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Carl B. Schreck

The hemoglobin patterns of juvenile coho salmon, Oncorhynchus kisutch, were determined by high pH polyacrylamide gel electrophoresis throughout parr-smolt transformation. Hemolysates separated in January displayed one major hemoglobin (Hb) fraction and seven minor Hb fractions. A reduction in the number of minor Hb fractions occurred in May, but these returned by July to a pattern originally exhibited in the winter. Similar shifts in relative proportional abundances of minor and major Hb fractions were observed concurrently; a decrease in relative proportions of major Hb fractions occurred in April and June while proportions of minor Hb fractions increased in June. An inverse relationship existed between relative proportional abundances of minor and major Hb fractions. Changes in other physiological factors indicated fish were undergoing parr-smolt transformation in the spring. Further signs of freshwater adaptation and desmoltification were coincident with a reversion of the Hb system to a pattern exhibited prior to the smoltification process. Changes in the Hb system thus appear to be concurrent with parr-smolt transformation of coho salmon.

L-thyroxine (T_4) administered in the diet (50 µg/g diet) to zero age coho salmon for 65 days tended to suppress the production of minor Hb fractions as seen by a reduction in their numbers and lowered proportional abundances. Plasma T_4 levels were the same for treatment and control groups. Immersion of coho salmon in L-thyroxine (10 µg T_4/100 ml H_2O) significantly elevated plasma T_4 levels, but did not alter the hemoglobin pattern from that observed in the control group.

Multiple Hemoglobin Variations in Coho Salmon, *Oncorhynchus kisutch*, During Parr-smolt Transformation

by

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MULTIPLE HEMOGLOBIN VARIATIONS IN COHO SALMON, ONCORHYNCHUS KISUTCH, DURING PARR-SMOLT TRANSFORMATION

INTRODUCTION

A current management problem faced by salmon hatcheries in the Pacific Northwest is the low survival of salmon to adulthood resulting in a poor contribution of these fish to the commercial fisheries and a low adult return to the hatchery. This problem is attributable, in part, to the inability to determine the correct time of release of high "quality" smolts - i.e. of salmon that are functionally capable of undergoing both migration and the osmoregulatory changes associated with sea-water entry. In preparation for their oceanic existence, salmonids must undergo parr-smolt transformation which entails profound biochemical, physiological, and morphological changes (Hoar, 1976). The physiological status, therefore, of anadromous salmon is important in determining the most effective time of their release, and is critical to the performance and survival of hatchery salmon during and after their seaward migration.

Among the factors currently under consideration as indicators of migratory readiness and seawater survival potential are increases in gill (Na+K)-ATPase activity (Zaugg, 1972) and plasma T_4 concentration (Dickhoff et al., 1978), and variations in plasma sodium levels after an osmotic challenge (Clarke and Blackburn, 1978). In evaluating these patterns however, one must take into account variations due to environmental factors, hatchery practices, and stock differences (Wedemeyer et al., 1980).

The occurrence of a sequential development of electrophoretically distinct multiple hemoglobin components within the erythroctyes has been observed in numerous migrating species of fish: Atlantic salmon, Salmo salar (Koch et al., 1964), coho salmon, Oncorhynchus kisutch (Giles and Vanstone, 1976), and the European eel, Anguilla anguilla (Rizotti, 1977). Functional differences between these observed hemoglobin variants have also been noted in these species (Westman, 1970; Giles and Randall, 1980; Weber et al., 1976). Alterations in the relative rate of synthesis of these functionally unique hemoglobins has been postulated as an adaptive strategy which enables fish to adapt to changes in environmental conditions such as oxygen tension, temperature, and salinity in order to meet changing metabolic requirements (Powers, 1980).

Utilizing starch gel electrophoresis, Vanstone et al. (1964) observed a total of six major hemoglobin fractions and sixteen minor fractions in coho salmon, during development from the fry to adult stages. Two distinct patterns, composed of ten different hemoglobin fractions (three major and seven minor) were later found to be associated with fry and pre-smolt stages of coho salmon (Giles, 1973). Giles and Randall (1980) described the physical characteristics of coho salmon "fry" hemoglobin, (the major fractions designated A_6 through A_8) and found the oxygen equilibria of these major fractions were greatly affected by changes in pH, temperature, and PCO_2. However, the "adult" hemoglobins, which constituted all the other minor fractions present, exhibited only slight changes in the oxygen affinity properties with variations in these parameters.

The function and control of the developmental changes in the synthesis of different hemoglobin types is unclear. Variations in the observed hemoglobin profile with relative increases in length and maturity in Atlantic salmon, Salmo salar (Koch et al., 1966) suggests these changes are associated with the relative rate of development of these fish. Manipulation of environmental factors such as dissolved oxygen concentration, temperature, and salinity by exposing fry and pre-smolt coho salmon was ineffective in inducing changes in the hemoglobin pattern (Giles and Vanstone, 1976). Since it appears that the formation of different hemoglobins produced in the red blood cells is not directly responsive to changes in the immediate environment, but rather is related more strongly to ontogenetic development, the hemoglobin profile may be of potential value as an indicator of the individual's physiological status.

The purpose of this study, therefore, was to examine the ontogenetic variations of multiple hemoglobin synthesis involved during parr-smolt transformation and to determine whether this physiological phenomenon can be related to smoltification by comparing changes in the hemoglobin pattern to other physiological factors indicative of the smolting process.

MATERIALS AND METHODS

Animal Maintenance

Underyearling coho salmon, used to monitor the hemoglobin system at various times throughout parr-smolt transformation, were obtained from Eagle Creek National Fish Hatchery, Oregon in December, 1981. Fish were reared under a natural photoperiod, in 0.7 cubic meter fibre glass circular tanks supplied with fresh wellwater (10-13° C) at Smith Farm, Oregon State University, Corvallis, Oregon. Fish were fed Oregon Moist pellets (OMP) twice daily to satiation at 0900 and 1500 hrs.

Sampling Procedures

Fifteen juvenile coho salmon (Eagle Creek stock) were sampled twice monthly (except once during January and July) and hemoglobin profiles determined. At sampling, all animals were stunned by a blow to the head, measured and weighed. Blood was withdrawn from severed caudal artery into heparinized capillary tubes and microcapillary tubes. Blood samples were centrifuged at 700 g for 5 min. at 0° C in a Beckman TJ-6R refrigerated centrifuge. The plasma supernatant was removed and frozen at -20° C until hormone thyroid analysis. The remaining erythrocytes were then prepared for hemoglobin analysis. The microcapillary tubes were centrifuged after sampling at 11,500 rpm for 5 min. to determine hematcrit values.

Hemolysate Preparations

Fish hemoglobins are known to be unstable (Riggs, 1981). Therefore alterations in the patterns and concentrations obtained by electrophoresis depend on the storage and treatment of fish hemolysates and the methods used to separate them. Hemolysates were prepared by methods adapted from Fyhn et al. (1979). The erythrocytes were washed 3 times with cold 1.7% NaCl in 1mM Tris buffer and lysed for 1 hr with 3 volumes of 1 mM Tris solution (pH 8). A one tenth volume of 100 mM phenylmethyl sulfonyl fluoride (PMSF) and 1 M NaCl was added to the lysed cells to inhibit enzymatic degradation of the hemoglobin macromolecules. This preparation was centrifuged with a Beckman microfuge at 8,800 g for 15 min. at 4° C. The purified hemoglobin supernatant was removed and one eighth volume of solution composed of 0.1 M KCN and 0.1 M $K_3Fe(CN)_6$ was added to the supernatant to convert hemoglobin to a more stable derivative, cyanomethemoglobin according to Braman et al. (1977). This helped minimize the formation of methemoglobin, which forms upon oxidation of hemoglobin and can obscure the results by producing additional bands on the gel. This preparation was stored at 4° C and then subjected to electrophoresis within 24 hrs. Hemoglobin preparations left for longer periods of time were found to result in poor resolution as seen by increased trailing and smearing in each column of the gel.

Electrophoresis

Polyacrylamide slab gel electrophoresis was conducted in a miniature electrophoresis chamber (Idea Scientific Co., Corvallis,

Oregon) according to Davis (1964) and Ornstein (1964). A 7.5% (pH 8.9) resolving gel, approximately 6.5 cm high, and 3% (pH 7.2) stacking gel, approximately 1.5 cm high, gave the best resolution. A 10 µl aliquot of each hemolysate was diluted with equal amounts of 10% glycerol and of Bovine Serum Albumin (0.006 mg/l) in upper buffer and was gently mixed just prior to loading. The gel was pre-electrophoresed for 1 hr at 150 V at 4° C. After loading 10 µl of diluted hemolysate in each well, the gel was run at 60 V at 4° C for 15 min. The voltage was then increased to 150 V for an additional 5 hrs. The lower voltage enhanced stacking as samples entered the upper gel, thus increased the resolution of the gel by making the hemoglobin bands sharper.

The gels were stained with 0.25% Coomassie Blue G-250 solution and destained by diffusion as described by Davie (1982). The use of a concentrated acetic acid/methanol/water destain (1:2:5) for the first 60 minutes followed by a regular destain (7% acetic acid, 5% methanol) was found to help wash out excess background stain. Comparisons between Coomassie Blue stain and O-dianisidine (Dietz et al., 1971), a benzidine stain specific for hemoglobin, confirmed that the hemolysate separated by the above procedure was not contaminated by plasma proteins and that the bands observed on the gels were hemoglobins.

To distinguish each hemoglobin component from run to run, R_X values were calculated as the ratio between the migration distance of the hemoglobin component, X, and the migration distance of Bovine Serum Albumin (BSA), a marker protein with which the samples had been mixed. Estimations of R_X values for a sample hemolysate using

identical replicates was consistent from column to column with a mean standard error of ± 0.001.

Relative concentrations of each hemoglobin component were determined by scanning each individual column of the stained polyacrylamide gel at 560 nm using a Beckman spectrophotometer with an attached Gilford densitometer 220. The resulting scan tracings revealed individual peaks. In some cases, extrapolation of adjacent peaks to baseline was necessary. Areas contained under the absorption peaks were estimated by tracing peaks onto Albanene prepared tracing paper (100% rag) and weighing cut pieces representing individual peaks on a Metler analytical balance, as suggested by Broyles et al. (1979). When the method was compared to results obtained from areas estimated with an Ultra-sonic SN 20044 electronic digitizer on the same identical gel, no significant differences between the two methods were found (Student's t-test; $\alpha = 0.05$). The areas for each peak were then expressed as percentage of the total area of all the peaks obtained for a particular track in a gel. In this way, the relative proportional abundances of each hemoglobin component in a sample could be determined. This method helped eliminate variations in gel loading by pipetting error and variations in plasma concentrations between small and large fish. The polyacrylamide gels for two sample times had poor resolution so were omitted from the results presented.

Plasma Thyroxine and Gill (Na^+K)-ATpase sample analysis

The radioimmunoassay procedure described by Dickhoff et al. (1978) and its modifications by Specker and Schreck (1982), were used to

determine plasma thyroxine concentrations in individual fish. Each assay was done on duplicate 10 µl aliquots.

Gill (Na+K)-ATPase activity was measured using methods described by Johnson et al. (1977) for tissue homogenates and by Bradford (1976) for protein analysis. Since degradation of this particular enzyme using these methods has been observed after 2 weeks (personal communication, Richard Ewing, Oregon Department of Fish and Wildlife, Corvallis, OR), determination of enzyme activity were performed within 10 days of sampling.

Saltwater challenge tests

The seawater challenge test described by Clarke and Blackburn (1977) was used to assess osmoregulatory ability during parr-smolt transformation. Forty coho salmon were taken each month from February to July, 1981, from the same group of fish utilized in the smoltification study. They were divided into two groups of 20 each: a control group placed in a 20 L plastic bucket containing aerated static fresh well water, and a group transferred into an identical bucket containing saltwater (30.4-31.1 $^{o}/_{oo}$. Instant Ocean). This salinity range was similar to that used by Clarke and Blackburn (1977). A water bath maintained water temperatures at 11-12° C. After a 24 hour period in the buckets, fish were killed by a blow to the head, measured (forklength-FL), weighed, and blood samples were taken from severed caudal arteries. Blood was centrifuged and plasma analyzed for sodium concentrations by use of a flame emmission

spectrophotometer (Perkin-Elmer). Samples were always taken between 1030 and 1200 hrs.

Statistical Analysis

All proportional data (R_X, relative proportional abundances) were converted by arcsine transformation before analysis to stabilize the variance and correct for lack of normality. Other data (plasma thyroxin levels) was transformed to $\log_{10}$ if required, to meet assumptions of the statistical test. Bartlett's test was used to determine equality of sample variances (Sokal and Rohlf, 1969). Randomized block design with single classification (ANOVA) for unequal sample sizes (Sokal and Rohlf, 1969) was utilized to analyze transformed data. If the main effects were significantly different, contrasts among means were made using Student Newman Keuls Multiple Comparisons Test (SNK) for unequal sample sizes (Zar, 1974). The level of significance was set at $P < 0.05$ for all statistical test mentioned. Means and standard error values in results and graphs represent data before transformations.

RESULTS

R_X values

R_X values for each hemoglobin fraction were not always identical month to month. A range of R_X values corresponding to a certain fraction was determined by graphing all values and locating peaks depicting the various hemoglobin fractions. The fractions were then categorized and labeled according to their R_X values after all data had been collected (Table 1).

The separation of fish hemolysates by high pH polyacrylamide gel electrophoresis revealed the presence of two to three major hemoglobin fractions (S_3, S_2, and S_1), which represented more than 80% of the total hemoglobin produced, and eight minor hemoglobin fractions (S_4 - S_{11}) (Fig. 1).

Inconsistencies in the R_X values for a particular fraction from month to month, may have resulted in part due to slight variations in phenotypes of the fish sampled. It is less likely that they resulted from variations in electrophoretic separating procedures, scanning speed of the spectrophotometer or sample size.

Changes in hemoglobin fractions

Abrupt changes in the occurrence of different hemoglobin fractions began to take place in April (Fig. 2) with a drop in the relative amounts of major fractions, S_2, and the minor fractions, S_4 and S_5. An appearance of a new major fraction, S_3, and two minor fractions S_{10} and S_{11}, occurred about this time. Later in June, major

Table 1. Mean R_X values ± S.D. for each hemoglobin fraction observed for coho salmon sampled during February-July, 1982. The number of times the R_X value was observed during the period is in parenthesis.

Fraction	$\overline{X}$ ± S.D.
S_1	0.35 ± 0.01 (17)
S_2	0.37 ± 0.01 (20)
S_3	0.43 ± 0.01 (59)
S_4	0.49 ± 0.01 (30)
S_5	0.56 ± 0.02 (31)
S_6	0.61 ± 0.02 (80)
S_7	0.67 ± 0.02 (91)
S_8	0.73 ± 0.02 (99)
S_9	0.79 ± 0.02 (35)
S_{10}	0.86 ± 0.01 (71)
S_{11}	0.93 ± 0.01 (3)

Figure 1. Electrophoresis pattern of hemoglobin fractions (S_1 - S_{11}), distinguished by their mean R_X values ± S.E. for coho salmon sampled throughout smoltification.

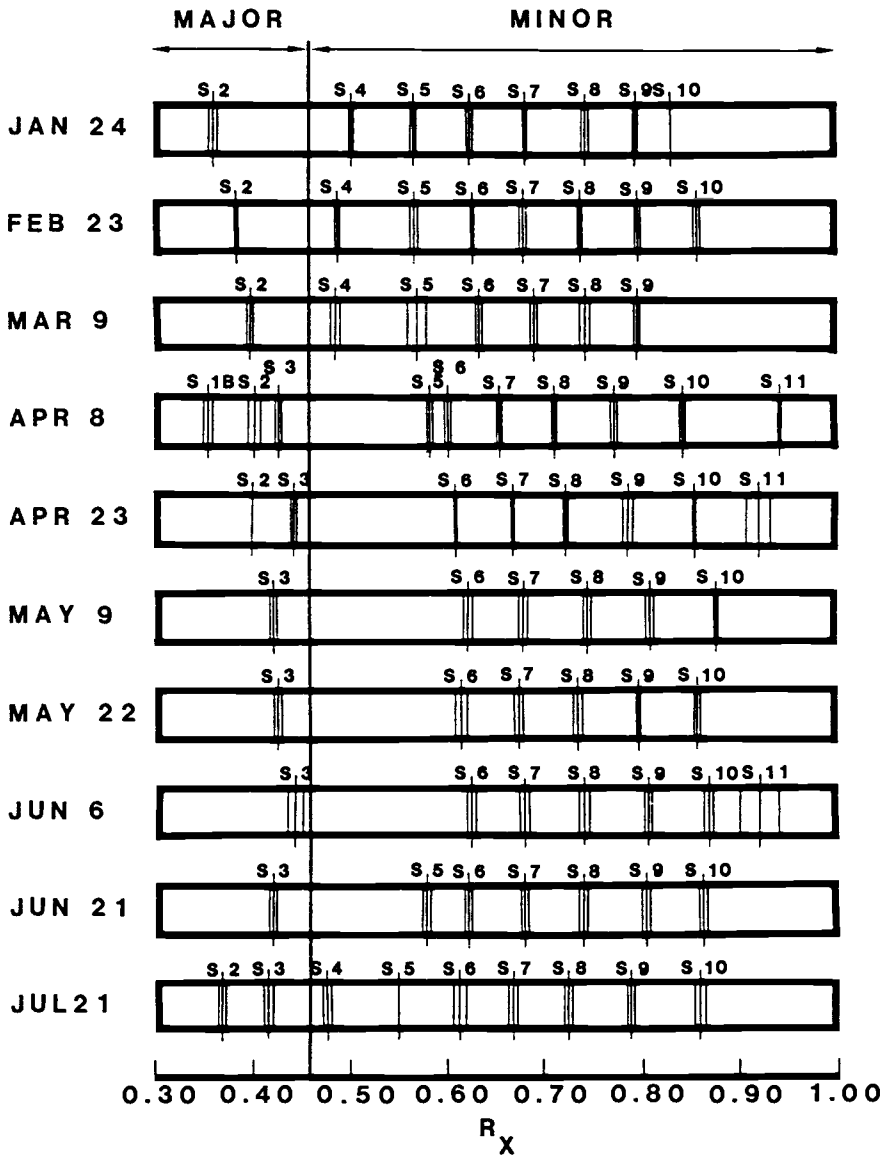
MAJOR
MINOR
JAN 24
FEB 23
MAR 9
APR 8
APR 23
MAY 9
MAY 22
JUN 6
JUN 21
JUL21
0.30 0.40 0.50 0.60 0.70 0.80 0.90 1.00
R X

Figure 1.

Figure 2. The frequency (%) of occurrence of various hemoglobin fractions (S_2 - S_{11}), i.e. the number of individuals exhibiting a particular fraction divided by the total sampled, for coho salmon throughout smoltification. Sample size for each data was as follows: Jan. 24, n = 11; Feb. 23, n = 15; Mar. 9, n = 11; Apr. 8, n = 13; Apr. 23, n = 15; May 9, n = 7; May 22, n = 4; Jun. 6, n = 9; June 21, n = 12; and July 21, n = 12. "Approximate period of change" indicates the period when most changes took place in the Hb profiles.

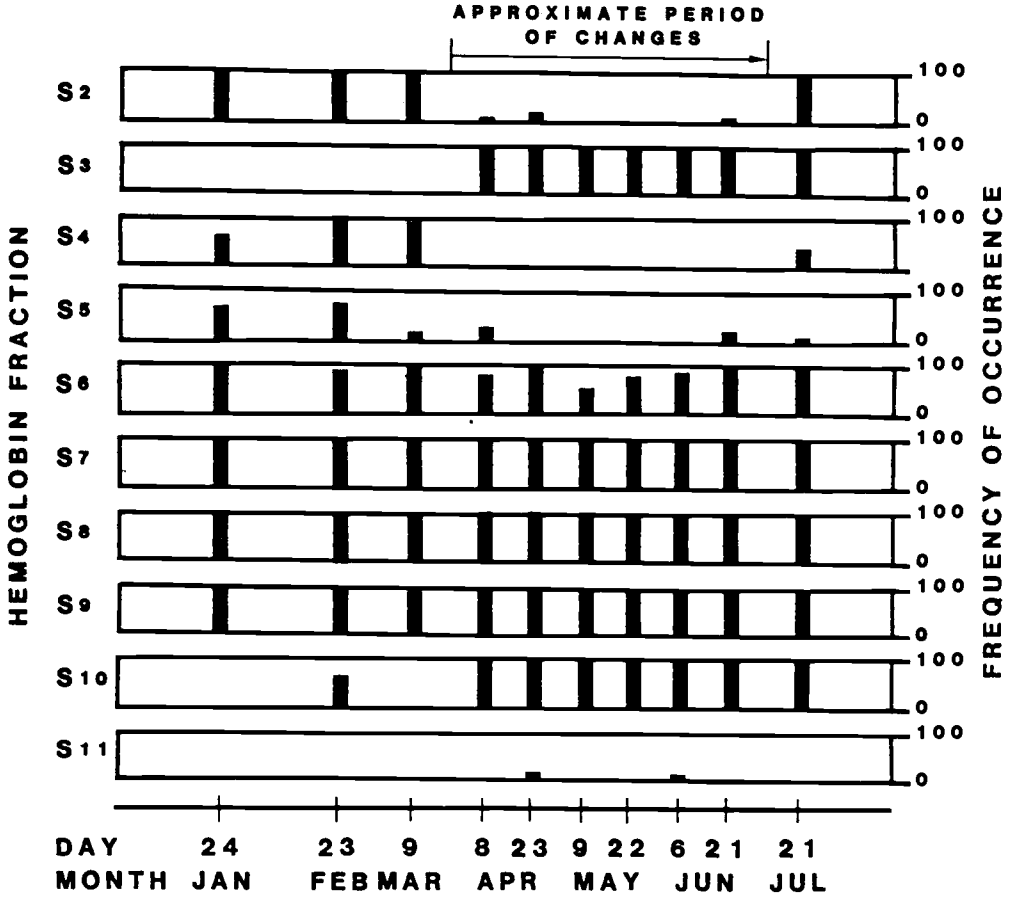
APPROXIMATE PERIOD
OF CHANGES
S2
S3
S4
S5
S6
S7
S8
S9
S10
S11
100
0
HEMOGLOBIN FRACTION
FREQUENCY OF OCCURRENCE
DAY 24 23 9 8 23 9 22 6 21 21
MONTH JAN FEB MAR APR MAY JUN JUL

Figure 2.

hemoglobin fraction, S_2, and minor fractions, S_4 and S_5, reappeared. The least number of fractions was observed during May (one major and five minor fractions), whereas the greatest number appeared in the months of February (one major and seven minor fractions) and July (two major and seven minor).

Quantitative analysis of each fraction revealed an apparent inverse relationship between the proportional abundances of major and minor hemoglobin fractions over the smolting period (Fig. 3 and Fig. 4). In order to compare the major and minor components, the relative proportional abundances of fractions, S_3, S_2, and S_1 were pooled together. The mean total proportional abundance calculated for these major fractions exhibited a significantly lower value on June 6 as compared to all other sample times (ANOVA, $F = 3.35 > F_{0.025(8,89)} = 3.04$ followed by SNK). Variations over time were also observed for a number of minor fractions always present in the fish; S_{10} ($F = 6.03 > F_{0.001(7,88)} = 3.92$), S_9 ($F = 7.16 > F_{0.001(7,88)} = 3.52$), S_8 ($F = 7.78 > F_{0.001(8,34)} = 4.36$), S_6 ($F = 4.03 > F_{0.005(8,23)} = 3.83$). The highest mean proportional abundance for these four minor fractions was exhibited on June 6 (SNK). Increased mean abundances were also noticed on May 22nd in the S_{10} fraction and March 9 in the S_6 fraction (SNK).

An evaluation of the absolute mean concentration of all the minor Hb fractions (estimated by the weight of the paper representing the minor peaks) indicated peak values on June 6 for these fractions. This further supports the thought that variations in the relative proportional abundances of minor hemoglobins were due to actual

Figure 3. Relative proportional abundances of observed minor hemoglobin fractions (S_4 - S_{11}, as defined in Fig. 1) represented as a percent of the total hemoglobin present for each individual (mean ± S.E.) in coho salmon throughout smoltification. Figures in parenthesis indicate significance after ANOVA was performed to analyze the changes in abundances over the sample time. (0.05)-significance at $\alpha < 0.05$ and (NS) - No significance at the 0.05 level. Those mean values that were significantly different (SNK) for each fraction over time are marked with an asterisk.

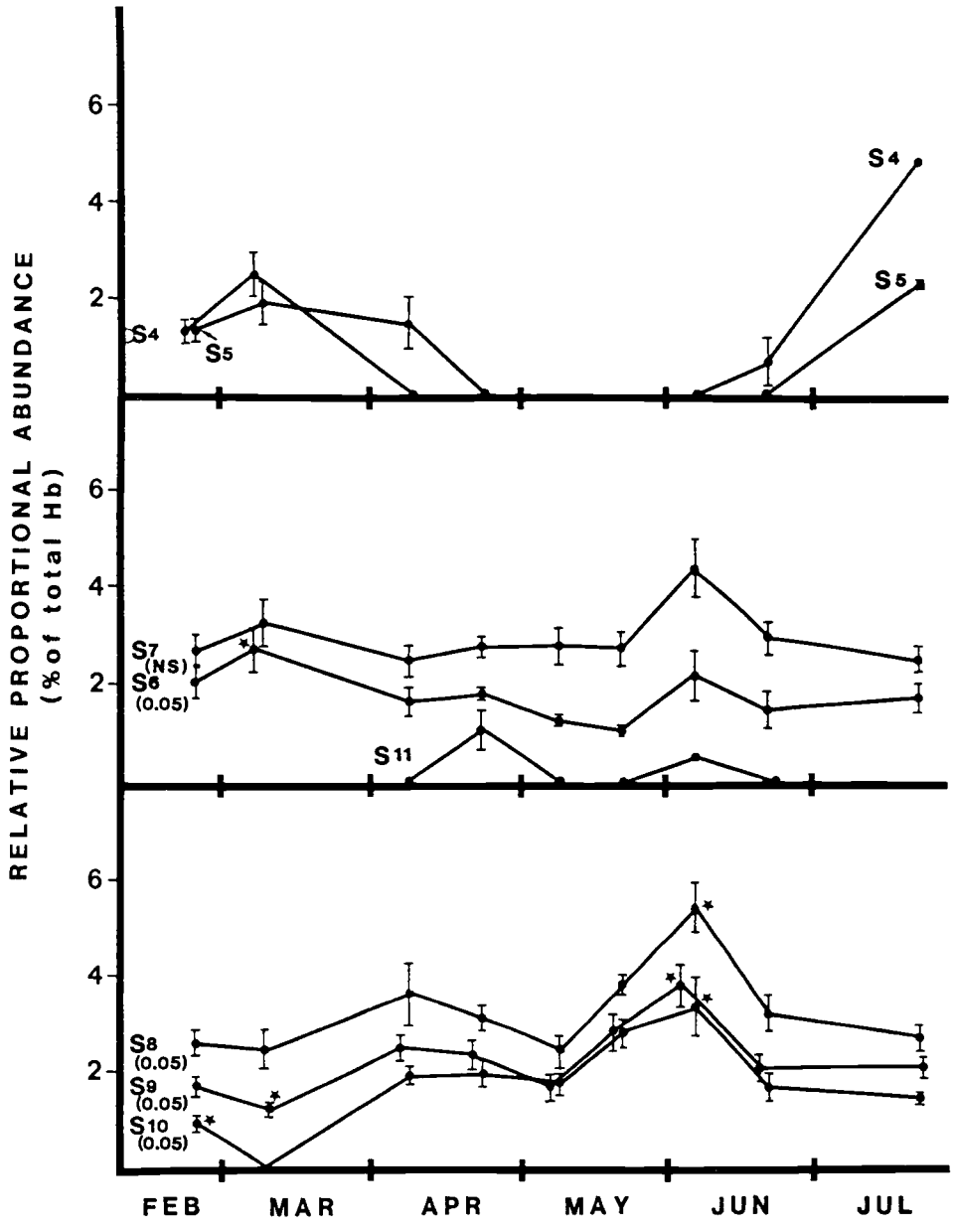
RELATIVE PROPORTIONAL ABUNDANCE
(% of total Hb)
6
4
2
S4
S5
S4
S5
S7
(NS)
S6
(0.05)
S11
S8
(0.05)
S9
(0.05)
S10
(0.05)
FEB
MAR
APR
MAY
JUN
JUL

Figure 3.

Figure 4. Relative proportional abundances of observed major hemoglobin fractions (S_3, S_2, and S_1, as designated in Fig. 1) represented as the total hemoglobin present for each individual coho salmon (mean ± S.E., n above the S.E. bar) throughout parr-smolt transformation. Figures in parenthesis indicate significance after ANOVA was performed to analyze the changes in abundances over the sample time. (0.05)-significance at $\alpha < 0.05$. Those mean values that were significantly different (SNK) are marked with an asterisk.

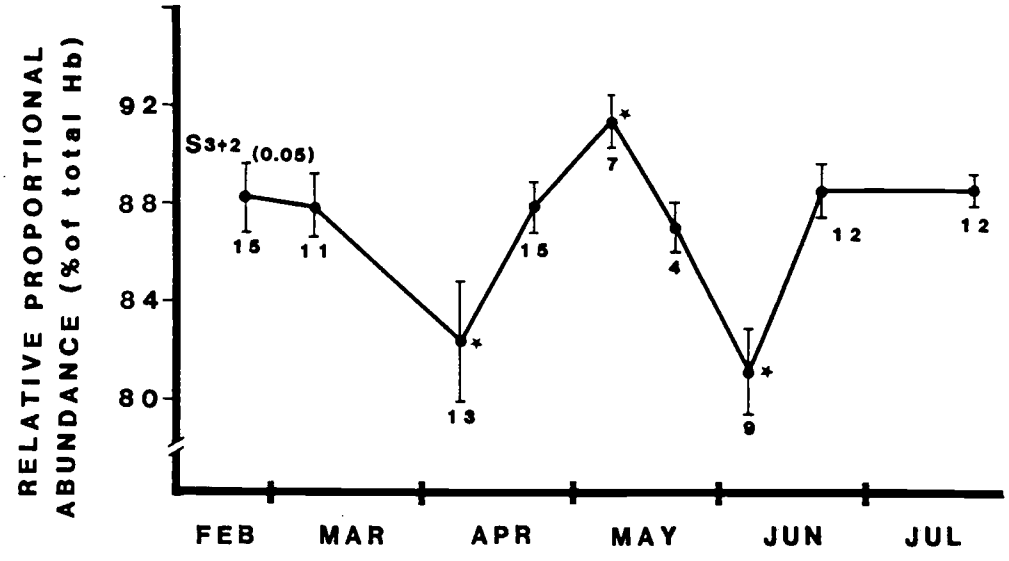
RELATIVE PROPORTIONAL ABUNDANCE (%of total Hb)
92
88
84
80
S3+2 (0.05)
15
11
13
15
7
4
9
12
12
FEB
MAR
APR
MAY
JUN
JUL

Figure 4.

changes in the concentration of these hemoglobins and not as a result of proportional decreases of the major hemoglobins.

Changes in hematocrit values

Hematocrit values were significantly different during parr-smolt transformation period ($F = 9.96 > F_{0.001(6,71)} = 4.63$; Fig. 5), increasing from 43.0 ± 0.02 percent (n = 15) on March 9 to 46.2 > 0.9 percent (n = 15) on April 23, then decreasing to 40.0 ± 1.4 percent (n = 12) on July 21st (SNK). Although values between April 23 to June 21st were similar (SNK), the highest mean hematocrit value observed on June 6th corresponded to the greatest relative proportional abundances of the four minor hemoglobin fractions.

In order to compensate for any variation caused by increases in the hematocrit, a correction factor was used to standardize the data. Thus when hematocrit values were taken into account the relative proportional abundances and absolute concentration (estimated by the weight of the paper under the respective peaks) of minor and major hemoglobins were still found to vary significantly over the smolting period. This indicated changes in the proportion of hemoglobin fractions were not solely a result of changes in the hematocrit values.

Changes in growth parameters

Increases in weight and fork length (FL) for the test fish were similar to those of coho salmon reared in production hatcheries (personal communications, J. Holway, Eagle Creek National Fish Hatchery). Mean weight for fish sampled May 9th decreased from the

Figure 5. Hematocrit values (mean ± S.E., n below the S.E. bar) obtained from coho salmon sampled throughout smoltification. Mean values that were significantly different (SNK) are marked with an asterisk.

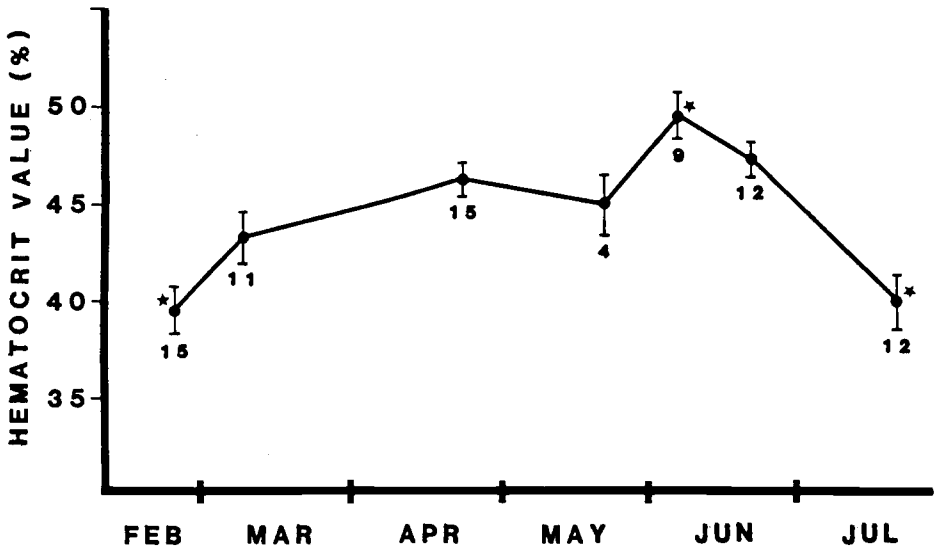
HEMATOCRIT VALUE (%)
50
45
40
35
15
11
15
4
9
12
12
FEB
MAR
APR
MAY
JUN
JUL

Figure 5.

previous sample date, but were not significantly different (student-t-test, $P < 0.05$). A gradual decrease in condition factor (CF = wt g x 10^{-2}/fork length cm^3) from 1.14 ± 0.018 (10^{-2} x g/cm^3) (n = 15) in February to 0.962 ± 0.019 (10^{-2} x g/cm^3) (n = 15) in April, corresponds to decreases in total relative proportional abundances of the pooled major hemoglobin fractions S_3, S_2 and S_1 (Fig. 6 and Fig. 4). The values for CF and the major hemoglobin abundances increased later at the end of May and early June. Condition factor was correlated to the major hemoglobin abundances on the following sampling dates: March 9 ($R^2 = 0.53$); May 9 ($R^2 = 0.50$); May 22 ($R^2 = 0.85$); however, no significant correlations between the two parameters were found ($R^2 = 0.04$) over the entire sample period.

Effects of seawater challenge test

Following saltwater challenge in March, the mean plasma concentration of sodium was 170.3 ± 2.7 mM/l (n = 20) (Fig. 7), a level typical of coho smolts (Clarke and Blackburn, 1977). No significant changes were found for samples obtained from March through May. Mortalities and lethargic behavior began to be noticed in response to saltwater challenges on June 22 and July 22. In addition, the surviving fish sampled on July 22 were found to have significantly higher plasma sodium concentrations after undergoing the saltwater challenge in relation to previous months (ANOVA followed by SNK: $F = 3.50 > F_{0.025(5,79)} = 2.71$), suggesting a decrease in performance and ability to osmoregulate effectively in saltwater.

Figure 6. Changes in condition factor (mean ± S.E., n below the S.E. bar) calculated from coho salmon sampled throughout parr-smolt transformation.

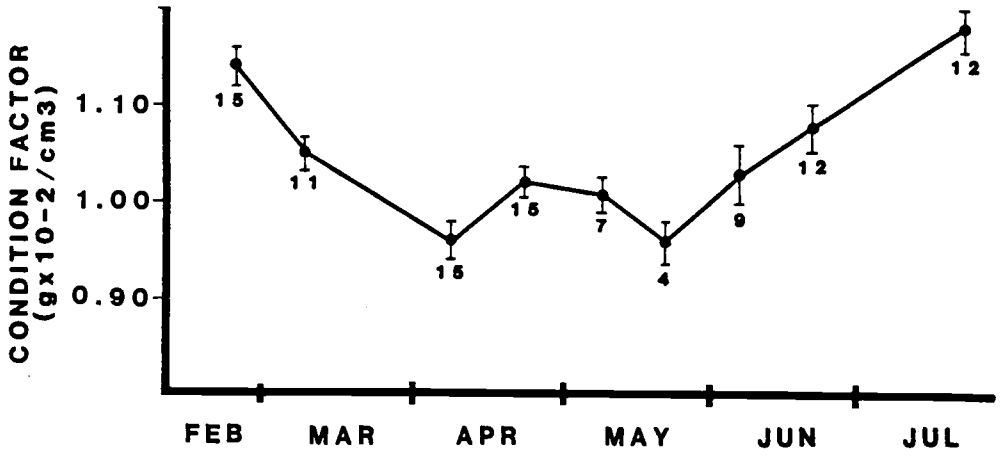
CONDITION FACTOR
(gx10-2/cm3)
1.10
1.00
0.90
15
11
15
15
7
4
9
12
12
FEB
MAR
APR
MAY
JUN
JUL

Figure 6.

Figure 7. Mean plasma sodium levels ± S.E. (n at the top of the S.E. bar) of coho salmon challenged in saltwater for 24 hrs. compared to freshwater controls during parr-smolt transformation. * Six mortalities were observed in June and fifteen mortalities occurred in July for the saltwater challenged group.

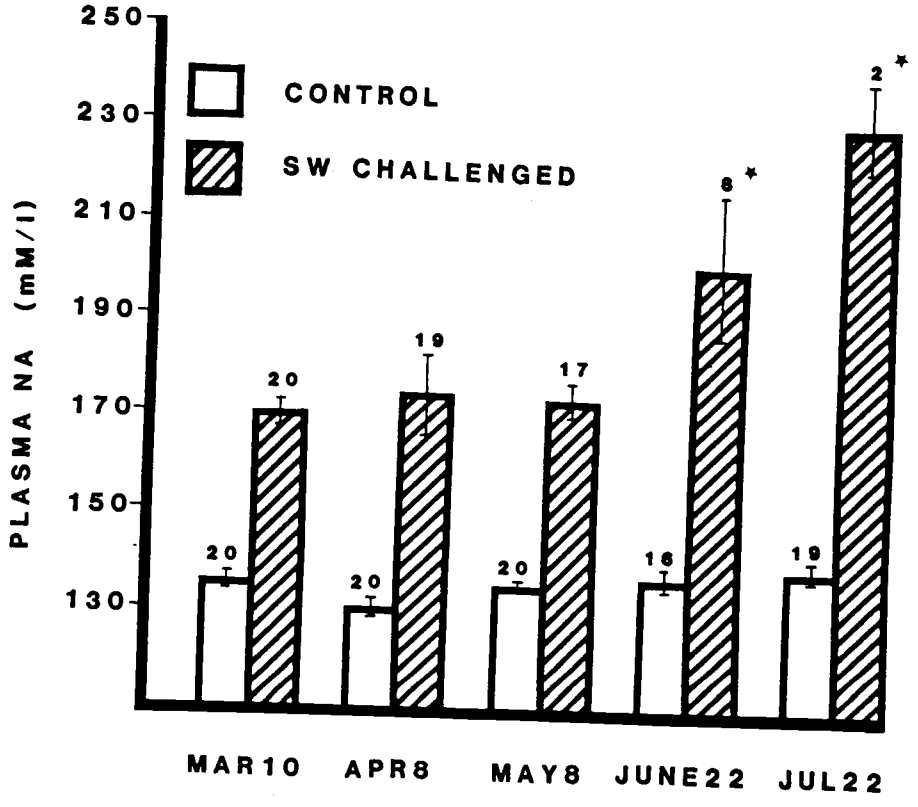
250
230
210
190
170
150
130
PLASMA NA (mM/l)
CONTROL
SW CHALLENGED
20
20
19
20
17
20
8
16
2
19
MAR10
APR8
MAY8
JUNE22
JUL22

Figure 7.

Changes in other smoltification indicators

The concentration of T_4 in the plasma for fish utilized for hemoglobin analysis changed significantly with time ($F = 20.10 > F_{0.001(7,86)} = 3.84$). Levels of plasma T_4 significantly increased from 2.99 ± 0.36 (n = 15) on February 23 to 6.65 ± 0.81 ng/ml (n = 11) on March 9 (SNK). A slight decrease from the peak level observed in June was seen again in July.

Gill Na-K-ATPase activity monitored monthly by Patino (1984) were elevated from March 9 and May 7 (ANOVA followed by SNK; $F = 5.12 > F_{0.001(8,120)} = 3.55$) (Fig. 8).

Figure 8. a) Plasma thyroxine concentrations (mean ± S.E., n below the S.E. bar) and b) Gill (Na+K)-ATPase enzyme activity (mean ± S..E, n below the S.E. bar) observed in coho salmon sampled throughout parr-smolt transformation.

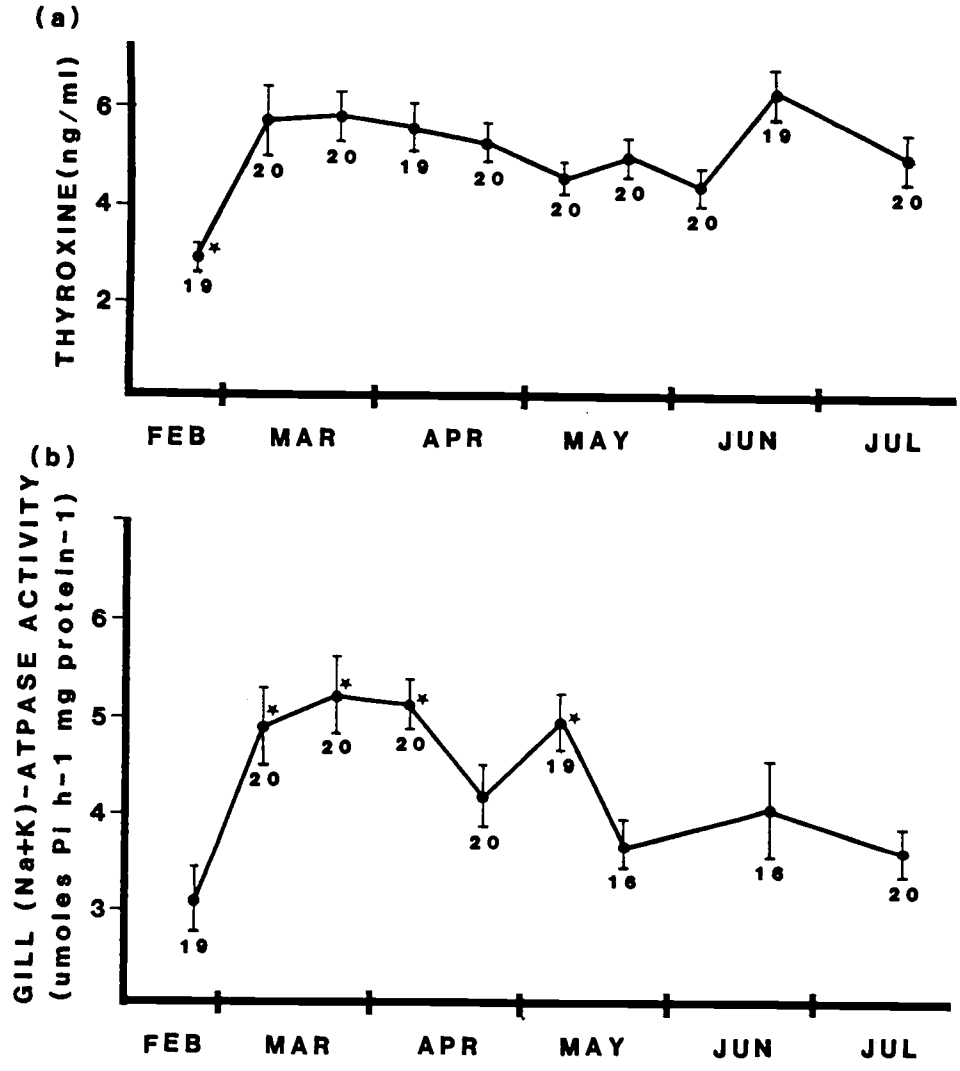
(a)
THYROXINE(ng/ml)
6
4
2
19
20
20
19
20
20
20
20
19
20
FEB
MAR
APR
MAY
JUN
JUL
(b)
GILL (Na+K)-ATPASE ACTIVITY
(umoles Pi h-1 mg protein-1)
6
5
4
3
19
20
20
20
20
19
16
16
20
FEB
MAR
APR
MAY
JUN
JUL

Figure 8.

DISCUSSION

There appeared to be a definite variation in the pattern of hemoglobins for coho salmon during the period associated with parr-smolt transformation. The number of hemoglobin fractions exhibited tended to decrease from January, where 8 fractions were noticeable, until May, at which time a total of 6 fractions, one major hemoglobin (S_3) and five minor hemoglobin fractions (S_6 - S_{10}), were evident. By June, this trend reversed and various hemoglobin fractions began to reappear, indicating an increase in the complexity of the hemoglobin system at this time.

In contrast to these findings, Giles (1973) described an increase in the numbers of minor hemoglobin fractions in coho salmon from five fractions in February to seven fractions in March. Thereafter, the number of minor fractions and major fractions remained constant into adulthood. Differences in hemoglobin seperation procedures or the frequency with which the samples were taken during this period could account for the discrepancies in the two studies.

Quantitative alterations in the hemoglobin system were shown by increases in the relative proportions of minor hemoglobin fractions, S_7, S_8, S_9, and S_{10}, with levels peaking at the beginning of June. This was followed by a decline in abundances of these fractions to levels experienced in early spring, possibly indicating desmoltification. Reciprocal changes in the relative pooled abundances of the major fractions, S_1, S_2 and S_3, also accompanied the changes in the four minor fractions. Comparable electrophoretic

patterns obtained in May from coho salmon reared at Eagle Creek National Fish Hatchery (see Appendix I; Fig. 1) and from Big Creek Hatchery sampled the previous year (See Appendix II; Figs. 1 and 2) were consistent with these findings, although in the latter group the appearance of the hemoglobin shift occurred slightly later in June rather than in May. Analysis of the hemoglobin system of these stocks further supports the central conclusion that there is an inverse relationship between proportional abundances of major and minor hemoglobin fractions over the smolting period.

Giles and Vanstone (1976) using different electrophoretic and staining procedures indicated the continued presence of three major hemoglobin fractions and seven minor fractions in presmolt coho salmon through to adulthood (11-36 mos.). Temporal changes in the relative concentrations of specific hemoglobin fractions were evident in their data, a finding qualitatively similar to what I observed. They noticed a decline in the relative abundances of the major fractions (labeled, A_6, A_7, A_8) with age of coho salmon until their seawater residence. Four minor fractions (called A_1, C_1, C_2, C_3) identified in Giles study (1976) were found to increase at approximately the same age (11-16.5 mos.) as fractions S_7, S_8, S_9, S_{10} identified in my study.

It thus seems that a shift in hemoglobin pattern may be associated with the period during which we believe parr-smolt transformation takes place. The other physiological parameters evaluated to reflect smoltification also appeared to undergo changes during this period. However, sharp peaks denoting clear shifts in physiological status were not evident in any of these measurements. Apparent signs of

external silvering of the body, darkening of the dorsal region, and decreased condition factor were first observed in April for coho salmon used in the present study. These changes coincided with the reduction in the numbers of hemoglobin fractions and decreases in the proportional amounts of minor hemoglobin fractions. Further, decreases in condition factor were correlated to increases in the pooled relative proportions obtained for major hemoglobin fractions, S_3 and S_2, during the months of March and May. Plasma thyroxine concentrations appeared to increase in March and remained the same until late June at which time a slight elevation was observed. Similarly, maximum levels of gill (Na+K)-ATPase activity were observed in March and declined in late May, but never attained levels found earlier in February. Following the decline in levels of (Na+K)-ATPase activity and prior to the small thyroxine peak present in late June, a shift in the relative proportions of major and minor hemoglobin fractions produced was apparent. By mid-June these proportional abundances began to revert back to proportions seen earlier in May. Patino (1982) found that the clearance rates of plasma cortisol levels in the same stock of fish used in the present study tended to be greatest during late April, while plasma levels of this hormone tended to increase in July.

Seawater challenge tests performed on these test fish failed to reveal any fluctuations in plasma sodium regulatory ability until June, indicating a decline in salinity tolerance. This reversion back to a physiological state experienced prior to parr-smolt transformation

suggests these fish were undergoing desmoltification, a process observed in fish retained in freshwater (Wedemeyer, 1980; Hoar, 1976).

The reversion in the production of the hemoglobin fractions to a pattern more typical of an earlier life history stage is also supported by findings of Koch et al. (1982) who found increased proportional abundance of the major "juvenile" hemoglobin fraction (Hb-A) after post-smolt Atlantic salmon were held in freshwater. In contrast, pre-smolt coho salmon allowed to migrate to seawater were shown to progressively decrease the proportional abundance of the major fractions while increasing proportions of the minor hemoglobin fractions over a 2 month period, after which the proportions remained constant for the rest of the life cycle (Giles, 1973).

The shifts in the physiological factors evaluated appeared to correspond with the general patterns for those characteristics described during smoltification. For instance, body silvering as a result of deposition of guanine and hypoxanthine in the sides and skin has been reported for salmonids undergoing parr-smolt transformation in the spring (Johnson and Eales, 1967; Staley, 1983). However, in hatchery fish the silvery appearance often develops prior to the development of other physiological characteristics necessary for marine survival (Wedemeyer, 1980). Decreases in condition factor caused by a reduction in total lipid content resulting from changes in metabolic rates has also been found to occur at the time of smolting (Vanstone and Markert, 1968).

Although no surges or peaks were apparent for the other physiological parameters monitored in this study, previous

investigations have reported that elevations in plasma thyroxine concentration were coincident with the time of parr-smolt transformation for coho salmon in freshwater (Folmar and Dickhoff, 1980; Specker, 1982). The completion of this seasonal thyroxine peak has also been suggested to be correlated to seawater survival (Folmar and Dickhoff, 1981; Dickhoff et al., 1982). In addition, high levels of (Na+K)-ATPase enzyme activity observed during this period have been correlated to increases in osmoregulatory capacity (Boeuf and Harache, 1982). Seawater challenge tests have also been used to predict salinity tolerance, as shown by the ability of salmonids to regulate their plasma sodium levels following a 24 hr. saltwater challenge (Clarke and Blackburn, 1977). Recent studies have reported that this test can only be used effectively on coho salmon to assess their osmoregulatory performance level and is not necessarily indicative of marine survival (Clarke, 1982).

Variations in the hemoglobin pattern may reflect the period associated with parr-smolt transformation. However, hemoglobin profiles may also be influenced by growth or size of the fish (Koch, 1966; 1982; Hasimoto and Matsuura, 1960), although it is not known if this is independent of ontogenetic events. Koch et al. (1964) noted an increase in the number of hemoglobin fractions exhibited by Atlantic salmon with increases in size. In addition, he found that as fish increased in size there was a corresponding reduction in the predominant hemoglobin fraction (labeled A_2). However, Koch (1964) also administered beef thyroid to fish and was able to accelerate the development of the hemoglobin pattern without increases in size.

Therefore, although the development of the hemoglobin pattern might be associated with size and/or sexual maturation, it is evidently influenced as well by other physiological factors involved in the developmental process.

In order to verify the existence of this hemoglobin shift seen during parr-smolt transformation, investigations must be conducted to determine the consistency of hemoglobin patterns within stocks from year to year and must assess the degree of variations found between stocks that might result due to rearing conditions and genotypic differences of the stocks. Additional work relating the shift in the hemoglobin profile typical of hatchery salmonids at the time of release to percentages of saltwater survival and adult returns must also be included to determine the potential use of this physiological phenomenon as a index of parr-smolt transformation.

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APPENDICES

Appendix I.

THE EFFECT OF REARING DENSITIES ON THE HEMOGLOBIN PROFILE OF COHO SALMON ONCORHYNCHUS KISUTCH

Juvenile coho salmon raised at three different densities, low, medium, and high, at the Eagle Creek National Fish Hatchery were sampled on May 4, 1982, to determine the hemoglobin pattern exhibited by each density group. The density factor (weight (lbs)/volume (ft^3)/length (in)) for low, medium and high density groups were 0.15, 0.30, 0.45, respectively. Separation of hemolysates by high pH polyacrylamide electrophoresis revealed all but two fish in all three density groups exhibited one major hemoglobin fraction, S_3, and five minor hemoglobin fractions, S_6-S_{10}. One fish from both the low and the medium density groups displayed additional hemoglobin fractions, S_5 and S_{11} not seen in the other fish (Fig. 9). No differences in the relative proportional abundances of hemoglobin fractions S_3, S_6 through S_{10} were observed between the groups. It was concluded from this study that no significant differences resulted in the hemoglobin profile for coho salmon reared under different rearing densities.

Figure 9. The frequency (%) of occurrence of various hemoglobin fractions (S_3 - S_{11}) - i.e. the number of individuals exhibiting a particular fraction divided by the total sampled, observed in Eagle Creek coho salmon raised at three different densities (see text for density factors) and sampled on May 4, 1982. Sample sizes are follows: High, n = 10; Med., n = 15; Low, n = 11.

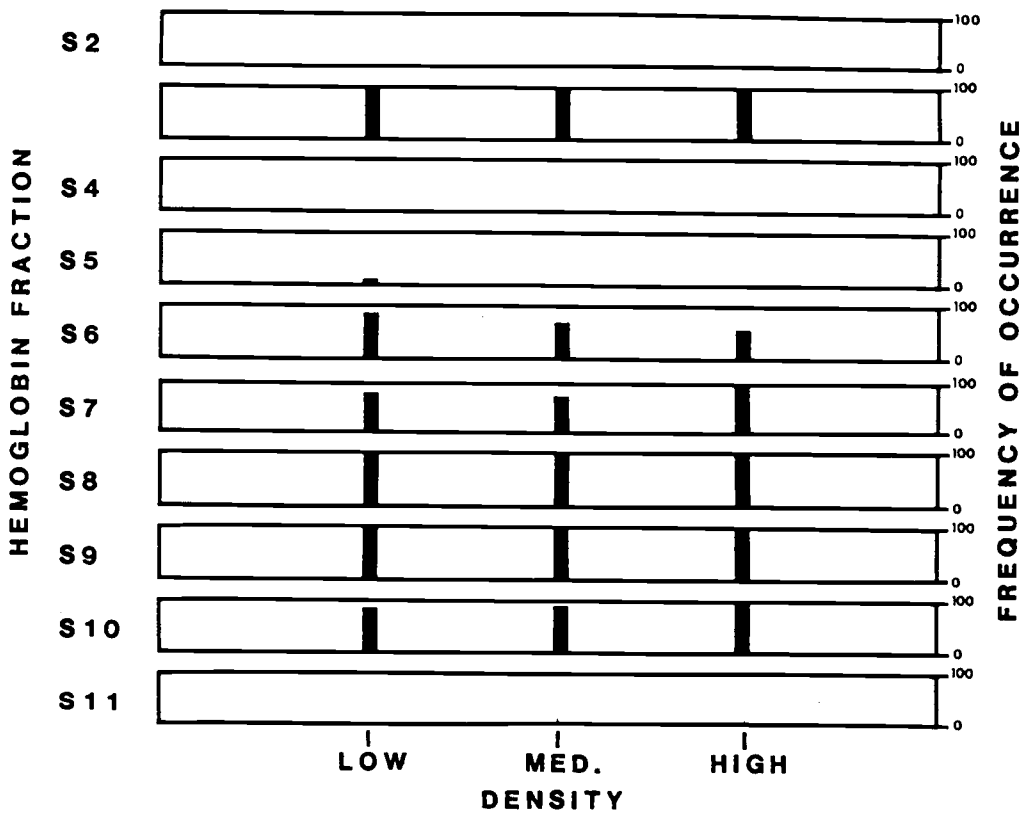
S2
S4
S5
S6
S7
S8
S9
S10
S11
HEMOGLOBIN FRACTION
100
0
FREQUENCY OF OCCURRENCE
LOW
MED.
HIGH
DENSITY

Figure 9.

Appendix II.

ASSESSMENT OF THE HEMOGLOBIN PROFILE OF BIG CREEK COHO SALMON DURING MAY AND JUNE 1981.

A preliminary study investigating the hemoglobin profile of coho salmon (Big Creek stock), obtained from the Oregon Department of Fish and Wildlife, Corvallis, Research Laboratory, was done during the months of May and June, 1981. This period corresponds to the time these fish are thought to undergo parr-smolt transformation, a developmental process that prepares salmonids for their seaward migration and ocean existence.

The objectives of this study were to 1) determine the hemoglobin profile for coho salmon using a revised assay to separate the multiple hemoglobins (described in methods section of the main paper) 2) determine if there were any qualitative or quantitative differences in hemoglobin fractions observed during May and June.

MATERIALS AND METHODS

Big Creek coho salmon were reared in 0.3 m^3 flow through circular tanks with fresh well water (10-13° C) at Smith Farm, Oregon State University, Corvallis, Oregon. Fish were fed Oregon Moist Pellets (OMP) at least twice daily to satiation.

Sampling procedures, hemolysate preparations, electrophoresis, and statistical analysis were as described previously in the main study using Eagle Creek coho salmon.

RESULTS

The separation of coho salmon hemolysates by high pH polyacrylamide gel electrophoresis revealed the presence of one major hemoglobin fraction, S_3 and six minor hemoglobin fractions, S_5 - S_{10}, throughout May and June (Fig. 10). Although no qualitative variations existed during the sample period, quantitative analysis, determined by the relative proportional abundances for each fraction, indicated significant decreases in proportions occurred June 30 for minor fractions and an increase was observed for the major fractions (ANOVA followed by SNK; S_3 - $F = 39.98 > F_{0.001(3,39)} = 6.55$; S_5 - $F = 14.02 > F_{0.001(3,39)} = 6.55$; S_6 - $F = 42.34 > F_{0.001(3,39)} = 6.55$; S_7 - $F = 44.13 > F_{0.001(3,39)} = 6.55$; S_8 - $F = 98.12 > F_{0.001(3,39)} = 6.55$; S_9 - $F = 46.74 > F_{0.001(3,39)} = 6.55$; S_{10} - $F = 3.60 > F_{0.001(3,39)} = 3.47$) (Fig. 2). An apparent peak in proportional abundances for minor fractions was observed June 4, but was only significant in fractions, S_9 and S_{10} (SNK). A reciprocal pattern in proportions was exhibited by the major fraction, S_3, showing lower proportions in early June. This inverse relationship between major and minor hemoglobin fractions existed throughout the sample period.

Figure 10. Frequency (%) of occurrence of various hemoglobin fractions, designated S_2 - S_{11}, i.e., the number of individuals exhibiting a particular fraction divided by the total sampled, observed in Big Creek coho salmon raised at Smith Farm, Oregon State University, Corvallis, Oregon, 1981. Sample size for each data was as follows: May 1, n = 13; May 26, n = 10; May 30, n = 12; June 4, n = 15; June 17, n = 4; June 30, n = 14.

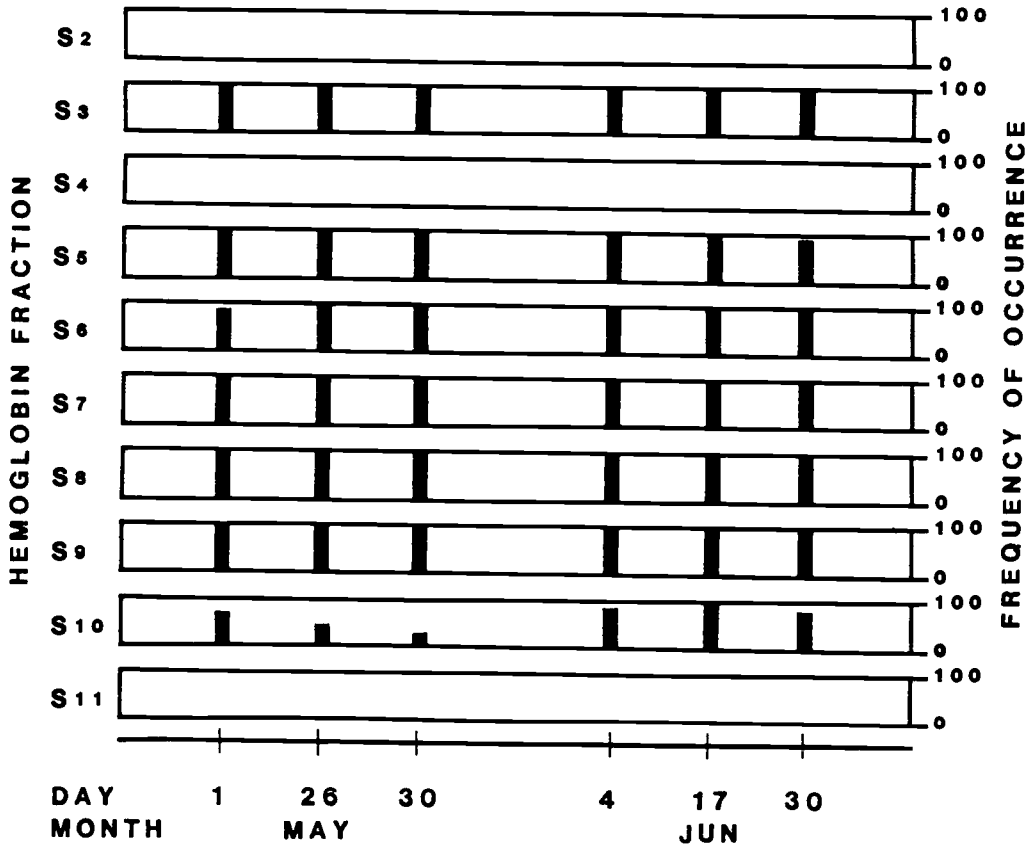
HEMOGLOBIN FRACTION
S2
S3
S4
S5
S6
S7
S8
S9
S10
S11
100
0
FREQUENCY OF OCCURRENCE
DAY
MONTH
1
26
30
4
17
30
MAY
JUN

Figure 10.

Figure 11. Relative proportional abundances of observed minor and major hemoglobin fractions (S_3 - S_{10}, as defined in Fig. 1 of the main paper) represented as the percent of the total hemoglobin present (mean ± S.E.) in Big Creek coho salmon sampled in May and June, 1981. Figures in parenthesis indicate significance after ANOVA was performed to analyze the changes in abundances over the sample time: (0.05) - significance at $P < 0.05$. Sample size for each date was as follows: May 26, n = 10; June 4, n = 15; June 17, n = 4; June 30, n = 14. Those mean values that were significantly different are indicated by an asterisk.

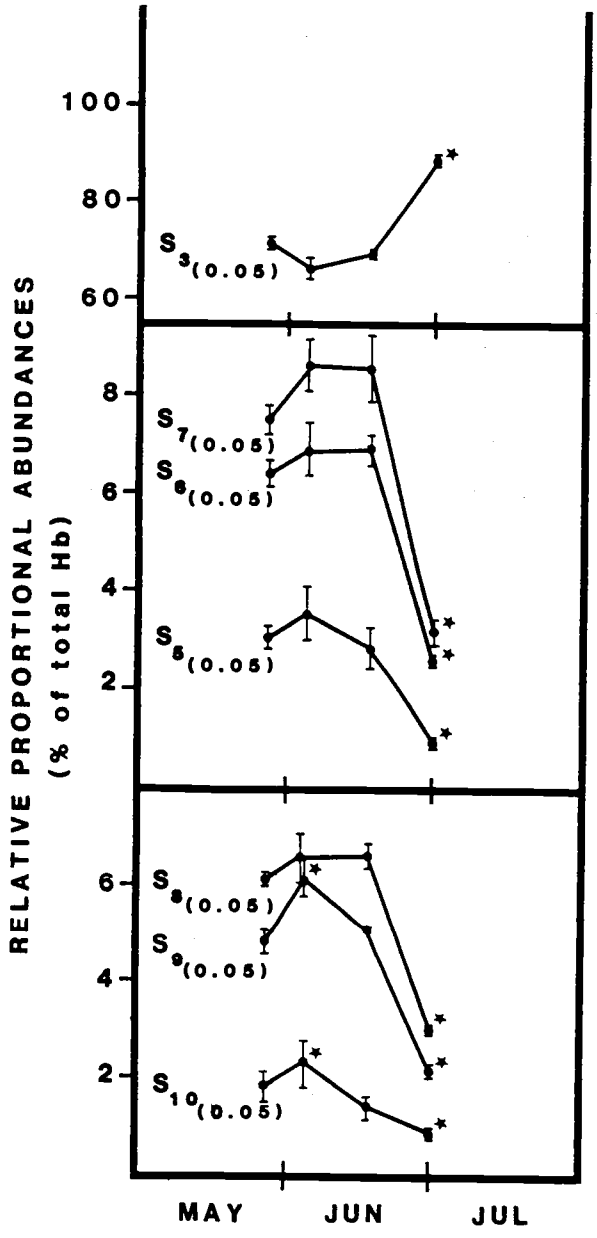
RELATIVE PROPORTIONAL ABUNDANCES
(% of total Hb)
100
80
60
8
6
4
2
6
4
2
$S_{3(0.05)}$
$S_{7(0.05)}$
$S_{6(0.05)}$
$S_{5(0.05)}$
$S_{8(0.05)}$
$S_{9(0.05)}$
$S_{10(0.05)}$
MAY
JUN
JUL

Figure 11.

Appendix III.

THE EFFECT OF L-THYROXINE ON THE HEMOGLOBIN PROFILE OF COHO SALMON, ONCORHYNCHUS KISUTCH

Transitions in the hemoglobin profile have been observed in amphibians during L-thyroxine induced metamorphosis (Forman and Just, 1981), a developmental process which resembles salmonid parr-smolt transformation. Prior to natural metamorphosis, amphibians experience a surge in thyroid hormone activity (Frieden and Just, 1970) somewhat similar to that occurring in the smoltification process of salmonids (Dickhoff et al., 1978, 1982). Potential similarities between the developmental processes of metamorphosis and parr-smolt transformation might lead one to suggest that alterations in multiple hemoglobin profiles in fish are under the same thyroid hormone control as amphibians. Evidence supporting this hypothesis was reported by Koch et al. (1964) who found young Atlantic salmon fed thyroid tissue in their diet produced an advanced hemoglobin pattern typical of older fish. Koch et al. (1964) further indicated the acceleration in the hemoglobin system was observed in all fish in the treated group independent of size, thus suggesting the changes in the hemoglobin profile could be triggered or induced directly or indirectly by thyroid hormones and are not necessarily associated with growth.

More recent studies have indicated prolonged oral administration of thyronine (T_3) at 12 ppm to yearling coho salmon initially results in a depression and later acceleration of the relative proportion of "adult" hemoglobin components from that seen in controls (personal

communications, Craig Sullivan, School of Fisheries, Univ. of Washington, Seattle, WA). In this case, thyroid hormones seem to inhibit or induce the expression of these particular hemoglobins associated with a later life history stage of the fish depending on the time the hormone is administered.

The goal of this study was to provide additional information as to the regulatory mechanisms involved in triggering the changes in the composition of multiple hemoglobins in the erythrocytes of coho salmon. Specifically, I evaluated the effects of exogenous thyroxine (T_4) adminstered via the diet and by immersion techniques on the hemoglobin profile.

MATERIALS AND METHODS

Animal Maintenance

Underyearling coho salmon (Big Creek stock crossed with Soleduc stock) were obtained from the Oregon Department of Fish and Wildlife, Corvallis Research Laboratory in October, 1981 and acclimated in 0.3 m^3 flow through circular tanks with fresh well water (10-13° C) for 3 weeks at Smith Farm, Oregon State University, Corvallis, Oregon. Fish were fed Oregon Moist Pellets (OMP) at least twice daily to satiation at 0900 and 1500 hrs until the initiation of the L-thyroxine administration experiments.

Sampling procedures, hemolysate preparations, electrophoresis were as described previously in the main study using Eagle Creek coho salmon.

Statistical analysis

All proportional data (R_X and relative proportional abundances) were converted by arcsine transformation before analysis to stabilize the variance and correct for lack of normality. Bartlett's test was used on data to determine equality of sample variances (Sokal and Rohlf, 1969).

To analyze the effects of treatments on the relative proportional abundances of hemoglobin fractions, a randomized block design, single classification, analysis of variance (ANOVA) for unequal sample sizes (Sokal and Rohlf, 1969) was done for each hemoglobin fraction and for each day sampled. Significant changes in weight, fork length, plasma thyroxine and hematocrit values for each group were also assessed with a preliminary ANOVA. If main effects were significant, contrasts among means were made using Student-Newman-Keuls Multiple Comparisons tests (SNK) for unequal samples sizes (Zar, 1974).

In the T_4 immersion experiment, contrasts were made between control and treatment groups using Student-t-test at $\alpha = 0.05$. Data from duplicate tanks were pooled together when similar as shown by Student-t-test at $\alpha = 0.05$.

The relationship between: 1) plasma levels of thyroxine and weight 2) plasma levels of thyroxine and FL 3) plasma levels of thyroxine and relative proportional abundances of major fraction, S_2 4) plasma levels of thyroxine and proportions of minor hemoglobin fraction, S_8 in each of the experimental groups was assessed by use of the simple correlation coefficient, r (Zar, 1975).

Thyroxine diet experiment

Coho salmon used in the thyroxine diet experiments were randomly distributed into four 0.3 m^3 recirculating tanks and acclimated for 3 weeks. On November 8, 1981, two groups of fish received thyroxine treated diets consisting of OMP supplemented with 50 µg L-thyroxine/g OMP and treatment continued for 65 days. Studies by Higgs et al. (1979) indicated this dosage resulted in approximately 4.49 µg/g/day of ingested hormone by treated fish and did not enhance growth. L-thyroxine (Sigma) was dissolved in 5 ml NaOH after which 95% ethanol was added so that the solution could be distributed over the food. Diets were made weekly and stored at -20° C after the ethanol was evaporated off. Prolonged storage of OMP treated with T_4 in this manner was not found to degrade thyroxine (Higgs et al., 1979). Control diets consisted of 1) untreated OMP (C-U) 2) OMP treated with the solvent, 95% ethanol (C-S).

Diurnal fluctuations in plasma thyroxine levels have been observed in salmonids (Higgs and Eales, 1973). Therefore, to maintain consistency, samples were taken between 0900 and 1045 hrs. At intervals of 8, 16, 33, and 65 days after the onset of the experiment, 5-10 fish from each treatment and control tank were sampled for hemoglobin and hormone analysis.

Thyroxine immersion experiment

Coho salmon used in the immersion experiment were randomly distributed in 4 aerated static brown containers (55 fish/100 L water)

partially submersed in a water bath to maintain the water temperature at 10-11° C. Beginning on November 8, 1981, two tanks were treated every other day with L-thyroxine to maintain a concentration of 10 μg T_4/100 ml in these tanks. This dosage has resulted in sustained plasma levels of brook trout (Salvelinus fontinalis) to 1-3 ng/100 ml (Eales, 1974). The other two tanks were used as controls and no hormone was added to the water. The application of hormone by immersion techniques continued for 60 days. 1 M NaOH (5 ml) used to dissolve the hormone did not significantly alter the pH of the treated water. To help minimize the amount of food debris and waste products, all tanks were cleaned by siphoning every other day out approximately 60 liters of water which was replaced with the same amount of fresh well water to maintain a volume of 100 liters. In this experiment, fish were fed to satiation on alternate days when no siphoning was taking place. Five to 10 fish were sampled between 0900 and 1045 hour on days 5, 16, 25, and 60 after the initiation of the thyroxine immersion experiment.

RESULTS

Thyroxine diet

Qualitative analysis of the hemoglobin fractions in each treatment group on January 12, after 65 days of feeding experimental diets indicated the majority of the fish (all but two) in the two T_4 groups, T-1 and T-2, were found to exhibit hemoglobin fractions (3 major and 5 minor fractions) as compared to the control group, C-U and C-S, (3 major and 6 minor fractions) (Fig. 12C). The control groups and 17%

Figure 12. Mean R_X values ± S.E. for various hemoglobin fractions (designated as S_1 - S_{10}) observed in zero age coho salmon after 8, 33, and 65 days of supplementing the diet with L-thyroxine (groups, T-1, T-2) as compared to controls fed untreated diet (C-U) and diet treated with solvent used to apply the hormone (C-S).

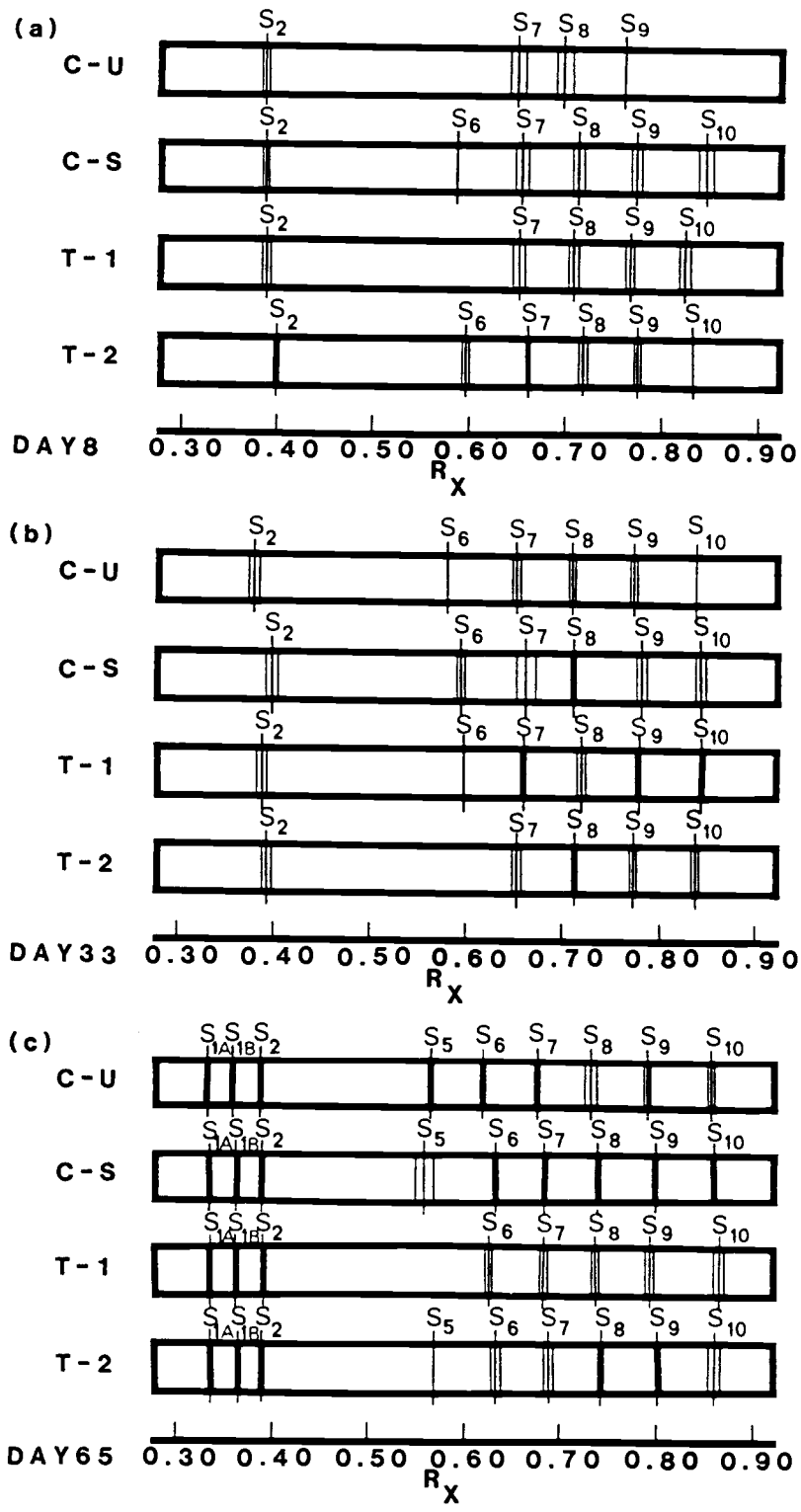
(a)
C-U
C-S
T-1
T-2
DAY8
0.30 0.40 0.50 0.60 0.70 0.80 0.90
R_X
(b)
C-U
C-S
T-1
T-2
DAY33
0.30 0.40 0.50 0.60 0.70 0.80 0.90
R_X
(c)
C-U
C-S
T-1
T-2
DAY65
0.30 0.40 0.50 0.60 0.70 0.80 0.90
R_X

Figure 12.

of the T_4 treated groups showed an additional minor fraction designated S_5 not seen in the other T_4 treated fish.

Thirty three days after initiation of the experimental diets, 88% of the T_4 group had only four minor hemoglobin fractions (S_{10}, S_9, S_8, S_7), whereas 50% of the control group exhibited five minor fractions (S_{10}, S_9, S_8, S_7, S_6) (Fig. 12B). All groups had one major fraction, S_2.

Considerable variation in the number of hemoglobin fractions observed in all 4 groups occurred 8 days after T_4 treatment. Although the majority of the fish in groups, C-S, T-1, and T-2, exhibited one major hemoglobin fraction and four minor fractions, the four minor fractions were not the same in all the groups as determined by their electrophoretic mobilities and calculated R_X values (Fig. 12A). The C-S and T-1 group exhibited minor fractions S_6, S_7, S_8, S_9, S_{10}, whereas the T-2 group exhibited S_6, S_7, S_8, S_9. The majority of the fish in the C-U group were found to have one major and two minor fractions (S_7 and S_8), even less than those groups listed above.

The quantitative analysis of data obtained from fish after 65 days of treatment revealed lower relative proportions of minor hemoglobin fractions, S_5, S_6, S_7, S_8, S_9 in T_4 treated groups than for both control groups (Fig. 13C). These variations in abundances among all groups were significant for fractions S_6 ($F = 4.07 > F_{0.001(3,23)} = 3.75$), S_7 ($F = 3.95 > F_{0.025(3,23)} = 3.75$), and S_8 ($F = 3.08 > F_{0.05(3,23)} = 3.03$).

The appearance of three major hemoglobin fractions (S_2, S_{1A}, and S_{1B}) was observed in all groups at this time. Due to the difficulty

Figure 13. Relative proportional abundances of observed hemoglobin fractions (S_1 - S_{10}, as defined in Fig. 1) represented as the percent of the total hemoglobin present (mean ± S.E., ratio above the bar represents n found to exhibit fraction divided by n sampled) in zero age coho salmon after a) 8 days b) 33 days c) 65 days of supplementing the diet with L-thyroxine (T-1 and T-2) as compared to controls fed untreated diet (C-U) and diet treated with solvent used to apply the hormone (C-S).

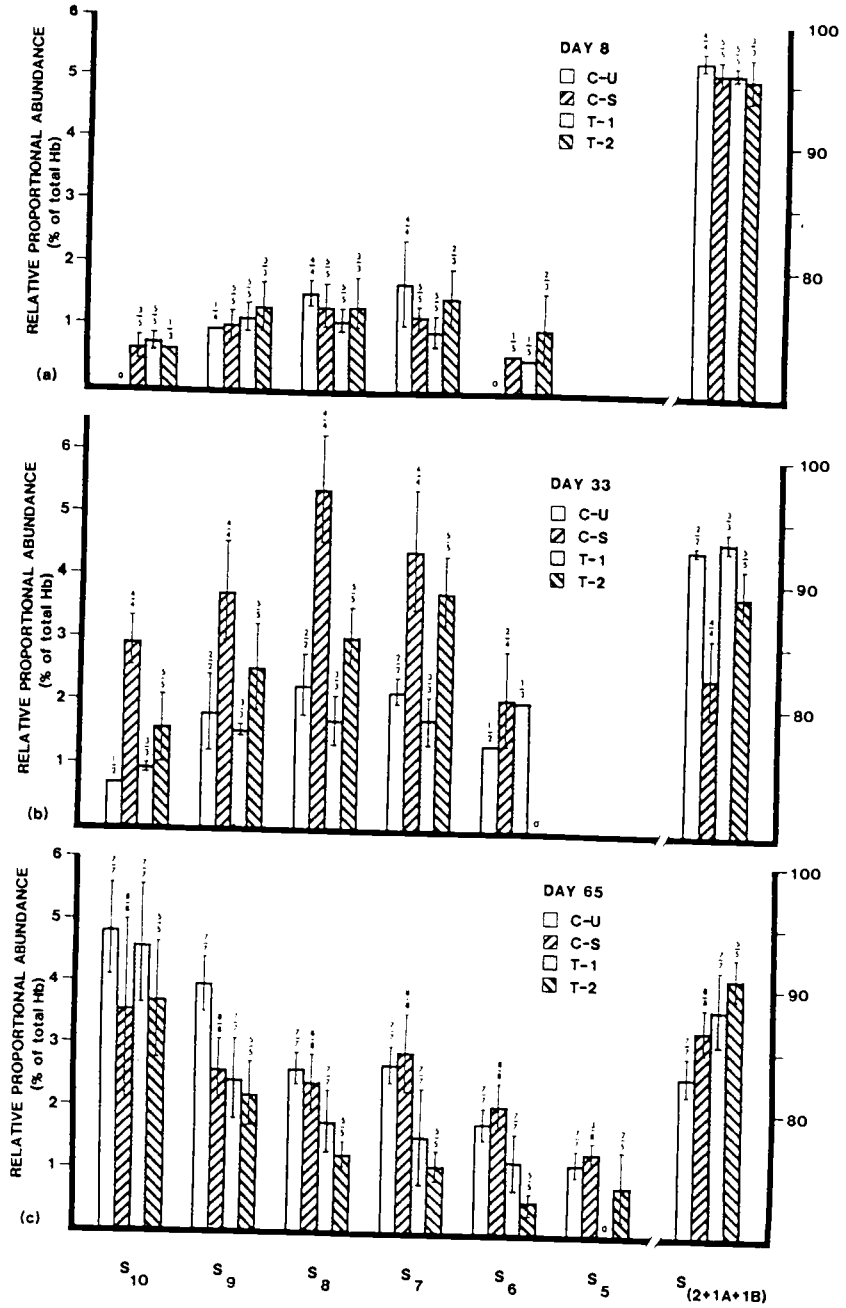
RELATIVE PROPORTIONAL ABUNDANCE (% of total Hb)
DAY 8
C-U
C-S
T-1
T-2
(a)
DAY 33
(b)
DAY 65
(c)
100
90
80
S_{10}
S_9
S_8
S_7
S_6
S_5
$S_{(2+1A+1B)}$

Figure 13.

of separating out the peaks resulting from the densitometer scanning of the three major fractions, it was necessary to combine the proportional abundances for these fractions into a pooled value, $S_{2 + 1A + 1B}$.

It was found through several trial runs, that the solution of PMSF used in the assay to inhibit enzyme activity had become inactive after dilution. The absence of this active PMSF led to three major fractions being present in this region of the gel, whereas in the presence of PMSF only one major fraction was observed. However, since all the blood samples were treated similarly in the assay and all groups exhibited the same three major hemoglobin fractions, the data was utilized for comparison among treatment groups.

The pooled relative proportional abundance for the major hemoglobin fraction, now designated $S_{2 + 2A + 1B}$ was significantly higher in the T-2 group than the control group, C-U ($\overline{X} = 0.91$, $n = 5$ vs. $\overline{X} = 0.83$, $n = 7$; $t = 2.18 > t_{0.02(10)} = 2.76$). However, test of significance by ANOVA showed no difference in the mean proportions of this fraction among the experimental diet groups.

On Dec. 11, 33 days after the start of the experimental diet, fish in the C-S group were observed to have significantly higher relative proportional abundances for minor hemoglobin fractions S_{10} and S_8 than those on the other groups (ANOVA followed by SNK: S_{10} - $F = 4.12 > F_{0.05(3,9)} = 3.86$ and S_8 - $F = 7.01 > F_{0.01(3,10)} = 6.55$) (Fig. 13B). An inverse relationship was shown between the proportional abundance of minor fractions and the major fraction among all groups. As a result of this relationship the C-S group also had the lowest

proportional abundance calculated for the major hemoglobin fraction, S_2 compared to groups, T-1, T-2, and C-U (ANOVA followd by SNK; $F = 4.85 > F_{0.025(3,10)} = 4.83$). The overall effect ethanol (the solvent used to distribute the hormone) had on treatment is unclear, since both the fractions revealed in the treated groups were not similarly effected.

The relative proportions of each hemoglobin fraction were not found to vary significantly among all four groups after only 8 days of experimental diets (Fig. 13A).

Average body weights of fish in the C-S group and T_4 treated groups, T-1 and T-2, were higher than mean weights observed for the control group, C-U, after 33 days (ANOVA followed by SNK; $F = 7.27 > F_{0.005(3,24)} = 5.52$) (Table 2). However, the mean condition factor was significantly higher in the C-S group only as compared to the mean value for T-2 and C-U ($\overline{X} = 1.35 \pm 0.03$, $n = 7$ vs $\overline{X} = 1.25 \pm 0.02$, $n = 7$; ANOVA followed by SNK, $F = 5.11 > F_{0.05(3,24)} = 3.01$). The next sampling date, 65 days after, revealed the fish in the T-2 group had a higher mean body weight than either the C-U group or T-1 group (ANOVA followed by SNK, $F = 4.25 > F_{0.05(3,36)} = 3.54$). Despite this difference in weights, the mean fork lengths and condition factor were similar for all groups of fish at this time.

The mean hematocrit levels taken at the end of the experiment were the same for all groups.

Feeding of T_4 did not significantly elevate plasma thyroxine levels (Fig. 14) after 65 days as compared to control fish. However, significant differences in thyroxine levels were revealed between the

Table 2. Sample numbers (n), weight, condition factor ($\bar{X}$ ± S.E.) of zero age coho salmon fed diet supplemented with L-thyroxin (50 ppm) over 65 days as compared to controls fed untreated diet (C-U) and diet treated with solvent used to apply the hormone (C-S).

Days after Initiation	Treatment	N	Weight (g)	Condition Factor (g/cm^3)	Hematocrit
8	C-S	5	17.7 ± 1.9	1.28 ± 0.06	
	C-U	5	19.8 ± 1.9	1.26 ± 0.06	
	T-1	6	18.2 ± 3.1	1.31 ± 0.06	
	T-2	4	15.5 ± 2.2	1.22 ± 0.07	
16	C-S	5	20.3 ± 2.0	1.40 ± 0.07	-
	C-U	5	20.3 ± 1.9	1.27 ± 0.04	
	T-1	5	17.0 ± 2.2	1.28 ± 0.08	
	T-2	5	20.4 ± 3.5	1.29 ± 0.05	
33	C-S	7	26.0 ± 1.9	1.36 ± 0.03	-
	C-U	7	15.6 ± 1.4	1.24 ± 0.02	
	T-1	7	21.0 ± 0.9	1.30 ± 0.02	
	T-2	7	25.2 ± 2.8	1.31 ± 0.02	
65	C-S	10	28.5 ± 1.7	1.28 ± 0.03	39.8 ± 1.8
	C-U	10	23.6 ± 2.5	1.12 ± 0.07	37.2 ± 1.3
	T-1	10	25.5 ± 1.9	1.24 ± 0.02	38.2 ± 1.6
	T-2	10	32.6 ± 1.4	1.18 ± 0.04	40.3 ± 1.5

Figure 14. Plasma thyroxine concentration (mean ± S.E., n above the bar) in zero age coho salmon fed a diet supplemented with T_4 after 5, 16, 33, and 65 days of treatment compared to controls fed untreated diet (C-U) and diet treated with solvent used to apply the hormone (C-S).

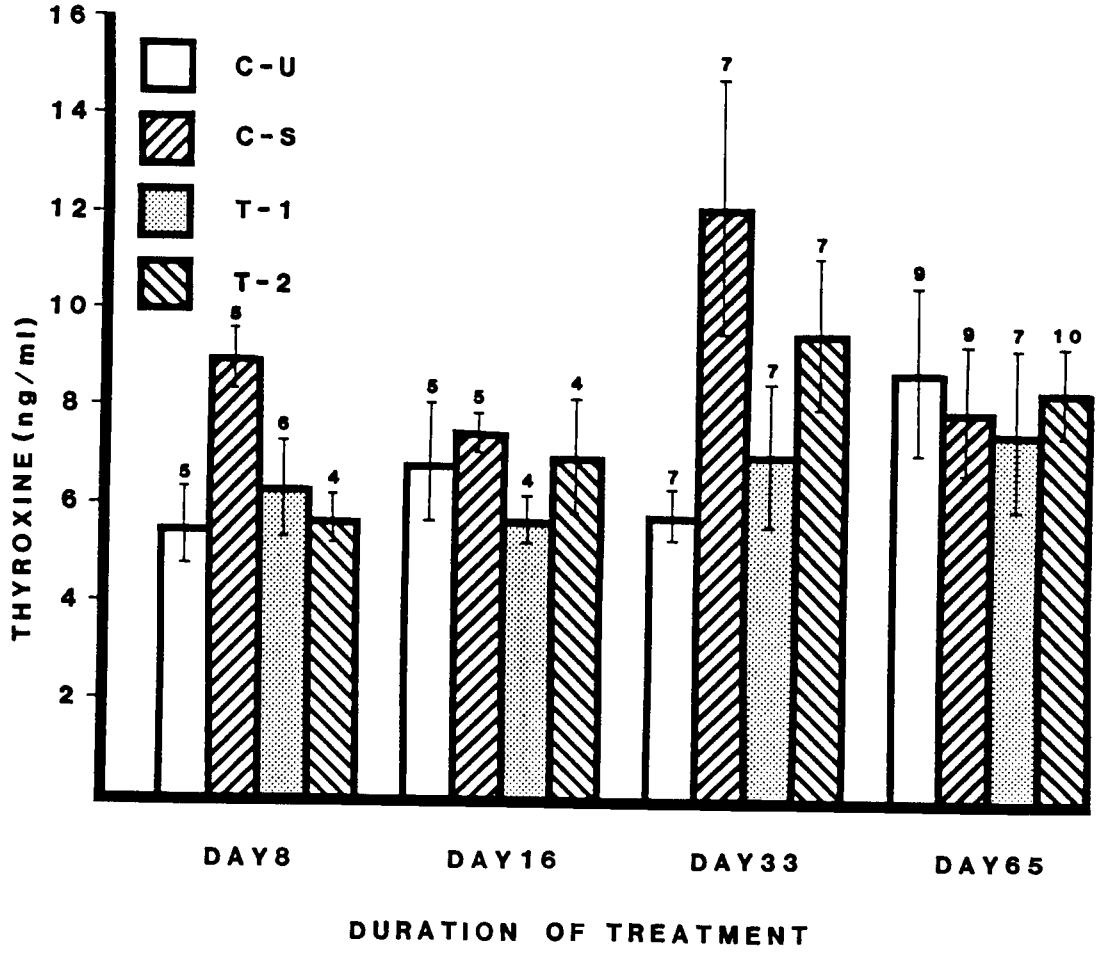
16
14
12
10
8
6
4
2
THYROXINE (ng/ml)
C-U
C-S
T-1
T-2
5
8
6
4
5
5
4
7
7
7
9
9
7
10
DAY8
DAY16
DAY33
DAY65
DURATION OF TREATMENT

Figure 14.

control groups, C-S and C-U, for earlier sampling dates. The mean plasma T_4 level was observed to be higher for the C-S group (8.99 ± 0.81 ng/ml; n = 5) than for the C-U group (5.52 ± 1.40 ng/ml; n = 5) 8 days after initiation ($t = 3.49 > t_{0.01(8)} = 3.36$) and 33 days after initiation (12.18 ± 2.6 ng/ml; n = 7 vs. 5.82 ± 0.49 ng/ml; n = 7). T_4 treated groups and the C-U group had similar mean plasma T_4 levels throughout the experiment (SNK) indicating treatment with ethanol only (C-S) may have an effect on thyroid hormone secretion.

Plasma thyroxin levels in the fish sampled after 65 days tended to be correlated with mean weight and forklength ($r = 0.88 > r_{0.05(2)\ 29} = 0.335$). This correlation was noticed 8 and 33 days after initiation of the experimental diets as well.

The relative proportions of major hemoglobin fraction S_2 and minor fraction S_8 obtained for T_4 treated fish tended to be correlated with plasma T_4 levels ($r = 0.88$ and $r = 0.93$, respectively $> r_{0.05(2)\ 9} = 0.67$). This relationship was also apparent for the C-U and C-S groups.

The relative proportion for major fraction S_2 and minor fraction S_8 were found to be inversely related with r value = -0.85 ($r_{0.05(2)\ 25} = 0.462$) for all groups of fish.

Thyroxine immersion

During the course of the thyroxine immersion study, mortalities began to occur starting January 2, 1981, in some of the tanks. The apparent reasons for these mortalities are unclear, although ammonia toxicity was most likely due to the use of a static system. As a result, fish were only sampled from the two healthy groups and no replicate tanks were used in the final sample period.

The resulting hemoglobin pattern observed for fish immersed in the thyroxin solution for 60 days did not differ much from that seen for the control group (Fig. 15B). Fish in both groups exhibited the major hemoglobin fraction S_2 and six minor hemoglobin fractions S_4, S_5, S_7, S_8, S_9, S_{10}. Hemoglobin fraction S_4 was only seen in 10% of the fish sampled from the controls and 33% from the T_4 treated groups. At the beginning of the T_4 treatment, the T_4 group did not exhibit the minor fraction S_6. However, all other fractions seen in the control were present. Only 50% of the fish in the control group produced this particular fraction as well. Perhaps these individuals exhibiting more hemoglobin fractions, hence producing a more advanced pattern, were developing at a faster rate than the rest of the population.

Relative proportions of the minor and major fractions were similar in the T_4 immersed group and the controls throughout the experiment (Fig. 16).

Mean weights, fork lengths, and condition factors remained the same for all groups of fish after 5, and 60 days of treatment (Table 3). A significantly higher mean body weight was found for the T_4 immersion group than for the control group after 25 days (14.1 ± 0.8, $n = 11$ vs. 15.6 ± 0.9, $n = 12$; $t = 2.11 > t_{0.05(21)} = 2.08$).

Immersion in T_4 did significantly elevate the concentration of thyroxine in the plasma after 5 days ($t = 3.58 > t_{0.05(9)} = 2.26$), 25 days ($t = 2.33 > t_{0.05(8)} = 2.31$), and 60 days ($t = 3.12 > t_{0.05(10)} = 2.23$) as compared to freshwater controls (Fig. 17).

Figure 15. Mean R_X values ± S.E. for various hemoglobin fractions (designed as S_2 - S_{10}) observed in zero age coho salmon immersed in T_4 treated water (10 µg/100 ml) after 5 and 60 days of treatment compared to freshwater controls receiving no thyroxine (CON).

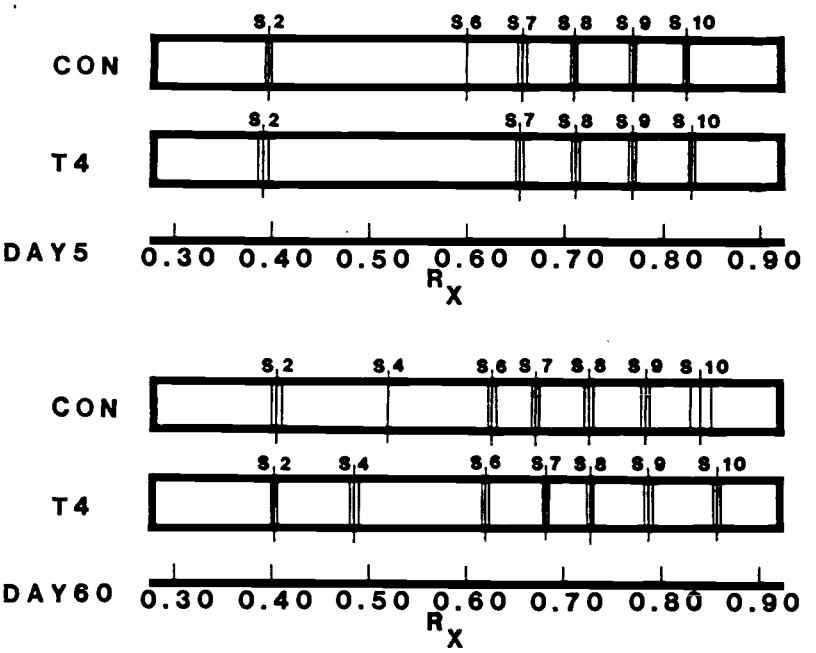
S,2
S,6
S,7
S,8
S,9
S,10
CON
T4
DAY5
0.30 0.40 0.50 0.60 0.70 0.80 0.90
R_X
S,2
S,4
S,6
S,7
S,8
S,9
S,10
CON
T4
DAY60
0.30 0.40 0.50 0.60 0.70 0.80 0.90
R_X

Figure 15.

Figure 16. Relative proportional abundances of observed hemoglobin fractions S_2 - S_{10}, as defined in Fig. 4) represented as the percent of the total hemoglobin present (mean ± S.E., ratio above the bar represents the number of fish found to exhibit fraction divided by the total number of fish) in zero age coho salmon immersed in T_4 treated water after a) 5 days and b) 60 days of treatment compared to freshwater controls receiving no thyroxine (CON).

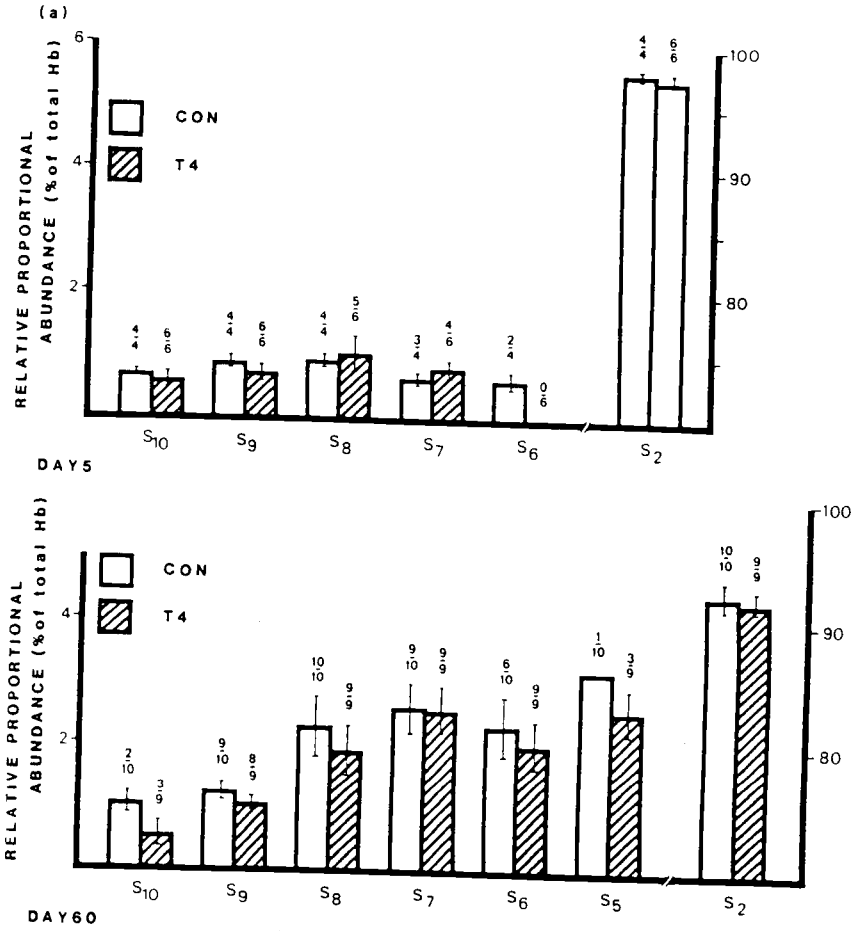
(a)
RELATIVE PROPORTIONAL ABUNDANCE (%of total Hb)
CON
T4
DAY5
S10
S9
S8
S7
S6
S2
DAY60
S5

Figure 16.

Table 3. Sample numbers (n), weight, condition factor ($\bar{X}$ ± S.E.) of zero age coho salmon immersed in T_4 treated water (10 µg/ml) 60 days as compared to freshwater controls receiving no thyroxine.

Days after Initiation	Treatment	N	Weight (g)	Condition Factor (g/cm^3)	Hematocrit
5	CON	9	13.6 ± 1.2	1.13 ± 0.02	-
	T_4	12	13.2 ± 1.2	1.21 ± 0.09	
16	T_4	10	14.7 ± 1.7	1.19 ± 0.04	
23	CON	11	14.1 ± 0.8	1.19 ± 0.02	
	T_4	12	15.6 ± 0.9	1.26 ± 0.03	
57	CON	13	17.9 ± 1.0	1.14 ± 0.01	
	T_4	15	18.2 ± 1.3	1.20 ± 0.02	
60	CON	35	21.3 ± 1.2	1.23 ± 0.02	34.4 ± 0.8
	T_4	28	19.8 ± 0.7	1.23 ± 0.02	29.8 ± 2.2 (n = 10)

Figure 17. Plasma thyroxine concentration (mean ± S.E., n above the bar) in zero age coho salmon immersed in T_4 treated water (10 µg/100 ml) after 5, 13, 25, and, 60 days of treatment compared to freshwater controls receiving no thyroxin (CON).

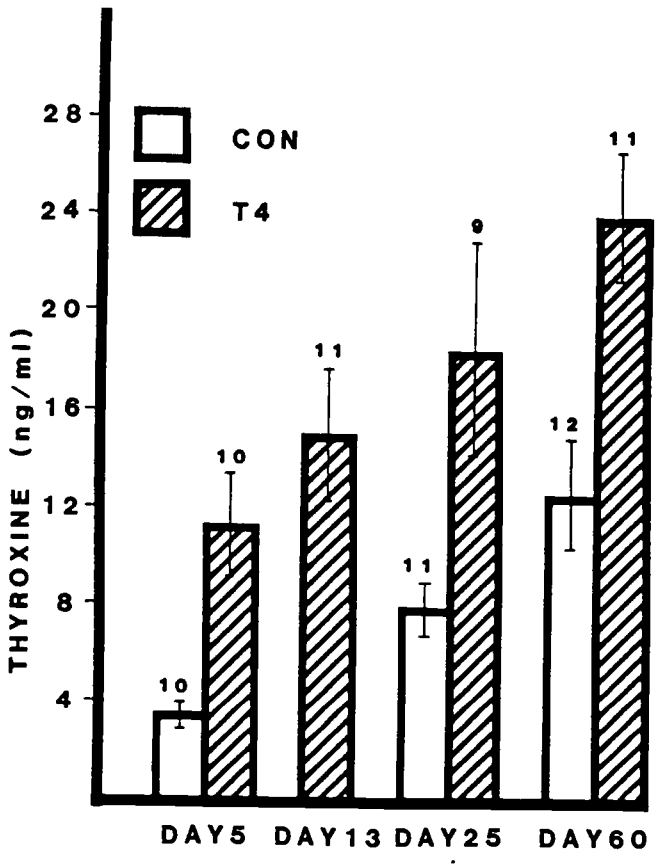
28
24
20
16
12
8
4
THYROXINE (ng/ml)
CON
T4
10
10
11
11
9
12
11
DAY5
DAY13
DAY25
DAY60

Figure 17.

DISCUSSION

Effects of thyroxine in the diet

Administration of T_4 to zero age coho salmon via the diet tended to reduce the number and lower the relative proportional abundances of minor hemoglobin fractions after 60-65 days of treatment in relation to controls. Prolonged T_4 treatment therefore, seemed to suppress production of these minor hemoglobin fractions that appear during normal development as seen in the control groups. These findings are contrary to observations of Koch et al. (1964), who fed 2 year old Atlantic salmon, Salmo salar, a diet consisting of 20% thyroid tissue and found they displayed an advanced hemoglobin pattern (seen by an increase in the number of hemoglobin fractions) relative to that expected for a particular size. For this particular species and age class therefore, thyroid application to the diet accelerated the progressive development of hemoglobin fractions. Other potential reasons for these conflicting observations, besides species and age differences, include variations in the duration of T_4 treatment and perhaps the type and amount of exogenous thyroid hormones getting into the fish. The latter point could explain differences found in the results for the T_4 treated groups in this study.

Discrepancies in the control group, C-U and C-S, after 33 days indicated the C-S group, fed OMP treated with the solvent used to apply the hormone, displayed an increase in the numbers and proportions of the minor hemoglobin fractions and decrease in the

proportions of the major "juvenile" hemoglobin fraction as compared to all other groups. Thus the treatment of OMP with solvent induced a more advanced hemoglobin pattern seen in fish 5.5 months older (see Fig. 1, 3, and 4, main text) during parr-smolt transformation period found in this species. It is interesting to note the highest plasma T_4 levels were also exhibited by fish in this solvent treated group. A direct correlation between the relative abundances of hemoglobin fractions and plasma T_4 levels was supported by the data for all groups.

It is unclear how ethanol affects the quality of the OMP, but studies done by Higgs et al. (1979) described differences in growth rate, food consumption, and condition factor between groups of coho salmon fed OMP treated with a low amount of solvent (5 ml alkaline 70% ethanol/kg) and OMP treated with a high level of solvent (45 ml/kg) after 112 days of treatment. The level used in this study was compared to the high solvent (50 ml/kg) although 95% ethanol was used instead of 70% ethanol.

If the solvent was responsible for producing increases in the endogenous T_4 levels in the plasma and accelerating the developmental process of hemoglobin production resulting in increases in the number and proportions of the minor hemoglobin fractions, one would also expect to see similar results in the T_4 treated groups, T-1 and T-2. Unfortunately, neither of these groups responded in the same manner as the C-S group, although the T-2 group did exhibit higher proportions of the minor fractions than either T-1 or C-U and had values that were closer to that found in the C-S group. One plausible explanation for

the inconsistencies between T_4 groups and the C-S group is that prolonged exposure to T_4 at the dosage of 50 µg/g could cause a surplus of endogenous T_4 in the plasma, possibly working as a negative feedback mechanism decreasing production of the circulating hormone. This explanation is further supported by histological data describing a depression in the thyroid epithelial cell height after 112 days of feeding T_4 treated OMP to zero age coho salmon at the same dosage used in this study (Higgs et al., 1970). At this dosage therefore, a sufficient amount of T_4 was absorbed or produced to cause a decrease in thyroid function consequently leading to decreased endogenous levels of thyroid hormones. This suppression of the thyroid function could be indirectly related to the suppression seen in the hemoglobin system of T_4 treated fish as described earlier. Similar inhibitions in the expression of various groups of hemoglobin fractions have been reported in yearling coho salmon fed diet containing low dosages of PTU, a synthetic goitrogen (2 mg/g) (Craig Sulllivan, unpublished data). Another explanation could be related to increases in plasma T_4 turnover rate. Since the span of time between the feeding of treatment diets and the actual sampling period was up to 18 hrs, this would allow enough time to elapse for endogenous T_4 levels to return to basal levels as observed at sampling time.

Both the C-S and T-2 groups were found to have larger mean weight and forklengths than the other groups indicating these fish were growing at a faster rate. This could account for the accelerated hemoglobin pattern observed for these fish. Perhaps more attention should be given in future studies to monitor the amount of treated

food fed to each tank instead of feeding fish to satiation, since growth and development are both critical factors in the hemoglobin system.

Although there might be a relationship between fish having higher endogenous T_4 levels and exhibiting advanced hemoglobin profiles, treatment of T_4 to the diet did not give consistent results and in fact showed suppression of normal hemoglobin production compared to controls. A more likely reason that is supported by the data is that those fish that had a faster growth rate as seen by higher weights and forklengths also exhibited higher T_4 levels in the plasma. As a result of developing faster than the majority of the fish in the population, these fish displayed an advanced hemoglobin profile.

Additional work using other application processes to sustain levels of plasma T_4 without using high levels of solvent and using various dosages < 50 µg T_4/g to prevent suppression of thyroid function are necessary. Once this is accomplished, one can further test whether increased T_4 levels in the plasma can accelerate the production of the hemoglobin fractions seen at a later developmental stage like that seen in the C-S group 33 days after initiation of treatment.

Effects of thyroxine immersion

Another application procedure that resulted in sustaining endogenous plasma T_4 levels was unsuccessful in inducing any significant changes in either the numbers of hemoglobin fractions displayed or their relative proportional abundances after 60 days.

From this study, one would conclude immersion by T_4 has no influence on accelerating hemoglobin production. However, one must realize the immersion technique has to be somewhat stressful to the animal, in that it is a static environment and the tendency for toxic wastes to build up are greater than with flow through systems. Additional handling of tanks during siphoning procedures and feeding only every other day can cause added stress that would alter normal developmental processes. The lower growth rates seen in the immersion study as compared to the diet study supports this idea. Narayansingh and Eales (1974) were able to eliminate problems associated with toxic waste build up experienced in my study by transferring fish every 24 hrs into identically treated tanks. Future studies using less stressful techniques such as that described must be designed to enable fish to grow and develop normally. Only after this is accomplished can one further test the relationship between T_4 and variations in the hemoglobin system.

In conclusion, the application of T_4 in the diet was found to inhibit the expression of minor hemoglobin fractions that appear during normal development. However, administration of T_4 by immersion techniques did not alter the hemoglobin pattern from that of controls.

Appendix IV.

ASSESSMENT OF THE HEMOGLOBIN PROFILE OF SPRING CHINOOK SALMON, ONCORHYNCHUS TSCHAWYTSCHA, DURING AUGUST TO OCTOBER, 1981

Spring chinook salmon, Oncorhynchus tschawytscha, appear to have five different migratory patterns within a population (Ewing et al., 1980). This makes it difficult to determine when to release these fish from the hatcheries. Scale analysis from returning adults, however, indicate peak migration, at least for Rogue River chinook salmon, occurs around mid August to September (Buckman and Ewing, 1982).

The multiple hemoglobin composition of salmonids has been found to undergo progressive changes during development (Koch, 1964, and Giles and Vanstone, 1976) perhaps indicative of the fish's physiological state. This might be further useful in helping determine an appropriate time of release of salmon from the hatcheries. Although the hemoglobin profile has been examined in other salmon, no studies have described this pattern in chinook salmon during parr-smolt transformation using polyacrylamide.

The objectives of this experiment were to determine 1) if there were any significant changes in the hemoglobin profile qualitatively or quantitatively, in chinook salmon during a period corresponding to parr-smolt transformation in this species and 2) to relate the time of these changes to data obtained by other investigators, concerning migratory readiness, saltwater survival, and yields of adult returns.

MATERIALS AND METHODS

Animal Maintenance

The Cole Rivers Hatchery spring chinook salmon used in this study were part of a growth rate study being conducted by Rick Birks, Oregon Department of Fish and Wildlife, Corvallis Research Laboratory, Oregon. Fish were reared in 0.7 m^3 flow through circular tanks under a constant growth regime in which fish were given approximately 34.6 grams of pelleted food/day or 1% of total/wt/tank/day. Fish were fed three times a day in this way. This type of feeding resulted in a linear growth pattern which corresponds to a growth pattern found for juvenile chinook salmon reared at the Cole Rivers Hatchery (personal communications, R. Birks, Oregon Department of Fish and Wildlife, Corvallis, Oregon). Water temperatures in the tank were maintained at 7.6° C throughout the sample period.

Sampling Procedure

At sampling, all animals were stunned by a blow to the head, measured, and weighed. Blood was withdrawn from the severed caudal artery into heparinized capillary tubes. Blood samples were kept on ice until they were centrifuged at 700 g for 5 min. at 0° C in a Beckman TJ-6R refrigerated centrifuge and the plasma supernatant was removed.

Hemolysate preparations, electrophoresis, and statistical analysis were as described previously in the main study using Eagle Creek coho salmon.

RESULTS

Separation of spring chinook salmon hemolysates by high pH polyacrylamide electrophoresis revealed the presence of eight hemoglobin fractions labeled T_1 through T_8, as determined by their calculated R_X values. Mean R_X values ± S.E. for the various hemoglobin fractions were as follows: $T_1 = 0.831 \pm 0.002$; $T_2 = 0.783 \pm 0.003$; $T_3 = 0.736 \pm 0.018$; $T_4 = 0.585 \pm 0.009$; $T_5 = 0.702 \pm 0.004$; $T_6 = 0.497 \pm 0.003$; $T_7 = 0.446 \pm 0.003$; $T_8 = 0.396 \pm 0.003$.

The majority of the spring chinook salmon sampled from August through to October, 1981, showed a hemoglobin pattern consisting of six hemoglobin fractions designated T_1, T_2, T_3, T_6, T_7, T_8. The appearance of hemoglobin fraction T_4 occurred on September 2 and was only seen in 45% of the fish sampled (n = 11) on this date. Another fraction T_5, appeared in 15% of the fish sampled (n = 13) on September 18, but was not noticed in the following sample dates.

Quantitative analysis of each fraction revealed highly significant variations in hemoglobin fractions T_1, T_2, T_3, T_6, T_7, over time (T_1 - $F = 8.65 > F_{0.001(3.52)} = 6.34$; T_2 - $F = 4.22 > F_{0.01(3.52)} = 4.20$; T_3 - $F = 5.57 > F_{0.005(3.52)} = 4.83$; T_6 - $F = 12.16 > F_{0.001(3.52)} = 6.34$; T_7 - $F = 5.15 > F_{0.005(3.52)} = 4.83$). Relative proportional abundances for the hemoglobin fractions were found to increase to peak values for T_1, T_2, and T_3 on October 6, whereas T_6 and T_7 exhibited decreases in their proportions on this date (SNK) (Fig. 18).

Mean forklength, weight, and condition factor were not significantly different during the sample period.

Figure 18. Relative proportional abundances of observed hemoglobin fractions ($T_1 - T_8$) represented as the percent of the total hemoglobin present (mean ± S.E.) in Cole Rivers spring chinook salmon sampled August - October, 1981. Figures in parenthesis: (0.05) - significance at $P < 0.05$ and (NS) - no significance at the 0.05 level. Mean values that are significantly different (SNK) are marked with the asterisk. Samples sizes for each date were as follows: Aug. 4, n = 20; Sept. 2, n = 12; Sept. 18, n = 11; Oct. 6, n = 13.

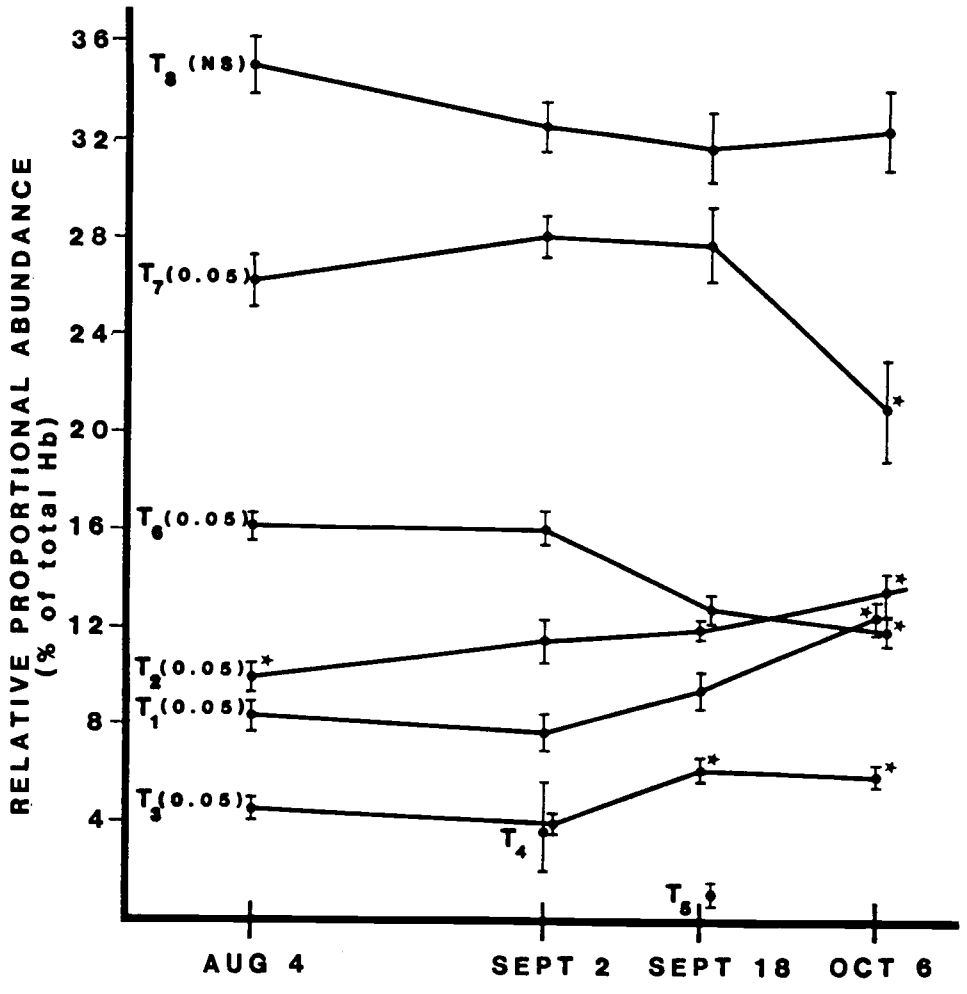
RELATIVE PROPORTIONAL ABUNDANCE
(% of total Hb)
36
32
28
24
20
16
12
8
4
T8 (NS)
T7(0.05)
T6(0.05)
T2(0.05)
T1(0.05)
T3(0.05)
T4
T5
AUG 4
SEPT 2
SEPT 18
OCT 6

Figure 18.

Gill Na-K-ATPase enzyme activity obtained from the same population of fish exhibited a single peak in September followed by a drop in October (personal communication, R. Birks, Oregon Department of Fish and Wildlife, Corvallis, Oregon).

DISCUSSION

A definite quantitative variation in the relative proportional abundances of hemoglobin fractions produced, was observed for laboratory reared spring chinook salmon during September and October. Three hemoglobin fractions, T_1, T_2, T_3, exhibited increased proportions while the hemoglobin fractions T_6 and T_7 showed decreased proportional values.

The timing of these significant changes in the production of the various hemoglobin fractions corresponds to the time when wild juvenile spring chinook salmon tend to migrate towards the ocean (Ewing and Birks, 1982).

Gill Na-K-ATPase activity observed for the fish used in this study began to decline at approximately the same period that proportional variations for the hemoglobin fractions appeared in September and October. Although these two physiological processes may be correlated, there is probably not a causal relationship, but they just happen to be exhibiting similar activities in response to the developmental process involving parr-smolt transformation.

Finally, to determine if the changes in the hemoglobin composition can be used to determine an optimum release time for hatcheries to utilize to maximize survival of spring chinook salmon to adulthood,

the hemoglobin profile, involving qualitative and quantitative variations, must be compared to hatchery releases, ocean entry of juveniles, and adult survival and return information for specific stocks of spring chinook salmon.