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THYROID-INDUCED CHEMICAL IMPRINTING IN EARLY LIFE STAGES AND ASSESSMENT OF SMOLTIFICATION IN KOKANEE SALMON: IMPLICATIONS FOR OPERATING LAKE ROOSEVELT KOKANEE SALMON HATCHERIES

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Prepared by:

Mary Beth Tilson
Allan T. Scholz
Ronald J. White
Heather Galloway

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

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EXECUTIVE SUMMARY

In 1991, two hatcheries were built to provide a kokanee salmon and rainbow trout fishery for Lake Roosevelt as partial mitigation for the loss of anadromous salmon and steelhead caused by construction of Grand Coulee Dam. The Sherman Creek Hatchery, located on a tributary of Lake Roosevelt to provide an egg collection and imprinting site, is small with limited rearing capability. The second hatchery was located on the Spokane Indian Reservation because of a spring water source that supplied cold, pure water for incubating and rearing eggs. The Spokane Tribal Hatchery thus serves as the production facility. Fish reared there are released into Sherman Creek and other tributary streams as 7-9 month old fry. However, to date, returns of adult fish to release sites has been poor. If hatchery reared kokanee imprint to the hatchery water at egg or swimup stages before 3 months of age, they may not be imprinting as 7-9 month old fry at the time of stocking. In addition, if these fish undergo a smolt phase in the reservoir when they are 1.5 years old, they could migrate below Grand Coulee Dam and out of the Lake Roosevelt system. In the present investigation, which is part of the Lake Roosevelt monitoring program to assess hatchery effectiveness, kokanee salmon were tested to determine if they experienced thyroxine-induced chemical imprinting and smoltification similar to anadromous salmonids.

Determination of the critical period for olfactory imprinting was determined by exposing kokanee to different synthetic chemicals (morpholine or phenethyl alcohol) at different life stages (fertilized egg, eyed egg, hatch, alevin, swimup, fry--various intervals, smolt), and then measuring the ability to discriminate the chemicals as sexually mature adults. Discrimination was deduced by conducting behavioral tests in a natural Y-maze, and performing coded wire tagging investigations involving the release of kokanee into Lake Roosevelt and recapturing them at egg collection sites scented with the chemical during their spawning migration. Whole body thyroxine content and blood plasma thyroxine concentration was measured to determine if peak thyroid activity coincided with imprinting or other morphological, physiological or behavioral transitions associated with smoltification. Indices of smoltification, including silvering, condition factor, salt water tolerance, osmoregulatory capability, gill Na^+-K^+ ATPase activity, intestinal water absorption (Jv), salinity preference and downstream migratory behavior, were monitored at intervals of two weeks to one month.

In 1992, thyroxine content in eggs of 1991 year class kokanee ranged from 6.5-10.5 ng/g body weight. Peak levels were observed at hatch (15.2 ng/g body weight) and swimup (22.1 ± 5.2 ng/g body weight), then declined rapidly in post swimup fry (to 0.5 ± 0.1 ng/g body weight). Circulating thyroxine levels in yearling (1990 year class) fish peaked in April and May, when fish were smolts (at 27.29 ng/ml serum). These fish were exposed to synthetic chemicals at various life stages, and odor discrimination tests were conducted in the autumn of 1993 with individuals that had become sexually mature. Fish were released into a stream below the confluence of two tributaries. The synthetic chemicals were metered into traps located on either arm approximately 100 meters upstream from the junction. A trap was also placed about 200 meters downstream from the release point.

Results documented chemical imprinting in kokanee salmon concomitant with elevated thyroxine levels. The group of fish that had the highest whole body thyroxine content (swimup stage) also had the highest percentage (88%) of fish that were attracted reliably to their exposure odor in behavioral tests. Recently hatched eggs and alevins also had relatively high thyroxine content and displayed accurate homing in behavioral tests (69% and 81% respectively). Additionally, smolts experienced elevated plasma thyroid levels and tended to be attracted (67%) to their exposure odor in the behavioral tests. In contrast, pre-hatch eggs and post-swimup fry had relatively low thyroxine content and did not evidence selective attraction to their exposure odor (ranges were 18-27% and 4-14% respectively). These results indicated that kokanee salmon imprint to chemical cues during two sensitive (or critical) periods at the alevin/swimup and smolt stages. It should therefore be possible to imprint fish to a synthetic chemical at the Spokane Tribal Hatchery, then decoy the adult fish to the Sherman Creek Hatchery for egg collection.

Results of 1993 investigations (with 1992 year class kokanee) confirmed the thyroxine data from the 1992 investigations (with 1991 year class kokanee). Whole body thyroxine content was 8.3 ± 1.1 ng/g body weight in kokanee eggs. Thyroxine content peaked at about the time of hatch (15.1 ± 2.1 ng/g body weight) and again at swimup (19.1 ± 0.4 ng/g body weight). In post swimup larvae, T_4 levels declined to less than 2 ng/g body weight. Plasma thyroxine concentration levels for yearling kokanee increased significantly in February and April. Basal levels were 11 .O ng/ml compared to 15.2 ng/ml at peak. Odor discrimination tests with these fish will be conducted during the fall of 1994.

A total of 299 fish were recovered from the kokanee fry (432,446) and residualized post-smolts (167,397) which were exposed to synthetic chemicals, coded wire tagged (CWT) and released into Lake Roosevelt in 1992 and 1993. Of the total 299 fish recovered, 295 (98.7%) fish had been stocked as residualized smolts and four fish (1.3%) had been stocked as fry. The relative percentage in each category was in contrast to the relative percentages of fry (72.1%) and smolts (27.9%) of the total CWT fish released. Thus, fish released as fry seemed to be missing from the reservoir.

Preliminary results of the coded wire tagging investigations also paralleled the results of olfactory discrimination experiments with respect to homing of fish exposed to synthetic chemicals at the smolt stage in 1992 and released as residualized smolts. In 1993, some fish became sexually mature at age 2. Of these, 87.5% of morpholine exposed fish recovered were collected in the morpholine scented stream and 81.3% of the phenethyl alcohol exposed fish recovered were collected in the phenethyl alcohol scented stream. Too few fish exposed to synthetic chemicals at other life history stages and released as fry were recovered to assess imprinting effectiveness. Failure of these groups to home and be recovered at locations where synthetic chemicals were metered into the reservoir was attributed principally to kokanee undergoing partial smoltification about one year after release, which resulted in emigration from the reservoir before reaching adult size.

Results of the smoltification experiments on yearling (1991. year class) kokanee in 1993 indicated that:

(1) Gill ATPase increased from 5.1 μmol . Pi mg protein-l . h^{-1} in January to 13.7 μmol . Pi mg $protein^{-1}$. h-1 in April, and condition factor decreased from 1.3 to 0.82 at this time.

(2) Silvering started to increase in February at the time of a thyroxine surge, and continued through the spring.

(3) Blood plasma osmolarity of fish held in freshwater was stable at 330 mOsm/l from January to July, but the osmolarity of fish held in sea water (30 ppt) was elevated from January through April at about 569 mOsm/l/l, then decreased in May and June to near isosmotic levels (344 mOsm/l).

(4) In salinity tolerance tests, 100% of the fish survived in 1, 10, and 20 ppt salt water during all months tested (January-July). In 30 ppt salt water,

0% of the fish survived from January through April compared to 90-100% survival from May through July.

(5) In salinity preference tests, fish showed a slight preference for concentrated sea water in February (53%), which declined to 23% by June.

(6) Fish displayed an increased tendency to migrate downstream in March (66%) and again in May (65%), following thyroxine surges.

(7) Intestinal water absorption (Jv) peaked in January then steadily declined.

The smolt assessment investigation also ascertained that smolts began to residualize by May or June as indicated by:

(1) Thyroxine peaked in February and April, then declined by July.

(2) Loss of parr marks and silvering began to increase markedly in February and peaked in May when 100% of the individuals exhibited silvery smolt coloration. Parr marks reappeared in June on about 60% of the individuals.

(3) Downstream migratory activity peaked in March and May at 65%, but by June, only 40% of the fish evidenced downstream migratory activity.

(4) Condition factor, which declined from 1.3 to 0.8 during the spring, began to increase in June to about 0.9.

(5) Intestinal water uptake peaked in January and was lowest in June.

(6) Gill Na^+-K^+ ATPase activity peaked in mid April at 13.7 $\mu mol \cdot$ Pi mg protein-l $\cdot h^{-1}$. The value then decreased to 7.0 $\mu mol \cdot$ Pi mg protein-l $\cdot$ h-1 in May and remained low in June.

(7) Osmoregulatory activity and salt water tolerance became evident in May. In June, fish still displayed the ability to osmoregulate and survive in full strength sea water.

These results suggested that kokanee underwent at least partial smolt transformation compared to other species of anadromous salmonids. These kokanee

experienced thyroxine peaks, increased silvering, increased downstream migratory activity and an increase in adaptation to salt water in the late winter and spring. If fish released from the hatchery as 7-8 month old fry experience similar transitions in Lake Roosevelt when they are 12-19 months old, these conditions may contribute to emigration and entrainment through Grand Coulee Dam, and loss of the fish from Lake Roosevelt. However, if these fish are held until they residualize, they would be more likely to stay in the reservoir.

Since the ratio of fry to smolts in coded wire tagging investigation was about 3:1 released and 1:99 recovered, these data provide conclusive evidence that fish planted as residualized smolts provide significantly better returns to both the creel and egg collection sites. In fact, fish planted as fry are essentially not contributing to either category. Releasing fish as residualized smolts could be an effective measure for enhancing Lake Roosevelt kokanee.

In order to release kokanee as residualized smolts instead of fry, we recommend the following measures:

(1) A new production well capable of delivering 2-4 CFS of additional flow be drilled at Spokane Tribal Hatchery to augment the existing water supply. Only about 100,000 kokanee can be reared to residualized smolt size at the Spokane Tribal Hatchery under present conditions. With the additional water, approximately 400,000 fish could be raised to that size. Estimated costs are about $250,000 for the well and water supply line plus $35,000-$50,000/year in feed.

(2) Develop a net pen program similar to that used for rainbow trout. Zero age kokanee could be stocked into net pens in October and held through the following July for release into the reservoir as yearlings (age 1+ post-smolts). If fish are held past their smolt stage in net pens, they may residualize and stay in the lake instead of migrating below Grand Coulee Dam. Estimated cost for purchase of net pens, anchoring systems and startup cost is $250,000 plus annual operating costs of about $65,000/year. We believe that these are relatively low costs for the potential biological benefits, especially considering that it could help to reduce conflicts with other potential water needs for power production and anadromous fish.

A final recommendation is that the fish passage facility at Rock Island Dam begin counting earlier in the year (perhaps January or February) in order to give a clearer picture of when kokanee are moving downstream. We know from creel surveys that kokanee are concentrated in the forebay of Grand Coulee Dam from about January to June, so water releases at any time during that period have the potential to entrain kokanee. At present, Rock Island counts do not commence until April 1, and substantial numbers of kokanee that migrate downstream before April 1 could be missed.

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1 .O INTRODUCTION

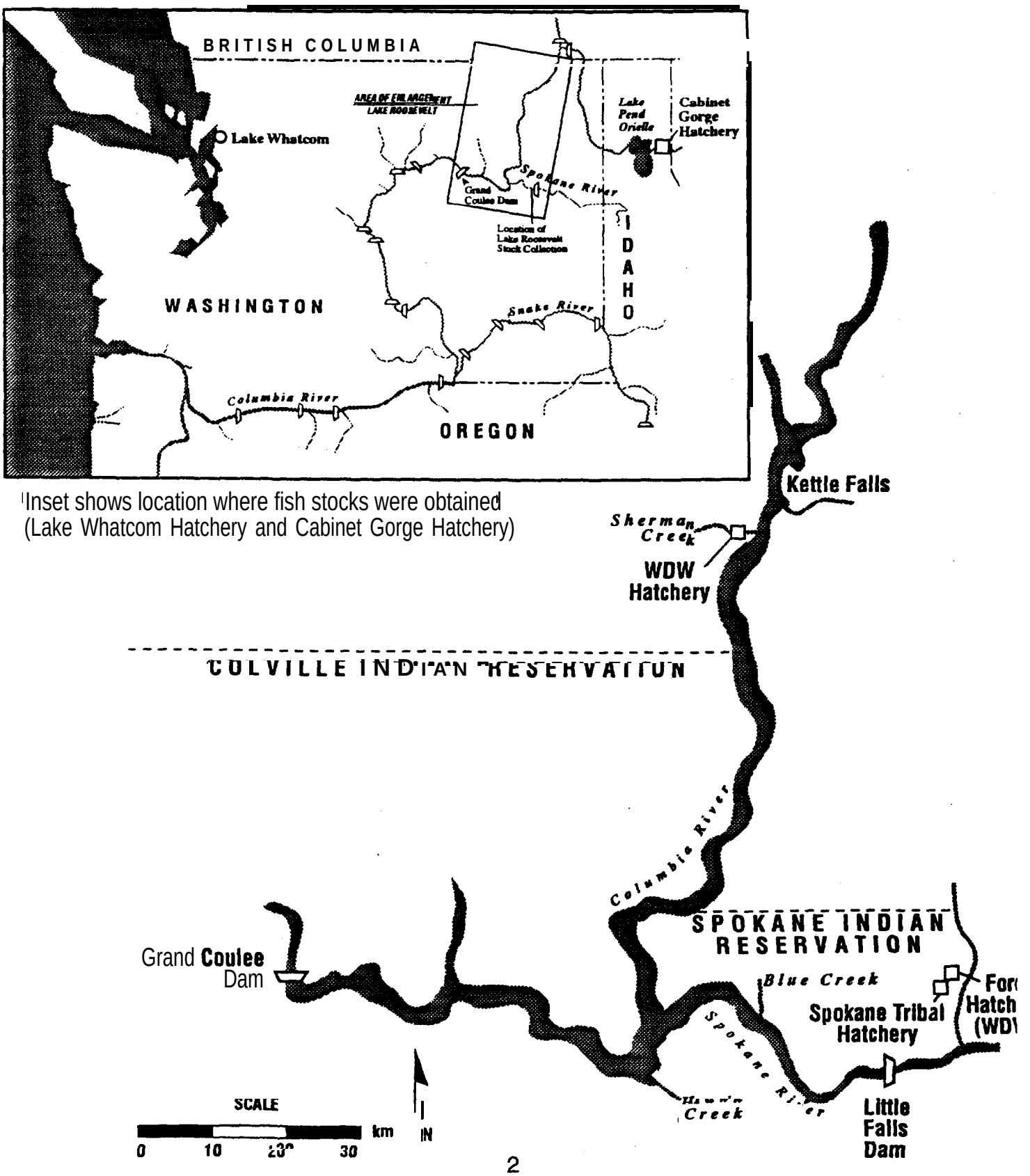
1.1 Lake Roosevelt Kokanee Hatcheries

In 1991, two kokanee hatcheries were built by Bonneville Power Administration (BPA) on Lake Roosevelt, which is the reservoir impounded by Grand Coulee Dam. Grand Coulee Dam had no fish ladder that allowed adults to return to natal spawning areas upstream from the dam. In its 1987 Columbia Basin Fish and Wildlife Program, the Northwest Power Planning Council (NPPC) approved the construction of the two hatcheries as a resident fish substitute measure to mitigate the loss of chinook salmon, coho salmon and steelhead trout that formerly ran into the blocked area above Grand Coulee Dam. The Power Council also directed BPA to fund a monitoring program to determine hatchery effectiveness. The most important objective of the monitoring program was to determine the best locations and times for stocking kokanee in terms of: (1) ensuring homing of adults to release sites for egg collection, and (2) reducing loss of juvenile kokanee salmon over Grand Coulee Dam.

One of the mitigation hatcheries was located at Metamooteles Springs on the Spokane Indian Reservation (operated by the Spokane Tribe) and the other on Sherman Creek near Kettle Falls, Washington (operated by the Washington Department of Fish & Wildlife--WDFW) (Figure 1). The Sherman Creek Hatchery is used as an imprinting site and egg collection facility. Eggs collected there are transferred to the Spokane Tribal Hatchery for incubation and rearing to the fry stage. At that stage (7-9 months old) they are stocked back in Lake Roosevelt at Sherman Creek and below Little Falls Dam on the Spokane River arm of Lake Roosevelt. At the time of this study, the Spokane Tribal Hatchery received kokanee eggs for rearing from the WDFW Lake Whatcom Hatchery in Bellingham, WA and from the Pend Oreille Cabinet Gorge Hatchery. Beginning in 1988, these fish were outplanted as fry at locations in Lake Roosevelt so that adult spawners would return for future egg takes in Sherman Creek and at Little Falls Dam (Figure 1).

At the present time, the hatcheries produce 2-3 million kokanee fry, and 100,000 residualized post-smolts released into Lake Roosevelt tributaries as noted above, as well as 500,000 rainbow trout for the Lake Roosevelt net pen program. The rainbow trout net pen program involves stocking 10 month old rainbow fingerlings (20 fish per pound) into net pens located in embayments at different locations on Lake

Figure 1. **Location of Lake Roosevelt kokanee hatcheries operated by Spokane** Tribe **and WOW.**



Roosevelt. The fish are reared in the net pens for 9 additional months, then released as post-smolts at 3-4 fish per pound. Individual fish grow to 1.5 pounds after 6 months in the reservoir and 3 pounds apiece after 18 months in the reservoir. In a given year, approximately 35 to 57% of the rainbow trout released from net pens have been harvested by anglers in Lake Roosevelt (Peone et *al.* 1990; Griffith and Scholz 1991; Thatcher et *al.* in press).

Monitoring and evaluation investigations indicated that the hatchery program has contributed significantly to increasing both the harvest rates of kokanee and rainbow trout in Lake Roosevelt, as well as the economic value of the Lake Roosevelt sport fishery. For example, creel surveys conducted by the U.S. Fish and Wildlife Service from 1980 to 1982 (pre-hatchery) estimated the annual harvest of kokanee at 300 to 1000 fish and the annual harvest of rainbow at 1000 to 3000 fish (Beckman et *al.* 1985). At that time the number of angler trips was approximately 80,000 per year and the economic value of the fishery was estimated at $2.8 million. From 1990 to 1992 (post-hatchery), harvest ranged from 8000 to 35,000 kokanee and 70,000 to 140,000 rainbow trout (Peone et *al.* 1990; Griffith and Scholz 1991; Thatcher et *al.* in press). During this period the number of angler trips ranged from 171,000 to 398,000 and the economic value ranged from $5.3 to $12.8 million. Thus, the Lake Roosevelt salmonid harvest has increased approximately 50 fold, angler use has increased by 5 times, and economic value has increased 5 fold since enhancement efforts were initiated.

However, monitoring and evaluation studies have also noted some significant problems with the kokanee program. For example, in the same year that 35,000 sub-adult kokanee were harvested, a loss of 25,000 sub-adult kokanee occurred through Grand Coulee Dam (Thatcher et *al.* in press). The magnitude of the loss was calculated from counts of Lake Roosevelt kokanee emigrants passing the Rock Island Dam fish passage facility, then backcalculating (based on a 15 percent loss- rate) to account for losses caused at each of the five dams located between Grand Coulee and Rock Island Dam. Another problem was that past releases had not produced any adults returning to the Sherman Creek stocking site. This led to two hypotheses: (1) kokanee experienced a parr-smolt transformation (smoltification) and were emigrating over Grand Coulee Dam, and (2) kokanee were not imprinting to the water of the stocking sites as fry and instead may be imprinting as eggs or newly hatched larvae at the Spokane Tribal Hatchery.

Anadromous salmonids undergo transitions in physiology, morphology and behavior, collectively termed parr-smolt transformation or smoltification, associated with their seaward migration. The smolt transitions include: (1) Changes in body shape to a slimmer, more streamlined fish, and body color, including a loss of parr marks and development of silvery coloration; (2) Adaptations to salt water. Parr are osmoconformers, unable to regulate blood osmotic concentration in full strength seawater (1000 mOsmol/l), so they typically experience 100% mortality in full strength seawater. During smoltification, the fish develop an outwardly directed active transport ion pump, (Na^+-K^+ ATPase) in the gill, which enables the fish to osmoregulate their blood ion concentration and increase survival in salinity tolerance tests with full strength seawater. In the ocean the fish develop a drinking reflex and imbibe seawater, which is absorbed across the intestine. The gill Na^+-K^+ ATPase pump then excretes the salt ions across the gills, so the net effect is retention of water which helps the fish to counter osmotic water loss; (3) Changes in behavior, including downstream seaward migration and development of a preference for seawater.

This process amounts to a metamorphosis, with the different morphological, physiological and behavioral transitions regulated by various hormones acting either alone or synergistically. Thyroid hormones appear to regulate silvering, downstream migratory activity and possibly some aspects of salt water adaptation. Cortisol and growth hormone appear to regulate gill Na^+-K^+ ATPase activity and intestinal water absorption.

In addition to this metamorphosis, smoltification is also a critical period for olfactory imprinting, when the juvenile fish form a permanent memory of the unique chemical characteristics of their water supply, which later serves as a cue for relocating the home river during the adult migration. The olfactory imprinting process appears to be induced by thyroid hormones. In coho salmon (*Oncorhynchus kisutch*), some stocks of chinook salmon (0. *tshwytscha*), and steelhead trout (*O. mykiss*), smoltification and olfactory imprinting typically occurs at 18 months of age.

However, not all species of Pacific salmon stay in their homestream for 1.5 years, then exhibit a distinct smolt stage like coho, chinook and steelhead. For example, pink (0. *gorbuscha* Walbaum) and chum (0. *keta*) migrate to the ocean immediately after emergence from redds. Anadromous sockeye (0. *nerka nerka* Walbaum), and their landlocked kokanee relatives (0. *nerka kennerlyi* Suckley), emigrate from their natal tributary to a freshwater nursery lake soon after swimup.

Eggs of both subspecies incubate for several months in gravel nests. After fertilization, eggs eye at about four weeks and hatch in about eight weeks. After hatching, the alevins remain under the gravel for about four more weeks until their yolk sacs are absorbed. They then emerge from the gravel, "swimup" into the water column, and emigrate immediately either upstream or downstream to the nearest lake. Sockeye remain in the lake for about a year, then smolt and migrate to sea. In contrast, kokanee stocks do not evidence a pronounced smolt migration (Ricker 1959; Seeley and McCammon 1966), so kokanee undergo incomplete smoltification and remain in the lake, where they grow to adult size. Both sockeye and kokanee exhibit natal homing to tributaries of lakes experienced only as embryos or larvae (Quinn et *al.* 1989), so if these fish utilize olfactory cues for homing, they must necessarily imprint during the egg, alevin or swimup stages.

1.2 Study Objectives

Kokanee are currently released from the Spokane Tribal Hatchery as 7-9 month old fry. If hatchery reared kokanee imprint to the hatchery water at egg or swimup stages before 3 months of age, they may not be imprinting as 7-9 month old fry at the time of stocking. In addition, if these fish undergo a smolt phase in the reservoir at 1.5 years old, they could migrate below Grand Coulee Dam and out of the Lake Roosevelt system. Despite their close association with the anadromous sockeye salmon, few imprinting or smoltification investigations have been done on kokanee salmon. Therefore, we conducted a battery of tests to determine if kokanee experienced thyroxine-induced chemical imprinting and smoltification similar to that of anadromous salmonids.

The imprinting and smolt assessment tests included:

(A) Determination of the critical period for olfactory imprinting in kokanee by exposing kokanee to different synthetic chemicals at different life stages, and then measuring the ability to discriminate to chemicals as sexually mature adults. Discrimination capability was deduced by conducting:

(1) behavioral tests in a Y-maze, and

(2) coded-wire tagging investigations involving the release of kokanee into Lake Roosevelt and recapturing them at egg collection sites scented with the chemical during their spawning migration.

(B) Measurement of whole body thyroxine content and blood plasma thyroxine concentration to determine if peak thyroid activity coincided with imprinting or other morphological, physiological and behavioral transitions associated with smoltification.

(C) Monitoring indices of smoltification, including:

(1) Morphological transitions
- (a) coloration changes (silvering and disappearance of parr marks), and
- (b) length/weight (condition factor indices).

(2) Physiological changes in
- (a) salt water tolerance,
- (b) osmoregulatory capability,
- (c) gill Na^+-K^+ ATPase activity, and
- (d) intestinal water absorption (Jv).

(3) Behavioral changes in
- (a) salt water preference, and
- (b) downstream migratory activity.

1.3 Background

Juvenile anadromous salmonids undergo changes in physiology, morphology and behavior which preadapts them to salt water. These changes signify a process termed smolt transformation (reviews by Hoar 1976; Folmar and Dickhoff 1980; Hasler and Scholz 1983; Barron 1986). Smoltification is also a "critical period" when juveniles imprint to the water of their home stream (Scholz et *al.* 1976; Hasler and Scholz 1983). Adults use this olfactory information to find their way back to the home stream during the spawning migration.

1.3.1 Changes in Morphology

Smolts are recognizable by their silvery appearance and slim, streamlined shape (Wedemeyer 1980; Gorbman et *al.* 1982; Winans 1984; Winans and Nishioka 1987). Juvenile salmonids have distinctive vertical black bars, or parr marks, on the sides of their bodies produced by clusters of melanophores in the integument. Parr marks help the fish to blend in with stream banks. The silvery layer which is evident on smolts is due to the deposition of two purines, guanine and hypoxanthine, produced by iridocytes. Both of these purines are present in parr, but there is a sharp increase in the ratio of guanine to hypoxanthine during smolt transformation (Robertson 1949; Johnston and Eales 1967, 1968, 1970). Guanine, deposited subcutaneously, covers the melanophores and masks the parr marks. These reflecting layers are thought to camouflage the fish in the ocean by reflecting the surface of the water, and therefore protecting against predators below them in the water column.

Body shape is reflected by the condition factor (K). In many cases, the condition factor drops as a salmonid smolts because the fish becomes more slender and streamlined (Hoar 1939; Fessler and Wagner 1969). Changes in silvering and condition factor were determined in this part of the study as possible indicators of smoltification.

1.3.2 Changes in Physiology

There are many ways anadromous salmon change physiologically in order to successfully adapt to salt water. These include changes in gill Na^+-K^+ adenosine triphosphatase (ATPase) activity, osmoregulatory ability and salt water tolerance. In anadromous salmonids, many preadaptive physiological changes occur during the parr-smolt transformation which prepare the fish for seawater while they are still in fresh water (McCormick and Saunders 1987; Hoar 1988; Specker 1988). Salinity tolerance develops during a fast-growing, fresh water phase of the smolting parr (Brown 1957). Glomerular filtration rate decreases, intestinal fluid uptake increases, and gill Na^+-K^+ ATPase activity increases in the springtime prior to seawater entry (Holmes and Stainier 1966; Zaugg and McLain 1970, 1972; Collie and Bern 1982). In fresh water, anadromous fish are hyperosmotic to the surrounding water. Therefore, they take on water passively and lose electrolytes through the gill membrane and

other permeable surfaces. In seawater the situation is reversed. The fish tend to loose water and gain ions. When the fish enters seawater, it prevents dehydration by drinking seawater, reducing urine flow and actively excreting the excess salts across the gill. For fresh water to be gained in the seawater adapted fish, sodium and chloride ions must be transported across the gut wall. This then produces a higher concentration in the gut, and allows water to be passively transported from the gut into the body fluids through osmosis (Utida et *al.* 1972).

1.3.2. I Intestinal Water Uptake

The intestine is a major site of salt and water balance in vertebrates. In some euryhaline fishes, intestinal absorption increases during adaptation to salt water and this process is mediated by corticosteroids (Collie and Hirano 1987). In coho salmon, the posterior intestine attains a rate of fluid absorption during smoltification in fresh water which is as high as the rate attained when they are adapted to salt water (Collie and Bern 1982). Veillette et *al.* (1993) showed that Atlantic salmon (*Salmo salar* L) smolts exhibited higher fluid transport rates across the posterior intestine than their parr stage cohorts. This suggests that the posterior intestine exhibits functional changes which are part of the changes occurring during smoltification. In this portion of the present investigation, intestinal fluid absorption was measured in the non-migratory kokanee salmon to determine whether kokanee showed a late spring increase in intestinal fluid absorption similar to the migratory coho and Atlantic salmon.

1.3.2.2 Gill Na^+-K^+ ATPase Activity

The increase in salt water may trigger Na^+-K^+ ATPase enzyme activities in the gut in order to transport Na^+ into the fish so that water would follow passively. In the gill, Na^+-K^+ ATPase increases in order to pump Na^+ out of the fish (Pickford et *al.* 1970; Zaugg et *al.* 1972; Zaugg and Wagner 1973). The orientation of the enzyme in the cell membrane appears to be situated so as to direct sodium ions inward in intestinal cells and outward in gill cells. Thus in the gill membrane, Na^+-K^+ ATPase serves as an outwardly directed active transport mechanism (Parry 1966; Conte 1966).

There have been numerous investigations demonstrating an increase in gill ATPase activity during the parr-smolt transformation (Zaugg and McLain 1970; Johnson et *al.* 1977; Buckman and Ewing 1982; Zaugg 1982b; Hasler and Scholz 1983; Ewing et *al.* 1979, 1984; Boeuf et *al.* 1985; Rondorf et *al.* 1989, Beeman et *al.*

1990; Birt et *al.* 1991; Franklin et *al.* 1992). The change in ATPase begins well before migration and peaks during the migratory phase and entry into seawater. In seawater, it rises somewhat after 4-5 days and stabilizes at the higher level, but if smolts remain in fresh water beyond the normal time of migration, the gill enzyme activity declines to the freshwater level (Zaugg and McLain 1970; Folmar and Dickhoff 1981). Thus, fish which remain in fresh water beyond the normal time of migration, readapt to fresh water and loose their migratory disposition. Such fish are termed “residualized” smolts.

The rise in gill Na^+-K^+ ATPase has been used frequently as an indicator of smoltification. In the present study it was necessary to determine if kokanee salmon exhibited an increase in gill ATPase activity at the time of the typical parr-smolt transformation similar to other salmonids. This would be an important component of the mechanisms used by salmon to transform into salt water fish.

1.3.2.3 Osmoregula tory Capability

Another indicator of smoltification and salt water adaptation which is characteristic of a smolt is the ability to maintain relatively low ion concentrations in the blood plasma despite high ion concentrations in a seawater environment. In both fresh water and seawater, smolts maintain serum osmotic concentration at about 300 mOsmol/l which is between the concentration of fresh water (25 mOsm/l) and seawater (1000 mOsm/l). The transfer of juvenile salmon to salt water is followed by changes in tissue electrolytes, then a brief transitory (<24 h) increase in blood serum osmolarity (to about 600 mOsm/l), and finally a subsequent stabilization near the previous levels (300 mOsm/l) (Eddy and Bath 1979). In some species of salmon such as pink and chum, the capacity to hypoosmoregulate develops in the alevin stages (Weisbart 1968). In other species, this ability develops later as yearlings or smolts (Folmar and Dickhoff 1980; Wedemeyer et *al.* 1980).

1.3.2.4 Salt Water Tolerance

With this ability to hypoosmoregulate comes an increased ability of the salmon to survive in, and adapt successfully to seawater. The adaptation of salmonids to seawater has ‘also been widely studied (Boeuf et *al.* 1978; Folmar and Dickhoff 1979; Hasler and Scholz 1983; Langdon and Thorpe 1984; Sweeting and McKeown 1987;

Franklin 1989), and many species of Pacific salmon are able to survive in full strength seawater during their smolt phase.

In this part of the study, the aim was to characterize whether yearling kokanee salmon would be able to regulate plasma ion concentrations and would therefore be able to survive in full strength seawater. This would not necessarily imply that fish would migrate over Grand Coulee Dam, but instead would only indicate salt water adaptation as an indicator of smoltification. A better indicator of entrainment potential through the dam would be salt water preference and downstream migratory behavioral tests.

1.3.3 Changes in Behavior

1.3.3. 1 Salt Water Preference

Houston (1956), Baggerman (1960b); Mclnerney (1964), and Conte et *al.* (1966) found that when given a choice between salt water and fresh water, smolts of several different species of anadromous species chose salt water. If kokanee showed a preference for salt water at specific times of the year, this preference may serve as an orientation mechanism (Mclnerney 1963, Fried et *al.* 1978), and may provide a disposition to emigrate out of the Lake Roosevelt system through Grand Coulee Dam. In this portion of the investigation, salt water preference behavioral tests were conducted to determine if kokanee showed a preference for salt water at the time of parr-smolt transformation similar to that of anadromous salmonids.

1.3.3.2 Downstream Migratory Behavior

Fresh water parr are territorial, aggressive and orient upstream. When the fish smolt, they cease territorial behavior and begin to migrate downstream. This migration is nocturnal in Atlantic, sockeye, coho salmon and steelhead smolts (Hoar 1958, 1976; Hasler and Scholz 1983). Salmon must remain in visual contact with the bottom in order to orient upstream into a water current (reviewed by Arnold 1974). Hoar suggested that while parr seem to remain near the bottom during both day and night, smolts remain near the bottom during the day but rise to the surface during the night. Smolts positioned higher in the water column lose visual contact with the bottom and are transported downstream (Saunders 1965).

There are differing thoughts about the mechanism of downstream migration. Some investigators believe that the movement downstream is strictly a passive displacement of the fish by the water current (Huntsman 1952; Hoar 1953). However, other investigators believe that the migration is directed and volitional (reviewed by Hartman et *al.* 1967; Chrisp and Bjorn 1978). Stasko et *al.* (1973) suggested that in many cases smolts display both types of behavior; orienting downstream and swimming faster than the current in low to moderate velocities, and orienting upstream and drifting downstream tail first in conditions of turbulent water.

Hartman et *al.* (1967) monitored traps that blocked the outlets in several sockeye nursery lakes. They found that smolts migrated nocturnally with the most intense activity occurring 3 to 4 hours after sunset. The majority of the fish traveled downstream at the same velocity as the current, suggesting passive displacement and drift. Ali and Hoar (1959) suggested that parr and smolts have different pigments in the retinas of their eyes. Parr had more porphyrhopsin which is more sensitive in low light conditions, while smolts contained more rhodopsin. Since porphyrhopsin is sensitive to wavelengths that penetrate under conditions of turbidity, parr may be able to maintain visual contact at night while smolts would not.

The downstream migration of most species of salmon lasts for approximately 4 to 6 weeks during the spring of the year (Hartman et *al.* 1967, Thorpe and Morgan 1978). Hartman et *al.* (1967) calculated the cumulative percentage of the total population of sockeye smolts and found that the majority of the migratory activity occurred over a 2-week period from May 9th to the 23rd.

If kokanee salmon exhibited an increase in downstream migratory behavior at certain times of the year, it may help to explain the loss of fish through Grand Coulee Dam. It would also be an important component to understanding the smolt transformation in kokanee. Therefore, downstream migratory tests were conducted at night to determine if kokanee displayed this behavior.

1.3.4 Neuroendocrine Regulation of Physiological Transitions

Salmonids have a seasonally changing physiology which is regulated by the neuroendocrine system. Hoar (1965) proposed that each springtime, salmonids undergo smolt transitions which preadapt them for life in the ocean. If they do not reach the ocean, the cycle is reversed and the physiology appropriate to life in fresh

water again appears. Hoar further hypothesized that these cycles are endogenous rhythms that can be resynchronized by external cues, especially the annual photocycle.

It is now well documented that the smolt transformation can be resynchronized by external factors such as photoperiod and lunar cycles (Zaugg and McLain 1972; Zaugg and Wagner 1973; Ewing et a/. 1979; Wedemeyer 1980; Grau et *al.* 1981) as well as temperature, discharge and water chemistry (reviewed by Thorpe and Morgan 1978; Higgs and Eales 1971, 1978; Youngson and Simpson 1984; Hoff nagle and Fivazzani 1990). Environmental cues such as daylength act on the pineal gland and the brain to signal the hypothalamus to release the hormones of the anterior pituitary gland. This causes endocrine organs such as the thyroid and interrenal glands to secrete hormones that act directly on target tissues. Other types of external cues detected by sensory neurons may also be integrated through the hypothalamic/pituitary axis. Many hormones are thought to be involved in the parr-smolt transformation (Hoar 1976, 1988; Folmar and Dickhoff 1980; Barron 1986; Leatherland 1982; Hasler and Scholz 1983). One of these is thyroxine (T_4), a thyroid, hormone.

1.3.4.1 Thyroid Hormones During Smoltifica tion

Previous investigations have suggested a stimulator-y effect of thyroid hormones on smoltification (reviewed by Leatherland 1982). Dickhoff et *al.* (1978) found a thyroxine hormone surge together with morphological changes in coho salmon. Dickhoff et *al.* (1982) found the thyroid surge was highly correlated with the ability to survive in seawater. Circulating levels of thyroid hormones have been shown to increase, or surge just prior to smolt transformation in the spring in several species of salmon and rainbow trout. This surge is associated with an increase in silvering, an increase in downstream migratory behavior and with olfactory imprinting (Osborn et *al.* 1978; Folmar and Dickhoff 1979; Dickhoff *et al.* 1982; Hasler and Scholz 1983; Scholz et *al.* 1985, 1992). A surge in T_4 concentration could be indicative of a critical period for imprinting or increased tendency to undergo smoltification and migrate downstream, so in this part of the investigation it was necessary to determine if there were changes in T_4 concentration in 1+ kokanee in order to compare these fish with other salmonids of the same age.

The relationship between downstream migratory activity and other physiological transitions is not the same in all cases. Seaward migration of salmonids usually coincides with the development of silvery coloration, although in some situations, distinct silvering may begin several months before the downstream migration begins. Likewise, salinity tolerance may increase before or after the start of the downstream migration. Hasler and Scholz (1983) suggested that various physiological and behavioral transitions are regulated independently by different hormones. Fluctuations in daylength generally synchronize the release of different hormones involved in smoltification and allow the morphological, behavioral and physiological transitions to occur at approximately the same time. Some hormones may also be responsive to fluctuations in other environmental factors (e.g., water temperature, water discharge), so the morpphological, physiological and behavioral transitions they regulate may occasionally be out of synchronization with the other transitions regulated by different hormones (Ewing 1979; Hasler and Scholz 1983).

1.4 Thyroid Hormones during Early Development

Thyroid hormones are known to be involved in the development of nervous systems and metamorphic events in a variety of vertebrates (Scholz 1980; Brown and Bern 1988; Brown *et al.* 1989) and in the imprinting period of coho salmon and rainbow trout (Hasler and Scholz 1983). Thyroid hormone levels influence the metamorphic events of a number of species of fishes during their development. For example, the change from freeliving ammocoete larvae to the parasitic adult form of the sea lamprey, the change from the pelagic, bilaterally symmetrical larvae to asymmetrical bottom dwelling adult form of flatfish, and the parr-smolt transformation in salmonids (Pickford 1957; lnui and Miwa 1985; Kobuke *et al.* 1987; Miwa and lnui 1987; Miwa *et al.* 1988; deJesus 1991; Yamano *et al.* 1991). In salmonids, transitions in levels of thyroid hormones may occur at several stages in development. For example, Tagawa and Hirano (1987) and Kobuke et *al.* (1987) both observed decreases in the thyroxine content of chum and coho salmon larvae after hatching. This disappearance of thyroxine after hatching suggested both that the thyroid hormone was being taken from the yolk into the tissues and then cleared from the fish, and that the newly developing thyroid follicles had not yet begun thyroid secretion. These results also suggested thyroid hormones are maternally acquired in salmon eggs, but at a certain time after fertilization the developing embryos begin to produce their own thyroid hormones (Kobuke *et al.* 1987; Sullivan *et al.* 1987; Tagawa and

Hirano 1987; Greenblatt et *al.* 1989). This was reflected in a peak of thyroid activity at the time of swimup and emergence.

An increase in thyroxine (T_4) levels generally occurs during smoltification in yearling coho salmon (Hoar 1976; Dickhoff et a/. 1978; Folmar and Dickhoff 1980; Hasler and Scholz 1983; Barron 1986). However, Dickhoff et *al.* (1982) also found an elevation in T_4 levels in zero-age fish during their first spring, but these levels were apparently not sufficient to stimulate emigration. In contrast, deJesus and Hirano (1992) found an increase in T_4 at the time of emergence in pink and chum salmon but decided it could be sufficient to stimulate the physiological and behavioral changes typically associated with downstream migration. Thus, increased thyroid activity appeared to be associated with smoltification and downstream migration. This makes sense in that coho do not migrate until they are yearlings wherease pink and chum salmon migrate as underyearlings.

In the present study, it was necessary to measure thyroxine levels in developing kokanee salmon eggs and larvae in order to determine if they were similar to other species of fish which showed changes in thyroid hormone content associated with development. If there was an increase in thyroxine content at this age, it could be associated with the migration to nursery lakes observed in the swimup fry of wild sockeye/kokanee stocks. It could also provide information about an additional critical period for olfactory imprinting that occurs prior to the smolt stage in species that migrate shortly after emergence.

1.5 Critical Period for Imprinting

It is not known exactly when kokanee salmon first imprint to their home water. Salmonids exhibit varying degrees and times of smoltification and imprinting. For example, pink and chum salmon are silvery soon after emergence and are able to enter salt water at that time. This is also the period in which they imprint (Barns 1976). Other species, such as coho salmon, chinook salmon and steelhead trout stay in fresh water for 12 to 18 months before migrating to the ocean, Hasler and Scholz (1983) found that coho salmon and steelhead trout imprinted during the smolt stage at approximately 16 to 18 months old. Sockeye salmon are an intermediate example of these two extremes. They migrate to a nursery lake soon after emergence and spend a year in the lake before smolting and migrating to the sea (Rounsefell 1958). Since sockeye and kokanee salmon migrate from natal streams as fry, they must imprint to

that water during their egg or alevin stage of development. Sockeye may experience a second sensitive period for imprinting at the time they smolt and emigrate from their nursery lake to the ocean. It is also possible that kokanee imprint during this time if they undergo partial smoltification.

Scholz (1980) suggested that the memory of natal stream odor may be permanently imprinted in coho salmon through thyroid hormones. There is evidence that thyroid hormones induce olfactory imprinting in salmonids (Scholz 1980; Hasler and Scholz 1983). Hasler and Scholz (1983) found that natural coho smolts exposed to synthetic chemicals from late April to early May, at a time when thyroid hormone levels were elevated, became imprinted to the chemicals and homed to them as adults; whereas natural presmolts exposed to these chemicals in February at basal thyroid hormone levels did not home to these chemicals. Injection of thyroid stimulatlng hormones into presmolts in February caused thyroid hormone concentrations to increase to smolt levels in February. These fish imprinted to the synthetic chemicals and homed to them in behavioral tests. In contrast, control fish which received saline placebo injections in February neither experienced elevated thyroid levels nor imprinted to the synthetic chemicals. Subsequent investigations revealed that thyroid hormones bind to receptors in the brain cell nuclei of steelhead trout (Scholz et *al.* 1985; White et *al.* 1990). This binding is thought to cause differentiation of neuron circuitry, which stimulates formation of permanent imprinted memories (Scholz et *al.* 1985). Lanier (1987) and Scholz *et al.* (1992) reported that, compared to natural presmolts or presmolts injected with saline placebo (both groups had basal thyroxine levels), both natural smolts and TSH injected presmolt steelhead (both groups had elevated thyroxine levels) experienced increased neuron size, axon and dendrite length, axon and dendrite branching, and increased number of synaptic connections.

In developing organisms, there is a critical period in which inducing chemicals cause permanent morphological changes in the organism. This means that there are certain times when the brain tissues may be more susceptible to chemical and/or physical influences, and neural pathways may change during these periods. In some salmonids, smoltification is such a critical period. In other salmon which migrate early out of their home stream (chum, pink, sockeye and kokanee), the critical period must be at or before swimup, since these species leave their home tributary at that time. The investigations described in Section 1.4 imply that thyroid hormones may be

sufficiently high in eggs or larvae to stimulate the imprinting process at these life stages. If so, hatchery reared kokanee may imprint to the hatchery water during early developmental stages and may not be able to imprint later to stocking sites. This may explain why few kokanee have returned to stocking sites in Lake Roosevelt as noted in our earlier reports (Scholz et *al.* 1993; Thatcher et *al.* in press).

In 1991, Scholz et *al.* (1992) initiated a preliminary study to determine the critical period for thyroxine induced olfactory imprinting in kokanee salmon. They found that T_4 concentrations in Lake Whatcom stock (1990 year class) kokanee were relatively high in eggs and alevins as compared to post-swimup fry, and the concentrations peaked at hatch and swimup. Then, in 1992, Scholz et *al.* (1993) repeated the study to determine if results from the previous year could be confirmed. The results from the 1993 study were consistent with the previous year. High thyroxine concentrations were found in eggs and alevins, with peaks at hatch and swimup followed by steadily declining levels in post-swimup fry. In addition to measuring T_4 levels, they initiated experiments to determine if kokanee could be imprinted to synthetic chemicals -- morpholine and phenethyl alcohol -- at different life stages. In 1992, zero-age and 1+ kokanee were exposed to synthetic chemicals. Most of these fish were marked and released in Lake Roosevelt in July and August 1992 as part of a field test described in Section 2.5. A portion of the fish from each group were retained at the Spokane Tribal Hatchery until August-October 1993. In this part of the study, controlled behavioral tests were conducted to determine if the fish imprinted to their exposure odor.

Thus, the present investigation attempted to determine three things. The first was to determine if kokanee salmon could be imprinted to synthetic chemicals. The second objective was to determine if kokanee salmon underwent smoltification, and if so, to what degree did it resemble the parr-smolt transformation of anadromous forms of salmon. The third objective was to discuss the implications of our findings on establishment of the kokanee fishery in Lake Roosevelt, and make recommendations about altering the operation of the Lake Roosevelt kokanee hatcheries to improve retention of kokanee, with concomitant enhancement of harvest opportunities,' as well as increasing returns of adults to egg collection sites.

2.0 METHODS AND MATERIALS

2.1 Rearing Conditions

Egg, larvae and juvenile kokanee salmon used for these investigations were incubated and reared at the Spokane Tribal Hatchery near Wellpinit, WA. Eggs were incubated in upwelling incubators placed on benches in hatchery raceways. Eggs and larvae remained in them through the swimup stage, when the fry swam voluntarily out of the incubator into the raceway. 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. 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 .O - 2.5 mm crumbles) and Silvercup size 1-4 mm crumbles. Yearling fish were fed Biodry 1000 pellets (3.0 - 4.0 mm) obtained from Bioproducts, Inc. Photoperiod was maintained at natural daylength as each raceway was partially exposed to natural conditions of light and weather.

2.2 Olfactory Imprinting Investigations

Our basic approach in ascertaining the critical period for olfactory imprinting was to monitor whole body thyroxine content from fertilized egg through fry stage (from 0 to 227 days post-fertilization), and plasma thyroxine content from fingerling to post-smolt stage (from 235 to 586 days post-fertilization) kokanee salmon. Simultaneously, the fish were exposed to either morpholine (C_4H_9NO) at 5 x 10^{-5} mg/liter or phenethyl alcohol ($C_8H_{10}O$) at 5 x 10^{-3} mg/liter at various developmental stages (Figure 2). This experiment was conducted twice, initially in 1992 and repeated in 1993.

2.2.1 1992 Imprin ting Investigations

In 1992, fish were exposed at the following life stages (determined by the number of days post-fertilization) :

Site a a e	Days Post Fertilization
(1) fertilized egg	***(O-30)***
(2) eyed egg	***(30-60)***
(3) hatching	***(53-63)***
(4) alevin	(60-90)

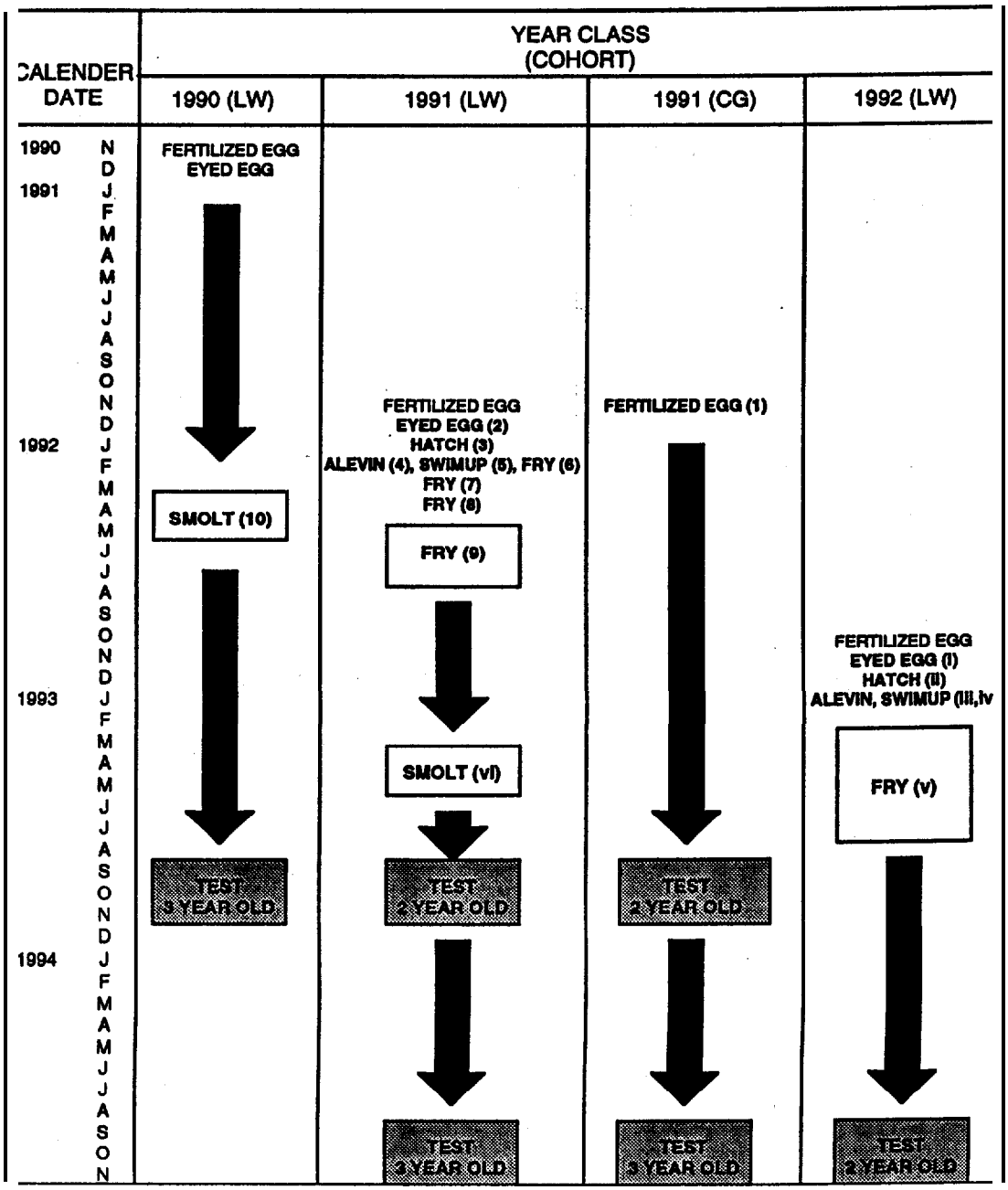
(5) swimup	(88-93)
(6) fry	(94-125)
(7) fry	(125-155)
(8) fry	(155-185)
(9) fry	(185-227)
(10) smolt	(481-541)

Fish in Group 1 were 1991 year class (age 0) obtained from the Idaho Department of Fish and Game Cabinet Gorge Hatchery stock located on the Clark Fork River near its confluence with the Pend Oreille Lake. Eggs were fertilized at Cabinet Gorge and transported immediately to the Spokane Tribal Hatchery. Fish in groups 2-9 were 1991 year class (age 0) larvae and fry obtained from Washington Department of Wildlife Lake Whatcom Hatchery stock. Eggs were fertilized at Lake Whatcom on 4 November 1991 and transferred as eyed eggs to the Spokane Tribal Hatchery on 6 December 1991. Fish in Group 10 were smolt stage fish originating from the 1990 year class (age 1+) at Lake Whatcom, that had been transferred to the Spokane Tribal Hatchery as eggs in 1990 and reared there since that time. Details about the first 1.5 years of life of these fish can be found in reports by Scholz et *al.* (1992, 1993).

Whole body thyroxine content was determined in twenty 1991 year class fish at approximately weekly intervals from day 0 to 134 days post-fertilization, then at biweekly intervals until 227 days post-fertilization. Blood plasma thyroxine was determined in twenty 1990 year class fish at biweekly intervals from fry to post-smolt stages at 235-586 days post-fertilization. Procedures were described by Scholz et *al.* (1992, 1993) and in Sections 2.7 - 2.10 of this report.

At each different interval one group of fish was exposed to morpholine and a second to phenethyl alcohol. Fertilized egg stage fish were exposed to phenethyl alcohol only because the number of eggs was insufficient to expose two groups. The fish were retained at the Spokane Tribal Hatchery for 15-20 months, without again being exposed to the odors, until they became sexually mature in the autumn of 1993 (1991 year class fish were two years old and 1990 year class fish were three years old) (Figure 2). Behavioral tests were then conducted, by releasing the fish in a stream below the confluence of two tributaries that formed a natural Y-maze, to determine which fish migrated upstream and selected the arm scented with their appropriate exposure chemical.

Figure 2. Experimental protocol for synthetic chemical Imprinting experiments. Kokanee salmon obtained from lake Whatcom (LW) or Cabinet Gorge (CG) were transferred to the Spokane Tribal hatchery as eyed eggs and recently fertilized eggs, respectively. Life stages of each year class in bold type are stages at which synthetic chemical Imprinting was attempted. Numbers **1-10** refer to the 10 life stages exposed to synthetic chemicals in 1992. Numbers I-vi were exposed in 1993. At each life stage one group was exposed to morpholine and a second group to phenethyl alcohol, with the exception of the fertilized eggs from CG (1) which were only exposed to phenethyl alcohol. Stages surrounded by boxes indicate the length of the exposure period.



2.2.2 1993 Imprinting Investigations

In 1993, fish were exposed at the following life stages (determined by the number of days post-fertilization):

Life Stage	Days Post Fertilization
(1) eyed egg	(44-64)
(2) hatching	(64-76)
(3) alevin	(70-94)
(4) swimup	(91-98)
(5) fry	(105-252)
(6) smolt	(470-530)

Fish in groups 1-5 were 1992 year class (age 0) fish taken from Lake Whatcom on 5 November 1992 and transferred as eyed eggs to the Spokane Tribal Hatchery on 15 December 1992 (Figure 2). Fish in group 6 were from the 1991 Lake Whatcom year class (age 1+) that had been retained at the Spokane Tribal Hatchery until the smolt stage.

In 1993, whole body thyroxine was determined in 20 zero age fish (1992 year class) at weekly intervals from 44 to 154 days post-fertilization, then at biweekly intervals until 252 days post-fertilization. Also, blood plasma thyroxine concentration was determined in 20 yearling fish (1991 year class) from pre-smolt to smolt stage at 228 (age 0) to 580 (age 1+) days post-fertilization. Procedures for thyroxine determination in both whole body and blood samples are described in Sections 2.7-2.13 of this report.

These fish were divided into ten groups (two groups from each stage) that were exposed to either morpholine or phenethyl alcohol. They are currently being held at the Spokane Tribal Hatchery for testing in 1994 (Figure 2).

2.3 Synthetic Chemical Imprinting Procedures

Details of the synthetic chemical imprinting procedure and methods for calculating steady state concentration of imprinting chemicals were described in a previous annual report (Scholz et *al.* 1993). Eggs and larvae were incubated at 9-10°C in upwelling incubators placed on benches in hatchery raceways. At the swimup stage, the fish swam voluntarily out of the incubator into the raceway. Odors were

delivered via peristaltic pump either into the incubator water supply lines or directly into the raceways following procedures described in a fish imprinting manual (Scholz et *al.* 1975). The odor delivery system is depicted in Figures 3 and 4.

Morpholine (C_4H_9NO) and phenethyl alcohol ($C_8H_{10}O$), metered at steady state concentrations of 5 x 10^{-5} mg/l and 5 x 10^{-3} mg/l respectively, were selected as imprinting chemicals because they had been used successfully to imprint several species of salmonids at behavioral detection thresholds of approximately 1 x 10^{-6} and 1 x 10^{-4} mg/liter respectively (Wisby 1952; Scholz et *al.* 1975; reviewed by Hasler and Scholz 1983). Additionally, neither chemical is found in natural waters, both are chemically stable, and both are highly soluble in water (Scholz et a/. 1975). Two imprinting chemicals were employed in this experiment so that one odor could act as a control for the other.

2.4 Odor Discrimination Test Procedure

Behavioral experiments were conducted in 1993 with kokanee exposed to synthetic chemicals in 1992. These were 2-year old 1991 year class Lake Whatcom and Cabinet Gorge fish, and 3-year old 1990 year class Lake Whatcom fish. A total of 847 fish were tested in 1993. In 1994, we are planning to repeat this experiment using fish that were exposed to synthetic chemicals in both 1992 and 1993, i.e., 1991 year class Lake Whatcom and Cabinet Gorge fish that will be 3-year old spawners, as well as 1992 year class Lake Whatcom fish that will be 2-year old spawners.

After synthetic chemical exposure was accomplished in 1992, 400 fish from each of the 17 groups exposed to synthetic chemicals as eggs, alevins, or fry, and 200 fish from each of the two groups exposed as smolts, were marked with a specific fin clip that distinctively identified the developmental stage and exposure chemical. These fish were combined in one raceway and held for 15 months until odor discrimination tests were conducted from September through November 1993 with individuals that had become sexually mature. At the time odor discrimination tests were conducted in 1993, the fish exposed to chemicals as zero age eggs, alevins or fry (1991 year class) were two years old fish and the fish exposed to chemicals as age 1+ smolts (1990 year class) were 3 years old. Mean lengths (±SD) were 345 mm (± 33 mm) and 423 mm (± 37 mm) for the two and three year old fish respectively. A total of 718 two year old and 129 three year old fish were tested in 1993. The remaining fish from the 1991 year class that did not become sexually mature in 1993, were

Figure 3. **Diagram of Incubation and Imprinting setup at Spokane Tribal kokanee hatchery.**

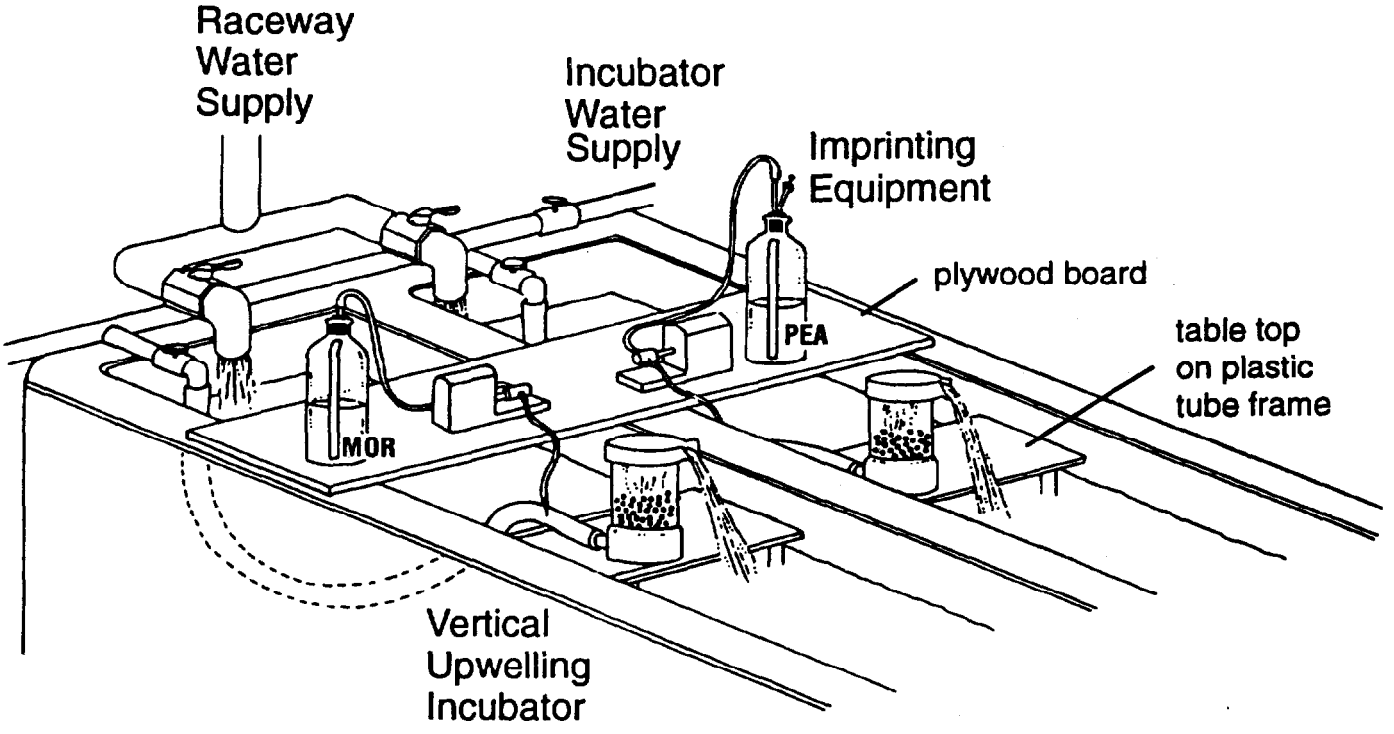
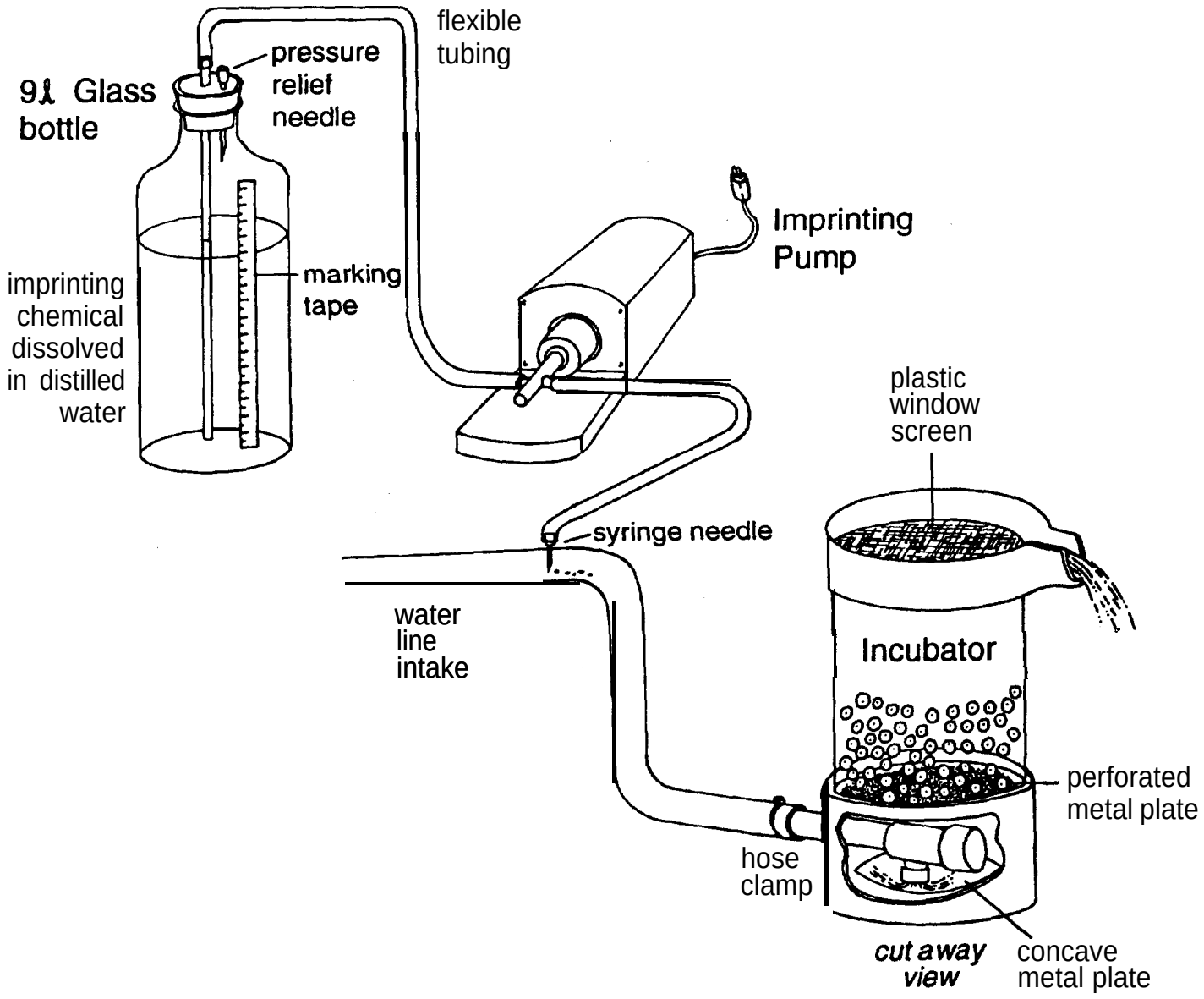


Figure 4. **Apparatus used for synthetic chemical imprinting of kokanee salmon.**



retained at the Spokane Tribal Hatchery and will be tested in the autumn of 1994. After synthetic chemical exposure was accomplished in 1993, 200 fish from each of the 12 groups exposed to synthetic chemicals were marked and were also retained at the Spokane Tribal Hatchery until they can be tested in the autumn of 1994.

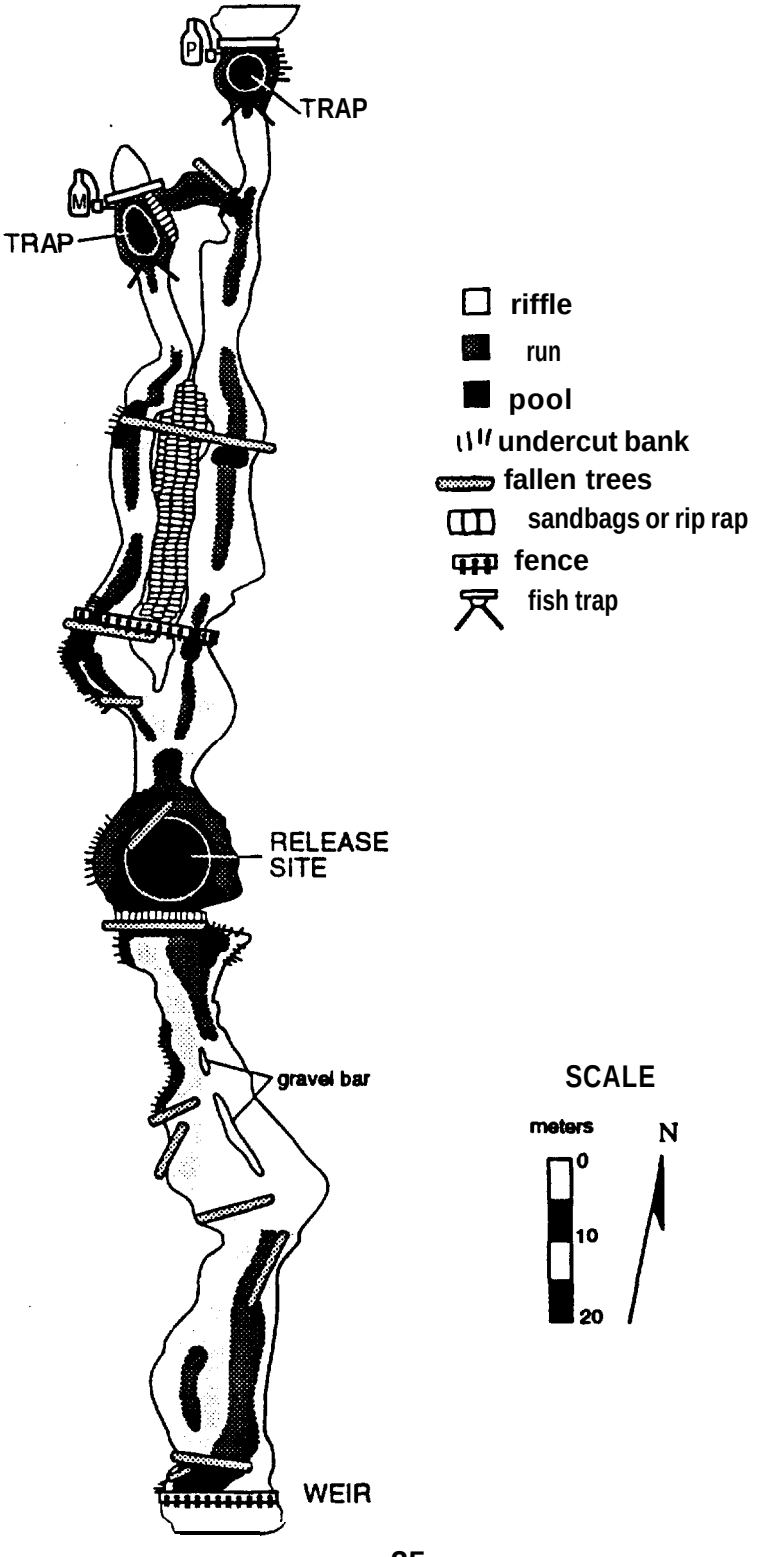
The fish retained at the hatchery for behavioral tests were not tested until they became sexually mature and evidenced a migratory disposition. Our intent was to mimic the physiological state and activate the upstream migratory drive of naturally spawning kokanee. Experimental fish were released daily, from September 22 to November 19, into a stream 50 meters below the junction of two tributary channels. Fish were randomly selected by dipnetting into the hatchery raceway which contained fish from all exposure groups. Morpholine and phenethyl alcohol were metered randomly into traps installed at each channel to attract fish (Figure 5). A response required upstream migration and selection of a channel scented with the correct exposure odor. Hence, this experiment simulated the kind of choice that must be made by naturally migrating adult fish.

Prior to, in the middle and at the end of conducting the experiments, Rhodamine B dye was released in the channels to estimate flow rates and thus determine the time it took for odors released at the traps to be cleared from each arm. The mean clearance times (± SD) were 5 minutes, 50 seconds (± 32 seconds) from one arm and 8 minutes, 56 seconds (± 39 seconds) from the second arm (n=3). Odors were periodically switched, at 2-5 day intervals, with morpholine metered into one channel on a total of 25 days and into the second channel on a total of 20 days. Phenethyl alcohol was metered into each channel in a reciprocal pattern. On nine days, no odor was delivered into either channel. A weir was set up about 100 meters below the release site to prevent fish from escaping downstream.

Each trap and the downstream weir were checked daily by two observers and the number of each type of fin clipped fish captured at each location was recorded. A blind procedure was used since, at the time the data were collected, neither observer knew what chemical treatment or developmental stage was designated by a particular fin clip.

Fish traps were constructed from rebar and angle iron. The upstream end of each trap blocked further upstream migration. The downstream end formed a V, with a narrow opening that directed the fish into the mouth of the trap, but not so narrow as to

Figure 5. Natural Y-maze used to conduct olfactory discrimination investigations. Bottles labeled M and P indicate where synthetic chemicals were metered into traps on each arm of the Y. The position of the release site and weir used to trap fish migrating downstream are also noted.



prevent the fish from volitionally exiting the trap. The reason for this mode of construction was that numerous conventional tagging studies, as well as sonic and radio-tracking investigations, indicated that homing salmon frequently ascend the wrong tributary at a stream juncture and subsequently backtrack below the confluence, then ascend the correct tributary (reviewed by Johnsen and Hasler 1980; Hasler and Scholz 1983). Thus, our traps were designed to allow the fish to find their way out of the trap and backtrack downstream, so a major assumption in the present study was that fish captured in a given trap were there of their own volition. The primary purpose of our traps was to make it easier to capture fish that had selected a particular channel, since nearly all of the fish in the channel migrated upstream to the trap. When the traps were checked, the trap opening was blocked off, preventing the fish from escaping downstream, and fish were dipnetted out of the trap.

For a fish to be classified as imprinted to their exposure odor, we required that three criteria be met:

(1) Downstream migration if the odor was absent;

(2) Upstream migration if the odor was present; and

(3) Selection of the tributary scented with the fish's exposure odor instead of the tributary scented with the alternate odor.

Fish meeting these criteria were classified as "imprinted" and those not fulfilling them were 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 a stream and downstream if morpholine was absent. These results indicated that the odor acted as a sign stimulus to induce a stereotyped behavior, i.e., swimming against a current (rheotaxis). Thus, Johnsen and Hasler (1980) concluded that the operational mechanism in selection of the home stream by olfactory orientation was positive rheotaxis if the odor was present and negative rheotaxis if the odor was absent.

We tested two hypotheses concerning the capture of fish in traps. The null hypothesis stated, "There is no difference in the distribution of fish exposed to different odors at a selected life history stage." This result would have indicated that the fish did

not imprint to their exposure odor at that development stage. This hypothesis was supported if:

(1) fish exposed to the synthetic chemicals were captured principally at the downstream weir in both odor present and odor absent trials, and

(2) fish migrating upstream with odors present were captured in traps scented with their exposure odor and alternate odor in about equal numbers, which would have indicated that the fish selected tributary channels randomly instead of being attracted to the channel scented with their exposure odor.

The alternative hypothesis stated, “There is a difference in the distribution of fish exposed to different odors at a selected life history stage.” This result implied that fish -did imprint to their exposure odor at that developmental stage. This hypothesis was supported if:

(1) fish exposed to the synthetic chemicals were captured principally in the downstream weir in trials with the odor absent and were captured in upstream traps if the odor was present,

(2) fish were captured more frequently in the trap scented with their exposure odor compared to the trap scented with their alternative odor, and

(3) fish switched their channel preference when the odors in the channels were switched.

For each life history stage a chi square test was used to determine if there was a significant difference ($P \leq .05$) between the distribution of morpholine exposed fish into the morpholine scented trap, phenethyl alcohol scented trap or downstream weir, and the distribution of phenethyl alcohol exposed fish into these traps. This would indicate if morpholine exposed fish were moving into the morpholine scented trap and phenethyl alcohol exposed fish were moving into the phenethyl alcohol trap. This statistical test was used both for conditions when odors were present and also when odors were absent to determine if there was a significant difference ($P \leq .05$) in the number of fish moving upstream or downstream under each type of condition.

2.5 Marking Fish for Release into Lake Roosevelt

In both 1992 and 1993, most of the fish exposed to the synthetic chemicals were released into Lake Roosevelt at Sherman Creek Hatchery or at Little Falls Dam to conduct a field test. A portion of each group released were tagged with distinctive coded wire tags. Tables 1 and 2 provide data regarding the stage exposed, the exposure odor, stocking location, stocking date, and total number of fish with coded wire tags released at each location. During the spawning season in 1993, morpholine (at a steady state concentration of 5×10^{-5} mg/l) was metered into the hatchery trap at Sherman Creek. Phenethyl alcohol (5×10^{-3} mg/l) was metered into the Spokane River at Little Falls Dam and a merwin trap was installed at that site. Electrofishing surveys were also conducted at both locations. The number of morpholine-exposed and phenethyl alcohol-exposed fish (marked with group specific coded wire tags) returning to each location was counted.

In 1993, groups of fish expected to return in these experiments included 1990 cohort kokanee that had been exposed as smolts in 1992 (returning as 3 year old spawners) and possibly a few 1991 cohort kokanee that had been exposed at egg, alevin, swimup or post-swimup fry stages in 1992 (returning as 2 year old spawners). However since most kokanee in Lake Roosevelt spawn at age 3 or age 4, the majority of these fish were not expected to show up in either the creel or at egg collection sites until 1994. In 1994, the following groups are expected to return: (1) 1990 cohort kokanee returning as 4 year old fish; (2) 1991 cohort kokanee returning as 3 year old fish; and (3) possibly a few 1992 cohort kokanee returning as 2 year old fish. In 1995, the following groups are expected to return: (1) 1991 cohort kokanee returning as 4 year old fish and (2) 1992 cohort kokanee returning as 3 year old fish. In 1996, 1992 cohort kokanee are anticipated to return as 4 year old spawners. In this report, we present the results of recoveries of coded wire-tagged fish in 1992 and 1993 (see Section 3.1.5).

For marking experiments, kokanee were dipnetted out of hatchery raceways and mildly anesthetized with a 50 mg/l concentration of tricaine methanesulfonate (MS 222), then coded wire tags (CWT) were injected into the rostrum using a model MK4 CWT machine (Northwest Marine Technology, Inc.), equipped with two different nose hoods specially fitted for fry and smelt-sized fish. With this device, we were able to successfully mark fry that weighed 1.7 g and were 59 mm in total length with less than 0.1% mortality. Lengths and weights of marked fry ranged from 59 to 94 mm and 1.7 to

Table 1. **Summary of kokanee salmon marked with coded wire tags (CWT) in 1992. Eyed egg to fry stages were from the Lake Whatcom 1991 cohort. Smolts were from the Lake Whatcom 1990 cohort.**

STAGE EXPOSED	EXPOSURE ODOR	#CWT MARKED	RELEASE LOCATION	DATE RELEASED	STAGE AT RELEASE
Eyed egg	MOR	7,367	Sherman Creek	Jul/Aug	Fry
	PEA	11,393	Sherman Creek	July	Fry
Hatch	MOR	22,222	Sherman Creek	July	Fry
	PEA	23,115	Sherman Creek	July	Fry
Alevin	MOR	11,441	Sherman Creek	July	Fry
	PEA	10,986	Sherman Creek	July	Fry
Swimup	MOR	8,370	Sherman Creek	July	Fry
	PEA	10,716	Sherman Creek	July	Fry
Fry (Feb)	MOR	20,194	Sherman Creek	June	Fry
	PEA	6,025	Shernian Creek	June	Fry
Fry (Mar)	MOR	9,798	Sherman Creek	July	Fry
	PEA	10,818	Sherman Creek	August	Fry
Fry (Apr)	MOR	11,445	Sherman Creek	July	Fry
	PEA	11,525	Sherman Creek	July	Fry
Fry (May-Jul)	MOR	6,838	Sherman Creek	July	Fry
	PEA	11,300	Sherman Creek	July	Fry
Smolt	MOR	7,501	Sherman Creek	-June	Smolt
	PEA	8,354	Sherman Creek	July	Smolt

Table 2. **Summary of kokanee salmon marked with coded wire tags (CWT) in 1993. Eyed egg to fry stages were from the Lake Whatcom 1992 cohort. Smolts were from the Lake Whatcom 1990 cohort.**

STAGE EXPOSED	EXPOSURE ODOR	#CWT MARKED	RELEASE LOCATION	DATE RELEASED	STAGE AT RELEASE
Eyed egg	MOR	10,961	Sherman Creek	June	Fry
	MOR	10,903	Spokane River	June/July	Fry
	PEA	10,721	Sherman Creek	June	Fry
	PEA	32,953	Spokane River	July	Fry
Hatch	MOR	7,988	Sherman Creek	June	Fry
	MOR	31,416	Spokane River	July	Fry
	MOR	22,026	Barnaby Creek	August	Fry
	PEA	7,988	Sherman Creek	June	Fry
	PEA	21,993	Spokane River	July	Fry
Alevi'n	MOR	10,938	Sherman Creek	July	Fry
	PEA	11,791	Sherman Creek	July	Fry
Swimup	MOR	10,908	Sherman Creek	July	Fry
	PEA	10,885	Spokane River	July	Fry
Fry (Feb-Jun)	MOR	10,802	Sherman Creek	June	Fry
(Feb-Jul)	PEA	10,896	Sherman Creek	July	Fry
Smolt	MOR	38,345	Sherman Creek	July	Smolt
	PEA	7,753	Sherman Creek	July	Smolt
	PEA	8,196	Blue Creek	May	Smolt

6.1 g respectively. Lengths and weights of residualized smolts ranged from 145 to 183 mm and 25.3 to 48.4 g respectively. Coded wire tagged fish were also given an adipose fin clip as an external identification mark. Marked fish were counted using a tally counter, then released back into hatchery raceways through a quality control. device (QCD) (Northwest Marine Technology, Inc.) equipped with a CWT detector. The fish were retained for approximately 30-45 days before release to estimate mortality rates and tag retention. Mortality rates were uniformly low (<0.1%) (D. Brown, personal comm.).

Tag retention was estimated by two methods. First, the QCD count was compared to the tally counter count to estimate the number of fish that were tagged successfully (>99% in most cases). Second, to account for the possibility that tags had been initially injected into the fish but had later been shed, a random sample of fish were rechecked after 30 days using the QCD counter. This was accomplished by randomly collecting 500 fish per each lot of approximately 10,000 fish that were marked and running them back through the QCD CWT detector. Each fish was enumerated using a tally counter before being placed into the CWT detector. Percent tag retention for each lot was determined by dividing the QCD count by the tally counter number. For each lot, the original QCD count obtained on the day the fish were tagged was multiplied by the percentage figure obtained after 30 days to estimate the number of fish in that lot that were released with CWT. For the 1992 tagging investigations, the mean percent tag retention after 30 days was 91.5%, and ranged from 84 to 97%. The relatively high variation was attributed to the relative inexperience of the fish tagging crew. For the 1993 tagging investigations, the mean percent tag retention after 30 days was 98.5%, and ranged from 97.5 to 99.3%. The relatively low variation was attributed to the expertise of the fish tagging crew, which was composed of the same individuals who accomplished the tagging work in 1992.

In Lake Roosevelt, creel clerks and fisheries technicians used hand held magnetic wands to check kokanee for CWT's and/or adipose clips. Heads of kokanee with adipose clips were cut off and sent to Spokane Tribal Hatchery, where CWT's were dissected out and examined with a dissecting microscope to determine the lot code. Percent tag retention of fish after release into Lake Roosevelt was estimated by dividing the number of fish bearing coded wire tags by the total number of fish examined that had adipose clips. In 1992 and 1993, a total of 99 adipose clipped fish marked in 1992 were recovered. Of these, 76 contained CWT's for a 76.7% tag

retention rate after approximately 6 to I8 months in Lake Roosevelt. In 1993, a total of 172 adipose clipped fish marked in 1993 were recovered. Of these, I62 contained CWT's for a 94. I % tag retention rate after approximately 3-6 months in the reservoir.

Fish exposed to morpholine and phenethyl alcohol at different life history stages and released into the reservoir at different locations were given different CWT lot codes. The number of fish from each lot returning to Sherman Creek near the head of Lake Roosevelt (morpholine scented) or Little Falls Dam on the Spokane Arm of Lake Roosevelt (phenethyl alcohol scented) was determined.

2.6 Whole Body and Plasma Sample Collection for Thyroxine

For determination of whole body thyroxine concentration, twenty eggs or larvae were anesthetized with tricaine methanesulfonate (MS 222), 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. These sampling procedures were replicated from December 1991 to July 1992 using fish from the 1991 year class, and again from December 1992 to July 1993 using fish from the 1992 year class. Twenty eyed eggs were taken at approximately weekly periods until I 15 days post fertilization. After the alevins had reached the swimup phase, samples were taken approximately every two weeks,

For determination of plasma thyroxine concentration from pre- and post-smolts, 20 yearling (I+) fish were collected twice per month from December 1991 through July 1992 (1990 year class). This sampling schedule was repeated from December 1992 through July 1993 (1991 year class). Sampling was performed at approximately the same time each day (I 100 to 1300) so that hormone levels at a particular time of day could be established. That way, the results could not be explained by diurnal fluctuations (Grau et a/. 1981). Blood was collected from the severed caudal peduncle using heparinized capillary tubes. The blood was centrifuged at about 2000 Relative Centrifugal Force (3500 RPM) for IO minutes. Plasma was pipetted off and stored at -80°C until the time of the T_4 assay.

2.7 T_4 Measurement

Two different methods were used for assessing T_4 concentration since the age groups consisted of eggs, larva, fry, and smolts. In the early stages of development

(egg, larva and small fry), T_4 levels were measured using a whole body extraction method described in Section 2.7.1 and 2.7.2. For the larger fish (>4.5 g), circulating T_4 blood plasma levels were measured according to the procedure described in Section 2.7.2.

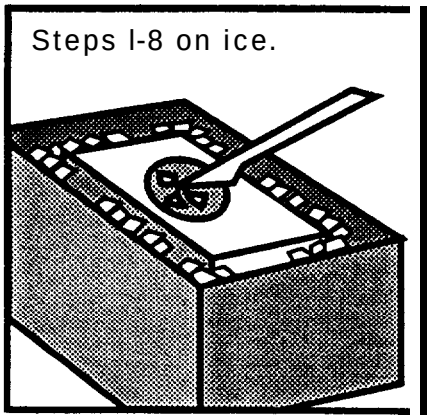
2.7.1 Whole Body Extraction

Thyroxine was extracted from whole fish and reconstituted using modifications of methods from Kobuke et a/. (1987) and Parker (1988) (Figure 6). Frozen fish were minced and placed in individual 15 X 85 mm boroscillicate glass culture tubes with 100% ice cold ethanol (ETOH) containing I mM 6-N-propyl-2-thiouracil (PTU; Sigma Chemical Company) at a µl volume equal to 2X's the mg weight of the fish. Propylthiouracil blocks the enzymatic conversion of thyroxine to triiodothyronine. Each fish was homogenized for 20 seconds with a Brinkman model Polytron 3000 tissue homogenizer at 20,000 RPM. PTU-ETOH of a µl volume of IX the mg weight of the fish was then added to the same tube and homogenized on ice for 20 additional seconds. The polytron blade was rinsed with tap water, distilled water and 95% ETOH between every sample. These sample tubes were vortexed for IO seconds and centrifuged for IO minutes at 3000 RPM using a Dynac centrifuge (Clay Adams, Inc.) refrigerated at 4°C. The supernatant was poured into a I Dram vial. The pellet was resuspended in 100% ETOH at a µl volume equal to I .5X the mg weight of the fish, and vortexed for IO seconds. This solution was again centrifuged at 3000 RPM at 4°C. The supernatant was combined with the first supernatant and dried in a vacuum oven at 60°C and 25 psi overnight. Samples were stored at 0°C until a radioimmunoassay could be performed (Appendix I). Whole body samples were reconstituted in 250 µl 95% ethanol (ETOH) and 250 µl 0.1 I M sodium barbital buffer (pH 8.6) (Figure 7, Appendix I). Each sample was then vortexed for 15 seconds. After transferring samples to I .5 ml plastic eppendorf snap cap vials, they were centrifuged at 4°C for IO minutes at 3000 RPM. Blood plasma samples from larger fish at this point were already centrifuged and ready for the radioimmunoassay.

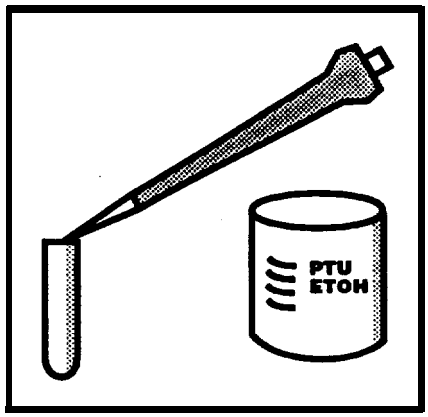
2.7.2 T_4 Radioimmunoassay

Each whole body or blood plasma sample was assayed in duplicate using a coat-a-count T_4 RIA kit (Diagnostic Products, Inc.) (Figures 8 and 9). To perform the radioimmunoassay (RIA), 25 µl of each kokanee sample and 1 ml of radiolabeled T_4 (^{125}I-T_4) were added to a tube that was coated with antibodies (Ab) which contained

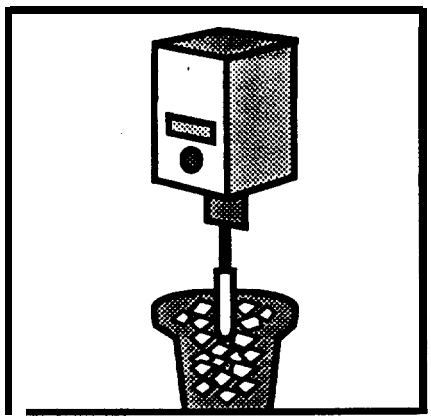
Figure 6. Procedures used for extracting and storing T_4 from fish samples.



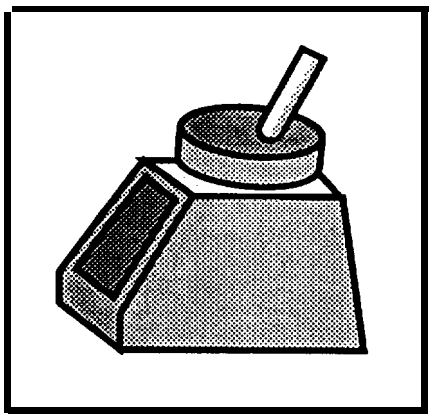
1 Mince sample with scaple in weighing boat on frozen cutting block. Put into 15 x 85 mm test tube.



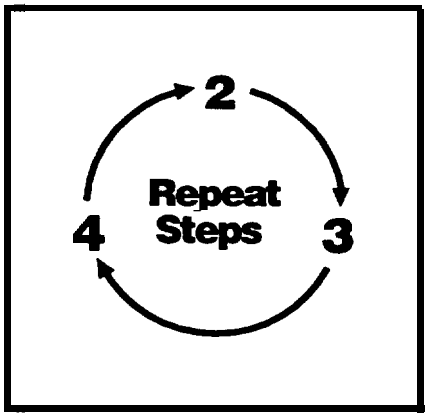
2 Add ice cold 100% ethanol containing 1.0 mM PTU [ETOH-PTU] to tube at a ul volume equal to 2 X the weight of the fish.



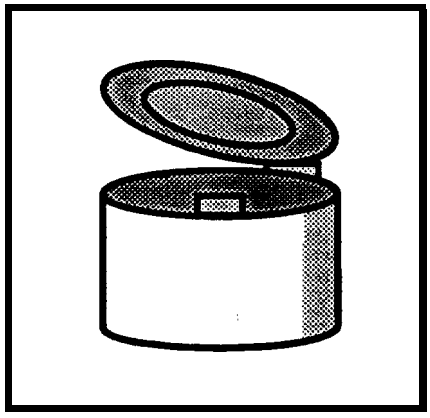
3 Homogenize for 20 sec to 1 min at 20,000 RPM using Brlnkman Instrument Polytron 3000 tissue grinder.



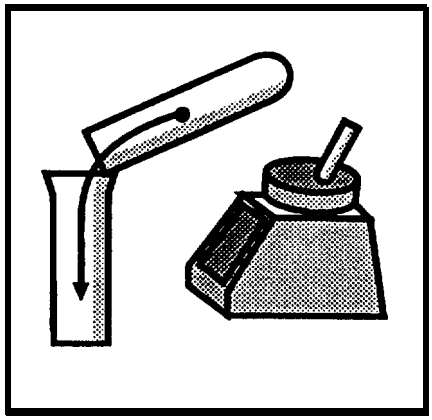
4 Vortex samples for 5 sec using a VWR Vortexer 2 and decant into centrifuge tube.



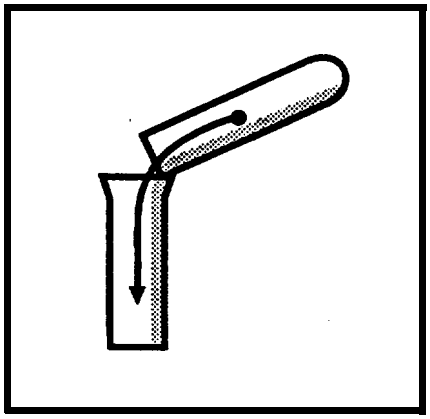
5 Repeat steps 2, 3 and 4 except use a volume of ETOH-PTU equal to I X weight of the fish, then vortex for 10 sec.



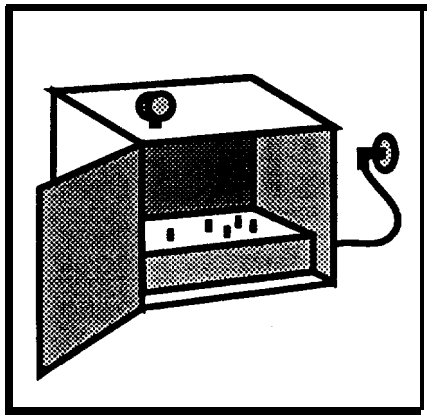
6 Centrifuge for 10 min at 3000 RPM using Clay Adams Dynac refrigerated centrifuge at 4°C.



7 Decant supernatent into a clean drying tube. Resuspend pellet in 100% ETOH (ETOH only, not ETOH-PTU) at a µl volume equal to I.5 X mg weight of the fish. Vortex for 10 sec and centrifuge at 3000 RPM for IO min at 4°C.

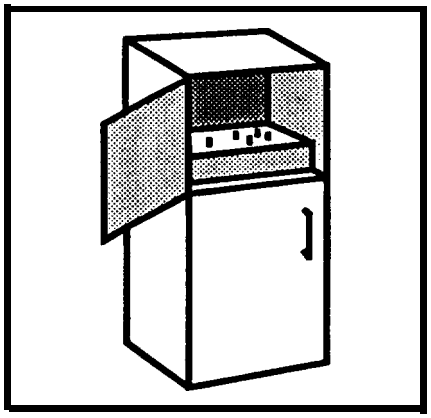


8 Pour supernatent in with first supematent. [The drying tube contains the extracted T_4].

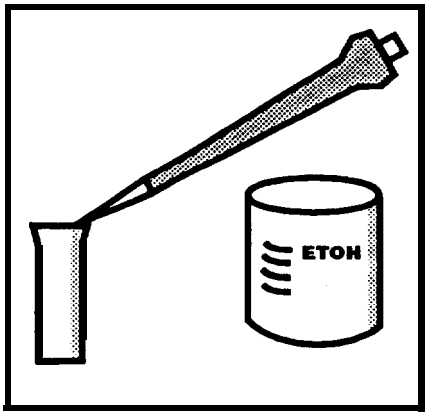


9 Dry in vacuum oven at 60°C and 25 psi until all liquid in the tube is evaporated (usually about 2 h to overnight), then store at O°C until assayed.

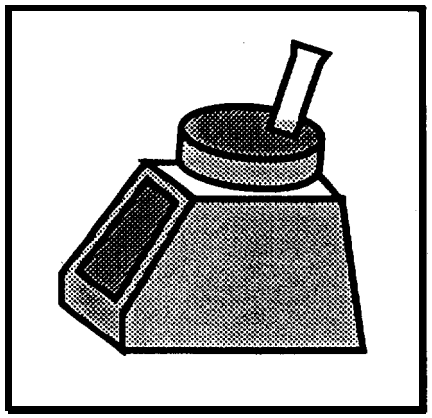
Figure 7. Procedures used to resuspend dried samples for hormone analysis.



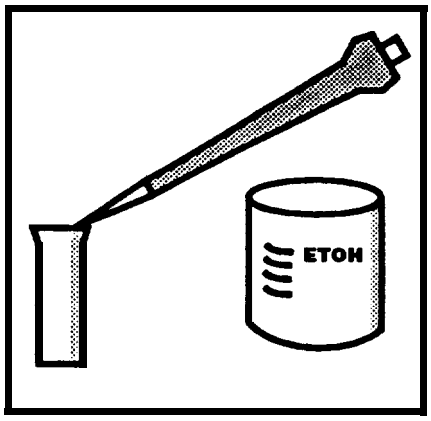
1 Remove dried samples from O°C freezer.



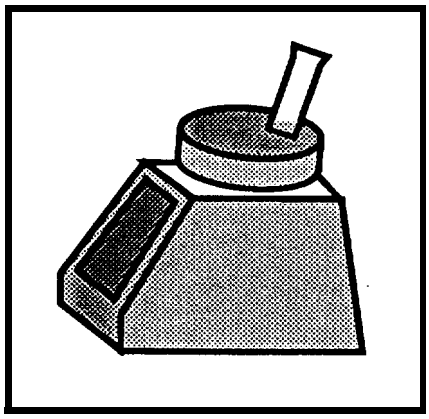
2 Add 250 µl 95% ETOH to each drying tube.



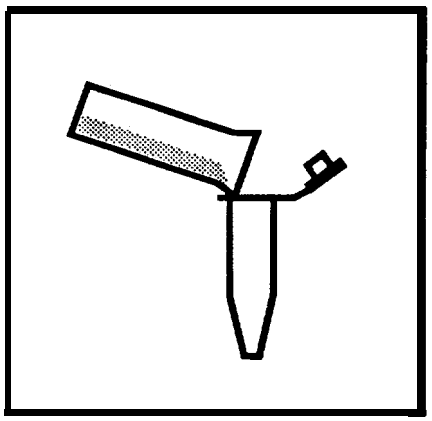
3 Vortex for 15 seconds.



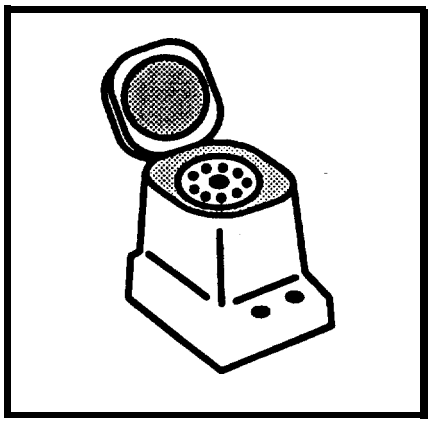
4 Add 250 µl of 0.11 M sodium barbital (pH 8.6) to each drying tube.



5 Vortex for 15 sec.



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



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

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

Figure 8. Radioimmunoassay procedure.

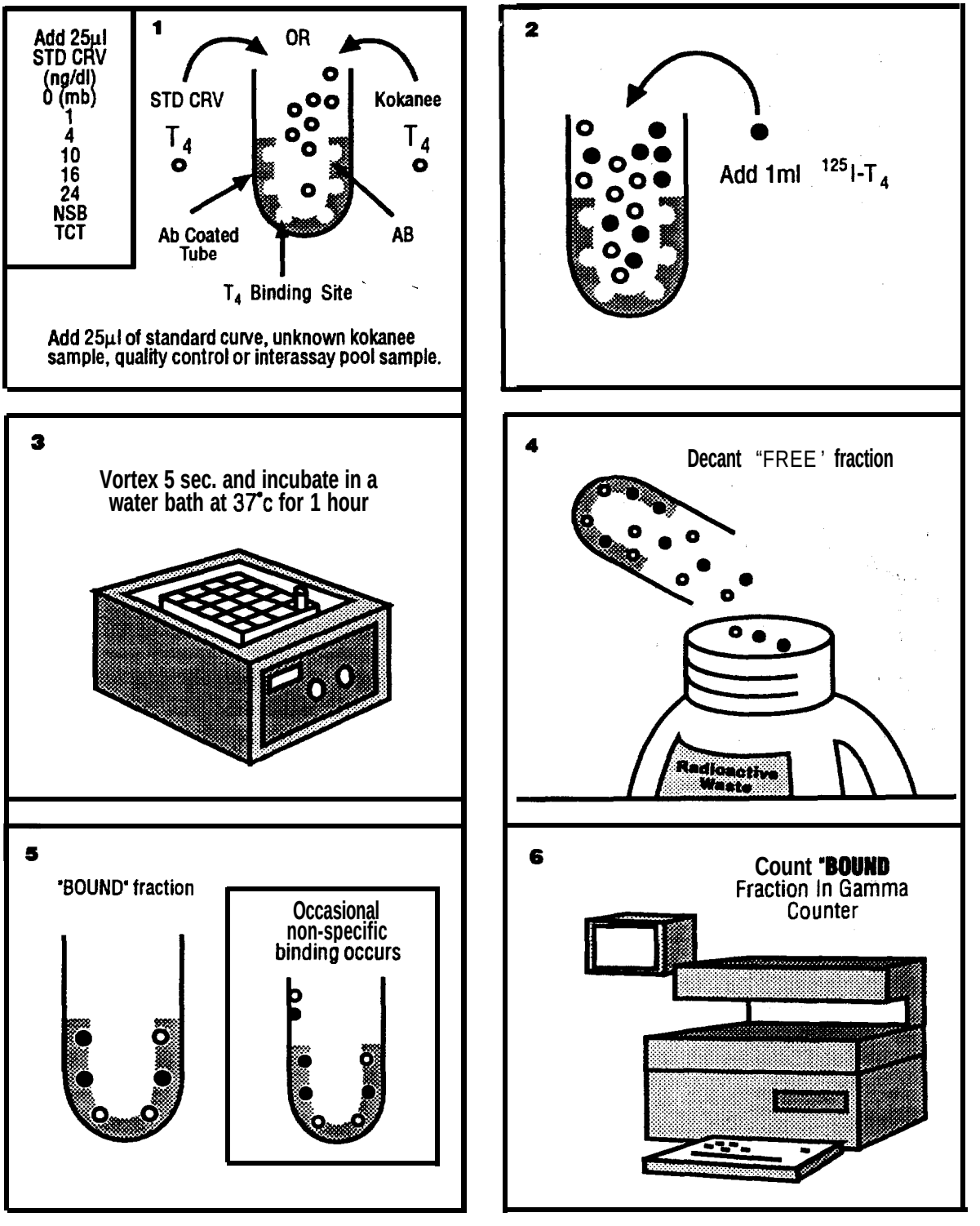


Figure 9. How the RIA technique works.

(A) **What happens in assay tubes containing high or low amounts of T_4?**

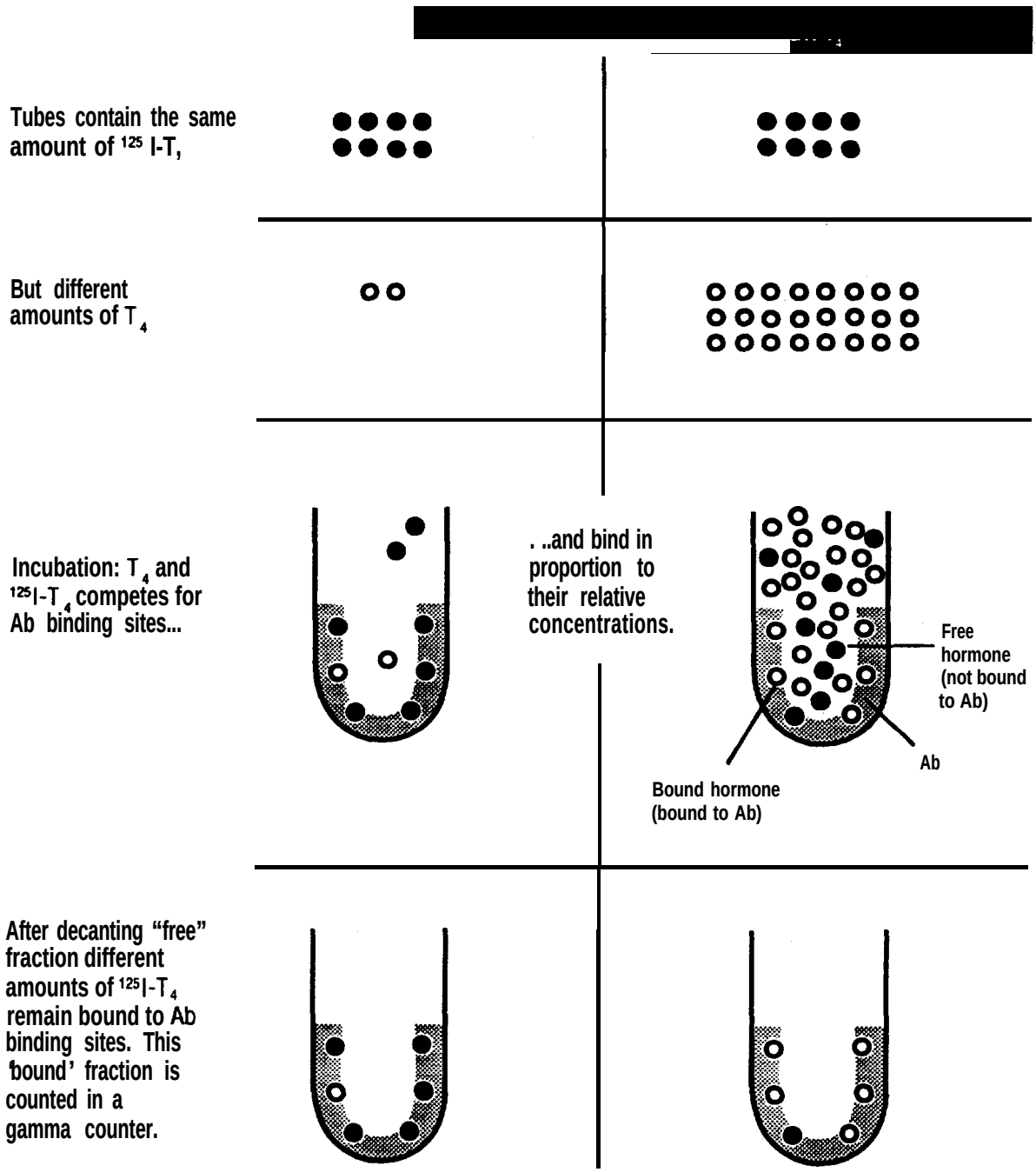
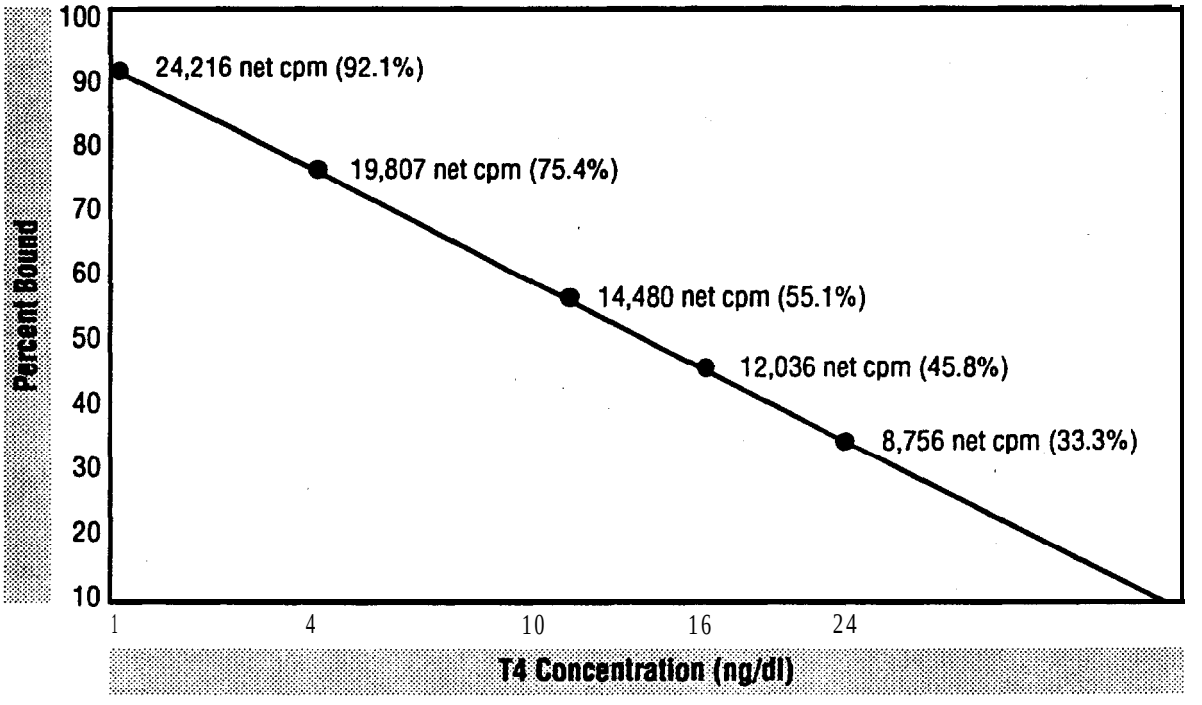


Figure 9. How the RIA technique works.

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



(C) Sample calculation to determine unknown concentration. Data were obtained as indicated in Fig 3b.

DATA

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

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

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

$$\textbf{Percent Bound} = \frac{15{,}922}{26{,}288} \times 100 = 60.8\%$$

Interpolate From Graph
60.8% Bound = 7.8 ng/dl

T_4 receptors. This procedure used a competitive binding technique where ^{125}I-T_4 and unknown T_4 sample compete for Ab binding sites, and bind in proportion to their relative concentrations. Therefore if the sample contained more nonradioactive T_4 than ^{125}I-T_4, there would be more non-radioactive T_4 bound than ^{125}I-T_4 (Figure 9). A standard curve was prepared with known concentrations of T_4 by pipetting 25 µl of 0, 0.5, 1, 4, 10, 16 and 24 µg/dl concentrations and 1 ml of ^{125}I-T_4 to Ab coated tubes. The actual concentrations of T_4 in kokanee samples were determined by comparison to the standard curve which was subjected to the same assay procedures.

Unknown kokanee and standard curve tubes were then vortexed for five seconds and incubated for 1 hour at 37°C to allow nonradioactive T_4 and ^{125}I-T_4 to compete for binding sites on the Ab. Equilibrium was obtained in 1 hour. The excess T_4 and ^{125}I-T_4 that did not bind to the receptors was decanted into a radioactive waste container and the remaining liquid in the tubes was blotted dry. Radioactivity of the remaining bound T_4 was counted for 1 minute using a programmable Cobra QC Model 85002 auto-gamma 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 = Radioactive counts per minute (cpm) of maximum binding tube; and

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

NSB, which is radioactivity that sticks to the test tube but not bound to binding sites, was determined by adding 1 ml ^{125}I-T_4 to two uncoated tubes which did not have T_4 antibody receptors. NSB tubes were decanted and remaining activity was considered nonspecific binding.

The Cobra biogamma counter was programmed to automatically determine the relative percent bound for each standard concentration and plot a log-logit graph of

the standard curve (% bound -v- standard concentration). The percent bound of each unknown kokanee sample was plotted on this standard curve and the corresponding T_4 concentration was determined.

2.8 Data Reduction

The total whole body T_4 content of individual fish samples was determined by multiplying the T_4 content of the 25 µl subsample by 20 (500 µl total sample volume divided by 25 µl subsample tested for T_4 content). This concentration was then converted to total ng T&ample. This number was divided by the weight of the fish to obtain the T_4 concentration of each fish in ng/g body weight. The mean T_4 (± SEM) was calculated for fish collected on the same sample date and plotted vs. age (days from fertilization). Analysis of variance was used to determine if there was a significant difference in T_4 concentration over time using STATVIEW II (Brainpower, Inc.) on an Apple Macintosh IIcx computer. Blood plasma T_4 concentration was measured in ng/ml from the RIA kit. The mean T_4 (±SD) was determined for fish collected on the same sample date and plotted vs. date collected. An ANOVA was used to determine if there was a significant difference in T_4 concentration over time.

2.9 Quality Control Procedures

Each T_4 samplewas assayed in duplicate to control for errors due to procedure. The mean percent error between each duplicate pair was calculated and the frequency distribution was plotted. Unknown quality assurance samples were analyzed at high, medium and low concentrations. The actual concentrations of these standards were obtained from Diagnostic Products, Inc. after all samples were run. Mean concentration (±SD) were then compared to the company's concentrations to determine assay reliability.

Distilled water was added to blank Ab coated tubes and incubated for 1 hour at 37°C to make sure the gamma counter was counting tubes properly. These blank tubes were decanted, blotted and counted like other tubes. If the counter was properly subtracting NSB counts from each sample and if the blotting procedure (when non-bound T_4 and non-bound radiolabeled T_4 were removed after incubation) did not contaminate any of the samples, these blank tubes should contain about 0 cpm (<50 cpm).

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

Six assays were performed because of the large number of samples involved. To test interassay accuracy, three different interassay pool (IAP) samples were inserted into each assay. If these were similar in concentration in all assays, the results could be compared. If not, results could not be compared. Also, the same standard curve samples were used in all assays. Mean values of IAP and standard curve samples were compared by calculating the percent error between assays.

2.10 Recovery Determination

Extraction efficiency was evaluated in two ways. In the first analysis, replicated tubes of minced eggs, alevins or fry were spiked with 0, 0.25, 0.5 and 0.75 ng of unlabeled thyroxine (n=24 fish, 2 at each concentration for each life stage). The samples were then subjected to the same extraction, reconstitution, and T4 assay procedures as unknown and standard curve samples. Mean (±SEM) for each set was calculated. Percent of T4 recovered was calculated by:

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

where: $T_4(\%R)$ = The mean percentage of T4 recovered;

$[T_4]sp$ = The mean T4 concentration measured in a particular set of replicate spiked samples;

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

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

$Wt(o)$ = Mean weight (mg) of the samples receiving 0 ng/dl T4; and

AMT = The mean concentration (amount) of T4 added to a particular spiked sample.

A linear regression was then plotted of T_4 recovered vs. T_4 added. The r^2 value determined consistency of recovery at various concentrations.

In the second analysis, approximately 11,000 - 12,000 cpm of ^{125}I-T_4 was added to each of 20 tubes (40 fish). After each step of the extraction procedure, the number of counts remaining was determined. In addition, five blank control tubes with 11,000 to 12,000 cpm of ^{125}I-T_4 added were counted to identify counts lost owing to radioactive decay. The percentage of T_4 recovered at each stage was calculated by the formula:

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

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

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

cpm_i = The mean initial number of counts put into the same tube; and

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

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

2.11 Smoltification Studies of Yearling Kokanee

To determine if yearling fish (12 - 20 months old) were going through a parr-smolt transformation, morphological, physiological and behavioral tests were

conducted in 1993 with age 1+ 1991 year class fish. Condition factor and silvering were used to determine if there were any changes in morphology. Changes in physiology were determined by testing for intestinal fluid transport (J_v), gill Na^+-K^+ ATPase activity, osmoregulatory capability and salt water tolerance. Behavioral tests included assessment of salinity preference and downstream migratory tendency.

2.11 .1 Condition Factor

Condition factor of yearling kokanee was determined by the equation below:

$$K_{TL} = (W/L^3) \times 10^5$$

where: K_{TL} = condition factor
W = weight of fish (g)
L = total length of fish (mm).

In salmonids, the condition factor drops as a fish smolts, because the fish becomes more slender and streamlined (Hoar 1939a; Fessler and Wagner 1969).

2.11.2 Intestinal Fluid Transport

Yearling fish used to measure intestinal fluid transport rate (Jv) were transported to living streams at Eastern Washington University at monthly intervals from January to June 1993. The fish were held in well water at 10-13°C for 2-5 days prior to sampling. Fluid absorption was measured in the middle and posterior intestines of 12 fish each month.

The fish were anesthetized with MS-222 and killed by a blow to the head. The body cavity was opened and the posterior intestine was cut free of the body wall (Figure 10). The intestine was then placed on ice until all fish were dissected. The maximum duration between removal of intestines from all fish and their placement in aerated physiological saline was 1 hour. The intestines were removed by gently tearing the mesenteries until they hung free from the peritoneal cavity. An effort was made to prevent the intestines from becoming contaminated with external body secretions. Once removed, the middle intestine was cut at the junction just posterior to the attachment of the pyloric caeca. Adhering mesenteries and blood vessels were then removed and the middle and posterior segments were separated at the prominent ileorectal valve. Their lumens were rinsed with Ringers solution (148 mM

Figure 10. Determination of intestinal water uptake: sac preparation.

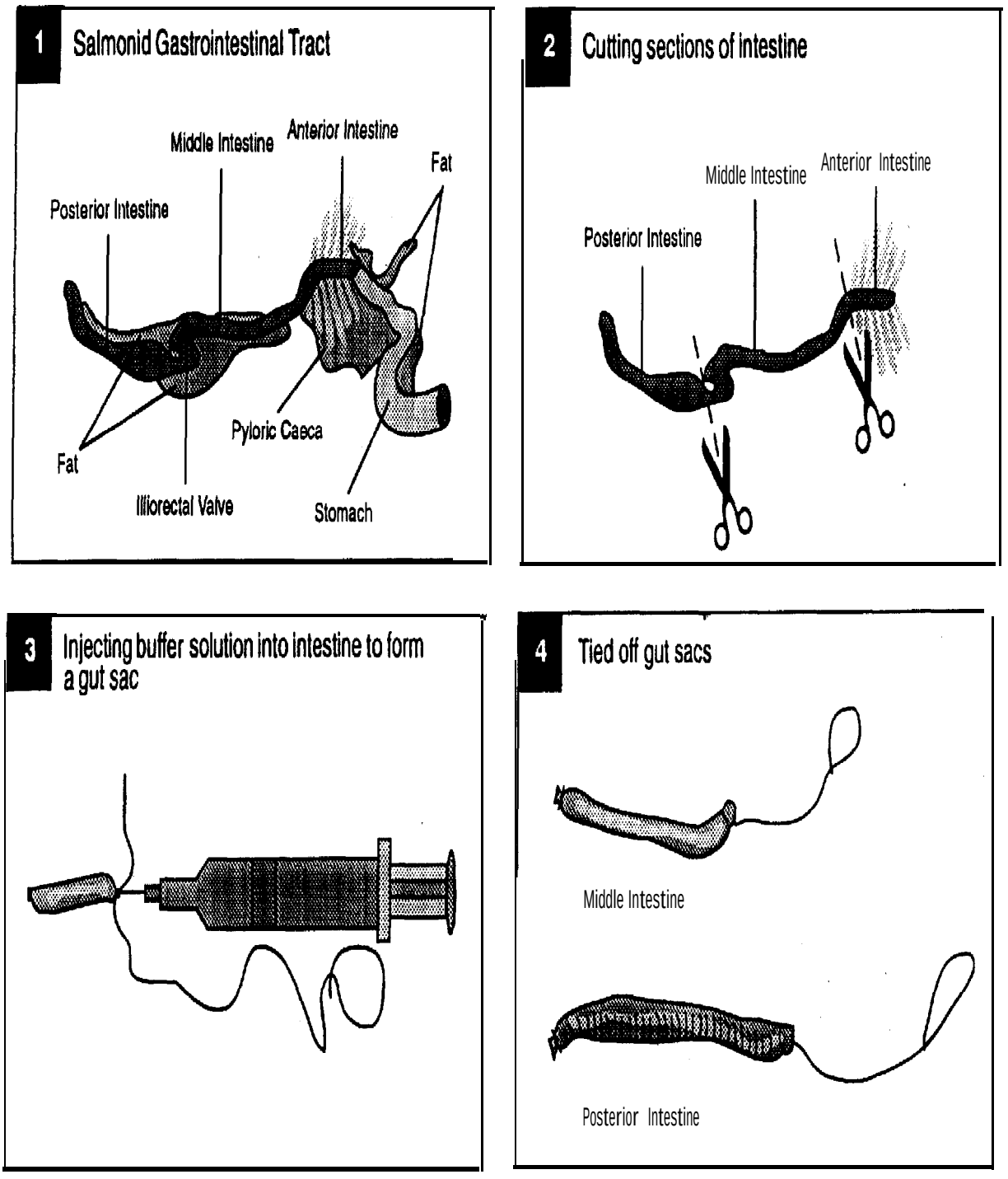
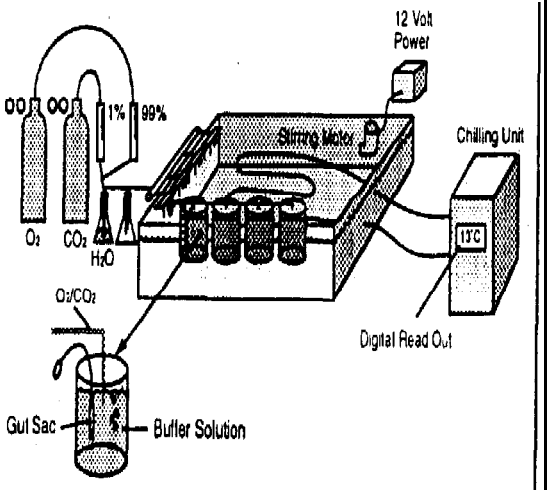


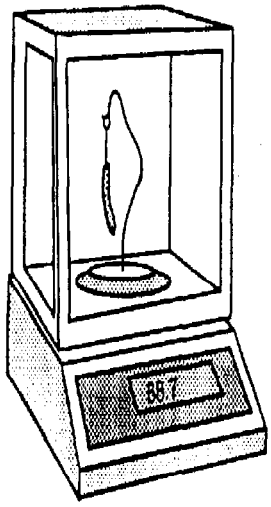
Figure 10. (Continued) Determination of intestinal water uptake: analysis of water transport.

5 Incubating intestinal gut sacs

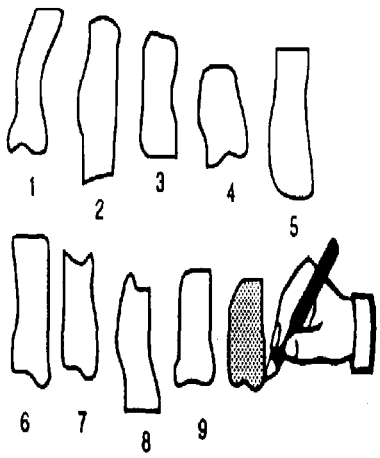


(Aerated gut sac in buffer solution)

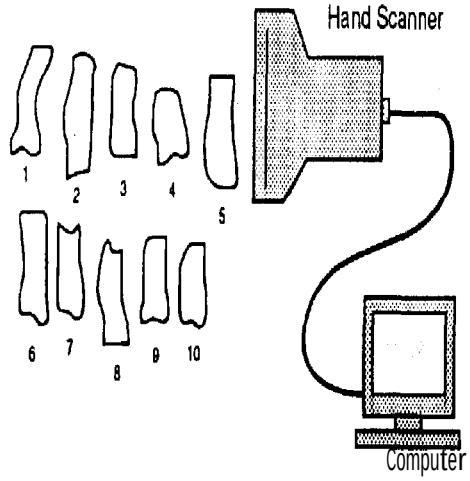
6 Regression of weight loss over time determines rate of transport



7 Size variability is standardized by determining intestinal surface area



8 Surface area determined by scanning intestinal perimeters into a computer



NaCl, 20 mM $NaHCO_3$, 2 mM K_2HPO_4, 10 mM glucose and $CaSO_4$, $MgSO_4$, KCl; 1 mM each) (Field et *al.* 1978), adjusted to pH 8.2 and 320 ± 10 mOsm/l. Collie and Bern (1982) have shown with histological examination that no gross morphological changes are apparent for up to 7 hours using this procedure. Osmolarity of buffer solutions was measured on a freezing point osmometer.

After rinsing out the contents of the intestines with buffered Ringer's solution, the most distal end was tied off with small size, lightly waxed, unflavored flossing nylon. A blunted hypodermic needle attached to a plastic syringe was then inserted into the other end of the intestine and a single tie of nylon was made about the intestine and needle. The intestine was then inflated with Ringer's solution until it began to slip off the shank of the needle (Figure 10). At this point the filling was stopped, the intestine was slid off the needle and the knot was pulled tight. Consistency in the procedure was developed in the last several years by doing many hundreds of Jv's [J = rate of transport of (v) = salts and water] associated with other studies (Kerstetter and White in press; Veillette et *al.* 1993). Despite possible variability in the pressures among the intestinal sacs, Collie and Bern (1982) have shown that nonevetted intestinal sacs from coho salmon do not have significant differences in the slope of sac weight loss with time for hydrostatic pressures between l-20 cm H_2O.

The gut sacs were then suspended in 35 ml of Ringer's of identical composition to that placed in the lumen; maintained at 13°C and bubbled vigorously with 95.5% O_2/0.5% CO_2, which maintained a pH of 8.2 (Figure 10). The sacs were pre-incubated 1 h prior to measurement of Jv. After pre-incubation the sacs were weighed to the nearest mg at 15 min intervals for 1 h. The slope of the regression line for sac weight loss plotted against time represented rate of water loss. This was normalized by dividing the surface area of the sac to give a Jv in $\mu l \cdot cm\text{-}2 \cdot h^{-1}$. Surface area was determined by spreading the sac serosal side down on water proof paper (J.L. Darling, Corp.), then outlining with 0.5 mm pencil lead (Figure 10). Using a Macintosh IIcx computer, the outline of the sac was then digitized with LIGHTNING SCAN and its surface area determined using the public domain software IMAGE (Figure 10). An ANOVA was used to document the change in Jv over time. Significant differences were reported by this method when $P \leq 0.05$.

2.11.3 Gill Na^+K^+-ATPase Activity

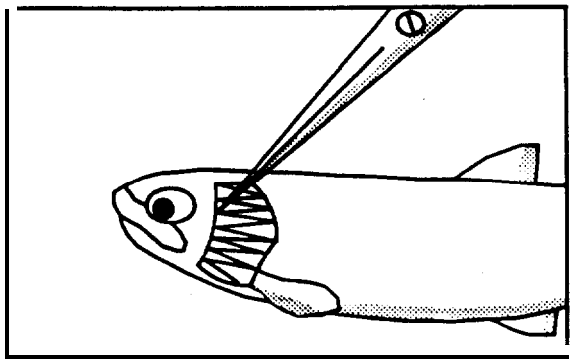
Gill Na^+-K^+ ATPase activities of yearling fish were determined using the method of Zaugg (1982a) (Figure 11, Appendix B). The recipes for chemical solutions used in this assay are recorded in Table 3. Samples of gill filaments from individual fish (n=20) were collected once a month from January 1993 through March 1993, then twice a month from April through June 1993. One ml of SEI (0.03 M sucrose, 0.02 M ethylenediamine tetraacetate, 0.1 M imidazole) was added to each gill sample and kept on dry ice during transportation from the hatchery to the Eastern Washington University (EWU) lab. Gill samples were stored at -80°C until the time of the assay.

Enzyme preparations were made by homogenizing thawed samples at 4°C and decanting the homogenate into a centrifuge tube placed in ice water. All homogenates were then centrifuged for 7 minutes at about 2000 Relative Centrifugal Force (3500-3900 rpm). The supernatant solutions were discarded and the pellets were manually resuspended in 0.5-l ml of SEID (SEI plus sodium deoxycholate) using a 10 ml glass tissue grinder. Homogenates were then centrifuged for 6 minutes and an aliquot (0.2 ml) was withdrawn for protein determination.

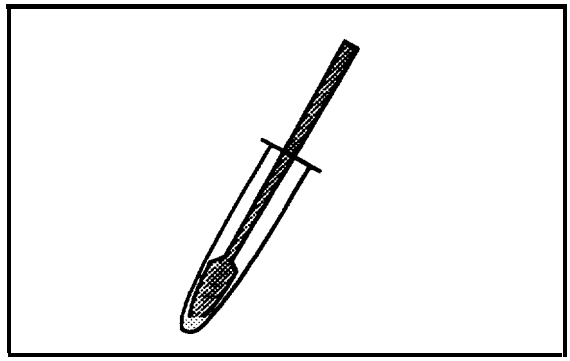
One 10 µl aliquot of each homogenate was placed into a 16 X 100 mm test tube on ice containing 0.65 ml of Solution A, and another 10 µl aliquot into a tube containing the same volume of Solution B which contained ouabain (See Table 3). Ouabain blocks potassium (K^+) binding sites therefore preventing coupled transport of Na^+ and K^+. This inactivates Na^+-K^+ ATPase but not other ATPases. Added to each of these tubes was 0.1 ml of 0.03 M Na_2ATP. These samples were then placed in a constant temperature water bath (37°C) for 10 minutes after which they were again placed on ice. Each unknown gill homogenate was allowed to react with ATP in Solution B (ouabain insensitive ATPase activity) and with ATP in Solution A (ouabain sensitive plus ouabain insensitive ATPase activity). The difference between the two levels of ATPase activity (A minus B) was the calculated Na^+-K^+ ATPase activity.

To determine the amount of phosphate that was released during hydrolysis of ATP, a colorimetric phosphate assay was performed. Samples were acidified with 1.85 ml of perchloric acid ($HClO_4$) to denature the protein, then 3.0 ml of 2-Octanol were added. Then 0.25 ml of ammonium molybdate reagent and 0.5 ml citrate reagent were added and the samples were shaken vigorously. Tubes were briefly centrifuged to separate the upper phosphate-molybdate containing layer. Absorbance of the

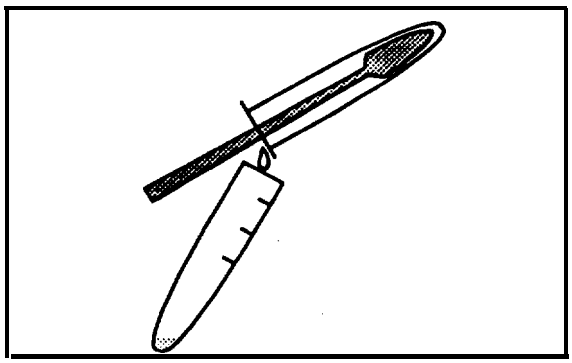
Figure 11. Procedures used for gill Na^+-K^+ ATPase determination. Methods taken from Zaugg (1982a).



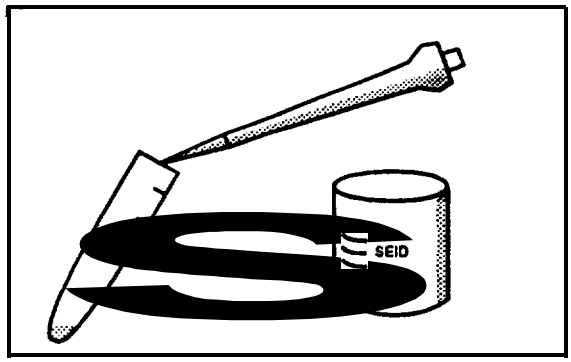
1. Gill filaments cut from both sides of fish, put into SEI and frozen (-80°c).



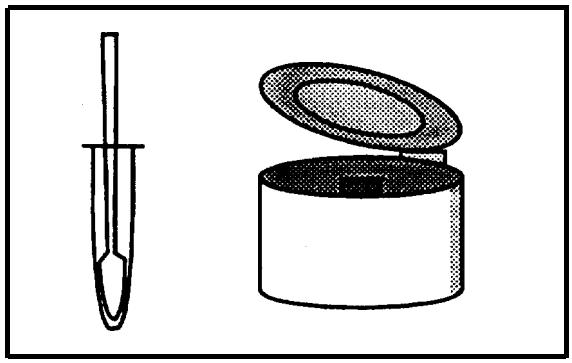
2. Manually homogenize thawed gill sample.



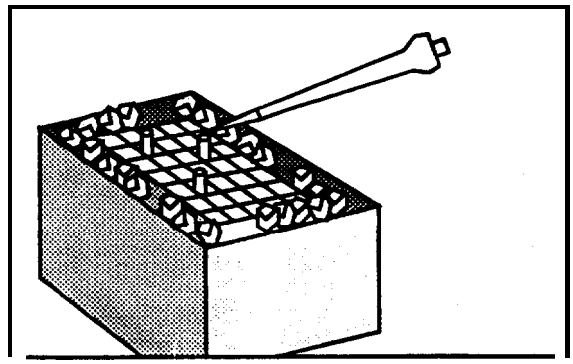
3. Decant homogenate into centrifuge tube on ice. Centrifuge 7 minutes at 3500 RPM.



4. Discard supernatant and suspend pellet in 0.5-l ml SEID.

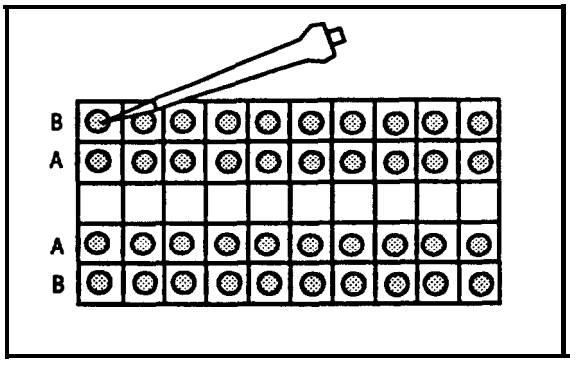


5. Homogenize and centrifuge for 6 minutes.

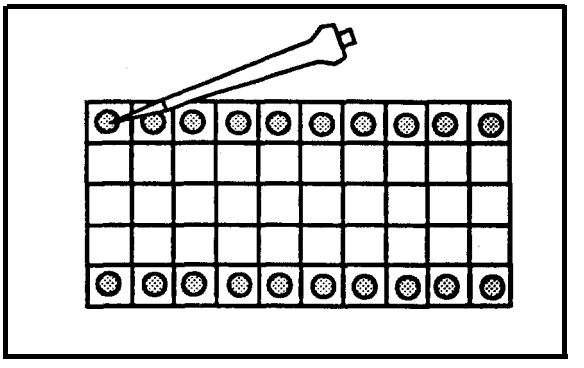


6. Pipet off liquid aliquot (~.2ml) into 13x100mm test tubes. This contains the enzyme Prep. Keep tubes in ice bath.

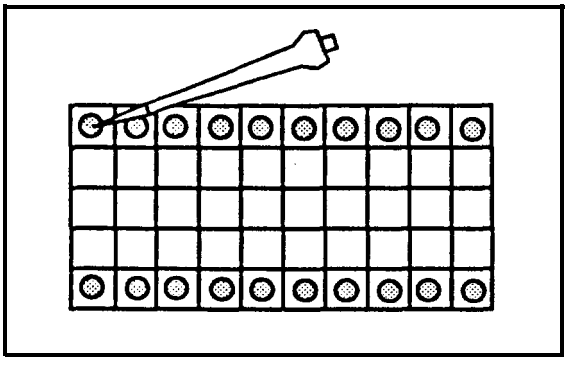
Figure 11. continued



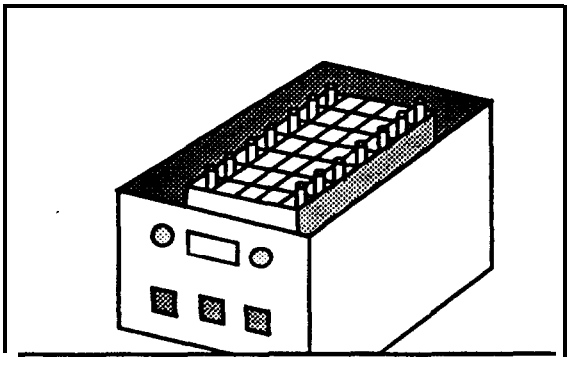
7. Add 10µl of each enzyme prep in 16x100mm test tube containing 0.65ml Stock A and another 10µl into Stock B. Keep tubes in ice bath.



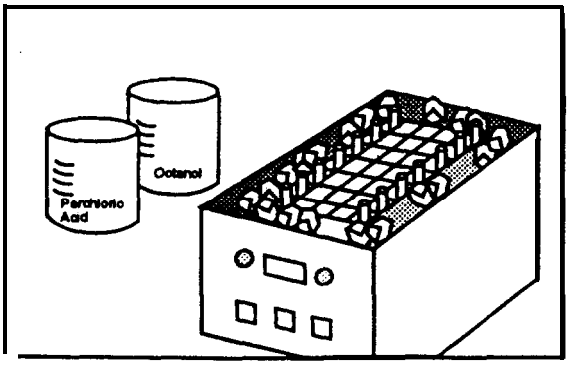
8. Add 25µl enzyme prep to 16x100mm test tubes containing lml water. Add 25µl SEID to blank. Set aside for protein determination.



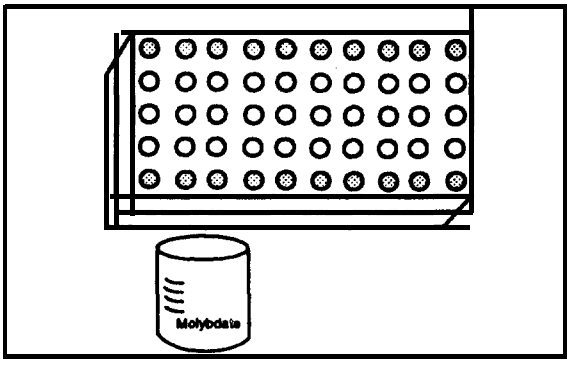
9. Add 0.1 ml Na_2ATP to each of the tubes in Step 7 and to reagent blanks.



10. Shake rack briefly before placing into constant temperature water bath (37°c) and shake for 1 minute of 10 minute period.

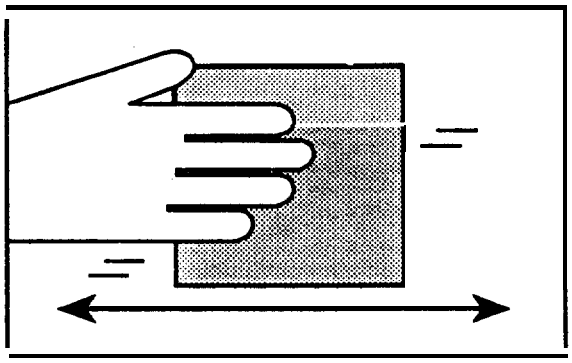


11. Place in ice bath and shake 1 minute. Add 1.85ml Perchloric acid, followed immediately by 3ml octanol.

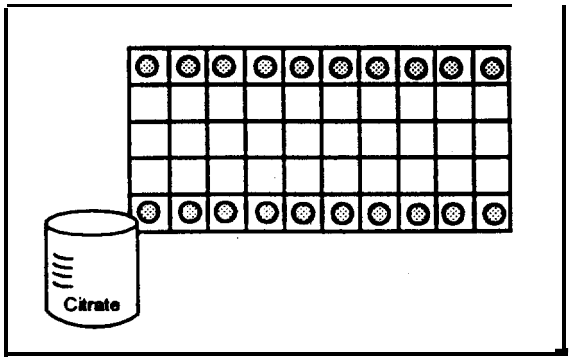


12. Transfer tubes to wooden rack with foam bottom and lid. Add 0.25ml Molybdate reagent to each tube.

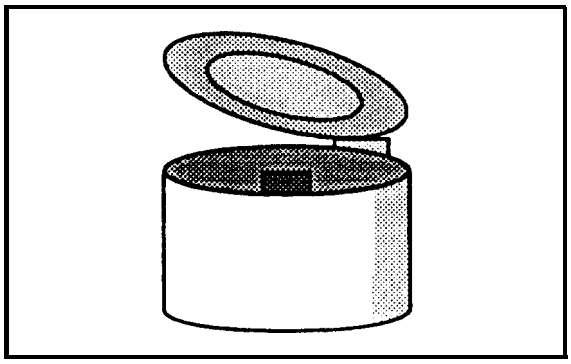
Figure 11. continued



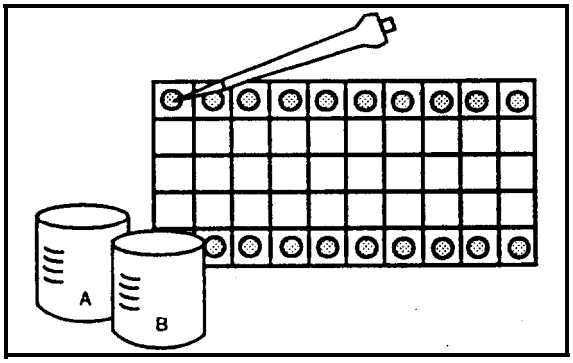
13. Place sheet of plastic wrap over top of tubes and place foam lid on rack. Shake vigorously for 30 seconds.



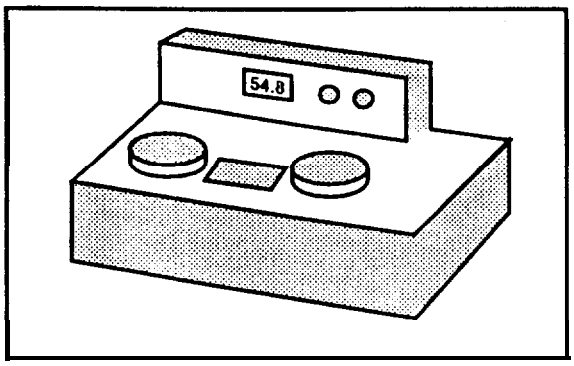
14. Discard saran wrap and add 0.5ml Citrate reagent. Repeat step P13.



15. Centrifuge tubes quickly.



16. For protein determination add 25µl of enzyme prep to each tube containing1 ml of dH20. Add 1 ml protein stock solution A, shake and let sit for 5min. Add 3ml stock solution B (Folins) and digest 1 hour.



17. Protein: Immediately read absorbance of proteins at 700nm with UV spectrophotometer. Phosphate: Read absorbance of octanol layer at 312nm with UV spectrophotometer.

Table 3. Recipe for chemical solutions for Na^+-K^+ ATPase assay taken from Zaugg (1982a).

SEI:

0.3 M reagent grade sucrose (102.7 g/L)
0.02 M disodium ethylenediamine tetraacetate (Na_2EDTA, 7.44 g/L); and
0.1 M imidazole (6.8 g/L);
all adjusted to a final pH 7.1 with HCI.

SEID:

SEI + sodium deoxycholate (0.1 g/l 00 ml)

STOCK A:

4.68 g $MgCl_2 \cdot 6\ H_2O$
9.07 g NaCl
5.6 g KCl
7.83 g imiditole
dissolved in a final volume of 1 L (including final adjustment of pH to 7.0 with HCI.

STOCK B:

0.42 g ouabain
added to 1 L of Solution A.

Na_2ATP (aqueous):

1.84 g/100 ml H_2O
adjusted to pH 7.0 with NaOH.

$HClO_4$:

0.95% (v/v)

AMMONIUM MOLYBDATE:

58.4 g $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$
dissolved in 200 ml of concentrated HCI, diluted to 1 L with distilled water.

CITRATE:

143 g citric acid·H_2O in 1 L final volume including adjustment to pH 2.9 with approximately 14 g NaOH.

yellow phosphate-molybdate complex containing octanol was determined with a Shimadzu UV160 spectrophotometer at 312 nm. The amount of phosphate hydrolyzed in unknowns was determined by comparing absorbance values to standards using known amounts of phosphate. Activities were presented as μmoles Pi . mg protein^{-1} . h^{-1} (Appendix A). Protein concentrations were determined by the method of Lowry et al. (1951) as modified by Miller (1959). Bovine serum albumin was used as a standard in this assay (Table 4).

Analysis of variance (ANOVA) was used to test for a difference between mean gill Na^+-K^+ ATPase enzyme activities for each group of fish over the sample period of six months. Significant differences were reported by this method when $P \leq 0.05$.

2.11.4 Seawater Survival

Seawater survival (salt water tolerance) was tested on yearling fish every month beginning in January 1993 and ending in July 1993. Once a month, 80 fish were transported from the Spokane Tribal Hatchery to Eastern Washington University (EWU) in garbage cans with air stones and kept at constant temperature. At EWU, fish were held in Living Streams at natural photoperiod. After a one to three day acclimation period in fresh water, fish were placed in three 500 L tanks with 10 parts per thousand (ppt) (n=20), 20 ppt (n=20) or 30 ppt (n=20) salinity. Twenty additional fish were kept in fresh water (Figure 12).

After 96 hours, percent survival was calculated and shown by the number of individual fish (n=20) alive after 96 h (Figure 12). The percent survival was calculated by the equation:

$$\% \quad S_{oc} = \frac{S \times 100}{N}$$

where S_{oc} = percent survival at a particular osmotic concentration,
s = number of fish alive after 96 hours,
N = initial number of fish in tank

Table 4. Recipe for Lowry protein determination.

STOCK 1

1 part Na-K tartrate
(10.0 g/500 ml)

1 part $CuSO_4$
(5.0 g/500 ml)

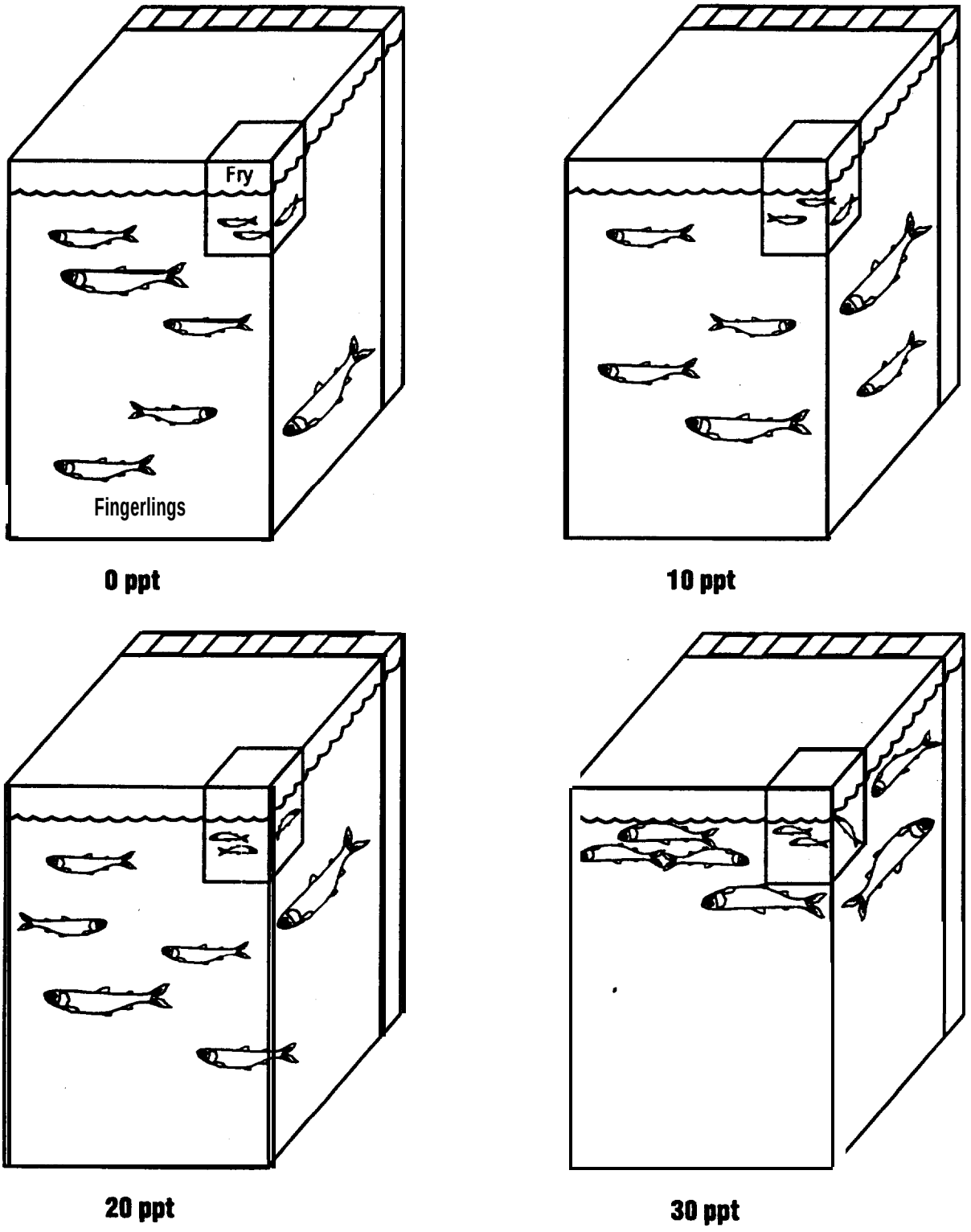
20 parts alkaline Na_2CO_3
(100 g Na_2CO_3 plus 20 g NaOH in 1 L final volume)

STOCK 2

1 part Folins Reagent (Sigma Chemical Co.)

10 parts distilled water

Figure 12. Salinity tolerance experimental setup. Blood plasma osmolarity was measured after 96 hours or when fish died.



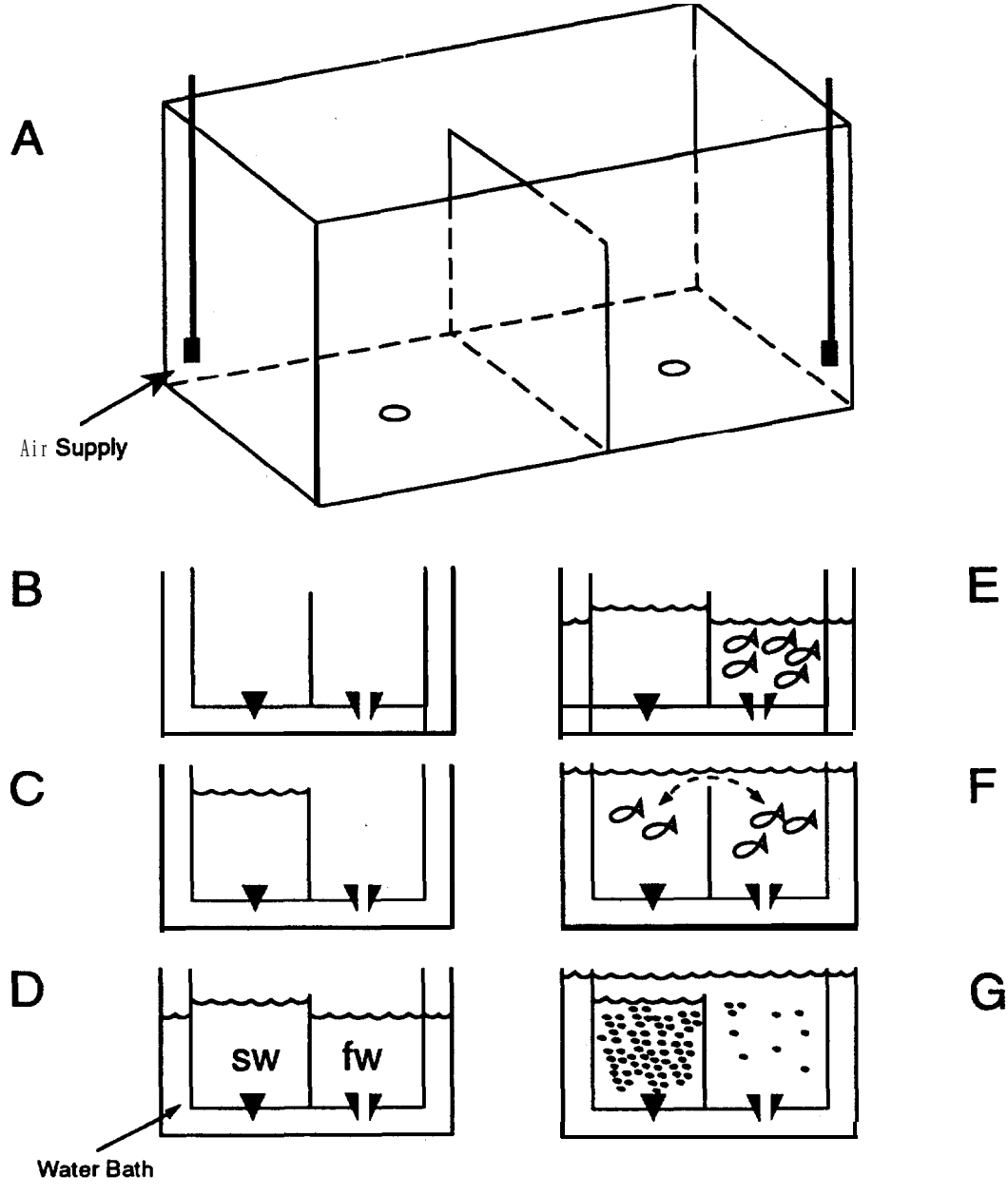
2.11.5 Osmoregulatory Capability

Osmoregulatory capability of fish used for salt water tolerance tests was determined after 96 hours or at the time of death. Blood samples were taken by severing the caudal peduncle and collecting blood from the caudal vein using heparinized capillary tubes. Plasma was immediately separated by centrifugation for 10 minutes at approximately 2000 Relative Centrifugal Force and stored at -80°C. Osmolarity was determined using a freezing point microosmometer by Precision Systems, Inc. The instrument was calibrated with a 100, 300 and 500 mOsm/l standard before each assay. If the values were within 2 to 3 mOsmol/l of each other, and the mean value had a S.D. of ±3 mOsmol/l, then the machine was considered calibrated. Samples were analyzed with these known standards supplied by Precision Systems, Inc., at regular intervals during the assay. An ANOVA was used to determine if there were any significant differences in mean monthly osmolarity. A t-test was used to determine if there were significant differences between mean osmolarity of fish held in fresh water and mean osmolarity of fish held in 10, 20 or 30 ppt salt water.

2.11.6 Salinity Preference

Salinity preference tests began in February 1993 and continued once a month through June 1993. Twenty five yearling fish were transported to EWU and held for at least 24 hours as an acclimation period. The salinity preference test followed the methods of Mclnerney (1964) and permitted young salmon to choose between fresh water and a particular concentration of seawater ranging from O-24 ppt. Three salinity preference test tanks were arranged side by side in a running water bath (Figure 13-A). An incomplete partition divided the tank into two compartments each containing an airstone and bottom center inlet. To begin the test, the inlets in each compartment were stoppered with rubber plugs, one solid and the other pierced by 1/4-inch diameter hole leaving the second compartment continuous with the surrounding water bath so that water could be added to the trough (Figure 13-B). Next the test seawater solution was poured into the sealed compartment to the level of the partition (Figure 13-C). Seawater concentrations were made by adding instant ocean salts to dechlorinated tap water to achieve osmotic concentration of 0, 8 and 24 ppt. Then the water bath was filled with fresh water and the unsealed compartment was flooded to within approximately 1/2-1 inch from the top of the partition (Figure 13-D). Five fish

Figure 13. Salinity preference tank and test procedure. Refer to text for explanation of figure.



were placed in the unsealed freshwater compartment with airstones and the fish were allowed to acclimate for 1 hour (Figure 13-E). After 1 hour, the air supply was turned off and the level of the surrounding water bath was again raised very slowly so that a freshwater "bridge" was formed across the partition allowing the fish to move freely between the two compartments (Figure 13-F). The depth of the bridge varied depending on the size of the fish. Figure 13-G diagrammatically shows the fresh water bridge and stratification layer with the less dense water on top. Two lights, and an observation mirror were mounted above the tank. The entire apparatus was surrounded with dark grey walls and curtains except for an eye-level slit through which observations could be made from a darkened background.

Observations were made once every 2 minutes by recording the number of fish in each of the two compartments for a period of 100 minutes. The seawater tanks were arranged in a random fashion, and the observations were taken without knowledge as to which compartments contained particular seawater concentrations. Evaluations of biological significance were based on an interpretation of the pattern of response to the various concentrations. If the percent preference was above 50%, a positive response (preference) to salt water was assumed. If the percent preference was below 50%, an avoidance to salt water was assumed. When a 50% preference was shown, it was assumed that there was no preference or avoidance. The response to freshwater was used as the baseline or control. Fish in the freshwater troughs should show approximately 50% preference, therefore spending equal amounts of time in each freshwater compartment.

2.11.7 Silvering

Coloration was visually determined at each sampling period for each fish. The classification scheme for coloration was as follows:

Parr: No silvering. Parr marks are clear.

Transition: Moderate degree of silvering. Parr marks are distinguishable but fish are starting to get silver.

Smolt: Parr marks absent. Silver layer very thick.

Data (monthly mean percentage $\pm$ S.D. of parr, transition or smolt) were analyzed using ANOVA. Significant differences were reported when $P \leq 0.05$. Data

was also regressed against T_4 concentration to determine if there was a correlation between the two variables.

2.11.8 Downstream Migratory Behavior

Yearling fish were picked up from the Spokane Tribal Hatchery and transported in plastic garbage cans to Eastern Washington University once a month from January through June 1993. These fish were transferred into Living Streams and kept at a temperature of 9-11 ° C. They were observed twice a month during this time. The Living Stream was visually divided into three equal parts by placing tape on the outside of the stream. At night, the percentage of fish in the upper, middle and downstream thirds of the tank were recorded every three minutes for a total of 21 minutes. The mean percent of fish in the downstream end was then determined for each sampling period.

Data (monthly mean percentage of fish in downstream third of tank) were analyzed using ANOVA to determine if there was a significant difference over time. In addition, a regression was run with thyroxine content to determine if there was a correlation between the two variables.

3.0 RESULTS

3.1 Imprinting Investigations

3.1.1 Thyroxine Content from 1992 Investigations

Results of experiments conducted in 1992 indicated that developing kokanee salmon (1991 year class) experienced elevated whole body thyroxine content at the time of hatch and again at swimup (Figure 14). Thyroxine content in eggs ranged from 6.5 - 10.5 ng/g body weight. Peak levels of 22.1 ± 5.2 ng/g body weight were observed at swimup (days 85-95 post-fertilization) then rapidly declined to 0.5 ± 0.1 ng/g body weight in older fry. 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) (Figure 14). At one month post-swimup their body weight had approximately doubled. They weighed about 0.5 g by 162 days post-fertilization, and 3.5 g by 248 days post-fertilization.

Circulating thyroxine levels in plasma of older fry to smolt stage fish (1990 year class) were low during summer, increased in autumn, decreased in early winter, and increased in late winter and spring (Figure 15). Peak levels were observed in April and May (Figure 15) when the fish were smolts (481-541 days post-fertilization). These 1.5 year old fish developed thick silver guanine plating over scales and displayed downstream orientation at night, which are characteristics of smolts.

3.1.2 Odor Discrimination Investigations in 1993

Behavioral tests were conducted in 1993 with the same fish which were described above. These tests were conducted in a natural Y-maze, with traps located at each arm and at a position downstream from the release point as described in Section 2.4 and Figure 5. Those fish, which were exposed to synthetic chemicals during the fertilized egg stage (O-30 days post-fertilization), were captured in traps scented with their exposure chemical only 17 percent of the time, the alternate chemical 15% of the time and at the downstream weir 67% of the time when odors were present (Tables 5,6, Figure 16). When the odor was absent, 92% were recovered at the downstream weir (Tables 5,6, Figure 16). Since their ability to discriminate odors was poor and the majority of the fish were captured at the downstream weir in both odor present and odor absent trials, this group was classified

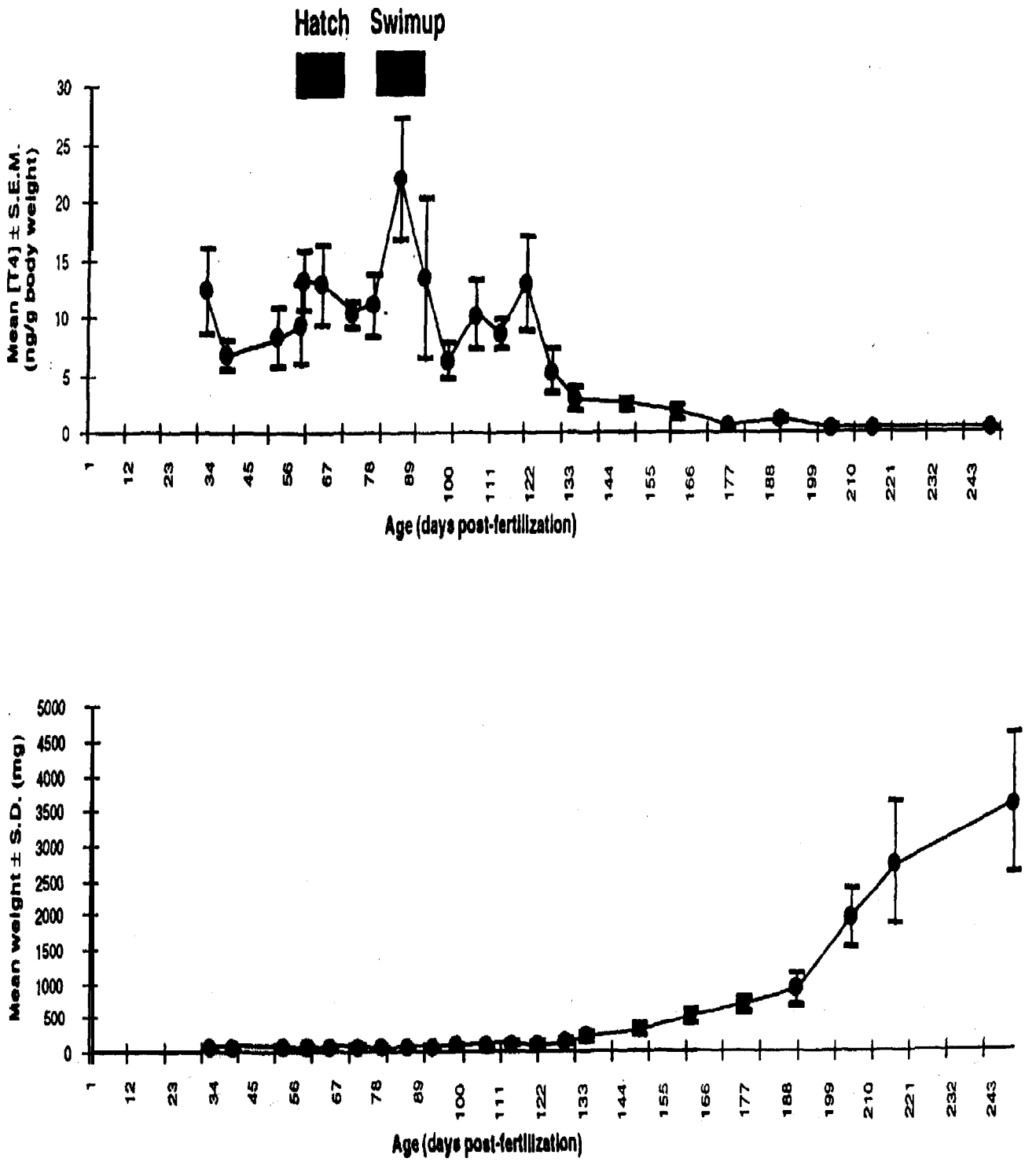


Figure 14. Whole body T_4 concentration and weights of 1991 brood, Lake Whatcom stock kokanee.

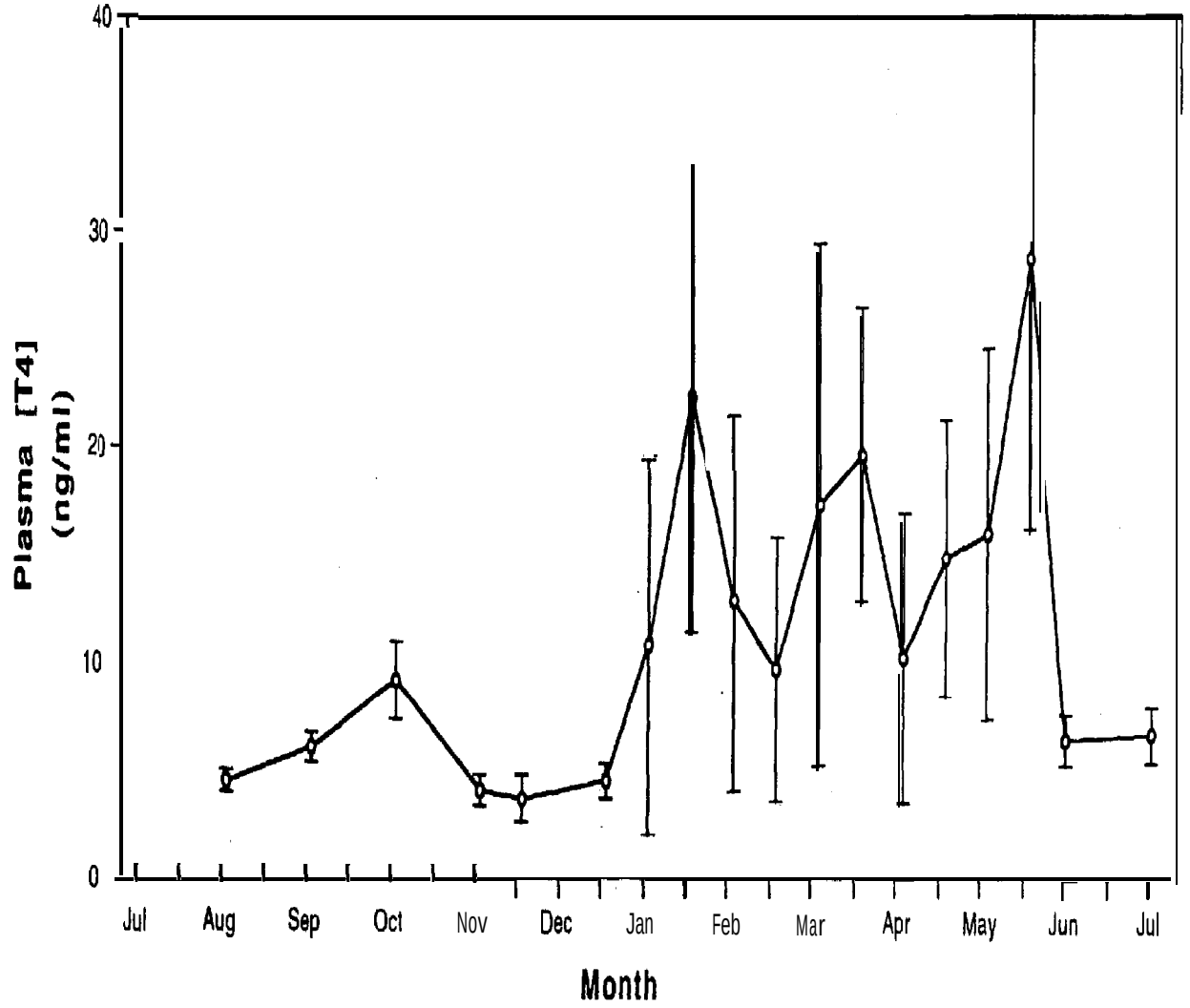


Figure 15. T_4 concentration in blood plasma of 1990 year class kokanee salmon from August 1991 to July 1992. Each point represents the mean ± S.D. of 10-22 fish.

Table 5. **Thyroxine content and percentage homing to synthetic chemicals of kokanee salmon exposed to either morpholine (MOR) or phenethyl alcohol (PEA) at different life stages. Behavioral tests were conducted to determine which fish migrated upstream to the arm with their exposure chemical and which migrated downstream instead. Percentages of fish captured in either MOR or PEA scented traps, or at a downstream weir, under odor present or odor absen conditions were based on the total number trials for each condition. The asterisk denotes blood serum concentration in ng/ml.**

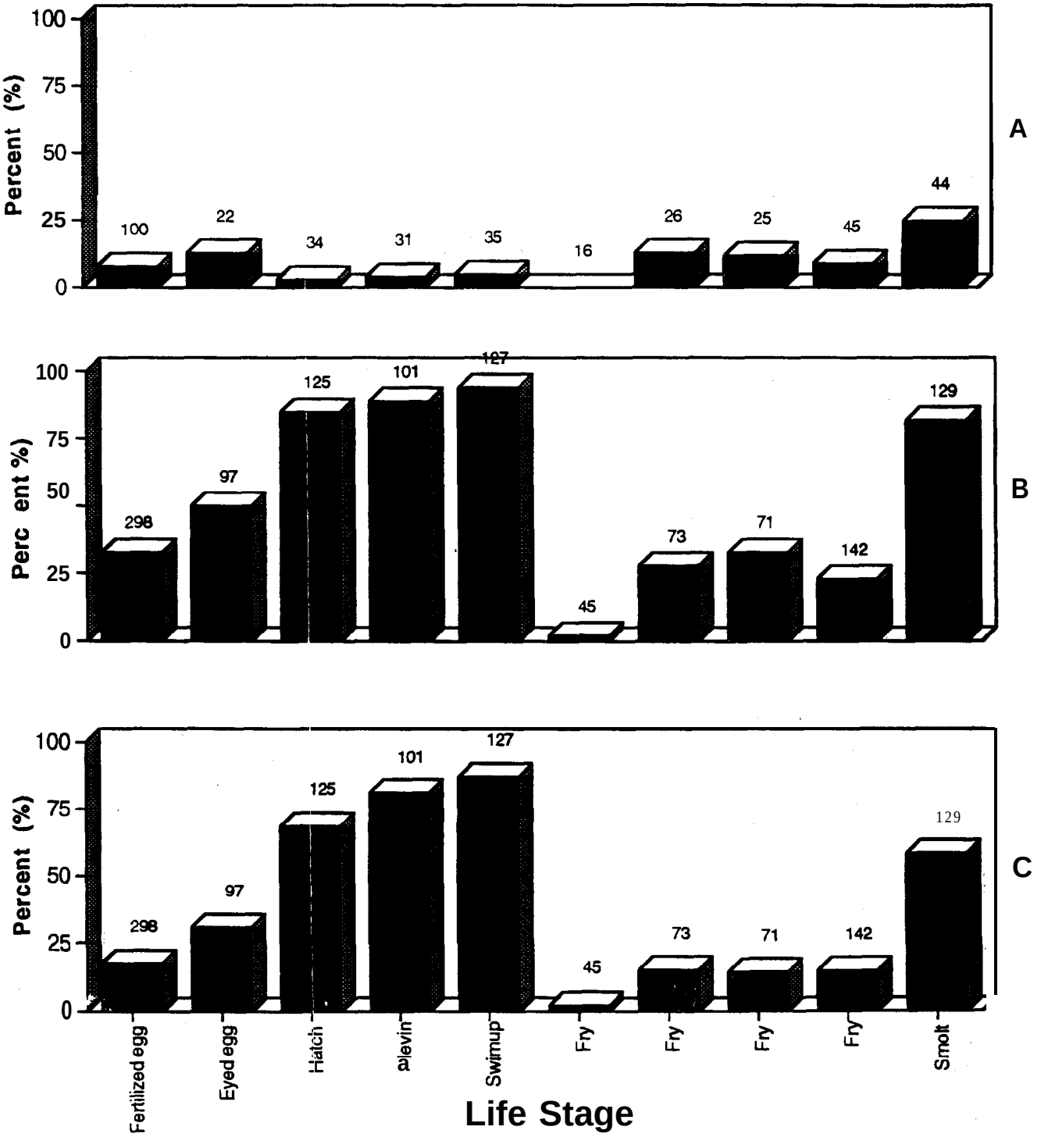
					Odor Present				Odor Absent			
Exposure Stage	Days Post= Fertilization	[T4]±S.D. (ng/g body weight)	Exposure Odor Chemical	#fish tested	# trials (n)	MOR Trap (%)	PEA Trap (%)	DOWN (%)	# trials (n)	Trap A (%)	Trap B (%)	DOWN (%)
Fertilized Egg	0-30	9.5 ± 1.7	PEA	242	298	15	17	67	100	3	5	92
Eyed Egg	30-60	9.5±2.5	PEA	36	49	16	33	51	12	8	8	83
			MOR	33	48	29	23	48	10	10	0	90
Hatch	53-63	13.1±2.5	PEA	31	66	14	67	20	18	1	11	78
			MOR	52	59	71	20	9	16	13	13	75
Alevin	60-90	17.5±3.9	PEA	35	53	8	81	13	14	0	0	100
			MOR	35	48	81	8	10	17	6	0	94
Swimup	88-93	22.1±5.2	PEA	25	68	4	87	9	19	5	0	95
			MOR	28	59	88	9	3	16	6	0	94
Fry	94-125	6.5±1.5	PEA	17	24	4	0	96	9	0	0	100
			MOR	14	21	0	0	100	7	0	0	100
Fry	125.155	7.8kl.0	PEA	30	35	3	17	80	16	0	6	94
			MOR	25	38	13	24	63	10	20	0	80
Fry	155-I 85	3.0±0.5	PEA	21	30	10	17	73	12	8	0	92
			MOR	22	41	17	22	61	13	8	8	85
Fry	185-227	1.0±0.1	PEA	52	62	16	21	63	23	0	13	87
			MOR	51	60	7	3	90	22	0	5	96
Smolt	481-541	20-28 *	PEA	65	68	27	57	16	22	14	14	73
			MOR	64	61	61	20	20	22	9	14	77

Table 6. **Statistical comparison of the number of kokanee salmon exposed to either morpholine (MOR) or phenethyl alcohol (PEA), captured in morpholine or phenethyl alcohol scented traps, or at a downstream wier, under odor present and odor absent conditions. Separate tests were made for each condition. The null hypothesis stated that there was no difference in the distribution of the two sets of fish exposed to different odors at a particular life history stage. The null hypothesis was rejected if p≤ .05, denoted by an asterisk. An asterisk signifies these groups of fish imprinted to their exposure odor.**

		Odors Present					Odors Absent				
Exposure Stage	Exposure Odor	# trials (n)	MOR Trap (#)	PEA Trap (#)	DOWN (#)	**Chi Square**	# trials (n)	Trap A (#)	Trap B (#)	DOWN (#)	**Chi Square**
Fertilized Egg	PEA	242	**45**	52	201	--	100	5	3	**92**	--
Eyed Egg	PEA	**49**	**8**	16	25	χ^2= 2.62	12	1	1	10	χ^2= 0.86**
	MOR	48	14	11	23	p=0.27	10	0	1	9	p=0.65
Hatch	PEA	66	9	44	13	χ^2=42.44	18	2	2	14	χ^2=0.04 **
	MOR	59	42	12	5	p<0.01*	16	2	2	12	p=0.98
Alevin	PEA	53	3	43	7	χ^2=63.42	14	0	0	14	χ^2=0.53 **
	MOR	46	39	4	5	p<0.01*	17	1	0	16	p=0.81
Swimup	PEA	**68**	3	59	6	χ^2=90.96	19	1	0	18	χ^2= 2.25**
	MOR	59	52	5	2	p<0.01*	16	1	0	15	p=0.33
Fry	PEA	24	'1	0	23	χ^2=0.52	9	0	0	9	χ^2=0.00 **
	MOR	21	0	0	21	p=0.99	7	0	0	7	p=1.0
Fry	PEA	35	1	6	28	χ^2=2.79	16	0	1	15	χ^2=1.51 **
	MOR	30	5	9	24	p=0.25	10	0	2	8	p=0.47
Fry	PEA	30	3	5	22	χ^2=0.93	12	1	0	11	χ^2=0.96 **
	MOR	41	7	9	25	p=0.63	13	1	1	11	p=0.62
Fry	PEA	82	13	17	52	χ^2=1.24	23	0	3	20	χ^2=3.84 **
	MOR	60	4	2	54	p=0.54	22	0	1	21	p=0.41
Smolt	PEA	**68**	37	12	12	χ^2=20.48	22	2	3	17	χ^2=0.14 **
	MOR	61	18	39	11	p<0.01*	22	3	3	16	p=0.93

** Chi square values were calculated for these groups even though the assumption stating: no more than 20% of expected values may De less than five, and no expected value may be less than 1, was violated.

Figure 16. Results of odor discrimination tests with adult kokanee salmon exposed to synthetic chemicals at different life stages. (A) Percentage that migrated upstream when the synthetic chemicals were absent; (B) Percentage that migrated upstream when synthetic chemicals were present; (C) Percentage that homed to exposure odor. The values displayed above the bars indicate the total number of trials for that particular life stage.



as “not imprinted” (Table 7). Fish exposed during the eyed egg stage (30-60 days post-fertilization) displayed a similar pattern and were also classified as “not imprinted,” (Tables 5,6,7, Figure 16).

When exposure odors were present, fish exposed to synthetic chemicals at hatching(53-63 days post-fertilization), alevin (60-90 days post-fertilization), and swimup (88-93 days post-fertilization) stages were captured in traps scented with their exposure chemical more frequently (at rates of 69%, 81% and 88% respectively) than either in traps scented with the alternate chemical (rates of 17%, 8% and 7% respectively) or at the downstream weir (rates of 15%, 12% and 6% respectively) (Tables 5,6, Figure 16). When exposure odors were absent, the majority of fish from all three groups were captured at the downstream weir (77%, 97% and 95% respectively) (Tables 5,6, Figure 16). Additionally, when phenethyl alcohol was switched to a different channel, 13 of 15 fish exposed to phenethyl alcohol at hatch, that had initially been attracted to the original phenethyl alcohol scented channel, were attracted to the opposite channel. When morpholine was switched to a different channel, 14 of 15 fish exposed to morpholine at hatch, that had initially been attracted to the original morpholine scented channel, were attracted to the opposite channel. Similarly, 12 of 15 fish exposed to phenethyl alcohol and 7 of 9 fish exposed to morpholine as alevins that were originally attracted to a channel scented with their respective treatment odor, were attracted to the opposite channel when the odors were switched. Also, 9 of 9 fish exposed to phenethyl alcohol and 10 of 10 fish exposed to morpholine at swimup, that were originally attracted to a channel scented with their respective treatment odor, were attracted to the opposite channel when the odors were switched. Thus, 65 of 73 fish (89%) were attracted to a new channel when their odors were switched into that channel. Since fish exposed at hatch, alevin and swimup stages all tended to swim upstream to the channel scented with their exposure odor when their exposure odor was present, selected the channel scented with their exposure odor with precision, switched channels when odors were switched, and swam downstream if the odor was absent, all three groups were classified as “imprinted” (Table 7).

The majority of fish exposed to synthetic chemicals as post-swimup fry in February, March, April or May-July (four different groups ranging from 125 to 227 days post-fertilization) were captured primarily at the downstream weir in both odor present (98%, 72%, 67% and 77% respectively) and odor absent (100%, 87%, 89% and 92%

Table 7. **Results from behavioral tests of kokanee salmon exposed to synthetic chemicals** (either morpholine or phenethyl alcohol) **at different life stages. Determinations of upstream and downstream migration when odors were present or absent, and ability to discriminate odors are listed for each life history stage.**

Exposure Stage	Days Post-Fertilization	Direction of swimming when odors were absent [1]	Direction of swimming when odors were present [2]	Did the fish select the arm scented with the exposure chemical? [3]
Fertilized egg	0-30	Down	Down	No
Eyed egg	30-60	Down	Up	No
Hatch	53-63	Down	Up	Yes
Alevin	60-90	Down	Up	Yes
Swimup	88-93	Down	Up	Yes
Fry	94-125	Down	Down	No
Fry	125-155	Down	Down	No
Fry	155-185	Down	Down	No
Fry	185-227	Down	Down	No
Smolt	481-541	Down	Up	Yes

[1,2] Criteria: When <75% of the individuals from a particular exposure stage tested displayed upstream migratory behavior, the group was classified as "Up"; when 75% displayed downstream migratory behavior, the group was classified as "Down".

[3] Criteria: Determination of odor discrimination was based on statistical significance (Chi Square test) at p=0.05. If fish selected the arm scented with the exposure chemical, they were considered imprinted.

respectively) conditions (Tables 5,6, Figure 16). Additionally, when odors were present, approximately equivalent numbers of fish from each group were captured in traps scented with the exposure odor and alternate odor (Tables 5,6). Thus, they appeared to be unable to discriminate their exposure odor and were classified as "not imprinted" (Table 7).

Fish exposed to synthetic chemicals as smolts tended to migrate upstream! (82%) if their exposure odor was present and downstream (76%) if it was absent (Tables 5,6). When odors were present 59% of the fish selected the channel with their exposure odor, compared to 23% that selected the alternate odor and 18% that migrated downstream (Tables 5,6, Figure 16). Thus, smolts were classified as "imprinted" (Table 7).

The group of fish that experienced highest whole body thyroxine content (swimup stage) also had the highest percentage of fish homing accurately in the behavioral tests (Figure 14,16). Recently hatched eggs and alevins also had relatively high thyroxine content and displayed accurate homing in behavioral tests (Figures 14,16). Smolts experienced elevated plasma thyroid levels and tended to home correctly in the behavioral tests (Figures 15,16). Pre-hatch eggs and post swimup fry had relatively low thyroxine content and exhibited poor homing to exposure odors.

These results indicated that fish exposed to morpholine or phenethyl alcohol at alevin/swimup and smolt life stages: (1) became imprinted to their treatment odor at that life stage, (2) retained the odor memory during the 15 month period 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. Some of the fish did not spawn in 1993, so they will be tested as 3 year old adults in the autumn of 1994. Additionally, behavioral investigations using the 1992 year class kokanee described in Section 3.1.4 will be repeated in the fall of 1994. These fish will be 2-year old spawners at that time.

3.1.3 CWT Returns

Recoveries of coded wire tagged fish exposed to synthetic chemicals at different life stages are recorded in Tables 8, 9, and 10. In 1992 and 1993, a total of 3,064,226 kokanee were released into Lake Roosevelt, of which 599,843 were tagged with CWT/finclips. Coded wire tag releases included 432,446 kokanee fry and 167,397

Table 8. Recoveries by location of kokanee salmon with coded wire tags from releases made in 1992. Recoveries are total number recovered from creel and fisheries surveys in 1992 and 1993[1].

STAGE EXPOSED	EXPOSURE ODOR	RELEASE LOCATION	LIFE STAGE AT RELEASE	TOTAL # CWT RELEASED	# CWT recovered at Sherman Creek (MOR scented)	# CWT recovered at Spokane River (PEA scanted)	# CWT recovered at Other
Eyed egg	MOR	Sherman Creek	Fry	7,367	1	0	0
	PEA	Sherman Creek	Fry	11,393	0	0	0
Hatch	MOR	Sherman Creek	Fry	22,222	0	0	0
	PEA	Sherman Creek	Fry	23,115	0	0	0
Alevin	MOR	Sherman Creek	Fry	11,441	0	0	0
	PEA	Sherman Creek	Fry	10,906	0	0	0
Swimup	MOR	Sherman Creek	Fry	8,370	0	0	0
	PEA	Sherman Creek	Fry	10,716	0	0	0
Fry (Feb)	MOR	Sherman Creek	Fry	20,194	3	0	0
	PEA	Sherman Creek	Fry	6,025	0	0	0
Fry (Mar)	MOR	Sherman Creek	Fry	9,798	0	0	0
	PEA	Sherman Creek	Fry	10,818	1	0	0
Fry (Apr)	MOR	Sherman Creek	Fry	11,445	0	0	0
	PEA	Sherman Creek	Fry	11,525	0	0	0
Fry (May-Jul)	MOR	Sherman Creek	Fry	6,838	0	0	0
	PEA	Sherman Creek	Fry	11,300	0	0	0
Smolt	MOR	Sherman Creek	Smolt	7,501	9	5	0
	PEA	Sherman Creek	Smolt	6,354	2	3	0

[1] Fiih released at fry stages were from Lake Whatcom (1991 cohort) and were recovered as P-year old adults in autumn 1993. Fish released as smolts were from Lake Whatcom (1890 cohort) and recovered as 2-year old fish in 1902 and 3-year old fish in 1993.

Table 9. Recoveries by location of kokanee salmon with coded wire tags from releases made in 1993. Recoveries are total number recovered from creel surveys, and fisheries surveys and monitoring egg collection sites in 1993[1].

STAGE EXPOSED	EXPOSURE ODOR	RELEASE LOCATION	LIFE STAGE AT RELEASE	TOTAL # CWT RELEASED	# CWT recovered at Sherman Creek (MOR scented)	# CWT recovered at Spokane River (PEA scented)	# CWT recovered at Other
Eyed egg	MOR	Sherman Creek	Fry	10,961	0	0	0
	MOR	Spokane River	Fry	10,903	0	0	0
	PEA	Sherman Creek	Fry	10,721	0	0	0
	PEA	Spokane River	Fry	32,953	0	0	0
Hatch	MOR	Sherman Creek	Fry	7,988	0	0	0
	MOR	Spokane River	Fry	31,416	0	0	0
	MOR	Barnaby Cree	Fry	22,026	0	0	0
	PEA	Sherman Creek	Fry	7,988	0	0	0
	PEA	Spokane River	Fry	21,993	0	0	0
Alevin	MOR	Sherman Creek	Fry	10,938	0	0	0
	PEA	Sherman Creek	Fry	11,791	0	0	0
Swimup	MOR	Sherman Creek	Fry	10,908	0	0	0
	PEA	Sherman Creek	Fry	10,885	0	0	0
Fry	MOR	Sherman Creek	Fry	10,802	0	0	0
	PEA	Sherman Creek	Fry	10,896	0	0	0
Smolt	MOR	Sherman Creek	Smolt	38,345	35	5	0
	PEA	Sherman Creek	Smolt	7,753	20	25	0
	PEA	Blue Creek[2]	Smolt	8,196	0	75	3[3]

1 Fish released at fry stages were from Lake Whatcom (1992 cohort). No recoveries of these fish are expected until they are age 2 in 1994. Fish released at the smolt stages were from Lake Whatcom (1991 cohort) and recovered as 2-year old fish adults in 1993. It is anticipated that additional fish from these lots will be recovered as 3-year old adults in 1994 and 4-year old adults in 1995.

2 Blue Creek is a tributary of the Spokane Arm of Lake Roosevelt located about 35 km downstream from Little Falls Dam.

3 All three fish recovered et other locations were collected in Hawk Creek.

Table 10. Recoveries by location of kokanee salmon with fin clips. These fish were exposed to synthetic chemicals in 1993, and held at the Spokane Tribal hatchery until July 1993, when they were released into the Spokane River as residualized smolts. Recoveries were made from September to November 1993.

LIFE STAGE	EXPOSURE ODOR	FIN CLIP	# RELEASED	LOCATION RELEASED	STAGE RELEASED	# RECOVERED AT: Sherman Creek (MOR scented)	Little Falls (PEA scented)	OTHER	PERCENT RETURN
Eyed egg	MOR	RP	325	Spokane River	Smolt	0	1	0	0.3
	PEA	LP	325	Spokane River	Smolt	0	0	0	0
Hatch	MOR	RV	325	Spokane River	Smolt	0	0	0	0
	PEA	LV	325	Spokane River	Smolt	0	5	0	1.5
Alevin	MOR	A-RV	325	Spokane River	Smolt	0	0	0	0
	PEA	A-LV	325	Spokane River	Smolt	0	8	0	2.1
Swimup	MOR	A-RP	325	Spokane River	Smolt	0	1	0	0
	PEA	A-LP	325	Spokane River	Smolt	0	9	0	2.8
Fry (Feb)	MOR	LV-RP	325	Spokane River	Smolt	0	0	0	0
	PEA	RV-LP	325	Spokane River	Smolt	0	0	0	0
Fry (Mar)	MOR	D-RV	325	Spokane River	Smolt	0	0	0	0
	PEA	D-LV	325	Spokane River	Smolt	0	0	0	0
Fry (Apr)	MOR	D-RP	325	Spokane River	Smolt	0	0	0	0
	PEA	D-LP	325	Spokane River	Smolt	0	0	0	0
Fry (May-Jul)	MOR	D	325	Spokane River	Smolt'	0	0	0	0
	PEA	A	325	Spokane River	Smolt	0	0	0	0

residualized smolts. A portion of these, including 416,705 fry and 70,149 smolts, were fish that had been exposed to either morpholine or phenethyl alcohol at different life history stages as part of our imprinting investigations (Tables 8,9,10).

A total of 299 CWT/finclipped fish were recaptured in 1992 and 1993, including 50 observed in randomized creel census or electrofishing/gill net surveys (179 unmarked kokanee were also observed in these surveys), and 244 recaptured at egg collection sites at Sherman Creek (morpholine scented) and Little Falls (phenethyl alcohol scented). Recoveries at egg collection sites included 211 fish from the imprinting study (Tables 8,9,10). Of the total 299 CWT recoveries, 295 (98.7%) fish had been stocked as smolts and four (1.3%) had been stocked as fry. The relative percentage in each category was in contrast to the relative percentages of fry (72.1%) and smolts (27.9%) in the total CWT fish released (599,843). Thus, fish released as fry seem to be missing from the reservoir.

Preliminary results of coded wire tagging investigations tended to corroborate the results of the olfactory discrimination experiments (see Section 3.1.3) with respect to homing of fish exposed to synthetic chemicals at the smolt stage and released as residualized smolts. In 1992, both morpholine and phenethyl alcohol exposed smolts were released at Sherman Creek. They were recovered as 2-year old and 3-year old sexually mature adult fish in 1992 and 1993 respectively. In 1992 and 1993, nine morpholine-exposed smolts were recovered as adult spawners at Sherman Creek (morpholine scented) compared to five recovered at Little Falls Dam (phenethyl alcohol scented), and two phenethyl alcohol-exposed fish were recovered at Sherman Creek compared to three at Little Falls Dam (Table 8). Too few fish exposed to synthetic chemicals at other life history stages and released as fry were recovered to assess imprinting effectiveness (Table 8). We do not believe that the failure of these fish to home was necessarily related to their failure to imprint to synthetic chemicals. The reason is that very few fish released as fry have been recovered anywhere in Lake Roosevelt. Instead, we believe that failure of most experimental groups to home and be recovered at locations where synthetic chemicals were metered into the reservoir was related to either their survival in the reservoir or emigration from the reservoir before reaching adult size.

In 1993, both morpholine and phenethyl alcohol exposed smolts were released at Sherman Creek (Table 9). In addition, one group of phenethyl alcohol exposed smolts was released in Blue Creek, a tributary to the Spokane River Arm of Lake

Roosevelt located about 35 km downstream from Little Falls Dam (Table 9, Figure 1). They were recovered as 2-year old sexually mature adult fish in 1993. Recoveries of fish exposed to morpholine during the smolt stages and released at Sherman Creek as residualized smolts, included 35 sexually mature adults at Sherman Creek (morpholine scented), and five at Little Falls Dam on, the Spokane River (phenethyl alcohol scented) and none at other locations (Table 9). Recoveries of fish exposed to phenethyl alcohol and released at Sherman Creek included 20 at Sherman Creek, 25 at Little Falls Dam and none at other locations. Recoveries of fish exposed to phenethyl alcohol and released at Blue Creek included none at Sherman Creek, 75 at Little Falls Dam and three at other locations. Thus, 87.5% of the morpholine-exposed fish were recovered in the morpholine scented stream, compared to 12.5% in the phenethyl alcohol scented river and 0% at other locations. In contrast, 16.2% of phenethyl alcohol exposed fish were recovered in the morpholine scented stream, compared to 81.3% in the phenethyl alcohol scented river and 2.4% at other locations. Additional recoveries of these fish are anticipated in 1994 and 1995 when they will be three and four years old respectively.

Table 10 provides data on recoveries of sexually mature 2-year old kokanee that were exposed to synthetic chemicals at different life history stages in 1992. These fish were given distinctive fin clips, and retained at the Spokane Tribal Hatchery until July 1993 when they were released into the Spokane River as residualized smolts, approximately 10 km downstream from Little Falls Dam. These were the same groups of fish used for olfactory discrimination experiments described in Section 3.1.2 . A total of 325 fish were released in each of 16 groups. These data indicated that fish exposed to phenethyl alcohol at hatch, alevin or swimup stages were recovered in higher numbers at a site scented with phenethyl alcohol than fish exposed to morpholine at the same stages.

CWT returns also provided data about the growth of kokanee after release into Lake Roosevelt. For example, fish released as zero age fry in June 1992 at a mean length of 71 mm grew to a mean length of 305 mm by November 1993 (age 2, n=4). Kokanee released as smolts (age 1+) in June 1992 at a mean length of 158 mm and a mean weight of 41 g grew to a mean length of 320 mm and weight of 347 g by September 1992 (age 1+) (n=2). One individual from this lot grew to 486 mm and 1,800 g by August 1993 (age 2+). Another individual released as an age 1+ smolt at a length of 190 mm and weight of 50 g in June 1992 grew to 520 mm and 1,284 g when

it was recovered in November 1993 at age 3. Kokanee released as age 1+ smolts in June 1993 at a mean length of 18 mm and weight of 32 g grew to a mean length of 350 mm and weight of 490 g by October 1993 (n=66). These observed growth rates confirm kokanee growth rates for Lake Roosevelt reported by Peone et *al.* (1990), Griffith and Scholz (1991) and Thatcher et *al.* (in press), based upon backcalculating growth from scales.

3.1.4 Thyroxine Content from 1993 Investigations

Results of experiments conducted in 1993 indicated that developing kokanee salmon (1992 year class) experienced elevated whole body thyroxine content at the time of hatch and again at swimup (Figure 17). At about 26 days before hatching (44 days post fertilization), kokanee salmon eggs contained a mean thyroxine concentration of 8.3 ± 1.1 ng/g body weight. Thyroxine concentration increased to a peak value of 15.1 ± 2.1 ng/g body weight about 7 days before hatching (n=lO; p=0.0001).

The period from hatch to reabsorption of the larval yolk sac was approximately 24-26 days. During this time, the $[T_4]$ significantly increased from 14.4 ± 2.2 ng/g at hatch to a peak of 19.1 ± 0.4 ng/g three days before swim-up (ANOVA, Fisher PLSD; n=10; p=0.0001). At swim-up (24-26 days after hatch or 94-96 days after fertilization) the $[T_4]$ was 8.9 ± 0.6 ng/g body weight.

Swim-up occurred between 92-97 days post fertilization. Within 10 days to 2 weeks after becoming free-swimming fry, there was a significant increase in mean thyroxine concentration (ANOVA, Fisher PLSD; n=10; p=0.0001). The $[T_4]$ increased from 8.9 ± 0.6 ng/g body weight to 12.1 ± 0.9 ng/g. From this point, $[T_4]$ steadily decreased to less that 2 ng/g body weight when fry were older than 126 days post fertilization (Figure 17).

There was no significant change in body weight during early development (ANOVA, Fisher PLSD; n=10; p>0.05) (Figure 18) . One week before hatch, the body weight was 56.1 ± 5.4 mg per fish. At hatch, the weight decreased to 42.8 ± 3.3 mg. After hatch, the weight rose again to 50.0 ± 8.3 mg before dropping to 40.8 ± 6.3 mg at swimup. At one month post swimup, body weight doubled. Fish weighed about 0.6 g, 167 days post fertilization, 1.2 g at 216 days post fertilization and 3.1 g at 252 days post fertilization.

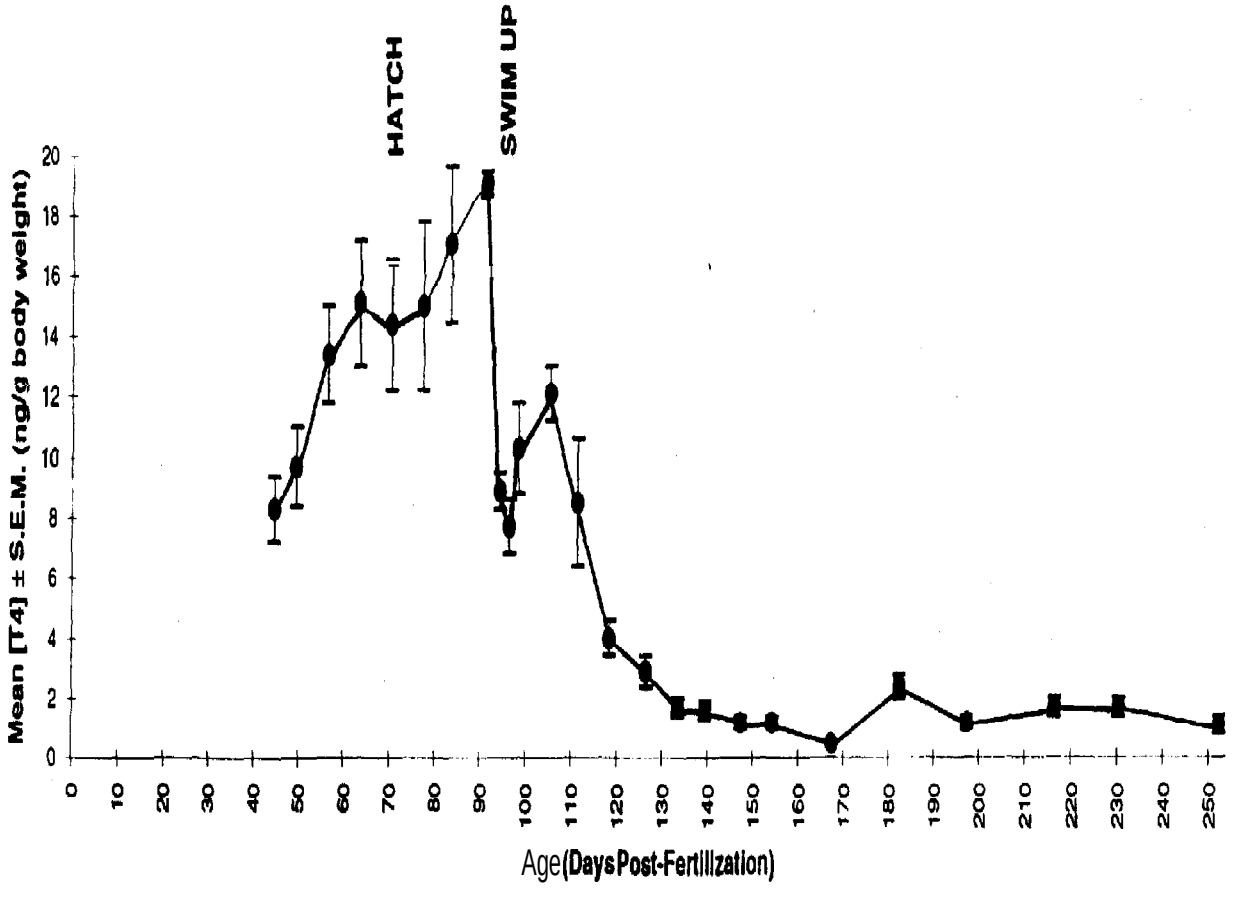


Figure 17. Whole body T_4 concentration of 1992 brood, Lake Whatcom stock kokanee salmon. Each data point represents the mean [T_4] (± SEM) of approximately 20 fish.

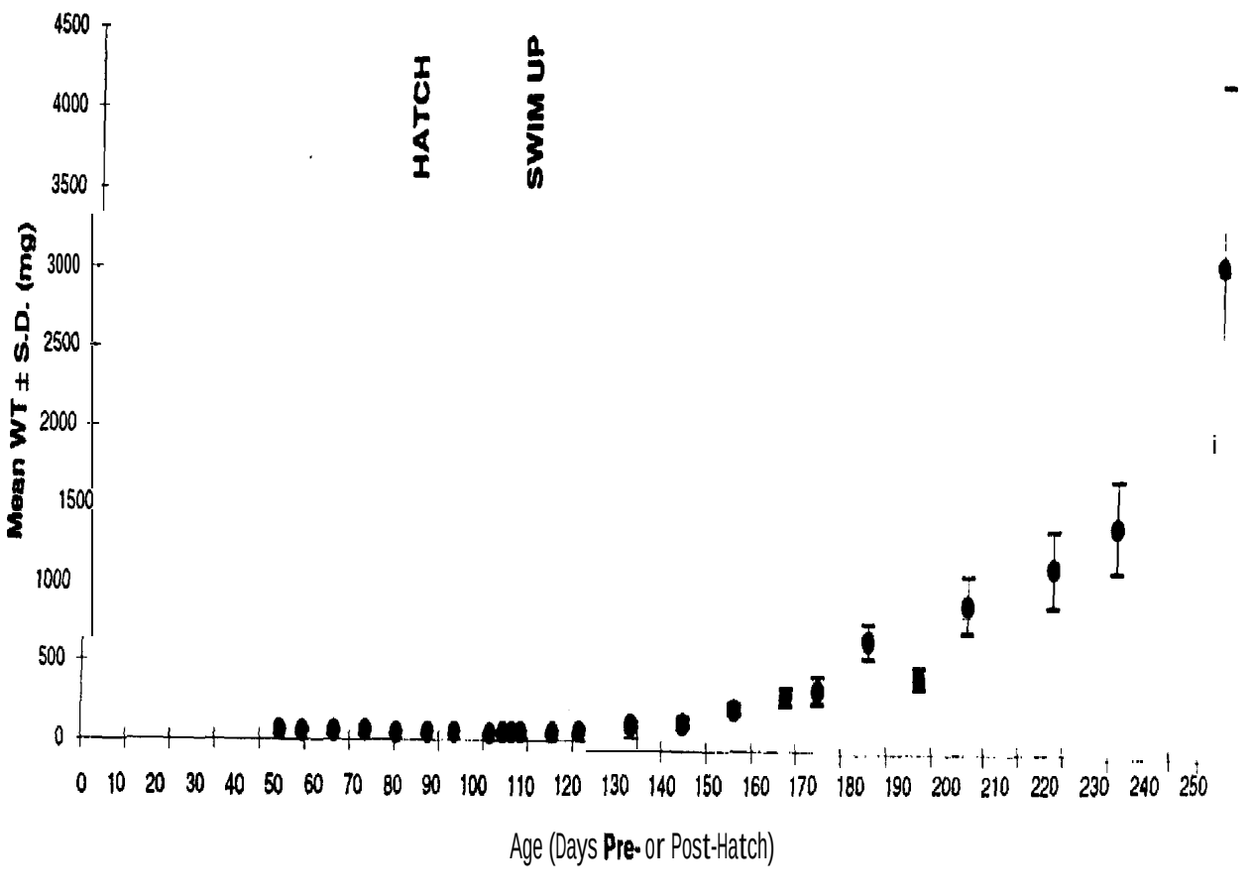


Figure 18. Whole body weights of 1992 year class, Lake Whatcom stock kokanee salmon. Each data point represents the mean weight (±SEM) of approximately 20 fish.

Plasma thyroxine concentration levels for yearling kokanee salmon are shown in Figure 19. On December 18, 1992 the mean T_4 concentration was 13.8 ± 1.3 ng/ml. The concentration dropped significantly to 11 .O ± 3.3 ng/ml on January 13, 1993 (ANOVA, Fisher PLSD; n=10; p=0.0001), and then rose significantly and peaked at 15.2 ± 0.9 ng/ml on February 10 (n=lO; p = 0.0001). The concentration decreased by March 23 to 10.6 ± 0.7 ng/ml but another significant peak was observed on April 20 at 12.2 ± 2.6 ng/ml (n = 10; p = 0.0001) (Figure 19, Table 11).

3.2 Quality Assurance Results for Radioimmunoassay (RIA)

Quality assurance results for radioimmunoassays conducted in 1992 were previously reported by Scholz et a/. (1993). Results of blind quality assurance samples for the RIA's in 1993 are recorded in Table 12. The actual concentration of the low T_4 blind sample was 2.3 ng/dl compared to a mean (±S.D.) measured value of 2.1 ± 0.4 ng/dl (n=6). The actual concentration of the medium T_4 blind sample was 7.3 ng/dl compared to a mean (± S.D.) measured value of 7.4 ± 0.7 ng/dl (n=6). The actual concentration of the high T_4 blind sample was 11.5 ng/dl compared to a mean (± S.D.) measured value of 11.8 ± 1.3 ng/dl (n=6).

Mean nonspecific binding for the six assays (n=12) was measured at 607 ± 211 counts per minute (cpm) or about 1 .1% of the 54,218 ± 6700 cpm measured in total count tubes (TCT). The blank tubes had a mean (±SD) of 7.2 ± 28 cpm. These counts are possible because only the mean background is subtracted from each tube, so a few counts are not 'considered out of the ordinary. Accuracy of pipetting 25 µl and 1 ml samples is recorded in Table 13. The mean cpm (± S.D.) of tubes that received 25 µl of ^{125}I-T_4 was 1445 ± 97 for a total error of 6.8%. The mean cpm of tubes that received 1 ml of ^{125}I-T_4 was 53,237 ± 393 cpm for a total error of 0.8%. A frequency distribution of percent error of duplicate samples measuring whole body [T_4] is presented in Figure 20. The mean percent error (±S.D.) of 245 duplicate samples was 9.3 ± 10% compared with the 6.8% error noted in pipetting the 25µl samples (Table 13). A frequency distribution of percent error of duplicate samples measuring blood plasma [T_4] is presented in Figure 21. The mean percent error of 154 duplicate samples was 1 .1 ± 0.8%.

Results of interassay pool (IAP) samples for each of the six assays are recorded in Table 14. Mean concentration (±S.D.) for IAP1 was 3.1 ± 0.3 ng/dl, IAP2 was 9.6 ± 0.2 ng/dl and IAP3 had a concentration of 13.5 ± 0.6 ng/dl. The percent error for all

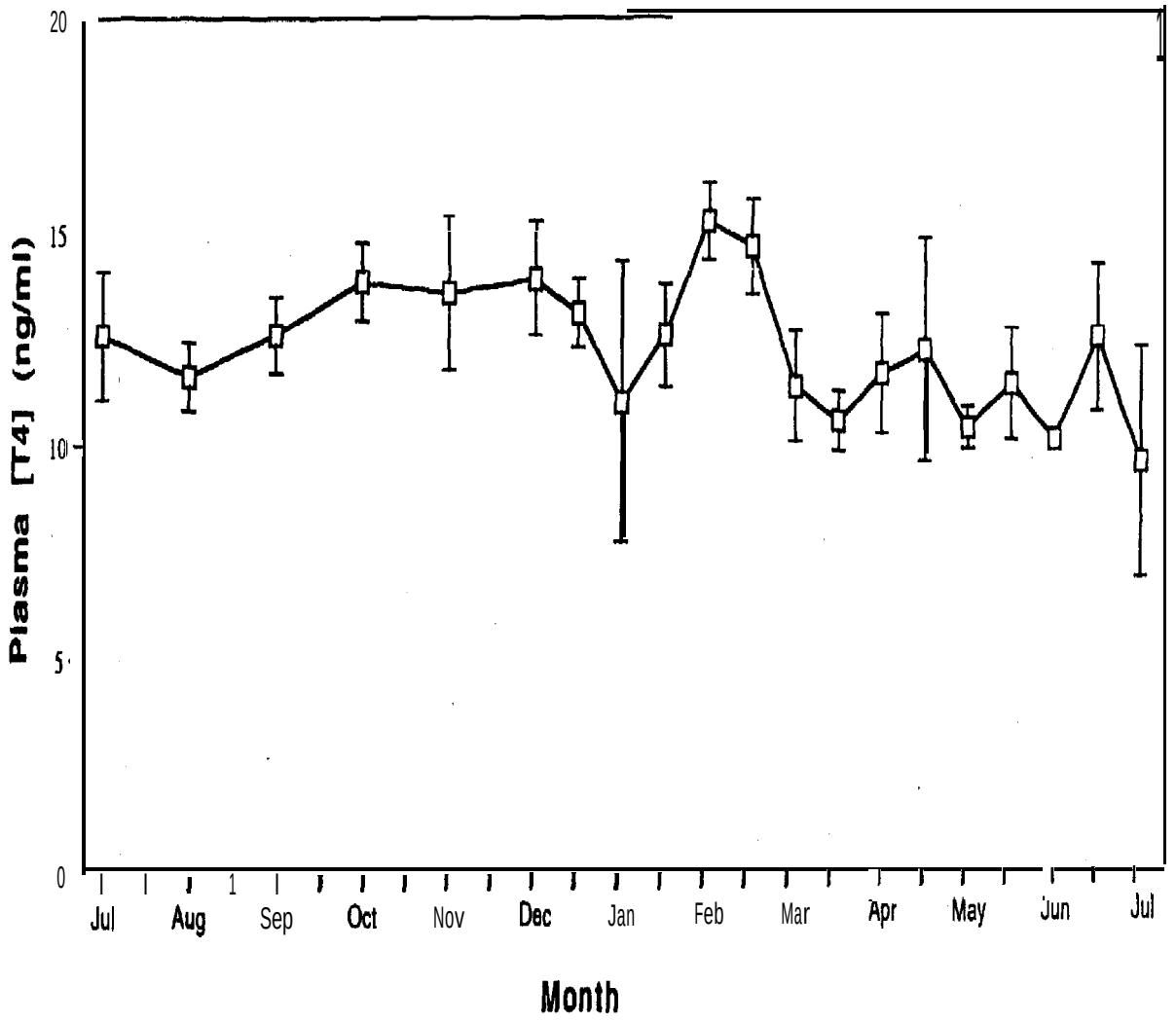


Figure 19. T_4 concentration in blood plasma of 1991 year class kokanee salmon from July 1182 to July 1993. Each point represents the mean ± S.D. of approximately 20 fish.

Table 11. Mean plasma T_4 concentration and number of samples significantly lower than mean [T_4] of date listed using ANOVA (alpha = 0.05). Samples range from December 1992 through June 1993.

DATE	MEAN [T_4] (ng/ml)	# of SAMPLES SIGNIFICANTLY LOWER THAN MEAN VALUE
Dec 18	13.9	8
Dec 30	13.1	7
Jan 13	11.0	0
Jan 26	12.6	4
Feb 10	**15.2***	**10**
Feb 23	**14.6***	**9**
Mar 10	11.4	0
Mar 23	10.6	0
Apr 7	11.7	1
Apr 20	12.2	3
May 5	10.4	0
May 20	11.7	1
Jun 2	10.1	0
Jun 23	12.5	4

* = signifies significant peak in [T4]

Table 12. Results of blind quality assurance samples. Measured concentrations were the mean ± S.D. of six kokanee assays. Actual and acceptable ranges were provided by Diagnostic Products Corporation.

Sample	Actual concentration (ng/dl)	Acceptable range (ng/dl)	Measured concentration (ng/dl)
Low	2.3	1.9-2.7	2.1 ± 0.4
Medium	7.3	6.2-8.4	7.4 ± 0.7
High	11.5	10.0-I 3.0	11.8 ± 1.3

Table 13. Pipettlng accuracy of 25 µl and 1 .0 ml samples. Counts = counts per minute of radiolabeled T_4. Percent error = standard deviation (S.D.) + mean counts.

	Assay #	Sample size (n)	Mean # of counts ± S.D.	Percent error (%)
25 µl	1	10	1688 ± 52	3.1
	2	10	1348 ± 62	4.6
	3	10	1369 ± 81	5.9
	4	10	1093 ± 114	10.4
	5	10	1653 ± 197	11.9
	6	10	1520 ± 77	5.1
Mean			1445 ± 97	6.8
1.0 ml	1	10	53,955 ± 686	1.3
	2	10	53,770 ± 442	0.8
	3	10	54,574 ± 361	0.7
	4	10	39,976 ± 366	0.9
	5	4	60,434 ± 85	0.1
	6	10	56,715 ± 420	0.7
Mean			53,237 ± 393	0.8

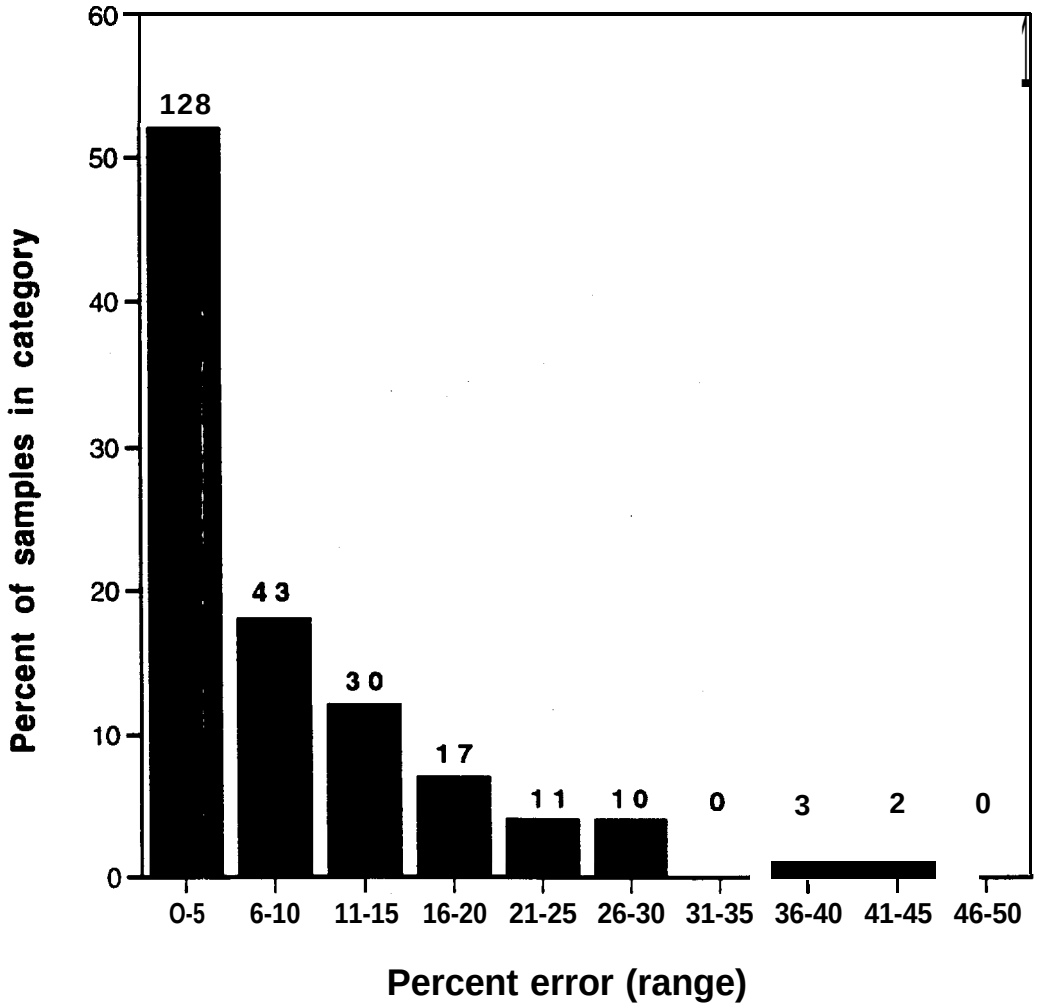


Figure 20. **Frequency distribution of percent error in duplicate zero-age whole body kokanee samples. Total sample size was 245 individuals. The number of samples in each category are noted above bars. These numbers were converted to percentages of the total sample size for plotting the frequency distribution.**

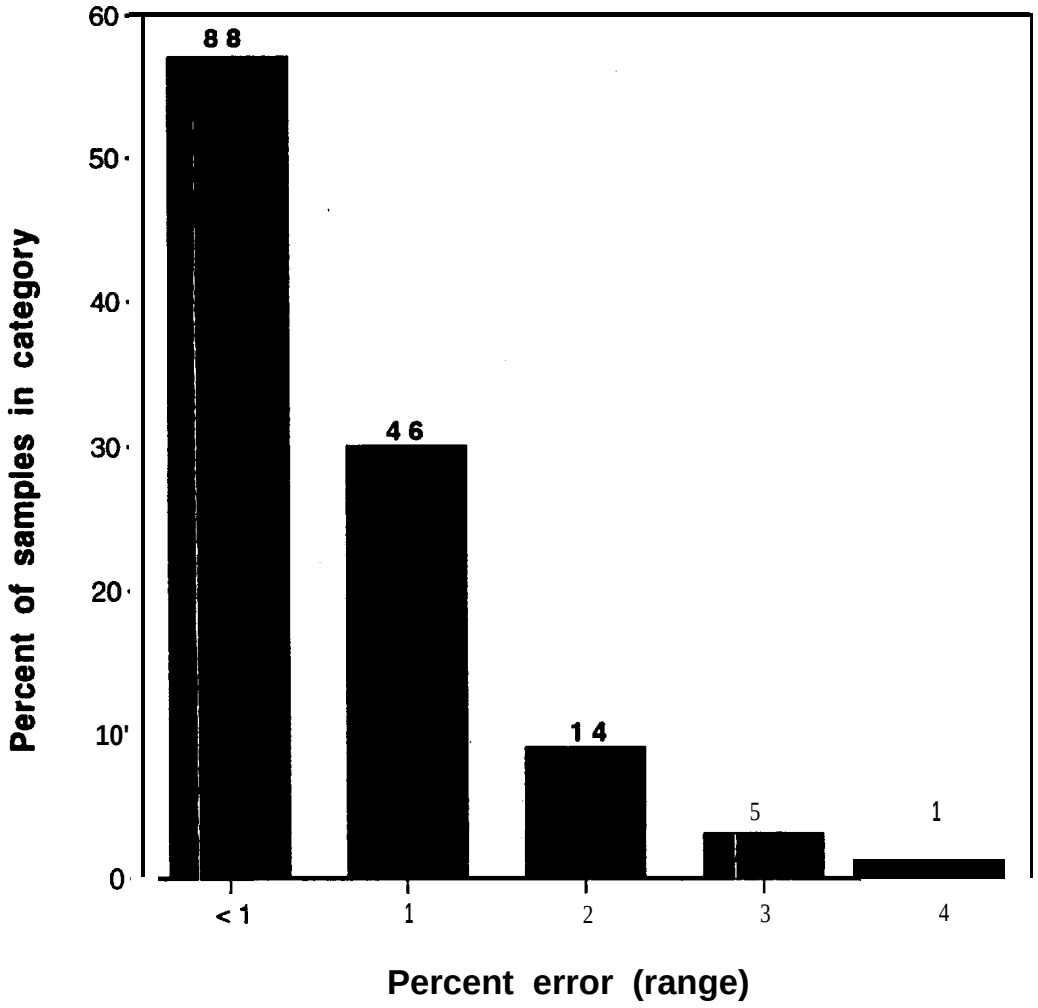


Figure 21. **Frequency dIstributIon of percent error in duplicate 1+ blood plasma samples from kokanee salmon. Total sample size was 154 individuals. The number of samples in each category are noted above bars. These numbers were converted to percentages of the total sample size for plotting the frequency distribution.**

Table 14. Mean (± S.D.) and percent error of three interassay pool (IAP) samples (ng/dl) run in six assays of kokanee salmon.

Assay	IAP 1	IAP 2	IAP 3
1	2.6	9.9	12.6
2	3.2	9.6	13.8
3	3.4	9.6	14.3
4	3.2	9.2	13.7
5	3.2	9.6	13.5
6	3.1	9.5	13.1
MEAN	3.1	9.6	13.5
S.D.	0.3	0.2	0.6
% ERROR	9 %	2%	4%

IAP samples was <10%. The mean concentration (±S.D.) for standard curve samples (ng/dl) are shown in Table 15. Concentrations between assays were reasonably uniform with the percent error ≤ 10%.

The actual values of the quality assurance samples were in close agreement to measured values. Blank tubes did not contain any significant contamination. Also, the error within duplicate samples was <10%. The relatively similar concentration and low standard deviation for IAP samples indicated that all the assays were comparable. These results indicated that the assay was reliable. Therefore T_4 concentration measurements were accepted as accurate for these kokanee salmon.

3.3 Recovery Determination

Details of recovery of T_4 from whole body samples were presented in 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.

3.4 Smoltification Investigations of Yearling Kokanee

Yearling (1+) kokanee salmon experienced thyroxine peaks and increased silvering during both years tested (1992 and 1993). Also, results of studies conducted in 1993 indicated that yearling kokanee experienced increased downstream migratory behavior and an increase in salt water adaptation at certain times of the year.

3.4.1 Smoltification of 1990 Year Class Kokanee

Results from thyroxine and silvering experiments with 1+ fish conducted in 1992 (1990 year class smolts) were discussed by Scholz et *al.* (1993) and summarized in Section 3.1.1 These results indicated a thyroxine peak in the winter (January) and again in the spring (May). Silvering and downstream migratory behavior was also noted in April and May (Scholz et *al.* 1993).

3.4.2 Smoltification of 1991 Year Class Kokanee

Results from thyroxine experiments with 1+ fish conducted in 1993 (1991 year class smolts) were discussed in Section 3.1.3.. These results were similar to the

Table 15. Mean (± S.D.) and percent error of standard curve samples (ng/dl) run In six assays of kokanee salmon.

Assay	0.5	1.0	4.0	10.0	16.0	24.0	
1	0.43	1.13	4.67	9.40	14.14	25.27	
2	0.51	0.98	3.98	10.03	16.44	23.54	
3	0.54	0.92	3.90,	9.79	16.19	24.80	
4	0.45	1.01	4.60	10.37	18.16	19.27	
5	0.55	0.87	4.11	10.37	15.47	24.26	
6	0.49	1.06	3.82	9.66	16.38	24.50	
MEAN	0.5	1.0	4.2	9.9	16.1	23.6	
S.D.	0.1	0.1	0.4	0.4	1.3	2.2	
% ERROF	10%	9%	9%	4%	8%	9%	

previous year's results in that there was a peak in the winter (February) and again in the spring (April).

3.4.2. 1 Silvering

Results of monthly visual observations of coloration are shown in Figure 22 and Table 16. The majority of fish observed in December were transition (>60%). In January, most of the fish observed were parr (80% and 90%). After January, silvering gradually increased each month through May when 100% of the fish sampled had developed silvery smolt coloration. In June, parr marks were present again, signifying a reversion back to parr. The number of fish in the transition phase increased in February following the increase in T_4 concentration in January. This increase in silvering continued throughout the sampling period (Figure 22).

Yearling kokanee salmon which were grouped as parr had a mean T_4 concentration ($\pm$ SD) of 12.7 $\pm$ 1.8 ng/ml. Fish grouped as transition exhibited a mean of 12.46 $\pm$ 2.1 ng/ml, and those grouped as smolts had a mean of 10.8 $\pm$ 1 .1ng/ml. There was a significant difference between parr and smolt and between transition and smolt (ANOVA; Fisher PLSD, p = 0.0001). These results indicated that parr had a higher mean T_4 concentration than smolts.

3.4.2.2 Downstream Migratory Behavior

Mean downstream migratory activities (% downstream) for yearling kokanee salmon from December 1992 to June 1993 are shown in Figure 22 and Table 17. There appeared to be two significant peaks in percent downstream behavior (ANOVA, Fisher PLSD, p = 0.0001). From January to March, the mean downstream migratory behavior increased significantly from 38% to 66%. It then dropped to 42% in late March before reaching a second peak of 65% at the beginning of May. The values dropped to 40-46% at the end of June. Downstream migratory activity increased following the thyroxine peak in February (Figures 19 and 22).

The fish did not appear to exhibit increased buoyancy, which is characteristic of smolts. They stayed near the bottom or middle of the living stream each time they were observed, suggestive of only slightly increased buoyancy. However, the downstream orientation of the fish did appear to change over time increasing from 29% on April 9 to 95% on June 30 (Table 17).

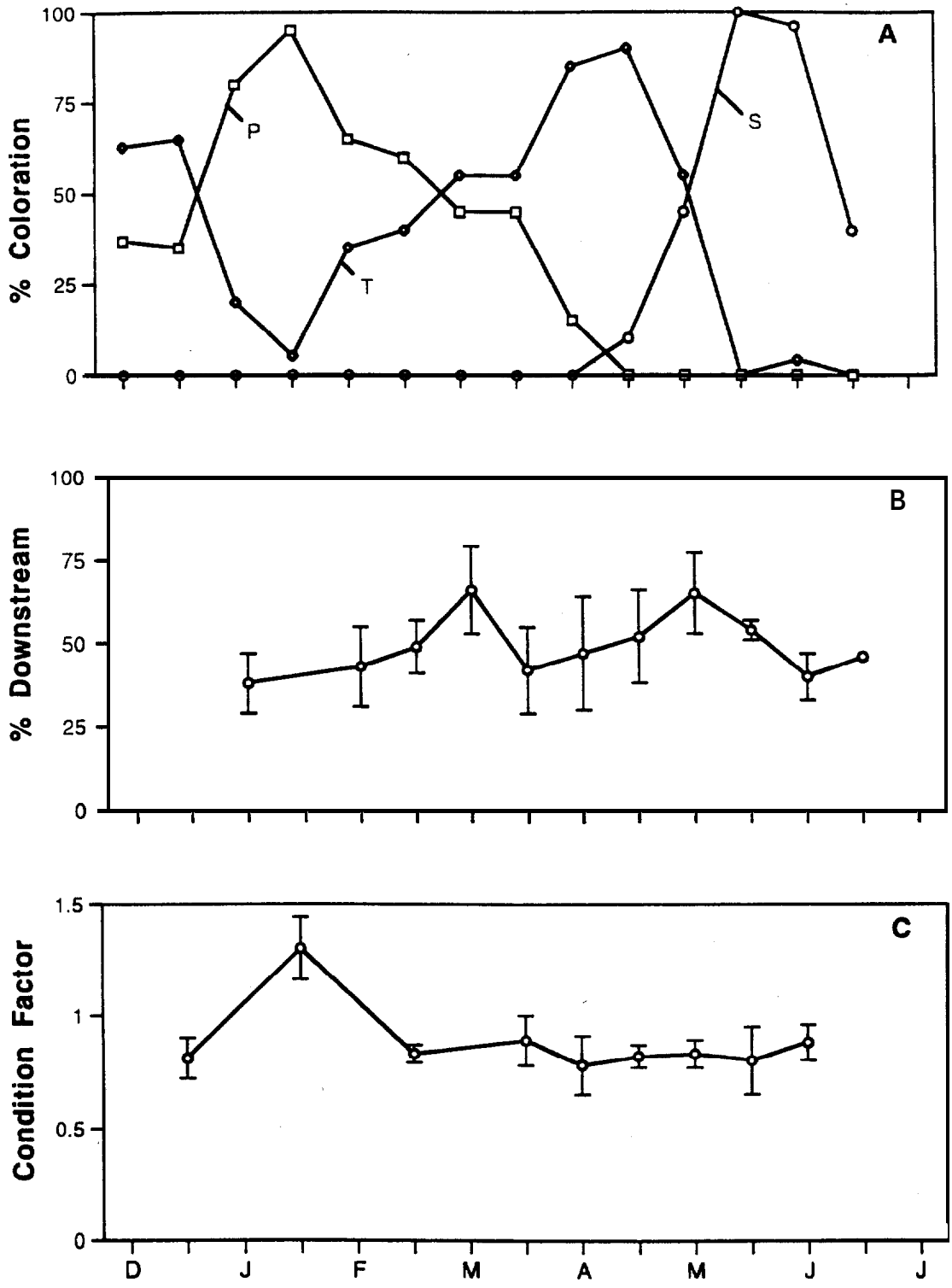


Figure 22 (A) Percentage of fish grouped as parr (P), transition (T), and smolts (S); (B) Percentage of fish in downstream third of tank; and (C) Condition factor (mean ± S.D.). Data represents yearling kokanee salmon sampled December 1992 through July 1993. Sample size (n)= 20 fish per data point.

Table 16. Total lengths (mm), mean weights (g), condition factor (K_{TL}), Na^+-K^+ ATPase activity (μmoles P_i · mg Protein-1 · h^{-1}) ($\pm$S.D.) of gill microsomes and percent silvering for yearling kokanee salmon collected during the study period (December 1992 - June 1993) at the Spokane Tribal Hatchery.

Date	n	Total Length (mm)	Weight (g)	K_{TL} [1]	ATPase (μmol P_i · mg Protein^{-1} · h^{-1})	% Silvering [2] P	T	S
12/30/92	15	108 ± 11	11 ± 4	0.83 ± .07	5.8 ± 3.2	35	65	0
1/13/93	16	116 ± 12	21 ± 8	1.30 ± .13	5.1 ± 2.0	95	5	0
2/10/93	19	118 ± 17	15 ± 7	0.83 ± .05	6.5 ± 2.2	65	35	0
3/10/93	19	117 ± 15	15 ± 7	0.88 ± .10	8.3 ± 3.7	45	55	0
4/7/93	18	129 ± 14	18 ± 6	0.79 ± .06	7.9 ± 3.5	15	85	0
4/20/93	19	141 ± 15	24 ± 8	0.82 ± .05	13.7 ± 7.8	0	95	10
5/5/93	19	153 ± 20	32 ± 14	0.84 ± .07	6.8 ± 5.2	0	55	45
5/20/93	19	162 ± 23	37 ± 21	0.81 ± .13	6.5 ± 3.4	0	0	100
6/2/93	16	168 ± 29	45 ± 27	0.87 ± .08	8.0 ± 2.3	0	4	96

1 K_{TL} = Condition factors based on total length.

2 P=parr
T = transition
S = smolt

Table 17. Mean percent of fish in downstream third of tank and number of samples significantly lower than mean percent for date listed using ANOVA (alpha=0.05). Each mean represents seven visual observations. Samples range from January 1993 through June 1993.

DATE	MEAN DOWNSTREAM MIGRATORY BEHAVIOR (%)	# of SAMPLES SIGNIFICANTLY LOWER THAN MEAN VALUE	MEAN DOWNSTREAM ORIENTATION (%)
Jan 16	38	0	30
Feb 5	43	0	0
Feb 24	49	1	20
Mar 8	**66***	**9**	36
Mar 21	42	0	35
Apr 9	47	0	29
Apr 22	52	2	72
May 4	**65***	**8**	91
May 13	54	3	78
Jun 12	40	0	94
Jun 30	46	0	95

* = signifies significant peak in percent of fish downstream,

3.4.2.3 Condition Factor

Average coefficients of condition (K_{TL}) for yearling kokanee salmon are shown in Figure 22 and Table 16. The mean condition factor (±S.D.) increased from 0.81 ± 0.09 in December to 1.3 ± 0.14 in January 1993. It then dropped to 0.83 ± 0.04 in February and remained consistent through June. This decline in K indicated that for a given fork length, fish weighed less in February through June than in January.

3.4.2.4 Intestinal Water Transport

Mean water transport (Jv) rate during the spring 1993 for both the middle and the posterior intestine of kokanee. (n=12 fish at each interval) are shown in Figure 23 and Table 18. Despite a four fold increase in body mass, the Jv for both the middle and posterior intestinal segments showed significant decreases with time (ANOVA, Fisher PLSD; middle p=0.0004 and posterior p=0.0115). The middle segment decreased steadily from 13.7 ± 3.2 $\mu l \cdot cm\text{-}2 \cdot h^{-1}$ to 4.4 ± 0.41 $\mu l \cdot cm\text{-}2 \cdot h^{-1}$ and the posterior segment decreased from 16.5 ± 4.1 $\mu l \cdot cm\text{-}2 \cdot h^{-1}$ to 4.9 ± 1.06 $\mu l \cdot cm^{-2} \cdot h^{-1}$.

3.4.2.5 Gill Na^+-K^+ ATPase Activity

Gill Na^+-K^+ ATPase levels for yearling kokanee are shown in Figure 23, Table 16 and 19. The average ATPase activity of gill microsomes increased significantly from 5.8 $\mu moles\ P_i \cdot mg\ Protein^{-1} \cdot hr^{-1}$ at the end of December to 13.7 in mid-April (ANOVA; Fisher PLSD, p=0.0001). The value then decreased to approximately 7.0 in May and stayed low through June.

There was no correlation between T_4 concentration and ATPase activity when a simple regression was run (r^2=0.005, p=0.51). There was also no correlation between condition factor and ATPase activity (r^2=0.024; p=0. 1528).

3.4.2.6 Salt Water Tolerance

Percent survival in salt water for yearling kokanee salmon is shown in Figure 23. There was 100% survival during all months tested for fish held in 10 ppt and 20 ppt seawater. Fish held in 30 ppt seawater, exhibited 0% survival from January

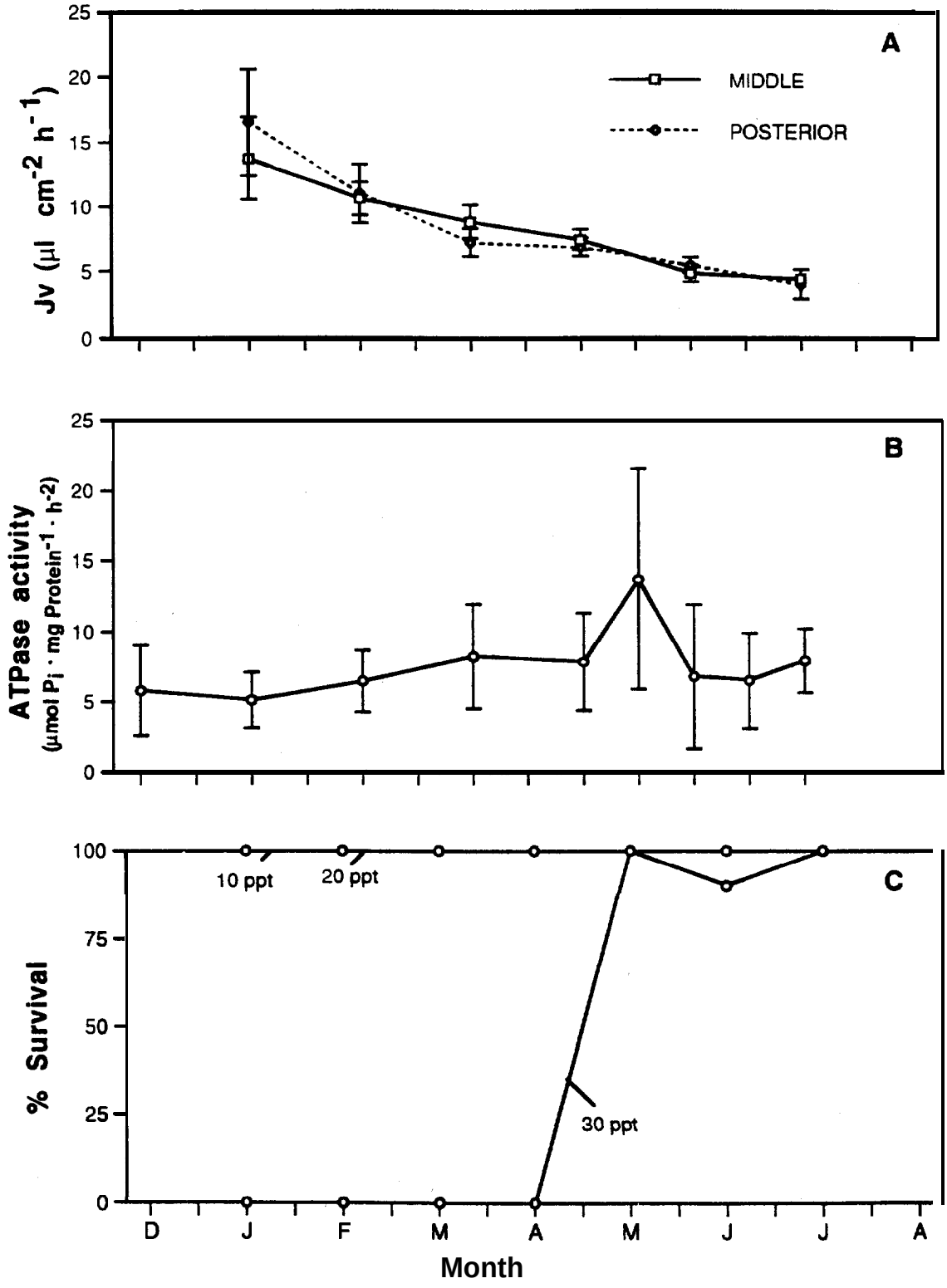


Figure 23. **(A) Intestinal water transport rate (J_V) (mean ± S.E.M., n=12); (B) Gill Na^+-K^+ ATPase activity (mean ± S.D., n=20);** and (C) Percent survival after 96 hours for yearling kokanee salmon sampled from December 1992 through July 1993.

Table1 8. Mean weight (±SD) (g), length (±SD) (mm) and intestinal water transport (±SEM) (µl . cm^{-2} . h-1) of middle and posterior intestine in kokanee salmon sampled from January to June 1993. Sample size = 12 fish each month.

Month	Weight (g)	Length (mm)	Middle Intestine Jv ($\mu l \cdot cm^{-2} \cdot h^{-1}$)	Posterior Intestine Jv ($\mu l \cdot cm^{-2} \cdot h^{-1}$)
JAN	9.8 ± 1.2	96 ± 0.4	13.7 ± 3.2	16.5 ± 4.1
FEB	12.7 ± 1.8	11.4 ± 0.4	10.6±1.3	11.0 ± 2.3
MAR	16.8 ± 1.6	12.7 ± 3.7	8.8±1.3	7.2 ±1.1
APR	21.1 ± 2.5	13.2 ± 0.5	7.4±0.8	6.8±0.7
MAY	44.2 ± 4.9	16.7 ± 0.6	4.8 ± 0.6	5.4 ± 0.6
JUN	43.5 ± 5.1	16.9 ± 0.6	4.4 ± 0.4	4.0 ±1.1

Table 19. Mean ATPase activity (μmol Pi . mg Protein-l . h^{-1}) from yearling kokanee salmon and number of samples significantly lower than mean ATPase of date listed using ANOVA (alpha = 0.05). Samples range from December 1992 through July 1993.

DATE	MEAN ATPase (μmol Pi . mg Protein" . h^{-1})	# of SAMPLES SIGNIFICANTLY LOWER THAN MEAN VALUE
Dec 30	5.8	0
Jan 13	5.1	0
Feb 10	6.5	0
Mar 10	8.3	1
Apr 7	7.9	0
Apr 20	**13.7 ***	8
May 5	6.8	0
May 20	6.5	0
Jun 2	7.9	0

. = signifies significant peak in ATPase values.

In May there was 100% survival and in June survival dropped to 90%. In July, survival rose again to 100%. Table 20 shows the osmolarity of the water taken from each saltwater tank in which fish were held during the experiment. In January the osmolarity of the full strength seawater was 1193 mOsm/l which is approximately 36 parts per thousand (ppt). In February, the osmolarity of the water was 1368 mOsm/l (about 41 ppt) and in April, osmolarity of the water measured 1410 mOsm/l on the osmometer (42 ppt). These high salinities were due to a malfunction of the salinity meter which was used to measure the salinity of the water. This was not detected until the water samples were tested in the osmometer at the same time as osmolarity of blood samples was determined.

3.4.2.7 Osmoregula tory Capability

Blood plasma osmolarity (mOsml/l) values for yearling kokanee salmon are shown in Table 21. Blood osmolarity levels of fish held in 10 parts per thousand (ppt) saltwater were not significantly higher than those of fish held in freshwater from January through March (t-test; pc 0.05). During April, plasma osmolarity levels of fish held in dilute saltwater were significantly higher than those held in freshwater (302 ± 6 mOsm/l -vs- 308 ± 3 mOsm/l). Although the statistical test showed significance, the biological difference of these values was probably not significant since fish in both dilute saltwater and freshwater had blood osmotic levels that were near isosmotic concentrations. From May through July, there was no significant difference in blood plasma osmotic concentration between fish held in 10 ppt saltwater and those held in freshwater Blood osmotic concentrations of fish held in 20 ppt saltwater were significantly higher than those held in fresh water during February (344 ± 14 vs 315 ± 11 mOsm/l). Again, these values were near isosmotic levels (330 mOsm/l) and therefore may not have been biologically significant. There was no significant difference in blood osmotic concentration during any other month.

Plasma osmolarity levels of fish held in full strength salt water (30 ppt) were significantly higher than plasma levels of fish held in fresh water (control) during every month tested, January to July (Table 21). However, blood osmotic concentration of fish held in 30 ppt declined significantly over time so that from May to July, it approached the level of the controls. By May, the blood osmotic concentration of fish held in full strength seawater was approximately uniform with blood osmotic concentration of fish held in fresh water (330 mOsm/l and 300

Table 20. Water sample osmolarity (mOsm/l) taken from three fish tanks (low, 10 ppt; medium, 20 ppt; and high, 30 ppt salinity) during salt water tolerance experiments in 1993. (-) indicates no sample taken.

MONTH	10 ppt (mOsm/l)	20 ppt (mOsm/l)	30 ppt (mOsm/l)
FEBRUARY	387	766	1193
MARCH		819	1368
APRIL	404	739	1410
MAY	428		926
JUNE	355	699	964
JULY	292	655	883
MEAN	373	714	**1124**

Table 21. **Mean blood plasma osmolarity (mOsm/l) ± SD of fish held in fresh water (control) compared with osmolarity of fish in three salt water concentrations (10, 20, 30 ppt) from January through July 1993. Means represent 10 samples (n=20 fish); t value and probability are given in parentheses below means.**

MONTH	FRESH WATER (0 ppt)	SALT WATER (10ppt)	(20ppt)	(30ppt)
January	325 ± 38	304 ± 4 (t=1.5; p=0.17)	360 ± 29 (k-1.9; p=0.08)	567 ± 26 * (t=-14.4; p=.0001)
February	315 ± 11	305 ± 4 * (t=2.7; p=0.01)	344 ± 14 * p=.0 (t=-5.2;	581 ± 25 * (k-30.4; p=.0001)
March	310 ± 7	310 ± 5 (t=0.18; p=0.86)	316 ± 14 (t=-1.12; p=0.28)	550 ± 33 * (k-22.5; p=.0001)
April	302 ± 6	308 ± 4 * (t=-2.6; p=0.02)	314 ± 10 * (t=-3.4; p=0.003)	579 ± 41 * (k-21.3; p=.0001)
May	304 ± 4	306 ± 3 (t=-1.1; p=0.33)	--	344 ± 24 * (t=-5.1; p=.0001)
June	--	309 ± 5	313 ± 12 (t=-1.2; p=0.28)	404 ± 60 ** (t=-5; p=.0001)
July	313 ± 5	309 ± 5 (t=1.6; p=0.13)	309 ± 4 (t=2; p=0.07)	335 ± 30 * (t=-2.2; p=0.045)

* = salt water blood plasma osmolarity is statistically different from fresh water (control) blood plasma osmolarity by unpaired, two-tailed t-test (alpha = 0.05).

** = blood plasma osmolarity of fish held in 30 ppt saltwater is statistically different from plasma osmolarity of fish held in 10 ppt saltwater by unpaired, two-tailed t-test (alpha = 0.05)

mOsm/l respectively). Although there was a statistically significant difference between the two groups, there was probably no biological difference. This was reflected in the survival of salt water fish for >96 h in salt water challenge tests.

Figure 24 shows mean blood plasma osmolarity levels for kokanee over a 7 month period from January through July. Osmolarity of fish held in freshwater were similar ranging from 302 ± 6 mOsm/l to 325 ± 38 mOsm/l. Although a significant difference was found between January and April (325 ± 38 mOsm/l vs 302 ± 6 mOsm/l; ANOVA; Fisher PLSD, p = 0.04), and January and May (325 ± 38 vs 304 ± 4 mOsm/l), these levels were near isosmotic levels and were probably not biologically significant. In 10 ppt (330 mOsm/l) seawater, plasma osmolarity levels ranged from 304 ± 4 to 310 ± 5 mOsm/l. There were no significant differences in mean plasma osmolarity during these months (ANOVA, Fisher PLSD; p > 0.05). Plasma levels in 20 ppt (660 mOsm/l) ranged from 309 ± 4 to 359 ± 29 mOsm/l. There was a significant decrease in blood plasma osmolarity from January to February and February to March (ANOVA, Fisher PLSD, p ≤ 0.05). Plasma osmolarity levels in full strength seawater (1000 mOsm/l) ranged from 581 ± 25 to 335 ± 30 mOsm/l. Levels remained elevated from January through April (> 550 mOsm/l), then decreased significantly in May (344 ± 24) signifying that the fish were osmoregulating, which is an indicator of smoltification (p = 0.0001). Blood plasma osmolarity then rose slightly in June but significantly decreased again in July to 335 ± 30 mOsm/l (ANOVA, Fisher PLSD; p = 0.0001) (Table 21).

Blood and environmental osmometry samples were run on two separate days. Standards were run at regular intervals throughout each assay to determine accuracy. Table 22 shows that on April 5, the mean (± SD) of the100 mOsm/l standard solution measured 98 ± 2, the 300 mOsm/l standard measured 304 ± 6 mOsm/l and the 500 mOsm/l standard solution measured 504 ± 7 mOsm/l. On July 20, the mean osmolarity of the 100 mOsm/l standard solution was 99 ± 2 mOsm/l, the 300 mOsm/l standard measure 297 ± 2 mOsm/l and the 500 mOsm/l standard measured 498 ± 1 mOsm/l. Thus, osmolarity of fish samples measured on separate days were comparable.

3.4.2.8 Salt water preference

3.4.2.8.1 General Observations

During the resting period, fish were not concentrated in any particular area, but were fairly quiet. Upon completion of the fresh water bridge, the fry typically nosed up

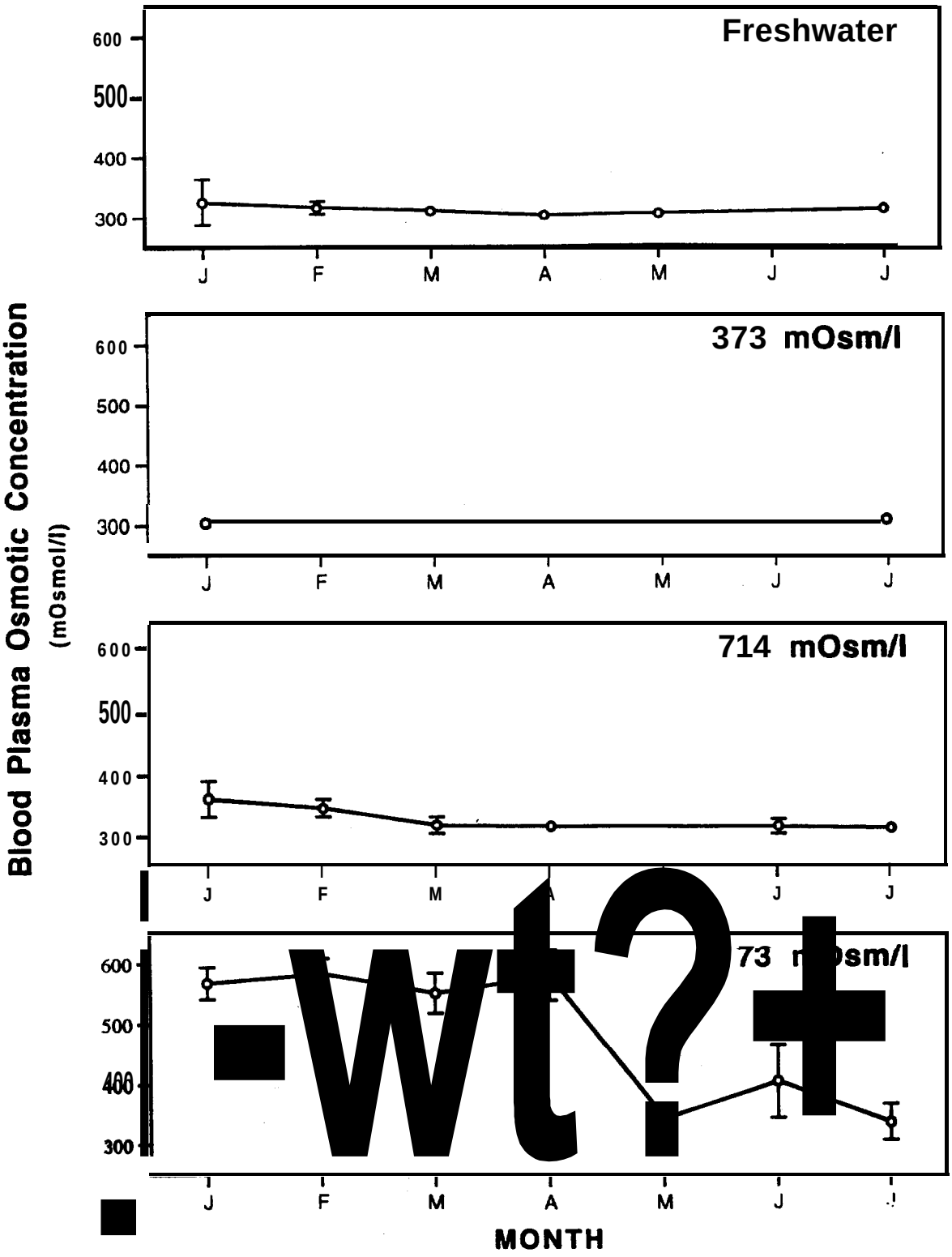


Figure 24. Blood plasma osmolarity (mOsmol/l) for kokanee salmon in four different salt water concentrations sampled from January through July 1993. Each data point represents the mean ± S.D. for 20 fish.

. Table 22. Osmolarity of osmotic concentration standards run on two dates (April 5 and July 20, 1993). Low (100 mOsm/l), medium (300 mOsm/l) and high (500 mOsm/l) standards were inserted between regular blood plasma samples at regular intervals throughout the run to determine accuracy of osmometer.

DATE	100 mOsm/ STANDARD	300 mOsm STANDARD	500 mOsm/ STANDARD	DATE	100 mOsm/ STANDARD	300 mOsm/l STANDARD	500 mOsm STANDARD
4/5/93	97	296	501	7/20/93	98	294	496
	98	302	503		100	296	498
	95	299	503		98	297	498
	97	306	502		101	301	498
	97	301	505		99	299	499
	101	302	504		99	296	497
	97	303	503		97	298	497
	98	306	507		96		496
	95	323	487		99		499
	95	303	512				
	100	301	505				
	101	299	503				
	98	307	519				
	100	305	504				
		304	504				
		306					
MEAN	98	304	504		99	297	498
SD	2	6	7		2	2	1

to the bridge and sometimes schooled and swam rapidly back and forth from fresh water to salt water. This behavior usually lasted about 10 to 20 minutes. Then activity decreased following the break up of schools and usually, individual fish remained in their tank of choice.

3.4.2.8.2 Dilute Seawater (8 ppt)

It appeared that in February and March there was an avoidance of dilute seawater. During these months only 22% and 24% of the fish preferred salt water. In April there was a slight preference to seawater (51%). In May and June there was an avoidance to dilute seawater (47 and 8% respectively preferred salt water) (Figure 25, Table 23).

3.4.2.8.3 Concentrated Seawater (24 ppt)

Fish showed a slight preference to concentrated seawater in February (53%). During March, April and May fish exhibited an avoidance to salt water. During these months, 43, 49, and 41% of the fish preferred salt water. In June, fish showed a strong avoidance to salt water. Only 23% preferred salt water (Figure 25, Table 23).

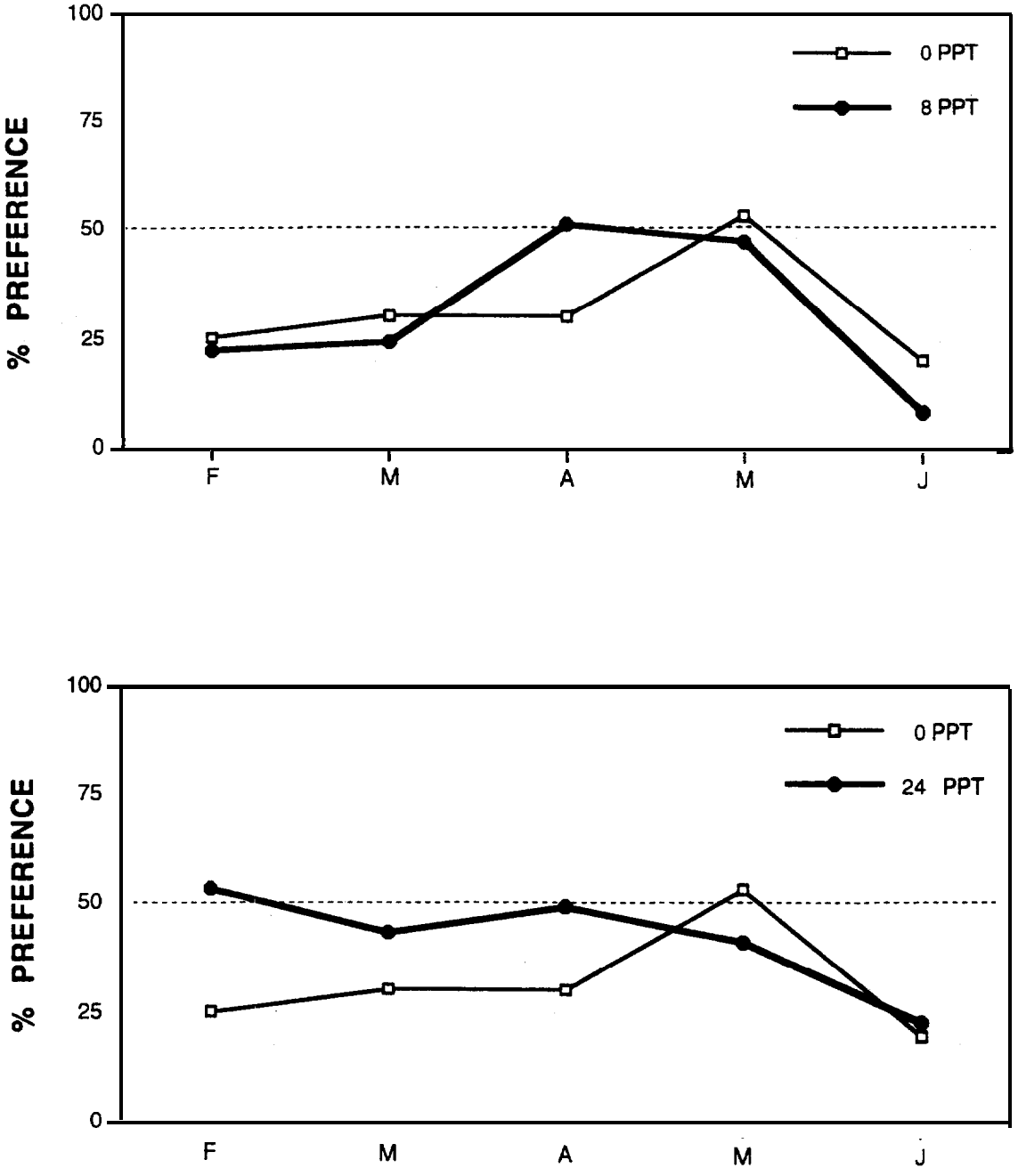


Figure 25. Salt water preference (% preference) for yearling kokanee salmon from February 1993 to June 1993. (A) Percent preference in control tank (0 ppt) and dilute salt water (8 ppt); (B) percent preference in control tank (0 ppt) and concentrated salt water (24 ppt).

Table 23. Percent of yearling kokahee salmon that preferred salt water after 100 minutes of observation. Sample period ranged from February to June 1993.

	PERCENT PREFERENCE (%)				
	FEB	MAR	APR	MAY	JUN
control (0 ppt)	25	30	30	53	20
dilute (8 ppt)	22	24	51	47	8
concentrated (24 ppt)	53	43	49	41	23

4.0 DISCUSSION

This study attempted to answer five major questions: (1) Could the results of previous investigations by Scholz *et al.* (1992, 1993), which indicated thyroxine peaks at hatch and swimup, be replicated? (2) Are thyroxine surges related to the critical period for olfactory imprinting? (3) Can kokanee salmon be artificially imprinted to synthetic chemicals and, if so, at what age? (4) Do kokanee undergo a smolt transformation and, if so, to what degree does their transformation resemble that of anadromous species? (5) How is this information related to the establishment of the kokanee fishery in Lake Roosevelt?

4.1 T_4 Concentrations of 0 age and Yearling Kokanee

In this study, as well as previous investigations by Scholz et *al.* (1992, 1993), the same pattern of thyroxine hormone (T_4) fluctuations was observed during early development of kokanee salmon. Therefore, the results of the present, study replicated previous work. In eggs and alevins, T_4 concentration was relatively high. During the periods around hatch and also around the time of swimup, T_4 concentrations peaked. In all cases, concentrations dropped to very low levels in post swimup fry. Another trend observed was that as T_4 concentration peaked, weight loss was observed. Scholz et al. (1993) suggested that since the T_4 peak coincided with rapid weight loss, it may not actually surge, but may be because of a shedding of membranes or tissues that do not contain T_4, therefore showing greater amounts of T_4 per body weight. These results suggest that T_4 is retained in the larvae, possibly by being bound to nuclear receptors in target cells. In the brain, T_4 could stimulate imprinting processes, such as formation of synaptic connections that could enhance formation of permanent olfactory memory (Scholz *et al.* 1985, 1992; White *et* a/. 1990).

Hasler and Scholz (1983), and Scholz *et al.* (1985, 1992, 1993) suggested that thyroxine surges are correlated with imprinting in yearling coho and steelhead trout. If this is true, the relatively high T_4 content of recently hatched eggs and alevins at the time of complete yolk sac absorption observed in this study may indicate that imprinting is occurring at hatch and swimup in underyearling kokanee salmon. Also, T_4 surges were observed in yearlings that evidenced smoltification in the present study. This may indicate a second critical period for imprinting in yearlings.

4.2 Imprinting Investigations

The olfactory hypothesis for salmon homing states that: (1) each stream contains a unique odor bouquet that is detectable by fish, **(2)** the fish form an imprint (ie., permanent memory) to this distinctive odor prior to emigrating from their natal tributary, and (3) adults recall the memory of the odor and utilize it as a cue for relocating their homestream during the spawning migration (Hasler and Wisby 1951). It is well documented that coho salmon (*Oncorhynchus kisutch* Walbaum) and steelhead trout (0. *mykiss* Walbaum) imprint to chemical cues at a critical period (approximately 16-18 months of age) during the smolt stage, and later use this information as a cue to relocate their homestream during the spawning migration. Evidence stems from field experiments in which coho salmon, steelhead trout and brown trout (*Salmo trutta* L.), that were exposed to synthetic chemicals (either morpholine or phenethyl alcohol) as smolts, were attracted as three year old adults into rivers scented with their appropriate exposure chemical at 92-96% accuracy (Cooper and Scholz 1976; Scholz et al, 1976, 1978a,b; Cooper et *al.* 1976; Hasler et *al.* 1978; Hasler and Scholz 1985).

The chemical imprinting process appeared to be activated by thyroid hormones. The proposed mechanism of action requires binding of thyroid hormones to nuclear receptors in neurons (Scholz et a/. 1985; White et *al.* 1990). This was indicated by experiments in which radiolabeled thyroid hormones injected into steelhead trout *in vivo* later accumulated and were retained in the brain cell nuclei (Scholz et *al.* 1985). Also, *in vitro* competitive binding assays and Scatchard plot analysis performed on isolated steelhead brain nuclei demonstrated the presence of saturable, high affinity triiodothyronine receptors (Bres and Eales 1986; White et *al.* 1990). Hormone binding was thought to activate transcription of genes that code for nerve growth proteins, which promote neuron differentiation, including arborization of axons and dendrites, and formation of synaptic connections. Effectively, this neuron differentiation hardwires of pattern of neuron circuitry that allows for the permanent **storage** of the imprinted olfactory memory (Scholz et *al.* 1992). We envision that the pattern of interconnections stores the olfactory memory in a manner similar to how electrical connections in a computer stores a memory on the hard drive. The imprinted odor, by binding to specific receptors in the olfactory epithelia, activates the memory in the same fashion that a finger striking a particular sequence of keys on the keyboard accesses the stored computer memory.

Golgi silver stain impregnation investigations revealed that neurons of presmolt steelhead trout, with basal levels of thyroid hormones, were poorly differentiated; whereas smolts exhibited well differentiated neurons following elevation of thyroid hormones (Lanier 1987; Scholz et *al.* 1992). Moreover, presmolts injected with TSH experienced elevated plasma thyroid levels, followed immediately by the same type of neuron differentiation observed in smolts; while in saline injected presmolts, which did not have elevated thyroid levels, neuron differentiation did not occur until the normal time (Lanier 1987; Scholz et *al.* 1992). Cardiac conditioning experiments conducted with Atlantic salmon (*Salmo salar* L.), identified two sensitive periods within the smolt stage where olfactory learning and memory retention occurred concomitant with thyroid activity (Morin et *al.* 1989a, b). Since formation of a long-term olfactory memory was associated with only one age group, Morin et *al.* concluded that their findings, "*suggested that thyroid hormones may be exerting a very specific thyroid brain effect rather than a general effect on the fish's nervous system*." Collectively, these results supported the hypothesis of thyroid-induced olfactory imprinting in smolt stage salmon.

However, not all species of Pacific salmon stay in their homestream for 1.5 years, then exhibit a distinct smolt stage like coho, steelhead, and Atlantic salmon. For example, anadromous sockeye, and their landlocked relatives the kokanee salmon, emigrate from their natal tributary to a nursery lake soon after swimup. Sockeye remain in the lake for about a year, then smolt and migrate to sea. Kokanee undergo incomplete smoltification and remain in the lake, where they grow to adult size. Both sockeye and kokanee exhibit natal homing to tributaries of lakes experienced only as embryos or larvae (Quinn et *al.* 1989), so if these fish utilize olfactory cues for homing, they must necessarily imprint during the egg, alevin, swimup or early fry stages.

Recently, several investigators have reported that whole body thyroxine content fluctuates in developing eggs and larvae of several species of *Oncorhynchus,* including sockeye and kokanee (Kobuke et *al.* 1987; Tagawa and Hirano 1987, 1990; Greenblatt et *al.* 1989; Leatherland et *al.* 1989; Tagawa et *al.* 1990; deJesus *et al.* 1992; Scholz et *al.* 1992, 1993). Typically, thyroxine content was relatively high in eggs, decreased in post-hatch alevins, then increased in swimup larvae. The disappearance of thyroxine after hatching suggested that it was being taken from the yolk into tissues and then cleared from the fish (Tagawa and Hirano 1990). Thyroid follicles in chum salmon (0. *keta*), another species that emigrates soon after emergence, were found to increase in size and number during yolk absorption, so it

was concluded that the increase in thyroxine content at the time of emergence (i.e. swimup stage) was owing to increased production of thyroxine by the embryonic thyroid gland (Tagawa and Hirano 1990). In comparative studies of all five species of Pacific salmon native to North America, sockeye eggs and larvae had the highest thyroxine content (Letherland *et al.* 1989). The implication of these studies was that thyroid hormones may be sufficiently high 'in sockeye/kokanee eggs or larvae to stimulate olfactory imprinting at those developmental stages.

4.2.1 Olfactory Discrimination Experiments

Results of the present investigation documented chemical imprinting in kokanee salmon concomitant with elevated thyroxine levels. The group of fish that had the highest whole body thyroxine content (swimup stage) also had the highest percentage of fish that were attracted reliably to their exposure odor in behavioral tests. Recently hatched eggs and alevins also had relatively high thyroxine content and displayed accurate homing in behavioral tests. Additionally, smolts experienced elevated plasma thyroid levels and tended to be attracted to their exposure odor in the behavioral tests. In contrast, pre-hatch eggs and post-swimup fry had relatively low thyroxine content and did not evidence selective attraction to their exposure odor. These results indicated that kokanee salmon imprint to chemical cues during two sensitive (or critical) periods during development, at the alevin/swimup and smolt stages.

4.2.2 Coded Wire Tag (CWT) Investigations

In general, preliminary results of coded wire tagging investigations with kokanee exposed to synthetic chemicals at the smolt stages, then released into Lake Roosevelt as residualized smolts, paralleled the results observed in olfactory discrimination experiments. For smolts released in 1992, 64.3% of the morpholine exposed fish were recaptured as sexually mature adults in the morpholine-scented stream compared to 35.7% in the phenethyl alcohol-scented stream. In contrast, the pattern of recoveries for fish exposed to phenethyl alcohol was reversed, with 40% recaptured in the morpholine scented stream and 60% recaptured in the phenethyl alcohol scented river. For smolts released in 1993, 87.5% of the morpholine-exposed fish were recaptured as sexually mature adults in the morpholine scented stream, compared to 12.5% in the phenethyl alcohol scented stream and none captured at other locations. In contrast, the opposite was true for fish exposed to phenethyl

alcohol, with 16.2% recaptured in the morpholine scented stream, 81.3% recaptured in the phenethyl alcohol scented river and 2.4% recaptured at other locations.

Additional information was obtained from CWT investigations that will prove useful for employing adaptive management techniques to enhance Lake Roosevelt kokanee. For example, in 1992 and 1993 a combined total of 3,064,226 kokanee were released from the Spokane Tribal Hatchery into Lake Roosevelt. Of these, 599,843, or 19.6%, were marked with coded wire tags and adipose fin clips. In 1993, a total of 229 kokanee were observed in randomly conducted creel census surveys or electrofishing and gill net surveys on Lake Roosevelt. Of these, 50, or 21.8%, bore coded wire tags or adipose fin clips. Since the percentage of marked to unmarked fish recaptured. (21.8%) was remarkably uniform with the percentage of marked to unmarked fish released (19.6%), these data suggest that the majority of kokanee in Lake Roosevelt are of hatchery origin.

Kokanee releases into Lake Roosevelt in 1992 and 1993 totaled 599,843 CWT/fin clipped fish, including 432,446 (or 72.1%) planted as fry and 167,397 (or 27.9%) planted as residualized smolts. Recoveries of tagged fish included those recovered in randomly conducted creel census and electrofishing/gill net surveys and those recovered in monitoring egg collection sites at Sherman Creek and Little Falls Dam, which were scented with morpholine and phenethyl alcohol respectively. A total of 299 CWT/fin clipped fish were recaptured in 1992 and 1993, including 4 fish (1.3%) that had been released as fry and 295 (98.7%) that had been released as smolts. Thus, the ratio of fry to smolts was about 3:1 released and 1:99 recovered. These data provide conclusive evidence that fish planted as residualized smolts provide significantly better returns to both the creel and egg collection sites. In fact, fish planted as fry are essentially not contributing to either category. Releasing fish as residualized smolts could be an effective measure for enhancing Lake Roosevelt kokanee.

There are two potential explanations for the poor rate of recovery of kokanee released as fry. The first might be high mortality rates caused by walleye predation at the time of stocking. The second is loss of kokanee from the reservoir.

There is some evidence that walleye predation does occur when kokanee fry are released. On July 14, 1992 the Washington Department of Wildlife set a gill net in the embayment at Sherman Creek for 15 hours, and captured one chinook salmon

and nine walleye. The chinook stomach contained two kokanee fry. The contents of the walleye stomachs averaged nine kokanee fry (range 1-23 kokanee per walleye) (Vail 1992). A hook and line sample at the same location on July 24, 1992 captured five walleye and two squawfish. Stomach contents averaged 1.6 kokanee per walleye (range O-3) and 1 per squawfish (Vail 1992). Also, in August 1992, 12 walleye were collected during electrofishing surveys, conducted as part of the Lake Roosevelt Monitoring Program, from the mouth of Sherman Creek in the vicinity of the hatchery fish ladder. Stomachs from four of these contained kokanee bearing coded wire tags. Thus, predation at the time of stocking does occur.

However, we do not believe that predation is the principle factor accounting for the disappearance of kokanee between the fry and adult stages for three reasons. First, stomach contents were analyzed from 928 walleye randomly collected in Lake Roosevelt as part of the Lake Roosevelt monitoring Program from 1988-1992 (Peone *et* a/. 1990, Griffith and Scholz 1991, Thatcher et *al.* in press). The principle fish species eaten by walleye were Cottidae (sculpins), followed by Percidae (yellow perch), Cyprinidae (minnows), and Catastomidae (suckers), with Salmonidae (kokanee, rainbow, whitefish) a distant fifth. Only 24 of the 928 walleye examined had confirmed salmonids in their gut. From 1991 to 1993, stomach contents of 245 walleye were examined and 14 of these contained a total of 16 salmonids (K. Underwood, pers. comm.). Thus, it appeared that although walleye may occasionally consume kokanee opportunistically at release sites, they are generally not an important predator on kokanee.

The second reason we believe walleye predation on kokanee is not significant is that walleye and kokanee fry occupy different environments in Lake Roosevelt. Kokanee typically emigrate to open water in the pelagic zone soon after stocking because they are rarely encountered in shoreline electrofishing surveys conducted in the. embayments where they are released (Peone *et al.* 1990, Griffith and Scholz 1991, Thatcher *et al.* in press). In the pelagic zone they prey upon large pelagic zooplankton such as *Daphnia sp.*, which is reflected in diet analyses that indicate that *Daphnia* typically contribute in excess of 90% to the annual diet of kokanee based upon index of relative importance indices which combine the numerical and weight percentage of the diet (Peone *et* al. 1990, Griffith and Scholz 1991, Thatcher *et al.* in press). Their optimal foraging on large zooplankton contributes high benefits in relation to costs for handling prey, which is energetically efficient, accounting for the relatively high growth

rates of kokanee in Lake Roosevelt. In contrast, walleye in Lake Roosevelt are typically benthivorous as reflected by the prevalence of bottom dwelling sculpins, and benthic macroinvertebrates in their diets. Thus, kokanee and walleye clearly occupy different niches within the reservoir, which would tend to reduce predator-prey interactions between these species.

The third reason we believe walleye predation is not the principle factor accounting for the loss of kokanee is that there is evidence of substantial kokanee entrainment through Grand Coulee Dam. For example, in 1991 a loss of approximately 25,000 subadult kokanee was noted (Thatcher *et al.* in press). We believe that such losses are owing partly to fish released as fry undergoing smoltification the following year and emigrating from the reservoir during periods of drawdown and water releases that provide the water budget for anadromous salmonids. The present study confirmed that Lake Roosevelt kokanee (Lake Whatcom stock) undergo at least partial smoltification as discussed in Section 4.3 below. Releasing fish as residualized smolts should reduce problems with kokanee entrainment. In the event that walleye predation is a greater problem than we presently believe, releasing larger sized post-smolts would also likely reduce predation rates and improve survival.

4.2.3 Conclusions from Imprinting Experiments

The present study confirmed the hypothesis that thyroid hormone peaks are correlated with the critical period for imprinting in kokanee, and identified a second critical period at the alevin/swimup developmental stage. Results of the present investigation suggested that it should 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. Therefore, the first aspect of the Lake Roosevelt kokanee salmon monitoring program has been addressed: to determine the best times for stocking kokanee to ensure homing to egg collection sites.

4.3 Smoltification Investigations

The second aspect of the Lake Roosevelt Kokanee monitoring program was to reduce loss of juvenile kokanee through Grand Coulee Dam. Because of the significant loss that has been observed (Thatcher *et al.* in press), it was hypothesized that these fish were undergoing a parr-smolt transformation. The next part of this study

attempted to ascertain the validity of this hypothesis by comparing the degree of smoltification to other anadromous salmon.

For salmonids to undergo a smolt transformation they must rapidly change from a freshwater parr to a salt water adapted smolt. This metamorphosis includes numerous morphological, physiological and behavioral changes. The smolt becomes a slimmer, more streamlined fish and more silvery. As the fish changes to become salt water adapted, there are changes in intestinal water uptake, gill Na^+-K^+ ATPase activity, osmoregulatory capability and salt water tolerance. Behavioral changes include an increase in downstream migratory activity and a preference for salt water. Many hormones are involved in the smolting process including an increase in growth hormone, an increase in cortisol, and a decrease in prolactin (reviews by Hoar 1976; Folmar and Dickhoff 1980; Hasler and Scholz 1983; Barron 1986). In addition, thyroid hormones typically increase during the smolt period (reviews by Hoar 1976; Folmar and Dickhoff 1980; Hasler and Scholz 1983; Barron 1986).

Smolting appears to be controlled by an endogenous (internal) rhythm that is synchronized by the annual photocycle (Wagner 1974a,b; Hoar 1976; Hasler and Scholz 1983;), lunar phases (Grau *et al.* 1981, 1982) and possibly other cues such as ionic composition, organic odor characteristics, or stream flow. These cues stimulate hormonal releases which, in turn, are responsible for the various behavioral and physiological adaptations mentioned earlier. Of these, the annual photocycle appears to be the most important stimulus since smoltification usually occurs at about the same time every year as daylength increases during the spring. Within this period, other cues act to precisely synchronize hormone release. For example, Grau et a/. (1982) suggested that the lunar cycle acts through several endocrine systems including the thyroid, to set the timing of smoltification. They found that the thyroxine surges in the spring coincided with the new moon phase of the lunar cycle. Many other factors affect thyroxine dynamics as well as moon phases, for example temperature, water flow rates, and altering the chemical characteristics of water (Zaugg and Wagner 1973; Grau *et al.* 1981; Barron 1986; Specker 1988).

4.3.1 Smoltification in Age 1+ Kokanee

From information gathered in this preliminary investigation, it appeared that kokanee underwent at least partial smolt transitions compared to other species of anadromous salmonids. These kokanee experienced thyroxine peaks, increased

silvering, increased downstream migratory behavior and an increase in salt water adaptation at certain times of the year.

4.3.2 T_4 Concentrations

The occurrence of the thyroid peak is an important aspect of the smolt transformation. Results of the 1993 investigations were similar to the previous year. Plasma thyroxine levels increased significantly and peaked on February 10, 1993 at 15.2 $\pm$ 0.9 ng/ml. This peak did not coincide with a new moon (new moons were January 22 and February 21). However, there was a second peak of thyroxine April 20 (12.2 $\pm$ 2.6 ng/ml), which did coincide with a new moon. These peaks were similar to results observed by Scholz *et al.* (1993). In both studies, thyroxine peaked in the winter (January or February) and again in the spring (April - June), (Figure 26). The peak T_4 value from Scholz *et al.* (1993) was more pronounced (an increase of 6 times the basal level compared to 1.5 times the basal level in the present study). However, there was a significant peak in this study and it was comparable to other anadromous salmonids. Table 24 shows approximate basal, and peak T_4 concentrations from a number of investigations, and the ratio of the peak to basal level. Typical ratios of peak to basal T_4 concentration ranged from 1.8: 1 to 7:1. In the 1993 investigation, the ratio was 1.5:1 which was comparable to that found by other investigators. Also in the present study, the peak T_4 concentration was significantly different from basal levels. During the previous year (Scholz *et al.* 1993), there was a higher peak observed with the same stock of fish. In both investigations, blood plasma was sampled every two weeks. It may be that there was a more definite surge but that the amplitude of the peak in this study could have been understated by the sampling frequency (two weeks) such that the peak that was observed in the 1992 investigation could have been missed in the present investigation.

T_4 concentrations of kokanee salmon were generally lower than other species of salmon at the time of smoltification. However, in some of the other investigations, T_4 at smoltification was lower than the values observed in this study (Table 24). Therefore, T_4 could have been high enough in these kokanee salmon to trigger other aspects of smoltification such as downstream migratory behavior and silvering and physiological transitions associated with salt water adaptations.

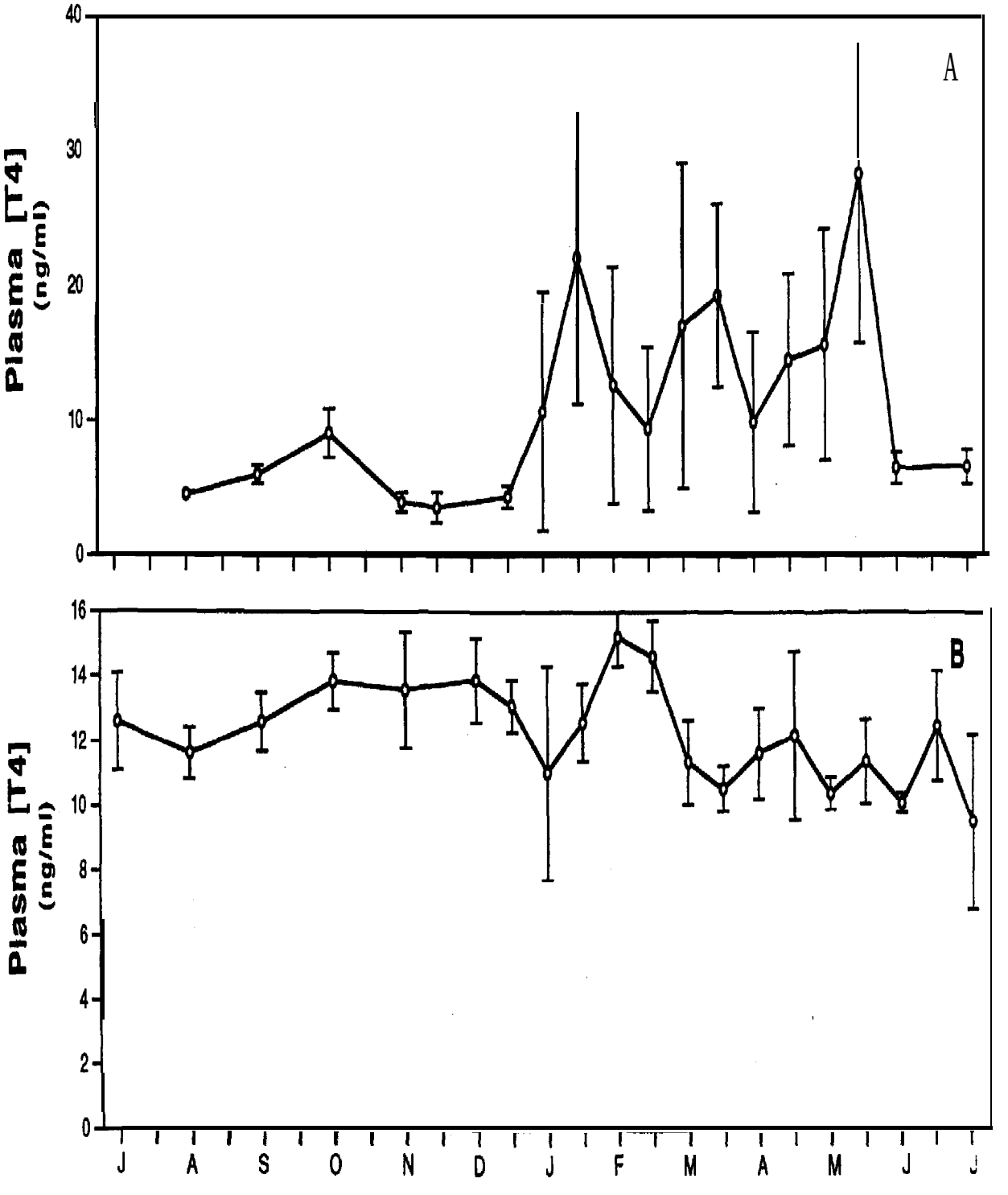


Figure 26. Thyroxine (T_4) hormone concentrations (mean + S.D.) from Lake Whatcom stock kokanee salmon (n=20). (A) 1990 year class; (B) 1991 year class.

Table 24. Comparison of T_4 concentration in yearling salmonids. Values are approximations, taken off graphs of previous investigations.

Species	Basal [T_4] (ng/ml)	Peak [T_4] (ng/ml)	Ratio	Source
C o h o	16	85	5:1	Dickhoff *et al.* (1978)
Coho	8	39	5:1	Dickhoff *et al.* (1982)
Coho	5	35	7:1	Grau *et al.* (1982)
Coho	10	73	7:1	Hasler & Scholz (1983)
Coho	4.8	8.5	1.8:1	Specker (1988)
Coho	6.5	11	2:1	Young *et al.* (1989)
Atlantic	4	26	7:1	Boeuff & Prunett (1985)
Atlantic	4	17	4:1	Prunett *et al.* (1989)
Steelhead trout	5.3	23	4:1	Scholz *et al.* (1985)
Rainbow trout	6	26	4:1	Osborn *et al.* (1978)
Kokanee	5	28	6:1	Scholz *et al.* (1993)
Kokanee	**10**	**15**	**1.5:1**	**Present study**

4.3.3 T_4 Concentrations and Silvering

Silvering in the skin is due to the deposition of two purines, guanine and hypoxanthine. Crystals of these substances are deposited in the skin layers of the fish. One purine is deposited beneath the scales and the other in the dermis of the muscles. In parr, both layers of purines are present, but these become thicker in smolts. The end result of this thickening is a silvery fish. The silvery appearance is thought to be an adaptation to the survival of the pelagic (ocean going) postsmolts in the marine environment (Hoar 1988).

Prior studies have shown that thyroid stimulating hormone (TSH) or treatment of thyroid hormones, induce purine deposition in salmonids (Robertson 1948, 1949; Chua and Eales 1971; Hasler and Scholz 1983). During both years of this study, it appeared that T_4 levels were high enough to stimulate an increase in purine deposition. In the 1993 investigation, the mean T_4 concentration in February was 15.2 $\pm$ 0.9 ng/ml. This was the highest mean level observed during the sampling period. This was also the time in which fish started turning silver. The number of fish in transition from parr to smolt increased from 5% in January, to 40% in February, 1993. Although T_4 concentration declined after February, silvering continued to increase into June. A second peak in T_4 concentration in mid April was accompanied by an increase in the rate of silvering, which resulted in the rapid change of fish from transitional coloration to silver smolt coloration by early May. These results were consistent with Hasler and Scholz (1983), who found an increase in T_4 concentration prior to silvering.

There was a significant difference in T_4 concentration between fish grouped as parr and those grouped as smolts and between those grouped as transition and smolt. These results showed parr with a higher mean T_4 concentration than smolts. These data conflicted with a study done by Hasler and Scholz (1983), who found T_4 concentrations increased from the parr stage to the smolt stage in coho salmon. They also found that T_4 concentration was correlated with the degree of silvering, and that parr had the lowest T_4 concentration. This difference may be owing to sampling frequency differences between the two investigations. In their study, T_4 and smolt coloration were measured only once per month compared to twice per month in the present study.

4.3.4 Condition Factor

The condition factor (K) was considered as a potential indicator of smolt transformation in yearling kokanee salmon. This coefficient of condition is a description of how fish add weight as they grow in length. It can be affected by variables other than smoltification. For example, fish health and food ration level could affect condition. In many cases, as a fish smolts, the condition factor drops because the fish becomes more slender and streamlined (Hoar 1939; Fessler and Wagner 1969). In the present study, when most of the fish were parr coloration (January 13), the condition factor was highest (1.3 ± 0.14). Then as the fish became more silvery in February, the condition factor decreased (0.83 ± 0.04). These data were similar to data collected by Johnston and Eales (1970), who found that parr had a higher (K) than fish classified as silvery parr or as smolts. These results suggest that kokanee change shape similar to other salmonids during smoltification (Gorbman *et al.* 1982; Winans 1984).

4.3.5 T_4 and Downstream Migratory Behavior

Several studies have suggested that increased thyroid activity may be involved with the induction of the seaward migration, as well as seawater adaptability and seawater preference (Hoar 1953; Baggerman 1960a,b, 1963; Eales 1963; Youngson *et al.* 1985). T_4 may bind to the retina and alter the composition of visual pigments, thereby decreasing the visual acuity of smolts in fresh water, eventually leading to their passive drift in water currents when fish fail to maintain visual contact with the bottom at night. Additionally, active downstream migration could result from activation of motor patterns generated by specific centers of neurons in the central nervous system. Scholz *et al.* (1985) found that hormones bound to specific triiodothyronine (T_3) receptors in the brain of steelhead trout at the time of smoltification. Also, Godin *et al.* (1974) found that pre-smolt Atlantic salmon treated with thyroxine, at a time when the thyroid gland was relatively inactive, showed increased downstream migratory activity compared to controls that were left untreated. Similarly, Hasler and Scholz (1983) found that pre-smolt coho salmon injected with thyroid stimulating hormone, in doses sufficient to elevate T_4 to physiological levels observed in smolts, displayed an increase in downstream migratory behavior compared to controls injected with a saline placebo.

In contrast, Birks *et al.* (1985) found that injections of T_4 decreased migration of steelhead trout and that an increased tendency to migrate occurred as the plasma thyroxine concentrations decreased. They suggested that there is little relationship between T_4 levels and migration tendency. However, it was not determined if the doses used were physiological or pharmacological, making it difficult to interpret the results of this investigation. (Administration of pharmacological doses of hormones frequently has the opposite effect as a physiological dose.) In addition, Ewing *et al.* (1984) found steelhead migrants had lower T_4 concentrations than non-migrants of the same populations. Thus, there is conflicting evidence about the role of thyroxine in stimulating downstream migration. In the present study, we accepted the premise, albeit with caution, that thyroxine induces downstream migratory activity.

Downstream migratory behavior in this study increased after the February 1993 peak of thyroxine and also after the April 1993 peak. These findings suggest that some kokanee experienced a high enough thyroid surge to trigger downstream migration. This pattern showed an increase in migratory behavior after a T_4 peak. However, the migratory disposition was never very high for these kokanee. The highest percentage exhibiting downstream orientation was 65% in March and May. In contrast, Hasler and Scholz (1983) found 95% of coho salmon showed downstream migratory behavior in the spring shortly after thyroxine surges using a test procedure similar to the one used for the present study. However, increases in migratory behavior in only a portion of the fish could explain why only some of the fish seem to emigrate below Grand Coulee Dam. If kokanee experience partial thyroxine induced migratory tendencies after they are released from the hatchery into Lake Roosevelt, it could account for the fish showing up at Rock Island Dam.

Another question which needs to be addressed is can lake conditions contribute to thyroxine surges which may trigger downstream migration in kokanee? Two components of rivers which change during spring runoff are water chemistry and velocity. The thyroxine surge which occurs at this time may be an adaptation which allows juvenile salmon to undergo a rapid smolt transformation and to use the increased stream velocity of spring runoff to speed their downstream migration. Previous reports have indicated that increased water flow stimulates thyroid function in salmonids which are not smolting (Higgs and Eales 1971). Youngson and Simpson (1984) found that T_4 concentration of non-emigrant Atlantic salmon remained relatively

unchanged even after an increase in water flow, while serum T_4 levels in emigrants became elevated.

Hoffnagle and Fivazzani (1990) found plasma thyroxine levels of landlocked chinook salmon increased significantly after exposing fish to lake water with a distinct chemical nature, but it did not increase after increasing water flow. Their results were consistent with previous investigations on the effects of novel water chemistry on smoltification (Specker and Schreck 1984; Grau et *al.* 1985; Lin *et al.* 1985; Nishioka *et al.* 1985) but they conflicted with those indicating a stimulator-y effect of increased water flow on plasma thyroxine levels (Youngson and Simpson 1984; Youngson *et al.* 1985). Hoffnagle and Fivazzani (1990) speculated that the thyroxine increases that were apparently related to an increase in flow rates might actually have been related to different water chemistry associated with higher flow. When novel water enters the system in Lake Roosevelt, it could stimulate T_4 surges which could, in turn, have the potential to stimulate downstream migratory behavior and emigration from Lake Roosevelt.

4.3.6 Salt Water Preference

4.3.6 1 Control Experiments

In the control experiments, when the fish had a choice between their original fresh water compartment and an alternate fresh water trough, they remained most of the time in the original compartment. Houston (1957) obtained similar results when he measured salinity preference of chum and pink fry, and coho smolts. This behavior was of concern because it did not represent a good control for the experiment. Fish in the fresh water troughs should have had no preference for either compartment (50% preference). However, it could be that this species has the ability to remember spatial relationships. Their preference for the original compartment may be the result of preference for “recognized” areas. Hoar (1956) suggested that chum and pink fry can rapidly learn direction in circular channels and retain this knowledge for some time. In the present experiments on kokanee yearlings, even though the compartments were the same size and color, there may have been visual cues in the form of airstones or tubes. There have been no known salinity preference studies done on kokanee. If a species did exhibit territoriality (such as coho fry), recognition of an area would be very important and this territoriality would tend to produce scattered distributions (Hoar 1954). Hoar found that sockeye smolts did not seem to show aggressive territoriality

and therefore, they were not dispersed as much as coho fry and would tend to stay in the first compartment. Sockeye salmon and kokanee salmon are a schooling fish (Hoar 1953) which may be the reason they stayed in the first compartment.

4.3.6.2 Dilute Seawater

The preference of fish observed in the dilute (8 ppt) salt water, followed a pattern similar to the control (0 ppt) preference. It appeared that the fish preferred the fresh water to the dilute salt water. In April, the salt water preference was 51%. These data suggested that there was no preference, or maybe a slight preference for dilute salt water. Since kokanee are normally fresh water residents, it seems anomalous that any would prefer salt water. It could be that these fish were attracted to a medium in which they were required to do the least amount of osmotic work. If this was the case, it was surprising that there was not a stronger preference for these fish to dilute salt water since the concentration of dilute salt water in this experiment was close to the isosmotic level for fresh water fish. However, after the freshwater bridge was in place, fish tended to swim back and forth for about 10-20 minutes, after which activity quieted down and fish remained in the tank they occupied at that time. It could be that some of the fish which never ventured out of the first compartment were not attracted to the seawater.

4.3.6.3 Concentrated Seawater

In February, kokanee showed a slight preference to concentrated seawater (24 ppt). This positive preference during February corresponded to the peak in thyroxine in February. Hoar (1952) found that thyroxine increased the rate of swimming in salmon. This increase in thyroxine in February may have stimulated increases in movement to a different compartment. However, kokanee salmon during this month did not appear to be more active than they were in other months, and the percent preference for 24 ppt was very slight (53%). This preference may not be a significant change in behavior. These kokanee did not respond as strongly to salt water as other species of anadromous salmon. Baggerman (1960b); Houston (1957) and Mclnerny (1963, 1964) reported a definite salt water preference for sockeye and other salmon as yearlings. The lack of salt water preference in kokanee salmon may be one of the reasons kokanee do not typically migrate downstream as yearlings.

4.3.7 Salt Water Adaptation

Four types of tests were performed to assess salt water adaptation in yearling kokanee salmon: intestinal water transport (J_v), determination of gill Na^+-K^+ ATPase activity, salt water tolerance and osmoregulatory ability.

4.3.7.1 Intestinal Water Transport

The first test to determine if kokanee yearlings adapted to salt water was measurement of intestinal fluid absorption potential (Jv). The ability of fish to regulate blood-serum concentration when placed in salt water comes from activation of Na^+-K^+ ATPase in the gill and gut. In the gut, Na^+-K^+ ATPase pumps sodium ions into the cells surrounding the lumen, which triggers the osmotic uptake of water.

In the present study, intestinal water transport decreased steadily from January (17 µl . cm-* . h^{-1}) through June (5 µl . cm^{-2} . h-1). Water absorption in June was about one third of that in January. This steady decrease may be indicative of the loss in migratory life history. The low fluid transport would be consistent with the need to reduce water intake in a fresh water environment. This fluid transport pattern for the kokanee salmon was quite different from that reported for the migratory species of *Oncorhynchus* and *Salmo* in that it showed no preadaptive increase during the late spring in 1+ fish. In most species of anadromous salmonids, Jv values increase during the spring rather than decrease (Collie and Bern 1982; Veillette et *al.* 1993). Normally, intestinal transport and gill ATPase increases coincide in the spring. In the present study, while gill ATPase increased in the spring, Jv decreased. These two parameters were out of phase with each other. This may be one of the factors which sets these kokanee apart from anadromous species. If this phenomenon is generally applicable to kokanee and if the physiological differences are related to genetics, it may indicate the futility of selectively breeding endangered Snake River sockeye with redfish lake kokanee as a component of the recovery strategy. Crossing these stocks may cause permanent genetic damage to the anadromous sockeye. A better strategy might be to introduce another stock of anadromous sockeye.

4.3.7.2 Na^+- K^+ A TPase Activity

The second test to assess salt water adaptation in kokanee was measurement of gill Na^+-K^+ ATPase activity. In the gill, Na^+-K^+ ATPase pumps sodium ions in an outward direction resulting in osmoregulation of blood osmotic concentration.

Changes in gill Na^+-K^+ ATPase activity during smoltification may be regulated by hormonal factors. Evidence has been presented for the activation of Na^+-K^+ ATPase by thyroid hormones in teleosts (Dickhoff et *al*. 1977; Folmar and Dickhoff 1978, 1979; Folmar et *al*. 1980a). However, there is little data which unequivocally demonstrates that thyroid hormones have a direct effect on osmoregulatory processes. In the present study, no correlation was found between T_4 and ATPase. Hasler and Scholz (1983) also found little correlation between T_4 and Na^+-K^+ ATPase, salt water tolerance or osmoregulatory capability, suggesting that T_4 does not play an important role in osmoregulation compared to other osmoregulatory hormones. Cortisol, prolactin and growth hormone have been implicated in osmoregulation in smolting salmonids (Specker 1982; Prunett and Boeuf 1985; Sweeting et *al*. 1985).

Numerous investigators have studied gill Na^+-K^+ ATPase levels in salmon smolts. Table 25 shows the ratio of increase from basal levels to peak levels during smoltification observed in those studies. Smolts typically show an increase in ATPase enzyme activity of 2 to 5 times the initial level observed in parr. Nearly all of the work based on Zaugg's method (7 out of 10 investigations) indicated a 2:1 ratio of peak to basal ATPase levels. The mean basal level of all of the studies was 7.8 µmol Pi • mg Protein-l . h^{-1} (n=15), while the mean peak level was 19.1 µmol Pi . mg Protein-l • h^{-1}. In the present study the level of ATPase increased significantly from January to April from a basal level of 5.8 to a peak of 13.7 µmol Pi • mg Protein-1 • h^{-1}, exhibiting an increase of 2 times the initial level. Therefore, gill Na^+-K^+ ATPase in kokanee appeared to be as fully functional as in anadromous species. This therefore allowed them to survive in the salt water challenge test for 96 hours.

4.3.7.3 Osmoregula tory Capability

The third test to determine if kokanee were adapting to salt water was to measure osmoregulation. Previous investigations have shown that Atlantic or coho salmon smolts placed in full strength salt water were able to regulate blood-serum concentration while parr were not (Houston 1960, 1964; Parry 1960; Potts 1970; Boeuf et *al*. 1978; Hasler and Scholz 1983). It appeared from this study that kokanee were able to regulate plasma ion concentrations in the spring of the year. From January to April, blood plasma osmolarity was significantly elevated in fish held in full strength seawater (about 550-650 mOsm/L) compared to fish held in fresh water (about 310 mOsm/L), indicating an inability to regulate serum osmotic concentrations. However, osmolarity of fish held in full strength seawater dropped significantly in May to 344 ±

Table 25. Comparison of Na^+-K^+ ATPase activities of yearling salmonids. Values are approximations, taken off graphs of previous investigations.

Species	Basal ATPase (µmol Pi/mg Prot/h)	Peak ATPase (µmol Pi/mg Prot/h)	Ratio	Source
Chinook	12	22	2:1(a)	Zaugg & McLain (1972)
Chinook	3.5	8	2:1 (b)	Ewing et *al.* (1979)
Spring chinook	3	12	4:1 (b)	Buckman & Ewing (1982)
Spring chinook	6	14	2:1 (a)	Beeman et *al.* (1990)
Spring chinook	0.5	5.5	11:1(b)	Franklin et *al.* (1992)
Fall chinook	16	26	2:1 (a)	Rondorf et *al.* (1989)
Coho	12	24	2:1 (a)	Zaugg & McLain (1972)
Coho	12	35	3:1 (a)	Zaugg (1982)
Coho	8	23	3:1(b)	Hasler & Scholz (1983)
Atlantic	5	20	4:1 (a)	Boeuff et a/. (1985)
Atlantic	10	37	4:1 (a)	Birt et *al.* (1991)
Atlantic-landlocked	10	22	2:1(a)	Birt et *al.* (1991)
Steelhead trout	16	28	2:1 (a)	Zaugg & McLain (1972)
Steelhead trout	2	6	3:1(b)	Ewing *et* al. (1984)
Sockeye-landlocked	1.5	5	3:1 (b)	Franklin et *al.* (1992)
Kokanee	7	14	**2:1(a)**	**Present study**

(a) = methods followed procedure developed by Zaugg (1982a)
(b) = methods followed procedure developed by Ewing and Johnson (1978)

24 mOsm/L , indicating the ability to regulate serum osmotic concentration. Serum osmotic concentration of fish held in fresh water was uniform from January to July (about 310 mOsm/L).

4.3.7.4 Salt Water Tolerance

The fourth test to determine if kokanee could adapt to salt water was the salt water tolerance test. In this procedure, fish were tested by direct transfer from fresh water to 10, 20 or 30 ppt seawater and percent survival after 96 h was determined. In 10 ppt, 100% of the fish survived for 96 hours every month (January through July). In 20 ppt seawater, 100% of the kokanee survived until June when only 80% survived. In full strength salt water (30 ppt) there was 0% survival (100% mortality) from January through April, but in May, June and July, there was 100% survival. Our study indicated that kokanee can survive and osmoregulate in salt water, but it did not show exactly when this starts to occur because of problems in controlling salt concentration from February to April. Due to a malfunction of the salinity meter, the seawater during March and April was made hypersaline (36-42 ppt; 1193 - 1410 mOsm/L) and it could be that if the seawater was 30 - 35 ppt (900 - 1000 mOsm/L), these fish could have osmoregulated and survived. The osmolarity of the full strength seawater in May was 28 ppt (926 mOsm/L) salinity. During this month there was 100% survival. This shows that these fish were able to osmoregulate enough to survive in full strength seawater. Likewise, in June, the osmolarity of the full strength seawater was 964 mOsm/l (30 ppt) and there was 90% survival during this month. In July, the seawater osmolarity was 883 mOsm/l (27 ppt) and there was 100% survival.

Although smolts are able to survive in salinities of 35-40 ppt (McCormick and Saunders 1987; Johnson and Clarke 1988; Clarke et *al* .1989), results of this study showed that fish were not smolted enough to withstand those salinities. Saunders and Henderson (1970, 1978) reported that when juvenile Atlantic salmon were challenged to high salinity (35-40 ppt), it proved to be an effective means of demonstrating additional salinity tolerance that is undeveloped before smoltification. In the present study, fish died in early spring. It is not certain whether these fish died because they could not osmoregulate at all, or if they died because of the unusually high salinities. During the months of May, June and July, when the full strength seawater was between 27 and 30 ppt, kokanee did survive. Further tests must be done to determine exactly when this osmoregulation starts to occur.

All four tests (determination of gill Na^+-K^+ ATPase activity, salt water tolerance and osmoregulatory ability) which were performed to determine if kokanee could adapt to salt water indicated that kokanee were able to osmoregulate about as well as other anadromous salmonid species. However, J_v values were out of phase with other osmoregulation parameters signifying that kokanee do not experience all of the physiological attributes of anadromous salmon.

4.3.8 Residualization of Smolt Transitions

Many species of anadromous salmonids, particularly steelhead/rainbow, become residualized if they do not reach salt water within about 60 days after the onset of smoltification. These residualized smolts stop their migration, their osmoregulatory systems readjust to fresh water, and they remain in the river until spawning. Thus if kokanee experience a similar phenomenon, entrainment losses could be reduced if kokanee are released as residualized smolts, instead of presmolt fry or fingerlings.

The present study confirmed that kokanee smolts began to residualize by May or June. This was indicated by:

(1) Thyroxine peaked in February and April, then declined by July.

(2) Loss of parr marks and silvering began to increase markedly in February and peaked in May when 100% of the individuals exhibited silvery smolt coloration. Parr marks reappeared in June on about 60% of the individuals.

(3) Downstream migratory activity peaked in March and May at 65% in behavioral tests. By June, only 40% of the fish evidenced downstream migratory activity.

(4) Condition factor, which declined from 1.3 to 0.8 during the spring began to increase in June to about 0.9.

(5) Intestinal water uptake peaked in January and was lowest in June.

(6) Gill Na^+-K^+ ATPase activity peaked in mid April at 13.7 $\mu mol\ P_i \cdot$ mg Protein-l $\cdot\ h^{-1}$. The value then decreased to 7.0 $\mu mol\ P_i$. mg Protein-l $\cdot$ h-1 in May and remained low in June.

(7) Osmoregulatory activity and salt water tolerance became evident in May. In June, fish still displayed the ability to osmoregulate and survive in full strength seawater.

Thus, June reductions in six of the seven smolt transition indicators suggested that this stock of kokanee began to residualize by June. Particularly noteworthy, their behavior (i.e., downstream migratory activity and salt water preference) appeared to revert back to the presmolt condition. Thus, kokanee released in July as residualized smolts might be more likely to remain in the reservoir than those released as fry or presmolt fingerlings. Therefore, to maximize kokanee retention in Lake Roosevelt, we strongly recommend that fish be retained until they are residualized smolts and released after July 1.

4.4 Management Implications

From the information gathered from this report, it was concluded that kokanee salmon exhibit smoltification comparable to that of other salmonid species. Results indicated that kokanee salmon displayed characteristics of smolts but that the degree of smoltification was not as pronounced as in other species of salmon. Also, some smolt characteristics occurred at a different time of the year compared to other salmon species. For example, in kokanee, thyroxine surges and silvering occurred earlier in the year than typically reported for the smolts of anadromous species. Table 26 lists attributes of typical anadromous salmonid smolts and compares those attributes to kokanee salmon. For example, anadromous salmon typically experience a surge of thyroxine, an increase in silvering and a decrease in condition factor. Kokanee salmon experienced all of these transitions. Both anadromous salmonids and kokanee salmon exhibited an increase in gill Na^+-K^+ ATPase and the ability to osmoregulate in full strength salt water.

The only parameters of smoltification which were measured in this investigation which were not similar to anadromous salmon were salinity preference and J_v. Anadromous salmon typically show a definite preference for salt water in the spring. Kokanee showed only a slight preference at the time of smoltification and the length of time they maintained this preference was very brief. This, as well as the J_v decreases could be some of the factors that set kokanee apart from sockeye salmon. In many systems, sockeye and kokanee live sympatrically. However, during the spring,

Table 26. Comparison of anadromous salmonId smoltIfIcatIon and kokanee salmon smoltIfIcation.

Anadromous Salmon	Kokanee
T_4 surges in the spring.	T_4 surges in winter and spring.
Increase in silvering at the time of smoltification.	Increase in silvering in winter.
Increase in downstream migratory behavior in spring	Increase in downstream activity, but not as definite as other species.
Increase in gill ATPase in spring	Increase in gill ATPase in spring.
Condition factor decreases	Condition factor decreases
Osmoregulation occurs during spring.	Osmoregulate as well as other salmon.
Salt water preference in spring is very definite	No definite preference for salt water.
J_V increases in spring	J_V decreases.

sockeye migrate to the ocean while kokanee do not. It could be that the preference for salt water is the directional cue needed to stimulate downstream migration.

Another factor measured was downstream migratory behavior. Usually, species of anadromous salmonids exhibit a definite increase in downstream migratory behavior during the smolt transformation. Kokanee salmon did exhibit this increase, but it was not as definite as other species. Also, kokanee experienced this behavior in the winter as well as the spring. These results may have implications for managing Lake Roosevelt kokanee salmon.

Lake Roosevelt has many intermittent, as well as perennial, rivers and streams that run into it. In October and November, many tributaries that had been dried up from July to October begin to flow again. This introduces new water chemistry into the reservoir. If kokanee were released as fry in July (O-age), they would be in Lake Roosevelt for 5 or 6 months before detecting any change in water chemistry. Then, in fall, when novel water is introduced, it may trigger a T_4 surge that would have the potential to stimulate downstream migration. In other natural lake and reservoir systems that have kokanee populations, when new water enters the lake in the winter or spring, kokanee may experience a T_4 surge owing to the introduction of novel water as is typical of other salmonids (Specker and Schreck 1984; Grau et *al.* 1985; Lin et *al.* 1985; Nishioka et *al.* 1985). However, if the system does not have a flow rate sufficiently high to displace fish downstream, the fish would not have the directional cues necessary to cause migratory behavior. In Lake Roosevelt, kokanee may be displaced downstream at such times because of the relatively high flushing rate compared to other systems. The average water retention time for Lake Roosevelt is approximately 40 days (range typically 15-66 days) (Griffith and Scholz 1990; Thatcher et *al.* In press). This is the number of days required for a particle of water to travel from the head of the reservoir to Grand Coulee Dam, a distance of about 140 miles (232 km). In the late spring, water retention time in Lake Roosevelt is typically less than 30 days and frequently less than 20 days. At these times the reservoir behaves more like a river than a lake.

An example of a different reservoir system in which there is a kokanee fishery is Lake Koocanusa which is the reservoir formed by Libby Dam, in Montana and British Columbia. Kokanee reproduce naturally in Lake Koocanusa. Although some kokanee entrainment occurs in this system, they are not as prone to emigrate as in Lake Roosevelt. Lake Koocanusa has a water retention time ranging from 40-200

days (Scholz et *al.* 1986), meaning the water does not flow as fast as in Lake Roosevelt. This means Lake Roosevelt behaves more like a run-of-the-river reservoir than a storage reservoir and has a higher flushing rate than Lake Koocanusa. This difference in flow may be one of the factors that separates the behavior of Lake Roosevelt kokanee from the behavior of Lake Koocanusa kokanee.

The kokanee from the Spokane Tribal Hatchery are normally released into Lake Roosevelt as zero age fry in July of their first year. Given this, it seems likely that kokanee released as fry into the reservoir would undergo at least partial smoltification with a concomitant high potential to be flushed from the reservoir during the year following release (exactly when has still not been pinpointed). High flow rates (low retention time) would exacerbate this problem.

In 1991, a loss of about 25,000 subadult kokanee was reported based upon counts at Rock Island Dam. During the months of March, April, May and June of that year, the average monthly water retention time in Lake Roosevelt was 25, 18, 19 and 29 days respectively (Thatcher et *al.* in press). Thatcher et *al.* found that harvest rates of kokanee in 1991 dropped to zero in June and July, and 93% of the total kokanee observed at Rock Island Dam were counted between May 20th and June 30th. However, counting did not begin until April 1 so it could be that more kokanee were passed Rock Island Dam in the winter or early spring, but were not counted. Also, creel records indicated that kokanee were concentrated in the forebay of Grand Coulee Dam between February and May 1991 (Thatcher et *al.* in press). Thus, the fish were in good position to be easily entrained as reservoir elevations dropped and water retention times decreased.

A valuable adaptive management lesson learned from our experiences with the rainbow trout net pen program in Lake Roosevelt may have application for managing, kokanee in Lake Roosevelt. Rainbow trout in Lake Roosevelt experienced a certain degree of smoltification (Peone et *al.* 1990; Griffith and Scholz 1991). In floy tagging studies conducted from 1986 to 1991, rainbow were released from net pens each month from March to July. Fish released in March and April were classified as smolts, and those released from mid May to July were classified as residualized smolts. From the total tags recovered, the percentage harvested in Lake Roosevelt and those emigrating below Grand Coulee Dam was determined (Table 27). Higher percentages of rainbow trout released in May, June and July were recovered in Lake Roosevelt (94.5, 93.9, 95% respectively) than those released in March and April (43

Table 27. Summary of recoveries of rainbow trout released from Lake Roosevelt net pens in different months. Percentages are based on the total number of fish released in that month between 1986 and 1991. The difference is probably related to the physiological state: smolt in March/April, residualized in May/June.

Month	Percentage of tagged fish recovered (%)	Relative percentage recovered in FDR (%)	Relative percentage recovered in Rufus Woods	Relative percentage recovered at Rock Island or McNary
Fall	2.1	90.9	6.1	3.0
March	0.6	43.0	10.0	57.0
April	2.8	66.4	16.8	16.8
May	6.8	94.5	4.7	0.8
June	15.5	93.9	6.1	0
July	7.0	95.0	5.0	0

Year(s) harvest	Average # stocked (X 1000)	# Angler trips (X 1000)	Average # harvested (X 1000)	Harvest/stock ratio
1990-1991[1]	241	284.8	76	0.3
1992[2]	323	291.4	148	0.5

1 Before 1992 fish were released from March-June.

2 After 1992 fish were released in May-June in an attempt to increase harvest in Lake Roosevelt.

and 66.4% respectively recovered in Lake Roosevelt). Similarly, the percentage of fish recovered at Rock Island or McNary Dam was high for fish released in March and April (57.0 and 16.8% respectively) compared with May, June and July (0.8, 0 and 0 respectively). This indicates that the smolts began to residualize after April and subsequently stayed in the reservoir better after that. Based on this information, the decision was made to release fish primarily in May and June, when possible, in an attempt to improve harvest rates in Lake Roosevelt. Before 1992, fish were released from March through June and the average number harvested was 76,000 fish (Table 27). After 1992, fish were predominantly released in May and June after they had residualized, and the average number harvested rose to 148,000 fish (Table 27). Fishing pressure was similar and the ratio of number of fish harvested to number of fish stocked increased after 1992, indicating that the increased harvest was related to management techniques of releasing residualized fish rather than fishing pressure or stocking rate. We believe that releasing kokanee after they have residualized could produce benefits similar to those reported for rainbow trout. They would be less likely to migrate out of the reservoir if they were residualized.

One problem with rearing kokanee to residualized smolt stage is that there is currently insufficient water at the Spokane Tribal Hatchery to increase the production of fingerling sized fish. The combined spring and well water production wells at the Spokane Tribal Hatchery have decreased from about 8-10 CFS in 1991 to about 5-6 CFS in 1993 (Buchanan 1993; Matt and Buchanan 1993). This quantity of water is sufficient to raise about 2.5 million kokanee fry, 500,000 rainbow fry and 100,000 kokanee smolts but the smolts have to be released by May or June. At flow rates of 8-10 CFS with this many fish, dissolved oxygen levels in the hatchery raceways were typically 7-8 mg/l. At flow rates of 5-6 CFS, dissolved oxygen levels with the same fish densities declined to about 5 mg/l in the raceways (Peone, personal comm.). At flow rates of 8 CFS, the Spokane Tribal Hatchery could rear about 400,000 kokanee smolts into July plus 2.5 million kokanee fry and 500,000 rainbow fry. At a flow rate of 10 CFS, about 600,000 to 800,000 smolt size fish could be reared in addition to the kokanee and rainbow fry. Thus, one possibility to rear more kokanee to smolt size is to develop a new hatchery well. Buchanan (1993) and Matt and Buchanan (1993) recommended a location for a new well about 3/4 mile southwest of the hatchery, that could produce about 2-4 CFS additional flow without affecting the existing spring or well discharge. The cost of the well and pump would be about $250,000. Additionally, about $25,000 to $50,000/year in extra food costs would be needed. We believe that

these are relatively low costs for the potential biological benefits, especially considering that it could help to reduce conflicts with other potential water needs for power production and anadromous fish.

Even if the well is added, only about 1/4 of the kokanee produced at the Spokane Tribal Hatchery could be reared to smolt-size. Since tag returns indicate virtually no returns from kokanee released as fry, it would be advisable to find a way to rear most of the remaining fry to residualized smolt size before release. One possibility would be to rear more kokanee at the Sherman Creek hatchery; however, this is not tenable because no additional water is available at the site. Another possibility would be to develop a kokanee net pen program similar to that used for rainbow trout. Zero age kokanee could be stocked into net pens in October and held through the following July for release into the reservoir as yearlings (age 1+ post-smolts). If fish are held past their smolt stage in net pens, they may residualize and stay in the lake instead of migrating over Grand Coulee Dam. Estimated cost for purchase of net pens, anchoring systems and startup cost is $250,000 plus annual operating costs of about $65,000/year. Again, this seems to be a relatively low cost for potential benefit. We recommend that both a new well and net pen rearing be pursued.

Relative efficiency of hatchery net pen rearing should be determined by comparing food conversion, disease rates, 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.

A final recommendation is that the fish passage facility at Rock island Dam begin counting earlier in the year (perhaps January or February) in order to give a clearer picture of when kokanee are moving downstream. We know from creel surveys that kokanee are concentrated in the forebay of Grand Coulee Dam from about January to June, so water releases at any time during that period have the potential to entrain kokanee. At present, Rock Island counts do not commence until April 1. Therefore, they could be missing substantial numbers of kokanee that migrate downstream before April 1.

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APPENDIX A

FLOWCHART OF PROCEDURE USED FOR PERFORMING RADIOIMMUNOASSAY ON EXTRACTED T4 SAMPLES.[1]

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

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

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

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

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

(e) **Foam decanting rack.**

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

(a) **25 µl eppendorf pipette with disposable tips;**

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

(c) **Water bath (37 °C);**

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

(e) **Vortexer;**

(f) **Assorted test tube racks;**

(g) **Gamma counter;**

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

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

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

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

Turn on the waterbath and adjust to 37°C. Vortex each T4 calibrator and ^{125}I-T_4 container for 10 seconds.

(3) Label assay tubes:

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

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

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

(d) Label 20 plain (uncoated) tubes 1 .O ml and 25 µl in replicates of 10 each.

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

(5) Add 1.0 ml ^{125}I-T_4 to each Ab coated tube and to the plain (uncoated) tubes marked TCT.

(6) Vortex each tube for 5 sec.

(7) Incubate racks of test tubes in waterbath for 1 hour @ 37°C. [Unknown kokanee sample T_4, calibrator T4, or unknown quality control sample T_4 (i.e., non-radioactive T4) and ^{125}I-T_4 compete for binding sites on the Ab. They bind in proportion to their relative concentrations. This is a reversible binding reaction. Equilibrium is achieved in 1 hour, at which point a portion of both non-radioactive T4 and ^{125}I-T_4 will be bound to the Ab (bound fraction) coated on the bottom of the tube and a portion of both non-radioactive T4 ^{125}I-T_4 will not be bound. The nonbound (free fraction) will remain in the liquid.

(8) Remove racks from waterbath. Remove TCT tubes and set aside. Decant free portion from remaining tubes. Pour liquid into liquid radioactive waste container. Place tubes (except for TCT tubes) into foam decanting rack. Turn the rack upside down on absorbent plastic backed paper and allow them to drain for two minutes, then strike the tubes sharply on the absorbent paper

several times to shake off all residual droplets. **Removing all visible moisture will greatly enhance precision.**

(9) Place each tube in the proper order in counting racks and count for 1 minute in the gamma counter. (Cobra QC Model B5002 Autogamma Counter, Packard Instrument Company).

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

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

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

APPENDIX B

FLOWCHART OF Na^+/K^+ ATPase ASSAY

I. Phosphate Assay

This assay procedure utilizes the method from Zaugg (1982a).

(1) Before starting:

(a) Turn on water bath and adjust to 37°C.

(b) Make up stock solution 1 and 2 for proteins.

(c) Set Citrate out of refrigerator, and set pre-made ATP and gill samples out of -80°C freezer to thaw while labeling tubes.

(d) Set wooden shaker rack and chemicals in order of use in a fume hood.

(e) Label test tubes for protein and enzyme homogenate.

PROTEIN & BLANKS = one tube for each sample plus one blank. Each tube will receive 1 ml of distilled water. Blank tube will receive 1 ml of distilled water plus a standard protein solution made up previously. (Blanks will be used to standardize spectrophotometer.)

GILLS = Use one plastic centrifuge tube for each sample.

ENZYME PREP & BLANK A & BLANK B = Label two test tubes for each gill sample. (i.e., IA & 1B). Also label two tubes Blank A and Blank B. Each tube will receive 0.65 ml Stock A or Stock B. Blanks will receive 0.65 ml Stock A or Stock B.

(2) Homogenize thawed gill samples in large glass homogenizer (10 strokes).

(3) Pour liquid into centrifuge tubes (keep pestle in). Centrifuge tubes are placed in ice bath.

(4) Centrifuge 8 minutes (3500 - 3900 rpm).

(5) Discard liquid and turn tubes upside down to drain: Wipe top of tube with Kimwipe.

(6) Add 0.5 ml SEID to pellet and shake whole rack to suspend pellet. Homogenize with small homogenizer 30 strokes. Pour liquid into same centrifuge tube it came from.

(7) Centrifuge 6 minutes.

(8) Pipet liquid off centrifuged alliquot. Pour into enzyme prep tubes.

(9) Keep enzyme prep in ice bath.

(10) Add 10 µl enzyme prep into each (A & B) with positive displacement micropipet. Wipe off tip after each use.

(11) Add 25µl enzyme prep in protein tubes and 25 µl SEID in blank tube.

(12) Add 0.1 ml(100µl) Na_2ATP to A's and B's and blank

(13) Shake for 1 minute after placing tubes and rack in warm water bath. Let stand for 9 more minutes.

(14) Transfer to ice bath and shake rack for 1 minute.

(15) Add 1.85 ml perchloric acid.

(16) Add octanol. Then place test tubes in specially designed wooden shaker rack.

(17) Add molybdate. Place a sheet of parafilm over tubes, place lid on rack and shake for 30 seconds.

(18) Let bubbles settle before adding 0.5 ml Citrate. Shake another 30 seconds with plastic wrap over tubes. (Citrate gets moldy so freeze in 1 L volumes. If it molds, filter, or use drop of carbon tetrachloride.)

(19) Centrifuge tubes quickly.

Phospate standard curve preparation.

Dilution (µmol)	phosphate standard* (µl)	Stock solution "A" (µl)
blank	0	760
.02	30.8	729.2
.04	61.5	698.5
.06	92.3	667.7
.08	123.1	636.9
.10	153.9	606.1
.12	184.6	575.4

- 0.65 µmol/ml phosphate in liquid form

  Then add: 1.85 ml H_3PO_4
  3.00 ml Octanol
  0.25 ml Molybdate
  0.50 ml Citrate

  Shake according to assay directions above, read octanol layer on spectrophotometer and plot µmol Pi - v - absorbance.

II. Protein Determination (Lowry method)

(1) Make up Stock 1 before assay. Do not mix up more than one day's worth of solution.

(2) Add 25 µl enzyme prep to a test tube containing 1 ml of distilled water.

(3) Add 1 ml Stock 1 solution. Shake rack.

(4) Let digest 5 minutes.

(5) Add 3 ml Stock 2. Shake rack.

(6) Let digest 1 hour. Read on spectrophotometer at 700 nm.

Protein standard curve preparation.

Dilution (µmol)	Protein stock* (µl)	H_2O (µl)
blank	0	1025
.01	10	1015
.02	20	1005
.04	40	985
.06	60	965
.08	80	945
.10	100	925
.12	120	905
.14	140	885
.06	160	865
.18	180	845
.20	200	825

* 1 mg/ml protein

III. Spectrophotometer Procedures

Read all proteins at 700 nm on the spectrophotometer before reading phosphates.

(1) Read protein blank.

(2) Set machine to zero.

(3) Run standard curve protein samples.

(4) Run protein samples.

(5) Rinse machine with distilled water, acetone, and another rinse of distilled water.

(6) Set spectrophotometer wavelength at 312 nm.

Read all phosphate samples at 312 nm.

(7) Run phosphate standard curve samples.

(8) Read all “A” phosphate samples.

(9) Read all "B" phosphate samples.

(10) Rinse machine with acetone and distilled water.

IV. To find Na^+-K^+ ATPase value:

Na^+-K^+ ATPase = μmol Pi . mg Protein-1 · h^{-1}

Phosphate and protein were determined in separate assays. Two solutions (A and B) were used to determine phosphate:

$$\mu\text{mol } P_{ia} = \frac{\text{absorbance of phosphate "A" samole}}{\text{slope of "A" standard curve}}$$

where: "A" = salt solution without ouabain (see text)

$$\mu\text{mol } P_{ib} = \frac{\text{absorbance of phosphate "B" samole}}{\text{slope of "B" standard curve}}$$

where: "B" = salt solution with ouabain (see text)

Protein was determined by the equation:

$$\text{mg Protein} = \frac{\text{absorbance of orotein samole}}{\text{slope of protein standard curve}}$$

Na^+-K^+ ATPase activity was determined by the equation:

Na^+-K^+ ATPase activity = "A" activity - "B" activity

where: "A" activity = (μmol P_{ia} · mg $Protein^{-1}$)15, and

"B" activity = (μmol Pib · mg $Protein^{-1}$)15