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Title: Interrenal and Metabolic Responses to Stress and their Modifying Factors in Juvenile Salmonid Fishes

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Carl B. Schreck

Genetic, ontogenetic, and environmental factors modified characteristic interrenal and glycemic responses to stress in juvenile salmonid fishes. During continuous confinement stress, plasma cortisol rose more quickly in chinook salmon, Oncorhynchus tshawytscha, acclimated to high rather than medium or low temperatures; hyperglycemia following either acute or chronic stress was also highest in this group. When chinook salmon were subjected to three handling stresses, separated by 3-h intervals, increases of plasma cortisol, glucose and lactate and decreases in plasma sodium were cumulative. Healthy chinook salmon appeared more able than diseased fish to elevate plasma cortisol after stress, but post-stress plasma glucose was higher in the unhealthy fish. Glycemic responses to stress in chinook salmon were related to diet type and to fasting. When rainbow trout, Salmo gairdneri, were exposed to pH 4.7 and then handled, the post-stress plasma cortisol elevation was more than twice that in those at ambient pH. Rainbow trout subjected to an acute disturbance daily for 10 wk exhibited habituation, evident from plasma cortisol and glucose levels after handling that were about one half of those in previously unstressed fish. The interrenal response to handling in coho salmon, Oncorhynchus kisutch, increased twofold during the normal period of smoltification. A comparison of chinook salmon, coho salmon, rainbow trout and brook

trout, Salvelinus fontinalis, subjected to identical handling stresses, suggested that there were species and stock differences affecting the magnitude of plasma cortisol elevations.

Rainbow trout fed with cortisol-treated feed exhibited reduced growth, condition factor, liver glycogen and circulating lymphocytes, and higher plasma glucose and hematocrit. The plasma cortisol elevation following handling was completely abolished in the cortisol-fed trout, indicating that continuous negative feedback of cortisol eliminated the fish's ability to initiate an interrenal stress response.

When forced to swim, mean oxygen consumption rate in stressed steelhead, Salmo gairdneri, was about twice that in unstressed fish and individual rates were positively correlated with plasma cortisol elevations. The metabolic cost of this stress was estimated to reduce the energy available for other performance components within the fish's scope for activity by about one quarter.

Interrenal and Metabolic Responses to Stress and their Modifying Factors in Juvenile Salmonid Fishes

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Contributions of manuscript coauthors are gratefully acknowledged and are outlined in the Preface; contributions of others to the individual studies are acknowledged at the end of each manuscript. For those papers submitted for publication, I appreciate the constructive comments of the anonymous journal referees.

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PREFACE

The results of this research program are presented as a series of manuscripts. Chapters II through VI, and Chapter VII in part, describe the effects of various genetic, ontogenetic and environmental factors on characteristic responses to stress of plasma cortisol (a primary response) and glucose (a secondary response), along with those of other physiological conditions, in juvenile salmonid fishes. Chapter II also includes a general review of the subject. Chapters VII and VIII report some consequences both of stress and of elevated plasma cortisol in the absence of stress. Results of additional experiments conducted in these subject areas were inconclusive and are presented in Appendices I and II. There is a common bibliography for all manuscripts and appendices.

In addition to my supervisor, Carl B. Schreck, the contributions of other authors of the manuscripts were as follows:

Chapter III. Linda A. Sigismondi carried out the plasma sodium and potassium assays.

Chapter IV. Richard D. Ewing and Alan R. Hemmingsen provided the fish and conducted the gill (Na+K)-ATPase determinations; Reynaldo Patino carried out the plasma thyroxine analysis.

Chapter V. Laurie G. Fowler formulated the diet treatments and provided the various diet-treated fish; Erika Plisetskaya conducted the plasma insulin assay and contributed to the discussion on that subject.

Chapter VI. Gary S. Weiner assisted in setting up the experiment and with the plasma sodium and potassium analyses.

Chapter VII. Lesley D. Barton carried out the blood cell counts and histological preparation of stomach tissues, and assisted with sample collections and on-site analyses.

Appendix I. Reynaldo Patino and J. Michael Redding assisted with both the design and carrying out of the experiments.

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I: GENERAL INTRODUCTION

Background

> "There are few concepts that have evoked as much discussion and disagreement as that of stress when applied to biological systems." (Pickering 1981a, p. 1)

> "A reliable measurement of stress is critical; however, a reliable, acceptable measurement of stress has not been found, perhaps because the concept is applied to so many different phenomena." (Moberg 1985, p. 28)

These quotes serve to illustrate the past difficulty in attempting to establish the exact nature and boundaries of stress in fish and its effects on general well-being. In a broad sense, one definition of stress physiology is the study of an animal's physiological, biochemical and behavioral responses to the various factors of the physical, chemical and biological environment (Yousef 1985). In this context, a stress factor is equated with any environmental factor and the study of stress becomes synonymous with environmental physiology (Yousef 1985). Although this concept is arguably valid, it is too wide in scope to have any operational value for those interested in investigating the causes and effects of stress. With specific reference to fish, Brett (1958) defined stress as a state produced by any environmental or other factor which extends the adaptive responses of an animal beyond the normal range, or which disturbs the normal functioning to an extent that, in either case, the chances of survival are significantly reduced. The latter part of this definition implies that all stress is

detrimental to the organism, which is not necessarily true.

A more widely accepted definition of stress is the nonspecific response of the body to any demand made upon it (Selye 1973a). In Selye's concept, a stressed organism passes through three distinct phases that he termed the General Adaptation Syndrome (GAS) (Selye 1936, 1950). The first stage is an alarm reaction, followed by a stage of resistance. The alarm phase is usually characterized by a rapid physiological response (Fig. I.1, A). Hypothetically, the magnitude of the response is a reflecton of the severity of the stress (Fig. I.1); although not rigorously tested, circumstantial evidence supports that view. During the second phase, the organism adapts to or compensates for the altered conditions causing the stress in order to regain homeostasis. This may be evident as a return of physiological conditions to the prestress state (Fig. I.1, B) or to an altered resting state (Precht 1958; Selye 1973b). If the stress is overly severe or long-lasting, compensation may not be possible and the organism enters the final stage of exhaustion, often indicated by physiological conditions remaining at or near the stressed level (Fig. I.1, C). In fish, stress-induced mortality would likely be associated with this phase. However, the GAS concept of the stress response is a generalized one and may not be applicable to all stressful situations (Schreck 1981, 1982), for example, the exposure of fish to a lethal dose of anesthetic (Strange and Schreck 1978) or an insidious toxicant (Schreck and Lorz 1978). In fact, Selye (1950) urged the preliminary nature of his model at the time, although it is still popular. Schreck (1981) concluded that a GAS-type response in fish is only elicited when they experience some form of fright, discomfort or pain.

More recently, as the role of corticosteroids during stress becomes clearer, the general applicability of the GAS concept has been questioned. Specifically, the finding that administration of corticosteroids <u>in vivo</u> has antiinflammatory and immunosuppressive effects contradicted the notion that all

Figure I.1. Three-dimensional graphic model demonstrating the increasing magnitude of physiological responses to stress dependent on the increasing severity and/or duration of the stress factor. "A" represents a peak stress response to a relatively mild disturbance and "B" indicates the return of the physiological condition to the prestress state. "C" represents persistence of the response resulting from a severe or prolonged disturbance that may be ultimately associated with death of the fish.

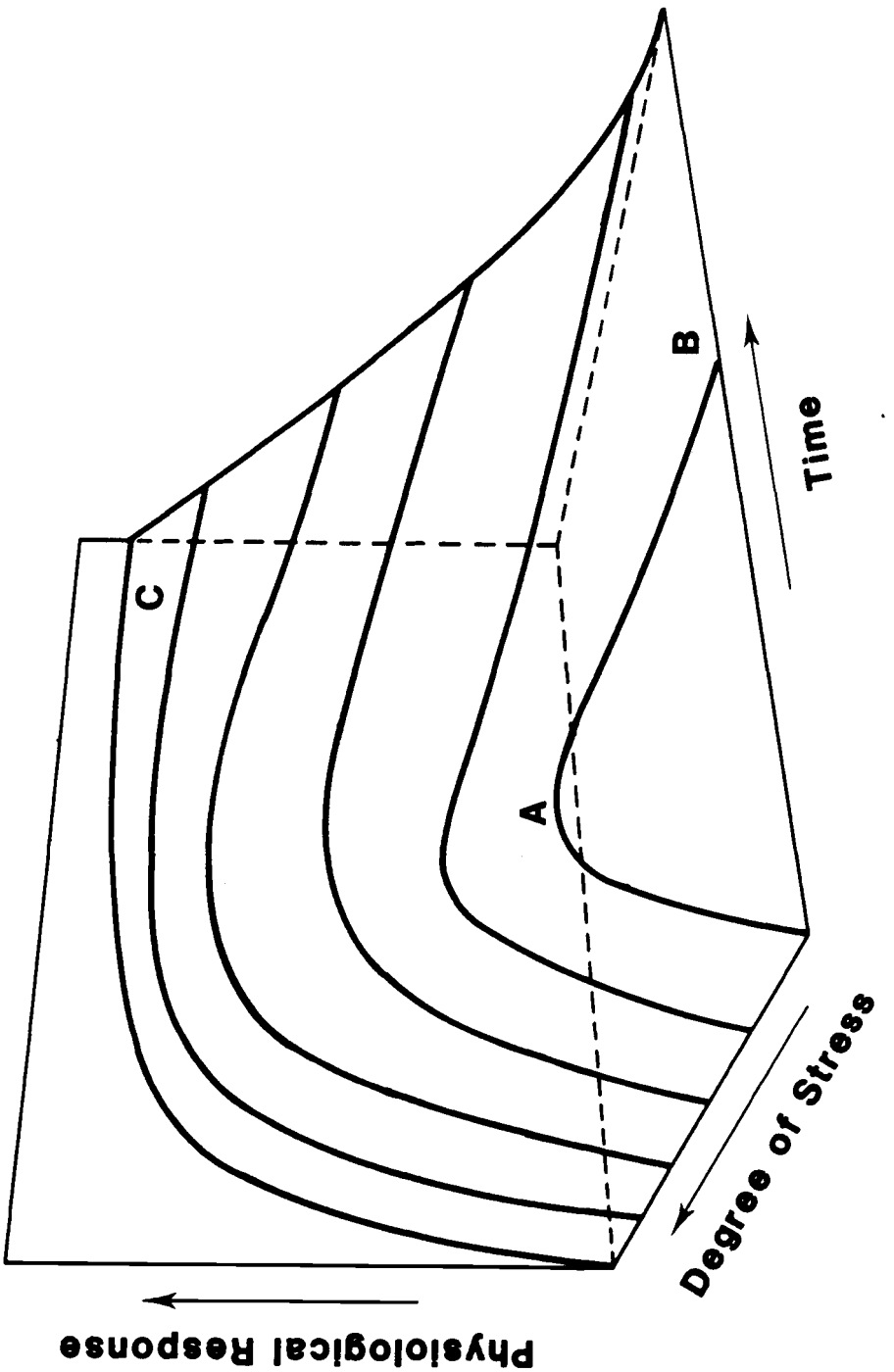

Figure I.1

physiological responses to stress are adaptive (Munck et al. 1984). Munck et al. (1984) proposed a new hypothesis whereby the role of corticosteroid secretions in response to stress, rather than being adaptive per se, is to protect the body from its own defense mechanisms during stress by suppressing those mechanisms. Regardless of the semantic argument whether or not such suppression constitutes an adaptive stress response, it is apparent that continued elevation of corticosteroids would be maladaptive, for example, by lowering disease resistance (Munck et al. 1984).

A more comprehensive model of stress has been presented by Moberg (1985). In this model, the stress response is divided into three categories: (1) recognition of a threat to homeostasis, (2) the stress response itself, and (3) the consequences of stress. The stress response and the consequences of stress represent adaptive and maladaptive phases, respectively, of the overall response of the organism. Each category is comprised of separate biological events that are initiated by perception of the stress factor by the central nervous system (CNS) and culminate with the development of a pathological condition if the change in biological function caused by the stress is severe or persistent enough. Moberg (1985) emphasizes the importance of the CNS in the organism's response to stress, both in perception of the stimulus and in organization of the biological response. As a good example of a change in biological function resulting from stress, Moberg (1985) cites the elevation of plasma corticosteroids. That is, corticosteroids are released during stress presumably to induce gluconeogenesis to increase glucose availability for metabolism. But increased gluconeogenesis is at the expense of lipid and protein metabolism, thus growth may be reduced. Furthermore, increased corticosteroid levels over time detrimentally affect immune responses and reproductive processes.

The elevation of plasma corticosteroids, i.e. cortisol, in fish in response to various types of stressful stimuli has been well documented (Barton and Toth 1980; Donaldson 1981; Schreck

1981; see Chapter II) and constitutes one of the major endocrine or primary responses to stress (Mazeaud et al. 1977). Cortisol is synthesized and released from the interrenal cells of the head kidney tissue and is controlled by negative feedback on the hypothalamic-pituitary axis (Fryer and Peter 1977). The other major endocrine response to stress is the secretion of catecholamines, primarily adrenaline (epinephrine), from chromaffin cells (Mazeaud et al. 1977; Mazeaud and Mazeaud 1981). Mazeaud et al.'s (1977) model implied that pathways of corticosteroid and catecholamine stress responses were distinct from each other. However, recent evidence reviewed by Axelrod and Reisine (1984) indicates that regulation of these stress hormones is more complex than earlier thought and that neuroendocrine control of both hormonal axes is interrelated.

Plasma glucose also rises in response to stress (Wedemeyer and McLeay 1981; see Chapter II) and is one of the many secondary responses to stress (Mazeaud et al. 1977); others include changes in plasma lactate, liver glycogen, hydromineral balance, and hematological features, such as hematocrit and circulating lymphocytes. Elevations in plasma glucose indicate mobilization of energy reserves such as tissue glycogen (Driedzic and Hochachka 1978) and reflect the degree of metabolic activity (Umminger 1977). Stress-induced changes in plasma cortisol and glucose tend to covary and circumstantial evidence suggests that this relationship is functional during stress in fish (Leach and Taylor 1980).

In addition to primary and secondary responses, there are also 'whole-animal' or tertiary responses to stress in fish (Wedemeyer and McLeay 1981; Wedemeyer et al. 1984). The most fundamental 'whole-animal' response to stress, but one of the least studied, is a change in the metabolic rate. Changes in metabolism or scope for activity have been suggested as possible methods for measuring stress in fish (Brett 1958; Wedemeyer and McLeay 1981) and other aquatic organisms (Bayne 1980). Most investigations with fish, however, have concentrated on measuring stress indicators or

changes in individual performances (Schreck 1981), such as osmoregulatory ability or swimming capacity, and have not directly measured changes in metabolic rate due to stress. This avoidance probably has been because of the difficulty of conducting such studies, both in terms of acquiring the necessary apparatus and the time required to achieve meaningful results.

Goals and Objectives

The ultimate goal of this research program was to contribute to the improvement of fisheries management practices through applied knowledge of the nature of physical stress in fish. This goal can be accomplished, in part, by: (1) improving the diagnostic value of physiological stress indicators currently in use, and (2) increasing the understanding of the consequences of stress. Since a major portion of both commercial and recreational fisheries management programs in North America involve salmonid fish (Salmoninae), this program focused only on juvenile salmonids. Specifically, the main objectives of this research were to: (1) assess the effects of selected factors on characteristic interrenal (primary) and glycemic (secondary) stress responses in juvenile salmonid fish, as determined by elevations in plasma cortisol and glucose, respectively, and (2) determine the effects of physical stress on the metabolic rate of juvenile salmonid fish. Additional objectives were to: (1) establish if a correlation exists between plasma cortisol and glucose elevations and corresponding stress-induced changes in the metabolic rate, and (2) further clarify the functional significance of elevated plasma cortisol during stress. Within the context of specific investigations, changes in certain other physiological conditions resulting from physical stress were also determined, including those in plasma lactate and sodium, liver glycogen and hematological characteristics.

II: A REVIEW OF FACTORS MODIFYING PLASMA CORTICOSTEROID AND GLUCOSE ELEVATIONS IN RESPONSE TO PHYSICAL STRESS IN FISH, WITH NEW DATA ON ACCLIMATION TEMPERATURE IN CHINOOK SALMON

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Oregon State University, Agricultural Experiment Station Technical Paper No. 7911.

Abstract

Experimental results and a review of the literature supported the hypothesis that various genetic, ontogenetic and environmental factors modify characteristic interrenal and glycemic responses of fish to physical disturbances. Juvenile chinook salmon, Oncorhynchus tshawytscha, acclimated to 7.5, 12.5 and 21.0°C, had similar cortisol elevations following a 30-s handling stress but plasma cortisol returned to resting levels more slowly in the low temperature-acclimated fish. Glucose increases in response to handling were much greater in the high temperature-acclimated fish than in the other two groups. In chinook salmon subjected to continuous confinement, plasma cortisol rose more quickly in those acclimated to the high temperature but peak levels in all three groups were similar. Both resting levels and stress-induced elevations in plasma glucose were highest in the high temperature-acclimated fish during confinement. Confinement-induced mortality was also highest in fish acclimated and confined at 21.0°C; there was less mortality of fish held at 12.5°C and none in those held at 7.5°C. Liver glycogen declined in all confined fish but the drop in glycogen levels was lowest in the low temperature-acclimated group.

A comparison of nine stocks of four juvenile salmonid species, subjected to identical 30-s handling stresses, suggested that there are genetic differences in stress responses in fish, although possible effects of early rearing history cannot be discounted. From our review of published literature, other stress response-modifying factors include time of day, external salinity, anesthesia, water quality, population density, light intensity and background color, nutritional state, health, and stage of development, as well as the fish's prior experience or exposure to stress. The findings indicate the importance of being familiar with both life histories and contingent environmental conditions when conducting stress investigations with fish. They also suggest

possible ways that stress can be managed for or how stress responses can be mitigated in order to improve fish's survivability and well-being.

Introduction

With both fundamental and applied researchers alike, there is a growing interest in the detrimental effects of stress in fish (see Pickering 1981b). This trend is particularly evident in fish culture where managers are concerned with the stress associated with physical disturbances such as handling, crowding or transport. This interest probably reflects the increasing awareness in fishery workers in general of the importance of fish quality, rather than simply quantity, in meeting management objectives.

Two often used stress indicators in fish are measurements of changes in plasma corticosteroids and glucose (reviews of Barton and Toth 1980; Donaldson 1981; Schreck 1981; Wedemeyer and McLeay 1981) and are good respective representations of the primary and secondary responses of fish to stress (Mazeaud et al. 1977). To date, there have been numerous investigations employing these two indicators in attempts to quantify the degree of stress resulting from various types of disturbances including capture, handling, confinement, transport and forced exercise. For general interest and to provide a convenient reference source for future work, we have summarized these investigations in Tables II.1 and II.2. About 45% of these studies have been conducted since the last and only major symposium on this topic (Pickering 1981b). Response trends are evident, but it is clearly apparent from Tables II.1 and II.2 that there is considerable variation in the magnitude of these stress-induced changes. Variations may be attributed to differences in the nature of the disturbance and the species of fish examined, as well as the influence of the fish's history and environment. Interpretation of response variations is further compounded in studies that have imposed a physical disturbance in combination with an additional stress factor.

For the measurement of corticosteroid and glucose elevations to have a wider clinical application than just being a research tool, either they must be relatively uniform over a wide

Table II.1. Chronological summary of published literature concerned with changes in plasma cortisol (or corticosteroids) in fish resulting from handling, confinement, exercise, or other types of physical disturbance. Values given, converted to ng/mL, are initial prestress levels and maximum values attained after imposition of the stress, as well as time to reach the maximum post-stress levels. (Decimal places were rounded to nearest unit; ca. values were estimated from graphs.)

Reference	Corticosteroid Level (ng/mL)			Physical Disturbance	Species
	Initial	Maximum	Time		
Leloup-Hatey 1960[a]	70–120	120–360	–	forced swimming	Cyprinus carpio
Hane et al. 1966	118[b]	ca. 450	48 h	continuous confinement	Oncorhynchus tshawytscha
Fagerlund 1967	21/25[c]	ca. 25	n.s.[d]	2–3 d moderate swimming	Oncorhynchus nerka
"	"	"	"	12–16 d " "	"
"	"	"	"	2.5 min restraint	"
"	"	147/284	30 min	30 min "	"
"	"	ca. 25/135	15 min	15 min agitation	"
"	"	240/383	3 h	3 h "	"
"	"	–/466	3 h	3 h transport with anesthetic	"
Hill and Fromm 1968	544	642	4 h	4 h forced swimming	Salmo gairdneri
Wedemeyer 1969	ca. 120	ca. 900	1 h	2 h mild agitation	"
Greswell and Stalnaker 1974	20[e]	35	–	transport and stocking	"
Porthe-Nibelle and Lahlou 1974	66	203	90 min	continuous mild agitation	Carassius auratus
Redgate 1974	10[f]	250	15 min	15 min restraint	Cyprinus carpio
"	43	260	2 d	pond-tank transfer	"
Spieler 1974	58	97	10–22 min	capture	Carassius auratus
Fryer 1975	73	93	15 min	60 s handling	"
Simpson 1975/76	ca. 10	ca. 200	0.125 d	30 min confinement and bag-tank transfer	Salmo gairdneri
Fuller et al. 1976	ca.50/40[c,g]	ca.100/120	3–72 h	retention in gill net	Coregonus lavaretus
"	37	539	55 h	seine-netting and continuous confinement	"
"	122	330	48 h	gill-netting and " "	"
Ilan and Yaron 1976	46	315	30 min	handling and injection	Cyprinus carpio
Mazeaud et al. 1977	ca. 30	ca. 90	5 min	5 min handling	Oncorhynchus kisutch

Table II.1. Continued

Reference	Corticosteroid Level (ng/mL) Initial	Maximum	Time	Physical Disturbance	Species
Strange et al. 1977	<10	ca. 100	20 min	tank-bucket transfer	Oncorhynchus tshawytscha
"	ca. 100	ca. 500	24 h	continuous confinement	"
"	ca. 20	ca. 140	20 min	" " at 23° C	Salmo clarki
"	"	ca. 170	70 min	" " at 9° C	"
Strange and Schreck 1978	ca. 5	ca. 75	30 min	tank-tank transfer	Oncorhynchus tshawytscha
"	"	ca. 50	1 h	" with anesthetic	"
Strange et al. 1978	<50	ca. 240	3.5 h	0.5 h confinement	"
"	"	ca. 500	12.5 h	continuous confinement (severe)	"
"	"	ca. 250	2 d	" " (moderate)	"
Barton et al. 1980	<2	ca. 43	15 min	handling and 90 s confinement	Salmo gairdneri
"	"	ca. 70	1 h	1 h agitation	"
"	"	ca. 213	16 h	continuous confinement	"
"	"	ca. 50	45 min	6 h transport	"
Leach and Taylor 1980	<20	ca. 470	1 h	continuous restraint (1)	Fundulus heteroclitus
"	ca. 50[h]	ca. 410	3 h	" " (2)	"
"	35	313	30 min	3 min handling	"
Pawson and Lockwood 1980	ca. 50[i]	ca. 280	ca. 5 h	trawl capture	Scomber scombrus
Specker and Schreck 1980	ca. 10	ca. 130	4 h	4 h transport - low density	Oncorhynchus kisutch
"	"	ca. 160	"	" - high "	"
"	"	ca. 140	12 h	12 h transport - low density	"
	"	ca. 170	"	" - high "	"
Strange 1980	50	>100	6 h	continuous confinement at 10° C	Ictalurus punctatus
"	25	225	3 d	" " at 20° C	"
"	"	150	1 d[j]	" " at 30° C	"
Strange and Schreck 1980	43	381	6 h	6 h continuous confinement	Oncorhynchus tshawytscha
"	31	188	"	" " in seawater	"

Table II.1. Continued

Reference	Corticosteroid Level (ng/mL) Initial	Maximum	Time	Physical Disturbance	Species
Tomasso et al. 1980	8	487	24 h	10 min confinement	Morone chrysops x saxatilis
"	"	ca. 180	6 h	2 h transport	"
"	"	ca. 160	6 h	" in salt	"
"	"	ca. 250	1 h	" with anesthetic	"
"	"	ca. 160	1 h	" in salt with anesthetic	"
Zelnik and Goldspink 1981	76	ca. 250	12 h	2 h forced swimming at 1 b.l./s	Salmo gairdneri
"	72	ca. 300	2 h	" at 2.6 b.l./s	"
	70	326	15 min	" at 5 b.l./s	"
Barton and Peter 1982	ca. 3	ca. 65	0.5 h	8 h transport	"
"	<2	ca. 35	8 h	" with salt	"
"	ca. 45	ca. 100	4 h	" in chilled water	"
"	"	ca. 85	"	" " " with salt	"
Davis et al. 1982	30	280	25 min	10 min net confinement	Morone saxatilis
"	"	ca. 360	"	25 min MS-222 incl. 10 min net confinement	"
"	"	ca. 350	"	" quinaldine incl. "	"
"	"	ca. 100	"	" etomidate incl. "	"
"	"	ca. 325	"	" salt incl. "	"
"	"	ca. 325	"	" " + MS-222 incl. "	"
"	"	ca. 370	"	" " + quinaldine incl. "	"
"	"	ca. 80	"	" " + etomidate incl. "	"
Pickering et al. 1982	ca. 25	ca. 130	2 h	handling and 2 min confinement	Salmo trutta
Davis and Parker 1983	ca. 35	ca. 205	6 h	6 h confinement	Salmo gairdneri
"	ca. 20	ca. 70	"	"	Salmo salar
"	ca. 15	ca. 55	18 h	"	Salvelinus namaycush
Limsuwan et al. 1983	20	87	30 min	10 min confinement	Ictalurus punctatus
"	"	44	24 h	" with etomidate	"
Redding and Schreck 1983	ca. 5	ca. 330	1 h	continuous confinement	Oncorhynchus kisutch
"	"	ca. 270	2 h	" in isotonic medium	"
"	ca. 20	474	24 h	" in seawater	"

Table II.1. Continued

Reference	Corticosteroid Level (ng/mL) Initial	Maximum	Time	Physical Disturbance	Species
Swift 1983	42[i]	57	2 h	enclosure-tank transfer	Scomber scombrus
"	"	69[k]	24 h	15 min confinement	"
"	156	73	4 d	moderate continuous confinement	"
Tomasso et al. 1983	8	57	6 h	continuous confinement	Ictalurus punctatus
Carmichael et al. 1984a	15	ca. 100	0.5 h	0.5 h net confinement	Micropterus salmoides
"	ca. 15	ca. 125	48 h	48 h "	"
"	"	ca. 70	3 h	3 h confinement at 10° C	"
"	ca. 20	"	1 h	" at 23° C	"
"	7	85	0.5 h	0.5 h "	"
"	"	46	"	" after brief hypoxia	"
"	"	131	"	" after CO_2 exposure	"
"	"	66-70	"	" after NH_3 exposure	"
Carmichael et al. 1984b	ca. 15	ca. 200	30 h	30 h transport (22° C)	"
"	"	ca. 140	48 h	" (20° C)	"
"	"	ca. 110	30 h	" " with salt, anesthesia, additives (fasted 36 h)	"
"	ca. 10	ca. 120	6 h	" " " "	"
				" (fasted 72 h)	"
Davis et al. 1984	29	ca. 55	9 h	6 h net confinement at 5° C	Ictalurus punctatus
"	25	ca. 30	12 h	" at 10° C	"
"	5	ca. 55	6 h	" at 15° C	"
"	8	ca. 60	"	" at 20° C	"
"	12	ca. 95	"	" at 25° C	"
"	13	ca. 95	"	" at 30° C	"
"	12	ca. 95	"	" at 35° C	"
Nichols and Weisbart 1984	ca.40-80	ca. 190	6-7 min	5 min chasing	Salmo salar
Pickering and Stewart 1984	4[m]	8.5	20 d	continuous confinement	Salmo trutta
Ainsworth et al. 1985	51	309	2 h	tank-transfer plus 2 h confinement	Ictalurus punctatus

Table II.1. Continued

Reference	Corticosteroid Level (ng/mL) Initial	Maximum	Time	Physical Disturbance	Species
Barton et al. 1985a	4	ca. 90	1 h	30 s handling in air - parr	*Oncorhynchus kisutch*
"	39	ca. 150	"	" - smolts	"
Barton et al. 1985b	9	ca. 140	1 h	30 s handling - pH 6.6	*Salmo gairdneri*
"	23	ca. 340	1 h	" - pH 4.7	"
Gilham and Baker 1985	53	123	30 min	daily injection (X3) - white background	"
"	32	288	"	" - black "	"
"	5	108	15 min	15 min emersion and handling	*Anguilla anguilla*
"	2	78	"	" - partial hypophysectomy	"
"	1	7	"	" - total "	"
Barton et al. 1986	61	182	0.5 h	single 30-s handling	*Oncorhynchus tshawytscha*
"	30	296	4 h	two 30-s handlings	"
"	32	476	7 h	three "	"
"	24	299	12 h	" " (diseased fish)	"

[a] as cited in Love (1970)

[b] 17-hydroxycorticosteroids

[c] males/females

[d] not significantly different from initial values

[e] means of pooled values for different experimental periods

[f] minimum pre-transfer value reported

[g] seine-netted and sampled immediately after capture

[h] maximum value for unstressed controls throughout experiment

[i] whole blood values

[j] 100% mortality reported at 2 d

[k] maximum value reported after initial

[m] corresponding value for uncrowded fish at 20 d

Table II.2. Chronological summary of published literature concerned with changes in plasma glucose in fish resulting from handling, confinement, exercise, or other types of physical disturbance. Values given, converted to mg/dL, are initial prestress levels and maximum levels attained after imposition of the stress, as well as time to reach the maximum poststress levels. (Decimal places were rounded to nearest unit; ca. values were estimated from graphs.)

Reference	Glucose Level (mg/dL)			Physical Disturbance	Species
	Initial	Maximum	Time		
Black 1957	75	152	4 h	15 min strenuous exercise	Salvelinus namaycush
Black et al. 1960	69	115	12 h	"	Salmo gairdneri
Chavin 1964	29	60	1 d	tank-tank transfer	Carassius auratus
"	"	93	25 min	handling and injection	"
Hammond and Hickman 1966	ca. 70	ca. 120	8 h	15 min forced swimming - unconditioned	Salmo gairdneri
"	"	ca. 100	24 h	" - conditioned	"
Black and Tredwell 1967	50-75	ca. 200	28 h	physical injury by descaling	"
Nakano and Tomlinson 1967	ca. 80	ca. 120	15 min	2-120 min chasing and tail grasping	"
Leibson and Plisetskaya 1969	ca. 50	ca. 170	26-28 h	capture and transport	Lampetra fluviatilis
Chavin and Young 1970	26[a]	54	1 d	pond-aquaria transfer	Carassius auratus
"	30	103	1 h	net capture	"
Houston et al. 1971	68	126	24 h	handling, anesthesia, and surgery	Salvelinus fontinalis
Wardle 1972	15-25	120	8 h	trawl capture	Pleuronectes platessa
Wedemeyer 1972	ca. 60	ca. 230	3 h	tank-tank transfer	Oncorhynchus kisutch
"	"	ca. 190	"	" "	"
Umminger and Gist 1973	53	263	2 h	brief handling and injection at 10° C	Carassius auratus
"	37	121	"	" at 20° C	"
"	26	75	"	" at 30° C	"
Wendt and Saunders 1973	ca. 60	ca. 90	15 h	15 min exercise at 0.2° C	Salmo salar
"	ca. 35	ca. 70	2 h	" 6.5° C	"
"	ca. 40	ca. 105	"	" 15° C	"
"	ca. 120	ca. 175	1 h	" - unconditioned	" -2-yr old
"	ca. 45	ca. 60	"	" - conditioned	" "
"	ca. 40	ca. 80	2 h	" - unconditioned	" -1.5-yr old
"	"	ca. 65	"	" - conditioned	" "
"	ca. 100	ca. 70[b]	5 h	22 h simulated transport	"

Table II.2. Continued

Reference	Glucose Level (mg/dL) Initial	Maximum	Time	Physical Disturbance	Species
Miles et al. 1974	89	139	6 h	brief handling and marking	Esox masquinongy
"	100	125	26 h	23 h retention and 3 h transport	"
Fletcher 1975	ca. 40	ca. 120	5 h	capture	Pseudopleuronectes americanus
Larsen 1976	ca. 23[c]	ca. 130	2 h	injection + 30 min anesthesia	Lampetra fluviatilis
Soivio and Oikari 1976	106	ca. 265	1 h	(30 s handling and retention)X3-freshwater	Esox lucius
"	54	ca. 100	"	" " - brackish water	"
Spieler and Nickerson 1976	30	44	6 d	20 s handling	Carassius auratus
"	"	47	1 d	" with anesthetic	"
Wydoski et al. 1976	ca. 70	ca. 140	4 min	5 min hooking and forced exercise	Salmo gairdneri
Casillas and Smith 1977	ca. 55	ca. 70	5 h	2 min "	" - hatchery
"	ca. 65	ca. 85	10 min	" "	" - wild
Ince and Thorpe 1978	ca. 70	ca. 95	30 min	injection while under anesthesia	"
Murat et al. 1978	ca. 45[c]	ca. 90	9 h	single injection	Cyprinus carpio
Perrier et al. 1978	67	165	4 h	2 min hooking and forced exercise	Salmo gairdneri
Rush and Umminger 1978	24	44	6 h	10 s handling - unconditioned	Carassius auratus
"	29	33	3 d	" - conditioned	"
Aldrin et al. 1979	63	73	24 h	3 h transport	Oncorhynchus kisutch
Pasanen et al. 1979	ca. 40	ca. 230	24 h	seining and marking	Coregonus albula
Harman et al. 1980	89	146	<2 h	40 min chasing with hypoxia	Aplodinotus grunniens
Leach and Taylor 1980	50-75	ca. 125	6 h	continuous restraint	Fundulus heteroclitus

Table II.2. Continued

Reference	Glucose Level (mg/dL) Initial	Maximum	Time	Physical Disturbance	Species
Specker and Schreck 1980	ca. 55	ca. 100	4 h	4 h transport - low density	Oncorhynchus kisutch
"	"	ca. 95	"	" - high "	"
"	"	ca. 75	12 h	12 h transport - low density	"
"	"	ca. 110	"	" - high "	"
Strange 1980	45	ca. 100	12 h	continuous confinement at 10° C	Ictalurus punctatus
"	28	ca. 250	"	" at 20 and 30° C	"
Schneider et al. 1981	ca. 59	ca. 88	29.5 min[d]	forced swimming (2.5 b.l./s) at 12° C	Salmo gairdneri
"	"	ca. 79	"	" " at 17° C	"
"	"	ca. 92	"	" " at 22° C	"
"	"	ca. 106	8.8 min	" " at 27° C	"
Soivio and Nikinmaa 1981	ca. 43[c]	ca. 52	24 h	6 h transport	"
Zelnik and Goldspink 1981	ca. 50	ca. 80	15 min	2 h forced swimming	"
Morata et al. 1982	72[e]	108	60 min	continual repeated forced activity	"
Pickering et al. 1982	ca. 110	ca. 140	4 h	handling and 2 min confinement	Salmo trutta
Carmichael et al. 1983	ca. 95	ca. 345	2.5+ h	crowding, handling and 2.5 h transport	Micropterus dolomieui
Limsuwan et al. 1983	44	130	30 min	10 min confinement	Ictalurus punctatus
"	"	57	"	" with etomidate	"
Nikinmaa et al. 1983	ca. 140	ca. 220	14 h	14 h transport	Salmo trutta
"	"	ca. 90	"	" in salt water	"
Swift 1983	61[c]	81	0.25 h	enclosure-tank transfer	Scomber scombrus
"	"	126[b]	120 h	15 min confinement	"
"	91	84[b]	2 h	moderate continuous confinement	"

Table II.2. Continued

Reference	Glucose Level (mg/dL) Initial	Maximum	Time	Physical Disturbance	Species
Carmichael et al. 1984a	53	ca. 135	0.5 h	0.5 h net confinement	Micropterus salmoides
"	ca. 50	ca. 140	3 d	48 h "	"
"	ca. 37	ca. 75	3 h	3 h " at 10° C	"
"	ca. 55	ca. 140	2 h	" at 23° C	"
"	61	124	0.5 h	0.5 h "	"
"	"	137	"	" " after brief hypoxia	"
"	"	194	"	" " " CO_2 exposure	"
"	"	200-279	"	" " " NH_3 exposure	"
Carmichael et al. 1984b	ca. 75	ca. 450	30 h	30 h transport (22° C)	"
"	ca. 50	ca. 350	24 h	" (20° C)	"
"	"	ca. 200	"	" with salt, anesthesia, additives	
"	ca. 120	ca. 150	150 h	" " " " (fasted 36 h)	"
				(fasted 72 h)	"
Cliff and Thurman 1984	ca. 90[e]	ca.>300	24 h	10 min struggling and 60 min confinement	Carcharhinus obscurus
Fletcher 1984	ca. 21	ca. 40	2 h	1 min aerial emersion	Limanda limanda
Barton et al. 1985b	85	ca. 130	6 h	30 s handling - pH 6.6	Salmo gairdneri
"	258	ca. 290	12 h	" - pH 4.7	"
Schwalme and Mackay 1985a	ca. 79[e]	ca. 238	2 h	30 s aerial emersion X3	Esox lucius
Schwalme and Mackay 1985b	ca. 42[c]	ca. 118	6 h	1.4 min hooking and 7 min transport	"
Woodward and Smith 1985	51	57	15 min	2 min agitation - untrained	Salmo gairdneri
"	"	65	60 min	" " - trained	"
Barton et al. 1986	55	84	3 h	single 30-s handling	Oncorhynchus tshawytscha
"	42	134	6 h	two 30-s handlings	"
"	57	204	12 h	three "	"
"	67	334	"	" " (diseased fish)	"

[a] minimum value reported 4 d after transfer
[b] maximum value recorded after initial
[c] whole blood values
[d] mean time of exercise trial
[e] values converted from molar units

range of conditions or researchers must be aware of the possible differences caused by additional stress and non-stress factors. These include acclimation conditions, age and size of the fish, time of day and year, and nutritional status, to name a few. In contrast to a growing body of literature that documents the effect of these factors on resting plasma corticosteroid and glucose levels, there are fewer reports that demonstrate their influence on stress responses in fish. Moreover, knowledge and manipulation of certain factors, demonstrated to actually reduce the responses to stress in fish, may allow managers to implement and assess various stress mitigation practices in order to meet their objectives more effectively.

One purpose of this paper was to briefly review previous studies dealing with possible genetic, ontogenetic and environmental factors that may modify corticosteroid and glucose changes in response to physical disturbances. In the context of this review, the other purpose was to present some new results on: (1) the effects of acclimation temperature on stress-induced plasma cortisol and glucose elevations in juvenile chinook salmon, Oncorhynchus tshawytscha, and (2) possible differences in these changes among various juvenile salmonid species and stocks subjected to an identical handling stress.

Acclimation Temperature Influence on Stress Responses

Methods and Materials

For both experiments, juvenile Cedar Creek stock fall chinook salmon, hatched and maintained at the Oregon Department of Fish and Wildlife Corvallis Research Laboratory, were acclimated to experimental temperatures of 7.5°C, 12.5°C and 21.0°C as follows. In two groups of duplicate tanks of fish acclimated to 12.5°C, water temperature was either

continuously increased or decreased by 1°C/d until the final temperature was reached, after which the fish were held at that temperature for an additional 3 wk for each experiment. A third group of fish in duplicate tanks remained at 12.5°C. The temperatures selected represented the range of temperatures that juvenile chinook salmon might be expected to encounter in rivers during seaward migration (Schreck et al. 1985) and the high temperature was below the lethal threshold value (Coutant 1972, cited in NASNAE 1972). Water temperature was continuously controlled to within ±0.5°C by mixing heated and chilled aerated well water through three-way valves using Honeywell ® programmable, cam-operated, pneumatic temperature controller-recorder units (Minneapolis-Honeywell Regulator Co., Brown Instruments Div., Philadelphia, Pennsylvania). Details of the experimental temperature apparatus are described by Golden (1978).

Throughout the acclimation period and both experiments, the fish were held at a density of about 6 g/L in 1-m-diameter tanks containing 350 L and having an inflow of 10 L/min. The fish were fed daily with Oregon Moist Pellets (OMP) at about 1.5% body weight/d up to, but not during, the experiment.

After acclimation at the final temperatures, fish were stressed and sampled in the following ways:

1. Acute Stress Experiment: Fish [35 ±1.2 g (mean ±SE)] from duplicate tanks for each temperature were subjected to a single handling stress by holding them in the air in a net for 30 s and returning them to the tank. Fish were removed for samples before and 1, 3, 6, 12 and 24 h after the stress.

2. Chronic Stress Experiment: Fish (54 ±2.5 g) from duplicate tanks for each temperature were captured and subjected to continuous confinement by being placed and then kept at a density of about 200 g/L in a live-cage immersed in the tank. Fish were removed for samples before and after 3, 6, 12, 24, 48, 72 and 120 h of confinement.

Fish were removed from the tanks and immediately placed in 200 mg/L tricaine methanesulfonate (MS-222) for subsequent blood and liver sampling according to the protocol of Barton et al. (1986, Chapter III). Plasma cortisol was determined by a ^{3}H-radioimmunoassay (Foster and Dunn 1974) modified by Redding et al. (1984b) for use with salmonid plasma. Plasma glucose was measured by the colorimetric procedure of Wedemeyer and Yasutake (1977) using Sigma® premixed 6% o-toluidine reagent (Sigma Chemical Co., St. Louis, Missouri). Characteristics of these two assays are given in Barton et al. (1986). Liver glycogen was assayed following the phenol-sulfuric acid method described by Montgomery (1957). Comparisons among temperature treatments at specific times and within treatments through time were made by one-way analyses of variance (ANOVA) followed with Duncan's new multiple-range tests at the 5% level (Steel and Torrie 1980). Cortisol and glucose data were first converted to logarithmic values to reduce variance heterogeneity (Bartlett's test, Snedecor and Cochran 1967).

Results and Discussion

(a) Acute Stress

Peak cortisol 1 h after the stress was similar in the three groups (Fig. II.1). The only significant differences in post-stress plasma cortisol were at 6 and 12 h, where titers were highest in the low temperature group (Fig. II.1). This suggests that a higher rate of cortisol clearance may have occurred at the higher temperatures. For example, in European eels, Anguilla anguilla, Leloup-Hatey and Hardy (1976) observed that metabolic clearance rate of cortisol increased in spring and summer months and attributed this change to the seasonal increase in temperature.

Plasma glucose in response to handling was substantially higher in the high temperature group than in fish from the other two temperatures (Fig. II.2); the peak concentration at 6 h

Figure II.1. Mean plasma cortisol (ng/mL ±SE, $\underline{N}$=12) in juvenile chinook salmon acclimated to low (7.5°C), medium (12.5°C) and high (21.0°C) temperatures and subjected to a 30-s handling stress. Values represent pooled data from duplicate tanks. Changes through time were significant for all treatments (ANOVA, $\underline{P}$<0.01). For a single time, a letter "a" accompanying a value indicates that extreme treatment values within that time group are different from each other but not from the intermediate value; a letter "b" indicates that that treatment value is different from both other values (ANOVA followed with Duncan's new multiple-range test at the 5% probability level).

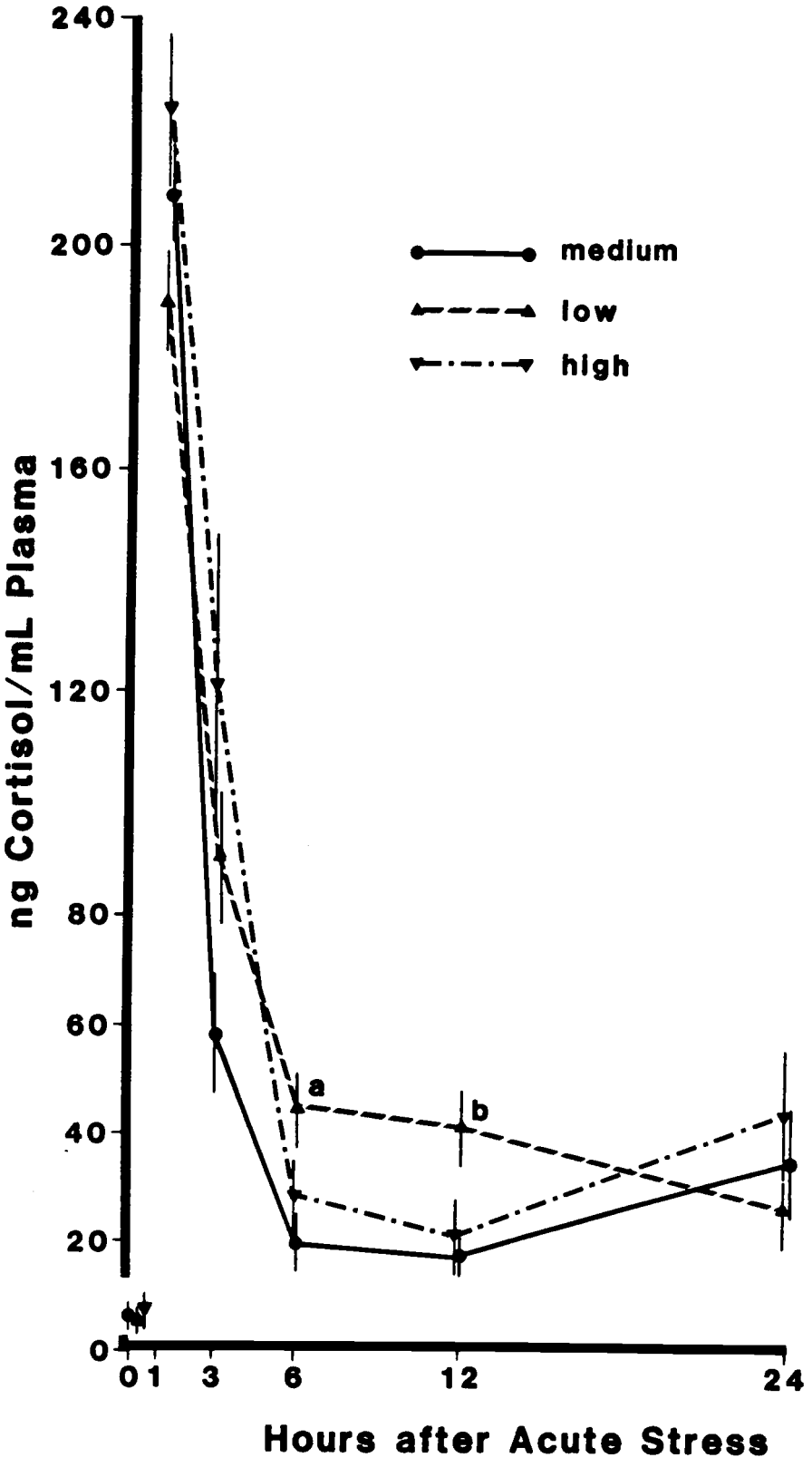

240
200
160
120
80
60
40
20
0
ng Cortisol/mL Plasma
medium
low
high
a
b
0 1
3
6
12
24
Hours after Acute Stress

Figure II.1

Figure II.2. Mean plasma glucose (mg/dL ±SE, $\underline{N}$=12) in juvenile chinook salmon acclimated to low (7.5°C), medium (12.5°C) and high (21.0°C) temperatures and subjected to a 30-s handling stress. Values represent pooled data from duplicate tanks. Changes through time were significant for all treatments (ANOVA, $\underline{P}$<0.01). Refer to Fig. II.1 for explanation of letters accompanying values.

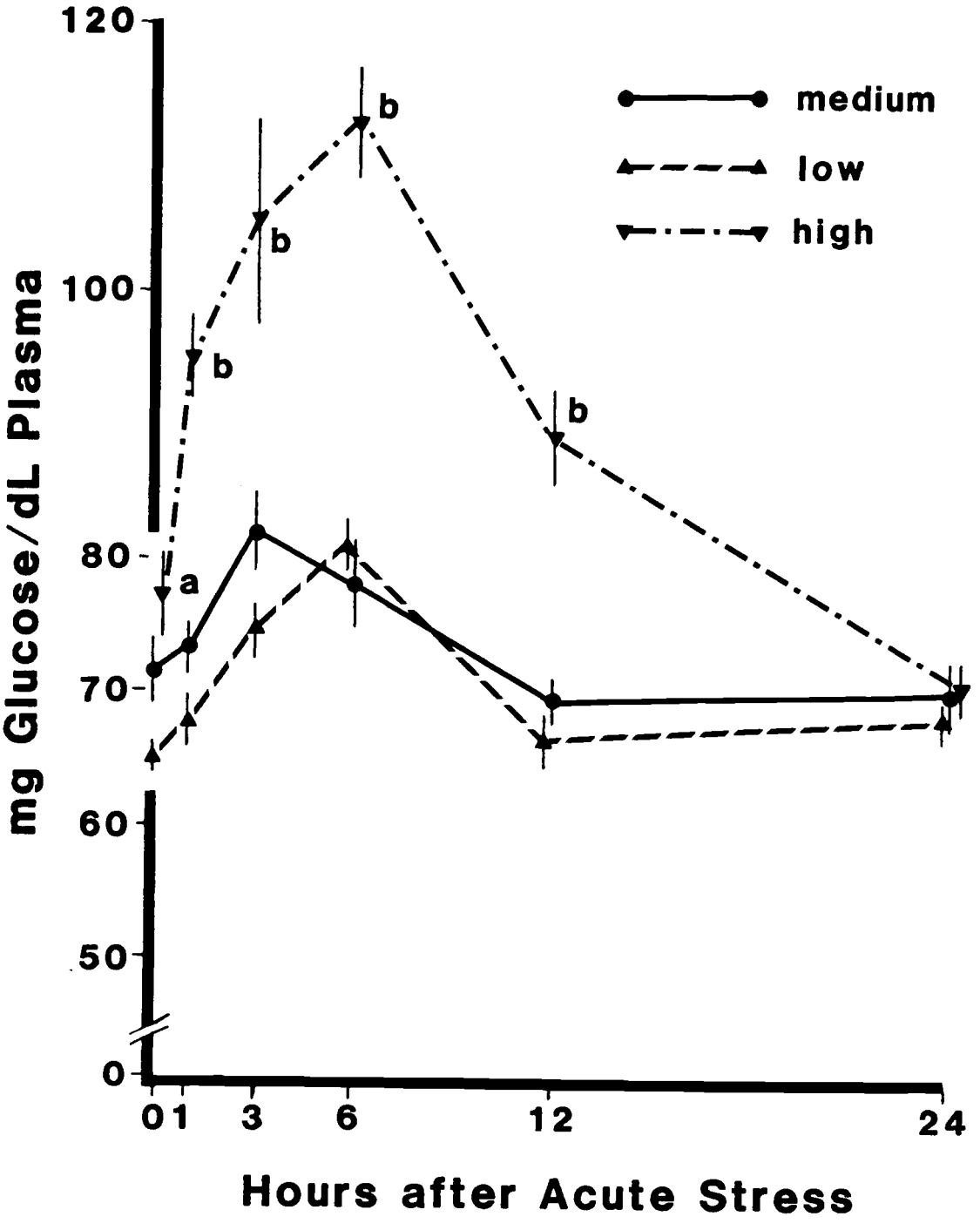

120
100
80
70
60
50
0
mg Glucose/dL Plasma
medium
low
high
a
b
b
b
b
0 1
3
6
12
24
Hours after Acute Stress

Figure II.2

represents a relative response of more than double those in fish from the two lower temperatures. Plasma glucose in fish returned to resting levels within 12 h at the medium and low temperatures but remained elevated at 12 h in the high temperature group (Fig. II.2). Similarly, elevation of plasma glucose in rainbow trout, Salmo gairdneri, following a 5-min 'hook and line' stress was most pronounced in fish held at 20°C and was significantly delayed in those held in 4°C water (Wydoski et al. 1976). Umminger and Gist (1973) also found a differential response of plasma glucose to handling stress in goldfish, Carassius auratus, acclimated to different temperatures but in their study, glucose responses and acclimation temperatures were inversely proportional. Those authors attributed this to the high temperature being closest to their preferred temperature so the fish were less easily stressed. In our experiment, the medium acclimation temperature was closest to the final preferendum of 11.7°C for small chinook salmon (Coutant 1977). Since blood glucose and standard metabolic rates are positively correlated in vertebrates generally (Umminger 1977), our results suggest that the higher glucose response to handling is a reflection of the greater metabolic response to stress in the fish acclimated to the high temperature. Changes in liver glycogen through time were not significant, although differences were evident among treatments at various times following the stress (Fig. II.3) suggesting a greater reduction in glycogen reserves in fish handled at the medium and high temperatures than that in the low temperature-acclimated fish.

(b) Chronic Stress

Plasma cortisol reached the peak level in response to continuous confinement more quickly in the high temperature group, e.g. by 6 h, as compared to the other two groups that had their maximum cortisol elevation at 12 h (Fig. II.4). All groups exhibited significantly elevated plasma cortisol for the duration

Figure II.3. Mean liver glycogen (mg/g ±SE, $\underline{N}$=10) in juvenile chinook salmon acclimated to low (7.5°C), medium (12.5°C) and high (21.0°C) temperatures and subjected to a 30-s handling stress. Values represent pooled data from duplicate tanks. Changes through time were not significant for any of the treatments (ANOVA, $\underline{P}$>0.05). Refer to Fig. II.1 for explanation of letters accompanying values.

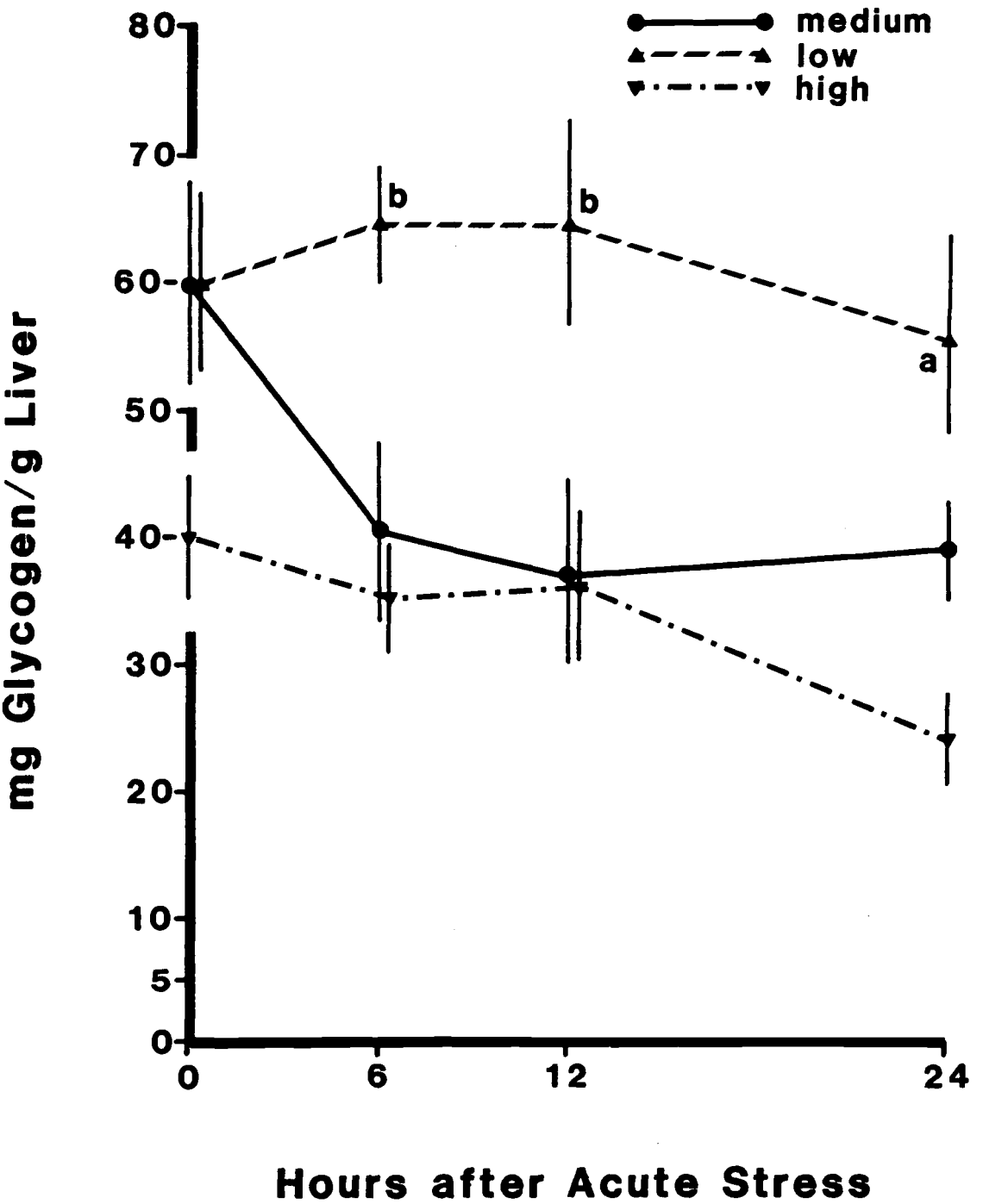

medium
low
high
80
70
60
50
40
30
20
10
5
0
b
b
a
mg Glycogen/g Liver
0
6
12
24
Hours after Acute Stress

Figure II.3

Figure II.4. Mean plasma cortisol (ng/mL ±SE, N=12) in juvenile chinook salmon acclimated to low (7.5°C), medium (12.5°C) and high (21.0°C) temperatures and subjected to continuous confinement at 200 g/L in a live-cage. Values represent pooled data from duplicate tanks. Changes through time were significant for all treatments (ANOVA, $P<0.01$). Refer to Fig. II.1 for explanation of letters accompanying values.

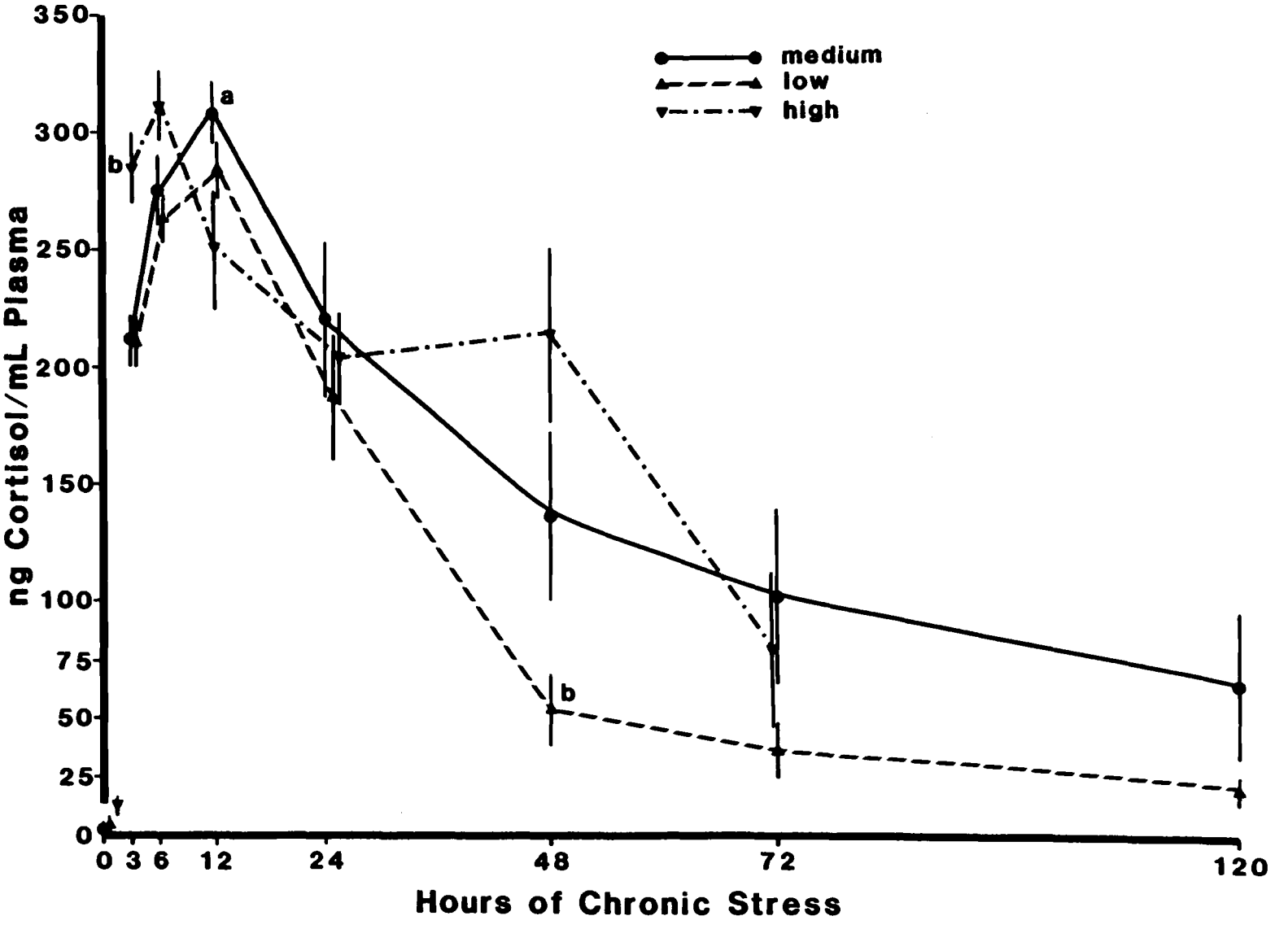

medium
low
high
ng Cortisol/mL Plasma
350
300
250
200
150
100
75
50
25
0
a
b
b
0 3 6 12 24 48 72 120
Hours of Chronic Stress

Figure II.4

of confinement although there was a general reduction in levels with time (Fig. II.4). The high temperature-group trial was terminated at 72 h because of high mortality. Other authors have also studied the influence of acclimation temperature on interrenal responses of fish to continuous confinement. In channel catfish, Ictalurus punctatus, plasma cortisol increased more rapidly (Strange 1980) and to a greater degree (Davis et al. 1984) in fish from higher temperatures when stressed by confinement. Carmichael et al. (1984a), however, observed no difference in corticosteroid elevations during confinement of largemouth bass, Micropterus salmoides, acclimated to 10 or 23°C. In the other study on salmonids, Strange et al. (1977) found that when juvenile cutthroat trout, Salmo clarki, acclimated to either 9 or 23°C, were subjected to netting and continuous confinement, both groups exhibited similar initial plasma corticosteroid increases but levels decreased after 70 min in the high temperature-acclimated fish.

By hour 24 of confinement, it was apparent from the data that fish were of two distinct groups based on bimodally distributed cortisol titers, i.e. fish with plasma cortisol still elevated and those with cortisol returning to resting levels. By arbitrarily choosing a threshold level at the approximate midpoint of the distribution to divide the two groups, we separated data from those fish with high plasma cortisol from those with recovering cortisol concentrations and compared numbers of fish with high cortisol from the three temperature groups. Between 24 and 72 h, the greatest number of fish with high cortisol came from the high temperature-treatment group (Fig. II.5); total proportion of live fish in the samples having chronically elevated plasma cortisol from 24 h to the end of the experiment was 58%, 41% and 17% from the high, medium and low temperature groups, respectively.

Mortality was also highest in the high temperature group. Fish from the medium temperature exhibited some mortality at 12 and 24 h but higher mortality in the high temperature group occurred at

Figure II.5. Mean plasma cortisol (from Fig. II.4) and percent mortality in juvenile chinook salmon acclimated to low (7.5°C), medium (12.5°C) and high (21.0°C) temperatures in the first 72 h of continuous confinement. Values represent pooled data from duplicate tanks. From 24 h onward, data for each treatment were separated into low and high value groups (see text). Division of groups was based on an apparent bimodal distribution of values and separated by an arbitrary threshold level (AT). Number of individuals comprising each high value point accompanies that value on the graph. Percent mortality of fish present at each of the sample times is indicated by a histogram.

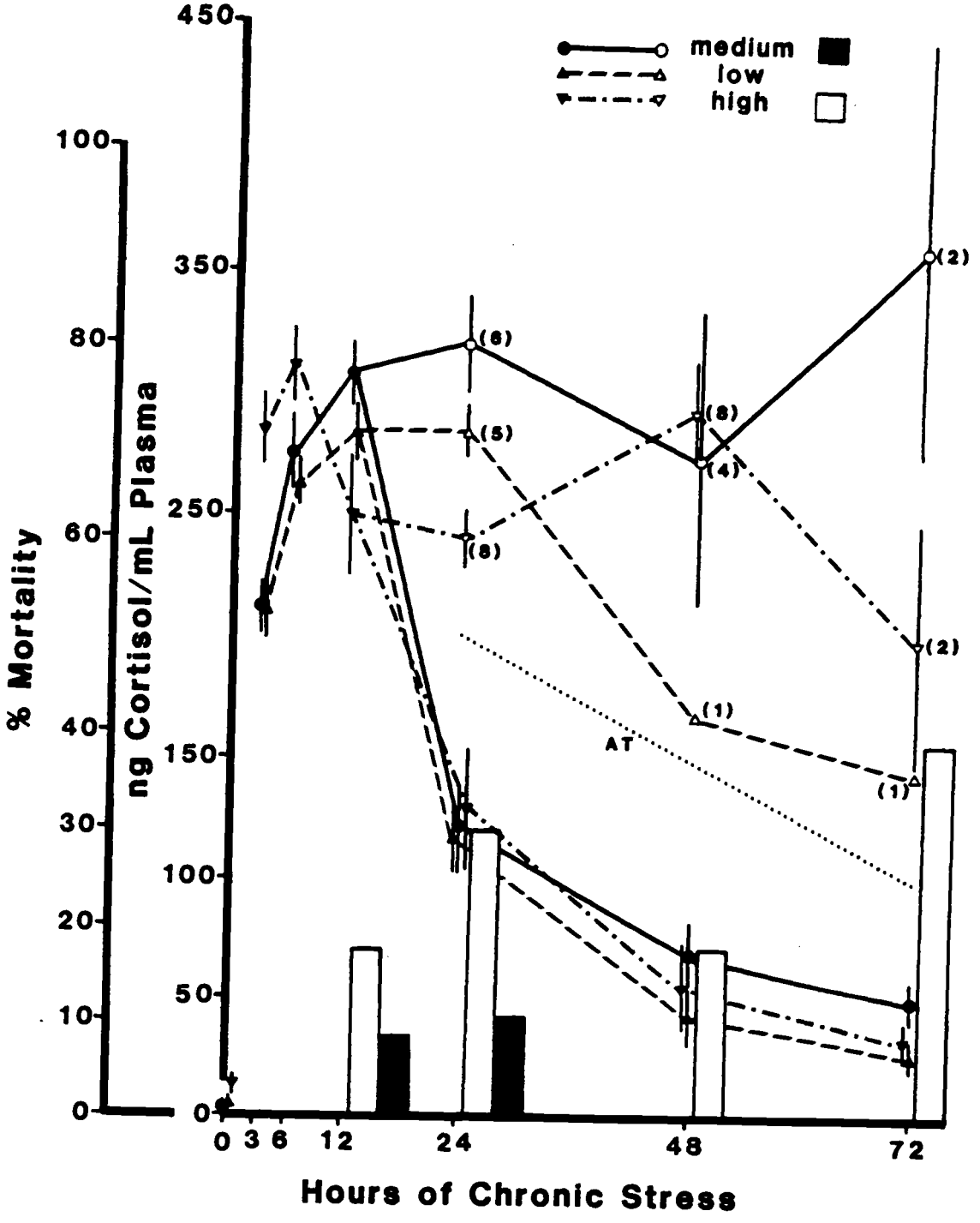

medium
low
high
% Mortality
ng Cortisol/mL Plasma
Hours of Chronic Stress
AT
(6)
(5)
(8)
(8)
(4)
(2)
(1)
(1)
(2)

Figure II.5

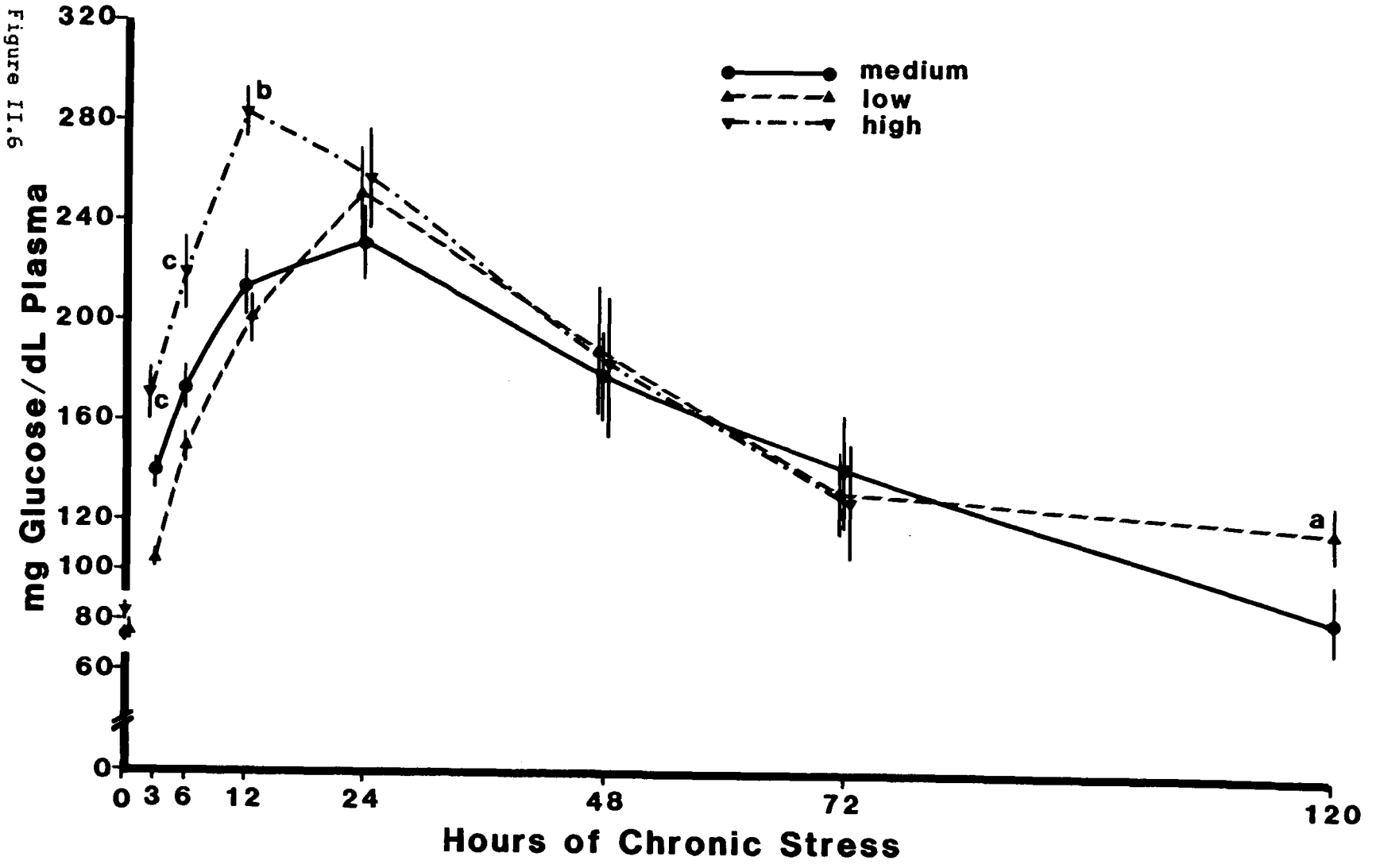
all sampling times after 12 h (Fig. II.5). By 72 h, only a few fish at the high temperature remained alive for sampling whereas no mortality occurred in the low temperature group throughout the experiment. Although we did not measure cortisol from dead fish, these results indicated a positive association between fish with chronically elevated plasma cortisol and those that were likely to die during continuous confinement.

Plasma glucose also rose more quickly in fish from the high acclimation temperature than in the other two groups in response to confinement but by 24 h, levels for all three temperature groups were similar (Fig. II.6). Plasma glucose exhibited a declining trend towards the end of the experiment; although it was still significantly elevated in all groups at 72 h, it had returned to the prestress level after 120 h in the medium-temperature group (Fig. II.6). Our finding of a greater glucose elevation in high temperature-acclimated fish within the first 12 h of confinement is consistent with those for channel catfish (Strange 1980) and largemouth bass (Carmichael et al. 1984a) and, like the acute stress response, may reflect a higher metabolic response of the fish to continuous confinement at the warmer temperature. By 24 h, plasma glucose from all three temperature groups was the same and declined at the same rate thereafter, except that it remained higher in the low temperature group at 120 h (Fig. II.6). In contrast, plasma glucose was elevated after 1 d in 20°C-acclimated, confined channel catfish but was near resting levels in low and high temperature groups (Strange 1980). Strange's (1980) results suggested that stress-elevated glucose decreased again as the fish approached death, since he observed a positive relationship between degree of mortality during chronic confinement and acclimation temperature as we did.

At the beginning of confinement, liver glycogen was similar in all groups, but after 12 h of continuous confinement it had declined by 45% in the low temperature group, 86% in the medium temperature group and 69% in the high temperature group (Fig.

Figure II.6. Mean plasma glucose (mg/dL ±SE, N=12) in juvenile chinook salmon acclimated to low (7.5°C), medium (12.5°C) and high (21.0°C) temperatures and subjected to continuous confinement at 200 g/L in a live-cage. Values represent pooled data from duplicate tanks. Changes through time were significant for all treatments (ANOVA, $P<0.01$). Refer to Fig. II.1 for explanation of letters "a" and "b" accompanying values; a letter "c" accompanying a value indicates that all three treatment values within that time group are different from each other (ANOVA followed with Duncan's new multiple-range test at the 5% probability level).

Figure II.6


320
280
240
200
160
120
100
80
60
0
mg Glucose/dL Plasma
medium
low
high
a
b
c
c
0 3 6 12 24 48 72 120
Hours of Chronic Stress

II.7); these declines were significant. After 48 h of continuous confinement, liver glycogen had returned to preconfinement levels in all groups (Fig. II.7). The greater drop in glycogen at the two higher temperatures reflects a higher rate of conversion of stored glycogen to glucose in response to the confinement stress and supports our finding of a greater glucose stress response in the high temperature group.

Review and General Discussion

Environmental Factors

(a) Temperature and Time of Day

From the foregoing results and discussion, acclimation temperature appears to have a variable effect on the magnitude of plasma corticosteroid elevations in response to disturbance in fish. Differences in peak levels were seen in channel catfish (Davis et al. 1984) but not in two other species (Carmichael et al. 1984a; present paper). Acclimation temperature does, however, increase the rate at which plasma levels increase in response to stress and also the subsequent rate of decline (Davis et al. 1984; present paper). It is tempting to speculate that the more rapid increase and decrease of plasma cortisol during stress is due to temperature-dependent increased rates of secretion and clearance, respectively, of the steroid. However, possible but undetermined physiological differences in fish from different acclimation temperatures, such as differences in sensitivity or responsiveness of the hypothalamic-pituitary axis, preclude the ability to make this conclusion.

Peak stress-induced increases in glucose are more profoundly affected by acclimation temperature than cortisol and post-stress plasma levels have been either positively (Wydoski et al. 1976; Carmichael et al. 1984a; present paper) or negatively

Figure II.7. Mean liver glycogen (mg/g ±SE, $\underline{N}$=10) in juvenile chinook salmon acclimated to low (7.5°C), medium (12.5°C) and high (21.0°C) temperatures and subjected to continuous confinement at 200 g/L in a live-cage. Values represent pooled data from duplicate tanks. Changes through time were significant for all treatments (ANOVA, $\underline{P}$<0.01). Refer to Fig. II.1 for explanation of letters accompanying values.

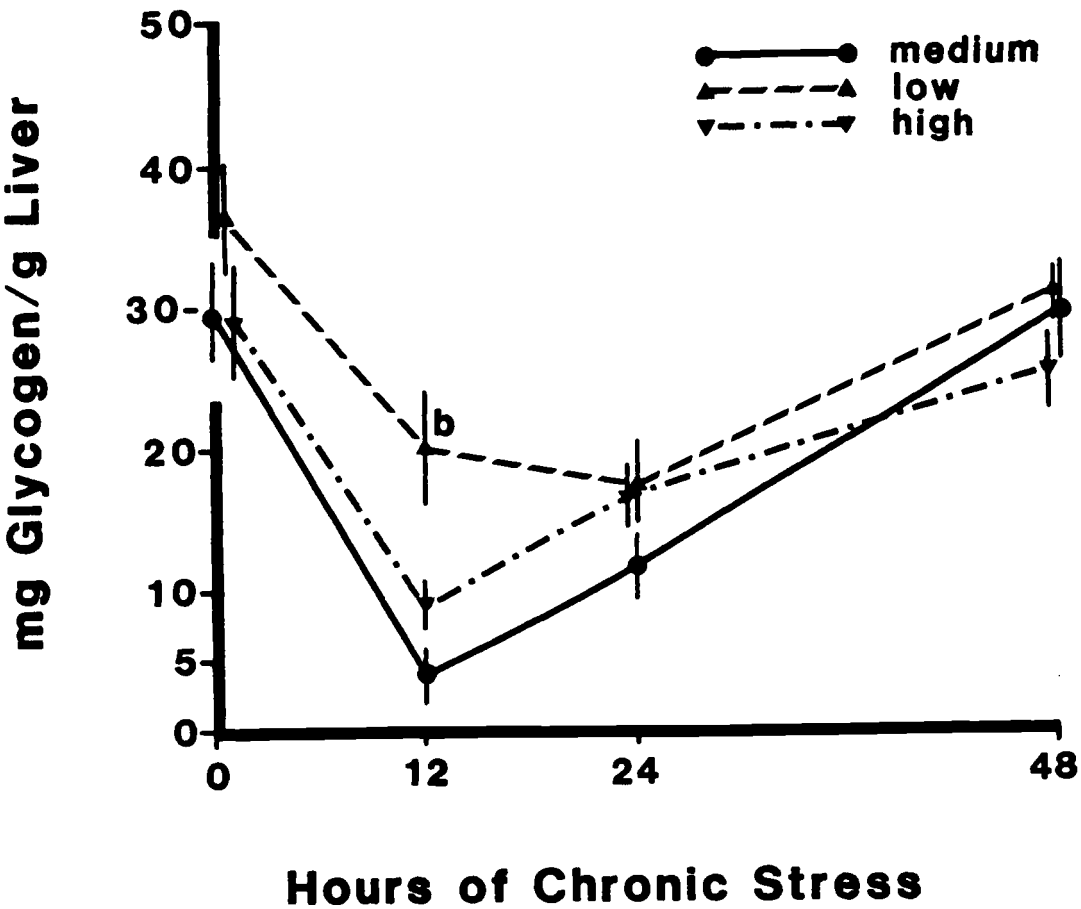

50
40
30
20
10
5
0
mg Glycogen/g Liver
medium
low
high
b
0
12
24
48
Hours of Chronic Stress

Figure II.7

(Umminger and Gist 1973) correlated with temperature. Similarly, Wendt and Saunders (1973) reported temperature-related differences in plasma glucose following exercise; Atlantic salmon, Salmo salar, in the summer (15°C) exhibited a higher postexercise glucose peak than in the late fall (6.5°C) or winter (0.2°C). A positive correlation with temperature is understandable since metabolic rate increases with temperature in fish in general, the Q_{10} being about 2.3 but also variable (Brett and Groves 1979). Such an interpretation may be complicated by the departure of the experimental acclimation temperature from the species' preferred temperature, as Umminger and Gist (1973) concluded.

Few investigators have examined the effects of a temperature change occurring concurrently with another physical disturbance on plasma cortisol and glucose. In an attempt to assess the efficacy of using chilled water, Barton and Peter (1982) found that the plasma cortisol increase in rainbow trout following 4 h of transport was additive on levels already elevated from 24 h of prior exposure to 1°C. Schneider et al. (1981) examined the effects of a sudden temperature change combined with an exercise stress on plasma glucose; in rainbow trout acclimated to 12°C, they observed an apparent additive effect on glucose elevations at 22°C but failed to do so at 27°C.

A number of investigations have attempted to establish presence or absence of diurnal rhythms in resting plasma corticosteroids (summarized by Spieler 1979; Davis et al. 1984), but there have been only a few concerned with the possible effect of time of day on the magnitude of either interrenal or glycemic stress responses. Both Davis et al. (1984) using channel catfish and Barton et al. (1986) using chinook salmon found that plasma corticosteroid changes in response to physical disturbance were similar regardless of time of day even though resting concentrations in both investigations were highest near midnight. Similarly, time of day did not effect the magnitude of

corticosteroid increases in largemouth bass from net confinement (Carmichael et al. 1984a).

In largemouth bass, Carmichael et al. (1984a) and in chinook salmon, Barton et al. (1986) both observed that the greatest glucose elevation after disturbance occurred in the late afternoon, although resting levels in chinook salmon were highest at night. These few studies do not constitute conclusive evidence, but do suggest that, while there is little diurnal influence on interrenal responses to physical stress, glycemic responses to such disturbances may be more pronounced during daylight. This may correspond with a higher level of activity, such as was observed in goldfish during the light portion of the photoperiod (Spieler and Noeske 1984), and may be related to a diurnal periodicity in control of insulin release by the pineal gland (Delahunty and Tomlinson 1984).

(b) Salinity and Anesthesia

Both the external salinity and treatment with anesthetics have an influence on the magnitude of corticosteroid and glucose stress responses and have often been used together to alleviate the stress associated with fish transport (Carmichael et al. 1984b; Wedemeyer et al. 1985). Redding and Schreck (1983) observed that in coho salmon, Oncorhynchus kisutch, during continuous confinement, plasma cortisol was reduced much more rapidly in those fish placed in isotonic water as compared to those in fresh water or seawater, regardless if they were previously acclimated in fresh water or seawater. Similarly, following the onset of severe confinement of juvenile chinook salmon, the elevation of plasma cortisol was about twofold higher at 6 h in fish held in fresh water than in those in salt water (Strange and Schreck 1980). Specker and Schreck (1980) conversely found that both plasma cortisol and glucose elevations were generally similar in coho salmon following transport when allowed to recover in either fresh water or seawater. Although reduced corticosteroid titers imply a

lower secretory rate, lower levels may also have resulted from increased clearance of the steroid from circulation. Using ^{3}H-cortisol as a tracer, Redding et al. (1984a) found that juvenile seawater-acclimated coho salmon exhibited a higher metabolic clearance rate of cortisol than those from fresh water. They also showed that accumulation of the tracer in the liver, gill and gall bladder was also higher in the salt water-acclimated fish.

When salt was added to test water, the resultant plasma glucose elevation from handling was reduced or eliminated in coho salmon and steelhead, Salmo gairdneri (Wedemeyer 1972). Wendt and Saunders (1973) observed that plasma glucose in Atlantic salmon smolts exercised for 15 min was still elevated after 15 h of recovery in fresh water but returned to normal levels relatively quickly in fish transferred to seawater. Both initial glucose and the subsequent increase from handling was greater in northern pike, Esox lucius, held in fresh water than those in brackish water (Soivio and Oikari 1976). In a simulated transport study, Carmichael et al. (1984b) found that the magnitude of the glucose stress response to transport was considerably reduced when near-isotonic salt concentrations were used in the hauling medium. Similarly, brown trout, Salmo trutta, transported in 0.6% salt water showed a much reduced hyperglycemia than fresh water-transported fish (Nikinmaa et al. 1983). Although the addition of salt, with or without anesthetics, has had positive results during transport of fishes, the use of salt alone may not necessarily ensure post-release survival (Strange and Schreck 1978).

Strange and Schreck (1978) reported that the interrenal response of chinook salmon to a severe confinement stress was attenuated when the fish were pretreated with 50 mg/L MS-222, even though this dosage of the anesthetic itself is stressful (Strange and Schreck 1978; Barton and Peter 1982). Limsuwan et al. (1983) found that both plasma corticosteroid and glucose elevations from a 10-min netting and confinement stress were significantly reduced

when channel catfish were first anesthetized with 3 mg/L etomidate. In hybrid striped bass, Morone chrysops X M. saxatilis, plasma corticosteroids were reduced 24 h following a 10-min confinement stress when fish were pretreated with either 25 mg/L MS-222 or 10 g/L salt, as compared with those that received no pretreatment (Tomasso et al. 1980). Using a preload and an in-transit treatment of MS-222, in combination with a near-isotonic salt concentration and anti-bacterial treatments, Carmichael et al. (1984b) were successful in significantly attenuating the transport-induced hyperglycemia of largemouth bass as well as reducing mortality from 88% to 5%. Similarly, a mild dose of MS-222 with or without the use of various salts reduced the hyperglycemia associated with transporting juvenile chinook salmon (Wedemeyer et al. 1985).

However, Barton and Peter (1982) were not able to reduce the cortisol increase resulting from truck-tank loading by prior treatment with MS-222 although in that instance, it was not clear if the post-stress cortisol elevation was induced by the handling or the anesthetic. Also, the use of quinaldine or MS-222, and/or salt, was not effective in reducing confinement-induced increases in plasma corticosteroids in striped bass, Morone saxatilis, but the use of etomidate was (Davis et al. 1982). Fagerlund (1967) observed a relatively high plasma cortisol elevation in sockeye salmon, Oncorhynchus nerka, transported with 2-phenoxyethanol as an anesthetic, but a comparative control transport was not conducted. By itself, 2-phenoxyethanol is acutely stressful to fish (Barton and Peter 1982). Spieler and Nickerson (1976) reported that goldfish anesthetized with methylpentynol and then handled had significantly elevated plasma glucose compared to no difference in those that were handled only, concluding that methylpentynol is not a suitable anesthetic for physiological studies.

(c) Previous Physical Disturbance

Prior exposure to another physical disturbance may have a strong influence on the magnitude of corticosteroid and glucose stress responses. Barton et al. (1986) found that when individual 30-s handlings were repeated at 3-h intervals in juvenile chinook salmon, both plasma cortisol and glucose post-stress elevations were cumulative. Cumulative increases in plasma glucose were seen by Carmichael et al. (1983) in smallmouth bass, Micropterus dolomieui, following various phases of a transport operation. Schreck et al. (1985) also observed cumulative increases in plasma cortisol in outmigrating Columbia River chinook salmon as they passed progressively through stages of a by-pass and collection facility at a major dam. Yet when fish are subjected to chronic stress, such as continuous confinement, plasma cortisol tends to become elevated to a plateau level and remain there until either compensation is achieved or the stress factor is removed (Schreck 1981). The cortisol titer following stress is regulated by negative feedback on the hypothalamic-pituitary complex (Fryer and Peter 1977). Brief intermittent disturbances that allow short recoveries in between may affect this regulatory mechanism differently than a continuous or chronic stressing agent (Barton et al. 1986) and thus may affect the magnitude of plasma cortisol levels following physical disturbance. Further work is needed to determine the effect of varying recovery times following stress both on fish's sensitivity and on primary and secondary responses to subsequent stress factors.

Repeated disturbance over a long time may result in habituation that is manifested by a lowered stress response. Juvenile rainbow trout subjected to a single, acute physical disturbance once a day for 10 wk habituated to the stress, evident as cortisol and glucose elevations that were about one half of those in previously undisturbed fish (B.A. Barton and C.B. Schreck, unpublished, Chapter VII). Rush and Umminger (1978) found that 3 wk of habituation to handling abolished the stress-induced plasma

glucose increase in goldfish. Similarly, some authors have noticed a lower elevation of glucose in response to forced exercise in fish that were preconditioned as compared to unconditioned fish (Hammond and Hickman 1966; Wendt and Saunders 1973). However, Woodward and Smith (1977) observed that conditioned rainbow trout elicited a higher glucose increase from a 2-min handling disturbance than unconditioned fish, which they attributed to a difference in post-stress epinephrine levels. Reasons for such differences are unclear, but in addition to severity and duration, novelty of the stimulus may have an important influence on the extent of fish's responses to stress, as pointed out for mammals (Levine 1985).

(d) Other Environmental Factors

Other environmental influences have been shown to affect stress-induced response-recovery patterns in fish. Reduced water quality alone will stimulate elevations of plasma corticosteroids and glucose (e.g. Tomasso et al. 1981a,b; Brown et al. 1984), but when acting in concert with physical disturbance, there may be an additive effect on both responses. For example, when Carmichael et al. (1984a) subjected largemouth bass to continuous confinement, plasma glucose doubled under control conditions but quadrupled in confined fish pre-exposed to 0.2 mg/L un-ionized ammonia. They also noted that pre-exposure to 135 mg/L carbon dioxide resulted in a greater hyperglycemia from confinement than in control fish. Rainbow trout, exposed to pH 4.7 for 5 d first, exhibited a plasma cortisol increase of more than twice that in control fish when subjected to brief handling (Barton et al. 1985b, Chapter VI). In that study, glucose changes in response to handling were masked by the already elevated levels induced by the low acidity.

Other external physical conditions influence the extent of stress responses. Specker and Schreck (1980) observed that both plasma cortisol and glucose tended to be higher in coho salmon transported at a high density (12 g/L) than in those at a low density (4 g/L), although the differences were neither large nor

consistent. Similarly, Schreck et al. (1985) reported that there was a trend for post-loading and post-transport cortisol titers to be lower in juvenile chinook salmon transported at low densities than those at high densities, but results varied among transport protocols tested.

The presence of darkness from shading or overhead cover appears to enhance the rate of recovery after physical disturbance. When steelhead parr were subjected to continuous confinement under either light or dark conditions, the decline of plasma cortisol towards resting levels was much quicker in fish held in the dark than in those exposed to light regardless of whether the fish were first acclimated to light or dark (J.M. Redding and C.B. Schreck, unpublished). A more rapid decline of plasma cortisol was seen in outmigrating chinook salmon when allowed to recover in covered vs. uncovered raceways following the stress experienced from by-pass and collection at a dam (Schreck et al. 1985). Also in chinook salmon, Wedemeyer et al. (1985) noted that the degree of hyperglycemia induced by a hauling stress challenge was reduced in fish held in covered tanks as compared to those in tanks illuminated with ambient light. Paradoxically, Gilham and Baker (1985) found that rainbow trout adapted to a dark background had a much greater increase in plasma cortisol after handling, as well as higher resting levels, than those adapted to a light background. They also showed that α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) titers were higher in black background-adapted, stressed trout and suggested a number of pathways whereby α-MSH or other hypothalamic-pituitary peptides may modulate ACTH and subsequently cortisol secretions in stressed fish.

The physiological status of the fish will also affect responses to physical stress. The nutritional state, manipulated both by diet contents and by prior food deprivation, affected post-stress hyperglycemia in juvenile chinook salmon (B.A. Barton, C.B. Schreck, L.G. Fowler, and E. Plisetskaya, unpublished, Chapter

V). In that investigation, fasted fish elicited lower glucose increases from handling and confinement, and within diet treatments, those fish on a high lipid ration exhibited the greatest change; neither type of diet nor fasting had a significant effect on the elevation of plasma cortisol. The presence of a chronic disease infection limited the ability of chinook salmon to evoke cumulative interrenal responses to brief repetitive handlings whereas the post-stress hyperglycemia in those fish was much higher (Barton et al. 1986).

Genetic and Ontogenetic Factors

(a) Species and Stock

The difficulty in making species comparisons for corticosteroid and glucose stress responses is that there are probably no instances where experimental procedures employed among published studies are exactly alike (e.g. Tables II.1 and II.2). In Table II.3, we compared plasma cortisol and glucose responses of stocks of juvenile chinook salmon, coho salmon, rainbow trout and brook trout, Salvelinus fontinalis, to an identical 30-s handling disturbance using the same sampling and assay protocol as Barton et al. (1986). Most groups were reared and acclimated on a similar water supply and under similar environmental conditions, and fed OMP, at either the Oregon State University Smith Farm facility or the Oregon Department of Fish and Wildlife Research Laboratory, Corvallis, Oregon (facilities share the same ground water system); exceptions were those groups held at the Abernathy Salmon Culture Technology Center, Longview, Washington, and the Western Fish Toxicology Station, Corvallis (Table II.3, footnotes). The trend was for the Pacific salmons to exhibit higher plasma cortisol elevations to handling than the rainbow or brook trout, but plasma glucose increases showed no species-related pattern. For all groups, the magnitude of the stress-induced glucose elevation at 3 h was positively correlated ($P<0.05$) with the

Table II.3. Mean plasma cortisol and glucose (±SE, N=10-12) in different species and stocks of healthy, acclimated juvenile salmonid fish over 24 h following an identical, single 30-s handling stress. Size, stock, rearing location and temperature (and data source) are indicated numerically in parentheses and listed in footnotes.

Species	Before Stress	Hours after Stress				
		1	3	6	12	24
		Cortisol (ng/mL)				
Chinook salmon (1)	61 ±29	159 ±26	114 ±42	13 ±3	44 ±13	57 ±23
" (2)	41 ±9	204 ±17	56 ±12	58 ±13	96 ±15	64 ±9
" (3)	6 ±2	209 ±8	58 ±11	20 ±6	18 ±4	35 ±10
" (4)	9 ±3	135 ±11	84 ±11	28 ±3	16 ±5	36 ±10
" (5)	7 ±1	186 ±13	135 ±12	-	26 ±5	40 ±7
Coho salmon (6)	31 ±9	200 ±13	72 ±14	24 ±5	17 ±6	23 ±7
" (7)	30 ±8	175 ±27	84 ±16	26 ±4	38 ±9	-
Rainbow trout (8)	9 ±3	136 ±15	48 ±8	37 ±13	43 ±11	5 ±2
" (9)	3 ±1	36 ±5	17 ±7	8 ±3	14 ±3	7 ±3
Brook trout (10)	4 ±2	77 ±9	26 ±8	2 ±1	4 ±2	4 ±2
		Glucose (mg/dL)				
Chinook salmon (1)	55 ±2	70 ±3	84 ±7	70 ±4	62 ±5	64 ±3
" (2)	78 ±3	100 ±5	106 ±8	105 ±7	101 ±5	99 ±5
" (3)	72 ±3	73 ±2	82 ±3	78 ±3	69 ±2	70 ±3
Coho salmon (6)	83 ±3	103 ±6	121 ±3	119 ±7	110 ±8	91 ±4
" (7)	78 ±3	101 ±7	119 ±5	107 ±7	85 ±3	-
Rainbow trout (8)	85 ±10	111 ±11	122 ±9	133 ±18	130 ±17	77 ±4
" (9)	74 ±2	96 ±2	108 ±10	95 ±5	74 ±5	69 ±1
Brook trout (10)	72 ±1	89 ±4	98 ±5	91 ±5	85 ±5	75 ±3

Table II.3 continued - footnotes

(1) 17 g; Trask River, OR stock; reared at Oregon State University Smith Farm Research Facility (OSU) in 12°C well water (from Barton et al. 1986, Chapter III)

(2) 33 g; Abernathy Creek, WA stock; reared at Abernathy Salmon Culture Technology Center in 12°C well water (from B.A. Barton, C.B. Schreck, L.G. Fowler, and E. Plisetskaya, unpublished, Chapter V)

(3) 35 g; Cedar Creek, OR stock; reared at Oregon Department of Fish and Wildlife Corvallis Research Laboratory (ODFW) in 12.5°C well water (temperature experiment, present paper)

(4), (5) 21-22 g; Rogue River, OR stock; reared at ODFW in 12°C well water (new data)

(6) 36 g; Big Creek Hatchery, OR stock; reared at ODFW in 12.5°C well water (new data)

(7) 25 g; Willard Hatchery, WA stock; reared at OSU in 12°C well water (new data)

(8) 52 g; Willamette River, OR stock; reared at Western Fish Toxicology Station, Corvallis in 17°C well water but acclimated in soft water (from Barton et al. 1985b, Chapter VI)

(9) 44 g; Willamette River, OR stock; reared at OSU in 11.5°C well water (new data)

(10) 26 g; Metolius River, OR stock; reared at OSU in 13°C well water (new data)

resting level (y=1.42x-0.74, r=0.82, N=8); there was no correlation between 1-h post-stress cortisol values and resting levels (r=0.44, N=10) nor between 1-h cortisol and 3-h glucose levels (r=0.06, N=8).

In response to a 6-h confinement stress, Davis and Parker (1983) found that plasma corticosteroids in juvenile rainbow trout rose more quickly, attained higher levels and were more prolonged after removal of the stress factor than in either Atlantic salmon or lake trout, Salvelinus namaycush. The rainbow trout were larger in their investigation, but they were 6 mo younger than the other two species and the Atlantic salmon were undergoing smoltification. Casillas and Smith (1977) compared wild and hatchery strains of rainbow trout and found that, while both strains exhibited a glucose response to a 'hook and line' stress, recovery from hyperglycemia was more rapid in the wild trout. Wydoski et al. (1976) also noted that hatchery rainbow trout elicited a significantly higher plasma glucose elevation during recovery from a 5-min 'hook and line' stress than wild trout did. Schreck (1981) summarized the post-stress changes in plasma corticosteroids during chronic stress from a number of studies with different juvenile salmonids. Although all groups of fish exhibited the same general response pattern, the variance in the data indicated that there was considerable variation among the investigations that presumably could be attributed to species or stock. Barton et al. (1986, Chapter III) also found that the magnitude of cumulative responses to repeated disturbances was different between two coastal stocks of chinook salmon even though the patterns of responses were the same.

These studies suggest that, at least for salmonids, there are species and stock differences in interrenal and glycemic stress responses to physical disturbance. However, it is clear that other factors such as early rearing history or acclimation conditions possibly affected species' sensitivity or responsiveness to stress. For example, in the rainbow trout comparisons, both results were

from the same stock of fish but sizes and acclimation temperatures were different (Table II.3, footnotes).

(b) Stage of Development

Responses to stress are also influenced by ontogenetic or developmental factors. Barton et al. (1985a, Chapter IV) found that, from 18-g coho salmon parr in March to 36-g smolts in July, there was a twofold increase in the corticosteroid response to handling. Using the same handling disturbance, similar increases in both plasma cortisol and glucose responses to stress were evident between 5-g rainbow trout and 20-g fish 10 wk later in the same experiment (B.A. Barton and C.B. Schreck, unpublished, Chapter VII). A greater corticosteroid response to handling associated with bleeding was reported by Hane et al. (1966) in ocean-caught adult chinook salmon in comparison to those sampled during their upstream migration, even though in the latter group, resting corticosteroid titers were higher. These differences in response appeared to be related to the developmental state of the fish rather than size. Although size has been shown to influence resting levels (Singley and Chavin 1975), Barton et al. (1985a) found no correlation between size of fish and the magnitude of the post-stress cortisol level within any particular sample day throughout their study. Moreover, Carmichael et al. (1984a) compared two largemouth bass groups of the same age, but with one group twice as large as the other, and showed that there was no size effect on either corticosteroid or glucose increases during 48 h of confinement. Conversely, greater hyperglycemia following a 'hook and line' stress was reported by Wydoski et al. (1976) in 43-48-cm hatchery rainbow trout than in their 20-25-cm counterparts, but ages of the two size groups were not specified.

Most investigators have not attempted to separate stress responses on the basis of sex perhaps because many of the studies, notably using salmonids, have been conducted with immature fish. In one study using adults, Fagerlund (1967) observed sexual

dimorphism in sockeye salmon in the elevation of plasma corticosteroids following certain types of physical disturbance with the females exhibiting significantly greater changes; specific reasons for this difference were not given. Larsen (1976), however, found that while hyperglycemia resulting from handling plus anesthesia in river lampreys, Lampetra fluviatilis, was abolished more quickly in mature than in immature fish, there was no difference between sexes of the mature fish. Further, in yearling largemouth bass, sex of the fish had no effect on increases of plasma corticosteroids or glucose in response to confinement (Carmichael et al. 1984a).

Summary and Management Considerations

Schreck (1981) presented a conceptual model to describe how a fish's potential performance capacity, defined by its genotype, is limited by its environment to delineate its realized capacity that is, in turn, further reduced by stress. In this model, stress decreases fish's realized performance capacity by acting on any or all of the performance vectors that contribute to that capacity, for example, disease resistance or swimming stamina. The recently published literature and work in our laboratory provide empirical evidence to support this model as it relates to stress-induced elevations in plasma corticosteroids and glucose. Clearly, internal and external environmental conditions, developmental state, and possible genetic factors influence the magnitude of these responses to physical disturbance (Fig. II.8).

Stress-response modifying factors may be considered as belonging to three categories: those that are relatively benign, those that exacerbate primary and secondary responses to disturbance and those that reduce or eliminate these responses. Benign factors, such as species or stock, stage of development, time of day or nutritional state, may affect the degree of response

Figure II.8. Summary of factors shown to modify interrenal and glycemic responses to physical stress in fish, as determined by increases in plasma corticosteroids and glucose, respectively. Factors in parentheses are those that: (1) have a modifying effect that is relatively minor, or (2) appear to have a modifying effect but more conclusive evidence is still needed.

FACTORS MODIFYING STRESS RESPONSES

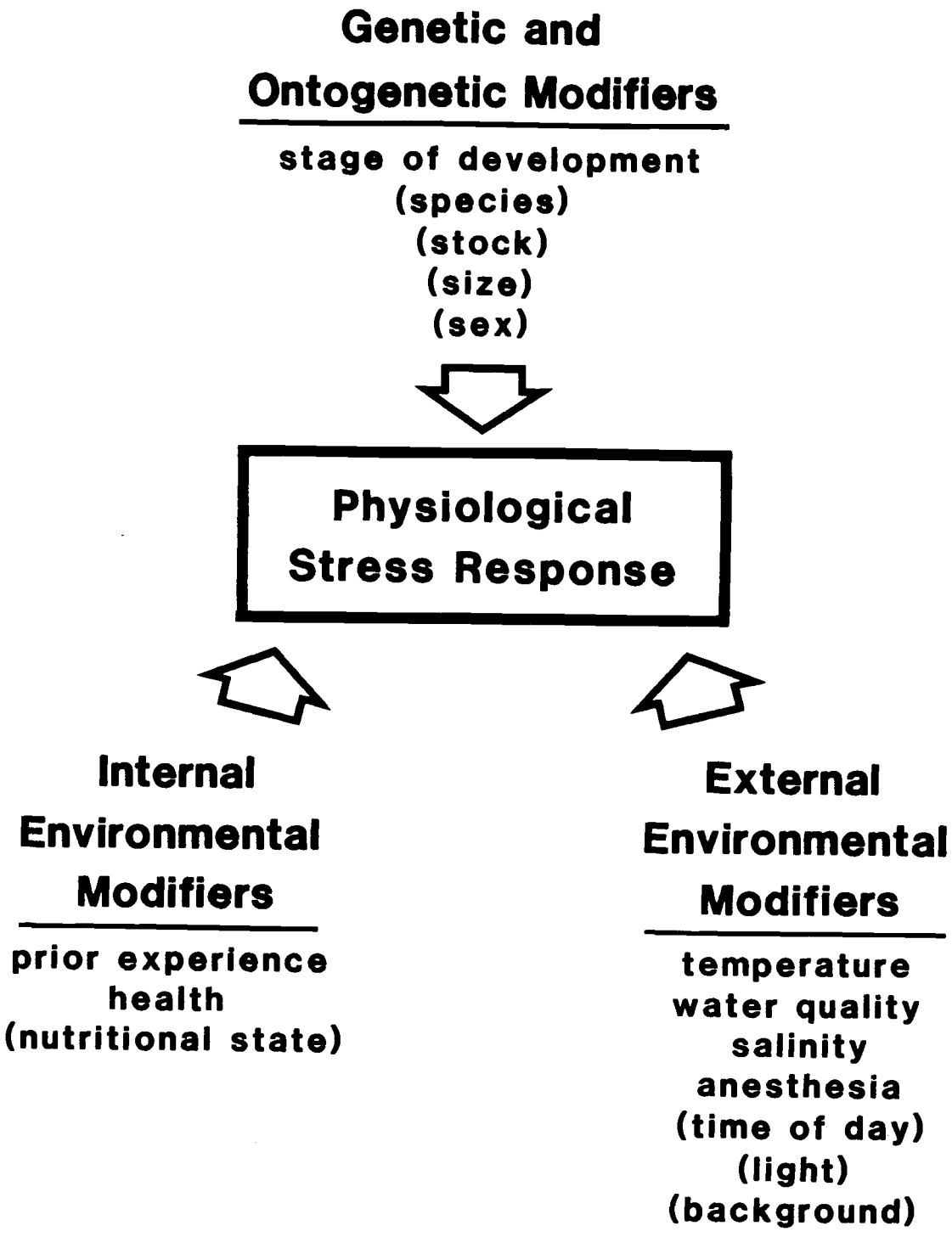


Figure II.8

to stress without necessarily being detrimental to the fish's well-being. Nevertheless, it is important that researchers and managers are aware of the differences when using these indicators to assess stress in fish and properly interpret their results. Response differences underscore the need for appropriate controls in stress studies as well as knowledge of the life history of the species in question.

Those factors that exacerbate interrenal or glycemic stress responses are additional stress factors and the resultant responses observed are often cumulative. Such factors include poor health, reduced water quality, excessively high or low water temperature, or overhead light. Cumulative stress will compromise the fish's realized capacity to perform by increasing its physiological load and thereby reducing its ability to cope with additional changes in its environment. It is practically impossible for fishery managers to eliminate stress in normal operations, for example, in aquaculture or fish stocking. However, stress can be managed for with relative ease. To this end, much could probably be accomplished by: (1) ensuring the fish are in the best possible health and condition, and (2) allowing a sufficient time period in a suitable environment for recovery between individual disturbances.

Other response-modifying factors can and are used by fishery managers to their advantage. The use of both salt and anesthetics reduces stress-induced increases in corticosteroids and glucose and has been shown to improve short-term survival. A simple practice, such as the use of overhead cover, may also prove to be a useful management tool to mitigate stress. Since physiological responses to stress are now well documented, future research needs to be directed towards the adverse effects of acute and chronic stress factors on long-term performance capacity, such as in changes in metabolic scope and immune response, and correlation of these changes with the popular stress indicators.

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III: MULTIPLE ACUTE DISTURBANCES EVOKE CUMULATIVE PHYSIOLOGICAL STRESS RESPONSES IN JUVENILE CHINOOK SALMON

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Abstract

The corticosteroid and hyperglycemic stress responses to multiple acute disturbances were cumulative in juvenile chinook salmon, Oncorhynchus tshawytscha. This was demonstrated by the stepwise pattern of increased plasma cortisol and glucose concentrations in fish subjected to a 30-s handling stress applied repeatedly at 3-h intervals over 6 h. The accumulation of physiological stress responses was substantiated by the resultant combined effects of these repeated disturbances on changes in concentrations of plasma lactate and of ionic sodium and potassium, and on the rate of decline of hepatic glycogen concentrations, all of which were greater than those that followed a single handling. Healthy fish appeared more able than diseased fish to elevate plasma cortisol after each of the three successive disturbances, but plasma glucose concentrations following the repeated handlings were higher in the unhealthy fish. As judged from plasma cortisol and glucose responses in fish subjected to a single 30-s stress at four different times in the day, there was little diurnal difference in sensitivity or responsiveness to handling.

Introduction

The notion that physiological responses of fish to stressful stimuli may be cumulative or compounded is intuitively accepted (Donaldson 1981; Wedemeyer et al. 1984) and is fundamental to a perception of the nature of stress and its effect on fish health. Acceptance is founded on circumstantial evidence in human medicine, and also in fish (Barton and Peter 1982; Carmichael et al. 1983; Adams et al. 1985; Barton et al. 1985b) but, to our knowledge, this notion has not been tested experimentally in fish to the extent of using the same acute disturbance repeatedly. The main objective of our experiment was to determine if fish exhibit their maximum physiological response to an initial severe acute stress or if they can respond further to successive stresses.

We evaluated primary and secondary responses (Mazeaud et al. 1977) in juvenile chinook salmon, Oncorhynchus tshawytscha, subjected to repeated acute handling stresses -- the primary or endocrine stress response on the basis of plasma cortisol concentrations, and the secondary or metabolic responses from concentrations of plasma glucose and lactate and of hepatic glycogen. As an indicator of ionoregulatory ability during stress, we also measured plasma ionic sodium and potassium concentrations. In addition, we compared plasma cortisol and glucose responses in healthy and unhealthy fish subjected to the same multiple disturbances. We also examined the influence of time of day on these responses in fish given a single handling stress. The type of handling used was representative of disturbances that are well known to evoke characteristic response-recovery patterns in both primary and secondary physiological responses of fish to stress (reviews of Donaldson 1981; Schreck 1981; Wedemeyer and McLeay 1981).

Methods and Materials

Experimental Design

For the multiple stress experiments, juvenile fall chinook salmon from three Oregon coastal stocks were maintained at the Smith Farm research facility, Oregon State University, in 0.6-m-diameter circular, flow-through tanks containing 100 L and receiving 4 L/min of 12±1°C aerated well water. Fish were held at densities of 9-13 g/L (depending on experiment) and fed daily with Oregon Moist Pellets at a rate of about 1.5% body weight/d. The fish were kept under a natural photoperiod and were acclimated for at least 2 wk before each experiment.

Duplicate groups of Trask River stock chinook salmon (mean weight, 17 g) were subjected to three degrees of stress: (1) single handling, (2) two handlings separated by a 3-h interval, or (3) three handlings, each 3 h apart. The handling stress consisted of netting the fish from the tank, holding them in the air for 30 s, and returning them to the tank for recovery. In each instance, fish were collected only once for blood samples just before each disturbance, and at 0.5, 1, 3, 6, 12, and 24 h after the final handling. Additional tanks of unstressed fish established as controls were either sampled or sham-sampled (disturbed with a net but not removed) at the same times to assess the effect of sampling on remaining fish.

Duplicate groups of fish from the Cedar Creek stock (mean weight, 22 g) and the Salmon River stock (31 g) were subjected to three consecutive 30-s handling stresses separated by 3-h intervals for comparison with the Trask River fish. The Salmon River fish had a chronic fin rot condition accompanied by an infection of the coldwater disease bacterium Cytophaga psychrophila (Bullock and Snieszko 1970). Fish were sampled just before each disturbance, and at 1, 3, 6, and 24 h after the third handling.

To evaluate the possibility of a diurnal difference in the

responses to stress that could affect interpretation of results from the previous experiments, we used juvenile Rogue River stock fall chinook salmon hatched and reared at the Corvallis Research Laboratory, Oregon Department of Fish and Wildlife. For the experiment, 20 fish (mean weight, 26 g) were held in each of eight tanks containing 100 L to acclimate for 3 wk under similar conditions as those in the multiple stress experiments (see above); a 14-h-light:10-h-darkness photoperiod to match prior rearing conditions was maintained with fluorescent lighting.

Duplicate groups of fish were subjected to a single 30-s handling stress at each of four different times in the day: 0030, 0630, 1230, and 1830 hours. At each time, fish were collected only once for blood samples just before the disturbance and at 1 and 3 h after handling. During dark periods, stresses were applied and samples were obtained with the aid of a 7-W red darkroom lamp as the only lighting.

Sampling and Analysis

To obtain plasma, we removed fish from the tanks by one or two passes of a hand net, taking as much care as possible not to physically agitate fish remaining in the tanks. Sampled fish were immediately placed in 200 mg/L tricaine methanesulfonate (MS-222) and were completely anesthetized in <1 min. The fish were then serially removed from the anesthetic solution and bled from the caudal vasculature with an ammonium-heparinized 0.25-mL Natelson capillary tube after the caudal peduncle was severed. Total time to obtain all samples after initial netting was <10 min. This sampling protocol has been previously found to be suitable for obtaining resting concentrations of plasma cortisol in juvenile salmonids (Barton et al. 1980, 1985a,b). Plasma was separated by centrifugation and stored at -15°C for future assay. Whole livers were removed, weighed and stored in chilled 30% KOH for later hepatic glycogen assay by the phenol-sulfuric acid method of

Montgomery (1957).

Plasma cortisol was determined by a ^{3}H-radioimmunoassay (Foster and Dunn 1974) modified for use with coho salmon, Oncorhynchus kisutch, by Redding et al. (1984b) and considered suitable for use with chinook salmon by the following criteria. Recovery of cortisol added to stripped plasma (endogenous steroids removed with charcoal), as determined over 24 assays, was >93%, but was 82% when recovered from unstripped chinook salmon plasma added to glass tubes containing four known cortisol concentrations in duplicate evaporated from ethanol ($r=0.998$). Data reported are unconverted values. Non-specific binding of chinook salmon plasma was 2%. Cross-reactivity at 50% displacement of labeled cortisol was 4% with cortisone, the other major salmonid corticosteroid (Idler 1971). The resultant curve from serial dilutions of a plasma pool from stressed chinook salmon containing about 300 ng/mL cortisol exhibited parallelism to about 30 ng/mL and appeared to converge with the standards curve at low concentrations. The minimum level of detectability, defined as the concentration two standard deviations from zero ($N=12$), was 1 ng/mL. In the range of high concentrations (e.g. >100 ng/mL), inter- and intra-assay coefficients of variation (CV=SD/mean) were 7% ($N=24$) and 3% ($N=8$), respectively. In the low range (e.g. <10 ng/mL), inter-assay CV was 15% ($N=21$).

Plasma glucose was measured by the colorimetric procedure of Wedemeyer and Yasutake (1977) modified by use of Sigma ® premixed 6% o-toluidine reagent; inter- and intra-assay CV's were 7% and 3%, respectively ($N=5$), and recovery of added glucose, as determined over 19 assays, was above 97%. Plasma lactate was assayed by fluorimetry (Passonneau 1974) and plasma ionic sodium and potassium concentrations were determined with a NOVA 1 sodium/potassium analyzer (Nova Biomedical, Newton, Massachusetts).

Data were treated by one-way analyses of variance (ANOVA) followed by Duncan's new multiple-range tests at the 5% probability

level (Steel and Torrie 1980). For analysis, cortisol and glucose data were transformed to logarithmic values because of heterogeneity among variances (Bartlett's test, Snedecor and Cochrane 1967).

Results and Discussion

Each application of an additional acute disturbance of the fish resulted in cortisol and glucose responses that were cumulative upon previous responses (Fig. III.1). When fish experience continuous stress, such as that from confinement, plasma cortisol concentration rapidly rises to a plateau and remains there rather than increasing indefinitely (Strange et al. 1978; Barton et al. 1980; Schreck 1981). In part, this is due to the negative feedback of cortisol on the hypothalamus, which suppresses adrenocorticotropic hormone (ACTH) output at the pituitary (Fryer and Peter 1977) and prevents cortisol in circulation from continuously increasing. This feedback mechanism may operate less effectively when fish are allowed a short recovery period between brief intermittent disturbances. The lack of an elevation in either plasma cortisol or glucose concentrations in control fish indicates that repeated sampling from the same tanks did not appreciably affect resting concentrations in remaining fish. This agrees with what was previously found for plasma cortisol in rainbow trout, Salmo gairdneri, (Barton et al. 1980, 1985b).

Although sustained increases in plasma glucose may be functionally related to elevated plasma cortisol during chronic stress in fish (Leach and Taylor 1980), acute rises in plasma glucose such as those seen in this experiment are probably mediated by catecholamines (Nakano and Tomlinson 1967; Mazeaud et al. 1977; Mazeaud and Mazeaud 1981) rather than by any effect of cortisol. Elevations in plasma glucose in response to stress or activity

Figure III.1. Mean (and 1 SE) plasma cortisol and glucose concentrations (N=12) in juvenile Trask River chinook salmon subjected to one or more 30-s handling stresses spaced (in the latter cases) 3 h apart. Solid arrows indicate applications of the second stimulus and the open arrow indicates application of the third. Means represent pooled data from duplicate tanks (open squares are values for unstressed controls). Statistical tests are based on ANOVA and Duncan's new multiple-range tests, P<0.05.

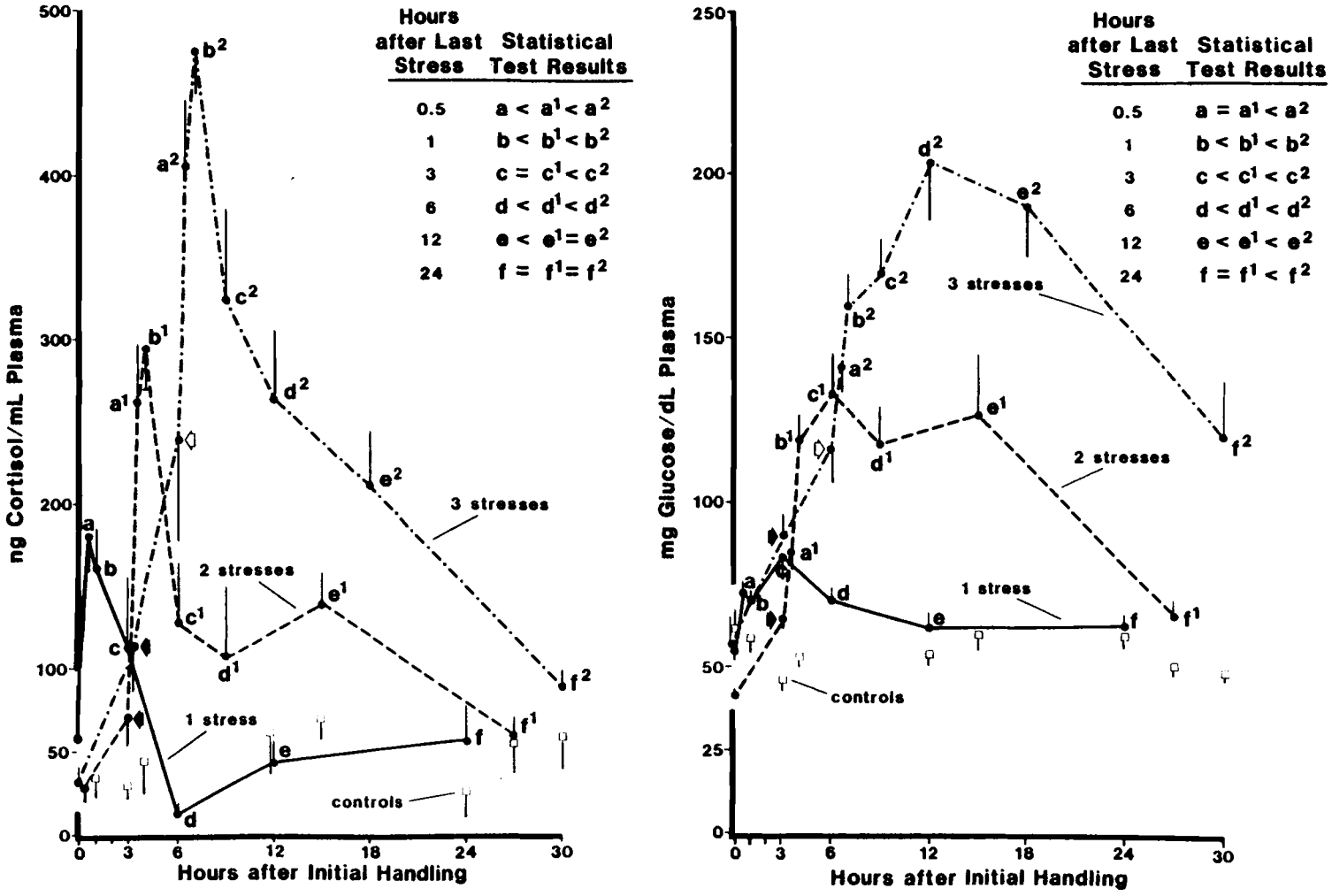
Hours after Last Stress
Statistical Test Results
0.5 a < a¹ < a²
1 b < b¹ < b²
3 c = c¹ < c²
6 d < d¹ < d²
12 e < e¹ = e²
24 f = f¹ = f²
ng Cortisol/mL Plasma
mg Glucose/dL Plasma
Hours after Initial Handling
3 stresses
2 stresses
1 stress
controls

Figure III.1

presumably reflect the metabolic response of the fish; there is a significant correlation between blood sugar concentration and standard metabolic rate in vertebrates in general (Umminger 1977). The cumulative increases in glucose may, therefore, indicate an increasing demand for energy in response to the combined effect of repeated disturbances. This presumed increase is consistent with our observations of a more rapid drop in hepatic glycogen from the prestress level to the minimum post-stress level measured after two or three repeated handling stresses than after a single disturbance. Specifically, 3 h after the second handling, hepatic glycogen concentrations had decreased significantly ($P<0.05$) from a prestress value of 37 ±8 (SE) to 5 ±3 mg/g ($N=6$); 0.5 h after the third handling, they had dropped from 30 ±6 to 5 ±2 mg/g ($N=6$). In contrast, the decline in hepatic glycogen from 15 ±4 to 9 ±4 mg/g 24 h after a single handling was not sigificant ($N=6$; $P<0.05$).

As judged by the magnitude of the maximum changes, the compounded responses of plasma lactate, sodium, and potassium to repeated handling were greater than those following a single disturbance (Table III.1). However, the responses did not demonstrate the temporal stepwise accumulation of changes exhibited by plasma cortisol and glucose. Moreover, concentrations of all three of these variables in the fish were still significantly altered 12 h after the third disturbance, but had returned to normal within 6 h after the single handling (data not shown). Previous studies have shown that plasma lactate increases (Soivio and Oikari 1976; Pickering et al. 1982) and, in fresh water, plasma sodium decreases (Redding and Schreck 1983; Barton et al. 1985b) and plasma potassium increases (Carmichael et al. 1983) following handling or confinement. In addition to confirming earlier findings, our results suggest that the combined effect of repeated handling on anaerobic metabolism and ionoregulatory performance was also cumulative.

Table III.1. Mean (±SE) concentrations of plasma lactate (N=10-12) and plasma ionic sodium and potassium (N=7-10) in juvenile Trask River chinook salmon before handling (combined mean from three experiments) and at the time of maximum change after one, two or three 30-s handling stresses separated by 3-h intervals. Percent changes from concentrations before any stress and recorded time to maximum change are indicated in parentheses. Means represent pooled data from duplicate tanks. Means within a column without a letter in common are significantly different (ANOVA and Duncan's new multiple-range tests, $P<0.05$).

Sampling Time	Lactate (mg/dL)	Sodium (meq/L)	Potassium (meq/L)
Before stress:	25 ±3^{z}	145 ±2^{z}	6.7 ±0.3^{z}
After one stress:	51 ±5^{y} (+104%) (0.5 h)	141 ±6^{z} (-2.8%) (0.5 h)	7.1 ±0.6^{z} (+6.0%) (0.5 h)
After two stresses:	52 ±8^{y} (+108%) (3 h)	136 ±2^{y} (-6.2%) (1 h)	7.5 ±0.5^{z} (+12%) (0.5 h)
After three stresses:	76 ±4^{x} (+204%) (0.5 h)	130 ±2^{y} (-10%) (12 h)	9.4 ±0.6^{y} (+40%) (0.5 h)

The plasma cortisol concentrations following the repeated stressors differed quantitatively between the two healthy chinook salmon stocks, Trask River and Cedar Creek, although both exhibited similar patterns of successive increases in response to the disturbances (Table III.2). This difference in corticosteroid response between the two healthy stocks suggests differences attributable to either genetic factors or prior rearing history. Additionally, it has been shown that ontogenetic factors in fish may modify the general pattern of corticosteroid stress responses (Barton et al. 1985a). In the unhealthy Salmon River stock fish, however, the plasma cortisol increase in response to the initial stress was highest followed by a lower relative response after the second handling, but the third handling evoked no further response (Table III.2). Also, plasma cortisol concentrations in the two healthy stocks appeared to be declining soon after they reached a peak 1 h following the final stress, whereas those in the unhealthy fish still remained high after 6 h (Table III.2). Fifty percent of the unhealthy fish stressed three times died within 3-6 h after the third handling compared with no mortalities in the other two groups.

Plasma glucose concentrations observed after the final handling were much greater in the diseased fish than in the healthy fish (Table III.2), suggesting a higher metabolic response in that group. Alternatively, the higher concentrations of plasma glucose evoked by the stressors in the diseased fish could indicate that these fish were preconditioned to respond in this manner by the chronic stress of continued infection. However, the high mortality observed among these fish after the final stress supports the view that the unhealthy fish were less capable than the healthy fish of coping with the cumulative stress resulting from the repeated disturbances.

The potential capacity of fish to respond to stress is limited by constraints of both the fish's internal and external environments (Schreck 1981). Our findings suggest that the

Table III.2. Mean (±SE) plasma cortisol and glucose concentrations (N=10-12) in two healthy stocks (Trask River and Cedar Creek) and an unhealthy stock (Salmon River) of juvenile chinook salmon after one, two or three 30-s handling stresses separated by 3-h intervals. Means represent pooled data from duplicate tanks. Means along a row not preceded by a letter in common, and means within a column not followed by a letter in common are significantly different (ANOVA and Duncan's new multiple-range tests, P<0.05).

Sampling Time	Trask River stock	Cedar Creek stock	Salmon River stock
		Cortisol (ng/mL)	
Before all stresses:	z_{32} ±9^{w}	z_{28} ±6^{w}	z_{24} ±8^{w}
Just before second stress:	z_{112} ±25^{v,u}	y_{43} ±4^{vu}	x_{176} ±24^{v}
Just before third stress:	z_{240} ±62^{u,t}	y_{81} ±17^{u,t}	z_{246} ±13^{v,u}
Hours after third stress: 1	z_{476} ±25^{s}	y_{197} ±16^{s}	x_{269} ±15^{v,u}
3	z_{325} ±55^{t,s}	y_{143} ±21^{t,s}	z_{261} ±11^{v,u}
6	z_{264} ±41^{t}	y_{114} ±17^{t}	z_{299} ±17^{u}
24	z_{89} ±19^{v}	y_{40} ±14^{w,v}	174 ±91*
		Glucose (mg/dL)	
Before all stresses:	z_{57} ±8^{w}	y_{72} ±2^{w}	y_{67} ±3^{w}
Just before second stress:	z_{90} ±7^{v}	z_{107} ±3^{v}	y_{152} ±15^{v}
Just before third stress:	z_{117} ±11^{v}	y_{149} ±10^{u,t}	x_{282} ±20^{u,t}
Hours after third stress: 1	z_{160} ±10^{u}	z_{155} ±6^{t,s}	y_{261} ±18^{u}
3	z_{170} ±11^{u}	z_{190} ±14^{s}	y_{283} ±19^{u,t}
6	z_{204} ±18^{u}	z_{190} ±20^{s}	y_{334} ±22^{t}
24	z_{121} ±17^{v}	z_{114} ±5^{v,u}	251 ±92*

* N=2 because of high post-handling mortality; values were not included in the analysis.

capacity of the diseased fish to elicit a corticosteroid response to stress reached its limit after the second handling because no further significant increase in plasma cortisol was evident. That plasma cortisol was still maximally elevated after 6 h precludes the possibility that increased clearance may have accounted for the lack of a further cortisol increase after the third handling in the unhealthy fish.

Some of the differences in changes in plasma cortisol and glucose in fish subjected to a single handling at different times of the day were significant (Table III.3) but small in comparison to those in fish after two and three handling stresses (Fig. III.1). An identical experiment carried out with coho salmon yielded very similar results (B.A. Barton and C.B. Schreck, unpublished, Appendix III). Generally, both plasma cortisol and glucose responses of the fish stressed at different times were similar to those evident within the first 3 h in fish handled only once in the multiple stress experiment. Although plasma cortisol was the same in all four time groups at 1 h post-stress, it remained higher at 3 h in the 0630-h group than in the other three groups (Table III.3). Resting plasma glucose was highest in the 0030-h group of fish but the relative change in concentration in response to handling was greatest in the 1830-h group (Table III.3). In view of the relatively minor differences in stress responses among fish from the four times tested, it appears unlikely that the comparatively large cumulative increases in plasma cortisol and glucose in the fish handled two and three times in succession would be due to a diurnal fluctuation in sensitivity or responsiveness to stress.

Acknowledgments

We thank Alec Maule and Jane Linville for conducting the lactate assay; Alec Maule, Samuel Bradford and Lesley Barton for

Table III.3. Mean (±SE) plasma cortisol and glucose concentrations (N=12) in juvenile Rogue River chinook salmon after a single 30-s handling stress at four different times in a 24-h period. Means represent pooled data from duplicate tanks. Means along a row without a letter in common are significantly different (ANOVA and Duncan's new multiple-range tests, P<0.05).

Sampling Time	Time of Day			
	0030 h	0630 h	1230 h	1830 h
		Cortisol (ng/mL)		
Before stress:	50 ±7^{z}	21 ±3^{y}	12 ±2^{y}	25 ±7^{y}
Hours after stress: 1	185 ±9^{z}	194 ±6^{z}	191 ±8^{z}	180 ±9^{z}
3	117 ±10^{z}	153 ±15^{y}	94 ±8^{z}	87 ±9^{z}
		Glucose (mg/dL)		
Before stress:	80 ±4^{z}	64 ±2^{y}	63 ±2^{y}	58 ±2^{y}
Hours after stress: 1	84 ±3^{z}	74 ±4^{y}	71 ±2^{y}	75 ±2^{y}
3	94 ±3^{z}	77 ±2^{y}	80 ±2^{y,x}	87 ±4^{z,x}

assisting with sample collections; Richard Ewing and Alan Hemmingsen for the use of Oregon Department of Fish and Wildlife facilities; Rich Holt for advice with disease diagnosis, and Michael Redding for assisting with the cortisol assay validation and for reviewing the manuscript. This investigation was supported with funds from Project 82-16, Contract No. DE-A179-82BP34797, Bonneville Power Administration. Mention of trade names or manufacturers does not imply U.S. Government endorsement of commercial products.

IV: CHANGES IN PLASMA CORTISOL DURING STRESS AND SMOLTIFICATION IN COHO SALMON

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Oregon State University, Agricultural Experiment Station Technical Paper No. 7232. Reprinted with permission from General and Comparative Endocrinolgy 59: 468-471 (1985);

Abstract

The cortisol stress response in juvenile coho salmon, Oncorhynchus kisutch, considered as the difference between resting and peak post-stress cortisol levels, increased from 80 ng/mL in March to 166 ng/mL in July, the period when smoltification normally occurs. Resting plasma cortisol levels also continually increased from 4 ng/mL in March to a maximum of 39 ng/mL in May, but then declined again to 3 ng/mL in July. The results indicate that there is an increased interrenal responsiveness to stress during the period of smoltification in coho salmon.

Introduction

The period of parr-smolt transformation (smoltification) in salmonids appears to be associated with increased corticosteroid secretion, as judged from at least three criteria: histological changes in interrenal tissue (Fontaine and Olivereau 1957, 1959; McLeay, 1975; Olivereau 1975; Nishioka et al. 1982), changes in plasma corticosteroid concentrations (Fontaine and Hatey 1954; Langhorne and Simpson 1981; Specker and Schreck 1982; Patino 1983), and changes in cortisol clearance dynamics (Patino 1983). The indications of heightened interrenal activity during smoltification suggest a potential role of corticosteroids in this process (Specker 1982).

Numerous studies with salmonids have also focused on the changes in plasma cortisol in response to various types of stressful stimuli (reviews by Donaldson 1981; Schreck 1981). We investigated the possibility of a differential interrenal responsiveness to stress during the period of smoltification in juvenile coho salmon, Oncorhynchus kisutch, using an acute handling stress as a representative stimulus. We considered the stress response to be the difference between the mean resting plasma cortisol concentration and the mean concentration determined 1 h after application of the handling stress; this level represents a significant response resulting from this type of stress in juvenile salmonids (B.A. Barton and C.B. Schreck, unpublished, Chapters II and III).

Methods and Materials

Coho salmon were obtained as eyed eggs from Big Creek Hatchery, Oregon, and reared under a natural photoperiod in 12°C aerated well water at the Corvallis Laboratory, Oregon Department of Fish and Wildlife. Three weeks before the first

sampling, about 315 14-mo-old parr were transferred to a 570-L circular fiberglass tank, for a resultant density of 0.55 fish/L. Water inflow rate to the tank was 19 L/min. Fish were fed a ration of 1.1-1.4% body weight per day with Oregon Moist Pellets before and throughout the study.

To obtain samples for normal resting levels of plasma cortisol, we rapidly netted fish from their home tank and completely anesthetized them in <1 min in a solution of 200 mg/L tricaine methanesulfonate (MS-222), buffered to pH 7.2 with imidazole. After immobilization, fish were serially removed from the anesthetic for sampling. A similar method was previously used with juvenile rainbow trout, Salmo gairdneri (Barton et al. 1980) and was verified for use for cortisol determination in coho salmon before the present study (Appendix IV). Each fish was weighed and fork length was measured. We then severed the caudal peduncle and removed blood from the caudal vasculature with an ammonium-heparinized 0.25-mL Natelson capillary tube. After centrifugation, plasma was stored at -20°C for future assay.

Fish in a second sample were then subjected to a severe, acute handling stress by holding them in the air in a net for 30 s and returning them to a 10-L live-cage immersed in the tank, at a density of 12-14 fish per cage. At 1 h after the stress, the fish were transferred directly from the live-cage to the anesthetic solution for blood sampling. A total of 10 unstressed and 10 stressed fish were obtained on each sampling date. Stress challenges and blood sampling were conducted at about the same time of day, at 2- or 3-wk intervals from mid-March to mid-July, 1983. Fish removed from the tank were replaced with marked fish (not used in subsequent samples) to maintain a constant number.

Plasma cortisol was determined by a ^{3}H-radioimmunoassay (RIA) described by Foster and Dunn (1974) and modified by Redding et al. (1984b) for use with salmonid plasma. Plasma thyroxine was measured by a ^{125}I-RIA using the method of Specker and Schreck

(1982). Specific activity of gill (Na+K)-ATPase was measured in homogenated tissue immediately after removal by the method of Johnson et al. (1977); data for unstressed and stressed fish were combined since no differences in activity were apparent. Data were analyzed with linear regression and one-way analyses of variance (ANOVA); differences in means were determined by using Duncan's new multiple-range test at the 5% level (Steel and Torrie 1980).

Results and Discussion

There were significant fluctuations (ANOVA, $P<0.01$) in normal resting plasma cortisol levels during the 4-mo study. Cortisol continually increased from a low level of 4 ng/mL in mid-March to a peak of 39 ng/mL in mid-May, and then declined to 3 ng/mL in mid-July (Fig. IV.1). On each sampling date, there was no trend in correlation between cortisol and fish size, suggesting that resting cortisol levels were not strictly size-dependent. The cortisol stress response increased from a low of 80 ng/mL in late March to a high of 166 ng/mL in mid-July (Fig. IV.1). This increase in response (mean post-stress level minus mean resting level) was significantly correlated with increasing mean fish weight through time ($P<0.05$; $r=0.81$, $N=8$). Similarly, the significantly increasing trend (ANOVA, $P<0.01$) of absolute post-stress cortisol concentrations (Fig. IV.1) was correlated with fish weight over the sampling period ($P<0.01$; $r=0.50$, $N=80$), although individual elevated cortisol levels did not consistently correlate with individual fish weights on any particular sampling date indicating that the stress response also was not strictly size-dependent.

Fluctuations in gill (Na+K)-ATPase activity and plasma thyroxine (ANOVA, $P<0.01$), as well as a rapid decline in condition factor (ANOVA, $P<0.01$) were indicative that these fish underwent smoltification during this period (Table IV.1).

Figure IV.1. Mean 1-h post-stress plasma cortisol ±SE, mean resting plasma cortisol ±SE, and cortisol stress response (mean post-stress level minus mean resting level) in juvenile coho salmon from mid-March to mid-July, 1983. Sample means (N=10), indicated by letter, are ranked left to right from lowest to highest. Means not significantly different (Duncan's new multiple-range test, $P<0.05$) share the same underline.

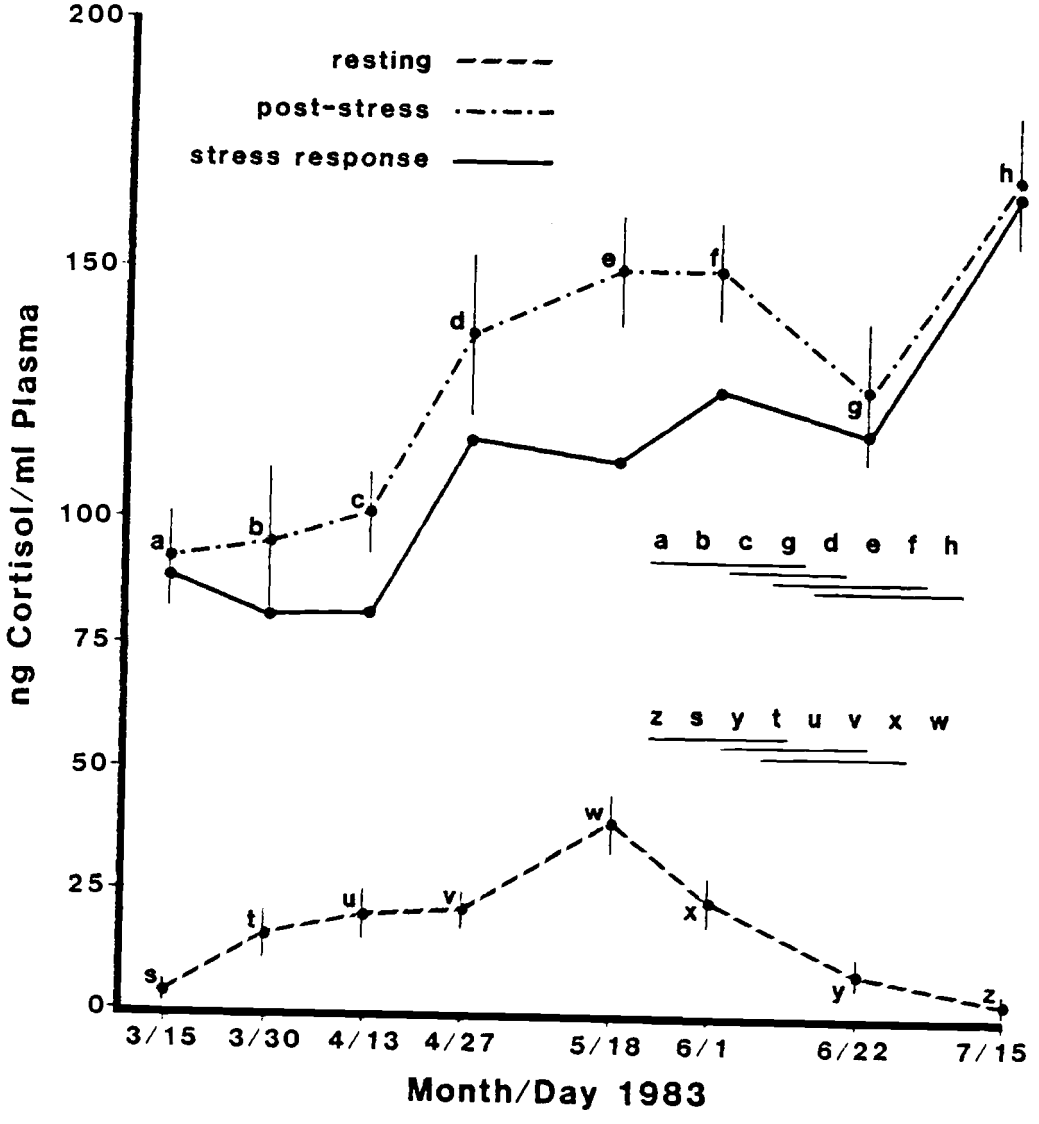
200
150
100
75
50
25
0
ng Cortisol/ml Plasma
resting
post-stress
stress response
a
b
c
d
e
f
g
h
s
t
u
v
w
x
y
z
a b c g d e f h
z s y t u v x w
3/15
3/30
4/13
4/27
5/18
6/1
6/22
7/15
Month/Day 1983

Figure IV.1

Table IV.1. Means ±SE for weight (N=20), condition factor (N=20), gill (Na+K)-ATPase activity (N=20), and plasma thyroxine (N=10) of juvenile coho salmon from mid-March to mid-July, 1983.

Date	Weight (g)	Condition factor (g X 100/cm^3)	Gill (Na+K)-ATPase activity (μmol Pi/hr/mg total protein)	Plasma thyroxine (ng/mL)
March 15	18.4 ±0.8	1.17 ±0.01	1.70 ±0.15	2.8 ±0.4
" 30	23.4 ±1.0	1.16 ±0.01	3.05 ±0.21	4.7 ±0.7
April 13	25.8 ±1.1	1.21 ±0.01	5.10 ±0.48	3.7 ±0.5
" 27	29.0 ±1.3	1.14 ±0.01	4.32 ±0.32	6.7 ±0.8
May 18	30.1 ±1.4	1.06 ±0.01	3.70 ±0.23	6.9 ±0.8
June 1	30.4 ±1.2	1.06 ±0.01	2.83 ±0.28	7.3 ±0.3
" 22	35.5 ±1.3	1.04 ±0.01	2.06 ±0.25	4.2 ±0.6
July 15	36.3 ±1.6	1.02 ±0.01	2.66 ±0.30	3.4 ±0.5

These changes in physiology and body form are characteristic of salmonids during smoltification (review by Folmar and Dickhoff 1980). Over the course of the experiment, resting cortisol and thyroxine levels were correlated ($P<0.01$, $r=0.32$, $N=80$) through time but not within individual fish. However, no correlation between plasma cortisol and gill (Na+K)-ATPase activity was evident ($r=0.08$, $N=80$).

Histological examinations have revealed evidence of increased interrenal activity over the smoltification period in both coho salmon (McLeay 1975) and Atlantic salmon, Salmo salar (Olivereau, 1975). Nishioka et al. (1982) observed ultrastructural differences within interrenal cells of coho salmon smolts that suggested high interrenal activity. The changes in interrenal hypertrophy indicated a temporally corresponding variation in the potential for cortisol secretion during smoltification, a presumption supported by our findings of an increased corticosteroid response to stress through time.

The increased cortisol response to stress during and after the smolting period could be interpreted as an indication that the ability of larger or older juvenile coho salmon to respond to stressful stimuli is generally greater than that of smaller fish, because of a possibly better developed interrenal secretory capacity. Alternatively, the sensitivity of developing juvenile salmon to physical disturbances may increase during and after smoltification. Therefore, in terms of the fish's awareness of stressful stimuli, the larger salmon may have been more severely stressed than the smaller parr.

The increase followed by a decline in resting plasma cortisol during the normal period of smoltification in coho salmon has not been previously observed. Specker and Schreck (1982) earlier demonstrated that cortisol in juvenile coho salmon was high in January, low in March, and then continually increased again to the end of May when their study terminated. Our results not only support the findings of Specker and Schreck (1982), but also extend

them by showing the subsequent decrease in cortisol in June and July.

In summary, we have demonstrated that there is an increased corticosteroid response to handling stress in coho salmon during smoltification, as well as showing that the previously reported rise in resting plasma cortisol is apparently transitory.

V: INFLUENCES OF DIET AND FASTING ON RESTING PHYSIOLOGICAL CONDITIONS AND STRESS RESPONSES IN JUVENILE CHINOOK SALMON

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Abstract

Juvenile chinook salmon, Oncorhynchus tshawytscha, reared on low, medium or high lipid diets for 18 wk, either were kept on their respective diets or were fasted for 20 d, and then subjected to either a 30-s handling stress only or to handling plus continuous confinement. Before the stress was imposed, differences due to diet content were apparent in continuously fed fish; fish fed the low lipid (high carbohydrate) diet had the lowest body weight and condition factor and the highest liver glycogen and plasma insulin. Compared with fed controls, 20 d of fasting caused a reduction in plasma cortisol, insulin and glucose, and liver glycogen, as well as in growth and condition factor. The greatest decline in plasma insulin in response to fasting occurred in fish reared on the low lipid ration. Plasma cortisol elevations in response to handling or to handling plus confinement were not appreciably affected by diet type or fasting. In fish that were only handled (in contrast to being handled and confined), post-stress hyperglycemia was greatest in fed fish that received the high lipid diet, and were generally lower in fasted than in fed fish. Liver glycogen declined after handling in the fed fish but did not change from the already low levels in 20-d fasted fish. The results indicate that prior feeding regimes and diet type used should be considered when one is interpreting both stress responses, particularly hyperglycemia, and resting physiological conditions in juvenile chinook salmon.

Introduction

The measurements of primary and secondary stress responses in fish have received considerable attention (reviews by Donaldson 1981; Schreck 1981; Wedemeyer and McLeay 1981) and are becoming increasingly popular among fishery workers as general physiological indicators of fish condition (Wedemeyer et al. 1984). Two such often used indicators are the elevations in plasma cortisol and glucose in response to physical disturbances. The state of the art of the use of these stress indicators is such that interpretation of results is still conditional on some prior knowledge of other factors that may modify the characteristic responses. It is common knowledge that nutritional factors profoundly effect the physiological state of fish. Our main objective was to determine the influences of various lipid levels in the diet and of fasting on plasma corticosteroid and glycemic levels of juvenile chinook salmon, Oncorhynchus tshawytscha, in response to handling and confinement. The second purpose of the experiment was to assess the effect of nutritive factors on selected indices (including plasma cortisol, insulin and glucose, and liver glycogen) of the resting conditions in this species.

Methods and Materials

Four replicate groups (per diet treatment) of Abernathy Creek stock fall chinook salmon were reared from the fry stage [1.63 ±0.02 (SE) g] on low, medium or high lipid diets at the Abernathy Salmon Culture Technology Center, Longview, Washington, as follows. The fish were fed with specially prepared Abernathy dry pellets four times per day; ingredients, nutrient contents and energy compositions of the three diets used are shown in Table V.1. Carbohydrate ingredients were varied inversely with added fish oil to keep the proportion of other constituents in the

Table V.1. Ingredients, nutrient content and energy composition of low, medium and high lipid diets used to feed juvenile fall chinook salmon for 18 wk before and during the experiment.

	Relative Lipid Content of Diet		
	Low	Medium	High
Ingredients (%)			
Herring meal	46.5	48.1	49.8
Stabilized tuna oil	1.2	7.3	13.3
Dried brewer's yeast	5.0	5.0	5.0
Milk nutrient concentrate	10.0	10.0	10.0
Blood meal	7.5	7.5	7.5
Wheat middlings	27.5	19.8	12.1
Vitamins and minerals[1]	2.3	2.3	2.3
Calculated Nutrient Content (%)[2]			
Protein	45.0	45.0	45.0
Lipid	7.0	13.0	19.0
Carbohydrate	28.6	23.4	18.3
Ash	9.7	9.6	9.5
Moisture	9.7	8.9	8.2
Energy Composition (kcal/100 g of diet)[3]			
Metabolizable energy	307	350	392

1 Composition: 1.5% regular vitamin premix no. 2, 0.1% regular trace mineral premix no. 1, 0.1% ascorbic acid and 0.6% choline chloride (60% variety). Premixes were similar to, but vary slightly in composition from, those in Fowler (1982).

2 Analyses of individual dietary ingredients used to calculate total nutrient content were conducted at the Abernathy Salmon Culture Technology Center, by methods described in Horwitz (1980).

3 Values calculated from Smith et al. (1980).

diet similar. Amounts fed were calculated by the method of Buterbaugh and Willoughby (1967), using a hatchery constant of 24 (metric). The fish were weighed every 2 wk and the feed was adjusted accordingly.

We reared 200 fish per tank under a simulated natural photoperiod, using fluorescent lighting, in circular fiberglass tanks (1.2 m in diameter and 0.6 m deep) containing 700 L of aerated 12°C well water and receiving a flow of 12 L/min. Analysis of a single sample yielded the following major water quality characteristics: dissolved oxygen, 89% saturation; pH, 7.7; total hardness, 90 mg/L as $CaCO_3$; total alkalinity, 76 mg/L as $CaCO_3$; and total dissolved solids, 180 mg/L. After 15 wk, some fish were removed from each tank for a different study, leaving 125 fish per tank for the present experiment.

After 18 wk, one group in duplicate from each diet regime was deprived of food (fasted) for 20 d (Fig. V.1). On day 20, all groups were subjected to a handling stress by first capturing them with a net and then holding them in the air in a perforated bucket for 30 s (Fig. V.1). Each group was then divided into two subgroups (Fig. V.1). One subgroup was allowed to recover in its home tank and the other subgroup was continuously confined during recovery at a density of about 100 g/L in a live-cage immersed in the home tank. From all treatment groups, fish were removed for blood sampling before the handling stress (0 h) and at 1, 3, 6, 12, 24 and 48 h after the onset of the stress. Liver samples from all groups were obtained before the stress and at 6, 12 and 24 h post-stress from the unconfined fish fed medium and high lipid diets.

Fish removed for sampling were immediately placed in a 200-mg/L tricaine methanesulfonate (MS-222) solution and completely anesthetized in <1 min. Fish were then weighed and fork length was measured for determination of condition factor (g X $100/cm^3$). The caudal peduncle was severed and blood was obtained from the caudal vasculature with a 0.25-mL ammonium-heparinized Natelson

Figure V.1. Treatment protocol used for experiments to determine effects of varying lipid levels in the diet and of 20 d of fasting on responses of juvenile chinook salmon to a 30-s handling stress followed by normal recovery or confinement.

EXPERIMENTAL TREATMENT PROTOCOL

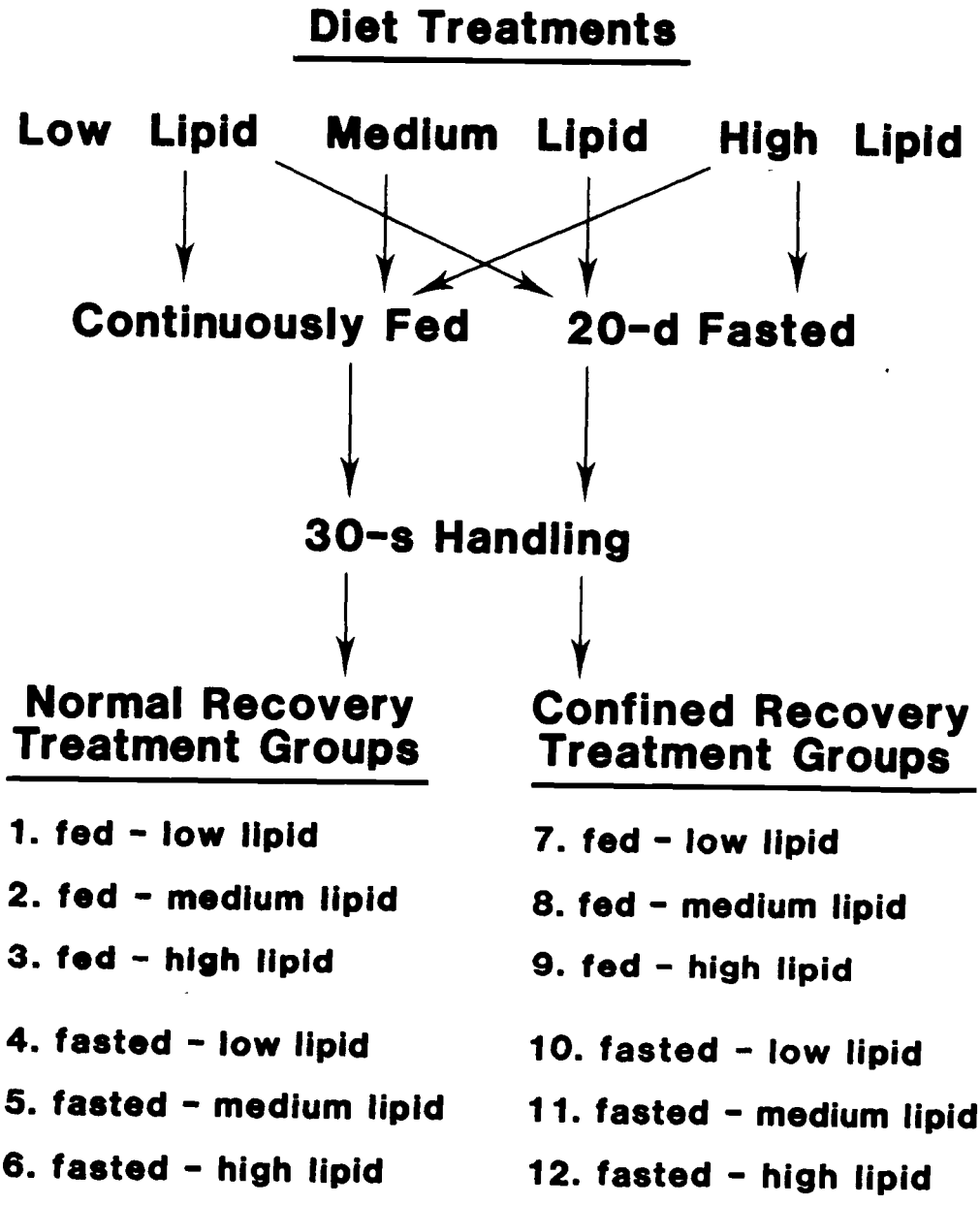

Figure V.1

capillary tube. Whole livers were removed, weighed to calculate hepatosomatic index (100 X liver wet weight/body wet weight), and stored in 1.5 mL of chilled 30% KOH solution for later glycogen determination. All sampling for each group was completed within 10 min. Plasma, separated from blood samples by centrifugation, was stored at -15°C for later analyses of cortisol, insulin and glucose.

Plasma cortisol was determined by using the radioimmunoassay (RIA) of Foster and Dunn (1974), as modified by Redding et al. (1984). Plasma insulin was measured by a recently developed, homologous salmon insulin RIA, in which coho salmon, Oncorhynchus kisutch, insulin was used as a standard and tracer (Plisetskaya et al. 1985, 1986). We analyzed plasma glucose by a colorimetric procedure (Wedemeyer and Yasutake 1977), using Sigma® premixed 6% ortho-toluidine reagent. Liver glycogen was determined by the phenol-sulfuric acid method of Montgomery (1957). Two-group comparisons were made by using Student's t-tests ($P<0.05$); multiple-group comparisons were made with two-way and one-way analyses of variance, followed by Duncan's new multiple-range tests at the 5% level (Steel and Torrie 1980). Correlation between liver glycogen and hepatosomatic index was made by linear regression.

Results

Resting Conditions

Within the fed group, juvenile chinook salmon from the high lipid diet were larger than those on the medium or low lipid diet (Fig. V.2). Collectively (i.e. three diet treatments combined), before the stress was applied, the weights, condition factors and hepatosomatic indices of fish fasted for 20 d were lower than those of their fed counterparts (Fig. V.2). Condition factors were

Figure V.2. Mean (±SE) weight (g, N=30), condition factor (g X 100/cm^3, N=30), hepatosomatic index (100 X liver weight/body weight, N=10) and plasma insulin (ng/mL, N=4-6) in fed and 20-d fasted juvenile chinook salmon after receiving low, medium and high lipid diets. Means represent pooled data from duplicate treatments. Significance ($P<0.05$) is designated by asterisks (*) as follows: (1) between fed and fasted fish, group with asterisk (diet treatments combined) indicates lower value than other group; (2) among diet types for each group, single asterisk indicates difference from extreme value but not middle value and double asterisk indicates difference from both other values within that group.

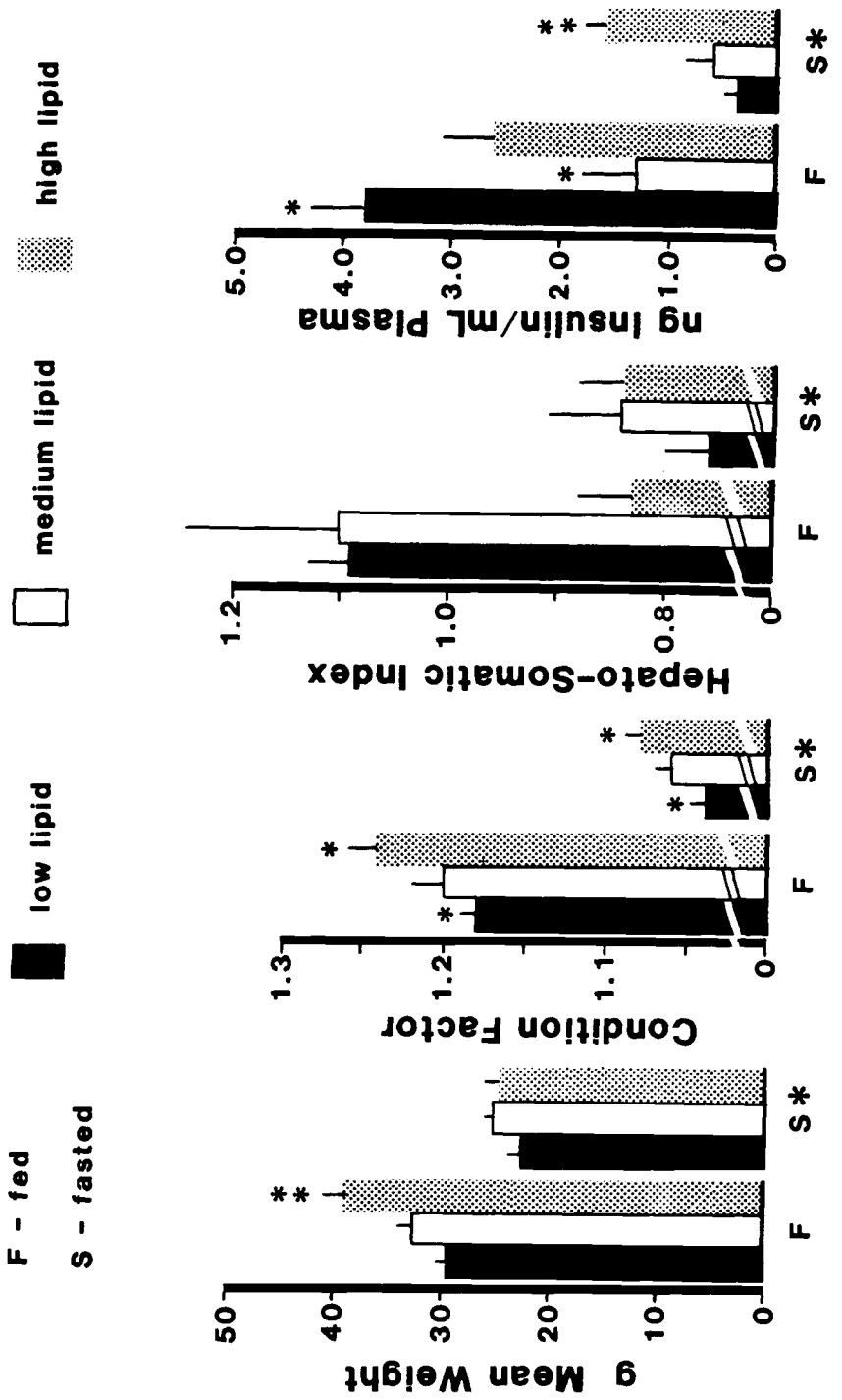
F - fed
S - fasted
low lipid
medium lipid
high lipid
g Mean Weight
Condition Factor
Hepato-Somatic Index
ng Insulin/mL Plasma
F
S*

Figure V.2

highest in fish from the high-lipid treatment in both fed and fasted groups (Fig. V.2). Hepatosomatic indices were greater in fed fish than in fasted fish (Fig. V.2), but differences within either group due to diet were not significant. High lipid-fed fish also had higher levels of stored body lipids; proximate analyses of fish after 18 wk of feeding before fasting showed a lipid content on a wet weight basis of 4.4%, 6.8% and 9.3% for fish fed low, medium and high lipid diets, respectively (L.G. Fowler, unpublished).

Plasma insulin was highest in fish fed the low lipid diet and lowest in those from the medium lipid-diet group (Fig. V.2). However, the 89% decrease in plasma insulin levels after fasting was significant in the low lipid-diet fish, but the 54% and 38% declines in insulin from fish in the medium and high lipid groups, respectively, were not (Fig. V.2).

In unstressed fed fish, liver glycogen was highest in the low lipid group but was lower and similar in the other two diet groups (Fig. V.3). The 20-d fasting period resulted in an overall decline of about 80% in liver glycogen content to levels that were similar for fish from all three diet treatments (Fig. V.3). The reduction in liver glycogen resulting from fasting paralleled that observed for the hepatosomatic index between the fed and fasted groups (Fig. V.2). Overall, liver glycogen and hepatosomatic index displayed a significant positive correlation ($r=0.40$, $\underline{N}=168$).

Resting levels of plasma cortisol were lower in fish after 20 d of fasting than in fed fish (Fig. V.4, initial values). Similarly, plasma glucose concentrations were lower in fasted fish than in fed fish (Fig. V.5, initial values). No differences in resting (initial) plasma cortisol or glucose atttributable to the diet treatments in either the fed or fasted fish groups were evident.

Figure V.3. Mean (±SE) liver glycogen (mg/g, N=9-10) in fed and 20-d fasted juvenile chinook salmon after receiving low, medium and high lipid diets and subjected to a 30-s handling stress (medium and high lipid treated fish only). Means represent pooled data from duplicate treatments (see Figure V.2 caption for explanation of asterisks indicating significance).

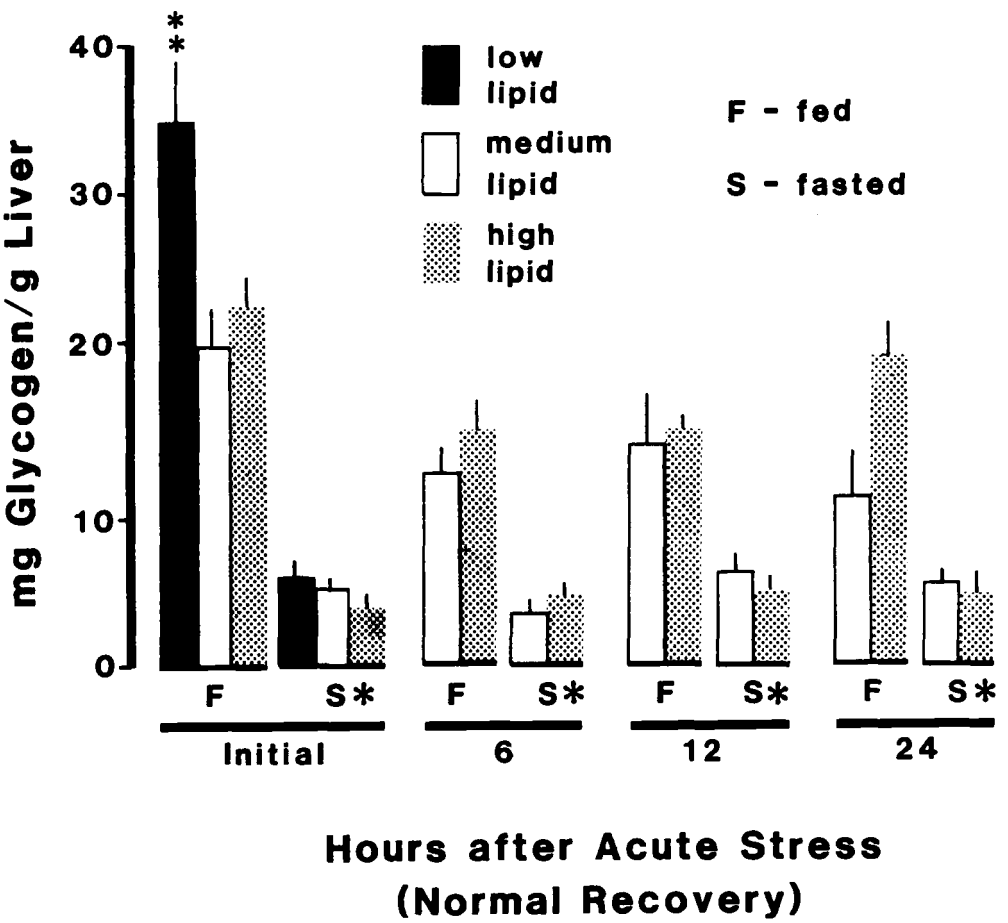
mg Glycogen/g Liver
40
30
20
10
0
**
low lipid
medium lipid
high lipid
F - fed
S - fasted
F
S*
Initial
6
12
24
Hours after Acute Stress
(Normal Recovery)

Figure V.3

Figure V.4. Mean (±SE) plasma cortisol (ng/mL, N=9-11) in fed and 20-d fasted juvenile chinook salmon after receiving low, medium and high lipid diets and subjected to a 30-s handling stress. Means represent pooled data from duplicate treatments (see Figure V.2 caption for explanation of asterisks indicating significance).

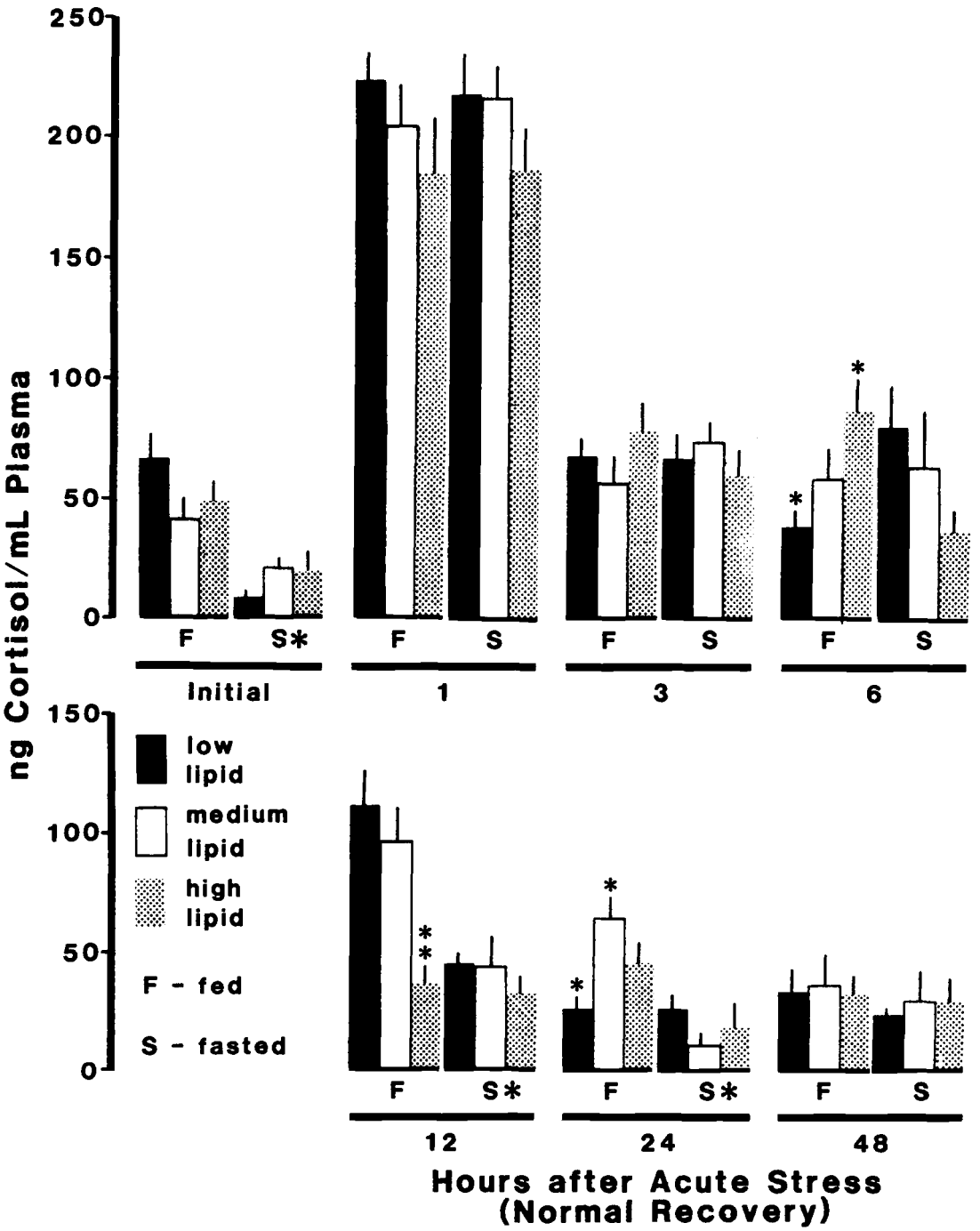
ng Cortisol/mL Plasma
250
200
150
100
50
0
F
S*
Initial
1
3
6
low lipid
medium lipid
high lipid
F - fed
S - fasted
12
24
48
Hours after Acute Stress
(Normal Recovery)

Figure V.4

Figure V.5. Mean (±SE) plasma glucose (mg/dL, N=9-11) in fed and 20-d fasted juvenile chinook salmon after receiving low, medium and high lipid diets and subjected to a 30-s handling stress. Means represent pooled data from duplicate treatments (see Figure V.2 caption for explanation of asterisks indicating significance).

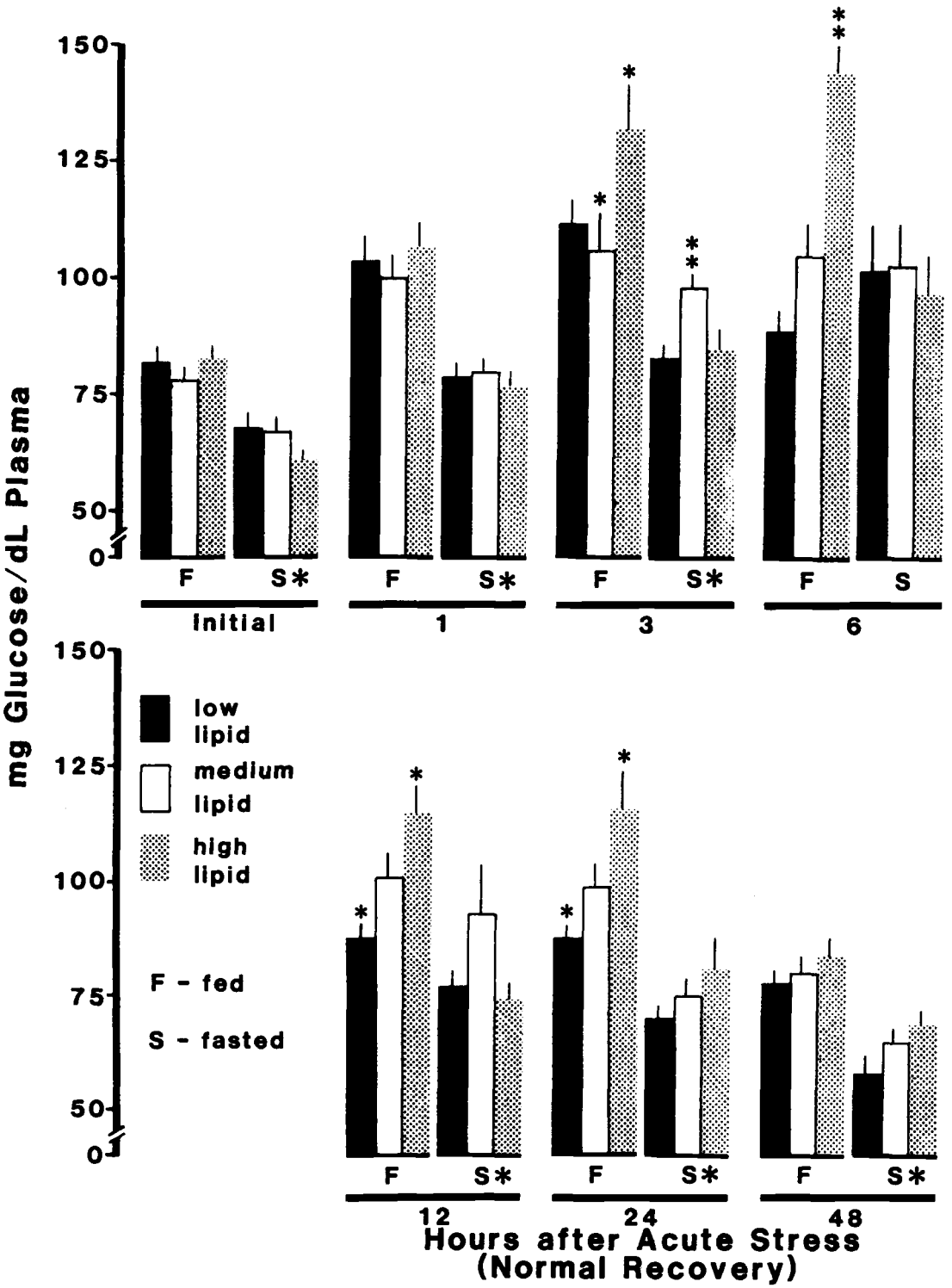
mg Glucose/dL Plasma
150
125
100
75
50
0
F
S*
S
Initial
1
3
6
low lipid
medium lipid
high lipid
F - fed
S - fasted
12
24
48
Hours after Acute Stress
(Normal Recovery)

Figure V.5

Responses to Stress

(a) Handling with Normal Recovery

In fish fed medium and high lipid diets, liver glycogen declined by 6 h in those subjected to the handling stress (F=2.75; df 3,71), but there were no differences between diet treatments (Fig. V.3). Liver glycogen levels in fasted fish did not change as a result of handling (Fig. V.3).

In fish allowed to recover under normal conditions, plasma cortisol rose rapidly following the 30-s handling stress and then declined (fed: F=70.4; df 6,193. fasted: F=105; df 6,189). Responses were similar in both fed and fasted fish (Fig. V.4), except for a subsequent further increase in cortisol in the fed fish from the low and medium lipid-diet groups at 12 h. In fed fish, cortisol was higher in the high lipid group at 6 h and in the medium lipid group at 24 h than in the low lipid group at these times (Fig. V.4). Differences in plasma cortisol responses among the diet treatments in fasted fish were not significant.

Both fed and fasted fish exhibited hyperglycemia in response to handling (fed: F=20.3; df 6,193. fasted: F=17.6; df 6,189) and, collectively, plasma glucose was higher in the fed fish than in fasted fish at all times after the stress except at 6 h (Fig. V.5). Among diet types for fed fish, hyperglycemia was greater in the high lipid group than in the other two groups at 6 h and greater than in the low lipid group at 3, 12 and 24 h post-stress (Fig. V.5). Differences among diet types in glucose responses were not significant in fasted fish.

(b) Handling with Continuous Confinement during Recovery

Fish subjected to continuous confinement sustained elevated plasma cortisol through time (fed: F=21.8; df 6,188. fasted: F=58.8, df 6,187); significant differences between fed and 20-day fasted fish were evident at 1, 3 and 48 h (Fig. V.6). Within the fed groups, differences resulting from diet type

Figure V.6. Mean (±SE) plasma cortisol (ng/mL, N=9-11) in fed and 20-d fasted juvenile chinook salmon after receiving low, medium and high lipid diets and subjected to a 30-s handling stress followed with continuous confinement. Means represent pooled data from duplicate treatments (see Figure V.2 caption for explanation of asterisks indicating significance).

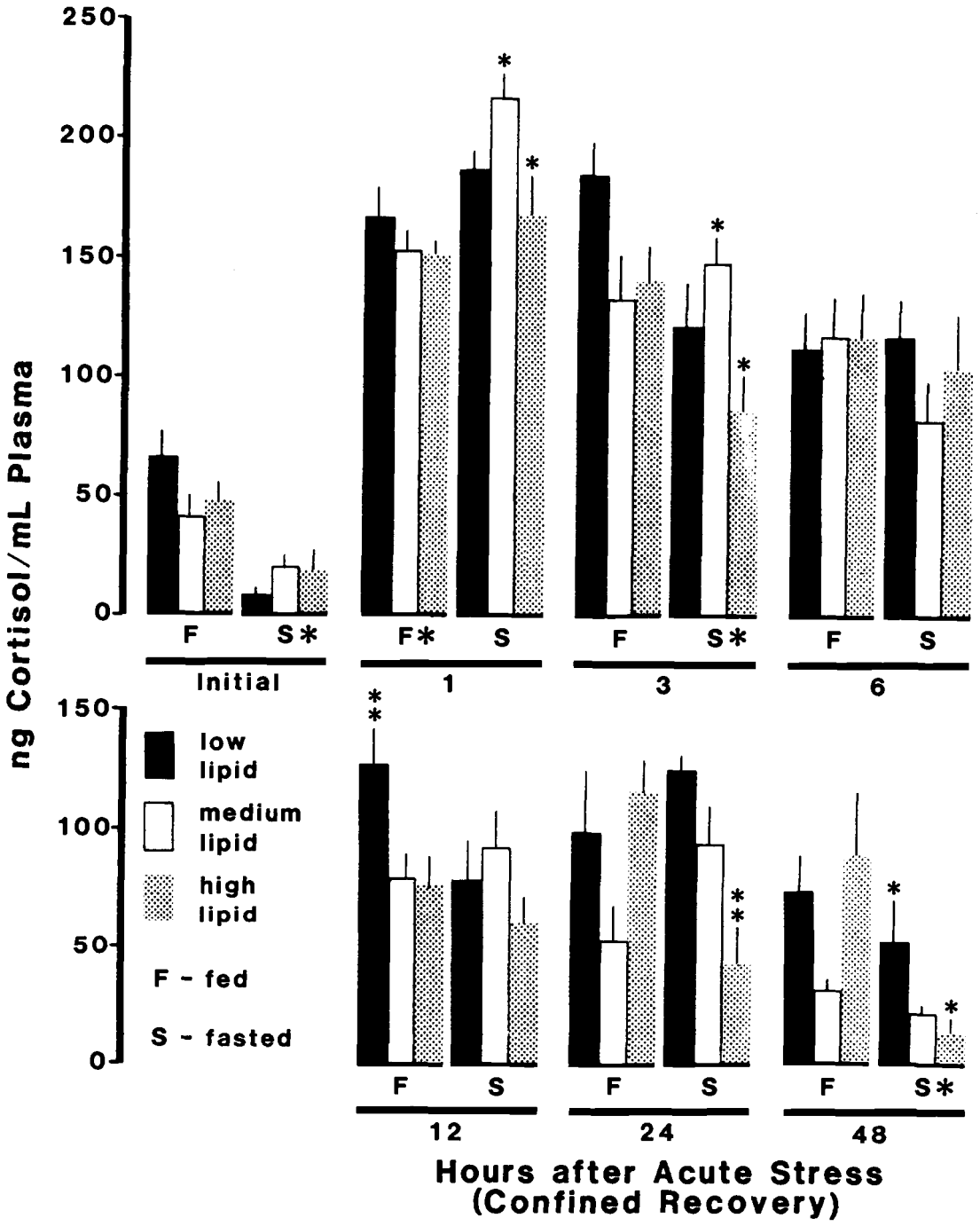
ng Cortisol/mL Plasma
250
200
150
100
50
0
F
S*
Initial
F*
S
1
F
S*
3
F
S
6
low lipid
medium lipid
high lipid
F - fed
S - fasted
F
S
12
F
S
24
F
S*
48
Hours after Acute Stress
(Confined Recovery)

Figure V.6

occurred only at 12 h, when plasma cortisol in fish fed the low lipid diet remained elevated above that in the other two groups (Fig. V.6). In the fasted groups, fish reared on the high lipid diet generally had the lowest plasma cortisol in response to the stress (Fig. V.6).

Overall, plasma glucose also remained high in continuously confined fish for up to 48 h after handling (fed: F=8.85; df 6,185. fasted: F=18.9; df 6,183), but was significantly lower in fasted fish than in fed fish at 1, 3, 12 and 48 h (Fig. V.7). Unlike the fish under normal recovery, which showed differences (Fig. V.5), the fed fish under continuous confinement showed no differences in plasma glucose attributable to diet type (Fig. V.7). In the fasted fish, however, differences in diet treatments were apparent at 3, 24 and 48 h (Fig. V.7); at these three times, plasma glucose was significantly lower in the low lipid group than in the medium lipid group.

Discussion

Resting Conditions

The high glycogen concentration in livers of resting fish fed a low lipid diet probably resulted from the higher content of carbohydrate in this diet (Buhler and Halver 1961; Phillips et al. 1966; Nagai and Ikeda 1971; Hilton 1982). Although this high level would not necessarily be considered abnormal, high liver glycogen has been shown to impair liver function (Hilton and Dixon 1982).

The higher plasma insulin observed in fish from the low lipid diet may also have been caused by the higher carbohydrate content in the diet. Despite the common belief that in fish, in contrast to mammals, insulin is mostly involved in regulation of protein and lipid metabolism (Patent and Foa 1971; Minick and Chavin 1972; Inui et al. 1975; Ince and Thorpe 1978b; Dave et al.

Figure V.7. Mean (±SE) plasma glucose (mg/dL, N=9-11) in fed and 20-d fasted juvenile chinook salmon after receiving low, medium and high lipid diets and subjected to a 30-s handling stress followed with continuous confinement. Means represent pooled data from duplicate treatments (see Figure V.2 caption for explanation of asterisks indicating significance).

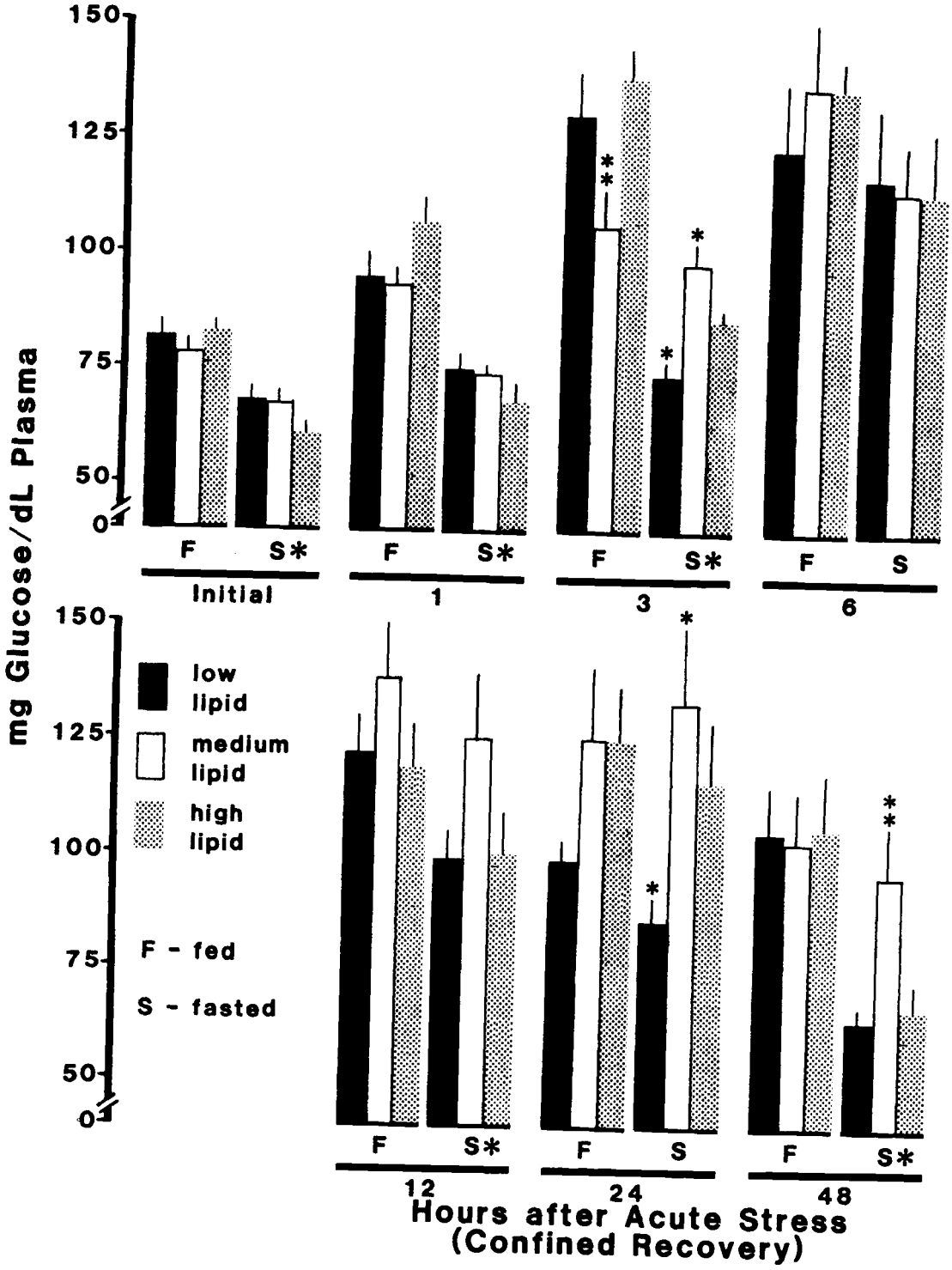
mg Glucose/dL Plasma
150
125
100
75
50
0
low lipid
medium lipid
high lipid
F - fed
S - fasted
F
S*
S
Initial
1
3
6
12
24
48
Hours after Acute Stress
(Confined Recovery)

Figure V.7

1979; Ablett et al. 1981a,b; Plisetskaya et al. 1984), glucose loading has been shown to induce insulin secretion in various fish species (Plisetskaya et al. 1976; Ince and Thorpe 1977; Ince 1979; Furuici and Yone 1981). The possible importance of insulin in promoting glycogenesis in fish (Plisetskaya and Leibson 1973) would explain the higher glycogen content observed in the fish with high plasma insulin. However, this is a tentative conclusion since past studies are equivocal; insulin treatment of fish has been found to both increase liver glycogen (Inui and Yokote 1975a; Ince and Thorpe 1976a; Cowey et al. 1977a) and to reduce it (Inui and Yokote 1975a; Lewander et al. 1976; Careiro and Amaral 1983; Saez et al. 1984).

In our experiments, there were no differences among diet treatments in either plasma cortisol or glucose in resting fed fish, although hyperglycemia has been previously observed in fish fed diets with a much higher carbohydrate content than the one we used (Cowey et al. 1977b; de la Higuera and Cardenas 1985). In contrast, Nagai and Ikeda (1971) found that blood glucose was lower in carp, Cyprinus carpio, after they were fed with a high carbohydrate diet.

In unstressed fed fish, those maintained on the high lipid diet gained the most weight over 18 wk and also had the highest condition factor. This relationship is understandable, since metabolizable energy was also 28% higher in the high lipid diet. This energy provided by additional lipids in the diet has a protein sparing effect that allows for increased deposition of body protein, and thus growth (Phillips et al. 1966; Reinitz et al. 1978; Bromley 1980; Watanabe 1982). Even when total energy values were similar, Edwards et al. (1977) found that growth and condition factor of rainbow trout, Salmo gairdneri, were inversely proportional to the percentage of metabolizable energy that was present as carbohydrate.

The decline during the 20-d fasting period in all of the physiological indices measured supports some earlier findings. For

example, Woo and Cheung (1980) noted that 66 d of food deprivation depressed plasma cortisol by 77% in the snakefish, Ophiocephalus maculatus. In juvenile presmolt coho salmon, Thomas et al. (1986) found that 2 wk of fasting reduced resting plasma cortisol concentrations by 30%. Fasting also inhibited the expected increase in resting plasma cortisol in Atlantic salmon, Salmo salar, during smoltification (Virtanen and Soivio 1985). Milne et al. (1979) observed, however, that changes in plasma cortisol in rainbow trout following fasting were not significant and Strange et al. (1978) found no difference in plasma cortisol between fed and fasted chinook salmon. Moreover, a doubling of plasma cortisol over 164 d of fasting was reported by Dave et al. (1975) in European eels, Anguilla anguilla.
The drop in plasma cortisol after fasting may result from a reduced availability of acetyl coenzyme A from glycolysis or of dietary cholesterol, the two sources of the cholesterol precursor for all steroids. Yet the data are controversial. In European eels, long-term starvation produced a significant drop in total plasma cholesterol (Larsson and Lewander 1973). But Ince and Thorpe (1976b) observed that total plasma cholesterol increased after fasting in northern pike, Esox lucius; they speculated that elimination of dietary cholesterol may have induced cholesterol synthesis to increase plasma levels.

A number of previous investigators have reported that fasting reduces circulating glucose in fish (Kamra 1966; Larsson and Lewander 1973; Ince and Thorpe 1976a; Milne et al. 1979; Woo and Cheung 1980), although this finding is evidently not universal (Tashima and Cahill 1968; Chavin and Young 1970; Nagai and Ikeda 1971; Ablett et al. 1981b; Fletcher 1984). The observed drop in liver glycogen was an expected response to food deprivation and has been demonstrated in a number of fish species (Wendt 1965; Inui and Oshima 1966; Swallow and Fleming 1969; Larsson and Lewander 1973; Ince and Thorpe 1976b; Milne et al. 1979; Woo and Cheung 1980; Hilton 1982). In contrast, Ablett et al. (1981b) observed no

changes in liver glycogen in rainbow trout fasted for 4 wk. Declines in condition factor and hepatosomatic index resulting from fasting, reported also by Larsson and Lewander (1973) and Woo and Cheung (1980), reflect the use of stored nutrient sources, particularly glycogen, to maintain blood glucose above critically low levels. This view is supported by the greatest net decline in liver glycogen observed after fasting in the fish fed the low lipid diet accompanying the large drop in hepatosomatic index.

Our finding of a drop in plasma insulin after 20 d of fasting agrees with those of Thorpe and Ince (1976) and Plisetskaya et al. (1986) who also noted declines of the same magnitude in plasma insulin of rainbow trout and coho salmon fasted for only 7 d. Similarly, plasma insulin was twofold lower in fasted goldfish, Carassius auratus, than in those that were fed (Patent and Foa 1971). The decline in circulating plasma insulin occurs also in naturally fasting lamprey, sturgeon and salmon in the course of upstream spawning migrations (Plisetskaya et al. 1976; Murat et al. 1981).

The large and significant decline in insulin after fasting in fish of the low lipid group, as compared with those in the two other treatment groups, is especially interesting. This decline indicates a differential response of insulin to fasting among groups fed the different diets, in spite of the limited sample sizes available. We are not aware of other investigations on fish that demonstrate this phenomenon, but Cowey et al. (1977b) observed differential blood glucose responses in rainbow trout to exogenous insulin treatment, depending on whether fish were fed high carbohydrate or high protein diets. The differential changes in plasma insulin among the diet groups after fasting may be related to the relative levels of liver glycogen present, and thus the dynamics of maintaining constant glucose levels from various energy pools during food deprivation. However, a clear interpretation of the significance of our in vivo findings is restricted by lack of knowledge of the other hormonal glucose regulators in fish,

such as glucagon, catecholamines, somatostatin and possibly cortisol, and their interactions with each other.

Responses to Stress

The responses to handling and confinement and subsequent returns towards resting levels of both plasma cortisol and glucose in the fed fish were typical of what has been previously reported in juvenile salmonids subjected to these stresses (Wedemeyer 1972; Strange et al. 1977, 1978; Barton et al. 1980, 1985b; Strange and Schreck 1980; Pickering et al. 1982; Redding and Schreck 1983). Similarly, the decline in liver glycogen in the fed fish after handling is a characteristic response to this type of disturbance (Nakano and Tomlinson 1967; Miles et al. 1974; Rush and Umminger 1978; Pasanen et al. 1979; Morata et al. 1982).

Even though resting levels of plasma cortisol were lower, fish were still capable of eliciting a characteristic cortisol elevation in response to handling after 20 d of fasting. The trend towards lower levels of plasma cortisol in the fasted fish after both brief and prolonged stresses suggests, however, that the fasted fish may have evoked a lesser interrenal response to stress than did their fed counterparts. To our knowledge, there are no other published reports documenting effects of fasting on corticosteroid changes in response to acute handling in fish, although Strange et al. (1978) observed similar plasma cortisol increases in fed and unfed chinook salmon during the first few days of moderate confinement. In chickens, it was recently shown by Rees et al. (1985a) that 60 h of food deprivation resulted in decreased corticosterone responses to exercise, as compared with fed controls, but resting corticosterone levels were higher in fasted than in fed birds.

The type of diet influenced the rise in cortisol in response to handling alone in both fed and fasted fish, but the inconsistency of the recovery patterns precludes a readily apparent

conclusion. Among the fish subjected to continuous confinement after handling, there was a trend towards decreased cortisol in fasted fish from the high lipid diet, particularly after 24 h. Inasmuch as the hypothalamic-pituitary-interrenal axis has been implicated in gluconeogenesis in fish (Storer 1967; Butler 1968; Swallow and Fleming 1969; Inui and Yokote 1975b; Lidman et al. 1979), the lower cortisol titers observed in the high lipid group after fasting may have reflected the mobilization of stored lipids for required energy through pathways independent of this axis. Visceral lipid, in particular, is suspected of being an important source of energy in fish during fasting (Ince and Thorpe, 1976b; Jezierska et al. 1982).

Both the type of diet and feeding versus fasting were important in determining the glucose responses to stress. Generally, hyperglycemia was not as high in fasted fish as in fed fish after either brief or prolonged stressors. A major source of elevated blood glucose during stress is catecholamine-induced glycogenolysis (Nakano and Tomlinson 1967; Mazeaud et al. 1977; Mazeaud and Mazeaud 1981). It follows that fish whose liver glycogen stores have been depleted by fasting may be less able than fed fish to elicit a glucose stress response to the same extent. Within the fed fish, the higher post-stress plasma glucose in the briefly stressed fish that received the high lipid diet may be significant; these fish may have been able to mobilize lipids more effectively than those fed low lipid rations, as an additional energy source during stress. Alternatively, higher plasma glucose levels following stress in fish fed the high lipid diet may have resulted from the higher caloric value of that ration. Of necessity, metabolizable energy was allowed to vary with the proportion of lipid and carbohydrate so that the protein content could remain constant (Table V.1).

The lack of a further decline in liver glycogen after handling in fasted fish, as compared with fed fish, also suggests that, after periods of fasting, the fish used sources other than

liver glycogen for energy. Our finding of a decline in liver glycogen in fed fish but not in fasted fish after the handling stress agrees with that of Nakano and Tomlinson (1967). These authors, as well as Aldrin et al. (1979), noted that the degree of post-stress hyperglycemia appeared to be directly related to glycogen levels. The changes in plasma glucose in fed and fasted fish subjected only to handling support that observation. However, the increase in plasma glucose after 6 h in fasted fish from all diet groups continuously confined after handling, up to levels comparable with those in the fed fish, indicates the possible use of an energy pathway other than glycogenolysis -- e.g. gluconeogenesis or mobilization of stored lipids. Since neither plasma proteins nor lipid metabolites were measured, we were unable to postulate which energy pathways were preferentially used to cope with the stress after fasting in the groups fed different diets. Comparisons with published literature are difficult since, as Ince and Thorpe (1976b) pointed out, various species use different strategies for energy production during fasting; some species use up glycogen or lipid stores to conserve protein whereas others convert body protein into energy to maintain glycogen reserves.

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VI: EFFECT OF PRIOR ACID EXPOSURE ON PHYSIOLOGICAL RESPONSES OF JUVENILE RAINBOW TROUT TO ACUTE HANDLING STRESS

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Oregon State University, Corvallis, OR 97331

Oregon State University, Agricultural Experiment Station Technical Paper No. 7199. Reprinted with permission from Canadian Journal of Fisheries and Aquatic Sciences 42: 710-717 (1985);

Abstract

Acid-stressed fish appear to be more sensitive to additional stressors than unstressed fish. When juvenile rainbow trout, Salmo gairdneri, were exposed to acidic conditions (pH 5.7-4.7) for 5 d, plasma cortisol was affected only slightly during the initial hours of exposure, but plasma glucose and hematocrit increased, and plasma sodium decreased. However, when fish held at pH 4.7 were subsequently subjected to a 30-s handling stress, post-stress plasma cortisol rose to a peak level of more than twice that in handled fish held at ambient pH (6.6). Effects of handling on plasma glucose or sodium were not apparent against levels already altered by the chronic acid exposure.

Judging by the corticosteroid response, we conclude that the acid-stressed fish were more sensitive to additional handling, even though they appeared to be physiologically normal after 5 d. Thus as a management consideration, when fish are stocked in acidified waters, care should be taken to avoid situations where the fish may encounter additional disturbances in the new environment. Plasma glucose and sodium were better indicators of chronic acid stress alone than plasma cortisol, but the greater cortisol response to handling at low pH may be a useful method of detecting increased interrenal activity during early stages of environmental acidification.

Introduction

The detrimental effect of acidic precipitation on fisheries resources is a global problem and is of paramount concern in eastern North America (reviews by Haines 1981; Haines and Johnson 1982). In addition to the direct effects on survival and reproductive success, the sublethal effects of acid stress on physiological mechanisms in fish are becoming better understood (reviews by Fromm 1980; Spry et al. 1981; Wood and McDonald 1982). By comparison, relatively little work has addressed the potentially compounding effects of chronic acid exposure and additional stressors, such as physical disturbance or strenuous exercise, on physiological responses in fish. Graham et al. (1982) reported that post-exercise changes in a number of hematological and other physiological conditions -- for example, plasma ion concentrations -- were more severe in acid-exposed rainbow trout, Salmo gairdneri, than in control fish when held in soft water. Graham and Wood (1981) had earlier found that severe exercise significantly increased the toxicity of H_2SO_4 to rainbow trout.

The suitability of both plasma cortisol and glucose as indicators of certain kinds of stress in fish is now well established (reviews by Donaldson 1981; Schreck 1981; Wedemeyer and McLeay 1981). Recently, Brown et al. (1984) found increases in plasma cortisol and glucose in rainbow trout, as well as changes in interrenal histology and plasma thyroid hormones, during a 21-d chronic exposure to acid. To evaluate the possibility that responses to other subsequent stressors are modified in rainbow trout stressed by acidification, we assessed the changes in cortisol and glucose resulting from an acute handling stress following a chronic exposure to low environmental pH. We also determined plasma sodium and potassium, and hematocrit as indicators of possible impairment of physiological performance after exposure of the fish to acid.

Methods and Materials

Experimental Design

Juvenile rainbow trout (Willamette River stock), weighing 51.7 ±1.9 g (mean ±SE), were reared and maintained in ambient flow-through well water at the Western Fish Toxicology Station, Environmental Protection Agency, Corvallis, Oregon. Twelve days before the experiment (in April, 1983), the fish were transferred for acclimation to covered, outdoor, 1.2-m-diameter circular tanks, each containing 350 L and receiving 4 L/min of aerated, treated (soft) well water with an ambient temperature of 17-19°C; pH was 6.6, alkalinity 12.6 mg/L as $CaCO_3$, total calcium 1.5 to 2.3 mg/L, and total hardness between 7.3 and 12 mg/L as $CaCO_3$. Quality characteristics of the well water routinely monitored were similar to those reported in detail by Chapman (1978). Softwater conditions were established and maintained by a reverse osmosis system (Purification Techniques Inc., Brielle, New Jersey). Based on proportional comparison with well water hardness at the time of the experiment (51-76 mg/L as $CaCO_3$), this system reduced concentration of water quality conditions by about 85%. A total of 120 fish per tank were held at a density of 18 g/L under a natural photoperiod and were fed daily to satiation with Oregon Moist Pellets up to and including the day before, but not during, the experiment.

For the experiment, aerated soft water was acidified with concentrated reagent-grade H_2SO_4, held at pH 4.0 (±0.1) with a Beckman Model 942 pH Monitor (Beckman Instruments, Fullerton, California) while vigorously reaerated to remove CO_2, and then diluted with softened well water to attain each experimental pH in separate mixing chambers. Fish were subjected to acidic conditions by changing the inflow supply over

to the acid-blended water through a single valve without disturbing the experimental tanks. From the ambient pH of 6.6, levels were within 0.3 pH unit of the final pH at 6 h and within 0.1-0.2 pH unit at 10 h. Final pH levels of 5.7, 5.2 and 4.7 were reached in 20 h. Treatment pH levels were automatically recorded every 3 h, and the diluter was manually adjusted as necessary to maintain exposure levels within ±0.1 pH unit. Duplicate tanks of fish for each pH, including ambient, were established for sampling; an additional two tanks with fish at ambient pH were left undisturbed as controls for the handling phase of the experiment.

After 2, 4, 6, 10 and 20 h, and 2, 3 and 5 d of acid exposure, five or six fish were quickly removed from each tank for blood sampling. At day 5, the fish were subjected to an acute handling stress by holding them out of the water in a bag seine for 30 s; the 5-d acid exposure samples also served as initial values for this phase of the experiment. At 1, 3, 6, 12, 24 and 48 h after handling, five or six fish were obtained from each tank for sampling. As controls, samples were simultaneously obtained from the previously undisturbed fish in the two additional tanks at ambient pH.

Sampling and Analysis

Sample fish removed from the tanks were immediately anesthetized in <40 s in a 200-mg/L concentration of tricaine methanesulfonate (MS-222). Individual fish were then taken serially from the anesthetic solution, the caudal peduncle was severed, and blood was obtained from the caudal vasculature with an ammonium-heparinized 0.25-mL Natelson capillary tube; total sample time per tank was <10 min. Plasma, separated by centrifugation, was stored at -20°C for future assay. Additional blood was also collected in heparinized microhematocrit tubes for immediate hematocrit determination.

Plasma cortisol was determined by the

^{3}H-radioimmunoassay described by Foster and Dunn (1974) and modified in our laboratory (Redding et al. 1984b). We assayed plasma glucose by an ortho-toluidine colorometric procedure modified from Wedemeyer and Yasutake (1977) using Sigma ® pre-mixed reagent. Plasma sodium (as Na^{+}) and potassium (as K^{+}) were measured with a self-calibrating NOVA 1 Sodium/Potassium Analyzer (Nova Biomedical, Newton, Massachusetts). We determined hematocrit as percent packed cell volume (%PCV) by direct measurement after centrifugation.

Since duplicated results were in agreement, data from each pair of tanks for each time and treatment were pooled. Homogeneity of variance was assessed using Bartlett's test (Snedecor and Cochran 1967) and, where appropriate (i.e. for cortisol and glucose), data were transformed to logarithmic values. One-way analyses of variance (ANOVA) were conducted both within treatments through time and among treatments for specific times. Significant differences among means were then determined by using Duncan's new multiple-range test at the 5% level (Steel and Torrie 1980). For ease of interpretation, arithmetic means and standard errors are presented.

Results and Discussion

Responses to Reduced Environmental pH

Plasma cortisol from fish held at pH 5.7-4.7 was elevated during the 5-d exposure compared with that from fish maintained at the ambient pH. However, significant differences from ambient were apparent only during the first 20 h of acid treatment (Table VI.1). At 10 h, cortisol was higher than at other times in the ambient pH group, and was not different from the test groups. Mudge et al. (1977) also reported a transient rise in plasma cortisol in brook trout, Salvelinus fontinalis, at 1-3 h during exposure to

Table VI.1. Responses of plasma cortisol, glucose and sodium, and hematocrit [as packed cell volume (PCV)] in juvenile rainbow trout during continuous exposure to reduced environmental pH. Values marked with an asterisk (*) indicate a significant difference from the ambient pH of 6.6 at that time (Duncan's new multiple-range test at 5%). Sample sizes (N) represent pooled data from duplicate treatments.

Time and pH	Cortisol (ng/mL; N=10) Mean (SE)	Glucose (mg/dL; N=8) Mean (SE)	Sodium (mmol/L; N=8) Mean (SE)	Hematocrit (%PCV; N=8-13) Mean (SE)
2 h: 6.6	16 (5)	86 (7)	151 (3)	46 (1)
5.7	39 (15)*	87 (6)	148 (3)	48 (2)
5.2	44 (15)*	94 (13)	154 (2)	48 (2)
4.7	27 (9)*	88 (14)	154 (2)	50 (1)
4 h: 6.6	6 (3)	92 (6)	149 (4)	48 (1)
5.7	49 (15)*	80 (3)	148 (4)	47 (1)
5.2	44 (10)*	95 (11)	153 (2)	49 (1)
4.7	50 (34)*	80 (3)	154 (1)	51 (1)
6 h: 6.6	10 (4)	99 (11)	152 (2)	47 (1)
5.7	44 (10)*	91 (6)	152 (2)	50 (1)
5.2	32 (9)*	84 (6)	152 (2)	50 (2)
4.7	33 (10)*	80 (4)	155 (2)	53 (1)*
10 h:6.6	34 (6)	93 (10)	149 (1)	49 (2)
5.7	29 (10)	90 (9)	149 (2)	50 (1)
5.2	58 (12)	99 (14)	153 (1)	50 (2)
4.7	55 (19)	92 (5)	151 (2)	51 (1)
20 h:6.6	16 (5)	74 (3)	149 (2)	48 (1)
5.7	47 (11)*	91 (12)	147 (2)	50 (1)
5.2	48 (6)*	109 (13)	152 (1)	53 (1)*
4.7	49 (12)*	89 (6)	143 (2)*	53 (2)*
2 d: 6.6	21 (9)	77 (6)	144 (1)	48 (2)
5.7	10 (3)	92 (10)	142 (1)	49 (1)
5.2	8 (2)	101 (7)	139 (3)	50 (1)
4.7	26 (8)	166 (21)*	138 (2)	53 (2)
3 d: 6.6	15 (6)	87 (9)	147 (2)	43 (1)
5.7	16 (8)	94 (8)	149 (2)	50 (2)*
5.2	12 (4)	110 (19)	150 (2)	50 (1)*
4.7	30 (7)	154 (19)*	143 (4)*	52 (2)*
5 d: 6.6	9 (3)	85 (10)	149 (2)	42 (1)
5.7	9 (7)	79 (5)	145 (3)	47 (1)*
5.2	11 (3)	143 (30)	143 (4)	46 (1)*
4.7	23 (9)	258 (42)*	130 (3)*	49 (1)*

pH 4.0, with titers returning to control levels within 24 h. Our findings of apparently recovered, or resting, plasma cortisol levels after 2-5 d of acid exposure are consistent with those of Brown et al. (1984) who found that water acidification did not increase plasma cortisol concentrations until day 8 of exposure. Moreover, Lee et al. (1983) showed no significant change in plasma cortisol of rainbow trout after 2- or 3-wk exposures to pH's as low as 4.2, but interim samples were not taken. The small transitory increase in plasma cortisol at 10 h in the ambient pH group may have resulted from increased activity around the tanks or from repeated sampling, although effects of these factors were not evident in the handling phase of this experiment, nor in earlier handling studies (Barton et al. 1980; Barton and Peter 1982).

A significant elevation in plasma glucose was evident commencing at 2 d in the pH 4.7 group and it was three times higher than in the ambient pH group at the end of the 5-d exposure (Table VI.1). These results corroborate those of Brown et al. (1984) and Lee et al. (1983), who both found that plasma glucose levels were higher in rainbow trout exposed to lower pH's.

Plasma sodium was significantly depressed in fish exposed to pH 4.7 for 20 h, 3 d, and 5 d (Table VI.1). Plasma potassium (data not shown) generally showed a declining trend at all pH's, from levels of about 4 to 5 mmol/L at the beginning of the experiment to about 1 to 2 mmol/L at the end of the experiment 7 d later. No differences from ambient pH in plasma potassium attributable to either the chronic acidity or to the subsequent handling stress were apparent. The decline in plasma sodium concentration in fish exposed to the low pH for 5 d was similar to the decline observed at pH 4.5 in adult rainbow trout after a 42-d exposure under the same experimental conditions (Weiner 1984) and confirms that which has been previously reported by others for juvenile salmonids (Lee et al. 1983; Saunders et al. 1983; reviews by Fromm 1980; Wood and McDonald 1982).

Hematocrits generally tended to be higher in the

acid-stressed fish and were significantly so at 6 and 20 h, and at 3 and 5 d (Table VI.1). However, we do not know if the differences evident at 3 and 5 d were due to the increased environmental acidity, or to the significant decline in hematocrit observed in the ambient pH group, possibly caused by some other factor. The tendency towards increased hematocrit in the acid-exposed fish agrees with earlier studies (Dively et al. 1977; Neville 1979; Milligan and Wood 1982; Saunders et al. 1983), and Milligan and Wood (1982) attributed the increase to erythrocyte swelling. Furthermore, they found a redistribution of body water, resulting in decreased plasma volume with no change in total body water.

Responses to Additional Handling Stress

All groups demonstrated a significant elevation ($P<0.01$) in plasma cortisol after being subjected to the acute handling stress (Fig. VI.1). However, the cortisol peak at 1 h in fish held at pH 4.7 was more than twice that in fish from the ambient pH. Plasma cortisol also tended to remain higher in all three acid-treated groups than in the ambient pH group for subsequent samples, with significant differences occurring at all sample times following handling (Fig. VI.1). Two fish died between 3 and 6 h after handling at pH 4.7. Our results support those of Brown et al. (1984), who concluded that chronic acid exposure stimulated activity of interrenal cells, as demonstrated by interrenal hyperplasia and increased size of nuclei after 4 d. By comparison, Mudge et al. (1977) found evidence of decreased cell and nuclear size, suggesting that activity of interrenal tissue decreased after acid exposure. However, their histological investigation was restricted to measurements taken after 3- to 24-h exposure periods only.

On the basis of the findings of Brown et al. (1984) of increased interrenal activity and our results from the subsequent handling stress, we speculate that clearance rate of cortisol may

Figure VI.1. Responses (mean ±SE) of plasma cortisol in juvenile rainbow trout subjected to a 30-s handling stress after 5 d of continuous exposure to various pH's. Values marked with an asterisk (*) indicate a significant difference from the ambient pH of 6.6 at that time (Duncan's new multiple-range test at 5%). Open squares are values for unhandled controls at ambient pH. Sample sizes of 10 fish represent pooled data from duplicate treatments.

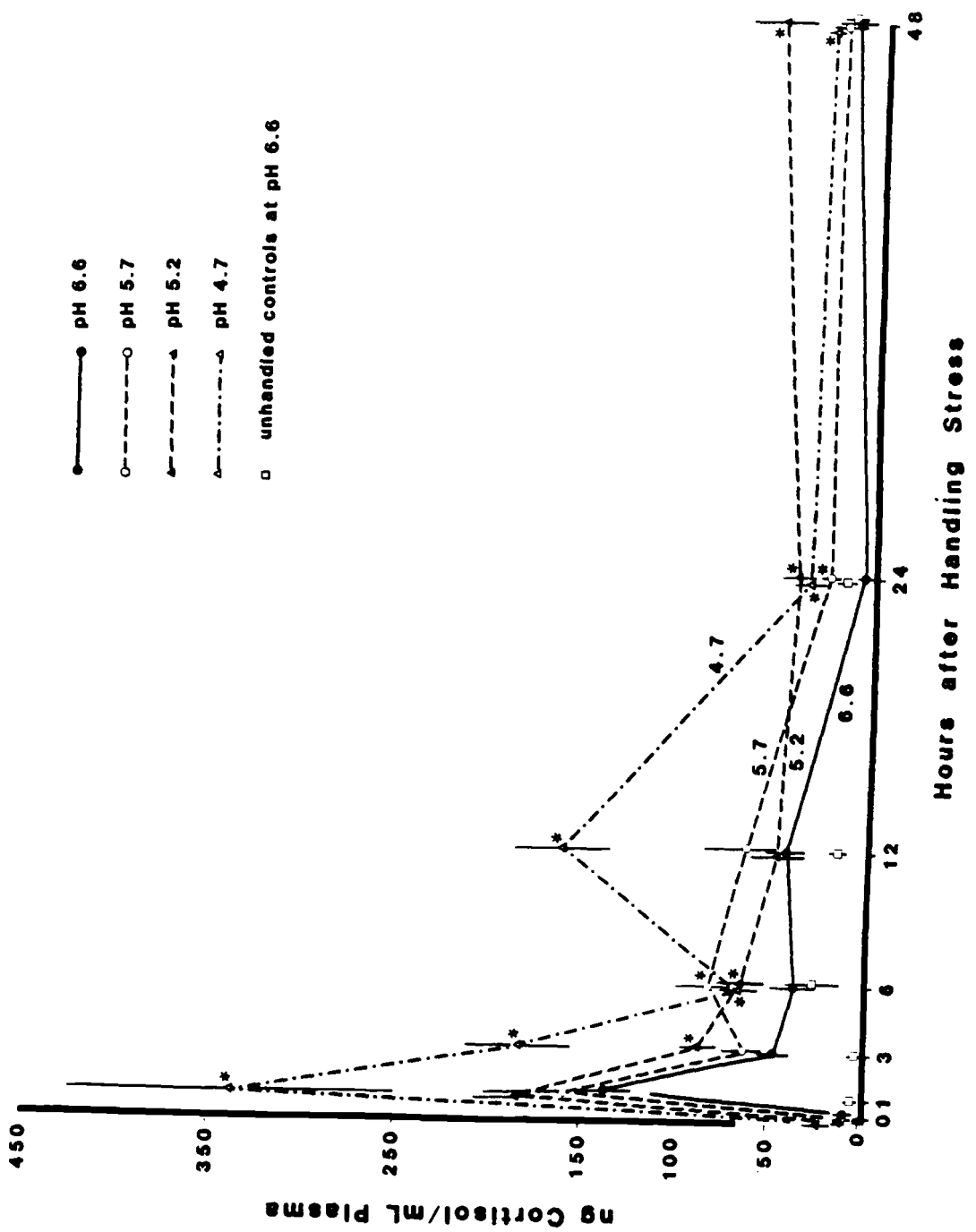
pH 6.6
pH 5.7
pH 5.2
pH 4.7
unhandled controls at pH 6.6
ng Cortisol/mL plasma
450
350
250
150
100
50
0
0 1 3 6 12 24 48
Hours after Handling Stress
4.7
5.7
5.2
6.6

Figure VI.1

also have increased during chronic acidification to compensate for the increased interrenal output, as a measure directed towards maintaining homeostasis. This would account for the relatively low cortisol titer after 5 d of acid exposure, since instantaneous plasma levels are a function of both rates of secretion and clearance. Thus, the hypothalamic-pituitary-interrenal axis may have been stimulated in response to chronic acid stress, but the differences in response -- i.e. at pH 4.7 as compared with ambient -- were not apparent until the fish experienced an additional, sudden plasma cortisol increase, caused by the 30-s handling stress. The pattern of rapid plasma cortisol elevation, followed by recovery, in response to handling was characteristic of what has been previously shown for juvenile salmonids (Strange and Schreck 1978; Barton et al. 1980; Pickering et al. 1982); this subject has been reviewed extensively by Donaldson (1981) and Schreck (1981).

After handling, there were significant transient increases in plasma glucose between 1 and 12 h (inclusive) in the pH 6.6 ($P<0.01$) and 5.7 ($P<0.05$) groups (Fig. VI.2). The response-recovery patterns were typical of those found in salmonids subjected to an acute physical disturbance (Nakano and Tomlinson 1967; Wedemeyer 1972; Wydoski et al. 1976; Casillas and Smith 1977; Perrier et al. 1978; Pickering et al. 1982). However, in the pH 5.2 and 4.7 groups, transient increases in plasma glucose caused by handling did not differ significantly from the elevated background levels caused by the chronic acid exposure. In the pH 4.7 group, plasma glucose continued to increase and reached a maximum 48 h after handling at the termination of the experiment (Fig. VI.2). This level was similar to the level that Brown et al. (1984) reported after a 21-d exposure to this pH. Plasma glucose in the pH 5.2 group also remained slightly elevated above that in the ambient pH group 48 h after handling (Fig. VI.2).

We suspect that the comparatively high glucose elevation we observed at 6-7 d in response to the acid stress was a combination of the following: the relatively soft ambient water, the

Figure VI.2. Responses (mean ±SE) of plasma glucose in juvenile rainbow trout subjected to a 30-s handling stress after 5 d of continuous exposure to various pH's. Values marked with an asterisk (*) indicate a significant difference from the ambient pH of 6.6 at that time (Duncan's new multiple-range test at 5%). Open squares are values for unhandled controls at ambient pH. Sample sizes of 8 fish represent pooled data from duplicate treatments. (See Fig. VI.1 for legend.)

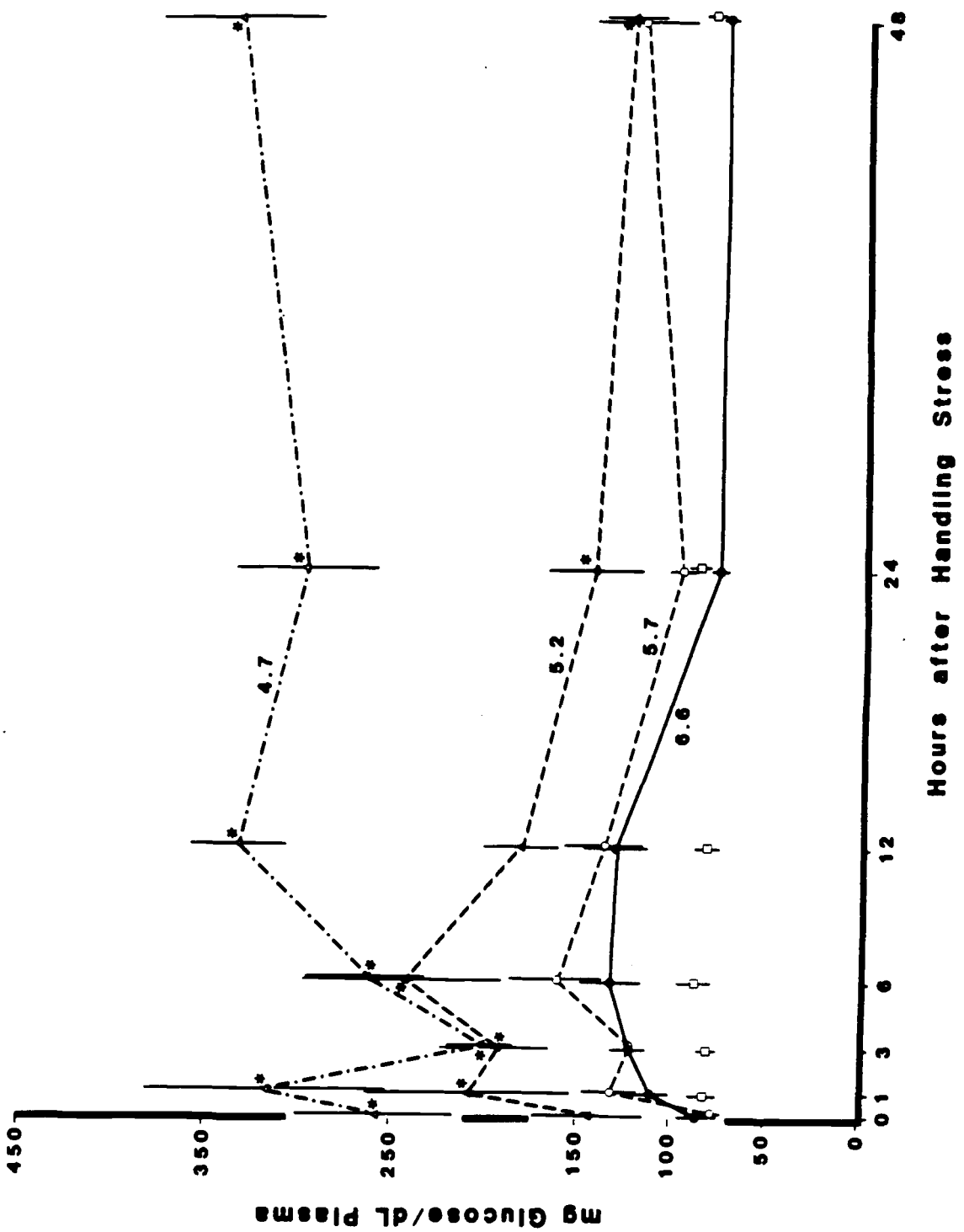
mg Glucose/dL plasma
450
350
250
150
100
50
0
0 1
3
6
12
24
48
Hours after Handling Stress
4.7
5.2
5.7
6.6

Figure VI.2

aggravating effect of the additional handling, and the relatively high ambient temperature we used. Recent evidence showed that the glucose response to a 30-s handling stress was three times greater in juvenile chinook salmon, Oncorhynchus tshawytscha, acclimated to 21°C than in those acclimated to 12 or 7°C (B.A. Barton and C.B. Schreck, unpublished, Chapter II). The apparently more rapid response of plasma glucose to the acid-stress alone in our fish than in those of Brown et al. (1984), may also have resulted from the relatively soft water we used. For example, Graham et al. (1982) found that physiological responses were more severe in acid-exposed trout subjected to strenuous exercise when held in soft water rather than in hard water, and post-exercise mortality doubled.

Leach and Taylor (1980) indicated that increased endogenous cortisol may have a functional role in sustaining elevated glucose levels in reponse to stress. Possibly, increased interrenal activity resulting from acid exposure, demonstrated histologically by Brown et al. (1984) and in our experiment by the secondary handling stress challenge, may be responsible for an increased cortisol output to maintain high levels of blood glucose as a readily available energy source. Mobilization of energy reserves would presumably be necessary to allow the fish to cope with the effects of chronic acid stress, such as by increasing metabolic or ionoregulatory activity.

All groups, including those held at ambient pH, showed decreases in plasma sodium after handling (Fig. VI.3); temporal changes were significant in pH groups 6.6 ($P<0.05$), 5.7 ($P<0.01$) and 4.7 ($P<0.05$). Plasma sodium in fish at pH 4.7, already depressed prior to handling, continued to decline significantly to the minimum level observed at 48 h post-handling (Fig. VI.3). Because of this trend, any transitory decreases that may have resulted from the additional handling stress were not apparent over reduced background levels.

The perturbations in plasma sodium within the first 24 h

Figure VI.3. Responses (mean ±SE) of plasma sodium in juvenile rainbow trout subjected to a 30-s handling stress after 5 d of continuous exposure to various pH's. Values marked with an asterisk (*) indicate a significant difference from the ambient pH of 6.6 at that time (Duncan's new multiple-range test at 5%). Open squares are values for unhandled controls at ambient pH. Sample sizes of 8 fish represent pooled data from duplicate treatments. (See Fig. VI.1 for legend.)

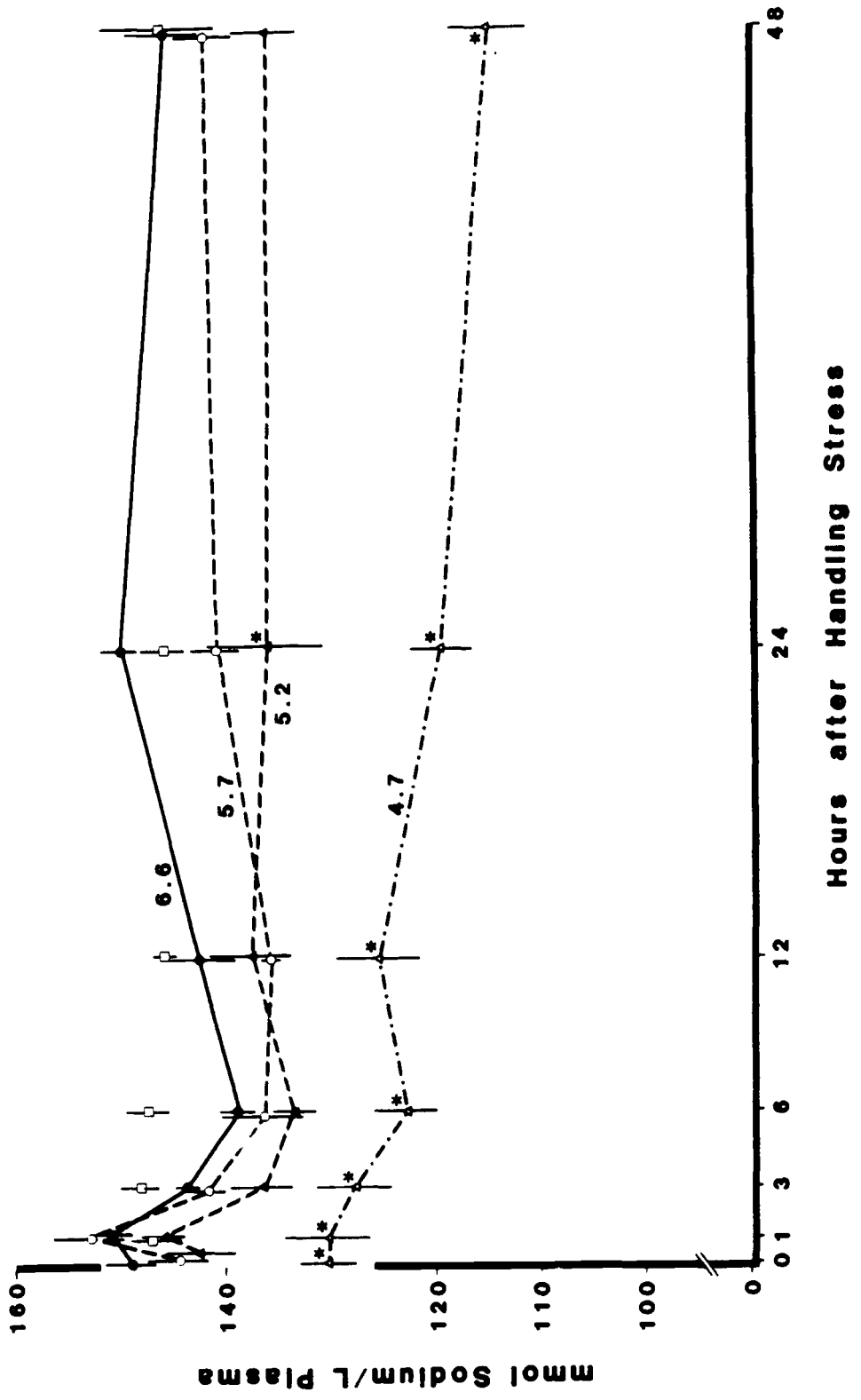
mmol Sodium/L Plasma
160
140
120
110
100
0
1
3
6
12
24
48
Hours after Handling Stress
6.6
5.7
5.2
4.7

Figure VI.3

after handling in pH 6.6 and 5.7 water are consistent with those which Redding and Schreck (1983) observed in juvenile coho salmon, Oncorhynchus kisutch, chronically stressed by confinement in fresh water. These results support the notion that under normal conditions, there is a short-lived reduction in ionoregulatory ability when fish are subjected to physical disturbances. The results differ from those of Graham et al. (1982), who observed an elevation in plasma sodium of rainbow trout after 6 min of strenuous exercise at pH 7.5 in both hard and soft water. Graham et al. (1982) attributed an increase in blood constituents to high intracellular lactate loading, causing water to move across the osmotic gradient and thus decreasing blood volume. Although there was a slight increase in plasma sodium 1 h after handling in the ambient and higher pH groups, we postulate that the 30-s handling stress we used was insufficient to sustain the lactate levels normally associated with severe exercise.

A transient increase in hematocrit in the pH 4.7 group 3 h after handling was significantly greater than that observed in the ambient pH group at that time (Fig. VI.4). Although hematocrits generally tended to be higher in lower pH groups after handling, differences among treatments at other times were not significant. There was also a significant increase in hematocrit over the 48-h post-handling period in the unhandled control group held at ambient pH (Fig. VI.4). Handling stress has been previously shown to increase hematocrit (Fletcher 1975; Soivio and Oikari 1976) and erythrocytic swelling (Soivio et al. 1977) in fish. In the handling phase of our experiment, only the combined stress of handling and acidity at pH 4.7 was sufficient to cause a significant increase in hematocrit above the pre-handling level.

Summary and Management Considerations

Acid-stressed fish appear to be more sensitive to

Figure VI.4. Responses (mean ±SE) of hematocrit [as packed cell volume (PCV)] in juvenile rainbow trout subjected to a 30-s handling stress after 5 d of continuous exposure to various pH's. Values marked with an asterisk (*) indicate a significant difference from the ambient pH of 6.6 at that time (Duncan's new multiple-range test at 5%). Open squares are values for unhandled controls at ambient pH. Sample sizes of 6-13 fish represent pooled data from duplicate treatments. (See Fig. VI.1 for legend.)

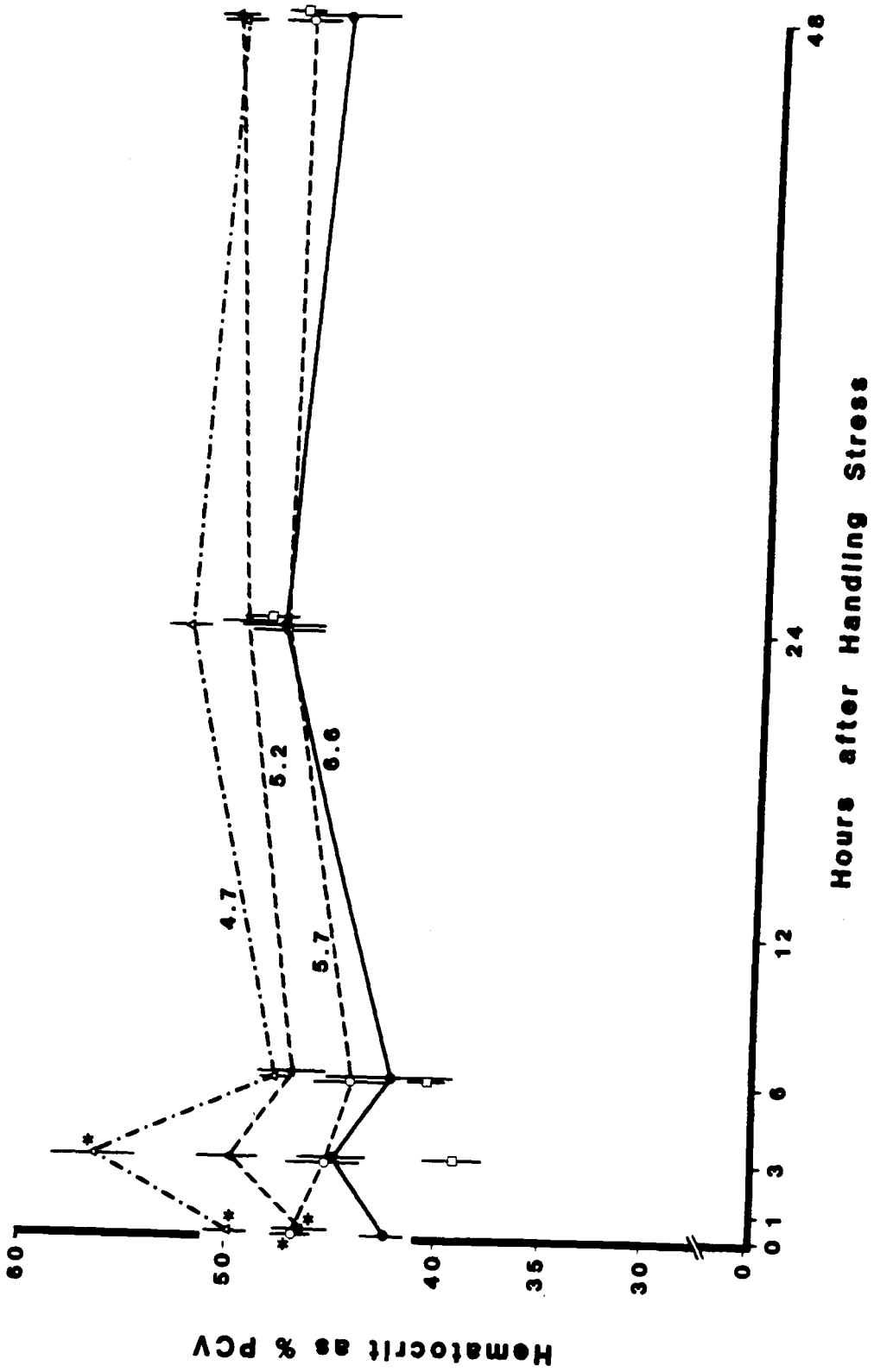
Hematocrit as % PCV
60
50
40
35
30
0
1
3
6
12
24
48
Hours after Handling Stress
4.7
5.2
5.7
6.6

Figure VI.4

additional handling stress than unstressed fish. When the rainbow trout in our experiment were subjected to the 30-s handling stress, the effect of prior acid treatment on interrenal activity was clearly apparent in the higher plasma cortisol response to handling at the low pH. However, any possible effects of handling on glucose or sodium were not apparent over background levels already affected by chronic acid exposure. Acid exposure alone altered plasma glucose, plasma sodium, and hematocrit levels significantly after 5 d, particularly at pH 4.7, but plasma cortisol was affected only during the initial period of exposure.

As a management strategy, fish stocking will probably be used more extensively in the future to provide short-term fisheries in waters that no longer support natural reproduction because of reduced pH. Since we have shown that acid-stressed fish could have an increased sensitivity to additional stressors, particularly at pH's below 5, fish stocked in acid waters may appear to be physiologically normal, with respect only to cortisol titer, within a few days after planting, when in fact they are not. Increased plasma glucose, depressed plasma sodium, and the observed mortality 3 to 6 h after handling in the pH 4.7 group support that view. Thus as a management consideration, extra attention should be given to ensure survival of fish stocked in acidic waters. Possible approaches towards that objective may include choosing suitable sites for stocking to avoid situations where the fish might encounter additional disturbances soon after release. As a further precaution, care should also be exercised to minimize the stress associated with transport and stocking (e.g. overcrowding, rough handling, poor water quality). In addition, selection of acid-tolerant stocks and pre-acclimation of fish could be considered (Flick et al. 1982).

In agreement with Brown et al.'s (1984) conclusion for glucose, our results showed that both plasma glucose and plasma sodium are more suitable as physiological indicators of chronic acid stress than plasma cortisol. However, a secondary acute

stress challenge, such as the 30-s handling stress, may be useful for determining possible increases in interrenal activity at an early stage of environmental acidification.

Acknowledgments

We sincerely thank Scott B. Brown, Freshwater Institute, Canada Department of Fisheries and Oceans, and Larry R. Curtis, Department of Fisheries and Wildlife, Oregon State University, for critically reviewing earlier drafts of this manuscript. We also thank Reynaldo Patino and Jane Linville, Oregon Cooperative Fishery Research Unit, for their technical assistance during the sampling phase of the study, and the staff at the Western Fish Toxicology Station, Environmental Protection Agency, Corvallis, Oregon for providing and maintaining fish and facilities for the experiment.

Mention of trade names or manufacturers does not imply U.S. Government endorsement of those commercial products.

VII: CHRONIC CORTISOL ADMINISTRATION AND DAILY HANDLING EFFECTS ON GROWTH, PHYSIOLOGICAL CONDITIONS, AND STRESS RESPONSES IN JUVENILE RAINBOW TROUT

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Abstract

Juvenile rainbow trout, Salmo gairdneri, were either fed cortisol or subjected to an acute physical disturbance daily for 10 wk to determine long-term effects on physiological and hematological conditions. In cortisol-fed fish, growth and condition factor, liver glycogen and circulating lymphocytes were reduced while resting plasma glucose and hematocrit were increased. In daily stressed fish, all features measured were similar to controls except that lymphocyte numbers were lower. After 10 wk, continuous cortisol feeding completely eliminated the plasma cortisol elevation following acute handling in these fish but the magnitude of the stress-induced glucose increase was not different. In fish stressed daily for 10 wk, the reduction in post-stress levels of both plasma cortisol and glucose after handling demonstrated the effect of habituation on these stress responses. We concluded that continuously elevated plasma cortisol from exogenous feeding had a profound long-term effect on juvenile rainbow trout but that daily stress-induced acute elevations of endogenous cortisol did not, except for a suppression of circulating lymphocytes.

Introduction

Biologists are familiar with the physiological responses of fish to stress (see Pickering 1981b) but mechanisms of action and the resultant consequences of stress on fish's overall performance capacity are not yet well understood. It is well established that plasma corticosteroids, which affect both metabolic and immunologic pathways, rise dramatically in fish in response to stressful stimuli, particularly those that have a sensory component (reviews of Donaldson 1981; Schreck 1981). The main objective of our investigation was to determine the possible consequences of a continually elevated cortisol titer in fish that would result from long-term chronic stress. We did this by examining the effects of chronic cortisol elevations in fish, but in the absence of other stressful stimuli. To do this, we fed cortisol to juvenile rainbow trout, Salmo gairdneri, and measured growth and condition, plasma cortisol, glucose and insulin, liver glycogen, hematocrit and circulating leucocyte ratios. In addition, we determined the effect of continued cortisol treatment on the ability of the trout to elicit characteristic interrenal and glycemic responses to an acute physical disturbance. Our second objective was to find out the long-term effects on fish of being subjected to continual acute stresses and how this treatment subsequently affects characteristic stress responses. We did this by determining the effect of repeatedly elevating endogenous cortisol, by applying an acute physical disturbance to the fish daily, on these same physiological and hematological features.

Methods and Materials

Juvenile Willamette River stock rainbow trout, 4-5 g in size, were obtained from the Western Fish Toxicology Station, Corvallis, Oregon, and transferred to the Oregon State University

Smith Farm research facility 10 d before the study for acclimation. Fish were held in 0.9-m-diameter circular tanks containing 327 L of aerated flow-through well water, with an inflow rate of 2.8 L/min. Flows were directed into the tanks perpendicular to the water's surface to reduce rotational current to a minimum; all tanks were cleaned weekly but at least 5 d before any sampling. During acclimation, fish were fed at 2% body weight (bwt)/d with Oregon Moist Pellets (OMP). For the experiment, duplicate tanks of 185 fish for each treatment were fed once daily between 0900-1000 h for 10 wk with either control or cortisol-treated OMP at 3% bwt/d. Extra care was taken by feeding slowly to ensure that all feed pellets were consumed. The experimental diet was prepared by dissolving crystalline cortisol (Sigma Chemical Co., St. Louis, Missouri) in 100% ethanol and then spraying the resultant solution on to the surface of the pellets (Pickering 1984) to produce a concentration of 100 mg cortisol/kg feed. The control diet was prepared using an equivalent amount of ethanol only. Feed pellets were air-dried for 1 h with occasional stirring to evaporate the ethanol and then refrozen. A third group of fish in duplicate tanks was fed the control diet, but was also subjected to an acute physical disturbance daily, 5-6 h after feeding, using one of the following three methods: (1) after capture, fish were held in the air for 30 s in a perforated bucket, (2) water was completely drained from the tanks and then allowed to fill at the normal inflow rate, or (3) fish were continually chased in the tanks with nets for 15 min. The particular stress-method used was varied randomly so that the fish would not become accustomed to a particular routine. During the 10-wk experimental period (October-December, 1984), fish were maintained under a natural photoperiod and water temperature ranged from a maximum of 13.0°C at the beginning to a low of 11.5°C at the end of the study.

At the beginning of the experiment and every 2 wk thereafter, 15 fish per tank were removed for length, weight and

condition factor (100 X g/cm^3) determinations and for blood sampling according the the following protocol. From the control and cortisol-fed groups, 5 fish per tank were removed before and at 1 and 3 h after feeding; from the daily stressed group, 5 fish per tank were removed before (6 h after feeding) and at 1 and 3 h after application of the stress. Feed rations were adjusted at each 2-wk interval in relation to the numbers and weights of fish remaining in the tanks in order to keep the relative ration constant.

Blood samples from the beginning of the study (0 wk) and at 4 and 8 wk were obtained for cortisol and glucose analyses. Cortisol was determined by a radioimmunoassay (RIA) (Foster and Dunn 1974) modified by Redding et al. (1984b) for use with salmonid plasma. Glucose was measured by the ortho-toluidine method (Wedemeyer and Yasutake 1977) using Sigma ® premixed reagent. Blood samples taken at 2 wk after onset of treatment were used for hematocrit, leucocrit and lymphocyte number determinations. Hematocrit, also measured after 6 wk, was determined as percent packed cell volume after centrifugation and leucocrit was assessed as percent "buffy" layer of the total blood column in the hematocrit tube (McLeay and Gordon 1977). Lymphocyte ratios were made from Giesma-stained blood smears by counting erythrocytes (average 1386 per slide, range 738-2024), lymphocytes and other leucocytes in 15 random fields per slide (i.e. per fish), using Yasutake and Wales (1983) as a reference. Liver samples for hepatosomatic index (100 X liver wet weight/body wet weight) and glycogen determination, and stomach tissues for histological examination were obtained after 10 wk. Whole livers were immediately placed in preweighed vials containing chilled 30% KOH and hepatic glycogen was later analyzed by the method of Montgomery (1957). Cardiac portions of stomachs from control and cortisol-fed fish were preserved in buffered 10% formalin. After histological preparation, slide-mounted sections, 6 μm thick and stained with hematoxylin and eosin, were qualitatively evaluated for evidence of tissue degradation or other abnormalities.

At week 10, the fish in control and cortisol-fed groups were each separated into two subgroups with one subgroup remaining in the 'home' tank. The fish remaining in all 'home' tanks were then subjected to the 30-s handling stress (described earlier). Blood samples for cortisol and glucose analyses were taken before any disturbance (initial) and at 1, 3, 6, 12 and 24 h after handling.

The second subgroups of fish from the control and cortisol-fed groups were placed in 100-L tanks and feeding was continued with their respective diets. After 2 d, both groups were fed with a single meal of 3% bwt with the cortisol-treated feed to characterize the profile of cortisol in the blood over 24 h post-feeding and the resultant effect on plasma glucose and insulin. Fish for blood samples were obtained just before feeding and at 1, 3, 6, 12 and 24 h after being fed. Plasma insulin was determined by Dr. E. Plisetskaya, University of Washington, using a RIA homologous for coho salmon, Oncorhynchus kisutch, insulin and displaying parallelism with rainbow trout insulin (Plisetskaya et al. 1986).

Cumulative percent increases in mean weight of fish were compared by linear regression of 2-wk growth increments in each tank; slope of regression represents the percent change in body weight per day for the growth period. Differences from control were assessed by comparing variances of slopes using Student's *t*-tests ($P<0.05$ and <0.01). Specific (instantaneous) growth rates were calculated as:

$$[(\ln \text{weight}_2 - \ln \text{weight}_1)/\text{days fed}] \times 100$$

(Ricker 1979). Multiple-point comparisons were made using one-way analyses of variance followed by Duncan's new multiple-range test at the 5% level (Steel and Torrie 1980); two-point comparisons were made with Student's *t*-tests ($P<0.05$). Because of variance heterogeneity (Bartlett's test, Snedecor and Cochrane 1967), cortisol, glucose and insulin data were transformed to logarithmic values for analysis.

Results and Discussion

Growth and Resting Physiological Conditions

Compared to the control group, relative growth in the cortisol-fed trout was reduced, but growth in the daily stressed fish was not different from controls (Fig. VII.1); the difference in slope between regressions for the control and the cortisol-fed fish was significant ($P<0.01$). Specific growth rate for the 10-wk period was lowest in the cortisol-fed fish (Table VII.1). All three groups exhibited reductions followed by increases in specific growth rates mid-way through the growth period (Table VII.1). This may reflect a normal cyclical variation in growth rates in rainbow trout of this size (Wagner and McKeown 1985). Davis et al. (1985) recently observed lower absolute growth and condition factor of cortisol-fed channel catfish, Ictalurus punctatus, using the same experimental protocol as we did. Although these authors attributed the reduced growth to the presence of cortisol in the diet, they were unable to demonstrate a concomitant increase in blood corticosteroids after feeding with cortisol. However, Davis et al. (1985) did find an increase in activity of hepatic tyrosine aminotransferase in cortisol-fed fish and concluded that increased corticosteroid-induced gluconeogenesis resulted in decreased growth. In contrast to our finding for daily stressed fish, Peters and Schwarzer (1985) reported significantly reduced growth of juvenile rainbow trout subjected to a daily, brief handling stress over 4 wk, but did not discuss whether or not this was a cortisol-mediated phenomenon.

The continual application of dietary cortisol to the juvenile rainbow trout also reduced the condition factor over the 10-wk period (Fig. VII.2). Robertson et al. (1963) found that cortisol treatment at pharmacological doses produced a rapid weight

Figure VII.1. Cumulative percent increase in mean weight (N=15 per replicate, two replicates per sample wk) of juvenile rainbow trout fed with either cortisol-treated or control feed, or normally fed and subjected to a daily acute stress, for 10 wk. Relative growth as % increase/d for each treatment is represented by the slope of line [** indicates significant difference (P<0.01) in slope from control].

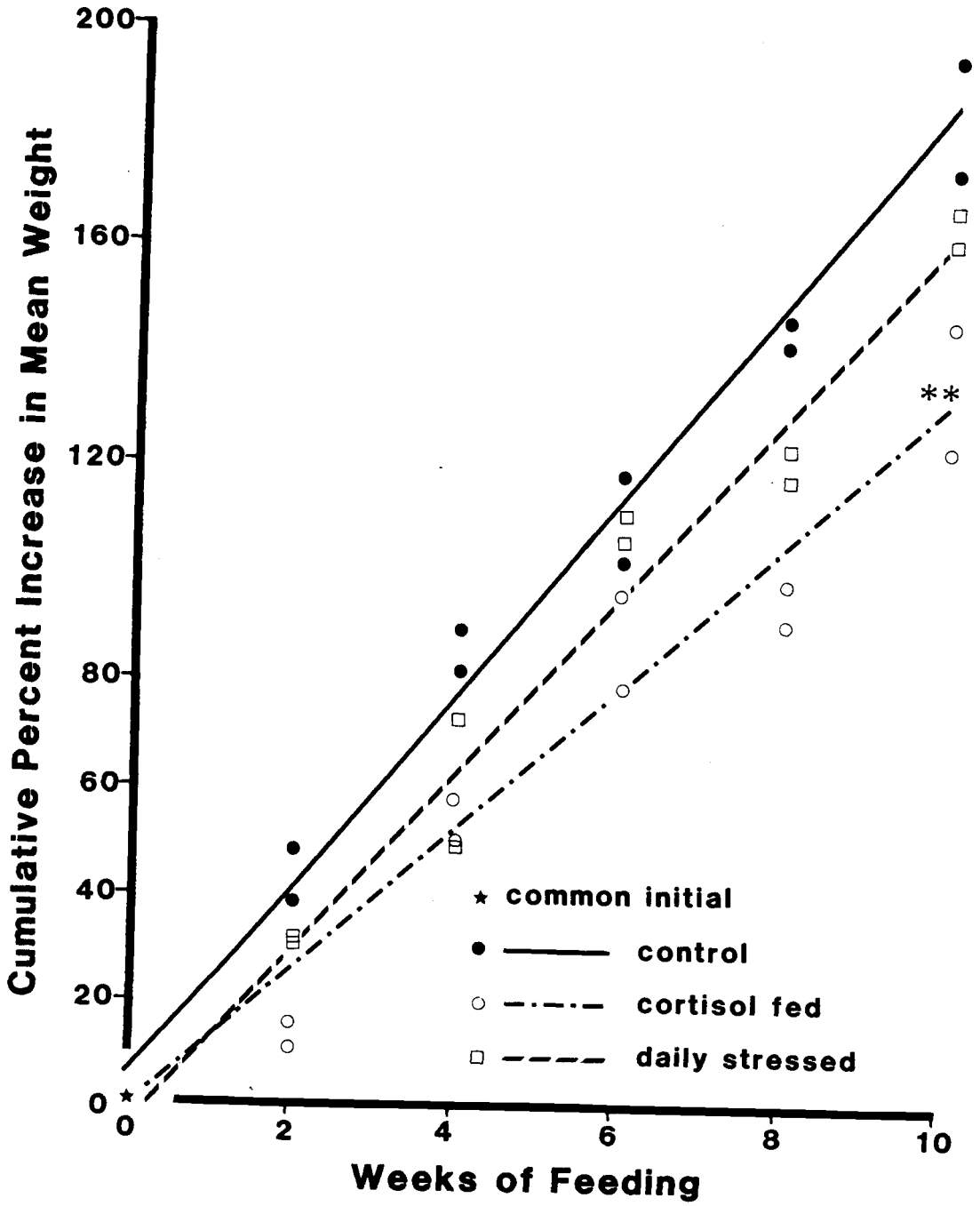

200
160
120
80
60
40
20
0
0
2
4
6
8
10
Cumulative Percent Increase in Mean Weight
Weeks of Feeding
**
★ common initial
● —— control
○ -·-·- cortisol fed
□ – – – daily stressed

Figure VII.1

Table VII.1. Specific growth rates (SGR) at 2-wk intervals and mean initial and final weights (g ±SE, N=30) of juvenile rainbow trout fed with either cortisol-treated or control feed, or normally fed and subjected to a daily acute stress, for 10 wk. Data represent pooled values from duplicate tanks per treatment.

	Treatment		
	Control	Cortisol Fed	Daily Stressed
Week 2:	2.52	1.36	1.59
Week 4:	2.59	2.02	2.32
Week 6:	1.59	1.98	2.63
Week 8:	2.20	0.49	0.89
Week 10:	2.40	2.31	2.60
SGR for 10-wk Period:	2.26	1.65	2.02
Initial Weight:	4.5 ±0.2	4.3 ±0.2	5.2 ±0.2
Final Weight:	21.4 ±0.7	13.4 ±0.5	21.0 ±1.0

Figure VII.2. Mean liver glycogen (mg/g ±SE, N=9-12), hepatosomatic index (±SE, N=12) and condition factor (±SE, N=12) in juvenile rainbow trout fed with either cortisol-treated or control feed, or normally fed and subjected to a daily acute stress, after 10 wk of treatment. Values represent pooled data from duplicate tanks per treatment [* indicates significant difference ($P<0.05$) from control].

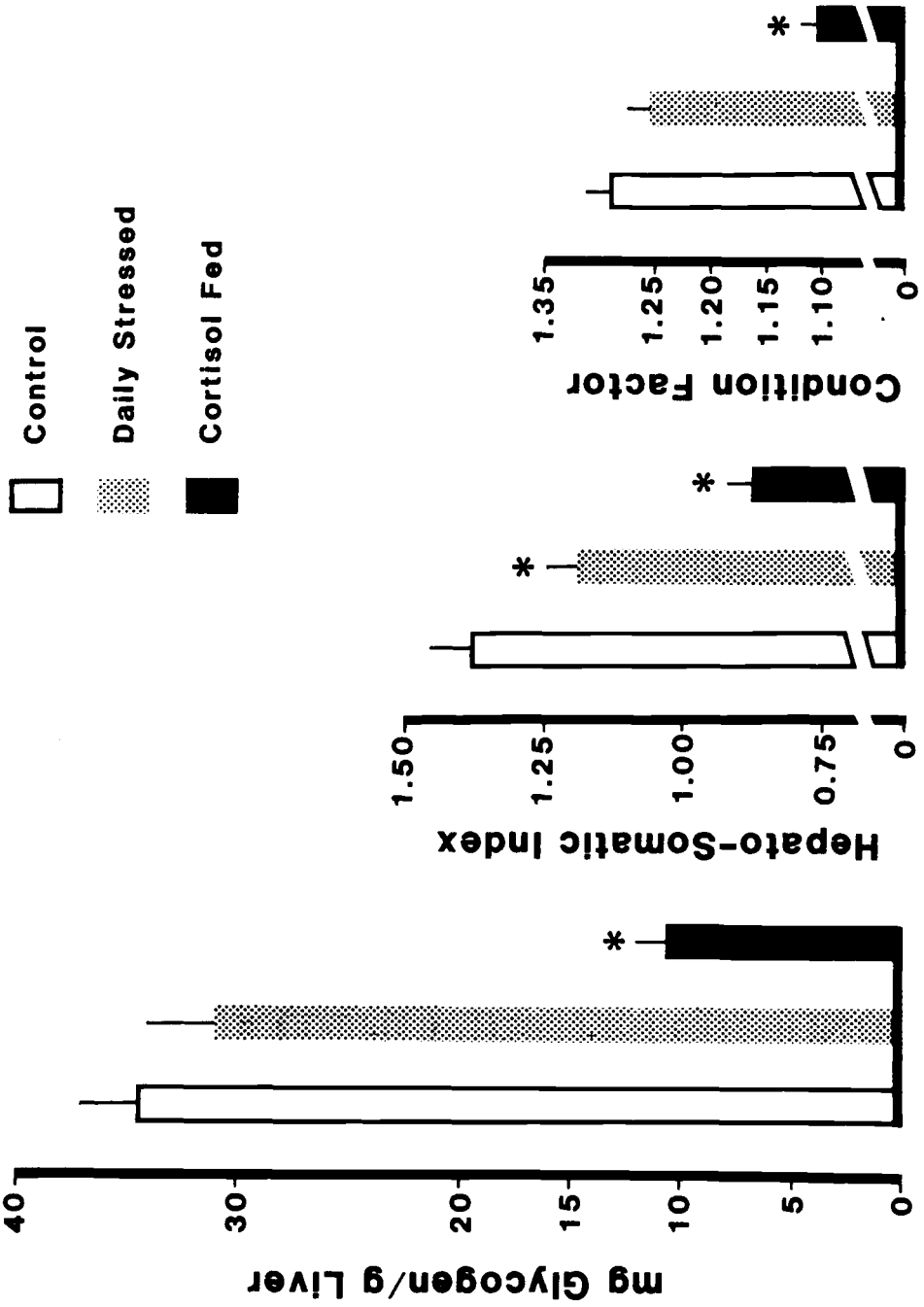

Control
Daily Stressed
Cortisol Fed
mg Glycogen/g Liver
40
30
20
15
10
5
0
Hepato-Somatic Index
1.50
1.25
1.00
0.75
0
Condition Factor
1.35
1.25
1.20
1.15
1.10
0
*

Figure VII.2

loss in juvenile rainbow trout and Pickering and Duston (1983) observed that both orally administered and implanted cortisol at physiological levels significantly reduced the condition factor of brown trout, Salmo trutta.

As Davis et al. (1985) pointed out, continuous stress may result in reduced growth by chronically elevating plasma cortisol and thereby shifting metabolism towards protein catabolism. Pickering and Stewart (1984) found that chronically crowded brown trout grew less than uncrowded controls fed the same ration. However, plasma cortisol initially rose but had returned to the level of the control group by 39 d in their experiment. They concluded that long-term growth suppression was not mediated by corticosteroids, but may have been caused by reduced food intake or decreased efficiency in food utilization relating to social interactions from crowding. For example, Strange et al. (1978) noted that juvenile chinook salmon, Oncorhynchus tshawytscha, stressed by high density confinement, refused food and, subsequently, grew less.

After 10 wk, liver glycogen and hepatosomatic index were much reduced in the cortisol-fed fish (Fig. VII.2); hepatosomatic index in daily stressed fish was also lower than in control fish. The large reduction in liver glycogen resulting from cortisol feeding is unusual in light of earlier investigations. Those studies reported increases in liver glycogen in fish after cortisol treatment (Butler 1968; Hill and Fromm 1968; Swallow and Fleming 1970; Inui and Yokote 1975; Lidman et al. 1979) or in fish with elevated endogenous plasma cortisol (Schmidt and Idler 1962). Other studies have indicated, however, that cortisol treatment can reduce liver glycogen in fish (Storer 1967; Ball and Hawkins 1976) or that there is an inverse relationship between endogenous plasma cortisol and liver glycogen (Peters et al. 1980; Paxton et al. 1984). Conflicting reports in the literature suggest that there are differences in cortisol-treatment effects on carbohydrate metabolism that may be due to physiological status of the fish,

dosages of cortisol used, and interactions of cortisol with the other glucoregulatory hormones. It is also possible that the low liver glycogen observed in the cortisol-fed fish resulted from a chronically increased metabolic rate, since cortisol injections have been found to increase metabolic rate in fish (Chan and Woo 1978) and there is a significant correlation between standard metabolic rate and blood sugar levels in vertebrates generally (Umminger 1977). An increased metabolic rate could also have contributed to the reduced growth in our cortisol-fed fish by shifting energy away from growth pathways (see Brett and Groves 1979).

As an alternative explanation for the reduced growth in our experimental fish, chronic cortisol administration possibly affected digestion efficiency at the gut wall rather than through biochemical action on metabolism. We observed a loss of appetite and aggressive feeding behavior in the cortisol-fed fish, even though all feed pellets presented to the fish were eaten. However, there was a noticeably higher accumulation of fecal matter at the bottom of the tanks of cortisol-fed fish, as compared with control tanks, suggesting poorer absorption of the food material in the gut. We also noticed qualitative differences in the cardiac stomach tissue between control and cortisol-fed fish (Fig. VII.3), characterized by apparent increases in gastric gland cell numbers and vacuolar spaces, and reductions in height of mucosal folds. However, a limited sample size precluded concluding that these changes were representative of all fish in the treated population and we were unable to resolve differences at the cellular level. Our observations of morphological alterations to cardiac stomach tissues in cortisol-fed trout are consistent with what previous authors have shown in fish with chronically elevated cortisol. In rainbow trout implanted with cortisol pellets, Robertson et al. (1963) reported necrosis of gastric glands and submucosa as well as a reduction in the number and height of mucosal folds. Similar cellular and tissue degeneration was found by Peters (1982) and

Figure VII.3. Cross-sections through cardiac stomach tissue of similarly sized control (A) and cortisol-fed (B) juvenile rainbow trout after 10 wk of treatment showing apparent reduction of mucosal folds and increases in gastric gland cell numbers and vacuolar spaces (magnification: 185X).

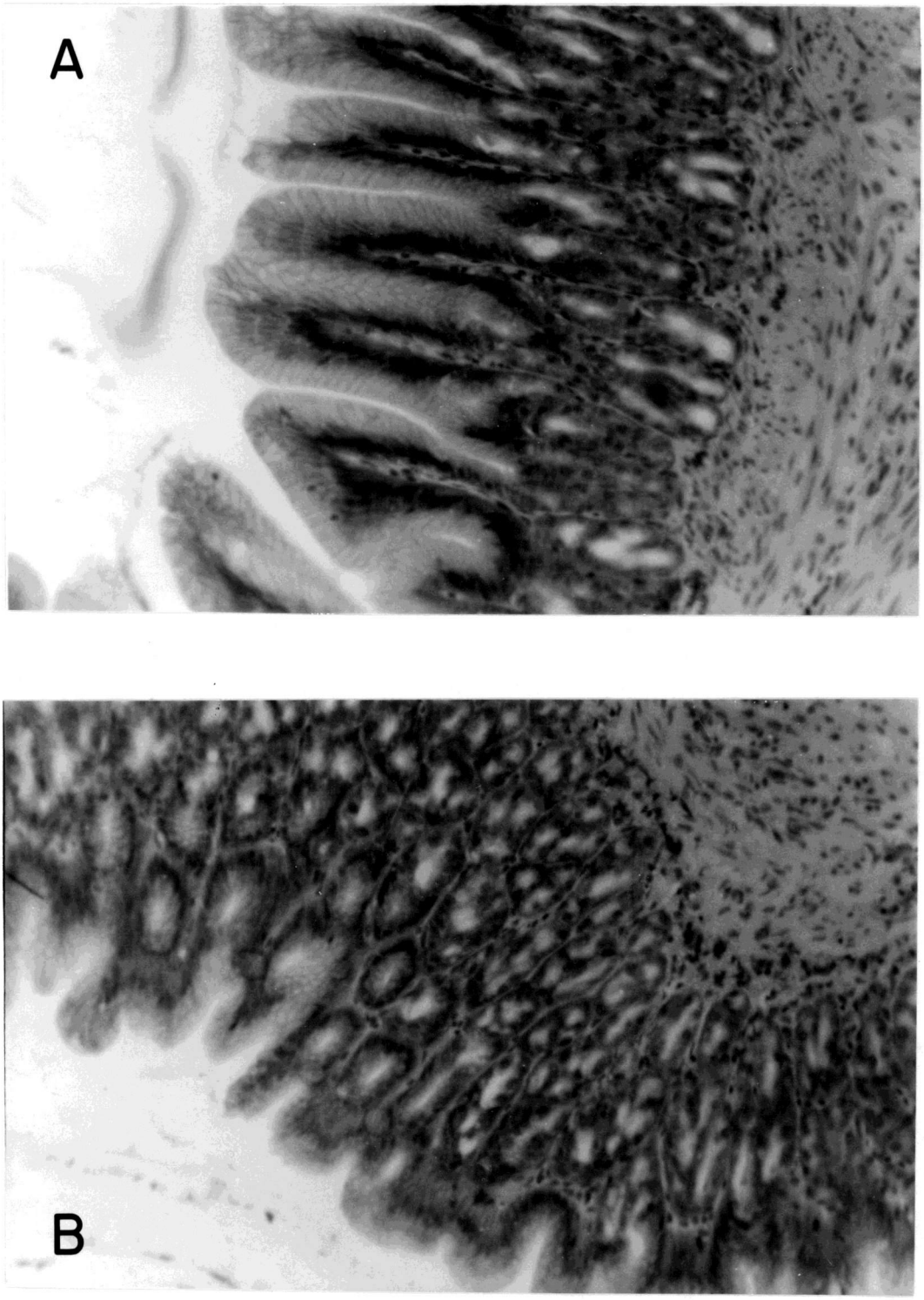


Figure VII.3

Willemse et al. (1984) in stomachs of European eels, Anguilla anguilla, under conditions of chronic stress. Peters (1982) further speculated that the resultant alterations in enzyme and acid secretion in the mucosa could interfere with normal protein digestion, and thus lead to reduced growth.

There was a noticeable increase through time in the levels of plasma cortisol in cortisol-fed fish at 1 h and 3 h post-feeding after 4 and 8 wk of treatment as compared to week 0 (Fig. VII.4). Fish stressed daily had lower 1-h post-stress plasma cortisol levels after 4 and 8 wk of treatment than at 0 wk, whereas plasma cortisol in control fish remained relatively unchanged as a result of feeding (except at 3 h in the week-8 group) (Fig. VII.4). The trend in higher cortisol titers at 1 and 3 h post-feeding in cortisol-treated fish was also evident at week 10 when compared to corresponding plasma levels in fish that had been previously unfed with cortisol (Fig. VII.5). We speculate that chronic cortisol treatment resulted in increased rate of absorption after feeding by altering physical absorption characteristics across the gut wall, perhaps through a pharmacological action. Cortisol is lipophilic and, as such, should pass relatively easily across the lipid matrix of cellular membranes. Cortisol-induced cellular degradation might enhance the rate of uptake of simple fat-soluble substances, such as cortisol, by increasing the proximity of the absorptive area to blood vessels. Cortisol levels were the same, however, at 6 and 12 h after feeding and were lower in the 10-wk cortisol-fed fish than in the control group after 24 h (Fig. VII.5), suggesting more rapid clearance in the treated fish. Chronically elevated plasma cortisol, administered using capsule implants, has been shown previously to increase clearance rate of corticosteroids in coho salmon (Redding et al. 1984a). Of interest was the straight line formed by declining post-feeding cortisol titers in the fish that were fed cortisol for 10 wk (Fig. VII.5) since, under normal conditions, clearance rate is non-linear (e.g. Nichols and Weisbart 1985; Nichols et al. 1985) and decreases with the concentration.

Figure VII.4. Mean plasma cortisol (ng/mL ±SE, N=10) in juvenile rainbow trout before (0 h) and after (1 and 3 h) feeding with cortisol-treated or control feed, and in daily stressed trout before and after a 30-s handling stress, at 0, 4 and 8 wk of treatment. Values represent pooled data from duplicate tanks per treatment [superscripts indicate significant differences ($P<0.05$) as follows: a/different from 0 h (initial) following treatment for that week, b/different from week 0 for that hour, c/different from weeks 0 and 4 for that hour].

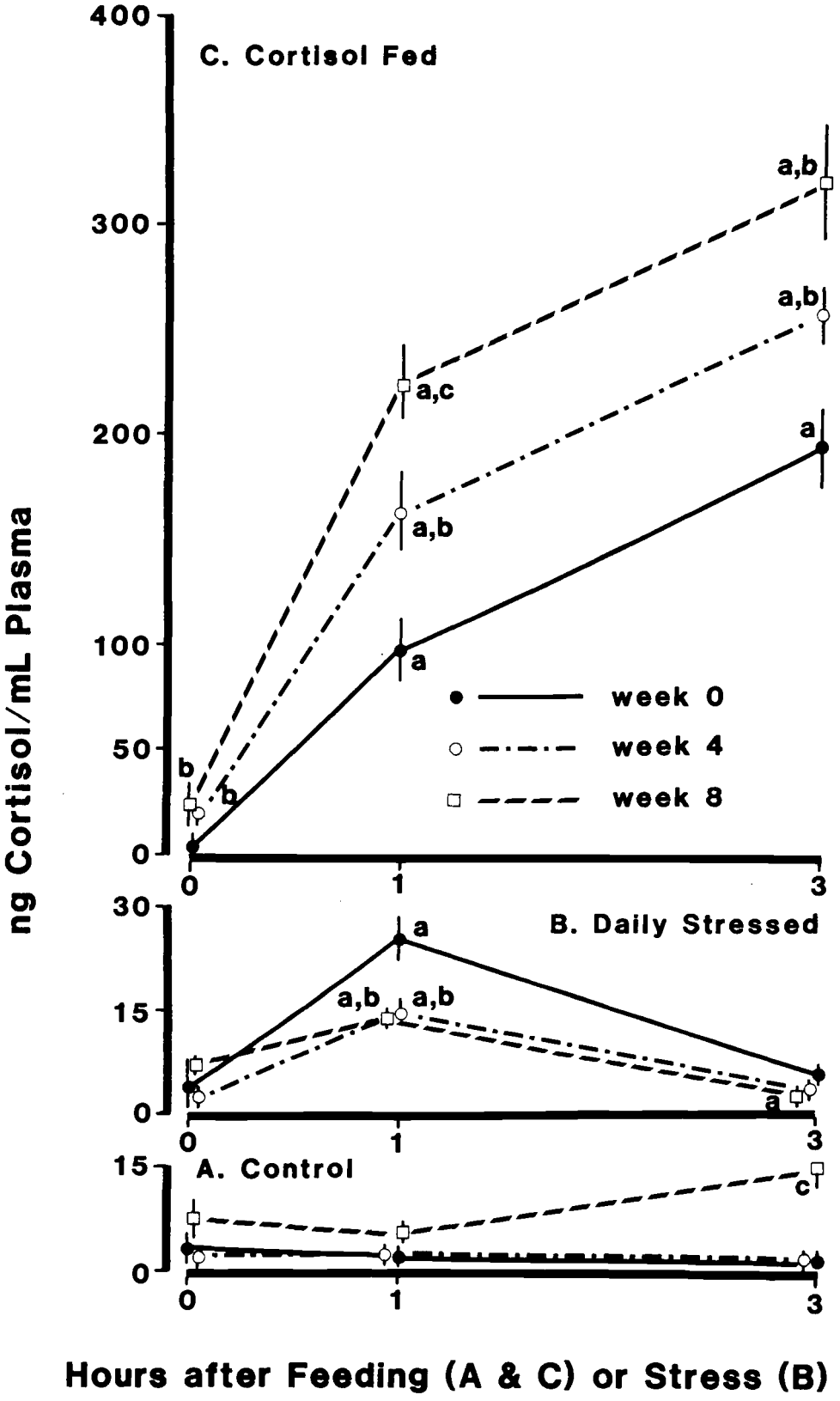

C. Cortisol Fed
B. Daily Stressed
A. Control
ng Cortisol/mL Plasma
Hours after Feeding (A & C) or Stress (B)
week 0
week 4
week 8
400
300
200
100
50
0
30
15
0
1
3
a,b
a,c
a
b
c

Figure VII.4

Figure VII.5. Mean plasma cortisol (ng/mL ±SE, $\underline{N}$=10) following a single meal of cortisol-treated feed in juvenile rainbow trout previously fed with either cortisol-treated or control feed for 10 wk. Values represent pooled data from duplicate tanks per treatment [superscripts indicate significant differences ($\underline{P}$<0.05) as follows: ^{a}/different from 0 h (initial) within that treatment, ^{b}/different from value for fish previously unfed with cortisol for that hour].

Figure VII.5

300
200
100
50
0
ng Cortisol/mL Plasma
previously unfed with cortisol
cortisol fed for 10 wk
a,b
a,b
a
a
a
a
a
a
b
a,b
0
1
3
6
12
24
Hours after Cortisol Feeding

Plasma glucose was elevated in cortisol-fed fish after 4 and 8 wk of treatment both before (i.e. 24 h after last feeding) and after feeding (Fig. VII.6). Glucose levels at weeks 0, 4 and 8 were similar to each other and exhibited the same 1-h post-stress increase (Fig. VII.6). Post-feeding plasma glucose in control fish remained relatively stable at 4 and 8 wk, but was lower at 0 h in the week-0 group than in later weeks and exhibited an increase at 3 h (Fig. VII.6). The glucose increases observed after feeding at week 0 were similar in control and cortisol-fed fish (Fig. VII.6). Plasma glucose in fish fed cortisol for 10 wk was characterized by a more rapid increase followed by a more rapid decline in concentration than what was found for control fish given the same relative cortisol-treated ration (Fig. VII.7). Judging from post-feeding levels at 1 and 3 h (A, Fig. VII.6) and at 6 h (prestress values - B, Fig. VII.6), feeding alone did not appear to alter plasma glucose appreciably.

Increased resting plasma glucose in cortisol-fed fish after 2 wk is consistent with previous investigations (Butler 1968; Patent 1970; Inui and Yokote 1975; Lidman et al. 1979; Leach and Taylor 1982). Many of the earlier studies applied high doses of cortisol by injection, thereby introducing the possibility that hepatic glycogenolysis, stimulated by stress-induced catecholamine secretion (Nakano and Tomlinson 1967; Mazeaud et al. 1977), may have also modified plasma glucose. An increase in plasma glucose following feeding with relatively low levels of exogenous cortisol was not observed by Davis et al. (1985). Our findings of higher resting plasma glucose with chronic cortisol feeding and the apparently cortisol-related transient change in glucose following a single cortisol meal both support its glucocorticoid role in teleosts and supports Davis et al.'s (1985) earlier conclusion that increased gluconeogenesis resulted in reduced growth. Moreover, our results clearly showed that dietary cortisol appeared in circulation at physiological levels relatively quickly after feeding. The concentrations of plasma cortisol we observed at 1 to

Figure VII.6. Mean plasma glucose (mg/dL ±SE, N=8-10) in juvenile rainbow trout before (0 h) and after (1 and 3 h) feeding with cortisol-treated or control feed, and in daily stressed trout before and after a 30-s handling stress, at 0, 4 and 8 wk of treatment. Values represent pooled data from duplicate tanks per treatment [see Fig. VII.4 for explanation of significance indicated by superscripts].

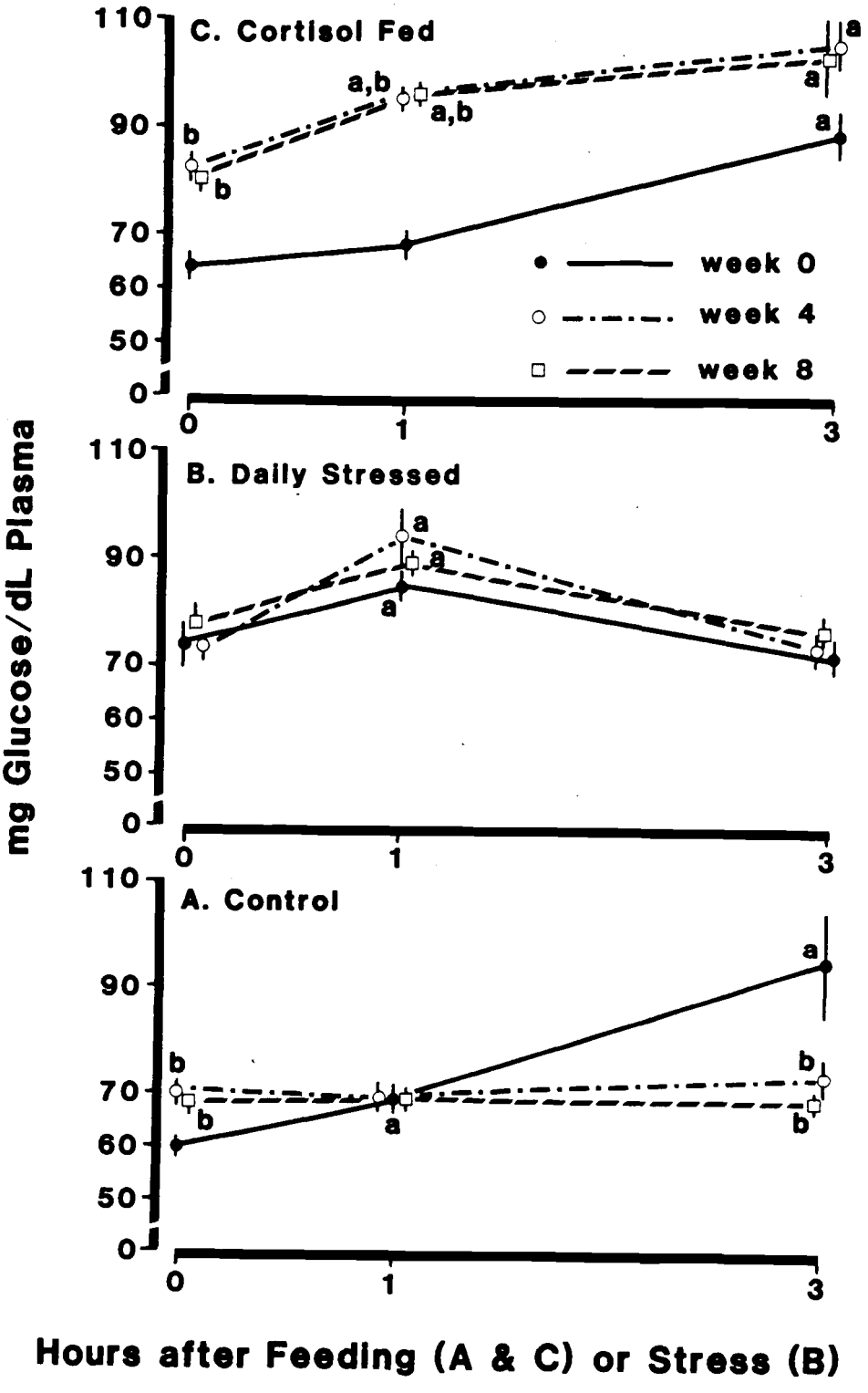

C. Cortisol Fed
B. Daily Stressed
A. Control
week 0
week 4
week 8
mg Glucose/dL Plasma
Hours after Feeding (A & C) or Stress (B)

Figure VII.6

Figure VII.7. Mean plasma glucose (mg/dL ±SE, N=10) following a single meal of cortisol-treated feed in juvenile rainbow trout previously fed with either cortisol-treated or control feed for 10 wk. Values represent pooled data from duplicate tanks per treatment [see Fig. VII.5 for explanation of significance indicated by superscripts].

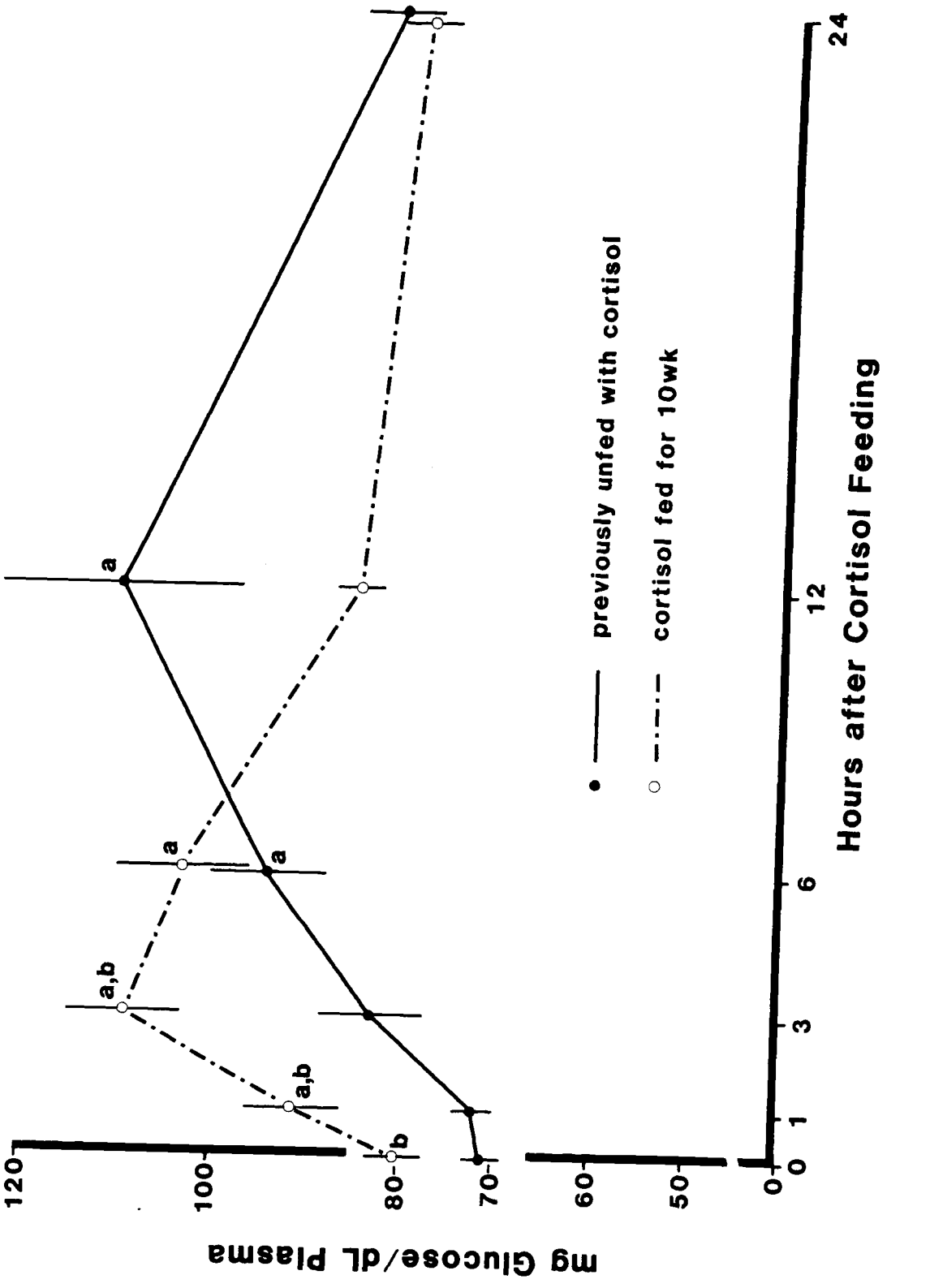

mg Glucose/dL Plasma
120
100
80
70
60
50
0
1
3
6
12
24
Hours after Cortisol Feeding
previously unfed with cortisol
cortisol fed for 10wk
a
a
a
a,b
a,b
b

Figure VII.7

6 h after feeding were similar to those found in juvenile salmonids following either a severe acute stress such as handling or chronic stress from confinement (Strange et al. 1977, 1978; Barton et al. 1980, 1985b, 1986; Strange and Schreck 1980; Pickering et al. 1982; Redding and Schreck 1983).

When compared with a representative control group from week 8, plasma insulin in the fish showed a greater increase after feeding with cortisol (Fig. VII.8). We believe that the presumed increase in plasma insulin in the cortisol-fed fish was a secondary response to the cortisol-induced elevated plasma glucose, rather than due to any direct effect of the cortisol. This is a tentative conclusion, however, since control data were not available at week 10 and resting levels of insulin in previously untreated fish at week 10 were also higher than at week 8. Various authors have shown that hyperglycemia in fish stimulates insulin secretion (Plisetskaya et al. 1976; Ince and Thorpe 1977; Ince 1979; Furuichi and Yone 1981). Although Munck et al. (1984) indicated in their review that corticosteroids may exert a suppressive action on insulin secretion in mammals (that could result in elevated plasma glucose), to our knowledge, this subject has not been studied extensively in fish.

Hematological Characteristics

After 2 and 6 wk, hematocrit was higher in cortisol-fed fish but was not appreciably altered in the daily stressed fish when compared with respective controls (Fig. VII.9). After 2 wk, lymphocyte ratios were 84% lower than control values in cortisol-fed fish and were 31% lower in daily stressed fish (Fig. VII.10). Neutrophil and thrombocyte (combined) ratios of 0 to 3 per 10^3 red blood cells (RBC) per fish were not consistently different among treatments, and means ±SE (N=12) were 1.04 ±0.29, 1.06 ±0.18 and 1.14 ±0.29 per 10^3 RBC for control, cortisol-fed and daily stressed fish,

Figure VII.8. Mean plasma insulin (ng/mL ±SE, N=5-9) following a single meal of cortisol-treated feed in juvenile rainbow trout previously fed with control feed for 10 wk. Representative control values were taken from fish fed a single meal of control feed following 8 wk of feeding with the same diet. Values represent pooled data from duplicate tanks per treatment [see Fig. VII.5 for explanation of significance indicated by superscripts].

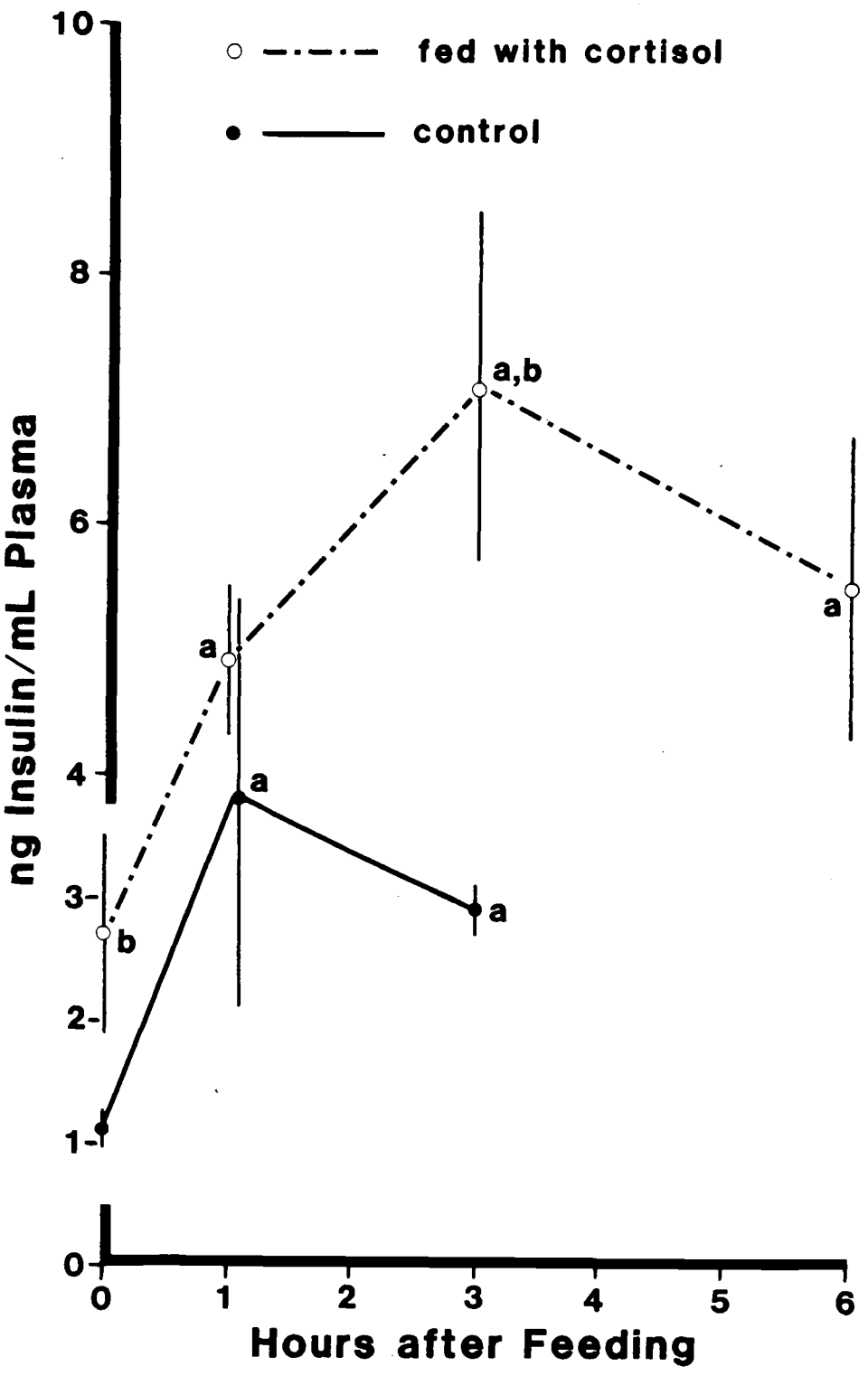

fed with cortisol
control
ng Insulin/mL Plasma
10
8
6
4
3
2
1
0
a,b
a
a
a
b
a
0
1
2
3
4
5
6
Hours after Feeding

Figure VII.8

Figure VII.9. Mean hematocrit (% packed cell volume ±SE, N=18-20) in juvenile rainbow trout fed with either cortisol-treated or control feed, or normally fed and subjected to a daily acute stress, after 2 and 6 wk of treatment. Values represent pooled data from duplicate tanks per treatment [* indicates significant difference ($P<0.05$) from control].

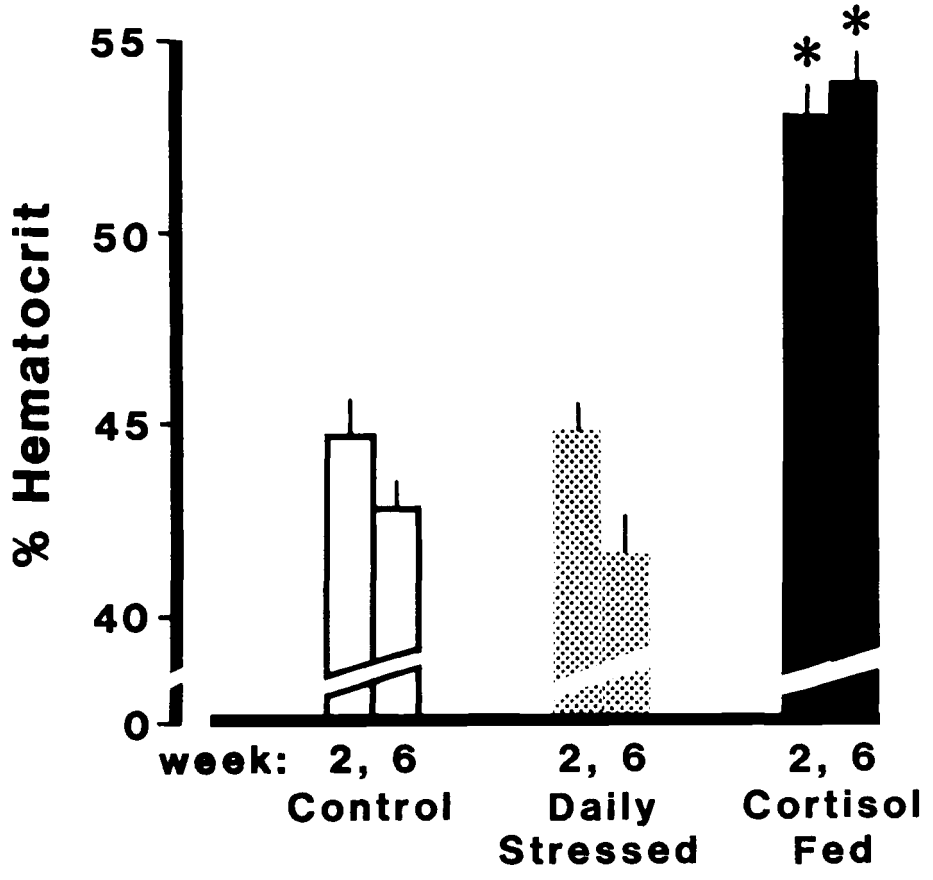

55
50
45
40
0
% Hematocrit
* *
week: 2, 6
Control
2, 6
Daily
Stressed
2, 6
Cortisol
Fed

Figure VII.9

Figure VII.10. Mean lymphocyte ratio [no. lymphocytes per 10^3 red blood cells (RBC) ±SE, N=12] and leucocrit (% of whole blood volume ±SE, N=17-19) in juvenile rainbow trout fed with either cortisol-treated or control feed, or normally fed and subjected to a daily acute stress, after 2 wk of treatment. Values represent pooled data from duplicate tanks per treatment [* indicates significant difference ($P<0.05$) from control].

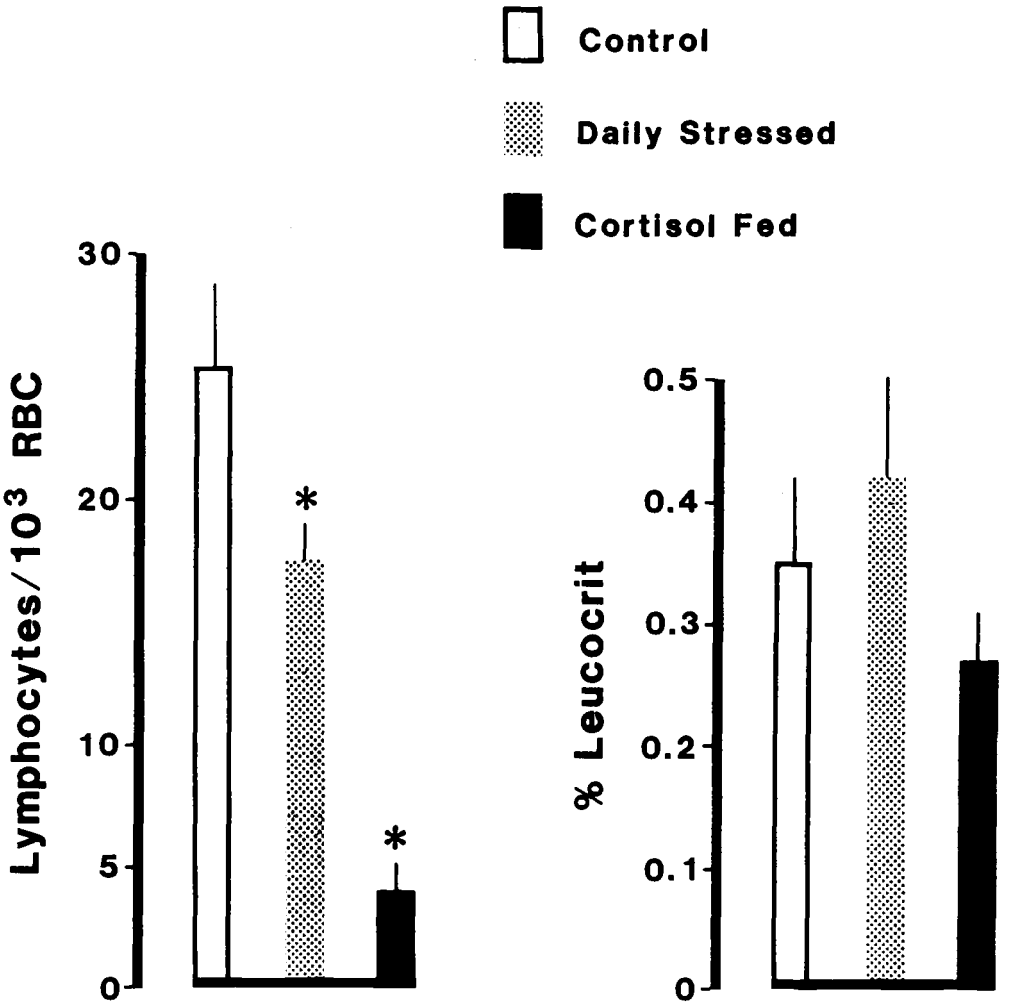


Figure VII.10

respectively. The apparent variations in leucocrit among the treatment groups were not significantly different (Fig. VII.10).

The higher hematocrit after 2 wk in the cortisol-fed fish, as compared to the untreated fish, is interesting in that this phenomenon has not been observed in fish that had plasma cortisol chronically elevated using implants (J.M. Redding and C.B. Schreck, unpublished) or that were treated with single or multiple cortisol injections (Johansson-Sjobeck et al. 1978; Leatherland 1985). An increase in hematocrit may be caused by an alteration in intra- or extracellular fluid or by proliferation in erythrocytes. Pickford et al. (1970) found that repeated injections of cortisol significantly increased the erythrocyte count in mummichog, Fundulus heteroclitus, although reasons were not given. We observed no evidence of increased hemopoietic activity in blood smears from cortisol-treated fish, that would be apparent as increased numbers of immature erythrocyte stages. This suggests that the increased hematocrit we observed possibly resulted from a cortisol-mediated decrease in extracellular fluid relative to blood cell volume. In adrenalectomized European eels, Chan et al. (1969) found that cortisol treatment increased the glomular filtration rate and urine production. Such a response from cortisol feeding could account for a reduced blood fluid volume and a resultant higher hematocrit.

A number of authors have also found that physical stress increases hematocrit values in fish (Soivio and Oikari 1976; Wells et al. 1984; Barton et al. 1985b) that may due to erythrocyte swelling accompanied by a redistribution of body fluids (Milligan and Wood 1982). However, there is no evidence to indicate that this effect is mediated by cortisol. Moreover, this phenomenon appears to be a transient response to acute disturbances since we saw no difference in hematocrit between the controls and the daily stressed fish. Similarly, Pickering and Pottinger (1985) found that a daily acute stress of exposure to malachite green had no long-term effect on hematocrit in brown trout.

The depression in numbers of circulating lymphocytes (but no change in neutrophils or thrombocytes) was caused by the dietary cortisol and confirms that which Pickering (1984) previously observed. Although there was a 22% increase in hematocrit, this alone (if due to hemopoiesis) cannot account for the over five-fold reduction in lymphocyte numbers. Pickering (1984) found that when brown trout were fed a single meal containing the equivalent amount of cortisol as we used, lymphocyte numbers were reduced by about 65% but returned to normal in 72 h. Our results suggest that lymphocyte numbers do not compensate, but, as long as an elevated cortisol titer is continually present, numbers of lymphocytes in the blood will probably remain low. This contradicts Pickford et al. (1970) who, after finding an increased white blood cell count in mummichog, concluded that the fish exhibited an adaptive response to chronic cortisol treatment. It has been previously shown that injection of cortisol into fish also reduces circulating lymphocyte numbers (McLeay 1973; Ball and Hawkins 1976) and that exogenous cortisol treatment increases the susceptibility of fish to various disease infections (Robertson et al. 1963; Roth 1972; Pickering and Duston 1983). Grimm (1985) recently found that physiological levels of cortisol suppressed the mitogen-induced proliferation of leucocytes from plaice, Pleuronectes platessa, cultured in vitro, suggesting that this may be an important part of the mechanism by which stress reduces disease resistance.

Of particular interest was the reduced numbers of blood lymphoctes in daily stressed fish after 10 wk of treatment. Various forms of stress have been demonstrated to reduce circulating lymphocyte or leucocyte numbers (Esch and Hazen 1980; Hlavek and Bulkley 1980; Peters et al. 1980; Pickering et al. 1982; Klinger et al. 1983), leucocrit (McLeay and Gordon 1977; Tomasso et al. 1983; Wedemeyer et al. 1983) and, possibly, lymphocyte function (Ellsaesser and Clem 1986). Pickering (1984) concluded that this stress-induced response is mediated by cortisol. A positive

association between environmental stress and outbreak of disease in fish is well established (Wedemeyer 1970; Snieszko 1974; Walters and Plumb 1980; Wedemeyer and Goodyear 1984). Our finding implies that the routinely handled fish may still have been less resistant to disease, even though growth and other physiological conditions appeared normal and they became accustomed to routine acute disturbances.

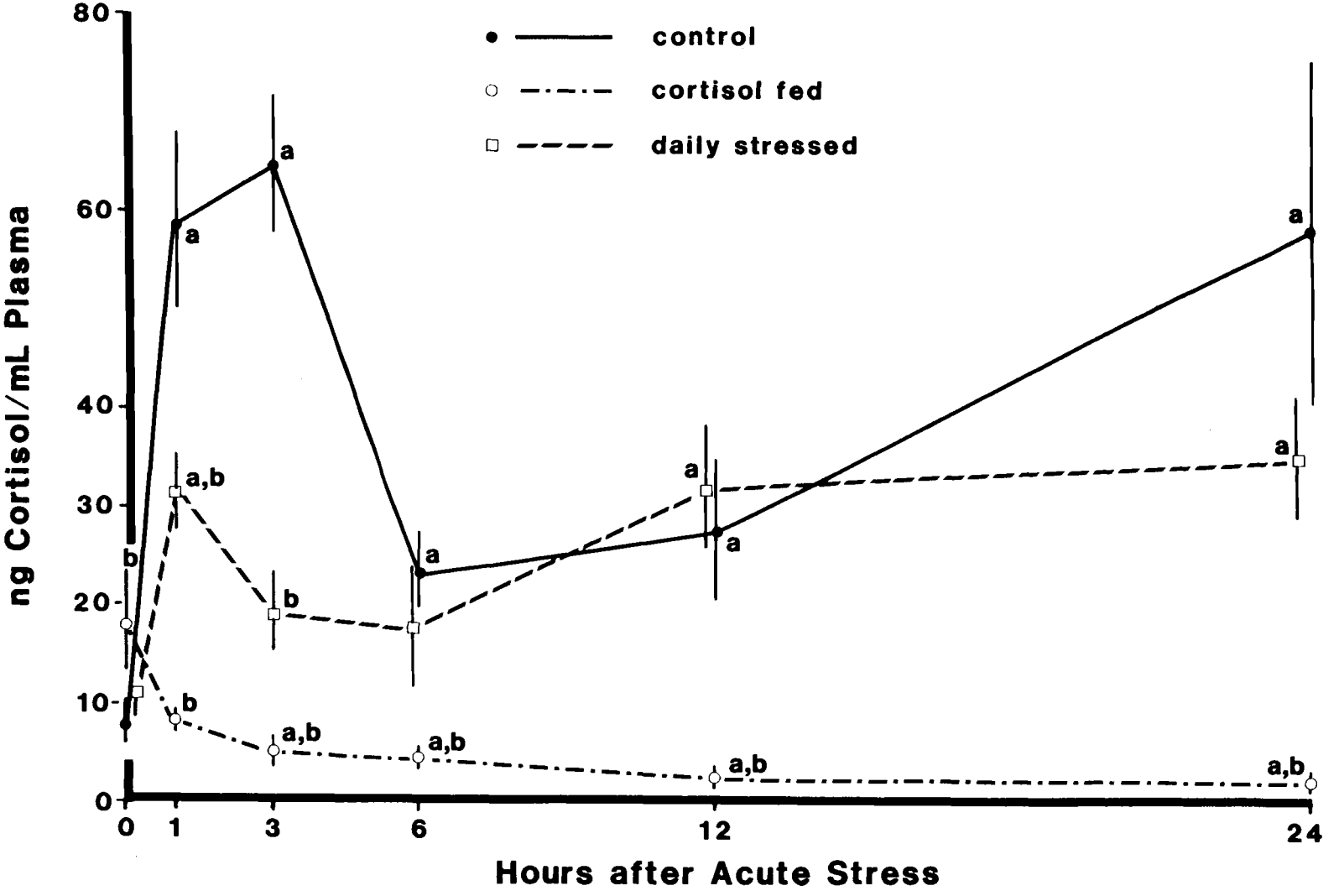
Our leucocrit values did not correlate with blood lymphocyte ratios and values were much lower than those previously reported for healthy salmonids (McLeay and Gordon 1977; Wedemeyer et al. 1983). Peters et al. (1980) found that a higher leucocrit occurred along with lower leucocyte counts following chronic stress in European eels and concluded that the increased leucocrit was caused by an increase in large granulocytes (≃ neutrophils). McLeay and Brown (1974) also reported an increase in circulating neutrophils in chronically stressed coho salmon without a long-term change in blood lymphocytes. However, the neutrophil count remained unchanged in our cortisol-fed fish which led us to conclude that leucocrit was not a suitable indicator of leucocyte depression in this experiment.

Stress Responses

When 10-wk cortisol-fed fish were subjected to the 30-s handling stress, plasma cortisol did not demonstrate any elevation in concentration as did that in the control group; from 3 h onward, post-stress cortisol levels in the cortisol-fed fish were lower than the initial (0 h) prestress level (Fig. VII.11). Plasma cortisol levels 1 and 3 h after handling were noticeably lower in the daily stressed fish than in controls (Fig. VII.11). Although both control and daily stressed fish evoked a stress response characterized by a rapid rise in plasma cortisol followed by a decline, titers in both groups rose again at 12 h and were still elevated after 24 h (Fig. VII.11).

Figure VII.11. Mean plasma cortisol (ng/mL ±SE, $\underline{N}$=12) following a 30-s handling stress in juvenile rainbow trout previously fed with either cortisol-treated or control feed, or normally fed and subjected to a daily acute stress, for 10 wk. Values represent pooled data from duplicate tanks per treatment [superscripts indicate significant differences ($\underline{P}$<0.05) as follows: $^{a/}$different from 0 h (initial) within that treatment, $^{b/}$different from control treatment for that hour].

Figure VII.11



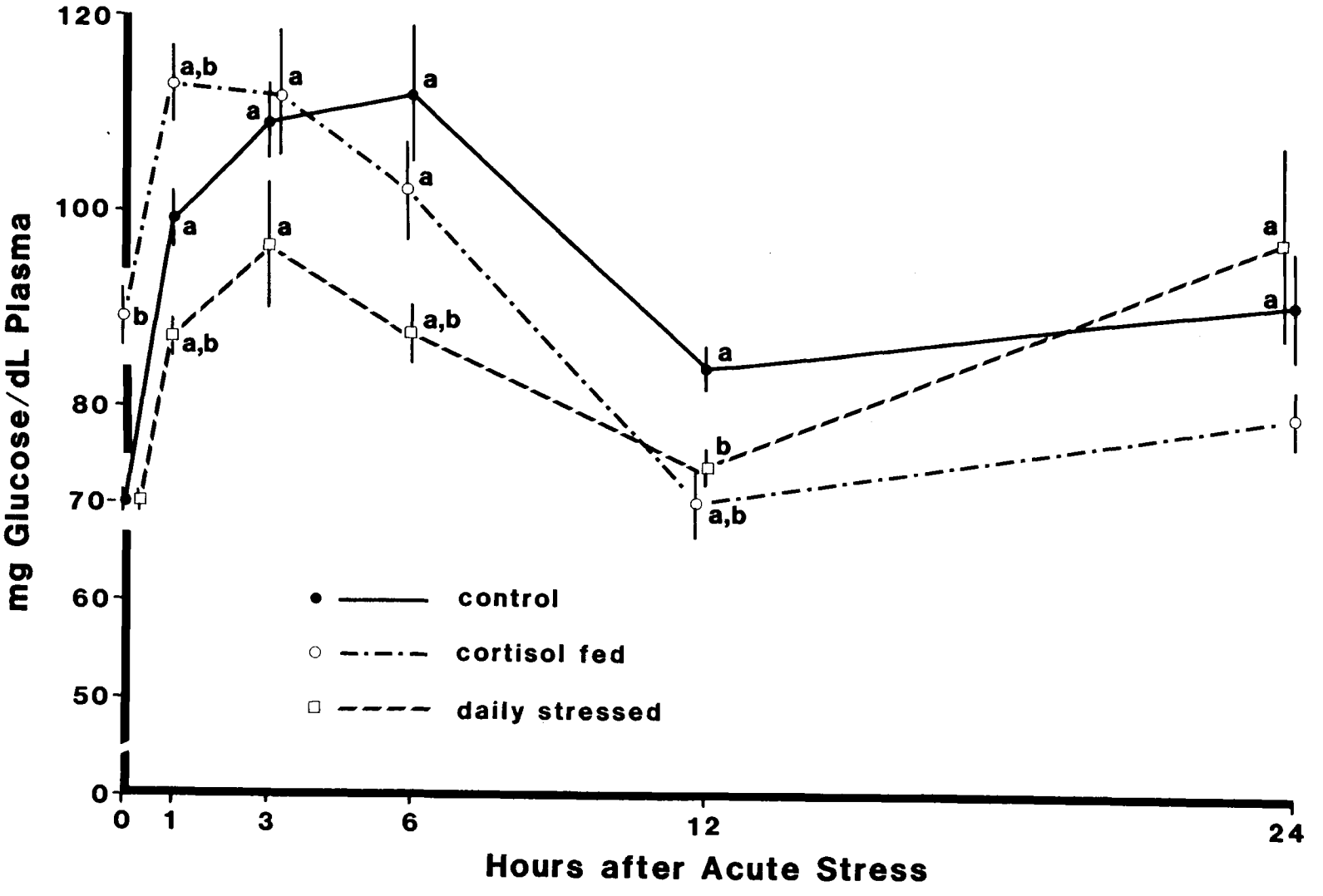
The chronic treatment with cortisol completely eliminated the ability of the fish to elicit an elevation in cortisol following handling. In fact, plasma cortisol declined following handling suggesting that residual cortisol in circulation was rapidly utilized peripherally or cleared as a result of the stress. Cortisol secretion from interrenal tissue in fish is regulated by negative feedback on the hypothalamus to inhibit synthesis or release of corticotropin-releasing factor (CRF) that, in turn, suppresses adrenocorticotropic hormone (ACTH) secretion from the pituitary (Fryer and Peter 1977). Although interrenal cells were not measured, continual negative feedback in the cortisol-fed fish may have caused an atrophy of these steroidogenic cells from the continued absence of stimulation by ACTH. Basu et al. (1965) found a dose- and time-dependent interrenal tissue atrophy was induced by cortisol injections in the cichlid, Tilapia mossambica, and those authors concluded that continued presence of the cortisol probably inhibited ACTH secretion.

Plasma glucose was elevated in response to handling in all three treatment groups (Fig. VII.12) but reached peak levels sooner in the cortisol-fed fish. However, initial (0 h) plasma glucose was highest in the cortisol-fed fish such that the relative increases in glucose were similar in cortisol-fed and control groups (Fig. VII.12). Plasma glucose 12 h after handling in the cortisol-fed group was both lower than the initial level and lower than in control fish at that time (Fig. VII.12). The response patterns of plasma glucose to handling were similar in control and daily stressed fish, but the magnitude of the elevation in the daily stressed fish was much less (Fig. VII.12).

Although resting plasma glucose was elevated, cortisol treatment did not appear to effect the hyperglycemic response to handling. Acute stress-induced plasma glucose elevations are mediated by catecholamines (Mazeaud et al. 1977; Mazeaud and Mazeaud 1981) and our finding supports the view that corticosteroids are probably not involved directly in this initial

Figure VII.12. Mean plasma glucose (mg/dL ±SE, N=11-12) following a 30-s handling stress in juvenile rainbow trout previously fed with either cortisol-treated or control feed, or normally fed and subjected to a daily acute stress, for 10 wk. Values represent pooled data from duplicate tanks per treatment [see Fig. VII.11 for explanation of significance indicated by superscripts].

Figure VII.12



response (Leach and Taylor 1980; Carmichael et al. 1984a; Barton et al. 1986).

Except for the secondary increases at 24 h, the responses to short-term handling in control fish were typical of those seen in juvenile salmonids, both for cortisol and glucose (Strange et al. 1977; Barton et al. 1980, 1985b, 1986; Pickering et al. 1982). However, the reduced plasma cortisol and glucose responses observed in the daily stressed fish clearly indicate the effect of preconditioning. A significant proportion of the normal stress response is thought to be due to the novelty of the stimulus (Levine 1985). We are unaware of other investigations on the effect of habituation on acute responses of cortisol to handling in fish, but daily exposure to malachite green eliminated the cortisol stress response in brown trout after 4 wk (Pickering and Pottinger 1985). Studies with rats conditioned to exercise indicated that the reduced corticosteroid response in conditioned animals was not from adrenal exhaustion, but due to adaptation of both the hypothalamic-pituitary axis (Frenkl et al. 1962) and the adrenal gland (Tharp and Buuck 1974). Similarly, Rees et al. (1983, 1985b) found that ducks, habituated to daily exercise stress and exercise plus handling, exhibited a reduced corticosterone response. Those authors concluded that this was caused by reduced endogenous ACTH secretion to the stimulus, since the cortisol response to exogenous ACTH was unchanged (Rees et al. 1985b).

Using a similar daily habituation protocol as we used, Rush and Umminger (1978) abolished the stress-induced hyperglycemic response in goldfish, Carassius auratus, in 3 wk. Woodward and Smith (1985) found that trained rainbow trout elicited significant hyperglycemia to a disturbance whereas untrained fish did not. They attributed this difference to a post-stress increase in epinephrine in trained fish that was not apparent in untrained fish. Their finding appears to be the opposite of what Rush and Umminger (1978) and we observed. However, in Woodward and Smith's (1985) experiment, trout were preconditioned by exercise training,

not by similar physical disturbances, suggesting the possible importance of the novelty factor in elicitation of a stress response in fish.

The maximum post-stress cortisol response was much lower in the 5-g fish at week 0 (Fig. VII.4) than it was in previously unstressed 21-g fish handled at week 10 (Fig. VII.11). A increase over time in the stress response was previously demonstrated in smolting coho salmon (Barton et al. 1985a) and may reflect an increased interrenal responsiveness to stress associated with juvenile development. Similarly, 1-h post-stress plasma glucose was lower in week-0 fish (Fig. VII.6) than in control fish stressed at week 10 (Fig. VII.12) suggesting that a change in fish's sensitivity or responsiveness to stress is a more general ontogenetic phenomenon. The subsequent elevations in both plasma cortisol and glucose 24 h after handling indicated that a delayed secondary stress response may have occurred in these fish. We have not observed this phenomenon before. An alternate and more likely explanation is that the fish may have been stressed again between 12 and 24 h without our knowledge (e.g. tanks disturbed).

Summary

In summary, we have shown that chronically elevated plasma cortisol had a profound long-term physiological effect on juvenile rainbow trout that was manifested as a reduction in growth and condition factor, circulating lymphocytes, and liver glycogen, and an increase in hematocrit and resting plasma glucose. The occurrence of changes commonly observed in chronically stressed fish that are similar to those in the cortisol-fed trout supports the view that long-term detrimental effects of stress in fish are, in large part, mediated by cortisol. The results also indicated that continuous negative feedback of cortisol on the hypothalamic-pituitary axis, possibly resulting from chronic

stress, may compromise a fish's ability to cope with additional stress factors by limiting the capacity to elicit an interrenal response to a stimulus. Fish subjected to daily handling clearly were able to habituate to the repeated acute disturbances, as evidenced by a reduction in interrenal and glycemic stress responses to handling over time. Although these daily handled fish showed no other long-term adverse physiological effects from this treatment, the reduction in circulating lymphocytes suggests that repeated routine handling of fish could lower their capacity to resist disease.

Acknowledgments

We thank C. Samuel Bradford for helping with sample collections, Erika Plisetskaya, University of Washington, for assaying plasma insulin, W. Tosh Yasutake, U.S. Fish and Wildlife Service, for examining slide mounts of stomach tissue, and the Western Fish Toxicology Station, Environmental Protection Agency, for providing the fish. Mention of trade names or manufacturers does not imply U.S. Government endorsement of commercial products.

VIII: THE METABOLIC COST OF ACUTE PHYSICAL STRESS IN JUVENILE STEELHEAD

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Abstract

A mild physical disturbance significantly increased the metabolic rate of juvenile steelhead, Salmo gairdneri, by 121%. Using a modified Blazka respirometer, steelhead were subjected to three, consecutive, mild 2-min disturbances, separated by 0.5-h intervals, and then forced to swim at 0.5 body lengths/s for 1 h. Mean (±SE) oxygen consumption in the stressed fish was 223 ±19 mg/kg/h compared with 101 ±9.7 mg/kg/h for unstressed controls given the same swimming challenge. There was no significant correlation between unit oxygen consumption and either fish weight (40-150 g) or water temperature (8-11°C) within the ranges used. Elevated plasma cortisol levels and oxygen consumption rates in stressed individuals were positively correlated (r=0.76). A simple model demonstrates how an acute physical stress may affect performance capacity in fish by reducing the energy available for other performance components within the fish's scope for activity by about one quarter.

Introduction

There is a large body of literature showing elevations in both plasma corticosteroids and glucose resulting from various types of physical disturbance (Donaldson 1981; Schreck 1981; Wedemeyer and McLeay 1981; Chapter II). These are often accompanied by changes in tissue glycogen and blood lactate, particularly if muscular activity is involved (Driedzic and Hochachka 1978). There is limited evidence to indicate that the correlation between rises in plasma cortisol and glucose in fish during stress is functional (Leach and Taylor 1980). Moreover, treatment of fish with exogenous cortisol has been shown to increase metabolic rate (Chan and Woo 1978). Since blood glucose and metabolic rate are positively correlated in vertebrates generally (Umminger 1977), this suggests that there is a metabolic cost associated with physical stress in fish that could be assessed by determining changes in metabolic rate.

One indirect method of determining metabolic rate in fish is by measuring oxygen consumption in a closed-system respirometer (Brett and Groves 1979). Many investigators of respirometry and swimming performance have long recognized the importance of a suitable adjustment period after fish are handled when loading their apparatus (Winberg 1956; Fry 1957; Beamish 1978). For example, Brett (1964) showed that restlessness or excitement in sockeye salmon, Oncorhynchus nerka, in the experimental apparatus could account for oxygen consumption rates of two to two-and-a-half times the true rate at low swimming velocities. In a study of arctic fish, Holeton (1974) found that it took about 48 h for metabolic rates to stabilize after capture and handling, and presumed that fright and oxygen debt were contributing factors to the approximately twofold increase in respiration rates. A 70% increase in oxygen consumption that took 3 to 5 h to return to routine levels after handling was reported by Saunders (1963) in starved Atlantic cod, Gadus morhua, held at 3°C.

The effect of handling and transfer on young carp, Cyprinus carpio, was an increase in oxygen consumption from 272 to about 400 mg/kg/h that took 3-4 d to recover (Korovin et al. 1982). Smit (1965) concluded from his goldfish, Carassius auratus, studies that excited fish had a much higher oxygen consumption rate than quiescent fish and emphasized the necessity of appropriate acclimation in such investigations. However, little work has been done to determine this metabolic response to a known severity of physical disturbance in the context of quantifying these stress effects on 'whole-animal' metabolism.

The objective of this experiment was to determine if there is a bioenergetic cost to fish associated with acute stress and whether this 'whole-animal' response correlates with established physiological stress indicators. This was accomplished by measuring the effect of acute physical disturbance on the metabolic rate, as represented by oxygen consumption, in juvenile steelhead, Salmo gairdneri. We considered the stress response to consist of both the sensory stimulation of neuroendocrine and physiological mechanisms and the resultant motor activity. We also compared stress-induced changes in metabolic rate with elevations in plasma cortisol and glucose. As indicators of anaerobic metabolism and osmoregulatory balance, plasma lactic acid and osmolality were also determined.

Methods and Materials

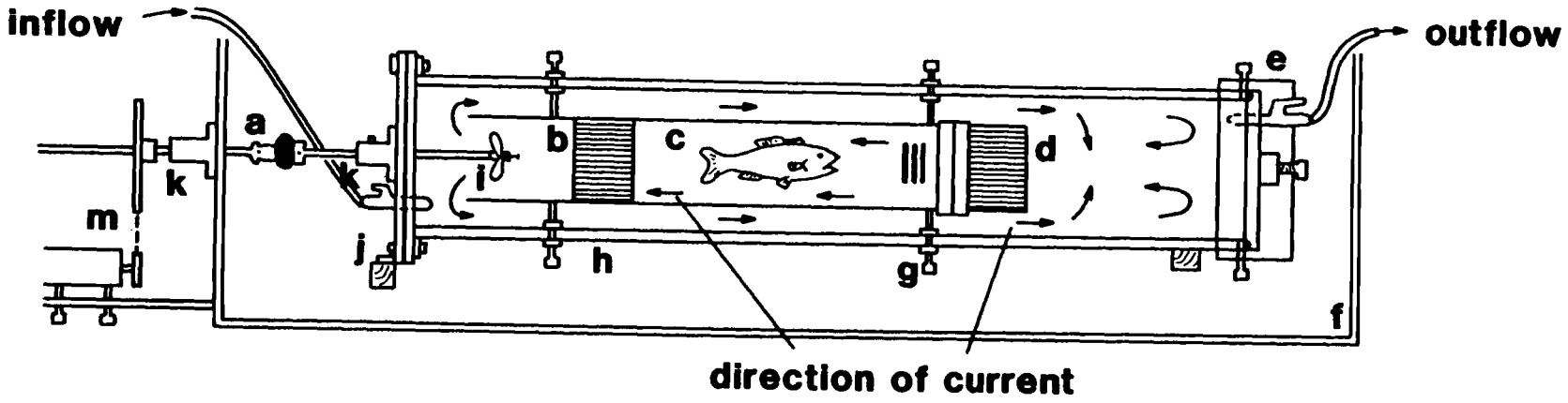
Experimental Apparatus

For the respirometry trials, two identical modified versions of the Blazka respirometer (Blazka et al. 1960) were constructed from clear acrylic tubing (Fig. VIII.1). Major modifications to Blazka's design were as follows: (1) the propeller was reversed on the driveshaft to pull, rather than push, the water

Figure VIII.1. Diagram of modified Blazka respirometer used to determine oxygen consumption of stressed and unstressed juvenile steelhead during a swimming challenge. See text for dimensions.

Figure VIII.1

CLOSED SYSTEM MODIFIED BLAZKA-TYPE FISH RESPIROMETER



a. driveshaft with detachable K-coupling

b. baffle

c. inner swimming chamber

d. removable baffle

e. removable lid

f. wooden box

g. set screw with O-ring seals

h. outer tube (acrylic)

i. 3-bladed propeller

j. hinge

k. sealed bearings

m. motor and pulley

through the swimming chamber, (2) the opposite end from the drive was extended to provide a mixing and reservoir area from which water was drawn, and (3) to produce a uniform flow, baffles were installed at both ends of the swimming chamber that consisted of 10-cm (upstream end) or 5-cm (downstream end) sections of plastic "drinking straws" filling the cross-sectional area of the inner tube and held in place with Vexar® plastic screen. The baffle at the end opposite the drive fitted into a plastic collar and was readily removable. Vertical black stripes were affixed to the sides of the upstream end of the swimming chamber to provide a visual cue for swimming orientation (Griffiths and Alderdice 1972).

Total volume of each unit was 15.5 L and the inner swimming chamber was 36 cm in length and 9.5 cm in diameter. The outer tube was 91 cm long and 15.2 cm in diameter. Each respirometer was contained within a waterproof plywood box filled with water that served as a barrier against possible air leakage or sudden changes in air temperature. The respirometers were mounted on hinges in the box and had detachable driveshafts so that they could be tilted 90° vertically for rapid loading and unloading and for stressing the fish. Fish were quickly unloaded by removing the entire inner tube, held in place with stainless steel set screws.

The current in each unit was generated by a 3-bladed bronze propeller connected to a 0.5-h.p. electric motor wired through a variable transformer. Current velocity (cm/sec) was matched with tachometer readings (rpm) by using a flowmeter (General Oceanics, Inc., Miami, Florida) suspended in an identical 'dummy' swimming chamber. A displacement correction was not made since the shape and size of the flowmeter (length 21 cm, cross-sectional area 8 cm^2) approximated those of the juvenile fish used in the trials. (Possible sublethal effects of ionic copper from the propeller were considered negligible since duration of actual respirometry tests was only 1 h.)

Respirometry Experiments

Juvenile Alsea River stock steelhead [92 ±7.3 g (mean weight ±SE)], hatched and reared at the Oregon Department of Fish and Wildlife Corvallis Research Laboratory, were transferred to 0.6-m-diameter holding tanks containing 100 L and receiving 3 L/min of flow-through aerated well water in a constant temperature laboratory at Nash Hall, Oregon State University, for at least 7 d of acclimaton. Rotational current (at surface) was about 0.1-0.2 body lengths (b.l.)/s and was just sufficient to keep tanks clean. The fish were held on a 12 L:12 D photoperiod and were fed once daily with Oregon Moist Pellets. Fish loaded into the respirometers were first quickly immobilized with 100 mg/L tricaine methanesulfonate (MS-222) and then weighed and measured. A number of trials were conducted to determine a suitable experimental protocol; the selected method was to first acclimate individual fish in the respirometer, receiving about 1.5 L/min flow-through water, without food for 3-4 d to assure a postabsorptive state (Beamish 1978), and then allow a swimming adjustment period at 0.2-0.3 b.l./s for 8 h before the trial.

The respirometry trial consisted of subjecting a fish to a swimming challenge of 0.5 b.l./s for 1 h. This mild challenge was imposed to reduce the variance in metabolic rate caused by spontaneous activity but without unduly stressing the fish. Single fish were used to eliminate the possibility of spurious results due to aggressive social interaction among fish when tested in groups (Brett and Sutherland 1965; Brett 1973). Times of day for swimming adjustment and respirometry trials were kept constant to minimize possible diurnal variation in activity due to entrainment to a feeding schedule and/or photoperiod (Brett and Groves 1979; Spieler and Noeske 1984). During acclimation and the trials, a curtain was left in place in front and upstream portions of the respirometers were partially covered with black plastic to avoid visually disturbing the fish (Kutty and Saunders 1973).

To compare stressed and unstressed fish (after acclimation and the swimming adjustment period), steelhead were subjected to the swimming challenge immediately following an acute physical disturbance, applied by standing the respirometer vertically on its hinged end and allowing the fish to struggle inside the inner chamber for 2 min. This disturbance was repeated three times at 0.5-h intervals just before the 1-h respirometry trial (Fig. VIII.2). The swimming adjustment velocity of 0.2-0.3 b.l./s was continued between the disturbances. A simultaneous trial was conducted in the second respirometer with unstressed fish as a control. Water samples were collected before each trial, flow-through water was then shut off, and, immediately after the 1-h respirometry trial, the water was turned on again and second water samples were taken for dissolved oxygen measurement. Dissolved oxygen was determined by the azide modification of the wet Winkler method (APHA 1980), using dilute titrant for greater resolution, and metabolic rate was expressed as oxygen consumption in mg/kg/h.

Fish were then quickly removed from the swimming chamber and immediately anesthetized in 200 mg/L of MS-222. The fish were killed with a sharp blow on the head and blood was collected from the caudal vasculature in ammonium-heparinized capillary tubes after severing the caudal peduncle; total time to obtain a blood sample was <2 min after initiating removal of the fish from the respirometer. To obtain representative control values, blood was also obtained from fish that had been: (1) maintained in the holding tanks with daily feeding for 10 d, (2) kept in holding tanks for at least 7 d as in (1) and then held in the respirometer for 3 d, and (3) held and acclimated as in (2) and then given the 8-h swimming adjustment.

Plasma, separated by centrifugation, was stored at -15°C for later cortisol, glucose, lactic acid and osmolality analyses. Plasma cortisol was determined by a radioimmunoassay (Foster and Dunn 1974) modified for use with

Figure VIII.2. Acclimation and experimental protocol used to determine oxygen consumption rates of stressed and unstressed juvenile steelhead during a 1-h swimming challenge at 0.5 b.l./s.

ACCLIMATION AND EXPERIMENTAL PROTOCOL

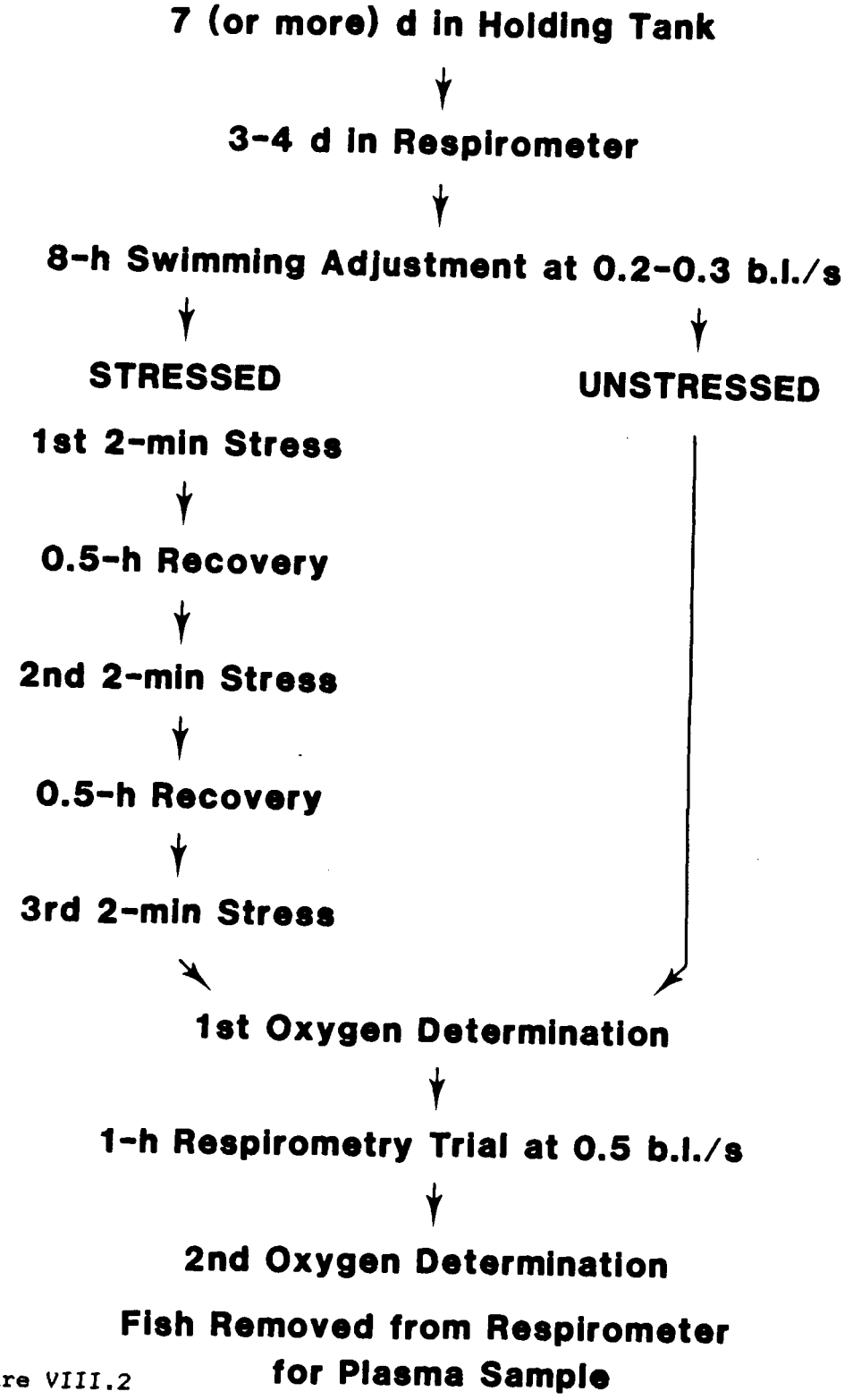


Figure VIII.2

salmonid plasma (Redding et al. 1984b). The resultant curve from serial dilutions of a plasma pool from stressed steelhead containing about 230 ng/mL exhibited parallelism with the standards curve. Recovery of cortisol from unstripped steelhead plasma was 79% when added to glass tubes containing four known cortisol concentrations in duplicate evaporated from ethanol (r=0.998); data reported are uncorrected values. Nonspecific binding of steelhead plasma was 3%. Plasma glucose was measured by the colorimetric procedure of Wedemeyer and Yasutake (1977) using premixed o-toluidine reagent (Sigma Chemical Co., St. Louis, Missouri). Additional details of cortisol and glucose assays are described in Barton et al. (1986, Chapter III). Plasma L(+)-lactic acid was assayed by fluorimetry using the method of Passonneau (1974) and plasma osmolality was determined with a Wescor Model 5100C vapor-pressure osmometer (Wescor, Inc., Logan, Utah).

Mean temperature was 9.9°C and ranged from 8.0 to 11.0°C during the trials. The lowest oxygen level recorded was 74% saturation so constant oxygen consumption rates were assumed (Beamish 1964b). Since there was no correlation between oxygen consumption and fish weight or temperature, as determined by multiple regression (see Results), mean consumption rates were compared by Student's t-test after confirming homogeneity of variance (Bartlett's test, Snedecor and Cochrane 1967) and similarity of mean weights of the two groups. Plasma constituent values were treated by one-way analysis of variance followed with Duncan's new multiple-range tests at the 5% level of significance (Steel and Torrie 1980); cortisol, glucose and lactic acid data were first converted to logarithmic values because of variance heterogeneity (Bartlett's test).

Results and Discussion

The mean oxygen consumption rate of the stressed steelhead, as determined from ten trials, was significantly higher ($P<0.01$) by more than twice that found in unstressed fish (Fig. VIII.3A). Although there was a trend in both groups for larger fish to have lower rates of oxygen consumption (Fig. VIII.3B), these relationships were not significant (unstressed: $r=0.14$, stressed: $r=0.35$). There was no correlation between oxygen consumption of either group and temperature. Both size and temperature have profound effects on the metabolic rate of fish (Brett and Groves 1979); increased size reduces unit oxygen consumption whereas elevated temperatures increase it. In this investigation, neither the size range (40-150 g) nor the temperature range (8-11°C) were apparently large enough for the influences of these two factors to be effective.

The more than twofold increase in oxygen consumption clearly indicates that the physical disturbance elevated the metabolic rate in juvenile steelhead and supports the observations of increased metabolic rates following capture and handling in earlier respirometry studies. The results also confirm that this increased level of metabolic activity was due specifically to the handling stress and not to some other factor, such as response to the swimming challenge, the experimental environment or nutritional state. The oxygen consumption rate in steelhead at 0.5 b.l./s was slightly higher than the standard consumption rates for juvenile salmonid fish of similar size and at a similar temperature and was expected with the mild swimming challenge imposed. Beamish (1964a) reported standard oxygen consumptions at 10°C of 81 and 79 mg/kg/h for 100-g brown trout, *Salmo trutta*, and brook trout, *Salvelinus fontinalis*, respectively. Holeton (1973) summarized standard oxygen consumptions, extrapolated for 100-g fish, for five salmonid species from ten investigations and found that this rate ranged from about 30 to 80 mg/kg/h at 10°C.

Figure VIII.3. A. Mean (±SE) oxygen consumption (mg/kg/h, N=10) during a 1-h swimming challenge at 0.5 b.l./s of unstressed juvenile steelhead and of those that were first subjected to three 2-min disturbances (stressed) separated by 0.5-h intervals (Fig. VIII.2) [** indicates sigificant difference from unstressed fish ($P<0.01$)]. B. Individual oxygen consumption values (mg/kg/h) and linear regression lines comparing unit oxygen consumption with fish weight for stressed and unstressed fish (regression coefficients not significant at $P=0.05$).

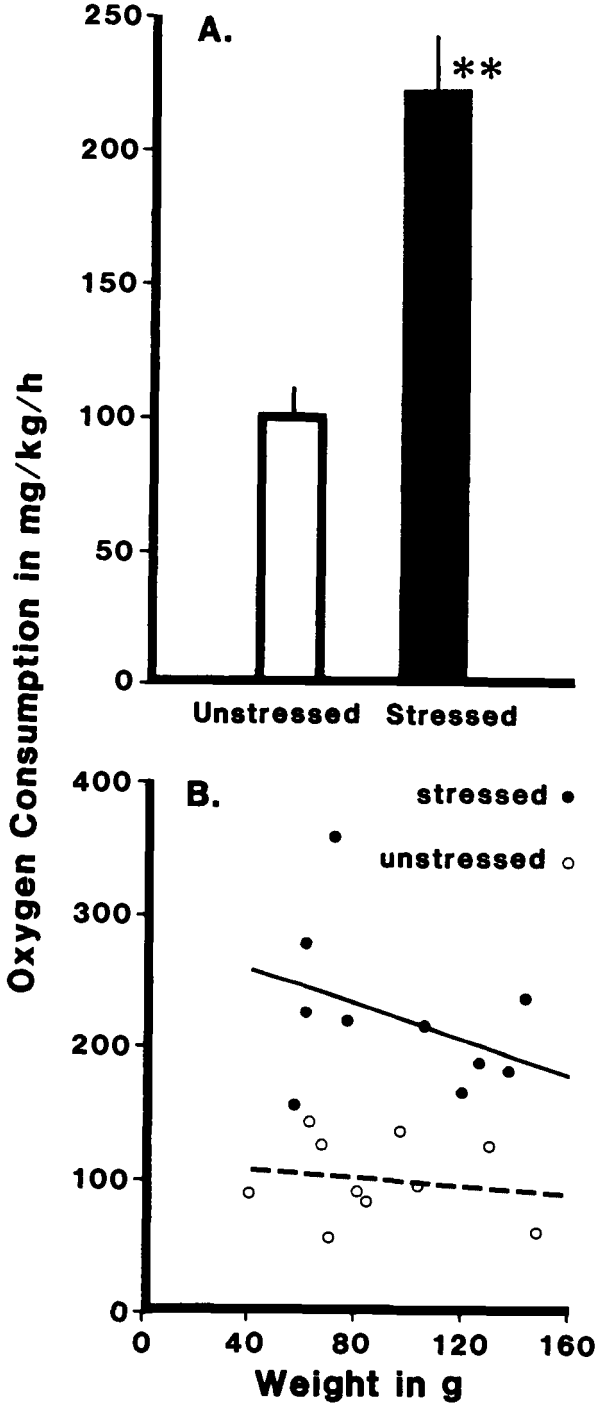


Figure VIII.3

A mean metabolic rate (N=57) of 89 mg O_2/kg/h for a number of species, mostly from temperate climatic zones, was extrapolated by Brett and Groves (1979) from data in Altman and Ditmar (1974, cited in Brett and Groves 1979).

Fish have a performance capacity, fixed genetically and by the environment (Schreck 1981), that is considered bioenergetically as their scope for activity, defined as the difference between the active (maximum) and standard (minimum) metabolic rates (Fry 1947, 1971). At 10°C, the standard metabolic rate represents approximately 10% of the active rate, estimated from Brett and Groves' (1979) summary for 100-g sockeye salmon. Thus, the scope for activity -- the energy available for performance -- is about 90% of a juvenile salmonid fish's energy budget. The difference of 122 mg/kg/h in oxygen consumption between stressed and unstressed steelhead in this experiment reflects the additional energy required by the fish to cope with the stress.

Rao (1971) extrapolated scope for activity of a 100-g rainbow trout, Salmo gairdneri, in fresh water and found it to be about 300 and 500 mg O_2/kg/h at 5 and 15°C, respectively. At 10°C, scope for activity based on Rao's (1971) calculations would be expected to be about 400-450 mg O_2/kg/h. This is similar to the experimentally determined scope for activity of 426-438 mg O_2/kg/h reported by Dickson and Kramer (1971) for hatchery and wild rainbow trout of variable weight at 10°C and is slightly less than the scope for activity of about 500 mg O_2/kg/h at 10°C estimated for 50-g sockeye salmon (Brett 1964). If results of Dickson and Kramer (1971) and Rao (1971) are representative of rainbow trout at that temperature, then we postulate that the metabolic cost required to compensate for the stress represents about one quarter of the fish's scope for activity, or energy budget beyond that required for standard metabolism. (This is based on our steelhead data from 8-11°C; mean 9.9°C.)

Plasma cortisol was higher in the fish subjected to the

2-min disturbances than in unstressed fish and in various prestress and control groups (Table VIII.1). The stress-elevated cortisol level observed was similar to that previously observed in Salmo spp. subjected to mild or brief disturbances (Strange et al. 1977; Barton et al. 1980, 1985b; Barton and Peter 1982; Pickering et al. 1982; Chapters II, VII) and confirmed that these fish were stressed. Cortisol titer was also slightly higher in the unstressed fish subjected to the respirometry trial than in tank-held control fish (Table VIII.1). This was likely caused by the stimulus of being forced to swim at 0.5 b.l./s for 1 h. Zelnik and Goldspink (1981) found that rainbow trout, forced to swim at velocities of 1 b.l./s and higher, had elevations in plasma cortisol after the onset of swimming, with the magnitude of the increases positively related to swimming speed. Other investigators have also observed forced exercise-induced increases in plasma cortisol in salmonids (Fagerlund 1967; Hill and Fromm 1968). The low cortisol levels found in prestress and resting fish taken from the respirometer indicated that neither the acclimation and swimming adjustment protocols used nor the method of fish removal from the apparatus altered plasma cortisol, as compared with the tank-held control value (Table VIII.1).

There was a significant ($P<0.05$) positive correlation between individual plasma cortisol values and oxygen consumption rates in the stressed steelhead ($r=0.76$, $N=10$; Fig. VIII.4); no such correlation was evident in unstressed fish. This finding suggests that individual variation among fish in sensitivity or responsiveness to stress, as indicated by the magnitude of the corticosteroid increase, is reflected in a corresponding metabolic cost to the fish. We are not aware of other studies that have attempted to establish this correlation between these acute responses before and our data do not imply a causal relationship between them, but in a related investigation, Chan and Woo (1978) observed a 79% increase over control fish in the oxygen consumption rate of hypophysectomized Japanese eels, Anguilla japonica,

Table VIII.1. Mean (±SE, N=10) plasma cortisol, glucose, lactic acid and osmolality in juvenile steelhead: (1) either stressed or unstressed and then subjected to a 1-h swimming challenge, and (2) from various stages of prestress acclimation (see footnotes for explanations of experimental and acclimation protocols). Means within a column without a letter in common are significantly different (Duncan's new multiple-range test, $P<0.05$).

Treatment Group*	Cortisol ng/mL	Glucose mg/dL	Lactic Acid mg/dL	Osmolality mOsm/L
Stressed fish:	71 ±11[a]	87 ±9[a]	37 ±5[a]	316 ±4[a]
Unstressed fish:	28 ±12[b]	77 ±12[a]	14 ±1[b]	312 ±3[a]
Prestress fish:	15 ±4[b,c]	66 ±3[a]	17 ±2[b,c]	313 ±3[a]
Resting fish:	9 ±3[b,c]	66 ±2[a]	21 ±4[b,c]	316 ±4[a]
Control fish:	8 ±3[c]	73 ±3[a]	21 ±2[c]	315 ±2[a]

*Stressed: acclimated and swimming-adjusted fish given three 2-min disturbances at 0.5-h intervals and then subjected to a 0.5-b.l./s swimming challenge.

Unstressed: acclimated and swimming-adjusted fish subjected to the 0.5-b.l./s swimming challenge only.

Prestress: fish acclimated in the respirometer for 3-4 d and then given an 8-h swimming adjustment period at 0.2-0.3 b.l./s.

Resting: fish acclimated in the respirometer for 3-4 d only.

Control: fish kept in laboratory holding tanks only with daily feeding for 10 d.

Figure VIII.4. Linear regression (y=127+1.36x) of individual oxygen consumption rates on corresponding plasma cortisol concentrations of juvenile steelhead after three 2-min disturbances (stressed), separated by 0.5-h intervals, and followed with a 1-h swimming challenge at 0.5 b.l./s.

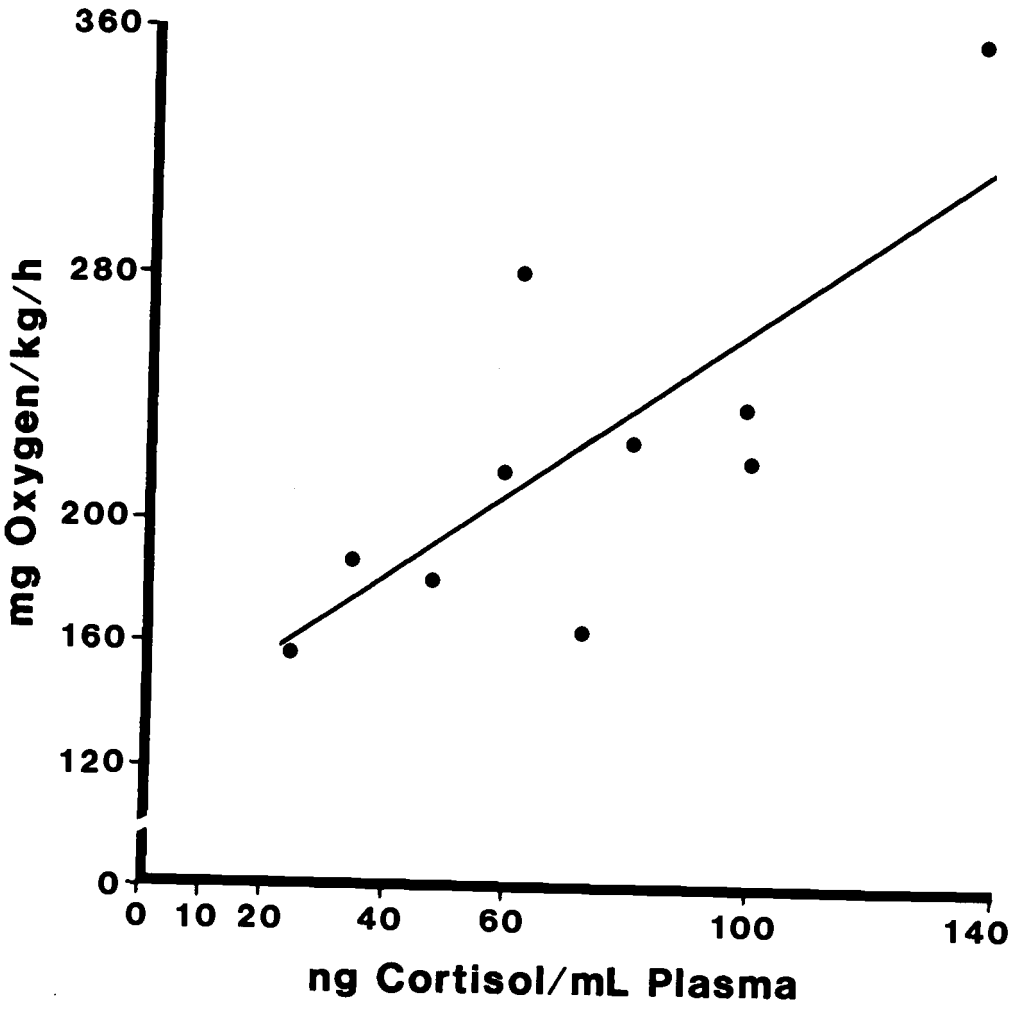


Figure VIII.4

9 h after injecting them with exogenous cortisol. Chan and Woo (1978) also indicated the possible functional relationship between cortisol and metabolism by reporting and discussing the elevations of both plasma glucose and activity of the hepatic gluconeogenic enzyme, glutamate-oxaloacetate transaminase, in the cortisol-treated eels.

Plasma glucose levels following the various treatments were not different from each other in our experiment, although there was a trend towards higher plasma glucose in the stressed fish (Table VIII.1). Also, we failed to observe a correlation between plasma glucose and metabolic rates of individually stressed fish. The lack of a larger glycemic response may be attributed to insufficient elapsed time from the onset of stress for significant hyperglycemia to appear in the samples. Based on our earlier studies (e.g. Table II.3 in Chapter II), plasma glucose appears to reach a peak in 3-6 h following this type of brief disturbance. Similarly, Pickering et al. (1982) found that a change in plasma glucose in brown trout was not evident until 4 h after a 2 min disturbance, although 2-h levels were higher than comparative control values. We also suspect that the lack of significance observed was due to the high degree of variance in plasma glucose values from stressed and unstressed fish. Both groups had similar high and low values (ranges: stressed 55-161 mg/dL, unstressed 54-165 mg/dL) that may have resulted as a differential glycemic response among individual fish to the 0.5-b.l./s swimming challenge, thus masking the stress response. [For example, if low and high values of each group are excluded, the resultant means of 81 ±3 for stressed and 69 ±6 mg/dL for unstressed fish are more different (t=1.94, df=14)].

Resting concentrations of plasma lactic acid in the various groups of unstressed steelhead (Table VIII.1) were similar, but slightly higher, to those previously reported for this species (Hille 1982) and the twofold elevation in lactic acid observed in stressed fish (Table VIII.1) is a characteristic response to

physical disturbances in fishes generally (Fraser and Beamish 1969; Soivio and Oikari 1976; Pickering et al. 1982; Schwalme and Mackay 1985a; Barton et al. 1986; and many others). There was no correlation between plasma lactic acid levels and metabolic rates of stressed fish. The increase in blood lactic acid above the resting state indicates that there was also an anaerobic metabolic response to the struggling and that a portion of the increased oxygen consumption in the stressed fish was possibly used to pay back the oxygen debt incurred (Beamish 1978). Plasma lactic acid in unstressed fish, also subjected to the 0.5-b.l./s swimming challenge, was lower than control values (Table VIII.1) suggesting that some circulating lactate may have been utilized as a glucose source or oxidized during swimming (Driedzic and Hochachka 1978).

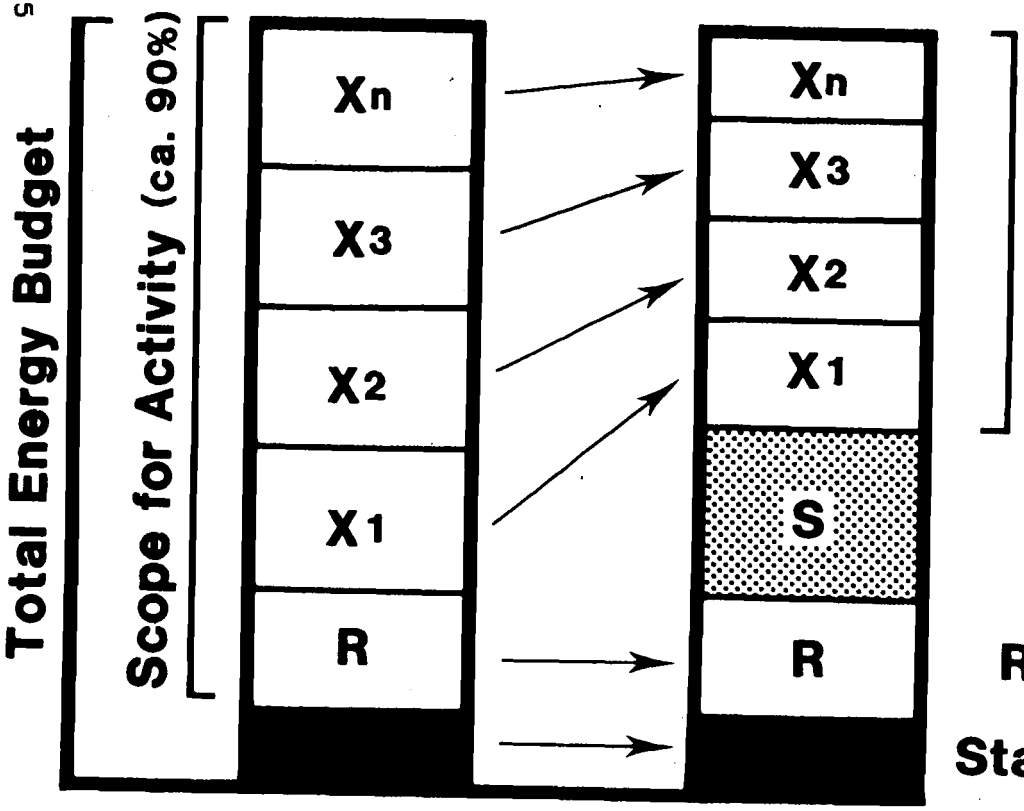
Plasma osmolality was the same in all of the treatment groups (Table VIII.1) and was similar to levels previously found in Salmo spp. in fresh water (Byrne et al. 1972; Jackson 1981). The results indicated that the disturbance used in our experiment was not severe enough to cause an osmotic imbalance in the steelhead within the time period measured.

In his discussion of smolt bioenergetics, Schreck (1982) proposed that stress is an additional energy-demanding load on the fish that would detrimentally affect subsequent performance. Present data support that notion and demonstrate that even a minor physical disturbance, like the one used, could significantly reduce a fish's scope for activity, leaving less energy available in stressed fish for some other possibly necessary performance component, such as seawater adaptation, disease resistance or swimming stamina (Fig. VIII.5). In our model, we have assumed that the 15% of the energy budget used for routine activity (Brett and Groves 1979) remains relatively constant, since a certain amount of energy above basal metabolism would presumably be required for the fish to continue to exist in its environment. This is unknown, however, and it is conceivable that routine activity may be reduced in stressed fish (e.g. stressed fish may cease feeding).

Figure VIII.5. Model based on present data and those of Brett and Groves (1979) to demonstrate how acute stress may affect fish's performance capacity by reducing energy availability for other performance components within their scope for activity.

Figure VIII.5

METABOLIC COST OF ACUTE PHYSICAL STRESS



Energy Components

Additional Performance Components

X1, X2, X3 . . . Xn

Stress (ca. 25% of SA)

Routine Activity (ca. 15%)

Standard Metabolism (ca. 10%)

In summary, we have shown that a physical disturbance elicited a more than twofold increase in the metabolic rate in juvenile steelhead. Moreover, the positive correlation between metabolic rate and plasma cortisol in stressed individuals substantiates the hypothesis that there is a metabolic cost associated with acute stress in fish and suggests that increased plasma cortisol may be a suitable quantitative indicator of the degree of stress experienced. Measurement of oxygen consumption is a direct method of evaluating the effects of stress on the bioenergetic capacity of fish and provides a basis of understanding of how other 'whole-animal' performances may be affected by stress. The nature of the stress applied in this investigation was relatively mild and is probably encountered routinely in fish hatchery and other management practices. It is therefore reasonable to assume that disturbances of greater severity or duration would impose an even greater energetic burden on scope for activity. Future research should be directed at determining the effects of increased severity and duration of the disturbance on metabolic scope and on recovery time to assess the degree of stress that fish can experience before their health or survivability is compromised.

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IX: SUMMARY

Evidence summarized from the literature (Chapter II) demonstrates how various genetic, ontogenetic and environmental factors can modify the magnitude of physiological stress responses. From the experiments conducted, it was shown that stage of development (Chapter IV), acclimation temperature (Chapter II), time of day (Chapter III), diet composition and fasting (Chapter V), exposure to low environmental acidity (Chapter VI), presence of disease infection (Chapter III) and prior experience to similar disturbance, either acutely (Chapter III) or habitually (Chapter VII), all had a major or minor effect on the elevations of plasma cortisol and/or glucose in response to handling. Furthermore, the responses to stress appeared to vary among species and stocks (Chapter II) but the influence of early rearing history cannot be discounted. The findings do not negate the value of plasma cortisol and glucose as indicators of stress. Rather, they point out the importance of being familiar with the fish's life history and contingent environmental conditions when carrying out stress investigations with fish and interpreting the findings. For example, a high glucose response to a stimulus, relative to a control group, could indicate either an increased sensitivity to stress (Chapters III and VI), suggesting that the fish is "more stressed", or a greater capacity to mobilize energy reserves in response to the stress because of nutritional state (Chapter V).

Within the context of the conceptual model presented by Schreck (1981), the ability to respond to stress may be also considered as an individual performance component. That is, the potential capacity of a fish to respond to stress is defined by its genetic makeup. The fish's realized capacity to evoke a stress response is further delineated by both its developmental stage and internal (e.g. nutritional state, health) and external (e.g. temperature, water quality) environmental conditions. The ability of fish to respond to stress is part of their adaptive capacity to

adjust to perturbations in their surroundings. However, compensation is not achieved without some metabolic cost.

In his classic paper, Fry (1947) provided a basis for description of factors affecting animal activity within its environment and defined the concept of scope for activity. Relative to Schreck's (1981) performance capacity model, stress further limits a fish's realized bioenergetic capacity by reducing the energy available within its scope for activity. Even a mild acute disturbance can reduce bioenergetic capacity by about one quarter (Chapter VIII). Other consequences of stress include depletion of glycogen reserves (Chapters II, III, V) and decreased immunocompetence through depression of circulating lymphocytes (Chapter VII). Some of these adverse consequences, such as reductions in lymphocyte numbers and somatic growth, and chronically elevated plasma glucose are probably mediated by cortisol (Chapter VII).

As our knowledge expands, it becomes increasingly clear that many important biological relationships are more complex and interrelated than earlier thought. The response of fish, or any organism, to stress is of this nature. It is not unreasonable to assume that when a fish is stressed, every physiological system is likely disturbed in some way. Figure IX.1 is an attempt to summarize the major aspects of the stress response as related specifically to changes in metabolism and to the data presented. This model represents a synthesis of previously published concepts (Mazeaud et al. 1977; Schreck 1981; Munck et al. 1984; Moberg 1985) and is supported in part, either definitively or circumstantially, by the experimental results previously discussed. It is understood that such a model is overly simple since it does not include the built-in feedback control systems, such as the negative feedback regulation of corticosteroid output, or the mechanistic components of the pathways, such as corticosteroid induction of gluconeogenic enzyme activity. Nor does it include the multitude of other primary and secondary stress responses and their possible

Figure IX.1. Important adaptive and maladaptive aspects of the endocrine-metabolic stress response pathway in fish. Increases in corticosteroids and catecholamines represent the primary response to perception of the stress factor; increases in blood glucose and metabolic rate represent the major metabolic secondary and tertiary responses, respectively.

MAJOR ASPECTS OF THE INTEGRATED ENDOCRINE-METABOLIC STRESS RESPONSE

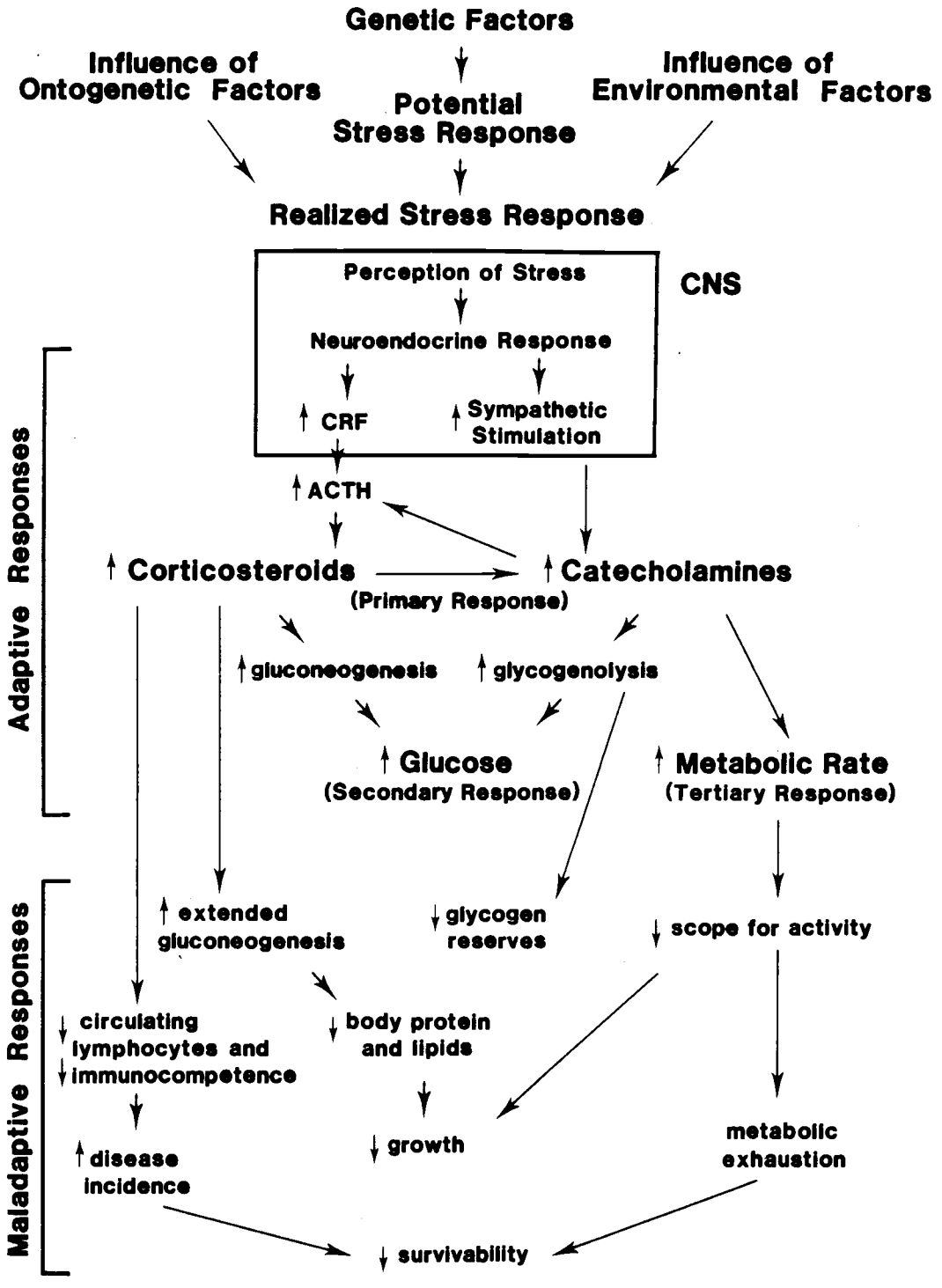

Figure IX.1

relationships to these pathways, such as the effects of catecholamines on gill vasculature and subsequently, water and ion balance. [Although not measured, catecholamines were included in this summary because of their integral role in carbohydrate metabolism (Mazeaud and Mazeaud 1981) and their interregulation with corticosteroids (Axelrod and Reisine 1984) during stress.]

When fish perceive a stressful stimulus, there is a change in biological function (Moberg 1985) as an adaptive response of the organism to compensate for the stress, according to the GAS-paradigm of Selye (1950). This response is characterized in Figure IX.1 by an increase in corticosteroids and catecholamines, followed by elevations in plasma glucose and in metabolic rate as energy reserves are mobilized and used to cope with the stress. However, there is a maladaptive component of the overall stress response that can be considered as the "cost of doing business". This is manifested by a reduction of energy availability in scope for activity, declines in glycogen reserves and decreases in circulating lymphocytes. If the stress factor is persistent, these changes could lead to reduced growth as a consequence of both continued gluconeogenesis and reduced metabolic scope, metabolic exhaustion as energy stores are depleted, and increased disease incidence resulting from reduced immunocompetence. It follows intuitively that the net result of severe or prolonged stress will be fish that are less capable of functioning optimally in their environment and, thus, have reduced chances of survival (Fig. IX.1).

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APPENDICES

APPENDIX I: EFFECT OF EXOGENOUS CORTISOL AND METYRAPONE ON SELECTED PHYSIOLOGICAL STRESS RESPONSES IN JUVENILE COHO AND CHINOOK SALMON

Introduction

The functional relationship between elevated plasma cortisol and hyperglycemia is still not clear in fish. Previous studies with fish using injections of exogenous cortisol have shown a stimulated increase in glucose levels, but results were not particularly conclusive (Butler 1968; Umminger and Gist 1973; Inui and Yokote 1975b; Lidman et al. 1979). For example, either repeated injections were required for a long time period (Butler 1968) or glucose stimulation may have been artifactual (Umminger and Gist 1973). In possibly the only study of the relationship of endogenous stress-elevated cortisol and hyperglycemia in fish, Leach and Taylor (1980) found in mummichog, Fundulus heteroclitus, that by blocking the characteristic interrenal response with metyrapone during short-term chronic stress, initial hyperglycemia was short lived and was significantly lower than untreated stressed fish after 6 h. They concluded that cortisol functions to sustain over time, elevations in plasma glucose initially stimulated by some other factor such as catecholamines.

The main objective of these experiments was to determine: (1) the influence of chronically elevated plasma cortisol resulting from an implant, and (2) the effect of suppressing the characteristic interrenal response with metyrapone, and of replacement with a single pulse dosage of exogenous cortisol, on the magnitude of the hyperglycemic response in juvenile salmonids subjected to acute or short-term chronic physical disturbances. Towards the second objective, we needed to determine the effective dosage of metyrapone, an inhibitor of cortisol synthesis, to use in an implant to suppress the characteristic stress-induced plasma cortisol increase. In addition, we determined the effect of blocking the stress-induced elevation of plasma cortisol on plasma ionic sodium and potassium in metyrapone-implanted fish, as well as the effect of a single injection of cortisol on plasma glucose and insulin in previously untreated fish.

Methods and Materials

General Methods

For the first experiment (Experiment 1), juvenile Willard Hatchery stock coho salmon, Oncorhynchus kisutch, were acclimated in 1.5-m-diameter circular tanks having an inflow of 6-7 L/min. For the second experiment (Experiment 2), juvenile Cedar Creek stock chinook salmon, Oncorhynchus tshawytscha, were acclimated in 350-L tanks receiving aerated 12°C well water at about 4 L/min. Before the third experiment (Experiment 3), juvenile Willard Hatchery stock coho salmon were held for acclimation in 100 L in tanks receiving the same water inflow as in Experiment 2. Fish from 'stock' tanks were implanted with cortisol or metyrapone according to the following protocol, modified from Pickering and Duston (1983). Crystalline form cortisol or metyrapone was first dissolved in 100% ethanol; the resultant solution was then dissolved in cocoa butter maintained in a liquid state at 40-45°C, such that the final ethanol concentration was 5% or 10% of the total volume of the implant.

To administer the implant, fish were first anesthetized in a 50-mg/L tricaine methanesulfonate (MS-222) solution. The fish were then treated by injecting 0.2 mL liquid cocoa butter containing the cortisol or metyrapone into the abdominal cavity, whereupon the cocoa butter rapidly solidified to form the implant. To determine the effective dosage of metyrapone to block the corticosteroid stress response, various predetermined concentrations of metyrapone were implanted into chinook and coho salmon. After 24 or 48 h of recovery in 100-L tanks, fish were subjected to a handling stress by holding them out of the water for 30 s. The fish were sampled 1 h after the stress to assess the amount of suppression of the characteristic cortisol response. For

Experiment 2, acclimated juvenile chinook salmon [mean weight (wt) 45.8 g] were tested with seven metyrapone dosages ranging from 0.02 to 20 mg in 5% or 10% ethanol. For Experiment 3, acclimated juvenile coho salmon (mean wt 59.7 g) were administered seven metyrapone dosages from 10 to 30 mg in 10% ethanol.

In all experiments, blood was obtained by rapidly anesthetizing sampled fish in a 200-mg/L MS-222 solution and then serially bleeding the fish into 0.25-mL capillary tubes after severing the caudal peduncle. Blood samples were centrifuged and plasma was stored at -15°C for future assay. Plasma cortisol was determined by radioimmunoassay (RIA) (Redding et al. 1984b), plasma glucose was measured by the ortho-toluidine method (Wedemeyer and Yasutake 1977), plasma insulin was assayed by Dr E. Plisetskaya, University of Washington, using a salmon-specific insulin RIA, and plasma ionic sodium and potassium were determined with a NOVA 1 Sodium/Potassium Analyzer (Nova Biomedical, Newton, Massachusetts).

Experiment 1: Cortisol Implant and Handling

Coho salmon (mean wt 25.2 g) received either a sham 90% cocoa butter-10% ethanol implant or one containing 0.4 mg of cortisol (16 mg/kg), and were then transferred to 100-L tanks having a water inflow of 4 L/min and held at a density of about 6 g/L. After 48 h, all fish were given a handling stress by holding them in the air for 30 s and returning them to the tanks. Samples for plasma cortisol and glucose were taken just before and 1, 3, 6 and 12 h after handling from duplicate tanks of treated fish.

Experiment 2: Metyrapone Implant and Handling

Chinook salmon (mean wt 52.1 g, acclimation density about 18 g/L) were injected with a 90% cocoa butter-10% ethanol implant containing 10 mg metyrapone or without metyrapone according to the

protocol previously described. Fish were transferred to 100-L tanks receiving a 4-L/min water inflow and held at a density of about 10 g/L. After 48 h, metyrapone-implanted fish were injected interperitoneally with 10 mg cortisol contained in 5% ethanol-95% Cortland's saline. A second group of metyrapone-implanted fish and the sham-implanted fish were given sham injections of ethanol-saline only. After 0.5 h, all fish were subjected to a 30-s handling stress and returned to the tanks. Samples for plasma cortisol and glucose were obtained before the injection and at 1, 3 and 6 h after the stress from duplicate tanks of treated fish.

Experiment 3: Metyrapone Implant and Handling and Confinement

Coho salmon (mean wt 53.4 g, acclimation density ca. 11 g/L) were implanted similar to those in Experiment 2 but with 30 mg metyrapone. After 48 h, fish were given cortisol or sham injections and then subjected to a 30-s handling stress using the same protocol as in Experiment 2. The fish were then confined in 10 L at a density of 80 g/L in perforated buckets immersed in 100-L tanks having a water inflow of 4 L/min for the duration of the experiment. Samples for plasma cortisol, glucose, ionic sodium, and ionic potassium were taken before injection and at 5, 10, and 20 h after handling from duplicate tanks of treated fish. Untreated and unhandled fish from an additional two acclimation tanks were sampled at those times as controls.

Concurrent with the experiment, a second group of acclimated, previously untreated coho salmon were given either a 10-mg cortisol (in Cortland's saline) injection or a sham (saline only) injection to compare the levels of plasma cortisol resulting from the dosage or from injection stress only after 1 and 5 h. Levels of plasma glucose and insulin were also determined from these fish.

Results

Effect of Implanted Cortisol

The presence of the cortisol implant elevated resting plasma cortisol about threefold but was effective in abolishing the characteristic cortisol elevation in response to the handling stress (Fig. AI.1). Resting levels of plasma glucose were higher in cortisol-implanted fish than in controls. However, chronically elevated plasma cortisol did not appear to enhance the hyperglycemia following acute handling within the first 6 h after the stress (Fig. AI.2). At 12 h post-stress, plasma glucose was higher but also variable in fish with cortisol implants (Fig. AI.2).

Determination of the Effective Metyrapone Dosage

The most effective dosage of metyrapone for suppressing the stress-induced increase in cortisol in juvenile chinook salmon was 10 mg/fish (about 200 mg/kg) as concentrations higher than that caused mortality (Table AI.1). Metyrapone doses higher than those in Table AI.1 were attempted but resulted in 100% mortality shortly after implantation. It is apparent that the concentration of ethanol used with the cocoa butter is also important since more effective stress response suppression was obtained with 10% than with 5% ethanol (Table AI.1).

In juvenile coho salmon, the most effective dosage of metyrapone for suppressing the plasma cortisol increase was 30 mg/fish (about 500 mg/kg) (Table AI.2). At this dosage, no mortality was observed. However, when this dosage was used on test fish in Experiment 3, close to 50% of the fish died; the experiment was carried out on the surviving fish.

Figure AI.1. Mean (±SE) plasma cortisol (ng/mL, N=9-10) in juvenile coho salmon that were either cortisol-implanted or sham-implanted and then subjected to a 30-s handling stress.

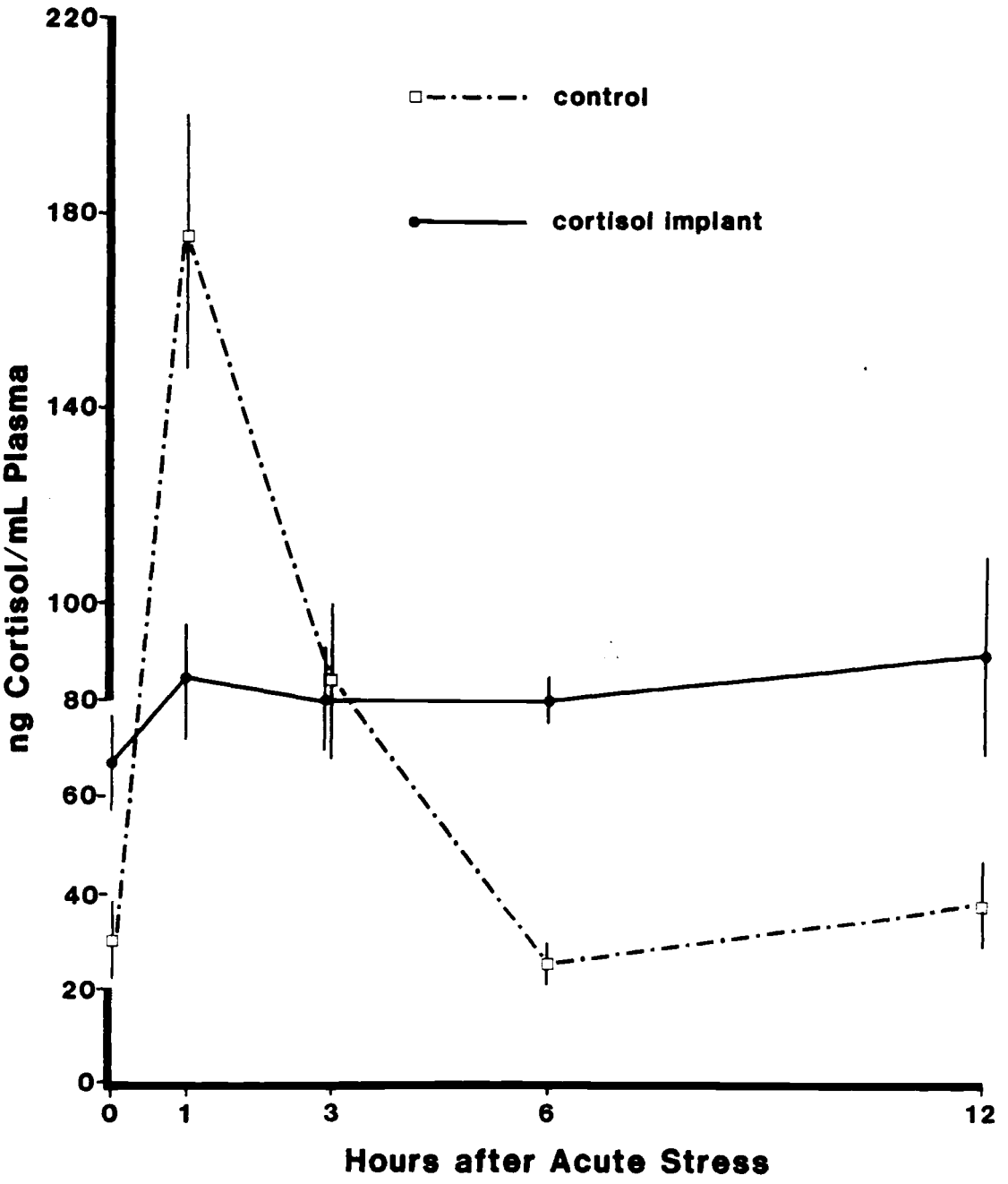

220
180
140
100
80
60
40
20
0
ng Cortisol/mL Plasma
control
cortisol implant
0
1
3
6
12
Hours after Acute Stress

Figure AI.1

Figure AI.2. Mean (±SE) plasma glucose (mg/dL, N=9-10) in juvenile coho salmon that were either cortisol-implanted or sham-implanted and then subjected to a 30-s handling stress.

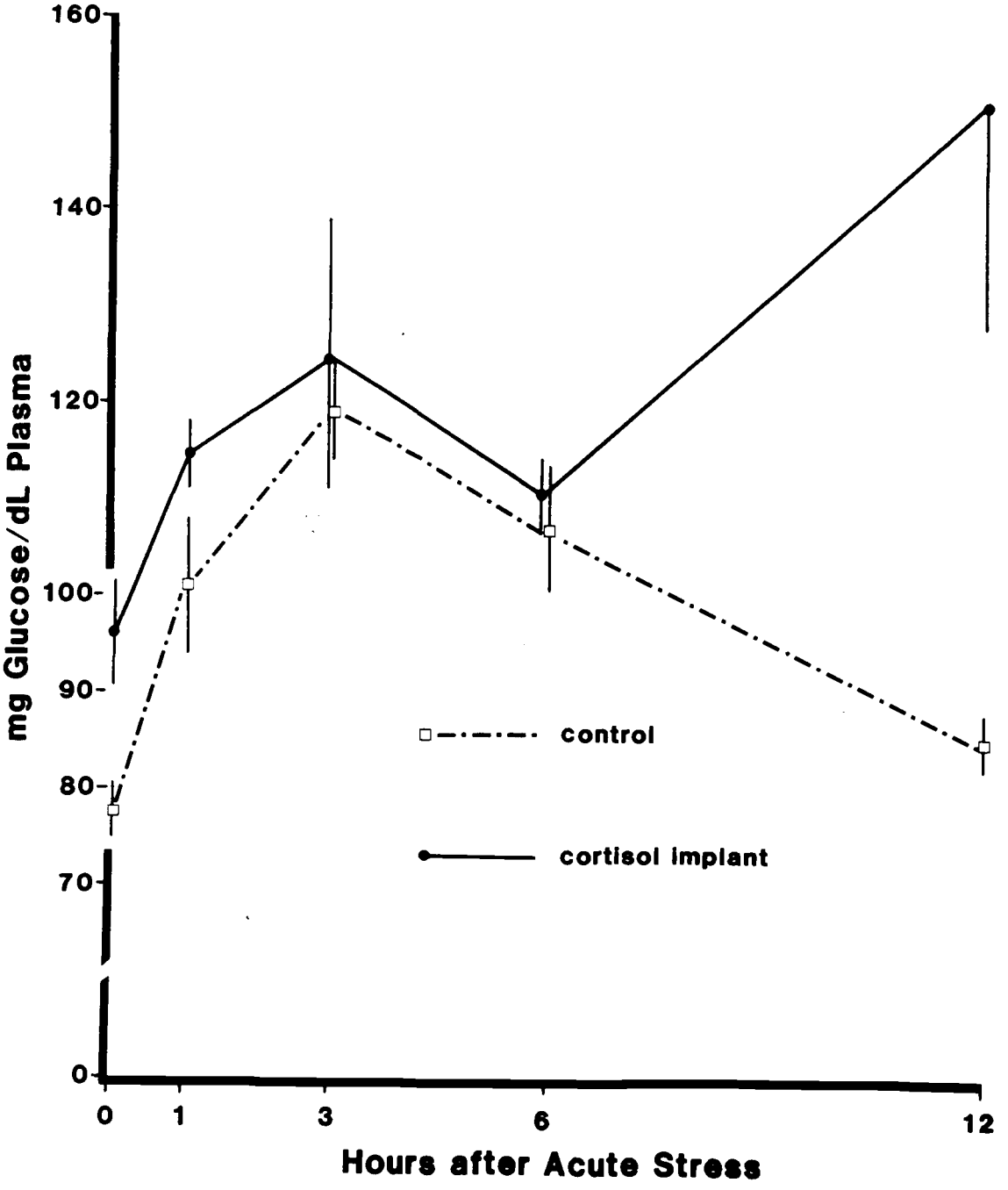

160
140
120
100
90
80
70
0
mg Glucose/dL Plasma
control
cortisol implant
0
1
3
6
12
Hours after Acute Stress

Figure AI.2

Table AI.1. Dosages of metyrapone in ethanol (EtOH) used in cocoa-butter implants in juvenile chinook salmon tested for Experiment 2 and resultant suppression of the plasma cortisol elevation in response to a 30-s handling stress at 1 h post-stress, expressed as percent response of control (dosages/kg were determined for those fish receiving that dosage only).

Metyrapone Dosage (mg) per Fish	Stress Response (% of Normal Response)
5% EtOH	100
0.02 mg in 5% EtOH (0.6 mg/kg)	91
0.2 mg in 5% EtOH (6.1 mg/kg)	98
2 mg in 5% EtOH (44 mg/kg)	72
5 mg in 5% EtOH (108 mg/kg)	49
10 mg in 5% EtOH (198 mg/kg)	44
10 mg in 10% EtOH (211 mg/kg)	32
20 mg in 5% EtOH (276 mg/kg)	32 (87% mortality)

Table AI.2. Dosages of metyrapone in ethanol (EtOH) used in cocoa-butter implants in juvenile coho salmon tested for Experiment 3 and resultant suppression of the plasma cortisol elevation in response to a 30-s handling stress at 1 h post-stress, expressed as percent response of control (dosages/kg were determined for those fish receiving that dosage only).

Metyrapone Dosage (mg) per Fish	Stress Response (% of Normal Response)
10% EtOH	100
10 mg in 10% EtOH (196 mg/kg)	55
12 mg in 10% EtOH (222 mg/kg)	50
14 mg in 10% EtOH (209 mg/kg)	62
17 mg in 10% EtOH (250 mg/kg)	47
20 mg in 10% EtOH (333 mg/kg)	31
25 mg in 10% EtOH (433 mg/kg)	28
30 mg in 10% EtOH (500 mg/kg)	16

Suppression of the Interrenal Stress Response

Using the most effective dosage determined (Table AI.1), the cortisol level in juvenile chinook salmon (Experiment 2) in response to handling was 62% lower in metyrapone-treated fish than in sham-implanted fish at 1 h post-stress, but resting levels of cortisol were notably higher (Fig. AI.3). The metyrapone-implanted, cortisol-injected fish had cortisol levels about 1.5 times that of the sham-treated fish, but rates of clearance appeared to be similar (Fig. AI.3). Similarly in juvenile coho salmon (Experiment 3), levels of plasma cortisol were not changed in metyrapone-treated fish but were three times higher than resting levels in sham-treated fish at 5 h post-stress (Fig. AI.4). Unlike plasma cortisol in the chinook salmon which returned to resting levels within 6 h following acute handling, cortisol in the coho salmon was still elevated at 20 h in response to continuous confinement. This response was effectively suppressed in the metyrapone-treated coho salmon at 5, 10, and 20 h (Fig. AI.4).

However, in each of the experiments, glycemic responses to the stress were similar for the three treatments. In Experiment 2, plasma glucose levels in the chinook salmon 6 h after acute handling were the same (Fig. AI.5). Likewise in Experiment 3, the patterns of plasma glucose changes in coho salmon during 20 h continuous confinement following the handling stress were the same (Fig. AI.6).

Of interest were the cortisol levels in previously untreated coho salmon at 1 h and 5 h after a single 10-mg cortisol injection (Table AI.3). At both times, cortisol levels were about three times higher than those at equivalent times in chinook salmon from Experiment 2 (Fig. AI.3) even though the injection concentrations were the same. There was no additional effect of exogenous cortisol in these coho salmon on the normal hyperglycemic response to the stress of handling and injection within the first 5

Figure AI.3. Mean (±SE) plasma cortisol (ng/mL, N=10) in juvenile chinook salmon that were: (1) metyrapone-implanted and cortisol-injected, (2) metyrapone-implanted and sham-injected, and (3) sham-implanted and sham-injected, and then subjected to a 30-s handling stress.

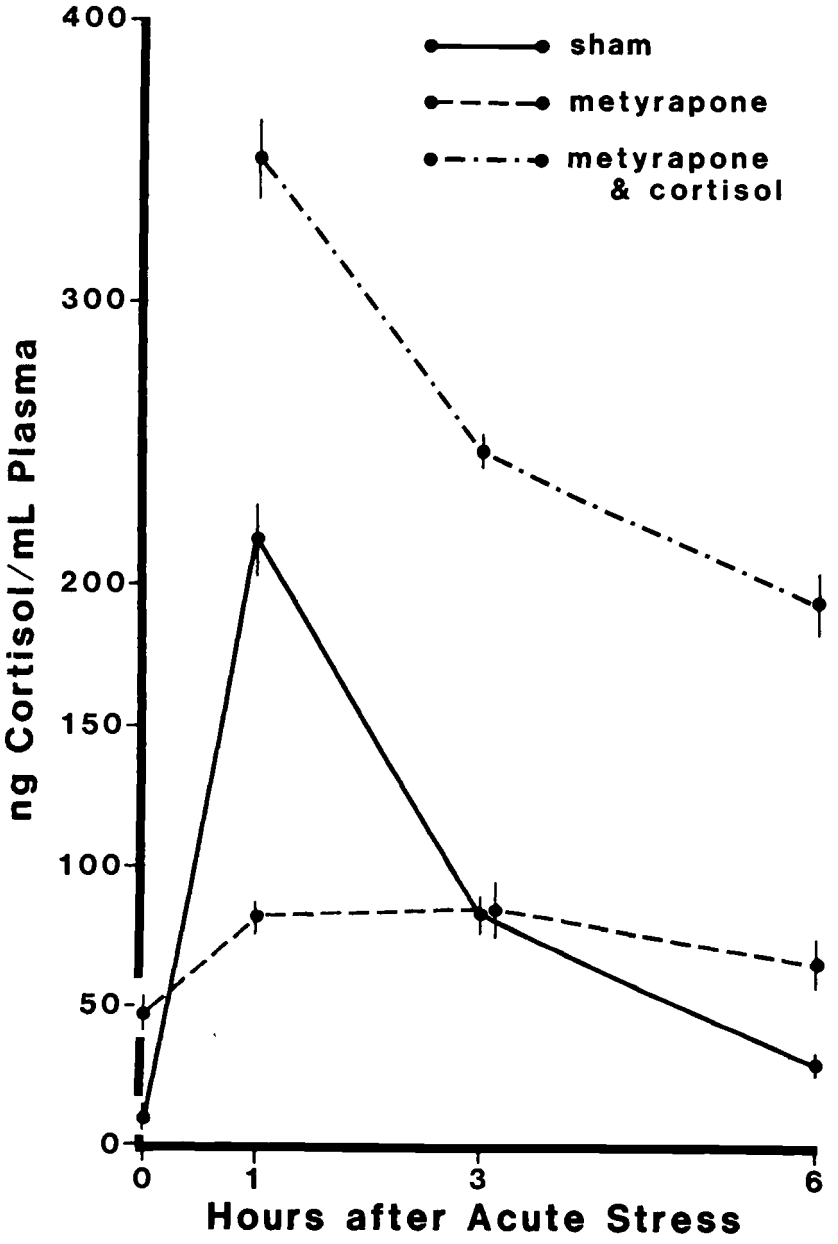

400
300
200
150
100
50
0
ng Cortisol/mL Plasma
sham
metyrapone
metyrapone
& cortisol
0
1
3
6
Hours after Acute Stress

Figure AI.3

Figure AI.4. Mean (±SE) plasma cortisol (ng/mL, N=10) in juvenile coho salmon that were: (1) metyrapone-implanted and cortisol-injected, (2) metyrapone-implanted and sham-injected, and (3) sham-implanted and sham-injected, and then subjected to a 30-s handling stress followed by 20 h of continuous confinement. (Note: vertical scales in upper and lower portions of the graph are different.)

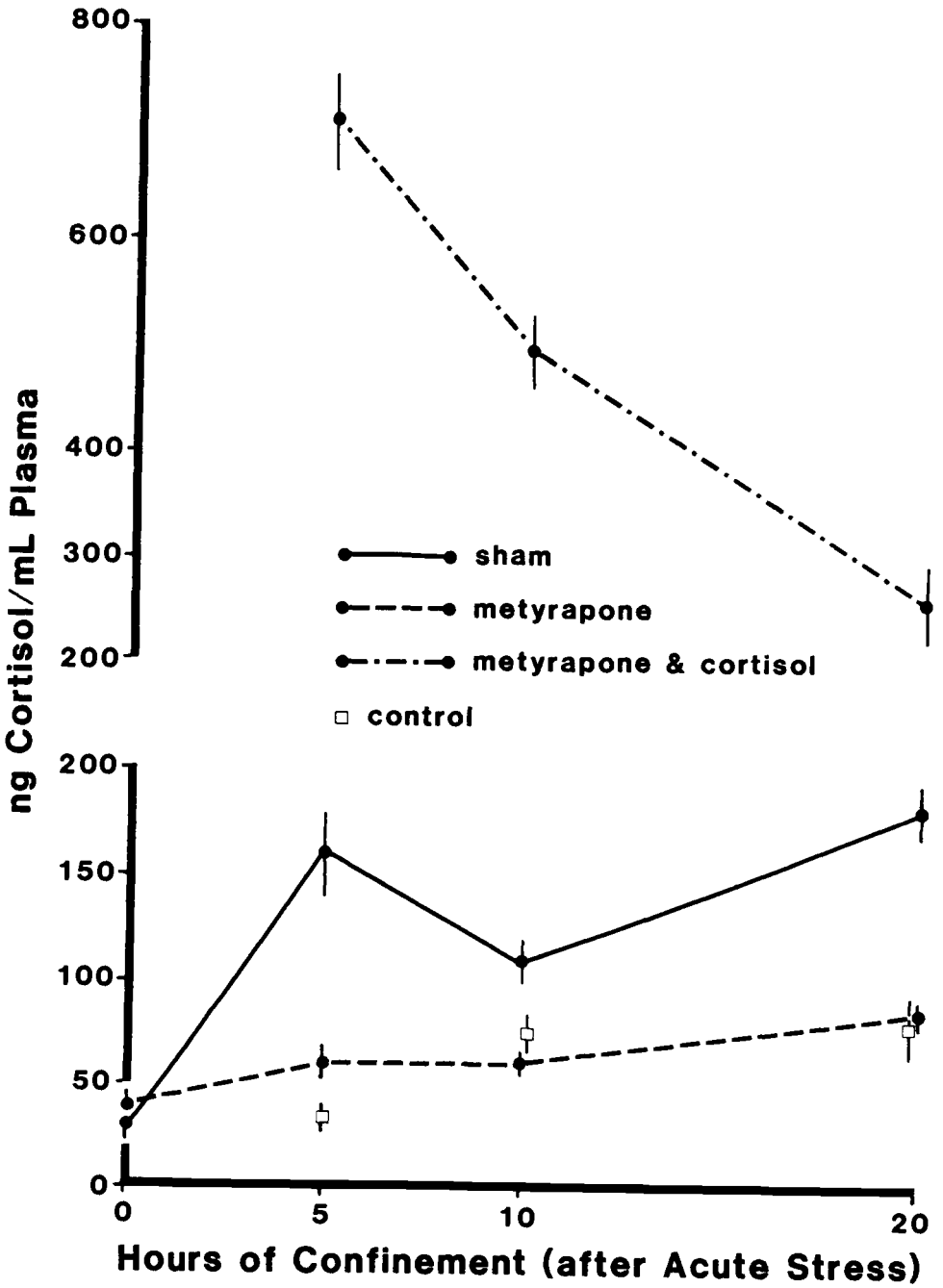

800
600
400
300
200
ng Cortisol/mL Plasma
sham
metyrapone
metyrapone & cortisol
control
200
150
100
50
0
0
5
10
20
Hours of Confinement (after Acute Stress)

Figure AI.4

Figure AI.5. Mean (±SE) levels of plasma glucose (mg/dL, N=10) in juvenile chinook salmon that were: (1) metyrapone-implanted and cortisol-injected, (2) metyrapone-implanted and sham-injected, and (3) sham-implanted and sham-injected, and then subjected to a 30-s handling stress.

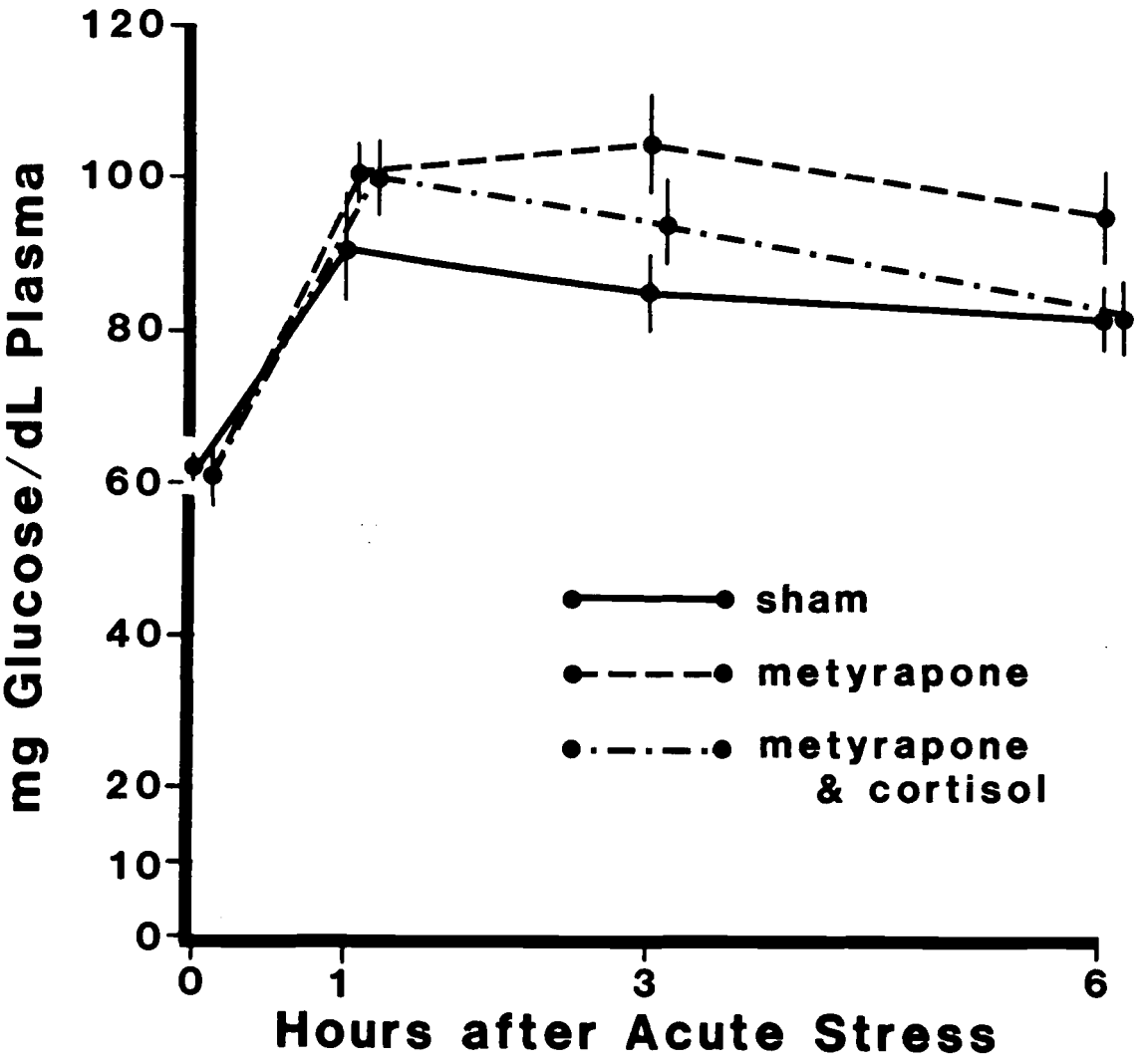

120
100
80
60
40
20
10
0
mg Glucose/dL Plasma
0
1
3
6
Hours after Acute Stress
sham
metyrapone
metyrapone
& cortisol

Figure AI.5

Figure AI.6. Mean (±SE) levels of plasma glucose (mg/dL, N=10) in juvenile coho salmon that were: (1) metyrapone-implanted and cortisol-injected, (2) metyrapone-implanted and sham-injected, and (3) sham-implanted and sham-injected, and then subjected to a 30-s handling stress followed by 20 h of continuous confinement.

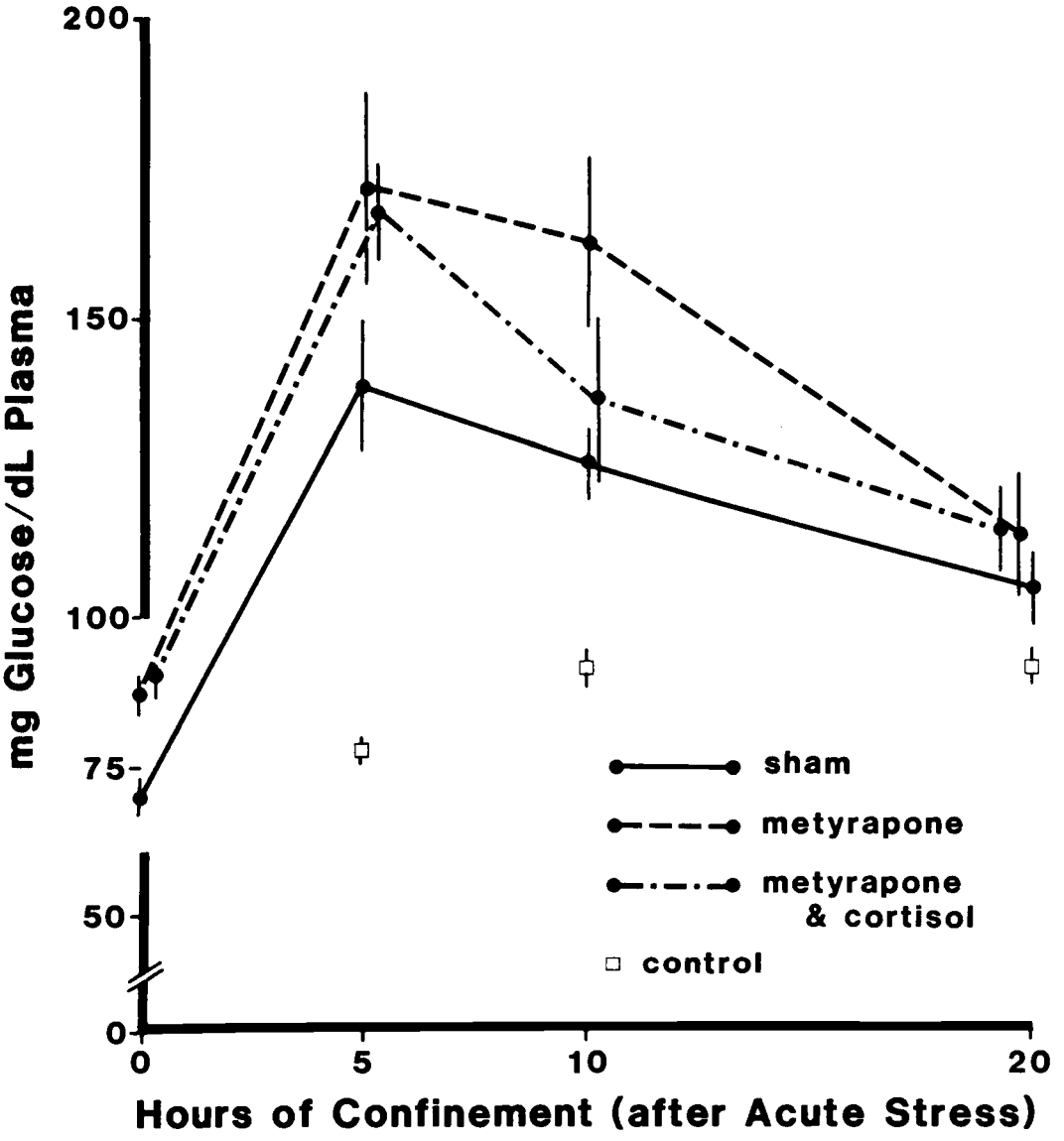

200
150
100
75
50
0
mg Glucose/dL Plasma
sham
metyrapone
metyrapone
& cortisol
control
0
5
10
20
Hours of Confinement (after Acute Stress)

Figure AI.6

Table AI.3. Mean (±SE) plasma cortisol (ng/mL, N=8-10), glucose (mg/dL, N=8-10), and insulin (ng/mL, N=7-10) in juvenile coho salmon following an injection of either 10 mg of cortisol in 10% ethanol-90% Cortland's saline or ethanol-saline only (sham).

		Cortisol Injection	Sham Injection
Before injection:	cortisol	19 ±7.1	19 ±7.1
	glucose	76 ±7.0	76 ±7.0
	insulin	1.5 ±0.3	1.5 ±0.3
1 h post-injection:	cortisol	1113 ±130	216 ±20
	glucose	99 ±4.5	105 ±4.6
	insulin	2.1 ±0.4	2.0 ±0.4
5 h post-injection:	cortisol	651 ±28	147 ±9.4
	glucose	103 ±7.7	132 ±13
	insulin	1.5 ±0.3	1.0 ±0.1

h after treatment (Table AI.3). In fact, plasma glucose in cortisol-injected fish appeared to be lower. Likewise, plasma insulin levels were not affected by the added cortisol (Table AI.3).

Neither blocking endogenous cortisol nor replacement with exogenous cortisol had any demonstrable effect on plasma ionic sodium or potassium in juvenile coho salmon. There was a depression in plasma ionic sodium in all three treatment groups in response to the combined stress of handling and continuous confinement which was most apparent at 10 h (Table AI.4). The rise in plasma ionic potassium was most evident at 5 h after handling, but results in general were more variable than those for sodium (Table AI.4).

Conclusions

The suppression of the characteristic cortisol elevation in response to handling in the cortisol-implanted coho salmon was similar to what was observed in cortisol-fed rainbow trout, Salmo gairdneri, (Chapter VII). This result is further evidence of the regulation of cortisol secretion by negative feedback on the hypothalamic-pituitary-interrenal axis (Fryer and Peter 1977). This finding also supports the earlier conclusion (Chapter VII) that the ability of fish to initiate an interrenal response to an acute stress is much reduced or even eliminated in fish that may have had cortisol titers already elevated from chronic stress.

The elevation in resting glucose in cortisol-implanted coho salmon were also similar to what was observed in rainbow trout continuously fed with cortisol in the diet (Chapter VII) and supports the notion that cortisol has a glucocorticoid function in salmonid fish. The delayed elevation of plasma glucose at 12 h in the cortisol-implanted fish over that in control fish suggests that

Table AI.4. Mean (±SE) plasma ionic sodium (Na^+) and potassium (K^+) (in mmol/L) in juvenile coho salmon that were: (1) metyrapone-implanted and cortisol-injected, (2) metyrapone-implanted and sham-injected, and (3) sham-implanted and sham-injected, and then subjected to a 30-s handling stress followed by 20 h of continuous confinement ([a]N=20; [b]N=13; all others, N=10).

		(a) Metyrapone /Cortisol	(b) Metyrapone /Sham	(c) Sham /Sham	Control
Before treatment:	Na^+	155 ±1.3[a]	155 ±1.3[a]	155 ±1.8	155 ±1.8
	K^+	3.6 ±0.2	3.6 ±0.2	3.4 ±0.1	3.4 ±0.1
5 h post-treatment:	Na^+	148 ±1.3	150 ±2.2	152 ±1.5	156 ±1.6
	K^+	4.6 ±0.3	4.1 ±0.2	4.2 ±0.3	4.4 ±0.2
10 h post-treatment:	Na^+	146 ±2.1	147 ±1.1	147 ±2.5	155 ±2.5
	K^+	4.3 ±0.2	4.5 ±0.3	3.9 ±0.2	3.7 ±0.2
20 h post-treatment:	Na^+	152 ±2.4	148 ±1.1	148 ±1.5	156 ±1.1[b]
	K^+	4.0 ±0.3	3.6 ±0.2	4.1 ±0.2	4.3 ±0.2

cortisol may have had a sustaining effect on the initial rise in glucose in response to handling. However, the data were variable at that time which makes this conclusion speculative.

Conversely, the results from both stress experiments with metyrapone-implanted chinook and coho salmon suggest that cortisol did not enhance or sustain catecholamine-induced elevations of plasma glucose during acute or short-term chronic stress. Alternatively, if cortisol did effect levels of stress-elevated plasma glucose, it did not do so in a dose-dependent fashion nor act at a level that was detectable with our experimental protocol. In coho salmon, cortisol did not appear to enhance or otherwise effect levels of plasma ionic sodium or potassium within the 20-h time frame investigated. Neither did exogenous cortisol appear to alter levels of plasma insulin in coho salmon, at least within the 5-h post-treatment period. The slight rise in plasma insulin in both groups at 1 h was likely in response to elevated levels of plasma glucose.

Provided the dosages are high enough, metyrapone implants with cocoa butter appear to be an effective method of blocking production of endogenous cortisol during stress. However, levels needed to achieve the desired effect are close to the threshold level of mortality. Moreover, the effective dosages (and lethal levels) do not appear to be consistent and thus would have to be determined on an individual experiment basis.

APPENDIX II: RECOVERY TIME FROM ACUTE HANDLING STRESS IN JUVENILE CHINOOK SALMON

Synopsis

In Chapter III, when fish were subjected to identical handling stresses at 3-h intervals following an initial handling stress, the resultant plasma cortisol and glucose elevations were cumulative. The objective of this series of experiments was to determine how the recovery interval after an identical handling stress affects the increases of plasma cortisol, glucose and lactate in fish in response to a second identical stress.

In the initial experiment, juvenile Trask River stock chinook salmon, Oncorhynchus tshawytscha (17.2 g), held and acclimated as described in Chapter III, were subjected to two, identical 30-s handling stresses spaced 1 and 12 h apart. Experiments were conducted using fish from duplicate tanks and pooling the results (N=12). Sampling and analyses in this and subsequent experiments were carried out according to the protocol in Chapter III. Plasma cortisol, glucose and lactate were determined at 0.5, 1, 3, 6, 12 and 24 h after the second stress as well as just before application of both stresses.

The cortisol elevation after the second stress was additive upon cortisol levels elicited from the second stress when spaced 1 and 3 h apart (Fig. AII.1); results from Chapter III for two stresses spaced 3 h apart are included in these graphs for comparison. However, when fish were allowed 12 h to recover, the second stress resulted in a maximum post-stress cortisol concentration similar to that evoked by two stresses 1 h apart (Fig. AII.1). This phenomenon was not evident for plasma glucose, where levels responded to the second stress in a similar manner after 1, 3 or 12 h of recovery from the initial stress (Fig. AII.2). Plasma lactate elevatons following a second stress were similar to that observed for cortisol. That is, after a 12-h recovery period between stresses, there was a much greater relative increase in lactate (Fig. AII.3) than there was to the second stress after a 1- or 3-h recovery period.

Figure AII.1. Mean (and 1 SE) plasma cortisol (ng/mL, N=11-12) in juvenile Trask River chinook salmon subjected to two 30-s handling stresses spaced 1, 3 or 12 h apart. (Solid arrows indicate application of the second stress; open squares represent unhandled controls.) Sample means, indicated by letter, are ranked below left to right from lowest to highest; means not significantly different ($P<0.05$) share the same underline (Duncan's new multiple-range test).

0.5 h:	a^1	$a\ a^2$	6 h:	$d^1\ d^2\ d$	
1 h:	$b^1\ b^2\ b$		12 h:	$e^2\ e^1\ e$	
3 h:	c^1	$< c^2\ c$	24 h:	$f\ f^1$	$< f^2$

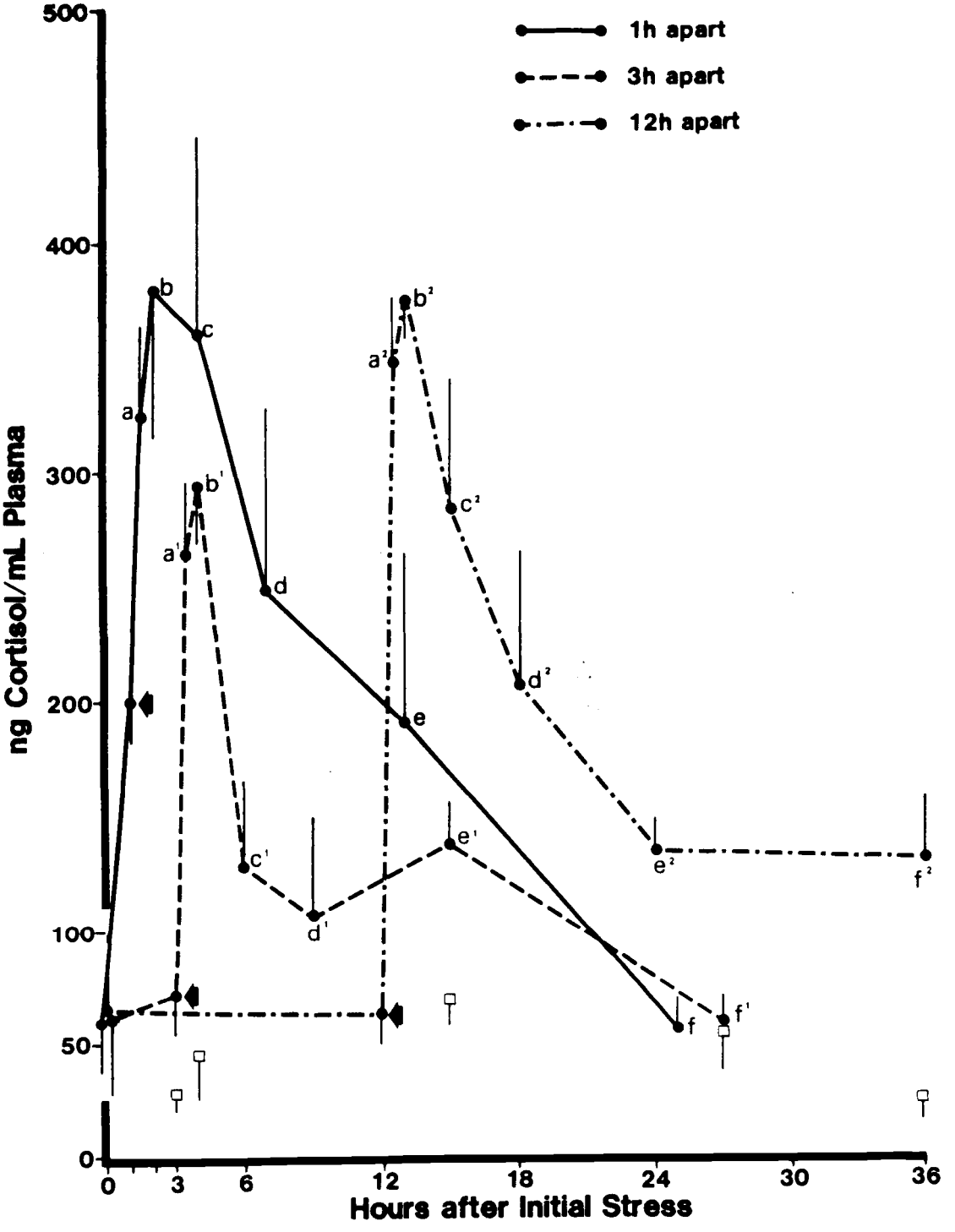

1h apart
3h apart
12h apart
ng Cortisol/mL Plasma
Hours after Initial Stress
500
400
300
200
100
50
0
0
3
6
12
18
24
30
36
a
b
c
d
e
f
a'
b'
c'
d'
e'
f'
a²
b²
c²
d²
e²
f²

Figure AII.1

Figure AII.2. Mean (and 1 SE) plasma glucose (mg/dL, N=11-12) in juvenile Trask River chinook salmon subjected to two 30-s handling stresses spaced 1, 3 or 12 h apart. (Solid arrows indicate application of the second stress; open squares represent unhandled controls.) Sample means, indicated by letter, are ranked below left to right from lowest to highest; means not significantly different ($P<0.05$) share the same underline (Duncan's new multiple-range test).

0.5 h:	a	a^1	a^2	6 h:	d	d^2	d^1
1 h:	b^2	b	b^1	12 h:	e	e^2	e^1
3 h:	c^2	c^1	c	24 h:	f^1	f^2	f

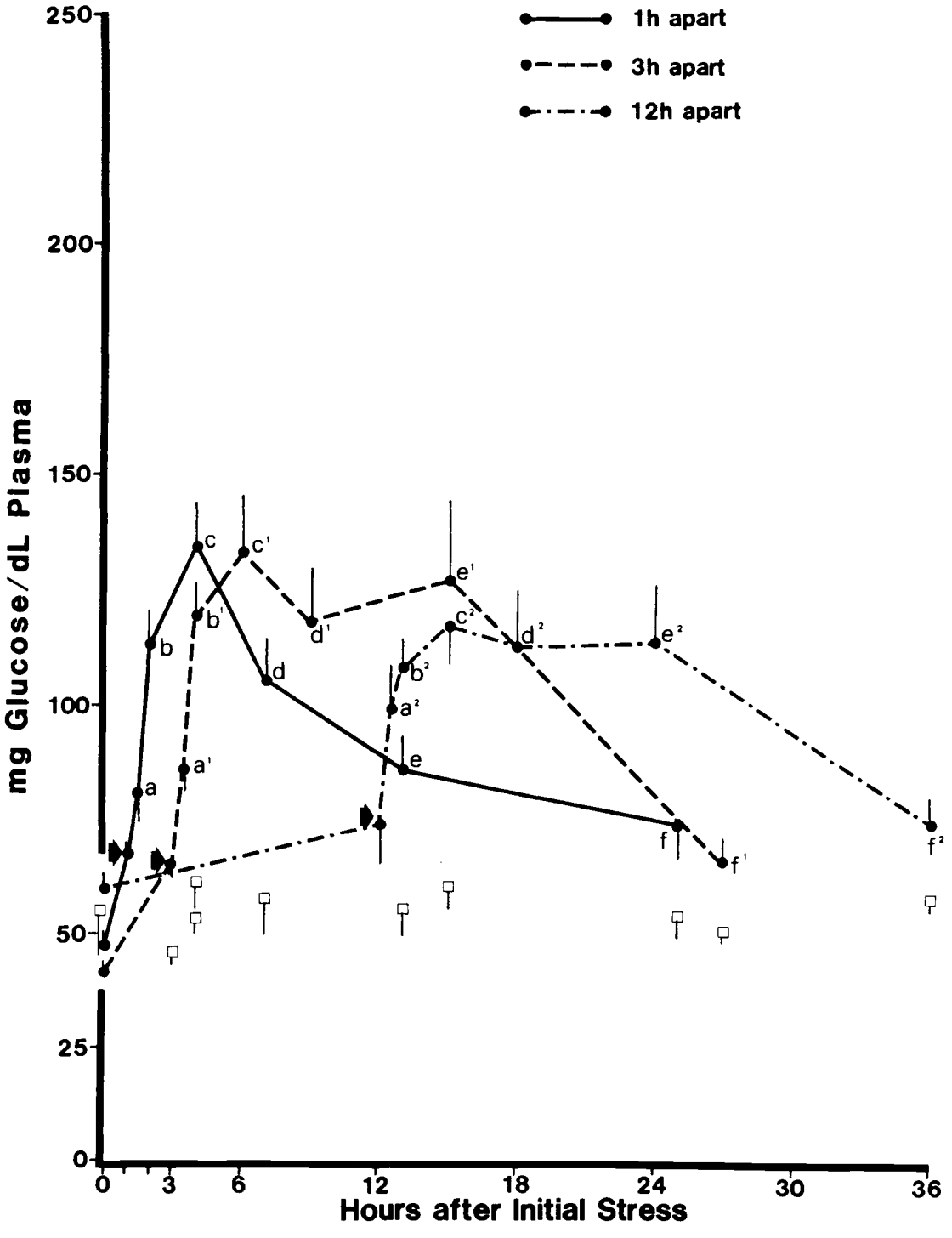

1h apart
3h apart
12h apart
mg Glucose/dL Plasma
250
200
150
100
50
25
0
0
3
6
12
18
24
30
36
Hours after Initial Stress
a
b
c
d
e
f
a'
b'
c'
d'
e'
f'
a²
b²
c²
d²
e²
f²

Figure AII.2

Figure AII.3. Mean (and 1 SE) plasma lactate (mg/dL, N=10-12) in juvenile Trask River chinook salmon subjected to two 30-s handling stresses spaced 1, 3 and 12 h apart. (Solid arrows indicate application of the second stress; open squares represent unhandled controls.) Sample means, indicated by letter, are ranked below left to right from lowest to highest; means not significantly different ($P<0.05$) share the same underline (Duncan's new multiple-range test).

0.5 h:	$a^1 < a^2 \quad a$	6 h:	$d \quad d^1 \quad d^2$
1 h:	$b^1 < b^2 < b$	12 h:	$e^1 \quad e^2 \quad e$
3 h:	$c^2 \quad c \quad c^1$	24 h:	$f^1 \quad f^2 \quad f$

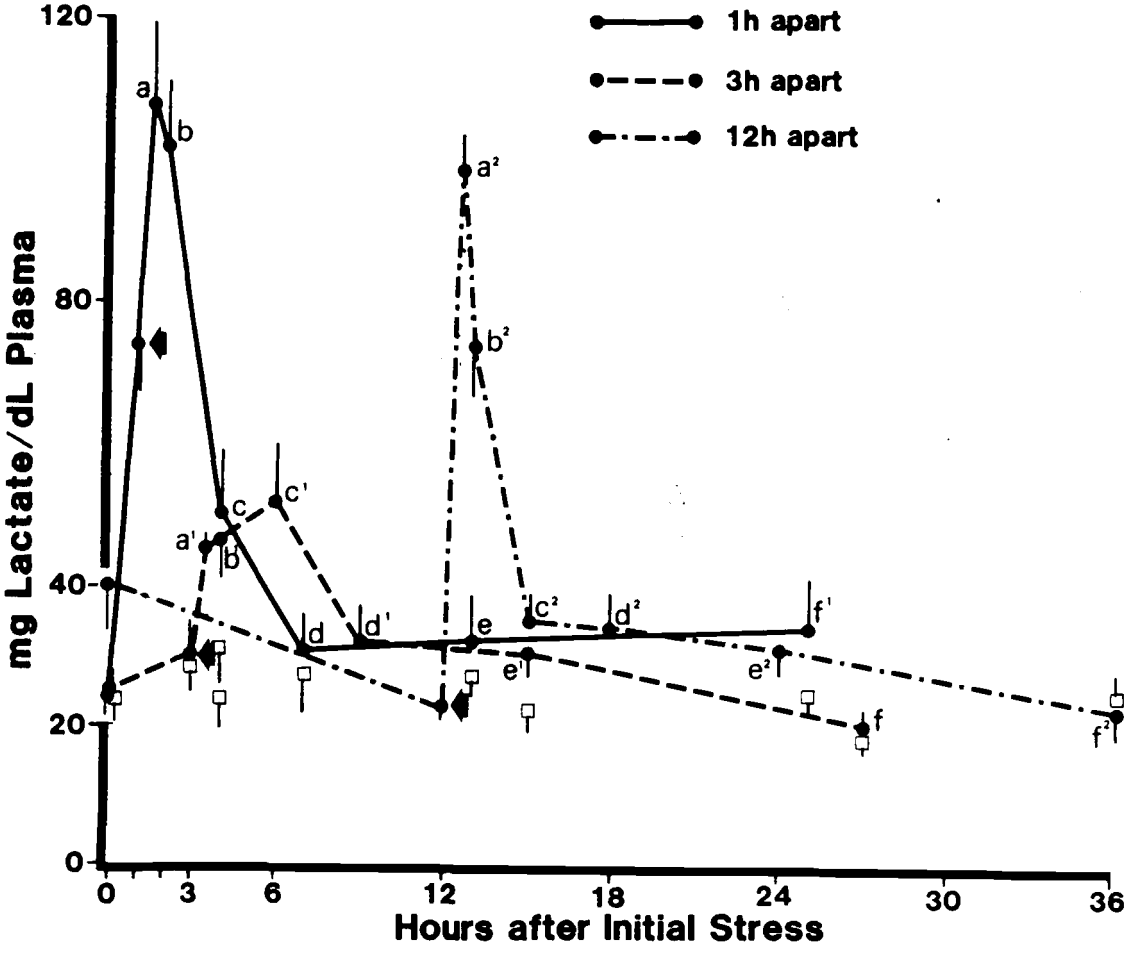

1h apart
3h apart
12h apart
mg Lactate/dL Plasma
120
80
40
20
0
0
3
6
12
18
24
30
36
Hours after Initial Stress
a
b
c
c'
a'
b'
d
d'
e
e'
a²
b²
c²
d²
e²
f
f'
f²

Figure AII.3

The results suggest that the recovery time between stresses may have affected both the magnitude and timing of multiple responses and that recovery from a single, acute stress may take considerably longer than interrenal and glycemic responses would indicate. When the magnitude of the cortisol response to the second handling (difference between peak post-stress and resting levels) was compared with the recovery time allowed between the two disturbances, this difference became greater with time (Fig. AII.4). This trend indicates that there may be a post-stress period during which fish are more sensitive to stress than they normally would be in a resting state. This could be an important factor to consider when making recommendations about optimum allowable recovery time for fish that have been subjected to physical disturbances, such as handling in a fish culture facility. In an attempt to clarify this trend, four experiments were conducted to determine the minimum recovery time necessary for the characteristic cortisol elevation in response to a second, similar acute stress to return to those levels normally associated with the response to a single acute handling stress.

In the first of these, juvenile Salmon River stock chinook salmon (20.5 g), acclimated in 100-L tanks at about 20 fish per tank, were subjected to a 30-s handling stress and returned to their home tank. At 3, 6, 12, 18, 24, 36 and 48 h after the initial stress, duplicate groups of fish were given a second 30-s handling stress. Samples of fish for assay of plasma cortisol were obtained just prior to the application of the second stress at each time and 1 h after the second stress for each of the handled groups. A separate group of fish (in duplicate), that was also stressed, was sampled before the stress and at 1, 3 and 6 h to establish a representative single acute stress response pattern for this stock of fish. In addition, two groups of fish remained untreated to serve as a control and to establish appropriate resting values for the remainder of the experiment. One group (in duplicate) was sampled at 0, 12 and 36 h; the second group (in

Figure AII.4. Difference in plasma cortisol in juvenile Trask River chinook salmon between two 30-s handling stresses relative to the recovery time between application of the stresses. (Solid squares are values extrapolated from Fig. AII.1; open squares are values extrapolated from Fig. III.1, Chapter III. Points on the Y-axis are 1-h post-stress values from a single stress.)

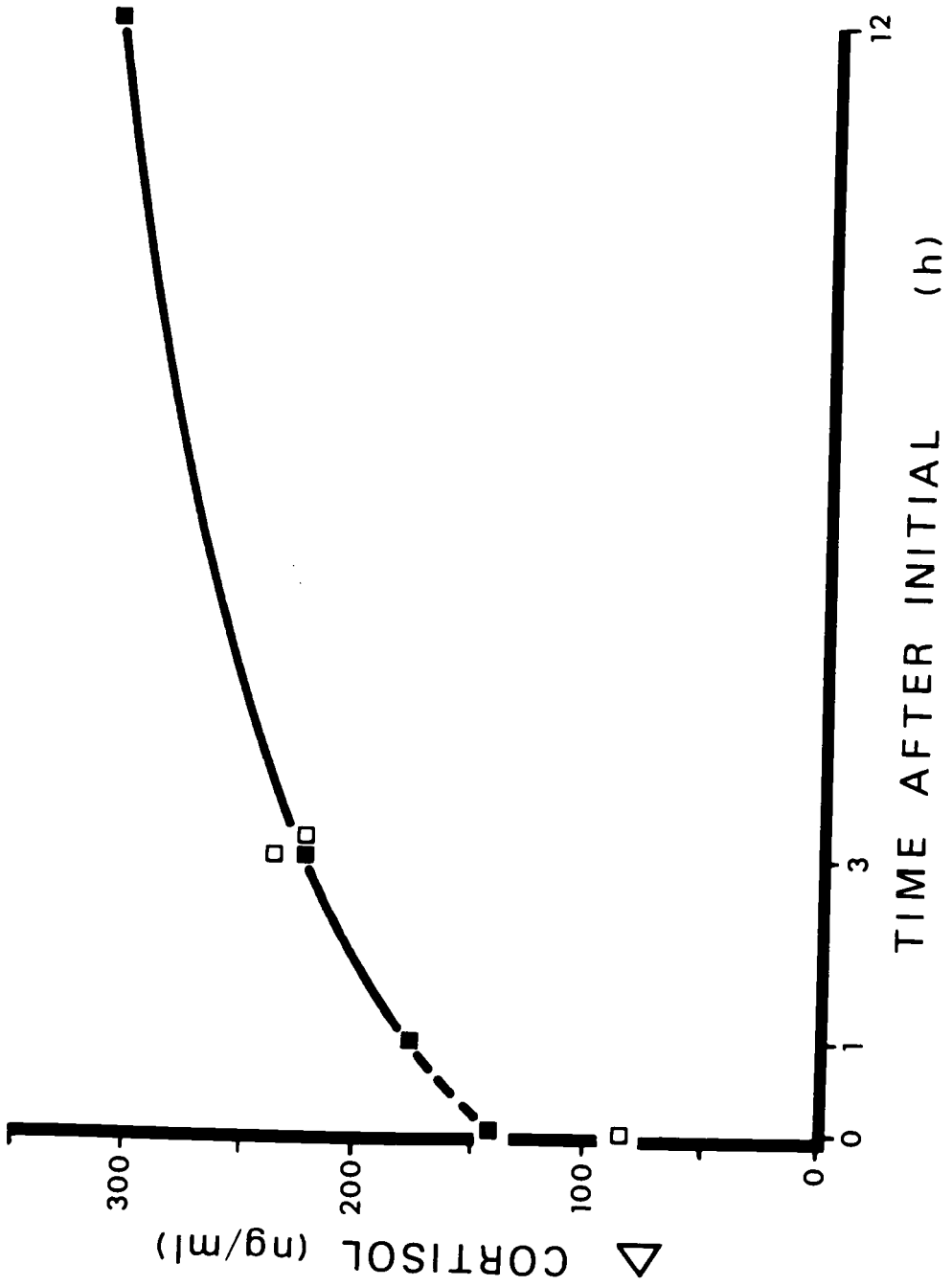


Figure AII.4

duplicate) was sampled at 0, 24 and 48 h.

Fish subjected to the single stress exhibited the characteristic increase in plasma cortisol (Fig. AII.5). But in fish subjected to the second handling stress, the 1-h post-stress response was similar in all instances to that level associated with the single stress, regardless of the time after the first stress that the second stress was applied or the cortisol titer at the time of the second stress (Fig. AII.5). Thus, there was no evidence of a critical period in these fish following the initial stress. These fish were in generally poor condition, having 'tail rot', and they were later diagnosed as being infected with the cold water disease bacterium, Cytophaga psychrophila. It was subsequently determined that the pattern of expected cortisol responses to handling was significantly altered in these diseased fish (Chapter III), so results were considered as non-representative.

A second experiment was carried out using juvenile Cedar Creek stock chinook salmon (15.9 g). Acclimation and experimental protocols were the same as in the earlier experiment except that 18- and 36-h recovery times were deleted. It was clear from looking at the difference between prestress and post-stress plasma cortisol for the different recovery times that there was a trend for the interrenal response to increase to 12 h and then decrease by 24 and 48 h (Table AII.1). However, there was a high level of variability in the data that was particularly evident in fish from control tanks [e.g. mean (±SE) resting cortisol: 82 ±18 ng/mL, N=20] indicating that these fish either had insufficient time to acclimate (9 d) or were already in a stressed state. Accordingly, these results were considered unreliable.

Third and fourth experiments were conducted using juvenile Rogue River stock chinook salmon (20.7 g), acclimated for 3-4 wk at 20 and 25 fish per tank, respectively, in the same manner as the first two experiments. The third experiment was identical to the second, whereas in the fourth experiment, samples of fish were

Figure AII.5. Mean (±SE) plasma cortisol (ng/mL, N=9-10) in juvenile Salmon River chinook salmon just before and 1 h after a second 30-s handling stress. Times on X-axis are number of recovery hours since the initial 30-s handling stress. Solid line indicates the response determined for a single 30-s handling.

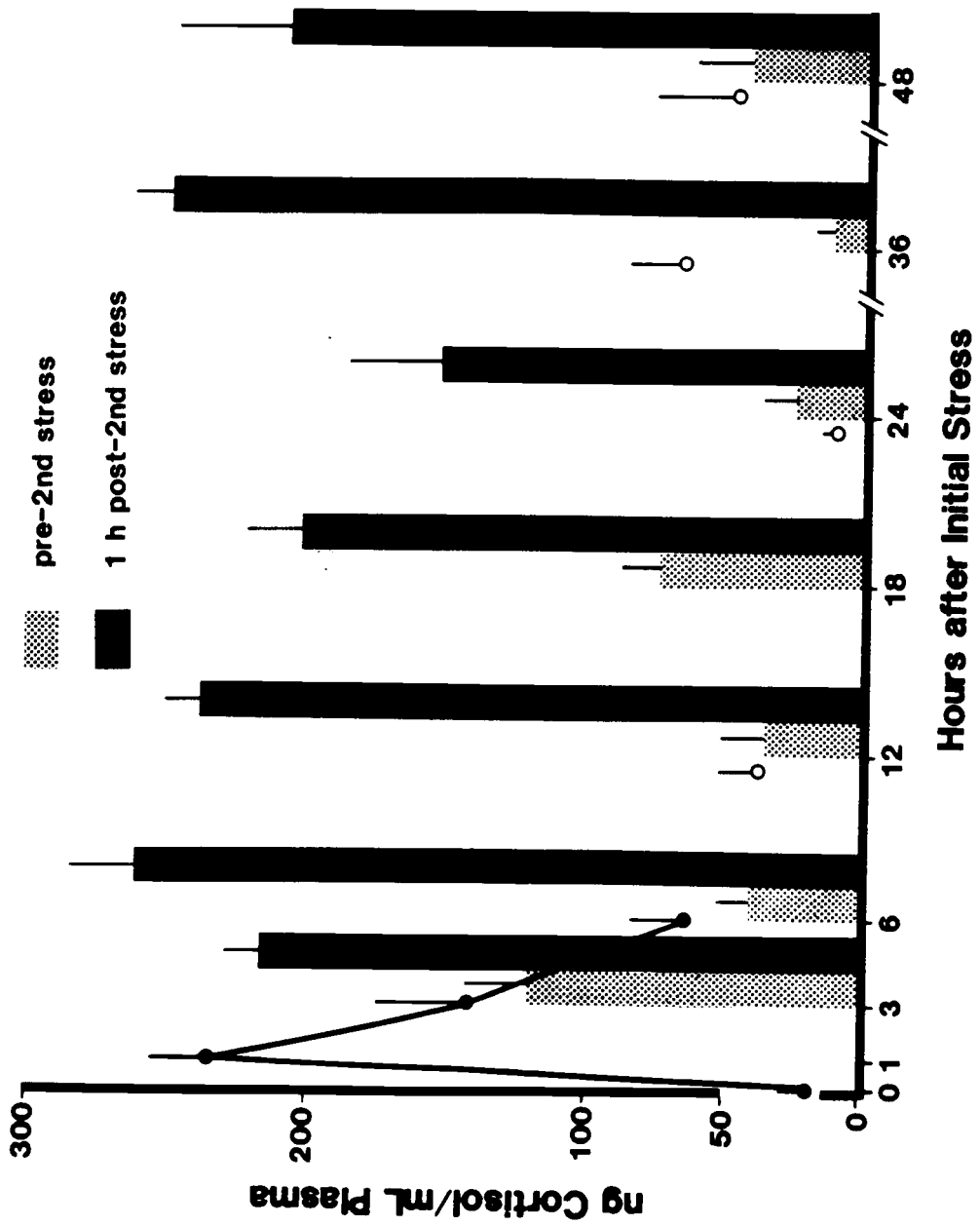

pre-2nd stress
1 h post-2nd stress
ng Cortisol/mL Plasma
300
200
100
50
0
0 1 3 6 12 18 24 36 48
Hours after Initial Stress

Figure AII.5

Table AII.1. Mean (±SE) plasma cortisol (ng/mL, N=10) in juvenile Cedar Creek chinook salmon just before and 1 h after a second 30-s handling stress. Recovery time indicates the number of hours since the initial 30-s handling.

Recovery Time after Initial Stress	Plasma Cortisol		
	Just before Second Stress	1 h after Second Stress	Difference between before and 1 h after Second Stress
3 h	96 ±28	204 ±32	108
6 h	78 ±43	207 ±25	129
12 h	46 ±11	228 ±21	182
24 h	78 ±37	162 ±22	84
48 h	91 ±32	149 ±20	58

obtained at 3 and 6 h as well as 1 h after the second stress.

In the third experiment, there appeared to be a greater response to the second stress after 24 h than at the other recovery times (Fig. AII.6). However, in the fourth experiment, plasma cortisol elevations from the second handling were the same after 12, 24 and 48 h of recovery as they were following a single handling stress (Fig. AII.7).

The results from these experiments suggest that there may be a critical post-stress recovery period but the experimental procedures employed were inadequate to quantify further this period of possibly increased sensitivity or responsiveness.

Figure AII.6. Mean (±SE) plasma cortisol (ng/mL, N=10) in juvenile Rogue River chinook salmon just before and 1 h after a second 30-s handling stress. Times on X-axis are number of recovery hours since the initial 30-s handling stress. Solid line indicates the response determined for a single 30-s handling.

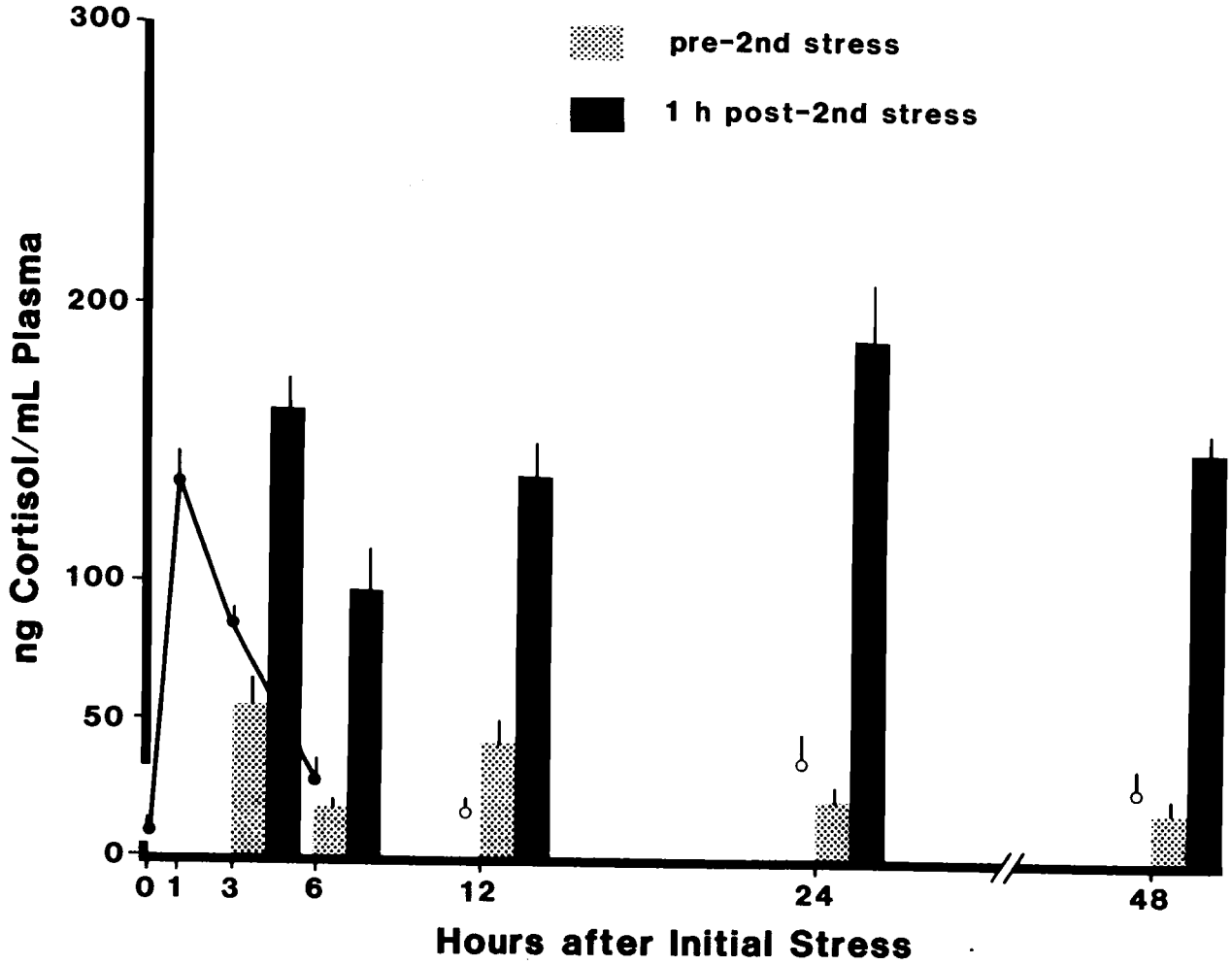

pre-2nd stress
1 h post-2nd stress
ng Cortisol/mL Plasma
300
200
100
50
0
0 1 3 6 12 24 48
Hours after Initial Stress

Figure AII.6

Figure AII.7. Mean (±SE) plasma cortisol (ng/mL, N=12) in juvenile Rogue River chinook salmon after a single 30-s handling stress (solid line) and at 1, 3 and 6 h after a second 30-s handling stress (dotted lines) at various times of recovery following the initial stress.

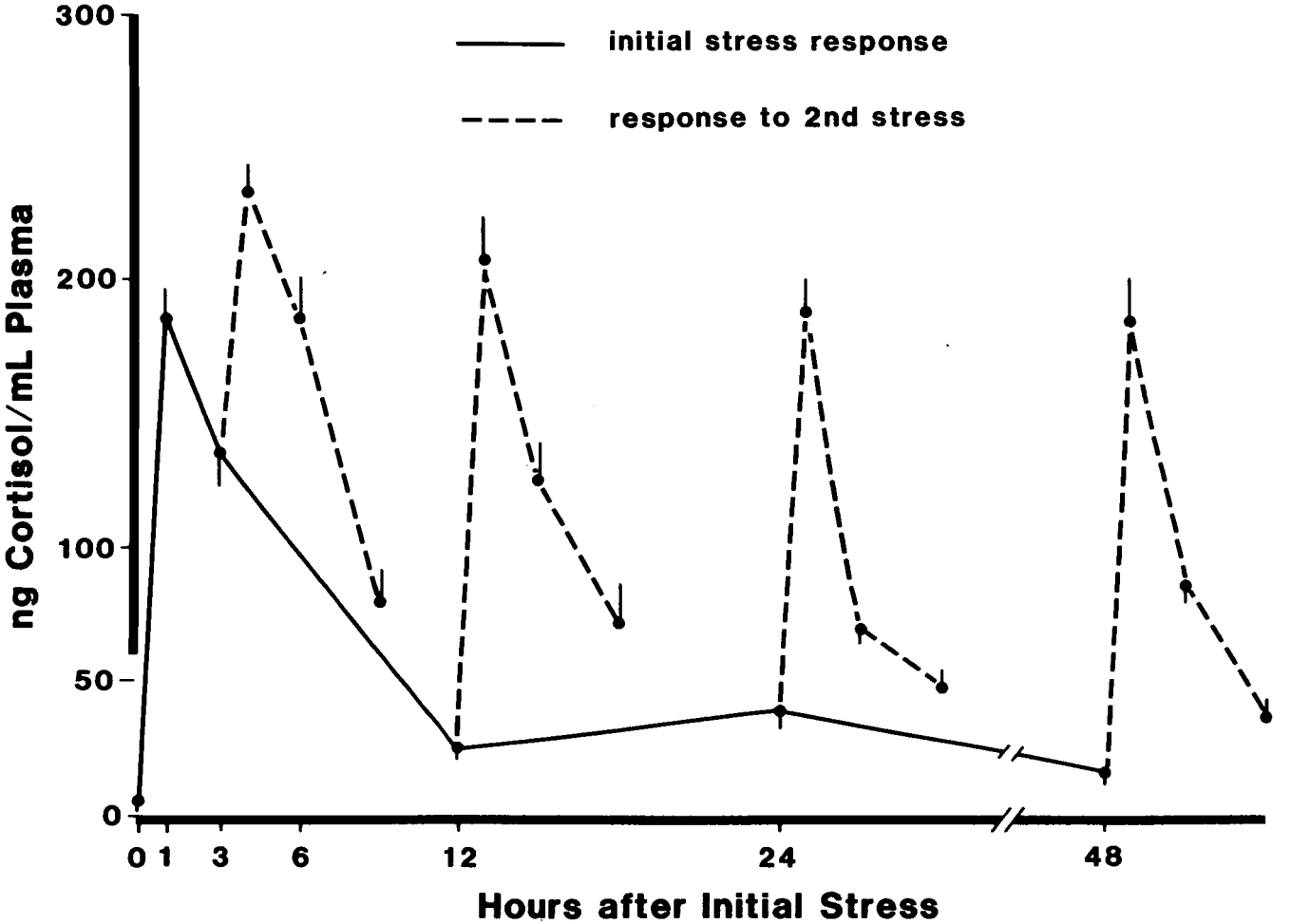

initial stress response
response to 2nd stress
ng Cortisol/mL Plasma
300
200
100
50
0
0 1 3 6 12 24 48
Hours after Initial Stress

Figure AII.7

APPENDIX III: DIURNAL DIFFERENCES IN PLASMA CORTISOL AND GLUCOSE ELEVATIONS IN RESPONSE TO ACUTE HANDLING IN JUVENILE COHO SALMON

Synopsis

In Chapter III, slight but significant differences in both plasma cortisol and glucose elevations in response to stress were apparent in juvenile chinook salmon, Oncorhynchus tshawytscha, handled at different times of the day. The objective of this experiment was to determine if there is a diurnal difference in the magnitude of plasma cortisol and glucose increases following acute stress in juvenile coho salmon, Oncorhynchus kisutch.

Juvenile Big Creek Hatchery stock coho salmon (mean weight 34.6 g) were acclimated for over 3 wk in 350-L circular tanks at a density of 5 g/L (50 fish per tank). Tanks received 6 L/min aerated well water at 12.5°C and fish were fed a ration of about 1.5% body weight once daily with Oregon Moist Pellets. At four times in the day: 0030, 0630, 1230 and 1830 h, fish in one tank (per time) were subjected to a handling stress by holding them in the air in a perforated bucket for 30 s and returning them to the tank. Fish were removed for samples for plasma cortisol and glucose determinations just before and 1 and 3 h after the stress. During dark periods, samples were taken with the aid of a 7-W red darkroom lamp.

In all instances, plasma cortisol and glucose exhibited characteristic elevations to the brief handling. The 1-h post-stress cortisol level at around midnight (0030 h) was significantly higher ($P<0.05$) than that in the early morning (0630 h) and in the evening (1830 h) (Fig. AIII.1). As well, glucose levels in response to the stress were lowest in the early morning (Fig. AIII.2). The results indicated that there were diurnal differences in both cortisol and glucose responses to acute physical disturbances in coho salmon but that these differences were slight. The pattern of diurnal variation was not the same as that in juvenile chinook salmon (Chapter III). Further experimentation and replication is necessary to conclude whether or

Figure AIII.1. Mean (±SE) plasma cortisol (ng/mL, N=12) in juvenile coho salmon just before and 1 and 3 h after a 30-s handling stress, applied at 0030, 0630, 1230 and 1830 h.

Figure AIII.1

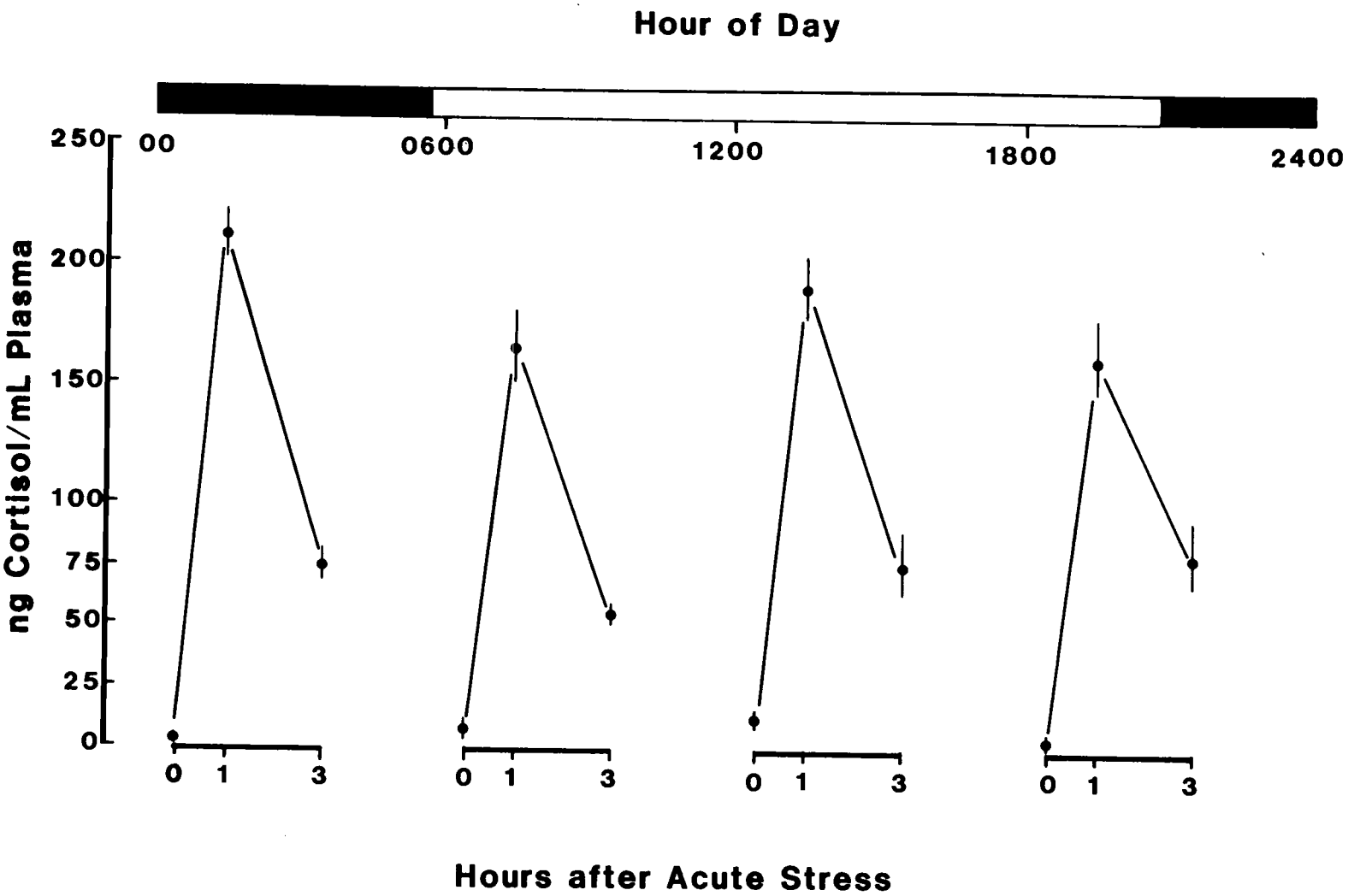
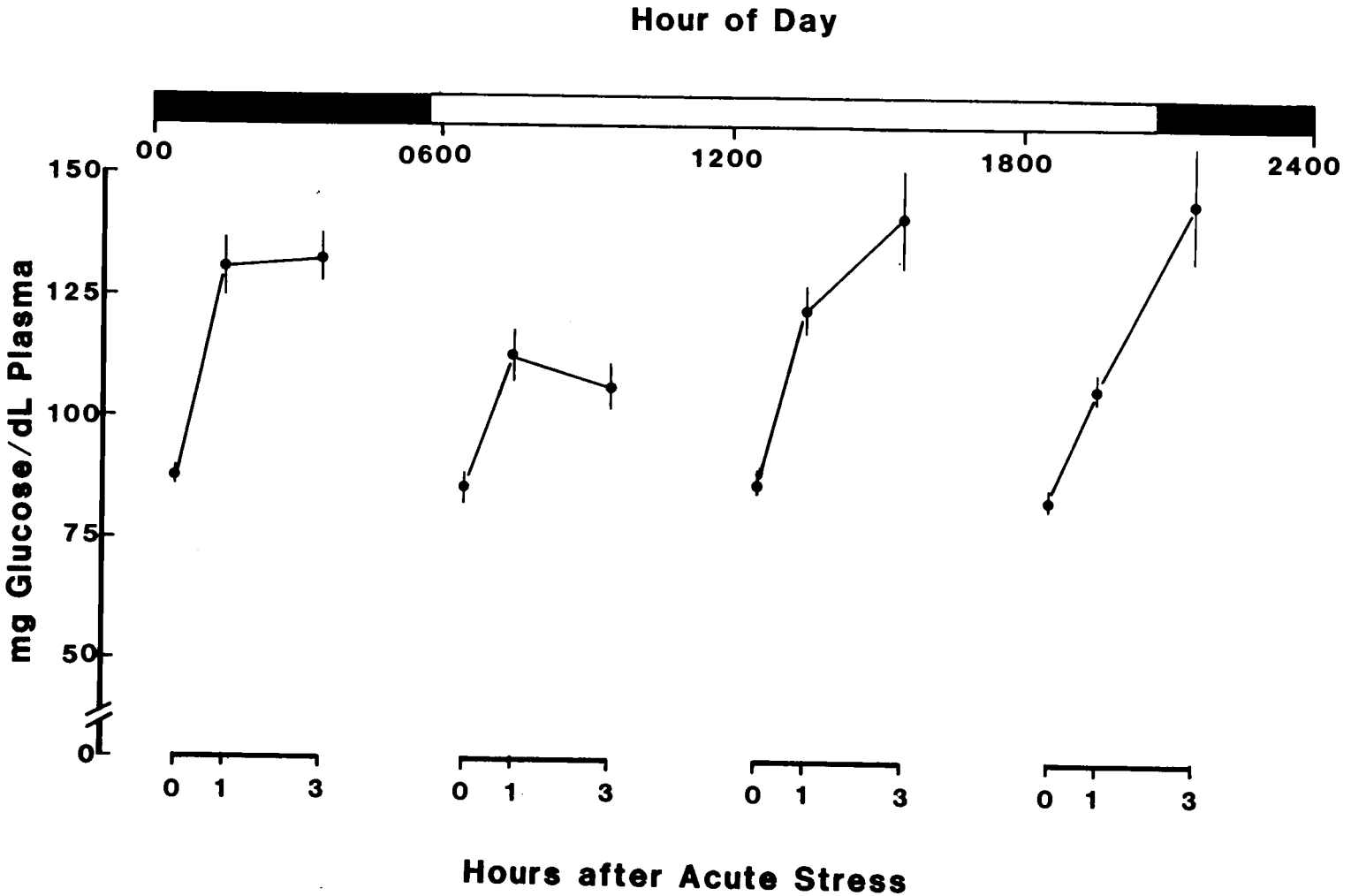


Figure AIII.2. Mean (±SE) plasma glucose (mg/dL, N=12) in juvenile coho salmon just before and 1 and 3 h after a 30-s handling stress, applied at 0030, 0630, 1230 and 1830 h.

Figure AIII.2



not the diurnal differences observed constitute a general phenomenon or simply represent random variation in characteristic cortisol and glucose responses to stress.

APPENDIX IV: VALIDATION OF A SAMPLING TECHNIQUE FOR OBTAINING PLASMA CORTISOL MEASUREMENTS IN STRESS/RECOVERY STUDIES IN FISH

Synopsis

To obtain representative levels of plasma cortisol in both laboratory experiments and field monitoring programs, it is necessary to employ a sampling method that does not itself artificially elevate circulating plasma cortisol. The purpose of the following experiments was to validate a simple technique for obtaining blood samples for plasma cortisol measurement by radioimmunoassay.

In three separate experiments using different salmonid species in each, samples of fish were dip-netted from their home tank and immediately immersed in a 200-mg/L solution of tricaine methanesulfonate (MS-222), either unbuffered, or buffered with 0.025 M sodium phosphate (Na_2HPO_4 and NaH_2PO_4) or 0.002 M sodium imidazole. Immobilization occurred within 30-40 s of immersion. Fish were removed, one at a time, to obtain blood (methods in Chapter III) from each anesthetic treatment alternately until all fish were sampled.

In the first experiment using juvenile fall chinook salmon, Oncorhynchus tshawytscha (14.7 cm, 33.9 g), there was no significant difference ($P<0.05$) in mean plasma cortisol between fish sampled from unbuffered MS-222 (pH 6.3) and MS-222 buffered with sodium phosphate (pH 7.3). However, plasma cortisol in fish from unbuffered MS-222 (25 ±9.7 ng/mL, $N=15$) exhibited considerable variation in individual levels within each treatment group. Such variation among individual fish appears typical of juvenile chinook salmon held in captivity, as judged from results of other experiments with this species and stock (Chapter III).

No trend-through-time increase in plasma cortisol was apparent in fish remaining in the anesthetic solution for the duration of the 46-min sampling period. No difficulty was encountered in obtaining an adequate blood sample after this time.

In the second experiment, juvenile coho salmon, Oncorhynchus kisutch (11.7 cm, 18.2 g), had mean plasma

cortisol levels of <1 ng/mL (N=10, both groups) when anesthetized and held in either unbuffered MS-222 (pH 6.2) or phosphate-buffered MS-222 (pH 7.4). Many of the individial fish in both groups had non-detectable cortisol concentrations (= zero) that may be indicative of low plasma cortisol in juvenile coho salmon at the parr stage for that time of year (February). In March, coho salmon parr had plasma cortisol levels of about 3 ng/mL (Chapter IV). No increase in plasma cortisol was evident in fish held in the anesthetic solution for 32 min, the duration of the experiment.

In the third experiment, juvenile rainbow trout, Salmo gairdneri (16.4 cm), had mean plasma cortisol levels of 18 ±3.4 and 16 ±2.9 ng/mL (N=12) when anesthetized and retained in unbuffered MS-222 using soft water (pH 3.9) or imidazole-buffered MS-222 (pH 7.3), respectively. No increase through time in plasma cortisol was apparent in fish held for the 33-min duration of the experiment.

The results indicated that normal resting values of plasma cortisol can be obtained by immediately immobilizing all the fish for subsequent sampling in 200 mg/L of MS-222, regardless if the final solution is buffered to neutrality or not. Furthermore, the length of time that the fish remain in the anesthetic solution after initial immobilization does not appear to affect the final results. Although lower dosages of MS-222 have been shown to be stressful to fish (Strange and Schreck 1978; Barton and Peter 1982), apparently the dosage of 200 mg/L is sufficient to eliminate the sensory stimulus required to elicit a stress response or to arrest the secretion of cortisol from the interrenal tissue into circulation, or both.