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Iris Knoebl for the degree of Master of Science in Fisheries Science presented on May 2, 1995. Title: Characterization and the Effects of Stress on Glucocorticoid Receptors in the Brains of Chinook Salmon (*Oncorhynchus tshawytscha*)

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Abstract approved: _____

Carl B. Schreck

The effects of stress on the brains of salmon may have important implications in light of the extremely high glucocorticoid levels experienced by migrating and spawning adults and the stressful procedures to which salmon smolts are exposed in hatcheries. The identification and characterization of glucocorticoid receptors in salmon brains is a first step in elucidating the effects of stress and high glucocorticoid levels on the brain. We identified glucocorticoid binding sites in non-adrenalectomized juvenile chinook salmon (*Oncorhynchus tshawytscha*) brain cytosol using the synthetic glucocorticoid triamcinolone acetonide (TA) and for cortisol, the naturally occurring teleost glucocorticoid. The binding sites had high affinity and low capacity for the ligands. Binding characteristics for [^{3}H] TA were typical of glucocorticoid receptors (K_d = 0.85 ± 0.13 nM, B_{max} = 22.4 ± 2.97 fmol/mg protein, n=7). The binding sites had a lower affinity for

cortisol (K_d = 4.54 ± 0.06 nM, B_{max} = 25.40 ± 2.20 fmol/mg protein, n=2) and exhibited specificity for glucocorticoids. TA, cortisol and dexamethasone (a synthetic glucocorticoid) displaced [^{3}H] TA most effectively. Corticosterone, cortisone and RU28362 (a synthetic "pure" glucocorticoid) were not potent competitors nor were sex steroids. The synthetic steroid RU38486 competed strongly at low concentrations but did not entirely inhibit TA binding at higher concentrations. Binding sites in nuclear extracts were not detected nor was binding to DNA-cellulose. These binding characteristics are consistent with published data on glucocorticoid binding sites in other salmonid tissues.

In addition, the effect of acute stress on the binding sites was examined. In three separate trials, juvenile chinook salmon were stressed by confinement for either 0.5 hr or 3 hr. The plasma cortisol levels increased in response to stress compared to control fish which were not stressed. The number of glucocorticoid binding sites available to bind [^{3}H] TA in saturation binding studies decreased as the duration of stress increased. The affinity of the binding sites did not change in response to acute stress. Comparison of total binding and non-specific binding data indicated that the reduction in the number of binding sites was due to a decrease in total binding, not to an increase in non-specific binding.

We conclude that glucocorticoid binding sites exist in chinook whole-brain cytosol, and these binding sites respond to acute stress.

Characterization and the Effects of Stress on Glucocorticoid Receptors in the Brains of Chinook Salmon (*Oncorhynchus tshawytscha)*

by

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Iris Knoebl, Author

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CONTRIBUTION OF AUTHORS

Martin S. Fitzpatrick. Provided guidance in the design of experiments and analysis of the data and provided editorial comments on the manuscript.

Carl B. Schreck. Provided guidance in the design, implementation and completion of the project and provided editorial comments on the manuscript.

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I. GENERAL INTRODUCTION

The exposure of a vertebrate animal to stressful stimuli results in a cascade of events including the elevation of plasma glucocorticoids, which in turn act on target tissues to elicit a response. In mammals, these target tissue responses, which are mediated by receptors, have been well studied (Burnstein and Cidlowski, 1989). In non-mammalian vertebrates such as fish, many similarities to the mammalian stress response exist, including the release of high levels of glucocorticoids into the blood (Barton and Iwama, 1991). Research is just beginning, however, into the details of glucocorticoid receptors and their characteristics within target tissues.

In teleosts, cortisol is the major circulating glucocorticoid and receptors for glucocorticoids have been reported and characterized in intestine (DiBattista *et al.*, 1983, 1984), gills (Sandor *et al.*, 1984), liver (Chakraborti *et al.*, 1987) and leukocytes (Maule and Schreck, 1990). Also well characterized are some of the harmful effects of chronic stress and elevated plasma cortisol in fish, such as depressed immune response (Maule *et al.*, 1989), suppressed reproductive function (Carragher *et al.*, 1989) as well as reduced growth rate (Barton *et al.*, 1987).

Investigations into the role of glucocorticoids and glucocorticoid receptors in the brain of teleosts are only beginning, however, much work has been done on mammalian brains. It has been shown that two distinct types of glucocorticoid receptors are present in rat brains (Reul and de Kloet, 1985). Type I receptors have a high affinity for mineralocorticoids (for

example aldosterone) and glucocorticoids and have a lower affinity for the synthetic glucocorticoid dexamethasone. Type II receptors, also known as the "classical" glucocorticoid receptors, have a high affinity for dexamethasone, a lower affinity for glucocorticoids and an even lower affinity for aldosterone. Within the rat brain there is regional variation in density of these two receptor types. The type II receptors have a uniform density throughout the brain and have a concentration about 10 times higher than type I receptors throughout most areas of the brain. The exception is within the hippocampal-septal system where there is an extremely high concentration of type I receptors (Reul and de Kloet, 1985).

In mammalian brains, the hippocampus is part of the limbic system. It is involved in memory and learning where it appears to be essential to the acquisition and consolidation of learning. In teleosts, the limbic system is part of the forebrain (telencephalon). It integrates species specific behavior such as maintaining the proper balance of aggression, sexual behavior and parental behavior (Aronson and Kaplan, 1968). The telencephalon of fishes seems to play a role in conditioned response learning and avoidance response. Previously learned responses are eliminated when the telencephalon is removed, although these responses can be relearned in some fish (Gleitman and Rozin, 1971).

In the mammalian brain, the hippocampus, with its high concentration of glucocorticoid receptors, mediates a negative feedback mechanism. Destruction of the hippocampus leads to hypersecretion of glucocorticoids (Sapolsky *et al.*, 1984) as the organism is no longer able to mediate the negative feedback mechanism.

Glucocorticoid receptors within the hippocampus are down-regulated in response to stress, however there is a different response to acute versus

chronic stress (Sapolsky *et al.*, 1984). Sapolsky (1984) found that acute stress reduced the number of receptors on rat hippocampal neurons, but the concentration of receptors returned to normal after about one week. Chronic stress, such as exposure to corticosteroids for a period of three months, also reduced the number of receptors in the hippocampus, but there was no recovery even after many months. These rats also had reduced ability to terminate glucocorticoid secretion after additional stress. Chronic stress causes not only down-regulation of receptors on the neurons of the hippocampus, but loss of neurons themselves. Several studies have shown neurotoxicity of glucocorticoids (Sapolsky, 1985, 1986).

Prolonged exposure of hippocampal neurons to glucocorticoids may have an effect on aging. There is evidence in rats of progressive loss of glucocorticoid receptors and neurons in the hippocampus due to exposure to glucocorticoids over a lifetime (Sapolsky *et al.*, 1985). Because of the role the hippocampus plays in memory formation and retrieval as well as neuroendocrine regulation, this neuron loss may account for some of the debilitating effects of aging. In rats, these effects of aging can be prevented by adrenalectomy at mid-age and can be accelerated by prolonged exposure to high circulating levels of glucocorticoids (Sapolsky *et al.*, 1984, 1985).

The neurotoxic effects of acute or prolonged, high level glucocorticoids may have implications in fish. Salmon hatcheries are economically important in the Pacific Northwest both to supplement dwindling natural salmon runs and to provide fish for sport and commercial fishermen. However, intensive salmon culturing practices expose fish to extremely stressful situations at several critical developmental stages. Such handling procedures as grading, transporting and artificial stripping may cause acute stress, while an environment with poor water quality (Smart, 1981) and the stress of social

interactions due to high stocking densities (Schreck, 1981) may promote chronic stress.

As yet, no work has been done to investigate the effects of stress and high glucocorticoid levels on the brains of fish, but there may be similarities to the mammalian response. In Pacific salmon, cortisol levels increase during their spawning migration and are at extremely high levels during spawning (Hane and Robertson 1959) at which time the fish can no longer regulate their plasma cortisol levels. These fish die after spawning. There may be a relationship between this inability to control cortisol levels and a reduced number of cortisol receptors or receptor containing neurons in the brain similar to that observed by Sapolsky (1985) in aged and chronically stressed rats. It is possible that a regulatory mechanism involving a structure like the mammalian hippocampus exists in teleosts.

In light of the effects of stress on rat neurons containing glucocorticoid receptors, further information regarding functional consequences of stress in the brains of fish would be useful to both hatchery managers and researchers in further elucidating the effects of stress on fish. The release of hatchery salmon smolts into rivers is an extremely stressful event. At that time they are vulnerable to predators and must also imprint on the release site. The stress experienced at this time may interfere with the ability of the smolt to avoid predators or to imprint, and it may be less likely to survive. If the aim of salmon hatcheries is to produce and release smolts which are healthy and fit for survival, the possible effects of stress on the brain should not be overlooked.

Glucocorticoid receptors in the brains of salmonids have yet to be fully characterized and identified. The identification and characterization of cortisol receptors in the brains of teleosts is a first step in determining how

the teleost brain responds to stress and the accompanying elevation of circulating cortisol. In chapter I we identified and characterized glucocorticoid binding sites in chinook salmon (*Oncorhynchus tshawytscha*) whole brain cytosolic fractions according to the criteria for receptors suggested by Clark and Peck (1977). These criteria state that receptors must have finite binding capacity, high affinity as well as specificity for the hormone, tissue specificity and a correlation to a biological response. In chapter II we performed experiments to determine whether acute stress had an effect on the binding sites characterized in chapter I. We exposed fish to confinement stress for 0.5 hr and 3 hr and report the effects of acute stress on the number of binding sites and binding affinity.

II. CHARACTERIZATION OF A GLUCOCORTICOID BINDING SITE IN THE BRAINS OF CHINOOK SALMON, *Oncorhynchus tshawytscha*

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II. CHARACTERIZATION OF A GLUCOCORTICOID BINDING SITE IN THE BRAINS OF CHINOOK SALMON, *Oncorhynchus tshawytscha*

Introduction

The effects of chronic stress on the brain may have implications for salmon, whose plasma glucocorticoid levels become extremely high during migration and spawning (Hane and Robertson 1959) and may be a contributing factor in their programmed senescence and death (Robertson and Wexler, 1960). The identification and characterization of glucocorticoid receptors in the brain of salmon is a first step in determining how the brain responds to stress and the accompanying elevation of circulating glucocorticoids. Glucocorticoid receptors have been localized in rat brains (Gerlach and McEwen, 1972; McEwen et al., 1976) and chronic stress has been linked to the loss of neurons containing glucocorticoid receptors (Sapolsky *et al.*, 1984a) which may lead to hypersecretion of glucocorticoids (Sapolsky *et al.*, 1984b).

In teleostean fishes, cortisol is the major circulating glucocorticoid and cortisol receptors have been reported and characterized in intestine (DiBattista *et al.*, 1983, 1984), gill (Sandor *et al.*, 1984), liver (Chakraborti *et al.*, 1987) and leukocytes (Maule and Schreck, 1990). Cortisol receptors in the brain of salmonids have yet to be fully characterized and identified. Chakraborti *et al.* (1987) reported finding no cortisol receptors in the brain of brook trout (*Salvelinus fontinalis*). In a paper describing glucocorticoid binding sites and down-regulation by dexamethasone (a synthetic glucocorticoid) in the liver of rainbow trout (*Oncorhynchus mykiss*), Lee *et al.* (1992) found that dexamethasone treatment also resulted in down-regulation of

glucocorticoid binding in the brain, however the binding sites were not fully characterized. We investigated glucocorticoid binding sites in the brains of chinook salmon (*O. tshawytscha*) using the synthetic glucocorticoid triamcinolone acetonide (TA), which has demonstrated high affinity binding to glucocorticoid receptors in other salmonid tissues (Sandor *et al.*, 1984; Maule and Schreck, 1990). Our primary objectives were to determine if saturable binding sites exist within the chinook brain, describe the binding characteristics, and determine the location within the brain cells.

Materials and Methods

Animals. Juvenile chinook salmon (1+ years old) were maintained at Oregon State University's Fish Performance and Genetics Laboratory at Smith Farm, Corvallis, Oregon. The fish were kept in 1 m circular tanks supplied with flow-through, pathogen free well-water at 12-13.5° C and fed daily *ad libitum* with a commercial fish diet (BioProducts).

Experimental protocol and tissue preparation. In order to determine binding characteristics at basal, unstressed levels, fish used in these experiments were as unstressed as possible to keep endogenous plasma cortisol levels low (because of the nature of the interrenal tissue in teleosts (Nandi, 1962), adrenalectomy is impossible). The fish were quickly netted and immersed in a lethal dose (200 mg/liter) of tricaine methane-sulfonate (MS-222) buffered with sodium bicarbonate (500 mg/L). At each sampling, only 4 to 6 fish were collected to ensure that brains were removed as quickly as possible after the fish were anesthetized. Blood samples were taken from the caudal vein for later analysis of plasma cortisol content to assure that the fish were not stressed. The brains were quickly removed, wrapped in aluminum foil, frozen between two blocks of dry ice and stored at -80° C until assayed. Preliminary experiments showed no difference in binding site number or affinity between cytosol prepared from frozen brains and cytosol from fresh unfrozen brains. For each assay, 4 to 6 brains were pooled, the number depending on the assay being done and amount of cytosol required. The protein content of the cytosol was between 2 and 5 mg/ml.

To obtain the cytosolic fraction, brains were homogenized in TEMS buffer (10 mM Tris-HCl, 1 mM EDTA, 12 mM monothioglycerol, 10 mM sodium molybdate and 10% (w/v) glycerol, all at pH 7.4), sonicated for several seconds and centrifuged at 1750 x g for 15 min. The supernatant was incubated with one-half volume of TEMS buffer containing 5% charcoal and 0.5% dextran (5% DCC) for 10 min to remove endogenous steroids. After centrifuging (1750 x g, 20 min) to remove the charcoal, the liquid fraction was centrifuged at 100,000 x g for 1 hr to obtain the cytosolic fraction (supernatant). To obtain the nuclear fraction, the pellet from the first 1750 x g centrifugation was washed three times in 10 vol wash buffer (10 mM Tris-HCl, 3 mM $MgCl_2$, 2 mM monothioglycerol, 250 mM sucrose, pH 7.5). The nuclear pellet was extracted in 3 vol extraction buffer (50 mM Tris-HCl, 1 mM Na_2 EDTA, 12 mM monothioglycerol, 700 mM KCl, 30% v/v glycerol, pH 7.5) for 1 hr, vortexing every 15 min followed by centrifuging at 100,000 X g for 1 hr. The supernatant was stripped of endogenous steroid by incubating with one-half volume 5% DCC for 10 min and centrifuged for 20 min at 1750 x g. Tissues were kept on ice at all times except during incubation.

Chemicals and reagents. [6,7-^{3}H] Triamcinolone acetonide ([^{3}H]TA, 43.8 Ci/mmol) and [1,2,6,7-3H] hydrocortisone ([^{3}H] cortisol, 85.5 Ci/mmol) were purchased from DuPont NEN (Boston, MA). Budget-Solve scintillation cocktail (Research Products International, Mt. Pleasant, IL) was used for scintillation spectrophotometry. RU38486 and RU28362 were gifts from Roussel Uclaf, Romainville, France. Radioinert TA, cortisol and all other steroids and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Binding kinetics and determination of Kd and Bmax. The binding rate of [^{3}H] TA was determined at three concentrations by incubating 0.100 ml aliquots of cytosol in duplicate with 0.05 ml of either TEMS buffer to determine total binding or 500 times molar excess TA to determine non-specific binding. Association kinetics experiments were performed at incubation temperatures of 2° C and 12° C. Reactions were stopped at specific times by putting the tubes on ice and adding 0.5 ml of 2.5% dextran-coated charcoal (2.5% DCC) (2.5% charcoal, .25% dextran in TEMS buffer). The tubes were centrifuged at 1750 x g for 20 min after which 0.5 ml of the supernatant was added to 5 ml scintillation cocktail for scintillation spectrophotometry. Specific binding was determined as the difference between total binding and non-specific binding at each time point and temperature.

Cytosolic saturation binding studies were conducted by incubating 0.1 ml aliquots of cytosol in duplicate with increasing concentrations (.03 nM - 7 nM) of [^{3}H] TA or [^{3}H] cortisol in the presence (non-specific binding) or absence (total binding) of 500 times molar excess TA. The assay tubes were allowed to incubate overnight (12 - 15 hr) at 2° C. After incubation, 2.5% DCC was added to separate bound from unbound ligand. After 5 min the tubes were centrifuged at 1750 x g for 20 min and 0.5 ml of the supernatant was added to 5.0 ml scintillation cocktail and quantitated using a liquid scintillation counter. Binding data were analyzed to obtain dissociation constants (K_d) and maximum number of sites (B_{max}) per milligram of protein based on nonlinear regression of the untransformed specific binding data (GraphPad Prism, GraphPad Software, Inc., San Diego, CA).

To confirm non-specific binding by using an unlabeled competitor structurally different from TA, saturation assays were also performed using [^{3}H] TA (0.1 nM - 14.2 nM) with and without 500 times molar excess of

unlabeled dexamethasone, cortisol and TA plus dexamethasone on the same cytosol pool.

To determine the dissociation rate, cytosol was incubated overnight (12-15 hr) at 2° C with 5.7 nM [^{3}H] TA in the presence (non-specific binding) and absence (total binding) of 500 times molar excess of unlabeled TA. Dissociation was initiated by the addition of 10 μM of unlabeled TA and incubation at 2° C for an additional 0 - 6 hr. The reactions were stopped and bound and unbound ligand was separated by the addition of 2.5% DCC followed by centrifugation at 1750 x g for 20 min. The supernatant (0.5 ml) was added to 5.0 ml scintillation cocktail and quantitated using a liquid scintillation counter. The first-order dissociation rate constant (k_{-1}) was calculated from the log concentration of the bound ligand vs. time using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

Steroid Binding Specificity. The steroid specificity of the binding sites was determined by incubating 0.1 ml cytosol aliquots in triplicate with 5 nM [^{3}H] TA and a range of competitor concentrations from 0 to 1000 times molar excess. Steroid competitors included synthetic glucocorticoids (dexamethasone and RU28362), natural glucocorticoids (cortisol, cortisone, corticosterone) and non-glucocorticoid steroids (RU38486, 17α-hydroxyprogesterone, testosterone, progesterone and estradiol). Because a mineralocorticoid receptor is known to exist in mammalian brains, aldosterone was also used, although it is not generally known to occur in teleosts (Henderson and Kime, 1987). The assays were incubated overnight at 2° C and were repeated on two different cytosolic pools for each competitor. Maximum specific binding was determined by subtracting nonspecific binding for TA (at 1000 times molar excess) from total binding (no competitor). The data are

expressed in percent of maximum specific binding displaced by a competitor (specific binding in the presence of a competitor divided by the maximum specific binding) and also in K_i values.

DNA-cellulose chromatography. Cytosol (1.0 ml) was incubated overnight at 2° C with 5 nM [^{3}H] TA and either 500 times molar excess TA or without TA. Protein was then precipitated by adding saturated ammonium sulfate in TEMG (TEMS minus Na_2 molybdate) buffer to obtain a 50% saturated solution. The saturated solution was stirred on ice for 1 hr and then centrifuged at 10,000 x g for 20 min. The supernatant was removed and the pellet washed once with 0.5 ml of column buffer A (TEMG plus 0.01 M NaCl and 0.02% w/v bovine serum albumin). The pellet was resuspended in 5 ml column buffer A. The sample was then added to a column (Bio-Rad polypropylene minicolumn) containing 0.4 g calf thymus DNA-cellulose which had been incubated overnight at 2° C in TEMG buffer. The sample was allowed to flow momentarily to penetrate the column. The flow was stopped and the column was incubated for 15 min at 22° C and then 45 min at 2° C to allow the sample to adsorb to the DNA. The column was washed with 12 ml column buffer A, then 8 ml of column buffer B (same as buffer A but with 0.4 M NaCl) and finally 8 ml of column buffer C (same as buffer A but containing 2.0M NaCl). Fractions (0.5 ml) were collected, combined with 5.0 ml scintillation cocktail and counted on a liquid scintillation counter to determine radioactivity.

Protein determination. The method of Bradford (1976) using bovine serum albumin (RIA-grade, Sigma) as the reference protein was used to determine protein content.

Plasma cortisol determination. Plasma cortisol was measured by a [^{3}H] cortisol radioimmunoassay reported and validated by Redding *et al.* (1984).

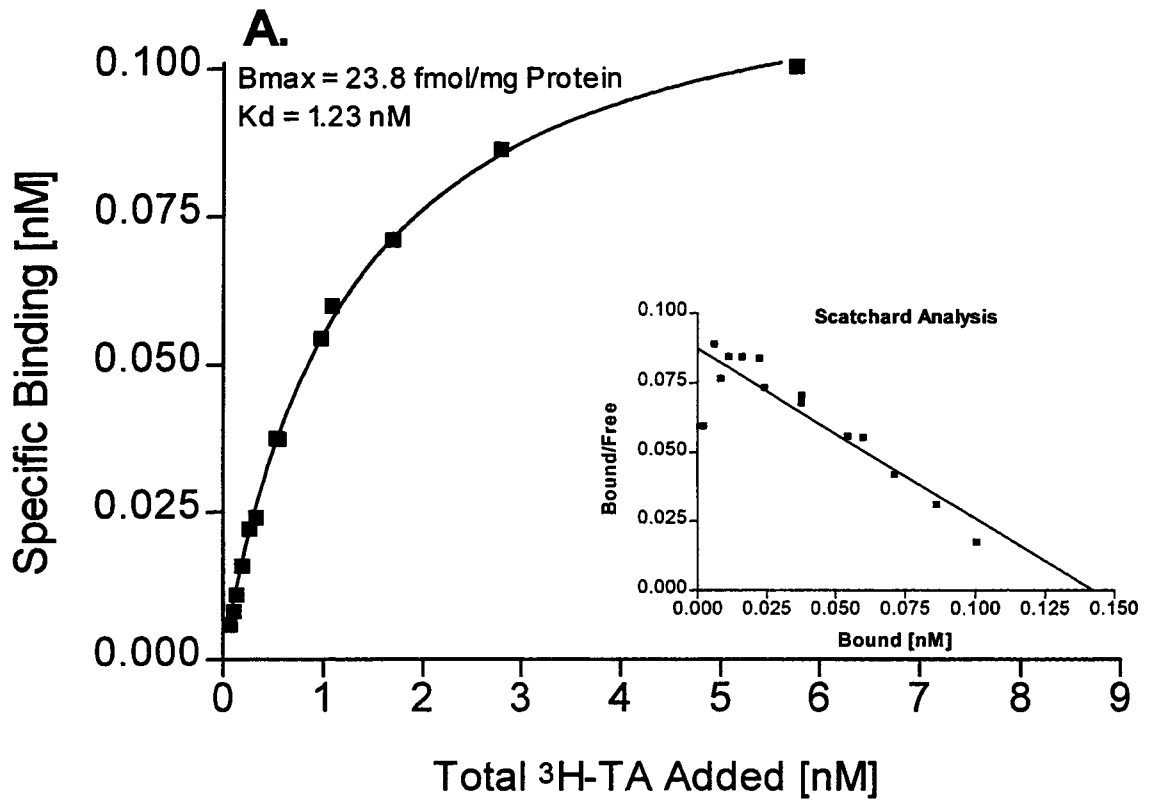
Results

Binding kinetics and determination of K_d and B_{max}. Maximum specific binding was reached at all [^{3}H] TA concentrations between 6 and 10 hr of incubation and binding remained stable at 2° C up to 24 hr (data not shown). The samples incubated at 12° C reached maximum specific binding more quickly (6 hr) but after 10 hr, maximum specific binding was the same at both incubation temperatures. Maximum specific binding decreased slightly for samples incubated at 12° C after 24 hr incubation. All subsequent assays were incubated overnight (12-15 hr) at 2° C to allow the reactions to reach equilibrium.

Saturation binding assays using [^{3}H] TA showed a single class of high affinity, low capacity binding sites (K_d = 0.85 ± 0.13 nM, B_{max} = 22.4 ± 2.97 fmol/mg protein, n=7 assays). Saturation assays using [^{3}H] cortisol plus unlabeled TA also demonstrated a single class of binding sites with the same low capacity but an affinity about five times lower than for [^{3}H] TA in the same cytosol pool (K_d = 4.54 ± 0.06 nM, B_{max} = 25.40 ± 2.20 fmol/mg protein, n=2 assays). Representative binding curves are shown in Fig. 2.1. The binding reaction is completely reversible (Fig. 2.2) with a dissociation rate of 0.011 nM/min and $t_{1/2}$ = 62.4 min.

Saturation assays performed to verify non-specific binding using 500 times molar excess of unlabeled dexamethasone, cortisol or TA plus dexamethasone showed little difference in dissociation constants or maximum number of binding sites (Table 2.1). No specific binding in the nuclear fraction was detected in either of two saturation assays performed (data not shown). Plasma cortisol content for fish used in all experiments was 14.5 ± 3.0 ng/ml (n= 81).

Figure 2.1 Specific binding determined by saturation analysis in chinook whole-brain cytosolic extracts. (A) Cytosolic extracts were incubated with increasing concentrations of [^{3}H]-triamcinolone acetonide (TA) in the presence (non-specific binding) and absence (total binding) of 500 times molar excess unlabeled TA. (B) Cytosolic extracts were incubated with increasing concentrations of [^{3}H]-cortisol in the presence (non-specific binding) and absence (total binding) of 500 times molar excess unlabeled TA. Specific binding is calculated as the difference between total binding and non-specific binding. Each data point is the mean of two replications within the same assay. The inset for each graph is a Scatchard plot of the transformed specific binding data. These data are representative of experiments performed to compare the specific binding of TA and cortisol on the same cytosolic pool. Protein content of the cytosol was 5.17 mg/ml.


A.
Bmax = 23.8 fmol/mg Protein
Kd = 1.23 nM
Specific Binding [nM]
Total 3H-TA Added [nM]
Scatchard Analysis
Bound/Free
Bound [nM]

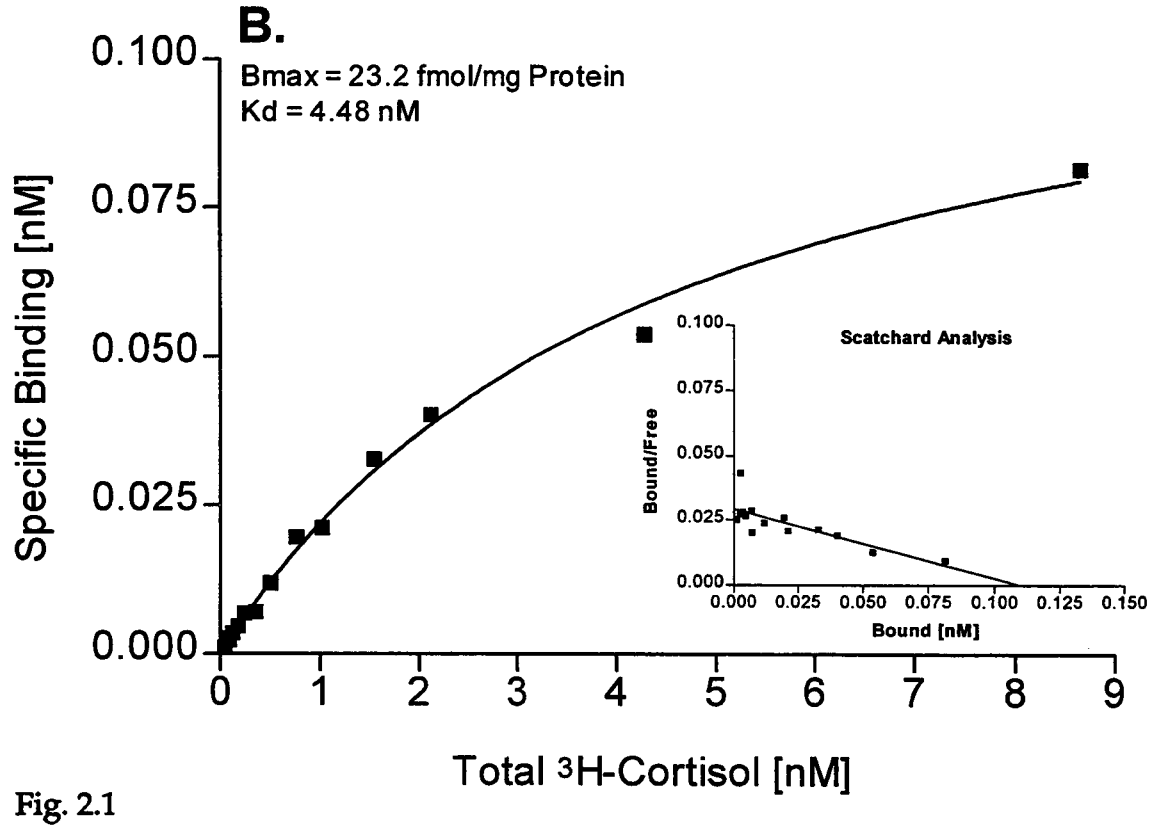

B.
Bmax = 23.2 fmol/mg Protein
Kd = 4.48 nM
Specific Binding [nM]
Total 3H-Cortisol [nM]
Scatchard Analysis
Bound/Free
Bound [nM]

Fig. 2.1

Figure 2.2 Dissociation of specific binding determined by incubating chinook whole-brain cytosolic extracts with 5.7 nM [^{3}H] TA in the presence (non-specific binding) and absence (total binding) of 500 times molar excess unlabeled TA. Dissociation was initiated by the subsequent addition of 10 μM unlabeled TA and continued incubation for an additional 0 - 6 hr. Specific binding is calculated as the difference between total binding and non-specific binding. The first-order dissociation rate constant (k_{-1}) was calculated from the log concentration of the bound ligand vs. time using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

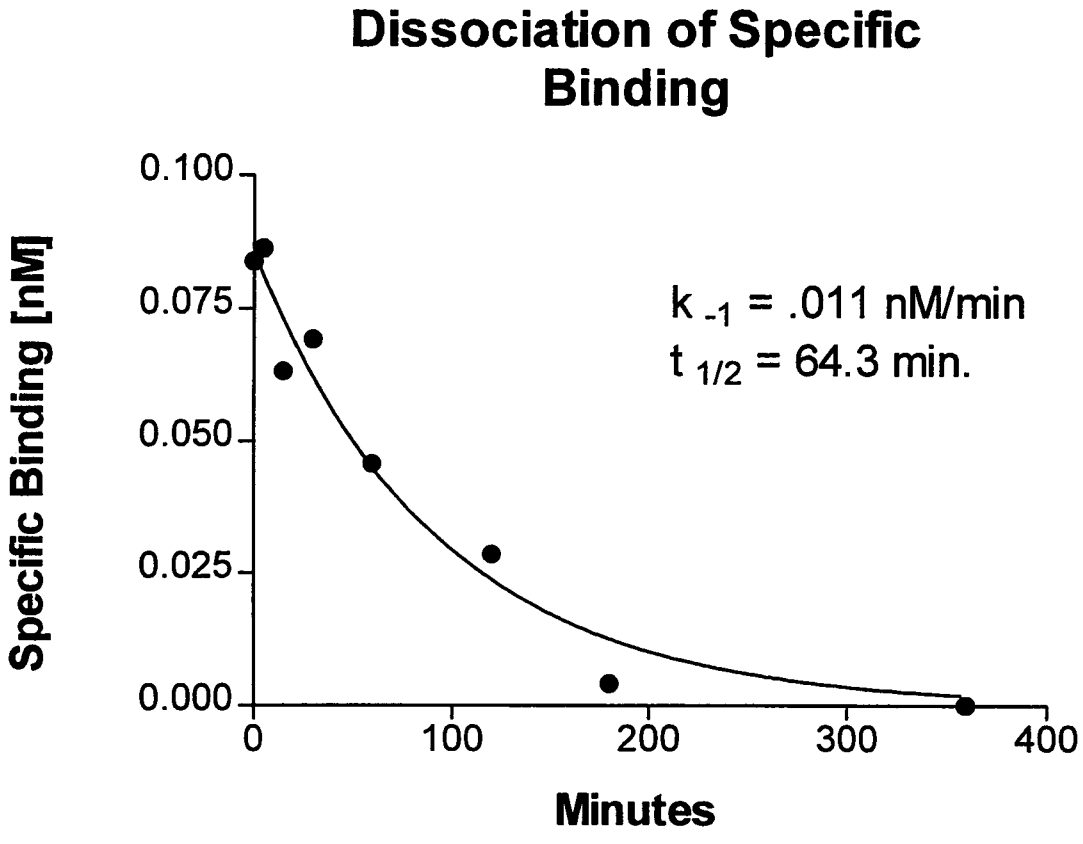

Dissociation of Specific Binding
Specific Binding [nM]
0.100
0.075
0.050
0.025
0.000
0
100
200
300
400
Minutes
k -1 = .011 nM/min
t 1/2 = 64.3 min.

Fig. 2.2

Table 2.1 Binding characteristics of ^{3}H-triamcinolone acetonide (TA) using different corticoids as non-specific binding competitors. These data represent single saturation binding assays performed using the same cytosol pool and 500-fold molar excess of either TA, dexamethasone (Dex), cortisol or TA plus Dex as unlabeled competitors. Values for K_d and B_{max} were determined via non linear regression using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). Protein content of the cytosol was 3.55 mg/ml.

Competitor	K_d [nM]	B_{max} (fmol/mg. pr)
TA	1.19	16.90
Dex	1.70	19.15
Cortisol	1.24	16.90
TA plus Dex	0.82	14.37

Steroid Binding Specificity. Cortisol and the synthetic glucocorticoid dexamethasone competed most strongly for the binding sites displacing 50% of the TA binding between 1 and 10 times molar excess and almost completely eliminating specific binding at 100 fold excess concentration (Fig. 2.3). The synthetic antiprogestin, glucocorticoid receptor antagonist RU38486 displaced approximately 50% of the specific binding between 0.1 and 1 fold molar excess but failed to completely inhibit specific binding even at 1000 times excess in one of the two assays performed. Further studies using RU38486 will be required to determine its competitive efficacy.

Corticosterone and the synthetic "pure" glucocorticoid RU28362 displaced at least 50% specific binding at concentrations between 10 and 100 times molar excess and almost completely eliminated specific binding at 1000 times excess concentration. A concentration of over 100 times molar excess was required for 17α-hydroxyprogesterone to compete for at least 50% specific binding and aldosterone and cortisone required 1000 times excess to attain 50% binding inhibition. Testosterone, estradiol and progesterone did not reach 50% inhibition even at 1000 fold excess concentration. The inhibition constant (K_i) values are shown in Table 2.2.

DNA-cellulose chromatography. No specific DNA binding was detected in either of two DNA-cellulose chromatography assays.

Figure 2.3 Specificity of glucocorticoid binding in chinook whole brain cytosol. Cytosolic extracts were incubated (in duplicate) with 5 nM [^{3}H] triamcinolone acetonide (TA) in the absence (total binding) or presence of .01, 1, 10, 100 and 1000 times molar excess of unlabeled competitor. Specific binding was calculated as the difference between total binding and binding in the presence of 1000 times excess unlabeled TA. The percentage of [^{3}H]-TA still bound after competitor displacement is expressed as the difference between total binding and binding in the presence of competitor divided by the specific binding. Each data point is the mean of 2 assays except data points for TA which are the mean of 4 assays. (A) Competition with natural corticoids. (B) Competition with synthetic corticoids. (C) Competition with other steroids.

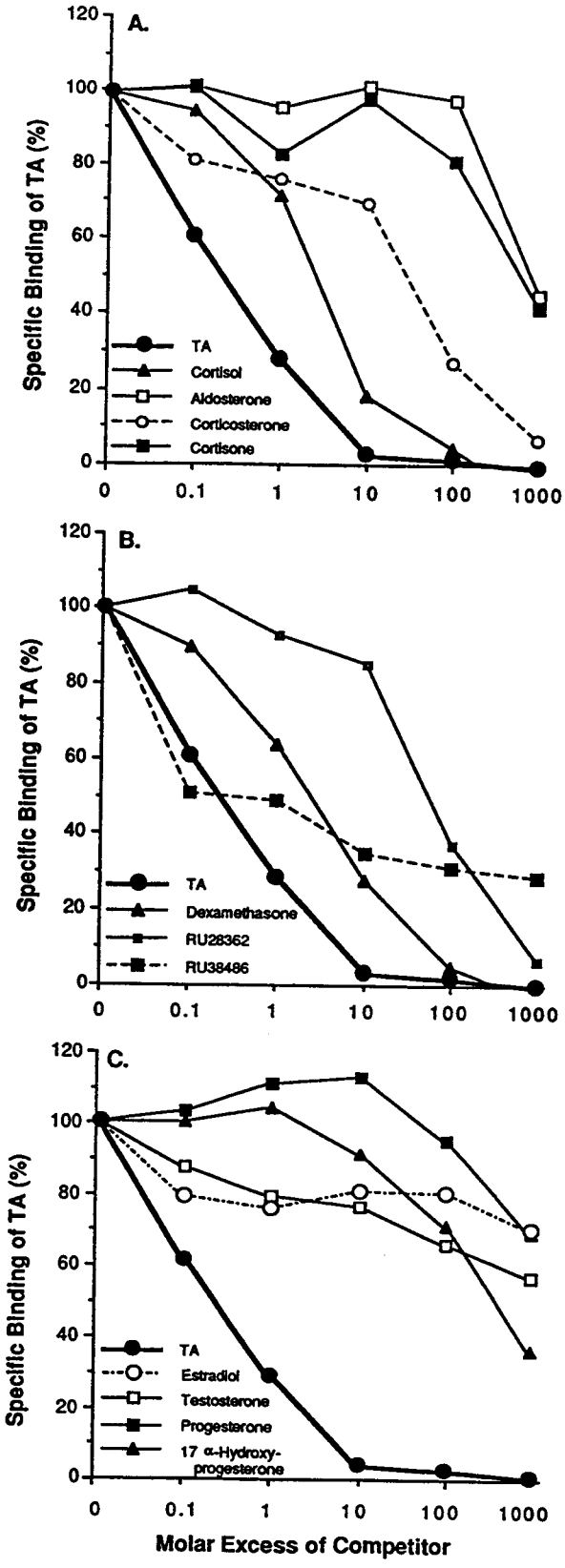

A.
Specific Binding of TA (%)
120
100
80
60
40
20
0
TA
Cortisol
Aldosterone
Corticosterone
Cortisone
0
0.1
1
10
100
1000
B.
Specific Binding of TA (%)
TA
Dexamethasone
RU28362
RU38486
C.
Specific Binding of TA (%)
TA
Estradiol
Testosterone
Progesterone
17 α-Hydroxy-progesterone
Molar Excess of Competitor

Fig. 2.3

Table 2.2 K_i values for steroid competitors shown in Figure 2.2. These values were determined via non-linear regression of percent specific binding data using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

Competitor	K_i [nM]
TA	0.036
Dexamethasone	0.431
Cortisol	0.471
Corticosterone	4.95
RU28362	11.3
17 α-Hydroxyprogesterone	73.5
Cortisone	124
Aldosterone	136
Testosterone	162
Estradiol	371
Progesterone	419

Discussion

These data demonstrate the existence of high affinity, low capacity glucocorticoid binding sites within chinook whole brain cytosolic extracts. Binding was completely reversible. The maximum number of binding sites we report are lower than reported in other salmonid tissues (Pottinger *et al.*, 1994; Lee *et al.*, 1992; Pottinger, 1990; Chakraborti and Weisbart, 1987; Chakraborti *et al.*, 1987; Sandor *et al.*, 1984) but are similar to the number reported by Lee *et al.* (1992) in rainbow trout brain. The dissociation constant (K_d) for glucocorticoid receptors in salmonid brains has not been previously reported, however the K_d of the binding sites in the chinook brain is similar to dissociation constants reported using [^{3}H] dexamethasone in the hippocampi of rats (Sapolsky *et al.*, 1984) and is consistent with accepted dissociation constants for steroid hormones. It is important to note that the fish used for these binding studies were not adrenalectomized. Elimination of endogenous free steroid was accomplished after tissues were removed and homogenized. Although the fish used in our experiments were unstressed and had low plasma cortisol titers at the time of death, some of the binding sites would have been occupied and activated by endogenous cortisol at that time. Therefore the actual number of binding sites within the brain may be higher than we report, however because no free cortisol is present in the cytosol preparations, the dissociation constant should not be affected.

Synthetic glucocorticoids such as TA and dexamethasone are known to have a higher affinity than cortisol for glucocorticoid receptors. To verify that [^{3}H] TA was binding to the same population of binding sites as cortisol, we compared binding between [^{3}H] cortisol and [^{3}H] TA in the same cytosol pool. As expected, the K_d was lower for TA (i.e. showed higher affinity), but

the number of binding sites was the same for both steroids, suggesting that TA and cortisol bind to the same population of binding sites.

We performed binding assays comparing the use of unlabeled TA, dexamethasone, cortisol and TA plus dexamethasone to determine if there was any difference in non-specific binding and thus a difference in binding characteristics. In order to determine accurate binding properties of receptors, non-specific binding to such components as other cellular constituents and glassware must be defined. Weiland and Molinoff (1981) recommend the unlabeled competitor used in the non-specific binding study be structurally different from the labeled ligand Our studies indicated no difference in non-specific binding characteristics between the competitors.

The steroid specificity characteristics of the binding sites in the brain are similar to those reported for glucocorticoid receptors in other salmonid tissues (Pottinger, 1990; Chakraborti *et al.*, 1987; Chakraborti and Weisbart, 1987; Lee *et al.*, 1992; Sandor *et al.*, 1984). TA competed most effectively with ^{3}H-TA for the binding sites, followed by dexamethasone and cortisol. Of the natural glucocorticoids cortisol displaced ^{3}H-TA binding the best, corticosterone was less effective, cortisone was the weakest competitor. RU38486 displaced ^{3}H-TA binding to 50% at low concentrations, but failed to displace specific binding completely at higher concentrations. RU28362 and the sex steroids were not potent competitors.

The mineralocorticoid aldosterone was not a potent competitor for the brain glucocorticoid binding site. Neither the presence nor the synthesis of aldosterone has been reported in teleosts, however we chose to test the possibility of mineralocorticoid competition for binding in the chinook brain in light of differences found in corticosteroid receptors in mammalian brains. Two distinct types of corticosteroid receptors are present in rat brains

(Veldhuis *et al.*, 1982; Reul and de Kloet, 1985). Type I (or mineralocorticoid) receptors have a high affinity for mineralocorticoids (for example aldosterone) and glucocorticoids and have a lower affinity for the synthetic glucocorticoid dexamethasone. Type II receptors, also known as the "classical" glucocorticoid receptors, have a high affinity for dexamethasone, a slightly lower affinity for glucocorticoids and even lower affinity for aldosterone. It is not known whether teleost brains show any similarities to mammalian brains regarding glucocorticoid receptor type or distribution and we did not attempt to distinguish between type I or type II receptors in these experiments. The inability of aldosterone to compete effectively in our experiments does not rule out the possibility of the presence of type I receptors in teleost brains as endogenous cortisol may occupy high affinity type I sites. In rats, type I receptors are believed to be partially occupied by basal corticosterone levels (Reul and de Kloet, 1985; Cascio *et al.*, 1989; Spencer *et al.*, 1990).

The subcellular localization of the unliganded glucocorticoid receptor is unresolved. The traditional view was that unliganded receptors were located in the cytosolic compartment of target cells and after binding to appropriate ligands, became activated and moved into the nuclear compartment to bind to DNA. Brink *et al.*, (1992) suggested that glucocorticoid receptors, both liganded and unliganded, reside in the nuclear compartment of mammalian cells. Additionally, a corticosteroid receptor, which may have an effect on the regulation of behavior, has been identified in neuronal membranes of an amphibian brain (Orchinik, *et al.*, 1991). We found no detectable specific binding in the nuclear fraction nor on DNA-cellulose. Previous studies of DNA- cellulose binding of gill and intestinal glucocorticoid receptors in the American eel (*Anguilla rostrata*) also gave

negative results (DiBattista *et al.*, 1983; Sandor *et al.*, 1984). We will undertake further attempts at DNA-cellulose binding with modifications of our technique and another DNA-cellulose source.

It is possible that binding sites may not be present in the nuclear preparation due to our extraction technique, or that binding sites may be present but unable to bind with radiolabeled TA because they are already occupied. The inability to detect specific binding in nuclear extracts is similar to findings for glucocorticoid receptors by some researchers (Pottinger *et al.*, 1994, Lee *et al.*, 1992, Porthe-Nibelle and Lahlou, 1984). Pottinger *et al.* (1994) report specific binding sites for glucocorticoids in intact liver nuclei of rainbow trout but at much lower levels than estradiol binding in the same preparations. Cortisol receptor activity has been reported in nuclear extracts from liver (Chakraborti and Weisbart, 1987) and gills of brook trout (*Salvelinus fontinalis*) (Weisbart *et al.*, 1987; Chakraborti *et al.*, 1987); however the affinity of these receptors is an order of magnitude lower than that for cytosolic receptors.

Nuclear binding sites in our preparations may have been occupied or activated by endogenous cortisol. There is evidence that, unlike other steroid receptors, activated type II glucocorticoid receptors in the brains of mice cannot rebind glucocorticoids even if the steroid dissociates from the receptor (Chou and Luttge, 1988).

Clark and Peck (1977) suggested the criteria required for hormone binding sites to be considered receptors are finite binding capacity, high affinity as well as specificity for the hormone, tissue specificity and correlation to a biological response (e.g., ability to bind to DNA). We have demonstrated the existence, in chinook whole-brain cytosolic preparations, of high affinity, low capacity glucocorticoid binding sites whose binding

characteristics are consistent with glucocorticoid receptors found in other salmonid tissues. With the exception of the ability to bind to DNA, we have demonstrated that these binding sites have most of the characteristics of classical receptors. The function and exact location of binding sites within the teleost brain, how they respond to elevated glucocorticoid levels associated with stress and senescence, and whether similarities exist with receptors found in the mammalian brain remains to be determined.

III. THE EFFECTS OF ACUTE STRESS ON THE NUMBER AND BINDING AFFINITY OF GLUCOCORTICOID BINDING SITES IN THE BRAINS OF CHINOOK SALMON, *Oncorhynchus tshawytscha*

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Introduction

The existence of high affinity, low capacity glucocorticoid binding sites in chinook salmon whole brain cytosolic preparations using synthetic glucocorticoids and cortisol has been documented (Knoebl *et al.*, in review). However it has not yet been determined whether these binding sites are affected by stress. Stress has been shown to reduce the number of glucocorticoid binding sites and change the affinity of the sites in the liver of rainbow trout (*Oncorhynchus mykiss*) (Pottinger *et al.*, 1994) and in gills of coho salmon (*O. kisutch*) (Maule and Schreck, 1991). In rats, both acute and chronic stress reduce the number of glucocorticoid receptors in hippocampal neurons (Sapolsky *et al.*, 1984), however chronic stress permanently reduces receptor number. Several studies have shown a neurotoxic effect of glucocorticoids on hippocampal neurons (Sapolsky, 1985, 1986). In mammals, the hippocampus (part of the limbic system) is involved in memory and learning and appears to play a role in acquisition and consolidation of learning. In teleosts, the limbic system is a part of the forebrain (telencephalon). It integrates species specific behavior such as maintaining the proper balance of aggression, sexual behavior and parental behavior (Aronson and Kaplan, 1968) and seems to play a role in conditioned response learning and avoidance response. Previously learned responses are eliminated when the telencephalon is removed, although these responses can be relearned in some fish (Gleitman and Rozin, 1971).

In order to determine the implications of stress on fish brains we investigated whether exposure to acute stress had any effect on glucocorticoid binding sites. This is a first step in demonstrating whether fish brains have a similar response to stress as mammalian brains. In these experiments we determined whether the number of glucocorticoid binding sites and the binding affinity were affected by acute stress in whole brain cytosol of exposed chinook salmon (*O. tshawytscha*). We used the synthetic glucocorticoid [^{3}H] triamcinolone acetonide ([^{3}H] TA), which has a high affinity for glucocorticoid receptors and was used in previous experiments (Knoebl *et al.*, in review). The fish were exposed to 0.5 hr and 3 hr confinement stress in order to determine whether the duration of the acute stress was a factor and to begin to understand how quickly any effects on the binding sites occur.

Materials and Methods

Animals. Juvenile chinook salmon (1+ years old, 50-250 g) were maintained at Oregon State University's Fish Performance and Genetics Laboratory at Smith Farm, Corvallis, Oregon. The fish were kept in 1 m circular tanks supplied with flow-through, pathogen free well-water at 12-13.5° C and fed daily *ad libitum* with a commercial fish diet (BioProducts).

Experimental protocol. This experiment was performed three times. In each trial, one group of fish serving as controls were removed from the holding tank by dip net and immediately immersed in a lethal dose (200 mg/L) of tricaine methane sulfonate (MS-222) buffered with sodium bicarbonate (500 mg/L). Immediately afterward, fish from the same holding tank were netted, held out of water for 30 sec and placed into confinement chambers. The confinement chambers were perforated 20 L plastic buckets which allowed a continuous flow of fresh water. These confinement buckets were suspended in 1 m circular tanks with continuously flowing fresh water. One group of treatment fish (from a single confinement chamber) were killed by a lethal dose(200 mg/L) of buffered MS-222 after 0.5 hr confinement, and another group (from a single confinement chamber) after 3 hr of confinement. All fish were weighed and a sample of blood was taken from the caudal vein to determine plasma cortisol content to asses the level of stress. The brains were removed and processed as described below. Each trial was performed in the morning so that the endogenous cortisol due to diurnal rhythm would be similar in all experiments. Experiments using rainbow trout show a diurnal cortisol peak at night and lower plasma cortisol levels during the day (Rance *et al., 1982*).

Trial 1. This trial was performed in April of 1993 using 50-150 g fish. A group of 12 fish served as controls. Groups of 10 fish were placed into each confinement chamber. The depth of the water in each chamber was just enough to allow the fish to swim upright but not cover their dorsal fins. Both the density of fish and the low water level restricted the movement of the fish. The treatment groups were sampled after 0.5 hr and 3 hr. The severity of this stress was sufficient to cause death in a third group of treatment fish confined for 24 hr.

Trial 2. In March 1995 the experiment was performed using 100-250 g fish and a less severe confinement stress. In this trial, a group of 4 fish served as controls and 4 fish were placed into each confinement chamber. The water level in the confinement chambers was about 30 cm which was enough to completely cover the fish and allow some room to move about the confinement chamber. The treatment groups were sampled after 0.5 hr and 3 hr of confinement.

Trial 3. In April of 1995 the same procedure as Trial 2 was repeated using chinook from the same source.

Tissue preparation and assay methodology. After lethal anesthetization, blood was collected from the severed caudal peduncle and centrifuged to obtain plasma. The plasma was frozen at -80° C until analyzed for cortisol content. Immediately after blood sampling, the brains from all fish were quickly removed. In Trail 1, the brains were immediately placed into ice-cold TEMS buffer, processed as previously described (Knoebl *et al.*, in

review) to obtain the cytosolic fraction which was frozen at -80° C until assays were performed. In Trials 2 and 3, immediately after removal, the brains were frozen on dry ice and stored at -80° C to be processed at the time the assays were performed. Previous experiments showed no difference in results between frozen cytosol or cytosol prepared from frozen brains. Briefly, processing of whole brains to obtain the cytosolic fraction involves homogenization with TEMS buffer, incubation with dextran-coated charcoal (DCC) to strip endogenous steroids, followed by ultracentrifugation for 1 hr at 100,000 x g. The supernatant is referred to as the cytosolic fraction or cytosol. In each trial the brains from each treatment group were pooled to obtain a sufficient quantity of cytosol, with a protein content of 3.5 to 4.8 mg/ml, to perform saturation binding assays. The cytosol from the control group of Trial 1 was diluted with excess buffer during processing and had a lower protein content at 2.2 mg/ml.

Saturation binding analyses using [^{3}H] TA were performed as previously described (Knoebl *et al.*, in review) to determine binding characteristics. Cytosolic fractions were incubated with increasing concentrations of [^{3}H] TA (0.01 to 6 nM) with (non-specific binding) and without (total binding) 500 times molar excess of unlabeled TA. Specific binding was defined as the difference between total binding and non-specific binding at each [^{3}H] TA concentration. Binding data were analyzed by nonlinear regression of the untransformed specific binding data using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA) to obtain dissociation constants (K_d) and maximum number of binding sites (B_{max}).

Plasma cortisol determination. Plasma cortisol was measured by a [^{3}H] cortisol radioimmunoassay reported and validated by Redding *et al.* (1984).

Protein determination. The method of Bradford (1976) using bovine serum albumin (RIA grade, Sigma) as the reference protein was used.

Chemicals and reagents. [6,7-^{3}H] Triamcinolone acetonide ([^{3}H] TA, 43.8 Ci/mmol) and [1,2,6,7-^{3}H] hydrocortisone ([^{3}H] cortisol, 85.5 Ci/mmol) were purchased from DuPont NEN (Boston, MA). Budget-Solve scintillation cocktail (Research Products International, Mt. Pleasant, IL) was used for scintillation spectrophotometry. Radioinert TA and all other steroids and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

In all trials, plasma cortisol levels increased as the duration of confinement increased (fig. 3.1), indicating the fish were stressed. The severity of the stress imposed during trial 1 is reflected in the much higher plasma cortisol level of the confined fish compared to cortisol levels for trials 2 and 3. The concentration of binding sites available to bind [^{3}H] TA was substantially reduced with increased confinement stress (Table 3.1). In Trial 1, the concentration of binding sites for the controls and for the 0.5 hr treatment remain unchanged (19.4 vs. 17.9 fmol/mg protein) but decreased to 8.1 fmol/mg protein, less than one half of the control number, after 3 hr stress (fig. 3.2A). In Trial 2 (fig. 3.2B) and Trial 3 (fig. 3.2C) the concentration of binding sites decreased as the duration of confinement stress increased. An increase or decrease in the K_d's corresponding to stress is not apparent although in all cases the binding sites maintain a high affinity for [^{3}H] TA (Table 3.1).

In order to ensure that the decrease in specific binding was due to decreased total binding, not increased non-specific binding, we compared the total binding and non-specific binding within each trial. In all trials total binding decreases with increased duration of confinement stress (fig. 3.3). The non-specific binding for all treatment groups in Trial 2 do not differ from each other except for some variation for controls at the highest [^{3}H] TA concentration. In Trials 1 and 3 the non-specific binding data for the 0.5 hr and 3 hr stress groups within each trial do not differ. However, in these two trials the non-specific binding for the controls is higher than the stress groups within each assay.

Figure 3.1 Plasma cortisol content as determined by radioimmunoassay using [^{3}H] cortisol. Duration of stress refers to the time fish were contained within a confinement chamber. Trial 1 was performed in April 1993 (Control n=12, 0.5 Hr stress n=9, 3 Hr stress n=11). Trial 2 was performed in March 1995 (all groups n=4). Trial 3 was performed in April 1995 (all groups n=4).

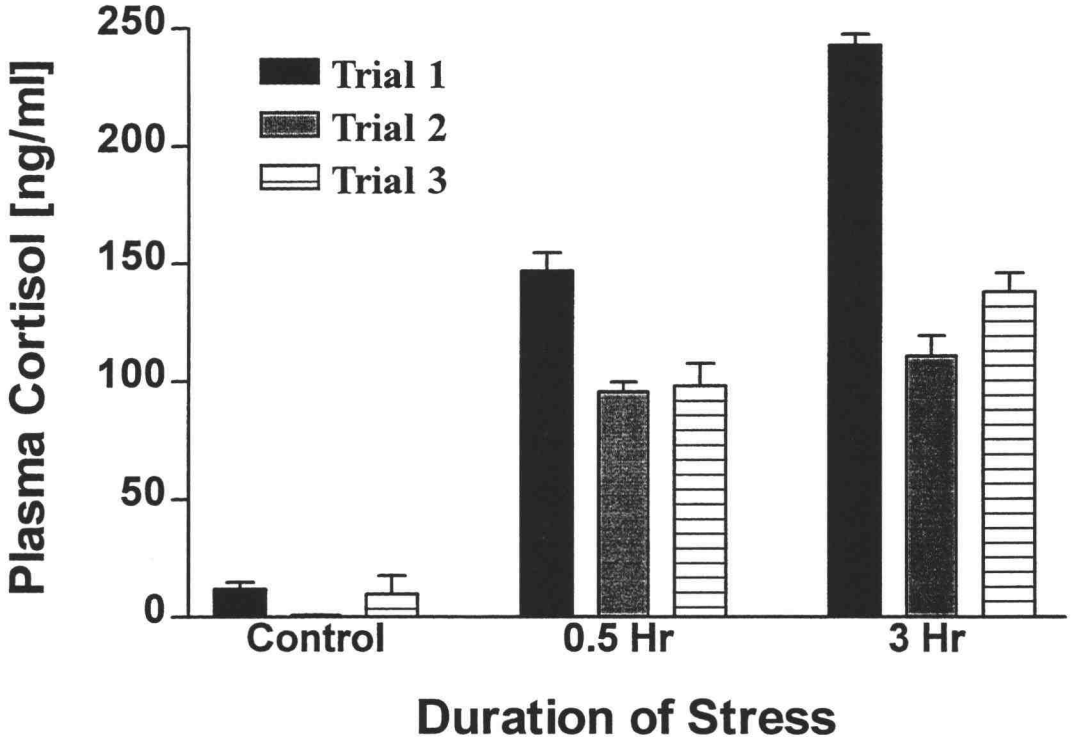
250
200
150
100
50
0
Plasma Cortisol [ng/ml]
Trial 1
Trial 2
Trial 3
Control
0.5 Hr
3 Hr
Duration of Stress

Fig. 3.1

Figure 3.2 Specific binding as determined by saturation analysis in pooled chinook whole-brain cytosolic extracts. Cytosolic extracts were incubated with increasing concentrations of [^{3}H] triamcinolone acetonide (TA) in the presence (non-specific binding) and absence (total binding) of 500 times molar excess unlabeled TA. Specific binding is calculated as the difference between total binding and non-specific binding and is shown as fmol/mg protein. The Scatchard replot of the specific binding data is shown beside each saturation analysis. (A) Trial 1 was performed in April 1993. (B) Trial 2 was performed in March 1995. (C) Trial 3 was performed in April 1995.

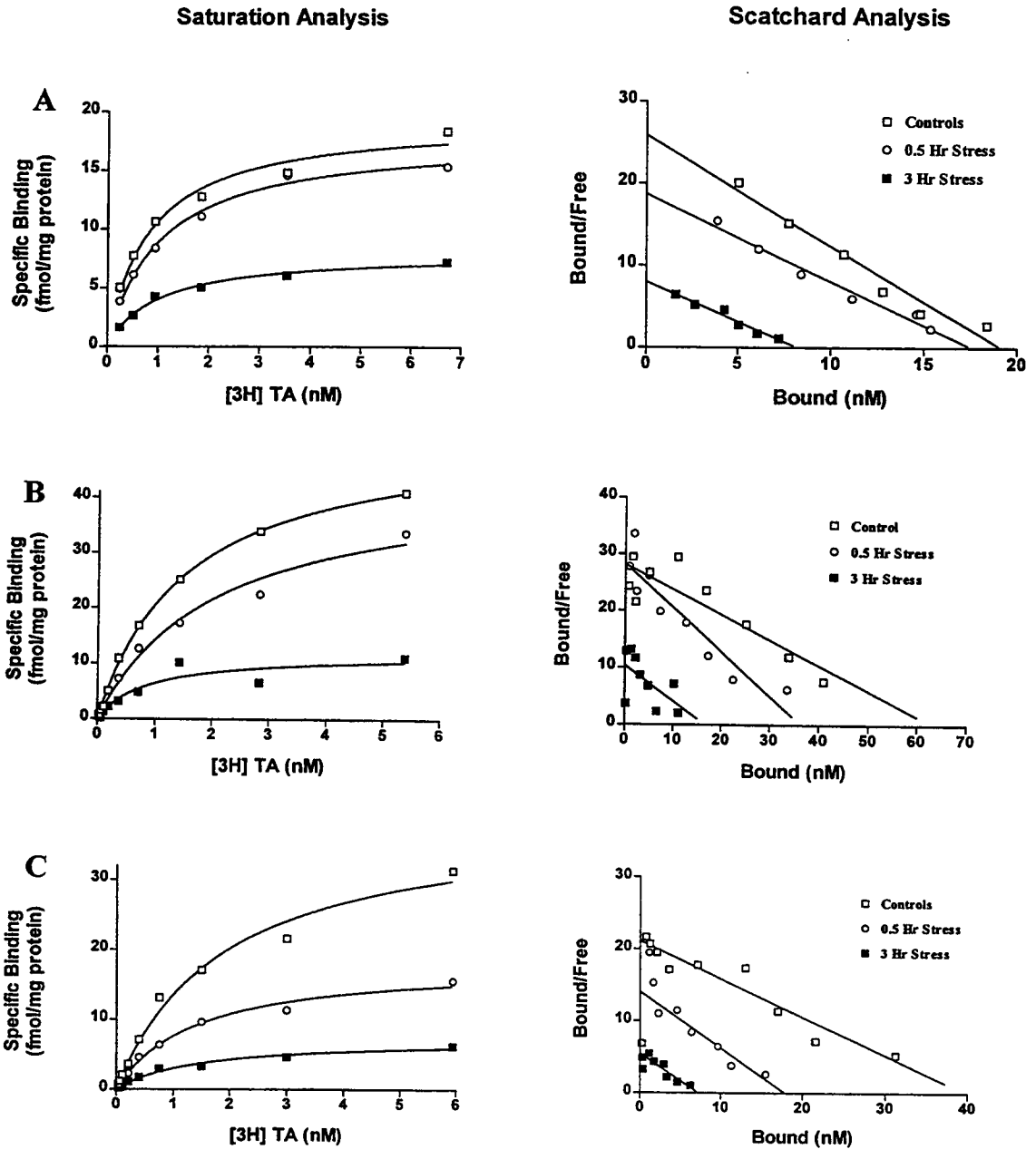
Saturation Analysis
Scatchard Analysis
A
Specific Binding (fmol/mg protein)
[3H] TA (nM)
Bound/Free
Bound (nM)
Controls
0.5 Hr Stress
3 Hr Stress
B
Specific Binding (fmol/mg protein)
[3H] TA (nM)
Bound/Free
Bound (nM)
Control
0.5 Hr Stress
3 Hr Stress
C
Specific Binding (fmol/mg protein)
[3H] TA (nM)
Bound/Free
Bound (nM)
Controls
0.5 Hr Stress
3 Hr Stress

Fig. 3.2

Table 3.1 Number of available glucocorticoid binding sites (B_{max}) and binding affinity (K_d) as determined by saturation analysis in pooled chinook whole-brain cytosolic extracts. Cytosolic extracts were incubated with increasing concentrations of [^{3}H] triamcinolone acetonide (TA) in the presence (non-specific binding) and absence (total binding) of 500 times molar excess unlabeled TA. Specific binding is calculated as the difference between total binding and non-specific binding and is shown as fmol/mg protein. (A) Trial 1 was performed in April 1993. (B) Trial 2 was performed in March 1995. (C) Trial 3 was performed in April 1995.

	Trial 1		Trial 2		Trial 3	
	Kd	Bmax	Kd	Bmax	Kd	Bmax
CONTROL	0.80	19.4	1.56	52.8	1.91	39.4
0.5 HR STRESS	1.01	17.9	2.10	44.2	1.39	18.3
3 HR STRESS	0.98	8.1	0.83	11.8	1.41	7.3

Figure 3.3 Total binding and non-specific binding data from saturation binding analyses of pooled chinook whole-brain cytosolic extracts. Cytosolic extracts were incubated with increasing concentrations of [^{3}H] triamcinolone acetonide (TA) plus 500 times molar excess unlabeled TA to obtain non-specific binding and without unlabeled TA to obtain total binding. (A) Trial 1 was performed in April 1993. (B) Trial 2 was performed in March 1995. (C) Trial 3 was performed in April 1995.

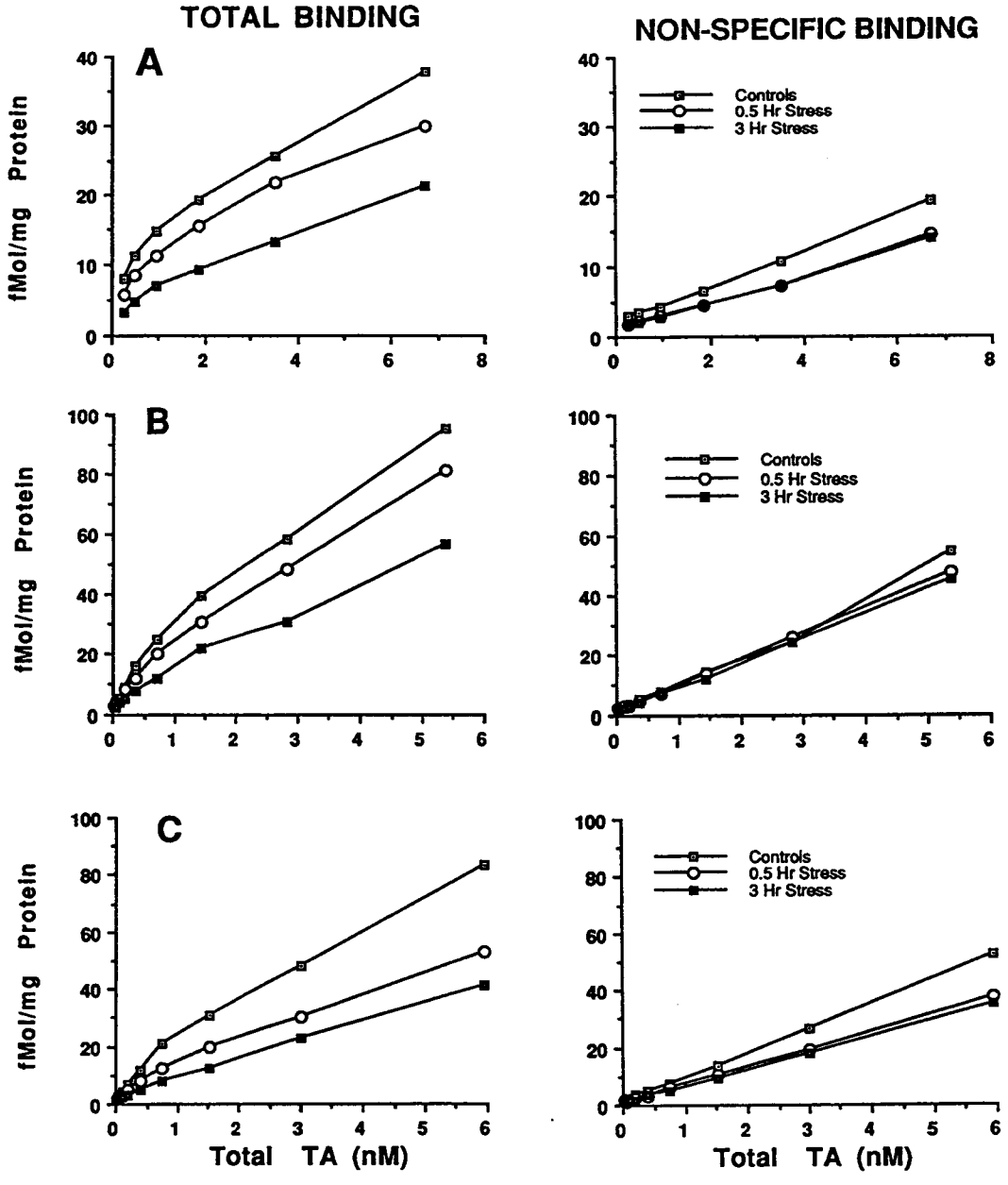
TOTAL BINDING
NON-SPECIFIC BINDING
A
B
C
fMol/mg Protein
Controls
0.5 Hr Stress
3 Hr Stress
Total TA (nM)

Fig. 3.3

Discussion

It is apparent from the results of these experiments that 3 hr of acute stress raises plasma cortisol but reduces from one to about five-fold, the concentration of glucocorticoid binding sites available. The decrease in ^{3}H-TA binding after stress is probably due to occupation and activation of receptors by endogenous cortisol, not to a reduction in actual cellular receptor concentration (down-regulation) which, in mammals, is believed to reach a minimum after 24 or more hours of glucocorticoid exposure (Svec and Rudis, 1981; McIntyre and Samuels, 1985). If activated glucocorticoid receptors in teleosts cannot rebind to another ligand, as in mammalian receptors (Chou and Luttge, 1988), only unactivated receptors will bind ^{3}H-TA in *in vitro* binding experiments. We did not measure the tissue cortisol content before processing and stripping of endogenous free steroids. However in control fish, a plasma cortisol level of about 10 ng/ml (28 nM) is a sufficiently high concentration to allow occupation of a significant portion of cortisol receptors which have a K_d of 4.5 nM (Knoebl *et al.*, in review).

Spencer *et al.* (1990) used rats 14-24 hr after adrenalectomy as indicators of *in vivo* steady-state receptor levels to compare receptor levels in intact unstressed, stressed and dexamethasone treated animals. Their studies indicated a decrease in type I receptor binding (using ^{3}H-aldosterone) and type II binding (using ^{3}H-dexamethasone) in both unstressed and stressed animals in various brain regions when compared to receptor numbers in adrenalectomized animals. They reported a 20% reduction in B_{max} of type II receptor binding in the hippocampus of intact, unstressed rats compared to adrenalectomized rats but no change in K_d. Rats subjected to 1 hr

confinement stress had a decrease in type II binding of about 40% and the K_d increased five-fold compared to adrenalectomized rats.

Cortisol has a five-fold lower affinity for the cytosolic binding sites than TA (Knoebl *et al.*, in review) and we allowed sufficient incubation time (overnight at 2° C) in our saturation assays for exchange of ^{3}H-TA with endogenous cortisol bound to unactivated receptors. The brain cytosol was also stripped of endogenous free steroid during processing, however we could not perform experiments using an adrenalectomized "control" group. Adrenalectomy is not possible in chinook salmon due to the location of the interrenal tissue in salmonids (Nandi, 1962). Consequently, the B_{max} values we obtained in these experiments may be lower than the true concentration of binding sites in the cytosol because some of the binding sites may have been activated by endogenous cortisol at the time the fish were killed.

The binding affinity in these experiments did not change as a result of the confinement stress. Maule and Schreck (1991) reported no change in affinity for glucocorticoid receptors in leukocytes of kidney and spleen or in gills after acute stress. However, a decline in the affinity of glucocorticoid receptors in other salmonid tissues after chronic stress has been reported (Maule and Schreck, 1991; Pottinger *et al.*, 1994).

Since specific binding is determined by the difference between total binding and non-specific binding, we examined whether the decrease in specific binding with increased stress was due to decreased total binding or increased non-specific binding within each experiment. In all trials total binding decreases in relation to the duration of stress. The non-specific binding component does not increase with stress, indicating the decrease in specific binding with increased stress is due to changes in the total binding.

These results indicate a reduction in the number of unbound or unactivated glucocorticoid binding sites in the brains of stressed chinook salmon. As the plasma cortisol levels increase, the high affinity binding sites most likely became increasingly bound and activated *in vivo* by the steroid, making them unavailable to bind with ^{3}H-TA in the *in vitro* binding studies. Acute stress and the rise in cortisol does appear to affect the number, but not the affinity, of the glucocorticoid binding sites previously identified and characterized (Knoebl *et al.*, in review).

IV. SUMMARY

The work presented in this thesis is a first step in elucidating the effects of stress on the brains of fish. We have characterized high affinity, low capacity glucocorticoid binding sites in non-adrenalectomized juvenile chinook salmon brain cytosol. In addition, we have demonstrated that acute stress affects the binding sites by lowering the number of sites available to bind steroid after exposure to acute stress. Our studies indicate no effect on binding affinity after acute stress.

The results of this research may begin to provide basic scientific information as to whether or not a parallel exits between the response to stress of mammalian and teleostean brains. The toxic effect of chronic stress on hippocampal neurons has been documented in mammalian brains (Sapolsky, 1985, 1986). Harmful effects of chronic stress and elevated plasma cortisol in fish, such as depressed immune response (Maule *et al.*, 1989), suppressed reproductive function (Carragher *et al.*, 1989) and reduced growth rate (Barton *et al.*, 1987) have also been documented. Further information regarding the functional consequences of stress in the brains of fish would be useful to both hatchery managers and researchers in explaining the effects of stress on fish.

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