

AN ABSTRACT OF THE THESIS OF

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Title: Physiology of Interrenal Function in Juvenile Coho Salmon

(Oncorhynchus kisutch) and Effects of Hatchery Rearing Practices

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Carl B. Schreck

Periodic determinations of plasma hormone levels suggested that changes in interrenal activity are unrelated to changes in thyroidal or gonadal activity during development (smoltification) of juvenile coho salmon (Oncorhynchus kisutch). Moreover, in an experiment where fish were reared under various conditions of crowding and water inflow rate, it appeared that changes of interrenal activity were also unrelated to changes in gill Na/K-ATPase activity during smoltification. This finding placed some doubt on current hypotheses regarding the significance of interrenal tissue vis-a-vis osmoregulatory organs in fresh water during smoltification, since these hypotheses require a close positive correlation between changes in their activities. Also, the relationships I observed among the experimental rearing conditions (crowding and water inflow levels), water quality (oxygen and total dissolved ammonia levels), and the physiological status of the fish (plasma cortisol, thyroxine and triiodothyronine levels, and gill Na/K-ATPase activity) suggested that crowding stress itself is an important factor by which high rearing density affects the physiology of coho salmon. Lowering the rearing density from a high to a low level two weeks before sampling affected

plasma cortisol but not plasma thyroxine or gill Na/K-ATPase.

The main steroid secreted by unstimulated or ACTH-stimulated interrenal cells in vitro was cortisol. However, the main plasma corticosteroids were cortisol and cortisone, and the levels of both steroids increased during stress or seawater exposure. I concluded that cortisone arises from the peripheral transformation of cortisol and that a clarification of its significance is needed for an adequate understanding of corticosteroid physiology in teleosts.

An increase in extracellular osmolality or sodium levels within the physiological range slightly enhanced the spontaneous release of cortisol by interrenal cells in vitro, but it did not affect the steroidogenic response of the cells to ACTH. Potassium enhanced steroid secretion only when elevated to pharmacological levels; changes within the physiological range of this ion did not have modulatory effects on ACTH-induced steroidogenesis. Thus, in contrast to findings in other classes of vertebrates, changes in the levels of plasma monovalent ions or osmotic pressure may not be important factors directly regulating interrenal function in coho salmon.

Treatment of interrenal cells with forskolin enhanced cortisol secretion in vitro, and hydrolysis-resistant ATP analogs depressed the response of the cells to ACTH. Analogs of cAMP, but not of cGMP, enhanced steroid secretion. Thus, as in most other vertebrates, cAMP is a major mediator of ACTH-induced steroidogenesis in interrenal cells of coho salmon. However, in contrast to previous findings in other vertebrates, phosphatidylinositol and cholera toxin did not affect interrenal steroidogenesis in coho salmon.

Physiology of Interrenal Function in Juvenile  
Coho Salmon (Oncorhynchus kisutch)  
and Effects of Hatchery Rearing Practices

by

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I dedicate this dissertation to my family: To Teresa, to our son Reynaldo, and to the little one now in Teresa's womb.

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My major professor is coauthor of chapters II through VI. In Chapter III, Mr. Joe Banks was a principal designer of rearing methods for the experimental fish at the production hatchery; Dr. Wally Zaugg

helped in the sampling and did the ATPase measurements. I recognized Mr. Banks and Dr. Zaugg as coauthors of Chapter III. In Chapter IV, Dr. Mike Redding gave significant advice and also helped in all experiments for this chapter, and thus I granted him coauthorship. I received significant help from Mr. Sam Bradford in my experiments of Chapter VI and he is therefore coauthor of this chapter.

Finally, I would like to acknowledge in a very special way the role of my wife, Maria Teresa, who throughout all my years as Ph.D. student provided me not only her unfaltering love and moral and financial support, but also her excellent technical assistance and encouraging company during many field trips.

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PHYSIOLOGY OF INTERRENAL FUNCTION IN JUVENILE  
COHO SALMON (ONCORHYNCHUS KISUTCH)  
AND EFFECTS OF HATCHERY REARING PRACTICES

I: GENERAL INTRODUCTION

Background

The endocrine system has been long recognized as a very important chemical link between the environment and the biological functions of an organism (Hoar, 1965). Knowledge of this system, therefore, is a pivotal step towards understanding the mechanisms by which individual organisms adapt to their environment as well as the mechanisms by which they undergo substantial biological transformations in response to environmental signals.

Much attention has been focused recently on the endocrine regulation of development of juvenile salmon, especially the developmental stage known as smoltification (e.g., see reviews by Barron, 1986, and Specker, 1988). During smoltification, stream-dwelling juvenile anadromous salmonids transform into migratory (seaward-going) individuals capable of surviving and growing in seawater; i.e., of adapting to seawater. The process of transformation comprises a complex variety of physiological, morphological and behavioural changes (Hoar, 1976).

The interrenal cells of the head kidney have been implicated as part of this endocrine regulation of smoltification and seawater adaptation of salmonids. The products of the interrenal cells, the

corticosteroids, may be involved in smoltification. Much of the evidence for this view comes from histological observations of interrenal cell activity (Fontaine and Olivereau, 1957, 1959; McLeay, 1975; Komourdjian et al., 1976) as well as from measurements of circulating levels of corticosteroids during smoltification (Langhorne and Simpson, 1981; Specker and Schreck, 1982; Barton et al., 1985; Virtanen and Soivio, 1985). As reviewed by Specker (1982), some researchers believe that a major function of cortisol in smoltification is the "preadaptation" of the freshwater fish to seawater residence by inducing the activity of seawater osmoregulatory mechanisms while the fish are still in freshwater (see also Richman and Zaugg, 1987; and discussion below). Also, Maule et al. (1987) suggested that cortisol may serve to avoid tissue damage during putative tissue changes that occur during smoltification by suppressing immune function. Langhorne and Simpson (1986), however, suggested that the increased interrenal activity seen in smolting fish may not be a "cause" of smoltification, but the "effect" of stressful changes in the osmoregulatory status of the fish during this time. Therefore, in contrast to the view espoused by Specker (1982), Langhorne and Simpson (1986) in essence proposed that cortisol serves to "readapt" the smolting salmon to the freshwater environment if they do not reach, or are not exposed to, seawater at the appropriate stage of development.

The evidence supporting a role for corticosteroids in the seawater adaptation of teleosts (e.g., Maetz, 1969; Johnson, 1973), although perhaps clearer than in the case of smoltification, is not

well established for all species of teleosts studied to date. Richman et al. (1987) suggested that cortisol-replacement treatment can partially ameliorate the deleterious effects of hypophysectomy--which reduces endogenous plasma cortisol levels by eliminating the source of ACTH--on the seawater adaptability of juvenile coho salmon (Oncorhynchus kisutch). Also, Richman and Zaugg (1987) reported that exogenous cortisol enhanced gill chloride cell number and Na/K-ATPase activity in intact, freshwater coho salmon prior to smoltification (before the smoltification-related increase in plasma cortisol). On the other hand, Langdon et al. (1984) observed no effects of exogenous cortisol on gill chloride cell size and number or gill Na/K-ATPase activity in Atlantic salmon (Salmo salar) juveniles at a similar stage of development as the coho in Richman and Zaugg's (1987) study. Also, Redding et al. (1984) observed a decrease in gill Na/K-ATPase in yearling coho salmon after treatment with cortisol in June. Other similar discrepancies among various studies have been noted previously (e.g., Richman and Zaugg, 1987; and references therein).

It is well established that the brain-pituitary-interrenal axis is a major pathway for the control of interrenal function in fishes (Donaldson, 1981). It is unclear in salmon, however, how the activity of this axis is regulated by the environment during smoltification either under natural circumstances or under artificial conditions such as those found in hatcheries. Although conditions of crowding or water quality may affect smoltification of hatchery salmon (Hosmer et al., 1979), the mechanisms by which these conditions may influence the

physiological development of the fish, including its interrenal function, are unknown.

The role of extrapituitary factors (extracellular and intracellular), including other hormones, in the regulation of interrenal function of teleosts is also not well established. Seawater adaptation or stress, for example, produce large changes in the ionic and osmotic levels of plasma in fishes (Eddy, 1981; Redding and Schreck, 1983; Hegab and Hanke, 1984), but the effects of these changes on interrenal physiology are unknown. Moreover, although the levels of sex steroids in plasma may change during smoltification (Sower et al., 1984), no information is available to determine if a relationship between gonadal and interrenal activities exists in juvenile salmon as it clearly exists in the case of adult salmon during maturation (Donaldson and Fagerlund, 1972; and references therein). Finally, it is unclear whether cortisol, virtually the only corticosteroid which has been studied in teleosts, is the single most prominent--or biologically significant--corticosteroid in these fishes and particularly in salmonids (e.g., Idler and Truscott, 1972; Weisbart and McGowan, 1984).

#### Purposes of Dissertation

A fuller picture of interrenal function and regulation in coho salmon would benefit not only the comparative endocrinologist, who may use this information to discern evolutionary patterns of interrenal function in vertebrates; but also the fish physiologist and

developmental biologist, who may gain further insights and answer some of the pending questions on the role of the interrenal tissue in juvenile salmon physiology and development. Moreover, and on a more practical (applied) level, knowing how the environment affects the physiological development of coho salmon juveniles may help in the establishment of better hatchery techniques for salmon culture. The practice of salmon aquaculture involves artificial rearing of the fish from fertilization to smolting (salmon ranching) or harvest (net-pen rearing), and has become an important tool for the preservation of anadromous salmonid runs. These runs have significant commercial as well as esthetic values throughout (mainly) the Northern Hemisphere.

Therefore, the immediate objective of this dissertation is to better understand the regulation of interrenal function in juvenile coho salmon, from both a developmental and a routine physiological perspective; and the ultimate goal is to enhance the quality of hatchery-produced coho salmon--i.e., its survivability to adulthood--by attempting to find, based on physiological data, better ways of rearing these fish.

#### Organization of Dissertation

The results of my thesis are presented in Chapters II through VI and in the Appendix. These chapters were prepared in manuscript form, and therefore each contains a rather restricted, although self-explanatory, introduction to its specific subject of study; a

description of the methods used and of the results; and a discussion of the results within the context of each study. Finally, an overall conclusion using both a developmental and an evolutionary approach is presented in Chapter VII.

Chapter II (Patino and Schreck, 1986) is a study of the relationship between sex steroids and smoltification, and of the relationships among these hormones, thyroxine and corticosteroids in coho salmon during this period. I had intended to pursue this line of investigation further; but the results of Chapter II were largely negative and, therefore, in the remainder of my dissertation I focused more on the area of interrenal physiology. Chapter III (Patino et al. 1986b), IV (Patino et al., 1987), V (Patino and Schreck, 1988), and VI (Patino et al., 1986a), and the Appendix consist of studies that seek to establish some basic facts about the control of interrenal function in juvenile coho salmon; namely, the relation of interrenal function to the external environment (social and physical) as well as the internal environment (extracellular and intracellular). The underlying theme of these chapters is interrenal physiology, while their overall purpose, as previously noted, is to understand interrenal function during development and environmental challenge.

II: SEXUAL DIMORPHISM OF PLASMA SEX STEROID LEVELS IN  
JUVENILE COHO SALMON DURING SMOLTIFICATION

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## Abstract

Concentrations of plasma sex steroids, cortisol, and thyroxine were measured by radioimmunoassay in hatchery coho salmon (Oncorhynchus kisutch) during winter and early spring. Mean plasma 11-ketotestosterone (11-KT) and estradiol levels fell into two distinct categories: 11-KT was 181-373% higher in males than in females, and estradiol was 109-143% higher in females than in males. No changes in plasma levels of estradiol in fish of both sexes, or in levels of 11-KT in males, were evident during spring when plasma thyroxine and cortisol were markedly elevated indicating that the fish were undergoing smoltification. Although plasma 11-KT in females appeared to be lower in late April than in February, it showed no correlation with plasma thyroxine or cortisol in these individuals. Our finding of sexual dimorphism in  $17\alpha$ - $20\beta$ -dihydroxy-4-pregnen-3-one was inconsistent between stocks of fish and among sampling dates, thus making interpretation of the results difficult. However, no relationship between this steroid and plasma thyroxine or cortisol was observed. Therefore, plasma levels of sex steroids do not seem to be related to the changes in plasma thyroxine or cortisol observed during smoltification of coho salmon.

## Introduction

Smoltification of salmonids is characterized by a series of physiological, morphological, and behavioral changes that prepare the fish for seawater residence (Hoar, 1976). The physiological changes observed during smoltification include, and perhaps are controlled by, several endocrinological transformations such as increases in activities of the thyroid (see Folmar and Dickhoff, 1980) and interrenal (see Specker, 1982) glands. Growth (Komourdjian et al., 1976), urophyseal (Nishioka et al., 1982) and corpuscles of Stannius (Aida et al., 1980) hormones may also be involved in smoltification.

Sex steroid hormones have recently been the subject of several studies related to smoltification; however, the results obtained from these studies were conflicting. Nagahama et al. (1982), who studied amago salmon (Oncorhynchus rhodurus) during smoltification, reported that plasma testosterone levels were low in immature males and that plasma estradiol was undetectable (less than 30 pg/ml) in females. Moreover, Ueda et al. (1983) found that  $17\alpha$ - $20\beta$ -dihydroxy-4-pregnen-3-one ( $17\alpha$ - $20\beta$ -OH-P), a steroid involved in final maturation in teleosts (Young et al., 1983), did not seem to undergo changes during the early period of smoltification in immature male amago salmon. In contrast, Sower et al. (1984) reported that estradiol levels in plasma from unsexed juvenile coho salmon (O. kisutch) significantly increased twice during smoltification: once in early April (from 94 to 142 pg/ml) and again in late May-June (from 80 to 219 pg/ml). Furthermore, Sower et al. (1984) found that the fluctuations in plasma

estradiol closely preceded the changes observed in plasma thyroxine, and suggested that (1) estradiol may have a regulatory influence on plasma thyroxine, or (2) these two hormones respond to the same environmental cues during smoltification.

The purpose of our study was to characterize the plasma levels of sex steroids (11-KT, estradiol and  $17\alpha$ - $20\beta$ -OH-P) in male and female hatchery coho salmon during the last few months before their release from the hatchery. We also measured plasma cortisol and thyroxine, two putative smoltification "markers", to enable us to relate our findings to the process of smoltification.

#### Materials and Methods

Animals. Yearling coho salmon from Fall Creek Hatchery (Oregon Department of Fish and Wildlife) and Eagle Creek (Oregon) National Fish Hatchery (U.S. Fish and Wildlife Service) were used for this study.

Experimental design. In 1983, we conducted a preliminary study at Fall Creek to compare plasma thyroxine and cortisol profiles during smoltification of fish reared in a raceway ( $82\text{ m}^3$ ) with those of fish held in a large rearing pond ( $768\text{ m}^3$ ). In 1984, fish were sampled only from the rearing pond at Fall Creek (fish from the raceway were not available) and from a raceway ( $45\text{ m}^3$ ) at Eagle Creek. Rearing densities ( $\text{kg}/\text{m}^3$ ) at the time of release (first week of May) were about 7 at the Fall Creek pond, 21 at the Fall Creek raceway, and 22 at the Eagle Creek raceway (fish are released from the pond at Fall

Creek in mid-March and again in mid-April before final release in early May). In 1984, the midday water temperature (measured at the time of sampling, 11 am-1 pm) at Fall Creek was 2-3 C in January, but remained between 8 and 10 C thereafter; at Eagle Creek, the water temperature was 4.5-7 C throughout the sampling period. The fish at both hatcheries were fed commercial fish diet daily.

Blood samples were collected at approximately 2-week intervals. Fish were quickly taken with a dip-net from the lower, middle, and upper sections of the raceways; at the Fall Creek pond, only fish from the lower end were collected. The fish were immediately killed in a solution of the anesthetic tricaine methanesulfonate (200 mg/l), and blood was taken into heparinized capillary tubes from the severed caudal peduncle. The plasma was separated by centrifugation and stored at -15 C for later hormone analyses (thyroxine, cortisol and  $17\alpha$ - $20\beta$ -OH-P for all samples; estradiol for the Fall Creek and 11-KT for the Eagle Creek samples). In 1984, the sex of the fish was determined by visual inspection of the gonads.

From beginning to end of the sampling period in 1984, mean fork lengths and weights of the fish changed from 12.7 to 14.1 cm and 23.3 to 30.3 g at Eagle Creek, and from 11.1 to 15.6 cm and 16.2 to 40.7 g at Fall Creek.

Radioimmunoassays. Plasma cortisol was directly measured in  $10 \mu$ l of plasma as described by Redding et al. (1984) with two modifications: (1) the amount of  $1,2,6,7$ - $^3$ H-cortisol was reduced to  $0.02 \mu$ Ci/tube to increase the sensitivity of the assay; and (2) standards were made in juvenile coho "stripped" plasma (Abraham et

al., 1977) to improve the linearity of and parallelism between the standard curve and untreated plasma dilutions at low (less than 50 pg/tube) cortisol concentrations (Brown et al., 1984). Plasma estradiol was determined (in 25-50  $\mu$ l of plasma) according to the methods of Sower and Schreck (1982). In assaying levels of 11-KT (in 25  $\mu$ l) and  $17\alpha$ - $20\beta$ -OH-P (in 15-30  $\mu$ l) we used a system similar to that used to measure androgens by Sower and Schreck (1982) and modified by Fitzpatrick (1985). Thyroxine (in 10  $\mu$ l) was assayed according to Dickhoff et al. (1978). All radioimmunoassays were performed on individual plasma samples except for the  $17\alpha$ - $20\beta$ -OH-P assay, which required the pooling of equal volumes of plasma from 2-3 fish for some of the samples collected in January-March. However, plasma pooling was done according to sex. The sensitivities (Abraham et al., 1977) of the steroid radioimmunoassays (pg/tube) were 5-10 for cortisol, about 1 for 11-KT, less than 1 for estradiol, and about 2 for  $17\alpha$ - $20\beta$ -OH-P. The crossreactivity of the 11-KT antibody with testosterone was less than 2% (Fitzpatrick, 1985), and that of the  $17\alpha$ - $20\beta$ -OH-P antibody with cortisol was less than 0.01%

Statistical analyses. The effects of sex and season on plasma hormone levels were determined with the regression approach to 2-way ANOVA (sex X sampling date). If significant effects of sampling date on plasma levels of sex steroids were found, the means (separated by sex) were compared with the Student-Newman-Keuls test, and their correlations with plasma thyroxine or cortisol were also determined using Pearson's correlation coefficients. The  $17\alpha$ - $20\beta$ -OH-P values

determined on pooled plasma samples were not used in the statistical analysis. However, these values were assigned to each sample that contributed to the pool and used to estimate the mean value for the respective experimental group (shown in the figures). An alpha level of 0.05 was chosen to judge the significance of statistical differences. We used the Statistical Package for the Social Sciences (Version 9.0; Northwestern University) for all statistical analyses.

### Results

Mean concentrations of plasma 11-KT (Fig. II-1a) and estradiol (Fig. II-1b) during coho salmon smoltification fell into two very distinct categories: male salmon had markedly higher (181-373%) 11-KT levels than females ( $P \leq 0.001$ ) and females had higher (109-143%) levels of estradiol than males ( $P \leq 0.034$ ). Main effects of sampling date on plasma 11-KT levels ( $P \leq 0.044$ ) were suggested by the overall ANOVA, and the multiple range test indicated that the levels in females were slightly lower at the last sampling date (April 27) than at the second and third samplings (February 17 and 29). However, correlation analysis showed no relationship between 11-KT in females and plasma thyroxine or cortisol. The levels of 11-KT in males, or levels of estradiol in both sexes, did not change during our sampling period.

A sexual dimorphism in plasma  $17\alpha$ - $20\beta$ -OH-P titers was apparent early during the sampling period in Eagle Creek salmon, with males having higher levels than females ( $P \leq 0.003$ ; Fig. II-1c). Moreover,

Figure II-1. (a) Plasma thyroxine, cortisol, and 11-ketotestosterone levels (solid triangles, males; open triangles, females) in yearling coho salmon from Eagle Creek National Fish Hatchery; (b) plasma thyroxine, cortisol, and estradiol levels (solid squares, males; open squares, females) in yearling coho salmon from Fall Creek Hatchery; (c) mean plasma  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ( $17\alpha-20\beta$ -OH-P) levels (see text) in yearling coho salmon from Eagle Creek National Fish Hatchery (solid circles, males; open circles, females) and Fall Creek Hatchery (stars, male and female combined values). Bars represent standard errors of the mean, and numbers in parentheses, the number of individuals analyzed. Samples were collected in 1984.

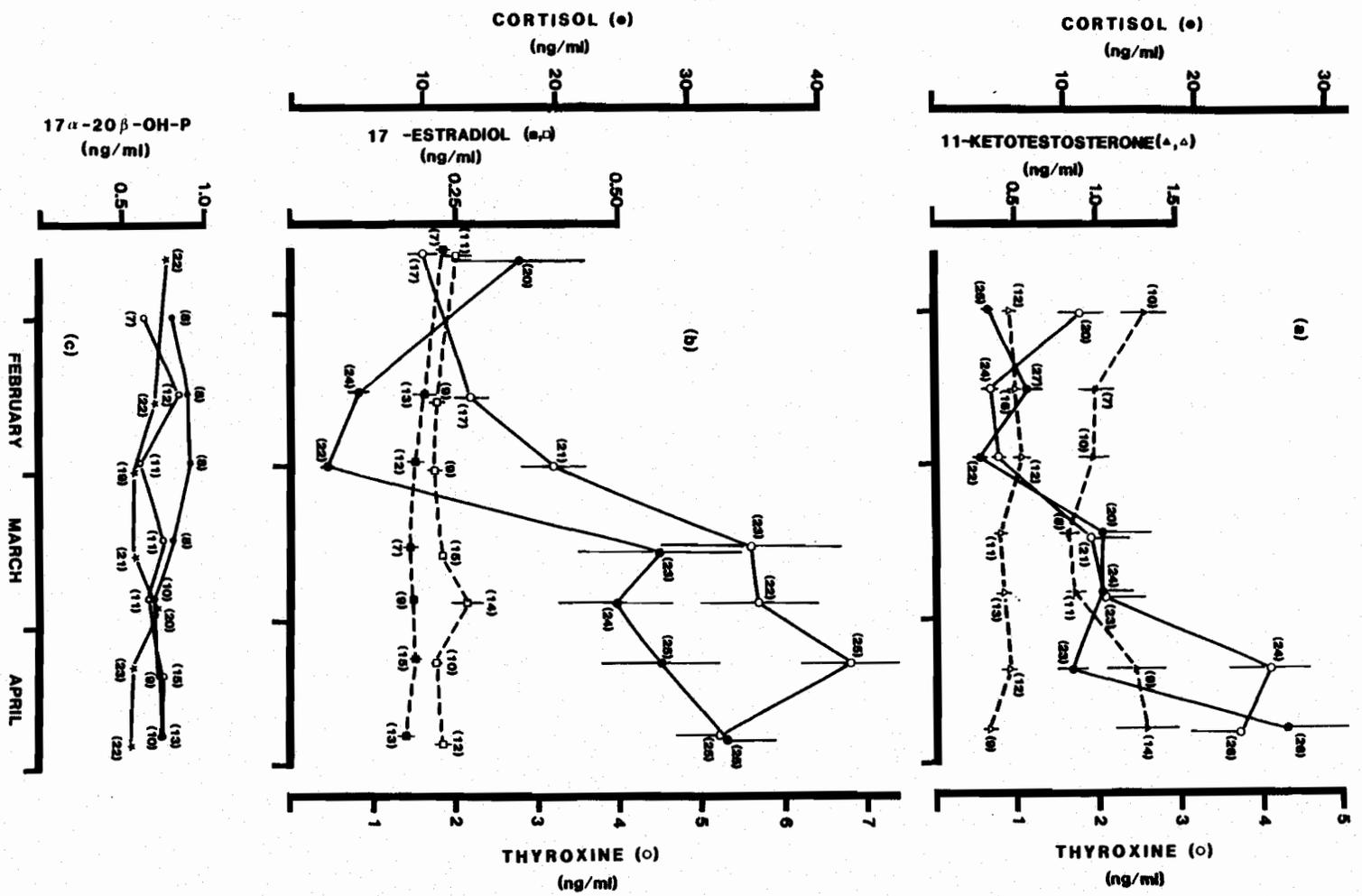


Figure II-1

although no main effects of sampling date on  $17\alpha$ - $20\beta$ -OH-P were observed, there was a significant interaction between sex and sampling date ( $P \leq 0.01$ ), again suggesting that the sexual dimorphism found for this hormone was dependent on the sampling date. Multiple range analysis showed that  $17\alpha$ - $20\beta$ -OH-P declined in Eagle Creek males (not in females) from February through April ( $P \leq 0.05$ ; see also Fig. II-1c), but no correlation with plasma thyroxine or cortisol was observed. No sexual differences or seasonal fluctuations in the plasma levels of  $17\alpha$ - $20\beta$ -OH-P were apparent in Fall Creek salmon (Fig. II-1c).

Plasma cortisol and thyroxine did not differ between male and female salmon. The levels of these hormones were generally low in winter and increased in spring ( $P \leq 0.001$ ; Figs. II-1a, b, II-2). However, high mean values of cortisol were occasionally seen in early winter (Figs. II-1b, II-2).

At Fall Creek, the plasma thyroxine profiles were similar in fish reared in the raceway and pond in 1983, although the absolute levels may have been lower in fish from the raceway (Fig. II-2). In contrast, the timing and profile of plasma cortisol changes in fish from the raceway differed from those of fish in the pond (Fig. II-2). These findings may indicate, at least in terms of plasma cortisol levels, either that the characteristics of the salmon population from the raceway differed from those of the pond, or that samples collected from the pond were biased due to our sampling technique (no clear trends were observed, however, among fish collected from different sections of the raceways). The patterns of plasma thyroxine and

Figure II-2. Plasma thyroxine and cortisol in yearling coho salmon from Fall Creek Hatchery reared in a raceway (dashed lines) or a large rearing pond (solid lines). Bars represent standard errors of the mean, and numbers in parentheses, the number of individuals analyzed. Samples were collected in 1983.

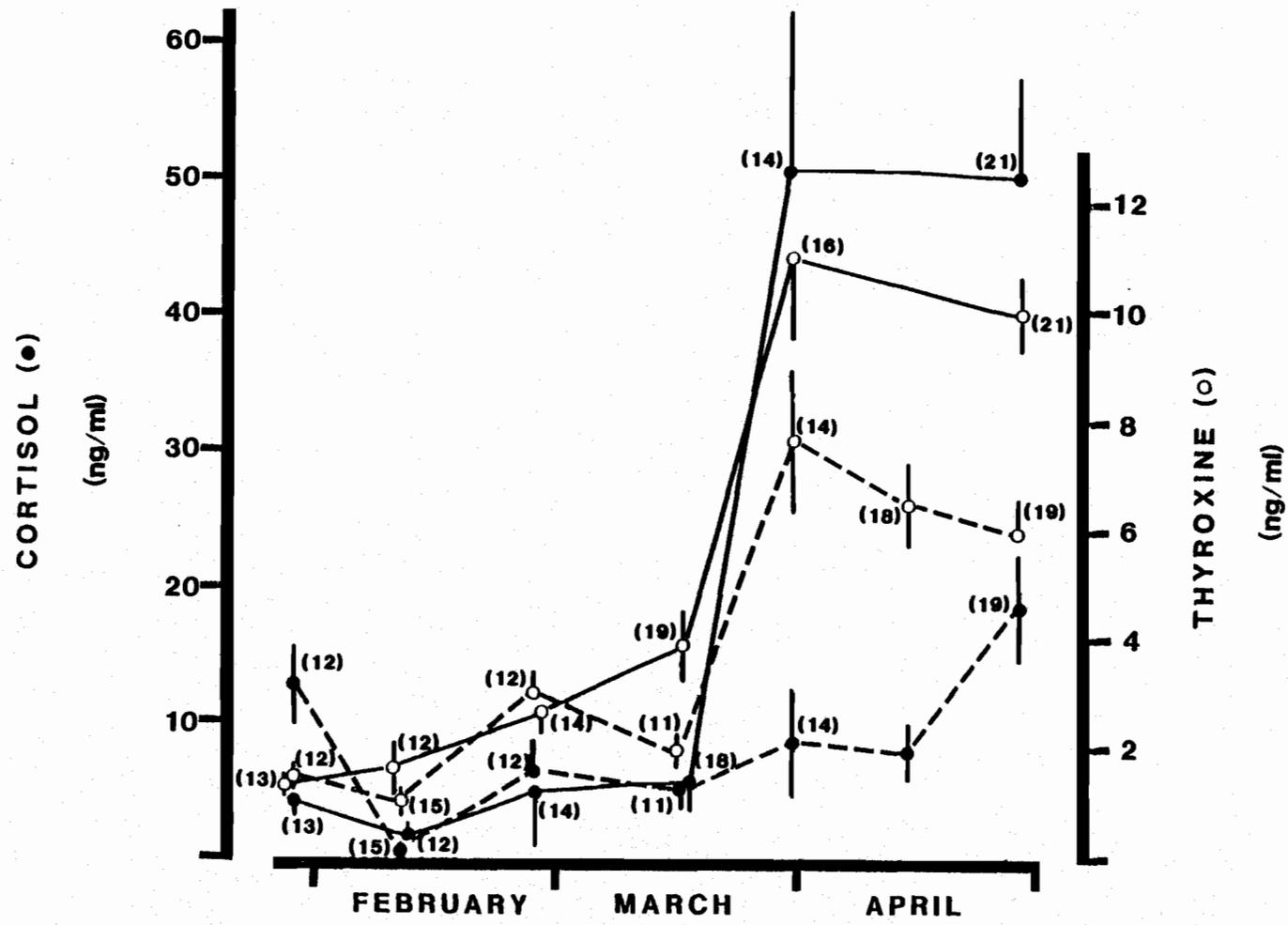


Figure II-2

cortisol levels in fish from the pond were consistent from year to year (Figs. II-1b, II-2).

### Discussion

The results of our study showed that sexual dimorphism of plasma androgens and estrogens can be detected in juvenile coho salmon at least as early as the latter part of their fresh water phase. Sexual dimorphism of plasma androgens has been also found in young rainbow trout (Salmo gairdneri) and brook trout (Salvelinus fontinalis) about 4-5 months before functional maturity (Sangalang et al., 1978); and in juvenile rainbow trout after gonadotropic hormone treatment (Crim et al., 1982).

The significance of the sexual dimorphism of plasma sex steroid levels at this early stage of coho salmon development is uncertain. However, it is well known that sex steroids can greatly influence and even reverse the sex of the early gonad in fishes (Yamamoto, 1969; Schreck, 1974; Hunter and Donaldson, 1983). These findings led to the hypothesis that sex steroids are the natural inducers of gonadal differentiation (Yamamoto, 1969). More recently, in vitro experiments with the gonads of rainbow trout fry have shown that sexual dimorphism of gonadal steroidogenesis does indeed appear at a very early age (100 days post-fertilization; van den Hurk et al., 1982). Thus, it seems likely that sexual dimorphism of plasma sex steroid levels may be recognized in coho salmon even younger than those used in our experiment, and could be related to the early sexual development of the fish.

In contrast to Sower et al. (1984), we found no change in plasma estradiol levels of yearling coho salmon during April. Similarly, plasma 11-KT in male coho salmon did not show seasonal changes. In female salmon, however, the levels of 11-KT appeared to be lower in late April than in February, although this conclusion may be equivocal since it is based on our findings on only one sampling date. It is possible that we would have observed the changes in plasma estradiol found by Sower et al. (1984) if we had monitored our fish a few weeks longer; the largest plasma estradiol changes in their fish occurred in late May and June, and our fish were released from the hatcheries in early May. Another possibility is that differences in the stock of fish or rearing conditions affect the pattern of potential sex steroid changes during smoltification. On the other hand, we found no relationships between plasma levels of estradiol (or other sex steroid) and those of thyroxine or cortisol, even during spring when the latter were markedly elevated indicating that our fish were smolting. Thus, our results failed to confirm the positive correlation between changes in plasma estradiol and thyroxine observed by Sower et al. (1984), and do not support the suggestion of a possible regulation of plasma thyroxine by estradiol during smoltification of coho salmon.

Nagahama et al. (1982) found low levels of sex steroids (androgens in males and estrogens in females) in immature amago salmon during smoltification in the fall. Moreover, they found that plasma estradiol in females increased from March through May only after

smoltification had occurred . Thus, it appears that in amago salmon (Nagahama et al., 1982) and coho salmon (present results), increases in plasma thyroxine during smoltification are not necessarily associated with changes in plasma sex steroid levels. However, both fishes may show elevations in plasma estradiol during spring (Nagahama et al., 1982; Sower et al., 1984). On the other hand, high plasma androgen levels may inhibit the process of smoltification in precocious male salmonids (Aida et al., 1984).

The inconsistency of our findings regarding  $17\alpha$ - $20\beta$ -OH-P levels in plasma of juvenile salmon may indicate that the sexual dimorphism (males having higher levels than females) observed during winter in Eagle Creek salmon was an artifact of the low sample sizes (since pooled samples were not included in the statistical analysis, the sample sizes were as low as 4 per sampling date early during the experiment). However, when the mean values (which included all samples) were graphically compared (see Fig. II-1c), a similar trend was observed. Moreover, the in vitro secretion of  $17\alpha$ - $20\beta$ -OH-P (determined by radioimmunoassay) by the testes of juvenile coho salmon (Oregon Aqua-Foods stock) in response to partially purified salmon gonadotropin (SG-G100) appeared to be higher than that by the ovaries (R. Patiño, M.S. Fitzpatrick, and C.B. Schreck, unpublished). Thus, it seems possible to us that sexual dimorphism of this plasma steroid occurs in juvenile coho salmon depending perhaps on genetic or environmental factors.

In conclusion, we demonstrated a consistent sexual dimorphism of plasma 11-KT and estradiol levels in juvenile coho salmon prior to and

during smoltification. Moreover, plasma levels of sex steroids were not related to plasma thyroxine or cortisol during smoltification.

#### Acknowledgments

We thank M. Teresa Patiño, Martin S. Fitzpatrick, and David Oberbillig for their excellent technical assistance during sampling at the hatcheries; and the Oregon Department of Fish and Wildlife and U.S. Fish and Wildlife Service for providing the fish. The antisera for 11-KT and 17 $\alpha$ -20 $\beta$ -OH-P were generous gifts from Dr. Y. Nagahama and Dr. A.P. Scott, respectively. The funds for this study were partly provided by the NOAA Office of Sea Grant, Department of Commerce, under Grant No. NA81AA-D-00086.

III: EFFECTS OF REARING CONDITIONS ON THE  
DEVELOPMENTAL PHYSIOLOGY OF SMOLTING COHO SALMON

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## Abstract

We determined the effects of rearing conditions on the physiological development of coho salmon Oncorhynchus kisutch during smoltification. Combinations of various levels of both rearing density and water inflow rate were used in this study. The experiments were performed at a production hatchery and at our laboratory.

High rearing density resulted in reduced levels of plasma thyroid hormones in the fish, but no effect of water inflow rate on these hormones was recognized. Although rearing density and water inflow rate affected plasma cortisol and gill Na,K-ATPase activity, their effects on these physiological variables seemed to vary through time producing different developmental patterns of physiology during smoltification. Measurements of plasma corticosteroid clearance and in vitro activity of interrenal cells suggested that rearing density affected plasma cortisol levels primarily by altering the submaximal activity of the interrenal cells. Lowering the rearing density of fish from high to low 2 weeks before sampling resulted in cortisol levels similar to those of fish reared at low density throughout the experiment; however, plasma thyroxine and gill Na,K-ATPase activity were not affected. The relationships observed among experimental rearing conditions, water quality, and physiological status of the fish suggested that crowding stress itself may be an important factor by which high rearing density affects the physiology of coho salmon.

## Introduction

Hatchery practices such as high rearing density can affect the physiological status of juvenile anadromous salmonids (Fagerlund et al., 1981, 1983; Schreck et al., 1985). Consequently, the process of smoltification, which is characterized by a variety of marked changes in behaviour, morphology, and physiology (Hoar, 1976), may also be affected. (Among the physiological changes occurring during smoltification are increased plasma levels of cortisol and thyroid hormones as well as gill Na,K-ATPase activity [e.g., Specker, 1982].) Indeed, the rate of return of adult coho salmon Oncorhynchus kisutch may be inversely correlated with the prior hatchery rearing density of juveniles (Fagerlund et al., 1983). Increasing rearing densities as well as decreasing water inflow rates also appear to reduce the rate of return of Atlantic salmon Salmo salar (Hosmer et al., 1979).

Poor water quality (Smart, 1981) and crowding stress (Schreck, 1981) have adverse consequences on the health of hatchery fish. However, the relative importance of these two factors as mediators of the deleterious action of high rearing densities on coho salmon (Fagerlund et al., 1983; Schreck et al., 1985) is unclear.

Identifying the major factors by which rearing density affects the fish is desirable because this information could be used to improve present hatchery practices. Moreover, it is of interest to know if fish could recover from the effects of high rearing density by a reduction in density ("thinning") shortly before release from the hatchery; for example, release of fish from a hatchery could be

staggered around the optimal time of release in order to allow some of the fish to recover from the effects of high density.

The hypothalamic-pituitary-interrenal axis may be important for the response and acclimation of fish to environmental (biotic or abiotic) stress (Donaldson, 1981; Schreck, 1981). However, the involvement of this axis in the physiological changes of juvenile salmonids under differing rearing conditions is unclear. Some studies have found no differences in plasma cortisol levels of salmonids reared under varying densities (Fagerlund et al., 1983; Schreck et al., 1985) whereas others have reported an inverse relationship between rearing density and plasma cortisol (Leatherland and Cho, 1985). Also, histological observations of interrenal cells have produced conflicting results (Fagerlund et al., 1981, 1983; Schreck et al., 1985). Research on the functional characteristics of the interrenal cells under different conditions of rearing may help elucidate their role during development of hatchery salmon.

At our laboratory, Schreck et al. (1985) studied the effects of rearing density on clinical indices of smoltification and "performance" of coho salmon. However, their study was performed at a constant water inflow rate and thus did not distinguish between the effects of water quality and those of social and other physical factors associated with crowding of the fish. Moreover, Schreck et al. (1985) sampled their fish only once, shortly before release from the hatchery and, therefore, no conclusions regarding the effects of rearing density on the pattern of physiological development of their fish could be made.

Here we have expanded the study of Schreck et al. (1985) by repeatedly sampling fish before their release from the hatchery, and by using water inflow rate as an additional rearing condition variable. Our objectives were (1) to monitor the effects of rearing density and water inflow rate on the developmental physiology of coho salmon during the last 2 months of hatchery rearing; (2) to determine the effects of rearing conditions on the secretory characteristics of interrenal cells; (3) to determine the physiological effects of thinning; and (4) to distinguish the effects of crowding stress (due to social factors and physical factors other than water quality) and water quality on the physiology of the fish.

#### Materials and Methods

Rearing sites and conditions. Experiments were performed at Willard National Fish Hatchery (NFH), of the U.S. Fish and Wildlife Service (Cook, Washington), and at Oregon State University (Corvallis) with coho salmon of the 1981 and 1982 brood years.

Juvenile coho salmon were reared under various test conditions at Willard NFH for 11 months (in 1982-1983 and in 1983-1984) until their release in early June. Outdoor raceways (volume, 36,000 l) were randomly stocked with 25,000 (low density), 50,000 (medium density--approximately the production density normally used at the hatchery), or 75,000 (high density) underyearling coho salmon, and each density was combined with water inflow rates of 757 (low inflow), 1,514 (medium inflow), or 2,271 (high inflow) l/min. All treatments were

conducted in two replicated raceways. Water temperatures were 6-8° throughout the sampling period. Fish were fed Oregon Moist Pellets several times a day at a total ration of 0.5% of body weight daily.

For the physiological analyses at Willard NFH, we used fish from the following treatments: high density-high inflow (HD-HI), high density-low inflow (HD-LI), low density-high inflow (LD-HI), low density-low inflow (LD-LI), and medium density-medium inflow (MD-MI). In 1983, we sampled fish from these experimental raceways between 1100 and 1300 hr. The fish were not fed while samples were being taken from the raceways.

To perform further tests on the effects of rearing conditions and thinning, we transported yearling coho salmon (1982 brood year) from each replicate of the HD-HI, HD-LI, LD-HI, and LD-LI treatments at Willard NFH to Smith Farm on 26 April 1984. The fish were placed in flow-through circular tanks (diameter, 0.91 m; volume, 155 l) at about their original hatchery densities (330 and 110 fish/tank for the high and low densities). For the experiments designed to ascertain the effects of thinning on the physiological status of the fish, a third HD replicate was set up for each of the high and low water inflow regimes with fish from the appropriate HD raceways at Willard NFH. The water temperature at Smith Farm was 12-13 ° throughout the experiment. To compensate for the higher temperature (and thus higher metabolic rate of the fish) and lower oxygen content of the water source at Smith Farm, we used greater water flows than at Willard NFH; the high and low inflow rates were 29.1 and 13.6 l/min, respectively. The fish

were reared under natural photoperiod and were fed Oregon Moist Pellets twice daily at a total ration of 1% of body weight daily. To alleviate a condition of bacterial kidney disease diagnosed in the fish at Willard NFH in 1984, the fish brought to Smith Farm were treated with erythromycin administered with the diet at a rate of 0.01% of body weight daily during the first 2 weeks of May. Based on the external characteristics and behaviour of the fish, they appeared healthy following treatment.

Physiological indices of smoltification. We sampled 10-12 fish/raceway (20-24/treatment) at Willard NFH on 13 and 25 April and 13 and 31 May 1983. Fish were quickly netted from the lower, middle and upper sections of the raceways and immediately killed by immersion in tricaine (200 mg/l). Blood from individual fish was collected from the severed caudal peduncle with an ammonium-heparinized capillary tube; plasma was separated by centrifugation and stored at  $-15^{\circ}$  for later hormone analysis. Gill filaments, for the determination of Na,K-ATPase activity, were collected from the same fish. Tissues were stored frozen in buffer (see Biochemical analyses) until assayed for enzyme activity.

Corticosteroid dynamics. For in vitro determination of cortisol secretion by the interrenal cells of fish at Smith Farm, 10 individuals/replicate (20/treatment) were sampled on 9-10 June 1984, killed by a blow to the head, and bled by severing the caudal peduncle. Fish from thinned groups (see below) were not used for these experiments. The head kidneys (containing the interrenal cells) were immediately placed in 8 ml of ice-cold incubation medium (Patino

et al., 1986a). The tissues were then minced and preincubated in medium for 4.5 hr at 13-14<sup>o</sup>, with one change of medium after 2 hr. Five samples/replicate (10/treatment) were then incubated in 3 ml of medium for 2.25 hr in the absence of ACTH to determine the spontaneous steroid secretory activity; the remaining five samples were incubated in the presence of a maximally effective porcine-ACTH concentration to determine the maximal steroidogenic capacity of the interrenal cells. Cortisol content of the incubation medium was later determined. Moreover, the plasma clearance rate of corticosteroids was determined in fish held at Smith Farm. We injected 111 kBq 1,2,6,7-<sup>3</sup>H-cortisol intracardially on 15 June, and 10 fish/replicate (20/treatment) were sampled for plasma radioactivity (mostly corticosteroid-linked: Patino et al., 1985) at 2, 4, and 8 hr after the injection.

Effects of thinning. On 29 May 1984, the third HD group for each inflow rate was thinned into duplicated LD groups of fish (excess fish were discarded from the experiment). On 11 June, 10 fish/replicate (20/treatment) from thinned and undisturbed control treatment groups were sampled, as previously described, for the determination of plasma hormone concentration and gill Na,K-ATPase activity.

Biochemical analyses. Determinations of cortisol in 10  $\mu$ l of plasma or 50  $\mu$ l of incubation medium were made by radioimmunoassay according to Redding et al. (1984; modified by Patiño and Schreck, 1986) and Patiño et al. (1986a), respectively. Plasma thyroxine (T<sub>4</sub>) and triiodothyronine (T<sub>3</sub>) were measured by the methods of Dickhoff et al. (1978). Gill Na,K-ATPase activity was determined according to

Zaugg (1982). Processing of samples and determination of plasma radioactivity after injection of  $^3\text{H}$ -cortisol were performed as described by Schreck et al. (1985).

Water quality. Dissolved oxygen and total dissolved ammonia-nitrogen ( $\text{NH}_3\text{-N}$ ) concentrations were determined in the water effluent of the raceways at Willard NFH and tanks at Smith Farm shortly before the fish were released from Willard NFH. Dissolved oxygen was measured with an electronic oxygen meter (Yellow Springs Instruments Company) for the Willard NFH samples, and by the Winkler technique (Carpenter, 1965) for the Smith Farm samples. The  $\text{NH}_3\text{-N}$  content of the water was determined by the automated phenate method described by Atlas et al. (1971).

Statistical analyses. For our statistical analyses we did not assume a control treatment. Our immediate interest in this study was to see if different rearing conditions produce fish of different physiological statuses. Comparisons between the physiological status of the fish and their smoltification "success" will be performed when the return data of tagged fish from Willard NFH are compiled and analyzed.

Physiological variables measured repeatedly during our sampling period (at Willard NFH) were analyzed by the regression approach to three-way analysis of variance (ANOVA; rearing density X water inflow rate X sampling date). Data obtained at only one period of time (at Smith Farm) were analyzed with the regression approach to two-way ANOVA (rearing density X water inflow rate). Results from the thinned groups were compared with those from their original HD treatments by a

regression approach to two-way ANOVA (density treatment X water inflow rate); if the effects of density treatment were significant, we concluded that thinning had a significant effect. Main and interaction (two- and three-way) effects of the various factors (density, flow, time) on the physiology of the fish were determined. In all of these analyses, we used the Statistical Package for the Social Sciences (version 9.0, Northwestern University).

The areas under the clearance curves of radioactivity following  $^3\text{H}$ -cortisol administration were calculated as described by Normand and Fortier (1970) and compared (high versus low density within the same water inflow and between the same densities across water inflow rates) by Student's  $t$ -tests for means with unequal variances (Snedecor and Cochran, 1980). The confidence level ( $P = 0.05$ ) was divided by the number of comparisons made (Bonferroni technique: Miller, 1981).

For the sake of simplicity and easier interpretation of the results, the MD-MI treatment at Willard NFH was not included in the statistical analyses. However, for the interested reader, the results for this group are shown in the appropriate figures and tables.

## Results

Final sizes of the fish were not affected by the experimental rearing conditions (J. L. Banks, unpublished data for several consecutive years). The final mean lengths and weights of the fish sampled by us were 13.2 cm and 25.4 g at Willard NFH, and 13.4 cm and 25.3 g at Smith Farm.

Physiological indices of smoltification. Plasma cortisol concentrations in coho salmon at Willard NFH varied significantly during the sampling period ( $P \leq 0.01$ ); they tended to be highest in May (Fig. III-1). They were affected by water inflow rate ( $P \leq 0.03$ ) but not by rearing density. Moreover, the effects of water inflow rate showed significant ( $P \leq 0.01$ ) two-way interactions with sampling date, indicating that the effects of rearing conditions were not uniform during our sampling period. For example, a large effect of water inflow rate on cortisol levels (higher in fish at HI than in those at LI) was observed on 31 May, but not before. No interaction effects of rearing density and water inflow rate were observed. The pattern of cortisol changes in fish at MD-MI was similar to those in fish at HI.

Plasma  $T_4$  (Fig. III-2) and  $T_3$  (Fig. III-3) in coho salmon also increased during our sampling period ( $P \leq 0.01$ ) at Willard NFH. High rearing density resulted in lower circulating concentrations of both of these hormones ( $P \leq 0.01$ ). No statistically significant interaction effects of rearing density and sampling date on plasma thyroid hormones were observed. However, for plasma  $T_3$ , the effects of density appeared to be more pronounced in April than in May. Neither plasma  $T_4$  nor  $T_3$  was affected by water inflow rate. The patterns of thyroid hormone changes in fish at MD-MI were similar to those in fish at LD.

Gill Na,K-ATPase activity increased through time (Fig. III-4;  $P \leq 0.01$ ). Main effects of rearing density ( $P \leq 0.01$ ) but not of water

Figure III-1. Plasma cortisol in yearling coho salmon reared at Willard National Fish Hatchery at high (HD), medium (MD), or low (LD) density, combined with a high (HI), medium (MI), or low (LI) water inflow rate. Samples were taken in 1983. Bars represent SEs of the mean values.

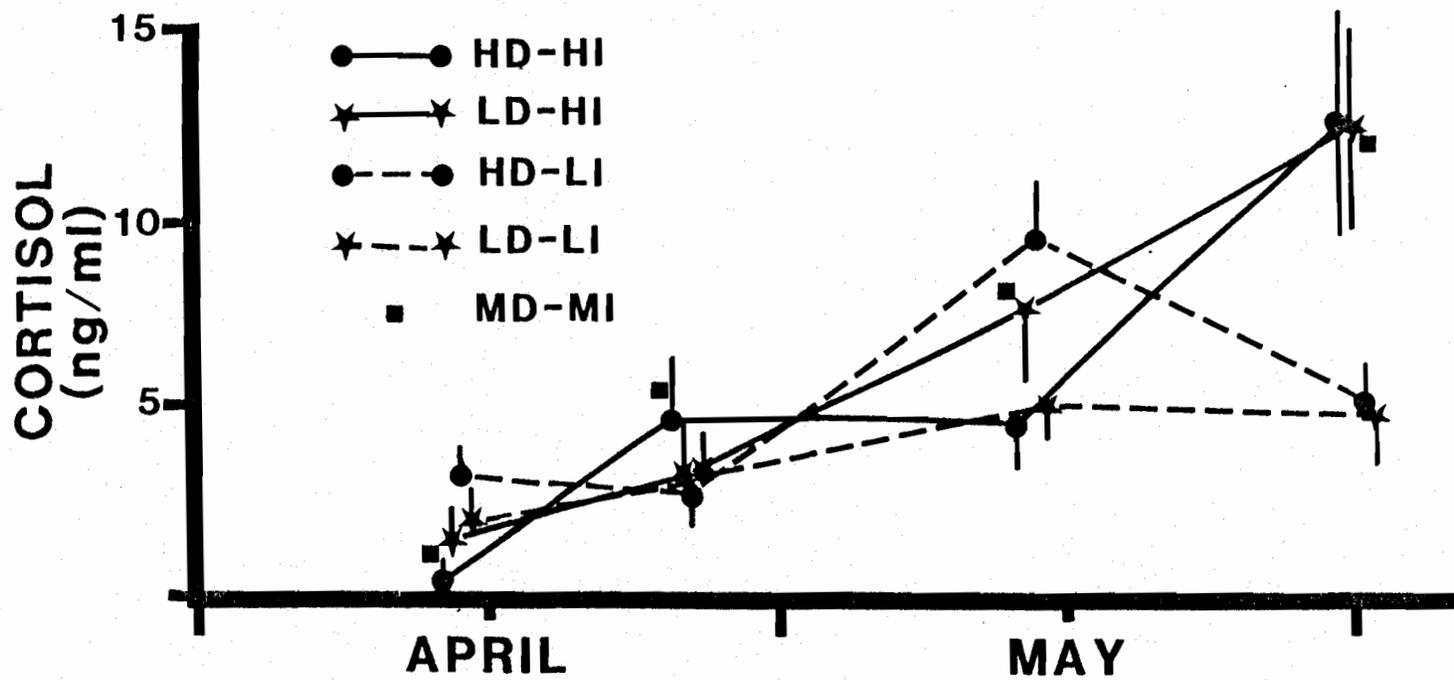


Figure III-1

Figure III-2. Plasma thyroxine in yearling coho salmon reared at Willard National Fish Hatchery at high (HD), medium (MD), or low (LD) density combined with a high (HI), medium (MI), or low (LI) water inflow rate. Samples were taken in 1983. Bars represent SEs of the mean values.

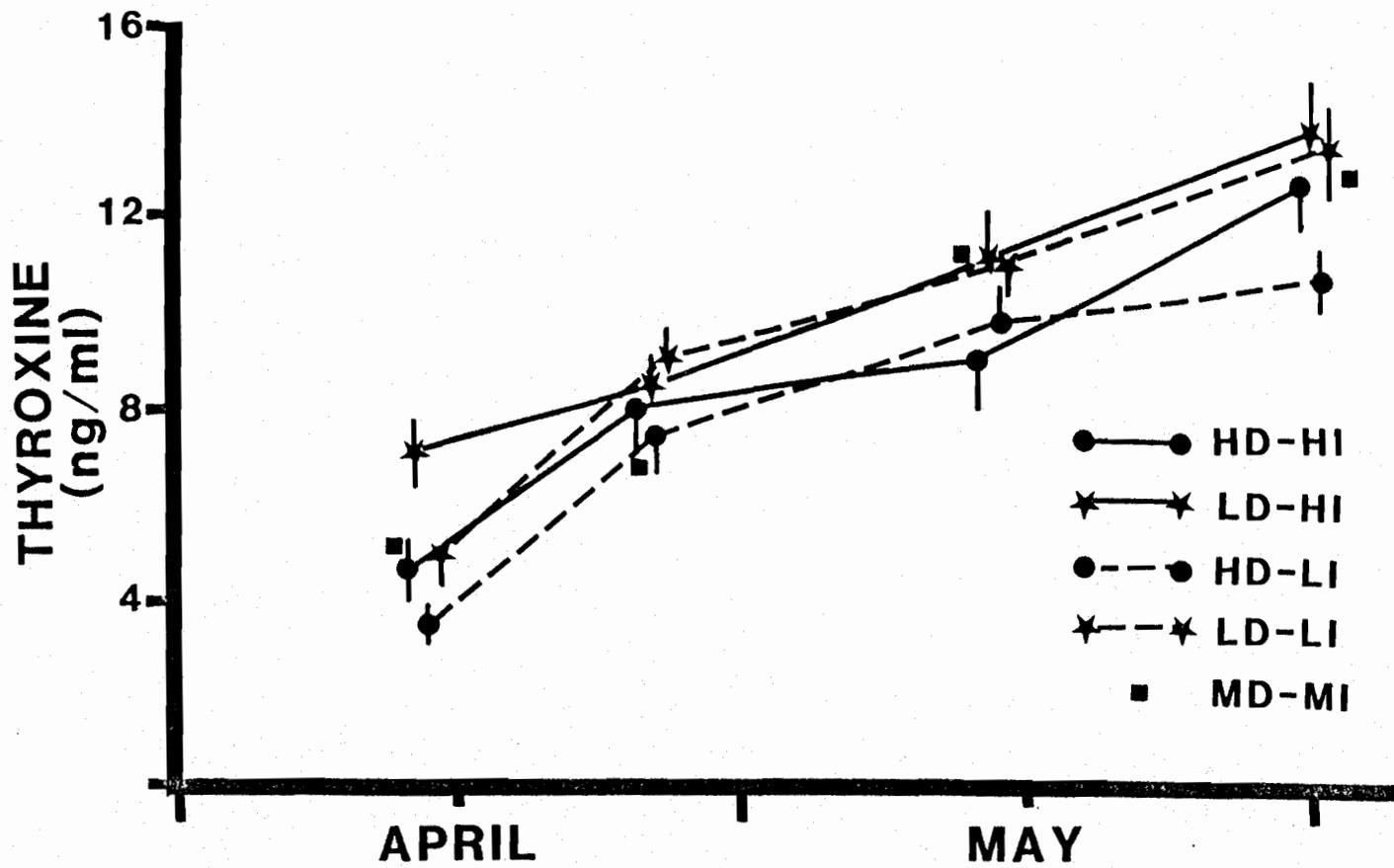


Figure III-2

Figure III-3. Plasma triiodothyronine in yearling coho salmon reared at Willard National Fish Hatchery at high (HD), medium (MD), or low (LD) density combined with a high (HI), medium (MI), or low (LI) water inflow rate. Samples were taken in 1983. Bars represent SEs of the mean values.

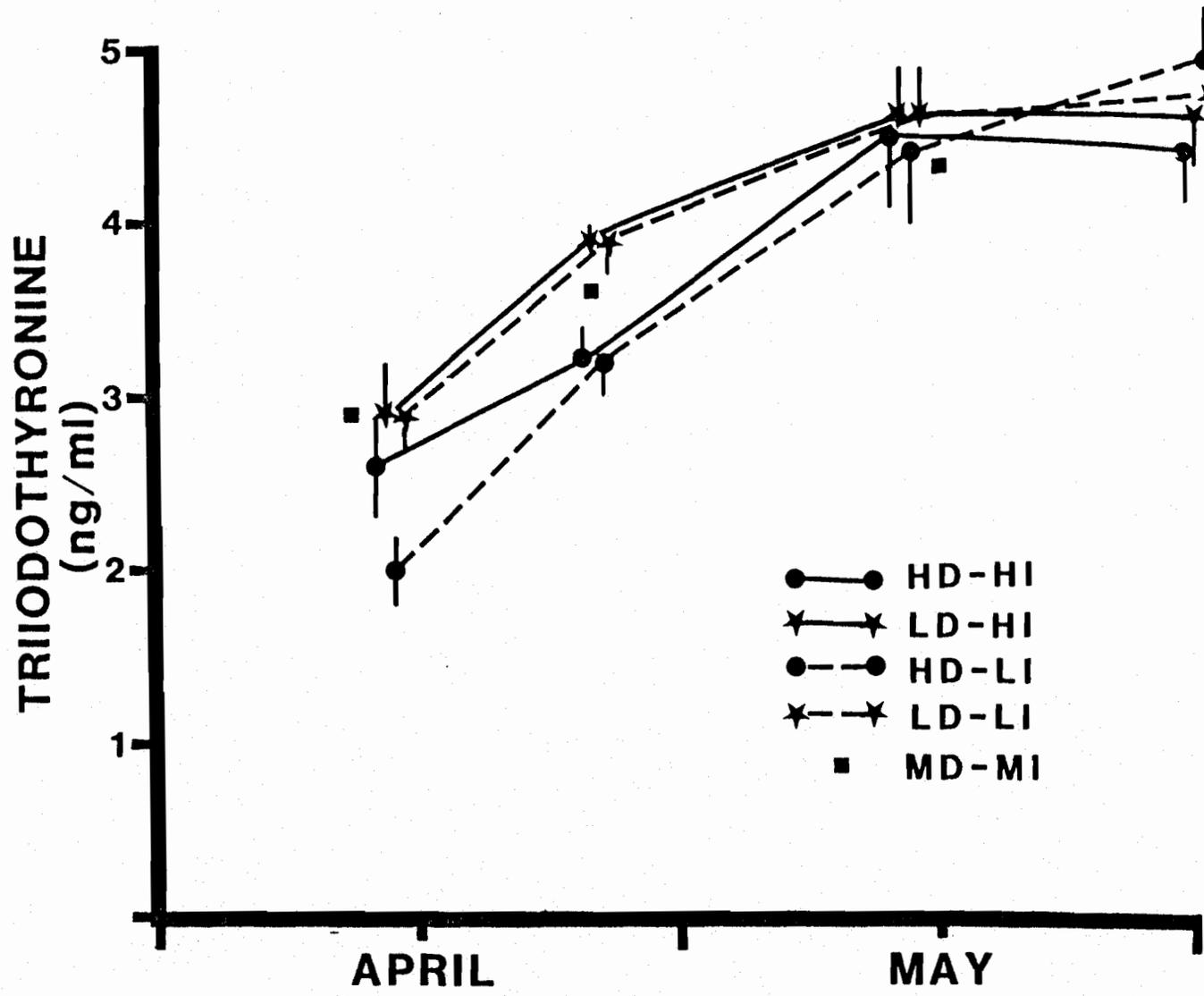


Figure III-3

Figure III-4. Gill Na,K-ATPase activity in yearling coho salmon reared at Willard National Fish Hatchery at high (HD), medium (MD), or low (LD) density combined with a high (HI), medium (MI), or low (LI) water inflow rate. Samples were taken in 1983. Bars represent SEs of the mean values.

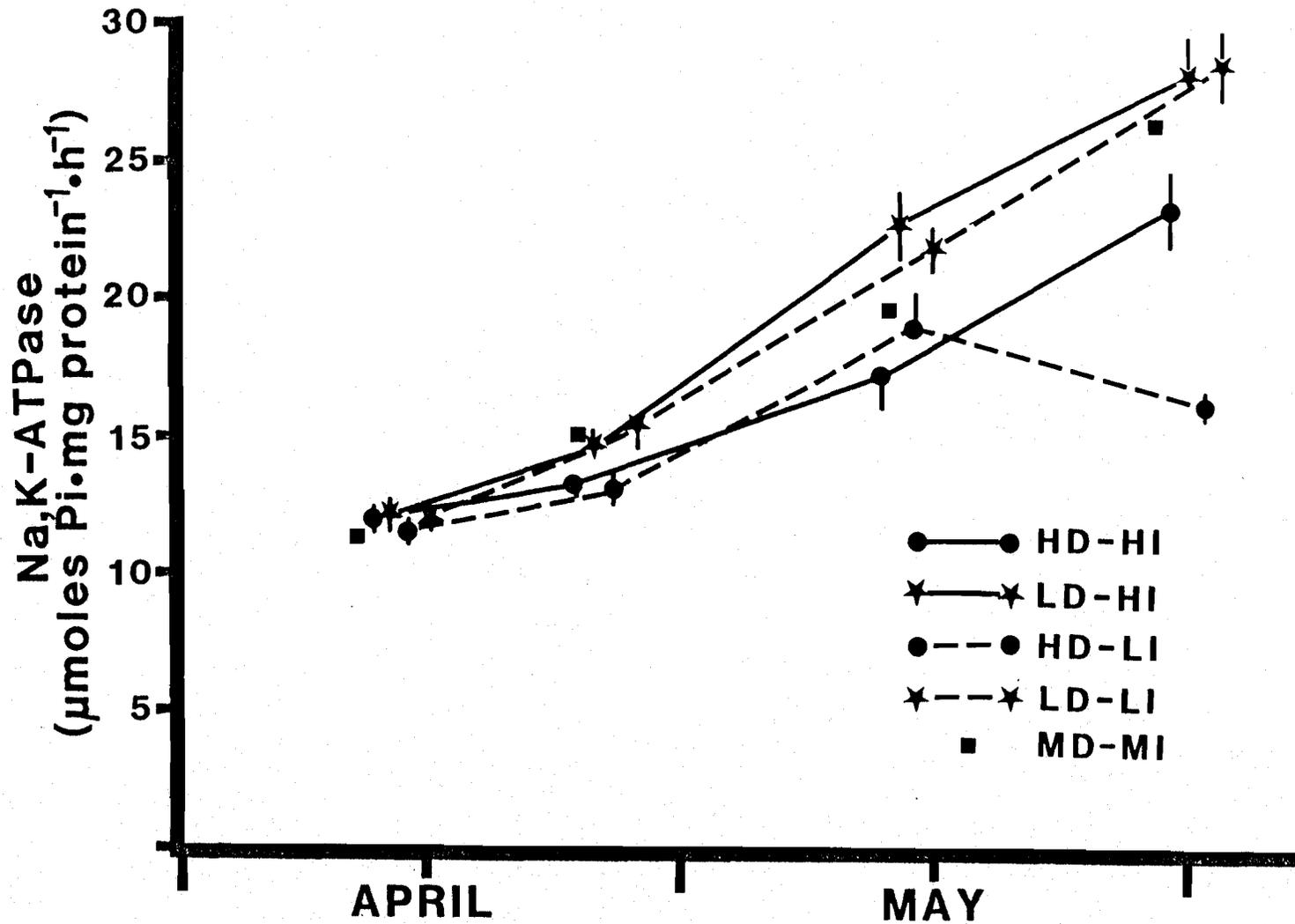


Figure III-4

inflow rate were observed. However, interaction effects of both rearing density and date and water inflow rate and date were recognized ( $P \leq 0.01$ ), indicating that both rearing variables had an effect on the pattern of development of the enzyme activity during our experiment. It appears that gill Na,K-ATPase activity in fish from the HD groups was generally lower than in fish from the LD groups during and after late April but not before. Moreover, a downward trend in gill enzyme activity was apparent at the end of the sampling period in fish from the HD-LI treatment. The pattern of gill Na,K-ATPase changes in fish at MD-MI was similar to those in fish at LD.

Corticosteroid dynamics. Resting corticosteroid output in vitro was higher in interrenal cells from coho salmon reared at HD than in those from fish reared at LD; no effects of water inflow rate on resting cortisol output were observed (Table III-1). On the other hand, the maximal secretory capacity of the interrenal cells was not affected by either rearing density or water inflow rate. The areas under the clearance curves of plasma radioactivity after  $^3\text{H}$ -cortisol administration were not significantly different among the experimental groups, indicating that clearance rates of plasma radioactivity were not affected by rearing conditions.

Effects of thinning. In coho salmon reared at Smith Farm at LD, plasma cortisol ( $P \leq 0.01$ ) and  $T_4$  ( $P \leq 0.01$ ), and gill Na,K-ATPase activity ( $P \leq 0.01$ ) were significantly higher than in fish held at HD (Figs. III-5, III-6). No effects of water inflow rate were observed.

Plasma cortisol was the only physiological variable that changed significantly ( $P \leq 0.01$ ) when rearing density was lowered about 2

Table III-1. Cortisol secretion (mean  $\pm$  SE; n=10) in vitro from the head kidney of juvenile coho salmon reared at Smith Farm at high (HD) or low (LD) density combined with a high (HI) or low (LI) water inflow rate. The head kidneys were incubated for 2.25 hr in the absence or presence of a maximally effective concentration of porcine-ACTH to determine their spontaneous and maximal secretory capacities, respectively. Values within a column without a letter in common are significantly different (two-way analysis of variance, rearing density X inflow rate; Student's t-test of pooled water-flow values,  $P \leq 0.02$ )

<u>Rearing conditions</u>	<u>Cortisol secretion (ng/head kidney)</u>	
	<u>Spontaneous</u>	<u>Maximal</u>
HD-LI	9.3 $\pm$ 2.6 z	155.0 $\pm$ 8.3 z
LD-LI	5.3 $\pm$ 1.3 y	168.0 $\pm$ 7.3 z
HD-HI	10.5 $\pm$ 2.2 z	172.0 $\pm$ 16.3 z
LD-HI	4.8 $\pm$ 1.3 y	141.8 $\pm$ 7.8 z

Figure III-5. Plasma cortisol and thyroxine levels in yearling coho salmon from Willard National Fish Hatchery reared at Smith Farm, Oregon State University. The fish were reared under high (HD) or low (LD) density combined with a high (HI) or low (LI) water inflow rate. Some HD fish were thinned into LD groups at their original water inflow rates (indicated by arrow) 13 days before sampling. Samples were taken in 1984. Thin bars represent SEs of the mean values.

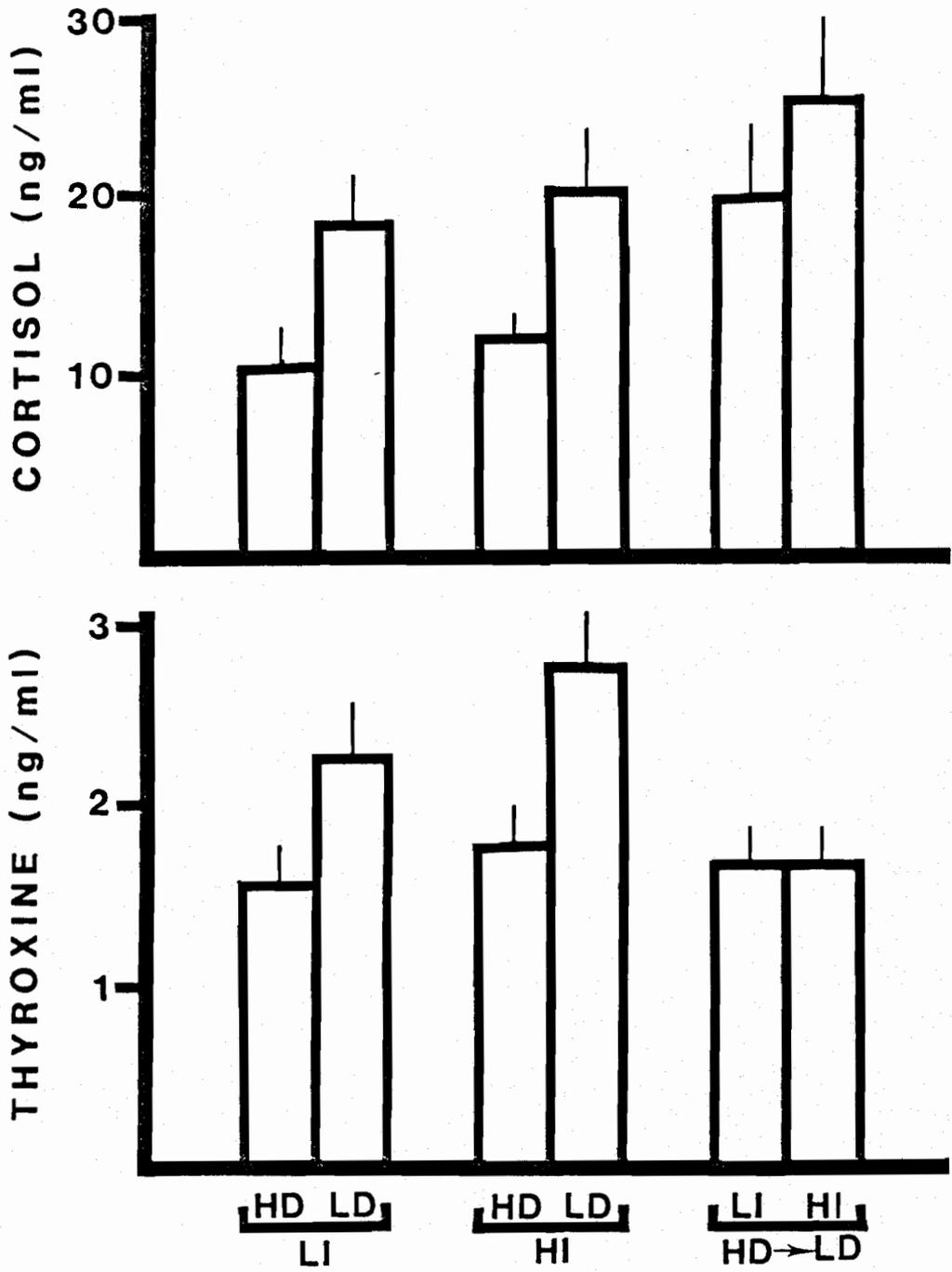


Figure III-5

Figure III-6. Gill Na,K-ATPase activity in yearling coho salmon from Willard National Fish Hatchery reared at Smith Farm, Oregon State University. The fish were reared under high (HD) or low (LD) density combined with a high (HI) or low (LI) water inflow rate. Some HD fish were thinned into LD groups at their original water inflow rates (indicated by arrow) 13 days before sampling. Samples were taken in 1984. Thin bars represent SEs of the mean values.

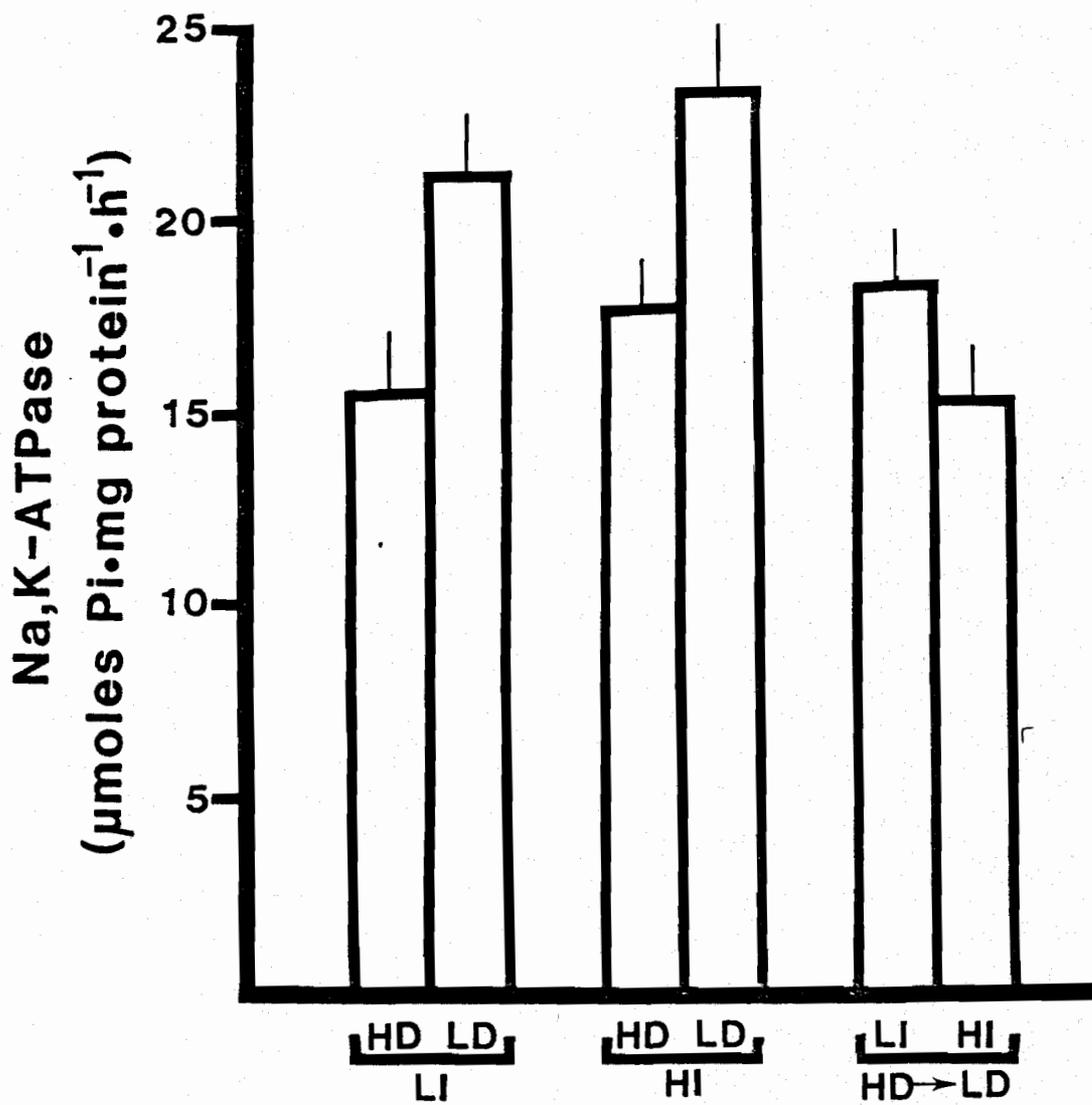


Figure III-6

weeks before sampling (Figs. III-5, III-6). Cortisol levels were higher in fish at the reduced density than in those at HD and were similar in fish at the reduced density and at LD.

Water quality. Dissolved oxygen and  $\text{NH}_3\text{-N}$  concentrations in the water effluent were related to the water inflow rate and rearing density of the fish at Willard NFH and at Smith Farm (Table III-2). However, dissolved oxygen concentrations were within the acceptable range for salmonids (Westers and Pratt, 1977). Total ammonia, when converted to approximate un-ionized ammonia concentrations (Thurston et al., 1977; the pH of the water at both Willard NFH and Smith Farm was about neutral), was also within safe levels recommended for fish (see Smart, 1981).

#### Discussion

Two variable hatchery conditions, rearing density and water inflow rate, clearly affected the physiological status of coho salmon in this study. Our sampling period at Willard NFH fell within the period of smoltification of coho salmon, as indicated by increased levels of various smoltification markers such as plasma thyroid (Dickhoff et al., 1978) and cortisol (Specker and Schreck, 1982; Barton et al., 1985) hormones as well as gill Na,K-ATPase activity (Zaugg and McLain, 1970; Zaugg and Wagner, 1973). The possible roles of these physiological changes during smoltification have been discussed previously (Folmar and Dickhoff, 1980; Specker, 1982; Barron, 1986; Langhorne and Simpson, 1986).

Table III-2. Water exchange rates (ER; volume/flow rate) and dissolved oxygen (DO) and total dissolved ammonia-nitrogen ( $\text{NH}_3\text{-N}$ ) concentrations of the water effluent at Smith Farm and Willard National Fish Hatchery (NFH). Juvenile coho salmon were reared at high (HD), medium (MD), or low (LD) density combined with high (HI), medium (MD), or low (LI) water inflow rate (the HI and LI at Smith Farm and Willard NFH were not the same). Some HD fish at Smith Farm were thinned into LD groups at their original water inflow rates (indicated by arrows) 10 days before the water was sampled. Values shown for water from Willard NFH are those measured in 1983 (water characteristics were similar from year to year: J.L. Banks, unpublished data).

Rearing conditions	Smith Farm			Willard NFH		
	ER(min)	DO(mg/l)	$\text{NH}_3\text{-N}$ (mg/l)	ER(min)	DO(mg/l)	$\text{NH}_3\text{-N}$ (mg/l)
HD-LI	11.4	7.3	0.11	47.6	8.6	0.24
LD-LI	11.4	8.5	0.05	47.6	10.4	0.12
MD-MI	----	---	----	23.8	10.6	0.10
HD-HI	5.3	8.1	0.06	15.9	10.7	0.12
LD-HI	5.3	9.0	0.03	15.9	11.2	0.06
HD->LD-LI	11.4	8.6	0.05	----	----	----
HD->LD-HI	5.3	8.9	0.03	----	----	----

Thyroid hormones in fish at Willard NFH were unaffected by water inflow rate (at least during our sampling period) but were generally lower in fish at HD (except for  $T_3$  in May, when no effects were apparent). On the other hand, water inflow rate affected cortisol levels and both rearing variables affected gill Na,K-ATPase activity. However, the specific effects of rearing conditions on cortisol and gill Na,K-ATPase varied over time thus producing different patterns of physiological development among the experimental groups of fish. An analogous phenomenon has been observed in juvenile anadromous salmonids when conditions of water temperature or photoperiod are varied (Wedemeyer et al., 1980).

In contrast to earlier results (Schreck et al., 1985) and to our present results at Willard NFH that showed no effects of rearing density on cortisol levels, we found that coho salmon reared at Smith Farm at LD had higher levels of this hormone than fish reared at HD. An inverse relationship between rearing density and plasma cortisol in rainbow trout Salmo gairdneri has also been reported (Leatherland and Cho, 1985). This apparent inconsistency in results obtained at our two experimental sites regarding the effects of rearing conditions on plasma cortisol (and gill Na,K-ATPase; effects of water inflow rate on this enzyme were seen at Willard NFH but not at Smith Farm) can be explained by our finding at Willard NFH that the effects of rearing conditions on the physiology of the fish appear to be neither unidirectional nor always evident throughout smoltification. Also, differences in rearing conditions between experimental sites such as

water temperature, shape and size of rearing container (raceway versus cylindrical tank), and routine hatchery procedures (cleaning, feeding, and so forth), and the fact that the fish at our laboratory were known carriers of bacterial kidney disease, may have affected the physiological status of the fish.

Plasma hormone levels depend on secretion as well as clearance rates. In vitro interrenal cell activity in the absence of ACTH was higher in Smith Farm fish held at HD than at LD. However, the maximal secretory capacity of these cells was similar in all treatment groups. Moreover, no differences in the overall clearance of corticosteroids were observed. (A slight positive relation between rearing density and corticosteroid clearance rate was observed by Schreck et al. [1985]; it is difficult to compare studies in which measurements were taken only once during smoltification because of possible variability in the effects of rearing conditions through this period.) Therefore, the higher cortisol levels in fish at LD were most likely caused by a higher submaximal stimulation (by ACTH or other factors) of their interrenal cells. We believe that this elevated in vivo secretion of cortisol in fish at LD does not necessarily indicate that they were under higher stress than fish at HD (a situation that would be paradoxical given the known deleterious effect of high rearing densities on salmonids; e.g., Fagerlund et al., [1983] and Schreck et al., [1985]). Instead, it may just reflect differences in patterns of physiological development during smoltification between the fish reared at LD and HD.

Two weeks of reduced density around the time of release from

Willard NFH was insufficient to influence the levels of plasma  $T_4$  and gill Na,K-ATPase activity in the fish reared at Smith Farm. On the other hand, plasma levels of cortisol in fish at the reduced rearing density became similar to those in fish reared at LD throughout the experiment. Moreover, a concurrent study on our Smith Farm coho salmon showed that the immunological characteristics differed between fish reared at HD and LD, and that the immunological characteristics of fish after thinning were similar to those of fish reared at LD throughout the experiment (Maule et al., 1987). However, the effect of reduced rearing density on "smolt" quality of coho salmon remains to be determined.

It seems unlikely to us that water quality alone was responsible for the effects of our experimental hatchery rearing variables on the physiology of juvenile coho salmon. For example, water inflow rate affected water quality as markedly as rearing density, and yet only rearing density influenced plasma hormone concentrations and gill Na,K-ATPase activity in fish at Smith Farm at the time of sampling. Also, rearing density, but not water inflow rate, affected thyroid hormone levels in fish at Willard NFH. Thus, it appears that another factor (or factors), such as crowding stress, may be at least partly responsible for the effects of high rearing density on the physiology of the fish. Social stress can markedly affect aspects of the physiology of juvenile coho salmon (Ejike and Schreck, 1980) and the European eel Anguilla anguilla (Peters, 1982). Fish size at high density was also suggested to influence stress level in a hatchery

situation (Fagerlund et al., 1981), although this phenomenon may not always be clear (Schreck et al., 1985).

Although oxygen and ammonia-nitrogen contents were similar in the water effluents of the LD-LI and HD-HI raceways, plasma cortisol levels in fish from these raceways differed on the last sampling date. Since no effects of rearing density on cortisol levels were observed at Willard NFH, some factor other than dissolved oxygen or ammonia, but related to water inflow rate, appears responsible for this finding. Water inflow rate at Smith Farm apparently had no effect on the physiology of the fish. However, given our finding of interaction effects of rearing conditions and date of sampling at Willard NFH, it is possible that an effect of water inflow rate on the fish could have been observed at times other than the single sampling time at Smith Farm. Meade and Herman (1986) suggested that unidentified water borne factors other than ammonia may be deleterious to fish under intensive culture conditions. In our study, however, it was not clear what factor other than water quality but associated with water inflow rate could have affected the physiology of the fish. The absence of statistical interaction effects of rearing density and water inflow rate on the physiology of coho salmon at both experimental sites also seems to rule out the possibility of a single, common pathway--i.e., water quality--in the mechanism by which these factors act.

In conclusion, our results suggested that varying levels of rearing density and water inflow rate affect the pattern of physiological development of coho salmon during smoltification. Therefore, the process of smoltification itself may be also affected.

A concurrent and continuing study by one of us (J.L.B.) will determine if the present experimental conditions affected the return rate of the adult fish. Rearing density seemed to affect plasma levels of cortisol by altering the submaximal stimulation of interrenal cells. Moreover, crowding stress may be a major factor responsible for the effect of high rearing density on the physiological status of the fish. Thinning the rearing density of the fish affected their levels of plasma cortisol but not those of plasma  $T_4$  or gill Na,K-ATPase activity. More research seems necessary to determine the value of thinning as a hatchery management option.

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IV: INTERRENAL SECRETION OF CORTICOSTEROIDS AND PLASMA CORTISOL  
AND CORTISONE CONCENTRATIONS AFTER ACUTE STRESS AND  
DURING SEAWATER ACCLIMATION IN JUVENILE COHO SALMON

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## Abstract

We determined the major corticosteroids secreted by interrenal tissue and those present in plasma of juvenile coho salmon (Oncorhynchus kisutch). Incubation medium of interrenal cells, unstimulated or stimulated with exogenous ACTH in vitro, and plasma of resting or acutely stressed salmon were extracted and qualitatively analyzed for steroid composition using high-performance liquid chromatography (HPLC). Concentrations of plasma cortisone and cortisol following an acute handling stress or exposure to seawater were quantitatively measured by radioimmunoassay (RIA). Cortisol was the only corticosteroid detected by HPLC in media after incubation of interrenal cells in the absence or presence of ACTH in vitro. However, both cortisone and cortisol were detected by HPLC in plasma sampled 1 hr after fish were acutely stressed by handling. Stress and seawater acclimation produced marked elevations in plasma levels of both steroids as determined by RIA and also resulted in long-lasting changes in the plasma cortisone:cortisol ratios. In resting fish, cortisone levels were similar or higher than cortisol levels. We concluded that cortisol is the primary steroid secreted by the interrenal cells of coho salmon, and that plasma cortisone arises primarily from the peripheral conversion of cortisol to cortisone. The relatively high levels of cortisone in resting fish, and its increase following stress and seawater acclimation suggest the possibility of a biologically significant role for this hormone.

## Introduction

Numerous studies have characterized the steroid secretion by the interrenal tissue of fishes. The interrenal tissue is homologous to the adrenal cortex of mammals. In teleosts, cortisol is usually the most important corticosteroid secreted by the interrenal, but other steroids such as cortisone, 11-deoxycortisol and corticosterone have been reported (Sandor, 1979). Cortisol is also the major corticosteroid in plasma of teleosts, although again a number of other steroids, notably cortisone, have been found (Idler and Truscott, 1972). Because of its preeminence, cortisol is virtually the only corticosteroid that has received any significant attention in physiological studies of teleosts.

Fagerlund (1970) reported measurable levels of cortisone in undisturbed juvenile coho salmon (Oncorhynchus kisutch) but could not detect cortisol. More recently, Weisbart and McGowan (1984) used a specific radioimmunoassay (RIA) to show that, in juvenile and adult Atlantic salmon (Salmo salar), cortisone--not cortisol--is the major plasma corticosteroid in fish at rest. A direct stimulatory role for cortisone in hepatic gluconeogenic activity in salmonids was reported by Freeman and Idler (1973). Leloup-Hatey (1974) suggested that a major function for cortisone in European eels (Anguilla anguilla) is to facilitate the entry of cortisol into target tissues.

Much of the evidence supporting a biological role of cortisol in fish has come from observations of its plasma dynamics during critical periods of environmental challenge (Henderson and Garland, 1980;

Donaldson, 1981; Schreck, 1981) or development (Idler and Truscott, 1972; Specker, 1982). Less is known regarding ontogenetic or environmentally induced changes in plasma levels of corticosteroids other than cortisol.

Fagerlund (1970) found no changes in plasma cortisone levels in juvenile coho salmon sampled after 30 min of continuous handling stress; however, the fish in his study were sampled only once after treatment. However, Schmidt and Idler (1962) and Fagerlund and Donaldson (1970) found that cortisone concentrations tended to increase with sexual maturation in adult sockeye salmon (Oncorhynchus nerka). Fagerlund and Donaldson (1970) also reported inconsistent changes in plasma cortisone following stress in adult salmon, which they attributed to degree of maturation or sex of the fish. There is little information regarding changes in plasma cortisone in teleosts during the critical early stages of seawater acclimation when a large increase in plasma cortisol may occur (Redding et al., 1984).

The aim of our present study was to characterize the interrenal secretion and plasma levels of corticosteroids in juvenile coho salmon. We determined (1) the major corticosteroids produced by the interrenal tissue in vitro, and (2) the major corticosteroids in plasma of fish at rest and their dynamics after acute stress and during seawater acclimation.

#### Materials and Methods

Animals. Juvenile coho salmon were obtained as eggs or yearlings

from hatchery stocks in Oregon and Washington. All fish except those held for seawater acclimation experiments, were reared under natural photoperiod in fresh water at 12-13<sup>o</sup>, in flow-through tanks at Smith Farm Hatchery, Oregon State University, and were fed Oregon Moist Pellets daily. The fish for the seawater acclimation experiments were transported from a production hatchery to the Hatfield Marine Science Center in Newport, Oregon, and reared in fresh water under natural photoperiod.

Interrenal secretory products and plasma corticosteroid composition. Two groups of 40 sub-yearling coho salmon (mean weight, 20 g) each were acclimated to 200-l tanks for 15 days before the experiment. On September 25, 18 fish were removed at once from one of the tanks, killed by a blow to the head, and bled into heparinized capillary tubes by severing the caudal peduncle; all fish were sampled within 5 min. The blood was pooled, and the plasma was separated by centrifugation and frozen until further analysis. The head kidneys from each fish were excised and placed in ice-cold incubation medium (Patiño et al., 1986a; the NaCl content was increased to 130 mM). Eighteen fish from the second tank were given an acute 30-sec handling stress (Barton et al., 1986) and sampled 1 hr later for blood and kidney tissue as described above.

Kidneys were pooled in two sub-groups of nine kidneys each in both control and stressed groups, and the tissue was processed as described by Patiño et al. (1986a). Briefly, the minced tissues were pre-incubated for 4 hr in 20 ml of medium; the medium was changed once after the first 2 hr. One sub-group per treatment was transferred to

10 ml of medium containing 50 mU/ml of porcine-ACTH (Sigma; 100 IU/mg) and the second sub-group to medium containing no ACTH. The tissue was incubated for 3 hr at 13-14°. After the incubation, 9 ml of medium were sampled and frozen until analysis.

Effects of stress on plasma levels of cortisone and cortisol.

Two replicate groups of sub-yearling coho salmon from the same population as in the previous experiment were acclimated to 200-1 experimental tanks as described above. On September 30, five fish from each tank were carefully netted and immediately killed by immersion in 200 mg/l 3-aminobenzoic acid ethyl ester (MS-222). The remaining fish in both tanks were then given a 30-sec handling stress, and five fish from each tank were sampled for plasma corticosteroids at 0.5, 1, 3, 6, 12 and 24 hr. The experiment was repeated in July 9 with yearling coho salmon of a different stock (mean weight, 26 g). The samples (n = 10 per sampling time) in this second experiment were taken only at 0, 1 and 3 hr from a single experimental tank.

Effects of seawater acclimation on plasma levels of cortisone and cortisol. These experiments were performed in conjunction with another study of the effects of cortisol on osmoregulation. Our fish had been injected intraperitoneally with 0.1 ml of cocoa butter 48 hr before the experiments started, but were otherwise untreated.

Two groups of 40 yearling fish (mean weight, 28 g) were transferred from the rearing tank (after the cocoa butter was injected) to their respective 100-1 tanks on April 15. After 48 hr, we quickly netted five fish from each tank and killed them with

anesthetic to collect blood samples as described earlier. The freshwater source to one of the tanks was then closed and the seawater source was opened with minimal disturbance to the fish. Ten fish from each tank were then taken after 4, 24, and 48 hr to collect blood samples. The freshwater and seawater temperatures were 13° and 12°, respectively.

The experiment was repeated on June 17; the same procedures were followed, except that fish (mean weight, 47 g) were not sampled 4 hr after the beginning of seawater exposure. The freshwater and seawater temperatures had risen to 18° and 16°, respectively.

High-performance liquid chromatography and radioimmunoassays. We extracted incubation media (9-ml) and plasma (1.2-ml) using 3-ml, low-displacement C<sub>18</sub> disposable columns mounted in a Baker-10 SPE™ System. The samples were loaded onto the columns as described by the manufacturer, washed with 2 ml (media) or 3 ml (plasma) of 20% acetone, and eluted with 0.6 ml methanol. The plasma extracts were filtered through 0.45 μm-disposable filters on a 1-ml glass syringe. The extracts were dried in a Speed Vac Concentrator (Savant) under a vacuum at 45°, and subsequently reconstituted in the mobile phase (see below) for injection.

For HPLC separation of steroids, we followed the methods of Huang *et al.* (1983) with slight modification. We used a reverse-phase (C<sub>18</sub>) 4.5- X 250-mm column and monitored the eluate with a 254-nm UV detector. The steroids were eluted with an isocratic mobile phase (1 ml/min) of water:methanol:acetonitrile:isopropanol, 55:32:6.5:6.5 (v/v), for 15 min, followed by a linear gradient (2%/min) of

water:methanol:butanol, 40:40:20, for 40 min. The sensitivity of this system is 10-15 ng (G. Feist and C.B. Schreck, unpublished) for our following reference steroids: aldosterone, cortisone, cortisol, 11-ketotestosterone, 11 $\beta$ -hydroxyandrostenedione, 11 $\beta$ -hydroxytestosterone, corticosterone, 11-deoxycortisol, 11-ketoprogesterone, androstenedione, testosterone, 17 $\alpha$ -hydroxyprogesterone, 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one, progesterone, and 20 $\beta$ -hydroxy-4-pregnen-3-one. We ran standards after every two or three sample injections to compensate for possible changes in solvent conditions.

We directly measured cortisol in 10  $\mu$ l of plasma, using the method of Redding et al. (1984) as modified by Patiño and Schreck (1986). We used the same general procedures for the cortisone RIA. Briefly, the amount of [1,2,6,7- $H^3$ ] cortisone was 0.01  $\mu$ Ci/tube, and the binding to the cortisone antisera (48 C/8; made by Dr. P. Vecsei, University of Heidelberg, and described by Weisbart and McGowan, 1984) in the "zero" tubes was kept near 40%. The incubation was performed under room temperature for 1.5 hr. At 50% displacement, the cross-reactivity of the cortisone antibody was less than 4% with cortisol, and less than 1% with progesterone and androstenedione. Diluting the plasma with steroid-free coho plasma (Abraham et al., 1977), or gradually increasing the plasma volume (from 10 to 50  $\mu$ l; corrected for the increasing non-specific binding) yielded curves parallel to the standard curve. The recovery rates of cortisone added to plasma at 5 to 100 ng/ml ranged from 90 to 120%. The intra-assay variability was less than 2% (n = 4) measured in low (12 ng/ml) or high (120

ng/ml) plasma pools; and the inter-assay variability was less than 4% (n = 3) measured in the high plasma pool. The lowest cortisone standard used in the assay, 5 pg/tube, gave counts significantly different from those of the "zero" tubes.

We used the radioimmunoassays to support the identification of peaks in HPLC-chromatograms of incubation medium extracts. Dilutions of the eluate fraction containing a peak with the same retention time as authentic cortisol gave a curve parallel to the standard curve in the cortisol RIA. Although cortisone was not detectable by HPLC (see Results), RIA analysis of the fraction corresponding to cortisone revealed minor quantities (ca. 1/200 that of cortisol) of this steroid in incubation medium extracts.

Statistical analyses. Values for plasma cortisone and cortisol in the experiments on stress and seawater acclimation were transformed to their natural logarithms, and analyzed by one-way ANOVA followed by Student-Newman-Keul's multiple range test. Cortisone:cortisol ratios were calculated for each fish, and analyzed as for plasma hormone levels. Data from replicate groups of stressed, subyearling fish were similar and thus were pooled. Plasma cortisol and cortisone concentrations and their ratio did not vary in freshwater control groups during the experiments on seawater acclimation; therefore, they were pooled within an experiment to evaluate the effects of seawater exposure. Paired-comparison  $t$  tests were used to evaluate the differences between cortisone and cortisol levels in undisturbed fish (Table IV-1). The alpha value chosen for statistical significance was 0.05.

Table IV-1. Plasma cortisol and cortisone (ng/ml; mean  $\pm$  standard error), weight of fish (g), and sample size (n) of undisturbed freshwater coho salmon from the various experiments of this study (see text)

<u>Fish</u>	<u>Weight</u>	<u>n</u>	<u>Cortisol</u>	<u>Cortisone</u>
Sub-yearling (September)	20	10	4.9 $\pm$ 2.6	7.7 $\pm$ 4.1
Yearling (July)	26	10	11.0 $\pm$ 3.2	15.7 $\pm$ 3.4
Yearling (April)	28	39	14.7 $\pm$ 1.6	39.2 $\pm$ 2.7** <sup>a</sup>
Yearling (June)	47	30	28.7 $\pm$ 6.8	44.9 $\pm$ 6.5**

<sup>a</sup> Significantly different from cortisol; paired  $t$  test,  $P \leq 0.001$

## Results

The only major peak detected by HPLC in extracts of incubation media from unstimulated or ACTH-stimulated interrenal tissue had the same retention time as authentic cortisol. However, peak height for the ACTH-stimulated tissue was about 70 times greater (350 vs. 5 absorbance units) than that of unstimulated tissue as expected from previous work (Patiño et al., 1986a). Similar stimulation by ACTH was seen in both stressed and unstressed groups. No peaks were observed in chromatograms of plasma extracts from undisturbed fish; this plasma had less than 8 ng of each steroid as determined by RIA before extraction. However, the plasma extract of fish sampled 1 hr after an acute stress showed two peaks with the same retention time as authentic cortisone and cortisol (Fig. IV-1).

Acute stress resulted in a marked elevation of cortisol concentration, a sharp peak occurring between 0 and 3 hr in both sub-yearling and yearling salmon (Figs. IV-2, IV-3). In contrast, plasma cortisone also increased after acute stress, but to a lesser degree than cortisol and, in both experiments, it remained elevated from 0.5 hr to the end of the experiment (Figs. IV-2, IV-3). At the end of the experiment, both cortisone and cortisol were still higher than their respective pre-stress values.

Juvenile coho salmon exposed to seawater in April and June showed increased concentrations of both cortisone and cortisol, and they remained elevated throughout the experiment (Figs. IV-4 and IV-5). In June, however, plasma cortisone rose to a greater degree than

Figure IV-1. HPLC chromatogram of steroids in plasma of sub-yearling coho salmon 1 hr after a 30-sec handling stress. Cortisone (E) and cortisol (F) were the only steroids detected. Media from in vitro incubations of unstimulated and ACTH-stimulated interrenal tissue showed a single peak corresponding to that of cortisol. Absorbance units at full scale, 254 nm; range, 0.001; chart speed, 0.5 cm/min.

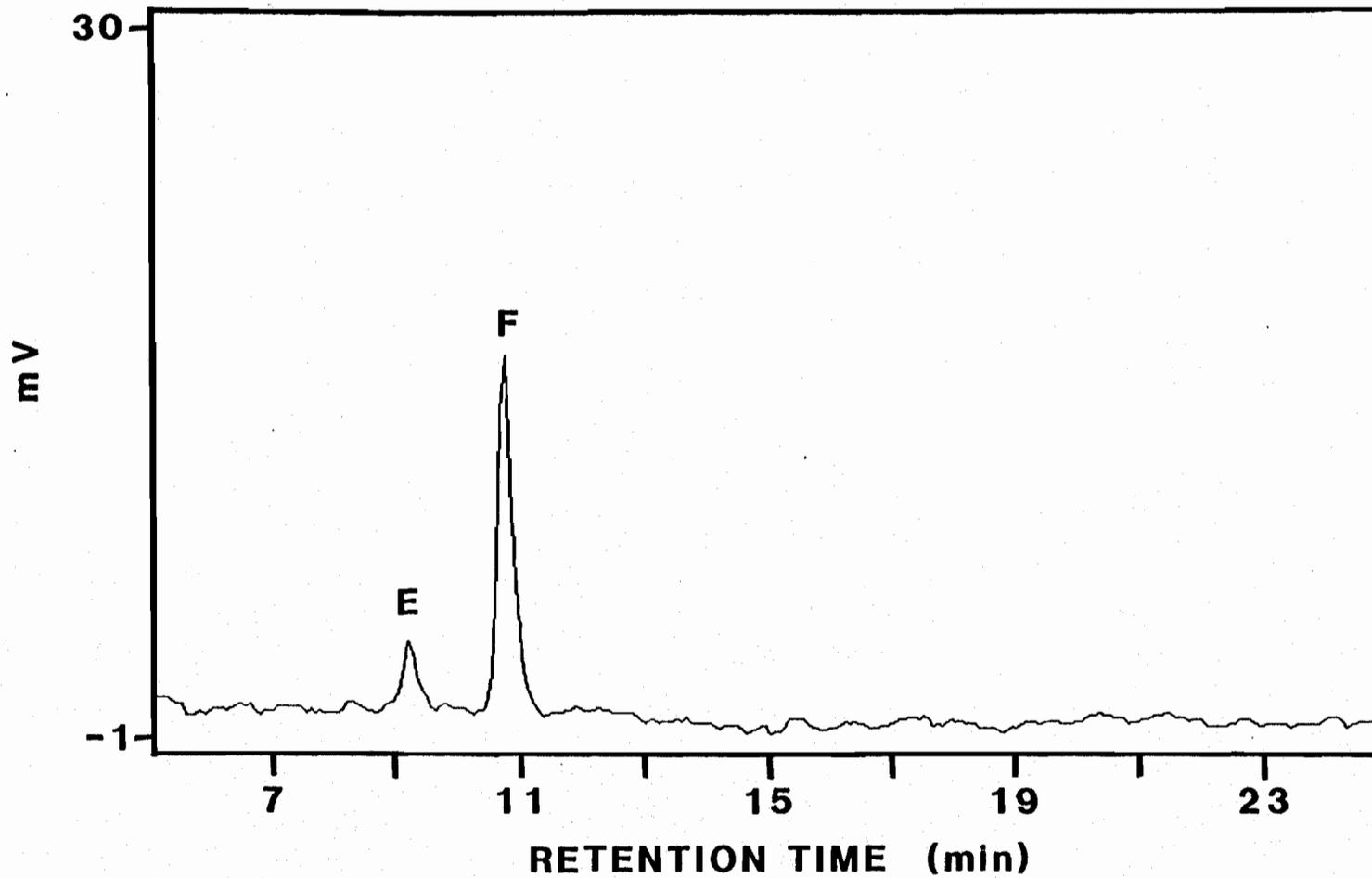


Figure IV-1

Figure IV-2. Plasma cortisol and cortisone (mean  $\pm$  standard error, n = 10) in sub-yearling coho salmon after a 30-sec handling stress. Points on a line associated with a common letter were not significantly different (Student-Newman-Keuls multiple range test).

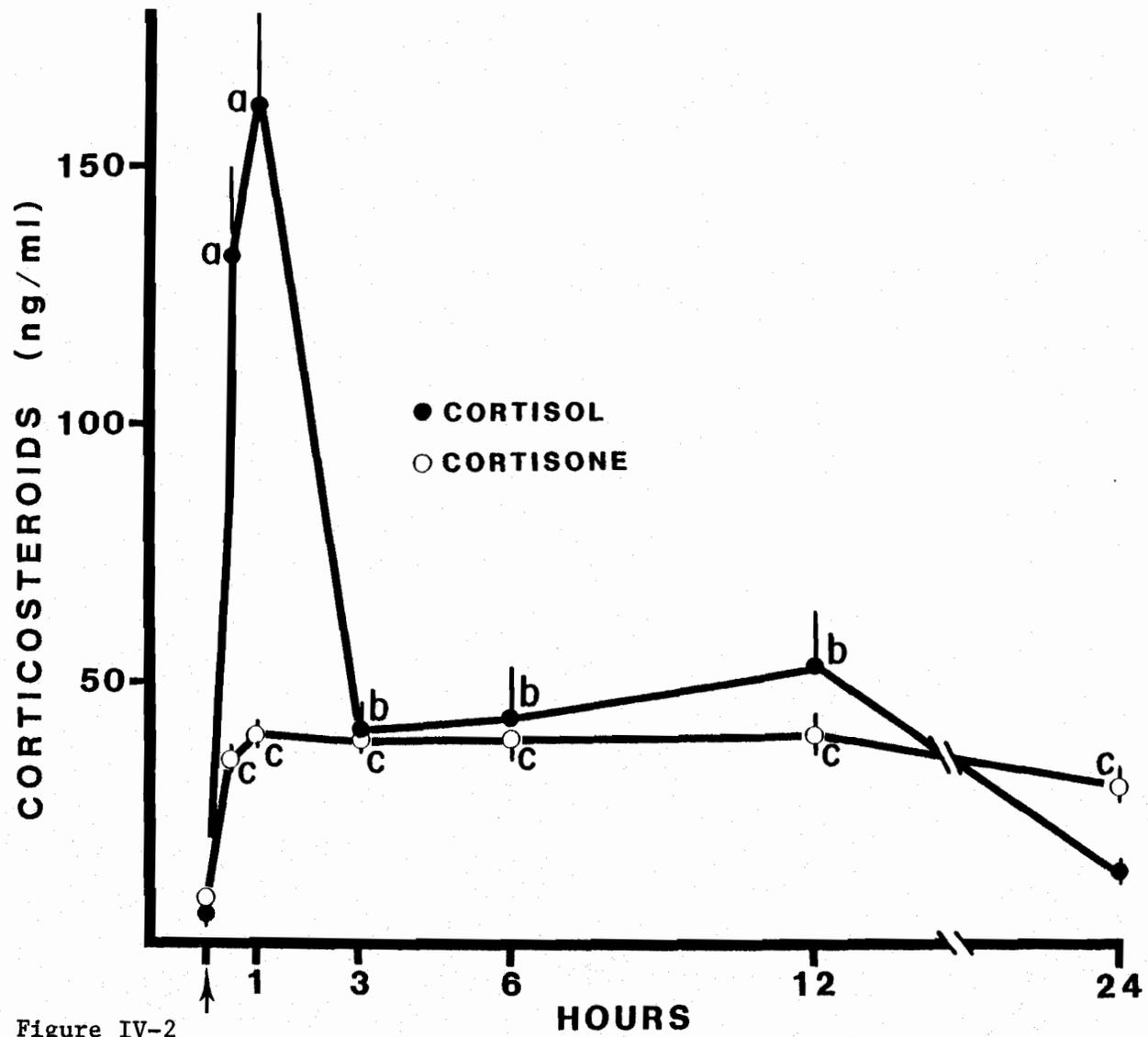


Figure IV-2

Figure IV-3. Plasma cortisol and cortisone (mean  $\pm$  standard error, n = 10) in yearling coho salmon after a 30-sec handling stress. Statistical significance as in Fig. 2.

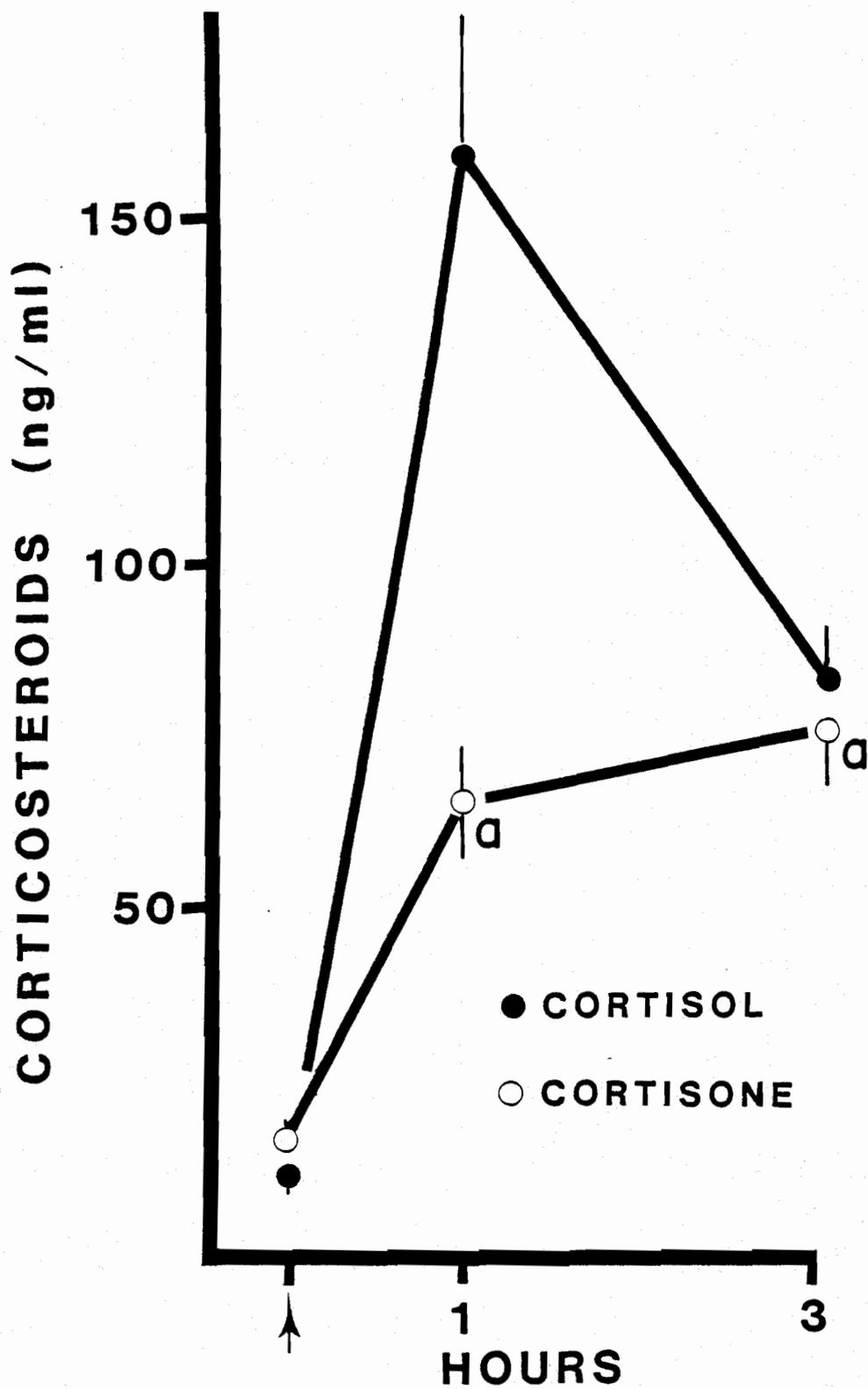


Figure IV-3

Figure IV-4. Plasma cortisol and cortisone (mean  $\pm$  standard error, n = 10) in yearling coho salmon after exposure to seawater (SW) in April. Control fish remained in fresh water (FW) throughout the experiment. Statistical significance as in Fig. 2.

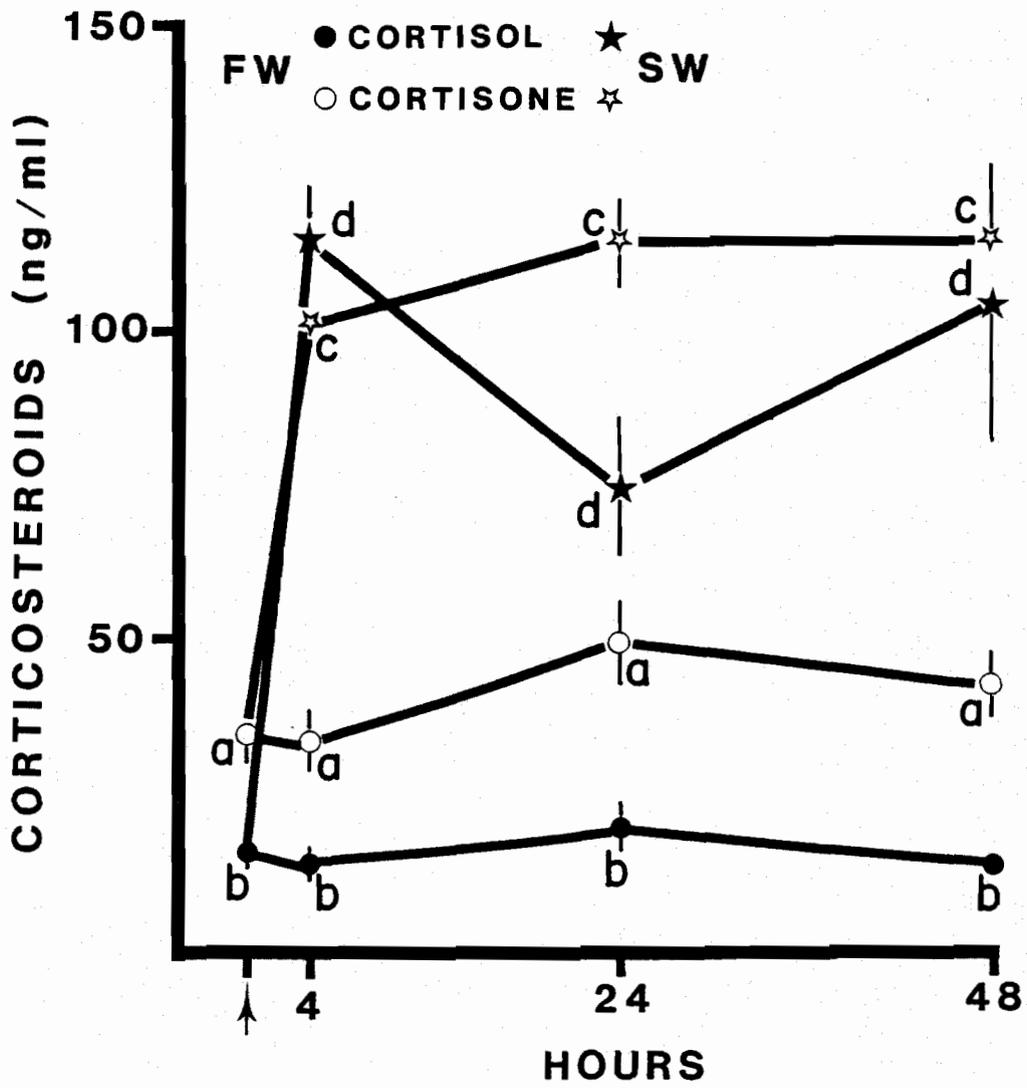


Figure IV-4

Figure IV-5. Plasma cortisol and cortisone (mean  $\pm$  standard error, n = 10) in yearling coho salmon after exposure to seawater (SW) in June. Control fish remained in fresh water (FW) throughout the experiment. Statistical significance as in Fig. 2.

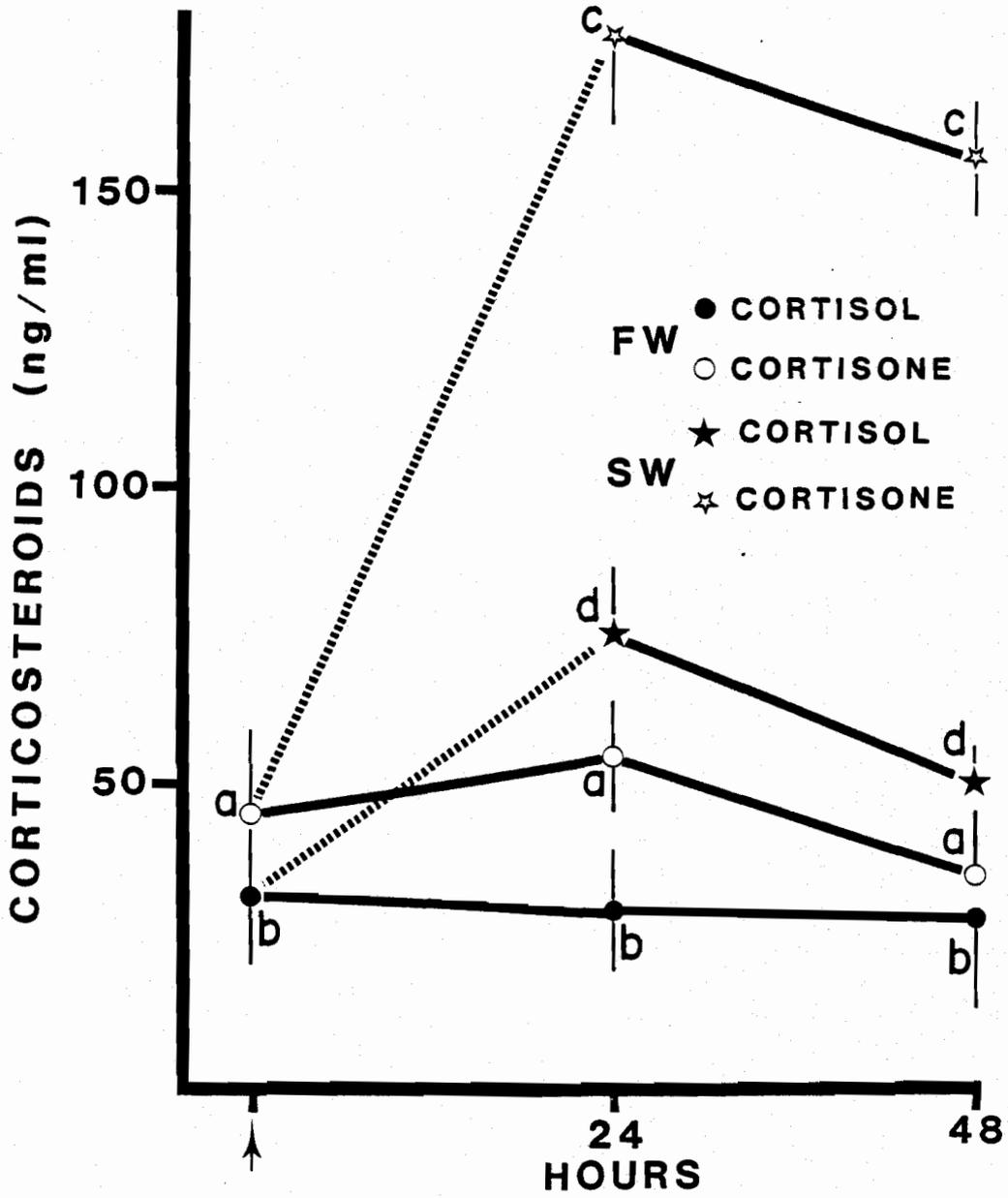


Figure IV-5

cortisol.

The cortisone:cortisol ratio of plasma showed a marked decline within 30 min after acute stress, returned to pre-stress levels by about 3 hr, and was higher than pre-stress levels at 24 hr (Fig. IV-6). After seawater exposure, the cortisone:cortisol ratio was lower than that in fresh water throughout the experiment in April, but was not different from that in fresh water in June (Fig. IV-7).

Plasma cortisol and cortisone concentrations were similar in sub-yearling and yearling fish reared at Smith Farm; however, in yearling fish reared at the Hatfield Marine Science Center, cortisone concentrations were significantly higher than those of cortisol (Table IV-1).

#### Discussion

Cortisol appears to be the major corticosteroid produced by the interrenal tissue of salmonid fishes, either from endogenous precursors (Nandi and Bern, 1965), or from exogenous radiolabeled precursors (e.g., Hargreaves et al., 1970; Sangalang et al., 1972). However, Nandi and Bern (1965) suggested that cortisone may also be secreted in lower but significant quantities by interrenal cells of rainbow trout (Salmo gairdneri). Moreover, Whitehouse and Vinson (1975) reported that significant amounts of cortisone, 11-deoxycortisol, and 17-deoxycorticosteroids were formed from exogenous precursors by the head kidney of Coregonus clupeoides. In the present study, however, by far

Figure IV-6. Cortisone:cortisol ratios (mean  $\pm$  standard error, n = 10) in sub-yearling or yearling coho salmon after a 30-sec handling stress. Statistical significance as in Fig. 2.

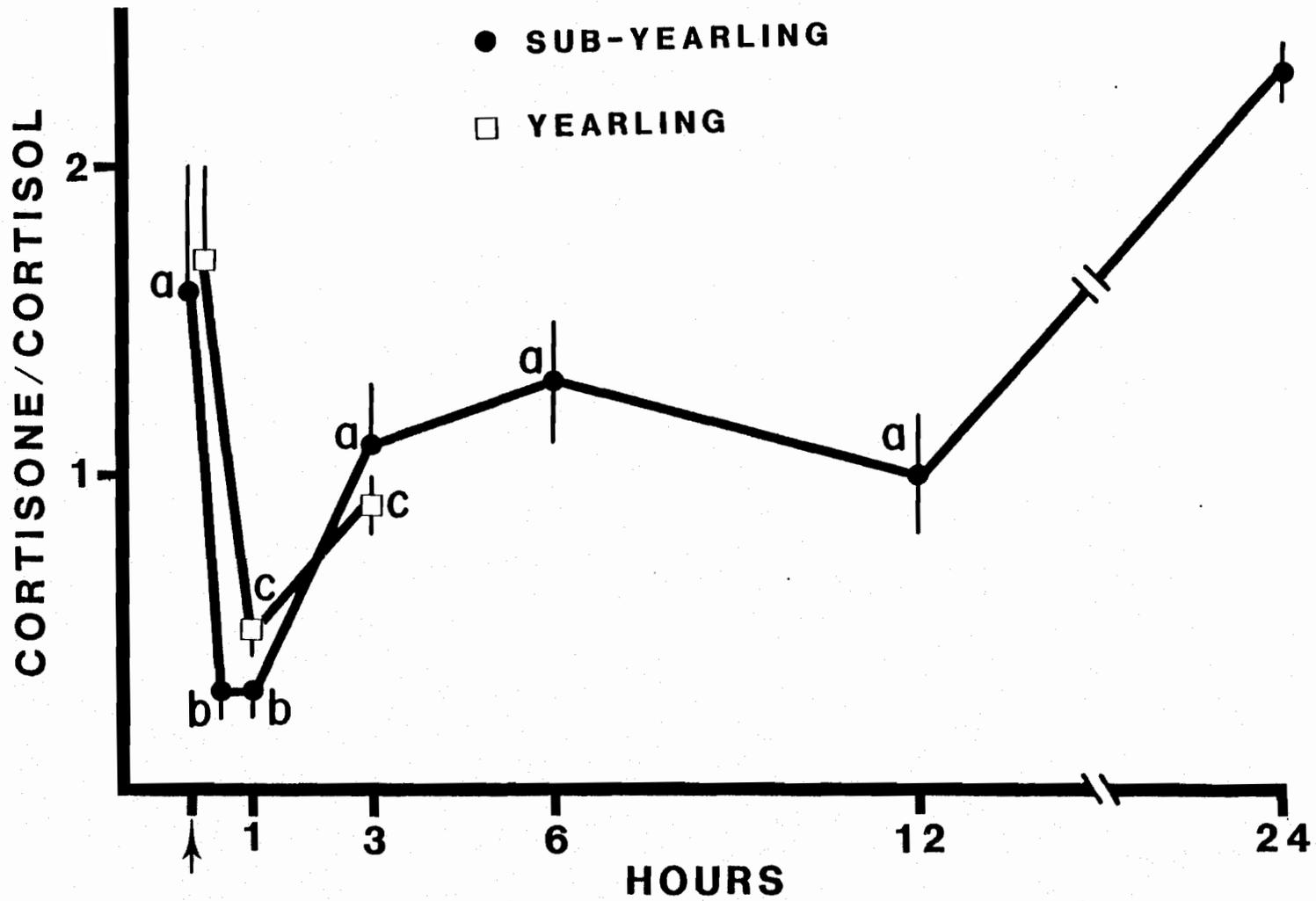


Figure IV-6

Figure IV-7. Cortisone:cortisol ratios (mean  $\pm$  standard error, n = 10) in yearling coho salmon exposed to seawater (SW) in April or June. Statistical significance as in Fig. 2.

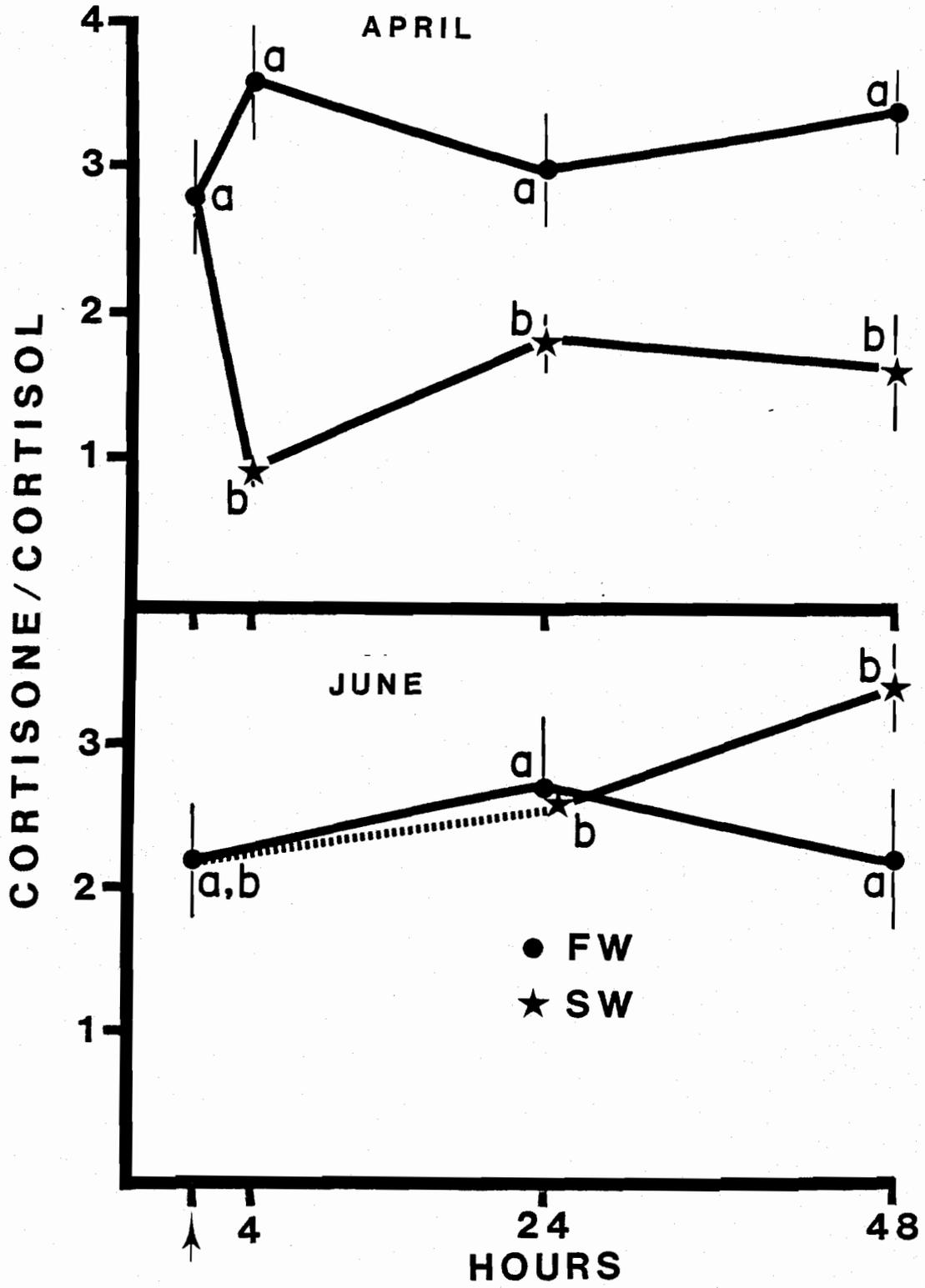


Figure IV-7

the major corticosteroid secreted by unstimulated or ACTH-stimulated interrenal tissue of coho salmon was cortisol. Although minute quantities of cortisone were also present in the incubation medium, it is uncertain that this steroid was secreted by the interrenal cells. Exogenous cortisol injected into the circulation of juvenile coho salmon is rapidly converted in vivo into plasma cortisone (Patiño et al., 1985). Moreover, various tissues of adult salmonids such as the spleen, heart, and gills seem able to convert cortisol into cortisone (Donaldson and Fagerlund, 1972). It is possible that some cortisol secreted by the interrenal cells was transformed into cortisone by the heterogeneous cell population of our head kidney preparations.

The biosynthesis of aldosterone in teleosts is a matter of controversy (Idler and Truscott, 1972; Sandor, 1979). Our study was not designed to detect small amounts of steroids in plasma or interrenal incubation medium. However, our results suggest that, if at all, endogenous aldosterone would be secreted in minuscule amounts by the interrenal cells of coho salmon. In comparison, interrenal tissue of an amphibian, Rana temporaria, secretes both aldosterone and corticosterone in approximately equal amounts, either spontaneously or in response to ACTH (Maser et al., 1982). Aldosterone has known physiological functions in amphibians, but its function in teleosts, if any, is unknown (Norris, 1985).

Patiño et al. (1985) found that cortisone is the major plasma metabolite of cortisol in juvenile coho salmon. Cortisone and cortisol together constituted 85 to 93% of total plasma radioactivity 4 hr after injection of radiolabelled cortisol. Moreover, plasma

cortisone does not appear to be converted to cortisol in salmonid fishes (Donaldson and Fagerlund, 1968). Thus, if cortisol is the only major product of the interrenal cells, cortisol and cortisone should be the principal corticosteroids in coho salmon plasma. Indeed, in the present study, these two hormones were the only corticosteroids detected by HPLC of plasma after stimulation of the interrenal tissue in vivo through stress. Furthermore, in plasma of undisturbed coho salmon in fresh water, the quantities of cortisone were similar to or higher than those of cortisol. This finding supports the previous observation of higher cortisone than cortisol levels in undisturbed coho salmon (Fagerlund, 1970) and in juvenile and adult Atlantic salmon (Weisbart and McGowan, 1984).

Plasma concentrations of cortisol and cortisone are affected by their binding and clearance kinetics. In adult sockeye salmon, the volume of distribution and plasma clearance rate of cortisol were higher than those of cortisone (Donaldson and Fagerlund, 1970; Fagerlund and Donaldson, 1970). In Atlantic salmon, the binding affinity for cortisone to plasma proteins was greater than that for cortisol (Freeman and Idler, 1971). Such differences, if true for yearling coho salmon, may contribute to high cortisone:cortisol ratios in plasma relative to the secretion of these steroids by the interrenal tissue.

We found that stress and seawater exposure in juvenile coho salmon resulted in rapid elevation of plasma cortisone. Moreover, the concentration of cortisone in plasma of undisturbed coho salmon is

appreciable. Therefore, as suggested by Weisbart and McGowan (1984), cortisone may have an important physiological role in salmonid fishes; however, these authors did not observe changes in either cortisol or cortisone concentrations in adult Atlantic salmon 24 or 48 hr after entry into seawater, contrary to our finding in juvenile coho salmon. Freeman and Idler (1973) showed that in vivo injections of cortisone, which was apparently not transformed into cortisol, increased glutamic-pyruvic transaminase activity in the liver of brook trout (Salvelinus fontinalis). Cortisone treatment decreased sodium ion and increased chloride ion in serum of hypophysectomized catfish, Ictalurus melas (Fortner and Pickford, 1982). However, Lee et al. (1986) reported that cortisol, but not cortisone, affected certain characteristics of a rainbow trout cell line in vitro. Transport of sodium and water by the intestine of goldfish (Carassius auratus) was stimulated by cortisol but unaffected by cortisone (Porthe-Nibelle and Lahlou, 1975). Studies addressing the possibility of specific receptors for cortisone are needed to clarify the role of this steroid.

The plasma corticosteroid profiles of yearling coho salmon in response to seawater differed significantly between the April and June experiments. The seasonal change in water temperature could be a factor responsible for this difference. The cortisone:cortisol ratio during seawater-induced cortisol secretion was lower than in freshwater controls in April, when the water temperatures were relatively low (12-13°), but no differences were observed in June, when the water temperature was higher (16-18°). Thus the rate of

conversion of cortisol into cortisone may have been faster in June due to the higher water temperatures. Such temperature dependent conversion rate of cortisol to cortisone has been observed in eels (Leloup-Hatey and Hardy, 1976). Also, Barton (1986) found that following an acute stress the return of plasma cortisol to its pre-stress levels was retarded in chinook salmon (Oncorhynchus tshawytscha) acclimated to low water temperatures. It is also possible that seasonal changes in the rate of plasma cortisol and cortisone clearance occur in juvenile anadromous salmonids, perhaps in relation to the developmental processes of smoltification as shown by Patiño et al. (1985). Interrenal secretion of cortisol also appears to change during the period of smoltification in coho salmon (Young, 1986).

Our results highlight a problem in studies suggesting a biological role for cortisol on the basis of its plasma levels: it appears that changes in plasma cortisol during the course of some physiological phenomena, at least in coho salmon, are accompanied by changes in the levels of its major plasma metabolite, cortisone. Also, biological effects ascribed to cortisol after in vivo treatment with this hormone may be equivocal, since cortisol is readily transformed into cortisone. In this regard, it is important to clarify the physiological significance of cortisone in teleostean fishes.

## Acknowledgments

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V: SPONTANEOUS AND ACTH-INDUCED INTERRENAL STEROIDOGENESIS  
IN JUVENILE COHO SALMON: EFFECTS OF MONOVALENT  
IONS AND OSMOLALITY IN VITRO

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## Abstract

We determined the in vitro effects of changes in extracellular monovalent ion levels and osmotic pressure on the spontaneous and ACTH-stimulated interrenal activity of coho salmon (Oncorhynchus kisutch). We used a perfusion system of incubation, and monitored interrenal activity by measuring the effluent cortisol content with radioimmunoassay. Increasing the medium osmolality with mannitol, from 206 to 290 or 353 mosmol, caused a minor enhancement (compared to the much greater effect of p-ACTH) in the spontaneous release of cortisol. A similar minor increase was observed when NaCl was elevated from 130 to 180 mM. On the other hand, no effect on the spontaneous release of cortisol was produced by increasing the KCl level from 3.2 to 9.6 mM, but was clearly increased when KCl was raised from 3.2 mM to a supraphysiological level of 27.2 mM. Ionic or osmolality changes, within the physiological range observed in coho salmon plasma, had no effects on the characteristics of interrenal secretion of cortisol in response to p-ACTH. If our results with interrenal cells in vitro are representative of the basic functioning of the cells in vivo, then one would have to conclude that changes in concentrations of plasma monovalent ions or in osmotic pressure may not play a significant physiological role in the direct regulation of interrenal steroidogenesis or corticosteroid release in coho salmon.

## Introduction

Stress in freshwater or seawater teleosts, or exposure of freshwater (euryhaline or stenohaline) teleosts to seawater causes marked changes in the levels of plasma monovalent ions ( $\text{Na}^+$  and  $\text{K}^+$ ) and osmolality (Eddy, 1981; Redding and Schreck, 1983; Hegab and Hanke, 1984). The changes in plasma corticosteroid concentrations observed under these situations may regulate osmoregulatory and other physiological processes responsible for adjusting the fish to the environmental challenge (Maetz, 1969; Johnson, 1973; Eddy, 1981; Schreck, 1981). Thus, the hydromineral regulatory function of corticosteroids in fish may be analogous (if not homologous) to their function in mammals where aldosterone--the principal mineralocorticosteroid in these animals--helps maintain the extracellular fluid volume and mineral balance (Yates et al., 1980).

Cortisol and cortisone are the major plasma corticosteroids in salmonid fishes at rest (Weisbart and McGowan, 1984; Patiño et al., 1987) and during stress or seawater acclimation (Patiño et al., 1987). Cortisol is the major corticosteroid secreted by unstimulated or ACTH-stimulated salmonid interrenal cells (Nandi and Bern, 1965; Patiño et al., 1987), while plasma cortisone appears to be formed by the peripheral transformation of cortisol (Idler and Truscott, 1963; Donaldson and Fagerlund, 1968; Patiño et al., 1985). It is not certain that aldosterone is produced by the interrenal cells of teleosts (Sandor, 1979). In contrast to mammals and amphibians, cortisol--in mammalian terminology, a glucocorticosteroid--is thought

to be the principal mineralocorticosteroid of teleosts (Norris, 1985). Cortisol in teleosts also has functions normally associated with glucocorticosteroids in mammals (Gorbman et al., 1983; Norris, 1985). However, little is known in fish regarding any direct effects that seawater- or stress-induced hydromineral imbalances may have on the spontaneous or stimulated corticosteroid secretion. In mammals, on the other hand, it has been well established that the level of extracellular ion content or osmotic pressure has a large influence on corticosteroid release by adrenocortical cells (e.g., Kaplan, 1965; Schneider et al., 1985; Robertson et al., 1984).

One study with rainbow trout (Salmo gairdneri) suggested that the spontaneous output of cortisol may not be significantly influenced by monovalent ions (Gupta et al., 1984). However, this study did not investigate the possibility of modulation of the stimulated output of corticosteroids by ions.

It is therefore evident that information on extrapituitary factors regulating interrenal function in teleosts, such as monovalent ions and osmotic pressure, would be of comparative value for research in interrenal (adrenocortical) physiology. Moreover, this knowledge would provide valuable insights into the role of corticosteroids in teleosts. The objectives of the present study were to determine the effects of  $\text{Na}^+$  and  $\text{K}^+$  concentrations and osmolality on the spontaneous and ACTH-stimulated cortisol secretion by the interrenal tissue of juvenile coho salmon (Oncorhynchus kisutch).

## Materials and Methods

Animals. Coho salmon were obtained as eggs or yearlings from hatchery stocks in Oregon. They were reared at Smith Farm Hatchery, Oregon State University, in flow-through circular tanks, and under natural photoperiod in fresh water at 12-13°. They were fed Oregon Moist Pellets daily.

Incubations. The interrenal tissue was sampled and processed as by Patiño et al. (1986a). The sex of the fish was determined and recorded at the time of sampling. For the osmolality experiments, the NaCl concentration for the control incubation was 85 mM, and the osmotic pressure was varied by addition of the inert sugar, d-mannitol (Sigma). The complete composition of the medium was described by Patiño et al. (1986a). The osmolalities (mosmol) of the media were 206 ( $\pm 1$ , standard error,  $n = 8$ ; no mannitol added), 290 ( $\pm 2$ ,  $n = 4$ ), or 353 mosmol ( $\pm 1$ ,  $n = 2$ ), as determined with a vapor pressure osmometer (Wescor, 5100 C). The tissues were started with a low osmolality in order to be able to manipulate the osmolality with mannitol. Preliminary experiments at our laboratory showed that the interrenal cells remained viable and responsive to ACTH for at least 24 hr under these conditions.

For the ion experiments, the NaCl and KCl concentrations for the control incubation medium were 130 mM and KCl 3.2 mM, respectively ( $289 \pm 1$  mosmol,  $n = 12$ ). Either the Na<sup>+</sup> or K<sup>+</sup> levels were varied by increasing the NaCl concentration to 180 mM ( $382 \pm 2$  mosmol,  $n = 5$ ) or the KCl concentration to 9.6 mM ( $299 \pm 2$  mosmol,  $n = 8$ ) or 27.2 mM

(337 mosmol,  $n = 1$ ). All the experimental osmolality and free-ion values (except  $K^+$  at 27.2 mM) used in this study fall within physiological levels observed in plasma of coho salmon under various conditions such as stress in fresh water or seawater, or during seawater acclimation (Redding and Schreck, 1983; and unpublished observations).

The minced tissues were preincubated for 2 hr in 8 ml of control medium at 13-14<sup>o</sup>, and were then transferred to a perfusion system for another 2-hr preincubation before the medium composition was changed. Individual interrenal tissues (head kidneys) were placed in vertical 1-cc plastic syringes (cut to a volume of 0.35 cc) and suspended with glass wool. Medium flowed into the syringe via silicon tubing and a Vacutainer (Becton-Dickinson) needle inserted through a plastic plunger. The out-flowing end of the syringe was connected to a peristaltic pump (Rainin Rabbit<sup>TM</sup>) and the effluent was collected with a micro-fractionator (Gilson, FC-80). The flow was set at 0.3 ml/min, and the fractions were separated every 6 min. The total void volume was 0.7 ml. Four separate interrenals could be simultaneously perfused with this set-up.

To determine the effects on the spontaneous output of cortisol, we collected two fractions (12 min) before the change in medium composition and ten fractions (1 hr) after the change. The medium for control interrenals was not changed. In some experiments, porcine-ACTH (Sigma; 100 IU/mg) was infused for 6 min, 1 hr after the change in medium conditions, into control and treated interrenals and 20 fractions (2 hr) were subsequently collected. The treatment continued

uninterrupted throughout the experiment.

In preliminary experiments, in which we tested the tissue responses to different doses of ACTH (Fig. V-1), ACTH at 5 mU/ml elicited a marked but submaximal interrenal response with a clear peak in activity. We selected this dose for subsequent experiments. We determined both the peak response rate (cortisol concentration/min in fraction with the highest level) and the total cortisol output (sum of cortisol content in all 20 fractions) in response to ACTH. The unstimulated (spontaneous) output of cortisol did not reach a stable level during our experiments. We do not think that this continuing decline in output was due to cell loss or steroid precursor depletion since, although the spontaneous output of cortisol at 7 hr post-sampling was 49% of the value at 5 hr (the normal time of ACTH exposure in our experiments), neither peak response rates nor total cortisol output differed when the tissues were exposed to ACTH at these two times (Table V-1). Moreover, preliminary results at our laboratory showed that the steroidogenic response of interrenal cells to a secondary stimulation with ACTH is similar to the response to the primary stimulation given 2 hr earlier.

Cortisol radioimmunoassay. Cortisol was directly determined in 200  $\mu$ l or 300  $\mu$ l of medium according to the method of Redding et al. (1984) as modified by Patiño et al. (1986a).

Statistical analyses. The spontaneous output of cortisol from head kidney tissue showed a non-linear correlation with the body weight of fish. However, we expressed the data in terms of percent

Figure V-1. Mean cortisol secretion rate of interrenal tissue of yearling coho salmon (mean weight, 41 g) in an in vitro perfusion system. The tissues were exposed to various doses (mU/ml) of porcine-ACTH for 6 min (during the collection of fraction number 1), and 20 sequential fractions of six minutes duration each were collected over a period of 2 hr. The sample sizes were 2, 4, and 2 for the 0.5, 5 and 500 mU/ml groups, respectively.

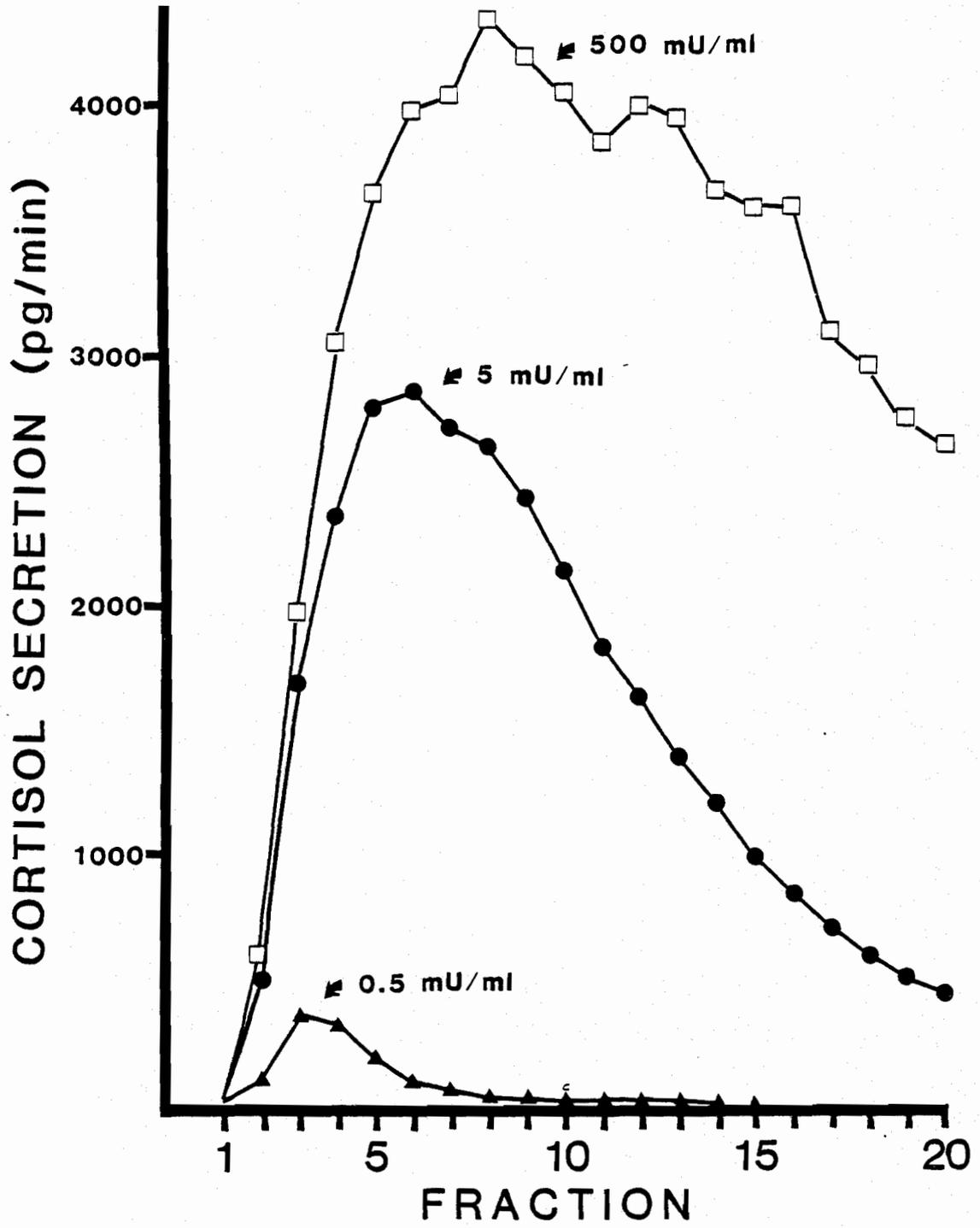


Figure V-1

Table V-1. Effects (mean  $\pm$  SE) of preincubation time on the spontaneous release of cortisol and on the peak secretion rate and total (2-hr) output of cortisol after a 6-min exposure to p-ACTH (5 mU/ml) by interrenal tissue of yearling coho salmon (mean weight, 68 g) in vitro. Differences in output between treatment times were determined with Student's t-test (\*\*, significantly different;  $P \leq 0.001$ )

Pre-incubation		Spontaneous	Peak	Total
<u>time (hr)</u>	<u>n</u>	<u>(pg/min)</u>	<u>(pg/min)</u>	<u>(ng)</u>
5	6	91 $\pm$ 9	4585 $\pm$ 439	297 $\pm$ 37
7	6	45 $\pm$ 5 **	4196 $\pm$ 692	249 $\pm$ 52

secretion of the fraction immediately preceding the treatment, since coefficients of variability were generally smallest with this transformation. We analyzed the results with nonparametric 2-way ANOVA for repeated observations. Main and interaction effects of treatment and fraction number were determined. If a treatment effect was ascertained, 1-way ANOVA followed by Duncan's multiple range test was used to determine differences among mean output rates within each fraction number (for this analysis, we used  $1/y$  transformations in order to homogenize variances).

Neither peak response rates nor total cortisol output following the 6-min ACTH exposure showed clear correlations with body weight within any given experiment, and coefficients of variability were not reduced by expressing the output in terms of percent of pretreatment values. These results were therefore expressed as pg cortisol/min (peak response rate) or as ng cortisol (total cortisol output). The log-transformed data were analyzed with the regression approach to 2-way ANOVA (treatment X sex). Only main effects were determined in these analyses.

We used an alpha value of 0.05 to judge the significance of statistical differences in our tests.

## Results

Increasing the medium osmolality with mannitol, from 206 to 290 or 353 mosmol, caused a slight but statistically significant increase in the spontaneous output of cortisol; the greater the increase in

osmolality, the longer the effect appeared to be (Fig. V-2). However, neither peak response rates nor total cortisol output after ACTH exposure were affected by medium osmolality (Table V-2). Since the sex of the fish did not affect the secretory characteristics of the interrenal cells in any of the present experiments, we combined the results for both sexes in the tables.

An increase in the NaCl concentration from 130 to 180 mM while holding  $K^+$  constant caused a slight increase in the spontaneous cortisol output, but an increase in KCl levels from 3.2 to 9.6 mM while holding NaCl constant had no such effect (Fig. V-3). These changes in  $Na^+$  or  $K^+$  had no effect on the ACTH-induced interrenal production of cortisol (Table V-3). A pharmacological increase in KCl from 3.2 to 27.2 mM markedly enhanced the spontaneous cortisol output, which was still higher than pretreatment levels at the end of the experiment (Fig. V-3).

### Discussion

This study suggests that there are significant differences in the extrapituitary control of corticosteroid secretion between salmon and other vertebrates previously studied.

In mammals (Schneider et al., 1985) and amphibians (Maser et al., 1982; Hanke et al., 1984), increases in extracellular  $Na^+$  levels or osmolality inhibited the spontaneous release of corticosteroids. However, the effect of  $Na^+$  on the spontaneous aldosterone secretion in mammals may not be specific to this ion, but may instead be associated

Figure V-2. Cortisol secretion rate (mean  $\pm$  SE) of interrenal tissue of yearling coho salmon (mean weight, 35 g) in an in vitro perfusion system. The osmolality (mOsmol) of the incubation medium was increased from the control level by addition of mannitol, beginning with fraction number 1, and the treatment continued uninterrupted for the duration of the experiment. Fractions were collected sequentially every six minutes. The medium conditions for the control group were not changed. Means within fraction numbers bearing a common letter were not significantly different. The sample size was four for each treatment.

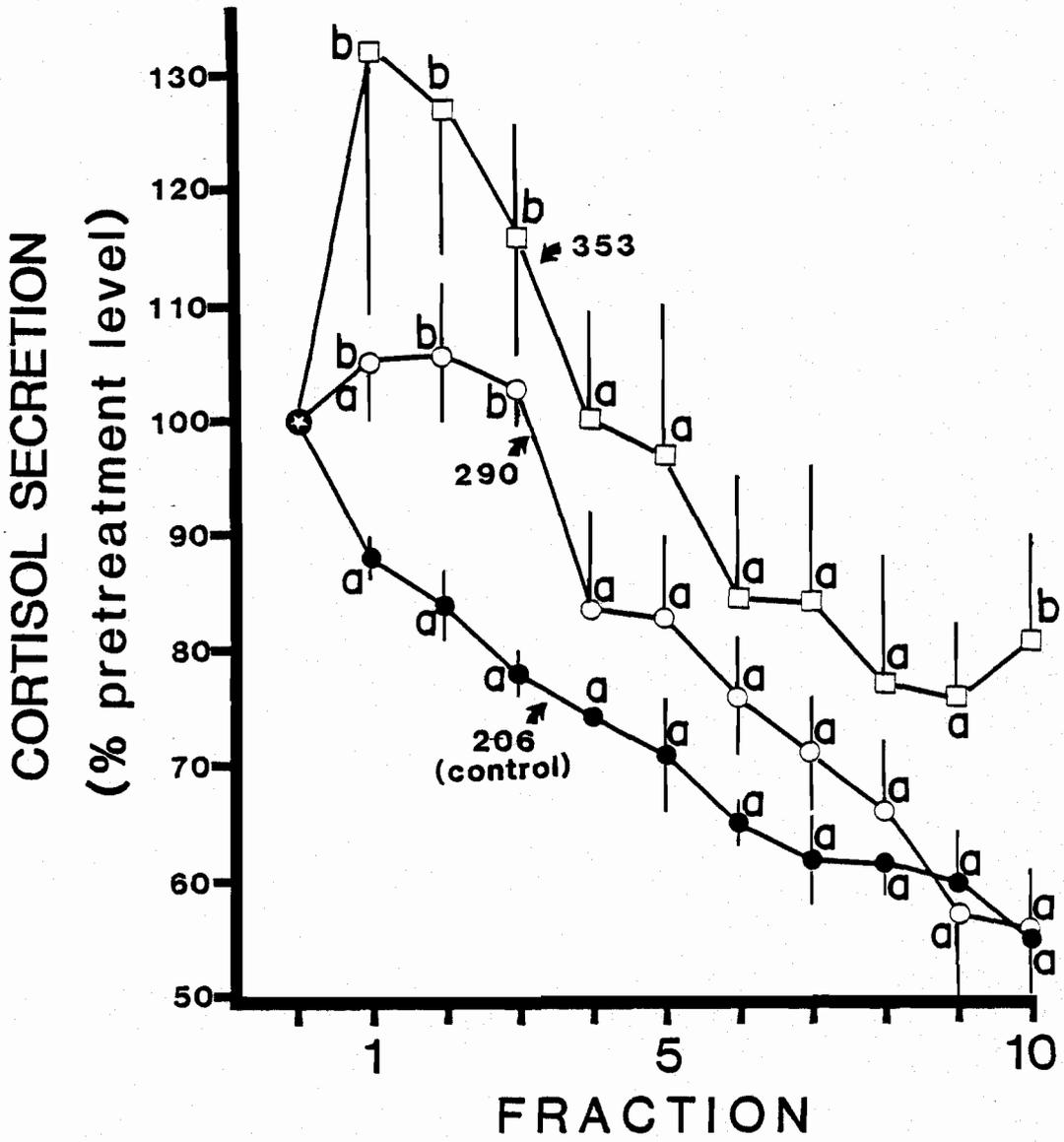


Figure V-2

Table V-2. Effects (mean  $\pm$  SE) of medium osmolality (manipulated with d-mannitol) on peak secretion rate and total (2-hr) output of cortisol after a 6-min exposure to p-ACTH (5 mU/ml) by interrenal tissue of yearling coho salmon (mean weight, 38 g) in vitro. No differences in interrenal response to ACTH were observed.

Osmolality (mosmol)	<u>n</u>	Peak (pg/min)	Total (ng)
206	10	3125 $\pm$ 360	174 $\pm$ 20
290	10	3784 $\pm$ 374	210 $\pm$ 22
353	8	3172 $\pm$ 435	196 $\pm$ 31

Figure V-3. Cortisol secretion rate (mean  $\pm$  SE) of interrenal tissue of yearling coho salmon (mean weight, 66 g) in an in vitro perfusion system. The ionic composition (mEq/l) of the medium was changed, beginning with fraction number 1, and the treatment continued uninterrupted for the duration of the experiment. Fractions were collected sequentially every 6 minutes. The medium conditions for the control group were not changed. Means within fraction numbers bearing a common letter were not significantly different. The sample sizes were 5, 6, 6, and 4 for the control, Na<sup>+</sup> 180, K<sup>+</sup> 9.6, and K<sup>+</sup> 27.2 groups, respectively.

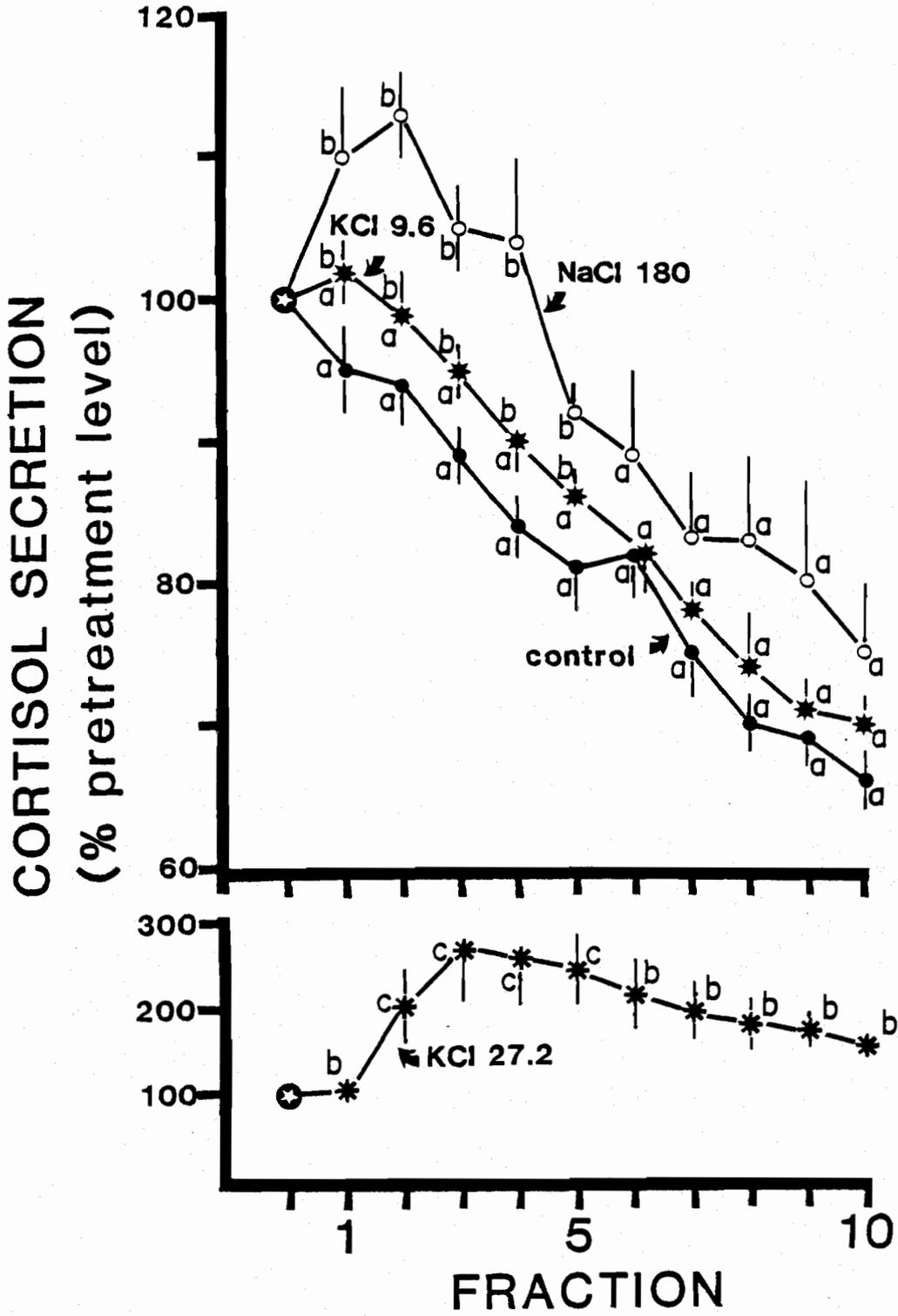


Figure V-3

Table V-3. Effects (mean  $\pm$  SE) of ionic composition of the medium on peak secretion rate and total (2-hr) output of cortisol after a 6-min exposure to p-ACTH (5 mU/ml) by interrenal tissue of yearling coho salmon (mean weight, 60 g) in vitro. The levels of Na<sup>+</sup> (as NaCl) and K<sup>+</sup> (as KCl) in the control medium were 130 mM and 3.2 mM, respectively. No differences in interrenal response to ACTH were observed.

<u>Ions</u> <u>(mM)</u>	<u>n</u>	<u>Peak</u> <u>(pg/min)</u>	<u>Total</u> <u>(ng)</u>
Control	13	4049 $\pm$ 147	247 $\pm$ 18
Na <sup>+</sup> : 180	10	4665 $\pm$ 364	268 $\pm$ 18
K <sup>+</sup> : 9.6	9	3570 $\pm$ 322	224 $\pm$ 26

with the changes in extracellular osmotic pressure accompanying the change in ion levels (Schneider et al., 1985). In the present study, an increase in  $\text{Na}^+$  caused a temporary increase--not a decrease--in the spontaneous output of interrenal cortisol, which in teleosts is considered the principal mineralocorticosteroid (Norris, 1985). Nevertheless, as in mammals, the small effect of  $\text{Na}^+$  on the spontaneous secretion of cortisol in coho salmon may be mediated by extracellular osmolality. We observed similar increases in interrenal cortisol output when the medium osmolality was raised either with NaCl or mannitol. Also, replacement of  $\text{Na}^+$  with choline in the incubation medium did not affect the spontaneous cortisol release from rainbow trout interrenal cells (Gupta et al., 1984).

In rainbow trout, changes in  $\text{K}^+$  levels within the physiological range seen in salmonids apparently produced no effect on the spontaneous output of cortisol in vitro (Gupta et al., 1984). Similarly, the spontaneous release of cortisol in coho salmon was not measurably affected by an increase in  $\text{K}^+$  (as KCl) from 3.2 mM to 9.6 mM; only when  $\text{K}^+$  was increased to a supraphysiological level of 27.2 mM was an effect on cortisol output evident. Similar results have been reported for the interrenal secretion of aldosterone and corticosterone in Rana temporaria (Maser et al., 1982). In mammals, on the other hand, relatively small increases in extracellular  $\text{K}^+$  levels clearly enhanced the spontaneous adrenal output of both aldosterone and glucocorticosteroids (Braley and Williams, 1977; Fredlund et al., 1977; Pham-Huu-Trung et al., 1979; Farese et al., 1980a; Lymangrover and Martin, 1981). Thus, it is questionable

whether  $K^+$  plays a significant physiological role in the spontaneous release of corticosteroids either in salmonids or Rana.

It is evident, however, that similar mechanisms for  $K^+$  action on corticosteroid secretion occur throughout the vertebrates since all species studied to date respond to increased--albeit sometimes supraphysiological--levels of  $K^+$ . The possible role of  $K^+$  in corticosteroid biosynthesis and secretion has been recently discussed by Foster et al. (1982), Robertson et al. (1984), and Kenyon et al. (1985b), among others.

In mammals, extracellular  $K^+$  and osmolality have significant modulatory effects on the angiotensin II-induced adrenal corticosteroid secretion (Kaplan, 1965; Fredlund et al., 1977; Schneider et al., 1985). Moreover, Fredlund et al. (1977) reported modulatory effects of  $K^+$  on ACTH-induced aldosterone secretion, and Lymanagrover and Martin (1981) and Robertson et al. (1984) observed modulatory effects of this ion on ACTH-induced glucocorticosteroid secretion. In fish, it is unclear whether the renin-angiotensin system is involved in the regulation of interrenal steroidogenesis (e.g., Henderson et al., 1976; Kenyon et al., 1985a; Takahashi et al., 1985). However, the present study showed that, unlike the situation in mammals, large changes within the physiological range of extracellular  $K^+$  or osmotic pressure had no modulatory effects on the in vitro interrenal response to ACTH in coho salmon.

As has been reported for mammals (Matthews and Saffran, 1973),  $Na^+$  in coho salmon did not influence the ACTH-stimulated

corticosteroid output from interrenal cells.

In conclusion, if our results with in vitro interrenal preparations reflect the functioning of these cells in vivo, it would appear unlikely that extracellular changes in levels of monovalent ions or osmotic pressure, such as those occurring during seawater acclimation or stress, play a significant role in the regulation of either the spontaneous or ACTH-stimulated cortisol secretion in juvenile coho salmon. A similar conclusion in rainbow trout regarding the effects of monovalent ions on the unstimulated interrenal release of cortisol is evident from the results of Gupta et al. (1984). Although the spontaneous release of cortisol in the present study was influenced by  $\text{Na}^+$  and mannitol by a mechanism apparently associated with osmotic pressure (increased osmotic pressure may have--by dehydration--resulted in a non-specific release of cortisol from interrenal cells), this effect was relatively minor (compared with the effect of ACTH) and appeared to be temporary; it was, moreover, the opposite of the effect observed in mammal and amphibian adrenocortical preparations under similar conditions. Significant differences thus appear to exist--particularly concerning the effects of extracellular osmolality--in the physiological mechanisms of control of interrenal (adrenocortical) function between coho salmon, on the one hand, and amphibians and mammals, on the other. Perhaps these differences are associated with the functions of the respective major corticosteroids in these groups of vertebrates. Hydromineral balance and intermediary metabolic activity are performed by the so-called mineralocorticosteroids and glucocorticosteroids, respectively, in

amphibians and mammals, whereas only one type of corticosteroid, the glucocorticosteroid, appears to serve both functions in teleosts (Gorbman et al., 1983; Norris, 1985). Differences in function may thus require differences in regulation. It remains to be established if the renin-angiotensin system is involved in the regulation of interrenal function of teleosts; if an involvement of this system is determined, the possible modulation of its effects on interrenal function by ions or osmolality would need to be evaluated.

#### Acknowledgments

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VI: ADENYLATE CYCLASE ACTIVATORS AND INHIBITORS CYCLIC  
NUCLEOTIDE ANALOGS, AND PHOSPHATIDYLINOSITOL: EFFECTS ON  
INTERRENAL FUNCTION OF COHO SALMON IN VITRO

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## Abstract

The role of cyclic AMP (cAMP) as mediator of ACTH action on interrenal steroidogenesis was evaluated in juvenile coho salmon (Oncorhynchus kisutch). Head kidneys (containing the interrenal cells) were incubated in the absence or presence of putative adenylate cyclase activators (forskolin and cholera toxin), ACTH combined with putative adenylate cyclase inhibitors (hydrolysis-resistant ATP analogs), dibutyryl cyclic (dbc) AMP, dbcGMP, or phosphatidylinositol. The cortisol content of the incubation medium was subsequently determined by radioimmunoassay. Forskolin markedly stimulated cortisol secretion by interrenal cells. Adenylate cyclase inhibitors depressed the steroidogenic response to ACTH. Dibutyryl cAMP, but not dbcGMP, enhanced steroid secretion. Thus, cAMP seems to be an important "second messenger" for ACTH action on salmon interrenal cells. In contrast to findings in mammalian adrenocortical cells, exogenous phosphatidylinositol and cholera toxin failed to stimulate corticosteroid secretion in salmon interrenal cells. However, it was unclear whether these negative findings were an artifact resulting from the use of kidney tissue fragments instead of isolated interrenal cells.

## Introduction

ACTH is a major hormone regulating adrenal (interrenal) corticosteroid production in all groups of vertebrates, from mammals (Yates et al., 1980) to fish (Donaldson, 1981). In mammals, a large number of studies have suggested that a "second messenger," cyclic AMP (cAMP), is an important intracellular mediator of ACTH action on adrenocortical steroidogenesis (Garren et al., 1971; Saez et al., 1981). Moreover, it was recently suggested that a major link between increased adrenal cAMP and steroid production in response to ACTH is a change in phospholipid metabolism. Treatment with ACTH increased adrenal inositol phospholipid content in vivo and in vitro, while cAMP had the same effect in vitro; and treatment of adrenocortical cells in vitro with inositol phospholipids enhanced the rate of steroid production by the cells (Farese et al., 1979, 1980b, c).

Little is known about the mechanism of ACTH action on interrenal cells of fish. The results of a single study suggested that cAMP is indeed a "second messenger" for the steroidogenic action of ACTH on the interrenal cells of a cichlid fish (Ilan and Yaron, 1980). In the crocodilian adrenal, however, cyclic GMP (cGMP), not cAMP, is elevated in response to ACTH (Honn and Chavin, 1975, 1977).

Substances that activate adenylate cyclase, such as forskolin (Seamon et al., 1981) and cholera toxin (Gill, 1977), may be useful probes to study the role of cAMP in hormone action. Likewise, substances that inhibit adenylate cyclase, such as some analogs of adenosine (Haslam et al., 1978) and ATP (Krug et al., 1973a,b), could

also provide valuable information.

We evaluated the role of cAMP in ACTH-induced steroidogenesis by interrenal cells of coho salmon, Oncorhynchus kisutch. Specifically, we determined the effects of potential adenylate cyclase activators (forskolin and cholera toxin) and inhibitors (hydrolysis-resistant ATP analogs), as well as those of cAMP and cGMP analogs on cortisol production by interrenal cells. Moreover, we also determined whether inositol phospholipids (phosphatidylinositol) have steroidogenic effects on interrenal cells. We monitored the steroidogenic activity of interrenal cells of coho salmon by measuring cortisol, since this appears to be the only major steroid secreted in response to ACTH or dibutyryl cyclic (dbc) AMP in vitro as determined by high-performance liquid chromatography of incubation medium extracts (R. Patiño, G. Feist, and C.B. Schreck, unpublished).

#### Materials and Methods

Experimental animals. Juvenile coho salmon from Eagle Creek (Oregon) or Willard (Washington) National Fish Hatchery of the U.S. Fish and Wildlife Service were used for this study. Yearling fish were transported from the hatcheries to Oregon State University (Corvallis, Oregon), where they were reared in freshwater at 12-13°, and fed Oregon Moist Pellets daily. Mean weights of the fish used in the different experiments ranged from 18 to 43 g.

Test substances. Porcine ACTH, dibutyryl cyclic (dbc) AMP, dbcGMP, cholera toxin,  $\alpha$ ,  $\beta$ -methylene-adenosine 5'-triphosphate

(Ap(CH<sub>2</sub>)pp),  $\beta$ ,  $\gamma$ -methylene-adenosine 5'-triphosphate (App(CH<sub>2</sub>)p), and L- $\alpha$ -phosphatidylinositol were purchased from Sigma Chemical Co. (St. Louis, MO); forskolin was obtained from Calbiochem-Behring (La Jolla, CA).

Incubation of interrenal cells. Fish were killed by a blow to the head and exsanguinated by severing the caudal peduncle, and their head kidneys (containing the interrenal cells) were removed and placed in 8 ml of ice-cold medium containing MgSO<sub>4</sub>, 1.25 mM; NaHPO<sub>4</sub>, 0.49 mM; NaCl, 85 mM; KCl, 3.22mM; CaCl<sub>2</sub>, 3.21 mM; Hepes, 2.38 g/l; glucose, 2.5 g/l; bovine serum albumin, 0.5 g/l; penicillin, 1 X 10<sup>5</sup> units/l; streptomycin, 100 mg/l; and phenol red. The pH was adjusted to 7.5 (This medium represents a slight modification of one developed by Dr. Robert Hard, Department of Zoology, Oregon State University.) The tissues were then cut into small fragments ( $\leq 1 \text{ mm}^3$ ) and preincubated at 13-14<sup>o</sup> for 4.5-5 hr with one change of medium after 2 hr.

Test substances were dissolved in incubation medium and, if necessary, the pH was adjusted to 7.5. Phosphatidylinositol was suspended in medium by sonication. Incubations were carried out in 1.5 or 3 ml of medium at 13-14<sup>o</sup>. At the end of 2.5 hr (or 5 hr for incubations with cholera toxin), fractions of medium were taken and frozen until assayed for cortisol content. Preincubations and incubations were performed under continuous shaking.

Cortisol radioimmunoassay. Cortisol was measured directly in 50  $\mu$ l of incubation medium following the procedures for plasma developed by Redding et al. (1984). The standards were dissolved in incubation medium.

## Results

Forskolin had a marked positive effect on cortisol production by the interrenal cells (Fig. VI-1). The smallest effective dose seemed to be 1  $\mu$ M, and the maximal response was obtained with 10  $\mu$ M. In contrast, cholera toxin at 10 or 100 nM failed to stimulate interrenal steroidogenesis (Fig. VI-2).

The ATP analogs used in this study reduced the effect of a maximally effective concentration of ACTH on interrenal steroidogenesis (Fig. VI-3). Concentration of Ap(CH<sub>2</sub>)pp and inhibition of steroid secretion were directly related. Moreover, App(CH<sub>2</sub>)p proved to be as effective as, or more effective than, Ap(CH<sub>2</sub>)pp in blocking the action of ACTH.

Steroidogenesis could be stimulated in the absence of ACTH by dbcAMP (Figs. VI-4, VI-5). However, the response to this cyclic nucleotide analog was biphasic: cortisol secretion first increased, then decreased, as the concentration of dbcAMP was increased. The turning point in the biphasic response appeared to occur at a dbcAMP concentration of about 1-10 mM. On the other hand, dbcGMP at a concentration of 1 or 10 mM had no effect on the steroidogenic activity of interrenal cells (Fig. VI-5).

Phosphatidylinositol at concentrations of 0.04 to 1 mM had no effect on cortisol secretion by the interrenal cells (Fig. VI-6).

Figure VI-1. Effects of increasing concentrations of forskolin on cortisol production by interrenal cells of coho salmon. The "1000" represents a saturated solution of forskolin. Values represented by bars bearing the same letter are not significantly different (one-way ANOVA and Duncan's multiple range test,  $P \leq 0.05$ ;  $n = 5$ ).

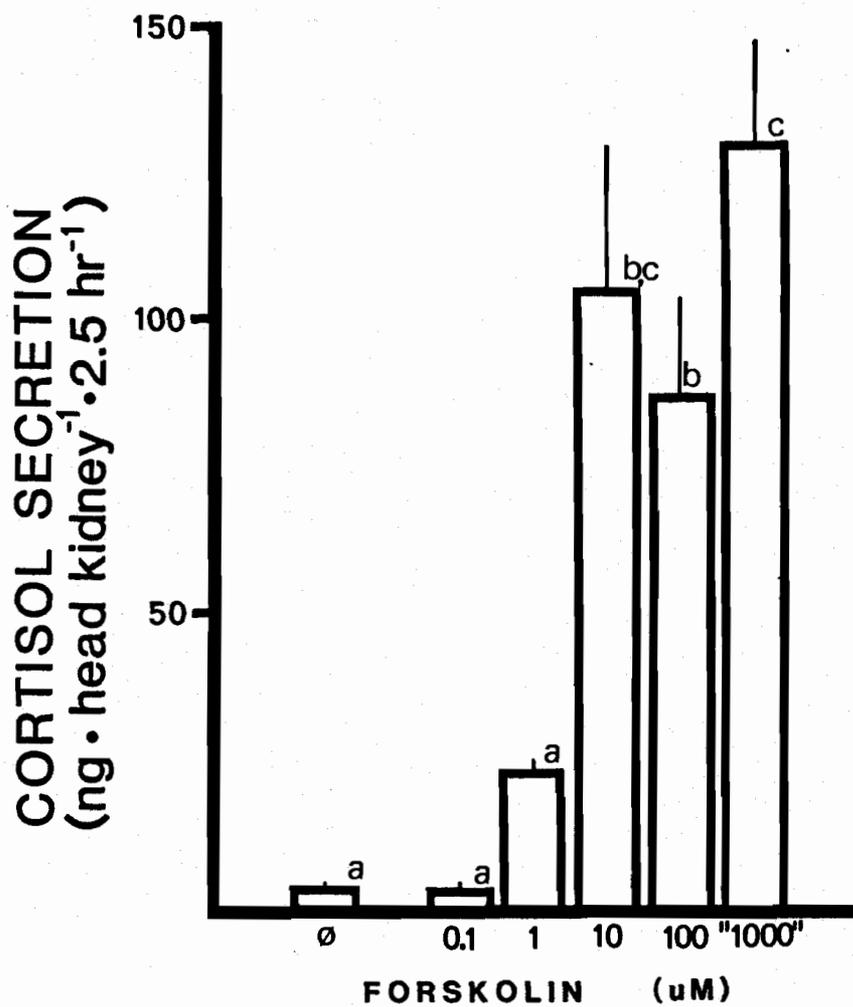


Figure VI-1

Figure VI-2. Effects of cholera toxin on interrenal steroidogenesis.

The three values did not differ significantly (one-way ANOVA,  $P \geq 0.05$ ;  $n = 5$ ).

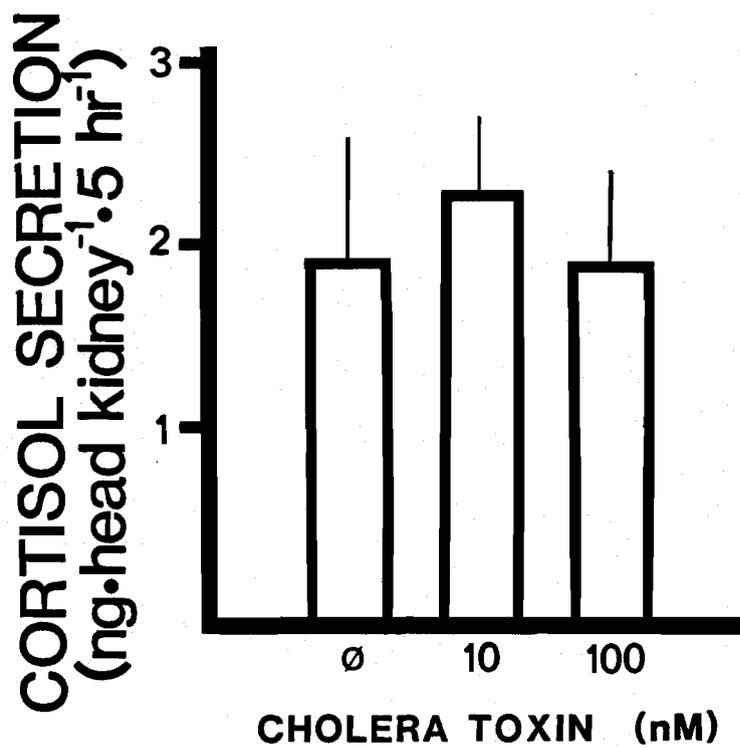


Figure VI-2

Figure VI-3. Effects of  $\alpha, \beta$ - ( $\alpha, \beta$ ) or  $\beta, \delta$ - ( $\beta, \delta$ ) methylene ATP analogs on ACTH-induced cortisol production by interrenal cells. A maximally effective ACTH concentration (ACTH<sub>m</sub>; 83 mU/ml) was used in combination with the ATP analogs. Values represented by bars bearing the same letter are not significantly different (one-way ANOVA and Duncan's multiple range test,  $P \leq 0.05$ ;  $n = 5$ , or 4 as indicated in parentheses).

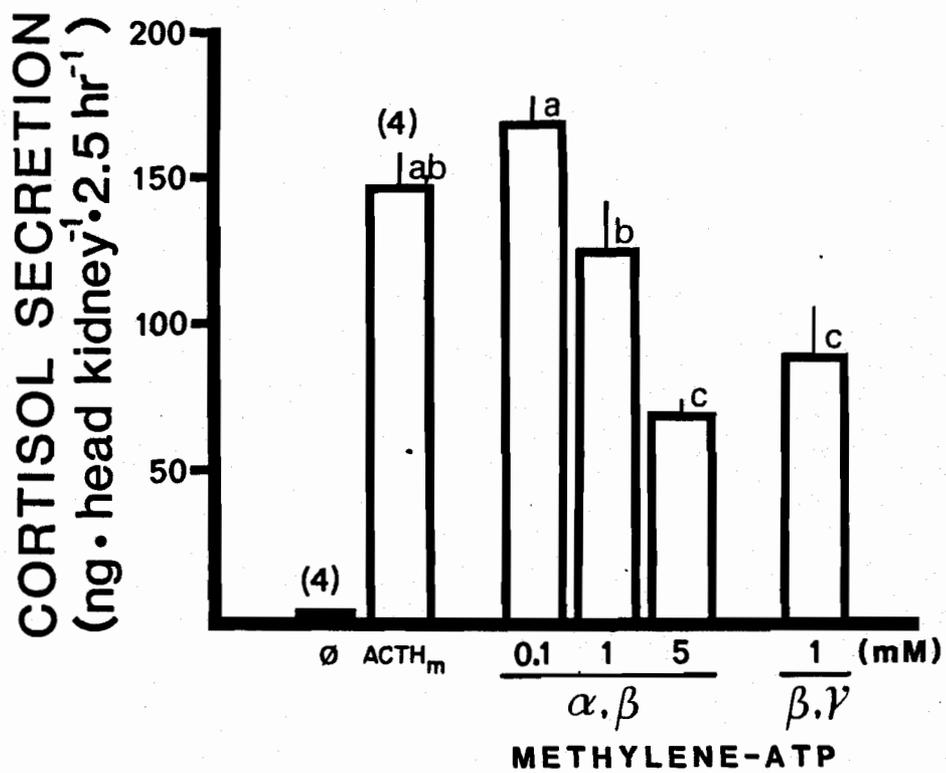


Figure VI-3

Figure VI-4. Biphasic steroidogenic response by interrenal cells to increasing concentrations of dbcAMP. Points accompanied by the same letter are not significantly different (one-way ANOVA and Duncan's multiple range test,  $P \leq 0.05$ ;  $n = 8$ , or 14 or 6 as indicated in parentheses).

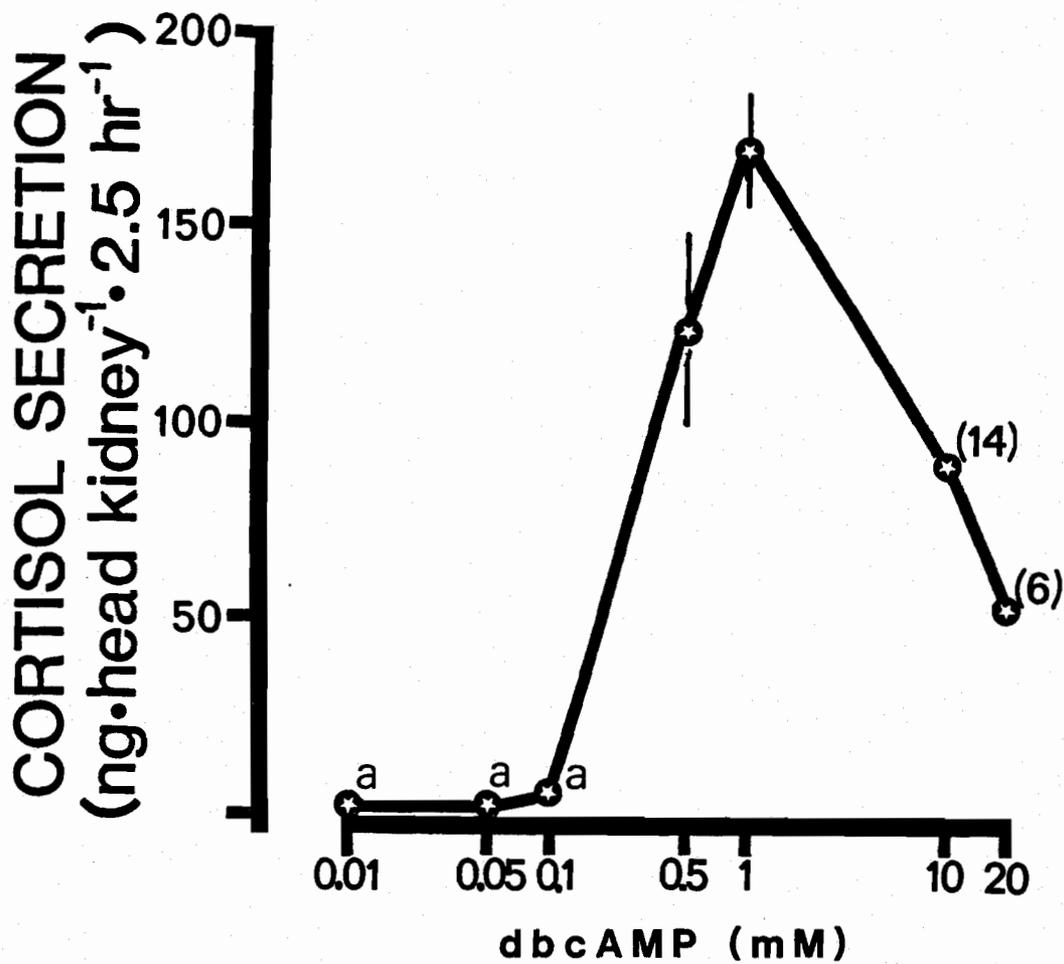


Figure VI-4

Figure VI-5. Comparison of effects of dbcGMP and dbcAMP on interrenal steroidogenesis. Control and dbcGMP bars bearing the same letter are not significantly different (one-way ANOVA,  $P \geq 0.05$ ;  $n = 6$ ). Cortisol secretion in response to dbcAMP was biphasic; dbcAMP-response bars bearing the same letter are not significantly different (one-way ANOVA and Duncan's multiple range test,  $P \leq 0.05$ ;  $n = 6$ ).

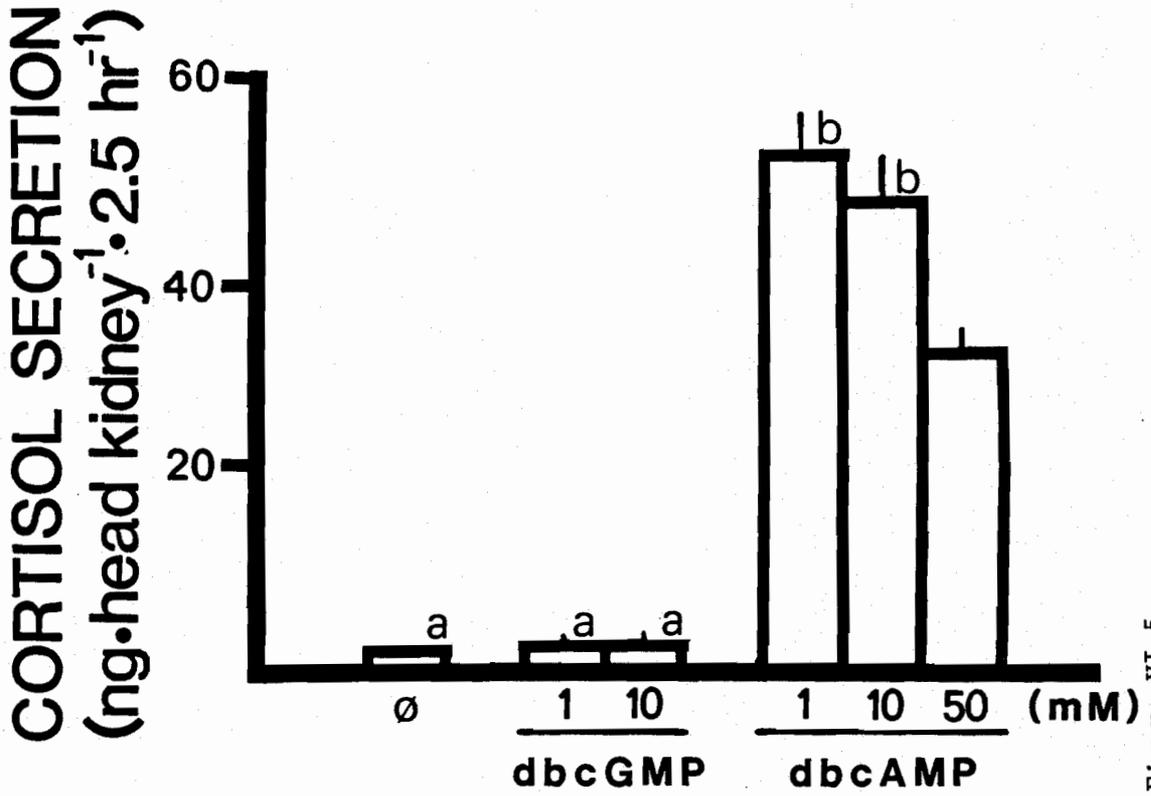


Figure VI-5

Figure VI-6. Effects of phophatidylinositol on interrenal steroidogenesis. The values represented by the four bars are not significantly different (one-way ANOVA,  $P \geq 0.05$ ;  $n = 6$ ).

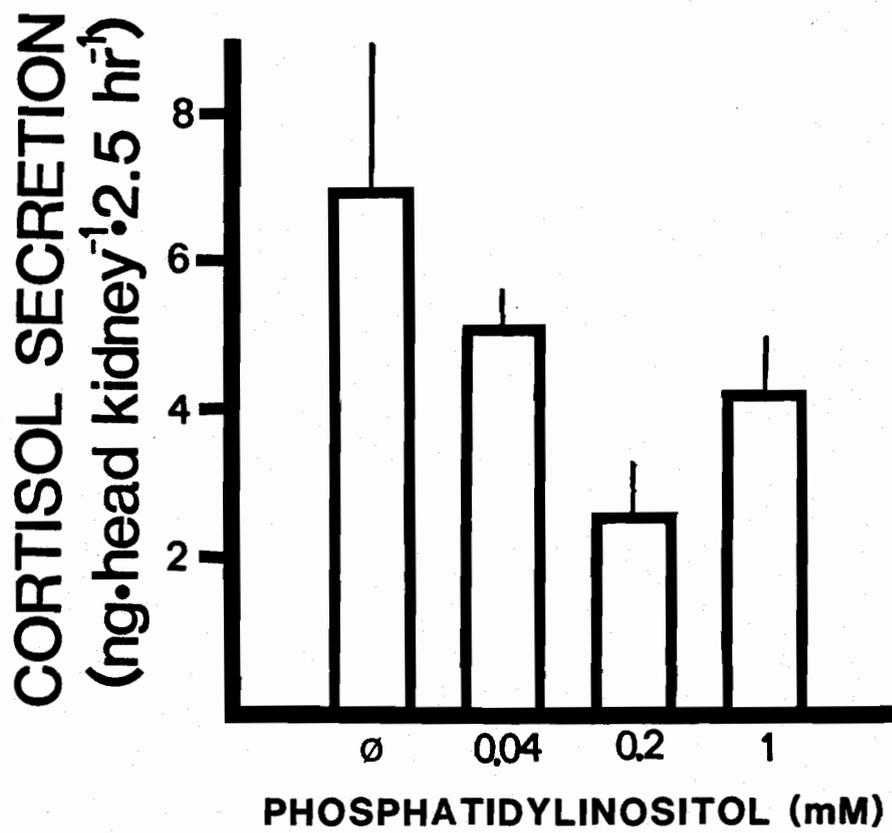


Figure VI-6

## Discussion

The results of our study support the notion of an obligatory role of cAMP as mediator of ACTH action on salmon interrenal steroidogenesis.

Forskolin, a reportedly potent adenylate cyclase activator (Seamon et al., 1981), was highly effective as a stimulator of steroidogenesis in interrenal cells of coho salmon. This finding itself strongly supports a role for adenylate cyclase and cAMP in the stimulation of interrenal steroidogenesis (Daly, 1984). Moreover, Ap(CH<sub>2</sub>)pp and App(CH<sub>2</sub>)p, which presumably inhibit adenylate cyclase activity (shown for Ap(CH<sub>2</sub>)pp in homogenized or intact rat cells in vitro by Krug et al., 1973a, b), were able to attenuate the steroidogenic response of interrenal cells to ACTH. Thus, our results provide indirect evidence suggesting that increases in endogenous cAMP levels enhance steroid production by interrenal cells, and that suppressed production of cAMP after ACTH stimulation also results in suppressed steroid synthesis. It should be noted, however, that the ATP analogs used in this study could potentially inhibit other cellular enzymes, and thereby confound interpretation of the results. Moreover, the effects of these analogs on adenylate cyclase may be more complex in salmon than in mammals: if App(CH<sub>2</sub>)p is used in conjunction with a submaximally effective dose of ACTH, its effect is biphasic, first enhancing steroidogenesis, at  $\leq 0.1$  mM, and then depressing it, at  $\geq 1$  mM (R. Patiño, C.S. Bradford, and C.B. Schreck, unpublished).

Some controversy developed recently concerning the importance of cGMP as "second messenger" of ACTH in mammalian adrenocortical cells; however, a review of the evidence suggested that cGMP does not play a major role in ACTH-induced steroid production by these cells (Schimmer, 1980). Nevertheless, in the crocodilian adrenal, cGMP was stimulated whereas cAMP was depressed by ACTH (Honn and Chavin, 1975, 1977). Furthermore, steroid production and the cAMP/cGMP ratio were highly correlated after ACTH stimulation (Honn and Chavin, 1977). We did not measure concentrations of endogenous cyclic nucleotides in the present study. However, dbcAMP, but not dbcGMP, was able to stimulate steroidogenesis. Thus, this finding supports our conclusion that in salmon interrenal cells, as in mammalian adrenocortical cells, elevations in cAMP (not cGMP) concentration may play a major role in mediating the steroidogenic action of ACTH. Cyclic AMP was also implicated in the regulation of corticosteroid production after treatment with ACTH of interrenals of a cichlid fish (Ilan and Yaron, 1980) and adrenals of a frog (Chavin et al., 1978).

Our finding of a biphasic steroidogenic response to dbcAMP by coho salmon interrenal cells was puzzling. We are unaware of similar findings in adrenocortical (interrenal) cells of any other animal when comparable methods (static incubations) and concentrations of dbcAMP were used. Perhaps concentrations of dbcAMP greater than 1-10 mM are toxic to interrenal cells of salmon and thus disrupt their normal functions. However, the cause of this biphasic response to dbcAMP remains unclear.

Cholera toxin is effective in activating adenylate cyclase and

enhancing cAMP production in most vertebrate cells; consequently, it has a steroidogenic effect on mammalian adrenocortical cells (Gill, 1977). In the present study, cholera toxin did not stimulate cortisol production in salmon interrenal cells, although concentrations of the toxin similar to those producing maximal effect on mammalian adrenocortical cells were used (e.g., Wolff et al., 1973; Palfreyman and Schulster, 1975). It is possible that the toxin did not reach the interrenal cells in sufficient concentrations to exert an effect since kidney tissue fragments, not isolated interrenal cells, were used in our study. On the other hand, it is also possible that the effect of this toxin on salmon interrenal cells is much reduced in comparison with its effects on mammalian adrenocortical cells.

Various hormones and neurotransmitters may use inositol phospholipids as mediators of their actions on cells. Synthesis (Farese, 1984) or turnover rate (Farese, 1984; Nishizuka, 1984) of these phospholipids may change in response to extracellular stimuli. Farese (1984) suggested that increased levels of inositol phospholipids, perhaps by de novo synthesis via the phosphatidate-polyphosphoinositide pathway, is an important "post-second messenger" event in the steroidogenic action of ACTH on adrenocortical cells. However, in contrast to the studies on mammalian adrenocortical cells, we failed to observe a steroidogenic effect of exogenous phosphatidylinositol on interrenal cells of coho salmon. As in the case of cholera toxin, it is unknown whether this finding was an artifact of our experimental protocol. However, it should be noted

that the highest concentration of phosphatidylinositol used in our study was five times greater than the concentration used by Farese et al. (1980b). Thus, unless this phospholipid was completely unable to penetrate the small kidney tissue fragments, it seems likely that our findings reflect the actual characteristics of the interrenal cells and not an artifact of methodology.

In summary, the results of our study suggested that cAMP is an important and obligatory intracellular mediator of the steroidogenic action of ACTH on the interrenal cells of coho salmon.

#### Acknowledgments

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## VII: CONCLUSION

I have presented the analysis of the results and the conclusions for the individual studies of my dissertation in previous chapters (see chapters II through VI). The ultimate goal of my dissertation (see Chapter I), to enhance the quality of hatchery reared coho salmon, was addressed in Chapter III. Here, I offer an overall summary and analysis of the salient results of my dissertation, and extend my previous conclusions using both developmental and evolutionary approaches.

## Interrenal Function and Developmental Physiology

At present, there are two very different views on the role of corticosteroids vis-a-vis osmoregulation during the developmental processes of smoltification. One view, recently reviewed by Specker (1982), suggests that increased interrenal activity causes important modifications in the osmoregulatory organs of smolting fish, such as in gill Na/K-ATPase activity. These changes would thus "preadapt" the freshwater individual to life in the ocean (Specker, 1982). On the other hand, Langhorne and Simpson (1986) proposed that the changes in corticosteroid levels are caused by, rather than being the cause of, the smoltification-associated transformations in gill Na/K-ATPase which in turn supposedly alter the osmoregulatory balance of the freshwater fish. The effects of changes in corticosteroid levels

would thus antagonize the effects of changes in gill Na/K-ATPase activity and hence maintain homeostasis (Langhorne and Simpson, 1986).

The available evidence is insufficient to determine which, if any, of the above views (hypotheses) is correct. However, one common aspect of both hypotheses is that they assume a close, positive association between changes in plasma corticosteroid levels and those in osmoregulatory mechanisms such as gill Na/K-ATPase activity. The results of my dissertation appear to place some doubt on this assumption and, consequently, on the two hypotheses themselves.

As I showed in Chapter III, gill Na/K-ATPase and plasma cortisol can be markedly affected by the rearing conditions of the fish during smoltification. But most importantly, the pattern of development of each physiological variable was affected differently by the rearing environment. I believe that this observation undermines the assumption that the change in one variable (whether cortisol or gill Na/K-ATPase) depends on the change in the other one, as suggested by Specker (1982) and Langhorne and Simpson (1986).

Specifically, I found that changes in gill Na/K-ATPase are not always accompanied by changes in plasma cortisol. For example, gill Na/K-ATPase steadily increased with time in low density fish reared at either low or high water inflow rate (see Chapter III). On the other hand, cortisol levels increased in the low density fish reared at high flow but not in those reared at low flow. Conversely, I found that changes in plasma cortisol seen after manipulation of the rearing conditions are not necessarily accompanied by changes in gill Na/K-ATPase activity. For example, at Smith Farm Hatchery (see Chapter

III), lowering the rearing density from high to low two weeks before sampling produced a significant change in plasma cortisol (up to a level similar to that seen in the control fish reared constantly at low density); however, gill Na/K-ATPase was unaffected. I believe that these observations (see also Chapter III for further examples) indicate that the changes in interrenal activity and gill Na/K-ATPase during smoltification are not only independent but also dissociated events.

It is thus tempting to suggest that the primary function of the changes in interrenal activity during the freshwater phase of smoltification is not osmoregulation (in either an active or reactive manner), but something else such as the regulation of the immune system (Specker, 1982; Maule et al., 1987) or of intermediary metabolism (Specker, 1982; Sheridan, 1986). Maule et al. (1987) observed clear correlations between interrenal activity and immune function in coho salmon during smoltification and after manipulation of the rearing conditions of the fish; and they concluded that corticosteroids may help avoid tissue damage during putative tissue changes at smoltification by suppressing the immune function of the fish. A similar role has been proposed for the changes in interrenal activity seen during metamorphosis of amphibians (e.g., Marx et al., 1985). I believe there is good evidence suggesting that corticosteroids are important for the seawater acclimation of euryhaline teleosts, including juvenile salmonids at smoltification (e.g., Richman et al., 1987); my arguments above refer only to the

role of changes in plasma corticosteroids during juvenile salmon development in fresh water.

Finally, the results of my dissertation (Chapters II and III) also showed that alterations in interrenal activity during coho salmon smoltification are independent of other endocrine events occurring at this time, such as the changes seen in the thyroidal and gonadal systems. In Chapter II, I showed that plasma cortisol levels increased while plasma sex steroid concentrations remained stable. In chapters II and III, I showed that the patterns of plasma cortisol and plasma thyroxine were affected differently by rearing conditions (see also Virtanen and Soivio, 1985, for similar conclusions regarding the relationship between plasma cortisol and thyroxine in Atlantic salmon). Lastly, in Chapter II, I showed that changes in thyroid activity were independent of sex steroid levels during smoltification.

It appears, therefore, that the various endocrine modifications documented during smoltification of coho salmon--or at least those investigated in this dissertation--are neither primarily linked in a "cause-effect" manner nor temporally associated in any specific fashion.

#### Interrenal Function and Evolutionary Patterns

It is well known that changes in interrenal activity in fishes may occur at defined stages of development or during certain types of environmental challenge (e.g., Idler and Truscott, 1972; Henderson and Garland, 1980; Donaldson, 1981; Schreck, 1981; Specker, 1982; and this

dissertation). These changes in interrenal activity may be to a large degree regulated by ACTH from the pituitary (Donaldson, 1981).

However, little is known about factors other than ACTH and about intracellular mechanisms controlling interrenal function in fishes.

The results of my experiments described in chapters V and VI highlighted both differences and similarities in interrenal physiology between coho salmon and other species of vertebrates previously studied. Perhaps the major difference that I found concerns the role of extracellular osmolality in the regulation of interrenal activity.

My findings in Chapter V suggested that variations in extracellular osmolality in coho salmon do not have any direct influence on interrenal activity, either under unstimulated conditions or after ACTH stimulation. The basal output of cortisol from interrenal tissue was only slightly enhanced by an increase of extracellular sodium or osmolality; and the output of cortisol after ACTH stimulation was not affected at all by these treatments.

In contrast to the present findings with coho salmon, alterations in extracellular osmolality levels have strong, inverse effects on the activity of the adrenocortical cell of mammals. In these animals, osmolality affects both the unstimulated or stimulated (by angiotensin II or ACTH) secretion of aldosterone (Schneider et al., 1984, 1985). Since aldosterone is the principal mineralocorticosteroid in mammals, the inverse relationship between extracellular osmolality and aldosterone secretion at the level of the adrenocortical cell may be an important means of maintaining the ion and fluid balance in

mammalian species (Yates et al., 1980; Schneider et al., 1985).

Teleosts are unique in that, in mammalian terms, a glucocorticosteroid (perhaps cortisol or cortisone, or both; see Chapter IV) appears to function as both mineralocorticosteroid and glucocorticosteroid (Norris, 1985; Gorbman et al., 1983). The presence of aldosterone in teleosts is questionable (Sandor, 1979). It is, therefore, tempting to speculate that the difference between mammals and coho salmon with respect to their extrapituitary regulation of adrenocortical function is related to their differences in adrenocortical secretory products or to their differences in the function of these products.

A description of interrenal function in amphibians may help illustrate my argument. The amphibian interrenal produces the two "mammalian" types of corticosteroids; namely, the mineralocorticosteroids (aldosterone) and the glucocorticosteroids (corticosterone/cortisol) (Norris, 1985). Moreover, in amphibians, aldosterone and corticosterone/cortisol serve primarily as mineralocorticosteroid and glucocorticosteroid, respectively (Norris, 1985). Finally, and again as in mammals, extracellular osmolality in amphibians appears to have a strong, inverse regulatory influence on the interrenal tissue, at least in in vitro conditions (Maser et al., 1982; Hanke et al., 1984).

Therefore, I propose that with the evolution of amphibians new mechanisms of interrenal regulation (e.g., extracellular osmolality) appeared as the interrenal cells began secreting two different "types" of corticosteroids or as the functions of these two types of

interrenal steroids diverged. Obviously, a major assumption I am making here is that my results with coho salmon can be extrapolated to other fishes; it would be interesting to know if this is true, particularly for the Sarcopterygii.

In this regard, it is also interesting to note that, in contrast to the situation in mammals, the secretion of mineralocorticosteroids and glucocorticosteroids in amphibians appears to be regulated simultaneously by the same factors. For example, angiotensin II (Perroteau et al., 1984), ACTH (Perroteau et al., 1984), osmolality (Hanke et al., 1984), and sodium (Maser et al., 1982; perhaps through its influence on osmolality), all affected the secretion of both types of corticosteroids from the amphibian interrenal in the same manner and to the same magnitude. In mammals, however, angiotensin II has very little effect on glucocorticosteroid production (e.g., Pham-Huu-Trung et al., 1981); and, conversely, ACTH has a relatively small effect on mineralocorticosteroid production, especially in those species where the renin-angiotensin system is known to be a strong stimulant of aldosterone secretion (Yates et al., 1980). Thus, it is also possible that regulatory factors specific to either mineralocorticosteroid or glucocorticosteroid secretion arose, in a phylogenetic sense, after the amphibians.

Therefore, considering the patterns of differences and similarities in the types of corticosteroids produced and in their regulation in the vertebrate species studied to date, it would appear that amphibians represent an intermediate condition of adrenocortical

cell function and regulation between fishes (coho salmon) and phylogenetically "older" classes of vertebrates.

The major intracellular mechanisms of regulation of interrenal steroid production in coho salmon are similar to those of most other species of vertebrates studied. I found that cAMP is an important and obligatory link between ACTH and steroidogenesis in coho salmon interrenal cells (Chapter VI). Moreover, I found that although cortisol is the only major steroid produced by unstimulated or ACTH-stimulated interrenal cells, significant amounts of cortisone are also present in plasma as a peripheral metabolite of cortisol (Chapter IV). I suggest there is a need to clarify the biological significance of plasma cortisone in order to adequately understand corticosteroid physiology in salmonids. It is unclear whether the renin-angiotensin system has any effect on interrenal steroidogenesis in fishes (e.g., Henderson et al., 1976; Kenyon et al., 1985a; Takahashi et al., 1985). A clarification of the role of this system in fishes would be valuable for characterizing the evolutionary pattern of interrenal function in vertebrates, if in fact there is a pattern.

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## APPENDIX

Effects of Chronic Confinement and Acute Handling Stresses on the  
In Vitro Response to ACTH of Coho Salmon Interrenal Tissue

Abstract

The effects of chronic stress by confinement and acute handling stress on the in vitro response of interrenal cells to ACTH were investigated in coho salmon, Oncorhynchus kisutch. Chronic stress markedly suppressed the ACTH-induced cortisol secretion from interrenal cells taken 24 hr after the onset of stress, but no differences between control and treatment groups were observed at 120 hr. Plasma cortisol levels in stressed fish were elevated at 24, but not at 120 hr.

Acute handling stress did not affect maximal secretory capacities of interrenal cells 9 or 24 hr after the stress. However, the sensitivity of the cells to ACTH was increased at 24 hr. Plasma cortisol in stressed fish was elevated at 1 hr, but had returned to resting levels 3 hr after the stress.

We concluded that stress, presumably by way of release of endogenous ACTH, may have long-term effects on the activity of the interrenal cells in coho salmon.

## Introduction

Several studies in mammals have shown that stress can affect the long-term regulation of the hypothalamic-pituitary-adrenocortical axis. For example, acute and/or chronic stress can affect the activity of adrenocortical cells (Lambert, Lammerant, and Kolanowski, 1983) and the pituitary gland (Young and Akil, 1985), assessed in vitro. Among the effects of stress or exogenous ACTH treatment in vivo on adrenocortical cells may be an enhanced in vitro capacity of adrenocortical cells to produce corticosteroids in response to ACTH (Kolanowski and Crabbe, 1974; Holmes et al., 1980; Llano et al., 1982; Lambert et al., 1983) and, as in the rat (Holmes et al., 1980) and the rabbit (Llano et al., 1982), a decreased sensitivity of adrenocortical cells to the peptide.

The general features of the hypothalamus-pituitary-adrenocortical (interrenal; HPI) axis in fish are similar to those of higher vertebrates (Donaldson, 1981). Depending on the type of stress, the response of the fish may be characterized by temporary or prolonged elevations in plasma corticosteroid levels (Donaldson, 1981; Schreck, 1981). In fish, however, the long-term effects of stress on the "control centers" (hypothalamus, pituitary, interrenal) along the HPI axis has received no attention.

We were interested in determining if the ACTH-control of interrenal activity in coho salmon, Oncorhynchus kisutch, is affected by stress. Specifically, we studied the effects of both prolonged and acute stimulation of interrenal cells by stress on the ability of

these cells to respond to further stimulation by ACTH in vitro. We used two types of stress in this experiment, chronic confinement and acute handling. The head kidneys, containing the interrenal cells (Oguri, 1960), were then removed and incubated in the absence or presence of various doses of ACTH, to obtain a dose-response curve.

#### Materials and Methods

Yearling coho salmon were obtained from Willard National Fish Hatchery (U.S. Fish and Wildlife Service) in spring, 1984, and reared at Smith Farm Hatchery, Oregon State University, in fresh water (12-13 C) flow-through circular tanks. The rearing density was less than 20 g/l. The fish were fed Oregon Moist Pellet daily, except during the experiments.

The incubation medium used in this study was described by Patiño et al. (1986a). Porcine (p)-ACTH (Sigma) was dissolved in medium, aliquoted and stored at -15 C; once thawed it was either used immediately or discarded.

The fish were quietly netted from the tanks, immediately killed by a blow to the head, exsanguinated by severing the caudal peduncle, and placed on ice. Individual head kidneys were removed into 8 ml of ice-cold medium within 15 min after sampling. The tissues were later minced and preincubated for 4.5 hr at 13-14 C. The medium was changed after 2 hr of preincubation to clean the tissues of blood and debris. This preincubation time was to allow the interrenal cells to reach a stable corticosteroid output (Ilan and Yaron, 1976, 1980; Rance and

Baker, 1981; Maser et al., 1982). The head kidneys were then transferred to 5-ml plastic culture dishes containing 3 ml of medium in the absence or presence of various doses of ACTH at 13-14<sup>o</sup>. Medium aliquots were collected after 2.25 hr of incubation and stored frozen. Preincubations and incubations were performed on a Junior Orbit Shaker (Lab-Line Instruments, Inc.) at 60 rpm.

For plasma cortisol analysis, blood was also collected when appropriate from the severed caudal peduncle using heparinized capillary tubes. The plasma was separated by centrifugation and stored frozen at -15 C. Medium (50  $\mu$ l) and plasma (10  $\mu$ l) samples were directly assayed for cortisol by radioimmunoassay according to Redding et al. (1984) and Patiño et al. (1986a), respectively.

#### Experimental Design and Results

Effects of chronic stress. On 9 July 1984, duplicate (treatment) groups of 45 fish each (mean weight, 26 g) were netted from the rearing (control) tank and confined in plastic buckets at a density of about 100 g/l. The buckets were placed in separate flow-through tanks and were well perforated to insure a continuous supply of fresh water. The number of treated fish sampled was always evenly divided between the two replicates. We sampled 24 fish each from both treatment and control groups 24 hr following the onset of confinement, for head kidney incubations; and collected blood from the first 10 fish of each group for plasma cortisol analysis.

In a separate experiment, two groups of 50 fish each were

acclimated to 100-l flow-through circular tanks for 2 weeks. We confined 25 fish from each tank in the buckets on 3 August; the remaining fish served as the controls for this experiment. Treatment and control fish (mean weight, 29 g) were sampled 120 hr later as described previously.

Stress by chronic confinement caused a marked elevation in plasma cortisol at 24 hr, but the hormone levels returned to resting conditions after 120 hr of confinement (Fig. AI-1).

Basal cortisol secretion (in the absence of ACTH from the medium) of interrenal cells of confined fish was higher than that of control cells at 24 hr after the onset of stress (Table AI-1). However, no differences in basal output of cortisol were observed between treatment and control interrenal cells at 120 hr.

Cortisol production in response to exogenous ACTH was significantly lower in interrenal cells from fish chronically stressed for 24 hr than in unstressed controls. However, the doses of ACTH used in this experiment did not cover the necessary range in order to ascertain differences in sensitivity between control and treated cells (there was no statistical difference in the response to the various doses of ACTH in the treated cells; Fig. AI-2). On the other hand, no differences in cortisol output or in the ACTH dose-response curves were observed between interrenal cells from control fish and fish confined for 120 hr (Fig. AI-3).

Effects of acute stress. Four (two control and two treatment) groups of 50 fish each were acclimated to 100-l tanks for 2 weeks. On

Fig. AI-1. Plasma cortisol levels in yearling coho salmon subjected to 24 or 120 hr of stress by chronic confinement (at 100 g/l), and 9 or 24 hr following an acute handling stress (held in the air for 30 sec). Double asterisks indicate significant differences from controls (Student-t test;  $P < 0.001$ ); NS, no significant differences ( $P > 0.05$ ). Other symbols are: C, control; CS, chronic stress; AS, acute stress. Bars represent standard errors;  $n=10$ .

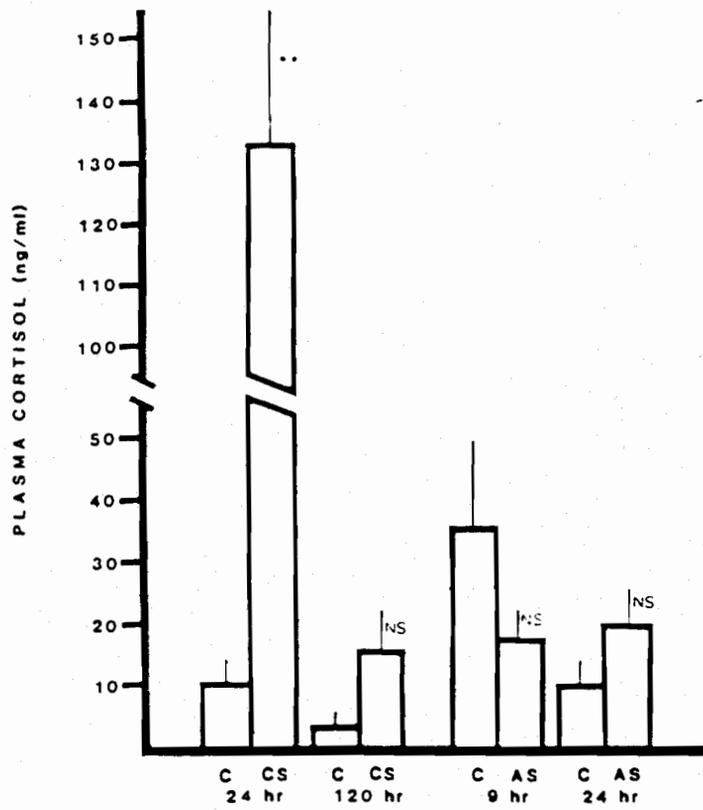


Table AI-1. Secretion of cortisol from interrenal cells of juvenile coho salmon subjected to 24 or 120 hr of chronic confinement, or 9 and 24 hr after acute handling. Incubation was performed in the absence of ACTH for 2.25 hr (see text for details).

Cortisol secretion (in absence of ACTH)				
(ng per head kidney per 2.25 hr)				
Time	Chronic confinement		Acute handling	
	Control	Treatment	Control	Treatment
9			5.13±1.46	2.19±0.66
24	5.65±1.37 <sup>a</sup>	15.56±3.75*	2.08±1.10	3.08±1.10
120	3.96±1.33	2.10±0.57		

<sup>a</sup>Mean ± standard error; n=4, except for the acute handling control at 9 hr where n=5.

\*Significantly different from control (Student-t test;  $P \leq 0.05$ )

Fig. AI-2. Net (ACTH-stimulated minus basal) cortisol secretion from the head kidney of yearling coho salmon after ACTH stimulation in vitro. The fish were confined at a density of 100 g/l for 24 hr before sampling (indicated in the figure). Two-way ANOVA (treatment X dose of ACTH) showed a significant difference in the response to ACTH between treated and control groups ( $P < 0.01$ ). Points on a curve followed by common letters are not significantly different (Duncan's multiple range test;  $P > 0.05$ ) and represent maximal cortisol secretion). Bars represent standard errors;  $n=4$ .

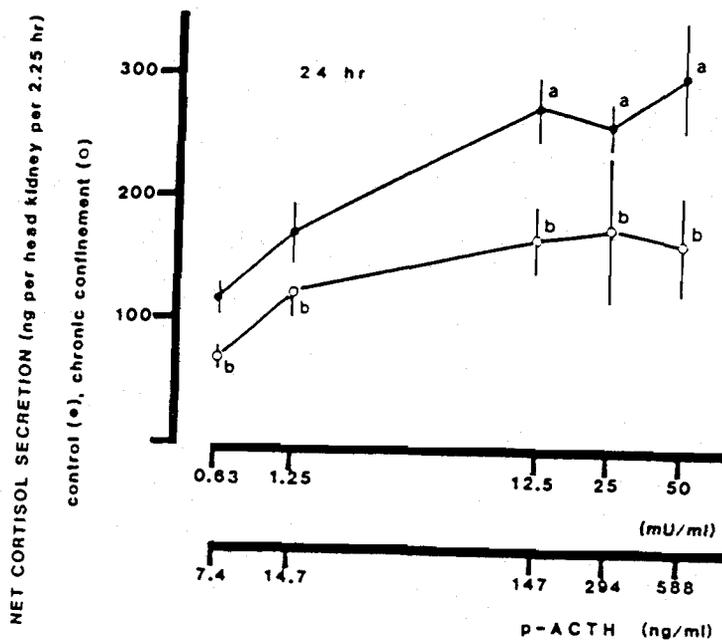
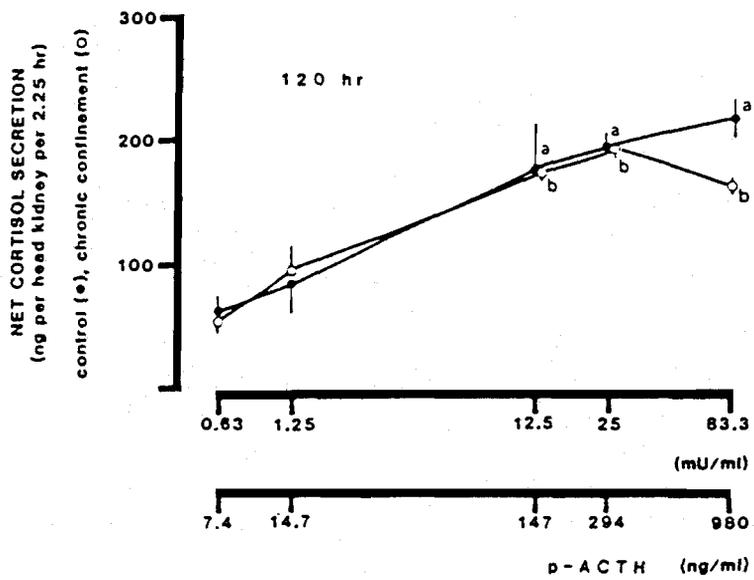


Fig. AI-3. Net (ACTH-stimulated minus basal) cortisol secretion from the head kidney of yearling coho salmon after ACTH stimulation in vitro. The fish were confined at a density of 100 g/L for 120 hr before sampling. Two-way ANOVA (treatment X dose of ACTH) showed no differences in the response to ACTH between the two groups ( $P > 0.05$ ). Points on a curve followed by common letters are not significantly different (Duncan's multiple range test;  $P > 0.05$ ) and represent maximal cortisol secretion. Bars represent standard errors;  $n=4$ .



the night of August 26, 25 fish from each treatment group were netted from their tanks, held in the air for 30 sec and then placed in identical tanks for recovery. We then sampled 24 treatment and 24 control fish (mean weight, 31 g) 9 hr later. On the morning of 27 August, the remaining 25 undisturbed fish from each treatment group were also stressed by suspension in the air for 30 sec, and 24 treated and 10 control fish were sampled 24 hr later. Tissue and blood samples were collected as previously described.

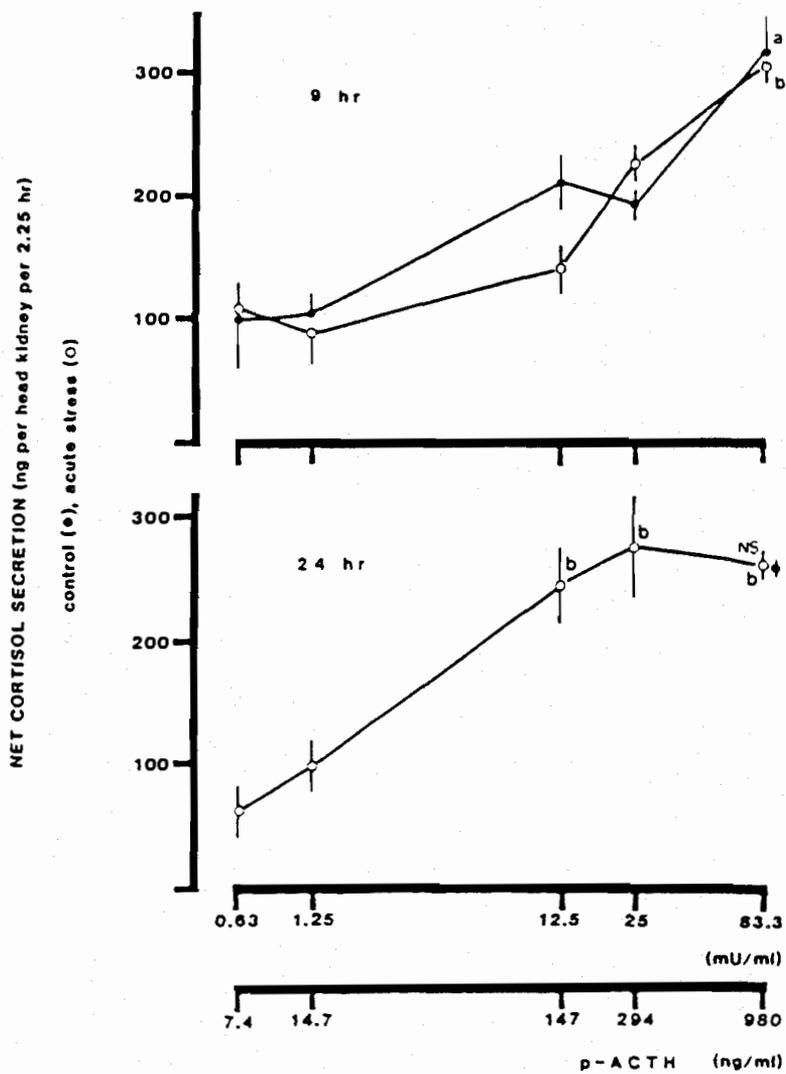
To characterize the dynamics of the circulating cortisol concentration after acute stress, we handled control fish in the same way as the treatment fish, and sampled 10 fish at 1 and 3 hr later, respectively, for plasma cortisol determinations on both days of the experiment.

The acute handling stress caused a transient elevation in plasma cortisol. The levels were close to 100 ng/ml at 1 hr post-stress, but had returned to resting values within 3 hr (data not shown). Resting plasma cortisol levels were also evident in the fish sampled 9 and 24 hr after the stress (Fig. AI-1).

There was no difference in basal cortisol secretion from interrenal cells of acutely stressed and control fish (Table AI-1).

The response to ACTH was similar in interrenal cells from control and stressed fish at 9 hr post-stress (Fig. AI-4). However, the ACTH dose-response curve of the interrenal cells from the treated fish was shifted to the left 24 hr after the acute stress compared to the control curve (Fig. AI-4).

Fig. AI-4. Net (ACTH-stimulated minus basal) cortisol secretion from the head kidney of yearling coho salmon after ACTH stimulation in vitro. The fish were held in the air for 30 sec, returned to their tanks, and then sampled at 9 and 24 hr following the acute stress. Two-way ANOVA (treatment X dose of ACTH) showed no differences in the interrenal response to ACTH between the two groups at 9 hr ( $\underline{P} > 0.05$ ). Points on a curve followed by common letters are not significantly different (Duncan's multiple range test;  $\underline{P} > 0.05$ ) and represent maximal cortisol secretion; NS indicates no significant difference from reference control value at the appropriate maximally effective ACTH dose (Student-t test;  $\underline{P} > 0.05$ ). Bars represent standard errors; n=4, except for single reference value at 24 hr where n=5.



## Discussion

Previous studies showed that stress or in vivo treatment with exogenous ACTH enhanced the capacity of adrenocortical cells to produce corticosteroids in vitro when samples were taken 12-24 hr after the end of the treatment in guinea pigs (Kolanowski and Crabbe, 1974; Lambert et al., 1983), rats (Holmes et al., 1980) and rabbits (Llano et al., 1982). We found that the effects of continuous stress by confinement on the secretion of cortisol by interrenal cells of coho salmon, assessed in vitro, differed according to the length of time between the onset of stress and sampling; however, no hyperreactivity of interrenal cells to ACTH was apparent at any time during this study.

Chronic stress by confinement resulted in elevated plasma cortisol levels in coho salmon during the first 24 hr of confinement (unpublished results from our laboratory showed that this effect can last for at least 48 hr). The in vitro maximal secretory capacity of the interrenal cells from treated fish was suppressed at 24 hr but returned to normal values by 120 hr. Although a complete ACTH dose-response curve was not available for treated samples collected at 24 hr, the results suggested a shift to the left in the curve, i.e., an increased sensitivity of interrenal cells to ACTH after 24 hr of confinement. However, no differences in sensitivity were observed after 120 hr.

Somewhat analogous results to ours regarding the capacity of adrenocortical cells to produce corticosteroids after prolonged ACTH

exposure were reported in an in vitro study in which mouse adrenal tumor cells were used. Morera and Saez (1977) showed that preincubation of adrenal tumor cells with ACTH for 2-14 hr reduced their capacity to respond to a secondary ACTH stimulation. It took the cells 24-48 hr to recover their normal responsiveness to ACTH (Morera and Saez, 1977; Tell et al., 1978). Furthermore, the extent of the ACTH-induced desensitization was related to the concentration and the time of exposure to ACTH (Morera et al., 1978).

Acute stress, like the one used in our study, is known to produce a marked but transitory elevation of plasma cortisol in salmonids, with concentrations returning to resting values within a few hours, as shown by us here and in previous studies (Schreck, 1981). However, even though the in vivo interrenal stimulation was transitory, and presumably so was the endogenous ACTH response, the interrenal cells appeared to be affected for a considerable period of time since their sensitivity to ACTH was enhanced in vitro 24 hr after the acute stress. Similar results to ours were obtained by Takahashi et al. (1985), who found that a single injection of exogenous ACTH in rainbow trout (Salmo gairdneri) enhanced, 12-14 hr later, the in vitro steroidogenic response of interrenal cells to ACTH. Since no maximally effective dose of ACTH was used in their study (Takahashi et al., 1985), it is not possible to compare their experiment to ours in terms of the effects of their treatment on the maximal capacity of the cells to produce cortisol.

In conclusion, stress in coho salmon, presumably by the action of endogenous ACTH, may have a prolonged influence on the responsiveness

of interrenal cells to ACTH. Moreover, the type of stress (chronic or acute) is important in determining the effects of stress on interrenal activity.

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