilization which is slightly earlier than for coho, most likely attributable to the fact that tilapia are a warm water species with accelerated development. The increase in steroidal content seen at hatch is most likely due to synthesis by embryos but may also be caused by metabolic processing by embryos of other maternal steroids not monitored by RIA.

It is interesting that the androgens showed similar patterns to one another during yolk sac absorption while E2 and DHP had similar trends to each other during this time. Possibly the androgens are acting in a concerted fashion to induce masculinization while the estrogens and progestins are functioning similarly to induce feminization. P4 when added to aquarium water (300 μg/l) and administered for 4 weeks from hatching, or from 43 DPF, significantly affected the sex ratio in favor of females in rainbow trout (van den Hurk and Slof, 1981), while administration of P4 incorporated into food (60 and 600 μg/g) had no effect on sex ratios (van den Hurk and Lambert, 1982).

The appearance of bimodal distributions of steroid content during early development may be an indication of sexual dimorphisms. Bimodality was particularly pronounced

for KT, which may give credence to van den Hurk and van Oordt's (1985) idea that 11-oxygenated androstenedione derivatives are particularly important in sustaining the differentiation and early development of the testes in rainbow trout. These authors found that administration of 11α-hydroxy-androstenedione resulted in transient masculinization of the gonad whereas administration of androstenedione did not. We have recently reproduced this finding in coho (unpublished data) which suggests that 11-position substituted androgens may indeed be important for initiating the formation of testes. It is interesting to note that bimodalities in sex steroid content during yolk sac absorption disappeared at the onset of feeding and did not reappear until approximately 40 days later. Preparatory in vitro experiments in this lab (Fitzpatrick) have also shown this to be the case with steroid secretions of coho gonads exhibiting bimodal patterns shortly after hatch which disappeared at the onset of feeding and did not return until later in development. Sexual dimorphisms of testosterone during early development has been documented in tilapia (Rothbard et al. 1987). Testosterone concentrations in 6-8 week old fry in bisexual populations showed bimodal distributions while interspecific crosses, characterized by 100% male progenies, showed unimodal distributions with regard to testosterone content. Sexual dimorphisms of steroids during later development have also been seen in plasma of coho, as during smoltification (Patino and

Schreck, 1985), and final maturation (Fitzpatrick et al., 1986).

Our histological analyses of developing gonads are in general agreement with those reported for coho by Goetz et al. (1979), and for other salmonids by Robertson (1953), Ashby (1957), Takashima et al. (1980), and van den Hurk and Slof (1981). At this level of resolution gonadal differentiation appears to occur dramatically in a one week period approximately 30 days after hatch. Up to this point, no clear distinctions between males and females could be discerned. The inability to identify sex before this period is problematic for research attempting to describe the process of sexual differentiation. The production and histological monitoring of unisexual populations may demonstrate subtle differences in gonadal differentiation which are presently unresolved. Correlation of hormonal events with histology of the differentiating gonad for monosex populations could provide invaluable information on the process of sexual differentiation.

Although we have shown that the steroidal milieu of developing coho salmon is indeed dynamic during the period of sexual differentiation, it is still impossible to conclusively determine whether dynamics in sex steroids are a cause of sexual differentiation or simply a result of it. Use of monosex populations and descriptions of other components of the endocrine system such as the hypothalamic-

pituitary-gonadal axis during early life stages will undoubtedly increase our understanding of this process.

HORMONAL CONTENT OF COMMERCIAL FISH DIETS AND OF YOUNG COHO SALMON, ONCORHYNCHUS KISUTCH, RECEIVING THESE DIETS

INTRODUCTION

Commercial fish diets are typically prepared from reproductively mature fish by-products and may consequently contain hormones. Hormones, such as steroids, are known to affect a wide variety of developmental and physiological processes in teleosts and if present in fish diets may have an effect on these processes. Thyroid hormones, such as thyroxine, are important for early growth and development in many vertebrate species (Dickhoff and Darling, 1983). Administration of sex steroids during early development results in varying degrees of sex inversion in a variety of teleostean fishes (see reviews by Yamamoto, 1969; Schreck, 1974; Donaldson and Hunter, 1982; Hunter and Donaldson, 1983). Androgens typically act as andro-inducers and estrogens as gyno-inducers. By virtue of this ability to cause sex inversion in the medaka, Oryzias latipes, Yamamoto (1962, 1969) advanced the theory that sex steroids are the natural inducers of sexual differentiation in fishes. Juvenile fish in the wild are not likely to be exposed to steroids in their diet (Sower and Iwamoto, 1985). Hatchery reared salmonids, however, may be exposed to relatively high levels of steroids from fish food throughout early development. The presence of sex steroids in commercial

fish diets may therefore have an influence on early developmental processes such as sexual differentiation.

We know of only one other study which has examined steroid levels in fish diets. Sower and Iwamoto (1985) found ng/gm feed quantities of testosterone in seven varieties of commercial salmon diets. Only two studies have examined endogenous steroid levels in teleosts during sexual differentiation (Rothbard et al., 1987; Feist et al., 1987).

Our objective was to determine steroid hormone and thyroxine levels in a number of commercial fish diets, to examine shelf life of steroid content of these diets, and to evaluate sex steroid profiles of two populations of developing coho salmon, Oncorhynchus kisutch, placed on either a "high" steroid level diet (Biomoist) or a "low" steroid level diet (casein based). Sex steroid content of developing coho was analyzed to determine possible effects of sex steroids on sex ratios, histology of the developing gonad, and hormone dynamics during the period of final sexual differentiation. Diets examined included two moist feeds, Biomoist starter #2 and Oregon Moist Pellets, a dry diet, Abernathy 1/8 inch pellets and an experimental casein based diet. Hormones measured included; testosterone, 11-ketotestosterone, androstenedione, progesterone, 17α-hydroxy-20β-dihydroprogesterone (17α,20β-dihydroxy-4-pregnen-3-one), 17β-estradiol, and thyroxine.

MATERIALS AND METHODS

Fish Diets. Biomoist starter #2 and Oregon Moist Pellets (OMP-3/32 and 1/8 inch) were obtained from Bioproducts, Inc., Warrenton, Oregon. Abernathy 1/8 inch pellets were obtained from Murray Feed Co., Washington. The casein based diet was obtained from Oregon State University's Department of Food Science and Technology, Corvallis, Oregon.

Fish Rearing and Sampling. Coho salmon gametes were obtained from Oregon Aqua-Foods hatchery in Springfield, Oregon, fertilized in November, 1985, and raised at Oregon State University's Smith Farm facility in Corvallis with a constant water temperature of 12^{o} C. At the onset of exogenous feeding, 60 days postfertilization (DPF), developing coho were placed on either Biomoist or casein diets. Fish from each diet (n=40) were sampled at 3, 10, and 17 days post onset of exogenous feeding (63, 70, and 77 DPF). Steroid levels were determined for 30 animals and 10 animals were fixed in Bouin's solution for histological analysis on each sampling date. An additional sample of 25 for each group was taken at 87 DPF. Steroid levels were determined for 15 animals and 10 were reserved for histology. Animals were fed 3-4 times daily to satiation.

Extractions. Diets (0.1 g each) and fish to be analyzed for whole body sex steroid content were sonicated in 1.0 ml of 0.1 N NaOH and extracted twice with 8 volumes

of diethyl ether. Tubes were vortexed vigorously for 30 seconds after addition of ether. Tubes were spun at 2000 g for 20 minutes after each ether extraction. The aqueous phase was removed by snap freezing in liquid nitrogen. Combined extracts were dried in a Speed Vac™ and stored at -20° C until analyzed for testosterone (T), 11-ketotestosterone (KT), androstenedione (A), progesterone (P4), 17α-hydroxy-20β-dihydroprogesterone (DHP), and 17β-estradiol (E2).

Extraction efficiencies for all steroids examined were determined at each sampling date (n=5 for fish, n=3 or 4 for diets) by addition of tritiated steroids to tubes containing whole fish or diet samples and 0.1 N NaOH. Spiked tubes were then sonicated and extracted. Spiked extracts were resuspended in 1.0 ml of assay media (phosphate buffered saline with gelatin -PBSG) and 0.5 ml were then counted on a scintillation spectrophotometer. Average extraction efficiencies for developing fish for T, KT, A, DHP, and E2 were 63.6, 57.2, 48.8, 65.6, and 57.9% respectively. Average extraction efficiencies for T, KT, A, P4, DHP and E2 for the Biomoist diet were 49.0, 56.0, 47.5, 40.5, 56.2, and 47.3% respectively. For the casein diet efficiencies were 57.3, 47.9, 65.9, 42.5, 62.7, and 61.8%, respectively. For the Abernathy diet efficiencies were 56.4, 49.1, 55.5, 56.0, 63.6, and 55.4% respectively. All assays were corrected for recovery.

Extracts of whole fish, and casein samples to be analyzed for sex steroid content were resuspended in 1.0 ml of PBSG and 0.1 ml aliquots removed for each steroid examined. Diet extracts of Biomoist and Abernathy were resuspended in 2.0 ml of PBSG and 0.1 ml aliquots assayed. Diets were stored for four months at -20° C after the first evaluation and then extracted and assayed for sex steroid content to determine shelf life of steroid levels in the diets.

Diets to be analyzed for thyroxine (T4) content were extracted in an identical manner as that for steroids except ethanol was used instead of diethyl ether. Extraction efficiencies were also determined in a similar manner. Extracts were resuspended in 1.0 ml of ethanol and 0.5 ml counted. Extracts to be analyzed for T4 content were resuspended in 1.0 ml ethanol and 0.1, 0.3 and 0.5 ml aliquots removed, dried and assayed. Average T4 extraction efficiencies (n=3) for Biomoist, Abernathy, OMP-3/32 inch, and OMP-1/8 inch pellets were 71.6, 61.9, 81.8, and 84.1% respectively.

Assays. Sex steroid content of diets and whole body fish extractions for T, KT, P4, DHP, and E2 were measured by RIA as described by Sower and Schreck (1982) and modified by Fitzpatrick et al. (1986). Androstenedione was analyzed using an anti-androstenedione-antiserum (AN6-22) obtained from Endocrine Sciences (Tarzana, California) and diluted 1:30. A slightly more concentrated charcoal solution (6.25

g charcoal and 4.0 g dextran/ liter PBSG) was used for all sex steroid assays to reduce non-specific binding. 1.0 ml of dextran coated charcoal was added to each tube for the T, KT, A, DHP, and P4 assays and 0.5 ml for the E2 assay. The lower limit of detection was 3.1 pg/tube for all assays except E2 which was 1.25 pg/tube. The intra-assay coefficient of variation for all steroid assays was less than 5%. Thyroxine content of fish diets was measured by RIA as described by Dickhoff et al. (1978). The lower limit of detection was 1.5 ng/tube.

All hormone concentrations measured via RIA were validated by confirming that serial dilutions were parallel to standard curves. Assays were further validated by accounting for addition of cold steroids to samples. We have also shown that this assay procedure is valid for steroid estimation in lipid-rich tissues by thin layer chromatographic separations followed by the RIA (Feist et al., 1988). Steroids present in quantities sufficient for detection by high-pressure liquid chromatography (HPLC) were also validated by correlating levels with those found by RIA. The concentrations of T, DHP, KT, A, and P4 in Biomoist were the only hormones which allowed for this technique.

High Pressure Liquid Chromatography. A modified method from Huang et al. (1983) was used for HPLC analysis. Samples for HPLC were prepared in the same way as that for RIA followed by an additional extraction with Baker-10 SPE

octadecyl (C18) cartridges (see Zief et al., 1982). The HPLC system consisted of an IBM LC/9533 Ternary Gradient Liquid Chromatograph, an IBM 9000 computer, two fixed wavelength UV detectors at 254 and 280 nm and a Hewlett-Packard 100 x 2.1 mm reverse phase (C18) column with a pore size of 5 μm. Steroids were eluted with an isocratic mobile phase (0.4 ml/min) of water:methanol:acetonitrile: isopropanol 62:28:5:5 immediately followed by a linear gradient of water:methanol:butanol 35:45:20 for 30 minutes. This solvent system allowed for the separation of 16 standard steroids and gave sensitivities of 5-10 ng per steroid.

Histology. Fish sampled for histological analysis were fixed in Bouin's solution, and embedded in paraffin. Serial sections (8-10 μm) were taken either transversely or sagitally. Sections were stained with hemotoxylin and eosin, permanently mounted, and examined by light microscopy. Descriptions of gonadal differentiation of coho salmon, outlined by Goetz et al. (1979), and of other salmonids, outlined by Robertson (1953), Ashby (1957), Takashima et al. (1980) and van den Hurk and Slof (1981), were used to identify stages of germ cell and gonadal differentiation.

Statistics. Data for sex steroid content of developing coho were analyzed by the nonparametric Mann-Whitney rank sum statistic. This statistic computes the significance of differences between medians of two populations after a

preliminary analysis of variance. The level of significance used for all tests was $P < 0.05$.

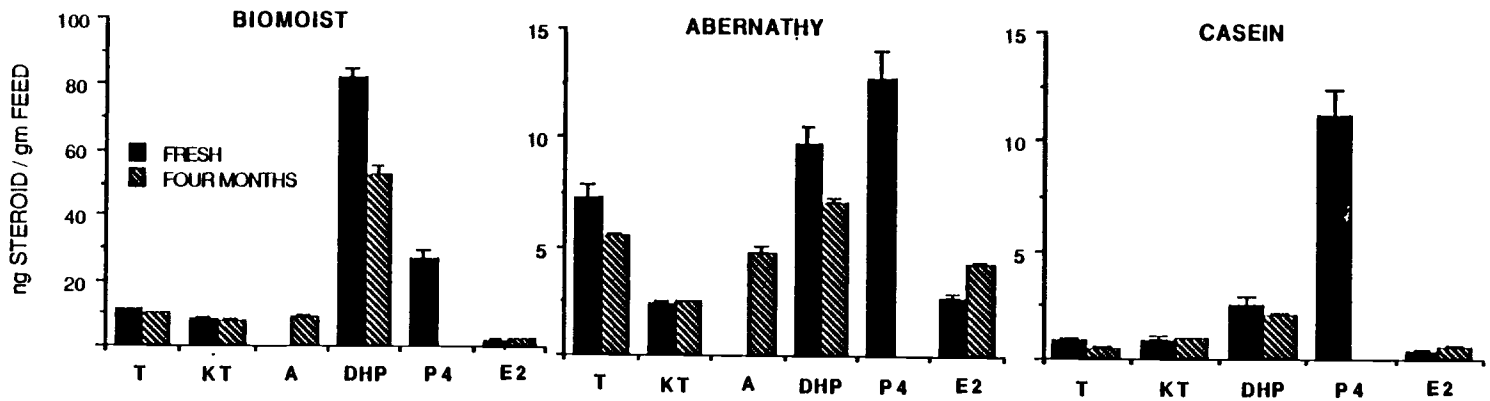
RESULTS

Sex Steroid Content of Diets. All of the fresh fish diets examined contained detectable levels of sex steroids (Fig. 13). DHP was by far the most prevalent steroid found in the Biomoist diet (mean value of 81.9 ng/g) and was also relatively high in the Abernathy diet (9.7 ng/g). T and P4 were also relatively abundant in both Biomoist (11.2 and 26.9 ng/g, respectively) and Abernathy (7.3 and 12.8 ng/g, respectively) diets. KT and A content of Biomoist (8.5 and 9.0 ng/g, respectively) was higher than that found in Abernathy feed (2.4 and 4.8 ng/g, respectively). E2 was present in the lowest concentrations of all steroids examined (2.2, 2.8, and 0.4 ng/g for Biomoist, Abernathy, and casein based diets respectively). The casein based diet contained comparatively low levels of all steroids examined except for P4 (11.2 ng/g).

All steroids examined were still present after four months of storage (Fig. 13). Generally, decreases in steroid content of these three diets was seen over time but shelf life of steroid concentrations varied considerably. Testosterone content decreased by an average of 12, 25, and 44% for Biomoist, Abernathy, and casein, respectively. Shelf life for DHP was less variable with mean decreases of

Figure 13. Concentration (ng/g) of sex steroids in three varieties of fish diets (Biomoist starter #2, Abernathy 1/8 inch pellets, and a casein based diet). Steroids include; testosterone (T), 11-ketotestosterone (KT), androstenedione (A), 17α-hydroxy-20β-dihydroprogesterone (DHP), progesterone (P4), and 17β-estradiol (E2). After the initial evaluation diets were stored for four months at -20° C until re-analyzed. Each value represents the mean ± the standard error for a sample size of 9 for the first evaluation and 3 for the second.

FIGURE 13.


BIOMOIST
ABERNATHY
CASEIN
ng STEROID / gm FEED
FRESH
FOUR MONTHS
100
80
60
40
20
15
10
5
T
KT
A
DHP
P4
E2

36, 27, and 23% respectively. KT content was relatively constant during the four month holding period. Slight increases in E2 during this period were probably due to higher non-specific binding in the second assay series. The largest percent decreases in steroid concentrations occurred for steroids initially present in the largest amounts.

Thyroxine. Thyroxine was also present in all diets examined (Fig. 14). Levels of thyroxine were statistically similar for all three diets.

Sex Steroid Content of Developing Coho. Sex steroid content of whole developing coho salmon placed on either the "high" level steroid diet (Biomoist) or the "low" steroid level diet (casein) are given in Fig. 15. Steroid content at 3 days post onset of feeding (63 DPF) is higher than at subsequent dates and a general decrease can be seen through time in all sex steroids examined.

Differences in steroid concentrations found in Biomoist and casein based diets are generally reflected in the steroid content of fish receiving these diets. Casein fed fish contained considerably less steroids than Biomoist fed fish for most steroids and sampling dates examined. The Mann-Whitney test statistic confirmed differences in steroid levels between fish receiving the two diets for T, KT, A, and DHP at 3 days post onset of feeding and for all five steroids examined at 10 and 27 days post onset of feeding. Differences seen at 17 days post feeding were not statistically significant. It should also be noted that a

Figure 14. Concentration (ng/g) of thyroxine in four varieties of commercial fish diets (Biomoist starter #2, Oregon moist pellets 3/32 and 1/8 inch, and Abernathy 1/8 inch pellets). Each value represents the mean ± the standard error for a sample size of 3.

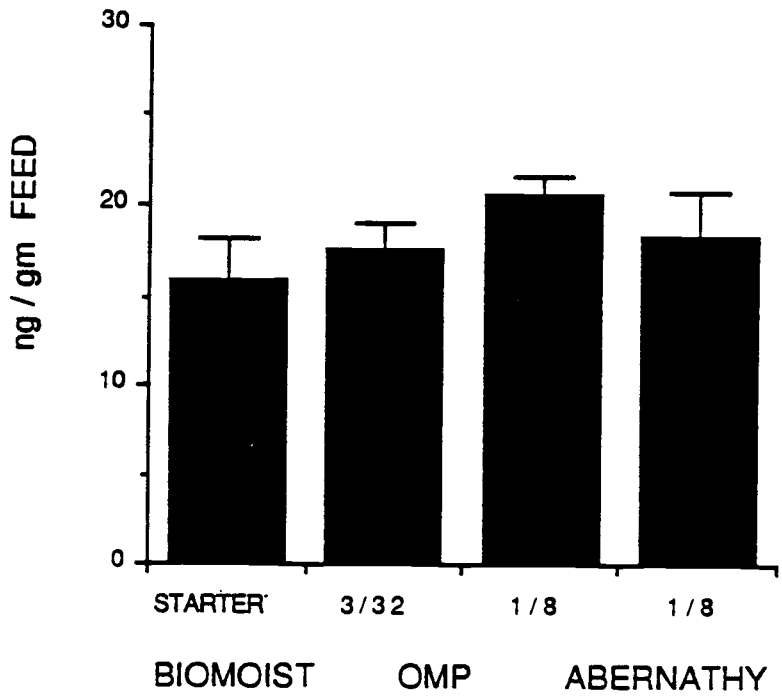


FIGURE 14.

Figure 15. Concentration (pg/gm tissue) of sex steroids in coho salmon fed two varieties of diets (Biomoist starter #2, and a casein based diet). Steroids include; testosterone (T), 11-ketotestosterone (KT), androstenedione (A), 17α-hydroxy-20β-dihydroprogesterone (DHP), and 17β-estradiol (E2). Each value represents the mean $\pm$ the standard error for a sample size of 27-30 for all dates except 27 days post-feeding where n=16. Stars indicate statistical significance between fish fed the different diets for $p < 0.05$.

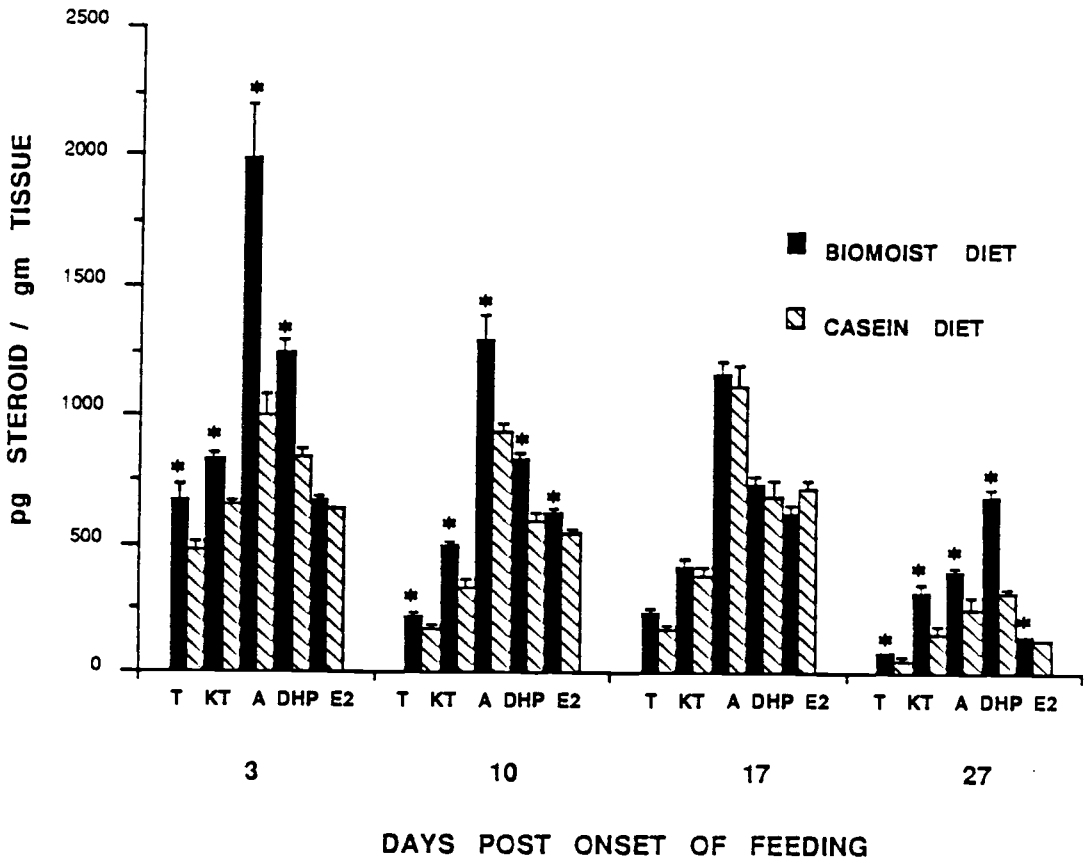

2500
2000
1500
1000
500
0
pg STEROID / gm TISSUE
BIOMOIST DIET
CASEIN DIET
T KT A DHP E2
T KT A DHP E2
T KT A DHP E2
T KT A DHP E2
3
10
17
27
DAYS POST ONSET OF FEEDING

FIGURE 15.

general decrease in the degree of difference between steroid content of fish placed on the two diets was evident as development progressed. That is, the difference in steroid content of Biomoist and casein fed fish is much greater at 3 days post feeding when compared to 10 days post feeding. By 27 days post feeding statistically significant differences had returned.

Histology. Gonads were evident at 63 DPF (3 days post onset of feeding) but appeared undifferentiated, consisting of a cyst like structure containing primordial germ cells. The gonads of developing coho remained in an undifferentiated state until 70 DPF. At this time slight differences could be seen among some animals. Some gonads contained larger and lighter staining gonocytes than others. It is suspected that these gonads were destined to become ovaries. Differences were very subtle, however, and no clear distinctions could be made. At 77 DPF a clear difference between males and females could be seen. Ovaries showed the typical lamellar structure and contained perinucleolar oocytes. In some cases oocytes were surrounded by follicular elements. Ovaries at 87 DPF appeared similar to those at 77 DPF.

Testes at 77 DPF appeared granular. In some cases testes contained blood vessels and a sperm duct. At 87 DPF sertoli-like cells and lobule and sperm duct formation was more complete. See Feist et al. (1988) for a more complete description of histology of developing gonads. It appeared

that gonadal differentiation occurred four to five weeks after hatch at 42 DPF.

No histological differences could be seen between Biomoist and casein fed fish with regard to gonadal differentiation or development. Sex ratios were similarly unaffected by diet, being essentially 1:1.

DISCUSSION

We have demonstrated the presence of sex steroids and thyroxine in several varieties of commercial fish diets. Sex steroid content of fish diets was reflected by whole body steroid content of developing coho receiving these diets. Differences in concentrations of the hormones in the diets apparently had no effect on sex ratios, histology of the developing gonad, or timing of sexual differentiation. Although steroidal content of diets did not affect sexual differentiation of coho, that the continual intake of hormones from fish diets throughout the hatchery or net-pen rearing phase may affect physiological processes seen later in development cannot be ruled out.

Juvenile salmonids in the wild generally feed on invertebrates and are unlikely to be exposed to levels of steroids contained in the the commercial diets (Sower and Iwamoto, 1985). Exposure to androgens later in development may play a role in the high incidence of male sexual precocity commonly found in salmonids in hatcheries and net-

pen rearing systems. Administration of androgens has induced the onset of precocious sexual development for Salmo gairdneri (Crim et al., 1982; Crim and Evans, 1983; Magri et al., 1985) and S. salar (Crim and Peter 1978; Dodd et al., 1978). Androgens are known to stimulate spermatogenesis in salmonids such as O. kisutch, (Fagerlund and McBride, 1977), O. tshawytscha (Schreck and Fowler, 1982), and S. gairdneri (Fagerlund and McBride, 1977; Sower et al., 1983). Androgens may also have an inhibitory effect on the smoltification process in salmonids (Ikuta et al., 1985). Exposure to high levels of anabolic steroids has been shown to have an adverse effect on initiation of entry into seawater and survival for O. kisutch (Fagerlund and McBride, 1975; Fagerlund et al., 1980). Administration of sex steroids and anabolic steroids to salmonids is also known to have varying effects on growth (Donaldson et al., 1978). Anabolic steroids, such as testosterone and 17α-methyltestosterone are currently being evaluated by hatcheries as possible growth inducers. However, microgram quantities of steroids per gram of feed appear to be needed to affect the above mentioned physiological processes.

Levels of thyroid hormones, such as thyroxine become dynamic during smoltification of salmonids and may play a role in this process (Dickhoff et al., 1978). Thyroid hormones have also been shown to effect growth rates of fish (see review by Donaldson et al., 1978). The presence of these hormones in fish diets may also have an effect on

these processes. Again, more research is needed to investigate the possible effects of hormones from diets on physiological processes seen after sexual differentiation.

The amount of sex steroids found in diets may be correlated to the amount of fish by-products incorporated into them. The casein diet contained the lowest levels of steroids of the diets examined; the only fish by-product present in this diet is 10% fish oil. The relatively high levels of P4 found is not surprising since the main constituent of this diet is a milk protein (casein-58%). Abernathy 1/8 inch pellets is comprised of 50% anchovy meal and 6% menhaden oil and contained considerably more sex steroids than the casein diet.

Starter diets and smaller pelleted feeds are generally comprised of a greater percentage of fish by-products than larger pelleted diets and consequently contain higher levels of steroids. Although the constituents of Biomoist starter #2 are a trade secret, Abernathy starter diets typically contain 15% more fish by-products than larger pellet diets. Smaller-pellet diets for OMP contain up to 25% more fish by-products than larger sized pellets. The Biomoist diet most likely contained more fish by-products than Abernathy, accounting for the relatively high levels of sex steroids found in the Biomoist pellets.

Thyroxine content of these diets with regard to pellet size is more or less constant. The lipophilic nature of the sex steroids explains this difference. Diets with larger

percentages of fish oil would be expected to contain a proportionately larger amount of sex steroids and concentrations of thyroxine, which is not generally lipophilic, probably does not depend on fish-oil content.

The relatively high concentrations of DHP found in the Biomoist and Abernathy diets is not surprising since this "maturation" steroid (Nagahama et al., 1983) would be expected to be high in reproductively mature fish by-products. Levels of testosterone found in all three diets examined is consistent with levels reported by Sower and Iwamoto (1985) who found levels of this hormone ranging from 0.4 to 7.0 ng/g feed.

That the steroid content of developing coho at 3 days post-feeding was generally higher than that of the three subsequent sampling dates is most likely attributable to two reasons. First, a small amount of yolk was present in fish at 3 days post feeding. Analysis of yolk has shown the presence of high levels of steroids (Feist et al., 1987). Second, fish sampled at 3 days post-onset of feeding were collected 3 hours after feeding while fish representing 10, 17, and 27 days post-onset of feeding were sampled 12 hours after their last feeding. Metabolic processing of steroids from diets probably plays a role in levels found in fish.

Cortisol content also appears to be substantial in Biomoist starter #2, Abernathy 1/8 pellets and OMP 1/8 and 3/32 inch pellets. Although assayed, cortisol levels have not previously been mentioned due to their low extraction

efficiencies (10.7, 9.1, 19.3, and 16.6% for Biomoist, Abernathy, OMP-3/32 and OMP-1/8 inch pellets respectively). Samples were extracted as previously mentioned and cortisol content determined by an assay described by Redding et al. (1984). Although extraction efficiencies were low, standard errors were small with regard to each diet (0.5, 0.6, 0.1. and 0.7% respectively). Cortisol was found at mean levels of 67.8 ± 8.2, 51.1 ± 5.2, 44.6 ± 0.7, and 35.1 ± 2.6 ng/g feed for Biomoist, OMP-3/32, OMP-1/8 and Abernathy 1/8 inch pellets respectively, when corrected for the extraction efficiencies. Although we place little confidence on the accuracy of these findings, we believe it is important to note the presence of this biologically active corticosteroid in the diets of hatchery salmonids. Cortisol is thought to play a role in the smoltification process of salmonids (Specker and Schreck, 1982). The continual intake of cortisol from fish diets during development may have an effect on this process. Further research is needed to investigate the possible effects of hormones from diets on physiological processes seen after sexual differentiation.

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