AN ABSTRACT OF THE THESIS OF

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Title: Cortisol and the Immune System of Juvenile Pacific Salmon During Stress and Development

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Abstract approved: _____
Carl B. Schreck

Plasma concentrations of cortisol, a glucocorticoid hormone that is immunosuppressive in mammals, increase during stress and smoltification of salmonids. There was a reduction in the ability of lymphocytes to generate antibody producing cells (APC) in vivo concurrent with increased plasma cortisol titers during smoltification of coho salmon (Oncorhynchus kisutch). Cortisol implants increased circulating cortisol concentrations, reduced immune response and increased fish mortality to vibriosis. Acute stress of chinook salmon (O. tshawytscha) in laboratory and field trials caused transient increases in plasma cortisol that were concurrent with decreased ability to generate APC in vitro 4 h after stress. However, 24 h after stress cortisol had returned to resting levels and immune response was enhanced. Disease challenges validated the APC assay as an accurate measure of immunocompetence as there was decreased survival at 4 h, but enhanced survival at 24 h after stress.

Fish that were acutely stressed or treated with cortisol had transient reductions in number of cells in spleen and peripheral blood and increases in thymus and anterior kidney. Chronically stressed fish responded in a similar manner, except that the number of cells in the anterior kidney was reduced throughout the experiment.

High affinity, low capacity glucocorticoid receptors (GR) were identified in gill and whole leukocytes from spleen and anterior kidney. Leukocytes from the anterior kidney had 2- to 3-fold more GR than did those from the spleen. The dissociation constants (Kd) and numbers of GR in leukocytes increased as a result of stress; the Kd of GR in gill of stressed fish also increased, but the number decreased. During smoltification, number and Kd of GR in anterior kidney leukocytes increased and Kd of GR in splenic leukocytes decreased with no change in number. Anterior kidney leukocytes were initially insensitive to cortisol added to cell cultures, but developed sensitivity concurrent with changes in GR.

Similar to a model proposed for mammals, I speculate that the functional significance of the immunosuppressive effects of cortisol in salmon may be to downregulate the immune system to avoid autoimmunity during recovery from stress, or as a result of tissue changes associated with smoltification.

Cortisol and the Immune System of Juvenile Pacific Salmon During Stress and Development

by

Alec G. Maule

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LaVon Mauer was invaluable in getting the dissertation into a form that was acceptable to the powers that be; and Adrian Hunter typed the original manuscript of what is now Chapter II of this dissertation. I have the highest regard for these women, who have difficult demanding jobs, but were always willing to help the two-finger typist.

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DEDICATION

This dissertation is dedicated to Ruth Maule, Judy Maule, and Amy Maule -- three generations, whose love and encouragement made it possible.

CONTRIBUTION OF AUTHORS

Dr. Stephen L. Kaattari helped in experimental design and helped to establish the use of the *in vivo* and *in vitro* hemolytic plaque assay to assess immune response in juvenile coho salmon (Chapters II & III). Dr. Ralph Tripp helped with experimental design and field work (Chapter III).

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

CORTISOL AND THE IMMUNE SYSTEM OF PACIFIC SALMON DURING STRESS AND DEVELOPMENT

I. GENERAL INTRODUCTION

The endocrine system provides a link between the integrative processes of the central nervous system and the physiological processes of organ systems throughout the body (Turner and Bagnara, 1976). Hormones secreted from endocrine cells can have effects at the level of the DNA in target cells (Ringold, 1985; Akerblom et al., 1988; Godowski et al., 1988; Schule et al., 1988) and, thus, regulate intracellular functioning critical to the life of the cell and organism (Leung and Munck, 1975; Homo et al., 1980; Kontula et al., 1986).

It is only recently that we have developed an appreciation of the interconnections between the endocrine and immune systems perhaps because of the mobile nature of immunocytes (Besedovsky and Sorkin, 1977; Ader, 1981; Blalock, 1984). It is generally accepted that physical and psychological stress can impair immune function and lead to decreased disease resistance (Monjan and Collector, 1977; Riley, 1981; Laudenslager et al., 1983). Furthermore, neuroendocrine responses appear to drive this stress-related immune dysfunction (Ader, 1981; Stein et al., 1985). The secretion of glucocorticoids, catecholamines and some neuropeptides increases in response to stress, and these hormones have wide ranging and sometimes opposing effects on

the immune system (Yu and Clements, 1976; Munck et al., 1984; Spangelo et al., 1985). Although there are many hormones that can effect the immune system (Berczi and Kovacs, 1987), considerable research has examined the influence of glucocorticoids (primarily cortisol and corticosterone) on the immune system (Haynes and Fauci, 1978; Dracott and Smith, 1979a, 1979b; Munck et al., 1984; Munck et al., 1987) because of their immunosuppressive effects. However, glucocorticoid treatment may enhance the immune response, depending on the concentration of the steroid and physical state of the animal (Cupps et al., 1984), timing of treatment and immune assay (Cupps and Fauci, 1982), or immune function assayed (Goodwin and Atluru, 1986).

The effects of hormones are generally mediated through specific receptors associated with the cell membrane, cytosol or nucleus (Agarwal, 1983; Roth et al., 1985; Plaut, 1987). The presence of specific glucocorticoid receptors (GR) in mammalian leukocytes is well established (Coffey and Djeu, 1986; Plaut, 1987). It has been generally reported that increased circulating glucocorticoids downregulate the number of GR in mammalian tissues (Schlechte et al., 1982; Shipman et al., 1983; Svec, 1985) by inhibiting the transcription of the GR gene (Rosewicz et al., 1988). It has also been shown that numbers of GR increased in leukocytes during proliferation (Crabtree et al., 1980a), differentiation (Hainque et al., 1987) and after antigen stimulation (Crabtree et al., 1980b). Moreover, some studies report correlations between the number of GR in leukocytes, the physiological status of the animal (eg., stressed, treated with hormone or pregnant), and the biological action of the hormone (Schlechte et al., 1982; Hirota et al., 1985b; Svec, 1985).

A large literature regarding fish physiology has developed (eg., Hoar and Randall, 1969, 1984; Ali, 1979) probably in response to economic needs of aquaculture. The endocrine systems in fish have been studied and it is known that the neuroendocrine response to stress in fish, such as Pacific salmon (Oncorhynchus spp.), is similar to that in mammals (Mazeaud et al., 1977; Pickering, 1981), and includes transient increases in concentrations of plasma cortisol (Strange and Schreck, 1977; Barton et al., 1985, 1986). In the experiments that follow, an operational distinction is made between acute stress and chronic stress. Although these terms are often associated with the duration of the stress, I was less concerned with duration than with physiological response either during or after the stress. Therefor acute stress is defined as being of less than 24 h duration and, experimentally, physiological measurements were taken after stress was terminated. Chronic stress was of greater than 24 h duration and continued throughout the experimental sampling.

Numerous physiological and biochemical changes accompany the developmental stage that prepares freshwater-adapted juvenile salmon for successful entry into the marine environment (i.e., smoltification; Hoar, 1976; Folmar and Dickhoff, 1981) including increased plasma cortisol titers (Specker and Schreck, 1982; Barton et al., 1985). There is not a general consensus of opinion about the role of increased cortisol in physiological responses during stress and smoltification (Specker, 1982; Langhorne and Simpson, 1986; Patino, 1988) and possible effects on the salmonid immune system have not been thoroughly examined (Pickering, 1981). However, stress can depress the numbers of circulating WBC in fish (Pickford et al., 1971;

McLeay, 1975b; Tomasso et al., 1983) and there is a connection between stress-induced elevation of plasma cortisol and susceptibility to disease (Pickering and Duston, 1983; Pickering, 1984; Pickering and Pottinger, 1985). The administration of glucocorticoids to fish can alter the morphology of immune organs (Chilmonczyk, 1982; Peters and Schwarzer, 1985), suppress immune function (Anderson et al., 1982), and reduce resistance to disease (Pickering and Duston, 1983). The facts that GR have been found in a variety of tissues in fish (DiBattista et al., 1983; Sandor et al., 1984; Chakraborti and Weisbart, 1987), and that cortisol treatment of brook trout (Salvelinus fontinalis) altered the number of GR in gills (Weisbart et al., 1987) suggest that cortisol effects are receptor mediated in fish as in mammals. The presence of specific GR has not been reported in leukocytes of fish or in tissues of Pacific salmon. Identifying specific GR in salmonid tissues would help elucidate the mechanisms of actions of that hormone in fish.

Goal and Objectives

The goal of the studies presented in this dissertation was to expand our understanding of the role of cortisol as an immunomodulator in Pacific salmon. The specific objectives of these experiments were to determine if there were changes in immune response or the distribution of immune cells of Pacific salmon concurrent with increased concentrations of plasma cortisol during stress or smoltification; to determine if long term or short term artificially elevated plasma cortisol titers had effects on the immune system of salmon similar to any seen during stress or smoltification; to

determine if there were specific glucocorticoid receptors in salmon leukocytes; and, if glucocorticoid receptors were identified, to determine if there were changes in receptor affinity or numbers associated with changes in plasma cortisol titers or immune response during smoltification and stress.

Organization of Dissertation

The results of experiments conducted to achieve the objectives listed above are presented in the form of manuscripts in Chapters II through VII of this dissertation. Each chapter contains an abstract, introduction, methods section, results section and discussion, and as such, there is unavoidable duplication of content. Chapter VIII is a summary of the work and a conclusion addressing the significance of the work.

II. CHANGES IN THE IMMUNE SYSTEM OF COHO SALMON (*ONCORHYNCHUS KISUTCH*) DURING THE PARR-TO-SMOLT TRANSFORMATION AND AFTER IMPLANTATION OF CORTISOL[1]

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ABSTRACT

The magnitude of the primary immune response of coho salmon (Oncorhynchus kisutch), as assessed by the production of splenic antibody secreting cells (plaque forming cells, PFC) after an injection of Vibrio anguillarum O-antigen, decreased during smoltification. This period was marked by increases in gill Na^+-K^+-adenosine triphosphatase (ATPase) activity and plasma thyroxine and cortisol titers. Numbers of leukocytes relative to erythrocytes in peripheral blood and splenic lymphocytes relative to fish body weight were also reduced. Fish reared at normal hatchery density (ca. 2 fish per L) appeared to have reduced rates of development and higher numbers of PFC than fish reared at one-third normal density. Moreover, in fish changed from normal density to low density 2 wk before sampling, ATPase activity and plasma thyroxine levels were equal to those in fish reared continuously at normal density, but plasma cortisol levels and PFC were equal to those in fish reared at low density. Fish with cortisol implants had higher plasma cortisol titers, reduced numbers of splenic PFC, splenic lymphocytes and circulating leukocytes, and greater mortality when fish were exposed to V. anguillarum.

INTRODUCTION

Smoltification of juvenile anadromous salmonids prepares them for successful entry into saltwater, and is marked by changes in behavior, morphology and physiology (Hoar, 1976; Folmar and Dickhoff, 1981). Included in these are changes in plasma levels of cortisol (Specker and Schreck, 1982; Barton et al., 1985), which has been shown to affect the mammalian immune system (Ader, 1981; Besedovsky et al., 1985; Blalock et al., 1985). The administration of corticosteroids to fish can alter the morphology of immune organs (Chilmonczyk, 1982; Peters and Schwarzer, 1985), suppress immune function (Anderson et al., 1982), and reduce resistance to disease (Pickering and Duston, 1983). Moreover, alteration in feeding behavior of salmon during smoltification and after release from a hatchery (Paszkowski and Olla, 1985) may alter the nutritional value of their diet, which may in turn alter immune competence (Blazer and Wolke, 1984a, 1984b). Developmental and environmental changes associated with smoltification may thus result in alterations in immune function of salmonids when they begin migrating to the ocean.

In the experiments discussed here, we assessed the immune system of juvenile coho salmon (Oncorhynchus kisutch) before and during smoltification to determine if there was a period of immunosuppression accompanying other physiological changes during smoltification. Furthermore, we examined the possible role of cortisol as an immunosuppressor by evaluating the effects of treatment with cortisol.

GENERAL METHODS

Four stocks of juvenile coho salmon were used during these experiments which were conducted during November to July of the year in which the fish underwent smoltification. All fish were held at either the Oregon State University Fish Disease Laboratory or Smith Farm Experimental Hatchery, Corvallis, Oregon. Both facilities are supplied with fish-pathogen-free well water that is 11-12^{o} C throughout the year. Fish were fed Oregon Moist Pellet at 1 - 2 % body weight daily, except on the day when experiments occurred.

The passive hemolytic plaque assay to Vibrio anguillarum O-antigen (VOA), as described by Kaattari and Irwin (1985), was used to assay the immune response. Briefly, we extracted O-antigen from V. anguillarum (LS-174) using the ethanol-incubation, acetone-drying method described by Anderson et al. (1979). Fish were anesthetized in tricaine methanesulfonate (MS-222) at a concentration of 50 mg/l and were injected intraperitoneally with 0.1 ml of a 100 mg/ml suspension of powdered antigen in phosphate buffered saline (PBS). Seventeen days after immunization, the fish were killed by a blow to the head and lymphoid organs were removed by dissection. Single-cell suspensions of individual organs were washed twice by centrifugation and resuspended in L-15 media. Lymphocyte viability, determined by trypan blue exclusion and hemacytometer cell counts, was always at least 90% and usually exceeded 95%. Cells secreting anti-VOA antibody (i. e., plaque forming cells, PFC) were enumerated using a modification of the Cunningham plaque assay (Cunningham and Szenberg, 1968). A mixture containing 0.1 ml of cell suspension, 0.025 ml of diluted steelhead (O. mykiss) serum, and 0.025 ml of a 10% dilution of

sheep red blood cells (SRBC) that had been coated with VOA was deposited into a slide chamber. The chambers were then sealed with wax and incubated for 1 to 3 h at 15° C. We counted PFC using 7 to 30 x magnification. At least two non-immunized fish were also assayed for PFC during each assay to determine nonspecific SRBC lysis. These controls ranged from 0 to 10 PFC per million lymphocytes, but never exceeded 4% of PFC in immunized fish.

Smears of peripheral whole blood were air-dried, fixed in ethanol, and stained with Geimsa stain. White blood cells (WBC) -- lymphocytes, polymorphonucleocytes, and thrombocytes -- were enumerated on the basis of descriptions by Anderson (1974) and Yasutake and Wales (1983).

Plasma cortisol and thyroxine (T_4) titers and gill Na^+-K^+-adenosine triphosphatase (Na^+-K^+-ATPase) activity have been shown to increase at the time when smoltification is believed to be happening (Zaugg et al., 1972; Dickhoff et al., 1978; Specker and Schreck, 1982). Although the functional significance of these changes has not been determined, we monitored these three variables in order to have a general idea of when smoltification occurred. On the same day that fish were immunized, other fish (n = 16 to 20) were rapidly netted from their home tank and immobilized in 5 liters of water containing 1 g MS-222, a dose that has been shown to inhibit stress-related increase in plasma cortisol titers (Strange and Schreck, 1978). After severing the caudal peduncle, we collected blood from the dorsal aorta into heparinized capillary tubes. After centrifugation, plasma was collected, stored at -20° C and later assayed for cortisol and T_4 using radioimmunoassays described by Redding et al. (1984) and

Dickhoff et al. (1978), respectively. Gill filaments were dissected from these fish and stored in sucrose buffer at -70° C. The Na^+-K^+-ATPase activity was determined for us by Dr. W. Zaugg (National Marine Fisheries Service, Cook, WA) by the method of Zaugg et al. (1972).

In two experiments, we implanted juvenile coho salmon with cortisol, using a molten (40° C) cocoa butter vehicle as described by Pickering and Duston (1983). Briefly, 4 mg cortisol was dissolved in 1 ml of cocoa butter, and 0.1 ml the mixture was injected intraperitoneally into each fish, resulting in a dose of about 0.01 mg cortisol per g fish body weight. This dose was previously found to elevate plasma cortisol to 60 $\pm$ 5 ng/ml for up to 7 wk. Control fish received injections of cocoa butter without cortisol.

Experiments in which data were collected during time series were subjected to analysis of variance (ANOVA) and, when significant differences were found, Duncan's Multiple Range test (DMR test) for pairwise comparisons. Data collected at one time were analyzed by *t*-test. Percentages were arcsine transformed and subjected to Chi-square analysis.

EXPERIMENTAL DESIGNS AND RESULTS

Smoltification and Immune Response

In 1984, we sampled juvenile coho salmon from the Cole River stock biweekly from 18 February (mean fish weight, 32 g) to 18 July (mean fish weight, 63 g). Gill Na^+-K^+-ATPase activity and plasma T_4 in these fish peaked on about 12 April (Figure 1A). After an early

Figure 1. Physiological and immunological measurements (mean + 1 SE) from coho salmon, 1984. (A) Gill Na^+-K^+-ATPase activity and plasma T_4, n = 16 to 20. (B) Plasma cortisol, n = 16 to 20, and number of plaque forming cells (PFC), n = 8 to 12. (C) Circulating white blood cells (WBC) as a percent of total circulating blood cells, n = 6, and number of splenic lymphocytes (X 10^7) relative to fish body weight, n = 8 to 12. Points marked (a) are significantly different from all other points on the same line; points marked (b) are significantly different from points marked (c) on the same line (Duncan's Multiple Range test, $P < 0.05$).

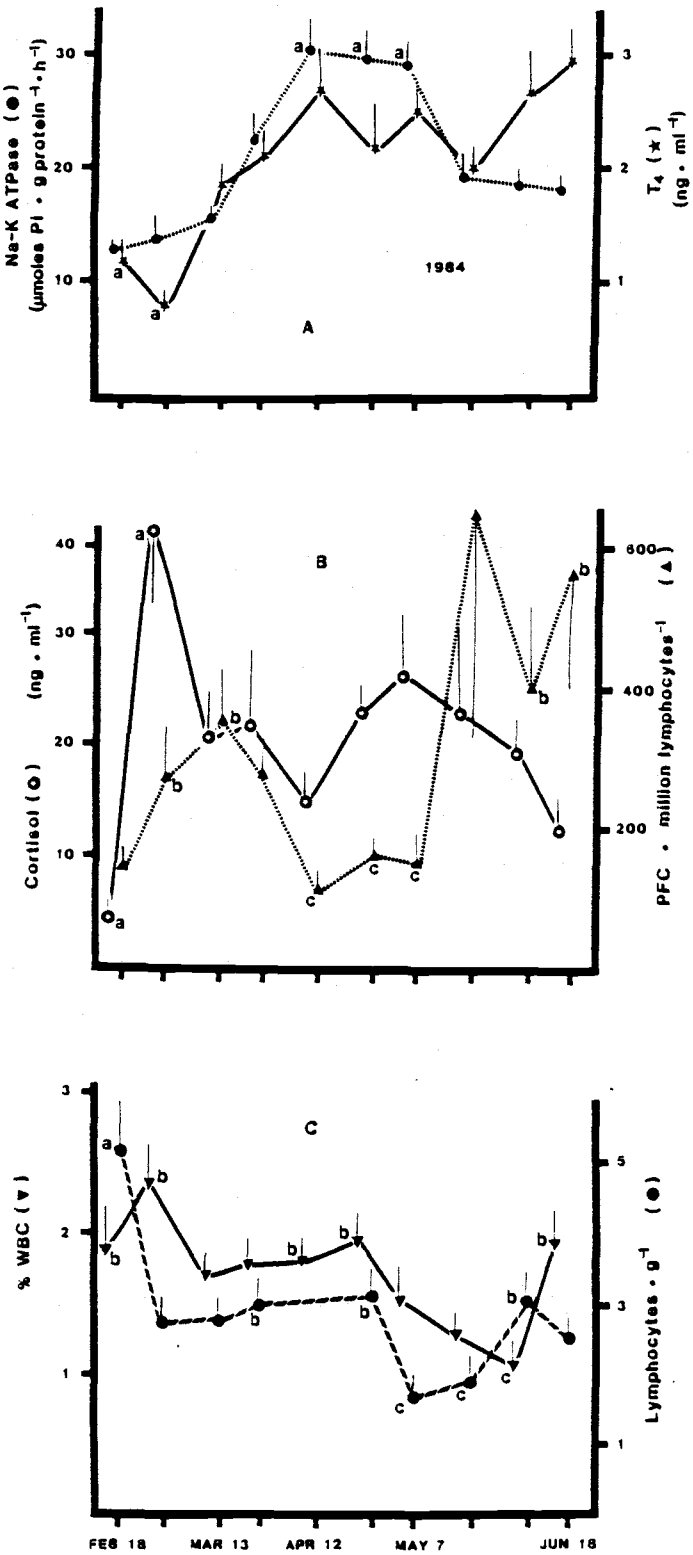
Na-K ATPase (●)
(μmoles Pi · g protein⁻¹ · h⁻¹)
T4 (★)
(ng · ml⁻¹)
1984
A
B
Cortisol (○)
(ng · ml⁻¹)
PFC · million lymphocytes⁻¹ (▲)
C
% WBC (▼)
Lymphocytes · g⁻¹ (●)
FEB 18
MAR 13
APR 12
MAY 7
JUN 18

Figure 1.

increase in plasma cortisol level, titers of this steroid increased significantly from initial values of about 5 to 25 ng/ml during late May and early June (Figure 1B). The number of PFC per million splenic lymphocytes was significantly depressed from 12 April to 7 May, as compared to the three sampling dates before and after this period (Figure 1B). There was also a significant decrease in circulating WBC relative to total circulating blood cells at the time when smoltification appeared to occur (Figure 1C). Even though fish body weight increased during this time, there was no change in absolute number of splenic lymphocytes. However, this relation translated into a significant decrease in splenic lymphocytes per unit of body weight at the same time as the relative number of circulating WBC decreased (Figure 1C).

In 1985 the same experimental design was used with coho salmon from Eagle Creek National Fish Hatchery. To determine if the kinetics of the immune response changed during smoltification, we measured splenic PFC from separate groups of fish, 13, 17, and 21 d after immunization, every 3 wk between 19 February (mean fish weight, 21 g) and 4 June (mean fish weight, 44 g). During this time, gill Na^+-K^+-ATPase activity and plasma T_4 and cortisol levels increased, peaking in late April to early May (Figure 2). Moreover, there was no apparent change in the kinetics of the immune response during this developmental period, as numbers of PFC decreased independently of the number of days after immunization (Figure 2B). We determined the sex of these fish based on gonad morphology and found no differences in response between sexes.

Figure 2. Physiological and immunological variables (mean + 1 SE) from coho salmon, 1985. (A) Gill Na^+-K^+-ATPase activity and plasma T_4, n = 15 to 20. (B) Plasma cortisol, n = 15 to 20, and numbers of plaque forming cells (PFC), n = 8 to 10. Points marked (a) are significantly different from all other points on the same line; points marked (b) are significantly different from points on the same line which are marked (c), (Duncan's Multiple Range test, $P < 0.05$).

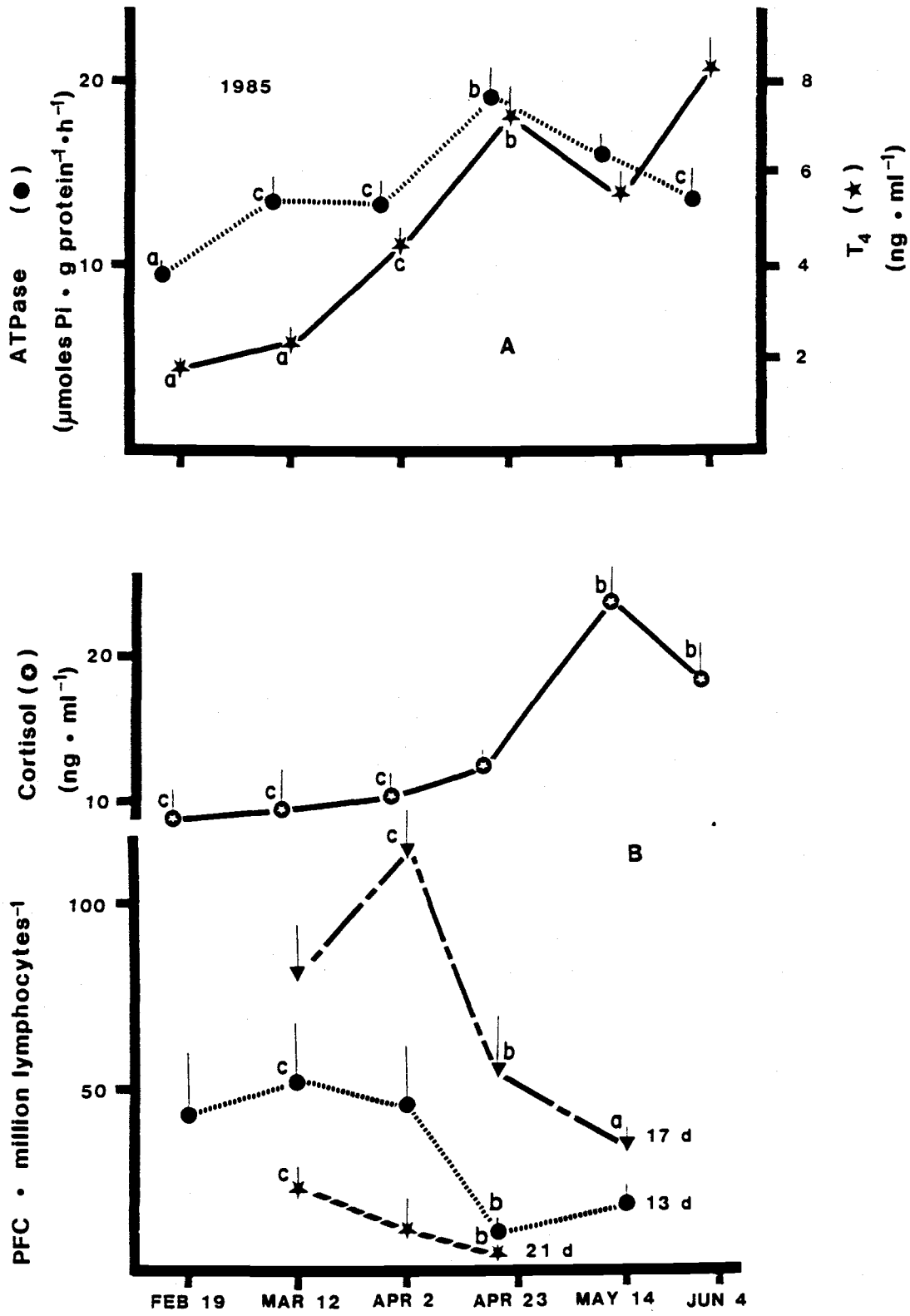
1985
ATPase (●)
(µmoles Pi • g protein⁻¹ • h⁻¹)
20
10
A
T4 (★)
(ng • ml⁻¹)
8
6
4
2
Cortisol (o)
(ng • ml⁻¹)
20
10
B
PFC • million lymphocytes⁻¹
100
50
17 d
13 d
21 d
FEB 19
MAR 12
APR 2
APR 23
MAY 14
JUN 4

Figure 2.

Rearing Conditions, Smoltification and Immune Response

To study the effects of rearing conditions on immune function, we worked in association with Patiño et al. (1986) who reported the effects of rearing density and water inflow rate on smoltification. Briefly, groups of Willard stock coho salmon were raised at high density (HD; 1941 fish per m^3) or low density (LD; 647 fish per m^3), in flow-through circular tanks (0.9 m in diameter and 0.17 m^3 in volume). Fish at each density were exposed to two water inflow rates, 2.0 and 1.0 l/min for high inflow (HI) and low inflow (LI), respectively. The rearing conditions had no effect on growth rates of the fish and at the end of the experiments mean fish weight was about 25 g. Each treatment in this 2 X 2 design was duplicated. To examine the effect of short-term reduction of fish density on the indices of smoltification and immune function, we reduced (thinned) fish density in duplicated HD-LI groups to LD 2 wk before immunization. In June 1984, when the Willard stock is usually released from the hatchery, Patiño et al. (1986) sampled fish for gill Na^+-K^+-ATPase activity, plasma T_4 and cortisol levels. Other fish from the same treatments were immunized with VOA, and PFC were determined 17 d later. The density at which fish were held affected all the variables tested; however, water inflow rate had no affect. Levels of the variables associated with smoltification were significantly lower in fish reared at HD than LD (T_4, ca. 1.5 versus 2.5 ng/ml cortisol, ca. 12 versus 20 ng/ml; Na^+-K^+-ATPase, ca. 16 versus 24 mmoles Pi/g protein /h; Patiño et al., 1986). Numbers of PFC were significantly greater in fish reared at HD than at LD (Figure 3). When fish density was changed, the effects were mixed. Reducing

Figure 3. Means (+ 1 SE) of plaque forming cells (PFC) from coho salmon raised at high (HD) or low density (LD) and high (HI) or low water inflow rate (LI). Fish in two HD groups were thinned to LD (HD-LD) 2 wk prior to sampling. Bars marked (a) are significantly different from others (*t*-test $P < 0.001$).

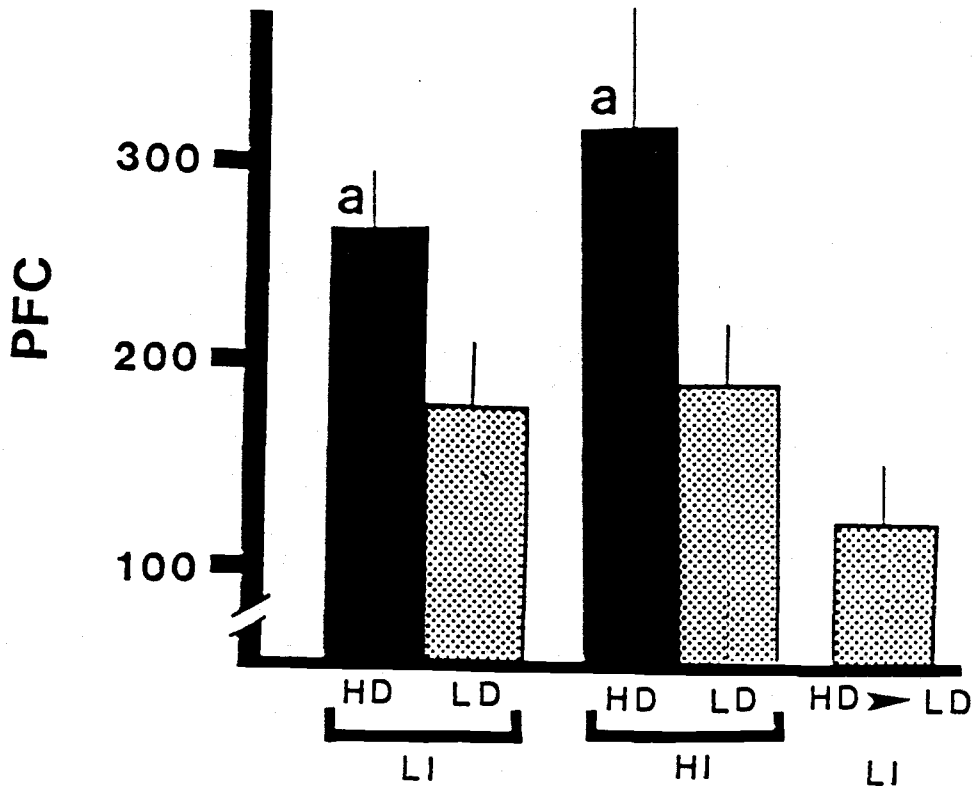

Figure 3.

fish density from HD to LD for 2 wk significantly increased plasma cortisol levels to about 20 ng/ml with a concomitant decrease in PFC to about 118 per million lymphocytes (Figure 3), but had no effect on gill Na^+-K^+-ATPase activity or plasma thyroxine titer (Patiño et al., 1986).

Exogenous Cortisol and Immune System

Results of our experiments during smoltification and because glucocorticoids have been shown to be immunosuppressive in fish (Anderson et al., 1982) suggested that the differences in PFC in response to VOA immunization might be linked to changes in plasma cortisol levels. To examine this link, six groups of 24 to 26 juvenile coho salmon (mean fish weight: 31 g) were acclimated to 64.5 L cuboidal aquaria that received a continuous supply of aerated, fish-pathogen-free well water at 15.0 +/- 0.5° C. Fish in one half of the groups received intraperitoneal injections of cortisol in cocoa butter and the rest received injections of cocoa butter (CB). Eleven days after injection, we exposed two groups of cortisol-implanted fish and two groups of CB-implanted fish to V. anguillarum (LS-174) using the waterborne infection method of Gould et al. (1978). An additional group from each treatment was treated similarly but was not exposed to the pathogen. Each disease-challenged group was exposed for 20 min to 1.5 million V. anguillarum cells per mL of water in 20 L. The bacterial cultures were grown at 18° C in Brain-Heart Infusion broth. Culture purity was determined by bacterial shape, motility, sensitivity to vibrostat 0/129 (2,4 diamino-6,7-di-isopropyl pteridine), and by rapid slide agglutination with V. anguillarum

Table 1. Mortality data and plasma cortisol (mean ± 1 SE) of juvenile coho salmon exposed to Vibrio anguillarum (LS-174), 11 d after they received an intraperitoneal injection of 0.4 mg cortisol in molten cocoa butter or cocoa butter.

Treatment replicates	Number of fish exposed	Mortalities (%)[a]	Mean time from exposure to death (d)	Mean plasma cortisol ($ng \cdot ml^{-1}$)[b]
Cortisol				
1	24	92	3.6	55.1 ± 7.8
2	24	75		
Cocoa butter				
1	25	44	3.3	5.1 ± 1.0
2	26	69		

[a] Treatments significantly different, $p < 0.01$; Chi-square test.

[b] n = 20 fish; treatments significantly different, $p < 0.01$; t-test.

antisera. Dead fish were removed daily during the 14 d test. Vibrio anguillarum was isolated from kidneys of all dead fish. Plasma cortisol was assayed in fish treated with cortisol or cocoa butter that were not exposed to the pathogen. Plasma cortisol levels were about 10 times higher in cortisol-implanted fish than CB-implanted fish and exposure to V. anguillarum resulted in significantly greater mortality in the cortisol-implanted than the CB-implanted groups (Table 1).

The effects of exogenous cortisol treatment on the number of PFC in response to VOA immunization and on the number and distribution of lymphocytes were also examined. We injected VOA into juvenile coho salmon from Eagle Creek National Fish Hatchery 7 d after cortisol or CB implantation, and 17 d later PFC were assayed using leukocytes from spleens and anterior kidneys. Kidney samples were collected in a way that ensured consistent sampling of tissue. Furthermore, one person dissected the kidney from all fish without knowing the source of the fish. Again, cortisol implants significantly elevated plasma cortisol levels, this time about 30 fold (Table 2). Mean cortisol level in the cortisol-implanted fish was only slightly lower than that of the peak seen in the same stock of fish during smoltification (Figure 2). The immune system was also affected, as the cortisol-implanted fish had significantly fewer PFC in the spleens and anterior kidneys, fewer splenic lymphocytes, and fewer circulating WBC than did the CB-implanted fish (Table 2). Cortisol treatment also caused a shift in the relative composition of circulating WBC (Table 2).

Table 2. Number of plaque forming cells (PFC), distribution of lymphocytes, and cortisol titers in juvenile coho salmon immunized with Vibrio anguillarum O-antigen. Seven days before immunization, fish received implants of 0.4 mg cortisol in cocoa butter or cocoa butter (CB). All values are mean ± 1 SE of 8 fish except for blood smears where sample size is 4 fish.

	Cortisol	CB
Plasma cortisol ($ng \cdot mL^{-1}$)	14.3 ± 1.9[a]	0.5 ± 0.4
Spleen		
Lymphocytes (million)	22.8 ± 4.7[b]	45.5 ± 9.4
PFC per spleen	77 ± 55[a]	298 ± 53
PFC per million lymphocytes	3 ± 1[a]	7 ± 1
Anterior kidney		
Lymphocytes (million)	19.0 ± 2.2	20.0 ± 1.0
PFC per anterior kidney	50 ± 27 [a]	135 ± 11
PFC per million lymphocytes	4 ± 3	7 ± 1
Peripheral blood		
Red blood cells (million per mL)	261.3 ± 31.3	242.6 ± 14.9
White blood cells (million per mL)	7.0 ± 1.0[b]	10.2 ± 1.9
Differential cell counts (%)		
Large lymphocytes	1.4 ± 0.5	1.8 ± 0.7
Small lymphocytes	86.7 ± 3.3[b]	78.0 ± 5.0
Polymorphonucleocytes	4.9 ± 1.2	9.8 ± 3.7
Thrombocytes	6.9 ± 2.1	10.4 ± 1.8

[a] Significantly different from controls; $p < 0.01$, t-test.

[b] Significantly different from controls; $p < 0.025$, t- or Chi-squared test

DISCUSSION

Our results have important implications relative to changes in immune competence and disease resistance of salmonids during smoltification and times of stress. Others have reported seasonal changes in the immune systems of fish, but these changes have been correlated to water temperature or sexual maturation (Yamaguchi et al., 1980; Wishkovsky and Avtalion, 1982; Honma and Tamura, 1984). We demonstrated that as coho salmon went through smoltification at a constant temperature, they became less able to generate a primary immune response to antigens of V. anguillarum, the causative agent of vibriosis (Figures 1 and 2). The accompanying reduction in relative numbers of splenic lymphocytes and circulating WBC (Figure 1D) suggests that general immune competence may have been impaired as well. This impairment occurred at a time when gill Na^+-K^+-ATPase activity, plasma T_4 and cortisol levels were all elevated. Rearing conditions, such as rearing density, which appear to alter the rate of smoltification (Patiño et al., 1986) also altered the ability of the fish to generate an immune response (Figure 3). Short-term changes in rearing density that affected plasma cortisol levels, but not gill Na^+-K^+-ATPase activity or plasma T_4 levels, also altered immune response, suggesting that of the three physiological variables measured as indices of smoltification, only plasma cortisol affected the immune system. Implantation of exogenous cortisol resulted in essentially the same changes in the immune system (i. e., decreased numbers of PFC, splenic lymphocytes, and circulating WBC, and reduced resistance to disease) as occurred during smoltification, again suggesting that this steroid may regulate the immune response of fish.

These findings are in agreement with those of McLeay (1975a), who found decreases in circulating WBC during stream residence of juvenile coho salmon, and suggested that these decreases were the result of adrenocorticosteroid activation during acclimation to coldwater, and smoltification. McLeay (1973a, 1973b) also reported reductions in circulating WBC in juvenile coho salmon following treatment with exogenous glucocorticoid and adrenocorticotrophic hormones. However, he found that the numbers of small lymphocytes were reduced in field studies (McLeay, 1975a) and laboratory studies (McLeay, 1973a, 1973b), whereas we found equal reductions of all WBC subpopulations during smoltification. These discrepancies may reflect differences in fish size or environmental factors among these studies.

Although we report changes in the immune system during smoltification, other investigators have reported that stress can depress the numbers of circulating WBC in fish (Pickford et al., 1971; McLeay, 1975b; Tomasso et al., 1983) and work by Pickering and Duston (1983), Pickering (1984), and Pickering and Pottinger (1985) has shown a connection between stress-induced elevation of plasma cortisol and susceptibility to disease. Pickering and Duston (1983) and Pickering and Pottinger (1985) reported increased plasma cortisol levels and increased susceptibility to several bacterial pathogens present in the water source, after brown trout (Salmo trutta) had been implanted with cortisol. However, Pickering and Pottinger (1985) did not find any effect of cortisol on numbers of circulating WBC. In our study, cortisol implanted fish had reduced disease resistance, accompanied by reduced numbers of circulating WBC and

splenic lymphocytes, but no reduction in the number of lymphocytes in the anterior kidneys. Moreover, the decrease in number of PFC per million lymphocytes in spleens and anterior kidneys of cortisol-implanted fish indicates that the observed immunosuppression was caused by mechanisms other than lympholysis alone.

Exogenous corticosteroid treatment in mammals can cause lympholysis, export of cells from thymus, spleen, and circulation, and sequestering of T-cells in bone marrow (Claman, 1972; Cohen, 1972; Fauci and Dale, 1975). There is considerable evidence that the B- and T- cell paradigm is also true in fish (Etlinger et al., 1976; Cuchens and Clem, 1977; Ruben et al., 1977; Sizemore et al., 1984; Miller et al., 1985, 1986, 1987) and that the anterior kidney in fish may be functionally similar to the bone marrow in mice (Zapata, 1979; Kaattari and Irwin, 1985). We report here that after long-term cortisol treatment of coho salmon, the numbers of antibody producing cells in the spleen and anterior kidney were reduced; however, the kidney showed no reduction in lymphocyte number although splenic lymphocytes and circulating WBC were reduced. This relation suggests that antibody producing cells were reduced in all organs, perhaps the result of suppressed T-cell function. Alternatively, another subpopulation(s) of leukocytes, not involved in the PFC response, was sequestered in the anterior kidney.

Although the functional significance of this apparent cortisol-mediated depression in immune competence during smoltification remains elusive, Munck et al. (1984) suggested that corticosteroids released during stress in mammals may function to prevent the immune system from generating autoimmunity and

consequently damage to body tissues. Similarly, we speculate that if there are tissue changes during smoltification, cortisol may suppress immune function to avoid damaging those tissues.

III. STRESS ALTERS IMMUNE FUNCTION AND DISEASE RESISTANCE IN CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*)

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ABSTRACT

We examined the effects of acute stress on the immune system and disease resistance of juvenile chinook salmon (Oncorhynchus tshawytscha) in laboratory and clinical trials. Immune function, as measured by the ability of lymphocytes from the anterior kidney to generate specific antibody producing cells (APC) in vitro, was depressed 4 h after stress, when plasma cortisol levels were highest. At the same time, resistance to the fish pathogen, Vibrio anguillarum, was also depressed. As compared with controls, plasma cortisol and APC of stressed fish were unchanged after 24 h, and disease resistance was enhanced as evidenced by higher survival rate and longer mean time to death of those fish that died. After 7 days, even though numbers of APC were depressed, plasma cortisol concentration and disease resistance did not differ from controls. This pattern was generally the same, independent of the type of stress applied: being held out of water in a dipnet for 30 s, manipulation during hatchery operations for 4 h, or transportation for 9 h. These and earlier findings suggest that similar endocrine-immune interactions operate in the mammalian and salmonid systems during acute stress.

INTRODUCTION

It is generally accepted that physical and psychological stress can impair immune function and lead to decreased disease resistance (Monjan and Collector, 1977; Riley, 1981; Laudenslager et al., 1983). Furthermore, neuroendocrine responses appear to drive this stress-related immune dysfunction (Ader, 1981; Stein et al., 1985). The secretion of glucocorticoids, catecholamines and some neuropeptides increases in response to stress, and these hormones have wide ranging and sometimes opposing effects on the immune system (Yu and Clements, 1976; Munck et al., 1984; Spangelo et al., 1985). The administration of glucocorticoids to animals or lymphocyte cultures usually depresses the immune response. However, glucocorticoid treatment may enhance the immune response, depending on the concentration of the steroid and physical state of the animal (Cupps et al., 1984), timing of treatment and immune assay (Cupps and Fauci, 1982), or immune function assayed (Goodwin and Atluru, 1986). Similar variability in the response of the immune system after stress has been reported. Gisler et al. (1971) reported that, compared to nonstressed controls, lymphocytes removed from mice 15 min after acute stress had an increased ability to generate antibody-producing cells (APC) to sheep red blood cells (SRBC) in vitro, but that the response was depressed 6 - 72 h after stress. Okimura and Nigo (1986) and Okimura et al., (1986a) demonstrated that acutely stressed mice had reduced numbers of splenic APC when immunized with SRBC, but when similarly stressed mice were immunized with trinitrophenylated (TNP)-Ficoll or TNP-lipopolysaccharide (LPS), splenic APC increased. This variability in response leads one to question whether a single

specific assay of immune function is an accurate index of immune competence, the final measure of which must be disease resistance.

We previously demonstrated that artificially elevating plasma concentrations of cortisol in Pacific salmon (Oncorhynchus spp.) led to a reduction in their ability to generate APC in vivo and their survival when exposed to the fish pathogen Vibrio anguillarum (Maule et al., 1987). We have also shown a direct action of cortisol on lymphocytes as the addition of physiological concentrations of cortisol to lymphocyte cultures reduced APC (Tripp et al., 1987). This cortisol inhibition was abrogated by the addition of supernatants from antigen-stimulated lymphocyte cultures (Tripp et al., 1987) or recombinant-interleukin-1 (Kaattari and Tripp, 1987) suggesting that cortisol is interfering with lymphokine production or activity. It has also been shown that acute stress results in the transient increase in circulating cortisol in salmon (Strange and Schreck, 1978; Barton et al., 1986), and that stress and cortisol administration can cause leukocytopenia in fish (Pickford et al., 1971; McLeay, 1973a). In the present study, our objective was to correlate changes in plasma cortisol concentration, ability of lymphocytes from the anterior kidney to generate APC in vitro and disease resistance in acutely stressed chinook salmon (O. tshawytscha).

MATERIALS AND METHODS

Experimental Animals

Juvenile spring chinook salmon (spring chinook salmon are distinguished from fall chinook salmon by virtue of the timing of their spawning migrations) were obtained from Eagle Creek (Oregon, U.S.A.) or Abernathy (Washington, U.S.A.) National Fish Hatcheries and were maintained at either the Oregon State University, Smith Farm Experimental Hatchery or Fish Disease Laboratory at Corvallis. Both facilities receive fish-pathogen-free well water at a constant temperature of 11-12^{0} C. Fish were fed Oregon Moist Pellet (OMP) commercial salmon food at a rate of 1 to 2 % body weight per day. Fish were acclimated to these conditions for at least 30 days before experiments were started. To separate genetic influences from possible environmental factors, we conducted clinical trials at the Warm Springs National Fish Hatchery, Warm Springs, OR, U.S.A. and the Oregon Department of Fish and Wildlife, Round Butte Hatchery, Madras, OR, U.S.A. The spring chinook salmon at these hatcheries were the same genetic stock and were fed OPM, but there were differences in water temperature regimens. Round Butte water was constant at 13^{0} C, while water at Warm Springs varied seasonally (6 - 21^{0} C) and diurnally (17 - 21^{0} C in summer).

Hormone and Immune Assays

Fish were sampled after rapid netting and transfer to a lethal concentration of tricaine methanesulfonate (200 mg/l). This dose of anaesthetic inhibits stress-induced increases in plasma cortisol in salmon (Barton et al., 1985). After fish were immobilized in

anaesthetic, we severed the tail and collected blood from the caudal vasculature into heparinized capillary tubes. Plasma was separated from the blood by centrifugation, stored at -20^{0} C, and later assayed for cortisol by a radioimmunoassay described by Redding et al. (1984).

The ability of lymphocytes to generate specific APC was assessed using the in vitro assay described by Tripp et al. (1987). Briefly, anterior kidneys were aseptically harvested and placed into tissue culture medium (TCM) composed of RPMI 1640 containing L-glutamine and bicarbonate (GIBCO, Grand Island, NY, U.S.A.) and supplemented with 10% (v/v) hybridoma-screened fetal calf serum (B. A. Bioproducts, Walkerville, MD, U.S.A.), 100 mg gentamicin sulphate/l (Sigma Chemical Co., St. Louis, MO, U.S.A.), 50 umol 2-mercaptoethanol/l (MCB Manufacturing Chemists, Inc., Cincinnati, OH, U.S.A.), 4.0 umol adenosine/l, 4.0 umol cytosine/l, 9.0 umol thymidine/l and 9.0 umole guanosine/l (Sigma). Lymphocytes were separated from surrounding tissue by gentle aspiration with a 1 ml syringe and tissue debris was allowed to settle to the bottom of the test tube. Supernatants containing cells were collected, and cells were washed by centrifugation and resuspended to 2 X 10^{7} cells/ml TCM. We transferred 0.2 ml cell suspension to wells of a 24-well, flat-bottom microculture plate (Corning Glass Works, Corning, NY, U.S.A.) and added 0.2 ml of either TCM (negative controls) or TCM containing the antigen TNP-LPS (0.4 ug/ml; Jacobs and Morrison 1975). Cell cultures were incubated at 17^{0} C in an airtight gasbox (C. B. S. Scientific, Del Mar, CA, U.S.A.) with blood-gas mixture (10% O_2, 10% CO_2 and 80% N_2) and were fed 50 ul of feeding cocktail (Tittle and Rittenburg 1978) every other day. After 9 days, cells were harvested, washed by

centrifugation, and resuspended in medium. Lymphocytes secreting anti-TNP antibodies were detected by Cunningham plaque assay (Cunningham and Szenberg, 1968). We mixed 0.1 ml lymphocyte suspension, 0.025 ml TNP-coated SRBC (Rittenberg and Pratt, 1969) and 0.025 ml diluted steelhead trout (O. mykiss) serum as a complement source and deposited the mixture in a Cunningham slide chamber. During incubation (2 h at 17° C), anti-TNP antibody became bound to TNP-SRBC and activated the complement cascade, resulting in lysis of the surrounding TNP-SRBC. Thus, lymphocytes secreting anti-TNP antibody caused a hole, or plaque in the surrounding TNP-SRBC. Plaques were counted using a low-power dissecting microscope, and were expressed in terms of APC per million lymphocytes; lymphocyte numbers were determined with a Coulter Counter (Model Z.M., Coulter Electronics, Hialeah, FL, U.S.A.).

Disease Challenge Tests

We tested disease resistance to the fish pathogen Vibrio anguillarum, a marine bacterium to which salmon in freshwater presumably had not previously been exposed. Duplicate treatment groups of fish were exposed to V. anguillarum (LS-174) using the waterborne infection method of Gould et al. (1978). Briefly, fish were exposed to 3 to 4 X 10^{10} V. anguillarum bacteria in 20 l of water for 20 min. Dead fish were removed at least every 12 h and we confirmed that death was caused by V. anguillarum by isolating the pathogen from their kidneys. Data were expressed as mean time to death (MTD) and percent total mortality.

Laboratory Experiments

To assess the effects of stress on immune response, we acutely stressed juvenile spring chinook salmon on two occasions (approximate weights: 25 and 40 g for experiments in January and March 1986) by holding them in a dipnet, out of water, for 30-60 s and sampled them 4 h, and 1, 2, 4, and 7 days after stress. In April and October 1986, we challenged similarly stressed fish (weights approximately 20 and 45 g) with V. anguillarum to determine if the pattern seen in the APC data reflected the ability of fish to resist disease. Groups of 25-40 fish were acclimated to 65-litre cuboidal tanks for 2 weeks. We acutely stressed three randomly selected groups of fish 7 days before exposing them to disease, and three groups 4 h or 1 day before exposure; three groups (controls) were not stressed. All groups were subjected to the exposure protocol but only two of three groups for each treatment actually received the pathogen. The third group served as controls for non-disease related mortalities (no fish in any of these groups died during the disease challenges). In the second experiment, we removed four fish from each tank before exposure and collected plasma for cortisol determination, and anterior kidney lymphocytes for APC assays.

Clinical Trials

We examined the effects of stressful handling during standard hatchery operations at Warm Springs and Round Butte hatcheries from May through October 1986 and 1987. Fish weights increased from about 5 g to 60 g during these trials. Hatchery operators mark or tag anadromous fish so that they can determine the proportion of juvenile

fish released that survive to adulthood and contribute to the fishery. Fish marking operations at the two hatcheries were similar, in that all of the fish in a raceway (about 300,000) were crowded to one end of the raceway, removed, anaesthetized, individually marked by hatchery workers, allowed to recover from the anaesthetic, and returned to a raceway at 20% of the previous density (i.e. 60,000 fish/raceway). At Warm Springs, 60,000 fish were handled in 4 h, compared with several hundred fish in 45 - 60 min at Round Butte. Workers at Warm Springs marked fish by clipping off the left or right pectoral fin, whereas workers at Round Butte clipped the adipose fin and inserted a microscopic coded-wire tag into the fish's snout. During four trials, we sampled 25 - 30 fish from raceways before stress, and 4 h, 1 day, 7 days and for several weeks after stress.

We collected emigrating juvenile fall chinook salmon from the Columbia River to assess the effects of stress on disease resistance. About 8 - 9 months after fall chinook salmon are spawned, the juveniles are released from hatcheries and migrate to the ocean. At McNary Dam, on the Columbia River, these emigrants are collected and transported by tank-truck or barge to a release site below Bonneville Dam, the last downstream dam on the Columbia River. Transported fish avoid the often fatal hazards associated with passage through four intervening hydroelectric dams, but the fish are exposed to stresses associated with collection and transportation (Maule et al., 1988). To assess the effects of this manipulation on disease resistance, we transported two groups of fish to the Mark O. Hatfield Marine Science Center (HMSC), Newport, OR, U.S.A. and exposed them to V. anguillarum. In each experiment, about 500 fall chinook salmon (weights were about

9 g in June and 24 g in August 1982) were transferred from the holding facilities at McNary Dam to a 760-litre tank mounted on a truck for transport to HMSC. The tank had a water recirculation system to aerate water and water temperature was maintained at ambient river-water temperature (16 $\pm$ 1^{o} C in June and 20 $\pm$ 1^{o} C in August) by adding ice as needed. Each transport run took 8-9 h and upon arrival at HMSC, 25 - 30 fish were stocked into each of 12 0.61-m diameter circular tanks. Duplicate tanks of fish were exposed immediately upon arrival, 1 day or 8 days after arrival. These fish were going through, or had gone through, a physiological transformation (smoltifcation) that would allow them to survive in the marine environment (Hoar, 1976). To control for an undetermined osmoregulatory preference, we exposed similar groups of fish to the pathogen in seawater and freshwater, as described above.

Data analyses

All numerical data were subjected to analyses of variance and, where significant between-group differences were found, we conducted pairwise comparisons, using the Duncan multiple range test at the $P < 0.05$ level. Although we present APC data in terms of percent of control, statistical analyses were done with the raw data. Percent mortality data from the disease challenges were analyzed using the G-test statistic based on the Chi-square distribution at $P< 0.05$ (Sokal and Rohlf, 1981).

RESULTS

Laboratory Experiments

Plasma cortisol was significantly elevated in stressed fish after 4 h but had returned to control levels by 1 day after stress (Figure 4). The APC decreased significantly 4 h after stress, apparently rebounded after 1 day, and then again decreased significantly 4 to 7 days after stress (Figure 4). The same patterns of plasma cortisol concentration and APC generation were seen in fish that were stressed before exposure to *V. anguillarum* (data not shown). The percent mortality caused by *V. anguillarum* reflects the pattern of APC response, in that fish exposed to disease 1 day after stress survived significantly better than those in any other treatment and fish exposed after 4 h suffered the highest percent mortality (Figure 5). Furthermore, of the fish that died, those that were exposed 1 day after stress were able to resist the lethal effects of the disease longer (significantly longer MTD in one experiment) than the non-stressed controls (Figure 5). Although the results of the APC assay suggested reduced immune competence 7 days after stress, this reduction did not equate with reduced disease resistance as these groups of fish survived at least as well as the non-stressed controls (Figure 5).

Clinical Trials

Results of four trials in hatcheries were similar in that plasma cortisol levels increased within a few hours after the stressful encounter and returned to levels equal to those in fish before stress within 24 h (Figure 6). The competence of lymphocytes from fish

Figure 4. Results from one of two experiments demonstrating the effects of 30-s stress on juvenile spring chinook salmon. Plasma cortisol (mean $\pm$ 1 SE; N = 8 - 12 fish) (top) and antibody producing cells (APC) generated by anterior kidney lymphocytes, as percent of controls (bottom). Control values (mean $\pm$ 1 SE) were 1429 $\pm$ 128 APC/ million cells. Stars indicate bars that are significantly different from controls, $P < 0.05$, G-test (top) or Duncan's multiple range test (bottom).

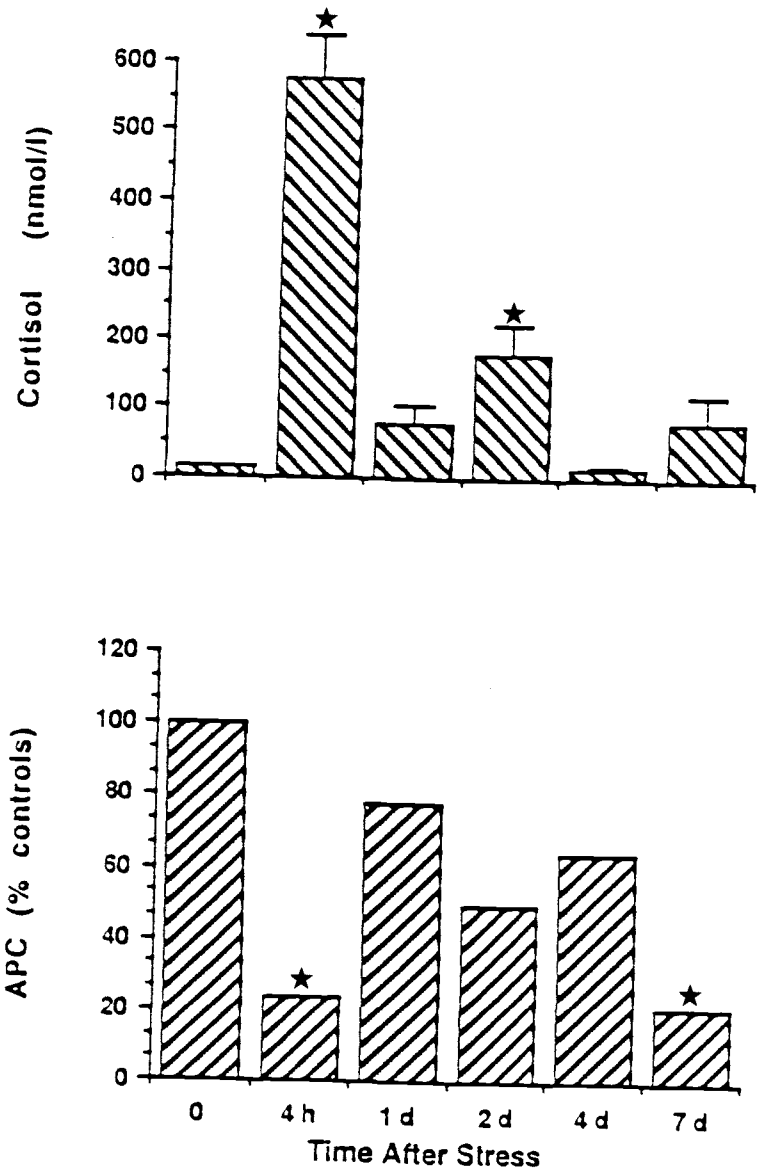
Cortisol (nmol/l)
600
500
400
300
200
100
0
APC (% controls)
120
100
80
60
40
20
0
0
4 h
1 d
2 d
4 d
7 d
Time After Stress

Figure 4.

Figure 5. Results of pathogenic challenges (*V. anguillarum*) of spring chinook salmon before and at various times after a 30-s stress. Plasma cortisol concentrations and APC data were similar to those in Figure 1. Mean percent mortality (top) and mean time to death (MTD) of fish that died in duplicate tanks (bottom) . Stars indicate bars that are significantly different from the value at Time = 0, $P < 0.05$, G-test (top) or Duncan's multiple range test (bottom).

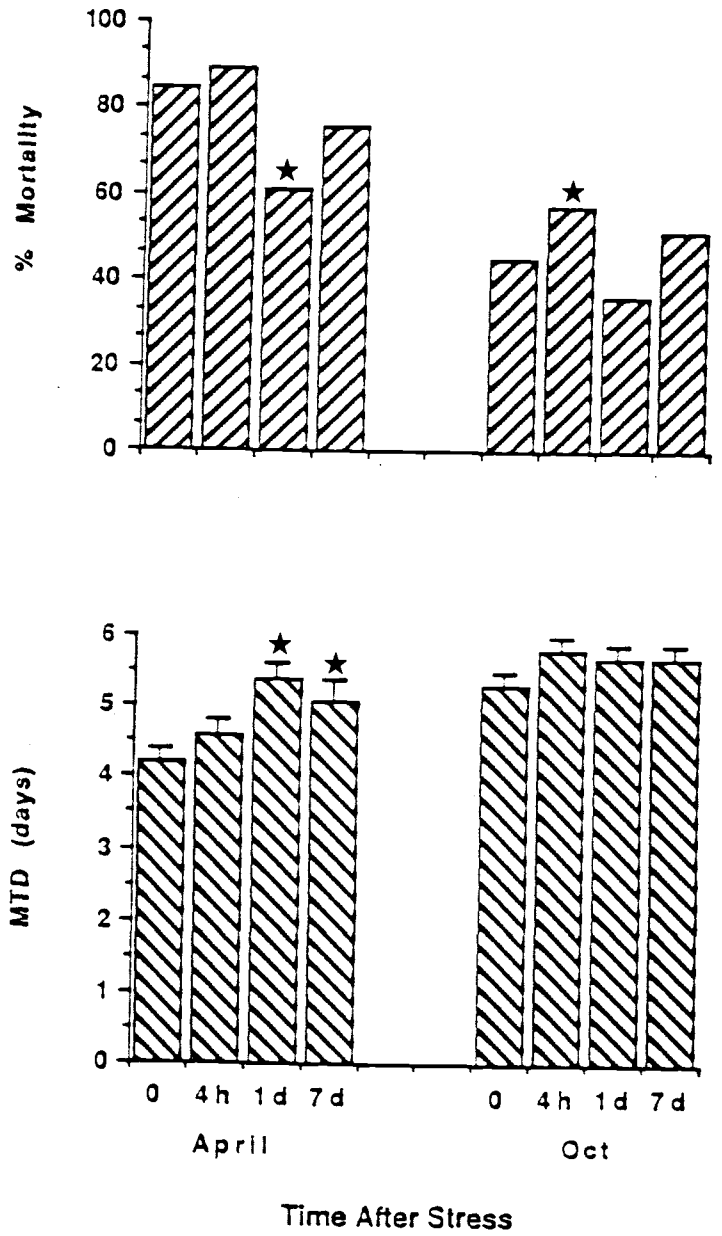
% Mortality
100
80
60
40
20
0
MTD (days)
6
5
4
3
2
1
0
0 4h 1d 7d
April
0 4h 1d 7d
Oct
Time After Stress

Figure 5.

Figure 6. Effects of stress of marking procedures at Warm Spring National Fish Hatchery in 1986. Similar results were obtained in 1987 and at Round Butte Fish Hatchery in 1986 and 1987. Plasma cortisol (mean $\pm$ 1 SE; N = 25 - 32 fish) in spring chinook salmon before (Time = 0) and at various times after marking (top). Antibody producing cells (APC) as percent of controls (bottom). Control value (mean $\pm$ 1 SE) was 460 $\pm$ 78 APC/million cells. Stars indicate bars that are significantly different from the value at Time = 0. $P < 0.05$, Duncan's multiple range test (top), or G-test (bottom).

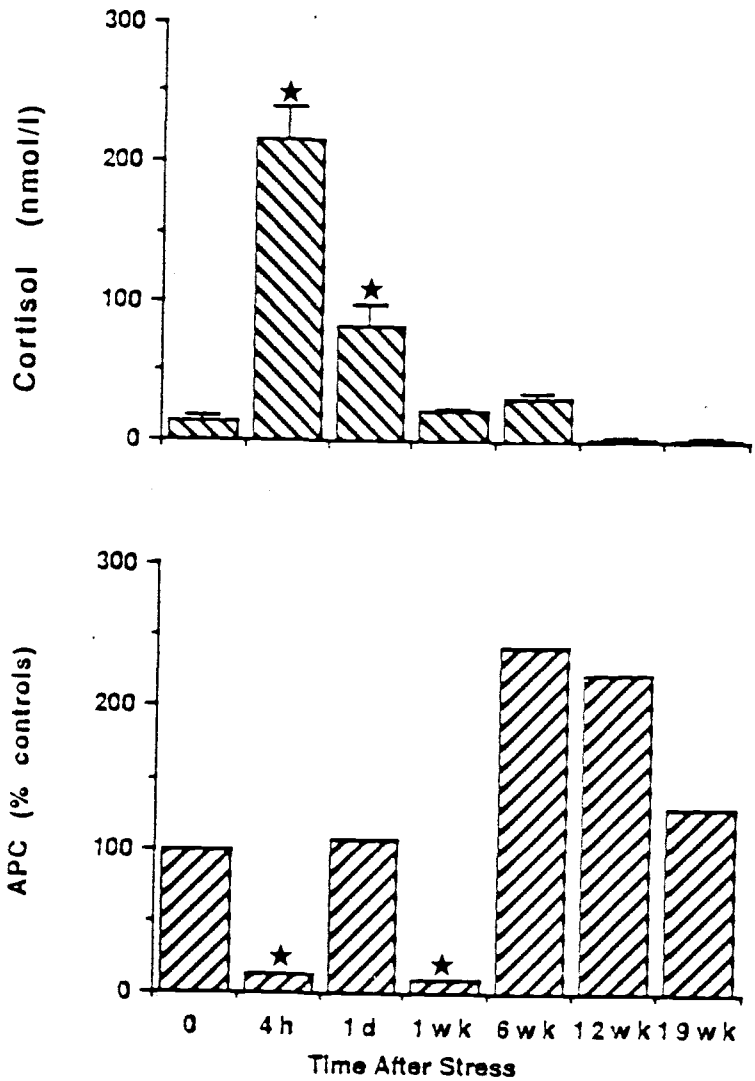
Cortisol (nmol/l)
300
200
100
0
APC (% controls)
300
200
100
0
0
4 h
1 d
1 wk
6 wk
12 wk
19 wk
Time After Stress

Figure 6.

stressed during hatchery operations was similar to that seen in laboratory experiments. The ability of lymphocytes to generate APC 1 day after stress did not differ significantly from that of fish sampled before stress. Number of APC was significantly reduced in three of four trials after 7 days, but in only one of four trials after 4 h. The differences between laboratory and clinical trials may be attributable to the need to sample fish in the middle of ongoing hatchery operations. Thus, we were unable to determine exactly how much time had passed since an individual fish had been marked. We continued to monitor these fish for up to 20 weeks after stress; numbers of APC were significantly higher 6 - 9 weeks after stress and, returned to levels equal to those in fish before stress by 17 - 20 weeks. As we indicated, numbers of fish in the raceways were reduced after marking to 20% of original density. However, after 20 wk mean fish weight had increased by at least 5 X, effectively returning biomass per raceway to that before marking.

The results of the disease challenges were consistent within the four trials. Survival was 4 - 18% higher in fish allowed to recover for 1 day than fish challenged immediately after transport or 8 days after transport (Figure 7). Additionally, the MTD of fish that died was 50 to 100% longer in groups challenged 1 day after arrival than in fish in either of the other two groups (Figure 7).

Figure 7. Effects of transportation on disease resistance of emigrating juvenile fall chinook salmon. (A) Mean percent mortality in duplicate groups of 30 fish exposed to V. anguillarum at various times after the stress of 9-h transportation. Pathogenic challenges were conducted in freshwater (FW) and seawater (SW). (B) Mean time to death (MTD) of fish that died. Stars indicate bars that are significantly different from the value at Time = 0; $P < 0.05$, G-test (A), or Duncan's multiple range test (B).

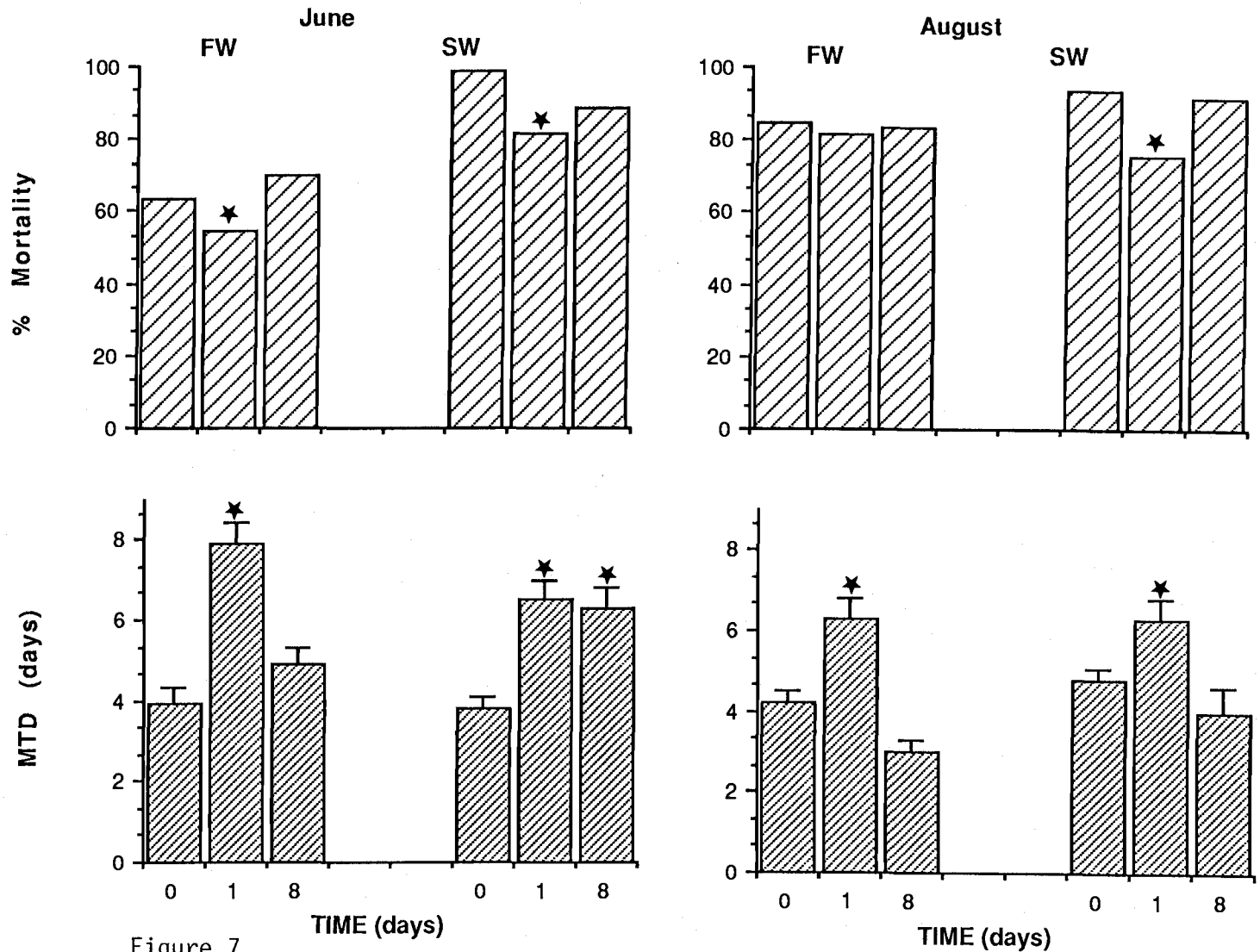
June
August
FW
SW
FW
SW
% Mortality
MTD (days)
TIME (days)
TIME (days)
0
20
40
60
80
100
0
2
4
6
8
0
1
8

Figure 7.

DISCUSSION

We have demonstrated that acute stress can have a transient, positive effect on immune function and disease resistance in salmon, as well as immunosuppressive effects. It is significant to note that the trends seen in this study were consistent throughout most of the experiments, despite the uncontrolled variability in using outbred stocks of fish at different stages of development, in various environmental settings, and after stress lasting from 30 s to 9 h. The fact that disease resistance was not depressed 7 days after stress indicates that, although the ability of anterior kidney lymphocytes to generate APC was depressed, other immune mechanisms were not affected or were strengthened at that time. In fact, the apparent stress-related resistance to this disease 1 day after stress must be the result of increased functioning of nonspecific immune mechanisms, because APC response to V. anguillarum O-antigen vaccination peaks in 16 - 17 days in vivo (Maule et al., 1987), whereas death from exposure to V. anguillarum occurred in 2 - 6 days (Figures 5 and 7). The positive change in immune response and improved disease resistance seen in salmon 1 day after stress may be the equivalent of the enhanced immune response seen in mice 15 min after stress (Gisler et al., 1971). The additional time (i.e., 1 day versus 15 min) required for the response in fish will be advantageous in elucidating the mechanisms involved.

We previously reported that cortisol implants reduced disease resistance and ability to generate APC in vivo (Maule et al., 1987) and that the addition of physiological concentrations of cortisol to cell cultures caused a dose-dependent reduction in APC in vitro

(Tripp et al., 1987). In those studies, however, exogenous cortisol was present throughout the 9 - 17 day incubation period required to develop the response. In mammals an acute cortisol treatment caused redistribution of lymphocytes (primarily regulatory T-cells) out of the circulation and into the bone marrow (Cupps and Fauci, 1982). This leukocytopenia reached a maximum by 4 h and returned to normal within 24 h. We have shown that, during the 3 - 4 months when coho salmon (O. kisutch) were undergoing smoltification, the number of circulating leukocytes and relative numbers of splenocytes decreased as resting plasma cortisol concentrations increased (Maule et al., 1987). Other data also demonstrated that, when coho salmon were acutely stressed or given a single feeding of cortisol-treated food, the numbers of cells in the spleen and circulation decreased, whereas cell numbers in the thymus and anterior kidney increased (Chapter IV, this volume). These alterations were evident within 3 h and persisted for 2 - 3 days. Thus, in the present study, the subpopulations of cells harvested from anterior kidneys of nonstressed control fish may have been different from the cells of stressed fish.

Studies with mice have also shown changes in functional populations of cells within lymphoid organs after stress. Okimura and Nigo (1986) reported that when they expressed their data in terms of anti-TNP APC per spleen, there was no significant difference between stressed mice and controls; however, when APC were expressed relative to number of cells in the spleen, stressed mice had significantly more APC than controls. This relation suggests that some cells not necessary for the anti-TNP response had been selectively removed from the spleens of stressed mice. Subsequently, Okimura et al. (1986a,

1986b) concluded that stress-induced increases in corticosteroids and catecholamines were acting to suppress the function of T-cells, thus suppressing the response of B-cells that require T-cell help for activation (T-dependent, T_D). At the same time, B-cells that did not require T-cell help (T-independent, T_I) were not affected or were augmented by the stress. Although there are functionally heterogeneous cells in the salmon immune system, the T_D - T_I paradigm has not been confirmed in salmon. It is known that the hormonal response of fish to acute stress is similar to that of mammals in that there is an immediate increase in circulating catecholamines followed by an increase in corticosteroids (Pickering, 1981). Furthermore, Tripp et al. (1987) and Kaattari and Tripp (1987) demonstrated that cortisol suppression of APC in salmon appears to be caused by inhibition of an interleukin-like (interleukin-1) molecule secreted by macrophage-like cells to activate antigen-specific B-cell precursors. In the present study, it appears that 4 h after stress, cortisol had a direct immunosuppressive effect, but that changes in immune function and disease resistance between 1 and 7 days after stress may be the result of other hormonally driven affects on the immune system. These findings suggest similar immune-endocrine interactions in the mouse and salmon.

IV. REDISTRIBUTION OF LEUKOCYTES IN BLOOD, THYMUS, SPLEEN AND ANTERIOR KIDNEY OF COHO SALMON (*ONCORHYNCHUS KISUTCH*) AFTER STRESS AND CORTISOL TREATMENT

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ABSTRACT

We examined the effects of acute and chronic stress, a single feeding of food containing cortisol and seven-day cortisol implants on the distribution of leukocytes in blood, thymus, spleen and anterior kidney of juvenile coho salmon, (Oncorhynchus kisutch). In acutely stressed and cortisol-fed fish, there were significant increases in numbers of leukocytes in the thymus and anterior kidney and significant decreases in blood and spleen within one day of treatment, followed by a return to control levels within 2 to 4 days after treatment. Changes in the numbers of leukocytes in the various organs of chronically stressed fish were similar to those in acute stress, except in the anterior kidney where cell numbers were decreased for the duration of the experiment. Although dietary cortisol resulted in elevated plasma cortisol titers and caused the same changes in leukocyte distribution as acute stress, there was no correlation between the magnitude or duration of elevated cortisol levels and leukocyte distribution. Furthermore, fish with cortisol implants had significantly elevated plasma cortisol levels seven days after treatment, but their leukocyte distribution did not differ from controls. These results suggest that factors in addition to cortisol are involved in the redistribution of leukocytes in salmon during stress.

INTRODUCTION

It is well documented that stress can affect the immune systems of homeotherms (Keller et al., 1981; Laudenslager et al., 1983) and poikilotherms, such as fish (Ellsaesser and Clem, 1986; Maule et al., 1989). A thread common to all vertebrates is that stress-induced increases in circulating glucocorticoids (primarily cortisol and corticosterone) alter a wide variety of immune functions (Tounefier, 1982; Munck et al., 1984; Garrido et al., 1987; Maule et al., 1987). Stress and treatment with glucocorticoids can alter the number and composition of circulating leukocytes by cytolysis (Homo et al., 1980; Ellsaesser and Clem, 1987) or redistribution of cells to other lymphoid organs (Fauci and Dale, 1975; Dracott and Smith, 1979; Steplewski and Vogel, 1986). Circulating leukocytes from stressed animals have been shown to be less immunologically responsive than those from nonstressed controls (Dracott and Smith, 1979b; Okimura et al., 1986a), suggesting that the immunocompetence of stressed animals is impaired. This interpretation ignored the possibility that immunologically competent cells might reside in other immune organs and that the organisms were still capable of resisting a pathogenic challenge, the true measure of immune competence.

Maule et al.(1989) have shown that the immunocompetence of juvenile chinook salmon (Oncorhynchus tshawytscha), as measured by the ability of lymphocytes from the anterior kidney to generate antibody producing cells (APC), was impaired at 4 h but was enhanced by 24 h after stress. Likewise, resistance to Vibrio anguillarum, a pathogenic marine bacterium, was impaired at 4 h and enhanced at day 1 after stress. However, by day 7 after stress, immunocompetence was

depressed but disease resistance was not different from that seen in nonstressed fish. It was suggested that the immune mechanisms involved in generating APC were not the same as those involved in resistance to V. anguillarum, and that those mechanisms were differentially affected by stress (Maule et al., 1989). It has been shown that cortisol can inhibit in vitro immune response by reducing the number of functional B cell precursors (Kaattari and Tripp, 1987), possibly by inhibiting secretion of a lymphokine (Tripp et al., 1987). It is possible that the reported changes in APC during the seven days after stress reflected changes in the distribution of leukocyte subpopulations among the various immune organs. The objectives of the present study were to determine if there were changes in the number of leukocytes in the blood, thymus, spleen or anterior kidney of juvenile coho salmon (O. kisutch) after acute or chronic stress and, if changes were detected, determine if short-term or long-term treatment with exogenous cortisol could cause similar changes. Acute stress was defined as stress that had a finite duration that ended before sampling began; chronic stress continued throughout the experiment and fish were sampled directly from the stressful conditions.

MATERIALS AND METHODS

Fish. In December 1985, juvenile coho salmon were transferred from the Eagle Creek National Fish Hatchery, Eagle Creek, Oregon to the Oregon State University Smith Farm Experimental Hatchery, Corvallis, Oregon where they were held in freshwater at a constant 11-12^{o} C and fed Oregon Moist Pellet at 1 - 2% body weight daily. By the time of these experiments the fish had gone through the parr-to-smolt transformation, the physiological, biochemical and behavioral changes associated with salmon migration to the ocean (Hoar, 1976). At the time of these experiments in August and October 1986, the fish weighed about 49 and 68 g, respectively.

Experimental design. Two weeks prior to each experiment, we transferred 22 fish to each of several 0.9 m-diameter, 155-L tanks. In order to simulate the effects of a transient increase in circulating cortisol without stress, we fed fish a single meal of food containing cortisol (100 mg/kg food) that had been dissolved in ethanol and sprayed on the food. Food for the control fish was sprayed with ethanol only and the ethanol was evaporated from both foods at room temperature. On the day of the first experiment, we fed fish in two tanks food containing cortisol and fish in four tanks food that had been treated with ethanol. Immediately after feeding, we stressed two tanks of fish that were not fed cortisol by suspending them in the air in a dipnet for 30 - 60 s. Fish in the remaining two tanks were nonstressed controls. We sampled seven fish from each treatment and controls after 3 h , and 1, 3, 7, and 10 days.

In the second experiment, we examined the effects of chronic stress on leukocyte distribution by transferring fish to 19-liter

buckets with holes drilled throughout. The buckets were suspended in the fishes' home tank to such a depth (about 5 cm) that fish were able to remain upright in the water, but could not swim and were in almost continuous contact with other fish (i. e., very crowded). The holes allowed water to circulate through the buckets, thus maintaining water quality. We sampled stressed and control fish 3 h, 2 and 9 days after initiation of stress. In order to examine the effects of chronically elevated plasma cortisol independent of chronic stress, we injected a group of fish with cortisol in a molten cocoa butter vehicle (Pickering and Duston, 1983; Maule et al., 1987). We also injected cocoa butter without cortisol into another group of fish, and had a third group of fish that served as undisturbed controls. The injection procedure constitutes an acute stress so we did not sample these groups until seven days after treatment.

Sampling, assays and data analysis. When sampling, we rapidly transferred fish to water containing 200 mg 3-aminobenzoic acid, ethyl ester (MS-222) /L , a dose that has been shown to inhibit increases in circulating cortisol in salmon (Strange et al., 1978; Barton et al., 1985). After the fish were anesthetized, we severed the caudal peduncle and collected blood from the caudal vasculature into heparinized capillary tubes. A sample of whole blood was diluted with phosphate buffered saline (pH 7.4) for cell counts and the remainder of the sample was centrifuged to separate plasma from cells. We stored plasma at -20^{0} C and later used a radioimmunoassay to determine cortisol concentrations (Redding et al., 1984). We dissected out the spleen, anterior kidney and both thymi and put them in test tubes containing measured volumes of RPMI-1640 medium. The

organs were gently aspirated with 1 ml tuberculin syringes to make single cell suspensions (Tripp et al., 1987) and cell counts were accomplished with a hemacytometer. We express cell counts as cells per organ or cells per ml whole blood. We did not know the ratios of the various leukocyte subpopulations in these cell suspensions. For purposes of total leukocyte counts, we assumed that the blood volume of each fish was 1 ml. We statistically analyzed data by analyses of variance and Duncan's multiple range test ($P < 0.05$) for pairwise comparisons between treatments and for time course variations within treatments.

RESULTS

The pattern of changes in plasma cortisol titers were similar for all of the treatment groups in that there were transient increases within 3 h of stress or cortisol feeding (Figures 8 and 9). However, the magnitude and duration of the responses differed between treatments. Three hours after an acute stress, fish had plasma cortisol levels that were 2- to 3-fold higher than controls, but these had returned to levels equal to control fish within 24 h. Chronically stressed fish and those fed cortisol had plasma cortisol titers 20- to 25-times higher than controls, and these did not return to levels equal to those in control fish until after at least day 2 (Figure 8). Based on other studies, we believe that plasma cortisol in fish from all the treatments may have reached their highest levels sometime just before or after our sample at 3 h (Barton et al., 1985).

In spite of the significant differences in cortisol titers between treatment groups, the changes in numbers of leukocytes in the various organs were very similar. In general there were decreases in leukocytes in the circulation and spleen (Figures 10 and 11) and increases in the anterior kidney (Figures 12 and 13) and thymus (Figures 8). There were, however, differences in how rapidly the changes occurred; number of leukocytes in the anterior kidney and spleen were significantly different from the controls by 3 h, but

Figure 8. Number of leukocytes (mean ± SE) in thymus and concentration of cortisol in plasma of control juvenile coho salmon and fish that were acutely stressed by being held out of the water for 30 to 60 sec, or were fed a single meal containing 100 mg cortisol/kg food. Sample sizes are 4 - 7 fish. Stars indicate points that differ significantly from controls at the same time; Duncan's multiple range test, $P < 0.05$.

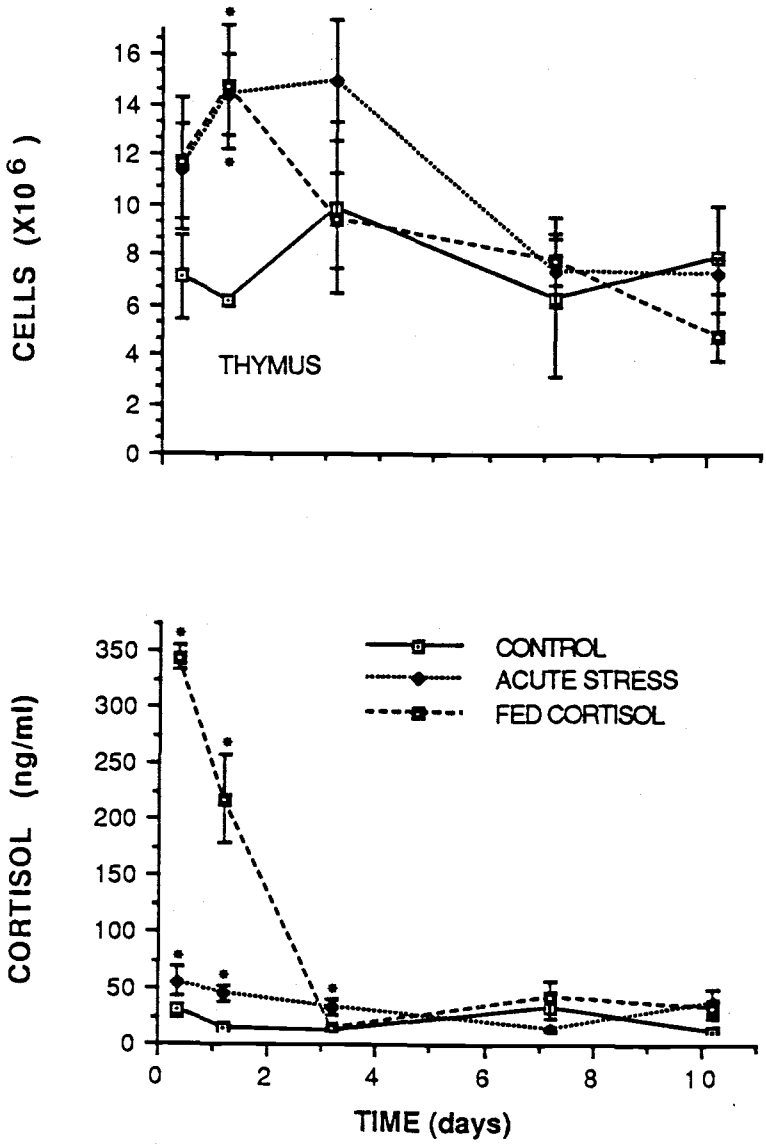
CELLS (X10 6)
16
14
12
10
8
6
4
2
0
THYMUS
CORTISOL (ng/ml)
350
300
250
200
150
100
50
0
0
2
4
6
8
10
TIME (days)
CONTROL
ACUTE STRESS
FED CORTISOL

Figure 8.

Figure 9. Number of leukocytes (mean ± SE) in thymus and concentration of cortisol in plasma of control juvenile coho salmon and fish that were chronically stressed by being crowded in very shallow water. Sample sizes are 4 - 7 fish. Stars indicate points that differ significantly from controls of the same time; Duncan's multiple range test, $P < 0.05$.

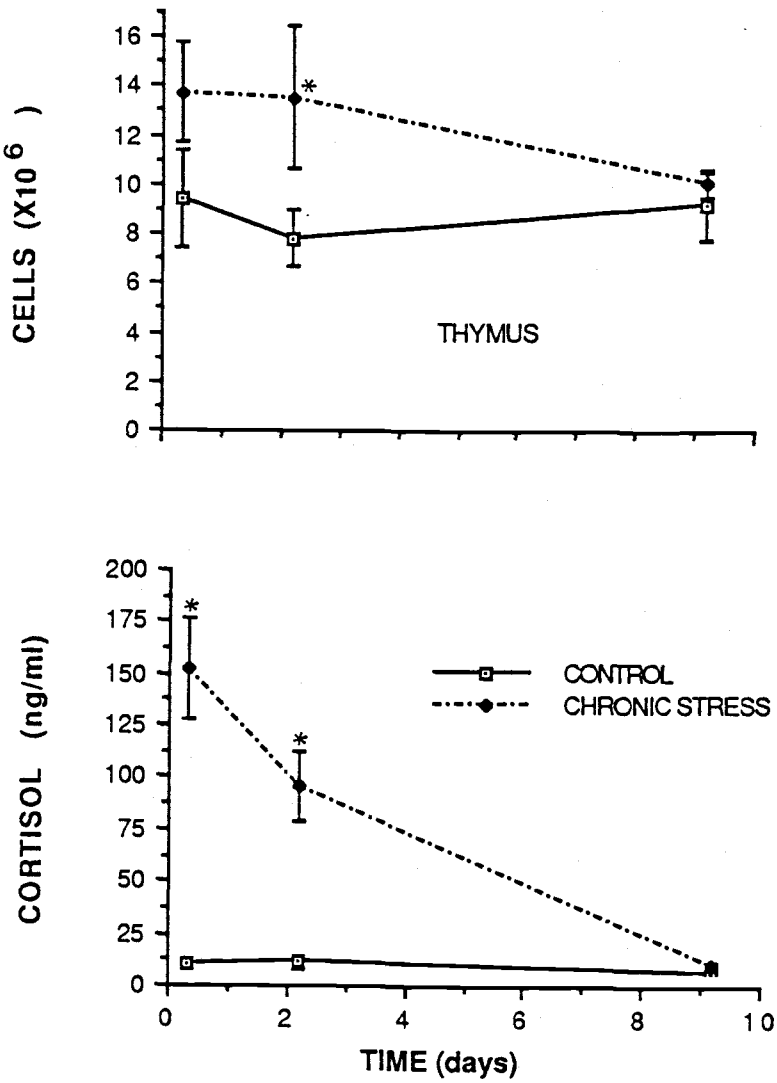
CELLS (X10 6)
16
14
12
10
8
6
4
2
0
*
THYMUS
CORTISOL (ng/ml)
200
175
150
125
100
75
50
25
0
*
*
CONTROL
CHRONIC STRESS
0
2
4
6
8
10
TIME (days)

Figure 9.

Figure 10. Number of leukocytes (mean $\pm$ SE) in blood and spleen of control juvenile coho salmon and fish that were acutely stressed by being held out of the water for 30 to 60 sec, or were fed a single meal containing 100 mg cortisol/kg food. Sample sizes are 4 - 7 fish. Stars indicate points that differ significantly from controls of the same time; Duncan's multiple range test, $P < 0.05$.

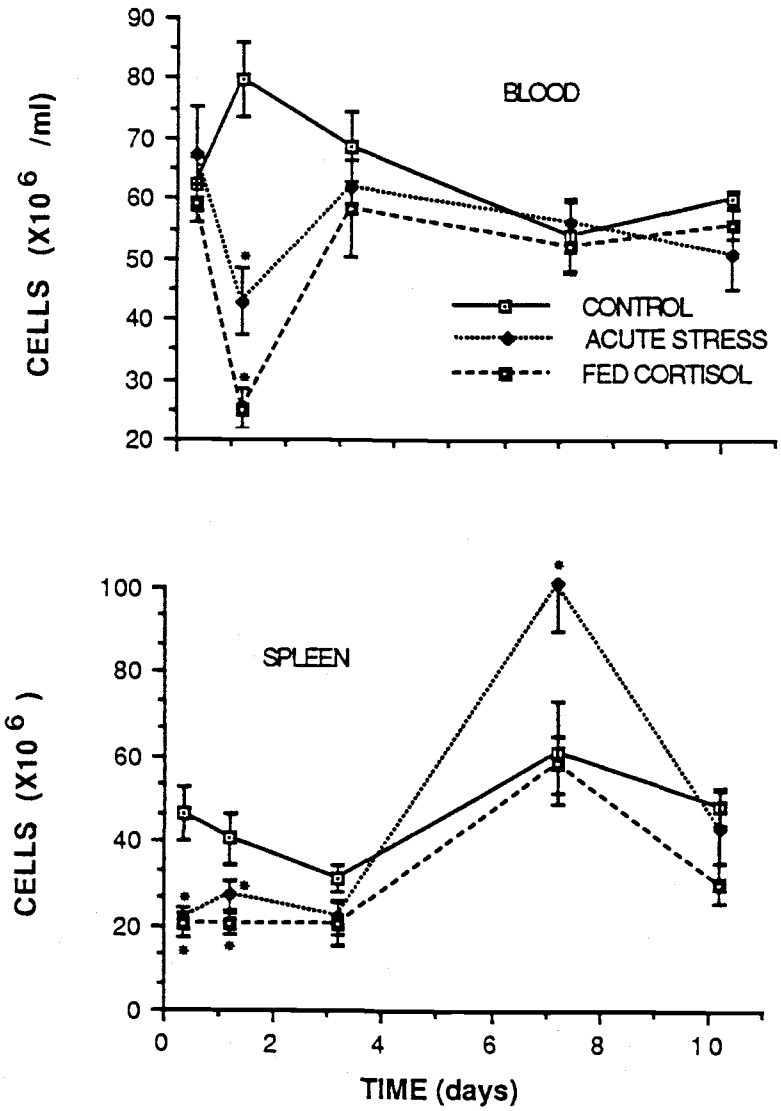
BLOOD
CELLS (X10 6 /ml)
90
80
70
60
50
40
30
20
CONTROL
ACUTE STRESS
FED CORTISOL
SPLEEN
CELLS (X10 6)
100
80
60
40
20
0
0
2
4
6
8
10
TIME (days)

Figure 10.

Figure 11. Number of leukocytes (mean ± SE) in blood and spleen of control juvenile coho salmon and fish that were chronically stressed by being crowded in very shallow water. Sample sizes are 4 - 7 fish. Stars indicate points that differ significantly from controls of the same time; Duncan's multiple range test, $P < 0.05$.

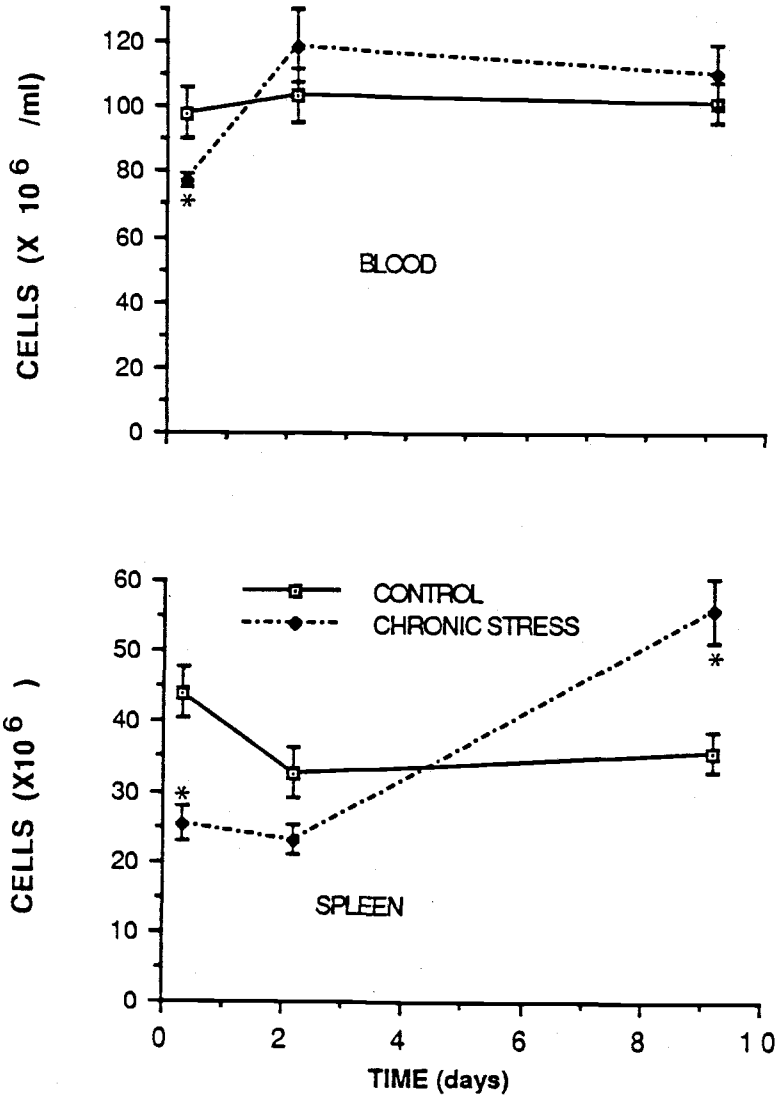
CELLS (X 10 6 /ml)
120
100
80
60
40
20
0
*
BLOOD
CELLS (X10 6)
60
50
40
30
20
10
0
CONTROL
CHRONIC STRESS
*
*
SPLEEN
0
2
4
6
8
10
TIME (days)

Figure 11.

Figure 12. Number of leukocytes (mean ± SE) in anterior kidney and total leukocytes in thymus, spleen, blood and anterior kidney of control juvenile coho salmon and fish that were acutely stressed by being held out of the water for 30 to 60 sec, or were fed a single meal containing 100 mg cortisol/kg food. Sample sizes are 4 - 7 fish. Stars indicate points that differ significantly from controls of the same time; Duncan's multiple range test, $P < 0.05$.

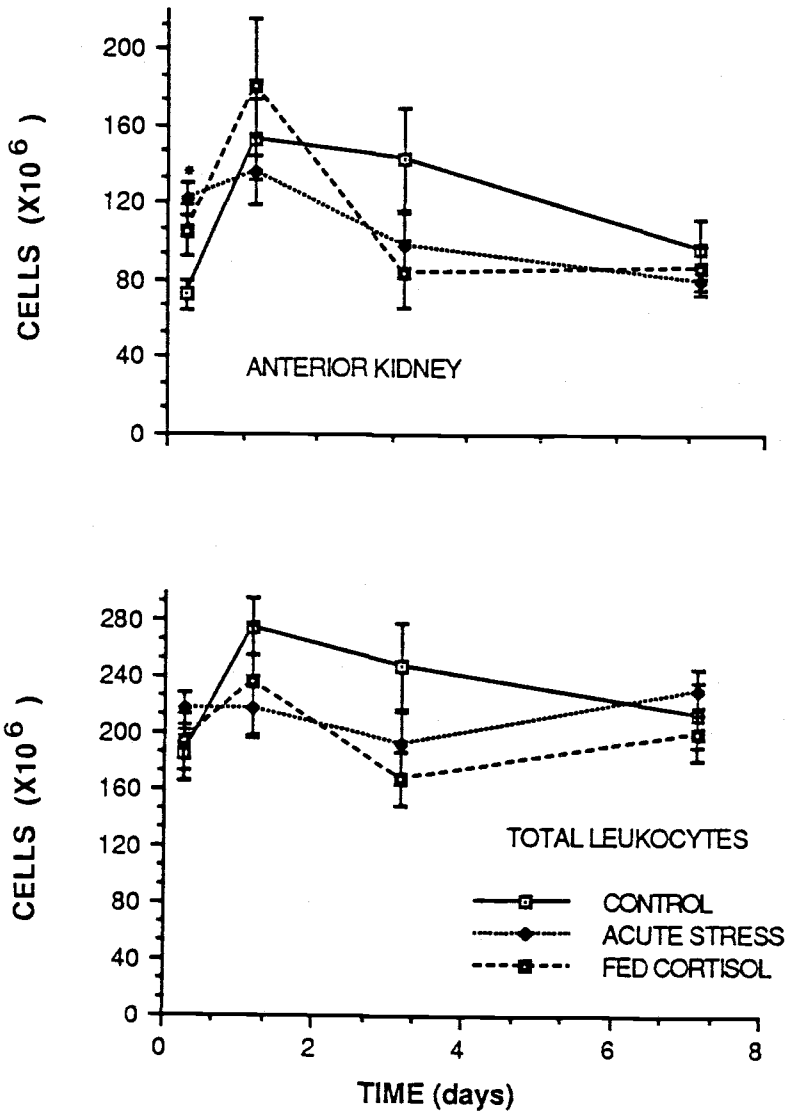
CELLS (X10^6)
200
160
120
80
40
0
ANTERIOR KIDNEY
CELLS (X10^6)
280
240
200
160
120
80
40
0
TOTAL LEUKOCYTES
CONTROL
ACUTE STRESS
FED CORTISOL
0
2
4
6
8
TIME (days)

Figure 12.

Figure 13. Number of leukocytes (mean ± SE) in anterior kidney and total leukocytes in thymus, spleen, blood and anterior kidney of control juvenile coho salmon and fish that were chronically stressed by being crowded in very shallow water. Sample sizes are 4 - 7 fish. Stars indicate points that differ significantly from controls of the same time; Duncan's multiple range test, $P < 0.05$.

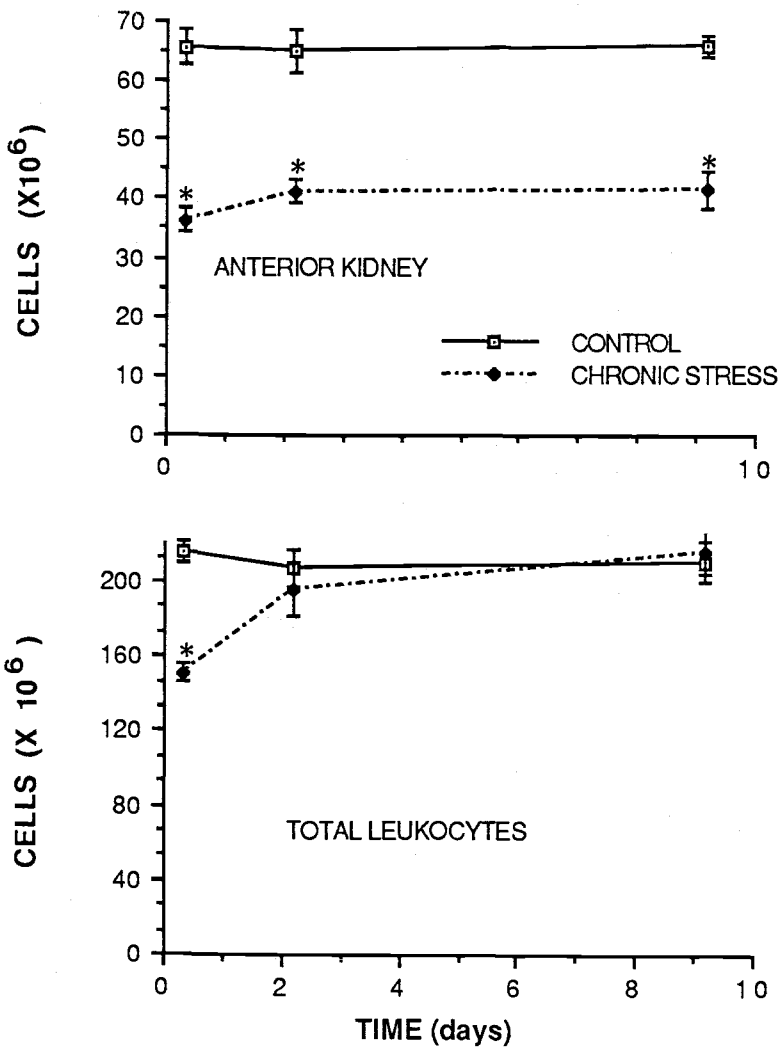
70
60
50
40
30
20
10
0
CELLS (X10^6)
ANTERIOR KIDNEY
CONTROL
CHRONIC STRESS
0
10
200
160
120
80
40
0
CELLS (X 10^6)
TOTAL LEUKOCYTES
0
2
4
6
8
10
TIME (days)

Figure 13.

those in the circulation and thymus were not significantly different from controls until day 1. These apparent differences in rate of change may not be biologically significant, but rather an artifact of our sampling schedule. Nonetheless, at sometime within the first day after treatment, leukocyte numbers in all organs were significantly different from controls and those numbers returned to control levels within 2 to 3 days of treatment. There were two exceptions to this general pattern; first, the number of cells in the anterior kidneys of chronically stressed fish decreased significantly within 3 h and remained significantly lower than controls through the day 9 sample (Figure 13). Second, the number of splenocytes unexpectedly increased from about 20 million cells at day 3 to 50 - 100 million cells/spleen at day 7 after stress or cortisol treatment (changes in chronically stressed fish occurred between days 2 and 9). Control fish had similar, but not statistically significant, increases in number of splenocytes (Figure 9); there were, however, unexplained significant increases in the number of leukocytes in the anterior kidneys of control fish at days 1 and 3 (Figure 12).

Fish that were implanted with cortisol in cocoa butter received an ongoing exposure to exogenous cortisol, as evidenced by the significantly elevated plasma cortisol titers seven days after treatment (Table 3). However, the leukocyte distribution in cortisol-implanted fish was not different from that in undisturbed controls. There were fewer leukocytes in the circulation of cortisol-implanted fish as compared to cocoa butter-implanted controls, but these vehicle controls were not significantly different from the undisturbed controls (Table 3).

Table 3. Number of leukocytes (X 10^6/organ) in various organs, total leukocytes in all four immune organs, and plasma cortisol concentrations (mean ± SE) seven days after juvenile coho salmon were implanted with cortisol in molten cocoa butter or with cocoa butter (CB-Implant). Also shown are the same variables for undisturbed control fish. Numbers in parentheses are sample sizes.

	Control		
	Undisturbed (4)	CB-Implant(6)	Cortisol Implant(7)
Thymus	5.9 ± 3.2	6.6 ± 1.4	8.6 ± 1.2[a]
Blood ($X10^6$/ml)	52.3 ± 5.9	66.3 ± 4.5	42.7 ± 5.5
Spleen	58.4 ± 12.2	93.0 ± 13.8	56.4 ± 16.3
Anterior Kidney	91.4 ± 15.6	114.9 ± 11.9	116.8 ± 17.2
Total	207.9 ± 23.2	280.8 ± 8.0	224.6 ± 29.7
Plasma Cortisol (ng/ml)	15.6 ± 5.8	6.2 ± 2.2	60.5 ± 20.8[a,b]

[a] Significantly different from CB-implant, Duncan's multiple range test, $P < 0.05$.

[b] Significantly different from Undisturbed, Duncan's multiple range test, $P < 0.05$.

The total number of leukocytes in the four immune compartments of the acutely stressed, cortisol-fed or cortisol-implanted fish were not significantly different from controls at any time after treatment (Figure 12; Table 3). However, there were significantly fewer total leukocytes in the chronically stressed fish as compared to controls 3 h after treatment (Figure 13).

DISCUSSION

We have shown that there were rapid, generally transient changes in the number of leukocytes in four tissues of the immune system in coho salmon following acute or chronic stress and cortisol treatment. However, there was no significant change in the total number of leukocytes in the four immune compartments combined after acute stress, cortisol feeding or cortisol implantation. These findings suggest that monitoring changes in number of leukocytes in tissues of the immune system is not an appropriate method for determining the immune status of Pacific salmon. Additional support for this finding comes from an earlier study that reported enhanced immune function and disease resistance 1 d after stress (Maule et al., 1989), while in the present study the number of leukocytes in the circulation was reduced 1 d after stress. Previous investigators have suggested that counts of circulating leukocytes can be used as an index of acute stress in fish (Pickford et al., 1971; McLeay, 1975; McLeay and Gordon, 1977). Our data support that suggestion, but we believe that additional physiological data are necessary before the significance or severity of the stress can be interpreted (Maule et al., 1988). Moreover, it would appear that leukocyte counts of chronically stressed fish, or those made when the length of time since an acute stress occurred is not known, are of little value.

Acutely stressed and cortisol-fed fish did not have significant changes in total number of leukocytes suggesting that changes in the number of leukocytes in the various immune compartments might be the result of redistribution of cells. Chronically stressed fish did have significantly fewer total leukocytes than controls 3 h after

treatment, but this number had returned to control levels within two days. Thus, some of the cells in chronically stressed fish may have been redistributed to sites other than the four immune compartments we examined. Chronically stressed salmon can attain ideal compensation (Precht, 1958) within 7 to 10 days, as evidenced by the return of plasma cortisol levels to control levels (Strange et al., 1978; Schreck, 1981). In the present study we can not determine if ideal compensation has occurred because of the scaling of plasma cortisol concentrations. However, the data suggest that compensation can also be detected in the distribution of leukocytes in immune organs, with the possible exception of the anterior kidney which had significantly fewer leukocytes than controls through nine days. Although the numbers of cells in the organs of fish implanted with cortisol did not differ from controls, in an earlier study (Maule et al., 1987) the number of cells in the circulation and spleens of implanted fish were significantly less than in controls. In that study, the fish were exposed to the exogenous cortisol considerably longer than in the present study, as sampling was done 24 days after treatment.

It appears that cortisol was directly involved in the change in numbers of leukocytes in the various organs, as feeding cortisol resulted in the same changes as did acute stress. Correlations have been shown between glucocorticoid receptor (GR) numbers and glucocorticoid bioactivity in mammals (Coffey and Djeu, 1986). Furthermore, there are variations in the number of GR in leukocyte subpopulations (Lippman and Barr, 1977; Distelhorst and Benutto, 1981; Katz et al., 1985) that may account for the differential redistribution of leukocytes during stress or glucocorticoid treatment

(Cohen, 1972; Rogers and Matossian-Rogers, 1982; Steplewski and Vogel, 1986). We have also found differences in numbers of GR in leukocytes from the spleen and anterior kidney of coho salmon (Chapter V, this volume) suggesting that the differential response of leukocytes from these organs may be mediated through GR. Although we have shown that cortisol can play an important role in the distribution of leukocytes in stressed salmon, there must also be other factors involved. Changes in plasma cortisol in the various treatments were similar, but levels in the cortisol-fed fish were higher for a longer time than levels in acutely stressed fish. However, the distribution of cells was the same. Alternatively, cortisol-implanted fish had significantly elevated plasma cortisol seven days after treatment, yet the distribution of leukocytes was not different from controls. Furthermore, fish in an earlier study had significantly different numbers of leukocytes in blood and spleen 24 days after receiving cortisol implants (Maule et al., 1987). This failure to find a clear relation between circulating cortisol titers and distribution of leukocytes suggests that stress-related redistribution of leukocytes is not driven solely by cortisol. In studies with rats (DeBlasi et al., 1986) and humans (Landman et al., 1984), it has been shown that stress and glucocorticoids can unmask B-adrenergic receptors on lymphocytes. Catecholamines are also secreted in response to stress and can act through B-adrenergic receptors to cause a redistribution of leukocytes from the circulation and spleen to the bone marrow (Landman et al., 1984). Catecholamines are also secreted in fish in response to stress (Mazuead et al., 1977). These similarities in stress-induced hormone secretion and GR suggest that similar

mechanisms may cause the change of leukocyte number in the tissues of the salmon and mammalian immune systems.

V. GLUCOCORTICOID RECEPTORS IN LEUKOCYTES AND GILL OF JUVENILE COHO SALMON (*ONCORHYNCHUS KISUTCH*)

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Corvallis, Oregon 97331 USA

ABSTRACT

We demonstrated that cytosol from the gill of coho salmon (Oncorhynchus kisutch) had saturable, high affinity, low capacity binding to radiolabeled [^{3}H]cortisol (Kd = 2.24 $\pm$ 0.28 nM, mean $\pm$ 1 SE; Nmax = 41.4 $\pm$ 7.4 fmol/mg protein) and radiolabeled [^{3}H]triamcinolone acetonide (TA; Kd = 0.38 $\pm$ 0.03 nM, Nmax = 37.8 $\pm$ 4.9 fmol/mg protein). Similarly, TA bound to cyctosolic fractions of leukocytes harvested from spleen (Kd = 0.32 $\pm$ 0.03 nM, Nmax = 8.3 $\pm$ 2.0 fmol/mg protein) and anterior kidney (Kd = 0.37 $\pm$ 0.03 nM, Nmax = 30.2 $\pm$ 5.2 fmol/mg protein), and to whole leukocytes from spleen (Kd = 0.30 $\pm$ 0.04 nM, Nmax = 445 $\pm$ 57 sites/cell) and anterior kidney (Kd = 0.40 $\pm$ 0.04 nM, Nmax = 1198 $\pm$ 180 sites/cell). The competition hierarchies of steroid competitors were the same for both ligands and all tissues (TA > cortisol > 17 -hydroxyprogesterone > cortisone > aldosterone > testosterone). The differences in ligand binding in leukocytes from spleen and anterior kidney are consistent with previously reported organ-dependent sensitivity of leukocytes to cortisol.

INTRODUCTION

It has been demonstrated that the ability of coho salmon (Oncorhynchus kisutch) to generate specific antibody-producing lymphocytes in vivo was reduced concomitant with increases in resting plasma cortisol concentrations during the parr-to-smolt transformation (Maule et al., 1987). Increases in plasma cortisol concentration after stress of chinook salmon (O. tshawytscha) have also been correlated with decreased immune response in vitro and decreased disease resistance (Maule et al., 1989). Furthermore, artificially elevating plasma cortisol by exogenous cortisol treatment caused reduced disease resistance and immune responsiveness (Maule et al., 1987), and the addition of cortisol to salmonid lymphocyte cultures caused a dose-dependent decrease in the ability of those cells to generate specific antibody-producing lymphocytes (Tripp et al., 1987). It appears that the addition of cortisol to salmonid leukocyte cultures suppressed the activation of B-cell (i. e. antibody-secreting cell) precursors by inhibiting the secretion or activity of an interleukin-like substance (Kaattari and Tripp, 1987; Tripp et al., 1987). Thus, it appears that cortisol can have direct effects on cells of the salmonid immune system, suggesting the presence of glucocorticoid receptors (GR) in those cells. The immunomodulatory effects of glucocorticoids and the presence of GR in mammalian leukocytes are well established (Coffey and Djeu, 1986; Plaut, 1987). Although GR have been found in a variety of tissues in fish (DiBattista et al., 1983; Sandor et al., 1984; Chakraborti and Weisbart, 1987), they have not been reported in immune cells of fish nor have they been reported in tissues of Pacific salmon

(*Oncorhynchus* spp.). The objective of the present study was to explore the possible existence of high affinity, low capacity GR in coho salmon and specifically locate GR in cells of the salmon immune system. Glucocorticoid receptors have been reported in gill of rainbow trout (*O. mykiss*; Sandor et al., 1984); therefore, we examined gill tissue from coho salmon to verify the presence of GR in this species.

METHODS AND MATERIALS

Fish. Yearling coho salmon (20 - 50 g body wt) from the Eagle Creek National Fish Hatchery, Eagle Creek, Oregon were acclimated to holding tanks at the Oregon State University Smith Farm Experimental Hatchery,, Corvallis, Oregon for at least 30 d. Fish were kept in circular tanks (about 1700 l) continuously supplied with well-water that was 11-12^{o} C throughout the year. Fish were fed Oregon Moist Pellet at 1 - 2% body wt daily except on days of experiments. At the time of sampling, we rapidly netted fish from a holding tank and transferred them to a bucket containing a lethal dose (200 mg/l) of 3-aminobenzoic acid, ethyl ester (MS-222). Such a dose has been shown to inhibit stress-related increases of plasma cortisol concentration in salmon (Strange and Schreck, 1978; Barton et al., 1986).

Tissue preparation. After we severed the caudal peduncle to bleed the fish, we removed gills, spleens, and anterior kidneys. We found it necessary to pool organs from up to 10 fish to obtain enough tissue for binding assays. All tissues were maintained at about 4^{o} C throughout the assays. For studies of receptors in gill cytosol, we removed gill filaments from gill arches and put them in ice-cold TEMS buffer similar to that described by DiBattista et al. (1983) composed of 10 mM Tris-HCl (all chemicals, except where noted, were the products of Sigma Chemical Co. St Louis, MO), 1 mM EDTA, 12 mM monothioglycerol, 20 mM sodium molybdate, and 10% v/v glycerol, all at pH 7.4. We used tissue culture medium (TCM), RPMI-1640 (B. A.

Products Co., Walkersville, MD) with 0.15 mM EDTA, pH 7.4 for assays with whole leukocytes.

To harvest cytosol from tissues, we disrupted cells with a motor-driven glass and Teflon homogenizer. We removed gross debris by centrifugation (2,000 x g, 20 min, 4^{0} C, TJ-6 refridgerated centrifuge; Beckman Instruments, Palo Alto, CA). The supernatant from this initial centrifugation was centrifuged at 100,000 x g for 60 min in an L8-70 ultracentrifuge with a type 40 fixed-angle rotor (Beckman Instruments). After this centrifugation, we carefully removed the supernatant and mixed two volumes of supernatant with one volume of TEMS containing 5% (w/v) Norit A charcoal (J. T. Baker Chemical Co., Phillipsburg, NJ) and 0.5% (w/v) dextran to remove endogenous steroids. We allowed these to incubate for 10 min before centrifuging (3,000 x g, 20 min) to separate the charcoal from liquid fraction. This series of centrifugations separated nuclei and membranes from the cytosolic fraction. Although the source of the receptors in the liquid fraction is not known (i. e., cytoplasm, nucleus or membrane), we refer to them as cytosolic receptors to distinguish them from whole cell receptors. We assayed protein content of the cytosolic fraction with Bradford reagent (Bradford, 1976) using bovine serum albumin standards and a model DB-G spectrophotometer (Beckman Instruments), and adjusted protein content to 4 - 6 mg/ml.

We harvested whole leukocytes from spleens and anterior kidneys by the same method used to prepare cell cultures to assess immune function (Tripp et al., 1987; Maule et al., 1989). Briefly, tissues in TCM were gently and repeatedly aspirated with a 1-ml tuberculin syringes to free cells from connective tissue. After allowing debris

to settle out, we removed the supernatant containing leukocytes and centrifuged it (300 x g, 10 min, TJ-6 centrifuge). We discarded the supernatant from this centrifugation, resuspended cells in TCM, and centrifuged a second time, before resuspending the cells to a concentration of 4 - 10 X 10^7 leukocytes/ml, based on cell counts using a hemacytometer. These cell suspensions contained red blood cells (1 - 2 X 10^6 cells/ml) and tissue debris, thus duplicating conditions of the cell culture experiments in which stress and the in vitro addition of cortisol suppressed the ability of lymphocytes to generate antibody-producing cells (Tripp et al., 1987; Maule et al., 1989). We were unable to detect specific GR binding in experiments with red blood cells or tissue debris. In a number of experiments, we processed duplicate tubes of spleen and anterior kidney leukocytes with a high concentration of radioinert steroid and counted cells at the end of the binding experiment. There were no significant changes in cell numbers during the experimental procedures.

Cytosol Binding Studies. We conducted binding and competition studies with [^{3}H]cortisol with a specific activity of 106 Ci/mmol (Research Products International, Mount Prospect, IL) and [^{3}H]triamcinolone acetonide (TA; 1,4-pregnadien-9 -fluoro-11ß,16 ,17 , 21-tetrol-3,20-dione-16,17 acetonide) with specific activity of 25.9 Ci/mmol (Amersham, Arlington Heights, IL). All studies were done with duplicate samples to measure total binding and nonspecific binding (NSB). Furthermore, each competition study and each binding study comparing cortisol and TA was done using a common pool of gill cytosol. To measure total binding of receptors in cytosolic

fractions, we added various volumes of radiolabeled steroid in absolute ethanol to test tubes. After the ethanol was evaporated (Speed Vac Concentrator, Savant, Inc., Farmingdale, NY) and 0.2 ml of cytosol was added, the resulting steroid concentrations ranged from 0.2 to 6.0 nM. Tubes for NSB were handled in the same way except that we also added various volumes of radioinert steroid in ethanol to tubes such that the addition of 0.2 ml of cytosol resulted in concentrations of radioinert steroids that were 100-fold greater than radiolabeled steroid. During the assays, we added 0.2 ml of cytosol preparation to each tube, vortexed them, and allowed them to incubate for 2 h, at which time we added 0.5 ml of TEMS containing 2.5% (w/v) Norit A charcoal and 0.25% (w/v) dextran. After 10 min, we separated the charcoal, containing unbound ligand, from bound ligand by centrifugation (3,000 x g, 20 min, TJ-6 centrifuge). We then added 0.5 ml of supernatant, containing bound ligand, to 5.0 ml scintillation cocktail (Budget-Solve, Research Products International) and counted the samples on a model LS 1800 liquid scintillation counter (Beckman Instruments). For steroid competition studies, we used the method described above except that we used concentrations of radioinert competitors (aldosterone, cortisol, cortisone, 17 -hydroxyprogesterone, testosterone, and TA) varying between 0.01 - 50,000 nM and 2.5 nM radiolabeled ligand (TA or cortisol).

Whole Leukocyte Binding Studies. We limited our study of GR in whole leukocytes to the use of TA because the cortisol and TA receptors appeared to be identical based on comparison of results from binding and competition studies with gill cytosolic GR. Furthermore,

the fact that GR have a higher affinity for TA than cortisol suggested that there would be less ligand-receptor dissociation during the ligand-diluting washes necessary to remove unbound steroid than if we used cortisol (Bennett and Yamamura, 1985). When total glucocorticoid binding in whole leukocytes was determined, we added enough TA in ethanol to tubes such that, after evaporation of the ethanol and addition of a set volume of TCM, a 0.05 ml aliquot contained the desired concentration of steroid. We started the assay by adding 0.1 ml TCM containing leukocytes to tubes containing 0.05 ml TCM with 100-fold excess radioinert TA and allowed it to incubate for 1 h. Leukocytes used to determine total binding were also incubated in TCM for 1 h. We then added 0.05 ml of TCM containing radiolabeled TA and allowed this to incubate an additional 2 h. Thus, the final incubation volume of each tube was 0.2 ml and contained 4 - 10 X 10^6 cells, 0.05 - 2.0 nM radiolabeled TA and 0 - 200 nM radioinert TA. We conducted competition studies with whole leukocytes using 0.5 nM radiolabeled TA and concentrations of radioinert steroids (aldosterone, cortisol, cortisone, 17- hydroxyprogesterone, testosterone, TA) varying between 0.1 - 50,000 nM. After the final incubations in whole leukocyte binding and competition studies, we added 0.5 ml TCM to each tube, centrifuged (300 x g, 10 min, TJ-6 centrifuge) to sediment the leukocytes and discarded the supernatants containing unbound steroid. We repeated this washing with 0.75 ml TCM, lysed the cells with 0.2 ml water, vigorously vortexed them, and added 0.4 ml absolute methanol. We then added 0.5 ml of this mixture to 5.0 ml scintillation cocktail and counted samples as described above.

Data Analyses. We used a computer software program (McPherson, 1985) to analyze binding data by subtracting NSB from total binding to get specific binding and obtain dissociation constants (Kd) and maximum number of sites (Nmax) per mg protein or per leukocyte based on Scatchard plot analyses (Scatchard, 1949). This program also calculated Hill coefficients for detecting cooperativity between interacting binding sites (Levitzki, 1978). We also used this program to analyze data from competition experiments to obtain concentrations of competitors that displaced 50% of ligand binding (IC50). We compared mean Kd and Nmax values using a t-test for small sample sizes (Ott, 1977).

RESULTS

Binding to Cytosol

We were able to detect saturable cortisol and TA binding in gill cytosolic fractions (Figures 14 and 15). Analyses of Scatchard plots suggest high affinity, low capacity binding for both ligands (Table 4). The affinity of the receptor was greater for TA (Kd = 0.38 $\pm$ 0.03 nM, all values presented in this manner are mean $\pm$ 1 SE) than for cortisol (Kd = 2.24 $\pm$ 0.28 nM) as would be expected for the

Figure 14. Binding of [^{3}H]cortisol to gill from coho salmon. (A) Total, specific, and nonspecific binding (NSB) of various concentrations of ligand to 0.20 ml cytosol containing 5 mg protein/ml. (B) Scatchard plot of data in (A), with accompanying dissociation constant (Kd), maximum number of receptors, and correlation coefficient (r). Bound/Free is the concentration of specifically bound ligand divided by concentration of free ligand at each concentration of radiolabeled ligand added. Hill coefficient (Hill coeff.) is the slope of the line of a Hill plot of data in (A).

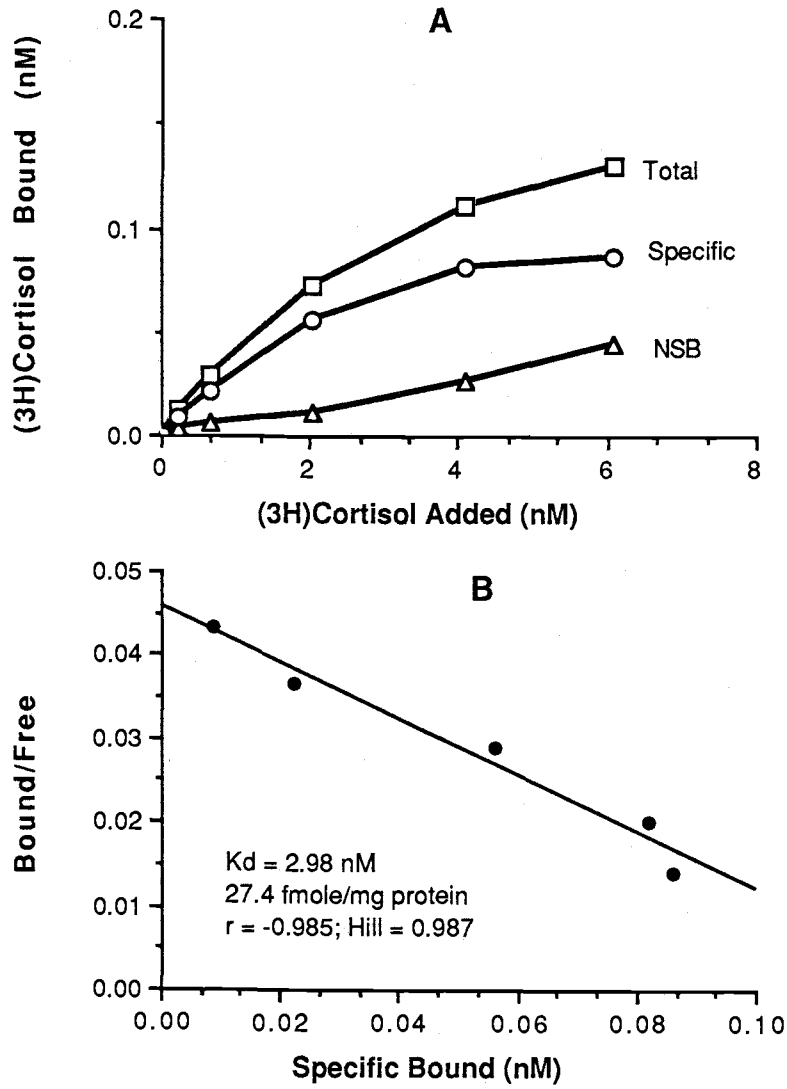
A
(3H)Cortisol Bound (nM)
0.2
0.1
0.0
0
2
4
6
8
Total
Specific
NSB
(3H)Cortisol Added (nM)
B
Bound/Free
0.05
0.04
0.03
0.02
0.01
0.00
Kd = 2.98 nM
27.4 fmole/mg protein
r = -0.985; Hill = 0.987
0.00
0.02
0.04
0.06
0.08
0.10
Specific Bound (nM)

Figure 14.

Figure 15. Binding of [^{3}H]triamcinolone acetonide (TA) to gill from coho salmon. (A) Total, specific, and nonspecific binding (NSB) of various concentrations of ligand to 0.20 ml gill tissue containing 5 mg protein/ml. (B) Scatchard plot of data in (A), with accompanying dissociation constant (Kd), maximum number of receptors, and correlation coefficient (r). Bound/Free is the concentration of specifically bound ligand divided by concentration of free ligand at each concentration of radiolabeled ligand added. Hill coefficient (Hill coeff.) is the slope of the line of a Hill plot of data in (A).

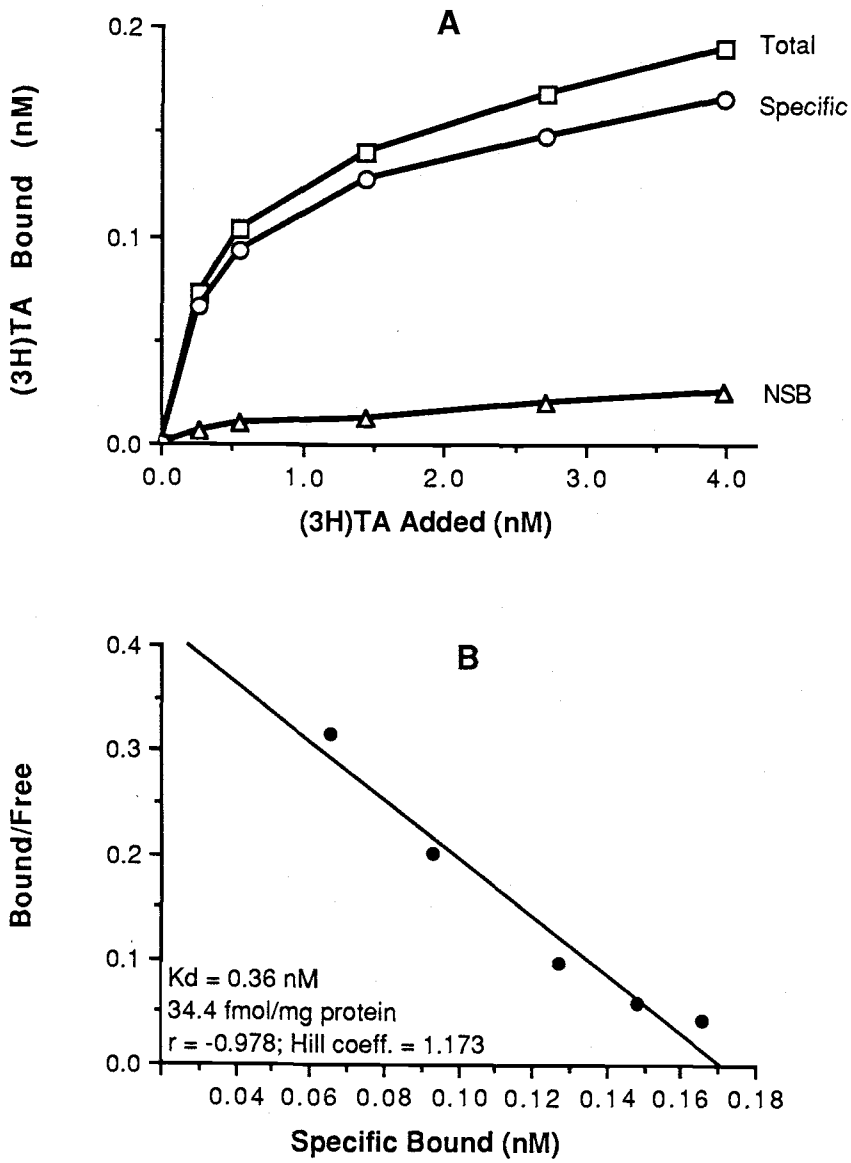
A
0.2
0.1
0.0
(3H)TA Bound (nM)
Total
Specific
NSB
0.0
1.0
2.0
3.0
4.0
(3H)TA Added (nM)
B
0.4
0.3
0.2
0.1
0.0
Bound/Free
Kd = 0.36 nM
34.4 fmol/mg protein
r = -0.978; Hill coeff. = 1.173
0.04 0.06 0.08 0.10 0.12 0.14 0.16 0.18
Specific Bound (nM)

Figure 15.

Table 4. Binding characteristics (mean ± 1 SE) of glucocorticoid receptors in cytosol and whole leukocytes in various tissues of coho salmon. Dissociation constants (Kd) and maximum number of receptors (Nmax) for [3H]cortisol and [3H]triamcinolone acetonide (TA) were derived from Scatchard plot analyses. Values with superscript in common are significantly different from each other (a,b,d: $P < 0.005$; c: $P < 0.05$; t-test).

Tissue	Ligand	Kd (nM)	Nmax	Hill Coefficient	N
			Cytosolic Binding		
			(fmol/mg)		
Gill	Cortisol	2.24 ± 0.28[a]	41.4 ± 7.4	1.007 ± 0.006	5
	TA	0.38 ± 0.03[a]	37.8 ± 4.9	1.109 ± 0.007	5
Leukocyte					
Spleen	TA	0.32 ± 0.03	8.3 ± 2.0[b]	1.024 ± 0.028	8
Anterior Kidney	TA	0.37 ± 0.03	30.2 ± 5.2[b]	0.980 ± 0.018	9
			Whole Leukocyte Binding		
			(sites/cell)		
Spleen	TA	0.30 ± 0.04[c]	445 ± 57[d]	0.994 ± 0.009	5
Anterior Kidney	TA	0.40 ± 0.04[c]	1198 ± 180[d]	0.980 ± 0.013	5

synthetic steroid. The fact that Nmax was about the same for both ligands (ca. 38 fmol/mg protein), suggests that they were binding the same receptor population. Hill coefficients were consistently around 1.0 suggesting a single class of receptors and no cooperativity. When conducting experiments with cytosol from spleen and anterior kidney leukocytes, we obtained saturable binding of TA (binding curves not shown) similar to those for gill cytosol. Scatchard plot analyses yielded Kd values for cytosolic GR from spleen and anterior kidney leukocytes that were not significantly different from each other or gill tissue (Table 4). However, Nmax of cytosolic GR from splenic leukocytes was significantly less than in cytosol from the other tissues (Table 4). The competition hierarchies of steroid competitors for gill cytosolic GR were the same for TA and cortisol (TA > cortisol > 17 -hydroxyprogesterone > cortisone > aldosterone > testosterone), however, IC50s were about 3-fold greater for TA as ligand than for cortisol (Table 5), reflecting the higher affinity of GR for TA.

Binding to Whole Leukocytes

We obtained saturable binding in whole leukocytes harvested from the spleen and anterior kidney with TA as ligand (Figures 16 and 17). The Kd for whole leukocyte preparations (0.30 $\pm$ 0.04 nM for spleen and 0.40 $\pm$ 0.04 nM for anterior kidney) were not significantly different from the corresponding Kd from cytosolic preparations, but they were significantly different from each other (Table 4). There were significantly more GR in anterior kidney leukocytes (1198 $\pm$ 180 sites/cell) than spleen (445 $\pm$ 57 sites/cell), similar to that seen in the cytosolic preparations (Table 5). Hill coefficients were

Table 5. Concentration of competitors necessary to inhibit 50% of the specific binding (IC50) by [3H]cortisol or [3H]triamcinolone acetonide (TA) to gill cytosol or whole leukocytes from spleen and anterior kidney (AK) of coho salmon.

	IC50 (nM)			
	Gill Cytosol		Spleen Leukocyte	AK Leukocyte
Competitor	[3H]Cortisol	[3H]TA	[3H]TA	[3H]TA
TA	1	2	16	17
Cortisol	6	17	62	77
17∝-HP[a]	183	670	2,300	3,420
Cortisone	987	3,000	13,000	62,000
Aldosterone	2,300	6,100	19,000	114,000
Testosterone	n. d.	n. d.	n. d.	n. d.

a - 17∝-hydroxyprogesterone

n. d.- no inhibition detected

Figure 16. Binding of [^{3}H]triamcinolone acetonide (TA) to whole leukocytes from spleens of coho salmon. (A) Total, specific, and nonspecific binding (NSB) of various concentrations of ligand to 10 X 10^6 leukocytes in 0.20 ml tissue culture medium. (B) Scatchard plot of data in (A), with accompanying dissociation constant (Kd), maximum number of receptors, and correlation coefficient (r). Bound/Free is the concentration of specifically bound ligand divided by concentration of free ligand at each concentration of radiolabeled ligand added. Hill coefficient (Hill coeff.) is the slope of the line of a Hill plot of data in (A).

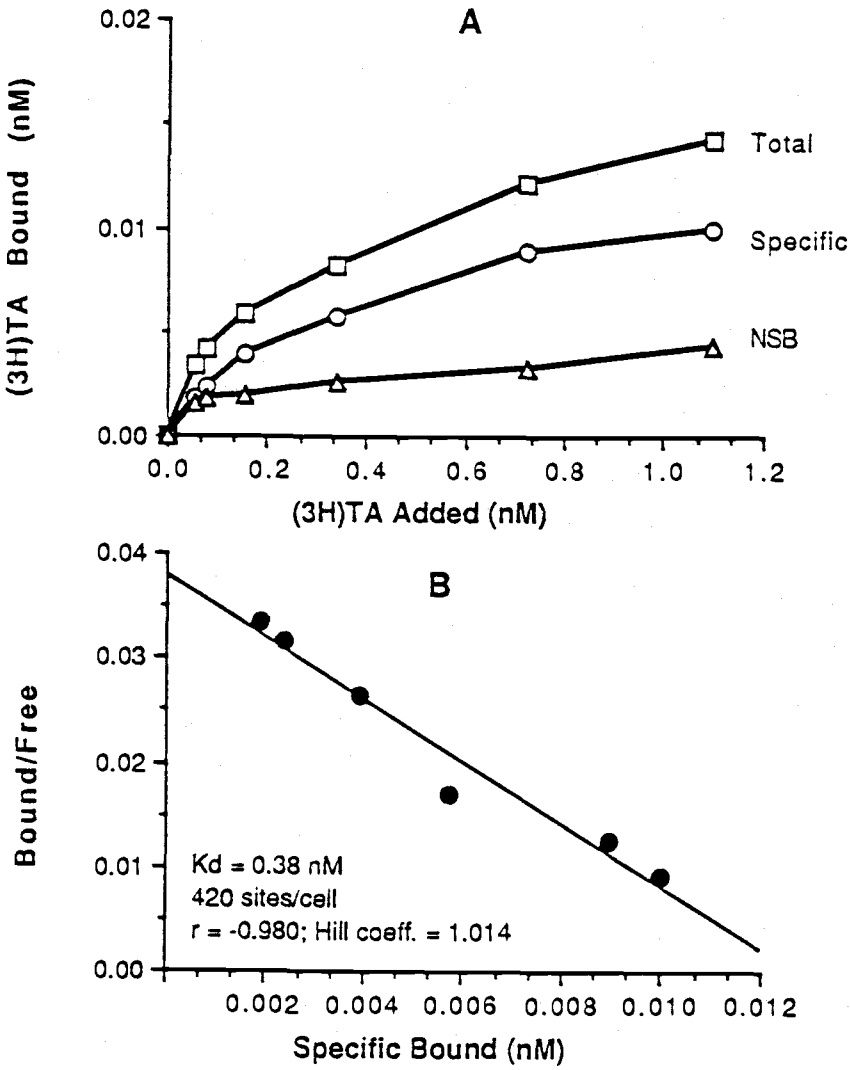
A
0.02
0.01
0.00
(3H)TA Bound (nM)
Total
Specific
NSB
0.0
0.2
0.4
0.6
0.8
1.0
1.2
(3H)TA Added (nM)
B
0.04
0.03
0.02
0.01
0.00
Bound/Free
Kd = 0.38 nM
420 sites/cell
r = -0.980; Hill coeff. = 1.014
0.002
0.004
0.006
0.008
0.010
0.012
Specific Bound (nM)

Figure 16.

Figure 17. Binding of [^{3}H]triamcinolone acetonide (TA) to whole leukocytes from anterior kidney of coho salmon. (A) Total, specific, and nonspecific binding (NSB) of various concentrations of ligand to 10 X 10^6 leukocytes in 0.20 ml of tissue culture medium. (B) Scatchard plot of data in (A), with accompanying dissociation constant (Kd), maximum number of receptors, and correlation coefficient (r). Bound/Free is the concentration of specifically bound ligand divided by concentration of free ligand at each concentration of radiolabeled ligand added. Hill coefficient (Hill coeff.) is the slope of the line of a Hill plot of data in (A).

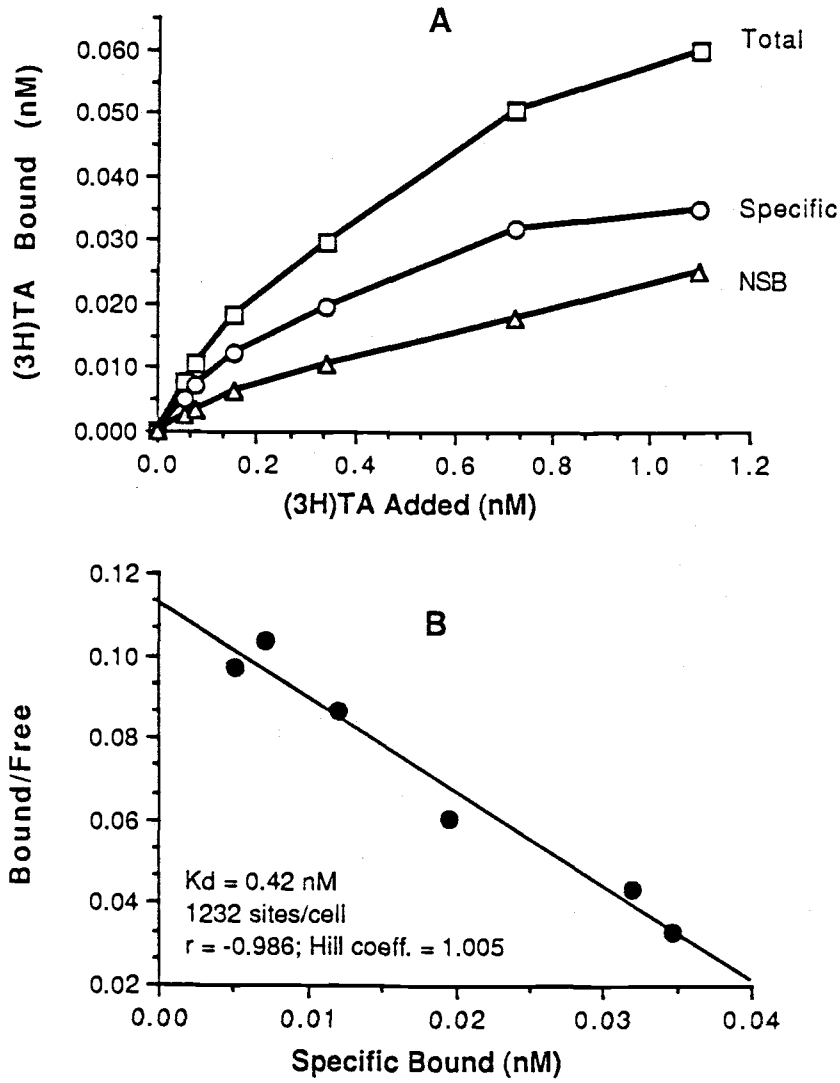
A
Total
Specific
NSB
(3H)TA Bound (nM)
0.060
0.050
0.040
0.030
0.020
0.010
0.000
0.0
0.2
0.4
0.6
0.8
1.0
1.2
(3H)TA Added (nM)
B
Bound/Free
0.12
0.10
0.08
0.06
0.04
0.02
Kd = 0.42 nM
1232 sites/cell
r = -0.986; Hill coeff. = 1.005
0.00
0.01
0.02
0.03
0.04
Specific Bound (nM)

Figure 17.

around 1.0 suggesting a single class of GR. The competition hierarchies of radioinert steroids' abilities to compete with TA for GR are the same for leukocytes from the two organs and were also similar to that in gill cytosol (Table 5). Higher IC50 for anterior kidney leukocytes may reflect the greater number of GR in these cells compared to splenic leukocytes.

DISCUSSION

In this study we have shown high affinity, low capacity binding of glucocorticoids in tissues of coho salmon and, for the first time, have shown glucocorticoid binding to leukocytes of fish. We used cortisol and TA as radioligands with gill cytosolic GR and found that GR have a higher affinity for TA, but the receptor numbers were the same whether TA or cortisol were used (Table 4). The inhibition by competitors was also similar for both ligands. For example, IC50 for the competitor 17 -hydroxyprogesterone is about one fifth that of cortisone which is half that of aldosterone, independent of whether the ligand is TA or cortisol (Table 5). Based on this circumstantial evidence, we believe that TA and cortisol detected the same population of GR.

The Kd values we obtained for gill cytosol using cortisol as ligand were similar to values reported for gill from brook trout (Salvelinus fontinalis; Chakraborti et al., 1987) and American eel (Anguilla rostrata; DiBattista et al., 1984), but Nmax were 5 to 7-fold greater in those species. This difference may reflect species differences or methodological differences in protein determination. The Kd that we obtained for gill GR using TA as ligand (Table 4) is considerably less than reported for American eel (2.85 $\pm$ 0.4 nM) or rainbow trout (1.43 $\pm$ 0.13 nM; Sandor et al., 1984). However, examination of data from competition experiments in those studies suggests that gill GR have 5 to 10- fold greater affinity for TA than for cortisol (Sandor et al., 1984; Chakraborti et al., 1987; DiBattista et al., 1984), the same relation we obtained when testing the two ligands using a single pool of cytosol. Moreover, our Kd

values for whole leukocytes are approximately the same as those reported for goat circulating leukocytes using [^{3}H]dexamethasone as ligand (0.27 $\pm$ 0.02 nM; Murakami et al., 1979), and that was more than 10-fold lower than Kd reported for various subpopulations of human leukocytes also using [^{3}H]dexamethasone (3.6 - 7.2 nM; Katz et al., 1985). These studies illustrate differences that could be interspecific or methodological.

The detection of specific GR in leukocytes suggests that the effects of cortisol seen in other studies (Kaattari and Tripp, 1987; Maule et al., 1987; Tripp et al. 1987; Maule et al., 1989) may be receptor mediated. Differences that we report in the binding of GR in leukocytes from spleen versus anterior kidney may be the result of differences in the proportions of leukocyte subpopulations residing in the two organs. The greater number of GR in leukocytes from the anterior kidney may be the result of the large proportion of macrophage-like cells in that organ (Zapata, 1979; Temmink and Bayne, 1987). Human monocytes (i. e., macrophages in peripheral blood) have twice the GR of other human leukocytes (Lippman and Barr, 1977). Another factor possibly contributing to the large number of GR in the anterior kidney is that in fish this organ is similar to the bone marrow of mammals (Zapata, 1979; Temmink and Bayne, 1987) and, as such, contains a high proportion of mitosing cells. It has been reported that human and rat leukocytes in the S and post-S phases of the cell cycle (i. e., just prior to mitosis) have 2 to 3-fold more GR than cells in the G_0 and G_1 phases (Crabtree et al., 1980b). The differences that we report in Kd may also be the result of varying leukocyte subpopulations in the two organs. Katz et al. (1985)

reported 2-fold differences of Kd in various leukocyte types in human peripheral blood. Our reported differences in GR binding are consistent with the findings of Kaattari and Tripp (1987) and Tripp et al. (1987) who reported organ-dependent sensitivity of leukocytes to cortisol and suggested that cortisol acted on anterior kidney leukocytes by suppressing the release of an interleukin-like substance from macrophage-like cells. We anticipate conducting experiments to examine GR in leukocyte subpopulations from various lymphoid organs.

VI. CHANGES IN AFFINITY AND NUMBER OF GLUCOCORTICOID RECEPTORS IN LEUKOCYTES AND GILL FROM COHO SALMON DURING STRESS AND CORTISOL TREATMENT

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ABSTRACT

We conducted a series of experiments in which juvenile coho salmon (Oncorhynchus kisutch) were acutely (30 s in a net out of water) or chronically (crowded in shallow water) stressed or fed a single meal containing cortisol. Glucocorticoid receptor (GR) binding was then determined in gill and whole leukocytes from spleen and anterior kidney using the synthetic hormone triamcinolone acetonide as radioligand. The GR in all tissues from chronically stressed fish had consistently lower affinities than controls; however, there were increased numbers of GR in whole leukocytes and decreased numbers in gill cytosol. Acute stress had no affect on GR in gill and did not affect the affinity of GR in anterior kidney leukocytes but did increase the number of GR. Acute stress reduced affinity and increased numbers of GR in splenic leukocytes, suggesting heterogeneity of response to stress. The results of feeding cortisol to fish suggest that the hormone may act to regulate its own receptor.

INTRODUCTION

Stress-induced increases in glucocorticoid secretion have been shown to have a wide variety of effects on the mammalian immune system (Baxter, 1976; Munck et al., 1984). These include cytolysis (Cohen and Duke, 1984), redistribution of leukocytes (Cohen, 1972; Rogers and Matossian-Rogers, 1982; Steplewski and Vogel, 1986) and decreased responsiveness of lymphocytes to *in vitro* antigenic and mitogenic stimulation (Dracott and Smith, 1979b; Okimura and Nigo, 1986; Okimura et al., 1986a). These effects appear to be mediated through specific glucocorticoid receptors (GR) in leukocytes (Coffey and Djeu, 1986).

The endocrine response of Pacific salmon (*Oncorhynchus* spp.) to stress, similar to that of mammals, is marked by a transient increase in plasma cortisol titers (Donaldson, 1981; Schreck, 1981). Acute and chronic stress, and cortisol treatment of juvenile coho salmon (*O. kisutch*) results in transient changes in number of leukocytes in immune organs (Chapter IV, this volume), and cortisol implants reduce disease resistance in the same species (Maule et al., 1987). Furthermore, chinook salmon (*O. tshawytscha*) have decreased immune response and disease resistance four hours after an acute stress, but by 24 h, immune response and disease resistance are enhanced (Maule et al. 1989). The addition of cortisol to chinook salmon lymphocyte cultures mimics the responses seen as a result of stress and can be eliminated by the addition of lymphokine(s) to the cultures (Tripp et al., 1987). We demonstrated the presence of specific GR in whole leukocytes and gill cytosol of coho salmon (Chapter V, this volume), suggesting that GR might be mediating the effects of stress and cortisol treatment in Pacific salmon. Our objectives in the present

study were to determine (1) if there were changes in the number or affinity of GR in leukocytes and gill of coho salmon during acute or chronic stress, and (2) if effects seen in GR could be attributable to the direct action of cortisol.

MATERIALS AND METHODS

Fish. Juvenile coho salmon parr were transported from the Eagle Creek National Fish Hatchery, Eagle Creek, Oregon and acclimated to conditions at the Oregon State University Smith Farm Experimental Hatchery, Corvallis, Oregon for at least 30 days. The fish were kept in circular tanks (about 1700 L) continuously supplied with well-water that was 11-12^{o} C throughout the year, and were fed Oregon Moist Pellets at 1 - 2% body wt daily except on days of experiments. We conducted experiments in February 1987 (presmolts, fish wt: 28.6 $\pm$ 1.1 g, mean $\pm$ 1 SE), March 1988 (smolts, fish wt: 131.5 $\pm$ 7.6 g) and December 1988 (smolts, fish wt: 78.3 $\pm$ 4.1 g). In July 1987, we conducted one experiment at the Oregon State University, Mark O. Hatfield Marine Science Center, Newport Oregon with juvenile coho salmon smolts (fish wt: 27.4 $\pm$ 1.6 g, mean $\pm$ 1 SE) from the Oregon Aqua Foods, Inc. fish hatchery at Springfield, Oregon. These fish were acclimated to dechlorinated tapwater (17-18^{o} C) for about two months prior to the experiment. Although these experiments were conducted under different conditions with different sized fish, we compared each treatment group against the controls for that experiment.

Experimental Conditions and Sampling. We acutely stressed fish by rapidly netting them and holding them out of water for 30 - 60 sec and then returning them to their tanks. We sampled the fish at various times thereafter. We chronically stressed fish by transferring them to 19 L buckets with holes drilled throughout. The buckets were suspended in tanks to a depth (about 5 cm) such that fish could remain upright but could not swim and were in almost continuous

contact with other fish. The holes allowed water to circulate into the buckets maintaining high water quality; fish remained in the buckets throughout the experiment. In order to simulate the effects of a transient increase in circulating cortisol without stress, we fed fish a single meal of food containing cortisol (100 mg/ kg food) that had been dissolved in ethanol and sprayed on the food. Food for the control fish was sprayed with just ethanol and the ethanol was evaporated from both foods at room temperature. In experiments to examine GR in leukocytes, we sampled fish at 3 - 4 h, 24 h and 7 days after treatment to coincide with the times used during our previous experiments in which the effects of similar treatments on the immune response and disease resistance were examined (Maule et al., 1989). At the time of sampling, we rapidly netted fish and transferred them to a bucket containing a lethal dose (200 mg/L) of 3-aminobenzoic acid, ethyl ester (MS-222). Such a dose of anesthetic has been shown to inhibit stress-related increases in plasma cortisol concentration in salmon (Strange and Schreck, 1978; Barton et al., 1986). After the fish were anesthetized, we severed the caudal peduncle and collected blood into heparinized capillary tubes, separated plasma by centrifugation, and stored it at -20^{0} C until determination of cortisol titer by radioimmunoassay by the method of Redding et al. (1984).

Glucocorticoid Receptor Assays. To obtain measurements of affinity and number of receptors, we performed binding experiments on tissues from treated and control fish at every sampling time; it was necessary to pool tissue from 5 to 10 fish. Tissue preparation and GR assays were the same as has been described (Chapter V, this

volume). We removed spleens and anterior kidneys and put them in ice-cold tissue culture medium (TCM), RPMI-1640 (B. A. Products Co., Walkersville, MD) with 0.15 mM EDTA (all chemicals, except where noted, came from Sigma Chemical Co., St Louis, MO.) at pH 7.4. We harvested leukocytes by gently and repeatedly aspirating tissue with 1-ml tuberculin syringes to free cells from other tissue. We then added 4 - 10 X 10^6 cells to duplicate tubes each for determining total and nonspecific binding (NSB). After incubating cells to be used for total binding in TCM and those for NSBs in TCM containing 100-fold excess radioinert triamcinolone acetonide (TA; 1,4-pregnadien-9 -fluoro-11ß,16 ,17 ,21-tetrol-3,20-dione-16,17 acetonide) for 60 min, we added sufficient radiolabelled TA (specific activity 25.9 Ci/mmol; Amersham, Arlington Heights, IL) so that the concentrations in the final volume of 0.2 ml were 0.05 to 2.0 nM. After an additional two hour incubation, the cells were washed twice in excess TCM to remove unbound hormone and lysed with 0.2 ml distilled water. We then added 0.4 ml 95% methanol and added 0.5 ml of the mixture to 5.0 ml scintillation cocktail (Budget-Solve, Research Products International, Mount Prospect, IL) and counted the samples on a model LS 1800 liquid scintillation counter (Beckman Instruments, Palo Alto, CA).

We removed gill filaments from gill arches and put them in ice-cold buffer (10 mM Tris-HCl, 1mM EDTA, 12 mM monothioglycerol, 20 mM sodium molybdate, and 10% v/v glycerol; pH 7.4) and disrupted the cells using a motor driven glass and Teflon homogenizer. After an initial centrifugation to remove gross debris, we centrifuged the tissue at 100,000 x g for 60 min in an L8-70 ultracentrifuge with a

type 40 rotor (Beckman Instruments). We added two volumes of supernatant to one volume of buffer containing 5% (w/v) Norit A charcoal and 0.5% dextran and then centrifuged to remove endogenous steroids. Although the source of GR in the liquid fraction after this series of centrifugations is not known (i. e., cytoplasm, nucleus, or membrane) we refer to them here as gill GR to distinguish them from leukocytes receptors. We assayed protein with Bradford reagent (Bradford, 1976) and bovine serum albumin standards and adjusted protein content to 4 - 6 mg/ml. We incubated samples for total binding in buffer and those for NSBs in 100-fold excess radioinert TA for 10 min and then added sufficient radiolabeled TA in buffer to result in 0.2 to 6.0 nM of hormone in a final volume of 0.2 ml. After two hours, unbound hormone was separated from bound with 0.5 ml of 2.5% (w/v) Norit A charcoal and 0.25% (w/v) dextran in buffer (10 min incubation) and centrifugation (3,000 g, 20 min). The radioactivity of 0.5 ml of this final supernatant was determined as previously described.

Data Analyses. We used a computer software program (McPherson, 1985) to analyze binding data by subtracting NSB from total binding to get specific binding and obtain dissociation constants (Kd) and maximum binding sites (Nmax) based on Scatchard plot analyses (Scatchard, 1949). When appropriate, we analyzed data by analysis of variance and Duncan's multiple range test for pairwise comparisons, at $P < 0.05$. We also did simple regression analyses to correlate mean

plasma cortisol concentration to Kd and Nmax of GR.

RESULTS

Plasma cortisol concentrations in fish that were acutely stressed, chronically stressed, or fed cortisol-treated food were significantly greater than controls within 4 h of treatment (Table 6). In acutely stressed fish, plasma cortisol concentrations returned to control levels within 24 h, but in fish that were chronically stressed or fed cortisol, plasma cortisol titers remained significantly higher than in controls until some time between 24 h and 5 - 9 days after treatment began (Table 6). Fish fed cortisol attained the highest plasma cortisol concentrations (336 $\pm$ 11 ng/ml; mean $\pm$ 1 SE), followed by chronically stressed (225 $\pm$ 17) and acutely stressed fish (102 $\pm$ 36). The plasma cortisol concentrations were correlated to changes in the Kd and Nmax of GR in leukocytes and gills; although, there were differential effects between organs (Table 7). There were rapid 2- to 3-fold increases in Nmax of leukocytes from spleen and anterior kidneys from acutely and chronically stressed fish and concurrent decreases (2- to 3-fold increased Kd) in the affinities of the receptors for TA, except in anterior kidney leukocytes from acutely stressed fish which were not different from controls (Figures 18 and 19). The receptor variables were returning toward control values by 24 h, except in splenic leukocytes of chronically stressed fish, in which the Kd continued to increase, similar to plasma

Table 6. Plasma cortisol concentrations (means ± 1 SE) of juvenile coho salmon that were not treated (Control), acutely stressed (Acute), chronically stressed (Chronic) or fed a single meal containing 100 mg cortisol per kg food (Fed) in separate experiments (Date). Sample sizes are in parentheses. Values marked with an asterisk differ significantly from controls of the same experiment and time; Duncan's multiple range test, $P > 0.05$.

	Plasma Cortisol (ng/ml)			
Date/Time	Control	Acute	Chronic	Fed
February 1987				
4 h	7 ± 3 (9)	30 ± 3* (8)	177 ± 21* (8)	
2 d	9 ± 2 (8)	5 ± 4 (8)	86 ± 19* (8)	
9 d	14 ± 12 (6)		4 ± 1 (7)	
July 1987				
4 h	5 ± 2 (5)		128 ± 10* (8)	
24 h	14 ± 5 (9)		98 ± 26* (6)	
5 d	1 ± 1 (6)		51 ± 19* (11)	
March 1988				
4 h	20 ± 7 (7)	102 ± 36* (8)	221 ± 28* (7)	
24 h	18 ± 5 (9)	21 ± 7 (8)	225 ± 17* (8)	
7 d	17 ± 6 (7)	16 ± 5 (7)	17 ± 6 (7)	
December 1988				
4 h	21 ± 6 (7)			336 ± 11* (7)
24 h	4 ± 1 (4)			208 ± 40* (7)
7 d	23 ± 9 (4)			33 ± 13 (7)

Table 7. Linear regression correlation coefficient (R) and level of significance (P) comparing mean plasma cortisol concentration to dissociation constant (Kd) and number (Nmax) of glucocorticoid receptors from coho salmon tissues. Comparisons for gill tissue from experiments with stressed fish (Stressed) and fish fed cortisol (Fed cortisol) were treated separately because values from control fish were different between experiments. Number of assays used in regression are in parentheses.

	Kd		Nmax	
Tissue	R	P	R	P
Gill				
Stressed (16)	0.531	0.033	-0.802	<0.001
Fed Cortisol (12)	0.640	0.023	-0.611	0.033
Leukocytes				
Spleen (9)	0.814	0.005	0.619	0.076
Anterior Kidney (9)	0.630	0.070	0.377	0.331

Figure 18. Maximum number (Nmax) and dissociation constant (Kd) of glucocorticoid receptors in splenic leukocytes from juvenile coho salmon that were acutely or chronically stressed, or were undisturbed (controls). Leukocytes from 7 to 9 fish were pooled for Scatchard plot analyses of binding data at each time for each treatment using triamcinolone acetonide as radioligand. Plasma cortisol concentrations for these fish are shown in Table 6, experiment of March 1988.

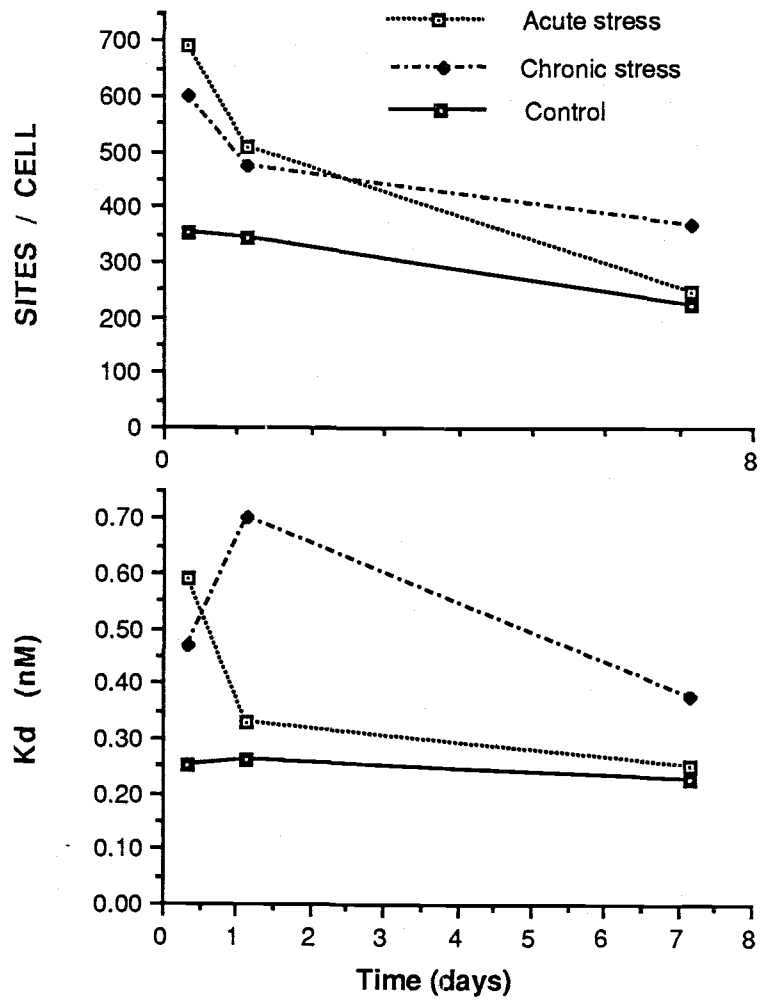
Acute stress
Chronic stress
Control
SITES / CELL
700
600
500
400
300
200
100
0
0
8
Kd (nM)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
1
2
3
4
5
6
7
8
Time (days)

Figure 18.

Figure 19. Maximum number (Nmax) and dissociation constant (Kd) of glucocorticoid receptors in anterior kidney leukocytes from juvenile coho salmon that were acutely or chronically stressed, or were undisturbed (controls). Leukocytes from 7 to 9 fish were pooled for Scatchard plot analyses of binding data at each time for each treatment using triamcinolone acetonide as radioligand. Plasma cortisol concentrations for these fish are shown in Table 6, experiment of March 1988.

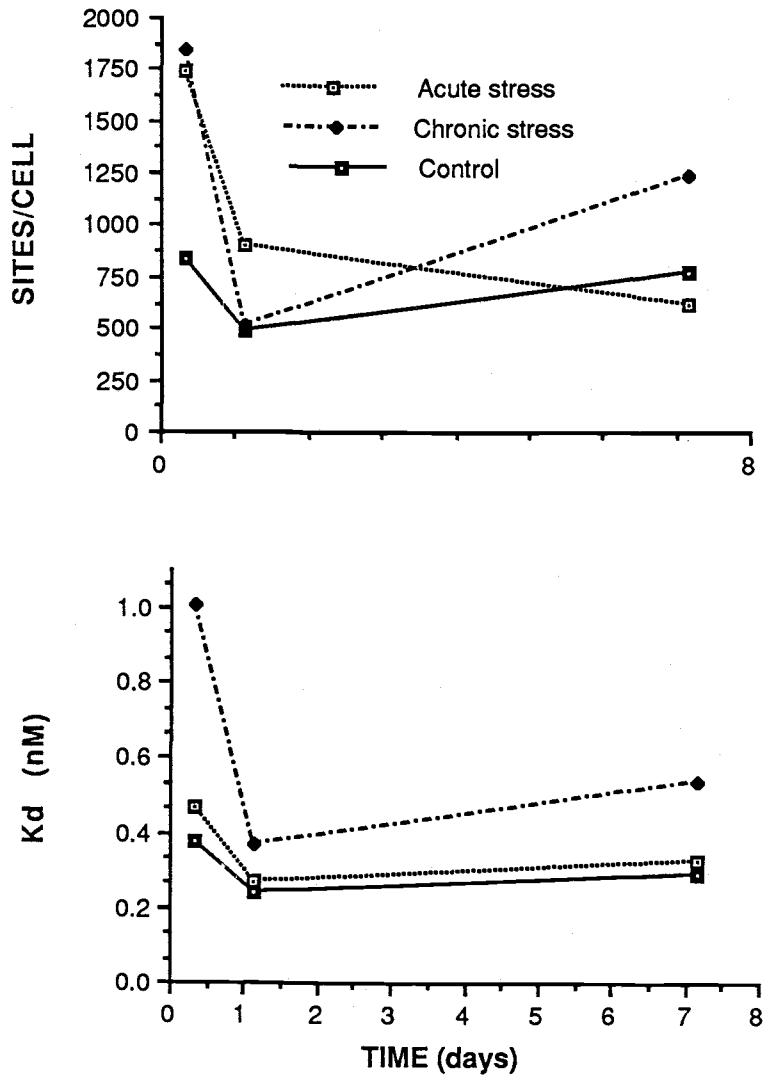
SITES/CELL
2000
1750
1500
1250
1000
750
500
250
0
0
8
Acute stress
Chronic stress
Control
Kd (nM)
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
7
8
TIME (days)

Figure 19.

cortisol concentration that was greatest at 24 h in this treatment (Table 6). By seven days after stress, GR variables in leukocytes of acutely stressed fish were the same as those in controls, but those in chronically stressed fish still appeared to be elevated over control values (Figures 18 and 19).

The Kd of GR from gills of chronically stressed fish increased similar to that seen in leukocytes; however, the number of GR in gill decreased in chronically stressed fish (Figure 20). There was no change in Kd or Nmax of GR from gills of acutely stressed fish (Figure 20). Feeding cortisol to coho salmon had effects on the GR variables in gill similar to those resulting from chronic stress in that there was a transient decrease in Nmax and increase in Kd (Figure 21). It should be noted that the Kd for controls were 2- to 3-fold higher than that of control fish in earlier experiments. The mean $\pm$ 1 SE of Kd for controls in this and a duplicate experiment conducted in November 1988 (1.11 $\pm$ 0.13 nM) was significantly different ($P < 0.05$, $n = 6$) than for controls in experiments conducted in February and July 1987 (0.35 $\pm$ 0.05 nM) suggesting seasonality in GR affinity. The Kd of GR in leukocytes from spleen and anterior kidneys of control fish in the cortisol-feeding experiment were the same as that seen in acute and chronic stress experiments (0.28 $\pm$ 0.04 nM; $n = 10$). However, Scatchard plot analyses of GR binding data from leukocytes of cortisol-fed fish had unacceptable correlation coefficients ($r > -0.50$). The binding curves indicated that there was an increase in NSB in leukocytes from cortisol-fed fish as compared to controls (data not shown). Similar high NSB previously found with gill cytosol were eliminated by preincubating tissue with dextran-coated charcoal to

Figure 20. Maximum number (Nmax) and dissociation constant (Kd) of glucocorticoid receptors in gills of juvenile coho salmon that were acutely or chronically stressed, or undisturbed (controls). Tissue from 7 to 9 fish were pooled for Scatchard plot analyses of binding data at each time for each treatment using triamcinolone acetonide as radioligand. Plasma cortisol concentrations for these fish are shown in Table 6, Experiments of February (A) and July 1987.

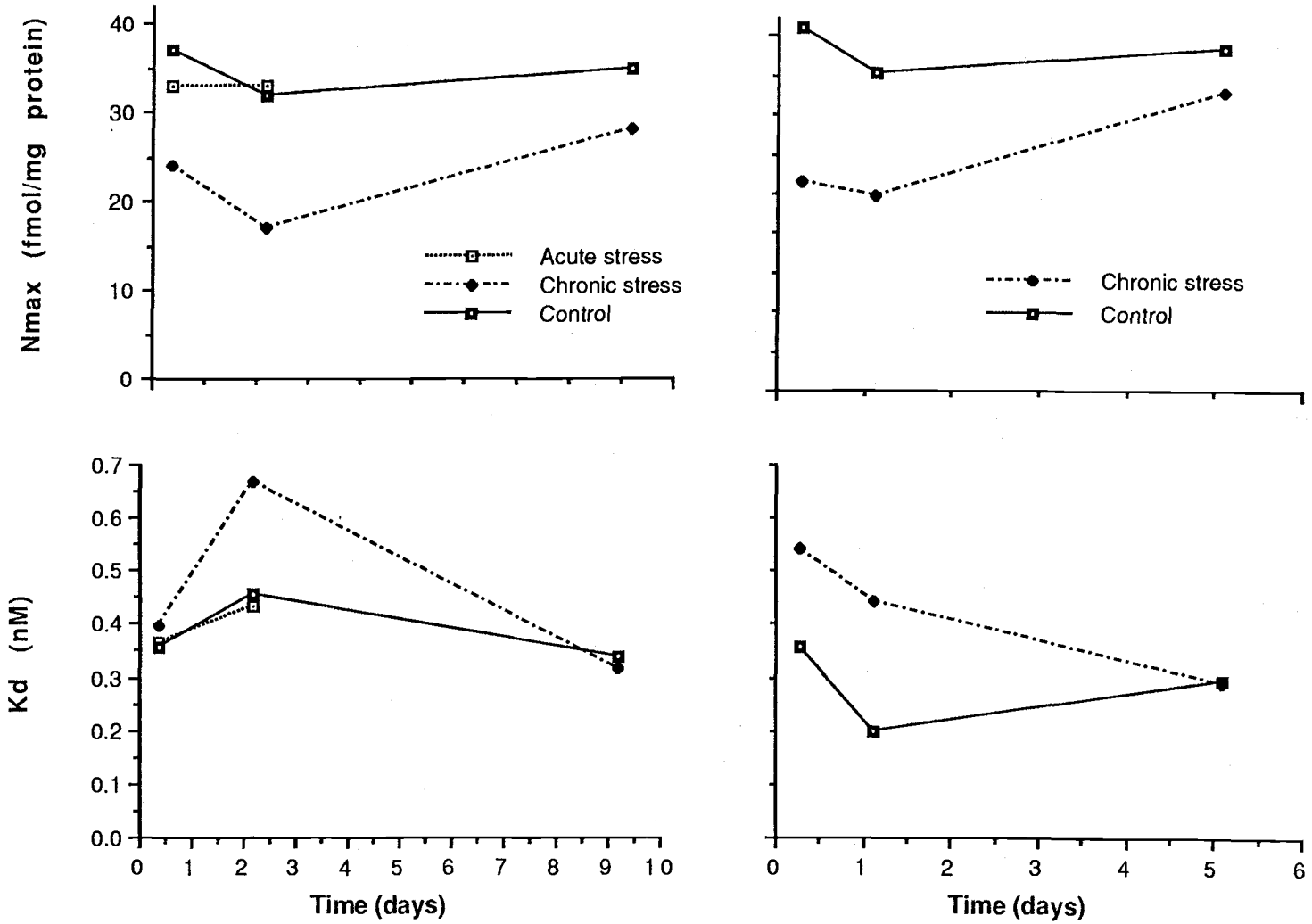
Nmax (fmol/mg protein)
40
30
20
10
0
Acute stress
Chronic stress
Control
Chronic stress
Control
Kd (nM)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 1 2 3 4 5 6 7 8 9 10
Time (days)
0 1 2 3 4 5 6
Time (days)

Figure 20.

Figure 21. Maximum number (Nmax) and dissociation constant (Kd) of glucocorticoid receptors in gills of juvenile coho salmon that were given a meal containing 100 mg cortisol per kg food or were undisturbed (controls). Tissue from 7 to 9 fish were pooled for Scatchard plot analyses of binding data at each time for each treatment using triamcinolone acetonide as radioligand. Plasma cortisol concentrations for these fish are shown in Table 6, experiment of December 1988.

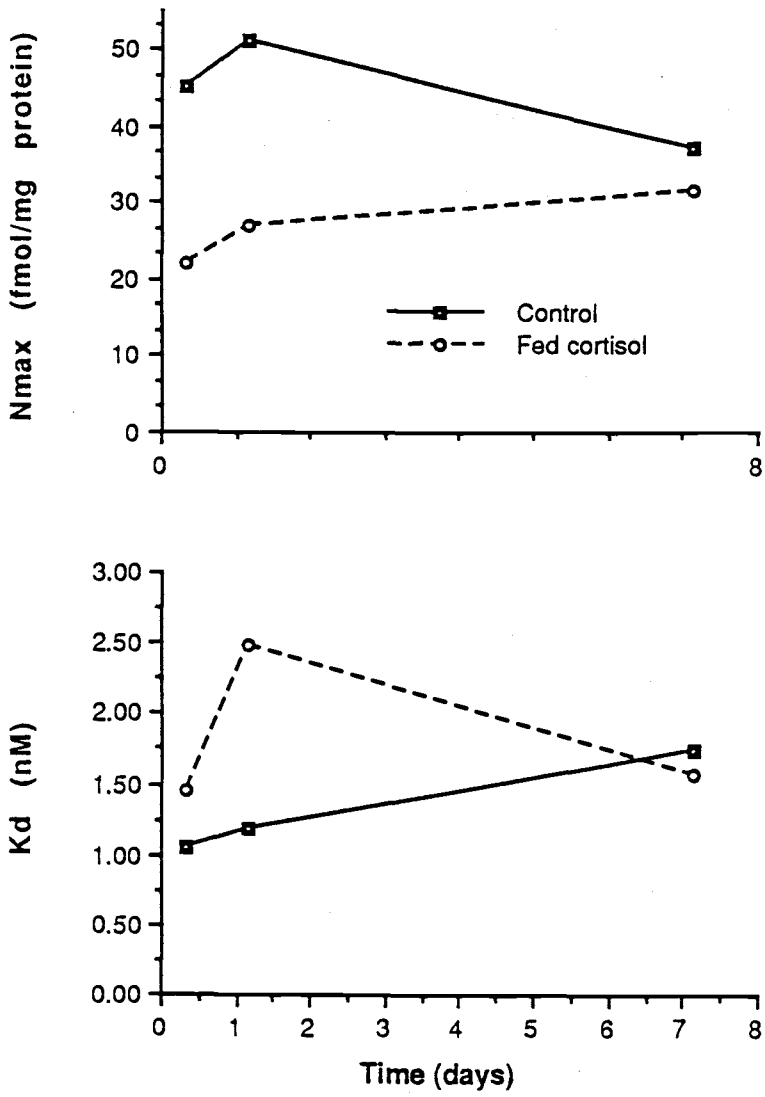
Nmax (fmol/mg protein)
50
40
30
20
10
0
0
8
Control
Fed cortisol
Kd (nM)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0
1
2
3
4
5
6
7
8
Time (days)

Figure 21.

remove endogenous steroids (Chapter IV, this volume), a procedure not possible with leukocytes as endogenous steroids are intracellular.

DISCUSSION

Chronic stress can apparently cause transient increases in number (Nmax) and concurrent decreases in affinity (i. e., increased Kd) of GR in leukocytes from spleen and anterior kidney of coho salmon. The GR in leukocytes and gill from chronically stressed fish had consistently lower affinities than that of controls; however, there were increased numbers of GR in leukocytes and decreased numbers in gill. Acute stress had no apparent affect on GR in gill, and while it did not affect the affinity of GR in anterior kidney leukocytes, it did increase the number of GR. Acute stress did reduce affinity and increase numbers of GR in splenic leukocytes, suggesting heterogeneity of response to stress between organs. It appears that these changes were at least partially caused by stress-induced increases in plasma cortisol titers, as the mean plasma cortisol concentrations were correlated significantly to Kd and number of GR in leukocytes and gills. Furthermore, cortisol treatment had the same effect on GR from the gill as did chronic stress. It has been generally reported that increased circulating glucocorticoids downregulate the number of GR in mammalian tissues (Schlechte et al., 1982; Shipman et al., 1983; Svec, 1985) by inhibiting the transcription of the GR gene (Rosewicz et al., 1988). However, when GR were detected by electrophoresis and immunoblotting rather than ligand binding there was an increase in GR in human T cells, suggesting that the high concentrations of endogenous glucocorticoids bound GR and removed them from detection by radioligand binding methods (Eisen et al., 1988). Our results support this conclusion, as radioligand binding assays failed in leukocytes from fish fed cortisol. However, when gill preparations

from the same fish were preincubated in dextran coated charcoal to remove endogenous cortisol, binding assays revealed results similar to those from chronically stressed fish.

The physiological response of the leukocytes to stress or cortisol treatment can effect GR binding. It has been reported that GR increased in proliferating (Crabtree et al., 1980a), differentiating (Hainque et al., 1987) and antigen-stimulated cells (Crabtree et al, 1980b). The changes that we detected in affinity of GR in leukocytes and gill suggest that there are either multiple GR genes, for which we have seen no evidence, or that stress altered the post-transcriptional processing of GR mRNA or post-translational processing of GR. Hirato et al. (1985a) used DEAE-cellulose chromatography to identify an hepatic GR that appeared after stress or glucocorticoid treatment. Binding studies with these isolated GR produced a curvilinear Scatchard plot, suggestive of multiple binding sites of low affinity (Hirato et al., 1985b). It has also been reported that mononuclear leukocytes from pregnant women contain a greater number of GR of a lower affinity than similar cells from nonpregnant women (Junker, 1983), suggesting that changes in the physiological state of an organism can alter affinity as well as numbers of GR.

A factor that may contribute to our results is the mobile nature of leukocytes. We previously reported that acute stress and cortisol feeding similar to the treatment used here caused decreases in the total number of leukocytes in the spleen and circulation and increases in the anterior kidney and thymus (Chapter IV, this volume). It has been reported that subpopulations of mammalian leukocytes are

differentially redistributed by cortisol treatment (Cohen, 1972; Rogers and Matossian-Rogers, 1982) and that subpopulations of leukocytes have GR with differing numbers and affinities (Lippman and Barr, 1977; Distelhorst and Benutto, 1981; Katz et al., 1985). Thus, we can not discount the possibility that changes in GR of leukocytes from the spleen and anterior kidney are the result of cells with fewer GR of higher affinity being redistributed out of the organs. However, we believe that this is not probable because there were increased numbers of cells in anterior kidney and decreased numbers in spleen (Chapter IV) while there was an increased number of GR in leukocytes from both organs. Furthermore, the affinity of GR from gill also decreased even though the cells in that tissue are sessile. The changes that we have observed in number of GR in gill from fish fed cortisol are in agreement with results of Weisbart et al. (1987). They reported that injection of cortisol into brook trout (Salvelinus fontinalis) caused a depletion of GR from gill cytosol that lasted for 72 h; at the same time, there was a gradual increase in nuclear GR until 48 h, followed by a sudden 4-fold increase by 72 h. Weisbart et al. (1987) suggested that this sudden increase in nuclear GR was from de novo synthesis, as maximum concentrations were attained at 60 to 72 h after treatment. Although we do not know if GR regulatory mechanisms are the same in salmonid gills and leukocytes, it is possible that our results with whole cells reflect similar GR regulatory mechanisms as the results of Weisbart et al. (1987), in that the net effect of increased exposure to cortisol was to increase GR in the whole cells.

Sapolsky et al. (1984) demonstrated that stress or corticosterone treatment resulted in site-specific changes in numbers of GR in the brains of rats. Our results and those of Weisbart et al. (1987) suggest that glucocorticoids regulate their own receptors in fish, similar to that demonstrated in mammals (Schlechte et al., 1982; Shipman et al., 1983; Svec, 1985); however, the specific changes (i.e., increased or decreased Nmax, or change in Kd) may be species and tissue specific.

VII. ORGAN-SPECIFIC CHANGES IN CORTISOL SENSITIVITY AND GLUCOCORTICOID RECEPTOR NUMBERS AND AFFINITY DURING DEVELOPMENT OF COHO SALMON

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ABSTRACT

We monitored *in vitro* immune responsiveness, sensitivity of leukocytes from spleen and anterior kidney to cortisol, and glucocorticoid receptor (GR) number and affinity in leukocytes and gill from coho salmon (*Oncorhynchus kisutch*) during the period of smoltification. Although immune responsiveness declined in leukocytes from both organs, there was organ-specific heterogeneity of response as splenic leukocytes generated more antibody-producing cells than leukocytes from anterior kidney. Splenic leukocytes were initially more sensitive to the suppressive effects of cortisol, and had fewer GR with increased affinity for the radioligand during smoltification than leukocytes from the anterior kidney. Leukocytes from the anterior kidney were initially insensitive to cortisol but developed sensitivity concurrent with an increased number of GR having a decreased affinity for the radioligand during smoltification. The changes in GR in leukocytes from anterior kidney were correlated to changes in plasma cortisol titers. Gill GR showed changes in number and affinity that were not correlated to changes in plasma concentrations of cortisol or T_4. These results substantiate earlier studies demonstrating suppressed immune response during smoltification and organ heterogeneity of leukocyte GR.

INTRODUCTION

Smoltification consists of the developmental changes that prepare the freshwater anadromous salmonid for successful entry into seawater (Hoar, 1976; Folmar and Dickhoff, 1981). This stage in development is marked by changes in behavior, morphology and biochemistry, including increased levels of circulating hormones such as cortisol (Specker and Schreck, 1982; Barton et al., 1985). It is well established that glucocorticoids can alter the functioning of the mammalian immune system (Ader, 1981; Munck et al., 1984), and we have demonstrated that similar relations exist between the endocrine and immune systems of Pacific salmon (Oncorhynchus spp.) during smoltification (Maule et al., 1987) and stress (Maule et al., 1989). We demonstrated that as plasma cortisol titers increased there was a concurrent decrease in immune response and disease resistance. Furthermore, cortisol added to in vitro cell cultures causes a dose-dependent decrease in immune responses, at least partially the result of decreased lymphokine activity (Tripp et al., 1987). Salmonid leukocytes and gills possess specific glucocorticoid receptors (GR) (Chapter V, this volume), and the numbers and affinity of these GR change in an apparent organ specific manner as a result of acute or chronic stress (Chapter VI, this volume).

In our earlier studies with coho salmon (O. kisutch) during smoltification, we examined changes in the ability of splenic lymphocytes to develop specific antibody-producing cells (APC) in response to in vivo inoculation with antigens from Vibrio anguillarum, a fish pathogen found in the marine environment (Maule et al., 1987). We now address objectives relative to changes in the endocrine -

immune interactions during smoltification of coho salmon: first, to determine if there was organ homogeneity of the immune response during smoltification using an in vitro assay with leukocytes from the spleen and anterior kidney; second, to determine if the sensitivity of leukocytes to cortisol changed during development; and, third, to determine if the affinity or numbers of GR in leukocytes changed concurrently with increased circulating cortisol during smoltification. A fourth objective was to determine if there were changes in the affinity or number of GR harvested with cytosol from gill tissue. Gills are a major osmoregulatory organ in fish and during smoltification cortisol may be involved in biochemical changes affecting permeability or ion transport (Hoar and Randall, 1984).

MATERIALS AND METHODS

Fish Care and Sampling. In December 1987 and 1988, juvenile coho salmon from the Eagle Creek National Fish Hatchery, Eagle Creek, Oregon, were transported to the Oregon State University, Smith Farm Experimental Hatchery, Corvallis, Oregon, and acclimated to conditions for at least 30 days. The immune system of fish is temperature (Avtalion, 1981) and diet dependent (Blazer and Wolke, 1984a, 1984b), so we kept these conditions as constant as possible, using circular tanks (about 1700 L) continuously supplied with well-water that was 11-12^{o} C throughout the year and a diet of Oregon Moist Pellets at 1 - 2% body wt daily except on days of experiments. Because we had a limited number of fish and had to pool tissue from 8-10 fish for each immune assay and GR assay, in 1987 we examined the sensitivity of leukocytes to cortisol and changes in gill GR; in 1988, we examined changes in GR in leukocytes from spleen and anterior kidney during smoltification. We conducted experiments every 2 - 3 weeks January (fish wt: 25 g) through July (fish wt: 90 g) 1987, and January (fish wt: 16 g) through September (fish wt: 90 g) 1988.

At the time of sampling, we rapidly netted fish and transferred them to a bucket containing a lethal dose (200 mg/L) of 3-aminobenzoic acid, ethyl ester (MS-222; all chemicals, except where noted, came from Sigma Chemical Co., St Louis, MO. U.S.A.). Such a dose of anesthetic has been shown to inhibit stress-related increases of plasma cortisol concentration in salmon (Strange and Schreck, 1978; Barton et al., 1986). After the fish were fully anesthetized, we severed the caudal peduncle and collected blood into heparinized capillary tubes, separated plasma by centrifugation, and stored it at

-20° C until determination of cortisol concentration by radioimmunoassay using the method of Redding et al. (1984). Plasma concentrations of thyroxine (T_4) have also been shown to increase during smoltification (Dickhoff et al., 1978; Maule et al., 1987) and may interact with cortisol (Young, 1986). Although the functional significance of the changes in T_4 are not known, we monitored plasma titers of this hormone as an index of smoltification in 1987 using a radioimmunoassay described by Dickhoff et al. (1978).

Immune System Assay. The ability of lymphocytes to generate specific APC was assessed using the in vitro assay described by Tripp et al. (1987). Anterior kidneys and spleens were aseptically harvested and put into tissue culture medium (TCM) composed of RPMI 1640 containing L-glutamine and bicarbonate (GIBCO, Grand Island, NY, U.S.A.) and supplemented with 10% (v/v) hybridoma-screened fetal calf serum (B. A. Bioproducts, Walkerville, MD, U.S.A.), 100 mg gentamicin sulphate/l, 50 umol 2-mercaptoethanol/l (MCB Manufacturing Chemists, Inc., Cincinnati, OH, U.S.A.), 4.0 umol adenosine/l, 4.0 umol cytosine/l, 9.0 umol thymidine/l and 9.0 umol guanosine/l. Lymphocytes were separated from surrounding tissue by gentle aspiration with a 1 ml syringe and tissue debris was allowed to settle to the bottom of the test tube. Supernatants containing cells were collected, and the pooled cells were washed by centrifugation and resuspended to 2 X 10^7 cells/ml in TCM. We transferred 0.2 ml cell suspension to wells of a 24-well, flat-bottom microculture plate (Corning Glass Works, Corning, NY, U.S.A.) and added 0.1 ml of either TCM (negative controls) or TCM containing the antigen TNP-LPS (0.8 ug/ml; Jacobs and Morrison 1975). We also added 0.1 ml of TCM

(negative controls and antigen controls) or TCM containing 4, 40, or 400 ng cortisol/ml, so that the final concentrations of cortisol were 0, 1, 10, or 100 ng/ml. We established 3 or 4 wells for each treatment depending on the number of cells available and incubated the cultures at 17° C in an airtight gasbox (C. B. S. Scientific, Del Mar, CA, U.S.A.) with blood-gas mixture (10% O_2, 10% CO_2 and 80% N_2). Every other day, cell cultures were fed 0.05 ml of feeding cocktail (Tittle and Rittenberg 1978) containing 0, 1, 10, or 100 ng cortisol/ml. After seven days, cells were harvested, washed by centrifugation, and resuspended in medium. Lymphocytes secreting anti-TNP antibodies were detected by Cunningham plaque assay (Cunningham and Szenberg, 1968). We mixed 0.1 ml lymphocyte suspension, 0.025 ml TNP-coated sheep red blood cells (Rittenberg and Pratt, 1969) and 0.025 ml diluted steelhead trout (O. mykiss) serum as a complement source and deposited the mixture in a Cunningham slide chamber. During incubation (2 h at 17° C), anti-TNP antibody became bound to TNP-SRBC and activated the complement cascade, resulting in lysis of the surrounding TNP-SRBC. These zones of lysis were counted using a low-power dissecting microscope, and were expressed in terms of APC per cell culture. Numbers of viable cells in the cultures at the end of the 7 days of incubation were determined using hemacytometer and trypan blue exclusion.

Glucocorticoid Receptor Assays. In order to obtain measurements of affinity and number of receptors, we performed binding experiments on tissues at every sampling time. It was necessary to pool 8 to 10 fish to obtain enough tissue for the assay. Tissue preparation and GR assays were the same as has been described (Chapter V, this volume).

We removed spleens and anterior kidneys and put them in ice-cold tissue culture medium, RPMI-1640 with 0.15 mM EDTA at pH 7.4. We harvested leukocytes by gently and repeatedly aspirating tissue in 1-ml tuberculin syringes to free cells from connective tissue. We then added 4 - 10 X 10^6 cells to duplicate tubes each for determining total and nonspecific binding (NSB). After incubating cells to be used for total binding in medium and those for NSB in medium containing 100-fold excess radioenert triamcinolone acetonide (TA; 1,4-pregnadien-9 -fluoro-11ß,16 ,17 ,21-tetrol-3,20-dione-16,17 acetonide) for 60 min, we added sufficient radiolabelled TA (specific activity 25.9 Ci/mmol; Amersham, Arlington Heights, IL) so that the concentrations in the final volume of 0.2 ml were 0.05 to 2.0 nM. After an additional two hour incubation, the cells were washed twice in excess medium to remove unbound hormone and lysed with 0.2 ml distilled water. We then added 0.4 ml 95% methanol and added 0.5 ml of the mixture to 5.0 ml scintillation cocktail (Budget-Solve, Research Products International, Mount Prospect, IL) and counted the samples on a model LS 1800 liquid scintillation counter (Beckman Instruments, Palo Alto, CA).

We removed gill filaments from gill arches and put them in ice-cold buffer (10 mM Tris-HCl, 1mM EDTA, 12 mM monothioglycerol, 20 mM sodium molybdate, and 10% v/v glycerol; pH 7.4) and disrupted the cells using a motor driven glass and Teflon homogenizer. After an initial centrifugation to remove gross debris, we centrifuged the tissue at 100,000 g for 60 min in an L8-70 ultracentrifuge with a type 40 rotor (Beckman Instruments). We added two volumes of supernatant to one vol of buffer containing 5% (w/v) Norit A charcoal and 0.5%

dextran and then centrifuged to remove endogenous steroids. Although the origin of GR in the liquid fraction after this series of centrifugations is not known (i. e., cytoplasm, nucleus, or membrane) we refer to them here as cytosolic receptors to distinguish them from receptors in whole cells. We assayed protein with Bradford reagent (Bradford, 1976) and bovine serum albumin standards and adjusted protein content to 4 - 6 mg/ml. We incubated samples for total binding in buffer and those for NSBs in 100-fold excess radioenert TA for 10 min and then added sufficient radiolabeled TA in buffer to result in 0.2 to 6.0 nM of hormone in a final volume of 0.2 ml. After two hours, unbound hormone was separated from bound with 0.5 ml of 2.5% (w/v) Norit A charcoal and 0.25% (w/v) dextran in buffer (10 min incubation) and centrifugation (3,000 g, 20 min). The radioactivity of 0.5 ml of this final supernatant was determined as above.

Data Analyses. We used a computer software program (McPherson, 1985) to analyze binding data by subtracting NSB from total binding to get specific binding and obtain dissociation constants (Kd) and maximum binding sites (Nmax) based on Scatchard plot analyses (Scatchard, 1949). We analyzed data by analysis of variance, Duncan's multiple range test for pairwise comparisons, at $P < 0.05$, and multiple regression analysis. When appropriate, data expressed as a percent of controls were analysed using the G-test statistic based on the chi-squared distribution at $P < 0.05$ (Sokal and Rohlf, 1981). We also used linear regression to compare mean plasma cortisol concentration to GR and APC data.

RESULTS

During the time when this stock of coho salmon normally undergo smoltification, there were significant changes in plasma concentrations of cortisol and T_4 (Figure 22). Although there were significant changes in plasma T_4, there was not a distinct increase suggestive of the period of smoltification as has been seen with this stock of fish in previous years (Maule et al., 1987). In 1987 and 1988, plasma cortisol titers began increasing in March and reached their highest values by the middle of April. There was another increase in plasma cortisol in June through July 1988 and the apparent beginnings of a similar pattern in the last samples in 1987 (Figure 22). There were significant changes in the number of APC generated by spleen and anterior kidney lymphocytes (Figure 23). The ability to generate APC was at its lowest between the middle of April through the first of June; by the middle of July, it appeared that APC were

Figure 22. Plasma cortisol and thyroxine concentrations (mean ± 1 SE) from coho salmon during smoltification in 1987 (A and B) and 1988 (C). Sample sizes were 10 to 20 fish. Points marked (a) are significantly greater than those marked (b), Duncan's multiple range test, $P < 0.05$.

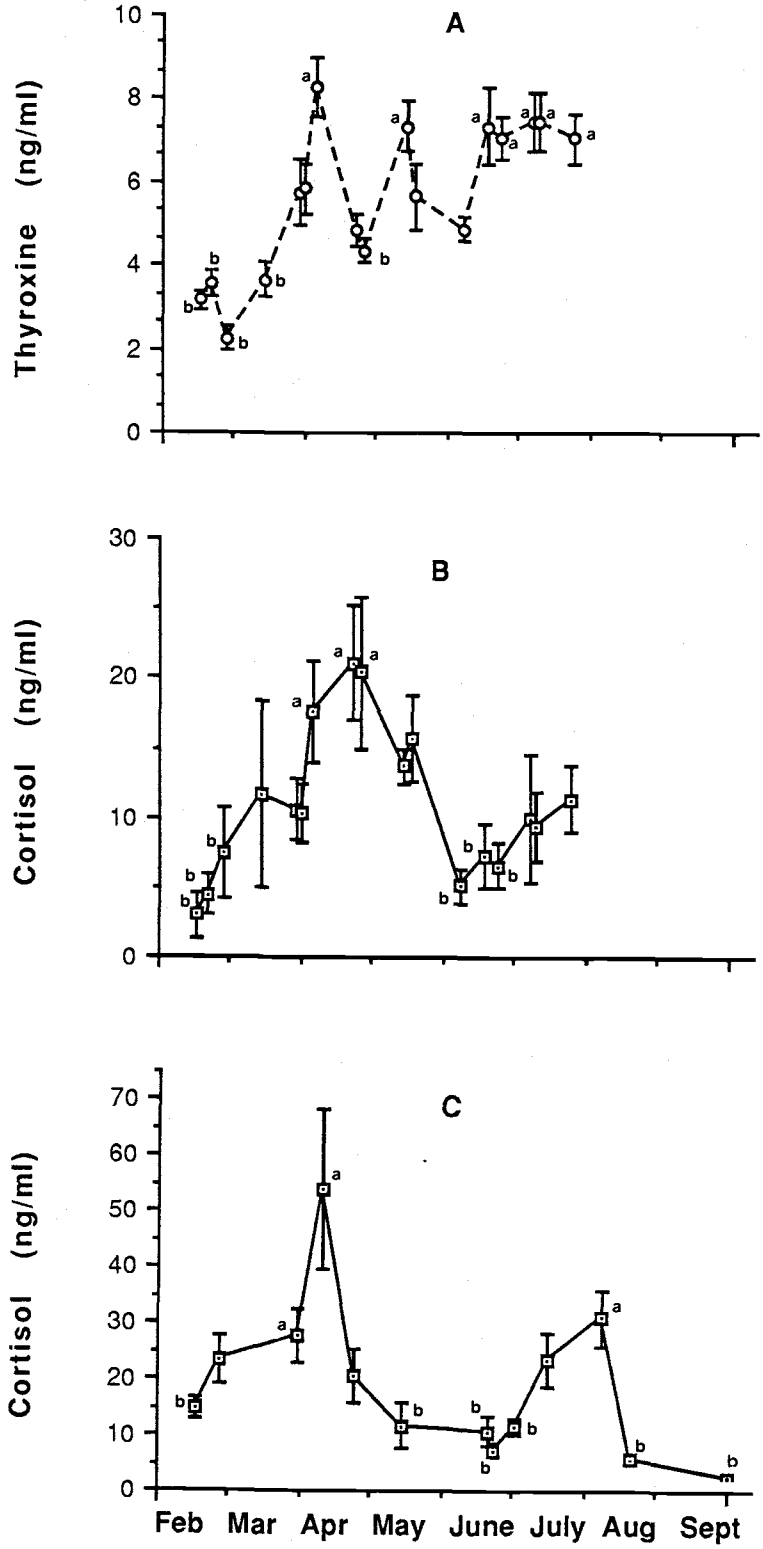
A
Thyroxine (ng/ml)
10
8
6
4
2
0
B
Cortisol (ng/ml)
30
20
10
0
C
Cortisol (ng/ml)
70
60
50
40
30
20
10
0
Feb
Mar
Apr
May
June
July
Aug
Sept

Figure 22.

Figure 23. Specific antibody-producing cells generated from lymphocytes from coho salmon at various times during smoltification in 1987. Points are mean $\pm$ 1 SE of 3 or 4 culture wells containing cells pooled from the spleens or anterior kidneys of 10 fish. Points with letters in common do not differ from each other, but do differ significantly from all other points on the same line, Duncan's multiple range test, $P < 0.05$.

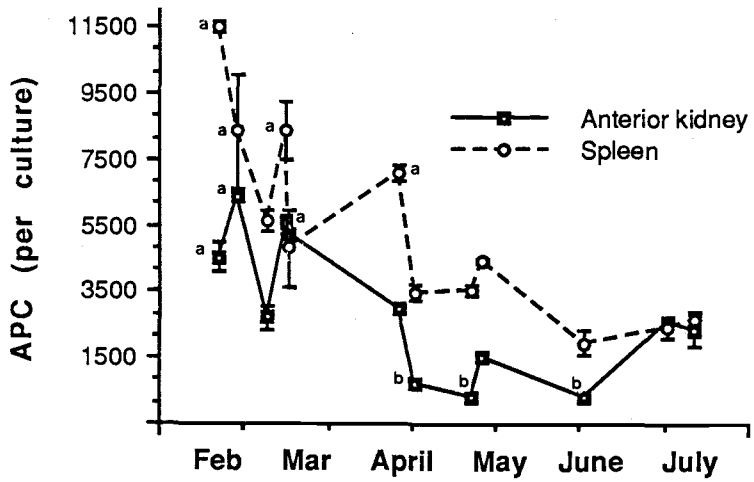
APC (per culture)
11500
9500
7500
5500
3500
1500
Feb
Mar
April
May
June
July
Anterior kidney
Spleen
a
b

Figure 23.

increasing (Figure 23). Regression analysis of the data through June revealed significant negative correlation between date and APC from spleen (coefficient of correlation = -0.836, $P < 0.005$) and anterior kidney (-0.798, $P < 0.01$). Lymphocytes from the spleen consistently generated more APC than did those from the anterior kidney. The data for anterior kidney include two assays in which no APC were detected; splenic cell cultures run concurrently, using the same reagents and assay conditions did generate APC (Figure 23) and the anterior kidney lymphocytes were viable at the end of the culture period. Plasma cortisol concentrations were not correlated to APC from either organ.

The addition of 100 ng cortisol per ml medium to splenic lymphocyte cultures usually reduced the number of APC to 40 to 99 % of controls (Figure 24). On one occasion (April 18) cortisol had no effect on splenic APC; thereafter, however, cortisol appeared to be increasingly suppressive (i.e., decreased relative to controls; Figure 24). Furthermore, the APC of splenic leukocyte cultures that contained 100 ng cortisol/ml (expressed as percent of controls) was correlated to mean plasma cortisol concentration ($R = 0.695$, $P = 0.036$). There was a sudden change in the cortisol sensitivity of lymphocytes from the anterior kidney during our sampling. Initially, the cells were totally insensitive, but developed sensitivity beginning in mid-April (Figure 24). By July, anterior kidney lymphocytes cultured with cortisol generated but 20 % of the APC of control cultures. These effects of cortisol in anterior kidney leukocyte cultures were not correlated to mean plasma cortisol concentration. There was no change in the cell viability at the end of the culture period that would explain the increased sensitivity to

Figure 24. The effects of 100 ng cortisol/ml medium on the ability of leukocytes to generate specific antibody-producing cells in vitro. Points are the percent of control of the mean APC in 3 or 4 culture wells containing pooled leukocytes from spleens or anterior kidneys of 10 coho salmon sampled at various times during smoltification in 1987. Mean control values are presented in Figure 23.

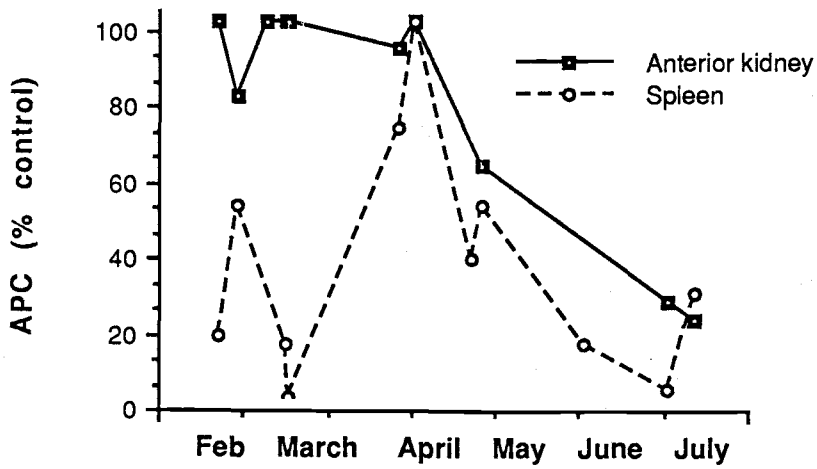
APC (% control)
100
80
60
40
20
0
Feb
March
April
May
June
July
Anterior kidney
Spleen

Figure 24.

cortisol. However, the two organs differed in the number of viable cells at the end of incubation. As a percent of controls, there were significantly fewer cells in anterior kidney lymphocyte cultures treated with 100 ng cortisol/ml (57.3 $\pm$ 3.5 %) than in similarly treated splenic cultures (92.4 $\pm$ 1.5 %; G-test, $P < 0.05$; $n = 10$.)

The affinity and number of GR in gill varied considerably during the course of the study (Figure 25); however, these changes were not correlated to mean plasma cortisol concentration (Table 8). The measurements of GR affinity and number in leukocytes in 1988 demonstrated organ heterogeneity similar to that seen in the immune assay (Figure 26). There were consistently 2- to 3-fold more GR in leukocytes from anterior kidney than in those from spleen and the number of GR in anterior kidney leukocytes increased at the beginning of April and declined gradually until June. The number of GR in splenic leukocytes remained relatively constant (Figure 26). Although the Kds were the same in leukocytes from the two organs when assayed in February, they diverged from March until June. During this time, GR in leukocytes from anterior kidney demonstrated higher Kd, while those in spleens remained low. Although there were some intra- and inter-organ changes in Kds from June through mid September, they were not of the magnitude seen earlier in the year (Figure 26), even in July when plasma cortisol titers were inexplicably elevated (Figure 22). Interestingly, the changes in GR in leukocytes from the anterior kidney, but not spleen, were correlated to mean plasma cortisol concentration (Table 8).

Figure 25. Number and dissociation constant (Kd) of glucocorticoid receptors harvested from gill in coho salmon sampled at various times during smoltification in 1987. Values were derived from Scatchard plot analysis of binding data using tissue pooled from 10 fish for each date.

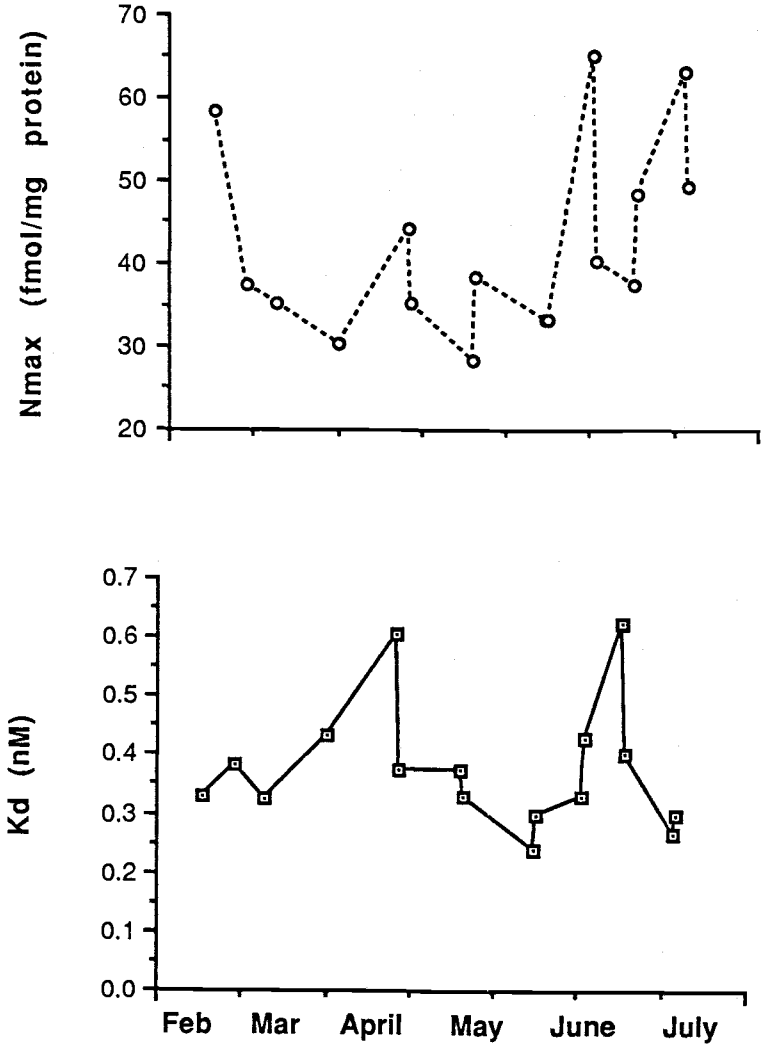
Nmax (fmol/mg protein)
70
60
50
40
30
20
Kd (nM)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Feb
Mar
April
May
June
July

Figure 25.

Figure 26. Number and dissociation constant (Kd) of glucocorticoid receptors in whole leukocytes from spleen or anterior kidneys of coho salmon sampled during smoltification in 1988. Values were derived from Scatchard plot analysis of binding data using cells pooled from 10 fish for each date.

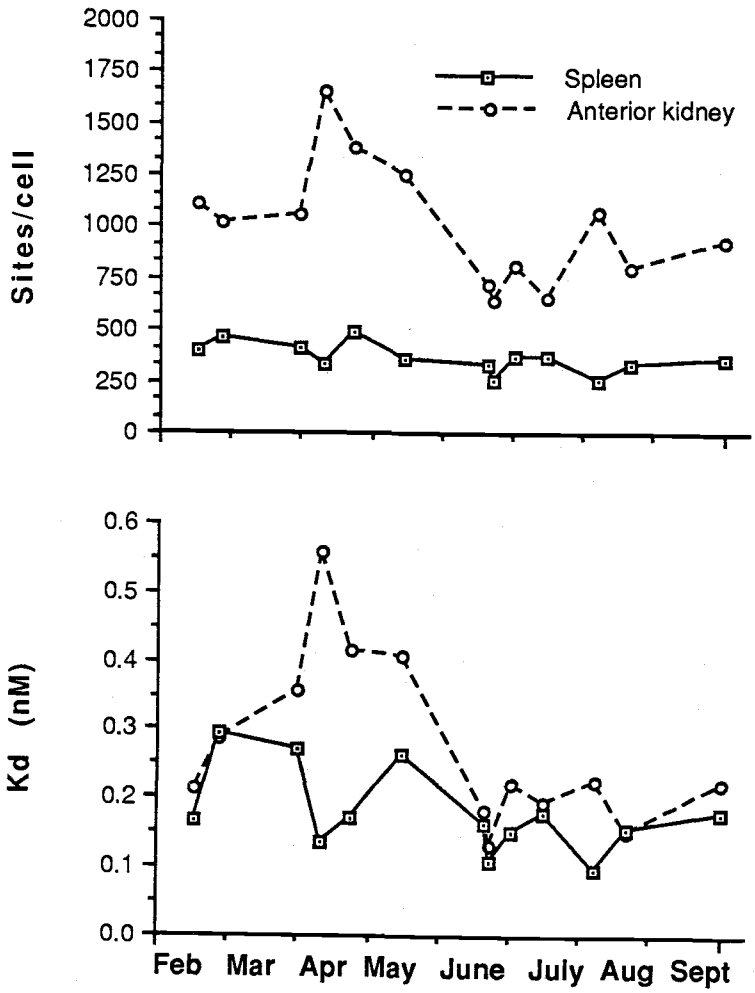
Sites/cell
2000
1750
1500
1250
1000
750
500
250
0
Spleen
Anterior kidney
Kd (nM)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Feb
Mar
Apr
May
June
July
Aug
Sept

Figure 26.

Table 8. Linear regression correlation coefficient (R) and level of significance (P) comparing mean plasma cortisol concentration to dissociation constant (Kd) and number (Nmax) of glucocorticoid receptors from gill (1987) and leukocytes (1988) during smoltification of coho salmon. Number of assays used in regression are in parentheses.

	Kd		Nmax	
Tissue/Year	R	P	R	P
Gill 1987 (16)	0.531	0.033	-0.802	<0.001
Leukocytes				
Spleen (13)	-0.027	0.933	0.034	0.915
Anterior Kidney (13)	0.706	0.005	0.675	0.010

DISCUSSION

In this study, using an *in vitro* assay, we have confirmed earlier work (Maule et al. 1987) by demonstrating reduced immune responsiveness during smoltification with lymphocytes from spleen and extended those observations to include reduced responsiveness of leukocytes from the anterior kidney (Figure 23). We have also demonstrated organ-specific changes in the sensitivity of lymphocytes to cortisol (Figure 24) and organ-specific changes in the affinity and number of GR in leukocytes (Figure 26) during smoltification of coho salmon suggesting a link between cortisol bioactivity and receptor function. Several researchers working with mammals have examined the correlation between GR and hormone bioactivity. Some have reported a positive relation (Omrani et al., 1980; Ranelletti et al., 1987) while others found no relation (Svec and Rudis, 1982; Grote et al., 1983). Lu et al. (1987) reported transient increases in number and Kd of GR in cytosol from rat pancreas beginning 10 d after birth. These changes occurred at the same time as increased activity in exocrine enzymes associated with dietary changes at weaning. Although other researchers have reported increased numbers of GR in the kidneys of chick embryos during development (Gendreau et al., 1987) and in livers of clawed toads (*Xenopus laevis*) during changes of season (Lange and Hanke, 1988), there were no changes in Kd and the observed changes in number of GR were not correlated with circulating glucocorticoids or biological activity. Although we found changes in the GR harvested from gill, the results were highly variable (Figure 25). There is an apparent change in Kd of gill GR in mid-April, the same time that anterior kidney leukocytes were developing cortisol sensitivity, but

it is based on a single assay and, thus, there is insufficient evidence to suggest biologically significant changes in GR in gill during smoltification. Furthermore, changes in gill GR were not correlated with plasma cortisol concentration.

In earlier work we demonstrated organ-specific changes in GR from leukocytes and gill in response to acute and chronic stress and cortisol treatment (Chapter VI, this volume). In that study, gill GR did not change in response to acute stress, but did have decreased affinity and numbers of GR when fish were chronically stressed or fed cortisol. In the latter treatments, plasma cortisol titers greatly increased (mean values > 200 ng/ml) and remained elevated beyond 24 h; acute stress resulted in plasma cortisol concentrations elevated to levels nearer to those reported during smoltification (mean values < 100 ng/ml). Thus, it appears that the differential changes in GR reported in these two studies were the result of cells in the various tissues having different thresholds of sensitivity to circulating cortisol. That is, the increased circulating cortisol during smoltification or acute stress was sufficient to activate GR regulatory mechanisms in leukocytes but not gill tissue, while higher plasma cortisol concentrations associated with chronic stress or cortisol treatment activated GR regulatory mechanisms in all three tissues.

Throughout most of our sampling, splenic leukocytes were sensitive to glucocorticoids and the sensitivity varied with endogenous cortisol. At highest endogenous cortisol concentrations, splenic leukocytes were least sensitive <u>in vitro</u> suggesting that the endogenous cortisol had already affected the leukocytes. Similarly,

anterior kidney leukocytes were not sensitive to cortisol added to the in vitro cultures at first, and so their sensitivity was not correlated with endogenous cortisol. At the same time endogenous cortisol was not correlated with splenic leukocyte GR, but was correlated with changes in anterior kidney leukocyte GR (Kd and Nmax). These relations suggest that splenic leukocyte GR did not change during smoltification because those cells were already sensitive to cortisol, while changes in anterior kidney leukocyte GR were involved in conferring sensitivity to those cells. In other words, development of sensitivity to cortisol was a receptor mediated process.

Although the changes in GR in anterior kidney leukocytes were correlated with changes in plasma cortisol titers, in July unexpectedly high plasma cortisol concentrations did not result in the same magnitude of changes in GR as seen earlier in the year. The failure of anterior kidney leukocyte GR to change in July may be because the cells had already developed sensitivity to cortisol, and also suggests that factors other than endogenous cortisol influence GR regulation. Lu et al. (1987) reported that although plasma concentrations of corticosterone increased just prior to changes in GR, there appeared to be other factors, perhaps hormonal, involved in GR regulation. A factor that might be involved in the changes in GR observed by us is the hormone prolactin. Junker (1985) reported that the affinity of GR in mononuclear leukocytes was lower in pregnant women, presumably with high prolactin titers, than in nonpregnant women. Schneider and Shyamala (1985) reported that mouse mammary epithelial cells required prolactin in the culture medium in order to express GR. Although we did not measure circulating prolactin

concentrations, others have found that during smoltification of coho salmon plasma prolactin titers increased gradually during March, preceding plasma cortisol increases, and decreased sharply in mid April (Young et al., 1989). Thus, it is possible that changes in circulating prolactin titers, as well as cortisol, are involved in the GR regulation seen in the present study.

VIII. SUMMARY AND CONCLUSIONS

In this dissertation, I have shown that the immune system of juvenile Pacific salmon was altered by developmental changes and stress, and that those changes were, at least in part, driven by increased plasma cortisol titers. During smoltification there was a decreased ability to generate APC *in vivo* (Chapter II) and *in vitro* (Chapter VII) and this was accompanied by decreased numbers of leukocytes in spleen and circulation. Similar decreased immune responses were seen within hours after acute stresses of various durations (i.e., 30 s to 9 h); however, by 24 h after those stresses the immune response was enhanced over what it had been before the stress (Chapter III). Perhaps most importantly, these changes in immune response correlated positively with the ability of fish to resist disease (Chapter III). Acute and chronic stress were also shown to temporarily alter the numbers of leukocytes in anterior kidney, spleen, thymus and blood (Chapter IV). Moreover, all of the effects seen during stress and smoltification could be duplicated by long- or short-term cortisol treatment (Chapters II and IV).

The presence of GR (which were shown to exist in salmon tissue and in leukocytes of fish for the first time) suggested that cortisol was having its effects via specific receptors (Chapter V) as has been shown in mammals (Coffey and Djeu, 1986). Furthermore, the GR in leukocytes from spleen and anterior kidney showed organ heterogeneity in cells from resting (Chapter V), stressed (Chapter VI) and smolting fish (Chapter VII). This further suggests that cortisol acts via GR in leukocytes, as there is a differential, organ-specific sensitivity

of leukocytes to cortisol in vitro (Tripp, 1988; Chapter VII). The GR in salmon were shown to be more responsive to stress and glucocorticoid treatment than those in mammals as there were changes in numbers and affinities of GR in spleen and anterior kidney leukocytes and gill (Chapter VI).

Among the most interesting results of these studies was the finding that leukocytes from the anterior kidney of presmolts were insensitive to cortisol added to cell cultures, but that sensitivity developed during smoltification (Chapter VII). Not only were there increased plasma cortisol titers concurrent with the change in leukocyte sensitivity, but there were also increased numbers of GR with decreased affinity for the ligand (Chapter VII). Furthermore, the fact that changes in in vitro leukocyte sensitivity to cortisol and leukocyte GR were correlated to endogenous cortisol titers in some tissues but not others, suggests that the development of sensitivity to cortisol is a receptor mediated process. To my knowledge, this correlation between changes in number and affinity of GR and change in sensitivity to glucocorticoid has not been shown in any other animal model, and suggests an excellent model for studying the regulation of receptors.

Another important finding is the correlation between stress-induced changes in plasma cortisol titers, immune response and disease resistance (Chapter II). The fact that disease resistance was enhanced shortly after stress, suggests that what seemed to be a maladaptive response (i.e., stress-induced immunosuppression) may have an adaptive component as well.

Conclusions

I strongly believe that science has not been done properly unless it creates more questions than it answers. Thus, in this section I will pose some questions, ranging from specific to general, and propose a model to help answer them.

There are several important questions relative to the regulation of GR during stress and smoltification of Pacific salmon. What factors drive the changes in GR, and are they the same during stress and smoltification? Plasma cortisol titers were correlated to GR during stress and smoltification (Chapters VI and VII) but when anterior kidney leukocytes were preincubated with cortisol for 3 h prior to GR assay, it had no effect on the number or affinity of the GR (Maule, unpublished data). This strongly suggests that some factor other than cortisol and external to the cells was necessary to cause the changes in GR. It seems reasonable that other hormones such as prolactin, plasma levels of which increase during the long process of smoltification (Young et al., 1989) and transiently during stress (M. Avella and C. Schreck, unpublished data), might act to sensitize leukocytes by increasing GR in anticipation of increased cortisol titers. As has been mentioned, prolactin was a necessary addition to medium for a mouse mammary epithelial cell line to express GR (Schneider and Shyamala, 1985); and, pregnant women with presumed elevated prolactin levels had increased numbers of GR of a decreased affinity, as compared to women who were not pregnant (Junker, 1983).

Another question is, what intracellular mechanisms are responsible for the changes in GR affinity? This question is related to the first, but has significance to gene regulation. It is

generally assumed that there is a single class of GR, even though GR may be present as oligmers prior to ligand binding (Rousseau, 1984). If this model is accepted, changes in receptor affinity must be the result of post-transcriptional or post-translational processing, which means that genes other than the specific GR gene are necessary to encode the factors responsible for processing the GR. A large number of factors modulate GR in the mammalian system (Noma et al., 1984; Carter-Su and Pratt, 1984; Bodine and Litwack, 1988). Furthermore, mRNA has been found in association with GR (Ali and Vedeckis, 1987; Kasayama et al., 1987) suggesting the possibility of spatially close synthesis of GR modulator(s). Another possible explanation for the reduced affinity of GR for the ligand is that the ligand itself has been modified. Even though this ligand modification was shown to occur on a tissue-specific basis (Funder et al., 1988), the fact that it occurs at all suggests that changes in ligand processing by target cells are possible. That is, under low hormone concentrations the hormone may not be altered by the cell, but with high hormone concentrations cellular processes may be initiated that alter the ligand so that it will not bind to the receptor with a high affinity. Thus, giving the appearance of receptors with a reduced affinity for the ligand. These findings effectively add several layers of complexity to the problem of identifying regulatory mechanisms of glucocorticoids and their receptors.

Based on the results of this dissertation and other studies, I propose the following model of glucocorticoid receptor regulation in coho salmon. In the resting state, GR are maintained by low levels of cortisol and other factors, as yet unidentified. During stress or

smoltification, levels of the factor(s) increase prior to or concurrent with increased cortisol titers. This factor causes an increase in intracellular GR either by releasing stored GR or increasing GR gene transcription. Although glucocorticoids have been shown to unmask certain membrane bound receptors (Landman et al., 1984; DeBlasi et al., 1986), a similar mechanism has not been shown for GR. Therefore, in this model, increased intracellular GR is the result of GR gene transcription. Post-transcriptional or post-translational processing may take one of two tracks. First, GR or mRNA might undergo maturational processes that require time so that the new GR, assayed within hours after transcription have not had sufficient time to be processed (i.e., are immature). Alternatively, new GR are processed differently, or not at all, as compared to GR in the resting fish. The second track seems most likely, as there are short term changes in numbers of GR as well as affinity. Thus, the factor presumably causes an increase in GR gene transcription to increase numbers of GR, but the post-transcriptional processing factors (perhaps the products of constitutive rather than inducible genes) do not increase. The timeframe within which I measured changes in GR (3-4 h) is well within that required for gene transcription and protein synthesis (Raacke, 1961). This model seems reasonable from an energetics view point as the cell would be able to respond to the transient increase in hormone concentration by increasing receptors, but would not expend energy or resources processing receptors that are needed temporarily. This model is consistent with reported multiple molecular forms of GR, some of which appear transiently after stress (eg., Hirato et al., 1985a, 1985b).

I speculate that the following mechanisms, based on the proposed model, were acting during the course of the experiments reported here. The initial increased cortisol was bound to the existing high affinity GR which was then bound to DNA. At the same time, GR gene transcription might increase the number GR, but these might not have been processed and remained closely associated with the nucleus. When gill cytosol was assayed after stress, DNA-bound GR were lost with the nuclear fraction, as were the majority of the new low affinity GR. Thus, the assay revealed fewer GR of a lower affinity as compared to nonstressed controls. Binding assays with leukocytes, however, revealed GR throughout the cell, most of which were new GR of low affinity. Through time, the increased cortisol concentration had a variety of effects on the cell (processes beyond the scope of this model) and, as hormone concentrations drop, low affinity GR were metabolized or otherwise disposed of, and post-transcriptional processing might then replenish the supply of high affinity GR, returning numbers and affinity of GR to their original status. Within this model, prolactin may be a regulatory factor.

A more general question posed by this research (and stated in Chapters II and VII) is, what is the functional significance of immunosuppression during smoltification and stress? It would seem counterproductive to suppress the immune system at times when the organism is going to enter a new environment, or face a new challenge. Extending the model proposed by Munck et al. (1984), the immune system may be downregulated after stress or during smoltification to avoid damaging tissues by autoimmunity. I will discuss this model relative to smoltification and refer the reader to Munck et al. (1984) for a

discussion on stress. During smoltification there are changes in color, body shape (Hoar, 1976) and osmoregulatory mechanisms (Zaugg et al., 1972), to name a few. These changes imply changes in gene transcription that, in the case of gill Na^+ K^+ ATPase activity, have effects on plasma membranes. Thus, it is possible that new cell-surface markers are exposed to which the immune system has not developed tolerance. The immune system must be downregulated during these tissue changes, and tolerance for new markers must develop. Thus, cortisol and the presumptive GR-regulatory factor(s) controlling GR could be mobilized to avoid autoimmunity. Within this model, I speculate that a GR regulatory factor is prolactin, although there are several other hormones the concentrations of which change during stress and smoltification (eg., epinephrine, growth hormone, adrenocorticotropic hormone; Pickford et al., 1971; Mazeaud et al., 1977; Young et al., 1989). As indicated previously, the mononuclear leukocytes from pregnant women have increased numbers of GR with a decreased affinity for ligand (Junker, 1983), the same changes as seen in smolting salmon (Chapter VII). Normally, the body would reject tissue with foreign cell markers such as those of the fetus, and so the immune system must be downregulated. Thus, as during pregnancy, prolactin may upregulate GR in leukocytes during smoltification to sensitize them to increased glucocorticoids. Furthermore, it is believed that basal secretion of prolactin is regulated by different mechanisms than is prolactin secretion in response to stress (Armario et al., 1987; Murai and Ben-Jonathan, 1987). Thus, within this model, increased basal secretion of prolactin during smoltification (Young et al. 1989) may have a role in increasing GR in anticipation of

increased cortisol secretion; but during stress, when increased plasma prolactin titers follow increased plasma cortisol titers (M. Avella and C. Schreck, unpublished data) prolactin may act to return GR to their original status, perhaps by regulating post-transcriptional processing. That this system is controlled by feedback loops is suggested by the finding that glucocorticoids downregulate prolactin gene transcription (Sakai et al., 1988).

I believe that in this dissertation I have attained the stated goal of expanding our understanding of the role of cortisol as an immunomodulator in Pacific salmon. However, a final question that must be addressed is, what does all of this mean to fisheries management? The understanding of physiological processes will allow managers to make decisions about when and where to conduct unavoidable fish manipulations, and may be used in a type of risk assessment when considering new management strategies. For example, the fact that smolts are immunologically compromised should be considered when deciding to transport fish for off-site releases. Furthermore, elucidating mechanisms by which the immune system is suppressed or enhanced may suggest ways to manipulate the processes, by therapeutic or prophylactic treatment, to enhance survival of fish to adulthood, the ultimate goal of fish biologists.

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