ARTIFICIAL IMPRINTING OF LAKE ROOSEVELT KOKANEE SALMON (ONCORHYNCHUS NERKA) WITH SYNTHETIC CHEMICALS: MEASUREMENT OF THYROXINE CONTENT AS AN INDICATOR OF THE SENSITIVE PERIOD FOR IMPRINTING TO OLFACTORY CUES

ANNUAL REPORT 1992

Prepared by:

Allan T. Scholz
Ronald J. White
Mary Beth Tilson
Stacy A, Horton

Upper Columbia United Tribes Fisheries Research Center
Eastern Washington University
Department of Biology
Cheney, WA 99004

Prepared for:

U.S. Department of Energy
Bonneville Power Administration
Division of Fiih and Wildlife
P-0. Box 3621
Portland, OR 97283-3621

Project Number 88-63
Contract Number DE-B 179-88BP9 18 19

SEPTEMBER 1993

ABSTRACT

In 1991, we intiated studies to determine the critical period for thyroxine-induced olfactory imprinting in kokanee salmon. In our preliminary investigation we found that thyroxine [T_4] levels of Lake Whatcom stock, 1990 year class, kokanee were relatively high in eggs and alevins as compared to post-swimup fry, and peaked at hatch and swimup. Here we report on follow-up studies conducted in 1992 designed to determine if our initial results could be replicated. Additionally, in 1992, we initiated experiments to determine if kokanee could be imprinted to synthethic chemicals--morpholine and phenethyl alcohol-- at different life stages.

In 1991, whole body thyroxine content [T_4] was measured in 460 Lake Whatcom stock kokanee and 480 Lake Pend Orielle (Cabinet Gorge) stock kokanee to indicate the critical period for imprinting. Lots of 20 kokanee eggs, alevins and fry from both stocks, reared at the Spokane Tribal hatchery, were collected at weekly intervals from November 1991 to August 1992 and assayed for T_4 content by radioimmunoassay. T_4 levels were monitored in Lake Whatcom stock, 1991 year class fish, from eyed egg (33 days post-fertilization) to fry (248 days post-fertilization) stages. T_4 concentration (± SEM) in eggs was 6.7 ± 1.3 ng/g body weight. T_4 peaked on the day of hatch at 13.1 ± 2.5 ng/g body weight, then declined to 10.3 ± 1.1 ng/g body weight in recently post-hatch alevins. T_4 peaked again at 22.1 ± 5.2 ng/g body weight during swimup, then steadily decreased to about 1 .0 ng/g body weight in 176-248 day old fry.

T_4 levels were monitored in Lake Pend Orielle stock, 1991 year class, fish from the day of fertilization (day 0) to 225 days post-fertilization. T_4 content of eggs was 9.5 ± 1.7 ng/g body weight and peaked on the day of hatch (day 53 post-fertilization) at 24.2 ± 4.5 ng/g body weight. After declining to 13.0 ± 2.9 ng/g body weight on day 81 post-fertilization, T_4 peaked a second time during swimup (88-95 days post-fertilization) at 24.3 ± 3.8 ng/g body weight. After swimup, T_4 concentration steadily declined to about 0.6 ng/g body weight in 225 day old post-fertilization fry. Thus, results of our 1992 investigations were consistent with our preliminary 1991 study. In all cases: (1) T_4 concentration was relatively high in eggs and alevins as compared to older fry; and (2) T_4 peaks occurred at hatch and swimup.

Blood serum T_4 concentration was measured in 9 month to 21 month-old Lake Whatcom stock, 1990 year class, kokanee from July 1991 to August 1992. T_4 concentrations were low in summer, peaked slightly in October, were low in early winter, then peaked several times between January and May 1992. Thus, the 1990 year class Lake Whatcom kokanee evidenced high T_4 activity from egg to swimup stages in their first year and in the winter and spring of their second year of life. The fish appeared to undergo smolt transformation between 16-18 months old.

In 1992, Lake Whatcom (1991 cohort) kokanee were exposed to synthethic chemicals-- 1,072,OOO to morpholine and 1,117,OOO to phenethyl alcohol-- at different life history stages: (1) eye to hatch; (2) hatch; (3) hatch to swimup; (4) swimup; and (5) post-swimup fry (in February, March, April and May-June). Additionally, Lake Whatcom (1990 cohort) kokanee were exposed to synthethic chemicals-- 36,000 to morpholine and 51,600 to phenethyl alcohol-- at age 16-18 months. Most of these fish were marked and released in Lake Roosevelt in July and August 1992 as part of a field test. A portion of the fish from each group was retained at the Spokane Tribal hatchery until August-October 1993, when behavioral tests will be conducted to determine if the fish imprinted to their exposure odor.

ACKNOWLEDGEMENTS

We gratefully acknowledge Tim Peone (Manager, Spokane Tribal Hatchery) for advice and coordination in all aspects of this study. Spokane Tribal hatchery personnel Del Brown, James Andrews Jayne Abrahamson and Darla Ford assisted with the rearing and collection of fish for this study. We thank all of the individuals who assisted with tagging kokanee. Coded wire tagging and fin clipping was accomplished by a Spokane Tribal Hatchery tagging crew composed of Diane Abrahamson, Eugene Abrahamson, Cyndi Day, Barbara Gunter, Shari Smallwood, Vina Timmons and Jo-Anne Wynne. Additional tagging of kokanee was accomplished by the Spokane Tribal Lake Roosevelt Monitoring Program crew composed of Janelle Griffith, Amy McDowell, Milo Thatcher, Rudy Peone, Sam Abrahamson, William Matt Jr., Charles Gallagher, Henry Etue Ill, Hunters High School FFA Students, and Dirk Christianson. Lori Hill (Upper Columbia United Tribes Fisheries Center) typed the manuscript. July MacMillion, Eastern Washington University, Instructional Media Center prepared the illustrations. Special thanks go to Fred Holm (Bonneville Power Administration, Division of Fish and Wildlife), who served as BPA project manager and critically reviewed this report.

This project was supported by a contract from the U.S. Department of Energy, Bonneville Power Administration (BPA), Division of Fish and Wildlife, Project No. 88-63, Contract No. DE-8179-888. Additional financial support for this project was provided by a grant from the U.S. Department of Interior, Bureau of Indian Affairs to the Upper Columbia Untied Tribes (UCUT) to fund the operation of the UCUT Fisheries Research Center at Eastern Washington University, Department of Biology.

[Disclaimer. Use of brand names in this report does not imply endorsement by BPA, Eastern Washington University, Upper Columbia United Tribes or the authors.]

TABLE OF CONTENTS

1 .0 INTRODUCTION

In 1991, we initiated investigations to determine the critical period for thyroid hormone-induced olfactory imprinting in kokanee salmon (Scholz et *a/.* 1992). The practical application of this investigation was to provide information that will improve homing of hatchery-reared kokanee back to release sites for egg collection. The aim is to enhance the operation of Lake Roosevelt kokanee hatcheries constructed by Bonneville Power Administration (BPA) as partial mitigation for the loss of anadromous salmonids caused by the construction of Grand Coulee Dam (NPPC 1987).

Two hatcheries were constructed: a production hatchery on the Spokane Indian Reservation, which has a spring water supply at constant temperature that is ideal for incubating and rearing kokanee, and an adult trapping facility operated by the Washington Department of Wildlife (WDW) at Sherman Creek, which will be used as an imprinting site and egg collection facility. The operation of the two hatcheries is coordinated by a steering committee composed of representatives of the Spokane Tribe, WDW, Colville Confederated Tribes and BPA. The hatcheries are capable of producing 8 million kokanee for outplanting into Lake Roosevelt.

At present, only about 2 million eggs are reared at the Spokane Tribal Kokanee Hatchery owing to a regional shortage of kokanee eggs. The egg source is from the Washington Department of Wildlife Lake Whatcom Hatchery located in Bellingham, WA. The Lake Roosevelt Management plan (Scholz et *al.* 1986) indicated that the Lake Roosevelt Hatcheries will be eventually supplied with eggs collected from adults outplanted at the WDW Hatchery located at Sherman Creek and at Little Falls Dam. Improved homing of adults back to egg collection sites should result in an increased egg take, so that the hatcheries can be operated at optimal efficiency and BPA ratepayers receive the maximum benefit for their investment.

Failure of kokanee outplanted as 6-8 month old fry to home to release sites as adults at both Lake Roosevelt and Pend Orielle Lake (planted from Cabinet Gorge Hatchery) have been noted (Scholz et *al.* 1992; Paragamian et *al.* 1991). We suspect that the reason for these failures is that either the fish were stocked at an inappropriate time for imprinting, or that they imprinted to the hatchery water supply at an early (egg or alevin) life history stage. Hence, the present study was conducted to better define the critical period for imprinting in kokanee.

1.1 Thyroid hormone-induced olfactory imprinting in salmonids.

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 16 month coho salmon and steelhead trout exposed to synthetic chemicals -- morpholine (5×10^{-5} mg/l) and phenethyl alcohol (5×10^{-3} mg/l) -- during the smolt stage, were attracted as 3 year old adults into rivers scented with the appropriate chemical with 92-96% accuracy (Scholz et a/. 1975, 1976, 1978a, 1978b; Cooper and Scholz 1976; Hasler et al. 1978; Johnsen and Hasler 1980). 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 and formed a permanent memory of the synthetic chemicals, then used this information as a cue for homing.

It seems likely that salmon with this ability would use it with the organic odor bouquet of natural water supplies. This idea was corroborated by Jensen and Duncan (1971) who transplanted marked, presmolt coho salmon from Leavenworth Fish Hatchery located on Icicle Creek, a tributary of the Wenatchee River, WA, to a spring-fed fish-holding facility located near Ice Harbor Dam on the Snake River. The fish were 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 trap when river water was pumped, but 399 fish were captured when spring water was pumped. These data indicate that the fish formed a permanent attraction for the spring water that they were exposed to for two days as smolts.

Olfactory imprinting in coho salmon *(Oncorhynchus kisutch)* and steelhead trout *(Oncorhynchus mykiss)* is activated by thyroid hormone surges that occur during the smolt stage (Scholz 1980; Hasler and Scholz 1983). Hasler and Scholz (1983) reported that presmolt coho salmon exposed to morpholine and phenethyl alcohol in February, when concentrations of thyroid hormones were at basal levels, did not imprint to the chemicals; whereas fish exposed to the chemicals in mid-April, during a

thyroid surge, did become imprinted to them. Moreover, fish injected with thyroid stimulating hormone (TSH) in February, and simultaneously exposed to morpholine and phenethyl alcohol, imprinted to the chemicals whereas control fish injected with a saline placebo did not imprint to the chemicals. These results indicated that thryoid hormones induced the chemical imprinting process because T_4 concentrations were observed to rise shortly before imprinting occurred in natural smolts. Additionally, TSH injections stimulated a T_4 surge that was comparable in concentration to the surge observed in natural smolts, but at an earlier time than normal. Fish exposed to the synthethic chemicals under these conditions became imprinted, whereas saline injected control fish did not experience a T_4 surge at the earlier time and did not imprint to the chemicals. The mechanism of action appears to require binding of thyroid hormones in brain cell nuclei (Scholz et a/. 1985; White et al. 1991) which stimulates transcription of genes that code for nerve growth factor (NGF) proteins. Activation of NGF causes neuron differentiation and wires a pattern of neuron circuitry that allows for the permanent storage of the imprinted olfactory memory (Lanier 1987, Scholz et *al.* 1985, 1992).

Some species of salmonids (e.g., pink -- *Oncorhynchus gorbuscha,* chum -- *Oncorhynchus keta,* and kokanee -- *Oncorhynchus nerka*) leave their homestream immediately after emergence from redds at the swim-up stage. Therefore, they must necessarily imprint during the egg or alevin stage. Thyroid hormone levels were noted to increase at the time of complete yolk sac reabsorption in pink (Sullivan et *a/.* 1983) and chum (Tagawa and Hirano 1987, 1989), as well as at hatching and swim-up in kokanee salmon (Scholz et *a/.* 1992). The implication of these studies is that thyroid hormones may be sufficiently high in eggs or newly hatched larvae to stimulate olfactory imprinting at those developmental stages.

1.2 Summary of 1991 T_4 concentration investigations in Lake Whatcom kokanee.

In 1991, we initiated investigations to determine the ontogenetic pattern of thyroxine fluctuations in kokanee eggs, alevins and fry to determine the critical period for imprinting. Thyroxine concentrations $[T_4]$ were measured in 487 Lake Whatcom stock and 70 wild Lake Roosevelt stock kokanee salmon. Eggs, alevins and fry, reared at the Spokane Tribal 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 whole body 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 1.25 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. If T_4 levels are an accurate guide for imprinting in kokanee, our preliminary results suggested that the critical period for imprinting in kokanee is at hatching or swim-up stages.

1.3 Objectives of 1992 investigations.

The objective of investigations conducted in 1992 included:

(1) Repeat the thyroid hormone study in Lake Whatcom stock kokanee to determine if our preliminary results could be replicated;

(2) Conduct a thyroid hormone study of Lake Pend Orielle kokanee from Cabinet Gorge hatchery to determine if the ontogenetic pattern of thyroid fluctuations observed in 1991 is a general pattern or stock specific pattern in kokanee; and

(3) Determine if kokanee salmon can be imprinted to synthethic chemicals. In this experiment kokanee salmon were exposed to either morpholine or phenethyl alcohol at different life stages-- fertilization to eyed egg, eyed egg to hatch, hatch, hatch to swimup, swimup, swimup to 1 month post swimup, fry (in February, March, April and May-July), and fingerlings (16-18 months old)-- so that the critical period for imprinting could be determined. Fish from each lot were tagged with group specific coded wire tags that identified the particular developmental stage when the fish was exposed to the synthethic chemical and odor treatment. Most of the

fish from each experimental group were stocked in Lake Roosevelt in July and August 1992. During the spawning migration of these fish in 1993 and 1994, morpholine will be metered into the Sherman Creek fish trap and phenethyl alcohol will be metered into a trap installed at a different site. The number of morpholine and phenethyl alcohol exposed fish returning to each trap and the number straying to other sites in Lake Roosevelt will be determined by conducting trapping and electrofishing operations. A small number of experimental fish from each group were retained in the hatchery until August 1993, when they will be behaviorally tested to determine if they recognized the odor they were exposed to at a particular stage in development. We will then determine if fish from stages exhibiting a positive response is correllated with high thyroid hormone concentration. In this report, we provide the details of the odor exposure portion of these experiments.

2.0 METHODS

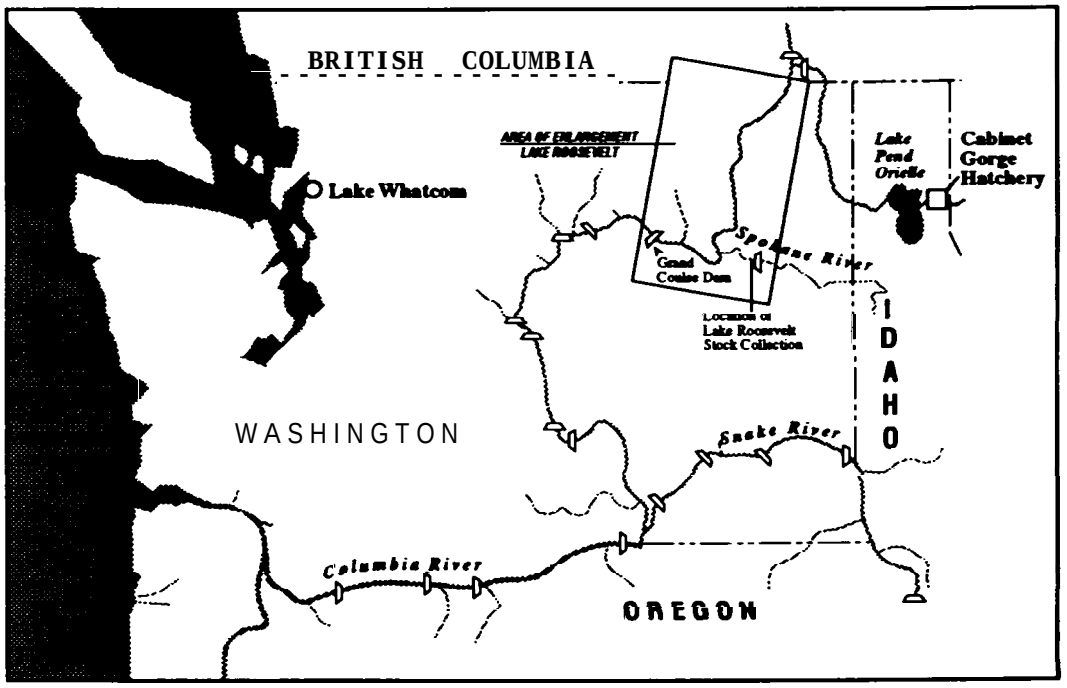
2.1 Fish stocks, rearing conditions and sample sizes.

Kokanee salmon were obtained from Lake Whatcom Hatchery (operated by Washington Department of Wildlife-- WDW) located near Bellingham, WA and Cabinet Gorge Hatchery (operated by Idaho Department of Fish and Game) located on the Clark Fork River near its confluence with Pend Orielle Lake (Fig. 1). Both stocks were reared at the Spokane Tribal Kokanee Hatchery located at Metamooteles Springs on the Spokane Indian Reservation (Fig. 1).

Thyroxine (T_4) levels were monitored in year two classes (cohorts) of Lake Whatcom stock kokanee. Zero-aged kokanee embryos were fertilized on 1 1-4-91 at Lake Whatcom Hatchery and transferred as eyed eggs to the Spokane Tribal Hatchery on 12-6-91. Mean whole body T_4 content ± SEM (ng/g body weight) was determined in 20 fish from this group at approximately weekly intervals from eyed egg commencing on 12-7-91 until 134 days post-fertilization, then at bi-weekly intervals through the time they were released in Lake Roosevelt in July and August 1992.

One year old (1 +) kokanee were spawned in December 1990 at Lake Whatcom Hatchery, transferred as eyed eggs to Ford Hatchery (WDW) in January 1991, and transferred again to the Spokane Tribal Hatchery in March 1991, where they were reared until most of them were released into Lake Roosevelt in July and August 1991. About 100,000 of these fish were retained at the Spokane Tribal Hatchery from July 1991 to July 1992 for an experiment to determine if release of 1.5 year old fingerlings instead of 8 month old fry could improve retention in Lake Roosevelt, angler harvest and return to stocking sites. Mean blood serum T_4 concentration ± SD (pg/ml plasma) was determined for 10-20 fish from this group at approximately bi-weekly intervals from August 1991 to July 1992. Whole body T_4 concentrations had previously been determined for these fish when they were eggs, alevins and fry from December 1990 to August 1991 (see Scholz et *a/.* 1992 and introduction of this report for details). These fish were tested at age I+ because we wanted to determine if they experience a thyroid surge at age 16-l 8 months, as is typical of many species of salmonids that smolt and migrate to the ocean in late spring. Smolt status was also determined by recording the presence or absence of parr marks and degree of silvering at each sample period.

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 Cabinet Gorge Hatchery)

Kettle Falls

Sherman Creek

WDW Hatchery

COLVILLE INDIAN RESERVATION

Columbia River

SPOKANE INDIAN RESERVATION

Grand Coulee Dam

Spokane Tribal Hatchery

Ford Hatchery (WDW)

Spokane River

Little Falls Dam

SCALE

km

0 10 20 30

N

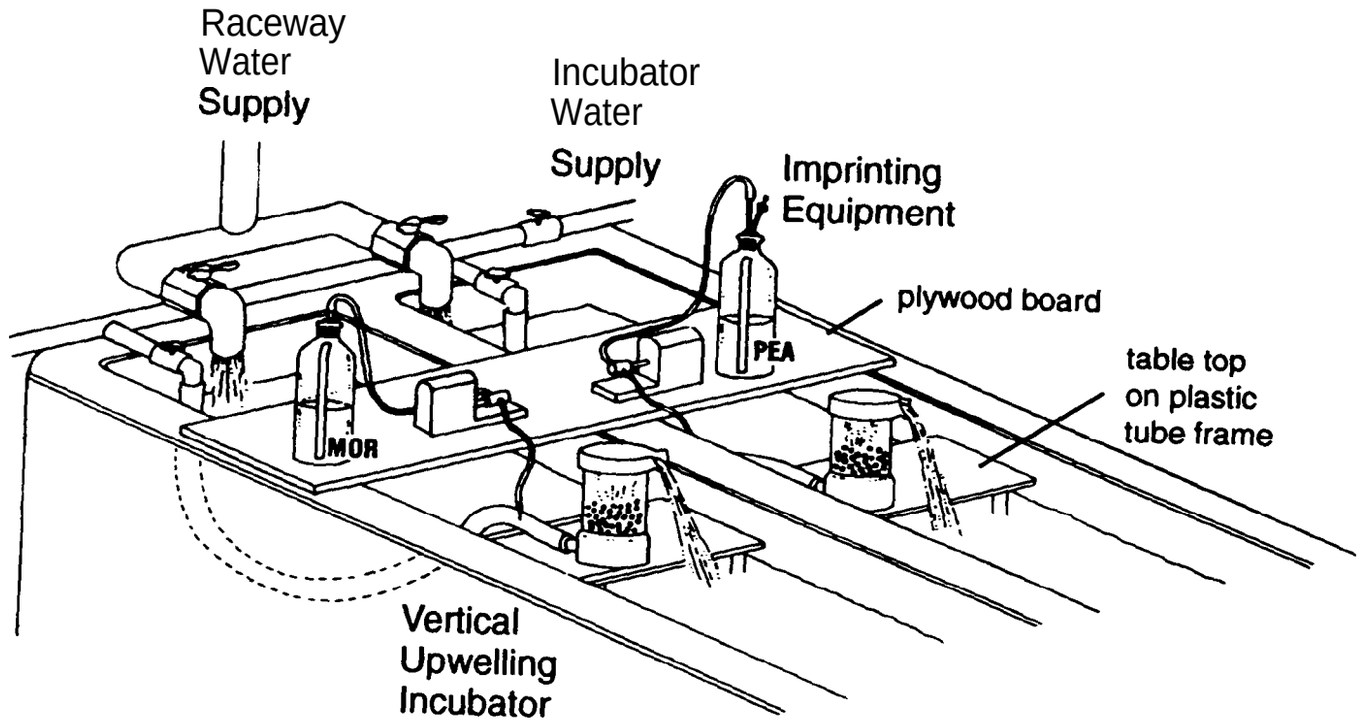
T_4 levels were also monitored in zero-age kokanee from Cabinet Gorge Hatchery that were progeny of Pend Orielle stock collected at Granite Creek. The eggs were fertilized on 12-2-91 at Cabinet Gorge Hatchery and transferred to the Spokane Tribal Hatchery on the same date. Mean whole body T_4 concentration ± SEM (ng/g body weight) was determined for 20 fish from this group at weekly intervals from the day of fertilization through 125 days post-fertilization, then at bi-weekly intervals through August 1992.

Eggs were incubated in upwelling incubators (Lake Whatcom eggs) or vertical-stacked tray incubators (Pend Orielle Lake eggs) placed on benches in hatchery raceways (Fig. 2). Eggs and larvae held in upwelling incubators remained in them through the swimup stage, when the fry swam voluntarily out of the incubator into the raceway. Fish in vertical-stacked tray incubators were manually transferred into the raceway after swimup. Water supply to the incubators was a well water supply at a constant temperature of 9-10°C. Water supply to the raceways was a combination of Metamooteles Springs water and well water at 8-11°C. Fish from all groups were maintained on natural photoperiod as each raceway was partially exposed to natural conditions of light and weather. After swimup, zero age fry were feed trained on Biodiet semi-moist mash (starter feed). Older fry were fed a combination of Biodiet semi-moist grower feed (1 .0 - 2.5 mm crumbles) and silvercup size 1-4 crumbles. One year old fingerlings were fed Biodry 1000 pellets (3.0 - 4.0 mm). All these feeds were obtained from Bioproducts, Inc.

2.2 Artificial imprinting with syn thethic chemicals.

Odor exposure followed the procedures described in a fish imprinting manual prepared by Scholz et *al.* (1975). Morpholine (MOR) (C_4H_9NO), a heterocyclic amine, was metered into one incubator or raceway at a steady state concentration of 5 x 1 O-5 mg/l; phenethyl alcohol (PEA) ($C_8H_{10}O$) was metered into the second incubator or raceway at a steady state concentration of 5 $\times 10^{-3}$ mg/l. Morpholine and PEA were selected as imprinting chemicals because they have been successfully used to imprint several species of salmonids (reviewed by Hasler and Scholz 1983). In salmonids, the behavioral threshold detection limits were approximately 1 x 10^{-6} mg/l for morpholine and and 1 $\times 10^{-4}$ mg/l for PEA (Scholz et a/. 1975). Additionally, neither chemical is found in natural waters; both are chemically stable and both are

Fig. 2 Diagram of incubation and imprinting setup at Spokane Tribal kokanee hatchery



highly soluble in water (Scholz et *a/.* 1975). Two imprinting chemicals were employed in this experiment so that one of the odors could act as a control for the other.

The odor delivery system is diagramed in Fig. 3. A 9 liter glass bottle was used to avoid imprinting chemicals reacting with plastic or metal containers. A syringe needle was punched through the cork fitting on the jug to allow air pressure to equalize in the jug as the solution was drained off. A piston pump or peristaltic pump was used to meter small quantities (10 ml/h) of the chemical solution into incubators Or raceways.

The steady state concentration for each imprinting chemical was calculated using the formula:

$$DC = \frac{SSC \times FR}{DR}$$

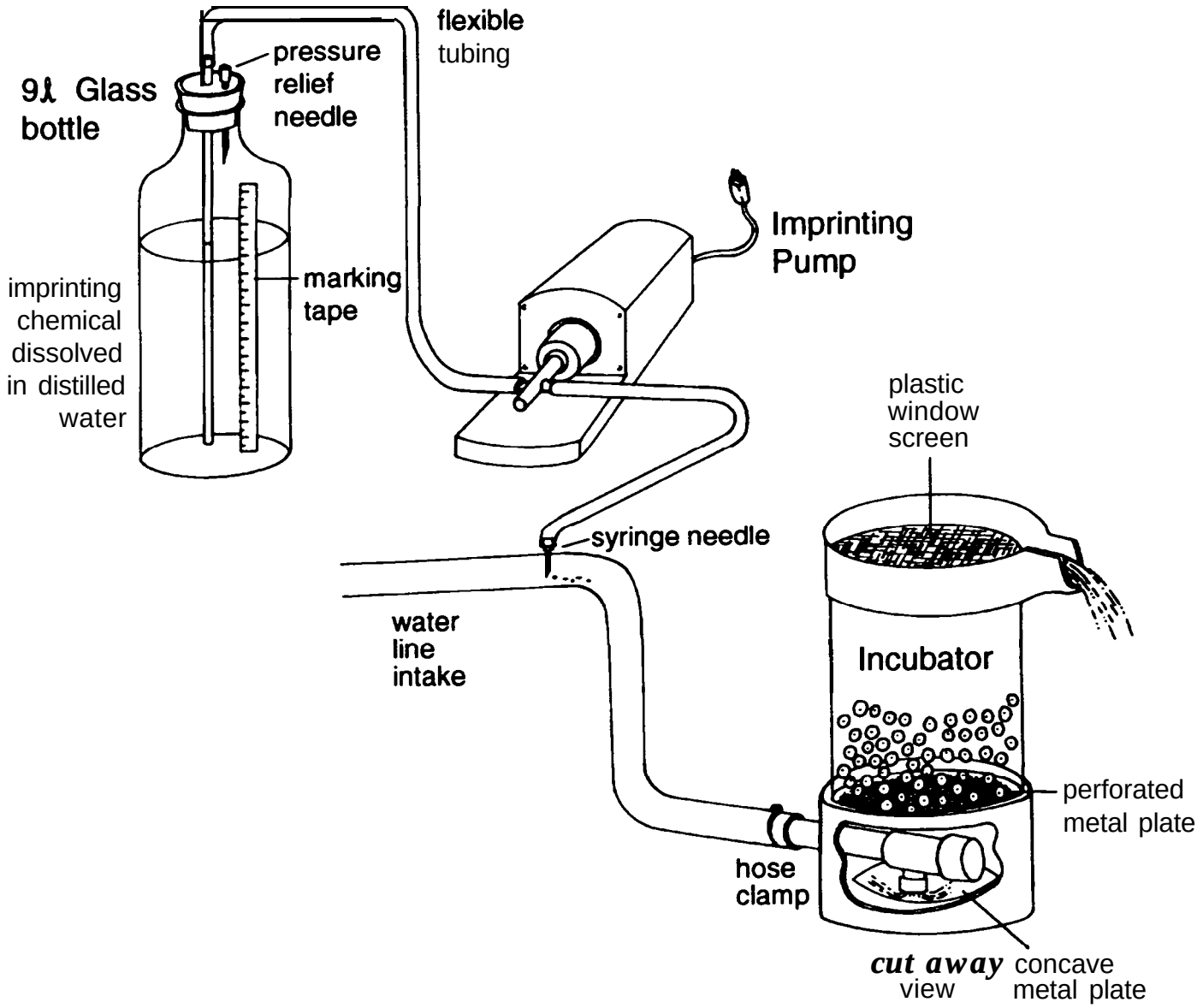
where: DC = Drip concentration (the amount of chemical added to the S/liter bottle) in g/l (or ml/l since 1.0 g/l of morpholine or PEA ≈ 1 ml/l).

s s c = Steady state concentration (the chemical concentration in the incubator or raceway) predetermined at 5 x 10^{-5} mg/l for morpholine and 5 x 10^{-3} mg/l for PEA. These values were converted into @l-

FR = Flow rate (the amount of water flowing through an incubator or raceway per unit time) in liters/sec. (The flow rate in gpm was converted to units of l/sec for this calculation); and

DR = Drip rate (or the rate at which the piston pump delivered fluid from the glass bottle into the incubator or raceway) in liters/sec. (Pumps were set to deliver 8-10 ml/hour, which was converted into units of l/sec for this calculation.)

Fig. 3 Apparatus used for synthetic chemical imprintiing of kokanee salmon.



Since the steady state concentration, flow rate and drip rate were predetermined values, the equation was solved for the drip concentration. Since the drip concentration was calcualted in mg/l, the calculated value was multiplied by the liter volume of the bottle (9 liters). This amount of chemical was added to the bottle, which was then filled to the 9 liter mark with distilled water. The output tube from the piston pump was positioned to meter the chemical into the incubator or raceway inflow stream so that the imprinting odors were thoroughly mixed into the incubator or raceway water supply (Fig. 3).

The hatchery well (258 feet deep) provided a 'neutral water source' during the exposure period (see discussion in Scholz et *a/.* 1975). Background odors in the different incubators and raceways were uniform, except for the addition of specific imprinting chemicals. Additionally, well water supplies do not usually contain an organic fraction that is detected by fish (Hasler and Wisby 1951), as evidenced by the fact that it is difficult to imprint fish to well water alone.

Since the purpose of this investigation was to define the critical period for imprinting in kokanee, the fish were divided into 17 separate lots and exposed to synthethic chemicals at different life history stages. The developmental stage, exposure dates and imprinting chemical used for each lot are noted in Table 1. (See Section 3.1.) The majority of the fish used for this experiment were from the Lake Whatcom 1991 year class. Each of the following developmental stages were exposed to morpholine and phenethyl alcohol: (1) eyed egg to hatch, (2) hatch (7 days pre-hatch to 7 days post-hatch), (3) hatch to swimup, (4) swimup (7 days pre-swimup to 7 days post-swimup), (5,6,7) fry (for about 30 days at monthly intervals from February through April) and (8) fry (for about 60-75 days from May to mid-July).

Additionally, one lot of 1991 year class Cabinet Gorge fish were exposed to phenethyl alcohol from the day of fertilization to the eyed egg stage. Also, two lots of Lake Whatcom 1990 year class (1.5 years old) fish were exposed to morpholine and phenethyl alcohol during their "smolt stage" i.e., at the time when anadromous sockeye would smolt. These 1.5 year old fish developed thick silver guanine plating, characteristic of smolts, during this period. The guanine deposition was sufficient to mask parr marks. The fish also exhibited downstream orientation in hatchery raceways, especially at night, according to fish culturists at the Spokane Tribal Hatchery.

A portion of the fish from each experimental lot were marked with a lot specific fin clip and retained at the Spokane Tribal Hatchery. These fish will be held until August 1993 when behavioral tests will be conducted to determine if imprinting occured at any developmental stages.

Most of the fish from each experimental lot were released in Lake Roosevelt in July and August 1992. A portion of each lot was marked with a lot specific coded wire tag that distinctively identified the developmental stage, exposure period, synthethic chemical, release date and release location. Experimental lot information is summarized in Table 1. (See Section 3.1 for details.) During the spawning migration of these fish (1993 and 1994 for 1990 year class Lake Whatcom fish, and 1994 and 1995 for 1991 year class Lake Whatcom fish), morpholine and phenethyl alcohol will be metered into traps installed at different sites in an attempt to attract fish that were exposed to the synthethic chemicals. One site will be the trap located at the Sherman Creek hatchery fish ladder, which will be scented with morpholine. The location of the phenethyl alcohol site will be selected in 1993.

2.3 Sample collection procedure for T_4 determination.

Details (including flowcharts and diagrams) of our procedures for collecting samples for T_4 determination, T_4 extraction and measurement of T_4 content by radioimmunoassay, were presented in our 1991 annual report (Scholz et *al.* 1992). In the present study, our methods generally followed those procedures. For egg and larval samples, 20 eggs or larvae were collected from both Lake Whatcom and Pend Orielle Lake groups. Sampled embryos, larvae and fry were anesthetized with 0.1 g/l tricane methanosulfonate (MS-222), blotted dry on absorbent paper, weighed to the nearest 0.1 mg using a Mettler AJ 100 analytical balance and placed into individually numbered vials (2 fish per vial, $n = 10$ vials). Fish were then quick-frozen on dry ice and stored at -80°C until T_4 was extracted and concentrations determined by radioimmunoassay.

One year old Lake Whatcom fish were sufficiently large to collect blood from the caudal vein using either heparinized capillary tubes or a 22 guage syringe needle. Blood was centrifuged for 10 min and the plasma fraction (contained T_4) pipetted into

a plastic sample vial. Plasma was then stored at -80°C until the time of radioimmunoassay.

2.4 T_4 Extraction Procedures

Thyroxine was extracted from eggs or whole fish and reconstituted using modification of methods from Kobuke et *al.* (1987) and Parker (1988). Frozen fish were minced and placed into individual 15 x 85 mm boroscillicate glass test tubes with ice cold ethanol (ETOH) containing 1 mM 6-N-propyl-2-thiouracil (PTU; Sigma) at a µl volume equal to 2 X's the mg weight of the fish. Propylthiouracil prevents degredation of T_4 by blocking its enzymatic conversion to triiodothyronine (T_3). Each fish was homogenized for 20 seconds with a Brinkman model Polytron 3000 tissue homogenizer at 20,000 RPM. Following homogenation, each sample was vortexed for five seconds and poured into a centrifuge tube. The test tube was then rinsed at a µl volume equal to 1 X the mg weight of the fish and homogenized for an additional 20 sec at 20,000 RPM. The polytron blade was rinsed with tap water, distilled water and ETOH between every sample. Samples were vortexed for 5 sec and added to the centrifuge tube. All the above procedures were performed on ice. Samples were centrifuged for 10 minutes at 3000 RPM at 4°C using a Dynac refrigerated centrifuge (Clay Adams, Inc.). The supernatant was poured into a 10 ml drying tube and the pellet resuspended in 100% ETOH at a µl volume equal to 1.5 X the mg weight of the fish, and vortexed for 10 seconds. This solution was again centrifuged at 3000 RPM at 4°C and the supernatant combined with the supernatant in the drying tube. Samples were dried in a vacuum oven at 60°C at 25 psi for 2-24 hours. The dried samples were stored at 0°C until T_4 content was assayed.

2.5 T_4 radioimmunoassay

A Diagnostic Products Inc., T_4 radiommunnoassay kit was used for determining T_4 concentrations. Dried samples from age zero fish were reconstituted in 250 µl 95% ethanol (ETOH) and 250 µl 0.11 M sodium barbital buffer (pH 8.6). Each sample was then vortexed for 15 seconds, transferred into a plastic eppendorf snap cap vial, and centrifuged at 3000 RPM for 10 minutes using an Eppendorf Model 5145C refrigerated centrifuge at 4.0°C. Serum samples from one year old fish were thawed slowly and vortexed for 5 sec. Reconstituted whole body and plasma samples thus prepared were pipetted into duplicate radioimmunoassay (RIA) tubes.

To perform the RIA, 25 µl of each kokanee 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. This procedure uses a competative binding technique in which the ^{125}I-T_4 and unknown T_4 sample compete for Ab binding sites, and bind in proportion to their relative concentrations. Therefore, samples that contain large quantities of T_4 will bind less ^{125}I-T_4 than samples that contain small quantities of T_4. A standard curve was prepared with known concentrations of T_4 ranging from 0 to 24 µg/dl by pipetting 25 µl of 0, 0.5, 1, 4, 10, 16 and 24 µg/dl standard concentrations and 1 ml of ^{125}I-T_4 into Ab coated tubes. The actual concentrations of T_4 in kokanee samples were determined by comparison to the standard curve samples, which were subjected to the same assay procedures.

Unknown kokanee and standard curve tubes were vortexed for five seconds and incubated for 1 hour at 37°C (the equilibrium point) to complete binding of non radioactive T_4 and ^{125}I-T_4 to Ab. The T_4 and ^{125}I-T_4 that did not bind to the antibody receptors was decanted into a radioactive waste container and the remaining liquid in the tubes was blotted dry on absorbent paper. Radioactivity of remaining bound T_4 was counted for 1 minute using a programmable Cobra QC Model B5002 auto-gama Counter (Packard Instrument, Co.).

Maximum binding (MB) of radioactivity was determined from the standard curve tubes containing 0 µg/dl T_4. Percent bound of each of the remaining standard curve and unknown samples was calculated using 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 counts = radioactive counts per minute (cpm) of maximum binding tube; and

NSB counts = nonspecific binding (cpm) i.e., radioactivity that sticks to the assay tube but not bound to antibody binding sites. This was determined by adding 1 ml T_4 to uncoated assay tubes that were subjected to the same procedures as

antibody coated assay tubes. The remaining activity was considered non-specific binding.

The gamma counter was programmed to determine the percent bound for each standard concentration and plot a log-logit graph of the standard curve (% bound -v- standard concentration). The program then compared the percent bound of unknown samples to this graph and interpolated their concentrations. The program automatically subtracted NSB from each standard curve and unknown sample before calculating percent bound.

T_4 concentration of blood sample from one year old fish was calculated by converting concentration from µg/dl to pg/ml serum using the formula:

$$T_4 = MC \times \frac{1000\ pg}{1\ \mu g} \div \frac{1\ dl}{100\ ml}$$

where: $[T_4]$ = T_4 concentration (in pg/ml serum);
MC = measured concentration (in µg/dl).

T_4 concentrations of whole body samples from zero age fish was calculated by the formula:

$$[T_4] = MC \times \frac{1\ dl}{100000\ \mu l} \times \frac{1000\ ng}{1\ \mu g} \times \frac{TS_{vol}}{SS_{vol}} \times \frac{1000\ mg}{SW} \times \frac{25}{1\ g} \quad \frac{\mu l}{sample} \times CF$$

where: $[T_4]$ = T_4 content (in ng/g body weight);
MC = measured concentration of the sample from RIA analysis (in µg/dl);
TS_{vol} = total volume of reconstituted samples (500 µl);
SS_{vol} = Subsample volume of aliquots used for performing the RIA (25µl);
SW = sample weight (in mg); and
C.F. = correction factor for extraction efficiency. This value was determined by spiking egg, alevin and fry samples with known amounts of T_4, subjecting them to the same extraction procedures described above, and determining the

percentage of the T_4 spike recovered. Details of the procedure and results are outlined in Scholz et *al.* (1992). The following percent recoveries were obtained: eggs (88.5%), alevins (91.7%) and fry (80.4%). The reciprocals of these numbers were used as correction factors to calculate T_4 concentration. These values were: eggs (1.13), alevins (1.09) and fry (1.24).

The mean ± standard error of the mean (SEM) T_4 concentration, as well as the mean ± standard deviation (SD) individual body weight, were calculated separately for zero age Lake Whatcom and Pend Oriellе Lake fish-- as well as one year old Lake Whatcom fish-- collected on the same sample date, using Statview II (Brainpower, Inc,) on an Apple Macintosh II cx computer. These values were plotted using an Excell (Microsoft, Inc.) spreadsheet and graph program.

2.6 Quality control procedures

The gamma counter was calibrated before each assay to check efficiency. Counter efficiency ranged from 80.4 to 80.7 percent. Background radiation was determined by counting two blank tubes at the front of each assay. The counter was programmed to automatically subtract this background level from each tube assayed. Also, each sample was assayed for T_4 in duplicate to control for procedural errors. The mean percent error ± 95% confidence interval (95% Cl) was calculated and a frequency distribution plotted. Quality assurance samples obtained from the assay kit manufacturer (Diagnostic Products) were assayed in the front, middle and end of the assay. Mean (± SD) and ranges in concentration were then compared to actual concentrations provided by Diagnostic Products, Inc. to determine assay reliability.

Effects due to delays in addition of reagents were tested by placing pairs of standard curve samples at the front and end of each assay. Significant difference between these samples were determined by calculating the Wilcoxon signed rank statistic using Statview II (Brainpower, Inc.).

During blotting to remove non-bound radiolabelled T_4 in the final step of the assay, the assay tubes were put into a foam rack, turned upside down on absorbent

paper, and allowed to drain for 2 minutes. The rack was then rapped sharply several times to shake out any drops of liquid remaining on the inside of the tube. It is possible that same radiolabelled T_4 'slopped' onto the outside of the assay tubes during this procedure. To determine the degree of such contamination, distilled water was added to six 'blank' tubes which were inserted randomly into the assay and subjected to all assay procedures except for adding radiolabelled T_4 . If no contamination occurred, these blank tubes should read either 0 or no more than a few cpm. These tubes also acted as a check to determine that the counter functioned properly e.g., automatically subtracted background counts. For example, since background gamma radiation is about 300 cpm in our laboratory, the blank tubes should read about 300 cpm if the counter was not subtracting the background properly. However, the tubes would read about 0 cpm if the counter subtracted background.

Because T_4 concentration was determined for a large number of samples, four assays were performed. To test interassay accuracy, three different interassay pool (IAP) samples were inserted in duplicate at three different positions into each assay. Also, the same known T_4 samples were used to construct the standard curve for all four assays. Mean values of IAP and standard curve samples were compared statistically by calculating the Wilcoxon signed-rank nonparametric test using Statview II software. Based on this test, if IAP and standard curve samples were not different at the $p = .05$ signifigance level, the results of the four assays were considered comparable.

3.0 RESULTS

3.1 Synthetic chemical imprinting experiments.

Data about the stock history, developmental stage (age) and calender date at time of odor exposure, total number exposed, imprinting chemicals used, tagging information (number tagged and tag type), release, date, and release location for each experimental lot used in the artificial imprinting experiments are recorded in Fig. 4 and Tables 1-3.

A total of 87,600 Lake Whatcom--1990 year class-- kokanee were retained at the Spokane Tribal Hatchery until they were about 18 months old before release in Lake Roosevelt at age 1+ in 1992 (Fig. 4). These fish were exposed to imprinting chemicals (36,000 to morpholine and 51,600 to PEA) as 16-18 month old smolts from March 15 to June 9, 1992. Most of these fish (35,342 morpholine-exposed and 51,587 PEA-exposed fish) were released in Lake Roosevelt at the Sherman Creek Hatchery from April 21, 1992 to June 9, 1992 as smolts or post-smolts (Table 1). Prior to release the fish were given an adipose fin clip **and tagged with a group specific coded wire tag** that identified their chemical treatment and release date (Table 1). Survival rates between January 1, 1992 and June 9, 1992 were 98.2% for the morpholine-exposed fish and 99.9% for the PEA-exposed fish (Table 2). A portion of the fish from each group (200 morpholine-exposed and 200 PEA-exposed fish) were given fin clips (right pectoral for morpholine and left pectoral for PEA) and retained at the Spokane Tribal Hatchery. These fish will be held until August-October 1993, when behavioral tests will be conducted to determine if the fish imprinted to their respective treatment odors.

Approximately 5000 Cabinet Gorge (Pend Orielle)-- 1991 year class-- kokanee were exposed to phenethyl alcohol from the time of fertilization through the eyed egg stage at the Spokane Tribal Hatchery in November and December 1991. All of these fish were retained at the Spokane Tribal Hatchery for behavioral tests.

Lake Whatcom-- 1991 year class-- kokanee were exposed to imprinting **chemicals at various developmental stages at the Spokane Tribal Hatchery. Exposure** stages included:

Table 1. Summary of synthetic chemical, treatment, exposure period, and marking of fish retained at the Spokane Tribal hatchery (STH) or released in Lake Roosevelt for artificial imprinting experiments.

Expenmental lot no	Source /brood year	Exposure period (development stage	Exposure dates	Synthetc chemical	Total exposed (#)	Fish retained at hatchery # marked	fin clip	Fish released in Lake Roosevelt Total number released	Total # with coded wire tags	Coded wire tag lot #	# in lot	Release date	Release location
1	co 91	Fert egg-Eyed egg	13/3/91-12/27/91	PEA	5.000								
2	LW 91	Eyed egg-Hatch	12/7/91-12/27/91	PEA	125,000	400	LP	98,393	11 393	62-51-27	11.393	7-06-92	Sherman Creek
3	LW 91	Eyed egg-Hatch	12/7/91-12/27/91	MOR	125,000	400	RP	113,367	7.367	62 51-28 62-51-44 62-51-44	3 192 2.060 2,115	7-06-92 7-27-92 8-03-92	Sherman Creek
4	LW 91	Hatch	12/7/91-12/27/91	PEA	197,000	800	LV	179,722	23,115	62-51-29 62-51-31 62-51-32 62-51-30	11.458 10,384 5.987 4,685	7-06-92 7-06-92 7-13-92 7-06-92	Sherman Creek
	LW 91	Hatch	12/27/91-1/4/92	MOR	125,000	800	RV	119,772	a.222	62-51-32 62-51-30 62-51-30	5,060 5,687 6,490	7-06-92 7-13-92 7-13-92	Sherman Creek
6	LW 91	Hatch-swimup	12/27/91-1/29/92	PEA	125,000	400	A-LV	88,868	10,868	62-51-33 62-51-33	9,092 1,776	7-13-92 7-20-92	Sherman Creek
7	LW 91	Hatch-swimup	12/27/91-1/29/92	MOR	150,000	400	A-RV	115 191	11,441	62-51-37 62-51-37	9,356 2,085	7-13-92 7-20-92	Sherman Creek
.	LW 91	Swimup	1/22/92-2/5/92	PEA	75,000	400	A-LP	63,716	10,716	62-51-35 62-51-35	7,351 3,365	7-13-92 7-20-92	Sherman Creek
9	LW 91	Swimup	1/22/92-2/5/92	MOR	75,000	400	A-RP	61,370	8,370	62-51-36 62-51-36 62-51-36	5.545 255 2,570	7-20-92 7-27-92 8-03-92	Sherman Creek
10	LW 91	FED Fry	1/29/92-2/26/92	PEA	125,000	400	LP-RV	109,025	8,025	62-51-34	8,025	7-13-92	Sherman Creek
11	LW 91	FEB Fry	1/29/92-2/26/92	MOR	125,000	400	RP-LV	117,656	20,194	62-51-24 62-51-25 62-51-26	5 . w 7.457 7.249	6-29-92 6-29-92 6-29-92	Sherman Creek
12	LW 91	MAR Fry	2/26/92-4/1-92	PEA	160,000	400	D-LV	157,318	10,818	62-51-39 62-51-39	4,265 6,553	7-27-92 8-03-92	Sherman Creek
13	LW 91	MAR FRy	2/26/92-4/1/92	MOR	160,000	400	D-RV	156,298	9,798	62-51-38 62-51-38	5,291 4.507	7-27-92 8-03-92	Sherman Creek
14	LW 91	APR Fry	4/1/91-4/29/92	PEA	160,000	400	D-LP	156,235	11,525	62-51-41 62-51-41	5,818 5,707	7-20-92 7-27-92	Sherman Creek
15	LW 91	APR Fry	4/1/92-4/29/92	MOR	160,000	400	D-RP	157,445	11,445	62-51-40 62-51-40	9,142 2,302	7-20-92 7-27-92	Sherman Creek
16	LW 91	MAY-JULY Fry	4/29/92-7/10/92	PEA	150,000	400	D	146,010	11,300	62-51-43	11,300	7-27-92	Sherman Creek
17	LW 91	MAY-JULY Fry	4/29/92-7/10/92	MOR	152,000	400	A	151,548	6,838	62-51-42	6,838	7-27-92	Sherman Creek
18	LW 90	Smolt	3/11/92-5/22/92	MOR	35,400	200	RP	35,342	35,342	65-51-14	35,342	6-09-92	Sherman Creek
19	LW 90	Smolt	3/11/92-5/22/92	PEA	51,587	200	LP	51,587	51,587	65-51-21	51,587	6-09-92	Sherman Creek

Clips
RP . right perctoral
LP . left pectoral
RV . right ventral
LV . left ventral
A . adipose
0 . dorsal

Source
CG 91 = Cabinet Gorge (Granite Creek) 1991 year class
LW = Lake Whatcom 1991 yearlings
LW 90 . Lake Whatcom fish 1990 year class hatched and reared at STH

Table 2. Survival of 1990 year class Lake Whatcom stock kokanee from January 1, 1992 to release in April to June 1992.

Chemical treatment	Initial # fry	# released	% survival
PEA	51,600	51,587	99.9
MOR	36,000	35,420	98.2
Total	87,600	86,929	99.2

Table 3. Survival of 1991 year class Lake Whatcom stock kokanee from egg (December 1992) to release (June-August 1992).

Chemical treatment	Life stage	Initial egg count at time of hatch	# released	% survival
PEA	Eye-Hatch	125,000	98,393	78.7
	Hatch	197,000	179,722	91.2
	Hatch-Swimup	125,000	88,868	71.1
	Swimup	75,000	63,716	85.0
	FEB Fry	125,000	109,025	87.2
	MAR Fry	160,000	157,318	98.3
	APR Fry	160,000	156,235	97.6
	MAY-JULY Fry	150,000	146,010	97.3
Sub-Total		1,117,000	999,287	89.4
MOR	Eye-Hatch	125,000	113,367	90.6
	Hatch	125,000	119,772	95.8
	Hatch-Swimup	150,000	115,191	76.7
	Swimup	75,000	61,370	81.8
	FEB Fry	125,000	117,656	94.1
	MAR Fry	160,000	156,298	97.6
	APR Fry	160,000	157,445	98.4
	MAY-JULY Fry	152,000	151,543	99.6
Sub-Total		1,072,000	992,642	92.6

Fig. 4 Flowchart of experimental procedures used to determine the critical period for chemical imprinting in kokanee salmon

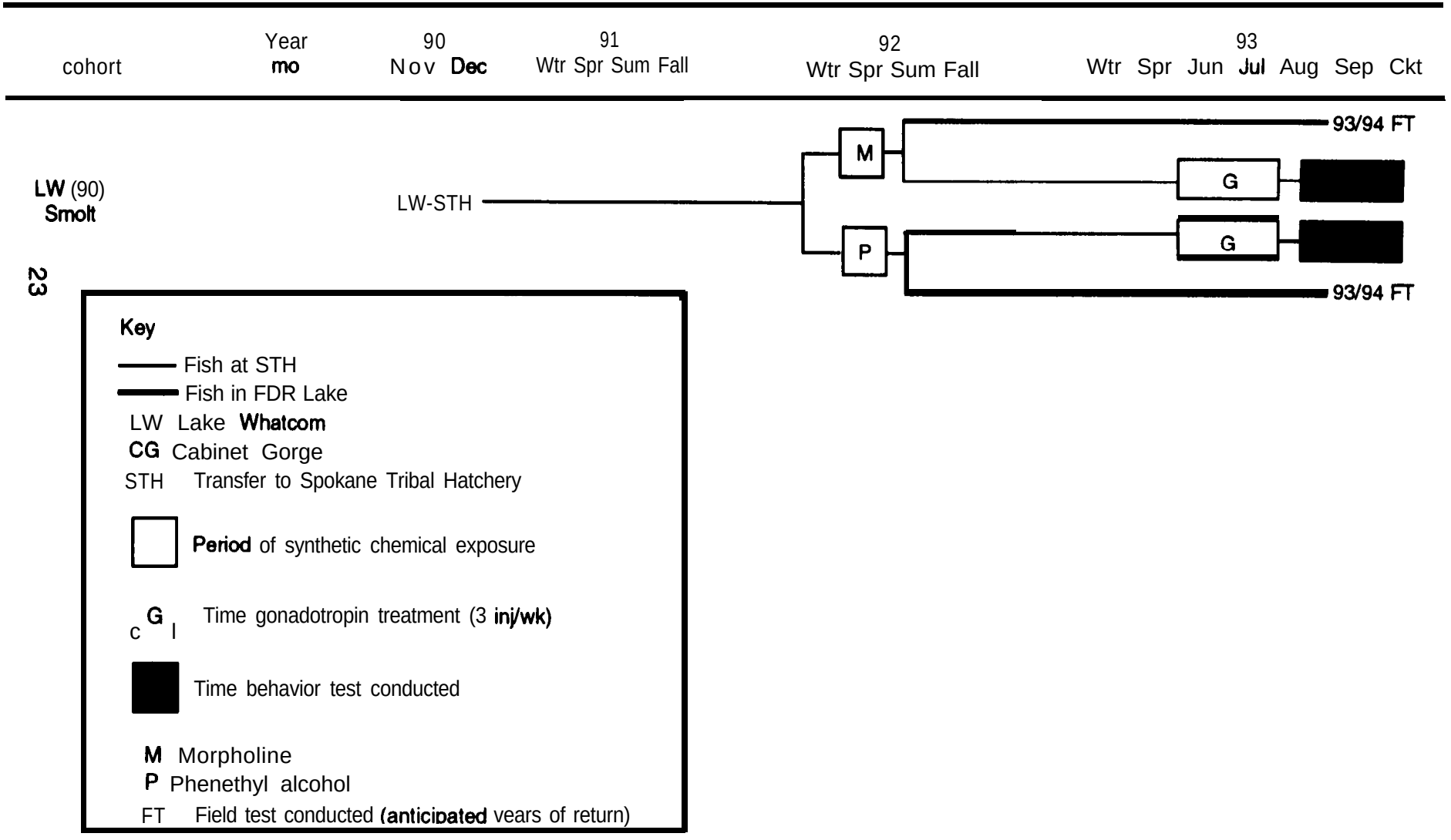


Fig. 4 continued

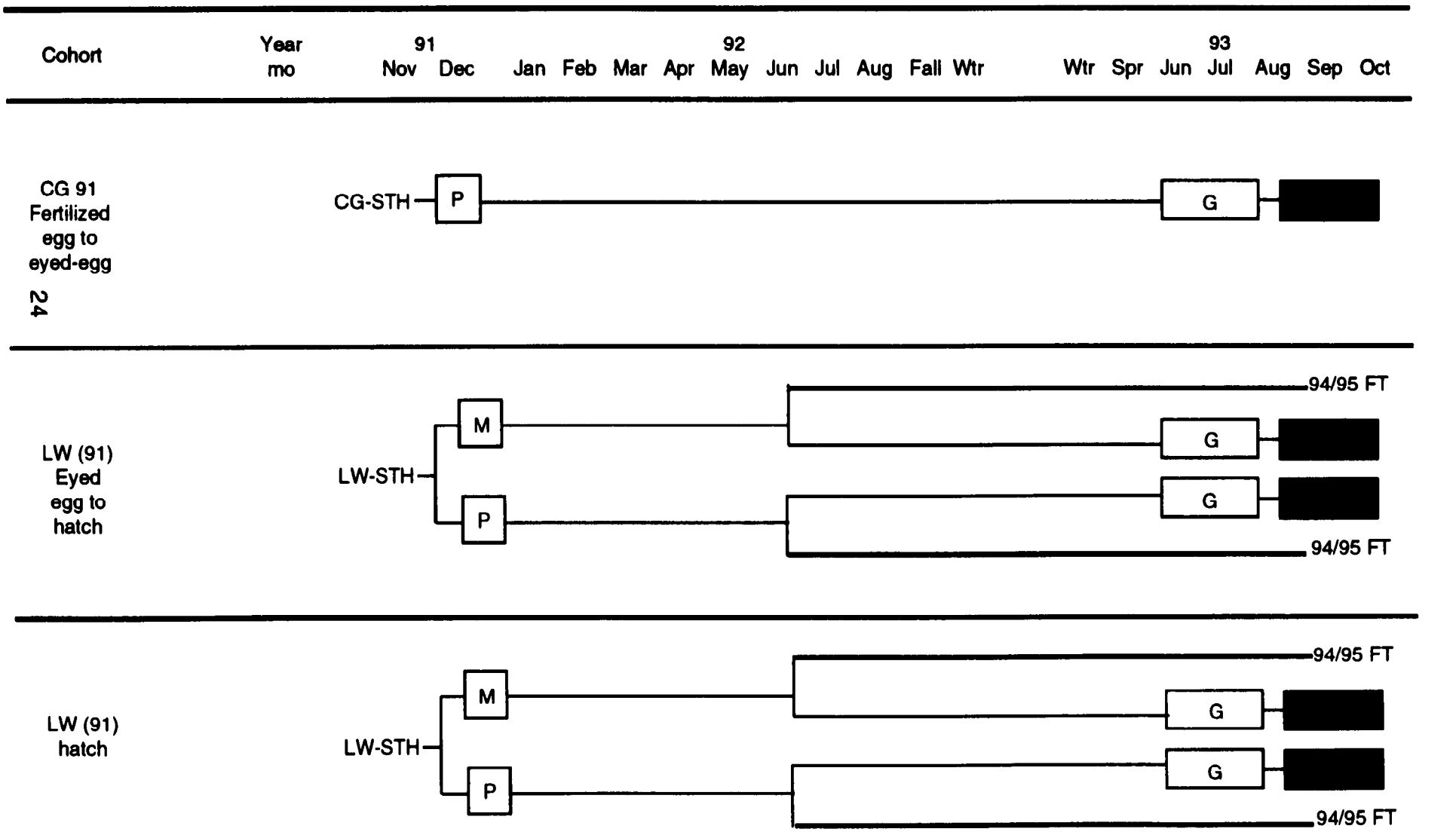


Fig. 4 continued

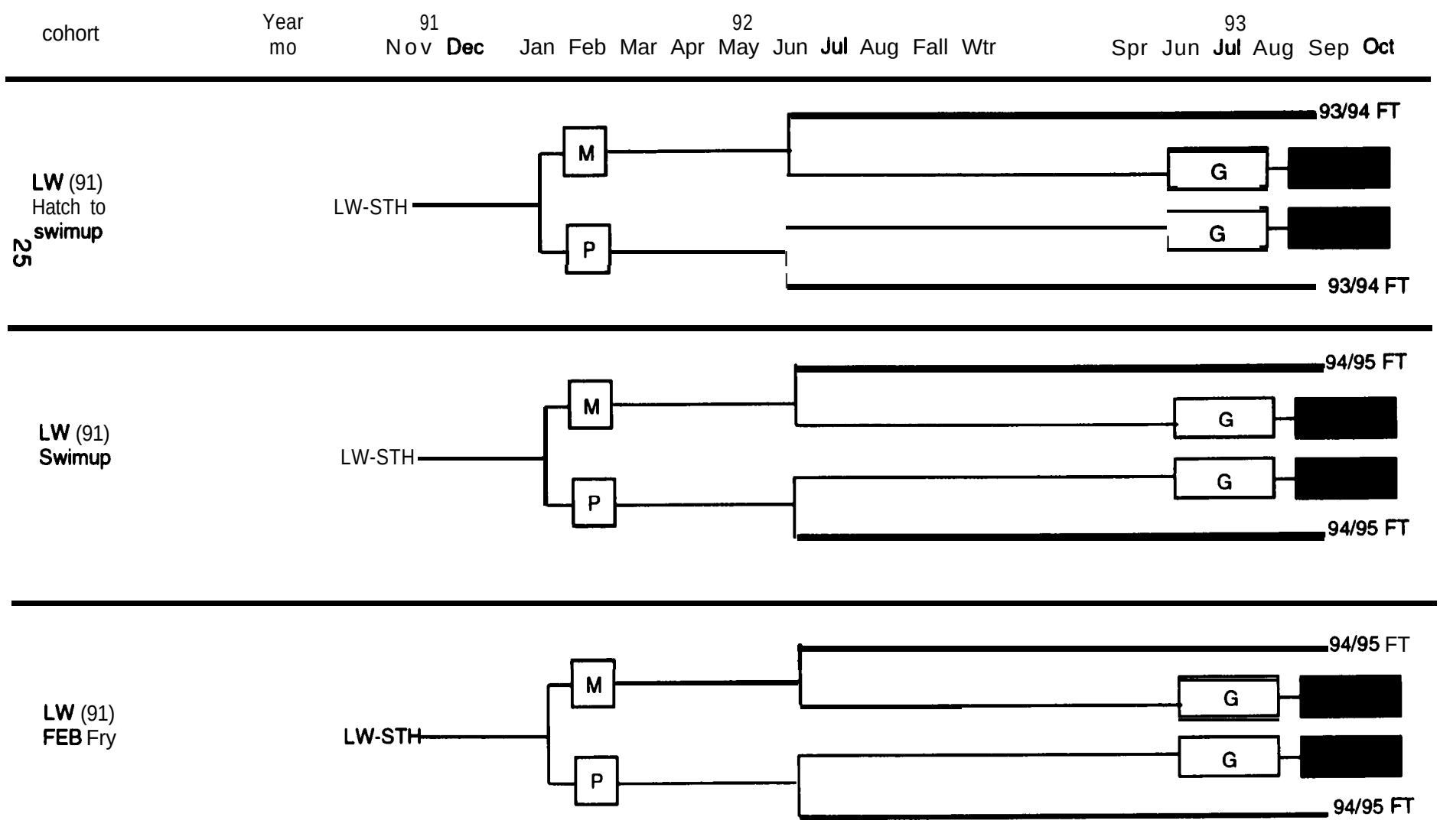


Fig. 4 continued

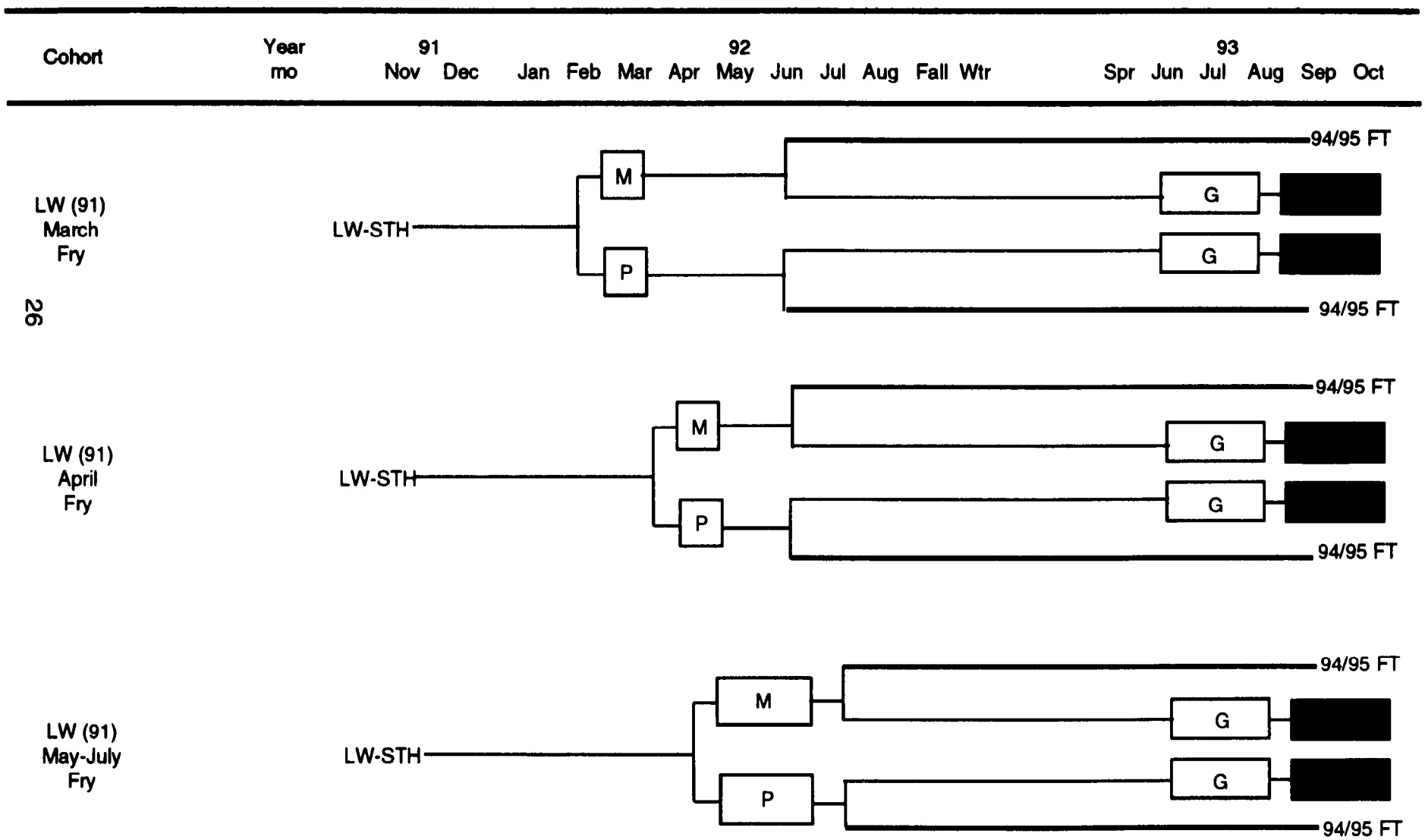
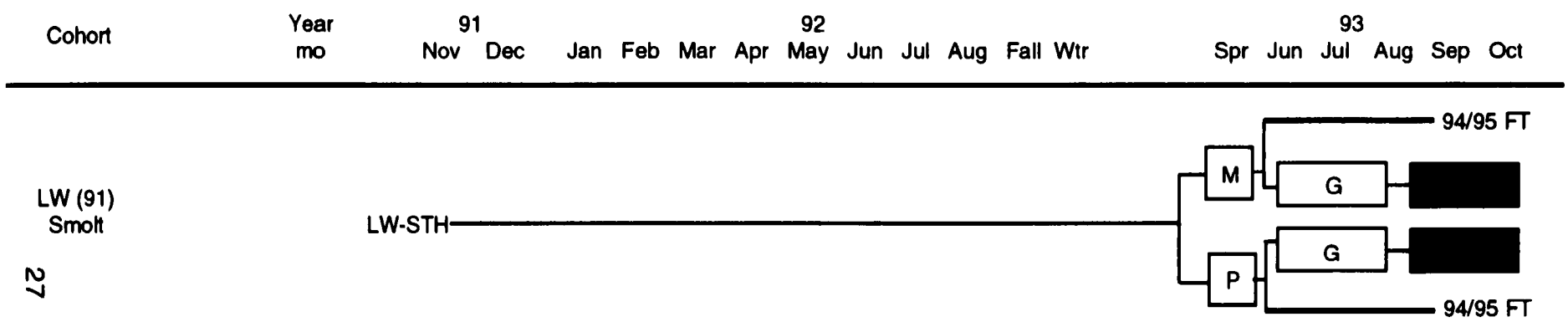


Fig. 4 continued



(1) Eyed egg to hatch *in December* 7997. Approximately 125,000 eggs were exposed to morpholine, with 113,367 released as fry (90.6% survival) at Sherman Creek Hatchery in July 1992. About 125,000 eggs were exposed to PEA, with 98,393 released as fry (78.7% survival) at Sherman Creek Hatchery in July and August 1992. A total of 7,387 of the morpholine-exposed fish and 11,393 of the PEA exposed fish were tagged with adipose fin clips and group specific coded wire tags prior to release (Table 1). A portion of the fish from each group (400 morpholine-exposed and 400 PEA-exposed fish) were given right pectoral (RP) and left pectoral (LP) fin clips respectively and retained at the Spokane Tribal Hatchery until August-October 1993, when behavioral tests will be conducted to determine if they imprinted to their respective chemicals.

(2) *Time of hatch in late December 1997 to early January 1992.* Approximately 125,000 eggs were exposed to morpholine, with 119,772 released as fry (95.8% survival) at Sherman Creek Hatchery in July 1992. About 197,000 eggs were exposed to PEA, with 179,222 released as fry (91.2% survival) at Sherman Creek Hatchery in July 1992. A total of 22,222 of the morpholine-exposed fish and 23,115 of the PEA exposed fish were tagged with adipose fin clips and group specific coded wire tags prior to release (Table 1). A portion of the fish from each group (800 morpholine-exposed and 800 PEA-exposed fish) were given right ventral (RV) and left ventral (LV) fin clips respectively and retained at the Spokane Tribal Hatchery until August-October 1993, when behavioral tests will be conducted to determine if they imprinted to their respective chemicals.

(3) *Alevin stage (hatch to swimup) from /ate December 7997 to /ate January* 7992. Approximately 150,000 alevins were exposed to morpholine, with 115,191 released as fry (76.7% survival) at Sherman Creek Hatchery in July 1992. About 125,000 alevins were exposed to PEA, with 88,868 released as fry (71 .1% survival) at Sherman Creek Hatchery in July 1992. A total of 11,441 of the morpholine-exposed fish and 10,868 of the PEA exposed fish were tagged with adipose fin clips and group specific coded wire tags prior to release (Table 1). A portion of the fish from each

group (400 morpholine-exposed and 400 PEA-exposed fish) were given adipose (A) + RV and A+LV fin clips respectively and retained at the Spokane Tribal Hatchery until August-October 1993, when behavioral tests will be conducted to determine if they imprinted to their respective chemicals.

(4) *Swimup stage in late January and ear/y February* 1992. Approximately 75,000 swimup fry were exposed to morpholine, with 61,370 released as fry (81.8% survival) at Sherman Creek Hatchery in July and August 1992. About 75,000 swimup fry were exposed to PEA, with 63,716 released as fry (85.0% survival) at Sherman Creek Hatchery in July 1992. A total of 8,370 of the morpholine-exposed fish and 10,716 of the PEA exposed fish were tagged with adipose fin clips and group specific coded wire tags prior to release (Table 1). A portion of the fish from each group (400 morpholine-exposed and 400 PEA-exposed fish) were given A+RP and A+LP fin clips respectively and retained at the Spokane Tribal Hatchery until August-October 1993, when behavioral tests will be conducted to determine if they imprinted to their respective chemicals.

(5) *Age 2-3 month old post-swimup fry in February 1992.* Approximately 125,000 fry were exposed to morpholine, with 117,656 released as fry (94.1% survival) at Sherman Creek Hatchery on June 29, 1992. About 125,000 eggs were exposed to PEA, with 109,025 released as fry (87.2% survival) at Sherman Creek Hatchery in July 1992. A total of 20,194 of the morpholine-exposed fish and 6,025 of the PEA exposed fish were tagged with adipose fin clips and group specific coded wire tags prior to release (Table 1). A portion of the fish from each group (400 morpholine-exposed and 400 PEA-exposed fish) were given RP+LV and LP+RV fin clips respectively and retained at the Spokane Tribal Hatchery until August-October 1993, when behavioral tests will be conducted to determine if they imprinted to their respective chemicals.

(6) Age 3-4 month o/d fry in March 7992. Approximately 160,000 fry were exposed to morpholine, with 156,298 released as fry (96.7% survival) at Sherman Creek Hatchery in July and August, 1992. About 160,000 fry were exposed to PEA, with 157,318 released as fry (98.3% survival) at

Sherman Creek Hatchery in July and August, 1992. A total of 9,798 of the morpholine-exposed fish and 10,818 of the PEA exposed fish were tagged with adipose fin clips and group specific coded wire tags prior to release (Table 1). A portion of the fish from each group (400 morpholine-exposed and 400 PEA-exposed fish) were given dorsal (D)+RV and D+RV fin clips respectively and retained at the Spokane Tribal Hatchery until August-October 1993, when behavioral tests will be conducted to determine if they imprinted to their respective chemicals.

(7) *Age 4-5 month o/d fry in* April 7992. Approximately 160,000 fry were exposed to morpholine, with 157,445 released as fry (98.4% survival) at Sherman Creek Hatchery in July, 1992. About 160,000 fry were exposed to PEA, with 156,235 released as fry (97.6% survival) at Sherman Creek Hatchery in July, 1992. A total of 11,445 of the morpholine-exposed fish and 11,525 of the PEA exposed fish were tagged with adipose fin clips and group specific coded wire tags prior to release (Table 1). A portion of the fish from each group (400 morpholine-exposed and 400 PEA-exposed fish) were given dorsal (D)+RP and D+LP fin clips respectively and retained at the Spokane Tribal Hatchery until August-October 1993, when behavioral tests will be conducted to determine if they imprinted to their respective chemicals.

(8) *Age 5-8 month o/d fry in May* to *July,* 7992. Approximately 152,000 fry were exposed to morpholine, with 151,548 released as fry (99.6% survival) at Sherman Creek Hatchery in July, 1992. About 125,000 fry were exposed to PEA, with 98,393 released as fry (78.7% survival) at Sherman Creek Hatchery in July and August, 1992. A total of 6,838 of the morpholine-exposed fish and 11,300 of the PEA exposed fish were tagged with adipose fin clips and group specific coded wire tags prior to release (Table 1). A portion of the fish from each group (400 morpholine-exposed and 400 PEA-exposed fish) were given D or A fin clips respectively and retained at the Spokane Tribal Hatchery until August-October 1993, when behavioral tests will be conducted to determine if they imprinted to their respective chemicals.

3.2 Whole body T_4 concentration in Lake Whatcom Stock kokanee- 1991 year class.

Lake Whatcom stock, 1991 year class, kokanee eggs weighed about 55-60 mg. These fish lost weight at the time of hatch (hatchlings weighed about 50 mg) and at swimup (47 mg) (Fig. 5). Yolk sacs were completely absorbed within a few days after swimup. At one month post-swimup their body weight had approximately doubled. They weighed about 0.5 g by 162 days post-fertilization, 1.0 g by 190 days post-fertilization, 2.0 g by 204 days post-fertilization, and 3.5 g by 248 days post-fertilization (Fig. 5).

In 1991 cohort Lake Whatcom stock kokanee, mean T_4 concentrations (± SEM) in eggs ranged from 6.7 ± 1.3 to 12.3 ± 3.7 ng/g body weight (Fig. 5). Mean T_4 concentration peaked on the day of hatch (60 days post-fertilization) at 13.1 ± 2.5 ng/g body weight (Fig. 5). After declining to 10.3 ± 1.1 ng/g body weight on day 73 post-fertilization, T_4 concentration peaked again at 22.1 ± 5.2 ng/g body weight at the time of swimup on day 86 post-fertilization, then steadily decreased to less than 1.0 ng/g body weight in fry older than 176 days post-fertilization (Fig. 5).

3.3 Whole body T_4 concentration in Pend Orielle (Cabinet Gorge) stock kokanee- 1991 year class.

Unfertilized eggs of Pend Orielle stock kokanee weighed about 46 mg. After water-hardening, fertilized eggs weighed about 55-60 mg (Fig. 6). The fish lost weight at the time of hatch (50 mg) but not at swimup (60.5 mg). Yolk sacs were completely absorbed at the time of swimup. Fish weighed about 100 mg on day 102 post-fertilization, 1 gram by day 165 post-fertilization and 6.7 grams by day 225 post-fertilization (Fig. 6).

In Pend Orielle stock kokanee, mean T_4 concentration (± SEM) in eggs ranged from 9.5 ± 1.7 to 16.7 ± 3.6 ng/g body weight (Fig. 6). T_4 concentration peaked at hatching at 24.2 ± 4.5 ng/g body weight (Fig. 6). After declining to 13.0 ± 2.9 ng/g body weight on day 81 post-fertilization, T_4 concentration peaked a second time during swimup (88-95 days post-fertilization) at 23.1 ± 6.3 to 24.3 ± 3.8 ng/g body weight (Fig. 6). After swimup, T_4 concentration declined to 0.6 ± 0.1 to 2.1 ± 1.3 ng/g body weight from 165 to 225 days post-fertilization (Fig. 6).

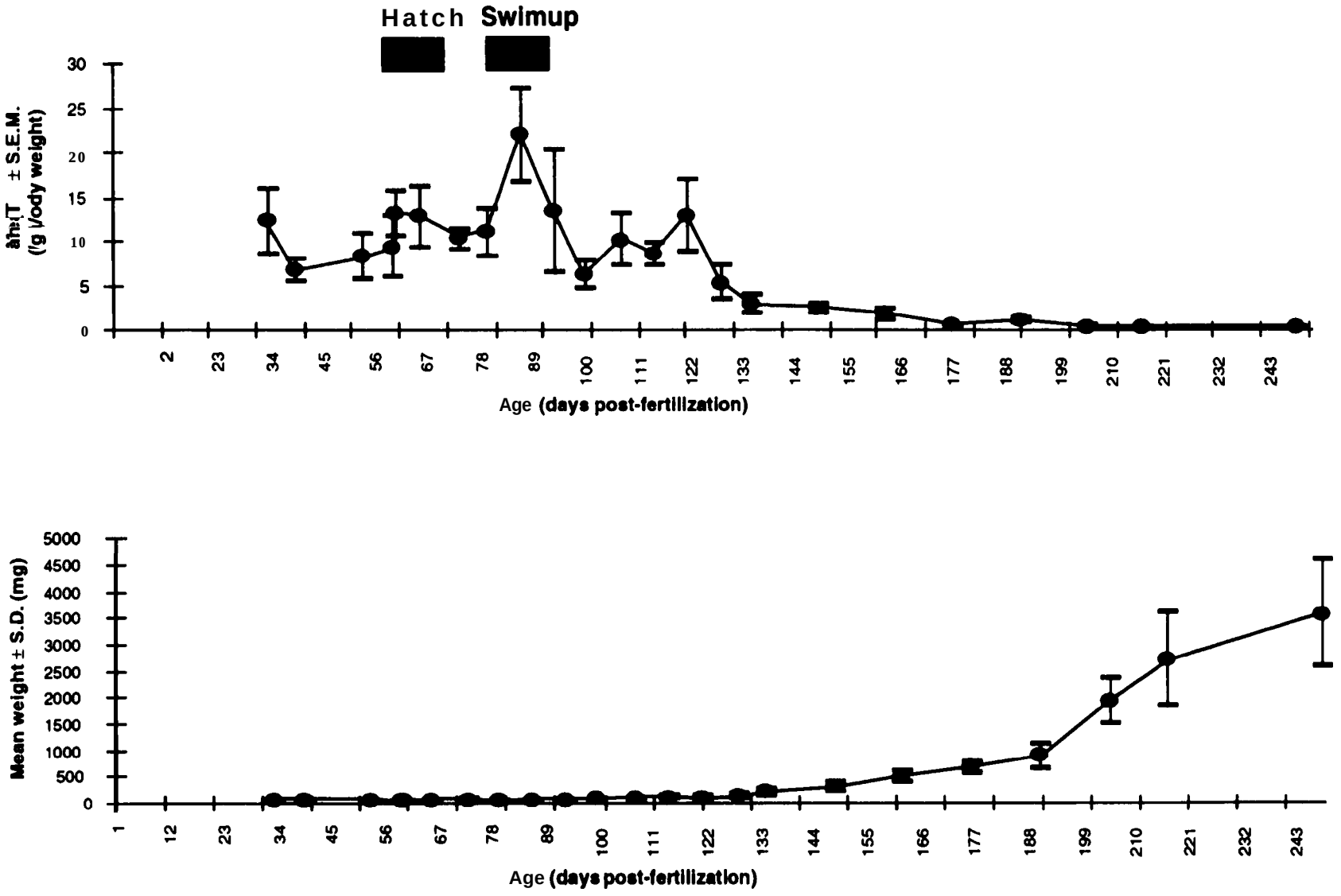
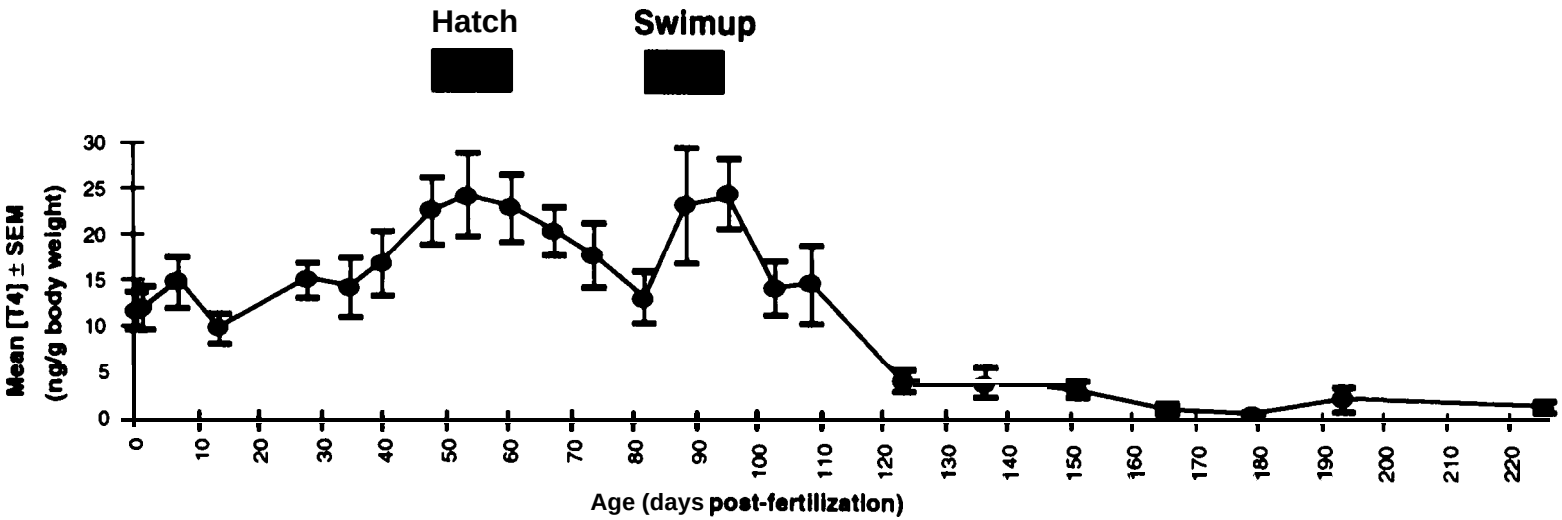


Fig. 5 Whole body T_4 concentration and weights of 1991 brood, Lake Whatcom stock kokanee



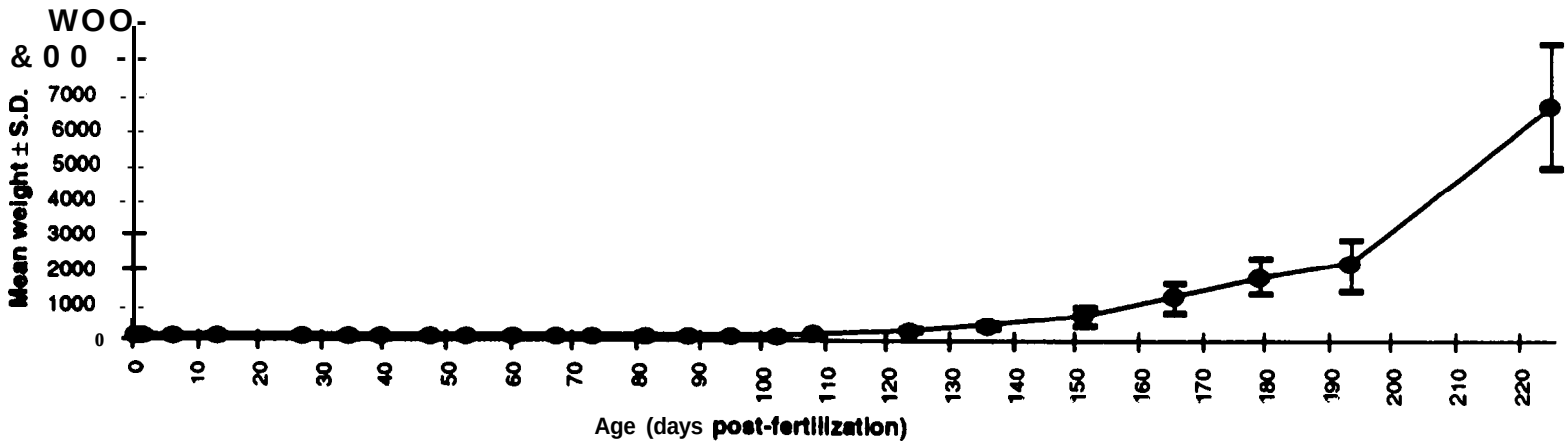


Fig. 6 Whole body T_4 concentration and weights of 1991 brood. Cabinet Gorge (Pend Orielle) stock kokanee

3.4 Blood plasma T_4 concentration in Lake Whatcom stock kokanee-- 1990 year class.

Blood serum T_4 concentration (pg/ml plasma ± S.D.) in 9 month to 21 month old Lake Whatcom stock (1990 year class) kokanee are presented in Fig. 7. T_4 concentrations were low during the late summer (about 5 pg/ml) and peaked in October (about 10 pg/ml). T_4 concentrations were low in November and December (about 4 pg/ml), then increased in January to about 2.2 pg/ml. T_4 concentration were fairly stable in February and March, ranging from about 10 - 17 pg/ml. T_4 concentration then fluctuated with several peaks at 20 to 28 pg/ml in April and May, before declining to about 6 pg/ml in June and July, and 4 pg/ml in August.

The 1990 year class Lake Whatcom kokanee evidenced some aspects of smoltification between age 12-18 months, including: (1) loss of parr marks and development of the distinctive silvery coloration that characterizes smolt transformation (Table 4); (2) decreased condition factor (Table 4); and (3) downstream orientation. The Spokane Tribal Hatchery manager noted 16-18 month old fish crowding at the downstream end of the raceways in the late evening and at night. These transitions were most pronounced in April and May. For example, by May only 0-15% of the fish retained parr marks, and most of the fish had evidenced subcutaneous deposition of thick silver guanine crystal flakes. However, several peaks of thyroxine activity were observed from January to May (Fig. 7) and these were all associated with a certain amount of silvering (Table 4). By July, most fish had reverted back to post-smolt or parr coloration.

3.5 Quality assurance results

Results of quality assurance samples are recorded in Table 5. 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 = 10). The actual concentration of the medium T_4 blind sample was 7.3 ng/dl compared to a mean (± 95% Cl) measured value of 6.9 ± 0.3 ng/dl (n = 10). The actual concentration of the high T_4 blind sample was 11.5 ng/dl compared to a mean (± 95% Cl) measured value of 10.5 ± 0.5 ng/dl (n = 10). Our measured ranges were within the acceptable ranges in 28 of 30 cases.

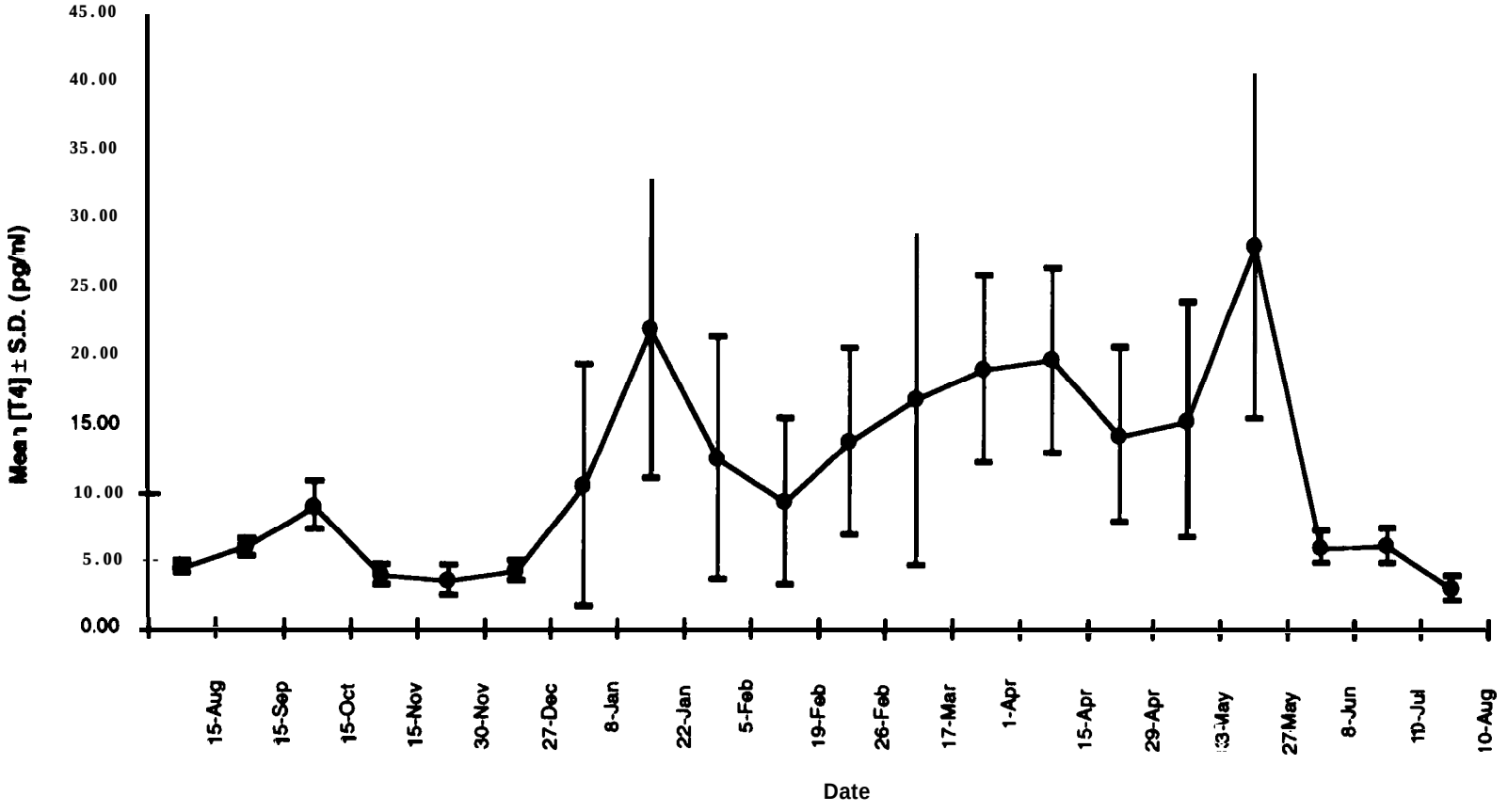


Fig. 7 T_4 concentration in blood serum of 1990 brood, Lake Whatcom stock kokanee from August 15, 1991 to August 15, 1992. Each dot point represents the mean · S.D. of 10-22 fish.

Table 4. Mean lengths, weight and condition factors of 8 to 22 month old Lake Whatcom, 1990 cohort, kokanee salmon. Also included is the percentage in parr, transition or smolt coloration, as well as those classified residualized (de-smolted) at each time interval.

Sampling date	Sample (size)	Mean length (mm ± SD)	Mean weight (mm ± SD)	Mean (±SD)	Coloration (%)			
					parr	transition	smolt	residualized
Jul 91	20		5.3 ± 1.4		100	0	0	0
Aug 91	20		7.4 ± 2.1		100	0	0	0
Sep 91	20		12.2 ± 3.4		100	0	0	0
Oct 91	20		12.2 ± 3.4		100	0	0	0
Nov 91	15	125.7 ± 15.5	21.4 ± 8.8	1.08 ± .04	100	0	0	0
Dec 91	20	125.8 ± 8.5	17.7 ± 4.0	0.88 ± .10	100	0	0	0
1-8-92	20	119.5 ± 13.2	15.1 ± 5.5	0.84 ± .09	80	20	0	0
1-22-92	21	118.9 ± 12.4	14.4 ± 4.4	0.83 ± .06	85	15	0	0
2-5-92	20	122.6 ± 12.7	16.4 ± 5.5	0.86 ± .07	90	10	0	0
2-19-92	20	121.1 ± 14.8	15.3 ± 6.4	0.82 ± .10	80	20	0	0
2-26-92	20	124.3 ± 16.2	17.3 ± 8.2	0.84 ± .09	65	30	5	0
3-17-92	20	124.8 ± 9.1	17.3 ± 4.1	0.87 ± .06	85	15	0	0
4-1-92	20	133.6 ± 17.2	22.0 ± 9.2	0.88 ± .09	5	35	60	0
4-14-92	20	149.1 ± 20.0	30.3 ± 13.7	0.86 ± .06	20	50	30	0
4-29-92	20	149.2 ± 18.3	29.5 ± 12.6	0.84 ± .06	0	85	15	0
5-13-92	11	158.5 ± 19.9	38.7 ± 19.1	0.91 ± .10	0	9	91	0
5-27-92	10	168.6 ± 16.6	46.0 ± 14.6	0.93 ± .06	0	30	70	0
6-8-92	10	183.1 ± 22.5	58.9 ± 25.0	0.91 ± .07	0	0	90	10
7-10-92	10	204.5 ± 26.6	90.8 ± 36.6	1.00 ± .08	0	0	30	70
8-10-92	5	211.6 ± 21.3	99.8 ± 31.3	1.03 ± .07	0	0	0	100

Mean nonspecific binding (± 95% Cl) for 16 replicate tubes was measured at 319 ± 33 counts per minute (cpm) (Table 6), compared to 69,762 ± 467 cpm measured in total count tubes (TCT). Fourteen of 16 blank tubes contained 0 cpm, one blank tube contained 3 cpm and one blank tube contained 4 cpm of radioactivity (Table 6). The two blank tubes containing radioactivity could have been contaminated during the blotting process when the nonbound fraction was decanted. Another possibility is that the background level in these tubes was slightly higher than the average background.

The accuracy of individuals who pipetted the 25 µl samples and 1.0 ml of radiolabelled T_4 is recorded in Table 7. The mean cpm (± 95% Cl) of 40 tubes that received 25 µl of ^{125}I-T_4 was 1,756 ± 63 for a total error of 5.9%. Coefficient of variation was 5.9. The mean cpm (± 95% Cl) of 40 tubes that received 1 .0 ml of ^{125}I-T_4 was 69,086 ± 304 for a total error of <1%. The coefficient of variation was 0.7.

Results of analysis of duplicate samples are presented in Table 8 and Fig. 8-10. The mean percent error (± 95% Cl) of 129 duplicate samples of Lake Whatcom kokanee (1990 cohort) blood was 1.6 ± 0.2% (Table 8, Fig. 8). The mean percent error (± 95% Cl) of 313 duplicate Lake Whatcom kokanee (1991 cohort) whole body samples was 8.4 ± 0.9% (Table 8, Fig. 9). The mean percent error (± 95% Cl) of 276 Lake Pend Orielle kokanee (1991 cohort) whole body samples was 8.6 ± 1.0% (Table 8, Fig. 10). Frequency distributions of the percent error of duplicate blood or whole body samples are recorded in Fig. 8-10.

Results and statistical comparisons of interassay pool (IAP) samples and standard curve concentrations for each of the four assays are recorded in Table 9. Mean concentration and 95% confidence intervals for four replicates of each IAP sample and four-six replicates of each standard curve sample in each of the four assays were remarkably uniform (Table 9). No statistically significant differences were detected for either IAP or standard curve samples (Table 9).

Results and statistical comparisons of standard curve samples used to check for drift between the start and finish of each assay are recorded in Table 10. 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

Table 5. Results of quality assurance samples. Measured concentrations were the mean ± 95% CI of 10 replicates for each sample. Actual concentrations and acceptable ranges were provided by Diagnostic Products Corporation.

Sample	Actual concentration (ng/dl)	Acceptable range (ng/dl)	Measured concentration--Observed mean ± 95% CI (ng/dl)	Observed range (ng/dl)
Low	2.3	1.9 - 2.7	2.3 ± 0.3	1.9 - 2.8
Medium	7.3	6.2 - 8.4	6.9 ± 0.3	6.5 - 7.5
High	11.5	10.0 - 13.0	10.5 ± 0.5	9.2 - 11.6

Table 6. Results of quality assurance procedures used to determine nonspecific binding and the degree of contamination.

Assay #	Non specific binding			Blank tubes	
	Sample Size (n)	Mean cpm	± 95%CI	Sample Size (n)	Counts
1	4	341	41	4	0,0,0,0
2	4	327	33	4	0,3,0,0
3	4	299	28	4	0,0,4,0
4	4	309	30	4	0,0,0,0
Mean	16	319	33	16	0 ± <1

Table 7. Pipetting accuracy of 25 µl and 1.0 ml samples. Counts = counts per minute of radiolabelled T_4. Percent error = standard deviation (S.D.) ÷ mean counts.

Sample	Assay #	Sample size (n)	Mean # of counts ± S.D.	Percent error (%)	Coefficient of variation
25µl	1	10	2049 ± 42	3.3	3.3
	2	10	1943 ± 49	4.1	4.1
	3	10	1439 ± 24	2.6	2.7
	4	10	1592 ± 136	13.7	13.8
Mean Error			1756 ± 63	5.9	5.9
1.0 ml	1	10	81.248 ± 320	<.1	0.6
	2	10	76,876 ± 402	<.1	0.8
	3	10	59,091 ± 273	<.1	0.7
	4	10	61,529 ± 219	<.1	0.5
Mean error			69,086 ± 304	<.1	0.7

Table 8. Percent error (± 95%CI) of duplicate kokanee salmon whole body and blood samples.

Stock (cohort)	Type of Sample	Population Statistics	
		# of duplicate samples (n)	Mean percent error (%) ± 95% CI
Lake Whatcom (90)	blood	129	1.6 ± 0.2
Lake Whatcom (91)	Whole body	313	6.4 ± 0.9
Lake Pend Oreille (91)	Whole Body	276	8.8 ± 1.0

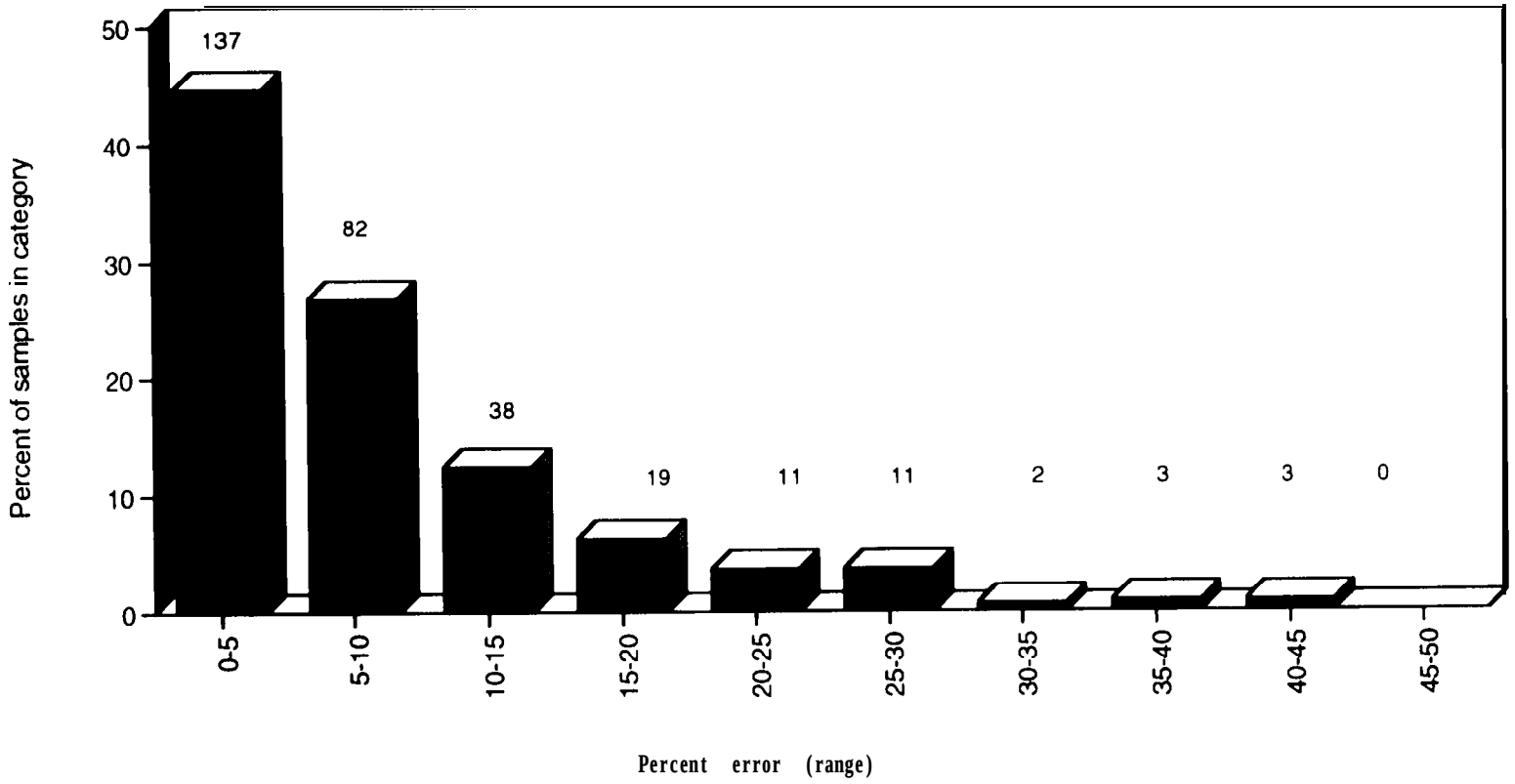


Fig. 8 Frequency distribution of percent error of duplicate Lake Whatcom, 1991 cohort, kokanee salmon egg and larvae samples. The total sample size (n) was 306 duplicate samples. The number of samples in each category are noted above the bar graph. These numbers were converted to percentages of the total sample for plotting the frequency distribution.

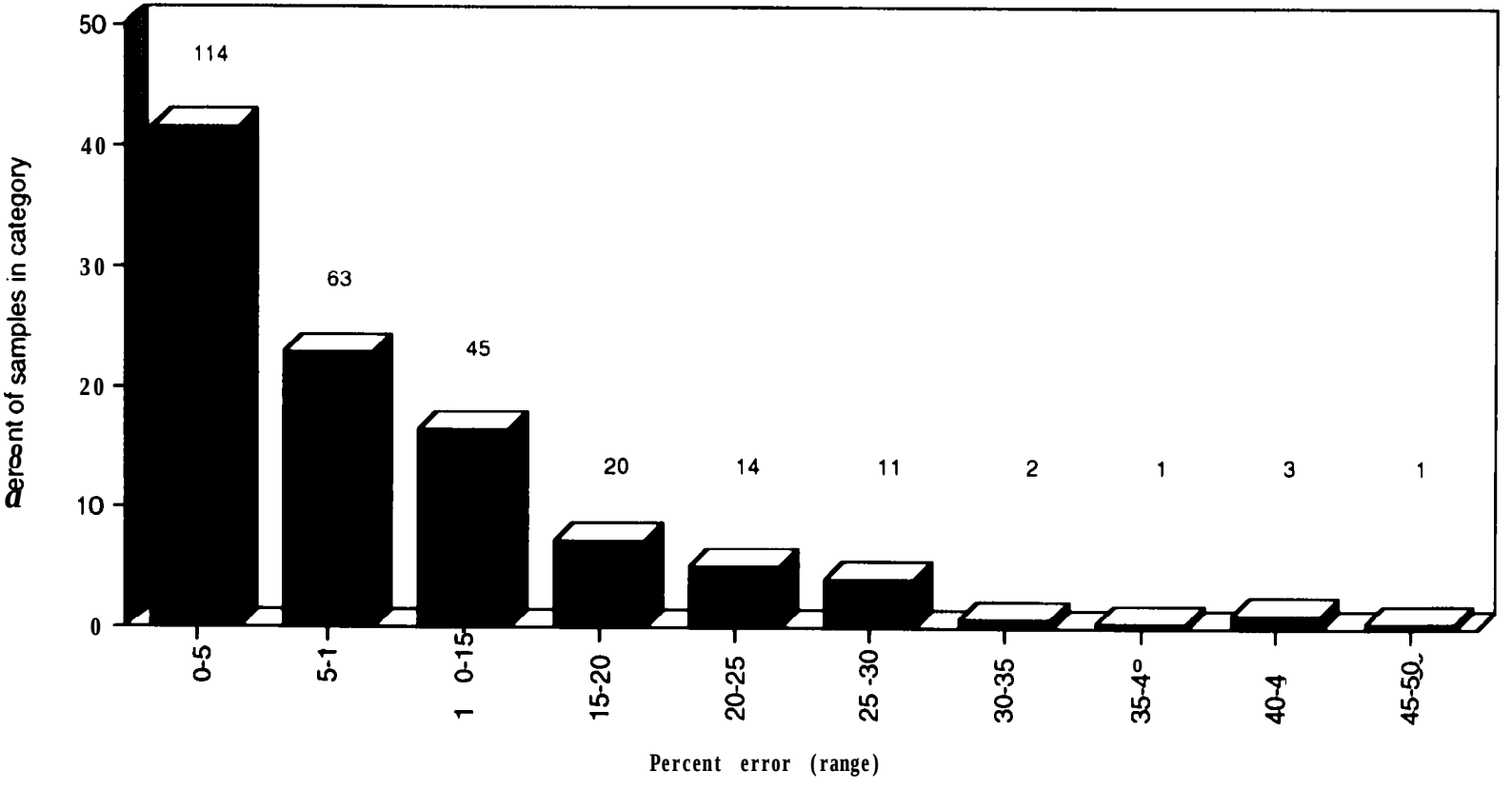


Fig. 9 Frequency distribution of percent error of duplicate Pend Orielle, 1991 cohort, kokanee salmon egg and larvae samples. The total sample size (n) was 274 duplicate samples. The number of samples in each category are noted above the bar graph. These numbers were converted to percentages of the total sample for plotting the frequency distribution.

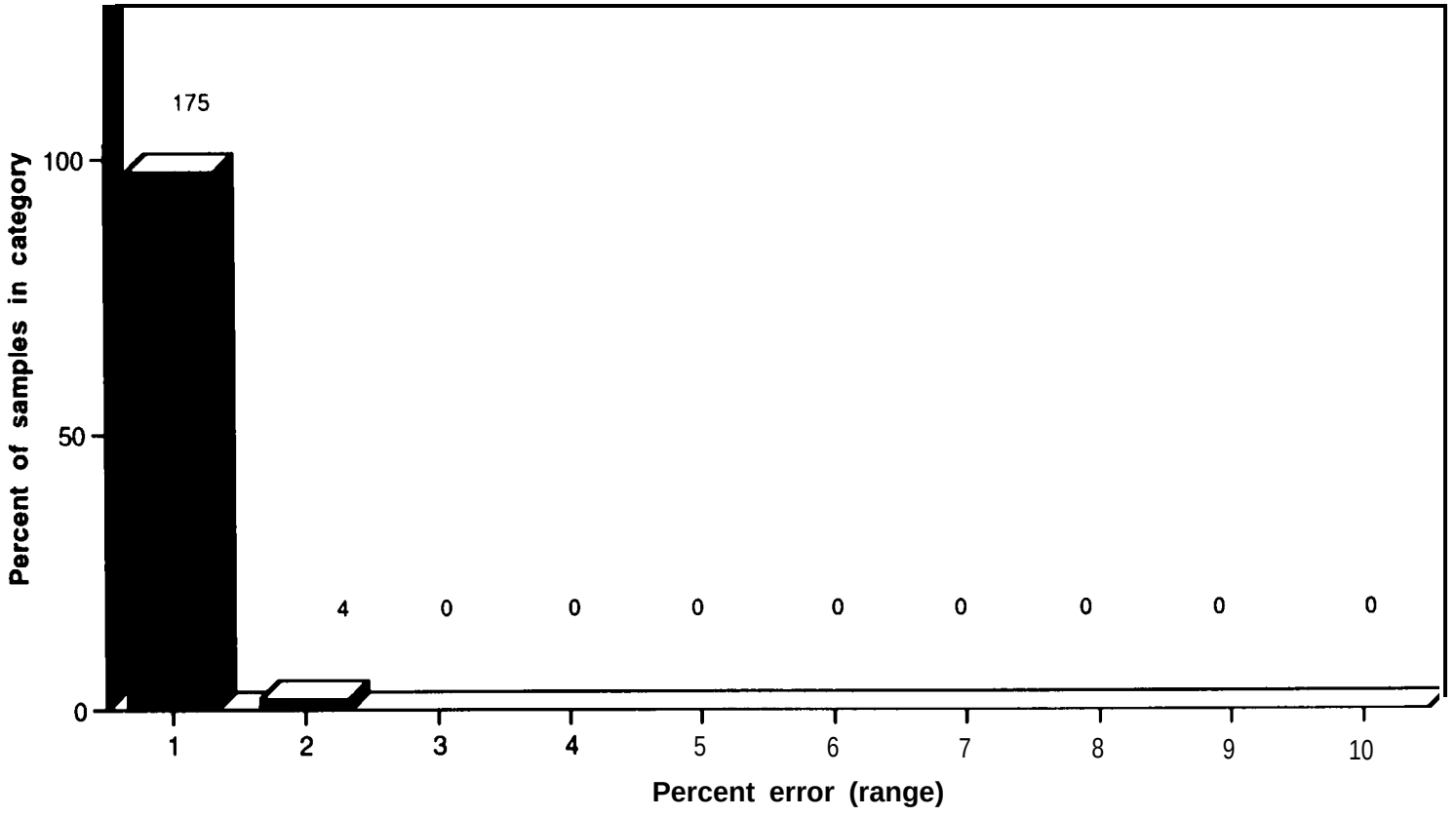


Fig. 10 Frequency distribution of percent error of duplicate Lake Whatcom, kokanee salmon blood samples. The total sample size (n) was 179 duplicate samples. The number of samples in each category are noted above the bar graph. These numbers were converted to percentages of the total sample for plotting the frequency distribution.

Table 9. **Statistical comparisons of mean values between interassay pool (IAP) and standard curve concentration (STDCRV) in four assays. Concentrations were in ng/dl. Sample size was six replicates of each IAP sample and each STDCRV sample per assay. Concentrations are presented as mean ± 95% confidence intervals (95% CI).**

Sample	First Assay (mean ± 95% CI)	Second Assay (mean ± 95% CI)	Thrid Assay (mean ± 95% CI)	Fourth Assay (mean ± 95% CI)	Wilcoxon signed rank comparisons with assay #			
					Assay #	2	3	4
IAP 1	2.4 ± 0.2	2.2 ± 0.3	2.6 ± 0.3	2.4 ± 0.4	1	z = 0 p = 1 ns	Z = 0 p = 1 ns	z = -0.47 p = .654 ns
IAP 2	6.9 ± 0.4	6.7 ± 0.6	7.0 ± 0.6	6.8 ± 0.9				
IAP 3	10.2 ± 0.8	10.8 ± 0.6	9.8 ± 0.9	11.2 ± 0.7	2		z = 0 p = 1 ns	z = -1.604 p = 0.1088 ns
					3			z=0 p=1 ns
STDCRV 0.5	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	1	z = 0 p = 1 ns	z = 0.94 p = 0.334 ns	z = -1.57 p = 0.114 ns
STDCRV 1.0	1.2 ± 0.2	1.1 ± 0.2	0.9 ± 0.1	0.9 ± 0.1				
STDCRV 4.0	4.0 ± 0.1	3.8 ± 0.2	4.3 ± 0.4	4.2 ± 0.3				
STDCRV 10.0	9.4 ± 0.5	9.9 ± 0.4	10.3 ± 0.5	10.6 ± 0.7	2		z = 1.49 p = .136 ns	z = -1.75 p = .079 ns
STDCRV 16.0	15.2 ± 0.6	15.9 ± 0.8	16.0 ± 1.0	16.9 ± 1.6				
STDCRV 24.0	23.3 ± 1.1	22.8 ± 1.4	23.2 ± 0.8	23.9 ± 1.4	3			z = -1.61 p = .104 ns

10). No statistically significant differences were recorded between the two sets of standard curve samples at the front and end of any of the four assays (Table 10).

3.6 Recovery determination

Details of recovery of T_4 from whole body samples was presented in our 1991 annual report (Scholz et *al.* 1992). Results indicated 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.

Table 10. Concentration of measured standard curve samples placed at the front and end of four assays to control for drift. Each number represents the mean value of a duplicate set of samples run at the front and end of each assay.

Assay No.	Placement in Assay	Concentration(ng/dl) 0.5	1.0	4.0	10.0	16.0	24.0	Wilcoxin signed rank statistic Z	P	S/NS
1	Front	0.4	1.1	4.1	9.9	15.7	23.6	-0.527	0.5982	NS
	End	0.6	1.3	4.0	9.6	15.6	24.0			
2	Front	0.5	0.9	3.8	10.3	16.9	23.2	-0.105	0.9165	NS
	End	0.4	1.1	4.0	9.9	15.4	25.0			
3	Front	0.5	0.9	4.6	10.2	15.4	22.7	-1.363	0.173	NS
	End	0.4	1.1	4.2	10.9	16.6	23.6			
4	Front	0.4	1.3	4.1	9.8	15.3	22.6	-1.572	0.1148	NS
	End	0.6	0.7	4.4	11.2	18.1	24.0			
Overall Mean ± 95% CI		0.49 ±0.05	1.05 ±0.13	4.15 f0.17	10.22 ±0.37	16.12 f0.68	23.59 ±0.52			

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 (1991 cohort), the concentration of the surge at the time of hatch (13.1 ± 2.5 ng/g body weight) was approximately 1.9 X (i.e., 199%) that of basal levels observed in recently eyed eggs (6.7 ± 1.3 ng/g body weight). After T_4 levels had declined to as low as 10.3 ± 1 .1 ng/g body weight in pre-swimup alevins, a second peak in T_4 concentration (22.1 ± 5.2 ng/g body weight) was observed at the time of swim-up (i.e., about a 214% increase). A similar pattern was observed in Lake Pend Orielle stock fish, with the concentration of the T_4 surge at the time of hatch (24.2 ± 4.5 ng/g body weight) approximately 2.54 X (i.e., 254%) that of basal levels observed in recently eyed eggs (9.5 ± 17 ng/g body weight). In the Pend Orielle stock, T_4 concentrations peaked a second time at swimup (24.3 ± 3.8 ng/g body weight), which was an increase of 187% over alevins shortly after hatch (13.0 ± 2.9 ng/g body weight). In both stocks, thyroxine levels declined to 0.5 to 2.0 ng/g body weight 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 in 9 of 10 replicates at the low concentration, 10 of 10 replicates at the medium concentration and 8 of 10 replicates of the high concentration. The percent error, and especially the upper range in error of duplicate samples from both Lake Whatcom and Lake Pend Orielle stock fish, was relatively high. However, the mean percent error (± 95% CI) of both sets of duplicate samples, 8.4 ± 0.9% for Lake Whatcom stock and 8.6 ± 1 .0% for Lake Pend Orielle stock, were well below the 187 to 254% 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 than aqueous solutions and, therefore, more difficult to pipette with uniform accuracy; and (2) a 5.9% error associated with pipetting 25 µl samples. The mean percent error (±95% CI) of duplicate samples (8.2 to 8.4%) could be largely explained by the 5.9% 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 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 4 hours, whereas those collected from older, larger fry were not completely evaporated until 8-24 hours. The difference was owing to the amount of PTU/ethanol solution used for extraction, since the volume used depended upon body weight. Low values of T_4 reported for the older fry (collected from April to July) could be partly related to decomposition of thyroid hormones in these samples owing to their relatively longer drying time. However, 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 100 fold difference between recently hatched eggs containing 13.1 to 24.3 ng/g body weight T_4 -v- 176 day old fry containing 1 .0 - 2.0 ng/g body weight T_4 .

4.2 Comparison of 1992 experimental results with 1991 results.

Our results with Lake Whatcom kokanee (1991 cohort) reported here were uniform with those collected with Lake Whatcom kokanee (1990 cohort) reported in our 1991 annual report (Scholz et ***al.*** 1992). Fig. 11 compares whole body T_4 fluctuations in both years. In both cases T_4 peaks occurred at hatch and swimup, and T_4 concentration levels declined to low levels in post swim-up larvae.

In 1991, whole body T_4 concentrations were measured in 487 Lake Whatcom stock kokanee. 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. Thus, both the pattern of T_4 fluctuations, as well as absolute concentrations, were uniform in both 1990 and 1991 year class Lake Whatcom kokanee.

Moreover, the magnitude of fluctuations at the time of hatch and swimup were similar for each of the two cohorts. For the 1990 cohort, the concentration at hatch (18.7 + 6.8 ng/g body weight) was approximately 1.7 X that of basal levels observed in eggs (12.5 ± 1.7 ng/g body weight). For the 1991 cohort, the concentration at hatch (13.1 ± 2.5 ng/g body weight) was approximately 1.9 X that of the basal level in eggs

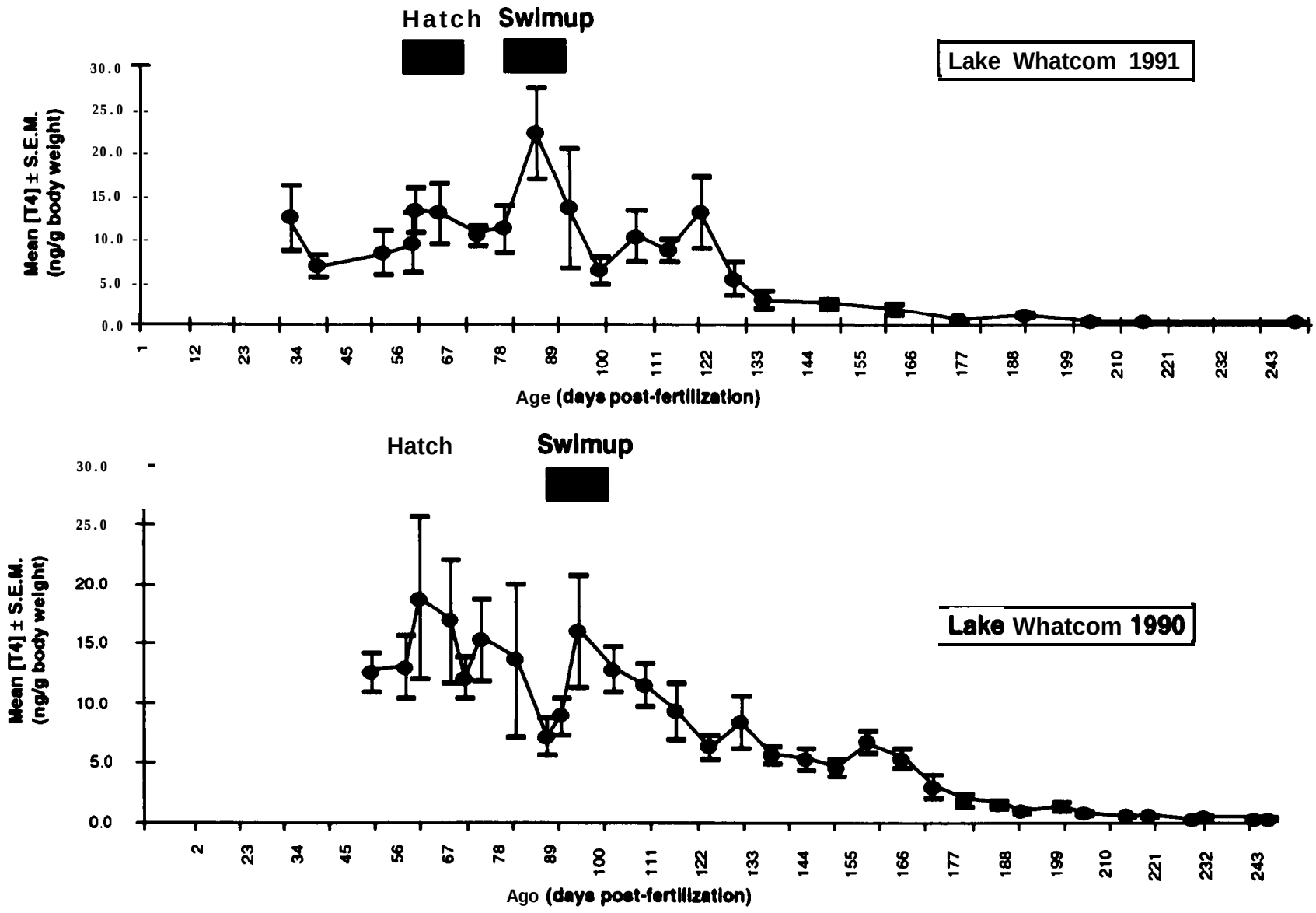


Fig. 11 Comparison of ontogenetic pattern of thyroxine content in two cohorts of Lake Whatcom stock kokanee reared at the Spokane Tribal Kokanee Ha

(6.7 ± 1.3 ng/g body weight). In the 1990 year class, T_4 levels increased 225% between post-hatch and swimup stages. In the 1991 year class T_4 levels increased 214% between post-hatch and swimup stages. Given these data, we conclude that the results of the present investigation replicated our preliminary results to a high degree.

4.3 Patterns of T_4 fluctuations in kokanee salmon eggs and larvae.

Results from the present study, as well as our previous investigation (Scholz et *al.* 1992) indicated a specific pattern of T_4 fluctuations during kokanee development. In the four groups examined-- 1990 and 1991 cohorts from Lake Whatcom, 1991 cohort from Lake Pend Orielle, and 1990 cohort from Lake Roosevelt-- the following results occurred:

(1) In all four cases, T_4 concentration was relatively high in eggs and alevins;

(2) In three cases, T_4 peaks occurred at hatch and swimup. (In the fourth case, T_4 concentration was not measured at hatch and swimup in Lake Roosevelt fish because of low numbers); and

(3) In all four cases, T_4 concentration were low in post swimup fry.

In the three cases where T_4 peaks occurred at hatch and swimup, weight loss was observed. For example, in 1990 year class Lake Whatcom kokanee, weight at the time of hatch was 47 mg compared to egg weights of 76 mg. In 1991 year class Lake Whatcom kokanee, weight at the time of hatch was 50 mg compared to egg weights of 55-60 mg. In 1991 year class Pend Orielle Lake kokanee, weight at the time of hatch was 50 mg, compared to egg weights of 55-60 mg. Thus, since the T_4 peak coincided with rapid loss of body weight, 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. 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 decreases.

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). Thyroxine levels remained at about the same concentration in pink, chum, and sockeye throughout this period, i.e., species which migrate shortly after emergence from redds, whereas T_4 levels in coho and chinook declined significantly. Transitory increases in 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.

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

(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 T4 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 concentration declines to very low levels.

Our results may indicate a critical period for imprinting in kokanee salmon at hatch or swimup, if thyroid surges in kokanee are correlated with imprinting as they are in smolt stage coho salmon and steelhead trout (reviewed by Hasler and Scholz 1983; Scholz et *al.* 1985, 1992).

4.4 Artificial imprinting investigations.

Currently, we are conducting studies to further delineate the critical period for imprinting in kokanee by exposing fish to synthetic chemicals at ten different life history stages: (1) fertilized to eyed egg; (2) eyed egg to hatch; (3) hatch; (4) hatch to swimup; (5) swimup; (6) post-swimup fry (February); (7) post swimup fry (March); (8) post swimup fry (April); (9) post swimup fry (May-July); and (10) smolts (age 16-18 months). (See Sections 2.2 and 3.1 of this report for details.) At each stage fish were exposed to either morpholine or PEA. We plan to conduct two types of tests to determine at which life stage(s) imprinting occurs.

First, most of the fish from each group were released into Lake Roosevelt at Sherman Creek Hatchery to conduct a field test. During the spawning season (August-November 1993 and 1994 for smolts released in 1991, and August-November 1994 and 1995 for fry released in 1991) morpholine (at a steady state concentration of 5×10^{-5} mg/l) will be metered into the fish ladder leading to the hatchery trap at Sherman Creek. Phenethyl alcohol (5 x 10-3 mg/l) will be metered into a trap installed at a different location to be selected-- probably either a trap installed in the Colville River, which is located across the reservoir from Sherman Creek or a trap at Little Falls Dam. The number of morpholine-exposed and PEA-exposed fish (marked with group specific coded wire tags) from each life stage group homing to each trap will be counted.

If, for fish exposed at a certain life stage, morpholine-exposed fish home preferentially to the morpholine-scented trap and PEA-exposed fish home to the PEA-scented trap, this result would indicate that fish-exposed at that particular life stage had imprinted to their respective odor. By comparing the relative rates of return of fish exposed at each life history stage, we hope to define the critical period(s) for imprinting.

For the second experiment, approximately 400 morpholine-exposed fish and 400 phenethyl alcohol-exposed fish from each life history stage were retained at the Spokane Tribal Hatchery for approximately 12-l 8 months after their exposure period before behavior tests were conducted. During the intervening period they were not again exposed the synthetic chemicals. Commencing in June 1993 after the summer solstice, we plan to inject gonadotropic hormone (3 times/wk x 12 weeks) to stimulate

gonad development in immature fish and bring them into a migratory disposition. Our intent is to mimic the physiological state and activate the upstream migratory drive of naturally spawning kokanee. Behavioral tests for odor discrimination will then be conducted from August to October 1993.

Odor discrimination tests will be conducted in a braided stream channel at Chamokane Creek adjacent to the Spokane Tribal Hatchery. The fish will be released below the junction of two channels, and morpholine or phenethyl alcohol will be metered randomly into one of the channels. A response will require upstream migration and selection of the channel scented with the correct treatment odor. Hence, this experiment will duplicate the kind of choice that must be made by naturally migrating adult fish. Prior to conducting experiments, Rhodamine B dye will be released at the point in each branch where morpholine of phenethyl alcohol will be introduced to map the odor trails downstream to the point where fish are released. Several morpholine and PEA-exposed fish from each exposure period, identified by an individually numbered floy tag or specific frequency radiotransmitter, will then be released about 100-150 meters below the confluence of the two channels.

Floy tagged fish will be recaptured in weirs that will be constructed in each branch channel about 50-100 meters upstream from the confluence, or in a fish trap located on the main channel about 250 meters downstream from the release point. Radio-tagged fish will be located with a directional antenna connected to a multi-channel receiver at intervals of 5 minutes and their tracks plotted on a topographic map of the stream. Morpholine, PEA or no odor (control) will be metered randomly into one of the channels and the number of morpholine-exposed and PEA-exposed fish from each treatment group captured in each trap will be determined. Each fish will be tested three times, once with its treatment odor present in one branch, once in the second branch, and once with the odor absent. For a fish to be classified as imprinted, we will require that three criteria be met:

(1) upstream migration if the treatment odor is present;

(2) selection of the tributary scented with the correct odor; and

(3) downstream migration if the odor is absent.

Fish meeting these criteria will be classified as 'imprinted' and those not fulfilling them will be classified as 'not imprinted.' Our criteria were derived from the work of Johnsen and Hasler (1980) who observed that sexually mature coho salmon, that had been exposed to morpholine during the smolt stage, migrated upstream if morpholine was present in the stream and downstream if morpholine was absent. These results indicated that the odor acted as a sign stimulus to release a stereotyped behavior, i.e., swimming against a current. Thus, Johnsen and Hasler (1980) concluded that presence of the odor evoked positive rheotaxis whereas absence evoked negative rheotaxis, which was the operational mechanism in selection of the home stream by olfactory orientation.

If floy tagged fish are random in their selection of the two branch channels, and if radio-tagged fish spend uniform amounts of time in each channel, the null hypothesis (*i.e.*, there is no difference in the behavior of fish exposed to different odors at a selected life history stage) would be supported. This result would imply that the fish did not imprint to their exposure odor at that life history stage.

If morpholine-exposed fish are captured in (or tracked into) the morpholine-scented channel when morpholine is present and in the downstream trap when morpholine is absent; if PEA-exposed fish are captured (or tracked into) the PEA-scented channel when PEA is present and in the downstream trap when PEA is absent; and if morpholine and PEA-exposed fish switch their channel preference when their odors are switched; the alternative hypothesis (*i.e.*, there is a difference in the behavior of fish exposed to morpholine and PEA at that selected life history stage) would be supported. This result would indicate that morpholine and PEA-exposed fish at a certain life stage: (1) became imprinted to their treatment odor at that life stage, (2) retained the odor memory during the 12-18 months period intervening between the time of odor exposure and the time that odor discrimination experiments were conducted, and (3) homed to the odor as sexually mature fish.

Results of the experiment should:

(1) Determine 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 correlated with the critical period for imprinting time in kokanee.

4.5 Management recommendations

In the present study 1+ year old kokanee appeared to undergo some of the behavioral and physiological transitions associated with smolt transformation. If 0+ fry planted in Lake Roosevelt undergo smoltification as 1+ fish in the reservoir, they may have a tendency to migrate out of the reservoir at that time. If so, it is possible that entrainment losses could be reduced or avoided if the fish are stocked as residualized post-smolts instead of as fry. Consequently, we recommend that three types of investigations be accomplished:

(1) Physiological and behavioral transitions should be defined in kokanee to determine if age 1+ kokanee undergo complete or partial smoltification. Tests should include determination of: (a) downstream migratory tendency; (b) degree of silvering and loss of parr marks; (c) development of salinity tolerance; (d) osmoregulatory capability; (e) fluctuations in gill Na+/K+ ATPase activity; (1) salt water preference; (g) change in intestinal water uptake (jv); and (h) change in condition factor. These investigations were initiated in 1993.

(2) Tag/recapture experiments should be conducted to compare angler harvest, amount of entrainment and homing to egg collection sites of fish released as age 0+ fry -v- fish released as age 1+ residualized post-smolts. In 1992, we initiated such an experiment by releasing 0+ fry and age 1+ fish into Lake Roosevelt. We plan to repeat this experiment in 1993. Tag recoveries will be made by anglers, by conducting electrofishing and gill net surveys in Lake Roosevelt, monitoring traps at egg collection sites,

and monitoring passage at counting stations at Rock Island and McNary Dams.

(3) Age 0+ kokanee fry should be placed in Lake Roosevelt net pens in October and held until the following July for tagging and release into the reservoir as age I+ post-smolts. The primary reason for conducting this study is that net pen rearing costs less that hatchery rearing (Peone et al. 1990). If fish have to be held until age I+ instead of age 0+ before release in Lake Roosevelt, it would probably be cheaper to rear them in net pens than at the hatchery. Therefore, efficacy of net pen rearing should be determined by comparing food conversion, survival rates, angler harvest rates, entrainment over Grand Coulee Dam and homing to egg collection sites of matched groups of kokanee, marked and released from hatcheries and net pens.

Our data indicate that different kokanee stocks and year classes evidenced a similar pattern of T4 fluctuations with peaks at hatching and swimup, followed by a subsequent decline in post swimup larvae. Thus, 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 swimup stages. Therefore, we recommend that for hatcheries using synthetic chemical imprinting, the best exposure period would be from 1 week pre-hatch to 1 week post-swimup. Since T_4 levels in post swimup larvae were low, we believe that it is unlikely that kokanee transplanted as 6-8 month old fry will site imprint to stocking sites.

LITERATURE CITED

Cooper J.C. and A.T. Scholz. 1976. Homing of artificially imprinted steelhead trout. Journal of the Fisheries Research Board of Canada 33: 826-829.

Hasler, A.D. and A.T. Scholz. 1983. Olfactory Imprinting and Homing in Salmon: Investigations into the Mechanism of the Imprinting Process. Zoophysiology, Vol. **14.** Springer-Verlag, Berlin, Heidelberg, New York, Tokyo. 134 pp.

Hasler, A.D. and W.J. Wisby. 1951. Discrimination of stream odors by fishes and relation to parent stream behavior. American Naturalist 85: 223-238.

Hasler, A.D., A.T. Scholz and R.M. Horrall. 1978. Olfactory imprinting and homing in salmon. American Scientist 66: 347-355.

Jensen, A., and R. Duncan. 1971. Homing in transplanted coho salmon. Progressive Fish-Culturist 33: 216-l 8.

Johnsen, P.B. and A.D. Hasler. 1980. The use of chemical cues in the upstream migration of coho salmon, Oncorhynchus kisufch, Walbaum. Journal of Fish Biology 17: 67-73.

Kobuke, L, J.L. Specker and H.A. Bern. 1987. Thyroxine content of eggs and larvae of coho salmon, Oncorhynchus *kisutch.* Journal of Experimental Zoology 242: 89-94.

Lanier, T.l. 1987. Correlation of 3,5, 3' triiodothyronine surges with cytological change in the neural tissues of steelhead trout (Salmo *gairdneri* Richardson). MS thesis. Eastern Washington University, Cheney, WA. 96 pp.

Leatherland, J.F., L. Lin, N.E. Down, and EM. Donaldson. 1989. Thyroid hormone content of eggs and early developmental stages of five *Oncorhpchus* species. Canadian Journal of Fisheries and Aquatic Sciences 46: 2140-2145.

Northwest Power Planning Council. 1987. Columbia River Basin Fish and Wildlife Program. Northwest Power Planning Council, Portland, OR.

Paragamian, V.L., V.L. Ellis and R. Gariss. 1991. Kokanee stock status and contribution of the Cabinet Gorge kokanee hatchery, Lake Pend Oreille, Idaho. Idaho Department of Fish and Game, Boise, ID. Annual Progress Report submitted to U.S. Department of Energy, Bonneville Power Administration, Division of Fish and Wildlife. Project No. 85-339. Contract No. DE-A179-85BP22493. 69 pp.

Parker, S.J. 1988. Thyroid Hormone Levels in Larval and Juvenile Striped Bass *Morone saxafilis:* Response to Changes in Salinity and Temperature. Ph.D. thesis. University of Rhode Island, Providence, RI. 147 pp.

Scholz, A.T. 1980. Hormonal Regulation of Smolt Transformation and Olfactory Imprinting in Coho Salmon. Ph.D. thesis. University of Wisconsin, Madison, WI. 363 pp.

Scholz, A.T., C.K. Gosse, J.C. Cooper, R.M. Horrall, A.D. Hasler, R.J. Poff and R.I. Daly. 1978a. Homing of rainbow trout transplanted in Lake Michigan: a comparison of three procedures used for imprinting and stocking. Transactions of the American Fisheries Society 107: 439-444.

Scholz, A.T., J.K. Uehara, J. Hisata and J. Marco. 1986. Feasibility report on restoration and enhancement of Lake Roosevelt Fisheries. In: Northwest Power Planning Council. Columbia Basin Fish and Wildlife Program--Applications for Amendments. Volume 3A; 1375-I 489.

Scholz, A.T., R.J. White, M. Muzi, and T. Smith. 1985. Uptake of radiolabelled triiodothyronine in the brain of steelhead trout *(Salmo gairdnen)* during parr-smolt transformation: Implications for the mechanism of thyroid activation of olfactory imprinting. Aquaculture 45: 199-214.

Scholz, A.T., R.J. White, V.A. Koehler and S.A. Horton. 1992. 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. Upper Columbia United Tribes Fisheries Center, Department of Biology, Eastern Washington University, Cheney, WA. Supplement report submitted to U.S. Department of Energy, Bonneville Power

Administration, Division of Fish and Wildlife, Portland, OR. Project No. 88-63. Contract No. DE-Bl79-88BP91819. 96 pp.

Scholz, A.T., R.M. Horrall, J.C. Cooper and A.D. Hasler. 1976. Imprinting to chemical cues: the basis for homestream selection by salmon. Science 196: 1247-1249.

Scholz, A.T., R.M. Horrall, J.C. Cooper and A.D. Hasler. 1978b. Homing of morpholine-imprinted brown trout *(Salmo trutta)* Fishery Bulletin (United States) 76: 293-295.

Scholz, A.T., R.M. Horrall, J.C. Cooper, A.D. Hasler, D.M. Madison, R.J. Poff, and R. Daly. 1975. Artificial imprinting of salmon and trout in Lake Michigan. Wisconsin Department of Natural Resources Fisheries Management Report No. 80: 46 pp.

Sullivan, C.V., W.W. Dickhoff, S.D. Brewer, and G.P. Johnston. 1983. Plasma thyroid-hormone concentrations and gill (Na+/K+) ATPase activities in post emergent pink salmon. Transactions of the American Fisheries Society 112: 825-829.

Tagawa, M. and T. Hirano. 1987. Presence of thyroxine in eggs and changes in its content during early development of chum salmon, *Oncorhynchus keta.* General and Comparative Endocrinology 69: 129-135.

Tagawa, M. and T. Hirano. 1989. Changes in tissue and blood concentrations of thyroid hormones in developing chum salmon. General and Comparative Endocrinology 76: 437-443.

White, R.J., A.T. Scholz, M.V. Baker and D.E. Liljegren. 1991. Detection of triiodothyronine (T3) receptors in isolated steelhead trout, *Oncorhynchus mykiss,* brain nuclei. Journal of Fish Biology 36: 783-785.