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Title: HORMONAL AND OSMOREGULATORY ASPECTS OF SMOLTIFICATION IN COHO SALMON, ONCORHYNCHUS KISUTCH

Abstract approved: Redacted for privacy

Carl B. Schreck

Coho salmon smolts (Oncorhynchus kisutch) were transported at low and high densities (12 and 120 g/L) for short and long periods (4 and 12 h). Because smolts can be transplanted directly to seawater, half of the fish in each treatment were transported to tanks containing seawater and half to tanks containing freshwater. Plasma corticosteroids and glucose were elevated at unloading in all groups. Corticosteroids were still above the resting levels 24 h later, whereas glucose had returned to basal levels at this time. Potential smoltification indicators such as plasma thyroxin concentration and gill Na-K-ATPase activity were not affected by transportation. Increased corticosteroids were correlated to increased mortality in transported salmon compared to acclimated control fish when subjected to a bioassay of stress—severe confinement. It is concluded that transportation induced stress in the fish regardless of hauling regimen, that increased corticosteroids may have potential as indicators of reduced performance capacity, that the greatest stress occurred during loading and the first few hours en route, and that transported coho salmon smolts seem to be equally fit for entry into freshwater or seawater.

Hormones of the hypothalamic-pituitary system probably mediate the environmental changes and endogenous rhythms that regulate the timing and physiological alterations of smoltification. Because thyroid hyperactivity is a major endocrine component of smoltification, yearling coho salmon were injected with mammalian prolactin (PRL) and thyrotropin (TSH) to determine their effect on plasma thyroxin concentrations. The response of plasma thyroxin to TSH is similar from January through May in coho salmon, suggesting that the thyroid does not change in sensitivity to TSH. A dose of 0.04 to 0.07 I.U. TSH is the minimum dose sufficient to significantly increase plasma thyroxin concentration. PRL (1 to 9 I.U.) depressed plasma thyroxin levels in coho salmon parr, smolts, and post-smolts.

Increased plasma thyroxin and gill Na-K-ATPase levels tentatively are considered indicative of smoltification, migratory readiness, and hence, seawater adaptability. In an experiment to consider a methodology which could be implemented at a culture facility to enhance the survival of ocean-going smolts and perhaps mitigate losses due to stunting, an abnormality of smoltification, coho salmon parr were maintained for 3 wk in water supplemented with sodium or calcium salts. Prolonged residence in sodium-supplemented freshwater increased plasma thyroxin levels and tended to elevate gill Na-K-ATPase activity. In contrast, acute exposure (24 h) to 75% seawater halved plasma thyroxin levels in coho salmon parr. Gradual acclimation to increased ambient salinity may accelerate changes in, or enhance, plasma thyroxin levels and gill Na-K-ATPase activity, and thus potentially improve the growth and survival of outmigrating smolts and reduce losses due to stunting.

Plasma corticosteroid levels were determined during smoltification and in response to mammalian PRL and TSH. The interrenal tissue, which synthesizes corticosteroids, becomes hyperactive during smoltification. Exogenous PRL and TSH have no effect on plasma corticosteroid levels at any time during smoltification. Plasma corticosteroid levels increase eight-fold between early April and late May in coho salmon, concurrent with increasing gill Na-K-ATPase and seaward migration. Generally, plasma levels of thyroxin and corticosteroids are related inversely. Thyroxin levels are maximum in early April, with the onset of silvering, and corticosteroids are at minimal concentrations at this time. Thereafter, thyroxin levels decline and corticosteroids increase.

Hormonal and Osmoregulatory Aspects of Smoltification in Coho Salmon, *Oncorhynchus kisutch*

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I. GENERAL INTRODUCTION AND MAIN FINDINGS

Despite the fact that the number of hatchery-raised coho salmon has almost doubled in the last decade, the number of commercial landings of coho salmon in Oregon was recently the lowest since 1961 (Gunsolus 1978). The release of fish of higher quality rather than in greater quantities may be the more effective means to mitigate the economic loss suffered by the fishery with the demise of the wild salmon runs. Most mortalities occur within the first year after smoltification, including migration and seawater entry. The general goals of this study were to develop culture practices which would increase the chances of salmon smolts reaching the ocean, growing, and surviving to the harvestable adult stage and to improve our understanding of the physiology of smoltification.

The first problem addressed was whether transportation reduced the survival potential of coho salmon smolts by altering their ability to make the transition from freshwater to seawater. Juvenile anadromous salmonids are routinely transported for various reasons, including the circumvention of dams. Three bioassays were used to discern whether transportation reduced the fish's capacity to survive. The performance of recently transported fish was compared to acclimated control fish. The bioassays included

(1) survival under severely crowded conditions in seawater vs. freshwater, (2) expression of latent kidney disease in seawater vs. freshwater, and (3) outmigration following release into a stream. Furthermore, I investigated (1) the effect that transportation has on potential physiological indicators of smoltification, such as plasma thyroxin levels and gill Na-K-ATPase activity, and (2) the effects that duration in transit and hauling density have on a transported fish's capacity to perform in the bioassays of survival potential.

I found that transportation reduced the ability of coho salmon smolts to survive in one bioassay, severe crowding. This reduced performance capacity was correlated with clinical assessments of stress, such as increased plasma concentrations of glucose and corticosteroids. Probably the most important finding of this first study was that direct entry into seawater following transportation did not adversely affect the coho salmon's performance in any of the bioassays of stress. This information suggests that transported coho salmon smolts are equally fit for entry into seawater or freshwater. The performance of salmon populations with regard to the transition from freshwater to seawater probably is linked with their degree of metamorphosis or variation among individuals in smoltification.

The investigations which followed from the first study focused on endocrine aspects of smoltification. There are few details of how hormones regulate the phenomenon of smoltification at present. Hoar (1965, 1976) suggested that the hormones of the adeno-

hypophysis will be found to mediate the environmental changes and endogenous rhythms that regulate the timing and physiological alterations of smoltification.

As early as 1939, Hoar observed that the activity of the thyroid increased during smoltification. Recently, the histological appearance of thyroid hyperactivity has been confirmed by measurements of increased plasma thyroxin concentrations during smoltification (Dickhoff et al. 1978). Baggerman (1960, 1962) credited the thyroid hormones with the change in preference from freshwater to seawater. Thyroxin also appears to be responsible for other components of smoltification, such as the silvering which is due to increased guanine deposition (Landgrebe 1941; Piggins 1962; Dodd and Matty 1964), and possibly changes in enzymes involved in hyposmotic regulation (Folmar and Dickhoff 1979).

The first question that I asked was, What is the cause of the increase in thyroxin that seems critical to smoltification? I approached this question by examining the response of the thyroid, in terms of changes in plasma thyroxin concentration, to two hormones of the adenohypophysis, thyrotropin (TSH) and prolactin (PRL), during the transformation from parr to smolt.

There were already two studies on the thyroxin response to TSH in other salmonids, the brook trout, Salvelinus fontinalis (Chan and Eales 1976) and the rainbow trout, Salmo gairdneri (Milne and Leatherland 1978). From these reports I was able to estimate an appropriate dose range of TSH to try in coho salmon. Two experiments were conducted in which various doses of TSH were injected and the

thyroxin response measured 24 h later. The thyroxin response to TSH in coho salmon was very similar to that in brook trout and rainbow trout. Doses of 0.04 and 0.07 I.U. TSH were found to significantly increase thyroxin and were evaluated as being physiological. From this information, I designed an experiment in which two doses of TSH, 0.01 and 0.05 I.U., were injected each day for two consecutive days at 28 d intervals from early January to late May. Had the peak in thyroxin been due to the development of increased sensitivity of the thyroid to TSH, I would have expected to see a change in the response to TSH through time. However, fish injected with a total of 0.1 I.U. TSH had similar thyroxin levels at all six sampling times, which were greater than the thyroxin titers in the control fish. Furthermore, the lower dose, 0.02 I.U. TSH, never significantly increased thyroxin levels relative to the titers in the control fish. Hence, I concluded from this study that the thyroid does not change in sensitivity to TSH. Perhaps, instead, thyroxin increases because the secretory activity of the thyrotropes, the pituitary cells which produce TSH, changes during smoltification, the peripheral metabolism of thyroid hormones changes, or the sensitivity of the hypothalamic-pituitary system to thyroxin changes.

PRL has long been recognized as the hormone responsible for the maintenance of hydromineral balance in, and thus survival of, teleosts in freshwater (Pickford and Phillips 1959). The possibility that PRL might affect thyroid activity was suggested by research on the physiology of amphibian metamorphosis and seemed hopeful on the basis of one study in a teleost.

Several aspects of amphibian metamorphosis correspond to salmonid smoltification. The similarities include a migration from freshwater to a dehydrating environment (seawater vs. land) during a transition from a juvenile stage to an adult stage, which is characterized by growth and gonadal maturation (after which amphibians, generally, and salmonids return to freshwater to breed). Also, metamorphic climax, like smoltification, is preceded by a surge in thyroid hormone secretion (Regard et al. 1978; Mondou and Kaltenbach 1979). These resemblences suggested possible evolutionary parallels between the endocrine modulation of smoltification and amphibian metamorphosis.

In amphibians, thyroxin-induced metamorphosis is antagonized by PRL (for review, see Frye et al. 1972; Licht et al. 1972; Eddy and Lipner 1975; Clemons and Nicoll 1977). It is unclear whether PRL inhibits the thyroid gland itself (Gona 1967, 1968) or interferes with thyroxin at the target tissues (Bern et al. 1967; Jaffe and Geshwind 1974) or both.

Among teleosts, there is one report that PRL inhibits thyroxin levels by acting as a goiterogen (Grau and Stetson 1977). Thus, it seemed probable that PRL might temper the secretory activity of the thyroid during smoltification. In fact, there is evidence that during smoltification in one species of Pacific salmon, Oncorhynchus masu, the PRL-producing eta cells of the pituitary become increasingly innervated by inhibitory neurons originating in hypothalamic nuclei (Zambrano et al. 1972). Bern (1978) suggested that the decreased activity of the eta cells is indicative of the inhibition of PRL secretion in preparation for seawater entry. Hence, my idea was:

If PRL antagonizes thyroid activity in salmon, the decrease in activity of the eta cells may be permissive to the enhanced production of thyroxin accompanying smoltification.

I found evidence to suggest that heterologous PRL can reduce thyroxin levels during both the parr and smolt phases of salmon development. This information indicates that the endocrine modulation of smoltification may find further parallels with the endocrine regulation of other developmental processes, such as amphibian metamorphosis.

As a consequence of the finding that PRL could inhibit thyroxin levels during smoltification, a further experiment was designed with the goal of considering a methodology which could be implemented at a culture facility to enhance the survival of ocean-going smolts and perhaps mitigate losses due to stunting, an abnormality of smoltification. Fish which have stunted fail to grow in seawater, resume a parr-like appearance, and suffer considerably mortality (Kennedy et al. 1976). Basically, I tested the effects of rearing coho salmon parr in water supplemented with sodium or calcium salts on plasma thyroxin and gill Na-K-ATPase levels. These two physiological variables were measured because they are considered tentatively as indicators of smoltification.

The hypothesis that increased ambient salinity might accelerate smoltification, or some components of smoltification, was derived from research on amphibian development. Low ambient salinity has been found to increase the rate of metamorphosis in bullfrog tadpoles, Rana catesbeiana (Ray et al. 1978). There was evidence to suggest

that the effects of increased ambient salinity on rate of metamorphosis were probably due to depressed concentrations of plasma PRL, which is anti-metamorphic.

Prolonged residence in sodium-supplemented water did increase plasma thyroxin levels and also tended to elevate gill Na-K-ATPase activity. I suggest that the gradual acclimation of smolting coho salmon to increased ambient salintiy may accelerate changes in, or enhance, plasma thyroxin and gill Na-K-ATPase activity and thus, potentially improve the growth and survival of outmigrating smolts and reduce losses due to stunting.

In my final study, I determined changes in the plasma concentrations of corticosteroids during smoltification. Although activation of the interrenal tissue, which elaborates corticosteroids, has been observed histologically during smoltification (Fontaine and Olivereau 1957, 1959; Olivereau 1962, 1975; McLeay 1975; Komourdjian et al. 1976), there was no information on actual changes in circulating levels of corticosteroids. Moreover, it was of interest to examine the general relationship among plasma corticosteroids, thyroxin, and gill Na-K-ATPase during smoltification.

Generally, increased gill Na-K-ATPase activity is considered critical to the development of salinity tolerance in salmonids (Zaugg and Wagner 1973; Zaugg and McLain 1976; Giles and Vanstone 1976) and indicative of migratory readiness (Zaugg and Wagner 1973; Lorz and McPherson 1976). Corticosteroids elevate gill Na-K-ATPase activity in eels, Anguilla sp. (Epstein et al. 1971; Doyle and Epstein 1972; Kamiya 1972) and coho salmon (Schreck and Ejike, unpublished).

There are also correlative data to suggest that thyroid hormones stimulate the activity of this enzyme (Folmar and Dickhoff 1979). Thyroid hormones causally elevate gill Na-K-ATPase *in vitro* in the nurse shark, *Ginglymostoma cirratum* (Honn and Chavin 1977). Hence, it seemed that a description of the actual changes in thyroxin, corticosteroids, and gill Na-K-ATPase during smoltification would help clarify the roles of thyroxin and corticosteroids in the development of salinity tolerance as well as generate useful questions about the physiology of smoltification.

I found that plasma levels of thyroxin and corticosteroids were related inversely. In early April, coincident with the onset of silvering, thyroxin was at a maximum and corticosteroids at a minimum. Thereafter, thyroxin declined and corticosteroids increased eight-fold by late May. These changes in thyroxin and corticosteroids were compared to data taken from Zaugg and McLain (1970, 1976) on gill Na-K-ATPase levels in coho salmon raised at the same temperature and which had parallel changes in growth patterns. Generally, gill Na-K-ATPase doubles in the second half of March in coho salmon raised at 10 C and peaks in May. If the assumption is made that these trends are representative and comparable, then thyroxin peaks about a month before enzyme activity peaks and the enzyme activity begins increasing while corticosteroids are still relatively low. This observation, with its inherent assumptions, leads me to speculate that thyroid hormones might have a role at least in the initiation of the increases in gill Na-K-ATPase activity that occur during smoltification. Corticosteroids, on the other hand,

may be involved in the maintenance of high Na-K-ATPase activity. The seaward migration of coho salmon begins in April, peaks in late May, and ceases by late June (see Conte et al. 1966; Lorz and McPherson 1976). Thus, corticosteroids may be increasing when the salmon enter seawater and elevated corticosteroid titers probably serve to maintain the gill Na-K-ATPase levels critical to seawater survival.

II. STRESS RESPONSES TO TRANSPORTATION AND FITNESS FOR MARINE SURVIVAL IN COHO SALMON SMOLTS

Introduction

Juvenile anadromous salmonids are transported for various reasons and this causes mortality that may be directly attributable to transportation (Ayles et al. 1976). The primary responses to handling and confinement, as perhaps incurred by fish during transportation, include increased levels of circulating catecholamines (Mazeaud et al. 1977) and corticosteroids (Mazeaud et al. 1977; Strange et al. 1977; Strange et al. 1978). Secondary effects of handling include fluctuations in blood chemistry, such as hyperglycemia, and indications of osmoregulatory dysfunction (Wedemeyer 1972). Such physiological changes in transported fish could alter their capacity to tolerate any second stress (e.g., those encountered after liberation into the wild) or alter their ability to adjust to direct entry into seawater.

The main objective of this study was to determine if transportation reduced the survival potential of smolting coho salmon (Oncorhynchus kisutch) by altering their ability to make the transition from freshwater to seawater. A transported fish was defined as being in a condition of stress if its capacity to perform in one of three bioassays was reduced compared to acclimated controls. The bioassays used to measure decreased performance included: (1) increased percent mortality after 24 h in a severely crowded live-cage in seawater vs. freshwater, (2) increased expression of latent disease in seawater vs. freshwater, and (3) decreased percent outmigration

following release into a stream.

I investigated (1) the effect that transportation has on potential smoltification indicators such as plasma thyroxin levels and gill Na-K-ATPase activity in fish transported to seawater vs. those transported to freshwater, and (2) the relationships that duration in transit and hauling density have to the degree of stress experienced by transported coho salmon. Clinical indicators of stress have never been adequately correlated to reduced performance (see Schreck and Lorz 1979). Therefore, accepted clinical estimates of stress, such as plasma concentrations of corticosteroids and glucose, were evaluated as indicators of actual reduced performance capacity.

Materials and Methods

The coho salmon smolts used in this study averaged 30 g and were obtained from Sandy Fish Hatchery, Oregon Department of Fish and Wildlife, Oregon. The four hauling regimens included low and high densities (12 and 120 g/L) and durations defined as short and long (4 and 12 h). The fish were transported in 200 L plastic cans that contained about 200 fish each; density was controlled by adjusting the amount of water in each can. Control groups were hauled at 12 g/L to Oregon State University's freshwater holding tanks in Corvallis and to the seawater holding tanks at the Marine Science Center in Newport on 17 April 1978. The fish used to assess the effects of transportation were transported to the seawater tanks on 1 May 1978, and to the freshwater tanks on 8 May 1978. They were held at less than 12 g/L in circular tanks with water flow at 4 L/min. Food was withheld the

day before transport, but thereafter all fish were fed to satiation daily with Oregon Moist Pellets.

Water quality in transit - Dissolved oxygen (Yellow Springs Instruments) remained near saturation (10.4 ± 3.9 mg/L) in all hauling cans. The ammonia concentration (Bausch and Lomb) remained between 2.0 and 3.4 mg/L in water in which fish were at a low density and between 6.3 and 10.6 mg/L in water in the high density group. The pH (Beckman) remained between 6.4 and 7.5. Water temperatures were maintained at 11.5 ± 2.5°C, with a notable exception that on 8 May the temperature increased above 15°C during the first 4 h in transit before it could be reduced and maintained with ice.

Effects of transportation on corticosteroids, glucose, thyroxin, and gill Na-K-ATPase activity - Thirty fish per treatment (two replicate tanks; 15 fish/tank), including the acclimated controls, were sampled at the time of unloading and on days 1,4, and 16 post transport (PT). For additional comparison, 30 hatchery fish were sampled at the hatchery on the two days on which the fish were hauled. Fish were captured with minimal disturbance. Blood was collected from the severed caudal artery in heparinized pipettes, centrifuged, and frozen at -20°C until analyzed.

Corticosteroids were assayed in 15 µL plasma according to Strange and Schreck (1978). Of the actual values determined by this assay, 80% were for cortisol and the rest were for other corticoids. Glucose was determined from a pooled (N = 10; 10 µL per fish) 100-µL plasma sample for each replicate, using the Harleco glucose reagent and

Standard Set Kit (Gibbstown, NJ). Thyroxin was determined in 25 µL plasma by using a radioimmunoassay described by Dickhoff et al. (1978). The coefficients of variation for the radioimmunoassay of coho salmon plasma were 13.1% intraassay (N = 10) and 21.8% interassay (N = 5) between 50 and 57% binding in the assay. The average recovery of thyroxin added to plasma was 105% (N = 4).

Gill filaments were taken from exsanguinated fish (N = 15) at all blood sampling times from one replicate of each treatment group transported to seawater holding tanks. Na-K-ATPase activity was determined by Dr. W. Zaugg (U. S. Fish and Wilflife Service, Cook, Washington).

Bioassays of stress - The potential stress imposed by the different hauling regimes and by direct entry into seawater was evaluated, in part, by subjecting the fish to a second stress, severe and prolonged confinement. At the time of unloading, 12 fish from each treatment (replicated), including the acclimated control fish, were placed in a perforated, 1.5 L live-cage, and mortality was recorded.

Postmortem bacteriological analyses were performed on each dead fish for 6 wk following transportation. Following the protocol of The American Fisheries Society (1974), I could detect vibriosis, furunculosis, enteric red-mouth disease, and bacterial kidney disease.

To determine the ability of these coho salmon smolts to migrate following release after transportation, I transported six groups of 150 smolts (average weight = 35 g) from Sandy Fish Hatchery, Oregon, on 23 May 1978 to Mill Creek, Polk County, Oregon, at three "levels"

of stress in duplicate, representing controls at "minimum stress" (2.25 h en route at 12 g/L), and experimental fish at "moderate stress" (6 h en route at 120 g/L) and "high stress" (9 h en route at 120 g/L). These six groups had been distinctly cold-branded on 28 April 1978. The fish were placed in the 200 L plastic cans at various times of the day so they could be liberated at the same time, but following different periods en route. Water temperature at the hatchery was 8.9°C; aeration was supplied by bubbling oxygen through air stones. The fish were released into Mill Creek, 5.6 km above a weir and downstream trap, at 1745 h. Water temperature in the stream was 9.4°C.

Water quality - Water quality in the hauling cans at the time of release of fish was consistent among groups, except for ammonia concentration, which averaged 0.8 mg/L, 8.4 mg/L, and 9.6 mg/L for the minimum, moderate, and high stress treatments, respectively. Dissolved oxygen ranged from 9.0 to 12.5 mg/L, temperature from 11.5 to 12.5°C, and pH from 6.4 to 6.7.

Results and Discussion

Transportation induced stress in coho salmon smolts under these experimental conditions. Initial moments of the capture-loading process may be the major effector of the stress response. All mortalities (1.9%) in transit occurred within 2 hr after loading. No mortalities were observed thereafter that could be attributed directly to transportation. The importance of the capture element of stress has been recognized by Miles et al. (1974) in the muskellunge (Esox

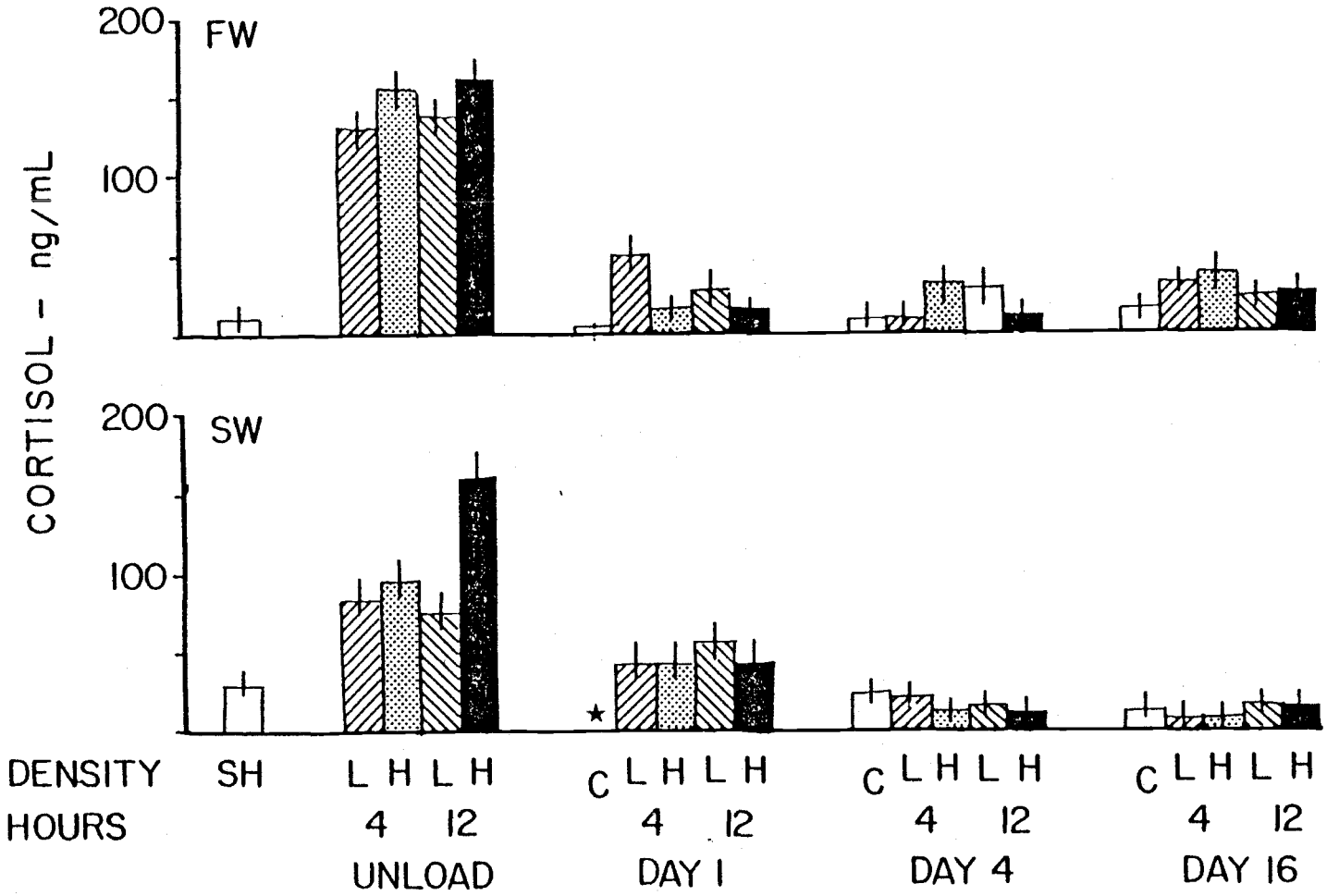
masquinongy) and by Barton et al. (1980) in rainbow trout (Salmo gairdneri).

Clinical indicators of stress - Clinical assessment of these transported smolts indicated that they were in a condition of stress. Plasma corticosteroid concentrations were markedly elevated in all treatment groups when the salmon were unloaded (Fig. 1). Differences in corticosteroid levels between fish transported to seawater and to freshwater are probably the result of a temporary temperature increase experienced during transit by those fish taken to freshwater. Temperature is a critical factor in reducing mortalities in transported fish (Horton 1955) and probably contributed to the fact that all treatment groups had equally high corticosteroids.

Of the coho salmon transported to seawater, corticosteroids were higher in the fish from the high-density, long-duration treatment than in fish from other treatment groups. Water ammonia levels were substantially higher in this treatment group. Although the data on en route mortality indicated that the first few hours, including loading, were the most stressful to the fish, these data from fish transported to seawater suggest that transportation continues to cause stress if the fish are maintained under adverse conditions.

Plasma corticosteroid levels returned to pretreatment concentrations within 24 h in the salmon placed in freshwater holding tanks, but remained slightly elevated in those placed in seawater. This slight difference could reflect a normal functional adjustment to osmoregulation in seawater, or additional stress of seawater. All groups exhibited "resting" levels by day 4 PT, a result in agreement

Figure 1. Plasma cortisol concentrations (mean ± one standard error) (two pooled replicates per treatment; 10-15 fish/pool) in coho salmon smolts following transportation. Fish were transported at 12 g/L (low density; L) or 120 g/L (high density; H) for 4 or 12 h and released into freshwater (FW) or seawater (SW) holding tanks. Samples were taken at Sandy Fish Hatchery (SH) before treatment, at the time of unloading (UNLOAD) and at days 1, 4, and 16 after transportation. Acclimated controls (C) were also sampled, except when not available (*).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UNLOAD DAY 1 DAY 4 DAY 16

Figure 1

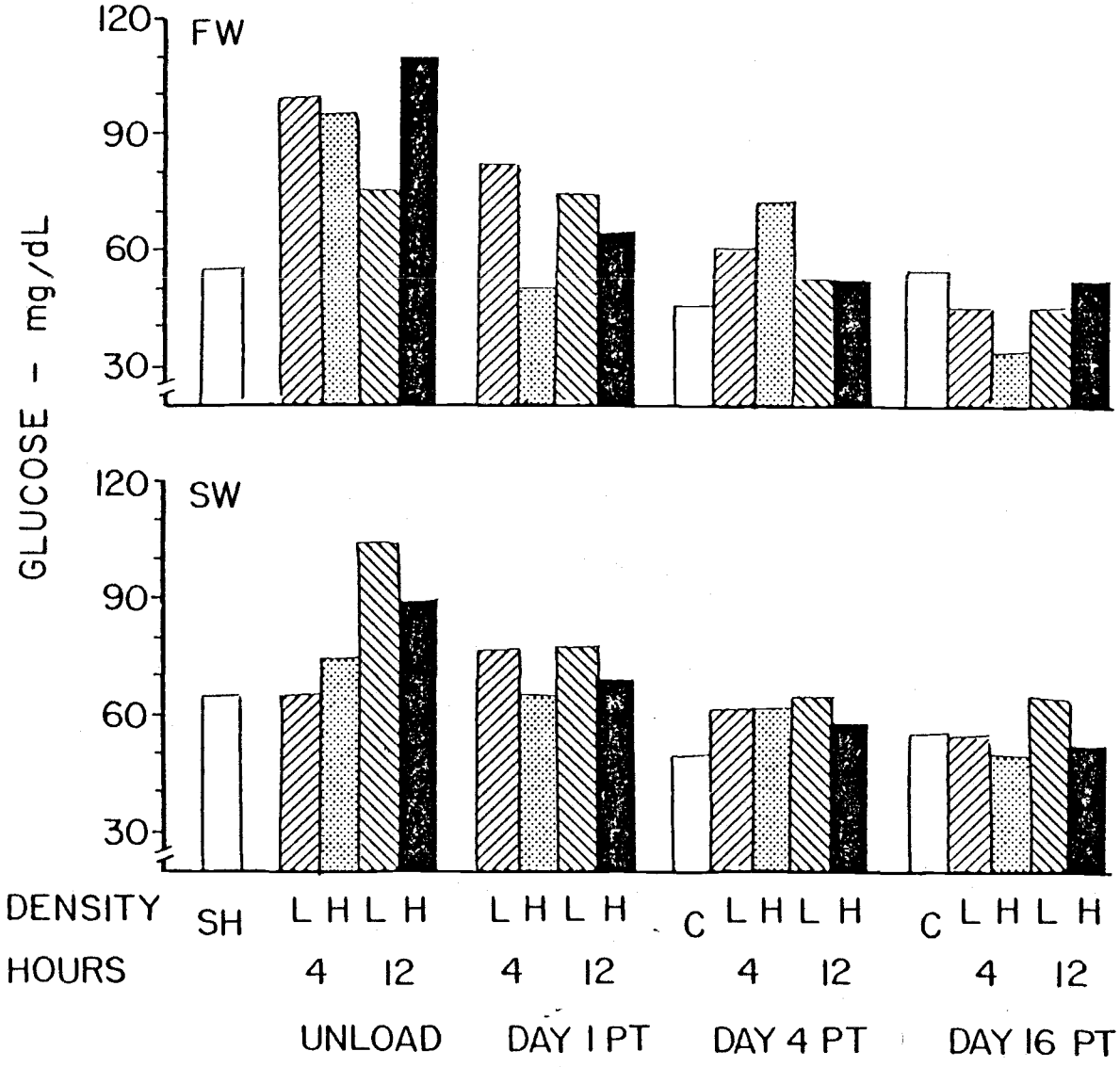
with the findings of Strange and Schreck (1978).

Glucose concentration increased generally during transportation (Fig. 2), as might be expected after handling stress (Wedemeyer 1972). Hyperglycemia was not as pronounced as that found by Wedemeyer, probably because these fish had not eaten for 2 d before sampling at unloading. Carbohydrate metabolism also changed in Atlantic salmon (Salmo salar) as a consequence of transportation (Wendt and Saunders 1973).

Changes in potential smoltification indicators - Transportation did not affect the concentration of circulating thyroxin. A transitory increase in circulating thyroxin following physical injury occurred in rainbow trout (Brown et al. 1978). A general decrease occurred through time, which is typical for coho salmon during this time of year (Dickhoff et al. 1978). Additionally, there was no trend in gill Na-K-ATPase activity among fish from the various treatment groups taken to seawater.

Performance of transported smolts in bioassays of stress - The increased mortality among transported coho salmon subjected to severe crowding compared to acclimated control fish supported the conclusion based on clinical tests that transported fish are in a condition of stress (Fig. 3). Many of the salmon in the 1.5-L crowding cages were dead within 24 h, whereas mortalities occurred in only one of the four acclimated control groups. Most fish were dead by day 4 PT; there were no differences among treatment groups. This bioassay of stress was found to be a fairly reliable indicator of degree of stress with

Figure 2. Plasma glucose concentrations (two pooled replicates per treatment; 10 fish/pool) in coho salmon smolts following transportation. Fish were transported at 12 g/L (low density; L) or 120 g/L (high density; H) for 4 to 12 h and sampled at unloading and 1, 4, and 16 d later. SH represents the mean glucose concentration in fish at Sandy Fish Hatchery on 1 May and 8 May before the treatment fish were transported to seawater (SW) or freshwater (FW), respectively.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SW
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C
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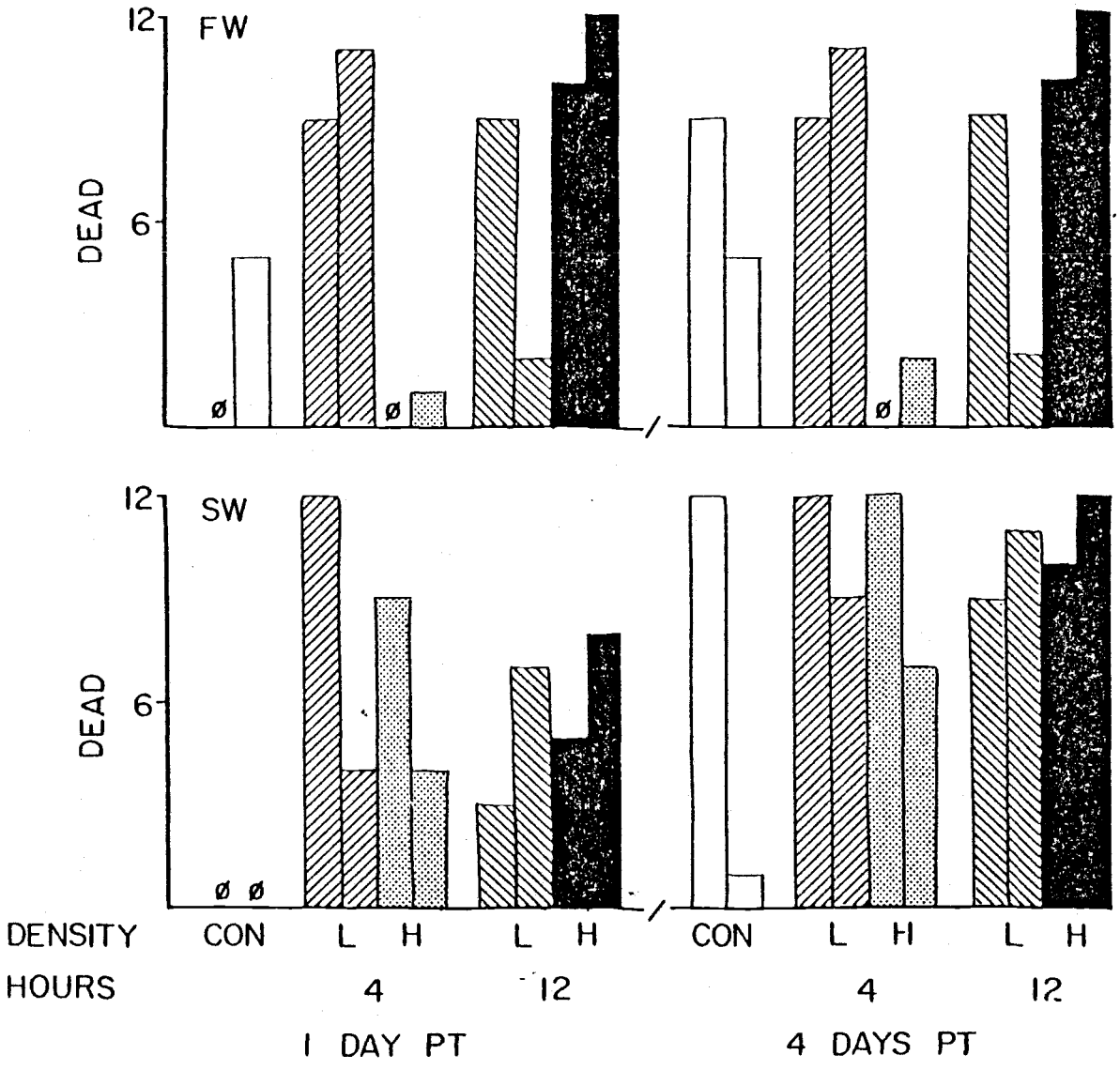
HOURS
4 12
4 12
4 12
4 12
UNLOAD
DAY 1 PT
DAY 4 PT
DAY 16 PT

Figure 2

Figure 3. Cumulative numbers of coho salmon that died during severe confinement in live-cages following transportation (PT). Fish were transported at 12 g/L (low density; L) or 120 g/L (high density; H) for 4 or 12 h. Twelve fish from each treatment were placed in live-cages in either freshwater (FW) or seawater (SW). Acclimated controls (CON) were placed in live-cages at the same time as those that had been transported. Ø indicates no deaths.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12
4
12
1 DAY PT
4 DAYS PT

Figure 3

chinook salmon (Strange and Schreck 1978). Coho salmon, however, persevere so much longer than chinook salmon that cause of death becomes confounded and the test loses sensitivity. Few fish died in the holding tanks during the first 6 wk PT, even though these fish were known carriers of bacterial kidney disease. Apparently, the transport regimen and elevated corticosteroids did not, in this instance, cause expression of disease.

The percent of fish migrating was not affected by the different transportation treatments. All fish schooled in the pool at the release site within minutes after release. None of the fish appeared disoriented. No fish were seen during visual inspection of the pool on subsequent days. A total of 42% of all fish released were captured in the downstream trap in eight days. However, the actual percentage of migrants may have been higher because the weir was not 100% efficient. On the day after release, it appeared as though the number of fish migrating was inversely related to the duration of transit (N = 12, 3, and 0 in 2.25, 6, and 9 hr hauling duration, respectively). Thereafter, however, capture frequencies of all treatment groups were similar. No migration was evident on days 7 and 8 and the weir was removed on day 9. On day 4 and thereafter, many of the fish captured had opaque eyes, and one fish was found impinged on the weir. Consequently, the fish captured toward the end of the period may have entered the trap passively.

In summary, transportation reduced the ability of coho salmon smolts to tolerate a second stress, severe and prolonged crowding. This reduced performance capacity and therefore condition of stress

was correlated with clinical assessments of stress such as increased circulating corticosteroids and glucose. Transportation did not increase expression of disease, change migration-disposition, nor affect potential smoltification indicators such as circulating thyroxin and gill Na-K-ATPase activity. None of the tests in this experiment discerned differences in degree of stress imposed by the different hauling regimes, although 98.9% of the fish that died in the first few hours en route were from the high density treatment group. Importantly, direct entry into seawater following transportation did not adversely affect the smolting coho salmon's performance in any of the bioassays of stress. This information suggests that transported smolting coho salmon are equally fit for entry into seawater or freshwater.

III. PLASMA CONCENTRATIONS OF THYROXIN IN RESPONSE TO EXOGENOUS THYROTROPIN AND PROLACTIN DURING SMOLTIFICATION IN COHO SALMON

Introduction

Juvenile anadromous salmonids undergo a developmental transformation from cryptic-colored, stream-dwelling parr to silvery, ocean-going smolts termed smoltification. A major endocrine component of smoltification is the hyperactivity of the thyroid (Hoar 1939, 1965, 1976; Baggerman 1960; Eales 1963), which results in large increases in plasma thyroxin levels (Dickhoff et al. 1978). Various aspects of smoltification have been credited to the action of the thyroid hormones. These include a change in preference from freshwater to seawater (Baggerman 1960, 1962), increased guanine deposition which causes the silvery appearance (Landgrebe 1941; Robertson 1949; Piggins 1962; Dodd and Matty 1964), and improved osmoregulatory capacity for seawater (Folmar and Dickhoff 1979; Collie and Bern 1980; Chapters IV and V).

Hoar (1965, 1976) has suggested that the hormones of the hypothalamic-pituitary system would be found to moderate the physiological changes associated with smoltification, including hyperactivity of the thyroid. Definite changes do occur in the activity and abundance of the various cell-types of the pituitary during the smoltification of Atlantic salmon, Salmo salar (Olivereau 1954) and the Pacific salmon, Oncorhynchus masu (Zambrano et al. 1972). However, very little work has been done on the effects of pituitary

hormones on plasma levels of thyroxin in teleosts. Chan and Eales (1976) and Milne and Leatherland (1978) have described the effects of mammalian thyrotropin (TSH) on thyroxin titers in the brook trout, Salvelinus fontinalis, and the rainbow trout, Salmo gairdneri. In addition to TSH, prolactin (PRL) has been reported to depress thyroxin in the euryhaline killifish, Fundulus heteroclitus (Grau and Stetson 1977).

My objective was to determine the effects of PRL and TSH on plasma thyroxin concentrations at various times throughout smoltification in the anadromous salmonid, Oncorhynchus kisutch.

Experimental Procedures and Results

Coho salmon were obtained from Sandy Fish Hatchery, Oregon Department of Fish and Wildlife. Fish used in experiments in 1979 were from 1977 brood year and were obtained from the hatchery in January 1979 as underyearlings. Fish used in experiments the following year were from the 1978 brood year and were obtained as eggs in January 1979. Both groups were subsequently reared in freshwater (10-12°C) under natural photoperiod. However, the group of fish used in experiment 2 was fed Oregon Moist Pellets and the rest were fed dry pellets. Food was withheld several days prior to and during the experiments to reduce variability in thyroxin levels, as suggested by Chan and Eales (1976). The experiments were conducted in 200-L tanks in which the fish were first acclimated for 7-10 d. In all cases the fish were randomly distributed into the tanks and the treatments were assigned to tanks using a random numbers table.

Coho salmon of this stock become smolts during the spring of their second year. During this time, gill Na-K-ATPase activity increases (personal communication, W. Zaugg, U. S. Fish and Wildlife Service, Cook, Washington), plasma thyroxin levels increase (personal communication, W. Dickhoff, University of Washington, Seattle, Washington), the parr marks disappear and the skin silvers (personal observation). As well, the fish migrate upon release (Specker and Schreck 1980).

Injection Preparation and Sampling Procedures

The tropic hormones, ovine PRL (oPRL from NIH-P-S13; 30 I.U. = 1 mg) and bovine TSH (bTSH from Sigma, St. Louis, Missouri; 0.73 I.U. = 1 mg), were dissolved in 0.6% NaCl-0.05% NaOH and injected intraperitoneally in 0.06 mL carrier. A crude preparation of salmon PRL (sPRL, a non-glycoprotein with MW = 25,000 extracted from chum salmon pituitaries) was supplied by Syndel Laboratories (Vancouver, British Columbia) and prepared in the same manner. Fish were injected between 1300 and 1500 h while anesthesized with tricaine methane-sulfonate, which has no apparent effect on plasma thyroxin levels in Salvelinus fontinalis (Chan and Eales 1976). Sampling occurred 24 h after the final injection. Fish were captured with minimal disturbance, stunned, weighed, and measured (fork-length = FL). Blood was collected from the severed caudal artery in ammonium-heparinized capillary tubes, centrifuged, and stored up to several months at -20°C until analysis.

Thyroxin Radioimmunoassay

The radioimmunoassay procedures used to determine the concentrations of plasma thyroxin were modified from those described by Dickhoff et al. (1978). Unextracted plasma was assayed in duplicates of 10 µl aliquots. The antibody was obtained from Endocrine Sciences (Tarzana, California) and used at 50-55% binding. High specific activity (700 mCi/mg) ^{125}I-labeled thyroxin (Industrial Nuclear, St. Louis, Missouri) was used at 15,000 dpm. The standards and all solutions were prepared in barbital buffer. The bound hormone was precipitated by polyethylene glycol during centrifugation, the supernatant removed and the pellet counted. Standard curves prepared in coho salmon plasma stripped of thyroxin (Larson et al. 1973) were parallel to those prepared in barbital buffer.

Statistical Analyses

Analysis of variance (ANOVA) was performed on the raw data, or when necessary to correct heteroscedasticity, on $\log_e$ transformations of the data. In cases of unequal sample sizes or missing cells, the regression approach to ANOVA was used (Nie et al. 1975). Insignificant interactions were included in the estimation of the error variance. Contrasts between group means were made using Student-Newman-Keuls (SNK) multiple range comparison test when preliminary ANOVA indicated significant main effects. Coefficient of condition (K) was calculated using the formula: $100\ W/L^3$, where W is the weight in grams and L is the FL in centimeters.

Experiment 1. The effect of dose of bTSH on plasma levels of thyroxin was tested twice. On March 15, 1979, coho salmon (mean weight = 16 g, mean FL = 12 cm) were injected once with the saline vehicle or 0.001, 0.01, 0.10, 0.25, 0.50, or 1.00 I.U. bTSH and sampled 24 h later. The experiment was repeated April 25, 1979, and the fish (mean weight = 19 g, mean FL = 13 cm) were injected with the saline vehicle or 0.001, 0.01, 0.04, 0.07, 0.10, or 0.25 I.U. bTSH. Both experiments included eight fish per group and a group of non-injected fish.

The concentration of thyroxin in the plasma was significantly affected by dose ($F = 23.62 > F_{0.001(9, 117)} = 3.38$) and time ($F = 21.90 > F_{0.001(1, 117)} = 11.4$) (Fig. 4). On March 15, 0.10 and 0.25 I.U. bTSH significantly elevated plasma thyroxin (SNK). The highest dose, 1.0 I.U. bTSH, caused a reduction in thyroxin titers relative to both 0.10 and 0.25 I.U. bTSH (SNK). On April 25, 0.04, 0.07, 0.10, and 0.25 I.U. bTSH elevated plasma thyroxin, but the effects were comparable among doses.

Experiment 2. This experiment was designed to test the effects of different doses of oPRL and sPRL on plasma thyroxin levels. Five different dose levels of sPRL and oPRL were used totaling 0.06, 0.18, 0.30, 0.42, and 0.54 mg, which corresponded to 1.8, 5.4, 9.0, 12.6, and 16.2 I.U. oPRL. Each injection also contained 0.025 I.U. bTSH, with the intention of elevating thyroxin so that the effects of PRL could be detected. One tank of eight fish was assigned to each treatment, with the exception that two tanks of eight fish were injected with only bTSH. For experimental control, eight fish were

Figure 4. Plasma thyroxin concentrations (mean ± one standard error, n = 8) in coho salmon injected with various doses of bTSH at two times during smoltification. CON = non-injected control fish. SAL = saline-injected control fish.

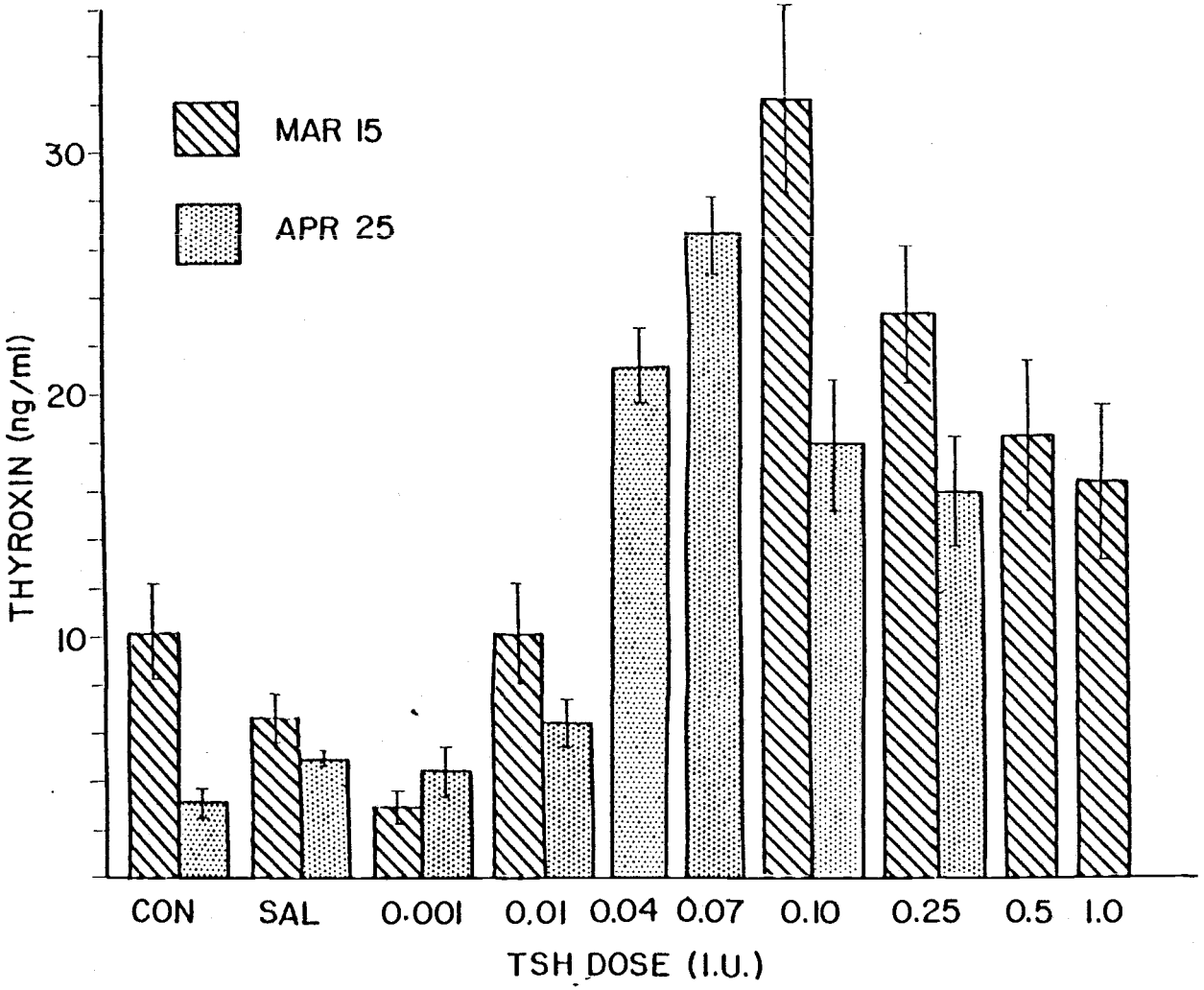
MAR 15
APR 25
30
20
10
THYROXIN (ng/ml)
CON
SAL
0.001
0.01
0.04
0.07
0.10
0.25
0.5
1.0
TSH DOSE (I.U.)

Figure 4

injected with only the saline vehicle and eight fish which were not injected were included in the sampling on February 22, 1980. The sex of each fish was determined at the end of the experiment. The fish averaged 34 g and 15 cm (FL) and K = 1.00 ± 0.02 (mean ± one standard error).

The data were analyzed as a 2 x 12 (sex x treatment) factorial experiment. The sex of the fish did not affect plasma thyroxin concentrations at any level of treatment ($F = 0.50 < F_{0.05(1, 96)} = 3.95$). Treatment with bTSH, sPRL, and oPRL affected thyroxin levels ($F = 2.76 > F_{0.01(11, 96)} = 2.43$) (Fig. 5). Comparison of mean thyroxin levels revealed only one significant difference which occurred between the control fish and bTSH-treated fish (SNK). However, the 90% confidence intervals about the means of three groups of oPRL plus bTSH-treated fish and three groups of sPRL plus bTSH-treated fish were lower than the 90% confidence interval about the mean of the bTSH-treated fish, suggesting a strong tendency for PRL to lower thyroxin levels.

Experiment 3. This experiment was performed to determine whether the effects of oPRL on circulating thyroxin levels were dose-related. Coho salmon (mean weight = 25 g, mean FL = 14 cm, 10 fish per group) were injected for two consecutive days (June 15-16, 1979) with a total of 1.8, 3.6, or 18.0 I.U. oPRL, or 0.05 bTSH plus 1.8, 3.6, 18.0 I.U. oPRL. Ten fish were injected for two days with the saline vehicle. Ten fish that were not injected were also sampled.

The effect of oPRL on the concentration of thyroxin in the plasma was dose-related in this experiment (Fig. 6). Both oPRL

Figure 5. Plasma thyroxin concentrations (mean ± one standard error, n at the base of the bar) in coho salmon parr injected for two consecutive days with bovine TSH plus various doses of ovine PRL or crude salmonid PRL. CON = saline-injected and non-injected control fish.

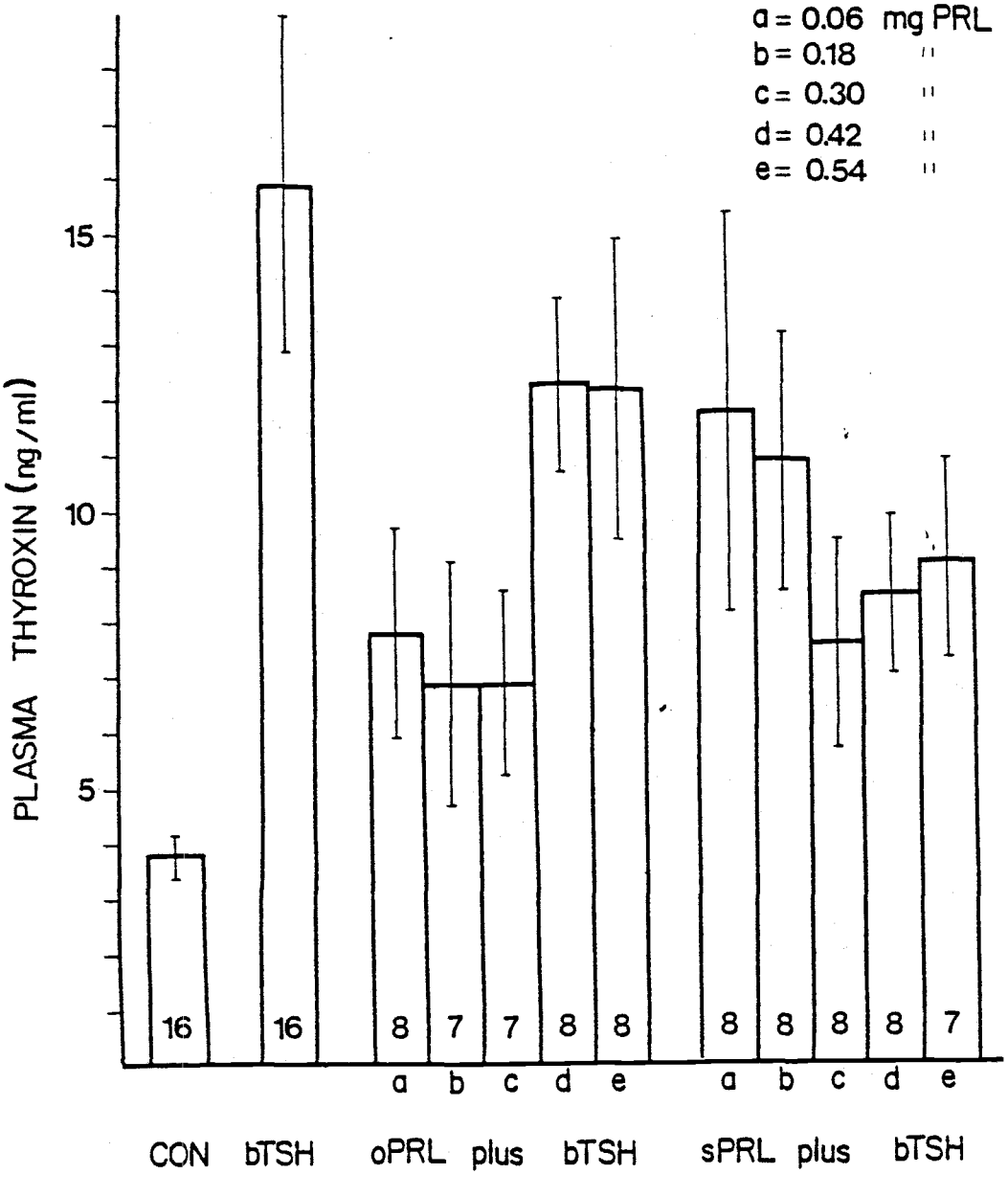
a = 0.06 mg PRL
b = 0.18 "
c = 0.30 "
d = 0.42 "
e = 0.54 "
PLASMA THYROXIN (ng/ml)
15
10
5
16
16
8
7
7
8
8
8
8
8
8
7
a
b
c
d
e
a
b
c
d
e
CON
bTSH
oPRL plus bTSH
sPRL plus bTSH

Figure 5

Figure 6. Plasma thyroxin concentrations (mean ± standard error; n = 10) in coho salmon smolts injected for two consecutive days with various doses of ovine PRL alone or in combination with bovine TSH. PREP = injection preparation. Dose (I.U.) is in parentheses. CON + SAL = pooled results from non-injected and saline-injected control fish.

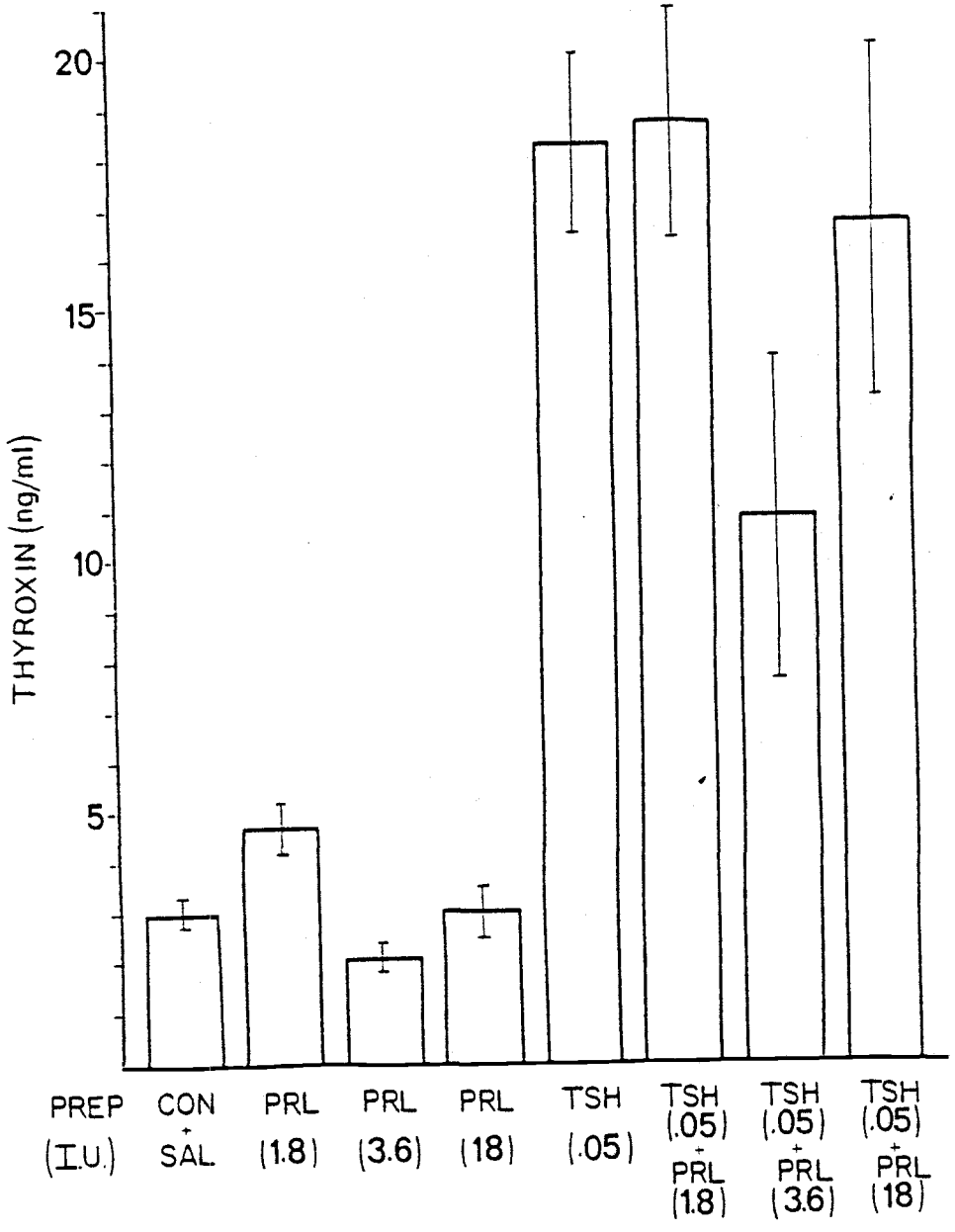
THYROXIN (ng/ml)
20
15
10
5
PREP (I.U.)
CON + SAL
PRL (1.8)
PRL (3.6)
PRL (18)
TSH (.05)
TSH (.05) + PRL (1.8)
TSH (.05) + PRL (3.6)
TSH (.05) + PRL (18)

Figure 6

($F = 7.30 > F_{0.01(3, 72)} = 4.08$) and bTSH ($F = 170.53 > F_{0.01(1, 72)} = 7.01$) had significant effects on plasma thyroxin. Fish that received 3.6 I.U. oPRL had lower thyroxin concentrations than those injected with 1.8 I.U. oPRL (SNK). Plasma thyroxin levels were lower in fish injected with 3.6 I.U. oPRL plus 0.05 I.U. bTSH than in fish injected with 0.05 I.U. bTSH alone or 1.8 I.U. oPRL plus 0.05 I.U. bTSH (SNK).

Experiment 4. This experiment was designed to test the effects of sequential injections of oPRL and bTSH on plasma thyroxin levels. Four groups of 30 fish each (weight = 18 g, FL = 13 cm) were injected on days 1 and 2 with (1) the saline vehicle, (2) 0.3 I.U. oPRL (each day), (3) 0.013 I.U. bTSH, or (4) 0.3 I.U. oPRL plus 0.013 I.U. bTSH. On both days 3 and 4, 10 fish from each of the four pretreatments were injected with (1) 0.3 I.U. oPRL, (2) 0.013 I.U. bTSH, or (3) 0.3 I.U. oPRL plus 0.013 I.U. bTSH. For experimental control, 20 fish were sampled on day 1 (May 7, 1979), 10 fish were sampled on day 4, and 10 fish were injected with the saline vehicle for four days and then sampled.

The data were analyzed as a 2 x 2 x 3 (the effects of bTSH and oPRL on days 1 and 2 x three treatments of days 3 and 4) factorial experiment. Although the effects of the treatments on days 3 and 4 were highly significant ($F = 18.14 > F_{0.001(2, 108)} = 7.41$), the effects of pretreatment on days 1 and 2 were insignificant ($F = 0.96$ for oPRL and $F = 0.61$ for bTSH). Singular comparisons between treatment groups for the marginal effects of treatment on days 3 and 4 were made using Student's t-test at the 5% level.

Ovine PRL (0.6 I.U. and 1.2 I.U.) substantially reduced the concentration of thyroxin in the plasma (from 4.0 ± 0.5 to 2.4 ± 0.3 ng/ml) ($t = 2.64 = t_{0.005(75)}$) (Fig. 7). Ovine PRL in combination with bTSH also reduced plasma thyroxin levels compared to bTSH-injected fish (from 7.8 ± 0.7 to 5.0 ± 0.7 ng/ml) ($t = 3.00 > t_{0.005(75)} = 2.64$).

Experiment 5. The following experiment was performed six times at intervals of 28 d beginning January 6, 1980. The purpose of this experiment was to investigate the response of plasma thyroxin levels to bTSH and oPRL during the transformation of coho salmon from parr to smolt. Coho salmon were injected each day for two consecutive days with (1) the saline vehicle, (2) 0.01 I.U. bTSH, (3) 0.05 I.U. bTSH, (4) 1.8 I.U. oPRL plus 0.05 I.U. bTSH, or (5) 9.0 I.U. oPRL plus 0.05 I.U. bTSH. Each treatment was replicated in two separate tanks with six fish per tank. Sampling included 12 fish that were not injected, so there was a total of six groups each month.

On the sixth run of this experiment (referred to as May 27b), additional treatments were included. For four consecutive days, the following preparations were injected each day: (1) 1.8 I.U. oPRL, (2) 9.0 I.U. oPRL, (3) 0.6 mg sPRL, or (4) the saline vehicle. Twelve fish were assigned to each treatment.

The increase in size of the coho salmon used in these experiments corresponded to growth in the hatchery with the exception of the fish on May 27 (Table 1). In May, the average weight of the salmon raised at Sandy Fish Hatchery is about 30 g (see Specker and Schreck 1980). However, the sample of fish used in the experiment May 27 tended to weigh less and be shorter than those of April 29 (Student's t-test at

Figure 7. Plasma thyroxin concentrations (mean ± one standard error; n = 10) in coho salmon smolts injected in various sequences with ovine PRL, bovine TSH, or both. The effects of the pretreatment injections on days 1 and 2 were insignificant.

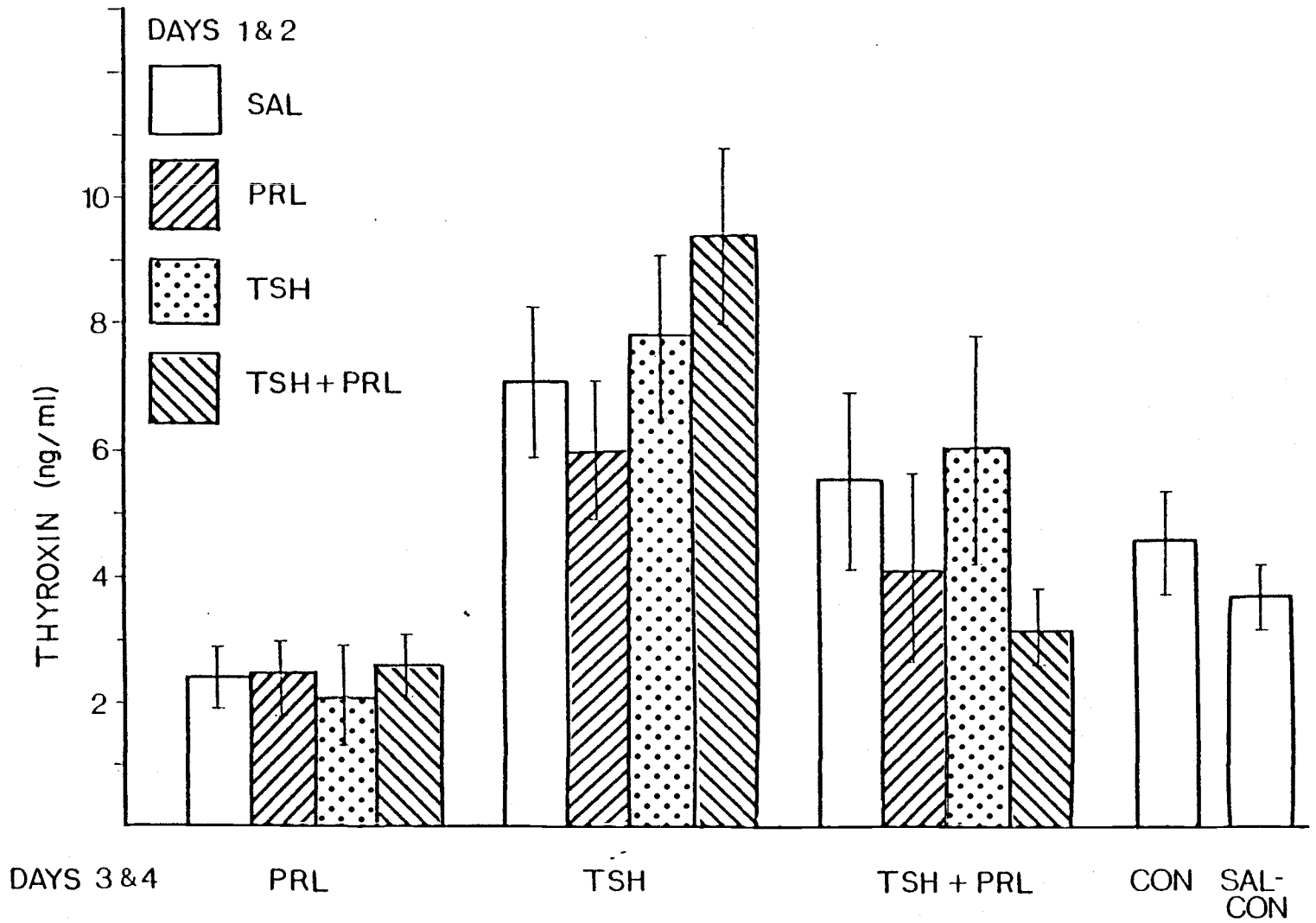
DAYS 1&2
SAL
PRL
TSH
TSH+PRL
THYROXIN (ng/ml)
10
8
6
4
2
DAYS 3&4
PRL
TSH
TSH + PRL
CON
SAL-CON

Figure 7

Table 1. The weight, fork-length, and coefficient of condition (mean ± one standard error; n in parentheses) of coho salmon sampled at 28 d intervals during smoltification.

Date (1980)	Weight (g)	Fork-length (cm)	Coefficient of condition
January 8	16.7 ± 0.5 (69)	11.7 ± 0.1	0.98 ± 0.03
February 5	23.9 ± 0.7 (72)	13.2 ± 0.1	1.02 ± 0.01
March 4	24.3 ± 0.7 (70)	13.3 ± 0.1	1.00 ± 0.02
April 1	27.7 ± 0.9 (71)	13.9 ± 0.2	1.00 ± 0.02
April 29	28.1 ± 0.8 (71)	14.1 ± 0.1	0.97 ± 0.02
May 27a	24.6 ± 1.0 (72)	13.7 ± 0.2	0.92 ± 0.01
May 27b	26.6 ± 1.6 (47)	14.1 ± 0.2	0.91 ± 0.08

the 10% level). Also, the coefficient of condition was lower for the sample on May 27 than at all other times (SNK). This probably resulted accidentally from culling of the fish from the same tank for each month's experiment.

The concentration of thyroxin in the plasma of the control fish increased from 1.6 ± 0.2 ng/ml (n = 22) on January 8 to 5.8 ± 0.2 ng/ml (n = 23) on April 1, and fell to 3.1 ± 0.5 ng/ml by May 27 (n = 24) (SNK following ANOVA with $F = 30.64 > F_{0.001(5, 135)} = 4.42$) (Fig. 8). These changes parallel those in hatchery stocks of coho salmon (Dickhoff et al. 1978).

Although there were significant interactions between time and treatment for plasma thyroxin concentrations ($F = 2.07 > F_{0.01(25, 387)} = 1.84$), the three groups of fish injected with 0.1 I.U. bTSH clearly had higher thyroxin titers (Scheffé's test) (Fig. 8). The mean thyroxin levels in the fish injected with 0.1 I.U. bTSH were similar at all times (SNK). The lower dose of bTSH (0.02 I.U.) evere elevated plasma thyroxin above the control groups (SNK).

Ovine PRL (3.6 and 18.0 I.U.) did not affect the plasma thyroxin response to bTSH, except on May 27. At this time the fish injected with 3.6 I.U. oPRL plus 0.1 I.U. bTSH had higher thyroxin levels than fish injected with either 0.1 I.U. bTSH or 0.1 I.U. bTSH plus 18.0 I.U. oPRL (SNK). However, the fish given four consecutive daily injections of 1.8 or 9.0 I.U. oPRL, or 0.6 mg sPRL at this same time had thyroxin titers comparable to the control fish ($F = 0.86$).

Figure 8. Plasma thyroxin concentrations (mean ± one standard error; n = 10 to 12) in coho salmon injected with bovine TSH and ovine PRL at 28 d intervals during smoltification.

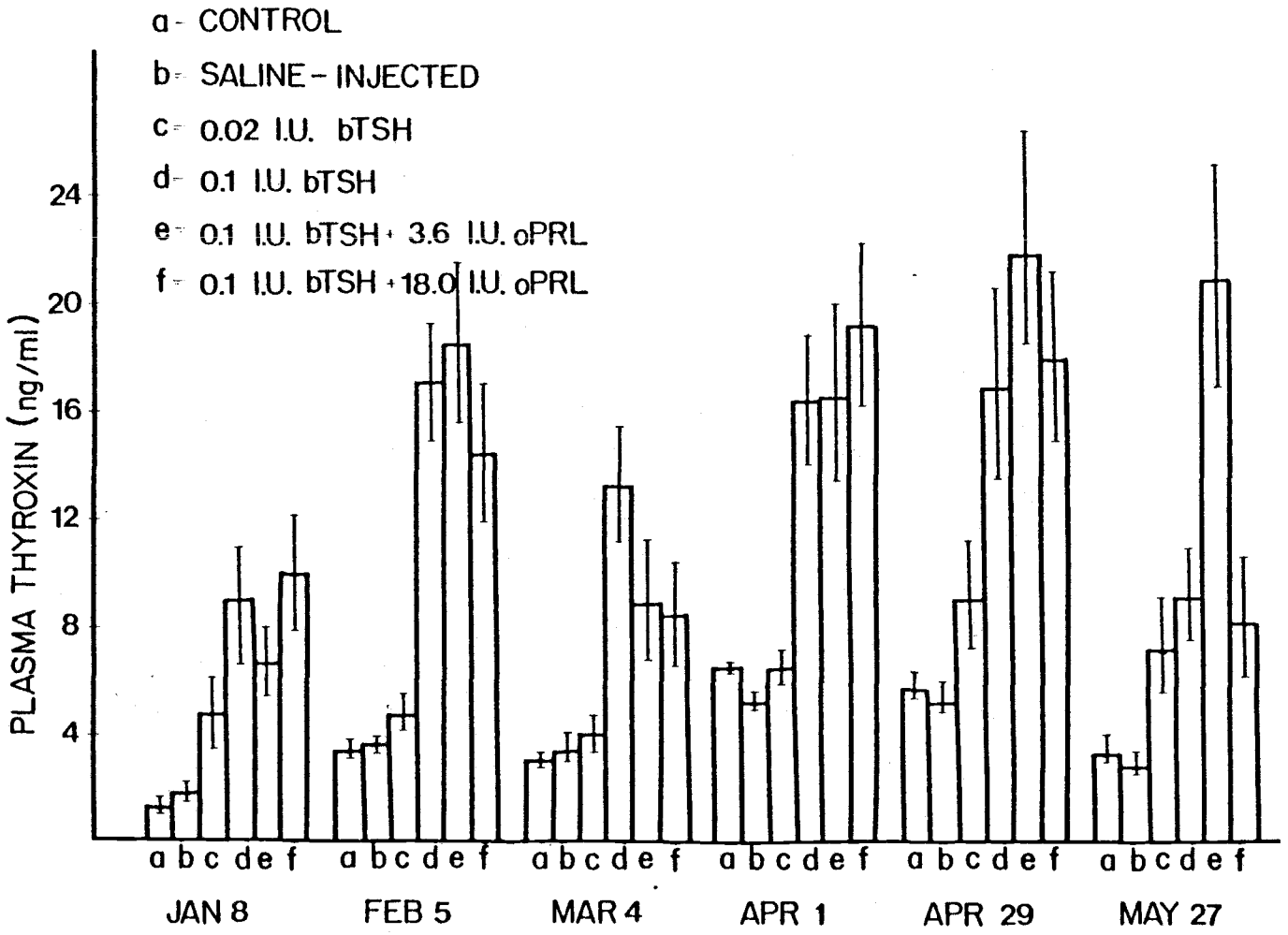
a- CONTROL
b- SALINE - INJECTED
c- 0.02 I.U. bTSH
d- 0.1 I.U. bTSH
e- 0.1 I.U. bTSH + 3.6 I.U. oPRL
f- 0.1 I.U. bTSH + 18.0 I.U. oPRL
PLASMA THYROXIN (ng/ml)
4
8
12
16
20
24
a b c d e f
JAN 8
FEB 5
MAR 4
APR 1
APR 29
MAY 27

Figure 8

Discussion

The concentrations of thyroxin in the plasma of coho salmon during smoltification were found to be similar to thyroxin levels in rainbow trout (Higgs and Eales 1973; Brown and Eales 1977; Leatherland et al. 1977; Milne and Leatherland 1978) and brook trout (White and Henderson 1977), and in coho salmon of some stocks (Folmar and Dickhoff 1979), but lower than levels in coho salmon of other stocks (Dickhoff et al. 1978). The changes I found in thyroxin levels during smoltification paralleled the trends previously reported for coho salmon (Dickhoff et al. 1978). Plasma thyroxin increased from about 1.6 ± 0.2 ng/mL in January to 5.8 ± 0.2 ng/mL in early April. By late May, thyroxin titers declined to 3.1 ± 0.5 ng/mL.

There was no evidence in these studies that the change in thyroxin levels during smoltification results from altered sensitivity of the thyroid to TSH. Coho salmon injected at 28 d intervals from January through April with 0.1 I.U. bTSH had similarly elevated plasma thyroxin levels at all times. The trend in the mean plasma thyroxin levels in 0.1 I.U. bTSH-injected fish was similar to the changes in thyroxin occurring in the control fish. At none of the six sampling times did 0.02 I.U. bTSH effect plasma thyroxin levels. The hyperactivity of the thyroid during smoltification may result from either greater amounts of TSH being secreted by the thyrotropes, as evidenced by histological signs of increased activity in the thyrotropes (Olivereau 1954; Bern 1978), an alteration in the

peripheral metabolism of thyroid hormones, or a change in the sensitivity of the hypothalamic-pituitary system to thyroxin, rather than the thyroid cells changing in sensitivity to TSH.

Chan and Eales (1976) demonstrated that a linear relationship existed between the total dose of bTSH injected per fish and the response of plasma thyroxin concentrations in the brook trout. The trout in their experiment had been injected on a dose per unit body weight basis. I interpreted this to mean that the thyroxin response was a function of the total dose, regardless of body weight, and did not adjust the dose for body weight.

The response of plasma thyroxin levels to various doses of bTSH was measured in coho salmon parr (March 15) and in presumptive smolts (April 25). The plasma concentration of thyroxin was higher in the control fish on March 15 (10 ng/mL) than in the control fish on April 25 (3.6 ng/mL). This difference in initial thyroxin levels was reflected generally at all doses of bTSH. Thus, no differences in the thyroxin response to bTSH were detected between parr and smolts, which concurs with the experiment conducted every 28 d.

The response of the thyroid cells to bTSH, in terms of plasma thyroxin concentration, was nearly identical to the response reported for brook trout (Chan and Eales 1976). In coho salmon, 0.04 I.U. bTSH was sufficient to cause elevated plasma thyroxin levels as measured 24 h later. Thyroxin levels declined as the dosage of bTSH exceeded the range of 0.07 to 0.10 I.U. This dose corresponds to about 3 µg/g body weight or about 50 µg per fish. Milne and Leatherland (1978) injected rainbow trout with 1.1 and 2.2 µg bTSH/g body weight, which

amounted to about 200 and 400 µg per fish. The lower dose (200 µg) caused a significant increase in thyroxin 9 h after the injection, but at 24 h plasma thyroxin levels were similar to those in the control fish. Plasma thyroxin levels in fish injected with the higher dose (400 µg) had elevated plasma thyroxin levels at 1 and 24 h. Thus, the temporal pattern of the thyroxin response to TSH changes at different doses. In my study, fish injected with 1.0 I.U. bTSH may have had lower thyroxin levels at 24 h due to thyroidal exhaustion.

Exogenous PRL depressed circulating levels of thyroxin in coho salmon in February, May, and June. This effect was observed whether oPRL was injected alone or in combination with bTSH. The inhibitory effect of oPRL on plasma thyroxin appeared in several experiments to be dose-related. The reason for this remains unknown, especially since at higher doses of oPRL there frequently was no effect on plasma thyroxin. As part of the procedure to determine the effects of oPRL on thyroxin titers, bTSH was frequently injected in all treated fish to raise the thyroxin levels in order to be able to measure any depression caused by oPRL. In the experiment in which oPRL was injected every 28 d from January through May, oPRL was injected with 0.1 I.U. bTSH. This dose of bTSH appears to have masked any effects of oPRL on thyroxin levels. Thus, the attempt to determine whether the effects of exogenous PRL on plasma thyroxin changed during the transformation from parr to smolt failed.

An apparent anomaly occurred in the experiment conducted on May 27, in that the fish injected with 3.6 I.U., but not 18.0 I.U., oPRL plus 0.1 I.U. bTSH had elevated thyroxin levels relative to the

fish injected with 0.1 I.U. bTSH alone. Although this apparent synergism between oPRL and bTSH may be, in fact, physiological, this seems unlikely on the basis of all the other instances when oPRL inhibited thyroxin titers. Also, in a concurrent experiment in which fish were injected with 1.8 or 9.0 I.U. oPRL alone, no change in plasma thyroxin occurred.

Previously, investigations on the role of PRL in regulating thyroid function in teleosts relied on metabolic and histological criteria and results were often conflicting. PRL appeared to stimulate the thyroid in the eel, Anguilla anguilla (Olivereau 1966, 1968) and the angelfish, Pterophyllum scalare (Osewold and Fiedler 1968). PRL was without effect on apparent thyroid secretory activity in the Indian catfish, Heteropneustes fossilis (Singh and Singh. 1976), the cichlid, Cichlasoma biocellatum (Mattheij et al. 1971), and the killifish, Poecilia latipinna (Higgins and Ball 1972).

Generally, exogenous PRL inhibited thyroxin levels in coho salmon in the parr and smolt stages. I was unable to detect differences in the effects of PRL on thyroxin levels due to development. In the only other studies in which the effects of exogenous PRL on plasma thyroxin levels were measured in teleosts, PRL was found to reduce thyroxin in F. heteroclitus (Grau and Stetson 1977) and was without effect in S. gairdneri (Milne and Leatherland 1978). Grau and Stetson (1977) provided evidence which indicated that oPRL acted as a goiterogen, lowering thyroid secretory activity. Milne and Leatherland (1978) injected 2 mg oPRL into rainbow trout.

The present study indicates that doses of oPRL in the range on 1 to 9 I.U. (about 0.03 to 0.30 mg) are sufficient to inhibit plasma thyroxin levels in coho salmon and that the dose used in rainbow trout was excessive.

IV. THE EFFECTS OF PROLONGED EXPOSURE TO SODIUM AND CALCIUM SALTS AND ACUTE EXPOSURE TO SEAWATER ON PLASMA THYROXIN CONCENTRATIONS AND GILL Na-K-ATPase ACTIVITY IN COHO SALMON PARR

Introduction

Juvenile anadromous salmonids undergo a developmental transformation from the parr to the smolt stage which is anticipatory to existence in the marine environment. The process of smoltification involves behavioral, morphological, physiological, and biochemical changes (Hoar 1976). A major endocrine event characterizing smoltification is the hyperactivity of the thyroid (Hoar 1939, 1965, 1976; Baggerman 1960; Eales 1963), resulting in large increases in plasma thyroxin levels (Dickhoff et al. 1978), which can affect behavior (Baggerman 1960, 1962), skin pigmentation (Landgrebe 1941; Piggins 1962; Dodd and Matty 1964), and growth (Higgs et al. 1977; Donaldson et al. 1979). A dramatic change associated with smoltification is the increase in gill Na-K-ATPase activity (Zaugg and McLain 1970, 1972; Zaugg and Wagner 1973; McCartney 1976; Giles and Vanstone 1976; Saunders and Henderson 1978), which peaks during the most active migratory behavior (Zaugg and Wagner 1973; Lorz and McPherson 1976; Ewing et al. 1979), and is generally considered critical to hydromineral balance in seawater (Epstein et al. 1967; Maetz 1969, 1971, 1974; Zaugg and McLain 1969, 1970, 1971, 1972; Jampol and Epstein 1970; Giles and Vanstone 1976).

The relationship of the thyroid to environmental salinity and osmoregulation in salmonids is not clear. Stunting, an abnormality of smoltification, occurs when some species of salmon are transferred

prematurely to seawater (Mahnken 1973; Kennedy et al. 1976). These fish fail to grow and resume a parr-like appearance. Histologically, the thyroid of "stunts" appears inactive (Clarke and Nagahama 1977; Bern 1978). However, measurements of plasma thyroxin in smolts transported to seawater at the normal time of release indicate concentrations which are either greater than (Folmar and Dickhoff 1979) or similar to (Specker and Schreck 1980) those of the salmon while still in freshwater.

The purpose of this study was to determine the effects of prolonged residence in freshwater enriched with either sodium or calcium and the effects of exposure to seawater on the plasma thyroxin levels of coho salmon, Oncorhynchus kisutch, parr. The hypothesis was that ambient sodium or calcium or both would accelerate the increase in thyroid activity characteristic of smoltification. The goal of this research was to consider a methodology which could be implemented at a culture facility to enhance the survival of smolts and perhaps mitigate losses due to stunting.

The hypothesis that increased ambient salinity might accelerate smoltification, or some components of smoltification, has been derived from research on amphibian development. Amphibian metamoppho-sis is a developmental transformation somewhat analogous to smoltification in that it is accompanied by a surge in thyroid activity (Regard et al. 1978; Mondou and Kaltenbach 1979) and prepares the animal for a desiccating environment. Low ambient salinity increases the rate of metamorphosis in Rana catesbeiana tadpoles (Ray et al. 1978). This probably occurs because elevated

salinity reduces plasma prolactin, which is anti-metamorphic (see Frye et al. 1973). Mammalian prolactin has been shown to depress plasma thyroxin levels in smolting coho salmon (Chapter III). Further, there is considerable evidence that in salmonids ambient salinity depresses prolactin cell activity (Leatherland and McKeown 1974; Leatherland and Lin 1975; McKeown and Hazlett 1975; Nagahama et al. 1977). Thus, high ambient salinity might be expected to depress prolactin, which in turn would enhance thyroxin levels and promote smoltification.

The relationship between plasma thyroxin and gill Na-K-ATPase is poorly defined for teleost fish. Thyroid hormones stimulate Na-K-ATPase in sharks *in vitro* (Honn and Chavin 1977), in tadpole epidermis (Kawada et al. 1969), and in a variety of mammalian tissues (Ismail-Beigi and Edelman 1970, 1971, 1974). In coho salmon smolts, a tentative positive correlation exists between plasma thyroxin and gill Na-K-ATPase during the 8 d following seawater entry (Folmar and Dickhoff 1979). A corollary purpose of this research, then, was to establish whether changes in gill Na-K-ATPase activity could be related to experimentally-induced changes in plasma thyroxin levels.

Materials and Methods

Experimental Conditions

Yearling coho salmon from brood year 1977 were obtained from Sandy Fish Hatchery, Oregon Department of Fish and Wildlife, in January 1979. They were maintained in freshwater (10-12°C) under natural photoperiod. The fish were fed dry pellets daily throughout the experiment.

On 3 February 1979, the fish (mean weight = 15 g, mean fork-length = 11 cm) were randomly distributed among six experimental tanks until there were 40 fish in each of the two control tanks and 30 fish in each of four treatment tanks. Water flow was set at 2 L/min. The fish were acclimated for 10 d. From February 13 to 27, chloride salts of sodium and calcium were delivered every 20 sec through automatic feeders into the treatment tanks to maintain the nominal concentration of calcium in the two tanks of hypercalcic water at 6 mM/L and the nominal sodium concentration in the two tanks of hypernatric water at 12 mM/L. From February 27 to March 6 these salt concentrations were doubled. The freshwater in the control tanks contained 0.5 mM Ca/L and 0.3 mM Na/L. Table 2 summarizes the environmental salinities encountered by each group of fish.

On February 26 and March 5, five fish from each tank (10 fish per group) were placed into static, aerated 20 L solutions of 75% seawater (Instant Ocean ®) and sampled 24 h later. Full-strength seawater was not used because two fish died in a pretest using 100% seawater.

Table 2. The nominal concentrations (mM/L) of sodium and calcium used in the experiment.

	Freshwater control	Hypernatric	Hypercalcic	75% seawater
Sodium	0.3	12-24	0.3	345
Calcium	0.5	0.5	6-12	7.5

Sampling Schedules and Procedures

Fish were sampled at the onset of the experiment (February 13) from the two control tanks (10 fish per tank) and at the end of each week from the control and treatment tanks (10 fish per tank on February 20 and 5 fish per tank on February 27 and March 6). Thus, sampling after 2 and 3 wks included fish from the control and treatment tanks plus the fish from 75% seawater.

The fish were captured with minimal disturbance, stunned, weighed, and the fork-length measured. Blood was collected from the severed caudal artery in ammonium-heparinized capillary tubes, centrifuged, and stored at -20°C for 2-4 mos. Gill filaments were cut from the first few gill arches, pulverized in a buffered solution, and frozen until Na-K-ATPase activity was assayed.

Sample Analysis

Thyroxin concentrations in plasma samples were determined using radioimmunoassay procedures described by Dickhoff et al. (1978). Two 10 µl aliquots of unextracted plasma were assayed using the antibody (Wiens Laboratories, Succasunna, New Jersey) at 50-55% binding, and high specific activity (700 µCi/mg) ^{125}I-labeled thyroxin (Industrial Nuclear, St. Louis, Missouri) at about 15,000 dpm. All solutions, including the standards, were made with barbital buffer. The bound hormone was precipitated by polyethyleneglycol during centrifugation, the supernatant removed, and the pellet counted. Curves based on standards made in coho salmon plasma stripped of thyroxin (Larson

et al. 1973) were similar and parallel to those from standards made in the barbital-buffered solution.

Gill Na-K-ATPase activity was measured by Dr. R. Ewing and R. Birks, Oregon Department of Fish and Wildlife Research Laboratory, using the whole homogenate method of Johnson et al. (1976), and the protein measured using a modification of the method of Lowry et al. (1951). Enzyme activity was not assayed for several months; therefore, more than 10% of the original activity was lost and the values reported are relative.

Statistical Analyses

The significance of changes in plasma thyroxin and gill Na-K-ATPase activity between February 13 and March 6 for the control group was assessed using single classification analysis of variance (ANOVA) for unequal sample sizes (p. 208, Sokal and Rohlf 1969). If appropriate, contrasts among the means were made using Student-Newman-Keuls multiple range comparison tests for unequal sample sizes (SNK) (p. 242, Sokal and Rohlf 1969).

The effects of the treatments during the experiment were analyzed as a 3 x 3 (group x time) factorial design, using the regression approach to ANOVA because of unequal sample sizes (Nie et al. 1975). Effects of seawater challenge on plasma thyroxin and gill Na-K-ATPase activity were analyzed as a 2 x 3 x 2 (time x treatment x seawater-challenged vs. not seawater-challenged) factorial design, using the regression approach to ANOVA. Insignificant second and third order interactions were included in the estimation of the error variance.

When preliminary ANOVA indicated significant main effects, single *a priori* contrasts between the control group and each treatment group were made using Student's t-test at the 5% level. Data from the replicated tanks were combined when similar (Student's t-test at the 5% level).

Results

The concentration of thyroxin in the plasma of the control fish in freshwater changed during the experiment ($F = 2.99 > F_{0.05(3, 48)} = 2.80$) (Fig. 9). Comparison of the mean thyroxin levels revealed no differences (SNK), however the 95% confidence interval for the mean thyroxin level on February 27 was above that for the fish sampled February 13. Thyroxin concentrations continued to increase until late March, when the thyroxin levels in these fish peaked at 8.4 ± 1.5 ng/ml (mean ± one standard error, n = 20). Titers then fell to 3.0 ± 1.2 ng/ml (n = 20) by mid-June. This pattern parallels changes in plasma thyroxin that occur in coho salmon in hatcheries during this period of development and time of year (Dickhoff et al. 1978).

Residence in hypernatric or hypercalcic water significantly affected plasma thyroxin levels ($F = 2.96 \doteq F_{0.05(2, 96)} = 3.09$); but among groups, thyroxin concentrations remained similar from February 20 to March 6 ($F = 0.14$). Thyroxin levels in fish from hypernatric water were higher than those in freshwater (4.79 ± 0.49 vs. 2.95 ± 0.35 ng/ml, $t = 2.28 > t_{0.05(63)} = 1.67$). Fish from the hypercalcic water had plasma thyroxin levels similar to the control fish (3.49 ± 0.44 ng/ml, $t = 0.42$).

A 24 h residence in 75% seawater altered plasma thyroxin concentrations ($F = 11.19 > F_{0.01(1, 90)} = 6.97$) (Fig. 10). There tended to be a difference among the groups ($F = 2.72 > F_{0.10(2, 90)} = 2.37$), which probably reflects both the higher thyroxin levels in fish from hypernatric water and the higher thyroxin levels in fish

Figure 9. Plasma thyroxin concentrations (mean ± one standard error; n at the base of the bar) in coho salmon parr residing in hypernatric or hypercalcic water compared to the control fish in freshwater.

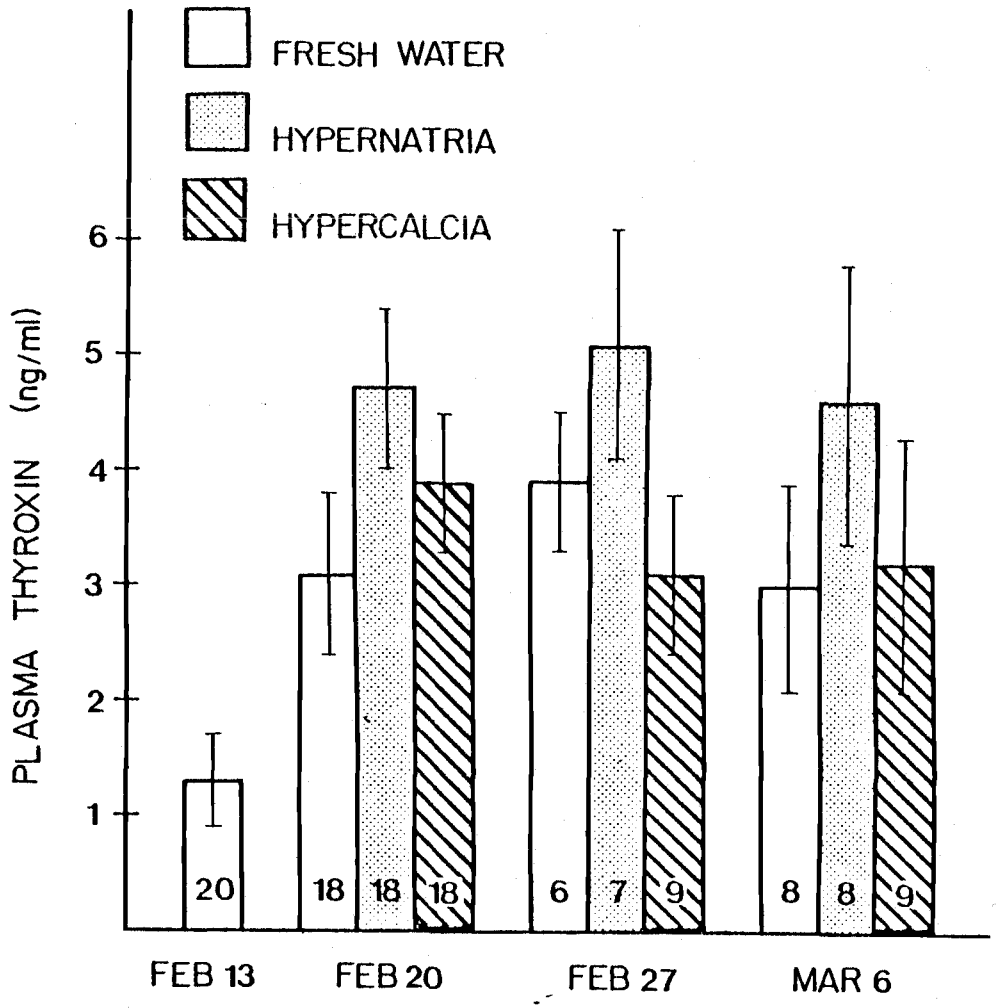
FRESH WATER
HYPERNATRIA
HYPERCALCIA
PLASMA THYROXIN (ng/ml)
1
2
3
4
5
6
20
18
18
18
6
7
9
8
8
9
FEB 13
FEB 20
FEB 27
MAR 6

Figure 9

Figure 10. A comparison between the plasma thyroxin concentrations (mean ± one standard error; n at the base of the bar) in coho salmon parr, which had resided in fresh-, hypernatric, or hypercalcic water for 2 or 3 wks (February 27 or March 6), before and after being subjected to 24 h in 75% seawater.

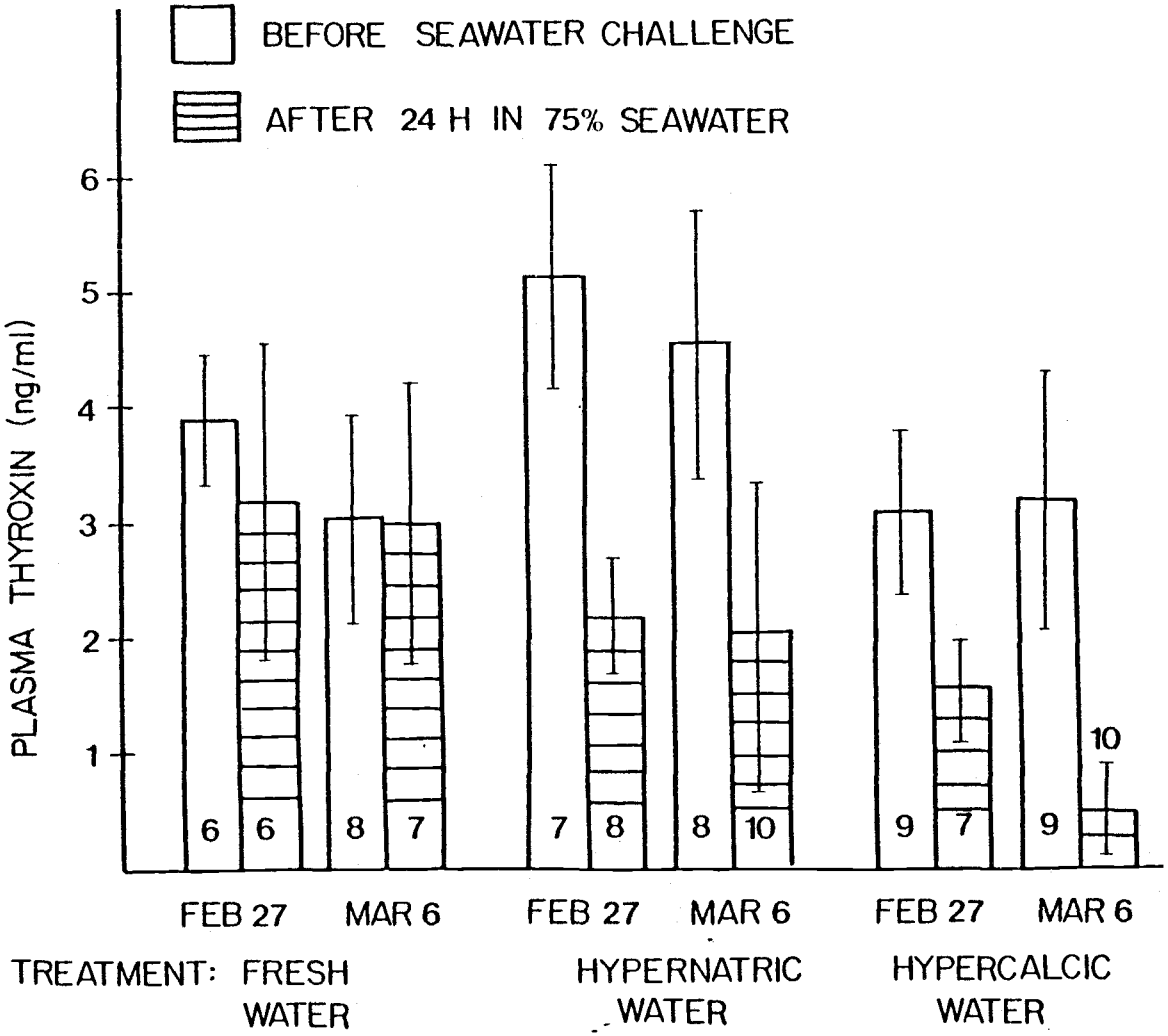
BEFORE SEAWATER CHALLENGE
AFTER 24 H IN 75% SEAWATER
PLASMA THYROXIN (ng/ml)
1
2
3
4
5
6
6
6
8
7
7
8
8
10
9
7
9
10
FEB 27
MAR 6
FEB 27
MAR 6
FEB 27
MAR 6
TREATMENT: FRESH WATER
HYPERNATRIC WATER
HYPERCALCIC WATER

Figure 10

from the control group following the seawater challenge test. There were no differences between data from tests conducted on February 27 or March 6 ($F = 0.74$), so comparisons between groups were based on data pooled from the two times. Exposure to seawater reduced thyroxin levels in fish from both hypernatric water (from 4.8 ± 0.8 ($n = 15$) to 2.3 ± 0.7 ($n = 19$) ng/ml, $t = 2.45 > t_{0.01(32)} = 2.45$) and hypercalcic water (from 3.1 ± 0.7 ($n = 18$) to 1.1 ± 0.3 ($n = 18$) ng/ml, $t = 2.79 > t_{0.01(32)} = 2.45$). Thyroxin levels in fish from the control group remained comparable before and after 24 h in seawater; however, three observations of concentrations greater than 20 ng/ml in seawater-challenged fish were not within four standard deviations from the mean and were thus considered outliers and eliminated to maintain homogeneity of variances.

Gill Na-K-ATPase activity changed in the control fish through time ($F = 3.32 > F_{0.05(3, 75)} = 2.73$), which is characteristic of coho salmon during smoltification (Zaugg and McLain 1970, 1972, 1976) (Fig. 11). Although comparison of the mean activity levels distinguished no differences (SNK), the lower bound of the 95% confidence interval for the mean enzyme activity of fish sampled February 27 was above the upper bound of the fish sampled February 13.

Seawater challenge had no effect on gill Na-K-ATPase activity ($F = 0.28$), so the data from before and after 24 h in seawater were pooled within each group. There was a highly significant effect of time on enzyme activity ($F = 11.89 > F_{0.001(2, 162)} = 7.21$). The effect of treatment may have been somewhat significant ($F = 2.30 \doteq F_{0.10(2,162)} = 2.34$). The Na-K-ATPase activity of the fish in

Figure 11. Gill Na-K-ATPase activity (mean ± one standard error; n at the base of the bar) of coho salmon parr residing in hypernatric or hypercalcic water compared to control fish in freshwater.

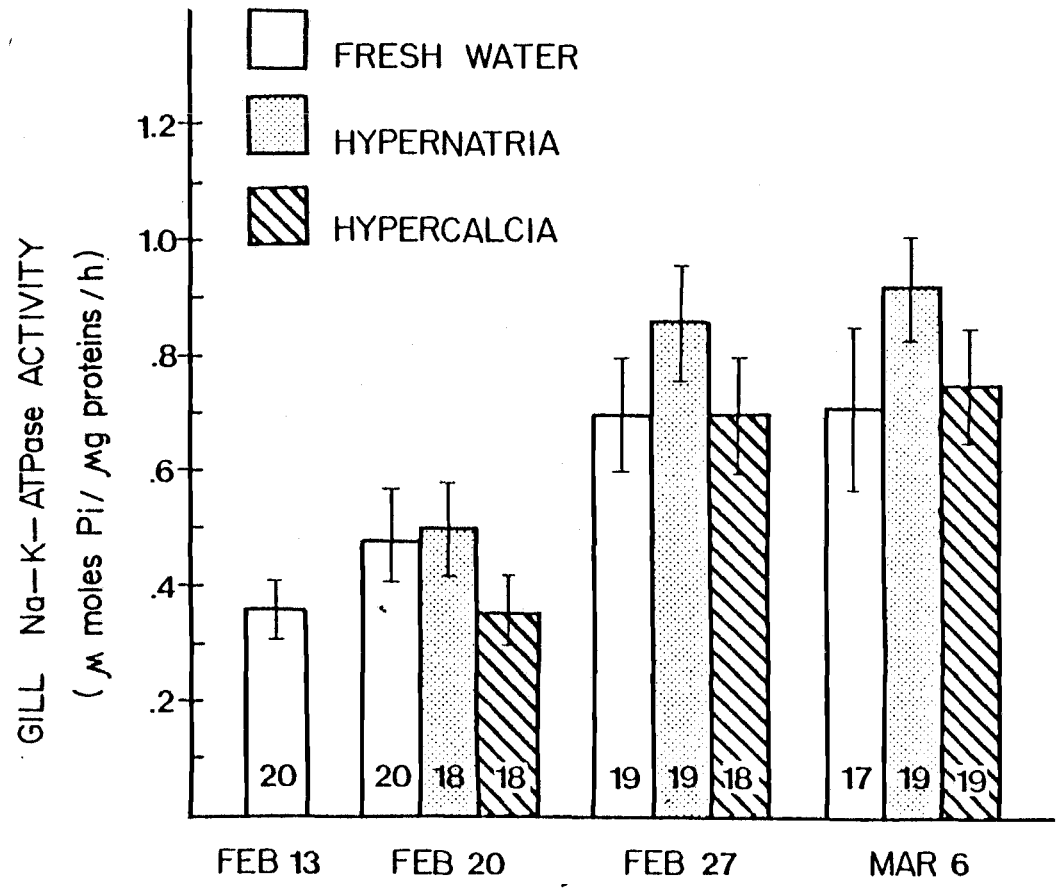
FRESH WATER
HYPERNATRIA
HYPERCALCIA
GILL Na—K—ATPase ACTIVITY
(μ moles Pi/ mg proteins / h)
1.2
1.0
.8
.6
.4
.2
20
20
18
18
19
19
18
17
19
19
FEB 13
FEB 20
FEB 27
MAR 6

Figure 11

hypernatric water tended to be higher than that of the control fish (0.773 ± 0.057 vs. 0.640 ± 0.065 μmoles Pi/μg protein/h, $t = 1.58 > t_{0.10(59)} = 1.30$), while the enzyme activity of the fish in hypercalcic water was comparable to that of the control fish (0.608 ± 0.056 μmoles Pi/μl protein/h, $t = 0.39$).

Gill Na-K-ATPase activity tended to be correlated with plasma levels of thyroxin ($r = 0.099 = r_{0.10(167)}$) (cf Figs. 9 and 11).

The average weights and fork-lengths of all groups of fish remained the same.

Discussion

In this study, the transfer of coho salmon parr to 75% seawater depressed plasma thyroxin levels. This finding concurs with the observation that premature transfer to seawater of coho salmon causes a hypothyroid condition, based on the histological appearance of the thyrotropes and thyroid cells (Clarke and Nagahama 1977; Bern 1978), as well as much of the early work which, based on metabolic and histological evidence, suggested that the thyroid is inhibited when teleosts enter seawater (see Pickford and Atz 1957).

The depression in thyroxin levels upon seawater entry is not due to increased peripheral utilization, since the turnover of radiolabeled thyroxin in the plasma is halved 24 h after transfer of coho salmon parr and smolts (personal communication, E. G. Grau, Department of Zoology, University of California, Berkeley). This information further suggests that the difference in thyroxin levels in fish from the control and treatment groups did not result from the treatment, and that the general trend for a decrease in thyroxin levels was typical. The depression in plasma thyroxin upon transfer to seawater could be due either to direct inhibition of the thyrotropes from increases in plasma sodium or osmolality, as evidenced by the histological appearance of these cells (Bern 1978), or due to increases in adrenocorticotropic hormone (ACTH) secretion. The latter possibility is suggested by the observations that cortisol, and therefore ACTH, increases with higher salinities in eels (Doyle and Epstein 1972; Forrest et al. 1973), and that ACTH is known to reduce the size of the thyrotropes in

the euryhaline stickleback, Gasterosteus aculeatus (Leatherland and Lam 1971).

In contradistinction, coho salmon parr maintained in freshwater in which ambient sodium levels were raised to 12 to 24 mM/L had greater plasma thyroxin concentrations than parr in freshwater. Thyroxin levels of parr in freshwater supplemented with 6 to 12 mM calcium/L were similar to those of control fish. The discrepancy between acute exposure to seawater and low chronic sodium levels probably results from the complexity of the factors which regulate thyroid secretory activity. The effects of environmental salinity are necessarily mediated through changes in plasma osmolarity or changes in the ionic composition of the plasma. Such changes in the plasma could alter either the thyroid directly or the peripheral metabolism of the thyroid hormones or both, as previously discussed. A further possibility is that ambient sodium altered the secretory rates of pituitary cells, thereby indirectly affecting thyroxin levels.

The original hypothesis of this research was that residence in hypercalcic or hypernatric water might elevate thyroxin levels as a result of the inhibition of prolactin. Prolactin injections can depress plasma thyroxin in coho salmon during smoltification (Chapter III), and are also known to reduce the size of thyrotropes in the euryhaline three-spine stickleback (Leatherland and Lam 1971). That sodium and not calcium affected thyroxin levels could be due to differences in the concentrations used, differences in the concentration of each particular ion relative to that which the fish typically would encounter, or in the mechanism of action of the ion. That

ambient salinity and prolactin cell activity are inversely related in teleosts has been amply demonstrated (Schreibman et al. 1973; Nagahama et al. 1973, 1977; Leatherland and McKeown 1974; Leatherland and Lin 1975; McKeown and Hazlet 1975). Changes in the sodium content of the environment are mediated through changes in plasma sodium concentration and plasma osmolality (Ball and Ingleton 1973; Nagahama et al. 1974, 1975; Wighman and Ball 1977). However, Wendelaar Bonga (1978) concluded from his work on the stickleback, Gasterosteus aculeatus, that plasma ionic calcium regulated prolactin cell activity. If the inhibition of the secretory activity of prolactin cells is the underlying cause for the apparent depression in thyroid levels, then the data presented herein tend to indicate that ambient sodium is more effective than calcium in the regulation of prolactin cells in coho salmon.

Alternatively, ambient sodium might have caused higher thyroxin levels in coho salmon parr by stimulating the release of ACTH and in turn cortisol. Cortisol, which increases with greater environmental salinity as mentioned, can prevent the release of prolactin into a stimulatory medium in vitro (Wigham et al. 1977). This possibility would support the original hypothesis, that prolactin suppresses thyroid activity. ACTH or cortisol may have affected the thyrotropes or thyroid directly, although ACTH has no effect on the thyroxin response to TSH in the brook trout, Salvelinus fontinalis (Chan and Eales 1976). However, ACTH does reduce the size of the thyrotropes in the stickleback (Leatherland and Lam 1971). This would result in a response such as that observed when the fish were placed in seawater.

If, in fact, ACTH does affect the thyrotropes in coho salmon, perhaps the difference in effects of acute exposure vs. chronic exposure to low sodium levels results from differences in the threshold of either the response of ACTH to a salinity change or the response of the thyrotropes and thyroid to ACTH and salinity changes.

This study demonstrated that residence in a hypernatric environment tended to elevate gill Na-K-ATPase activity. Furthermore, gill Na-K-ATPase activity and plasma thyroxin tended to be correlated. This concomitant increase in gill Na-K-ATPase with experimentally-elevated plasma thyroxin levels is the most convincing evidence to date that thyroxin plays a role in the induction of Na-K-ATPase activity in teleosts. Thyroid hormones are known to stimulate Na-K-ATPase in mammalian tissues (Ismail-Beigi and Edelman 1970, 1971, 1974), in tadpole epidermis (Kawada et al. 1969), and in the gills of nurse sharks *in vitro* (Honn and Chavin 1977), but have not been demonstrated to affect the enzyme activity of teleostean fish. Folmar and Dickhoff (1979) followed changes in gill enzyme activity and plasma thyroxin in coho salmon smolts for 8 d after transfer to seawater. Their data also suggest a positive correlation in that generally there was an increase in both variables, although daily variation was large and there was no statistical evaluation.

The possibility that elevated cortisol levels caused the increase in Na-K-ATPase cannot be ignored. Cortisol, which increases at higher salinities as mentioned, stimulates Na-K-ATPase activity (Doyle and Epstein 1972).

Gill enzyme levels can be depressed by prolactin, as demonstrated in the killifish, Fundulus heteroclitus, (Pickford et al. 1970). Thus, if the earlier hypothesis that ambient sodium suppresses prolactin cell secretory activity were true, then the removal of prolactin's tonic inhibition may have enhanced Na-K-ATPase activity.

Two sets of information suggest that this research may have implications for hatchery practices. First, marked increases in gill Na-K-ATPase activity are critical to the development of salinity tolerance that occurs during the smoltification of anadromous salmonids (Zaugg and Wagner 1973; Zaugg and McLain 1976; Giles and Vanstone 1976). Secondly, there is evidence to indicate that the fish survive and grow better in seawater if they are transferred approximately a month after the peak in thyroxin levels (personal communication, W. W. Dickhoff, Department of Zoology, University of Washington). It is at this time that their ability to absorb water across the posterior intestine peaks (Collie and Bern 1980), suggesting the optimization of Na-K-ATPase levels. In this experiment, the addition of sodium chloride elevated plasma thyroxin concentration and gill Na-K-ATPase activity in coho salmon parr. The possibility that gradual acclimation to increased ambient salinity would accelerate changes in plasma thyroxin and gill Na-K-ATPase and thus improve the osmoregulatory capacity, growth, and survival and reduce losses due to stunting of coho salmon smolts deserves further consideration.

V. CHANGES IN PLASMA CORTICOSTEROID CONCENTRATIONS DURING SMOLTIFICATION AND IN RESPONSE TO MAMMALIAN PROLACTIN AND THYROTROPIN IN COHO SALMON

Introduction

During smoltification, juvenile anadromous salmonids are transformed from stream-dwelling parr to smolts capable of osmoregulation in the marine environment. The physiological changes accompanying the transition from parr to smolt are anticipatory to seawater entry. For example, based on histological observations, the pituitary-interrenal axis is activated during the smoltification of Atlantic and Pacific salmon (Fontaine and Olivereau 1957, 1959; Olivereau 1962, 1975; McLeay 1975; Komourdjian et al. 1976). Corticosteroids, elaborated by the interrenal tissue, are generally considered critical to osmoregulation in seawater, whereas the hypophysical hormone prolactin (PRL) is critical to osmoregulation in freshwater (see Utida et al. 1972; Johnson, 1973; Bern 1975). This is demonstrated in the organ-cultured bladder of seawater-adapted Gillichthys mirabilis, where cortisol maintains a high permeability to water which is inhibited by PRL in a dose-dependent fashion (Doneen 1974; Doneen and Bern, 1974). Moreover, PRL favors increased ion uptake, whereas cortisol enhances water absorption with low ion uptake (Doneen and Nagahama 1973). Thus in a freshwater environment, the chronic presence of high levels of corticosteroids would seem to result in potentially detrimental shifts in membrane permeabilities.

The only available information on actual plasma corticosteroid levels during smoltification are derived from two plasma pools taken

from Atlantic salmon parr, Salmo salar, in December and smolts in March and April: corticosteroid levels were five times greater in smolts than in parr (Fontaine and Hatey 1954). The primary objective of the present study was to determine plasma corticosteroid concentrations during the smoltification of coho salmon, Oncorhynchus kisutch.

The second objective of this study was to determine the effects of the tropic hormones PRL and thyrotropin (TSH) on corticosteroid levels. Two seemingly contradictory sets of observations on the relationship between PRL and interrenal tissue function have been reported. In the case of the holostean bowfin, Amia calva (Hanson and Fleming 1979) and the killifish, Fundulus kansae (Fleming et al. 1971, 1973), exogenous PRL elevated plasma cortisol levels. However, in the case of the euryhaline fish, Gillichthys mirabilis, mentioned previously, the proposed model of bihormonal control of hydromineral balance would make a corticotropic role for PRL seem unlikely. Thus I hypothesized that PRL would either have no effect on, or possibly inhibit, corticosteroid levels. Further, the adenohypophysial hormone TSH has been reported to exert corticotropic effects in the euryhaline killifish, Poecilia latipinna (Ball and Hawkins 1976). Because thyroid hyperactivity and increased thyroxin levels are major endocrine components of smoltification (Hoar 1939; Baggerman 1960; Eales 1963; Dickhoff et al. 1978; Chapters III and V; see Hoar 1976), I hypothesized that TSH might have a role in the activation of the interrenal tissue observed during smoltification.

Corticosteroids are known to cause elevations in gill Na-K-ATPase in teleosts such as eels, Anguilla sp. (Epstein et al. 1971; Doyle and Epstein 1972; Kamiya 1972), and coho salmon (Schreck and Ejike, unpublished). Increased gill Na-K-ATPase activity is considered critical to the development of salinity tolerance in salmonids (Zaugg and Wanger 1973; Zaugg and McLain 1976; Giles and Vanstone 1976). However, there are also correlative evidence to suggest that thyroid hormones stimulate gill Na-K-ATPase in coho salmon (Folmar and Dickhoff 1979; Chapter V). Thyroid hormones causally elevate gill Na-K-ATPase activity in vitro in the nurse shark, Ginglymostoma cirratum (Honn and Chavin 1977). Thus the final objective of this study was to examine the relationship among plasma corticosteroids, thyroxin, and gill Na-K-ATPase activity during the smoltification of coho salmon.

Experimental Procedures and Results

Coho salmon eggs from the 1978 brood year were obtained from Sandy Fish Hatchery, Oregon Department of Fish and Wildlife. The fish were reared in freshwater (10-12°C) under natural photoperiod. The fish in experiment 1 were fed Oregon Moist Pellets and the fish in experiments 2 and 3 were fed dry pellets. Feeding was discontinued for several days prior to and during the experiments. There were no signs of illness in these fish at any time. Treatments were assigned to tanks using a random numbers table. The fish were acclimated for 7-10 d in the experimental tanks before the experiments began.

Coho salmon of this stock presumptively become smolts during the spring of their second year. At this time, gill Na-K-ATPase activity increases (personal communication, W. Zaugg, U. S. Fish and Wildlife Service, Cook, Washington), plasma thyroxin levels increase (personal communication, W. Dickhoff, Department of Zoology, University of Washington, Seattle, Washington), and the skin silvers and the parr marks disappear (personal observation). The hatchery usually releases the fish in May and the fish are known to migrate upon release (Specker and Schreck 1980).

Ovine prolactin (oPRL) was obtained as a gift from the National Institutes of Health (NIH-P-S13); salmon prolactin (sPRL), a non-glycoprotein of MW = 25,000, was donated by Syndel Laboratories (Vancouver, British Columbia); and bovine thyrotropin (bTSH) was purchased through Sigma (St. Louis, Missouri). The tropic hormones were dissolved in 0.6% NaCl-0.05% NaOH and injected intraperitoneally in 0.06 ml carrier. Fish were injected between 1300 and 1500 h while anesthesized with tricaine methanesulfonate. Sampling occurred 24 h after the second injection. The fish were captured with minimal disturbance, stunned, weighed, and measured (fork-length = FL). Blood was collected from the severed caudal artery in ammonium-heparinized capillary tubes, centrifuged, and stored at -20°C.

Corticosteroid concentrations were measured in 10 µl plasma samples using the competitive protein binding assay described by Strange and Schreck (1978). The coefficient of variation within and among assays was 14%. Over 75% of the values measured by this assay were for cortisol, the remainder for other corticosteroids.

Experiment 1. This experiment was designed to test the effects of different doses of oPRL and sPRL on plasma corticosteroid levels. Five different dose levels of sPRL and oPRL were used, totaling 0.06, 0.18, 0.30, 0.42, and 0.54 mg, which corresponded to 1.8, 5.4, 9.0, 12.6, and 16.2 I.U. oPRL. All treated fish received two consecutive daily injections (February 20-21, 1980) containing 0.025 I.U. bTSH per injection. There were eight fish per tank. Fish in two tanks received only bTSH. Otherwise the treatments were not replicated in different tanks. For experimental control, eight fish were injected with the saline vehicle and eight fish which were not injected were included in the sampling. The sex of each fish was determined at the end of the experiment.

To meet the assumptions of parametric statistics, the data were transformed to $\log_e$, which maintained homogeneity of variances (Bartlett-Box F = 0.28, p = 0.99 rather than F = 3.30, p < 0.001), and reduced kurtosis (from 4.66 to -1.52), and skewness (from 2.14 to 0.38). Because of unequal sample sizes, the regression approach to analysis of variance (ANOVA) (Nie et al. 1975) was used to analyze the transformed data.

This experiment was analyzed as a 2 x 12 (sex x treatment factorial design. The sex of the fish had no effect on corticosteroid levels ($F = 1.44 < F_{0.05(1, 69)} = 3.98$). Treatment with bTSH in combination with five different doses of oPRL and sPRL each did not affect plasma corticosteroids ($F = 1.81 < F_{0.05(11, 69)} = 1.93$) (Table 3).

Experiment 2. This experiment was conducted six times at 28 d intervals, beginning January 6, 1980, to examine changes in plasma

Table 3. The concentrations (95% confidence interval about the harmonic mean) of corticosteroids in the plasma of coho salmon treated with two consecutive daily injections totaling 0.05 I.U. bTSH or 0.05 I.U. bTSH plus 1.8, 5.4, 9.0, 12.6, or 16.2 I.U. oPRL, or 0.05 I.U. bTSH plus 0.06, 0.18, 0.30, 0.42, or 0.54 mg crude sPRL. Control fish were either not injected or injected with the saline vehicle.

Treatment	n	Plasma corticosteroids (ng/ml) Harmonic mean	95% confidence interval
Control	15	4	1 to 9
bTSH	12	2	- to 7
1.8 I.U. oPRL + bTSH	8	9	2 to 48
5.4 I.U. oPRL + bTSH	7	4	- to 18
9.0 I.U. oPRL + bTSH	5	2	- to 15
12.6 I.U. oPRL + bTSH	6	9	1 to 64
16.2 I.U. oPRL + bTSH	7	11	2 to 71
60 mg sPRL + bTSH	6	5	- to 43
180 mg sPRL + bTSH	7	22	3 to 180
300 mg sPRL + bTSH	7	22	6 to 88
420 mg sPRL + bTSH	8	2	- to 7
540 mg sPRL + bTSH	5	10	- to 134

corticosteroids and the response of corticosteroids to PRL and TSH during smoltification. Yearling coho salmon were injected each day for two consecutive days with (1) 0.01 I.U. bTSH, (2) 0.05 I.U. bTSH, (3) 1.8 I.U. oPRL plus 0.05 I.U. bTSH, (4) 9.0 I.U. oPRL plus 0.05 I.U. bTSH, or (5) the saline vehicle. Each treatment was replicated in two tanks and there were six fish per tank. Sampling included 12 fish that were not injected, so there was a total of six groups each month.

The data were transformed to $\log_e$ to reduce heteroscedasticity (from Bartlett-Box $F = 20.87$, $p < 0.001$ to $F = 1.00$, $p = 0.47$), kurtosis (from 4.45 to -1.41), and skewness (from 2.12 to 0.04). The effects of treatment and time were analyzed as a 6 x 6 (time x treatment) factorial design using the regression approach to ANOVA. Considerable tank-to-tank variability caused a significant second-order interaction ($F = 2.53 > F_{0.001(25, 364)} = 2.20$) which had no pattern with respect to treatment (Fig. 12). Treatment with bTSH and oPRL had no effect on plasma corticosteroids ($F = 1.52 < F_{0.05(5, 394)} = 2.24$) so the effect of treatment was discarded from the model.

Plasma corticosteroids changed significantly through time ($F = 13.15 > F_{0.001(5, 394)} = 4.20$). The trends in plasma corticosteroids were similar whether considering the fish from the control groups or all the fish sampled at each time. Contrasts between groups were made on means obtained from all the fish sampled at each time (Fig. 13). The fish sampled May 27 had higher mean corticosteroid concentrations than the fish at all other times (Student-Newman-Keuls multiple range comparison test = SNK).

Figure 12. Plasma corticosteroid concentrations (95% confidence interval about the harmonic mean, n = 10 to 12) in coho salmon injected with bovine TSH and ovine PRL at 28 d intervals during smoltification. Sampling error due to tank-to-tank variation resulted in a statistically significant interaction between time and treatment.

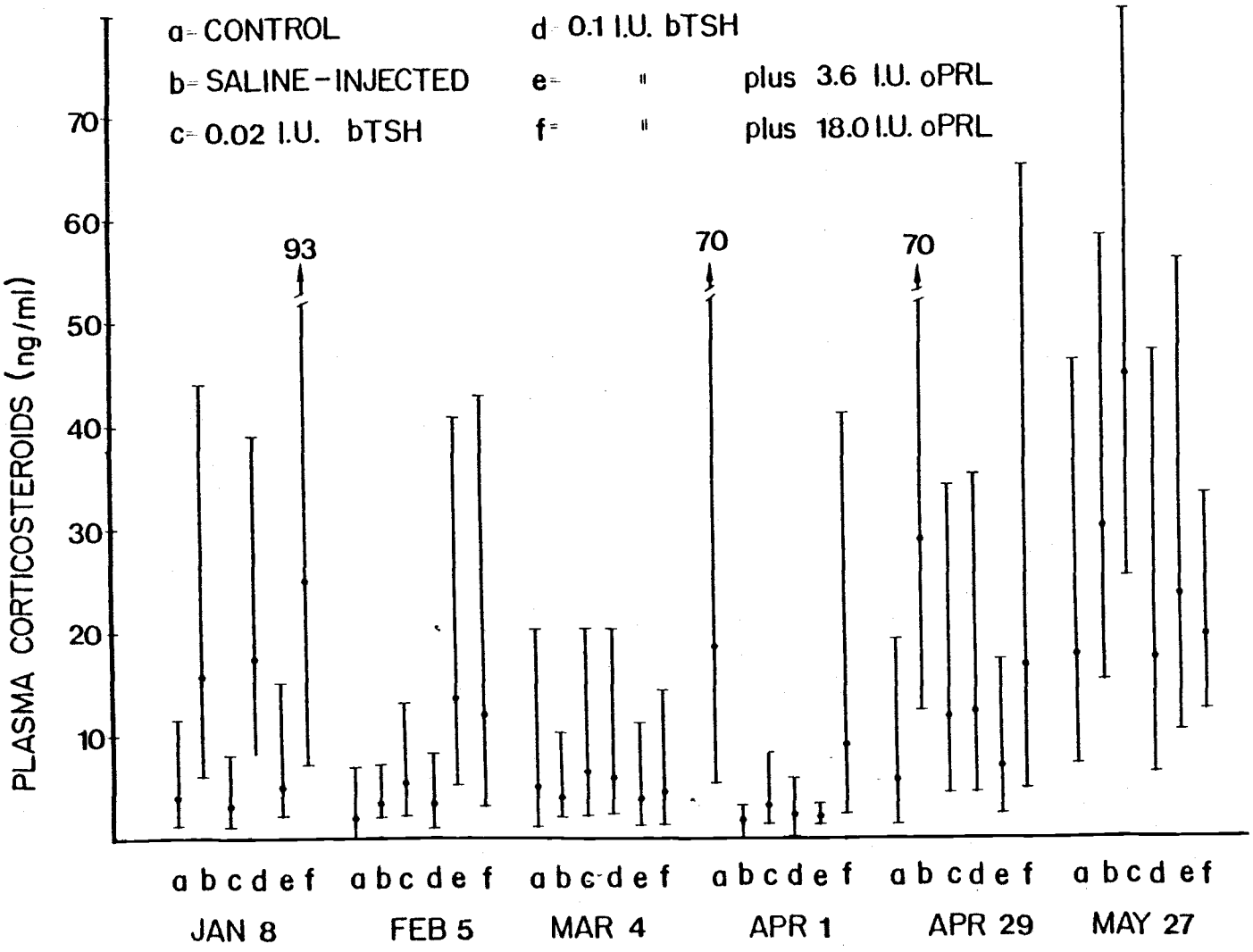
a- CONTROL
b- SALINE - INJECTED
c- 0.02 I.U. bTSH
d- 0.1 I.U. bTSH
e- " plus 3.6 I.U. oPRL
f- " plus 18.0 I.U. oPRL
PLASMA CORTICOSTEROIDS (ng/ml)
10
20
30
40
50
60
70
93
70
70
a b c d e f
JAN 8
FEB 5
MAR 4
APR 1
APR 29
MAY 27

Figure 12

Figure 13. A summary of changes in the concentration (95% confidence interval about the harmonic mean, n in parentheses) of corticosteroids in the plasma of coho salmon during smoltification. The solid circles represent the mean corticosteroid levels of the fish in experiment 2; the open circles represent the mean corticosteroid levels of all fish sampled in experiments 1 and 3 (February 22 and May 27b).

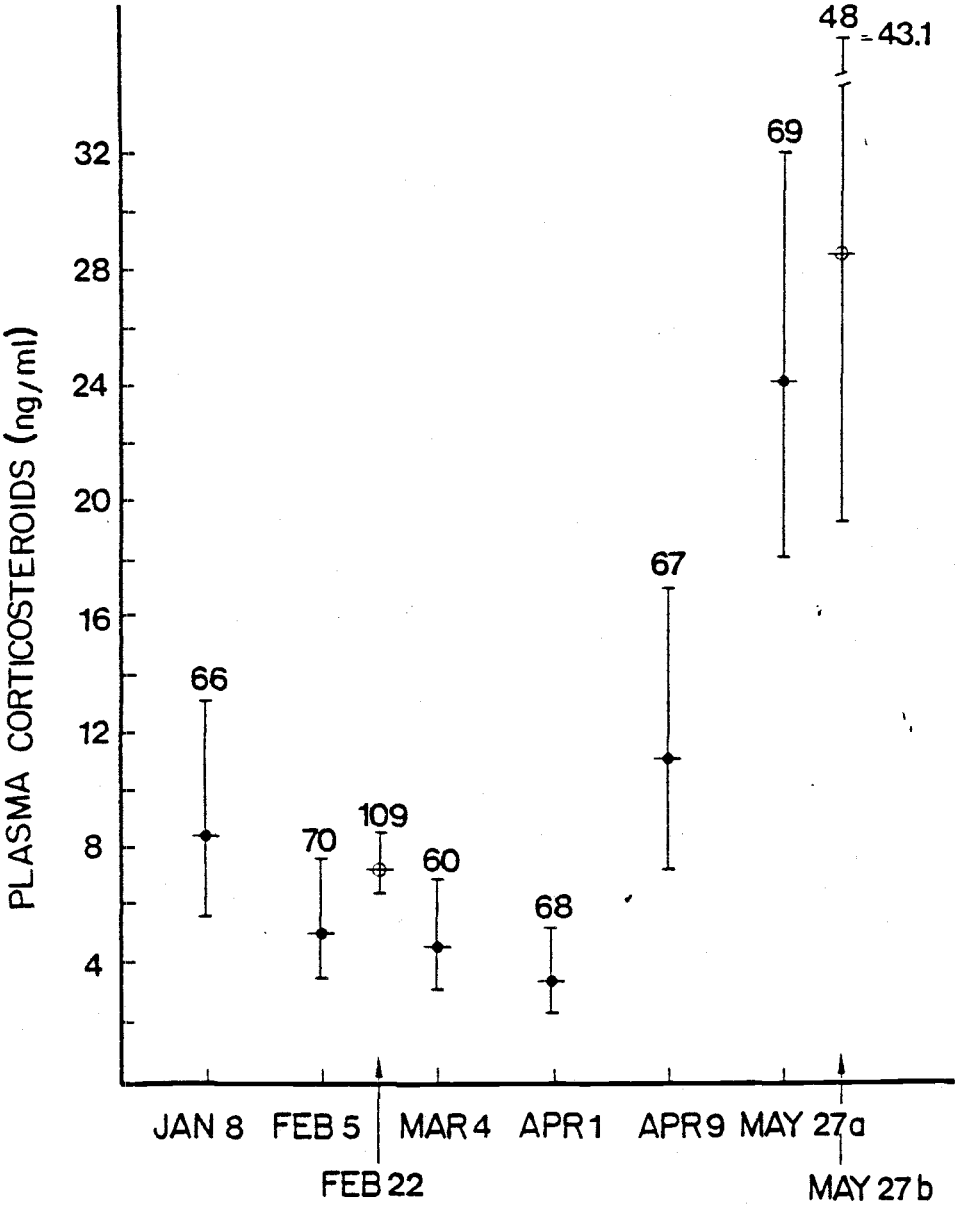
PLASMA CORTICOSTEROIDS (ng/ml)
4
8
12
16
20
24
28
32
66
70
109
60
68
67
69
48
=43.1
JAN 8
FEB 5
FEB 22
MAR 4
APR 1
APR 9
MAY 27a
MAY 27b

Figure 13

Corticosteroid levels of fish sampled April 29 were higher than those of fish sampled February 5 through April 1 (SNK). The fish sampled January 8 had higher corticosteroid levels than the fish sampled April 1 (SNK).

Experiment 3. Concomitant with the sixth run of the previous experiment (May 27a), four additional groups were included (referred to as May 27b). For four consecutive days, either (1) 1.8 I.U. oPRL, (2) 9.0 I.U. oPRL, (3) 0.6 mg sPRL, or (4) the saline vehicle were injected each day.

The data were transformed to $\log_e$ to reduce heterogeneity of variance (from Bartlett-Box $F = 8.78$, $p < 0.001$ to $F = 1.51$, $p = 0.21$), kurtosis (from 4.36 to -1.02), and skewness (from 24.03 to 1.06). These PRL treatments at this time of year had no effect on plasma corticosteroids ($F = 0.46 < F_{0.05(3, 43)} = 2.82$) (Table 4).

Table 4. The concentration (ng/mL, n = 12) of corticosteroids in the plasma of coho salmon following four consecutive daily injections totaling 7.2 or 36.0 I.U. oPRL, or 2.4 mg crude sPRL.

Treatment	Harmonic Mean	95% confidence interval
Saline	24.4	9.8 to 61.0
7.2 I.U. oPRL	24.8	9.8 to 62.8
36.0 I.U. oPRL	26.1	8.8 to 77.7
2.4 mg sPRL	43.3	24.8 to 75.6

Discussion

During smoltification in coho salmon, mean plasma concentrations of corticosteroids decreased between January and April from 8.4 to 3.5 ng/mL and increased to 26.0 ng/mL by the end of May. This finding corroborates the histological evidence for interrenal activation during smoltification (Fontaine and Olivereau 1957, 1959; Olivereau 1962, 1975; McLeay 1975; Komourdjian et al. 1976) and extends the work of Fontaine and Hatey (1954) on circulating corticosteroid levels. Plasma corticosteroid levels correspond very closely to the histometric indices of interrenal activity used by McLeay (1975), including (1) the most pronounced interrenal activation in smolts in late April, at which time his study ended, (2) moderately pronounced signs of interrenal activity in parr during the coldest winter months, and (3) indications of less interrenal activity in parr in late March.

McLeay (1975) found that interrenal hyperactivity of parr in the winter was associated with cold (4°C) water temperature. Cold-temperature acclimation elevated plasma cortisol in juvenile coho salmon (Allan 1971 in McLeay 1975). The fish in this study were maintained in well water with a relatively stable temperature ranging from 10 to 12°C. Thus temperature alone does not induce the observed changes, unless the pituitary-interrenal axis is sensitive even to the slight change in temperature that these fish experienced. This sensitivity is unlikely, considering that juvenile cutthroat trout, Salmo clarki, subjected to diurnal temperature cycles (13-23°C) exhibit

no substantial changes in plasma corticosteroids (Strange et al. 1977).

In these studies, neither sPRL, oPRL, nor bTSH, when injected alone or in combination, affected plasma corticosteroid levels. Exogenous PRL was also without effect on the interrenal tissue of the killifishes, Fundulus heteroclitus (Pickford and Kosto 1957) and Poecilia latipinna (Ball and Hawkins 1976), the sailfin molly, Mollienesia sp. (Ball and Ensor 1969), the European eel, Anguilla anguilla (Chan et al. 1968), and the starry flounder, Platichthys stellatus (Johnson and Clarke in Johnson 1973). PRL and corticosteroids exert opposing actions on membranes responsible for hydromineral balance, as previously discussed. In the majority of cases, including coho salmon, PRL does not stimulate or affect interrenal activity, as would be expected.

Figure 14 is a composite of mean corticosteroid levels (from Fig. 13), mean plasma thyroxin levels (from Fig. 8 in Chapter III, the same fish), and mean gill Na-K-ATPase activity (data taken from Zaugg and McLain 1970, 1976) in coho salmon held at 10°C during smoltification. Thyroxin and corticosteroid levels clearly are not correlated positively, suggesting in itself that TSH is not corticotropic in this instance. In fact, thyroxin and corticosteroids are related inversely in general: thyroxin increased from 1.7 to 5.9 ng/mL, while corticosteroids decreased from 8.4 to 3.5 ng/mL between January 8 and April 1. The fish began to silver in April. Between April 1 and May 27, thyroxin decreased to 3.1 ng/ml and corticosteroids increased to 26.0 ng/mL.

Figure 14. Changes in the mean concentrations (n = 60 to 70) of plasma corticosteroids in coho salmon during smoltification at 10°C are compared to mean plasma thyroxin concentrations (n = 24) (from Chapter III, same fish) and gill Na-K-ATPase activity (laboratory-held coho salmon also at 10 C, taken from Zaugg and McLain 1970, 1976).

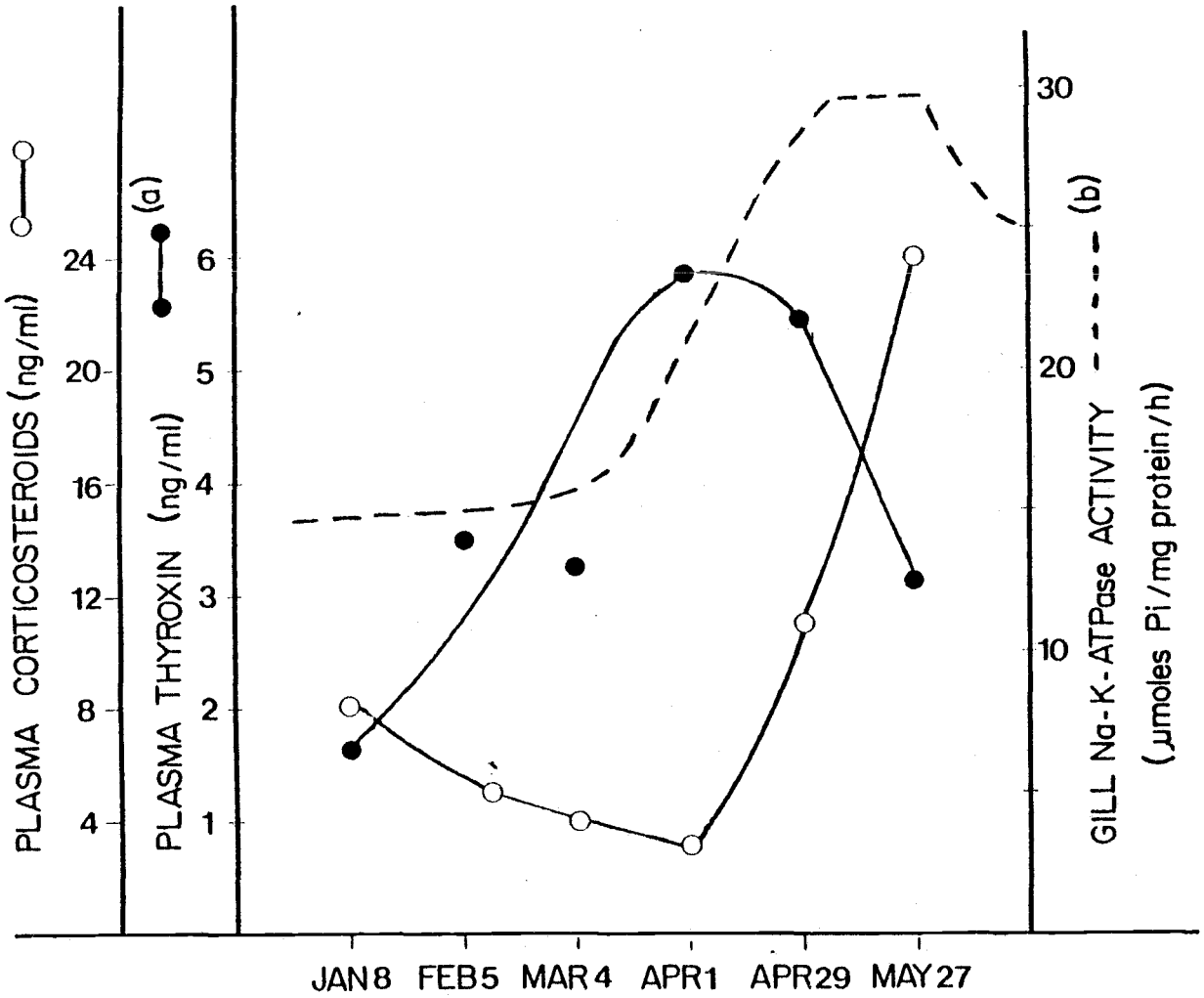
PLASMA CORTICOSTEROIDS (ng/ml)
PLASMA THYROXIN (ng/ml)
(a)
GILL Na-K-ATPase ACTIVITY
(µmoles Pi/mg protein/h)
(b)
24
20
16
12
8
4
6
5
4
3
2
1
30
20
10
JAN 8
FEB 5
MAR 4
APR 1
APR 29
MAY 27

Figure 14

The dynamics of plasma thyroxin and corticosteroid titers are particularly interesting with respect to changes in gill Na-K-ATPase activity during smoltification. The similarity in trends of thyroxin and gill Na-K-ATPase levels through time is striking. That thyroid hormones may increase Na-K-ATPase in teleost fishes has been suggested by correlative data in previous studies (see Introduction this Chapter). These data strengthen the contention that thyroid hormones have a role in increasing gill Na-K-ATPase activity during smoltification, because Na-K-ATPase activity repeatedly is found to double in the last two weeks of March (Zaugg and McLain 1970, 1976), during which time corticosteroids are still declining. However, corticosteroids may be critical to the maintenance of high Na-K-ATPase activity. The decline in gill Na-K-ATPase which occurs when salmonid smolts are retained in freshwater and revert to a parr-like appearance (Zaugg and McLain 1970) perhaps could be inhibited by injections of corticosteroids even in freshwater. On the other hand, the decline in gill Na-K-ATPase activity in parr-revertants probably reflects a decline in plasma corticosteroid levels.

To the preceding discussion must be added several caveats: First, although Zaugg and McLain (1976) demonstrated that temperature affects the rate of change in Na-K-ATPase activity in coho salmon, Ewing et al. (1979) suggest instead that the effects of temperature on enzyme activity are actually the result of different growth rates. Thus the data from Zaugg and McLain's work (1970, 1976) may not be directly comparable to this study even though the fish were raised at the same temperature; however, change in the coefficients of condition in the

fish in the present study and the fish in Zaugg and McLain's study (1976) were parallel (cf. Zaugg and McLain 1976 with Table 1 in Chapter III). Secondly, interpreting the physiological meaning of concentrations of circulating hormones can be problematic in that the kinetics are unknown. Low titers, such as observed for corticosteroids on April 1, may reflect an acceleration in tissue binding or clearance and thus increased physiological function. However, it remains common practice to assume that peripheral availability directly reflects the degree to which the hormone has a physiological role.

In conclusion, sPRL, oPRL, and bTSH have no effect on plasma corticosteroid concentrations during smoltification in coho salmon. Corticosteroid levels increase eight-fold in April and May in smolts retained in freshwater. During this time, gill Na-K-ATPase activity peaks and plasma thyroxin levels decline. The seaward migration of coho salmon begins in April, peaks in late May, and ceases by late June (Conte et al. 1966; Lorz and McPherson 1976). Thus corticosteroids are increasing when coho salmon enter seawater and elevated corticosteroid titers probably serve to maintain high Na-K-ATPase activity.

VI. SUMMARY

The endocrine system has an important role in the developmental changes that occur in anadromous salmonids in preparation for migration and existence in marine waters. The hormones of the hypothalamic-hypophysial system probably mediate the effects of environmental changes and endogenous rhythms on the timing and physiological alterations that occur during smoltification. Definite changes occur in the activity and abundance of the various cell-types of the adenohypophysis during smoltification (Olivereau 1954; Zambrano et al. 1972; Bern 1978). Histological indices suggest also that the thyroid cells (Hoar 1939; Baggerman 1960; Eales 1963) and interrenal tissue (Fontaine and Olivereau 1957, 1959; Olivereau 1962, 1975; McLeay 1975; Komourdjian et al. 1976) become more active during smoltification. The thyroid hormones and corticosteroids synthesized by these endocrine glands have been attributed with various changes that occur during this developmental transformation (see Chapters III and V). However, at the onset of the research presented herein, there was no information on the effects of any pituitary hormones on the levels of thyroxin or corticosteroids in the plasma of anadromous salmonids, nor on the actual concentrations of thyroxin and corticosteroids found in the plasma of salmonids undergoing smoltification.

Figure 15 summarizes the findings of my research and includes supportive evidence from other investigators. The PRL-producing cells of the adenohypophysis appear to become inhibited and the thyrotropes become active to a greater extent in smolts compared to parr

Figure 15. A model of the hormonal regulation of some aspects of smoltification. This model summarizes the research presented here, although undoubtedly many endocrines act in concert to mediate this developmental transformation. ↑ = an increase, ↓ = a decrease X = no effect, ⊕ = a stimulatory effect, ⊖ = an inhibitory effect.

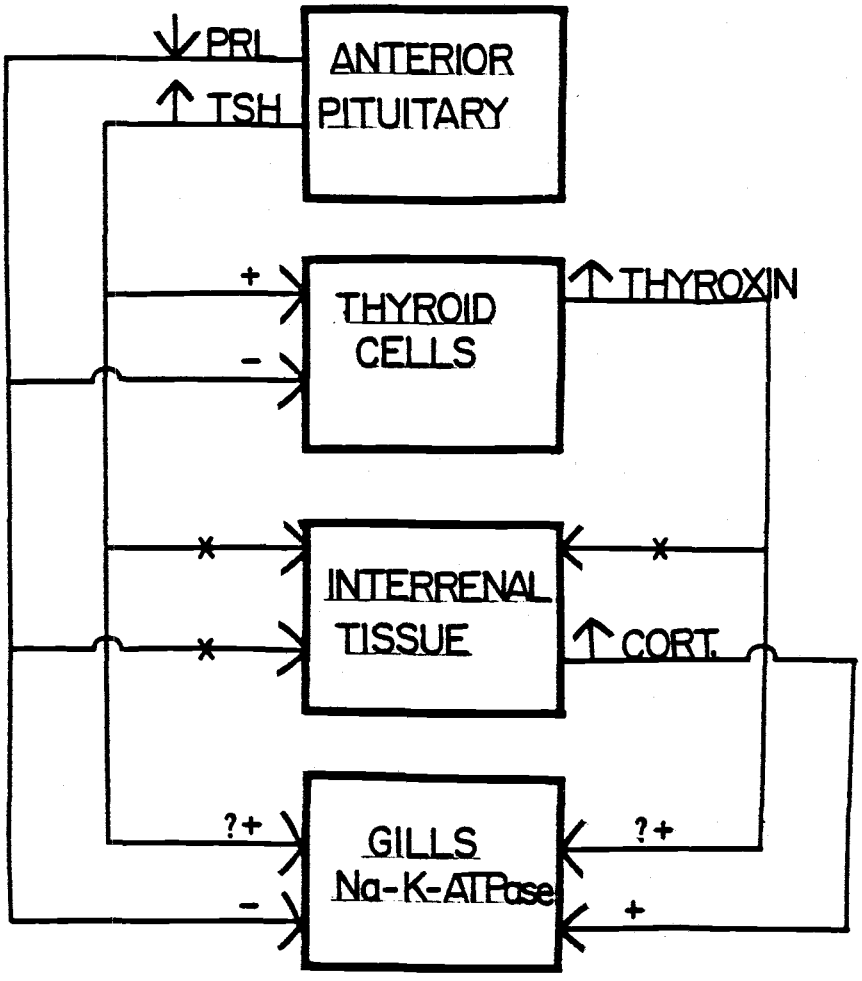
PRL
TSH
ANTERIOR PITUITARY
+
-
THYROID CELLS
THYROXIN
INTERRENAL TISSUE
CORT.
?+
-
GILLS Na-K-ATPase
?+
+

Figure 15.

(Olivereau 1954; Zambrano et al. 1972; Bern 1978). The production and secretion of TSH probably increases, which results in the increase in plasma thyroxin levels (data in Chapters III and IV; Dickhoff et al. 1978). Increased levels of TSH and thyroxin may induce or facilitate the increase in gill Na-K-ATPase activity, as evidenced by data presented in Chapters III, IV, and V. The apparent changes in PRL, TSH, and thyroxin production and secretion probably do not affect the increase in plasma corticosteroid levels, as indicated by data presented in Chapter V. Because exogenous PRL depresses plasma thyroxin levels (data in Chapter III) and can also depress gill Na-K-ATPase activity (Pickford et al. 1970; Epstein et al. 1980), the apparent reduction in PRL-cell activity may be permissive to the increases in thyroxin (data in Chapters III and IV) and gill Na-K-ATPase (references in Chapter V) observed during smoltification.

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