MEASUREMENT OF THYROXINE CONCENTRATION AS AN INDICATOR OF THE CRITICAL PERIOD FOR IMPRINTING IN KOKANEE SALMON (ONCORHYNCHUS NERKA): IMPLICATIONS FOR OPERATING LAKE ROOSEVELT KOKANEE HATCHERIES

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ABSTRACT

Previous investigations have determined that thyroid hormone surges activate olfactory imprinting in anadromous salmonid smolts. The mechanism of action appears to require binding of thyroid hormones to receptors in brain cell nuclei, which stimulates neuron differentiation and wires a pattern of neuron circuitry that allows for the permanent storage of the imprinted olfactory memory, In this study, thyroxine concentrations [T_4] were measured in 487 Lake Whatcom stock and 70 Lake Roosevelt stock kokanee salmon to indicate the critical period for imprinting. Eggs, alevins and fry, reared at the Spokane Indian Kokanee Hatchery, were collected from January through August 1991. Sampled fish were flash frozen on dry ice and stored at -80°C until T_4 was extracted and concentrations determined by radioimmunassay. T_4 levels were monitored in Lake Whatcom stock fish at approximately weekly intervals from 10 days before hatch to 185 days post hatch. Mean concentration ± SEM of 10-20 individual fish (assayed in duplicate) were determined for each time period. T_4 concentration peaked on the day of hatch at 16.8 ng/g body weight and again at swim-up at 16.0 ± 4.7 ng/g body weight. T_4 concentration was 12.5 to 12.9 ng/g body weight in eggs, 7.1 to 15.2 ng/g body weight in alevins, 4.5 to 11.4 ng/g body weight in 42 to 105 day old fry and 0.1 to 2.9 ng/g body weight in 112 to 185 day old fry. T_4 levels were monitored in Lake Roosevelt stock fish at approximately monthly intervals from the egg stage in January through the time they were released in July. T_4 concentrations were highest in eggs at 13.3 ± 2.8 ng/g body weight, then steadily decreased to 0.1 ± 0.1 ng/g body weight in older fry. Fry were released in Lake Roosevelt tributaries in July and August 1991, at about 170-180 days post hatching, in order to imprint them to those sites. The results of this study indicate that the time of release was not appropriate for imprinting. If T_4 levels are an accurate guide for imprinting in kokanee, our results suggest that the critical period for imprinting in kokanee is at hatching or swim-up stages.

ACKNOWLEDGEMENTS

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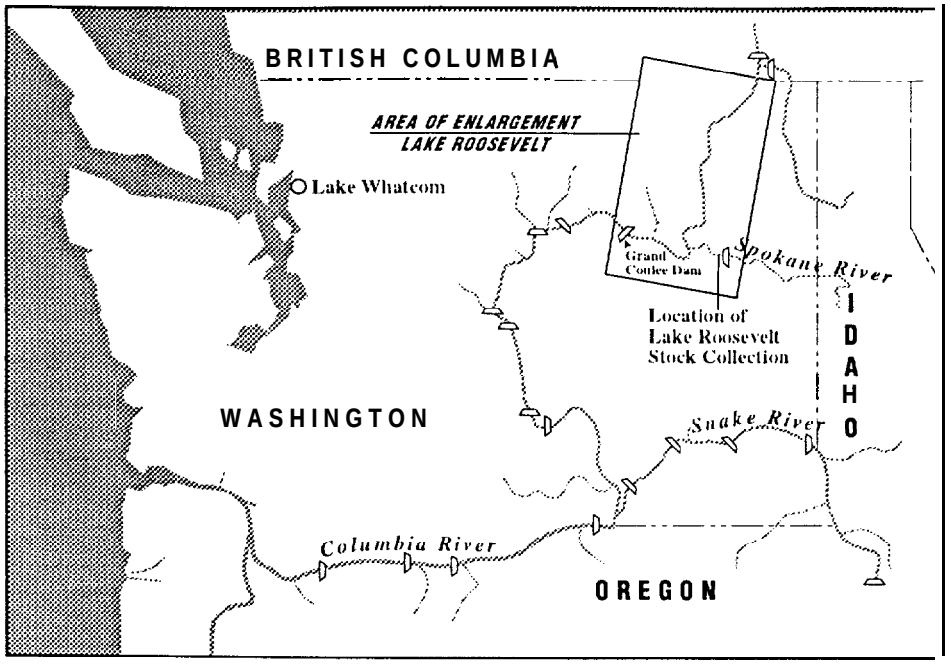
1 .O INTRODUCTION

In their 1987 Columbia River Basin Fish and Wildlife Program [1987 FWP--Section 903(g)(l)(C)], the Northwest Power Planning Council (NPPC) directed Bonneville Power Administration (BPA) to construct two kokanee salmon (*Oncorhynchus nerka*) hatcheries on Lake Roosevelt (NPPC 1987). At maximum production, the hatcheries will produce 8 million kokanee salmon for outplanting into Lake Roosevelt and 500,000 rainbow trout (*Oncorhynchus mykiss*) for the Lake Roosevelt net pen program. The aim of the hatcheries is to enhance the resident fishery in Lake Roosevelt as a partial replacement for the loss of anadromous salmon and steelhead trout from that region caused by the construction of Grand Coulee Dam. In 1939, Grand Coulee Dam blocked salmonids ascending above that point because it does not have a fish ladder.

1.1 *Lake Roosevelt kokanee hatcheries*

The Lake Roosevelt kokanee hatcheries are located at Metamooteles Springs on the Spokane Indian Reservation (operated by the Spokane Tribe) and Sherman Creek near Kettle Falls, WA (operated by the Washington Department of Wildlife--WDW) (Fig. 1). The operation of the two hatcheries is coordinated. The Sherman Creek hatchery will be used as an imprinting site and egg collection facility. Eggs collected there will be transferred to the Spokane Tribal hatchery for incubation and rearing to the fry stage before stocking back in Lake Roosevelt at Sherman Creek and other locations. The Spokane Tribal hatchery was constructed by BPA in 1990 and became operational in 1991. It currently receives kokanee eggs from the WDW Lake Whatcom hatchery in Bellingham, WA for rearing and collects kokanee eggs below Little Falls on the Spokane Arm of Lake Roosevelt. These fish are currently being outplanted at locations in Lake Roosevelt where it is hoped adult spawners will return for future egg takes, at Sherman Creek and at Little Falls Dam on the Spokane Arm of Lake Roosevelt. Construction of the Sherman Creek site was completed in 1991. It will become operational in Spring 1992.

Fig 1. Location of Lake Roosevelt kokanee hatcheries operated by Spokane Tribe and WDW



Inset shows location where fish stocks were obtained (Lake Whatcom Hatchery and Lake Roosevelt at Little Falls Dam).

The Power Council also directed BPA to fund a monitoring program to determine hatchery effectiveness [1987 FWP--Section (g)(l)(e)] (NPPC 1987). One objective of the monitoring program is to determine the best locations and times for stocking kokanee in terms of: (1) ensuring homing to release sites for egg collection; (2) reducing loss over Grand Coulee Dam; and (3) increasing harvest rates by anglers. The present study focuses on the first of these goals.

1 .2 *Critical period for imprinting: Implications for hatchery rearing of Lake Roosevelt kokanee.*

In anadromous salmonids, olfactory imprinting to homestream odors occurs during a narrow window, or critical period, at the time of smolt transformation, just prior to their seaward migration (reviewed by Ricker 1972; Hasler and Scholz 1983). If fish are transplanted from their natal tributary or hatchery into a different river before smolt transformation occurs, they will often adopt the river of release as their homestream and return there during the spawning migration. In contrast, if fish are transplanted from their natal tributary to a different one after smolt transformation occurs, they will not adopt the river of release but, instead, will return to the natal tributary. If fish are released from a hatchery after smolt transformation occurs, they either return to the hatchery rather than the stocking site (if the hatchery is in the vicinity of the stocking site) or stray into many streams (if they hatchery is located a great distance from the stocking site) (Ellis 1957, Peck 1970; reviewed by Ricker 1972, and Hasler and Scholz 1983).

Previous investigations have determined that thyroid hormone surges activate imprinting in coho salmon (*Oncorhynchus kisutch*) and steelhead trout (*Oncorhynchus mykiss*) smolts (Scholz 1980; Hasler and Scholz 1983). The mechanism of action appears to require binding of thyroid hormones to receptors in brain cell nuclei, which stimulates neruon differentiation and wires a pattern of neuron circuitry that allows for the permanent storage of the imprinted memory (Scholz *et* al. 1985; Lanier 1987; White *et al.* 1991).

1.3 *Study Objective: Measurement of thyroid concentrations in eggs and larvae as an indicator of the critical period for imprinting.*

Some species of salmon [e.g. pink (*Oncorhynchus gorbuscha*) and chum (*Oncorhynchus keta*)] do not exhibit a distinct smolt stage but instead leave the home stream for the ocean immediately after complete yolk absorption and emergence from redds. Anadromous sockeye salmon, as well as their landlocked kokanee relatives, typically migrate out of their natal tributary to a nursery lake soon after swimup. Sockeye salmon typically exhibit natal homing to tributaries of lakes experienced only as embryos or fry, not to the lake and its outlet experienced as smolts (Quinn *et al.* 1989). Therefore, these species must necessarily imprint during the egg or alevin stage.

Tagawa and Hirano (1987, 1989) measured thyroid hormones in the eggs and alevins of chum salmon and found a pronounced increase in thyroxine levels at about the time of swimup when their yolk sacs were completely absorbed. In post-emergent pink salmon maximum levels of thyroid hormone were detected at the time of complete yolk reabsorption (Sullivan *et al.* 1983). In contrast, Kobuke *et al.* (1987) determined that thyroid levels in coho salmon were highest in the egg stage and decreased in post-hatch larvae before increasing to very high levels in smolts. In this experiment, Kobuke *et al.* also noted that the thyroid gland of newly emerged coho fry was functional as evidenced by the fact that TSH treated fish responded by increasing thyroid hormone concentration as compared to saline treated controls. The implication of these studies is that thyroid hormones may be sufficiently high in eggs or newly hatched larvae to stimulate imprinting at those developmental stages. If so, hatchery reared fish may imprint in the hatchery during early developmental stages and not be able to imprint later to stocking sites.

The objective of the present study was to determine if these results could be extended to kokanee. Thyroxine [T_4] concentrations were measured in egg, larvae (alevin), and fry stages of 487 Lake Whatcom stock and 70 Lake Roosevelt stock kokanee salmon to indicate the critical period for imprinting.

2.0 METHODS

2.1 Fish stocks, rearing conditions and sample sizes.

Kokanee eggs, alevins and fry, reared at the Spokane Tribal Kokanee hatchery and Washington Department of Wildlife Hatchery located at Ford, WA were collected from January through August 1991. T_4 levels were monitored in Lake Whatcom stock fish at approximately weekly intervals from 10 days before hatch until they were released in July and August at about 185 days post hatch. Mean concentration ± SEM of 10-20 individual fish (assayed in duplicate) were determined for each collection period. T_4 levels were monitored in Lake Roosevelt stock fish at approximately monthly intervals from the egg stage in January through the time they were released in July and August at about 180 days post hatch. Mean concentration ± SEM of 10-15 individual fish (assayed in duplicate) were determined for each lot.

Lake Whatcom eggs were fertilized by Washington Department of Wildlife personnel (WDW) at the Lake Whatcom Hatchery in December 1990 and transferred as eyed eggs to the WDW Ford Fish hatchery located near Ford, WA (Fig. 1) on January 2, 1991. The fish were reared in baskets in fry rearing raceways at the Ford Hatchery until January 25 to February 5 1992 when they hatched. Fry were retained at Ford until the Spokane Tribal Hatchery was completed on April 10, 1991. Fry rearing was completed in raceways at the Spokane Tribal hatchery. Hatchery water temperature ranged from 9.0 to 10.5°C at the Ford Hatchery, and 10.1 to 11.5 at the Spokane Tribal Hatchery. Egg to fry survival rate was about 83%.

Approximately 10,000 eggs from wild Lake Roosevelt kokanee were collected and fertilized at Little Falls Dam (Fig. 1) by Spokane Tribal hatchery personnel in November 1990. Eggs were reared outdoors in Heath Trays at Metamooteles Springs, the principle water source for the Spokane Tribal Hatchery. The Heath Tray was positioned in the spring discharge creek. Water temperatures during egg incubation and alevin stages ranged from 5 to 8 °C. After hatching the eggs were transferred into hatchery

raceways. Water supply to the raceways was a combination of Metamooteles Springs water and well water. Water temperatures in the raceways ranged from 10.1 to 11.5°C. Egg to fry survival rate was approximately 80%.

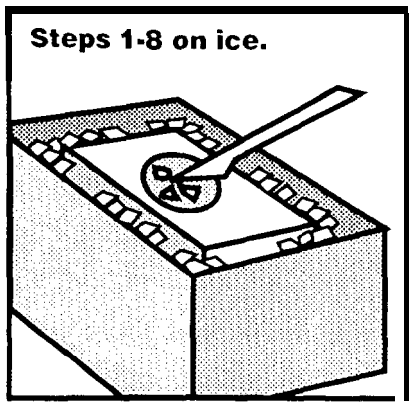
2.2 Sample Collection Procedures

Eggs and larvae were anesthetized with MS 222 (0.1g/l), weighed to nearest 0.1 mg using a Mettler AJ 100 Analytical balance and placed into individually numbered vials. Sampled fish were flash frozen on dry ice and stored at -80°C until T_4 was extracted and concentrations determined by radioimmunoassay. Samples were collected at approximately the same time each day, between 1100 and 1300 Pacific Standard Time, so that hormone levels at a particular time of day could be established and temporal trends determined independent of diurnal fluctuations in T_4 concentration.

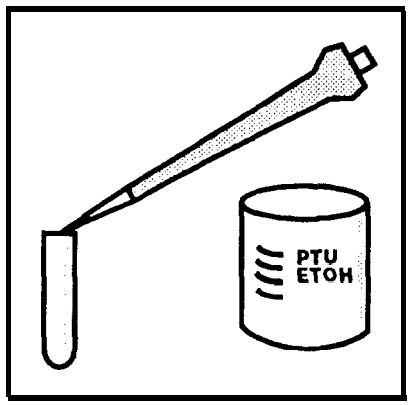
2.3 T_4 Extraction Procedures

Thyroxine (T_4) was extracted from eggs and larvae using procedures adapted from Parker (1988) (Fig. 2). Eggs or larvae were minced with scalpel in a plastic weighing boat on a chilled metal cutting block and placed into a 15 by 85 mm test tube. Ice cold 100% ethanol containing 1 .O mM 6-n-Propyl-2-thiouracil (ETOH-PTU) was added to the tube at a µl volume equal to 2 X the mg weight of the fish. The tissue sample was homogenized for 20 sec at 20,000 RPM using a Polytron 3000 tissue grinder (Brinkman Instruments, Inc.), then vortexed for 5 seconds and decanted into a centrifuge tube. The test tube was then washed with a µl volume of ETOH-PTU equal to 1X the mg weight of the fish, homogenized for an additional 20 sec at 20,000 RPM, vortexed for 5 seconds, and then transferred into the centrifuge tube. All the above procedures were performed on ice. The centrifuge tube was vortexed for 10 seconds to thoroughly mix the two subsamples, then centrifuged for 10 min at 3000 RPM using a Dynac refrigerated centrifuge at 4°C (Clay Adams, Inc.) The supernatent was decanted into a 10 ml drying tube. The pellet was resuspended in 100% ETOH at a µl volume equal to 1.5 X the mg weight of the fish, vortexed for 10 seconds, and centrifuged again for 10 minutes at

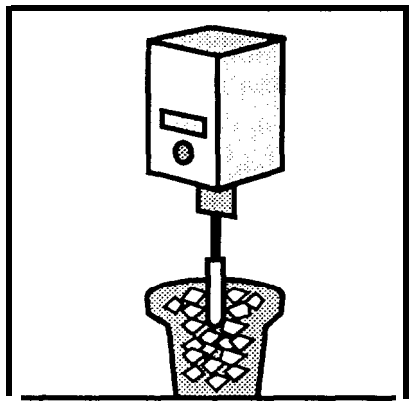
Fig 2. Procedures used for extracting and storing T4 from kokanee samples



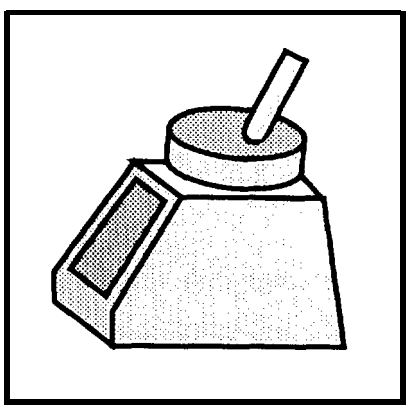
1 Mince sample with scaple in weighing boat on frozen cutting block. Put into 15 x 85 mm test tube.



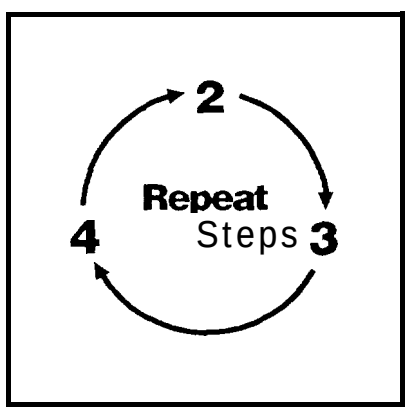
2 Add ice cold 100% ethanol containing 1 .0 mM PTU [ETOH-PTU] to tube at a ul volume equal to 2 X the weight of the fish.



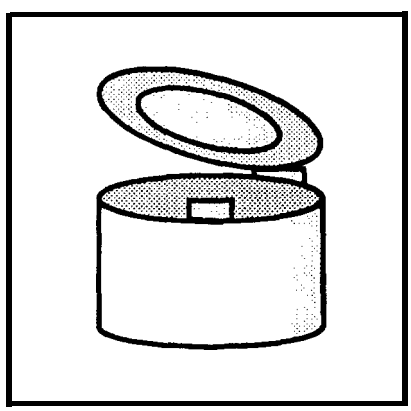
3 Homogenize for 20 sec to 1 min at 20,000 RPM using Brinkman Instrument Polytron 3000 tissue grinder.



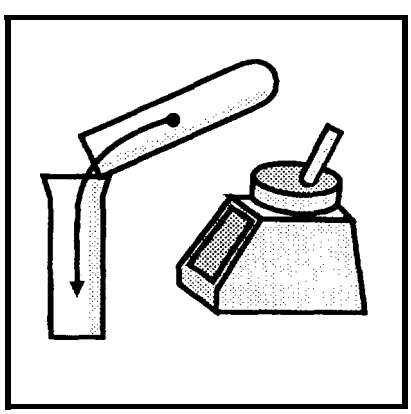
4 Vortex samples for 5 sec using a VWR Vortexer 2 and decant into centrifuge tube.



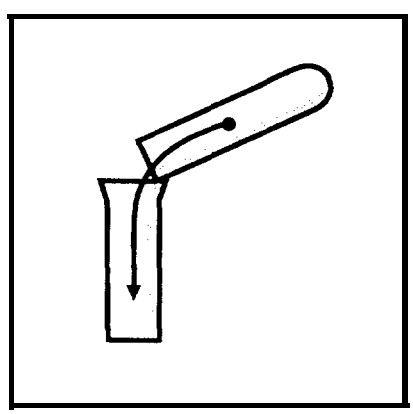
5 Repeat steps 2, 3 and 4 except use a volume of ETOH-PTU equal to 1 X weight of the fish, then vortex for 10 sec.



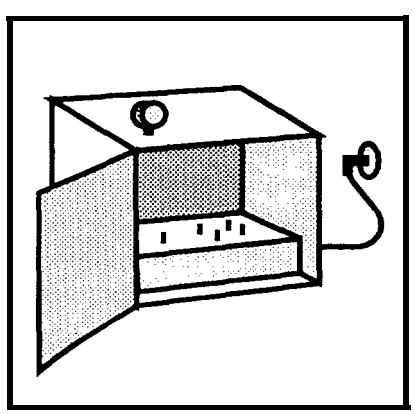
6 Centrifuge for 10 min at 3000 RPM using Clay Adams Dynac refrigerated centrifuge at 4°C.



7 Decant supernatent into a clean drying tube. Resuspend pellet in 100% ETOH (ETOH only, not ETOH-PTU) at a µl volume equal to 1.5 X mg weight of the fish. Vortex for 10 sec and centrifuge at 3000 RPM for 10 min at 4°C.



8 Pour supernatent in with first supernatent. [The drying tube contains the extracted T4].



9 Dry in vacuum oven at 60°C and 25 psi until all liquid in the tube is evaporated (usually about 2 h to overnight), then store at 0°C until assayed.

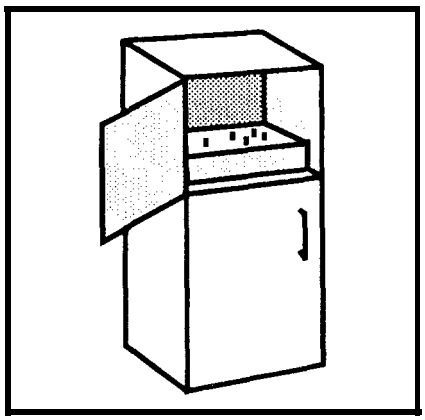
3000 RPM at 4°C. The supernatent was combined with first supernatent in the drying tube. The supernatent in the drying tube, which contained the extracted T_4, was evaporated in a vacuum oven at 60°C and 25 psi. Samples from eggs, alevins and fry under 500 mg were dry in about 1.5 to 2 hours. Fry ranging from 500 to 1000 mg were dried overnight. Fry greater then 1000 mg required about 24 to 48 h to dry. The dried sample was stored at 0°C until T_4 content was assayed.

2.4 T4 Radioimmunoassay

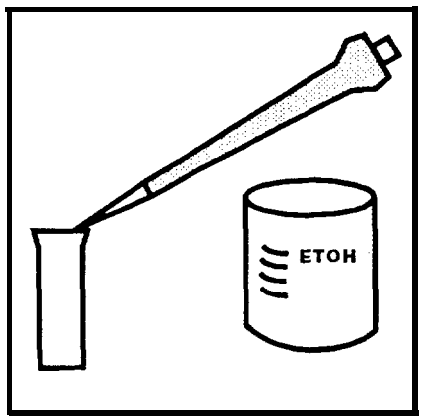
On the day radioimmunoassays (RIA) were performed, samples were resuspended in 250 µl 95% ETOH and 250 µl 0.11 M sodium barbital (pH 8.6) (Fig. 3). Each 500 µl sample was vortexed for 15 seconds after each solution was added. The sample was then transferred into a plastic eppendorf snap cap vial and centrifuged at 3000 RPM for 10 minutes using an Eppendorf Model 5415 C refrigerated centrifuge at 4.0°. Each sample was assayed in duplicate for T_4 content using a coat-a-count T_4 RIA kit (Diagnostic Products, Inc.) (Fig. 4, Appendix II). In this procedure 25 µl of an unknown sample and 1 ml of radiolabelled T_4 (^{125}I-T_4) were added to a tube coated with antibodies (Ab) that contained T_4 receptors. Thus, each tube contained the same amount of ^{125}I-T_4 but different amounts of unknown T_4. The procedure is based on competitive binding, i.e., ^{125}I-T_4 and unknown T_4 compete for Ab binding sites and bind in proportion to their relative concentrations. Hence, more ^{125}I-T_4 will bind to binding sites if the unknown sample contains less T_4 than if the unknown sample contains more T_4. Actual concentrations of unknown samples were determined by comparison to a standard curve with known concentrations of T_4 ranging from 0 to 24 ng/dl. Twenty-five µl aliquots of 0,1,4,10,16 and 24 ng/dl standard curve concentrations and 1 ml of ^{125}I-T_4 were added to Ab coated tubes and subjected to the same assay procedures as unknown kokanee samples.

Unknown and standard curve tubes were vortexed for 5 seconds, then incubated for 1 h @ 37°C to allow time for T_4 and ^{125}I-T_4 to compete for binding sites on the Ab. This is a reversible binding reaction. Equilibrium

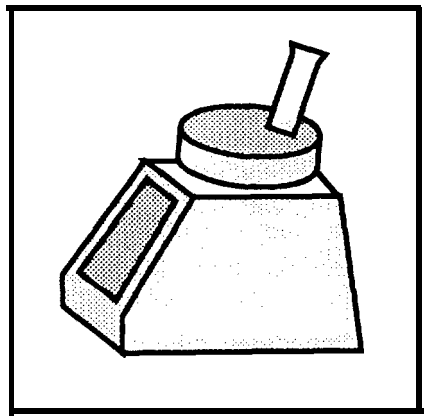
Fig 3. Procedures used to resuspend dried samples for hormone analysis



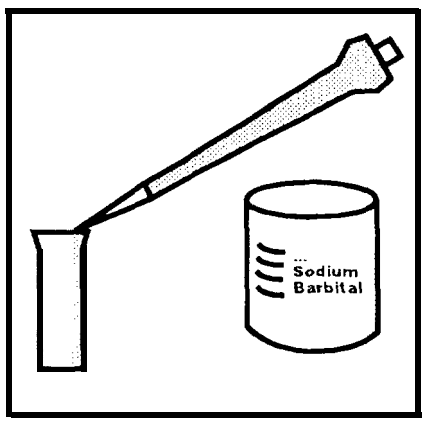
1 Remove dried samples from 0°C freezer.



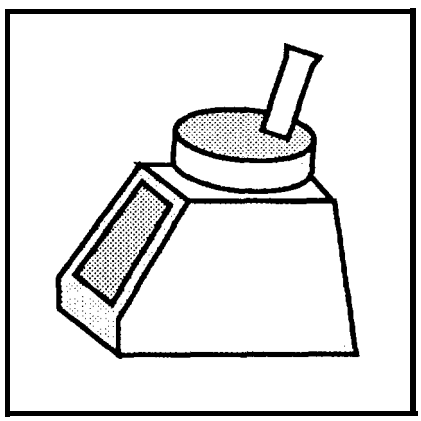
2 Add 250 µl 95% ETOH to each drying tube.



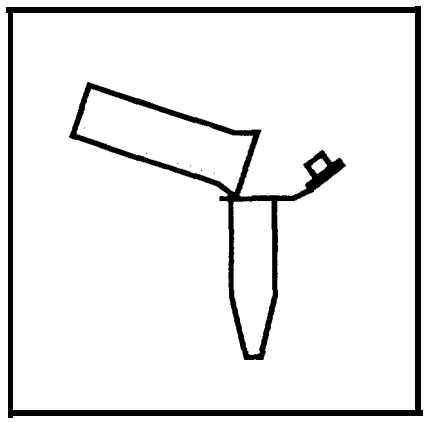
3 Vortex for 15 seconds.



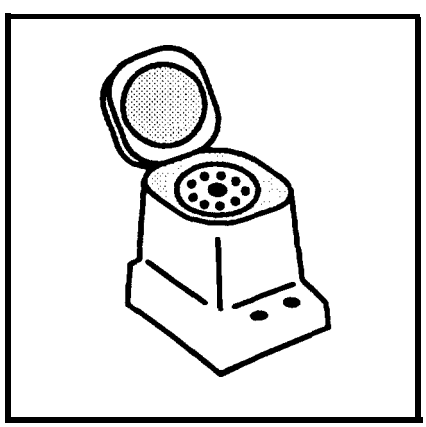
4 Add 250 µl of 0.11 M sodium barbital (pH 8.6) to each drying tube.



5 Vortex for 15 sec.



6 Pour resuspended sample into a 0.5 ml or 1 ml plastic Eppendorf snap cap vial.



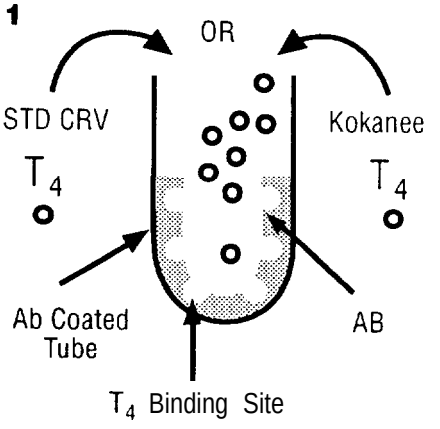
7 Centrifuge at 3000 rpm for 10 min using an Eppendorf Model 5415 C refrigerated centrifuge at 4°C.

◄ [This step clears any remaining particulate fraction from the solution by sedimenting it on the bottom of the tube. When pipetting samples into RIA tubes, make sure you don't put the pipette tip into this sedimented material because it interfers with the assay.]

Fig 4. Radioimmunoassay Procedure

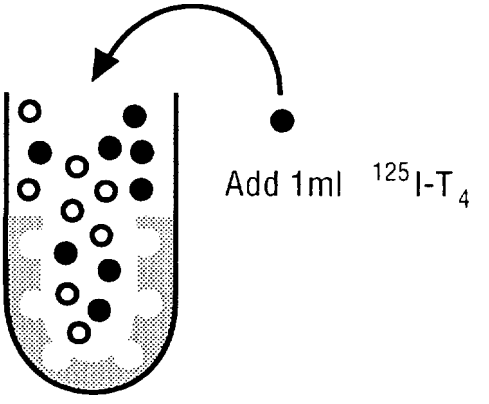
Add 25µl
STD CRV
(ng/dl)
0 (mb)
1
4
10
16
24
NSB
TCT

1



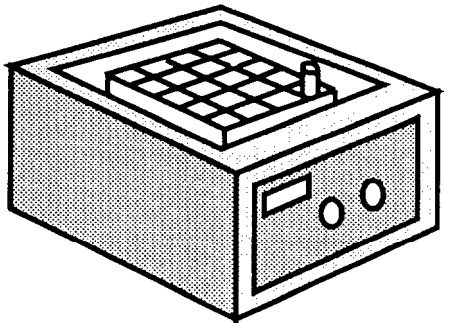
Add 25µl of standard curve, unknown kokanee sample, quality control or interassay pool sample.

2



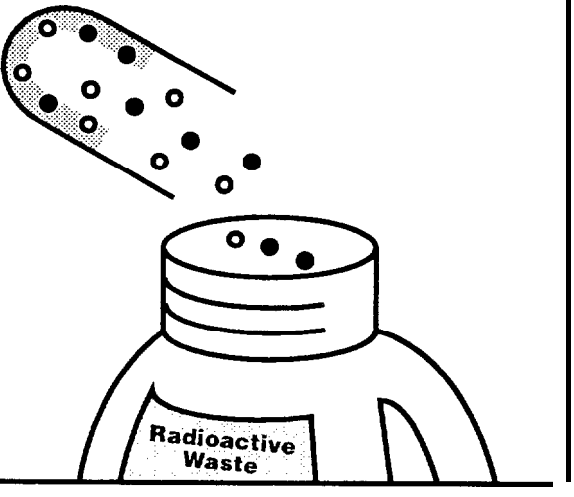
3

Vortex 5 sec. and incubate in a water bath at 37°C for 1 hour



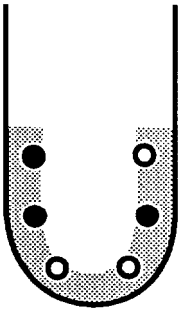
4

Decant "FREE" fraction

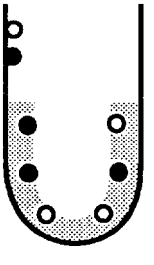


5

"BOUND" fraction

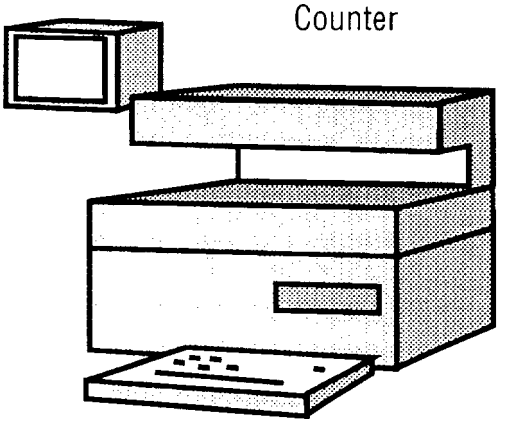


Occasional non-specific binding occurs



6

Count "BOUND" Fraction In Gamma Counter



is attained in about lh. The T_4 and ^{125}I-T_4 bind in proportion to the relative concentration according to the Law of Mass Action. Non-bound T_4 and non-bound ^{125}I-T_4 was discarded by pouring this liquid into a radioactive waste container and then blotting the remaining liquid out of the tubes. Radioactivity of the bound fraction remaining in the Ab coated tubes was counted for 1 minute using a programmable Cobra QC Model B5002 Auto-gamma Counter (Packard Instrument, Co.) (Fig. 5).

Maximum binding (MB) of radioactivity was determined from the standard curve tubes containing 0 ng/dl T_4. Percent bound of each of the remaining standard curve and unknown samples was calculated from the equation:

$$\text{Percent Bound} = \frac{\text{counts - NSB counts}}{\text{MB counts - NSB counts}} \times 100$$

where: Counts = Radioactive counts per minute (cpm) of either a standard curve or unknown tube;

MB = Radioactive counts per minute (cpm) of maximum binding tube; and

NSB = Nonspecific binding (cpm) (see below).

A computer program, contained in the counter, automatically determined the relative percent bound for each standard concentration and plotted a log-logit graph of the standard curve (% bound -v- standard concentrations). The program then examined the percent bound for each unknown sample and interpolated the concentration of the sample from the standard curve graph.

2.5 Data reduction

Total T_4 content of individual fish samples was determined by multiplying the T_4 content of 25 µl subsample by 20 (500 µl total sample volume ÷ 25 µl subsample tested for T_4 content) and converting this

Fig 5. How the RIA technique works

Fig 5 (a) What happens in assay tubes containing high or low amounts of T_4 ?

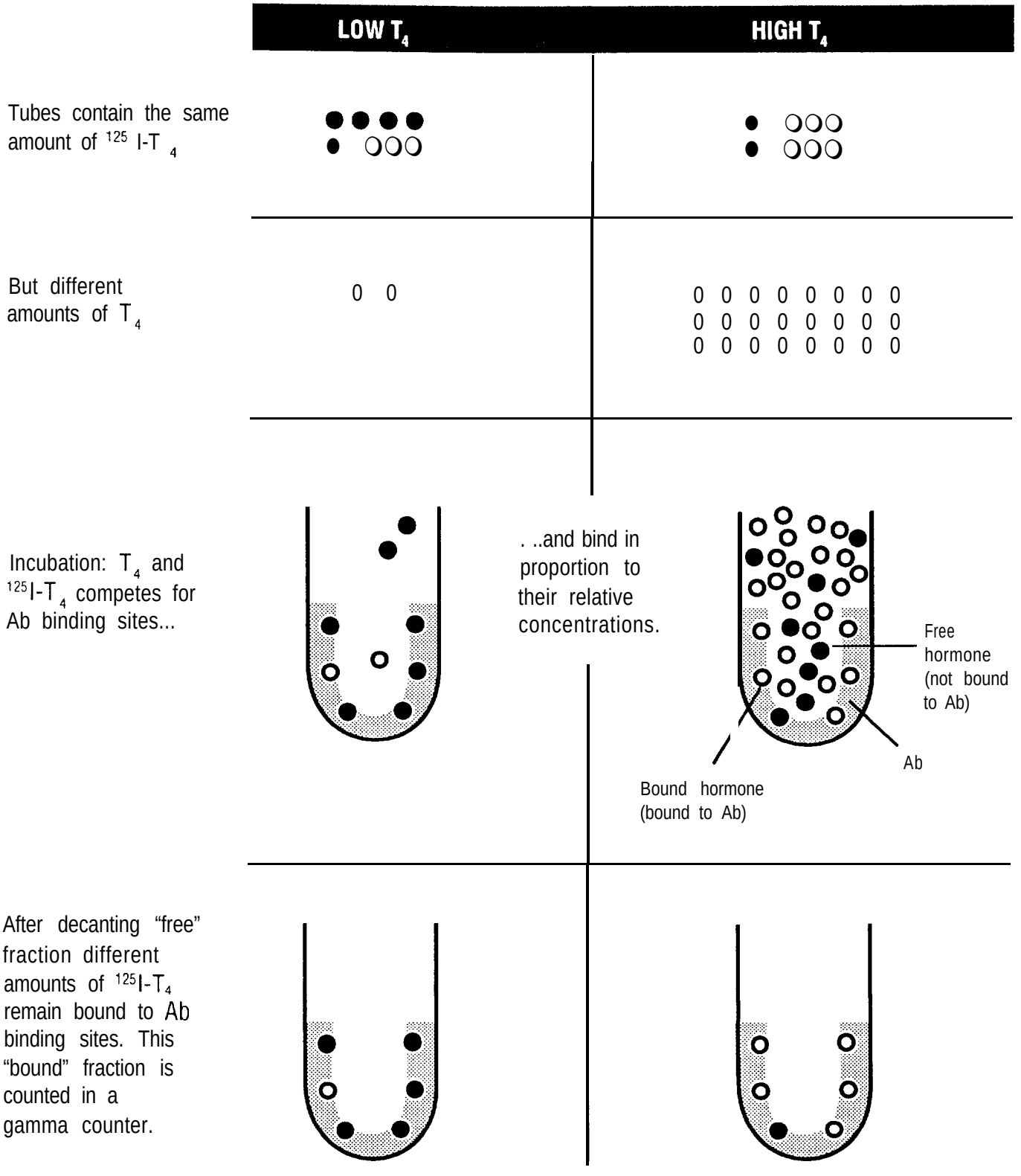


Fig 5. How the RIA Technique Works

Fig 5(b) Sample of log/logit standard curve plot. The plots and calculations were made automatically via computer program contained on a programable gamma counter. To determine the standard curve line the computer program fitted a linear regression line (using least squares) through a log/logit plot of percent bound (y axis) -v- standard curve concentrations (x-axis).

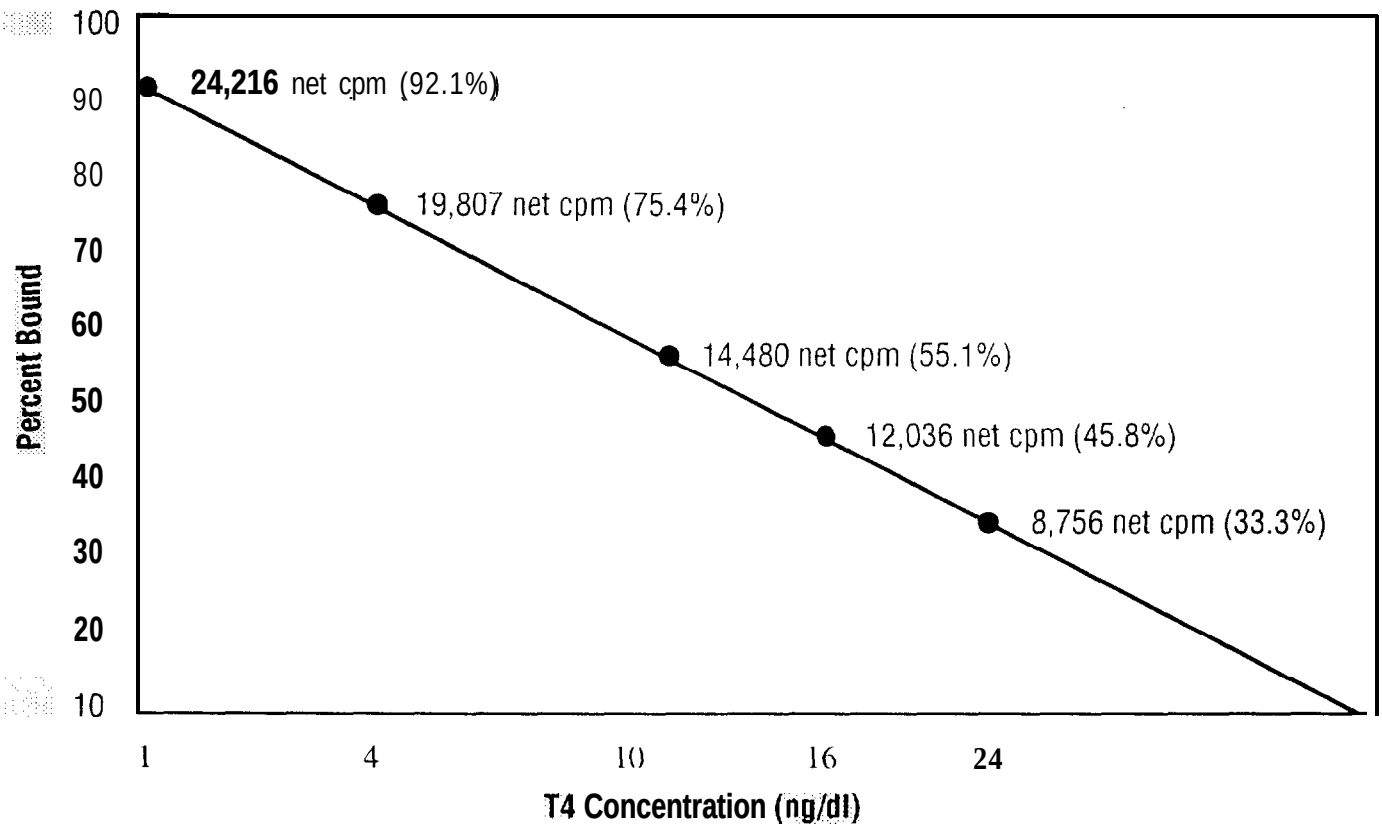


Fig 5(c) Sample calculation to determine unknown concentration. Data were obtained as indicated in Fig 5b.

DATA

Unkown Kokanee Sample (Contains 16,141 cpm)	NSBTube (Contains 219 cpm)

$$\textbf{Percent Bound} = \frac{\textbf{Net Counts x 100}}{\textbf{Net MB Counts (cpm)}}$$

where: Net Counts= Unknown tube count - NSB tube count (16,141 - 219 = 15,922)
Net MB Counts = MB tube count - NSB count (26,507 - 219 = 26,288)

$$\textbf{Percent Bound} = \frac{15{,}922}{26{,}288} \times 100 = 60{\cdot}8\%$$

Interpolate From Graph
60.8% Bound = 7.8 ng/dl

concentration to total ng T&ample. This number was divided by the weight of the fish to provide the T_4 concentration of each fish in ng/g body weight. A correction for extraction efficiency was made as described in Section 2.7 of this report. The mean ± (SEM) were calculated for fish collected on the same sample date and plotted -v- age (days from hatch), using Statview II (Brainpower, Inc.) on an Apple MacIntosh IIcx microcomputer.

2.6 Quality Assurance Procedures

Accuracy level of the assay was determined by:

(1) Blind analysis of quality control standards at high, medium and low concentrations. These samples were inserted in duplicate at the front, middle and end of our assay. After obtaining results from our assay, we received information about the actual concentration from Diagnostic Products, Inc. Mean measured concentrations (±SD) were then compared to actual concentrations to determine assay reliability.

(2) Nonspecific binding (NSB), i.e., radioactivity that sticks to the test tube but not bound to binding sites, was determined by adding 1 ml ^{125}I-T_4 to two plain test tubes that did not contain Ab (uncoated tubes). Tubes were incubated for lh @ 37° and radioactivity decanted into a radioactive waste container. The tubes were then blotted and remaining radioactivity counted. The amount of radioactivity remaining was considered non-specific binding. The program contained on the Biogamma counter automatically subtracted the mean NSB from each standard curve and unknown sample before calculating percent bound.

(3) Ten blank tubes, i.e., tubes that did not contain ^{125}I-T_4 or T_4 were assayed to ensure that the gamma counter was functioning properly. Distilled water was added to Ab coated tubes and incubated for lh @ 37°C. The tubes were decanted, blotted and counted like other tubes.

(4) All standard curve, unknown and quality control standards were assayed in duplicate to control for procedural errors. For each pair of samples the mean value was calculated and percent error from the mean determined. The mean percent error ± 95% confidence interval (95% Cl) for all samples was calculated using Statview II (Brainpower, Inc.) on an Apple IIcx computer. A frequency distribution was plotted of the percent error.

(5) To determine pipetting accuracy, individuals pipetting the unknown/standard curve samples and ^{125}I-T_4 samples respectively pipetted 10 replicate samples of 25 µl ^{125}I-T_4 o r 1 ml ^{125}I-T_4 into uncoated tubes. The amount of radioactivity in each sample was counted in the gamma counter. The mean (± 95% Cl) and coefficient of variation were determined for each set of samples.

(6) The T_4 radioimmonassay requires that reagents be added within a specified time frame so that samples at the front and back end of the assay are incubated for the same amount of time to insure that equilibrium binding is uniform. Therefore, because the number of samples was large, we performed two assays. To control for the possibility that different assay conditions contributed to the results, three different interassay pool (IAP) samples were inserted in triplicate into each assay. If IAP samples were uniform in concentration in both assays, then the results could be compared. If IAP samples were significantly different in concentration in the two assays, then the results could not be compared. Additionally, the same standard curve samples were used in both assays. If standard curve samples were uniform in concentration in both assays, then results could be compared. If standard curve samples were significantly different between the assays, then results could not be compared. Statistical comparisons were made between the mean values of IAP and standard curve samples in both assays by

calculating the Wilcoxon signed-rank nonparametric statistic using Statview II (Brainpower, Inc.) on an Apple MacIntosh II*cx* computer.

(7) To determine whether there was any drift or position effect due to delays in addition of reagents, pairs of standard curve samples were placed at the front and end of each assay. To determine if standard curve samples at the end of the assay were significantly different from the matched samples at the front of the assay a Wilcoxon signed rank nonparametric statistic was calculated using Statview II (Brainpower, Inc.) on a MacIntosh II*cx* microcomputer.

2.7 Recovery determination

To determine the efficacy of the extraction process in recovering thyroid hormones as well as transfer of samples into different types of tubes, two recovery systems were employed. A sample of 40 eggs, alevins, or fry was collected at one time interval. For the first type of recovery analysis, unlabelled thyroxine was added to the minced eggs, alevins or fry prior to homogenization. Replicates tubes each were spiked with 0, 5, 10 and 15 ng/dl T_4 . The samples were then subjected to the same homogenization, extraction, reconstitution and T_4 assay procedures as the unknown and standard curve samples. Mean ± SEM were calculated for each set of replicate samples. The percentage of T_4 recovered from each spiked tube was calculated by the formula:

$$T_4(\%R) = \left[T_4(sp) - \frac{([T_4](o) \times Wt(sp))}{(Wt(o))}\right] \div AMT$$

where: $T_4(\%R)$ = The mean percentage of T_4 recovered;

$[T_4]sp$ = The mean T_4 concentration measured in a particular **set of replicate spiked samples;**

$[T4]o$ = The mean T_4 concentration measured in the sample spiked with a concentration of 0 ng/dl T_4;

$Wt(sp)$ = Mean weight (mg) of a particular set of spiked samples

$Wt(o)$ = Mean weight (mg) of the samples receiving 0 ng/dl T_4; and

AMT = The mean concentration (amount) of T_4 added to a particular spiked sample.

The formula provides a correction for the different weight of each sample. A linear regression was plotted of amount of T_4 recovered -v- amount of T_4 added to each sample. The r^2 value was examined to determine the consistency of recovery at various spike concentrations.

The second type of recovery analysis was performed to determine where T4 was lost in the extraction process. The procedure involved spiking samples with a known number of counts per minute (cpm) of radiolabelled thyroxine and subject them to the same homogenization, extraction, and reconstitution procedures as used for unknown and standard curve samples. Approximately 5000 cpm of ^{125}I-T_4 was added to each of five tubes (10 fish). Prior to extraction the initial number of counts in each tube was determined. At each step of the extraction procedure the number of counts remaining was determined. Additionally, 5000 cpm of ^{125}I-T_4 was added to five blank control tubes to account for counts lost owing to radioactive decay. These control tubes were not extracted. The percentage of T_4 recovered at each stage was calculated by the formula:

$$T_4(\%R)s = \frac{cpm_s}{cpm_i} \times C.F.(s)$$

where: $T_4(\%R)s$ = The mean percentage of T_4 recovered at a particular step in the extraction process.

cpm_s = The mean counts per minute in a particular **tube at that step of the extraction process:**

cpm_i = The mean initial number of that same tube for sample decay: and

C.F.(s) = A correction factor applied at time s to account for the natural decay of the radioisotope. This was determined by dividing the counts of the control tubes at time(s) by their initial counts. The reciprocal of the mean value (decimal percent) was the value of the correction factor at time s.

The tissue content of thyroxine, as determined by RIA, was corrected for the extraction efficiency by dividing the RIA value of each unknown sample by the mean value (decimal percentage) calculated for the extraction.

RESULTS

3.1 T4 Concentration in Lake Whatcom Stock Kokanee

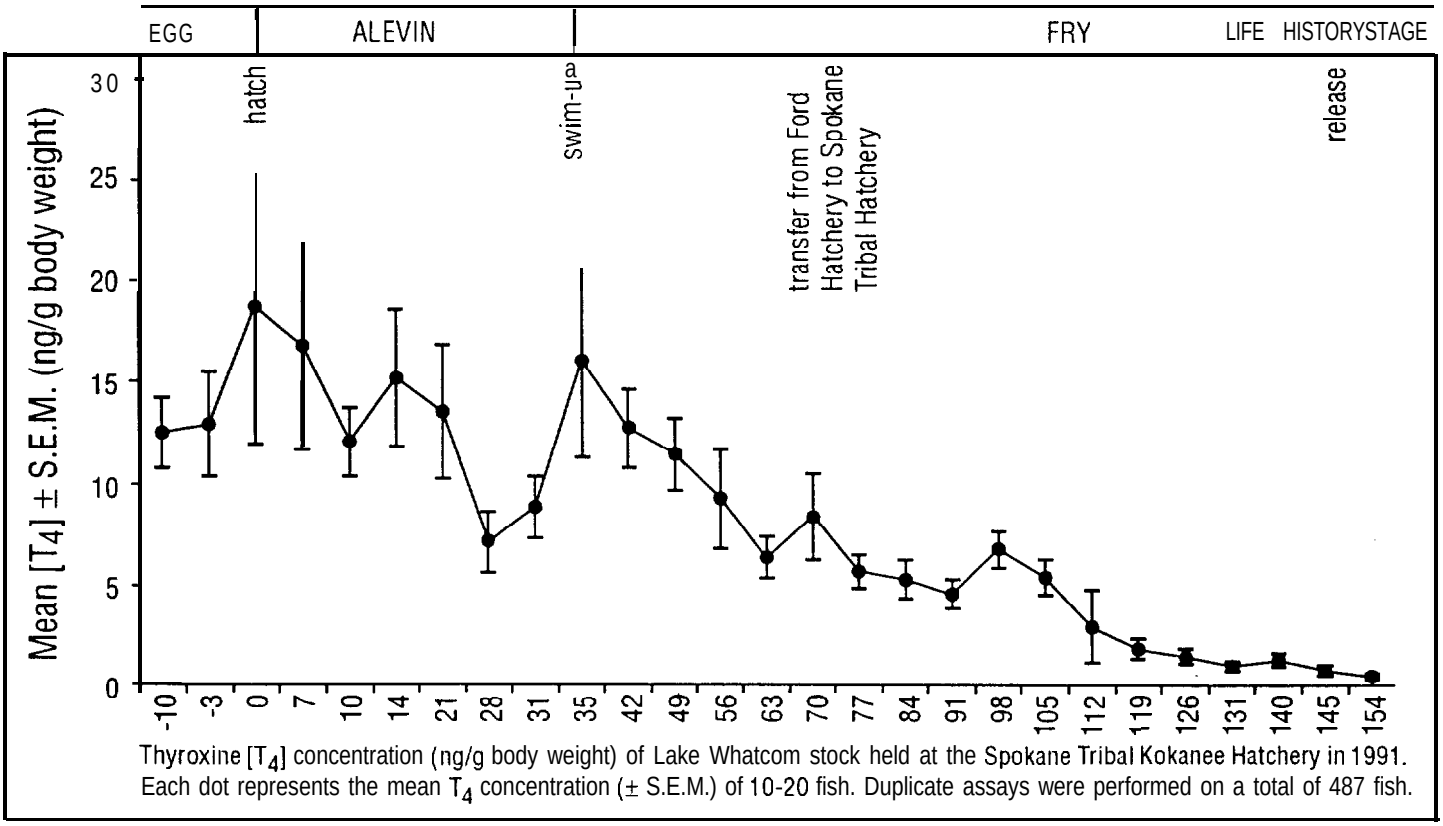
Lake Whatcom stock kokanee eggs weighed about 150 mg. These fish lost weight at the time of hatch (hatchlings weighed about 95 mg) and remained at a relatively constant weight from hatch to swimup (Fig. 6). Yolk sacs were completely absorbed within a few days after swimup. At two weeks post swimup their body weight had approximately doubled. They weighed about 0.5 g by 98 days post-hatch, 1 .O g by 126 days post-hatch, 2.0 g by 154 days post hatch, and 4.7 g by 168 days post-hatch (Fig. 6). Growth rates of post-hatch fish indicate that the fish were healthy.

In Lake Whatcom stock kokanee mean T_4 concentrations (± SEM) in eggs ranged from 12.5 ± 1.7 to 12.9 ± 2.6 ng/g body weight, respectively at 10 days and 3 days pre-hatch. (Fig. 6). Mean T_4 concentration peaked on the day of hatch 18.7 ± 6.8 ng/g body weight (Fig. 6). After declining to 7.1 ± 1.5 ng/g body weight on day 28 post-hatch, T_4 concentration peaked again at 16.0 ± 4.7 ng/g body weight at the time of swimup on day 35 post-hatch, then steadily decreased to less than 1 .O ng/g body weight in fry older than 126 days post-hatch.

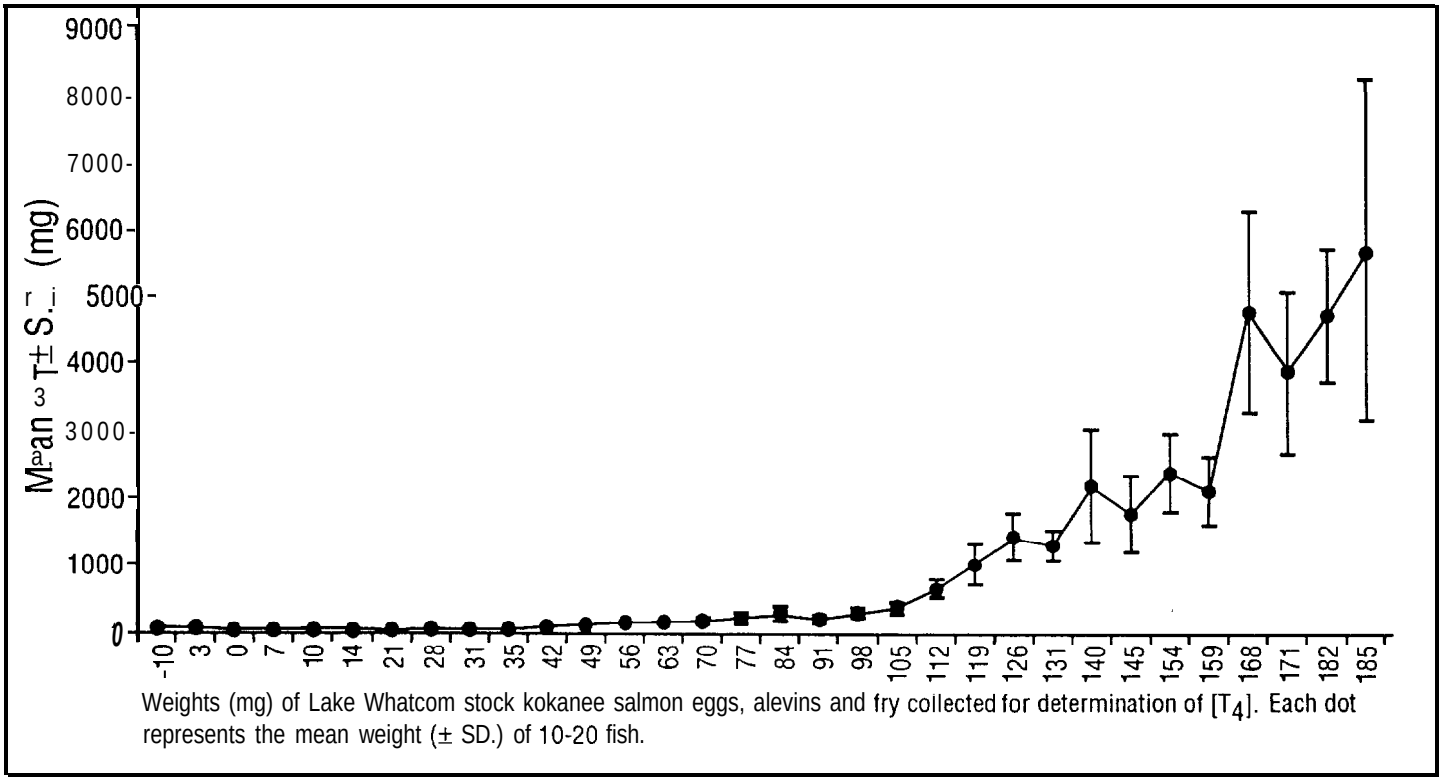
3.2 T_4 Concentration in Lake Roosevelt Stock Kokanee

Lake Roosevelt stock kokanee eggs weighed about 150 mg. The fish lost weight at the time of hatch and started to gain weight after swimup. Lake Roosevelt stock fish weighed approximately 0.3 g in March, 0.9 g in May and 4.6 g in July (Fig. 7). T_4 concentrations were highest in eggs at 13.3 ± 2.8 ng/g body weight, then steadily decreased to less than 1 .O ng/g body weight in fry older than 125 days post-hatch (Fig. 7). T_4 concentrations were not determined at either the time of hatch of swimup for Lake Roosevelt stock kokanee because very few eggs from this stock were collected.

Fig 6. T_4 concentration and weights from Lake Whatcom stock kokanee



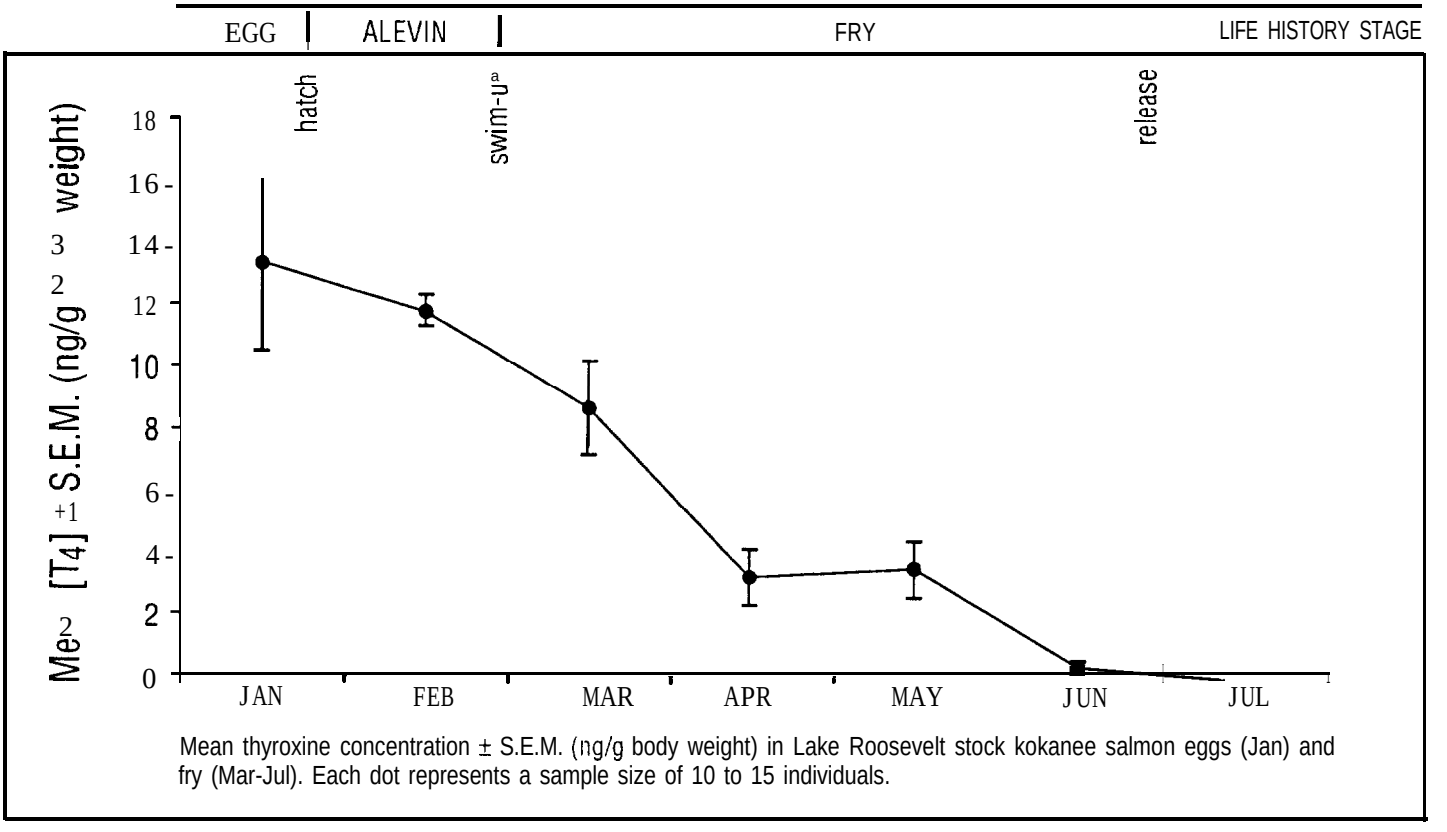
Thyroxine [T_4] concentration (ng/g body weight) of Lake Whatcom stock held at the Spokane Tribal Kokanee Hatchery in 1991. Each dot represents the mean T_4 concentration (± S.E.M.) of 10-20 fish. Duplicate assays were performed on a total of 487 fish.



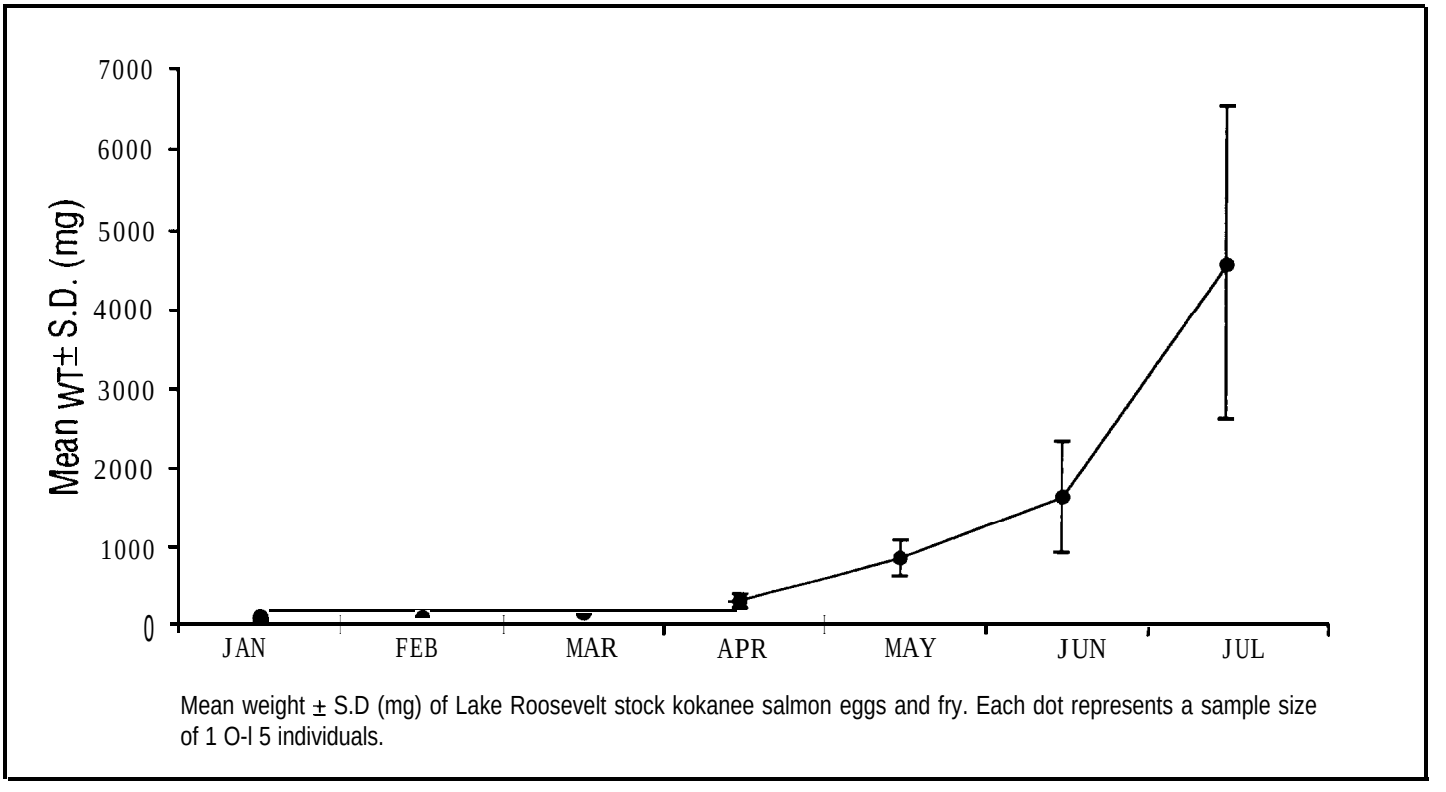
Weights (mg) of Lake Whatcom stock kokanee salmon eggs, alevins and fry collected for determination of [T_4]. Each dot represents the mean weight (± SD.) of 10-20 fish.

Age (Days Pre- or Post-Hatch)

Fig 7. T_4 concentration and weights from Lake Roosevelt stock kokanee



Mean thyroxine concentration ± S.E.M. (ng/g body weight) in Lake Roosevelt stock kokanee salmon eggs (Jan) and fry (Mar-Jul). Each dot represents a sample size of 10 to 15 individuals.



Mean weight ± S.D (mg) of Lake Roosevelt stock kokanee salmon eggs and fry. Each dot represents a sample size of 1 O-l 5 individuals.

Age (Days Pre- or Post-Hatch)

3.3 Quality Assurance Results

Results of blind quality assurance samples are recorded in Table 1. The actual concentration of the low T_4 blind sample was 2.3 ng/dl compared to a mean (± 95% Cl) measured value of 2.3 ± 0.3 ng/dl (n =6). The actual concentration of the medium T_4 blind sample was 7.3 ng/dl compared to a mean (± 95% Cl) measured value of 7.4 ± 0.5 ng/dl (n = 6). The actual concentration of the high T_4 blind sample was 11.5 ng/dl compared to a mean (± 95% Cl) measured value of 12.2 ± 0.5 ng/dl (n = 6).

Mean nonspecific binding (±95% Cl) for eight replicate tubes was measured at 298 ± 31 counts per minute (cpm) compared to 55,671 ± 451 cpm measured in total count tubes (TCT). Nine of ten blank tubes contained 0 cpm and one blank tube contained 2 cpm of radioactivity. The one blank tube containing radioactivity was probably contaminated during the blotting process when the nonbound fraction was decanted. The mean cpm (± 95% Cl) of ten tubes that received 25 µl of ^{125}I-T_4 was 1,355 ± 86 for a total error of 6.3%. Coefficient of variation was 10.4. The mean cpm (± 95% Cl) of seven tubes that received 1 .O ml of ^{125}I-T_4 was 53,601 ± 2,225 for a total error of 4.1%. The coefficient of variation was 5.6. A frequency distribution of percent error of duplicate kokanee samples is presented in Fig. 8. The mean percent error (± 95% Cl) of 476 duplicate samples was 6.8 ± 0.6%. The mean percent error of duplicate samples (6.8 ± 0.6%) was approximately uniform with the 6.3% error noted in pipetting the 25 µl samples.

Results and statistical comparisons of interassay pool (IAP) samples and standard curve concentrations for each of the two assays are recorded in Table 2. Mean concentration and 95% confidence intervals for three replicates of each IAP sample and four replicates of each standard curve sample were remarkably uniform (Table 5). No statistically significant differences were detected for either IAP or standard curve samples (Table 2).

Results and statistical comparisons of standard curve samples used to check for drift between the front and end of each assay are recorded in

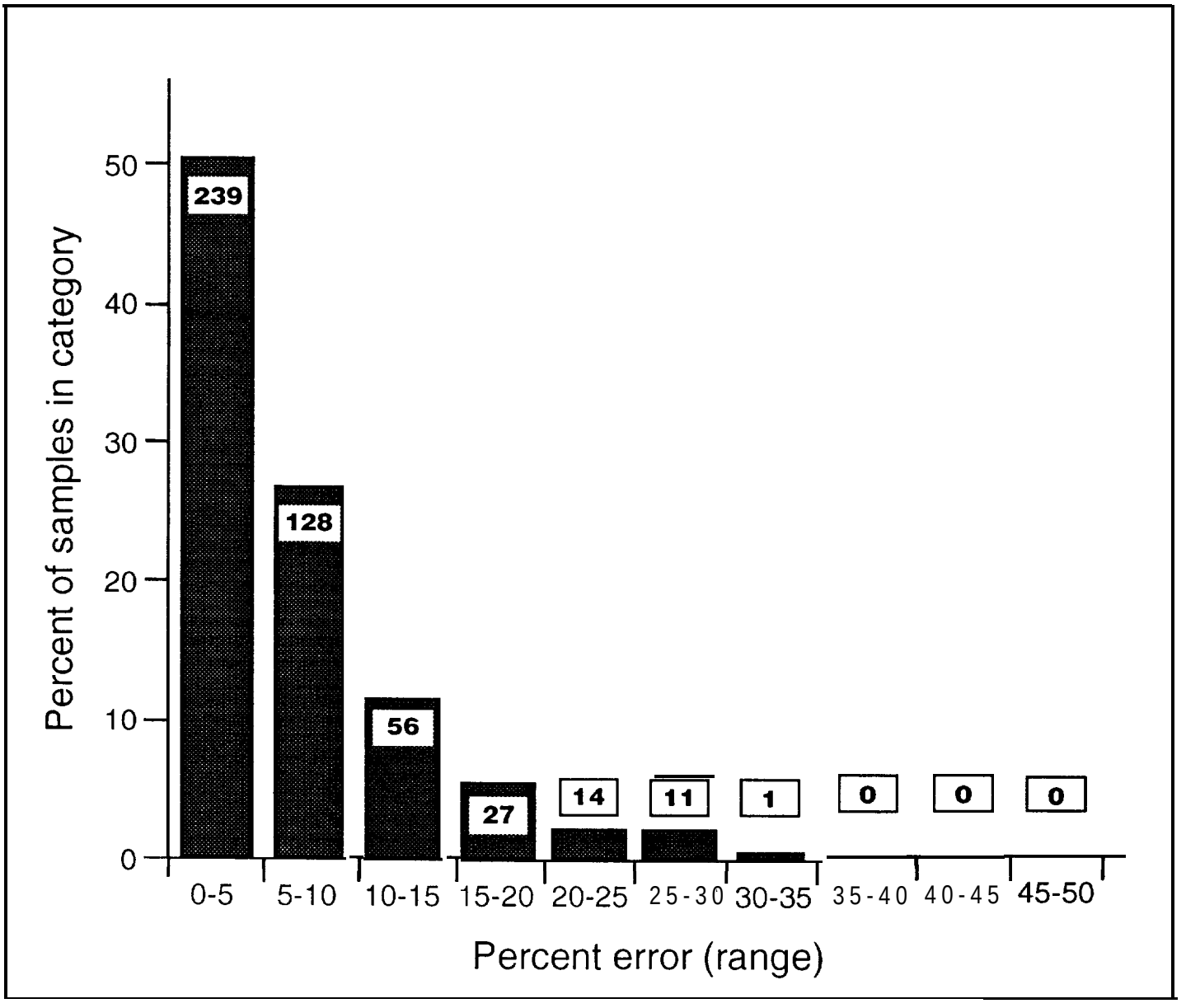
Table 1. Results of blind quality assurance samples. Measured concentrations were the mean ± 95% Cl of six replicates for each sample. Actual and acceptable ranges were provided by Diagnostic Products Corporation.

Sample	Actual concentration (ng/dl)	Acceptable range (ng/dl)	Measured concentration (ng/dl)
Low	2.3	1.9-2.7	2.3 ± 0.3
Medium	7.3	6.2-8.4	7.4 ± 0.5
High	11.5	10.0-13.0	12.2 ± 0.5

Table 2. Statistical comparisons between interassay pool (IAP) and standard curve concentration (ng/dl) in two assays of kokanee salmdn. Sample size was three replicates of each IAP and four replicates of each standard curve sample per assay. Standard curve samples refer to concentration in ng/dl.

Sample		First assay (mean ± 95% CI)	Second assay (mean ± 95% CI)	Wilcoxon Signed-rank statistic
IAP 1		2.4 ± 0.5	2.6 ± 0.9	z = -0.447
IAP 2		7.4 ± 0.8	7.4 ± 0.7	P = 0.655
IAP 3		12.3 ± 0.7	12.2 ± 0.3	NS
STDCRV	1.0	1.2 ± 0.1	1.1 ± 0.1	
STDCRV	4.0	4.3 ± 1.5	4.3 ± 0.7	z = o
STDCRV	10.0	10.6 ± 0.1	10.4 ± 1.0	P = 1.0
STDCRV	16.0	16.4 ± 1.1	16.6 ± 0.5	NS
STDCRV	24.0	23.5 ± 0.7	23.6 ± 0.9	

Fig 8. Frequency distribution of percent error in duplicate kokanee samples



The total sample size (n) was 476 individuals. The number of samples in each category are noted in white boxes. These numbers were converted to percentages of the total sample size for plotting the frequency distribution.

Table 3. Mean concentrations of duplicate samples were reasonably uniform at both ends of each assay. Moreover, no pattern, such as the concentrations of each end sample being lower than the corresponding front sample, could be detected for either assay (Table 3). No statistically significant differences were recorded between the two sets of marked samples at the front and end of either assay (Table 3).

3.4 Recovery determination

Recovery data from kokanee egg, alevin and fry spiked with 0.25, 0.5 and 0.75 ng exogenous T_4 is recorded in Table 4. Results indicate that percent recovery was 88.5% from eggs. 91.7% from alevins and 80.4% from fry. The reciprocals of these numbers were used as correction factors (multipliers) to calculate T_4 concentration in egg, alevin and fry samples. These recovery values were similar to those reported by other investigators, e.g., Kobuke et *al.* (1987) reported 83% in coho eggs. Regression plots (Fig. 9) indicated that the amount of T_4 recovered from each sample was linearly related to the amount of T_4 added to the sample (r^2 values were .987 for eggs, .990 for alevins and .995 for fry).

Recovery data from kokanee eggs, alevins and larvae spiked with 11,000 to 12,000 cpm ^{125}I-T_4 indicated that nearly all of the T_4 is lost in the extraction process (Table 5). For example, 70.4% of the initial counts (corrected for radioactive decay) were extracted in the supernatent from eggs, for a 29.6% loss. In comparison, 67.9% of the initial counts (corrected for radioactive decay) remained after the supernatent was dried then resuspended and transferred into centrifuge tubes, for an additional 2.5% loss. Radio-T_4 recovery analysis indicated a similar pattern in the recovery from egg, alevins and fry samples as those spiked with nonradioactive T_4, with more radio-T4 extracted from eggs and alevins than fry. The percentages of recovery were lower for radio-T4 than T_4 (respectively 67.9% -v- 88.5% for eggs, 66.5% -v- 91.7% for alevins, and 56.8% -v- 80.4% for fry). We attributed the lower recovery rates using ^{125}I-T_4 to intereference from chemical additives used in manufacturing the ^{125}I-T_4. Therefore, correction factors applied to

Table 3. Concentration of measured standard curve samples placed at the front and end of two assays to control for drift. Each number represents the mean value of a duplicate set of samples run at the front or end of each assay.

Assay No.	Placement in assay	Concentration (ng/dl) 1.0	4.0	10.0	16.0	24.0	Wilcoxon signed-rank statistic Z	P	S/NS
1	Front	1.0	3.5	10.5	17.0	23.8	-0.14	.893	NS
	End	1.3	5.1	10.6	15.8	23.1			
2	Front	1.0	4.2	10.9	16.6	24.9	-0.73	.465	NS
	End	1.1	4.6	9.9	16.8	22.7			
Overall Mean		1.1	4.3	10.5	16.6	23.3			
± 95% CI		0.1	0.7	0.4	0.5	0.9			

Table 4. Percent recovery for kokanee egg, alevin and fry samples spiked with nonradioactive T_4 at concentrations of 0.25, 0.50 or 0.75 ng/sample. Each line is the mean value of five applicable samples.

Stage	Sample weight (mg)	Total T_4 measured (ng)	Amount of T_4 added to sample (ng)	Amount of added T_4 recovered (ng)	Percent of spike recovered (%)
Egg	72	0.94	0	--	--
	81	1.25	.25	0.20	80.0
	72	1.35	.50	0.41	82.0
	66	1.55		0.69	92.0
Mean Percent Recovery (Egg)					88.5
Alevin	127	0.94	0	--	--
	124	1.15	.25	0.24	96.0
	110	1.25	.50	0.43	86.0
	128	1.65	.75	0.70	93.3
Mean Percent Recovery (Alevin)					91.7
Fry	937	1.3	0	--	--
	989	1.6	.25	0.20	80.0
	951	1.7	.50	0.40	80.0
	714	1.6	.75	0.61	81.3
Mean Percent Recovery (Fry)					80.4

Fig 9. Linear regression plots of amount of T_4 spike recovered –v– amount of T_4 added in kokanee samples

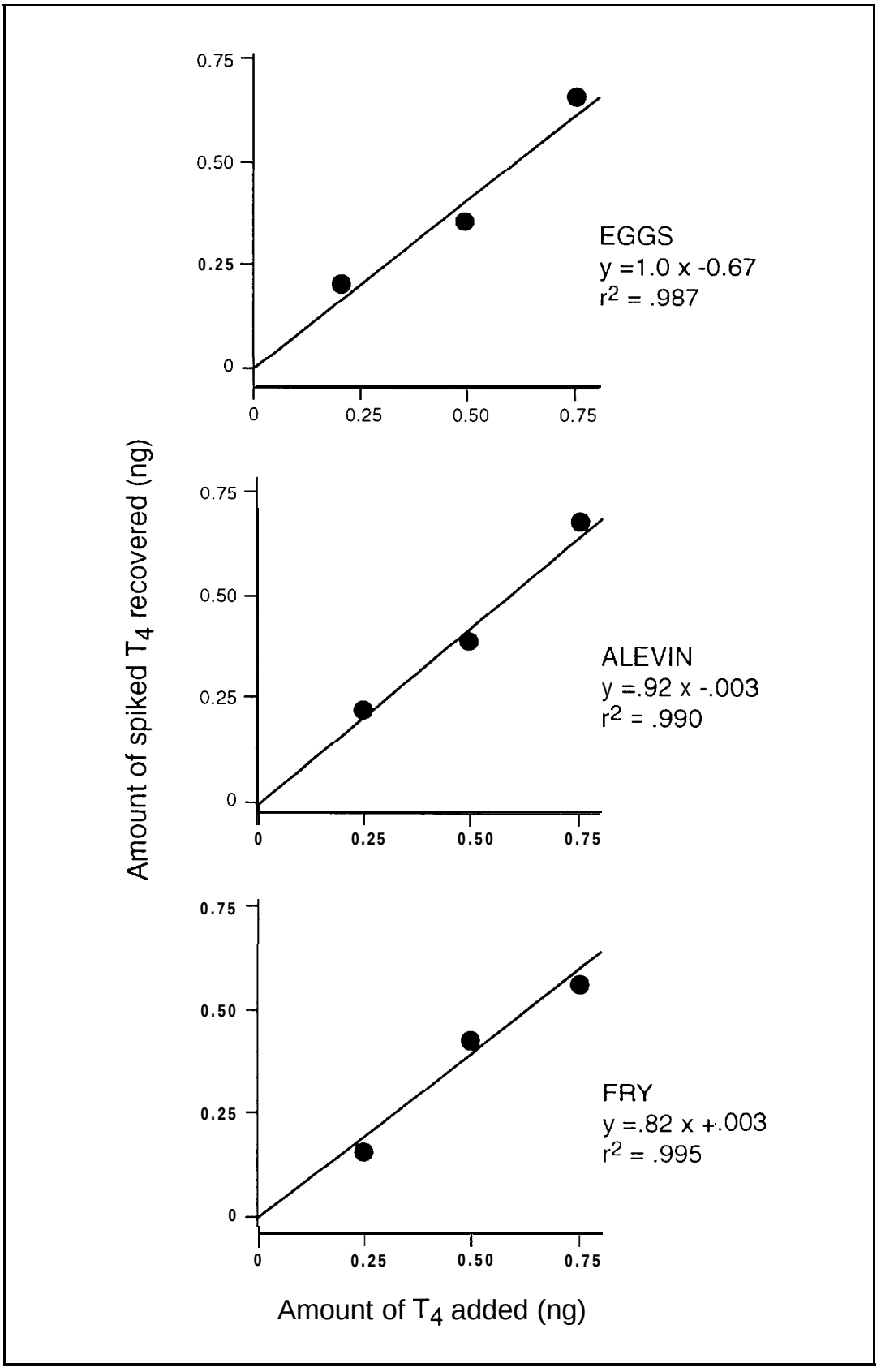


Table 5. Recovery of radiolabelled T_4 from kokanee egg, alevin and fry samples spiked with approximately 11,000 to 12,000 cpm ^{125}I-T_4.

Stage	Sample size (# lots of 2 fish/lot)	Mean initial counts (cpm)	Mean counts remaining after extraction (cpm)	Percent of counts remaining after extraction (%)	Mean counts remaining after resuspension (cpm)	Percent of counts remaining after resuspension (%)	Percent recovered (%)
Egg	5	12,285	8,549	70.4	8,263	67.9	67.9
Alevin	5	11,979	7,837	67.2	7,710	66.5	66.5
Fry	5	10,980	6,221	57.4	6,038	56.8	56.8
Initial	5	11,956	11,801	98.7	11,578	96.8	96.8

unknown samples were based upon the recovery samples spiked with non-radiolabelled T_4 , which did not contain chemical additives.

4.0 Discussion

4.1. Accuracy of experimental results

Our results indicated that developing kokanee salmon experience thyroid hormone surges at the time of hatch and at swimup. In Lake Whatcom stock kokanee, the concentration of the surge at the time of hatch (18.7 ± 6.8 ng/g body weight) was approximately 1.75 X (i.e., 175%) that of basal levels observed in eggs (12.5 ± 1.7 ng/g body weight.) After T_4 levels had declined to as low as 7.1 ± 1.5 ng/g body weight in pre-swimup alevins, a second peak in T_4 level (16.0 ± 4.7 ng/g body weight) was observed at the time of complete yolk sac absorption or swim-up stage (i.e., about a 225% increase). A similar pattern was observed in Lake Roosevelt stock fish, with the highest concentration (13.3 ± 2.8 ng/g body weight) found in eggs just prior to hatch. Hatchlings and swimup fry were not sampled for Lake Roosevelt stock fish. In both stocks, thyroxine levels declined to near non-detectable levels in post-swimup fry.

We believe that these results represent true fluctuations in T_4 concentration because results of quality assurance procedures indicated that the assay was reliable. Measured concentrations of blind quality control samples were within the acceptable range of actual concentration. The percent error, and especially the upper range in error of duplicate samples, was relatively high. However, both the mean percent error (± 95% Cl) of duplicate samples (6.8 ± 0.6%) and the range (0 to 32.5%) were well below the 175% to 225% fluctuations in concentration observed during T_4 surges. We suspect that the error noted in duplicate samples was owing primarily to two factors: (1) Dried samples were resuspended in ethanol solution. Ethanol solutions are less viscous that aqueous solutions and therefore more difficult to pipette with uniform accuracy; and (2) a 6.3% error associated with pipetting 25 µl samples. The mean percent error (± 95% Cl) of duplicate samples (6.8 ± 0.6%) was uniform with the 6.3% error noted in pipetting the 25 µl samples.

One factor that might have contributed error to our results is the different post-extraction evaporation time between smaller-sized eggs,

alevins, swimup fry, and recently post-swimup fry versus older fry. Samples collected from egg to recent post swim-up fry (through April) were generally evaporated within 2 to 24 hours, whereas those collected from older larger fry were not completely evaporated until about 48 hours. The difference was owing to the amount of PTU/ethanol solution used for extraction, since the volume used depended upon body weight. We are concerned that the extremely low values of T_4 reported for the older fry (collected from late May to late July) may be partly related to decomposition of thyroid hormones in these samples owing to their relatively longer drying time. However, T_4 concentrations had declined to near non-detectable levels in recent post-swimup fry samples collected in April and May, which were evaporated in the same time frame as eggs and alevins, so our T_4 concentrations for older fry may be accurate. Additionally, recovery analysis indicated that, although fry did have a lower recovery rate than eggs or alevins (80.4 -v- 88.5 and 90.4% respectively), the difference was not sufficiently great to explain a greater than 181 fold difference between recently hatched eggs containing 18.7 ng/g body weight T_4 -v- 182 day old fry containing 0.1 ng/g body weight T_4.

4.2 Comparison of experimental results to those from other investigations.

Our results are comparable to other investigations of thyroid hormone concentrations in eggs and larvae of other salmon species in terms of both concentration levels and temporal (ontogenetic) patterns of thyroid hormone concentration. In our study, T_4 levels in eggs and alevins ranged from 4.8 to 23.3 ng/g body weight with peaks at hatching and swimup (about 40 days post hatch), then declined to near nondetectable levels in older fry (180 days post hatch).

Appreciable quantities of thyroid hormones have been found in the eggs and larvae of a variety of telost fishes (Kobuke et *al.* 1987; Brown *et* al. 1988, 1989; Tagawa et *al.* 1990). Thyroxine and triiodothyronine (T_3) were detected in plasma of yolk-sac stages in a variety of salmonid species (Sullivan et *al.* 1987). In chum salmon eggs, Tagawa et *al.* (1990)

observed that whole body T_4 content was about 15 ng/g body weight and T_3 content was about 10 ng/g body weight.

Tagawa and Hirano (1987, 1990) observed a transient increase in whole body concentration of T_3 and T_4 in chum salmon at the end of yolk absorption, when larvae emerged from their gravel bed. In their study, T_4 concentration decreased from time of hatch to about 40 days post hatch, then peaked at the time of yolk reabsorption, after which T4 levels declined.

Grace *et al.* (1992) reported that significant amounts of thyroxine (T_4 = 20 ng/g) and triiodothyronine (T_3 = 10 ng/g), were present in fertilized eggs of chum salmon. Both T_3 and T_4 levels were stable during the egg stages, decreased during yolk sac absorption, then increased at emergence. Grace *et al.* (1992) observed only a slight increase in T_4 in chum salmon at the time of emergence but decided it could be sufficient to stimulate physiological and behavioral changes associated with swimup.

Letherland *et al.* (1989) measured thyroid hormone content of eggs and larvae of all five species of North American Pacific salmon (pink, chum, chinook, coho, and sockeye). They determined that sockeye had the highest T_4 levels of the five species studied. Moreover, sockeye larvae contained significantly more T_4 than any of the other species examined through 92 days post fertilization (i.e., the time of emergence and swimup). Triiodothyronine and thyroxine levels remained at about the same concentration in pink, chum, and sockeye throughout this period whereas T_3 and T_4 levels in coho and chinook declined significantly. Transitory increases in T_3 and T_4 levels were observed in sockeye at about the time of hatch and again at swimup if the decrease in body weight owing to yolk absorption was taken into account.

Our results differed from those reported by other investigators in one important respect. We observed a pronounced, transitory increase in T_4 concentration on the day of hatch, whereas other investigations observed either a small increase or no increase. There are two possible explanations for this discrepancy:

(1) In our investigation, we collected fish as they were hatching. The T_4 concentration measured in these fish was 23.3 ng/g body weight. T_4 concentration of kokanee from the same raceway collected one week later was 9.4 ng/g body weight, or approximately the same as observed in pre-hatched eggs so this surge was brief. We were unable to determine if other investigators collected fish at the exact time of hatch. If not, it is possible that they could have missed the peak.

(2) In our study, body weight of kokanee on the day of hatch (47 mg) was reduced 39% compared to eggs (76 mg). Thus, the T_4 peak coincided with rapid loss of body weight. Since T_4 concentration nearly doubled during this time when body weight was nearly halved, the peak may not represent a surge so much as preferential retention of T_4 or sheding of fluids and egg coat membranes that did not contain T_4. In other species, including anadromous sockeye, a decrease in body weight was not observed at the time of hatch. Instead the lowering of body weight was observed at the time of swimup, which was associated with a transitory increase in T_4 concentration. Thus, T_4 peaks were correlated with time of body weight loss both in our study and those performed by other investigators. These results suggest that T_4 is selectively retained in the fish undergoing body weight loss, possibly because it is bound to nuclear receptors in target cells or T_4 clearance rate is very low.

In our study a pronounced decrease in body weight was noted in kokanee at the time of hatch, whereas Leatherland *et al.* (1989) reported that body weight of the anadromous form (sockeye) of this species did not decrease until swimup. Difference in the pattern of early ontogenetic develop, in this case body weight loss at hatch -v- swimup, between potadromous (kokanee) and anadromous (sockeye) forms of *Oncorhynchus nerka* may be related to subsequent differences in physiology and behavior **between the two forms. [These differences should be investigated further** in view of plans to utilize Snake River kokanee stocks to help restore Snake River sockeye stocks that are currently listed as a Federal endangered species.]

In summary, our results and those obtained by other investigators indicate:

(1) T_4 is present in significant amounts in the egg and larval stages of a variety of salmonids, including both anadromous and resident forms of sockeye/kokanee (*Oncorhynchus nerka*);

(2) There is often a pronounced transitory peak in T_4 concentration at the time of swimup, especially in species such as sockeye and chum salmon that emigrate from their natal tributary shortly after emergence (there is also a transitory peak at the time of hatch in the case of kokanee); and

(3) Subsequent to swimup, T_4 levels decline to very low levels.

Our results may indicate a critical period for imprinting in kokanee salmon if thyroid surges in kokanee are correlated with imprinting as in other species of salmon.

4.3 Evidence supporting thyroid regulation of olfactory imprinting in salmonids: A homing mechanism independent of inherited genetic constraints.

Thyroid hormone surges are indicators of critical periods for metamorphosis and imprinting in egg and larval stages of a variety of vertebrates (reviewed by Hasler and Scholz 1983; Scholz *et al.* 1985). Hess (1973) reported that the critical period for imprinting in birds begins in the egg and extends through hatching, which is correlated with a surge of thyroid hormones (Thommes *et al.* 1977). Neonatal rats normally experience a surge of thyroid hormones between days 10 and 14 after birth; their brain neurons undergo differentiation and wiring of neruon circuits occurs at this time (Hamburgh 1968; LeGrand 1979). Brains of rats with either natural or experimentally induced hypothyroid conditions during the sensitive period undergo abnormal brain development, characterized by reduced arborization of neurons and fewer synoptic contacts (Hamburg 1968; LeGrand 1969). Such rats are deficient in learning and memory functions. Administration of thyroid hormones to

hypothyroid rats during the sensitive period results in normal brain development, and restores learning and memory functions (Hamburgh 1968; LeGrand 1979). Thyroid hormones have also been shown to stimulate metamorphosis in larval flatfishes and sea lampreys (e.g., Tanaka *et al.* 1990).

Hasler and Wisby (1951) proposed that salmon smolts imprint to the odor of their natal tributary and subsequently use the memory of the odor as cue to relocate their home stream during the spawning migration. Evidence supporting this olfactory imprinting hypothesis was reviewed by Hasler *et al.* (1978) and Hasler and Scholz (1983). Both the smoltification (Dickhoff *et al.* 1978, 1982a,b) and olfactory imprinting processes (Scholz 1980; Hasler and Scholz 1983) appear to be under thyroid control. During smoltification both physiology and behavior are influenced by thyroid hormones (Hoar 1939, 1976). For example, thyrotropin injections stimulated the silvery appearance (Robertson 1949), downstream migratory activity (Godin *et al.* 1974), and olfactory imprinting (Scholz 1980, Hasler and Scholz 1983) in presmolts.

4.3.1 Evidence for imprinting: transplantation and transportation experiments.

Studies concerning fish transplanted from their natal tributary to a different one before, during, or after the smolt stage, suggest that imprinting occurs during the smolt stage (reviewed by Ricker 1972). When Rounsefell and Kelez (1938) transferred marked, presmolt coho salmon from their native river to a different one, the fish returned as adults to the river into which they had been transferred. In addition, coho, chinook, sockeye and Atlantic salmon, and rainbow and brown trout, raised in a hatchery and transplanted before undergoing smolt transformation, returned to the river of release (Shapovalov and Taft 1954, Donaldson and Allen 1957, Lindsay *et al.* 1959, Carlin 1968, Shirahata and Tanaka 1969, Ellis 1970, Jensen and Duncan 1971, Aho 1975, Mahnken and Joyner 1975, **Mighell 1975, Vreeland et *al.* 1975, Wahle 1975, Heard and Crone 1976,** comprehensive review by Ricker 1972 and Hasler and Scholz 1983).

There is evidence that this process is rapid. Shirahata and Tanaka (1969) found that transplanted smolt-stage sockeye salmon returned to the stream of release. They believe that the time necessary for imprinting was less than 10 days since the fish had left the river by that time. Carline (1968) and Jensen and Duncan (1971) transplanted Atlantic salmon and coho salmon just as they began to smolt: the fish left the river within two days and returned to it during the spawning migration. Particularly illuminating was a study by Mighell (1975) who found that four hours was a sufficient period for holding coho smolts in a new stream to ensure homing to it.

In contrast, when Peck (1970) transplanted hatchery-raised coho salmon to a Lake Superior tributary several weeks after smolt transformation, the return to the stream of release was poor with many recoveries in other streams. Peck concluded that the fish may have spent the sensitive period in the hatchery, where the water supply was not connected with Lake Superior, and suggested that imprinting terminates soon after smolt transformation begins, thereby preventing fish from becoming imprinted to other tributaries during the course of their downstream migration.

Additional support for this view comes from a study by Stuart (1959) with brown trout at Dunalastair Reservoir in Scotland. In this land-locked population, the fish grow to maturity in the reservoir and spawn in the tributaries. Stuart transplanted one group of brown trout from their natal tributary to a different one before smolt transformation had begun, while a second group was retained through the smolt stage before being transferred. Fish transplanted before undergoing smolt transformation returned to the river of release to spawn, whereas those transplanted after smolt transformation returned to their natal tributary.

In efforts to improve stocking techniques to maximize the return of stocked fish, the time of release has been found to be prominent in achievement of success (Reimers 1979). There is evidence that releasing hatchery-raised fish outside a narrow window during smoltification reduces the return to the site of release. For example, Ellis (1957) found

that chinook salmon transplanted after smoltification in the hatchery returned to the river where the hatchery was located instead of to the stocking site. Additionally, King and Swanson (1973) and Winter (1976) found that the time at which transplanted fish are stocked might influence homing to the stream of release; straying of rainbow trout in Lake Superior was greater, for example, if the fish were released late (July-- as post-smolts) than if released early (April-- just before smolt transformation). Reingold (1975a, b) conducted similar experiments on steelhead trout in the Snake River system, Idaho, and obtained comparable results.

Smolt transportation studies by Ebel (1970), Ebel et *al.* (1973), Park (1975), Slatick et al. (1975), and Weber (1975), (reviewed by Collins 1976), are also consistent with the imprinting hypothesis. Studies were conducted in the Clearwater-Snake-Columbia river system in Idaho, Washington, and Oregon. Chinook, and rainbow smolts were collected at dams after they had migrated downstream 120-240 km from their natal tributaries in the Cleat-water and Snake rivers they were then marked and transported 570 km downstream by truck to a site below Bonneville Dam. During the spawning migration, fish were recovered predominantly at their natal tributary or hatchery; very few were recovered from the transplant site or other locations in the Columbia River system. Weber (1975) held fish at Dworshak Hatchery, on the Clear-water River, until they had completed the initial stages of smolt transformation, transported them 810 km to the lower Columbia River, and subsequently recovered them at the hatchery 18 months later. No fish were recovered at downstream monitoring stations. In similar experiments with Atlantic salmon, Mills and Shackley (1971) obtained comparable results. The experiments with fish transported downstream after smolt transformation indicates that the fish became imprinted to some factor in the water-- that is, fish found their way through the home river system and reached their natal stream without having used that route for the downstream migration.

4.3.2 Imprinting of salmonids to synthetic chemicals and natural odors.

It is well documented that smolt stage salmon and trout can imprint to a water source and subsequently use this information as a cue for homing during the spawning migration. Evidence stems from field experiments in which coho salmon (*Oncorhynchus kisutch*), steelhead trout (*Oncorhynchus mykiss*), and brown trout (*Salmo trutta*) exposed (i.e., artificially imprinted) to synthetic chemicals-- morpholine (at 5 x 1 0^{-5} mg/l) and phenethyl alcohol (at 5 x 10^{-3} mg/l)-- during the smolt stage, were attracted as adults into rivers scented with the appropriate chemical at 92-96% accuracy (Scholz, *et al.* 1975, Cooper and Scholz 1976, Scholz *et al.* 1976, 1978a, b, Johnsen and Hasler 1980, Sutterlin *et al.* 1982, reviewed by Hasler and Scholz 1983). Since the juvenile fish had never been exposed to the scented streams before they were attracted to them as adults it was concluded that they became imprinted to the synthetic chemicals, then used this information as a cue for homing. The experiments also indicated that the memory of the olfactory cues was learned and not a genetic memory.

It seems likely that salmon possessing this ability would use it with natural chemicals in the environment. Additional support for this conclusion comes from two studies which show that salmonids become imprinted to natural water. Jensen and Duncan (1971) transplanted presmolt coho salmon from Leavenworth Fish Hatchery on Icicle Creek, a tributary of the Wenatchee River in Washington, to a spring-fed fish holding facility approximately 125 km away on the Snake River. The fish were marked, held for 48 hours until they began to smolt, and then released into the Snake River. During the spawning migration, marked fish were recovered near the spring-water discharge 0.8 km downstream from the release point. No fish were recovered at Leavenworth Hatchery. To determine whether the fish were actually homing to the water in which they had been held as smolts, water from the holding facility was pumped through a floating trap. As a control, river water was pumped through the trap on alternate days. No fish entered the trip when river water was used, but 399 fish were captured when spring water was used. Thus, it

seems clear that spring water from the fish-holding facility was the orienting stimulus and that fish were able to learn the characteristics of the water within two days. In the second study, Stuart (cited in Scholz et *al.* 1978b) marked a group of young brown trout in one branch of a forked stream that flowed into the Dunalastair Reservoir, in Scotland. After the fish had migrated to the reservoir, all the water from the home fork was diverted into a new channel, and the original channel was maintained by water from the second fork. During the spawning migration, adult trout homed to the new channel in preference to the channel by which they had entered the reservoir.

4.3‘3. Thyroid activation of olfactory imprinting in salmonids.

Subsequent investigations into the mechanism of the imprinting process revealed that exposure of pre-smolt coho salmon and steelhead trout to synthetic chemicals-- morpholine and phenethyl alcohol-- in February, when the concentration of thyroid hormones were at basal levels, did not result in imprinting. Fish exposed to the same chemicals during the smolt stage, in mid-April shortly after experiencing a thyroid hormone surge, did become imprinted to the synthetic chemicals (Scholz 1980, Hasler and Scholz 1983). Moreover, experimental pre-smolts simultaneously exposed to one of the synthetic chemicals and injected with thyroid stimulating hormone (TSH) in February, in doses sufficient to elevate thyroid hormone concentrations to smolt levels by late February, formed a permanent memory to that chemical; whereas control fish injected with either adrenocorticotrophin (ACTH) or a saline placebo did not (Scholz 1980, Hasler and Scholz 1983).

In field experiments conducted when the fish were in spawning condition, the fish exposed to the synthetic chemicals (either as natural smolts in April or those administered TSH injections in February) exhibited positive rheotaxis (i.e., migrated upstream) if the odors were present and were able to choose accurately between two tributaries scented with morpholine or phenethyl alcohol 95% of the time (Scholz 1980, Hasler and Scholz 1983). If the odors were absent, the fish swam downstream. In contrast, the fish that had been exposed to the synthetic chemicals as pre-smolts in February, as well as those administered ACTH

or saline placebo injections in February, were random in their selection of the two tributary streams and usually did not migrate upstream even when the odors were present (Scholz 1980; Hasler and Scholz 1983). These investigations indicated that thyroid hormones induce the imprinting process because: (1) Thyroid hormones concentrations were observed to rise shortly before imprinting occurred in natural smolts, and (2) TSH injections allowed for imprinting at a time earlier than it would normally occur. Morin *et al.* (1989) reported that thyroid activity was correlated with olfactory learning during smoltification in Atlantic salmon and suggested that thyroid hormones also play a role in olfactory imprinting in this species.

4.3.4. Proposed Mechanism for thyroid activation of imprinting

For thyroid hormones to exert an effect on target tissue the hormone must first bind to a saturable receptor in the nucleus to form a hormone-receptor complex, so it was necessary to determine if salmonid brain neurons contained specific, high affinity saturable triiodothyronine (T3) nuclear receptors. This was accomplished by conducting *in vivo* and *in vitro* experiments (Scholz *et al.* 1985; White et 1990). *In vivo* experiments involved injecting a tracer dose of radioactive ^{125}I-T_3 either alone or with a IOO-fold excess of nonradioactive T_3 into the body cavity of steelhead smolts, then, in each case, measuring the amount of radioactivity that accumulated in isolated brain nuclei. Radioactive T_3 accumulated and was retained in the brain nuclei. In fish receiving nonradioactive T_3, the uptake of ^{125}I-T_3 was reduced by 90% when compared to fish receiving only radiolabelled T_3 (Scholz *et al.* 1985). This investigation also determined that different regions of the steelhead brain accumulated different amounts of ^{125}I-T_3. The diencephalon accumulated nearly twice as much radioactivity as other brain regions (Muzi 1984). The diencephalon is interconnected with the olfactory bulb (telencephalon) by extensive nerve fiber tracts and may serve to integrate olfactory function in teleosts (Kaplan and Aronson 1969, Aronson 1981). Oshima and Gorbman (1966) reported that odor evoked electroencephalograms (EEG) recorded from the olfactory bulb of fish were modified by thyroxine treatment.

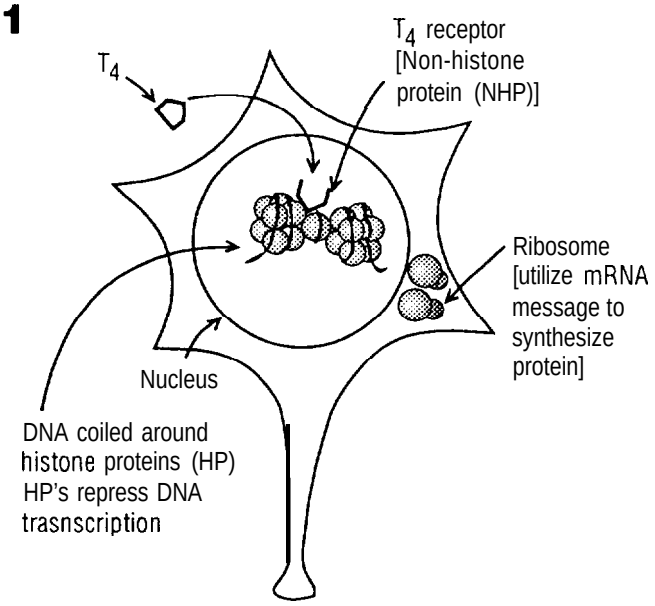
The presence of specific, high affinity, saturable T_3 receptors in neurons was detected by performing an *in* vitro T_3 competitive binding assay on nuclei isolated from the brains of 214 steelhead smolts. Scatchard analysis determined the binding affinity and capacity at 57.2 x 1 $0^9 M^{-1}$ and 10.8 pmol/mg DNA respectively (White *et* a/. 1990). Both affinity and capacity values indicated the presence of high affinity, saturable T_3 receptors.

It is thought that hormone receptor binding activates transcription of a segment of DNA, which is the gene that codes for nerve growth factor **(NGF),** a protein that promotes arborization of axons and dendrites, and development of synoptic contacts (reviewed by Scholz *et al.* 1985). Thus, thyroid hormones would induce a pattern of neural wiring that could account for the formation of a permanent long-term olfactory memory. The pattern of interconnections stores the olfactory memory in a manner similar to how electrical connections in a computer stores a memory on the hard drive. The imprinted odor, by binding to specific receptors in the olfactory epithelia, activates the memory in the same fashion that a finger striking a particular sequence of keys on the keyboard accesses the stored computer memory. (See Fig. 10).

Thyroid induced differentiation of brain neurons was observed in steelhead trout undergoing parr-smolt transformation (Lanier 1987). In these experiments, Golgi silver stain impregnation techniques were used to trace the development of dendrite and axon projections in neurons from the diencephalon region of pre-smolt and smolt stage steelhead trout. Lanier (1987) demonstrated that rapid neuronal proliferation occurred during the time of the thyroid surge. She found an increase in the number axons, dendrites and synapses, and also an increase in the size and length of the axons and dendrites in smolts collected in late April and May (when the thyroid surge occurred) as compared to pre-smolts collected from January to March (when thyroid hormones were at a basal levels). Moreover, pre-smolt steelhead, injected with sufficient TSH in February to cause thyroid hormone concentrations to rise to smolt levels, exhibited pronounced neuron proliferation compared to control fish injected with saline. Neuron differentiation included increases in both neuron size and

Fig 10. Proposed Mechanism for Thyroid Induced Activation of Olfactory Imprinting

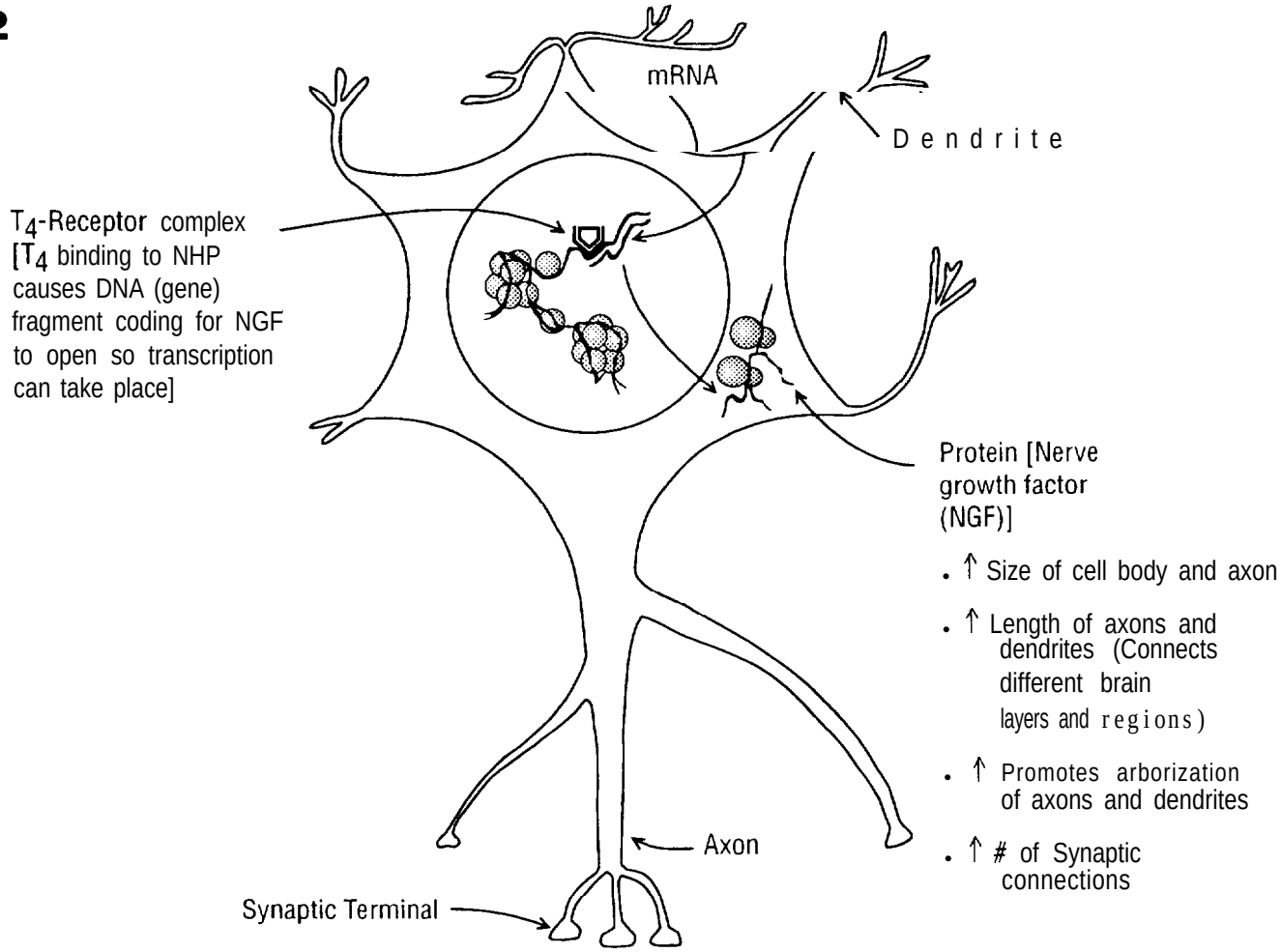
1



3

- Creates permanent wiring of neuron circuits. Pattern of interconnections stores the olfactory memory in a manner similar to how electrical connections in a computer stores a memory on the hard drive.
- The imprinted odor, by binding to specific receptors in the olfactory epithelia, activates the memory in the same fasion that a finger on striking a particular sequence of keys on the keyboard acccesses the stored computer memory.

2



- ↑ Size of cell body and axon
- ↑ Length of axons and dendrites (Connects different brain layers and regions)
- ↑ Promotes arborization of axons and dendrites
- ↑ # of Synaptic connections

synaptic contact number, as well as increased length and branching of axons and dendrites. This work is currently being re-examined by Scholz and White (Fig. 1 1), who found similiar results.

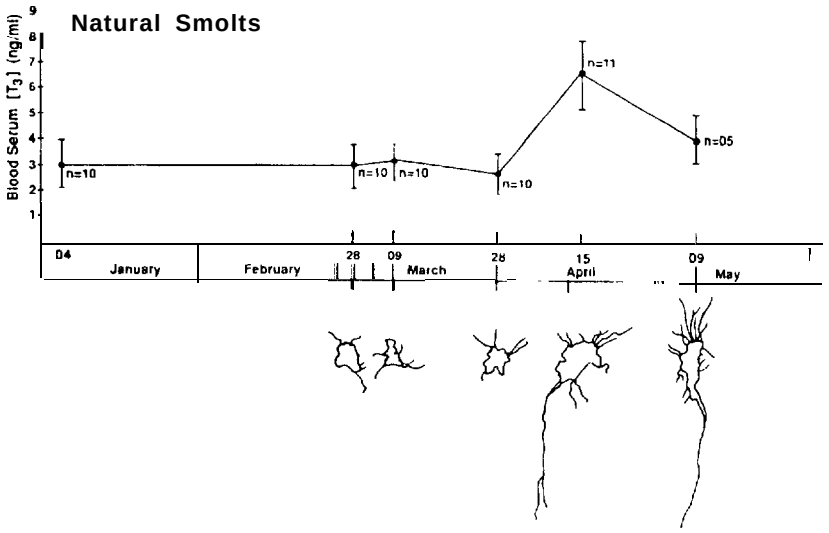
4.3.5 Sequential Imprinting

The above information implies that there is a single critical period, correlated with a thyroid surge, when salmon become imprinted. However, species such as sockeye must imprint at lease twice, first when they emigrate from their natal tributary to their nursery lake and second when they emigrate from their nursery lake to the ocean. Quinn *et al*. (1989) pointed out that if thyroid surges are correlated with imprinting, then "*the ability of salmon to learn odors on more than one occasion is not fully compatible with a single peak of thyroid hormone in the spring*."

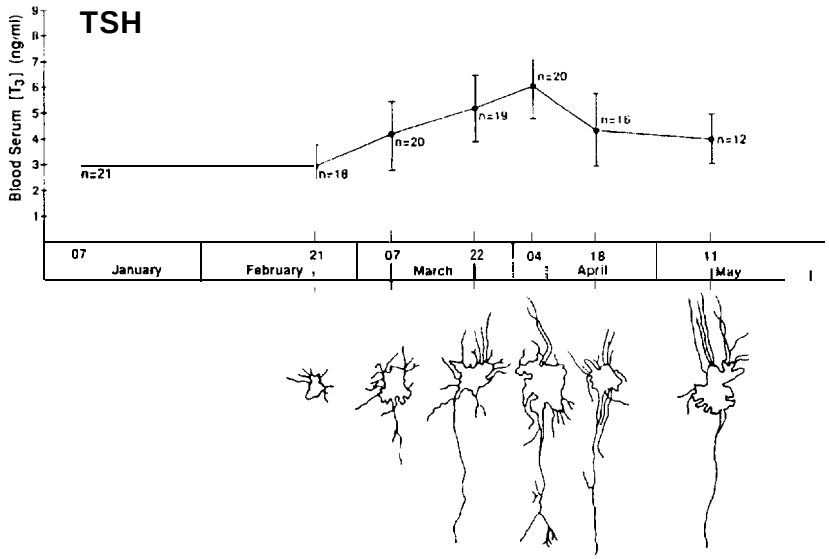
Quinn *et al.* pointed out that the solution to this problem may lie in the discovery by Dickhoff *et al*. (1982a) that exposure of coho salmon to different novel waters during the smolt stage induced transient peaks in thyroid hormone levels equal to the number of different waters tested. Other investigations have confirmed that brief peaks in thyroid hormone occured concentration in coho, chinook, and Atlantic salmon exposed to novel waters (Lin *et al*. 1985; Nishioka *et* a/. 1985), addition of saltwater (Specker and Schreck 1984; Nishioka *et al.* 1985) and alterations in flow, presumably because of increases in tributary odors owing to freshets (Nishioka *et al.* 1985, Youngson *et al*. 1986, 1992). Grau *et al*. (1981) noted that coho salmon experience multiple thyroid surges linked with the new moon phase of the lunar cycle. Thus, imprinting in salmon may be flexible, with multiple peaks in thyroid hormones associated with learning site-specific odors in sequential order during seaward migration. Quinn *et al* (1989) speculated that, *"if thyroid hormones are linked to olfactory learning, there may be feedback from migration to hormones, resulting in additional learning during migration. Exposure to novel waters (e.g., at the confluence of rivers) might induce elevated hormone levels and trigger learning of the water source as an olfactory way-point to be used during upstream migration years later."* The fish may also fix the position of the mouth of their home river system owing to as thyroid

Fig 11. Effect of thyroid hormones on stimulating neuron differentiation in presmolt and smolt stage steelhead trout

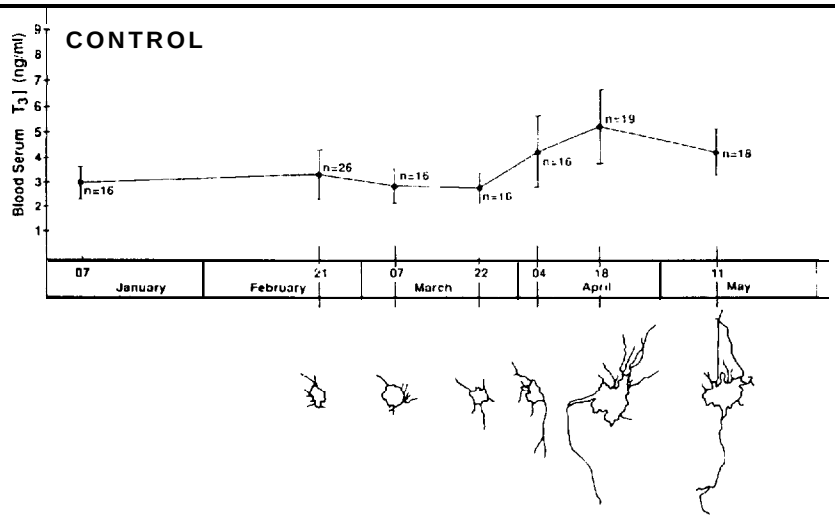
Neuron drawings were made by using a camera lucida attached to a phase contrast microscope. The image was focused in several planes so that the 3-dimensional neuron could be converted into a 2-dimensional drawing. (From A.T. Scholz and R.J. White, Eastern Washington University, Biology Department, Biology Department, unpublished data.)



Brain neuron development correlated with thyroid hormone surge in natural smolts during April.



Brain neuron development occurs earlier in presmolts injected with TSH in doses sufficient to cause thyroid hormones to rise to smolt levels in early March.



Brain neuron development occurs at the time of the thyroid surge in April in presmolts injected with a saline placebo to control for experimental artifacts.

surge at the time they first enter saltwater (See also Appendix I for additional discussion of stocking site imprinting.)

However, this idea is not completely consistent with the results of certain transplant experiments (e.g., Peck 1970) cited in Section 4.3.1. At present it is unknown if the multiple thyroid surges are, in fact, associated with sequential imprinting during the course of downstream migration. But it remains an attractive idea because it would be an elegant adaptation by the fish to be able to link exposure to novel water to thyroid surges, which, intern would trigger odor imprinting. Certain species like sockeye which experience thyroid surges as fry and smolts, must imprint at least twice-- as fry and again as smolts.

Thyroid surges at smolt release do appear to be linked to homing of adults. Chinook salmon from the Rakaia River, New Zealand exhibited cyclical surges of thyroxine apparently associated with lunar phasing with highest peaks occuring about 3-6 days before the new moon (Hopkins and Sadler 1987). Moreover, recoveries of adults, from 136 smolt releases from seven year classes released between 1979 and 1985, showed that the highest percent recoveries were from those releases made about 3-6 days before the new moon (Hopkins 1992). Nishioka *et al.* (1989) also observed that highest percent return of adult coho salmon to California hatcheries coincided with releases linked to thyroxine surges associated with the new moon.

4.3.6 Pheromones and Homing

A homing hypothesis that involves pheromones emitted by juvenile fish as a source for attracting adults has been advanced by several authors (reviewed by Horrall 1981 and Liley 1982). This idea was first **suggested by G.H. Parker (cited in Chidester 1924) who thought, *"it is possible that a certain race of fish may give off emanations that differ chemically from those of other races; hence, the return of individual races to their homestream could be attributed to their power to sense the familiar emanation. "***

In support of this idea White (1934) and Solomon (1973) reported that streams that had previously been barren of Atlantic salmon (*Salmo salar*) became attractive to migrating adults shortly after juveniles were transplanted into them. White (1934), for example, planted fry in a stream that previously had never contained salmon. Later that year adults migrated up the river for the first time in recorded history, suggesting that *"homing is influenced by population specific pheromones or metabolic products released by juveniles."* The major drawback with this evidence is that no information was available which could definitely prove that the adults were genetically related to the juveniles. An equally likely alternative is that adults could have been attracted into the stream by some generalized conspecific odor. The problem, if the latter interpretation is correct, is that generalized olfactory attractants cannot account for the site specificity involved in homing.

In electrophysiological tests, Dizon *et al.* (1973) noticed that toward the end of the spawning season adults ceased to respond to their imprinting odor, morpholine or phenethyl alcohol, and, instead, began to respond strongly to the odors of other salmon. A major point here is that the switch occured after most of the adults had already migrated up the homestream and into the home tributary. Thus, conspecific odors may not provide specific homing cues but instead may act as generalized attractants providing fish which have failed to home correctly with a mechanism for attracting them to sites with other spawning adults, thereby allowing for completion of their life cycle.

In a more advanced formulation of the pheromone hypothesis, Nordeng (1971, 1977) proposed: (1) populations or races of salmon in different streams emit pheromones that serve to identify distinctly fish from that particular river, (2) the memory of this population-specific pheromone is inherited, *i.e.,* stored in genetic memory, and (3) homing adults follow pheromone trails released by juveniles which reside in the stream, *i.e.,* the juveniles provide a constant source of population odor. Nordeng's hypothesis is supported by a variety of behavioral and electrophysiological data which suggests that attic char, *Salvelinus alpinus*, are able to discriminate between different populations of their

species on the basis of odor (Nordeng 1971, 1977; Doving *et al.* 1974, 1980; Selset (1980); Selset and Doving 1980). Also, Nordeng (1977) found that mature males, kept isolated for four years, homed to a river that contained other members of their own population.

Larkin (1975) and Selset (1980) speculated that residual pheromonal odors left from previous habitations by stock members might permit natal homing even in situations when there were no young remaining in the stream during the spawning migration. Such a mechanism would be important for stocks such as pink salmon that spawn only in odd years in southern British Columbia. Quinn et *al.* (1983) reported that water source preferences of adult coho salmon, *Oncorhynchus kisutch*, were apparently based in part on recognition of pheromones. Foster and Berline (1980) observed pheromone-mediated spawning site selection by lake trout, *Salvelinus namaycush*.

Even though evidence is accumulating that salmon may use pheromones as cues for homing, it is also clear from numerous successful transplant experiments that homing involves imprinted as well as genetic memory. Moreover, Selset and Doving (1980) in discussing their work on migratory char state: *"our results do not exclude olfactory imprinting, but make it likely that the possible imprinting would be to substances emanating from the fish and not from vegetation or minerals"*.

There is strong evidence that Pacific salmon can discriminate odors of different water sources without the aid of conspecific pheromones. Using reward (food) and punishment (electric shock) for conditioning, Hasler and Wisby (1951) trained groups of bluntnose minnows and coho salmon to discriminate between waters collected from two Wisconsin streams. After about 30-40 conditioning trails, when “reward” water was introduced into their test tank, the fish swam towards it prior to the reward being offered. When “punishment” water was introduced, they swam to the opposite end of the tank before the electric shock was administered. When their nasal sacs were cauterized, the trained fish were not able to discriminate between the waters, indicating that it is the characteristic odor of the water that is discernable by fish. In addition, they found that trained fish were not able to identify the sample

if the organic fraction was removed. Hasler and Wisby (1951) proposed that because of difference in soil, vegetation and faunal assemblages of their drainage basin, each stream would have a distinctive organic odor bouquet that salmon could imprint to. Since stock specific pheromones are organic odors they could form part of the odor complex. However, both the bluntnose minnows and coho salmon tested by Hasler and Wisby, were able to differentiate between the two Wisconsin streams in the absence of stock specific pheromones, because both stocks of fish were tested to novel waters that had never been occupied by other members of their stock. For example, the coho salmon were native to Washington State and flown to Wisconsin for the experiment. No coho salmon had ever been stocked in the two Wisconsin streams that provided source water for the odor discrimination tests. Clearly, these fish were able to discriminate the organic odors of the two streams even though stock specific pheromones were absent.

In the artificial imprinting work with coho salmon, rainbow trout, and brown trout reviewed here (see section 4.3.2.), it is unlikely that fish homed to pheromones. In all of these experiments, there were no young coho salmon present in the scented streams at the time the adults were attracted to them. Since warm summer stream temperatures preclude survival of juvenile salmon in Lake Michigan tributaries, all production occurs in hatcheries separate from the tributaries. The fish are then stocked into smolting ponds in the tributaries when they are about 17 months old. The fish are not stocked in the streams until just before smolt transformation and reside in them for only a brief time, usually from 2 to 4 weeks, before migrating to the lake. Although it is conceivable that the juveniles could impart their odor to the stream system by accidentally scraping off mucus on rocks, we feel this is an unlikely possibility in view of the short period of time the juveniles are actually present in the stream. Yet, adults home back to the tributary where they were stocked with great precision (Scholz *et al.* 1975, Scholz et *al.* 1978a). Also, since fish from identical genetic stock are planted in more than one Lake Michigan tributary, a significant amount of straying from the transplant site into other tributaries would be expected if the

pheromone hypothesis is correct. But not much straying occurs (Scholz et a/., 1975, 1978a)

In the one case there are no records of juvenile salmon ever being stocked into one stream used in some of the artificial imprinting experiments. Additionally, extensive electrofishing operations were conducted in the stream from late winter through late spring and again from late summer through late fall every year for three years and not one juvenile salmon was captured. Despite the fact that the stream was barren, morpholine -exposed fish were attracted to it when morpholine was present but not when morpholine was absent.

In the artificial imprinting experiments, differential responses were observed in the behavior of fish with different imprinting experiences, *i.e.,* morpholine-exposed fish homed to a morphlone scented stream and phenethyl alcohol exposed fish homed to a phenethyl alcohol scented stream. These fish were from the same spawning stock and randomly separated into groups for imprinting to different odors. The fish were stocked directly into Lake Michigan midway between the two streams scented with either morpholine or phenethyl alcohol. According to the pheromone hypothesis, our different experimental groups should have displayed uniform behavior, with equal numbers recovered in each test stream, because they were related. Consequently, the fact that different experimental groups behaved distinctively, by homing to the chemical that they had been exposed to the smolts, would seem to rule out the possibility that the fish homed to pheromones.

Additionally, Sutterlin et a/. (1982) found that Saint Croix River stock Atlantic salmon, reared as smolts at a marine site 12 km from their parent stream, returned to the marine site after having spent 13-25 months at sea. A group of smolts chemically imprinted with morpholine in salt water had the highest rate of return. Their results are difficult to explain in terms of the proposed pheromone hypothesis because no juvenile salmon from the Saint Croix River were present at the salt-water-imprinting site, and yet the experimental fish homed there. The experimental fish homed preferentially to this site even though their parent stream, with a population of Atlantic salmon presumably emitting

race-specific pheromones, was located only 12 km away. Sutterlin *et al.* (1982) concluded: "*If such pheromones are operative, it would appear that their influence can be over-ridden by other directive factors.*"

Collectively, these data illustrate clearly that salmon are able to home in the absence of pheromones. However, it remains an intriguing idea that, in natural populations, genetic or imprinted memory of pheromones, in addition to olfactory imprinting of the organic homestream odor bouquet, may provide homing cues for adult fish. Available evidence indicates that there are both genetic and imprinted components to migration and home-stream selection in salmonids (Barns 1976). Possibly, salmon may have redundancy mechanisms for orientation as has been reported in birds.

4.4 Genetic component of homing in salmonids.

Two important conclusions can be drawn from transplantation, transportation and olfactory imprinting studies reviewed in Section 4.3 of this report: (1) the memory of the home stream is not inherited, and (2) homing is connected with a period of rapid and irreversible learning of the cues that identify the home stream at the time the young salmon begin their downstream migration. In regard to the first point, however, Barns (1976) has argued that there is also a genetic component. To test this hypothesis, he transplanted pink salmon (*Oncorhynchus gorbuscha*) eggs from their original tributary (donor stream) to a second one (recipient stream). One group of donor stream eggs was cross-fertilized by males from the recipient stream; while the other group were pure bred donor stream fish transplanted into the recipient stream. Both groups were raised in the recipient stream and then marked before they migrated to the sea. About equal numbers of both groups left the recipient stream, but only about half as many from the pure donor stream stock as from the **hybrid stock returned to it. Barns concluded that,** *"imprinting alone brought back some of the pure donor stock,"* and *"addition of the local male genetic complement improved the return to the* **river of release."**

4.4.1 Failure of certain transplantation experiments

Transplantations of Pacific salmon carried out within a species natural area frequently fail to produce any new anadromous stocks (Withler 1982). For example, Vernon (1957), Hartman and Raleigh (1964), and Ricker (1972) noted that large-scale transplants of sockeye salmon fry to new areas, sometimes even within the same river system, were frequently unsuccessful in terms of initiating, augmenting or rehabilitating spawning runs to the recipient stream. Bowler (1975) concluded that, "*poorly matched donor stocks from different environments and different behavioral stocks, when transplanted to bolster depleted indigenous fish populations, may cause poor survival of both the donor stock and indigenous population. Transplanted fish that survive and spawn can contribute to gene pool contamination and dilution of indigenous fish populations.*"

Altukov and Salmenkova (1990) transplanted a genetically distinct hatchery donor stock of chum salmon eggs into a recipient stream with a genetically distinctive wild population and monitored allelic frequencies at isozyme loci over successive generations. Coefficients of return to spawn in the recipient stream were lower for the alien fish as compared to the native stock, and in succeeding generations the alien fish disappeared from the spawning runs (as determined by genetic analysis). Thus, Altukov and Salmenkova (1990) concluded that transplanting chum salmon eggs from hatchery populations, "*is ineffective as a means of establishing a new stock in territory already occupied by another (wild) stock, and the practice is deplored on grounds of conserving unique gene pools within species.*"

Sockeye salmon stocks, in particular, have genetic differences which effect egg size, rate of yolk absorption, time of emergence, **swimming performance and tendency to migrate (Wood and Foote 1990). Anadromous sockeye salmon and landlocked kokanee** stocks spawn sympatrically in locations across British Columbia, yet remain genetically distinct. To investigate the possibility that these differences are maintained by selection against hybrids of the two forms, Foote *et al.*

(1992) raised pure and reciprocal crosses of Shuswap River sockeye and kokanee in controlled hatchery conditions and tested their seawater tolerance. In the spring of their second year, sockeye were the first to show increased seawater adaptabilities, hybrid groups next, and kokanee last. The observed differences could not be attributed to tank effects, or to differences in size, sex, or state of maturity, so it was concluded that these differences in seawater adaptability were of genetic origin.

Sockeye fry emerge in the spring from gravel nests (or redds) and swim to a nursery lake. This river migration involves complex genetically inherited rheotactic and olfactory cues (Brannon 1972, Bodznick 1978). Raleigh (1967) collected water hardened sockeye salmon eggs from beach spawners, inlet tributaries and outlet tributaries at Karluk Lake, AK, and transported them to a fish hatchery at Lewiston, ID. The eggs were incubated at the hatchery and resulting fry tested in laboratory tanks. Fry exhibited responses to water currents consistent with those they would have had to make to reach Karluk Lake, i.e., fry developing from outlet eggs tended to swim upstream at a significantly greater frequency than fry developing from inlet eggs. Fry from inlet eggs swam downstream at a significantly greater frequency than fry developing from outlet eggs. Raliegh's test results demonstrated that inlet and outlet fry differed significantly in their direction and time of migration. Because the rearing and test environment was identical, these differences were concluded to be of genetic origin. Raleigh concluded that, when transplanting salmonid fry, it is important to choose a donar stock with appropriate innate responses that are matched to the requirements of the recipient environment to be seeded.

Brannon (1967, 1972) investigated genetic control of migratory behavior in newly emerged sockeye salmon fry into nursery lakes. Fertilized eggs from three stocks-- outlet streams, inlet streams and lake shoreline areas-- were transferred to a fish hatchery remote from their lakes of origin and incubated under controlled conditions very different from their natural environments. The alevins and fry were then tested in a laboratory apparatus to determine their preferred direction of migration and each stock responded to water current with the same

behavior pattern exhibited in its natural environment. Chilko River stock sockeye, which migrate upstream from the outlet stream into the nursery lake, exhibited a strong preference (82.3%) to swim upstream (positive rheotaxis). Francois Lake stock sockeye fry, which must migrate downstream from an inlet stream to the nursery lake, exhibited a strong preference (80.0%) to swim downstream (negative rheotaxis). Cultus Lake stock sockeye, which exhibit shoreline spawning in the Lake and require, therefore, no migration to reach the nursery lake, showed random rheotactic behavior.

Progeny of a hybrid stock comprised of Chilko stock crossed with Francois Lake stock fish were also tested. The percentage of fry exhibiting positive rheotaxis (48.4%) was uniform with those exhibiting negative rheotaxis (47.8%). These results clearly indicate that the sockeye fry have an innate tendency to orient to water currents, which will lead them to their nursery lake. Raleigh (1971) repeated Brannon's experiment and obtained similar results.

Controlled experiments with rainbow trout (*Oncorhynchus mykiss*) and cutthroat trout (*Oncorhyncus clarki*) indicated that fry migrations of these species from spawning areas to nursery lakes are also under innate control; progeny taken from outlet spawning stocks tended to move upstream and progeny taken from inlet spawning stocks tended to move downstream (Lindsey et al. 1959; Notthcote 1962; Raleigh and Chapman 1971; and Bowler 1975). Thus, inherited differences in genetic composition appear to affect patterns of development growth, physiology, and migratory behavior of salmonid stocks.

4.5 Evidence for celestial orientation and magnetic field detection in salmonids.

4.5.1 Magnetic field orientation by salmonid fry

Innate directional preferences in salmon appear to be related to their ability to detect and orient using either celestial cues (e.g., sun compass or polarized light -- Groot 1965, Dill 1971) or electromagnetic fields (Quinn 1980). To evaluate, the role of magnetic fields in sockeye

fry orientation, Quinn (1980) and Quinn *et al.* (1981) experimented with sockeye fry from the Cedar River (Lake Washington, WA) which migrate downstream in a northwesterly direction from magnetic north, and Chiko River (Chilko Lake, BC), which migrate upstream in due south direction from magnetic north, to reach their respective nursery lakes. Fry from each location were tested for directional preference in an experimental tank without any current flow and a magnetic coil that was capable of shifting the earths magnetic field 90° counter clockwise, so that magnetic north was pointed approximately west. When the magnetic coil was turned off fry from Cedar River oriented in a northwesterly direction and fry from Chilko River oriented in a southerly direction relative to magnetic north in the test tank. When tested during the day or night fry from these two different stocks, isolated from the natural environment, moved in compass directions corresponding to the directions which they would have to maintain to reach their respective nursery lakes. However, when the magnetic coil was switched on at night fry from both stocks shifted their mean direction preference by 90° counter-clockwise corresponding to the 90° counter-clockwise shift of the magnetic field in the test apparatus; Cedar River fry swam in a westerly direction (90° counter clockwise from north) and Chilko lake fry swam in an easterly direction (90° counter clockwise from south).

Quinn (1980) also reported, "*During the day, only fish tested in covered tanks displayed redirected movement in the altered magnetic field; those tested with a view of the sky showed geographically appropriate movement patterns despite the shifted field.*" Thus, Quinn's study provides evidence for both celestial and magnetic compass orientation in lake migrating sockeye salmon fry. During the day, celestial cues, possibly a sun compass orientation mechanism, appears to take precedence over electromagnetic cues. However, since many stocks of sockeye, including the Cedar River stock, migrate to their nursery lakes at night, electromagnetic cues may also play an important role. Collectively, Quinn's results imply that: (1) sockeye fry posses an innate (genetic) population specific component of directional preference that will direct them in the appropriate compass heading to reach their nursery

lake (see also Brannon *et al.* 1982) and (2) celestial and magnetic cues provide the orienting mechanisms.

Quinn and Groot (1983) tested chum salmon fry for compass direction preferences in circular experimental tanks equipped with a device to alter magnetic fields. The fry normally had to travel in a southwesterly direction to reach to ocean. The mean direction preferred by fry was 257° in uncovered tanks and 258° in covered tanks. If the magnetic field was rotated 90° counter-clockwise, fry directional preference switched to 284° in uncovered tanks and 292° in covered tanks. Although fry directional preference changed under the influence of an altered magnetic field, direction and magnitude of change in preference were not predictable i.e., fry should have changed their preference by about 90° in a counter clockwise direction for a new preferred direction of about 168°; instead they shifted from about 257° to 292°, about 35° in a clockwise direction. Thus, the results of this study are inconclusive concerning the ability of chum salmon to orient to magnetic fields, but shifting the field did alter the fishes' orientation.

4.5.2 Magnetic field orientation by salmonid smolts

Johnson and Groot (1963) and Groot (1965) reported that emigrating sockeye smolts originating from stocks of selected inlet tributaries in Babine Lake, a large, complex lake with several arms, in British Columbia had to make a circuitous journey to arrive at the outlet. Because stocks of fish from certain inlets had to make several complex turns, their compass bearing to the outlet had to switch several times during the course of their smolt migrations. Johnson and Groot (1963) provided evidence to show that:

(1) Smolts were not able to orient to downstream water currents within the Lake because the portion of the water column they were travelling in was effected predominantly by random, **wind generated currents, and;**

(2) Smolts were unable to use to either shoreline landmarks or lake bottom topography for orientation since they swam in the middle of the water column out of sight of the lake's shoreline.

Hence, this study suggested that sockeye smolts from each inlet posses an internal guidance mechanism of directional preference(s) that helps them to locate this outlet. Apparently, at the time of their smolt migration, this genetic program provides the fish with both azimuth (compass bearing) and distances that need to be travelled before making a required turn. Further, the genetic program apparently contains sequential information because the stock from one inlet must make four turns, including one turn of nearly 180°, to reach the outlet.

Groot (1965) tested Babine Lake fish in a circular orientation tank removed from the lake. Fish in the tank oriented in the same direction(s) for the precise time periods as fish in the lake, i.e., they displayed correct orientation in the same sequence as if they had been in the Lake; indicating that the sequential pattern of direction preference is innately controlled.

Groot (1965) presented evidence that Babine Lake sockeye detected sun and polarized light compasses. However, smolts were still able to orient properly even when the test arena was covered, effectively blocking both the sun and polarized light. Groot concluded that an undetermined orientation system operates in the absence of these cues and referred to this component as "type x" orientation.

Quinn and Brannon (1982) tested Babine Lake sockeye smolts trapped near the outlet in a round orientation arena equipped with an electromagnetic coil that rotated the magnetic field 90° counter-clockwise from north. With a view of the sky, the smolts oriented generally towards the lake's outlet when the magnetic coil was switched either off or on. When the test apparatus was enclosed under opaque covers, the smolts displayed a bimodal distribution when the coil was switched off, orienting towards or away from the axis of the outlet. In a field rotated 90° counterclockwise smolts oriented 56° counterclockwise

from the axis of fish tested in the normal field. Since fish in covered tanks changed from roughly north-south to west-east preferences with a 90° change in the horizontal component of the magnetic field, this experiment indicates that sockeye smolts can orient to magnetic fields. Quinn and Brannon concluded that Groot's (1965) "type x" orientation may have been related to detection of electromagnetic cues.

Orientation of other species of Pacific salmon also appears to be influenced by magnetic cues. Taylor (1986) reported that 18 month old chinook salmon (*Oncorhynchus tschawytscha*) that had been transplanted in New Zealand, oriented at 270° in their rearing troughs. Individual fish transferred into a circular test arena equipped with a Hemholtz coil, exhibited a mean unimodel orientation of 264° with the coil switched off. A 90° clockwise shift in the electron magnetic field caused the fish to shift their direction preference about 90° in a clockwise direction to a bimodel preference of 354°/174°. After restoration of the normal magnetic field, the mean axis of orientation reverted to 274°/94°.

4.5.3 Magnetic field orientation by salmonid adults

Quinn (1980) reasoned that if juvenile salmon posses the ability to use magnetic cues in association with a genetic program of directional preference, than it is likely that salmon adults also have a similar capability. Quinn speculated that the highly patterned adult migrations in the ocean are cued at least in part by magnetic field perception. Adult salmon feeding in the North Pacific Ocean, migrate south of the Aleutian chain in a counter-clockwise gyre. At specific times of the year and at particular geographic locations specific stocks break out of this pattern and swim to the mouth of the home river (Neave 1964), suggesting that patterned migration routes are used by adults reminiscent of the patterns reported for smolts during their emigration from large, complex lakes (Groot 1965).

4.5.4 Some factors complicating celestial and magnetic field orientation in salmon fry.

Several studies indicate that salmon fry orientation can be influenced by factors other than a rigid genetically programmed directional preference, especially odors and water current velocity. Brannon (1972) reported that changing water source can reverse the directional preference of different stocks of sockeye salmon fry. When fry were tested in the same source water used for incubation, regardless of origin, they responded in a manner appropriate to their homestream, but when tested in water different from the water source used for incubation, they always showed a negative rheotaxis.

Raleigh (1971) experimenting with sockeye fry, found that different inlet water sources did not affect the direction of migration of inlet fry but did affect the proportion of outlet fry that moved upstream. Bodznick (1978) tested the water source preference of sockeye fry from two different populations (Lake Washington, WA and Chilko River, BC) in a two choice Y-maze, with Lake Washington water (lake water) and well water as the alternate choices. Lake Washington stock fry reared in lake water strongly preferred lake water over well water. In three tests groups 74.6%, 77.7%, and 93.9% preferred lake water compared to 3.4%, 0%, and 0.7% preference for well water and 25.3%, 22.3% and 5.4% exhibiting no preference. In contrast Lake Washington stock fry reared in well water preferred well water at rates of 34.4%, 39.3%, and 41.9% in the three tests. Well water reared fry preferred lake water at rates of 39.3%, 35.5%, and 35.6%, and did not respond at frequencies of 26.3%, 25.2%, and 22.5%. Lake Washington stock fry not incubated in either Lake Washington water or well water, but instead incubated in Cedar River (inlet to Lake Washington), exhibited an 82.3% preference for lake water, 0.9% preference for well water and did not respond of a rate of 16.8%. Chilko Lake stock fry preferred lake water at a frequency of 81.9%, well water at 3.2% and did not respond 14.8% of the time.

Bodznick argued that two separate factors account for the behavior preferences of sockeye fry-- an apparently innate preference for lake

water as compared to well water and an attraction for a recently experienced water source over foreign waters. Bodznick believed that water source preference plays an important role in the guidance of sockeye fry migration from their incubation stream to a nursery lake. Presumably presence of the odor evokes positive rheotaxis of fry in outlet streams, which swim upstream to reach the lake odor; whereas the absence of the odor evoke's negative rheotaxis in fry, which swim downstream until they encounter the odor.

Quinn and Groot (1984) reported that orientation of chum salmon fry in a circular test arena is influenced by subtle changes in water current. The arena contained a central inflow stand pipe and eight exit ports, which emptied into eight fry collection traps so that directional preference of fry could be ascertained. Lots of 200 fry were released at night (after sunset) in the central portion or the circular tank near the standpipe and the number of fry in each collection trap, as well as the number remaining the the circular tank, was determined the following morning (before sunrise). At current flows of about 6 liters/minute/exit port, 91% of 15,978 fry exhibited a strong preference to swim in a unimodal southerly direction (mean preference = 182°), an appropriate direction to carry them to saltwater from their homestream. About 9% remained in the circular tank. When current flow was reduced to 3 liters/minute/exit port, fry distribution was distinctly biomodal with about equal numbers of fish orienting in a southerly (194°) and northernly (14°) directions. Only about 66% of the 6,373 fry tested at the low flow rate moved out of the circular tank. Collectively, these results indicate that fry orientation is motivated by an interplay of several factors.

4.6 Potential reasons for failure of unsuccessful transplants.

Most unsuccessful transplants did not fail in the sense that there were no fish returning to the new site, but rather in the long-term failure to form self-sustaining stocks at the new location (Blackett 1979, Helle 1981, Horrall 1981). Horrall (1981) listed potential reasons for the failures which occur in stock transplants, including:

"(1) stocking in locations with unsuitable spawning or nursery areas;

(2) stocking from gene tically inappropriate donar stocks;

(3) stocking during a non-imprintable period in the species life history;

(4) stocking without taking into consideration the possibility that pheromones may be utilized for homing, and that none are available at the stocking location unless they are specially provided."

One interesting facet of salmonid transplantation is that fish stocked a long distance from their native stream to the transplant site generally home with greater precision to the adopted stream compared to those transplanted within the same river system. For example, introduction of West Coast (Columbia River) salmonids into Great Lakes tributaries has resulted in excellent returns to the transplant site (Scholz *et al.* 1975, 1978); whereas transplanting salmonid species into different tributaries on the West Coast has met with more limited success (reviewed by Ricker 1972). We suspect that this difference may be, in part, related to inherited versus imprinted (learned) aspects of the fishes' orientation systems.

It seems clear from the above discussion that salmonids possess two distinctive orientation mechanisms (See also Appendix I.) The first is an innate genetic program of directional preference cued by celestial (sun compass, polarized light) navigation, geomagnetic fields and, perhaps, conspecific pheromones. The second is an imprinted olfactory memory of the homestream water, including an odor bouquet of the organic components and possibly population specific pheromones emanating from the fish stock themselves. Thus, it appears that the salmonids possess redundancy mechanisms for relocating the home tributary. The genetic mechanism may function primarily in juvenile migrations and the open water phase of the adult migration, whereas the odor imprinting may

function primarily in the upstream migration of adults to natal spawning tributaries. Horrall (1981) argued that innate programs are important in adapting to local environments "*but the capacity for imprinting (learning) has the great advantage of being able to store far more complex objects of behavior than the genetic program* (*Mayr 1974*) ."

One explanation for the relatively poor success of transplantation within the same river drainage is that the genetic and imprinted orientation systems no longer match; which might confuse the fish, resulting in poor returns to the transplant site and increased straying. In contrast, when salmonids are transplanted a long distance, since their genetic program is not closely matched to the new environment, the fish must ignore their innate mechanism (which may be nonfunctional if cued by geomagnetic fields) and rely solely on imprinting to relocate the transplant site.

4.7 Recommendations for managing Lake Roosevelt kokanee hatcheries.

Based on the above discussion, we recommend the following actions be taken in developing a management plan for the Lake Roosevelt kokanee hatcheries.

4.7.1 Identification of the critical period for imprinting

In the past two spawning seasons (1990 and 1991), no kokanee were recovered in Sherman Creek from plants of approximately 850,000 kokanee per/year made from 1988 to 1990. In those years, eyed eggs from Lake Whatcom stock kokanee spawned in December and initially incubated at the Lake Whatcom Hatchery, were transferred to Ford hatchery in January. Fish were hatched and reared at Ford Hatchery, then stocked at **Sherman Creek as about 210 day old fry in July in an attempt to imprint** them to that site. A similar strategy has been employed at the Cabinet Gorge hatchery (Pend Orielle, ID). Fry are either transplanted to Granite Creek (at about 200 days old) or exposed to synthetic chemicals (from 170 to 200 days old) at the hatchery for imprinting. Returns of spawning adults to both locations have been poor (Paragamian *et al.* 1991).

The results of the present study indicate that the time of release (or time of exposure to synthetic chemicals) was not appropriate for imprinting. If thyroxine levels are an accurate guide for imprinting in kokanee, our results suggest that the critical period is at hatching (about 60 days old) or swimup (about 90 days old) stages. Thyroxine levels in fry older than about 160 days post-fertilization were so low that T_4 was virtually nondetecteable, so it unlikely that fish older than 160 days post-fertilization could be imprinted. Kokanee stocked into both Sherman Creek and Granite Creek probably spent the sensitive period in the fish hatchery and were thus unable to imprint to the stocking site. Kokanee exposed to morpholine at Cabinet Gorge fish hatchery were probably also past the sensitive period. Kokanee in both Lake Roosevelt and Lake Pend Orielle, are not released from hatcheries until late June through mid July to coincide with the time of increasing food resources in these reservoirs (Peone *et al.* 1990, Griffith and Scholz 1990). At Lake Roosevelt fish cannot be stocked at a earlier time because reservoir operations for flood control, power production, and water releases for anadromous fish passage reduce water retention time, which flush kokanee over Grand Coulee Dam and prevent establishment of the zooplankton community (primarily cladocerans) upon which kokanee prey (Beckman *et al.* 1985, Peone *et al.* 1990, Griffith and Scholz 1990) Therefore a method must be found that will allow kokanee to be retained in hatcheries until the annual reservoir drawdown is accomplished, but form an imprint to locations where the fish are transplanted and to which they must later home for egg collection.

Currently (1992), we are conducting studies to further delineate the critical period for imprinting in kokanee by exposing fish to synthetic chemicals at the Spokane Tribal Hatchery for brief periods commencing with recently fertilized eggs. Lots of 10,000 kokanee were exposed to either morpholine or phenethyl alcohol at each of the following stages (or times):

(1) Recently fertilized egg to eyed egg (l-30 days post fertilization);

(2) Eyed egg to hatch (30-60 days post fertilization);

(3) Time of hatch (about 55-65 days post-fertilization-- to coincide with the first peak of thyroid hormones);

(4) Hatch to swimup (60-90 days post fertilization);

(5) Time of swimup (85-95 days post fertilization-- to coincide with the second peak of thyroid hormones);

(6) Fry (90-120 days post fertilization);

(7) Fry (120-l 50 days, post fertilization);

(8) Fry (150-180 days post fertilization);

(9) Fry (150-210 days post fertilization);

(10) Fry (210-240 days post fertilization); and

(1 1) 16 to 18.5 month old fingerlings to coincide with the time of smoltification (March to Mid May of their second year).

Each lot was marked with a group specific fin clip plus a distinctive coded wire tag.

We plan to conduct two types of tests to determine which group(s) become imprinted to the odor. First, a small number of fish will be retained at the hatchery and brought into early spawning condition via administration of gonadotropic hormone (Hasler and Scholz 1983). These fish will be tested in a y-maze to determine if they can select an arm scented with their treatment odor. Second, most of the fish will be released into Lake Roosevelt at Sherman Creek to conduct a field test. During the spawning migration, morpholine will be metered into a fish ladder leading to the hatchery trap at Sherman Creek. Phenethyl alcohol will be metered into a trap installed in the Colville River, which is

located across the reservoir from Sherman Creek. The number of morpholine and phenthyl alcohol exposed fish from each lot homing to each trap will be determined. Results of this study should:

(1) Indicate if it will be possible to imprint fish to a synthetic chemical at the Spokane Tribal Hatchery and decoy the adult fish to the Sherman Creek Hatchery for egg collection;

(2) Identify the critical period for imprinting; and

(3) Confirm if thyroid hormone peaks are a useful indicator of imprinting time in kokanee.

We believe that until the time of imprinting is firmly established, it would make sense to transfer about 80,000 eyed eggs from either the Spokane Tribal Hatchery or Lake Whatcom Hatchery to Sherman Creek Hatchery. This recommendation is based on our best available evidence that the probable time of imprinting in kokanee is at hatching or swimup. The fry would then imprint directly to Sherman Creek water. Our suggested number of 80,000 eggs is based on the following calculations:

(1)	80,000	eggs
	x 0.8	egg to fry release survival based on kokanee survival rates reported by Spokane Tribal Hatchery and Ford Hatchery managers.
	= 64,000	fry for release into Lake Roosevelt
(2)	64,000	fry released into Lake Roosevelt
	x .30	fry to adult survival in Lake Roosevelt
	= 19,200	spawning adults
(3)	19,200	spawning adults
	x .5	female to male ratio
	9,60	adult female spawners
(4)	9,600	adult female spawners
	x 1670	average fecundity of Lake Roosevelt kokanee based on data collected by Peone et *al* (1990).
	16,032,000	eggs produced by spawning females

(5)16,032,000 eggs
x .80 egg to fry release survival
12,825,600 fry for release into Lake Roosevelt and Banks Lake.

The resulting 12.8 million fry is very close to the maximum production level of 13 million kokanee fry programmed for these hatcheries (8 million for release into Lake Roosevelt and 5 million for release into Banks Lake). Assuming that sufficient space and water supply is available at the hatcheries, it might be prudent to initially incubate 100,000, instead of 80,000, eggs; which would produce approximately 20 million eggs and 16 million fry for planting into Lake Roosevelt and Banks Lake. Although this is about 3 million fry over the target production, it would allow for a 20% margin of error at the front end of the stocking program; to account, for example, for a 20% loss of kokanee over Grand Coulee Dam.

4.7.2 Selection of a genetically appropriate donor stock

Returns of kokanee to egg collection sites in Lake Roosevelt tributaries could be improved by selection of a genetically appropriate donor stock. We have concern that Lake Whatcom fish may not provide the best genetic match for Sherman Creek because in Lake Whatcom these fish migrate in an eastwardly direction to relocate their home tributary, whereas in Lake Roosevelt the most likely migration route will be in a northernly and then westerly direction. Therefore, we believe that other stocks should be located to provide source fish that might be better matched for Lake Roosevelt.

Potential donor stocks should have the following characteristics and migratory tendencies:

(1) Donor stock fry should have an innate preference to migrate upstream to a nursery lake (i.e., they should be from an outlet spawning stock). The reason for this recommendation is that such a tendency might cause the fish to orient upstream in Lake Roosevelt, which would aid in keeping them away from Grand Coulee Dam;

(2) The donor stock should spawn in tributaries where they must orient at 90° (East) to 180° (South) to reach the nursery lake as fry and 270° (West) to 360° (North) to relocate their natal tributary as adults. This is because fry leaving the Sherman Creek site must initially travel in a 90° then 180" to reach the principle feeding areas Lake Roosevelt, which have been shown to be from the confluence of the Spokane River to the forebay of Grand Coulee Dam (Beckman *et al.* 1985, Peone *et al.* 1990; Griffith and Scholz 1990). Adults returning to Sherman Creek will have to travel at azimuths of 360° then 270° to relocate Sherman Creek; and

(3) The donor stock should have a marked tendency to not undergo smoltification. The reason for this recommendation is that drawdowns in Lake Roosevelt frequently coincide with smolt migrations of anadromous Columbia River salmon (in part because water stored in Lake Roosevelt forms part of the water budget used to flush salmon to the ocean). Water retention time in Lake Roosevelt (which is typically 50-80 days) are reduced to 15-30 days during periods of drawdown, so the reservoir behaves more like a river than a lake during drawdown. If the donor stock has a tendency toward downstream smolt migration, they will likely be lost over Grand Coulee Dam.

We recommend that the Spokane Tribal Hatchery and WDW Sherman Creek hatchery mangers screen other potential kokanee donor stocks in Washington, Idaho, Montana, Oregon, Colorado and British Columbia utilizing the criteria listed above and attempt to obtain eggs from candidate stocks. Fish from candidate stocks should be marked with distinctive coded wire tags and adipose fin clips before release into Lake Roosevelt. Records should be kept of:

(1) The estimated harvest rates of each stock;

(2) The number of each stock returning to egg collection sites; and

(3) The number recovered below Grand Coulee Dam in Rufus Woods Reservoir and at, Rock Island Dam and McNary Dam counting facilities.

(4) Each stock should also be tested to determine if smoltification occurs. Test should be conducted to determine salinity preference, salinity tolerance, osmoregulatory capability and migratory tendency to orient downstream.

One potential donor stock would be a native stock of kokanee that spawn in Big Sheep Creek, a tributary that flows into the Columbia River just south of the international border. Big Sheep Creek is located about 65 km north of Sherman Creek, and, like Sherman Creek, flows into the Columbia River from the west. Therefore, as fry and adults these fish already migrate in directions to reach prime feeding areas and relocate their natal tributary, which would also be appropriate for individuals of this stock transplanted to the Sherman Creek Hatchery. Apparently, these fish do not have a pronounced tendency to be lost over Grand Coulee Dam since local residents report that they have spawned in Big Sheep Creek since the reservoir was closed (51 years ago), which indicates that a proportion of these fish has successfully resided in Lake Roosevelt.

An alternative approach would be to intentionally find a poorly matched donor stock from outside the Columbia Basin in an attempt to force fish to ignore innate directional preferences and rely solely on imprinted cues for relocating the home tributary. (See section 4.6 for rationale.) Stocks from a different latitude, e.g., Fraser or Skeena River, British Columbia or Alaskan stocks might be appropriate.

4.7.3 Provision for population specific pheromones that may be utilized for homing.

Provision should be made to ensure that population specific pheromones are present at the Sherman Creek Hatchery at the time spawning adults are expected to return. Therefore, we recommend that the Sherman Creek Hatchery retain about 10,000 Lake Whatcom stock kokanee year round to provide a source of pheromones in case the fish either form an imprint to, or posses a genetic memory of, pheromones.

4.7.4 Stocking at locations with suitable nursery areas.

Lake Roosevelt appears to contain suitable fry nursery areas and adult habitat for kokanee (Peone *et al.* 1990). It has been calculated that zooplankton levels are normally sufficient to support 5.9 million adult kokanee with an average weight of 0.5 kg (Jagielo 1984; Beckman *et al.* 1985, Scholz *et al.* 1986; Peone *et al.* 1990, Griffith and Scholz 1990). Stocking of 8 million hatchery raised kokanee fry is anticipated to produce about 3 million adults with an average weight of 1 .O kg (Scholz *et al.* 1986). However, we are concerned that future reservoir operations may be different than historical operation, owing in part to application of the federal Endangered Species Act to Snake River salmon. During the past decade, when zooplankton abundance estimates were made, drawdown occurred primarily from late March to mid May and refill occurred from mid-May to late June. Full pool was normally achieved between June 1 and July 1. This provided adequate water retention times after June 1 to retain and rebuild zooplankton populations, so the growing season lasted from about mid-June to October.

It is anticipated that future reservoir operations will be modified to better protect threatened and endangered species of Snake River salmon. The plan is to store water through April at Grand Coulee and release it in May and June to improve flows for passage of anadromous salmonids. The reservoir is not expected to refill until the end of July, which will reduce water retention times, prevent rebuilding of zooplankton populations until later in the year and reduce the length of the growing season by 1 .0 to 1.5

months. Kokanee growth rates and fecundity will likely be reduced, and loss over Grand Coulee Dam will be increased. Given these uncertainties, we recommend that about 10,000 kokanee be retained at each of the Lake Roosevelt Hatcheries to provide brood fish for future egg collection, in case insufficient numbers of spawners return to egg collection sites or kokanee fecundity (Peone *et al*. 1990) is lower than anticipated owing to poor growth rates.

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APPENDIX I.

NOTES ON OPEN WATER AND UPSTREAM PHASES OF ADULT SALMON MIGRATION

In the preparation of this report, several references were located that shed light on the orientation mechanism used for open water migrations of salmonids and their transition to the upstream migration stage. Quinn *et al.* (1989) tracked adult sockeye salmon returning to the Fraser River, British Columbia using depth sensing ultrasonic transmitters. Fish were collected on the northern approach route in Queen Charlotte strait, Johnstone Strait and Discovery Passage at the north end of Vancouver Island. Most of the fish swam southeast in a compass direction appropriate to reach the Fraser River. However, a substantial number of fish swam in the opposite direction (northwest--back toward to the open ocean).

In the north region near the entrance of the straits (about 200 km from the Fraser River) there were approximately equal numbers of observation of fish swimming homeward (n = 32) and away from home (n = 28). In the southern region (in the Strait of Georgia-- about 50-100 km from the Fraser River) most fish swam toward home river (n = 40) compared to only six swimming away from home. Fish in the north region apparently drifted on tidal currents, and occasionally swam into the flood currents away from the Fraser River, whereas those in the south region held position on the flood and stemed the ebb. [This type of behavior was also reported for adult sockeye salmon migrating towards the Skenna River British Columbia (Madison *et al.* 1972), sockeye and pink salmon migrating to the Fraser River on the southern approach route through the Strait of Juan de Fuca and San Juan Islands (Stasko *et al.* 1973, 1976), and chum salmon migrating in Outsuchi Bay, Japan (Scholz *et al.* 1972).] The difference between bimodal orientation of sockeye in the north and the unimodel orientation in the Strait of Georgia (Quinn *et a/.* 1989) may reflect a switch from orientation mechanisms used in open water to use of olfactory information as the fish approach their home river.

In the paper by Quinn *et al.* (1989), fish making progress in a homeward direction swam faster (79.2 cm/sec) than fish moving in the opposite direction (55.4 cm/sec). The fish did not follow the shoreline but instead remained in open water. Encounters with shorelines or islands apparently confused the fish, which first milled in the vicinity of the underwater shelf, then swam back to open water and resumed their initial course bearing.

Quinn *et al.* (1989) also reported that tracked sockeye appeared to frequently swimup and down in the water column. Doving *et al.* (1985) noted that Atlantic salmon swim up and down through the halocline and postulated that the fish were orienting to tidal currents by comparing odors in this region of steepest gradients. In contrast, anosmic fish did not confine their vertical movements to the vicinity of the halocline (Doving *et al.* 1985). Quinn *et al.* (1989) reported that vertical movements of individual sockeye tracked in relation to the thermocline (which, in the Strait of Georgia, corresponds to the halocline) indicated that the fish often swam near the depth of steepest gradients, but that many exceptions were noted.

Based on Monte Carlo computer simulation techniques, Saila and Shappy (1963) concluded migration of Pacific Salmon from open ocean feeding areas to the coastal vicinity of their home river is accomplished largely through random movement, with little homeward directed orientation. Their model was based on five assumptions:

(1) Swimming speed was assumed to be 4.6 km/h (110 km/day) at sea and 2.3 km/h (55 km/day) in coastal waters;

(2) Fish reached the appropriate coastline in 175 days, which was termed the "limit of endurance";

(3) The start point of the migration was placed 1200 nautical **miles (2224 km) due west of their goal;**

(4) Fish could relocate their home river if they arrived along the coast within 74 km of its mouth; and

(5) The proportion of salmon successfully homing to the vicinity of their home river was 10 to 20%.

Quinn and Groot (1984) determined the validity of these assumptions by comparing them to new information collected from tagging investigations. Quinn and Groot reported that assumptions concerning swimming speed, duration of the migration and return success appeared to be incorrect. Tagging studies indicated that sockeye salmon travel 40-60 km/day (not 110 km/day) in the open ocean, which is close to their theoretically most efficient speeds of 2.6 km/h. Sonic tracking studies indicate sockeye travel at about 2 km/h in coastal waters (Madison *et al.* 1972, Stasko et al. 1973, 1976) Quinn and Groot argued that, *"At efficient speeds of 2-3 km/h, it is clear that virtually no salmon would return at the simulated level of orientation [predicted by Saila and Shappy]. Thus, at slower, more realistic swimming speeds, more precise orientation is required to simulate return success."* In terms of the endurance assumption, tagging and tracking studies indicate that sockeye salmon can migrate long distances from the open ocean to coastal areas in far shorter times than the 175 days projected by Saila and Shappy. Neave (1964) reported that tagged sockeye travelled 1200 and 1600 km from the high seas to coastal areas in 27 and 35 days respectively. Quinn and Groot (1984) calculated that *"these travel times would be accomplished if the fish continuously swam 1.85 and 1.90 km/h respectively* ***on straight lines*** *(discounting currents)* ***for the entire journey"*** (emphasis added). Another sockeye salmon, tagged on the high seas and recovered off the mouth of the Columbia, River travelled 2200 km in 57 days (or 1.6 km/h assuming a straight line) (Quinn and Groot 1984). Thus, it is clear that the assumption of a 175 day endurance period is erroneous. These rapid migrations require precise orientation.

Quinn and Groot also believed that Saila and Shappy's estimate of 10-20% return success was incorrect owing primarily to poor interpretations of tag-recovery data. Quinn and Groot (1984) argued that, *"These errors resulted in substantially underestimating the extent of salmon homeward orientation. Thus, contrary to the original conclusion,*

any hypothesis concerning the high seas migrations of salmon must explain the strong orientation of these fishes in the open ocean."

We believe that Saila and Shappy's fourth assumption is also incorrect and that salmon must encounter the coast closer than 74 km in order to successfully locate the home river. Instead, we believe that fish must home more precisely, to within about 48 km of their home river. In experiments with Lake Michigan salmon artificially imprinted with synthetic chemicals, Scholz *et al.* (1975) reported that morpholine-exposed coho salmon stocked 13 km from a simulated homestream homed there but that those stocked 64 km away did not. Sonic tracking experiments indicated that fish stocked 64 km from the simulated homestream did not home to that site even if they encountered the stream during their migration. In the tracking experiments, salmon collected near their release site were displaced about 70 km along the Lake Michigan shoreline so that they would have to pass the simulated homestream in their migration back to the release site. All of the tracked fish (n = 14), migrated in a compass direction back toward the stocking site, following the Lake Michigan shoreline. Upon encountering the morpholine scented stream, the fish paused in their migration and some actually entered the stream. The fish remained in the vicinity of the simulated homestream for l-4 hours, but eventually all of the fish, even those that briefly entered the morpholine stream, continued their migration to the stocking site. These results indicate that the salmon return to a generalized region near the location where they were stocked before they will remain at a stream scented with an imprinting chemical.

Recoveries of fish in the Lake Michigan experiments indicated that coho salmon and steelhead trout will migrate to streams within about a 48 km radius of their original stocking location (Scholz *et al.* 1975). Carlin (1968) determined that Atlantic salmon migrated into streams within a 40 km radius of their release site, so a distance of 40-48 km may be equivalent to the area which a fish can search when migrating to its natal river, not the 74 km suggested by Saila and Shappy.

We interpret these results to mean that there are two phases to the spawning migration of salmonids-- an open water phase, during which the

fish relay primarily on celestial and magnetic cues coupled with a genetic program of directional preference (See section 4.5 of this report), and an upstream phase during which fish rely primarily on imprinted olfactory cues to locate the home river and natal tributary (See section 4.3 of this report), During the first stage, fish return to a generalized region within about 48 km of the location where they were originally stocked (in the case of stocked fish) or from the mouth of their home river (in the case of naturally migrating fish). Tracking experiments indicated that the fish must return to this region before they will enter and remain in a specific stream. Only after a fish arrives in this area will it search for a stream scented with the appropriate odor.

APPENDIX II

FLOWCHART OF PROCEDURE USED FOR PERFORMING RADIOIMMUNOASSAY ON EXTRACTED T_4 SAMPLES.'

(1) This T_4 assay procedure utilizes a coat-a-count T_4 RIA kit purchased from Diagnostic Products Corporation, Los Angeles, CA. The kit contains:

(a) 12 x 75 mm polypropylene test tubes coated with an antibody (Ab) that contains T_4 receptors;

(b) Radioactive T_4 [^{125}-T_4];

(c) T_4 calibrators (or standard curve), i.e., six standards containing 0, 1, 4, 10, 16 or 24 ng/dl T_4; and

(d) Three quality control samples of unknown low, medium and high concentration;

(e) Foam decanting rack.

In addition to the kit contents, the following items are needed.

(a) 25 µl eppendorf pipette with disposable tips;

(b) 1 ml pipette with disposable 1 ml tips;

(c) Water bath (37 °C);

(d) About 24 uncoated (without Ab) polypropylene test tubes 12 x 75 mm;

(e) Vortexer;

(f) Assorted test tube racks;

(g) Gamma counter;

(h) Supplies for handling radioactivity i.e., lab coat, disposable plastic gloves, absorbant paper with plastic back, liquid and solid radioactive waste containers, decontaminate, spill kit, and lab survey meter.

Make sure you have a sufficient quantity of all materials prior to initiating the assay. The assay must be performed in the radioisotope laboratory.

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[1]You must pass a test and be certified by the EWU Radiation Safety Officer before can handle radioisotopes. All radioisotope work must be performed in the certified radioisotope laboratories (Room 1862 and Room 188). Food or beverages are not allowed in these laboratories. Wear plastic gloves and protective clothing when handling radioisotopes.

(2) Perform baseline thyroid scan on yourself prior to handling any radioactivity. Perform a baseline "wipe test" prior to opening and handling radioactive containers. Turn on and program the gamma counter.

Turn on the waterbath and adjust to 37°C. Vortex each T_4 calibrator and ^{125}I-T_4 container for 10 seconds.

(3) Label assay tubes:

(a) Label Ab coated tubes in duplicate with each calibrator concentration: i.e., 12 tubes total, two each with 0, 1, 4, 10, 16 and 24 ng/dl. The 0 tube is the MB (maximum binding) tube since it contains no non-radioactive T_4 and will therefore bind the maximum amount of radioactive T_4 .

(b) label Ab coated tube in duplicate for each unknown kokanee sample, each unknown quality control sample, and interassay pool (IAP) samples. The IAP sample allows for comparison between assays performed on different dates.

(c) Label plain (uncoated) TCT (total count) tubes and two plain (uncoated) NSB (nonspecific binding) tubes in duplicate. NSB refers to radioactivity that sticks to tubes but is not bound to Ab. This amount is subtracted from each tube in the assay.

(d) Label 20 plain (uncoated) tubes 1.0 ml and 25 µl in replicates of 10 each.

(4) Pipet 25 µl of the 0 calibrator into each NSB and 0 Ab coated tubes. Pipet 25 µl of each remaining calibrator, unknown kokanee samples, unknown quality control sample and IAP samples into duplicate tubes. Use the same pipet tip for each duplicate sample but discard the tip before proceeding to a new sample. Pipet directly to the bottom of the tube because the Ab is coated on the bottom of the tube.

(5) Add 1 .O ml ^{125}I-T_4 to each Ab coated tube and to the plain (uncoated) tubes marked TCT.

(6) Vortex each tube for 5 sec.

(7) Incubate racks of test tubes in waterbath for 1 hour @ 37°C. [Unknown kokanee sample T_4, calibrator T_4, or unknown quality control sample T_4 (i.e., non-radioactive T_4) and ^{125}I-T_4 compete for binding sites on the Ab. They bind in proportion to their relative concentrations. This is a reversible binding reaction. Equilibrium is achieved in 1 hour, at which point a portion of both non-radioactive

T_4 and ^{125}I-T_4 will be bound to the Ab (bound fraction) coated on the bottom of the tube and a portion of both non-radioactive T_4 ^{125}I-T_4 will not be bound. The nonbound (free fraction) will remain in the liquid.

(8) Remove racks from waterbath. Remove TCT tubes and set aside. Decant free portion from remaining tubes. Pour liquid into liquid radioactive waste container. Place tubes (except for TCT tubes) into foam decanting rack. Turn the rack upside down on absorbent plastic backed paper and allow them to drain for two minutes, then strike the tubes sharply on the absorbent paper several times to shake off all residual droplets. **Removing all visible moisture will greatly enhance precision.**

(9) Place each tube in the proper order in counting racks and count for 1 minute in the gamma counter. (Cobra QC Model 85002 Autogamma Counter, Packard Instrument Company).

[Note: A computer program, contained in the counter, automatically subtracts NSB from each tube, determines the relative % bound for each standard concentration and plots the standard curve of % bound -v- standard concentrations. The program then examines the % bound for each unknown sample and interpolates the concentration of the sample from the standard curve graph. **For this program to work properly it is important that the TCT, NSB, MB, standard curve samples, unknown quality control samples and unknown kokanee samples be placed in the proper order in counting racks.** The proper order can be determined by entering the number of the protocol for a program named Kokanee T_4-RIA on the keyboard. Then press the function key for "tube display" (F7). The counter's computer screen will display the tubes in proper sequence. Make sure samples are placed into counter racks in this sequence.]

(10) Pipet 25 µl of ^{125}I-T_4 into each of the ten plain uncoated tubes marked 25 µl. Only the person who pipetted the 25 µl calibrators and unknown kokanee samples should do this. Pipet 1 .O ml of ^{125}I-T_4 into each of the ten plain uncoated tubes marked 1.0 ml. Only the person who pipetted the 1 .O ml of ^{125}I-T_4 into each assay tube should do this. Place these 20 tubes in a counting rack and count the amount of radioactivity in each tube. **This is a quality control**

procedure to determine the level of accuracy achieved by each individual pipettor in replicating the volume pipetted.

(1 1) Repeat wipe test to be sure that you have not contaminated any laboratory space or equipment. Between 8 and 24 hours after you performed the assay, repeat the thyroid scan on yourself.