To the Graduate Council:

I am submitting herewith a dissertation written by Colleen Caldwell Woodward entitled "Physiological Stress Response in Wild and Hatchery-reared Rainbow Trout". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Ecology.

Richard, J. Strange, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Provost
and Dean of The Graduate School

PHYSIOLOGICAL STRESS RESPONSES INCLUDING FREE AND PROTEIN-BOUND CORTISOL IN TWO STRAINS OF RAINBOW TROUT

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Colleen Caldwell Woodward

June 1988

Dedicated to a mooneye, Hiodon tergisus.
Long since passed, but not forgotten.

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ABSTRACT

The stress induced changes in plasma cortisol, glucose, and chloride were more extreme in wild rainbow trout (Salmo gairdneri) than in hatchery-reared rainbow trout subjected to severe short-term confinement in a net and electroshock. By 12 h of net confinement, plasma cortisol in wild fish increased from resting levels of 10 ng/mL to 480 ng/mL compared to hatchery-reared fish which increased from resting levels of 2 ng/mL to 155 ng/mL. Plasma glucose was higher in wild fish, increasing from resting levels of 55 mg/dL to 284 mg/dL compared to the increase in hatchery-reared fish from resting levels of 58 mg/dL to 196 mg/dL. Plasma concentrations of cortisol, glucose, and chloride were less altered in response to electroshock than they were in response to net confinement. One hour after galvano-narcosis, plasma cortisol concentrations in the wild fish peaked at 234 ng/mL and remained moderately elevated for 4 d; cortisol in hatchery-reared fish peaked at 70 ng/mL within 0.5 h of the electrical stimulus and then returned to resting levels within 1 h. No substantial changes in plasma glucose or plasma chloride occurred in either strain after galvano-narcosis.

Except for the initial disturbances, stress-induced changes in plasma cortisol, glucose, and chloride were only slightly varied throughout six weeks of long-term, moderate confinement for both strains. Distribution of plasma cortisol bound to a heat-labile carrier protein (corticosteroid binding-protein (CBP)) in both strains ranged

from 5 to 10% at prestress conditions. Cortisol bound to CBP increased to 32% in the wild fish and to 28% in the hatchery-reared fish by the end of con-finement; however, CBP in control hatchery-reared fish increased throughout the study. The distribution of plasma cortisol bound to a non heat-labile protein (albumin protein (Alb-P)) in both strains ranged from 35 to 45 % at prestress conditions, and fluctuated from 26 to 60 % throughout the confinement. The ratio of free to total cortisol concentration for both strains indicates that free cortisol is consistently and slightly less than one-half of total cortisol throughout the normal physiological range.

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CHAPTER I

INTRODUCTION

The growing concern for additional sources of food and increasing recreational demands has resulted in the expansion of fish production through aquaculture. Routine fish culture practices which result in handling, confinement, and water quality deterioration- to name just a few- potentially contribute to reduced growth, inhibited reproduction, and decreased survival. A major focus of fisheries research has been to delineate and minimize the deleterious effects common in intensive culture operations in order to insure the fishes well-being and ultimate marketability or survival upon release in the wild.

The Concept of Stress

The term stress can be translated into several contexual meanings when used at different levels of organization. To an ecologist, a stressor is a detrimental or disorganizing influence in an ecosystem. This influence is a variable which is foreign to that system or which is natural but occurs in amounts beyond the adaptability of the organism (Barrett et al. 1976; Odum 1985). To an ethologist, stress is the potential danger, which results in the activation and mobilization of the animal's reserves for coping with danger beyond their maximum efficiency (Archer 1979). And, to a physiologist, stress results in physiological compensation that is found toward one end of a continuum

of adaptive responses to internal or external stimuli (Greenberg 1986). Similarly, Selye (1973) stated stress is the sum of all the non-specific responses of the body to any demand made upon it. And regardless of the context, Pickering (1981) best summarized all biological definitions of stress having in common some type of stimulus that acts upon the biological system and results in a compensatory response. The term "stress" will be used to delineate a response in which the organism tries to maintain homeostasis against an external abiotic or biotic force. The challenge, or stimulus, will be designated as the stressor while stress will be the response to the stressor. An acute stressor means the stimulus will be removed before the response of the organism is complete. And a stressor which is long-term (chronic) and sublethal is a stimulus or a variety of stimuli that persists long after the organism has responded. The incipiency of a chronic stressor will result in the organism eventually adapting, but at a reduced level, where behavior, reproduction, and growth will eventually become impaired (Billard et al. 1981; Schreck 1981; Wedemeyer et al. 1984).

The General Stress Response

In the 1950's, Selye developed the first comprehensive theory which facilited the assessment of a generalized stress response called the General Adaptation Syndrome. The model describes an organism's response to a stressor in three phases: (1) the alarm or emergency reaction where there is an initial activation of the body's defense mechanisms characterized by the release of catecholamines and corticosteroids; (2)

the stage of resistance when the organism adapts to the stressor characterized by increased activity of the hypothalamic-pituitary adrenal axis and mobilization of energy reserves; and (3) the stage of exhaustion when the prolonged effects of stress are characterized by the suppresion of the immune system and thus increased susceptibility to disease.

These stress-induced physiological alterations have been recognized in fish as well (Wedemeyer 1970; Mazeaud et al. 1977; Wedemeyer and McLeay 1981). Current fisheries research has focused on the effects of intensive fish culture and management practices which include handling, hauling, and confinement. The primary stress response in fish, analogous to Selye's "alarm reaction" in higher vertebrates, has been characterized by an initial increase in plasma catecholamines (Nakano and Tomlinson 1967; Mazeaud et al. 1977), and elevated corticosteroids (Strange et al. 1977; 1978; Barton et al. 1980; 1986; Specker and Schreck 1980; Barton and Peter 1982; Pickering et al. 1982; Barton and Schreck 1987). Secondary responses in fish, analogous to Selye's "stage of resistance", has been characterized by changes in blood chemistry such as increased plasma glucose concentrations (Nakano and Tomlinson 1967; Houston et al. 1971; Wedemeyer 1972, 1976; Casillas and Smith 1977; Nikinmaa et al. 1983 ; Barton et al. 1987), and electrolyte flux (Wedemeyer 1972,1976; Nikinmaa et al. 1983). And subsequently, the tertiary response in fish, analogous to Selye's "stage of exhaustion", has been characterized by the suppresion of the immune system and increased susceptibility to diseases (Pickering and Duston 1983;

Pickering 1984; Pickering and Pottinger 1985; Pickering and Pottinger 1987; Maule et al. 1987). These studies show that physiological responses to typical culture and management practices are good indicators of the fish's "well-being" (Moberg 1985).

Strain Differences

The rainbow trout (Salmo gairdneri) is a popular sport fish as well as a popular research animal. In addition, it is the most commonly cultured and widely introduced of all salmonids (Wolf and Rumsey 1985). Although the effects of stress on the physiology of domesticated stocks of hatchery-reared rainbow trout has been thoroughly investigated, the paucity of information comparing the domesticated stocks to stocks of wild fish is conflicting. Mazeaud et al. (1977) believed domesticated fish have been selected over generations for specific traits such as a poor responsiveness to stressors prevalent in the hatchery environment. Genetic and environmentally induced adaptations to domestication results in the modified behavior of the captive animal to certain forms of repeated external stimuli (Price 1985). In contrast, Wydoski et al. (1976) demonstrated more severe blood chemistry disturbances in hatchery-reared rainbow trout than their wild counterparts when subjected to hook and line stress. And similarly, Casillas and Smith (1977) found that the wild rainbow trout recovered more quickly than the domesticated rainbow trout when subjected to hook and line stress.

Functional Aspects of Carrier Proteins

In order to obtain a better understanding of the dynamics of a steroid, it is important to take into account not only the measure of its total concentration (which does not necessarily reflect endogenous cortisol utilization), but to study the transport and metabolism of the steroid (Mills 1962). Extensive literature reviews concerning the physical and chemical properties of steroid-binding proteins in mammalian systems have accumulated in recent years (Burton and Westphal 1972; Wagner 1978; Ballard 1979; Siiteri et al. 1982; Westphal 1983; Englebienne 1985). Although the majority of available information is from mammalian systems and mammals are physiologically different from fish, the basic principles reviewed here are likely to be similar. The prevailing dogma of mammalian steroid hormone dynamics is that the steroid exists in either unbound or protein-bound fractions. In an active state, the unbound steroid is able to diffuse into the target cells. Steroids which are bound to proteins, such as the high affinity-low capacity binding protein (ie., corticosteroid-binding globulin- CBG) and the low affinity- high capacity protein (albumin), function to protect the steroid from metabolism and enzymatic degradation. In addition to protection against degradation, the protein-steroid complexes are similar to reservoirs where the inactive steroid constitutes an available pool for later use. The equilibrium dynamics of the free steroid fraction are reviewed by Englebienne (1985). As the free steroid is removed by the target tissue, the equilibrium between

the bound and free steroid fraction is disrupted. Albumin, because of its low affinity, will be the first to release its steroid to restore equilibrium between the bound and free steroid fraction. Thus, the binding proteins indirectly influence the biological activity of the target tissue by controlling the steroid's availability. Changes in the concentration of binding proteins will influence not only the amount of unbound plasma steroids, but also the rate of uptake by the tissues. Therefore, in order to more fully understand the dynamics of a steroid, it is important to evaluate its distribution between the bound and unbound fractions.

Corticosteroid Dynamics in Fish

Fish have two binding protein systems of varying affinity and capacity analagous to mammalian CBG and albumin (Freeman and Idler 1971; Idler and Truscott 1972). In the first system, similar to mammalian CBG, the binding protein has a high affinity for cortisol. The binding affinity ranged from 6.0×10^6 L/M in Atlantic salmon (Freeman and Idler 1971) to 1.4×10^7 L/M in rainbow trout (Fostier and Breton 1975). This is in contrast to the higher binding affinity in humans (84×10^7 L/M (Pugeat et al. 1984). The second binding system in salmonids, analogous to human serum albumin, exhibits a low affinity ($2.2 - 5.3 \times 10^4$ L/M, Seal and Doe 1965) and non-specific binding for most, if not all, endogenous steroids (Englebienne 1985). Although it is apparent that fish have two binding systems for corticosteroids, there is no evidence of the same high affinity mammalian corticosteroid binding-

protein. The binding capacity, protein-bound corticosteroid, ranges in fish from 30-55 % while the remaining steroid is free (Idler and Freeman 1968; Nichols and Weisbart 1985). This contrasts the binding capacity in humans where 83-98 % of the circulating corticosteroid is bound to proteins while the remaining steroid is free (Sandberg and Slaunwhite 1959; Robin et al. 1978; Dunn et al. 1981). Yet, salmonids have relatively high stress levels of cortisol concentrations (500 ng/mL, Strange et al. 1977; 650 ng/mL, Pickering and Christie 1981) when compared to humans (100-200 ng/mL, Freeman and Idler 1966). Research in which the dynamics of the corticosteroid binding-proteins and the free steroid during a stress response have not been observed in fish.

Objectives

The objectives of this research were to characterize the magnitude and duration of the primary and secondary physiological responses to stress in hatchery-reared and wild rainbow trout. Both stocks of fish were subjected to standard culture and management techniques of electroshock, acute net confinement, and moderate long-term cage confinement. Plasma cortisol was measured as an indicator of the primary physiological response to stress. Plasma glucose was measured as a secondary indicator of compensatory metabolic changes and plasma chloride was measured as an indicator of osmoregulatory dysfunction. Total cortisol concentration was evaluated with respect to changes in the distribution of free cortisol and cortisol bound to proteins during long-term confinement stress in both fishes.

CHAPTER II

PHYSIOLOGICAL STRESS RESPONSES IN WILD AND HATCHERY-REARED RAINBOW TROUT

INTRODUCTION

Physiological stress responses are well-documented in salmonids subjected to handling (Wedemeyer 1972; 1976; Strange et al. 1977; Barton et al. 1980; Pickering et al. 1982), confinement (Strange et al. 1978; Davis and Parker 1983), and electroshock (Schreck et al. 1976; Bouck et al. 1978). The similar response to a variety of stressors, expressed as changes in physiology, is associated with the General Adaptation Syndrome (Selye 1973). One primary physiological response to a stressor is the release of corticosteroids from the interrenal tissue which, in turn, induces secondary metabolic changes such as increases in plasma glucose (Leach and Taylor 1980) and osmoregulatory compensation (Nikinmaa et al. 1983; Redding and Schreck 1983).

Through generations of selection of rainbow trout, fish culturists have developed a fish that differs from its wild counterpart in behavior (Flick and Webster 1964) and physiology (Vincent 1960; Dickson and Kramer 1971). The primary objective was to compare and contrast the magnitude and duration of physiological stress responses in hatchery-reared and wild rainbow trout subjected to standard fish culture and management techniques such as electroshock and confinement in a net. Previous direct comparisons of the stress response between wild and

domestic fish involving hook-and-line stress measured only secondary metabolic responses (Wydoski et al. 1976; Casillas and Smith 1977). Cortisol, one of the major corticosteroids in teleostean fishes (Idler and Truscott 1972), was measured to evaluate the primary physiological response to stress. Plasma glucose was used as an indicator of the secondary metabolic responses and plasma chloride was used as an indicator of osmoregulatory dysfunction.

METHODS

Wild rainbow trout (117-185 mm total length TL) were collected from March through April by backpack electrofishing (220 V) from the Oconoluftee River and the Little River in the Great Smoky Mountain National Park, Tennessee (14-17 $^{\circ}$ C; 6.8-7.0 pH; conductivity 20-25 umos/cm). Domestic rainbow trout (160-211 mm TL) were obtained from Tennessee Wildlife Resource Agency's Buffalo Springs State Hatchery (18° C; 7.1-7.3 pH; conductivity 300 umos/cm). All fish were acclimated for 5-6 weeks under 12:12 photoperiod in circular flow-through tanks (890 L; flow rate 1.5 L/min) supplied with carbon-filtered tap water (15-16° C; dissolved oxygen 9.4 mg/L; 7.0 pH; conductivity 45-50 umos/cm). Both fish groups were maintained on daily rations of beef liver (all except hatchery-reared fish which were maintained on pelleted Purina trout chow before the electroshock experiment). Food was withheld from experimental and control fish 24 h before treatments.

Confinement stress was evaluated by placing 35 fish in (65 cm x 95

cm) dipnets immersed 5 cm below the surface. Sufficient water flow and aeration was provided to prevent additional stress through water quality deterioration and oxygen depletion. Five fish of each type were sampled prior to being placed in the net (time 0), and, at 0.5, 1, 3, and 12 h after net confinement. The dimensions of the net was adjusted as fish were removed to maintain a similar density. At 12 h, the remaining fish were released from the net and sampled at 1, 3, and 14 d during recovery. All fish were sampled once.

Electroshock stress was induced with a portable backpack electrofishing unit, used to deliver 110 V until all fish exhibited galvanonarcosis (< 5 s). Five fish were removed from the tank before recovering from paralysis (time 0) and bled. Fish were sampled at 0.5, 1, 3, and 12 h, 1, 2, 4, and 7 d after electroshock. Samples representing controls were collected from an adjacent tank that did not receive an electrical stimulus.

Fish were anesthetized in 100 mg/L of unbuffered tricaine methane sulfonate (MS-222), and all blood samples were collected within 5 min of capture. Blood was collected with heparinized capillary tubes after severing the caudal peduncle. Plasma was obtained by centrifugation and stored at -20° C until assayed. Total length (mm), wet weight (g), and sex were recorded for each fish.

Plasma cortisol was quantified by radioimmunoassay similar to the procedure for the assay of cortisol in unextracted plasma (Foster and Dunn 1974). Rabbit antiserum (Cambridge Medical Diagnostics, Billerica, Massachusetts) prepared against cortisol-3-bovine serum albumin was

freshly prepared before each assay from a 1:300 dilution previously stored at -80° C in phosphate buffer. Unknown plasma samples (10 uL) were diluted with phosphate buffer saline (0.1 M, 7.4 pH) containing 0.1% gelatin to give a final solution volume of 500 uL. A cortisol standard curve was similarly constructed with a range of 0.0-1.0 ng/mL (Sigma Chemical, St. Louis, Missouri). Tubes containing plasma unknowns and standards were heat treated (70° C, 1 h) in a water bath to denature serum binding proteins. Once tubes had cooled to ambient temperature, 100 uL [1,2^{3}H] cortisol (1.02 x 10^{6}DPM, New England Nuclear, Boston, Massachusetts) and 100 uL antibody were added, the mixture was vortexed and allowed to equilibrate for 16 h at 4° C. After equilibration, unbound steroid was removed by the addition of chilled Dextran-charcoal suspension (200 uL, 0.1% Dextran, 10% charcoal in 0.2 M phosphate buffer, 7.4 pH). The mixture was vortexed, incubated at 4° C for 20 min, and centrifuged (15 min, 2500 x g, 4° C). The supernatant was added to 4.0 mL of scintillation counting reagent (Scintiverse II), and counted on a beta liquid scintillation counter. Data were transformed to a log-logit scale. Cross reactivity (measured at 50 % displacement of bound [^{3}H] cortisol) was determined in our laboratory for nine steroids. The steroids giving greater than 0.1 % cross reactivity with the cortisol antibody were corticosterone (14 %), α-17 hydroxyprogesterone (2 %), and cortisone, a major corticosteroid in teleostean fish (0.4 %). The intra- and interassay coefficient of variation (CV) of cortisol measured in pooled samples of trout plasma was 5.8 % and 9.9 % (n=3), respectively. The accuracy of the RIA was

determined by recovery of exogenous cortisol in pooled rainbow trout plasma. The concentrations of cortisol added to the pooled plasma were 0, 50, 100, 300, 500, and 600 ng/mL and the amounts recovered were 25.1 ± 1.42 (X ± SE, n=4), 61.8 ± 0.84, 111.9 ± 5.17, 328.3 ± 16.5, 493.9 ± 22.56, 557.5 ± 18.4, respectively.

Glucose concentrations were determined on 10 uL of plasma using an assay (Sigma Chemical) based on glucose oxidase and peroxidase, with o-dianisidine as the chromagenic oxygen receptor. Chloride concentrations were determined on 5 uL of plasma using an assay (Sigma Chemical) based on the combination of chloride with mercuric ions to liberate thiocyanate ions that form a colored complex with ferric ions. Student's t-test ($P \leq 0.05$) was used to test for significant differences between control and experimental groups.

RESULTS

Confinement in a Net

Plasma cortisol concentrations in the wild rainbow trout were more than double (402 ng/mL ± SE 75.2) and triple (480 ng/mL ± 130.4) the cortisol values of the hatchery fish (155 ng/mL ± 20.2 and 155 ng/mL ± 15.0) by 6 and 12 h, respectively (Figure 1a). Resting levels for plasma cortisol concentrations in wild and hatchery-reared rainbow trout were 10 ng/mL (± 6.8) and 2 ng/mL (± 0.8), respectively. Plasma glucose levels increased to 284 mg/dL (± 86.4) from resting levels of 55 mg/dL (± 5.5) in the wild fish and remained elevated until release from the net at 12 h (Figure 1b). Plasma glucose in the hatchery-reared fish

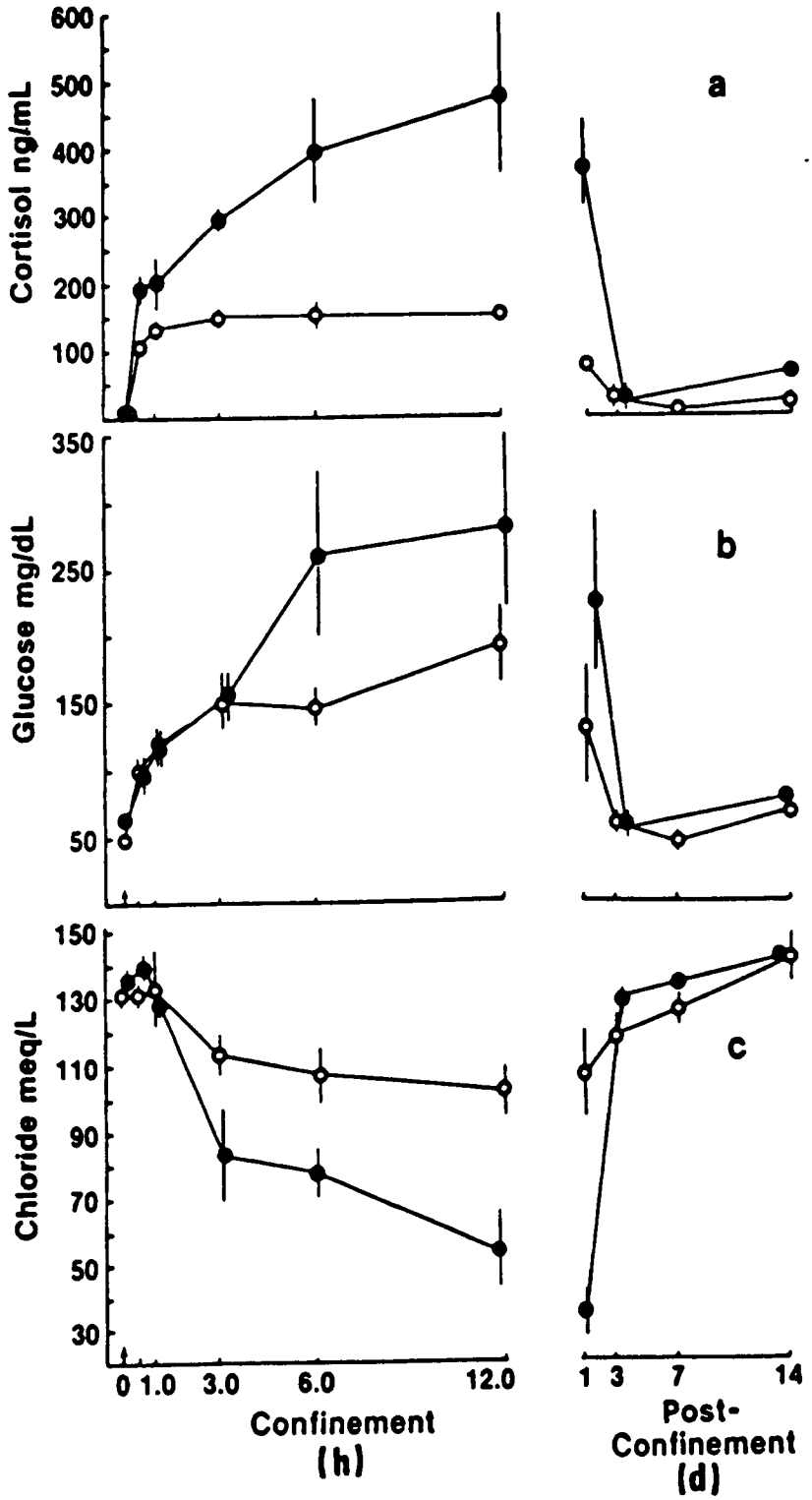

Figure 1. Concentrations of plasma cortisol (a), glucose (b), and chloride (c) during and after 12 h of confinement in a net for hatchery-reared (open circles) and wild rainbow trout (solid circles). Points are means (± SE) (n=5), except for wild fish on day 14 (n=1).

responded similarly (from 50 mg/dL ± 0.8 to 196 mg/dL ± 25.4), but to a lesser degree. In addition, the wild fish exhibited significantly depressed plasma chloride concentrations (from 135 meq/L ± 3.9 to 33 meq/L ± 4.1) that persisted until 24 h after removal from the net (Figure 1c). Plasma chloride concentrations decreased only slightly (from 132 meq/L ± 3.4 to 107 meq/L ± 13.0) in the hatchery-reared fish.

Fish were released from the net after 12 h to assess recovery. Three days later, plasma cortisol, glucose, and chloride had returned to resting levels in both groups. Hatchery-reared fish began feeding almost immediately after release from the net, whereas wild fish did not resume feeding until 3 days after release. Wild rainbow trout experienced 12.5 % mortality whereas no deaths occurred in the hatchery fish. As a result of mortality of wild fish, samples were not collected at day 7 during recovery. The sample at day 14 for wild fish consisted of one individual.

Electroshock

At time 0, electroshocked fish of both groups were not significantly different from control fish in plasma cortisol, glucose, or chloride (Figure 2a,b,c). Plasma cortisol in the wild fish peaked at 1 h (234 ng/mL ± 54.0) after electroshock and remained elevated above resting levels throughout recovery, although control values at day 1 and beyond were also slightly elevated. Plasma cortisol in hatchery-reared rainbow trout peaked at 0.5 h (70 ng/mL ± 7.5) and returned to resting levels at 1 h. Plasma cortisol in electroshocked fish was

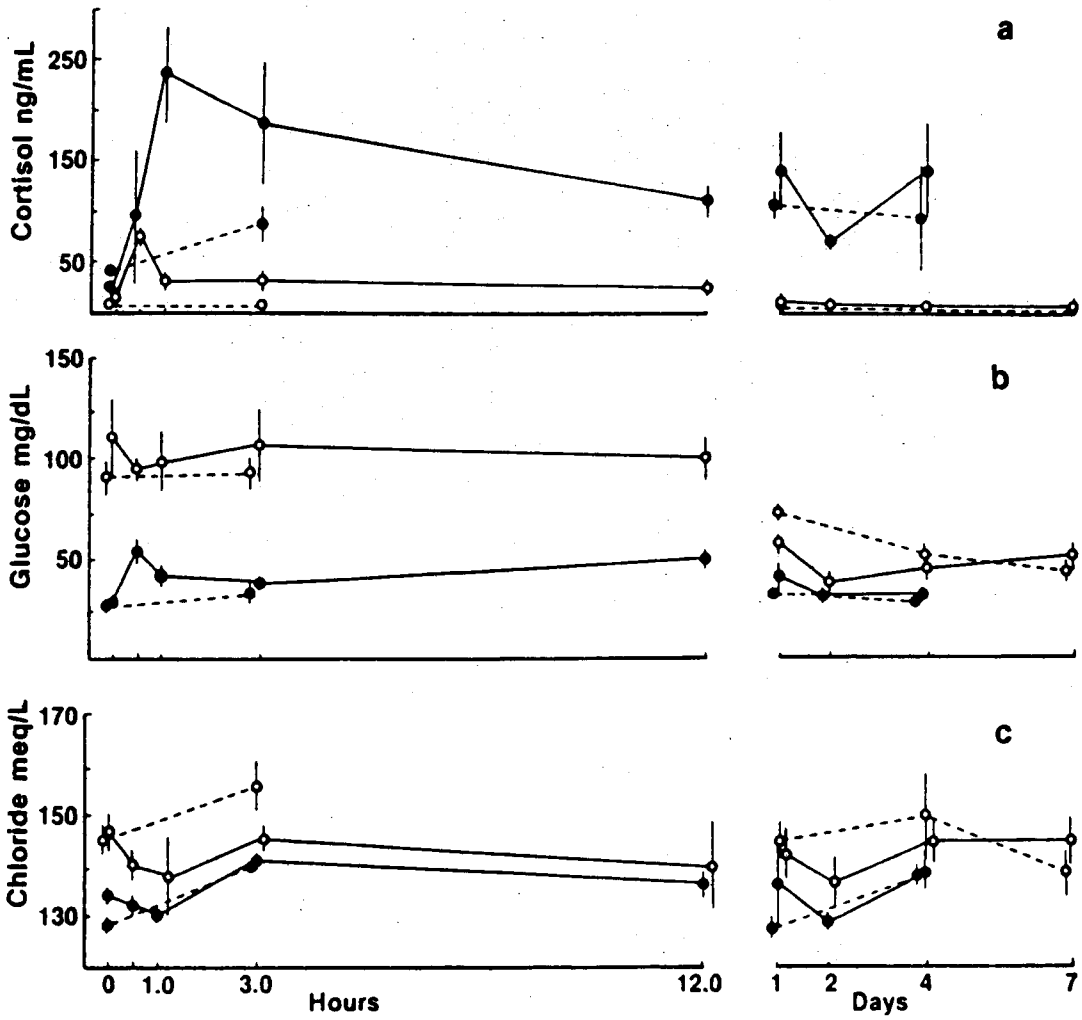

Figure 2. Concentrations of plasma cortisol (a), glucose (b), and chloride (c) immediately after electroshock stimulus (time 0) and for 7 days thereafter for hatchery-reared (open circles) and wild rainbow trout (solid circles). Dashed lines represent control fish not subjected to electroshock. Points are means (± SE) (n=5).

significantly different between wild and hatchery-reared fish at 1 h. Hatchery rainbow trout had consistently higher plasma glucose values than did the wild fish in both experimental and control groups. No significant changes were observed in plasma chloride. Some mortality (5.5 %) was exhibited in the wild fish, but none occurred in the hatchery-reared rainbow trout. Both groups of fish resumed feeding within day 1 of the electroshock treatment.

DISCUSSION

Distinctive physiological differences were found between wild and hatchery stocks of rainbow trout subjected to severe confinement and electroshock. During confinement, wild fish exhibited more pronounced changes in plasma cortisol, glucose, and chloride concentrations than did hatchery-reared fish. Despite mortality in the wild fish, the return of plasma values to prestress levels by 3 d post-confinement indicated that recovery was complete. Investigators who have measured changes in plasma cortisol (Wedemeyer and Chatterton 1970; Barton et al. 1980; Davis and Parker 1983), plasma glucose (Wedemeyer 1976; Casillas and Smith 1977), and plasma chloride (Wedemeyer 1972; Davis and Parker 1982) in hatchery-reared rainbow trout during net confinement have reported similar values to those found in this study for the domestic fish. The severe hypochloremia, in addition to the magnitude in elevation for cortisol and glucose in the wild fish, indicated that the wild fish were more susceptible to stress than were the hatchery-reared fish. No previous studies could be found that have shown mean plasma

cortisol concentrations in rainbow trout as high as were observed in this study for the confined wild fish.

As a result of galvano-narcosis, both types of fish exhibited elevated plasma cortisol within 30 min of the stimulus. Plasma cortisol returned to almost resting levels within the hour in the hatchery-reared fish but continued to increase in the wild fish and remained elevated above their baseline values throughout the remainder of the experiment. Leach and Taylor (1980) determined that electroshock induced plasma cortisol values in the mummichog, Fundulus heteroclitus, similar to those observed in this study for wild rainbow trout. Contrary to Schreck et al. (1976), the electrical stimulus did not elicit an immediate response in plasma cortisol or glucose for either group of fishes. Plasma cortisol in the control wild fish was slightly elevated after the 3 h sample. This may have been due to disturbances caused by periodic sampling from the same tank, with brief chasing perhaps inducing persistent elevated cortisol concentrations in the wild fish. Others have reported that such repeated sampling is not a sufficient stressor to induce blood chemistry changes (Barton et al. 1980; Barton et al. 1986), but the subjects were hatchery fish. The difference in plasma glucose concentrations between wild and hatchery-reared rainbow trout in the electroshock experiment might be related to the differences in nutritional status due to a greater percentage of carbohydrate in the pelleted feed fed hatchery-reared fish compared to the beef liver fed wild fish (Nakano and Tomlinson 1967; Barton et al. 1986).

Ionoregulatory mechanisms as measured by plasma chloride changes

were not affected in either fish stock as a result of the stimulus. Apparently, electroshock is not sufficiently severe to compromise osmoregulation. While mortality induced by electroshock may have been the result of fractured vertebrae, hemorrhaging, or respiratory failure (Spencer 1967; Schreck et al. 1976; Hudy 1985), it was not due to hemodilution which apparently killed the wild trout in the net confinement experiment.

Years of artificial selection have resulted in the divergence of growth rate (Reisenbichler and McIntyre 1977; Rosentreter 1977), ecology (Leider et al. 1986), and genetic variability (Allendorf and Phelps 1980) between cultured and wild fish. Results of this study indicate that primary and secondary differences in physiological responses to stress do, in fact, exist. However, in a comparison of wild and hatchery-reared rainbow trout subjected to hook and line stress, Wydoski et al. (1976) determined that hatchery fish developed more severe hyperglycemia, in contrast to these results. Wydoski et al. (1976) theorized that the wild fish were more physically fit and therefore more able to deal with the acute stressor than their hatchery-reared counterparts. In addition, Casillas and Smith (1977) determined that wild rainbow trout required less time to recover from hook-and-line stress than did hatchery fish. This was in contrast to the wild fish in the net confinement experiment which recovered almost as quickly from much greater physiological excursions as did the hatchery fish. The differences observed in this study for net confinement compared to the earlier hook-and-line studies may be due to the greater severity of net

confinement and its longer duration as a stressor.

Others who have found variations in plasma cortisol concentrations among salmonid stocks have attributed the differences either to life history or genetics (Barton et al. 1986). Intensively cultured fish are inadvertently and intentionally selected for their insensitivity to stressors. Specific traits that are desirable in hatchery systems, such as attenuated lipid mobilization and hyperglycemia, could be selected for in particular strains of rainbow trout (Mazeaud et al. 1977). Selections for increased growth rate, increased fecundity, and modification of behavior are routine in current hatchery practices.

Increased concentrations of plasma cortisol and glucose observed in this study for wild rainbow trout subjected to a severe stressor (net confinement) may be somewhat advantageous. Elevated levels of corticosteroids and blood sugar may allow the wild fish to cope with life-threatening situations such as predation, competition, and periodic perturbations in the environment that are absent in hatcheries. In a comparative analysis, the response of a species should be investigated before its use as an experimental animal. Based on the results of this study, it is recommended that investigators consider the origin of their fish stock in their interpretation of physiological responses to stress.

CHAPTER III

FREE AND PROTEIN-BOUND CORTISOL IN WILD AND HATCHERY-REARED RAINBOW TROUT SUBJECTED TO CHRONIC STRESS

INTRODUCTION

The Chronic Stress Response

The dynamics of the physiological stress response have undergone extensive evaluation in recent years. Since Selye's pioneering work of the General Adaptation Syndrome in the 1950's, the physiological stress response has been used in fish research as an index of the fish's ability to maintain homeostasis in the face of stressful environmental stimuli. One of the primary responses to stress is the increase in adrenocortical activity. Although cortisol is widely used as an indicator of the primary stress response in fish, it typically reflects severe short-term effects and is not a good indicator of chronic sublethal stress (Strange et al. 1978; Pickering and Stewart 1984; Pickering and Pottinger 1987). Increased cortisol concentrations feed back to the hypothalamus which results in a reduction of adrenocorticotrophic hormone (ACTH) output and consequently a reduction in the rate of synthesis and secretion of cortisol from the interrenal tissue (Donaldson 1981). Chronic stress is the result of persistent environmental stimuli in which the fish either adapts or succumbs. If the fish adapts to the stimulus, it will be able to sustain itself, but

at the cost of other physiological processes such as reduced growth (Pickering and Stewart 1984; Davis et al. 1985; Barton et al. 1987), reproductive inhibition (Andrews 1979), and decreased immune resistance and thus increased susceptibility to diseases and pathogens (Pickering and Duston 1983; Pickering and Pottinger 1985, 1987; Maule et al. 1987).

Physiological Aspects of Carrier Proteins

Virtually all mammalian species and many non-mammalian vertebrates possess specific protein systems for the transport of plasma steroids (Seal and Doe 1965; Martin 1980). Since their discovery in the 1930's it had been assumed the carrier proteins acted as a means of transport for the steroids to target cells. The steroid-protein complexes serve as inert reservoirs protecting the steroids from enzymatic action and metabolism, until by dissociation, the steroid becomes physiologically available at receptor sites of target cells (Sandberg and Slaunwhite 1959; Hoffman et al. 1969). In addition, carrier proteins function as buffers by mediating the steroids availability to the receptors (Slaunwhite et al. 1962; Tait and Burstein 1964); therefore, the biological activity of the plasma steroids is not dependent on its total concentration but rather on the concentration in its unbound state.

Cortisol is quantitatively the most important steroid of the corticosteroid-binding globulin (CBG) in man (Dunn et al. 1981), higher primates (Pugeat et al. 1984), guinea pig (Mickelson et al. 1981), and fish (Seal and Doe 1965; Freeman and Idler 1966; Idler and Freeman 1968; Idler and Truscott 1972; Goodman and Butler 1976). Although mammalian-

like CBG has not been identified in fish, two protein systems of varying affinity and capacity analagous to mammalian CBG and albumin have been demonstrated to occur in 20 species of fish (Seal and Doe 1965; Freeman and Idler 1966; Fostier and Breton 1975; Martin 1980; Nichols and Weisbart 1984; Nichols et al. 1985). The carrier protein, similar to CBG, has a high affinity but a low capacity for cortisol and is heat labile at 60 ^{o} C. The carrier protein, similar to human serum albumin, has a low affinity but high capacity and is not heat labile at 60 ^{o} C.

Little is known about the physiological mechanisms between binding proteins in fish and the cortisol stress response. While the salmonids have relatively higher stress-induced cortisol levels compared to man (Freeman and Idler 1966; Idler and Freeman 1968), the dynamics of the percent distribution of cortisol bound to CBG, and cortisol bound to albumin, are unknown as to whether the two binding systems provide protection for the fish at times of hypercorticism. Therefore, the ratio of free cortisol and protein-bound cortisol may have important implications in stress physiology research, especially since current methods of quantitation measure total steroid concentrations. Changes in total concentration may not neccesarily reflect changes in the free (physiologically active) fraction (Robin et al. 1978; Siiteri et al. 1982).

It was demonstrated in Chapter II that the wild and hatchery-reared rainbow trout exhibit distinctive physiological differences to severe short-term confinement and electroshock. One of the objectives of this study was to compare and contrast the magnitude and duration of the

stress response in wild and hatchery-reared rainbow trout subjected to chronic sublethal stress from long-term cage confinement and to evaluate the relationship between total plasma cortisol concentrations and the distribution of the binding-protein fractions in wild and hatchery-reared rainbow trout. Cortisol, the predominant corticosteroid in teleostean fishes (Idler and Truscott 1972), was measured to evaluate the primary physiological response to chronic stress and to evaluate changes with respect to the percent distribution of total cortisol bound to the CBG-like protein (CBP) and the albumin-like protein (Alb-P) and unbound (free) fractions. Plasma glucose was measured as an indicator of the secondary metabolic responses and plasma chloride was measured as an indicator of osmoregulatory dysfunction.

METHODS

Wild rainbow trout (126-275 mm total length, TL) were collected June 1987 by backpack elctrofishing (220 V) from the Little River in the Great Smoky Mountain National Park, Tennessee (16-18 $^{\circ}$ C; 6.8-7.0 pH; conductivity 20-25 umos/cm). Hatchery-reared rainbow trout (199-342 mm TL) were obtained April 1987 from Tennessee Wildlife Resource Agency's Buffalo Springs State Hatchery (18 $^{\circ}$ C; 7.1 pH; conductivity 300 umos/cm). All fish were acclimated 8 weeks in circular flow-through tanks (890 L; flow 1.0 L/min) supplied with carbon-filtered tap water (14-17 $^{\circ}$ C; dissolved oxygen 12 mg/L; 7.0 pH; conductivity 45-50 umos/cm); photoperiod was 12 h light:12 h darkness. Domestic rainbow trout were fed pelleted Purina trout chow and wild rainbow trout were fed daily

rations of beef liver. Food was withheld from experimental and control fish 48 h before treatments.

Confinement stress was evaluated by placing 100 fish into a small cage (40 x 55 x 70 cm) constructed of plastic pipe and 13 mm plastic mesh. The cage was suspended in the tank and adjusted as fish were sampled to maintain a consistent density of 1.7 kg/L at a flow rate of 4.2 L/min. The moderate confinement allowed the fish to move freely but maintained them in constant contact with one another. Sufficient water flow and aeration was provided to prevent additional stress through water quality deterioration and oxygen depletion. Five fish of each type were sampled before being placed into the cage (time 0), and at 6, 12 h, 1, 3, 6, 9±1, 12, 17±1, 22, 27±1, 32±1, 36, 40, 44, 49 and 56 d. As fish were dipnetted, care was taken to insure random sampling. Samples representing controls were collected from an adjacent tank in which fish were allowed to swim freely. Fish were sampled at 0900-1000 each sampling period to preclude diurnal and anticipatory feeding effects on plasma cortisol (Bry 1982; Pickering and Pottinger 1983). All fish were sampled once. Fish were anesthetized in 100 mg/L of tricaine methane sulfonate (MS-222), and all blood samples were collected within 5 min of removal from the cage. Blood was collected with a heparinized syringe at the base of the caudal tail. Plasma, obtained by centrifugation, was stored at -20 ^{o}C until assayed. Total length (mm), wet weight (g), and sex were recorded for each fish.

Plasma cortisol and plasma glucose were quantified as in Chapter II for unextracted fish plasma. Plasma chloride was quantified as in

Chapter II by colorimetric determination for the hatchery-reared fish. A cloudy precipitant was present in the plasma of the wild fish and would have interfered with the colorimetric determination. As a result, plasma chloride concentrations in the wild fish were determined by microtitration (Wedemeyer and Yasutake 1977). Duplicate samples of pooled fish plasma determined with the colorimetric and microtitration methods did not result in different chloride concentrations ($P < 0.005$; Product-moment correlation coefficient). The intra- and interassay coefficients of variation (CV=100 SD/mean, n=3) for the radioimmunoassay of plasma cortisol were 5.4 % and 9.9 %, respectively.

The percent distribution of cortisol bound to the heat labile CBP, bound to Alb-P, and free was determined by centrifugal ultrafiltration isodialysis developed by Hammond et al. (1980) for small volumes of plasma. The percent distribution of the binding protein is assessed based on the amount of radiolabeled steroid added in excess that binds to the protein. Thus the amount of the radiolabeled steroid attached to the binding site of the protein molecule will equal that of the protein at saturating conditions. Aliquots of [1,2^{3}H] cortisol (3×10^5 dpm; 51.9 Ci/mmole, New England Nuclear, Boston, Massachusetts) were added to 5 uL of [^{14}C] glucose (12×10^3 dpm; 271 mCi/mmole, ICN Radiochemicals, Irvine, California). Prior to the assay, [1,2^{3}H] cortisol was purified on a Sephadex LH-20 column and eluted with 5.0 ml of chloroform:ethanol (95:5). The [^{14}C] glucose, which does not bind to free steroids but monitors the movement of unbound components across the dialysis membrane, was stored frozen and prepared fresh prior to each assay.

Distilled water (500 uL) was dialyzed with radioisotopes in place of the plasma to determine the occurrence of non-specific binding of steroids to the membrane.

To determine the percent free cortisol, native plasma (not heat-treated) was added to aliquots of [^{14}C] glucose and [1,2^{3}H] cortisol, vortexed, and incubated in a water bath at 37 $^{\circ}$ C for 30 min, and an additional 30 min at ambient temperature (20 $\pm$ 5 $^{\circ}$ C). Duplicate aliquots (200 uL) were pipetted into isodialysis capsules which were prepared up to one week prior to the assay and stored in 0.02 % sodium azide solution at 4 $^{\circ}$ C. After incubation, the isodialysis capsules containing radioisotopes and plasma were placed into 6 mL glass scintillation vials containing three paper filter discs (diameter 13 mm; Whatman No. 1), and centrifuged at 750 x g for 1 h at 37 $^{\circ}$ C. Hammond and his associates determined that during centrifugation the three paper filter discs absorbed 32 $\pm$ 2 uL of the ultrafiltrate under the dialysis membrane. Therefore, after centrifugation, 30 uL of the remaining plasma were pipetted out of the isodialysis capsule and placed onto a paper filter disc within a duplicate scintillation vial. Distilled water (350 uL) was added to the duplicate vials of (i) the three filter discs with approximately 30 uL of the absorbed ultrafiltrate, and (ii) duplicate vials containing one filter disc with the 30 uL of the incubated radioisotopes and plasma pipetted from the isodialysis capsule. The vials were vortexed and 3 mL of scintillation counting reagent (Scintiverse II) were added and counted in a beta liquid scintillation counter adjusted for the simultaneous counting of [^{3}H] and [^{14}C]

isotopes. The data were corrected for background counts for [^{3}H] and [^{14}C] spill in the liquid scintillation counting system. The percent free cortisol was calculated following Hammond et al. (1980).

The same procedure was repeated in duplicate with plasma heated at 60 C for 1 h in a water bath. Heat treatment presumably destroys the CBP and additional cortisol is unbound. Because the albumin-steroid complex is unaffected by the heat treatment, the measure of cortisol bound by CBP can be calculated as the difference in binding (as measured in CPM) in the unheated and heat inactivated plasma:

$$\frac{\text{\% free (heated)} - \text{\% free (unheated)}}{\text{\% free (heated)}} \times 100 = \text{\% bound to CBP}$$

The intra- and interassay coefficients of variation of the percent distribution of free cortisol before and after heat-treatment measured in pooled fish plasma were 7.2 and 9.5 % (n=4), respectively. The calculated value of the free cortisol in ng/mL represents the product of total cortisol (ng/mL) multiplied by the percent free cortisol to get the concentration of unbound cortisol.

Plasma collected from wild rainbow trout was pooled to provide sufficient volume of plasma for the assay. Wild rainbow trout were slightly smaller in size and less in condition than the hatchery-reared rainbow trout. The data were subjected to least squares analysis of variance for unequal subclasses (Personal Computer, SAS 6th Edition). Dependent variables (total cortisol, free cortisol, cortisol bound to CBP, and Alb-P, plasma glucose, plasma chloride) were tested for

significant differences against treatment levels (confined vs control), and fish type (wild vs hatchery-reared) over time (day). Residual mean separation procedures were determined on the main effects using Least Square Means (Personal Computer, SAS 6th Edition). The percent cortisol bound to CBP of mature female, mature male, and immature hatchery-reared rainbow trout was subjected to one-way analysis of variance (Zar 1984). The Student's t was used to test for significance of the regression coefficient (slope) for the simple linear regression of free cortisol concentrations on total cortisol concentrations of both wild and hatchery-reared rainbow trout (Zar 1984).

RESULTS

Primary and Secondary Responses

Chronic confinement resulted in few differences in primary and secondary physiological stress responses between the wild and hatchery-reared rainbow trout. Prestress plasma cortisol concentrations in wild fish were 60 ng/mL ($\pm$ 15.1) (mean $\pm$ SE) and in hatchery-reared fish were 27 ng/mL ($\pm$ 3.4) (Figure 3a). Within day 1 of confinement, plasma cortisol levels increased to 166 ng/mL ($\pm$ 43.5) ($P < 0.006$) and to 57 ng/mL ($\pm$ 7.5) ($P < 0.006$) in wild and hatchery-reared rainbow trout, respectively. Plasma cortisol concentrations in wild fish decreased to prestress levels by day 6 of confinement, and did not return to prestress levels in the hatchery-reared fish until day 28 of confinement. Plasma glucose concentrations in wild rainbow trout

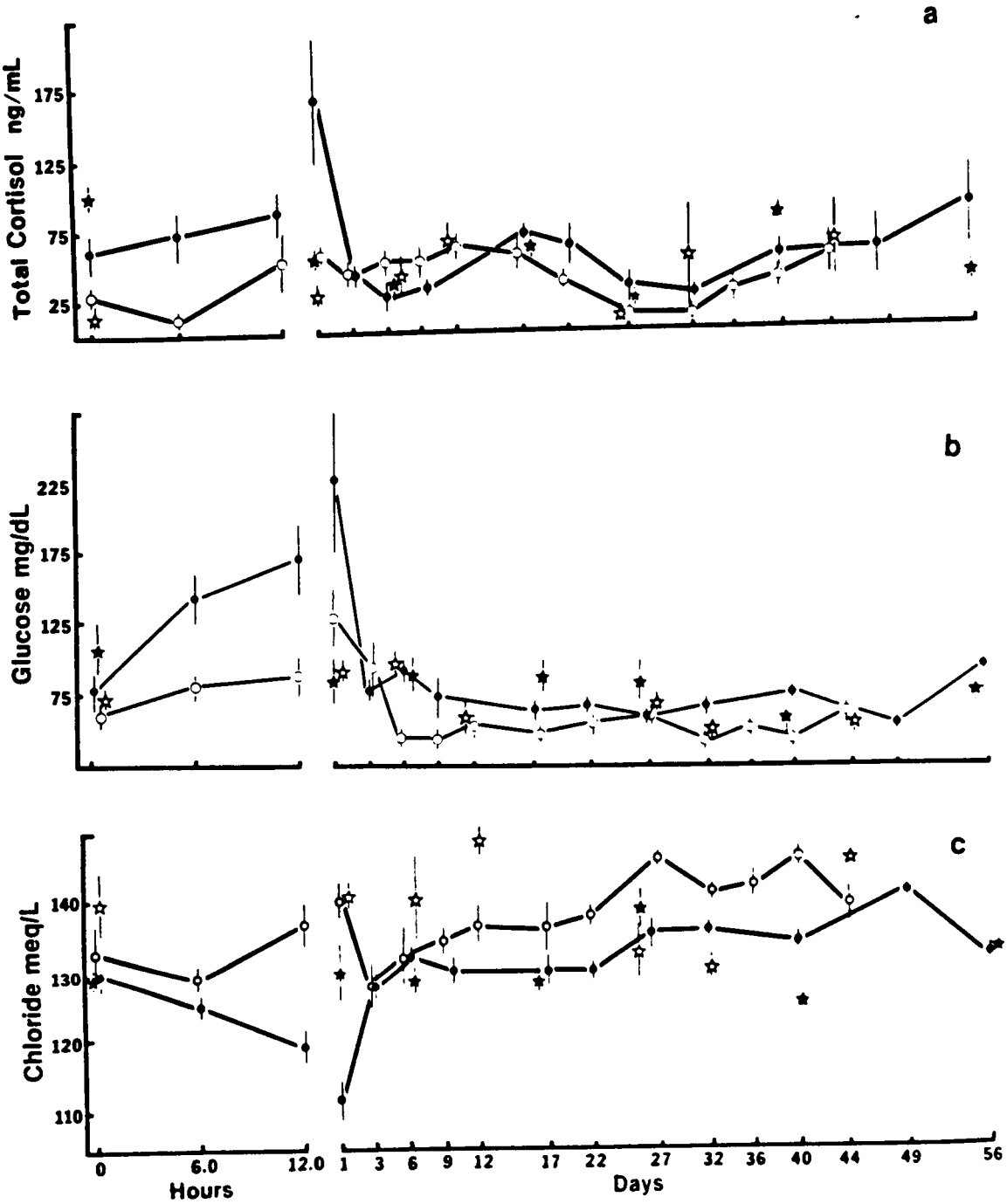

Figure 3. Concentrations of plasma cortisol (a), glucose (b), and chloride (c) of hatchery-reared rainbow trout subjected to long-term confinement (open circles), control hatchery-reared fish not subjected to confinement (open stars), and of wild rainbow trout subjected to long-term confinement (solid circles), control wild fish not subjected to confinement (solid stars). Points are means (± SE) (n=5).

increased from resting levels of 78 mg/dL (± 14.5) to 228 mg/dL (± 48.4) ($P < 0.006$) within day 1 of confinement; and plasma glucose concentrations in hatchery-reared rainbow trout increased from resting levels of 61 mg/dL (± 7.2) to 128 mg/dL (± 20.6) ($P < 0.1$) within day 1 of confinement (Figure 3b). Between day 3 and day 6, plasma glucose levels of both fish groups decreased to prestress levels and stayed down throughout the remainder of cage confinement. In addition, wild rainbow trout exhibited a decline in plasma chloride concentrations from resting levels of 131 meq/L (± 1.7) to 114 meq/L (± 4.4) within day 1 of confinement (Figure 3c) ($P < 0.001$). Conversely, plasma chloride values in hatchery-reared fish increased from resting levels of 133 meq/L (± 4.6) to 143 meq/L (±4.1) ($P < 0.1$) within day 1 of confinement, then decreased to prestress levels by day 3 ($P < 0.006$).

Three mortalities occurred within the first five days of confinement in the wild rainbow trout and four mortalities occurred as late as day 22 of confinement in the hatchery-reared fish. Extensive hemorrhaging was observed in abdominal viscera and gonadal tissue until day 6 in samples of wild fish and until day 22 in samples of hatchery-reared fish. Both experimental groups and their controls fed throughout the entire confinement study.

Distribution of Cortisol

Plasma from the wild rainbow trout (n=5) was pooled to provide a sufficient volume for both heated and unheated plasma in the percent distribution assay. The values for the distribution of unbound and

protein bound fractions of cortisol were highly variable in the wild fish throughout the confinement (Table 1). Values for free cortisol concentrations, the product of the percent free cortisol multiplied by total cortisol concentrations in wild rainbow trout, are presented in Table 2. The slope of the linear regression of free cortisol on total cortisol for wild fish was 0.36 and indicated that free cortisol remained slightly less than one-half the total cortisol throughout a range of normal concentrations ($P < 0.001$) (Figure 4a).

The free cortisol concentrations in hatcheryreared rainbow trout increased from resting levels of 13 ng/mL ($\pm$2.1) to 30 ng/mL ($\pm$ 4.3) by day 1 of confinement ($P < 0.05$) then decreased near prestress levels of 12 ng/mL ($\pm$ 4.7) by day 44 (Table 3). The slope of the linear regression of free cortisol on total cortisol for hatchery-reared fish was 0.45 ($P < 0.001$) (Figure 4b). The percent distribution of plasma cortisol bound to CBP in confined hatchery-reared rainbow trout increased from resting levels of 9 ($\pm$ 4.9) to 28 % ($\pm$ 6.0) by day 44 ($P < 0.1$) (Figure 5a). In addition, the distribution of plasma cortisol bound to Alb-P fluctuated throughout confinement, but by day 44 had decreased to 30 % ($\pm$ 8.4) from a time 0 value of 44 % ($\pm$ 1.9) ($P < 0.1$) (Figure 5a). The percent free cortisol fluctuated slightly throughout the confinement but stayed between 43 and 56 % (Figure 5a). The percent cortisol bound to CBP was significantly greater in non-disturbed, mature female hatchery-reared fish over non-disturbed mature males and immature fish ($P < 0.05$). Percent cortisol bound to CBP in mature female fish was 48 % ($\pm$ 7.8, n=5) compared to 19 ($\pm$ 6.8, n=10) and 16 % ($\pm$ 5.5, n=12) in mature male,

Table 1. Percent distribution of cortisol that is free, bound to CBP, and to Alb-P, in wild rainbow trout subjected to longterm confinement and control rainbow trout not subjected to confinement. All numbers are pooled plasma and represent mean concentration (n=5). (--) represent samples that were not run due to insufficient plasma volume. < 1 indicates below level of detection.

	CBP (%)		ALB-P (%)		Free (%)	
Time	Confined	Control	Confined	Control	Confined	Control
0.0(hour)	10	< 1	48	48	42	52
6.0	< 1		46		54	
12.0	< 1		41		59	
1 (day)	5	< 1	60	54	35	46
3	23		38		39	
6	2	--	36	--	62	50
9	< 1		44		56	
17	< 1	5	44	47	56	48
22	38	60	24	--	38	--
32	< 1		39		61	
40	35		26		38	
49	< 1		55		44	
56	32	< 1	--	62	--	38

Table 2. Total cortisol concentration and free cortisol concentration in confined wild rainbow trout subjected to long-term confinement and control wild rainbow trout not subjected to confinement. Numbers represent mean concentration (n=5).

	Total Cortisol (ng/mL)		Free Cortisol (ng/mL)	
Time	Confined	Control	Confined	Control
0.0(hour)	60	100	25	52
6.0	71		39	
12.0	86		51	
1 (day)	166	50	58	23
3	39		15	
6	26	38	16	19
9	33		19	
17	70	59	39	28
22	62		30	
27	31	22	12	13
32	26		16	
40	51		31	
49	55		25	
56	85	35	28	13

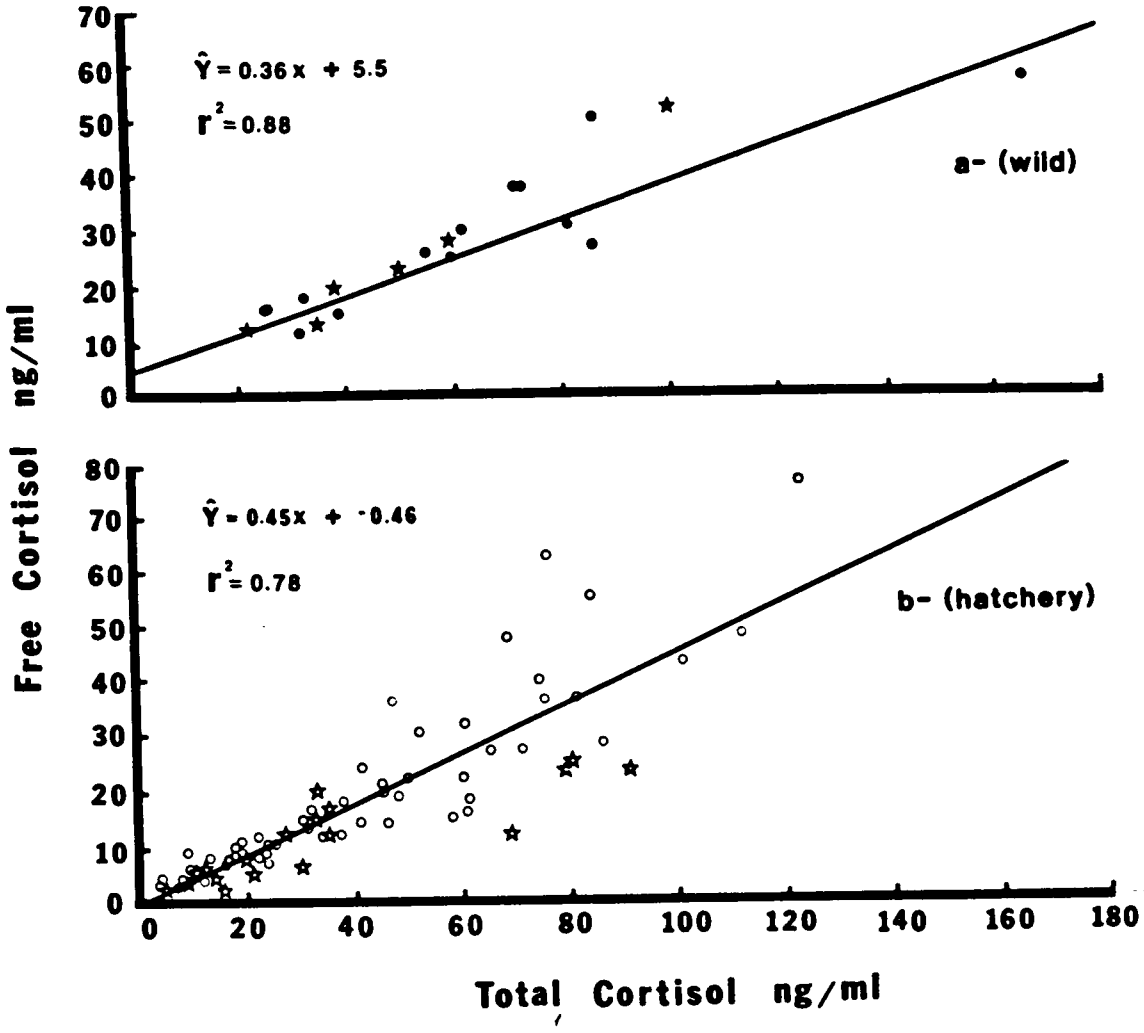

Figure 4. Free cortisol concentrations over total cortisol concentrations of (a) wild rainbow trout (solid circles) subjected to long-term confinement, control wild fish (solid stars) not subjected to confinement, and of (b) hatchery-reared rainbow trout (open circles) subjected to long-term confinement, control hatchery-reared fish (open stars) not subjected to confinement. Each point in (a) represents pooled samples of five fish and each point in (b) represents one fish.

Table 3. Total cortisol concentration and free cortisol concentration in confined hatchery-reared rainbow trout subjected to long-term confinement and control hatchery-reared rainbow trout not subjected to confinement. Numbers represent mean concentration (n=5).

Time	Total Cortisol (ng/mL)		Free Cortisol (ng/mL)	
	Confined	Control	Confined	Control
0.0 (hour)	27	23	13	7
6.0	11		5	
12.0	52		31	
1 (day)	57	26	30	14
3	42		14	
6	50	39	26	11
9	50		23	
12	60	64	32	20
17	53		23	
22	31		13	
27	12	12	5	3
32	13	54	6	5
36	29		11	
40	37		12	
44	52	62	12	15

Figure 5. Percent distribution of cortisol bound to CBP, bound to Alb-P, and free, in (a) hatchery-reared rainbow trout subjected to long-term confinement, and of (b) control fish not subjected to confinement. Lines are the (± SE) for each sample mean (n=5). Total cortisol of (a) confined fish (solid circles), and (b) control fish is illustrated over the percent distributions.

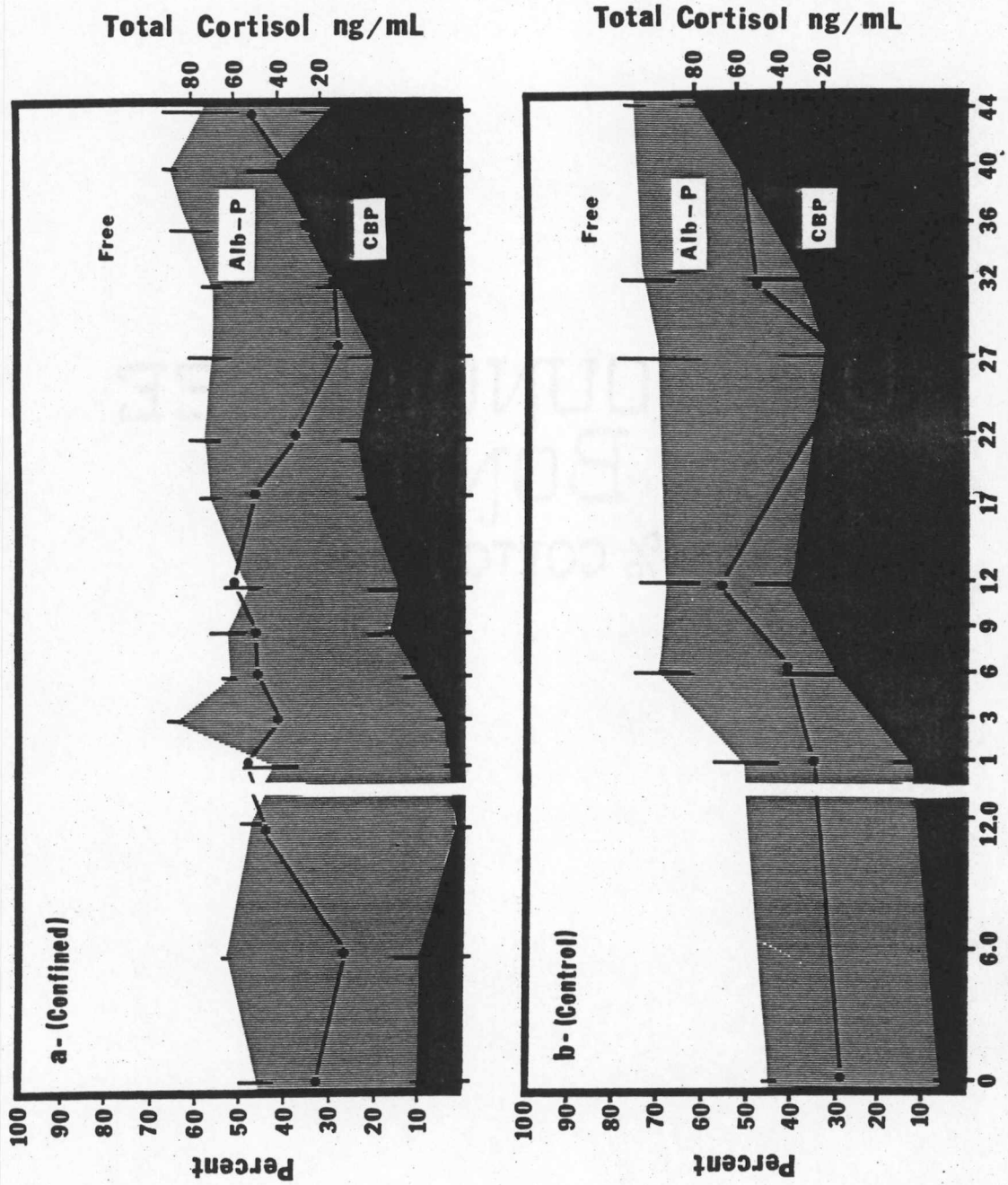
Total Cortisol ng/mL
80
60
40
20
a- (Confined)
Free
Alb-P
CBP
Percent
100
90
80
70
60
50
40
30
20
10
Total Cortisol ng/mL
80
60
40
20
b- (Control)
Free
Alb-P
CBP
Percent
100
90
80
70
60
50
40
30
20
10
0
6.0
12.0
Hours
1
3
6
9
12
17
22
27
32
36
40
44
Days

Figure 5

and immature fish, respectively.

The percent distribution of cortisol bound to CBP and Alb-P in the control group of the hatchery-reared fish responded similarly to the experimental group. While the percent free cortisol decreased from 55 % (± 5.0) at time 0 to 23 % (± 1.2) by day 44 (Figure 5b), concentrations of free cortisol doubled from 7 ng/mL (± 1.9) at time 0 to 14 ng/mL (± 8.0) by day 44 in the control fish (Table 3).

DISCUSSION

Long-term cage confinement resulted in few differences in the primary and secondary physiological responses to stress between wild and hatchery-reared rainbow trout (Figure 3 a,b,c). As was the case in Chapter II experiments, wild rainbow trout exhibited more extreme physiological responses in plasma cortisol, plasma glucose, and plasma chloride concentrations within day 1 of the confinement stress than did the hatchery-reared rainbow trout. These initial changes in the plasma constituents in wild fish resulting from chasing, dipnetting, handling, and confining are well-documented in salmonids (Wedemeyer 1972; Strange et al. 1978; Barton et al. 1980; Schreck 1981; Pickering et al. 1982). Although the severity of these stressors in the wild fish appeared to be no greater in magnitude than those imposed on the hatchery-reared fish, the results unequivocally indicate that physiological differences in the two strains do exist.

Other than the initial disturbances, chronic confinement did not have a marked effect on the long-term physiological responses in plasma glucose or plasma chloride for either fish type. The absence of metabolic disturbance and ionoregulatory dysfunction in rainbow trout subjected to long-term moderate confinement was also observed in salmonids by Wedemeyer (1976). He demonstrated that plasma glucose levels increased initially but returned to prestress levels within four days of the chronic stress in crowding densities similar to that used in this study (1.7 kg/L).

Previous studies on the physiological responses of long-term sublethal confinement in salmonids are varied. Fluctuations of total cortisol levels in this study for both wild and hatchery-reared rainbow trout are in contrast to reports of interrenal acclimation during chronic stress by Strange et al. (1978), Pickering and Stewart (1984), and Pickering and Pottinger (1987). Acclimation of interrenal tissue, and thus the diminished secretion of cortisol, did not occur in either the wild or hatchery-reared rainbow trout. The effect of the persistent crowding stimulus may have resulted in fluctuations characteristic of feedback activation and inhibition of the pituitary-interrenal axis in fish (Donaldson 1981). Although the cage was adjusted after each sampling, it is believed that the fluctuations of plasma cortisol represent the confinement stress rather than the periodic disturbance of the cage.

The results of chronic sublethal confinement on the percent distribution of cortisol bound to CBP, Alb-P, and free in wild rainbow

trout are varied and thus difficult to interpret. The plasma from the wild fish became viscous when subjected to heat-treatment during the assay which may have interfered with the dialysis across the membrane. In addition, pooling the plasma precluded individual analysis in which the influence of sexual maturity may have had an effect on the results of percent distribution in confinement as was observed in the hatchery-reared rainbow trout.

The amount of cortisol bound to CBP measured in this study for both wild and hatchery-reared rainbow trout was 5-10 % (37 $^{\circ}$ C) at time 0 which was similar to an earlier study in Atlantic salmon (Salmo salar) where Freeman and Idler (1966) observed 4-7 % (4 $^{\circ}$ C) with gel filtration. In addition, Idler and Freeman (1968) observed the percent of total protein-binding by equilibrium dialysis (CBG plus Albumin = 30-55 %, 4 $^{\circ}$ C) for five species of salmonids. Similarly, Nichols and Weisbart (1985) measured total protein-binding by equilibrium dialysis (44 %, 4 $^{\circ}$ C) in Atlantic salmon adapted in freshwater that was similar to the total protein-binding (45-58 %, 37 $^{\circ}$ C) found in this study for prestress levels for both confined and control hatchery-reared and wild rainbow trout. However, by the end of the study, cortisol bound to CBP and Alb-P in the control hatchery-reared fish had increased beyond the protein-bound levels of the confined fish to 70 %.

An increase in assay temperature of the steroid-binding protein complex results in decrease binding affinity in the mammalian CBG complex. Although the assay for this study was run at higher temperatures (37 $^{\circ}$ C) than the assay temperatures of 4 $^{\circ}$ C in Freeman and

Idler (1966) and Idler and Freeman (1968), Freeman and Idler (1966) demonstrated that fish plasma is less sensitive to an increase in assay temperature compared to mammalian CBG. Salmonids are poikilothermic which allows them to adapt to wide temperature ranges and may explain the slight change in the binding affinity of the steroid protein complex to higher assay temperatures (Freeman and Idler 1966; 1971).

The increase in the percent cortisol bound to CBP over time for control hatchery-reared fish is surprising. It is unknown whether this increase over time, and the resulting increase of total protein-bound cortisol to 70 %, is caused by some nonspecific effect that was not controlled for in either group, or the effect of sexual maturation. It is possible that with gonadal maturation an increase in levels of sex steroids occurred (Pickering and Christie 1981). Sandberg and Slaunwhite (1959) reported that administering estrogen caused an increase in the CBG levels and binding capacity of cortisol in humans. Burton and Westphal (1972) reported that the injection of progesterone into rats resulted in an increase in CBG activity. The mammalian studies were in direct contrast to previous observations in fish (Idler and Freeman 1968) where mature female salmonids had decreased levels of corticosteroid-binding proteins, while the immature fish consistently had more bound cortisol. Contradictory to Idler and Freeman (1968), this study indicates that the percent cortisol bound to CBP in hatchery-reared rainbow trout was greater in the mature females (48 %) than the mature males (16 %) and immature fish (18 %). Idler and Freeman (1968) and Idler and Truscott (1972) postulated that the decrease of binding

proteins in the migratory adult female sockeye salmon (Onchorhynchus nerka) was due to a general protein depletion (characteristic of the Cushings state) rather than the specific decrease in CBG.

Dunn et al. (1981) modeled changes in the dynamics of cortisol fractions in mammalian systems where a two-fold increase in total cortisol resulted in a two-fold increase in percent free cortisol and in a four-fold increase in free cortisol concentrations. Similarly, Nichols and Weisbart (1984) found that under normal physiological conditions in rainbow trout, the percent distribution of bound and free cortisol is directly proportional to the total concentration of cortisol. And similarly in this experiment, except for one time where total cortisol in the wild fish increased three-fold and free cortisol concentrations increased two-fold at day 1 of confinement (Table 2), the ratio of free cortisol concentration to total cortisol in the wild fish was 1:2.8 (Figure 4a). The ratio of free cortisol concentrations to total cortisol in the hatchery-reared rainbow trout was 1:2.2 over time (Figure 4b), until the end of the confinement where total cortisol continued to increase and free cortisol concentrations remained at prestress levels (Table 3). In this study, chronic confinement did not result in elevated total cortisol concentrations above the normal physiological- binding capacity determined for rainbow trout (362 ng/mL; Nichols and Weisbart 1984). During elevated cortisol levels, it is probable that the linear relationship of unbound and bound may be disrupted as cortisol begins to exceed the binding capacity of the binding proteins (Robin et al. 1978; Dunn et al. 1981).

It would be interesting to see the effect of high elevations of cortisol observed in Chapter II for the wild fish, on the ratio of free to total plasma cortisol concentrations. If the dynamics of corticosteroid protein-binding in fish systems is similar to mammalian systems, it is likely that an increase in total plasma cortisol (beyond the normal physiological concentration) would result in an increase in the free cortisol fraction. During times of stress, plasma cortisol would exceed the binding capacity of the carrier proteins and the free cortisol fraction would no longer be linear to the total plasma cortisol levels typically used as a primary indicator of the fish's "well-being".

CHAPTER IV

SUMMARY AND RECOMMENDATIONS

Summary

Wild rainbow trout exhibited more pronounced physiological changes to short-term severe net confinement than did hatchery-reared fish. The severe hypochloremia, in addition to the large elevation in plasma cortisol and plasma glucose, indicated that the wild rainbow trout were more susceptible to severe stress than the hatchery-reared fish. Electroshock elicited an increase in plasma cortisol within 30 minutes of the stimulus for both types of fish. Plasma cortisol decreased to prestress levels in the hatchery-reared fish but continued to increase in the wild fish where it remained elevated throughout the remainder of the experiment. Metabolic regulation as measured by plasma glucose changes, and ionoregulatory mechanisms as measured by plasma chloride changes, were not effected in either wild or hatchery-reared fish as a result of the electroshock.

Both wild and hatchery-reared fish exhibited only slight physiological changes throughout the moderate, long-term confinement in the cage. Wild rainbow trout exhibited more extreme physiological responses in plasma cortisol, plasma glucose, and plasma chloride within day 1 of the confinement stress than did the hatchery-reared fish. Other than the initial disturbances demonstrated in the wild fish, the chronic stress of the moderate long-term confinement did not have a

marked effect on the long-term physiological responses for either fish type. This was the first time of which the author is aware that the method of isodialysis has been applied to investigate changes in the distribution of plasma cortisol in fish over time. The method, developed by Hammond et al. (1980), was used to estimate the distribution of cortisol bound to a heat-labile protein - CBP (ie. similar to the high affinity- low capacity mammalian CBG), and the distribution of cortisol bound to a non heat-labile protein - Alb-P (similar to the low affinity-high capacity mammalian albumin binding protein) in small volumes of fish plasma. Plasma cortisol bound to CBP for both strains of fish was 5-10 % at prestress conditions. Cortisol bound to CBP had increased to 28 % in the hatchery-reared rainbow trout by the end of the confinement, however, the control fish responded similarly to the confined group as well. Fluctuations of CBP-bound cortisol in the wild fish were difficult to interpret. Alb-P-bound cortisol fluctuated throughout the study in both confined and control groups of wild and hatchery-reared rainbow trout. Curiously, cortisol bound to CBP was significantly greater in non-disturbed mature female hatchery-reared fish (48 %) compared to immature (19 %) and mature male fish (16 %) throughout the study. Similar to results by other researchers, the percentage of protein-bound cortisol for both wild and hatchery-reared fish ranged from 55 to 58 %. The remaining cortisol fraction was considered free and biologically available to target tissue. The ratio of free cortisol concentration to total cortisol in hatchery-reared and wild rainbow trout was 1:2.2 and 1:2.8, respectively.

Recommendations

Additional studies are needed in order to elucidate the changes in the percent distribution of cortisol bound to protein fractions. In future studies, it is suggested that additional physiological varibles be observed in order to ascertain reduced fitness indicative of chronic stress. Recently, Barton et al. (1987) showed decreased growth as a good indicator of reduced fitness from chronic stress; Pickering and Pottinger (1987) showed changes in the numbers of lymphocytes and thrombocytes as indicators of long-term stress; and this author observed hypertrophy of the spleen (personal observation-not published). It is suggested that immature hatchery-reared fish be used in order to eliminate the confounding and putative-influence of sexual maturity on the final analysis. In addition, the temperature of the binding-protein assays should be carried out similar to the temperature of acclimation in the fish. Finally, in order to fully understand the dynamics of the corticosteroid stress reponse, in addition to the percent distribution of cortisol free and bound to protein fractions, fish research needs to move toward identifying the physical and chemical properties of the binding-proteins, elucidating and measuring the free steroid fraction in plasma, and illustrating receptor number and affinity for the plasma steroids.

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APPENDIXES

APPENDIX A

RADIOIMMUNOASSAY FOR QUANTITATION OF CORTISOL IN FISH PLASMA

I. Glassware should initially be cleaned with Chromerge solution and rinsed three times with deionized water. Glassware exposed to radioactive reagents are soaked in Isoclean decontamination agent, and then taken through acid cleaning protocol.

II. Reagents

A. Assay Buffers - pH=7.4 (PBS-gA contains gel and PBS-A does not contain gel).

Sodium Chloride (58 g/mol.wt.)
Sodium Azide (65 g/mol.wt.)
Sodium Phosphate, dibasic (142 g/mol.wt)
Sodium Phosphate, monobasic (138 g/mol.wt.)
Knox gelatin (unflavored)

B. Sequence of additions:

1. Solution A (0.2 M) store separately at 4 $^{\circ}$ C
   - 27.6 g Sodium Phosphate (monobasic)
   - 1000 mL distlled water

2. Solution B (0.2 M) store separately at 4 $^{\circ}$ C
   - 28.4 g Sodium Phosphate (dibasic-anhydrous)
   - 1000 mL distilled water

3. Phosphate Buffer PBS-A (no gel)
   - 1.0 g Sodium Azide
   - 9.0 g Sodium Chloride
   - Solution A = 75 ml
   - Solution B = 425 ml
   - pH the buffer to get 7.4 (may need to add an additional 50-100 ml of Solution B to get desired pH)
   - Q and S to 1 liter by adding distilled water to obtain 1 liter of buffer

4. Phosphate Buffer PBS-gA (gel)
   - 500 mg Knox gelatin
   - 500 ml PBS-A (Phosphate Buffer no gel, pH=7.4)
   - heat the mixture to dissolve the gelatin
   - refrigerate at 4 $^{\circ}$C

III. Antibody Preparation- Cambridge Medical Diagnostics (Rabbit-anticortisol Cat No. 299-206R Lot No. M8521)

A. Antibody is stored as neat sera at -80 $^{\circ}$ C. To prepare antibody from neat sera for 1:100 dilution, take 0.05 uL of neat sera

(prepackaged by distributor) and add 5.0 ml of PBS-gA to neat sera to get the dilution of 1:100. Aliquot 300 uL, 100 uL, and 50 uL into tubes. Store the aliquots at -80° C.

B. Dilution of the aliquot antibody: If the desired antibody dilution for the assay is 1:3000, then 350 uL of the Ab is added to 10.15 ml of the PBS-gA Buffer to get a total volume of 10.5 ml to work with at 100 uL of Ab per tube (100 tubes). Antibody will have to be tested for binding specificity of the appropriate dilution (could vary with batches from the distributor).

IV. Standard Curve Preparation

Cortisol standard prepared from Hydrocortisone (Sigma No. 4001)

Solution A- 10 mg cortisol + 10 mL ETOH = 1 mg/mL
Solution B- 100 uL Soln A + 9.9 mL ETOH = 1 ug/100 uL
Solution C- 100 uL Soln B + 9.9 mL ETOH = 100 ng/mL
Solution D- 1000 uL Soln C + 9.0 mL ETOH = 1 ng/100 uL

Solution D is used for the preparation of the Standard Curve:

0.01 ng/mL = 1.0 uL Soln D
0.025 ng/mL = 2.5 uL "
0.05 ng/mL = 5.0 uL "
0.10 ng/mL = 10.0 uL "
0.25 ng/mL = 25.0 uL "
0.50 ng/mL = 50.0 uL "
1.00 ng/mL = 100.0 uL "

V. Assay Procedures

A. Preparation of Tubes

1. For racks which hold 50 tubes, set up 5 mL boroscilicate tubes in the following order:

Tubes 1-10 Standard Curve
11-50 Samples - 30 tubes will allow for 15 samples in duplicate.

Tube 1 (Total Count Tube) 500 uL PBS-gA + 100 uL Ab
Tube 2 (Non-specific Binding) 600 uL PBS-gA + no Ab
Tube 3 0.0 500 uL PBS-gA + 0 uL Standard + 100 uL Ab
Tube 4 0.01 " " + 1 uL Standard + " "
Tube 5 0.025 " " + 2.5 uL Stand + " "

Tube 6	0.05	"	"	+ 5.0 uL Stand	+	"	"
Tube 7	0.10	"	"	+ 10.0 uL Stand	+	"	"
Tube 8	0.25	"	"	+ 25.0 uL Stand	+	"	"
Tube 9	0.50	"	"	+ 50.0 uL Stand	+	"	"
Tube 10	1.00	"	"	+ 100.0 uL Stand	+	"	"

2. Dry standard curve tubes in a vacuum drying oven to remove ETOH for 25-30 min at 100° C. (Vac 25 Hg)
Note: It is best to prepare the standard curves the same day to prevent adhering of the radioisotope to the glass.

3. After drying, add 500 uL of the PBS-gA assay buffer to standard curve tubes and bring to 4° C.

4. VORTEX

B. Preparation of Unknown Sample Tubes

1. In a separate rack set up one 10 mL boroscilicate tube for each sample.

2. To each 10 mL tube add 2.5 mL PBS-gA (500 x dilution); for a 1000 x dilution when endogenous steroid levels are high use 5.0 mL of PBS-gA.

3. Add 10 uL of the sample plasma.

4. VORTEX

5. Aliquot 500 uL of the dilution into the appropriate duplicate tubes of 5.0 mL boroscilicate tubes previously set up and numbered in the standard curve rack.

C. Final Assay Procedures

1. Heat ALL tubes in the racks to 70° C water bath for 1 h.

2. Cool to room temperature.

3. Prepare the [1,2 ^{3}H] cortisol to 5000 CPM/100 uL
Add 100 uL of the [1,2 ^{3}H] cortisol to <u>ALL</u> tubes.

SEE APPENDIX E FOR PURIFICATION AND PREPARATION OF [^{3}H] CORTISOL

4. VORTEX

5. Add 100 uL of the Ab sera (1:3000) to all tubes except the Non-specific Binding Tube

6. VORTEX

7. Incubate from 6 to 16 hours at 4° C. (Usually place in ice in a refrigerator overnight)

8. Add 200 uL of Dextran-coated Charcoal (4° C) to all tubes except the Total Counts Tube- add 200 uL of PBS-A to this tube.

Note: Addition of the charcoal should not take more than five minutes from the beginning to the end.

To prepare dextran-coated charcoal:

0.6250 g powdered charcoal (Sigma) + 0.0625 g Dextran (Sigma) + 100 mL of PBS-A (NO-GEL). Note: Add dextran to buffer first.

9. VORTEX

10. Place in ice bath for 20 minutes.

11. Centrifuge at 2500 rpm for 15 minutes at 4° C.

12. Place tubes back on ice and pour off the supernatant into Scintillation Vials (wearing gloves).

13. Add 4.0 mL of Scintillation cocktail (Scintiverse II).

14. Mix and count for 1 or 2 minutes in beta liquid scintillation counter.

APPENDIX B

ENZYMATIC DETERMINATION OF GLUCOSE IN FISH PLASMA

Glucose Assay- Sigma Diagnostics, St. Louis Mo. Procedure No. 510

This procedure is based on an enzymatic method in which the glucose present in the blood is oxidized by Glucose Oxidase to Gluconic Acid and Peroxide. The addition of Peroxidase catalyzes the oxidation of Peroxide and an indicator- o-dianisidine. The intensity of the brown color (the oxidized o-dianisidine) is proportional to the intensity of the glucose concentration which will be measured with a spectrophotometer.

1. Tubes are labeled: Blank, Standard 40 mg/100 mL, 100 mg/100mL, and 200 mg/100 mL, sample 1, 2, 3 etc.....

2. a) To blank tube add 0.5 mL deionized water.
   b) To standards add:
      i. 10 uL of glucose standard (100 mg/100 mL) to the 40 mg/100 mL tube + 0.20 mL deionized water
      ii. 25 uL of glucose standard to the 100 mg/100 mL tube + 0.20 mL deionized water
      iii. 50 uL of glucose standard to the 200 mg/100 mL tube + 0.20 mL deionized water
   c) To samples add: 10 uL plasma sample + 0.20 mL deionized water.

3. VORTEX

4. To <u>ALL</u> tubes add 3.0 mL of the prepared Combined Enzyme Color Reagent.

5. VORTEX

6. Place in 37 $^{\circ}$ C water bath for 30 min.

7. After incubation, read absorbances on a spectrophotmeter at 460 nm adjusting periodically with the blank tube.

8. Standards are plotted and the samples are read off the standard curve.

APPENDIX C

MICROTITRATION OF CHLORIDE IONS IN FISH PLASMA

Chloride ions in plasma react with mercuric ions added from a microburet to form an insoluable mercuric chloride precipitant. The end point is indicated by the appearence of a violet-blue color when all of the plasma chloride ions are precipitated and the excess mercuric ions react with the indicator (diphenylcarbazone) (Wedemeyer and Yasutake 1977).

I. Materials and Reagents

A. Standard sodium chloride solution (100 meq/L): dissolve 0.584 g of sodium chloride in 100 ml of deionized water.

B. Mercury II nitrate monohydrate- Aldrich Chemical CO.
Mercuric nitrate titrant solution (0.02 N): dissolve 0.32 g of mercury (II) nitrate in 50-75 ml deionized water containing 1 ml of nitric acid.

C. Nitric Acid (0.03 N): dilute 0.2 ml concentrated (12 N) nitric acid in 100 ml of deionized water.

D. Phenylazoformic acid 2-phenylhydrazide mol.wt. 240.27 - Aldrich Chemical Co.
Diphenylcarbazone (0.1 %): dissolve 0.10 g of diphenylcarbazone in 100 ml of 95 % ethanol.

II. Procedure

A. 0.40 ml of nitric acid, 0.40 ml of indicator solution (diphenylcarbazone) and 50 uL of plasma are added to boroscilicate tube (5.0 mL).

B. Using a microburette, the titrant is added slowly while contents of the tube are swirled gently. The end point is the first permanent color of violet.

C. Titrate a 50 uL water blank and 25 uL, 50 uL, and 100 uL of the sodium chloride standards.

D. Once a standard curve is constructed, the plasma end points are read off the curve.

APPENDIX D

ASSAY PROCEDURE FOR DETERMINING PERCENT DISTRIBUTION OF CORTISOL FREE AND BOUND TO PROTEINS IN FISH PLASMA

I. Membrane Preparation

A. The membranes for the isodialysis capsules are prepared from dialysis tubing purchased from Fisher Scientific (Spectra/Por, M.W. cutoff 12,000-14,000 Daltons, Cat. No. D1614-12 Size:25 mm x 15.9 mm diameter, 50 ft/roll).

B. The dialysis tubing (cut into two foot sections) is boiled in 95 % ethanol under a hood for 30-45 minutes and rinsed extensively with distilled water to remove the ethanol.

C. The dialysis tubing is boiled twice in a 2-3 liter solution containing 5-10 mg of disodium EDTA and 5-10 g of sodium carbonate with a distilled water rinse between each boilings (Caution: if too much EDTA is added and not removed during the rinse, the EDTA will have an adverse effect on the binding of the protein which will result in inconsistant results.) Additional tubing can be stored up to a month in 0.02 % sodium azide solution at 4 $^{\circ}$C.

II. Capsule Preparation

A. The capsules are prepared in 3-4 cm sections from 12 x 75 mm boroscilicate disposible test tubes. The cut end is fire polished before use.

B. The rubber bands, made from latex rubber surgical tubing (1/8 i.d.) are cut in 3-4 mm sections.

C. The dialysis tubing cut in 3 cm sections are placed over one end of the capsule and the rubber band is rolled onto the tube and over the membrane with the help of an acrylic cone (Dr. James T. Murai, Department of Obstetric, Gynecology and Reproduction, University of California San Francisco, San Francisco, Ca).

D. The membrane is stretched tight over the end of the tube to remove all wrinkles and folds, and excess membrane is trimmed with a razor blade.

E. The capsules are prepared up to one week in advance and stored in a 0.02 % of sodium azide solution.

III. Cleaning Glass Capsules for Reuse

A. After the assay is complete, the plasma, membranes, and rubber bands are disposed and the capsules are rinsed once in distilled water, and twice in methanol.

B. Warm the tubes in a 0.1N solution of sodium hydroxide for two hours.

C. Pour off the sodium hydroxide and rinse twice with 0.1N of hydrochloric acid.

D. Rinse one time in distilled water and a final time in methanol.

E. An aliquot of the methanol is counted on a scintillation beta counter to detect any residual radioactivity. If radioactivity is present, then the tubes are rinsed again in methanol.

IV. Experimental Procedures

A. In preparation for an assay of 10 samples:

1. To 10 disposable test tubes [1,2 ^{3} H] cortisol (3×10^{5} dpm) dissolved in ethanol is added to each.

2. The ethanol is evaporated and 5 uL of [^{14} C] glucose (271 mCi/mmole, New England Nuclear, Boston, Massachusetts) (12×10^{3} dpm) is added to each tube.

3. To each tube 500 uL of plasma is added, and vortexed.

4. The tubes are incubated in a water bath for 30 min at 37 $^{\circ}$C, and an additional 30 min at room temperature (20 $\pm$4 $^{\circ}$ C).

B. Preparation of scintillation vials for plasma equilibrated with radiolabeled cortisol and glucose:

1. Label vials 1-10 in duplicate (1a 1b, 2a 2b, 3a 3b...).

2. To each vial add three paper filter discs (diameter 13 mm; Whatman No. 1).

3. Insert membrane covered isodialysis capsules (prepared earlier) into each vial.

4. Pipette duplicate aliquots (200 uL) of the equilibrated plasma into the isodialysis capsules.

5. The vials are capped and centrifuged at 3000 rpm (750 g) for 1 h at 37 $^{\circ}$ C.

C. Determination of the percent distribution of unbound steroid:

1. During centrifugation, a second set of scintillation vials are prepared in duplicate (1ap 1bp, 2ap 2bp, 3ap 3bp... p is for "plasma"). One filter disc is placed into each vial.

2. After centrifugation, the isodialysis capsules are carefully removed from the scintillation vials and 30 uL of the remaining ultrafiltrate is pipetted off and placed onto the filter disc of its respective "plasma" vial (ultrafiltrate from isodialysis capsule 1a is placed onto filter disc of vial 1ap).

3. Once ultrafiltrates have been added to their respective plasma vials, all vials, including those that contained the isodialysis capsule, should be placed in the proper order: 1a, 1ap, 1b, 1bp, 2a, 2ap, 2b, 2bp ...

4. Add 350 uL of deionized water into all scintillation vials and vortex.

5. Add 3 ml of scintillation fluid, and count on the Beta Scintillation counter- being sure to correct for the [^{14} C] and [1,2^{3} H] isotopes.

D. Determination of the percent distribution of bound steroid:

1. Prior to the assay, heat an additional set of the same 10 samples of plasma at 60°C in a water bath for 1 h.

2. Proceed with the same procedure for determination of the distribution of the percent cortisol unbound.

APPENDIX E

PURIFICATION PROCEDURE OF TRITIATED CORTISOL FOR RADIOIMMUNOASSAY AND PERCENT DISTRIBUTION ASSAY

A. The column is packed to 6 cm with (0.5 g) Sephadex LH-20 swelled 24 h in Chloroform:Ethanol (95:5).

B. Dry down ethanol from an aliquot of radiolabeled cortisol and resuspend with 200 uL of Chloroform:Ethanol solvent.

C. The tritiated cortisol and solvent mixture is added to the column and collected in 1 ml fractions.

D. In duplicate, 10 uL is removed from each fraction and added to 3 ml of scintillation liquid to be counted on a beta scintillation counter.

E. Fractions containing the desired counts are pooled and the solvent is dried down with nitrogen gas. The purified radiolabled cortisol is resuspended in 95 % ethanol for storage.

Note: Tritiated cortisol should be repurified at least one day before being used in the Percent Distribution Assay of Binding Proteins.

VITA

Colleen Caldwell Woodward was born in Houston, Texas on July 17, 1960. She attended elementary schools in that city and was graduated from St. Agnes Academy in May 1978. In August of 1978 she entered Texas A & M University, and in December 1982 received a Bachelor of Science degree in Wildlife and Fisheries. She entered Southwest Texas State University in the Fall of 1983, and in June 1985 received a Master's of Science degree in Aquatic Biology. The following September, she began her doctoral program at the University of Tennessee in Knoxville. She received her Doctor of Philosophy degree with a major in Ecology in June 1988.

She currently resides in Brunswick, Georgia, with her husband, Arnold G. Woodward, who is a marine biologist with the Georgia Department of Natural Resources.