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Title: CHANGES IN PLASMA CORTISOL CONCENTRATION OF JUVENILE SALMONIDS DURING STRESS

Abstract approved: Redacted for privacy

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I investigated changes in plasma cortisol concentration over time in juvenile salmonids subjected to various stressors that might be encountered in their normal life cycle. This work was directed at finding a general indicator for stress in fish that could be used be used to aid fisheries research and management. Plasma cortisol concentrations in juvenile chinook salmon (Oncorhynchus tshawytscha) netted and confined in a small live-cage rose from approximately 100 ng/ml to about 500 ng/ml in 24 h. Fish dip-netted into a bucket containing aerated water and sampled serially at 90-s intervals showed an increase in plasma cortisol concentration from less than 10 ng/ml to 100 ng/ml in 20 min. In juvenile cutthroat trout (Salmo clarki) acclimated to 13 C and subjected to a rapid increase in water temperature to 26 C plasma cortisol concentration increased from 20 ng/ml to 70 ng/ml in 25 min and remained elevated for more than 3 h, however, an increase in temperature from 12 C to 20 C elicited no change in cortisol. Fish acclimated to diurnal temperature cycles

(13-23 C) had no substantial changes in plasma cortisol concentration throughout the cycles. No dramatic changes in basal levels of plasma cortisol were noted as fish grew from 7 to 14 cm over a period of 5 months. Fish acclimated to very warm water (22 or 23 C) had the same initial cortisol concentration as fish acclimated to cool water (9 or 12 C), but the trout in warm water had more erratic changes in cortisol during confinement.

Mean plasma cortisol levels in juvenile chinook salmon increased from near 0 to about 200 ng/ml in response to 0.5 h of severe confinement, remained elevated for over 6 h after release, and returned to basal levels within 12.5 h. Fish subjected to severe, continuous confinement had increase in plasma cortisol to about 400 ng/ml during the first 1.5 h; little further increase occurred, and by 12.5 h mortality to the stressor reached 50%. In response to moderate confinement a definite but variable elevation in plasma cortisol occurred with a return to basal levels within 6 to 8 days as the fish acclimated to the stressor. A depression in gill Na+K ATPase was noted in juvenile salmon approximately 3 wk after acclimation to moderate confinement.

Brief anesthetization with 50 mg/l buffered tricaine methanesulfonate (MS-222) of yearling chinook salmon during mild handling resulted in plasma cortisol comparable to those in non-anesthetized controls. Prolonged exposure (180 min) to a depressing dose of

MS-222 (25 mg/l) elevated cortisol more than an immobilizing dose (50 mg/l), while 100 mg/l was lethal within 30 min Fish anesthetized (50 mg/l) during a severe 30 min handling stressor had substantially lower mortality to a second handling stressor after the fish were no longer anesthetized than untreated controls. Anesthetization during the first stressor also prevented the cortisol stress response evident in the control fish Anesthetic (with or without buffer) administered before initial capture was most effective at increasing survival during a second stressor, while anesthetic supplied after capture was slightly less effective. A 0.5% NaCl solution supplied after capture was less effective than any anesthetic treatment in increasing future survival, but was better than no treatment. The saline treatment did not attenuate the cortisol stress response.

Introduction of seawater (25 to 30 g/l dissolved solids) caused a slight, transient elevation of plasma cortisol in juvenile chinook salmon. When the fish were severely confined immediately after seawater introduction they had a significantly lower increase in cortisol and better survival than fish confined in fresh water.

Changes in Plasma Cortisol Concentration of Juvenile Salmonids during Stress

by

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CHANGES IN PLASMA CORTISOL CONCENTRATION OF JUVENILE SALMONIDS DURING STRESS

I. GENERAL INTRODUCTION

During the course of their existence all organisms encounter a wide variety of situations that tax homeostasis. Vertebrates have evolved a non-specific response (stress) to the various specific demands placed upon them. Selye (1936) first recognized the physical manifestations of stress in people (i.e. adrenal hypertrophy, atrophy of lymphoid tissue, gastrointestinal ulcers) and symptoms of stress subsequently became known as the General Adaptation Syndrome. We now recognize this gross evidence of stress as secondary or tertiary reactions to the primary neural and hormonal stress responses. Primary stress responses include increased stimulation of the sympathetic nervous system and elevated plasma concentrations of catecholamines and corticosteroids. The adaptive significance of increased corticosteroid levels during times of stress is not fully understood, however, the widespread effects of these hormones (e.g. increased blood glucose at the expense of protein reserves) probably work to help the organism survive a crisis situation possibly at the expense of future fitness.

Sublethal stress is of interest in fisheries because it potentially slows growth, reduces fecundity, and increases incidence of disease

in both cultured and wild stocks. While stress has always concerned fisheries personnel it has only recently been the object of systematic study. Much of the research on stress in fishes has been aimed at a method of assessing sublethal stress through changes in physiology with the goal being a stress indicator that would then be useful in work on the reduction of stress. Changes in a number of biochemical and hematological characteristics that represent secondary stress responses have been correlated with the presence of various stressors. Elevated blood glucose has been noted in fish stressed by handling (Houston et al. 1971a, Wedemeyer 1972, Umminger and Gist 1973, Miles et al. 1974), elevated temperature (Wedemeyer 1973), and electroshock (Schreck et al. 1976). Handling may lower plasma chloride concentration (Wedemeyer 1972, Umminger and Gist 1973, Miles et al. 1974), but the response is not always definitive (Houston et al. 1971a, 1971b). Adding electrolytes to the water has been shown to reduce hypochloremia and hyperglycemia in fish stressed by handling (Wedemeyer 1972). In freshwater fish hypochloremia may occur as a result of sodium excretion or water imbibition; a gain in weight following handling, suggesting water intake, has been noted (Stevens 1972), but in another case hyperchloremia occurred with no change in carcass water content (Umminger and Gist 1973). Hyperglycemia and hypochloremia are reflections of stress quite likely associated with the cortisol component of the General Adaptation Syndrome.

Increased hemoglobin may follow handling (Houston et al. 1971b) and rapid increases in ambient water temperature (Wedemeyer 1973). Changes in circulating leucocytes have been produced by cold shock (Pickford et al. 1971a); catecholamines and corticosteroids were implicated as factors controlling the leucocyte dynamics (Pickford et al. 1971b, 1971c).

Research into primary stress responses which might be more sensitive and less complex than secondary responses has centered on plasma corticosteroids, in particular cortisol which is the principal corticosteroid in teleosts (Schmidt and Idler 1962, Idler and Freeman 1965, Fagerlund and Donaldson 1970, Chavin and Singley 1972, Cambell et al. 1976). The literature on changes in cortisol during stress in fishes is discussed in the individual section introductions, however, some generalizations can be made here. Most studies have simply documented an increase in plasma cortisol after exposure to some stressor. Only a few experiments have included serial measurements over time to show changes in cortisol during stress and no investigations have followed cortisol dynamics to their completion, in other words, until recovery, acclimation, or death of the stressed fishes. The principal objective of this study was to determine cortisol dynamics in juvenile salmonids subjected to stressors that they might encounter during their life cycle.

The line of research presented here first documented the initial

cortisol responses to confinement (a physical handling-type stressor intended to parallel a variety of culture and management situations) and heat using juvenile chinook salmon (Oncorhynchus tshawytscha) and cutthroat trout (Salmo clarki) in short term experiments. Possible diurnal rhythmicity was also explored. Changes in cortisol during confinement and heat were then followed to completion in long term experiments. Changes in cortisol during anesthetization were determined and cortisol concentration employed to help evaluate the use of anesthetic to reduce stress and increase future survival. Cortisol dynamics during saltwater adaptation were also evaluated. Additionally, possible gross changes in basal levels during growth were checked. The data gathered during this research provides consistent and comparable information on which to base conclusions about the usefulness of plasma cortisol concentration as a stress indicator, as well as providing insight into some sublethal effects of stress and fruitful approaches toward the reduction of stress.

II. ACUTE CHANGES IN CORTISOL DURING CONFINEMENT AND HEAT

Introduction

The detection of stress that reduces fitness of fish or fish populations is of interest in fisheries, because it would allow an assessment of the impact of pollution and of research and management techniques. Elevation of plasma cortisol concentration appears to be in many vertebrates a generalized response to a variety of disturbances (Selye 1971, 1976); potentially, cortisol might be used as an indicator of stress in fish. Circulating cortisol concentrations in fish have been shown to increase in response to exposure to certain pollutants, such as copper (Donaldson and Dye 1975), chromium (Hill and Fromm 1968), pesticides (Grant and Mehrle 1973; Yaron and Ilan 1974) and a component of kraft mill effluent (Dye and Donaldson 1974). Research and management techniques that may result in increased cortisol levels in fish include electroshock (Schreck et al. 1976), antibiotic injection (McBride et al. 1975), unbuffered anesthetic (Wedemeyer 1969), formalin (Wedemeyer and Yasutake 1974), and handling-exercise-type stress (Hane et al. 1966; Donaldson and McBride 1967; Donaldson and Fagerlund 1970; Fagerlund and Donaldson 1970; Grant and Mehrle 1973; Nemeth and Jurani 1974; Spieler 1974; Mazeaud et al. (1977). While it is known that cortisol concentrations are elevated by certain

stressors, the relationship between time, stress and cortisol response is not well established, although reports by Chavin (1973) and Singley and Chavin (1975a) outlined the sequence of cortisol titers in goldfish (Carassius auratus) exposed to saline for various times. I am interested in determining how rapidly and to what magnitude circulating cortisol levels change after the onset of continuously applied stressors, that is, stressors producing either capacity or resistance adaptation as considered by Precht (1958).

Methods

The handling stressor consisted of netting and then confining the fish in either a live-cage or a bucket. Experimental temperatures were in normal, extreme, or lethal ranges for the test species. Fish sampled for cortisol analysis were killed by a blow to the head and bled into heparinized capillary tubes by severing the caudal peduncle. The time required to obtain blood samples from an entire group of fish was always less than 5 min. Blood was centrifuged and stored at -15 C until assayed.

I determined plasma cortisol concentration in each sample using a competitive protein binding assay adapted from Murphy (1967). Plasma (10-25 µl) was extracted twice with redistilled ethanol. After the combined extracts were evaporated to dryness, 1.0 ml of 0.5% human male serum (aqueous solution) in which corticoid binding sites had been "saturated" with ^{3}H-cortisol was added and allowed to react for 5 min at 45 C. The samples were then placed in an ice bath and

80 mg of Florisil (magnesium silicate) was added to remove unbound corticoids. I determined tritium activity in 0.5 ml aliquots from each sample. Duplicate cortisol standards were processed along with the unknowns and cortisol concentration determined graphically. I have shown that the cortisol concentration measured in this manner is abou about twice the cortisol value obtained when cortisol is partitioned-out of the crude extract by thin layer chromatography on silica gel using a dichloromethane:methanol:water (150:9:0.5) solvent system (Fagerlund 1970) (Fig. 1). Part of this discrepancy is due to a loss of 35 percent of the cortisol (determined by radio-tracer methods) during our chromatography purification. The small remaining activity is probably cortisone, although it has only slight affinity for human serum, and other steroids.

Experimental Design and Results

Immediate Response to Confinement

On 4 November 1975, about 50 12-14 cm juvenile chinook salmon were dipnetted from a holding tank (8 C) into a 10-liter bucket supplied with vigorous aeration. Thirty serial samples were taken at about 90-s intervals. The cortisol concentrations increased in a linear fashion from less than 10 ng/ml to near 200 ng/ml in less than 20 min (Fig. 2). During this period, the rate of increase was

Figure 1. Cortisol as purified by thin layer chromatography (uncorrected for a 35% loss during TLC) versus cortisol determined directly on extract in split plasma samples. Dashed line indicates regression: $y=0.48x-7.80$, $r^2=0.84$.

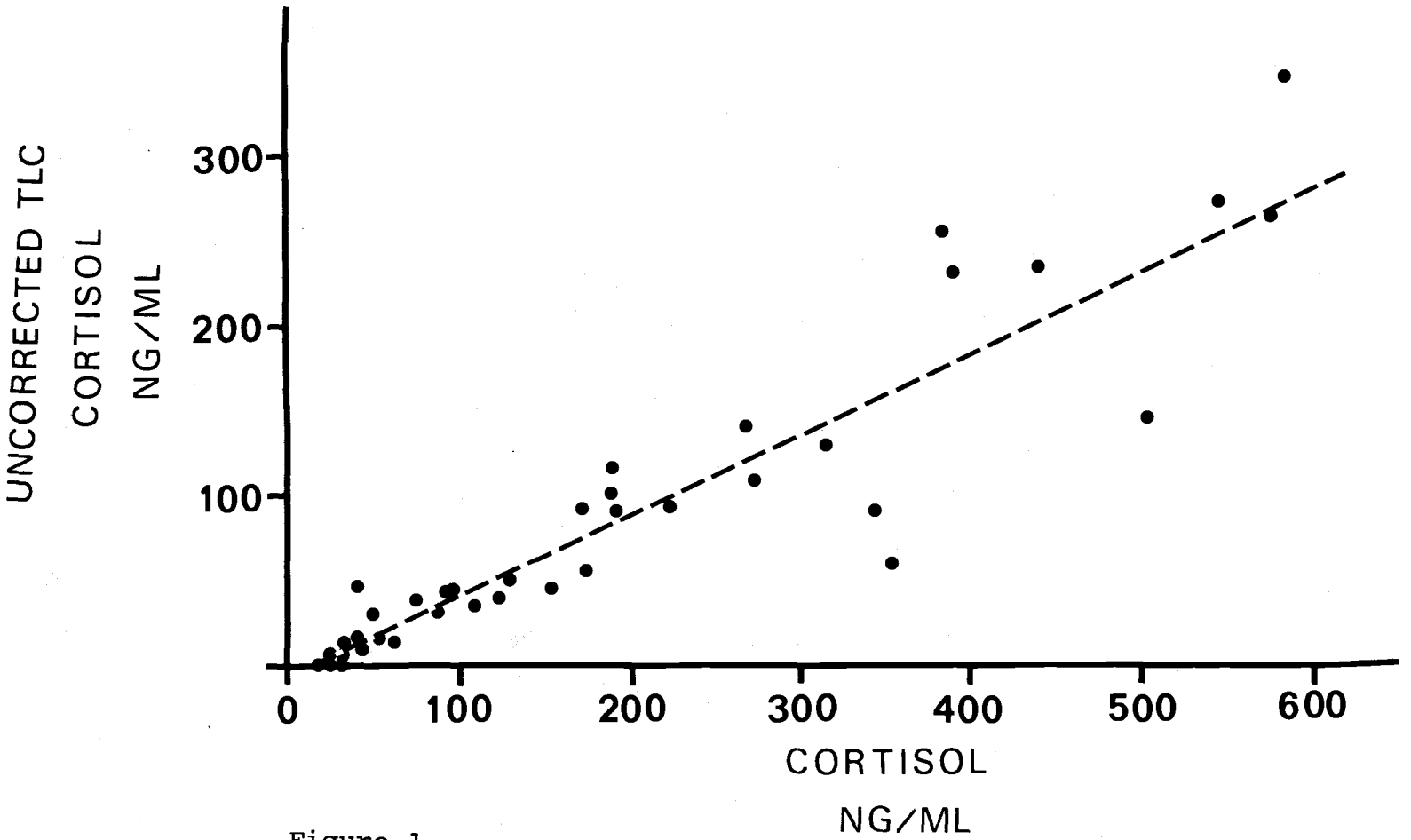
UNCORRECTED TLC
CORTISOL
NG/ML
300
200
100
0
100
200
300
400
500
600
CORTISOL
NG/ML

Figure 1

Figure 2. Plasma cortisol concentration over time of juvenile chinook salmon placed in an aerated bucket and sampled at 90-s intervals. Each point represents one fish. The illustrated linear regression for the first 16 samples indicates a rate of increase of 5 ng/ml per min.

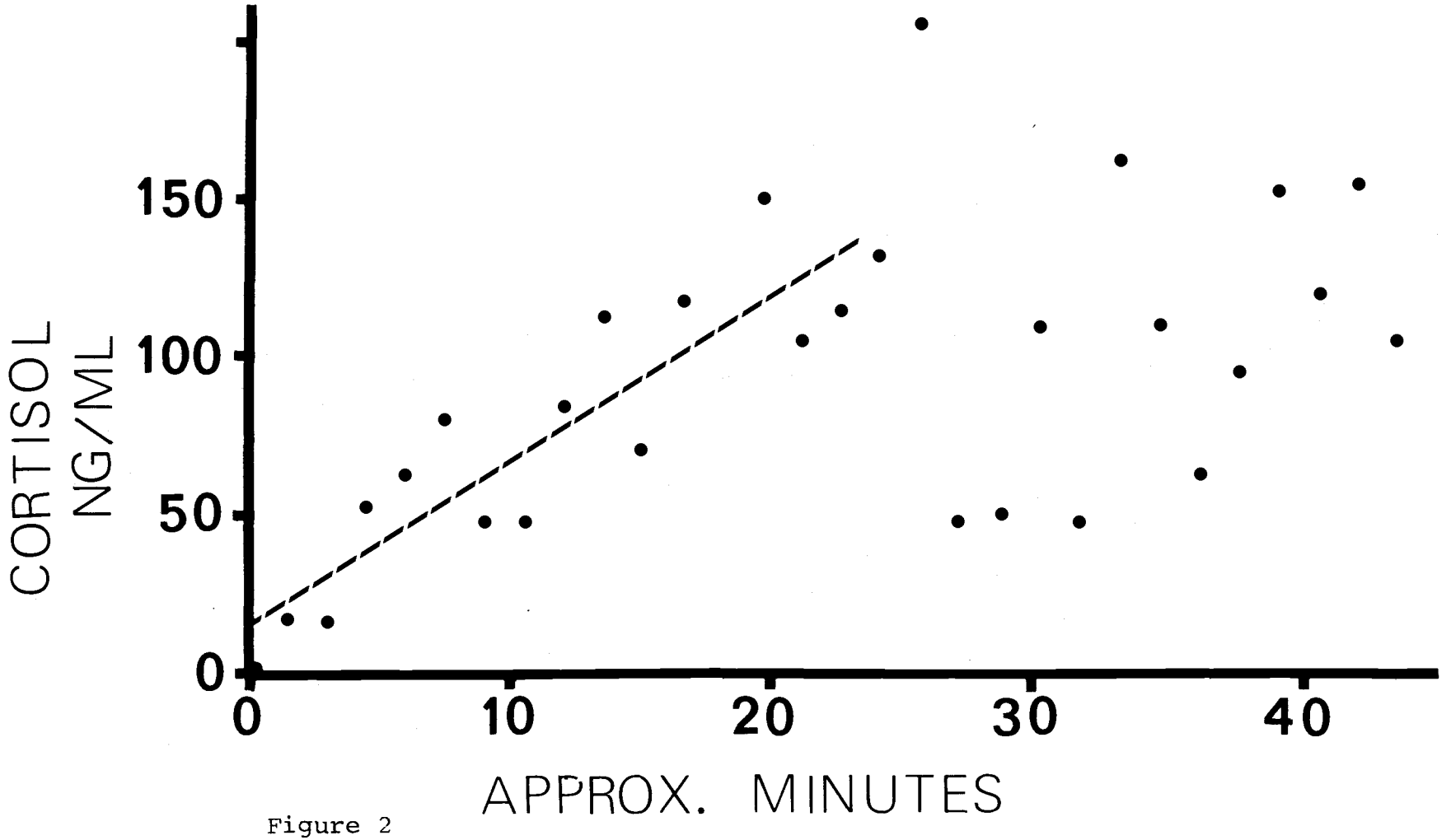

Figure 2

about 5 ng/ml per min; after the initial rapid rise, the rate slowed substantially. No fish died as a result of the stressor.

Response to Further Confinement

About 100 juvenile chinook salmon (14-17 cm long) were acclimated for 2 weeks in two identical 2 m circular tanks at 11 C. At time 0, or 1045 h, 20 October 1975, I dip-netted the experimental fish into a small live-cage (0.25 m x 0.25 m x 1.00 m) suspended in their tank. Groups of fish were taken from the live-cage and the control tank at 0, 1, 6, 12, 18, 24 and 48 h. Fish netted and then confined in a live-cage had a continuous increase in cortisol concentration from about 100 ng/ml to about 500 ng/ml in the first 24 h; at 48 h the concentration had fallen to about 250 ng/ml (Fig. 3). Cortisol concentration in the controls fluctuated below 100 ng/ml. There was no overlap in 95% confidence intervals of experimental and control salmon after time 0. The degree of stress involved in this trial is indicated by cumulative mortality in the caged fish, which reached 20% in 48 h (Fig. 2).

Thermal Shock

Six cutthroat trout (13 cm) were acclimated to a constant temperature of 13 C in each of six 140-liter, temperature controlled tanks. At time 0, or 0745 h, 23 February 1976, the temperature

Figure 3. Plasma cortisol concentration (lower panel) of juvenile chinook salmon confined in a small live-cage (solid line) and unconfined (broken line). Short horizontal lines indicate standard errors for means of eight individual values. Cumulative mortality of the confined fish is shown in the upper panel.

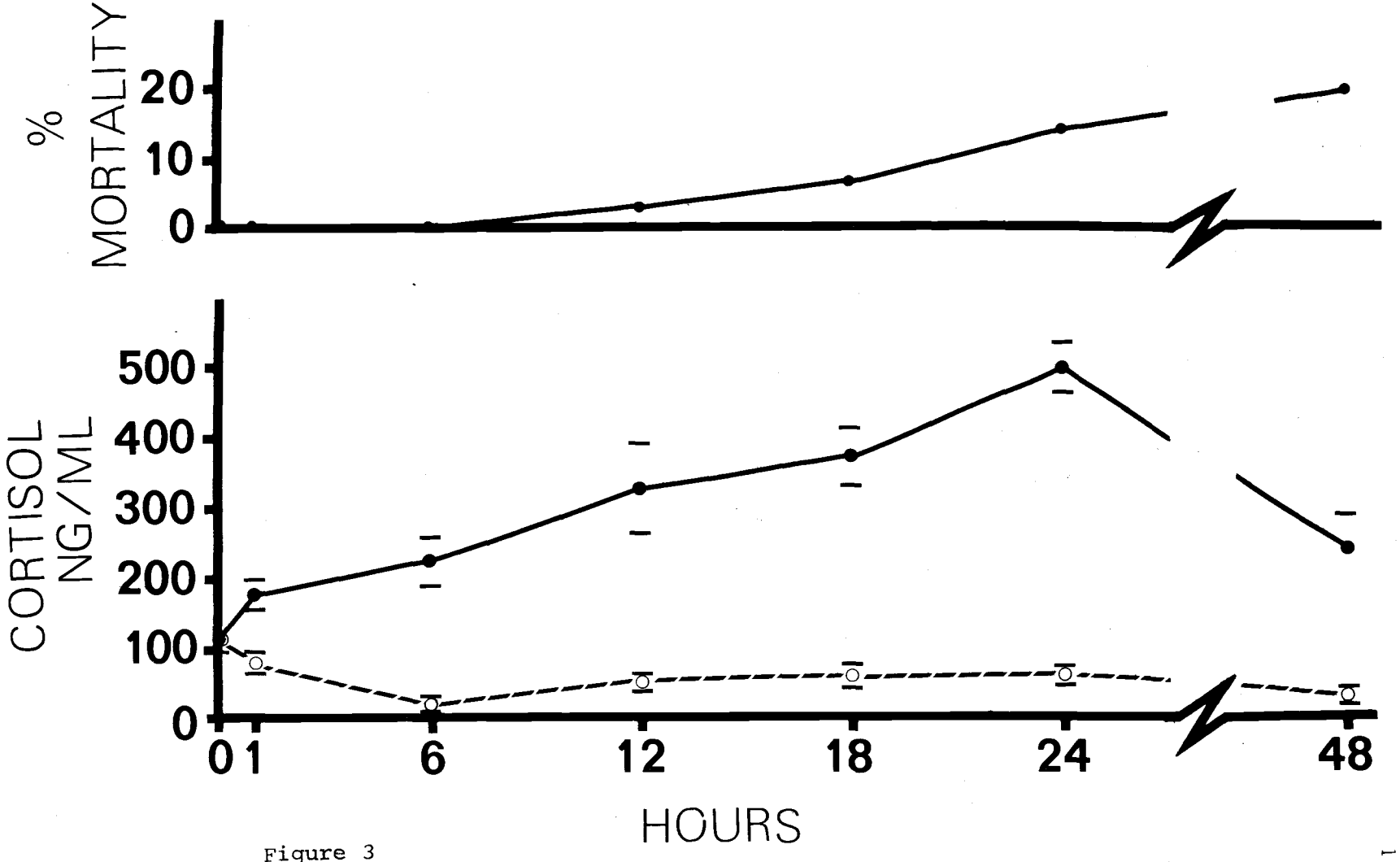
% MORTALITY
20
10
0
CORTISOL NG/ML
500
400
300
200
100
0
0 1
6
12
18
24
48
HOURS

Figure 3

was increased to 26 C in 20 min, and this temperature was maintained for the duration of the experiment. Fish were sampled from a different one of the 6 tanks at 0, 25, 55, 85, 165, and 210 min; the last sampling time was approximately one-half of the median survival time (6 h) for this severe heat stress. Plasma cortisol concentrations increased from about 20 ng/ml to about 70 ng/ml in 25 min and except for a dip at 60 min, remained at this level for more than 3 h with no further increase (Fig. 4).

Diurnal Temperature Fluctuations

Groups of 17 cm cutthroat trout were acclimated in 140-liter tanks to three different diurnal temperature regimes well within the zone of tolerance for cutthroat trout. In one tank, the 24 h temperature cycle ranged from 13 C at 0300 to 23 C at 1500 and back to 13 C at 0300. In the second tank, the temperature was offset by 12 h. A control tank (C-1) was held constant at 13 C. Three other control tanks (C-2) held constant at 13 C were sampled once each during the experiment to reveal any effect of repetitive sampling at 6-h intervals on the fish held in control tank C-1. I sampled six fish from each of the fluctuating temperature tanks and the control tanks at 6-h intervals through a 24-h period, beginning at 1500 on 12 May 1976. There was no substantial difference in plasma cortisol levels between any of the groups (Fig. 5). Fish under all conditions maintained relatively

Figure 4. Plasma cortisol concentration over time (lower panel) of juvenile cutthroat trout acclimated to 13 C and subjected to a rapid increase in water temperature (upper panel) to 26 C. Short horizontal lines indicate standard errors for means of six individual values.

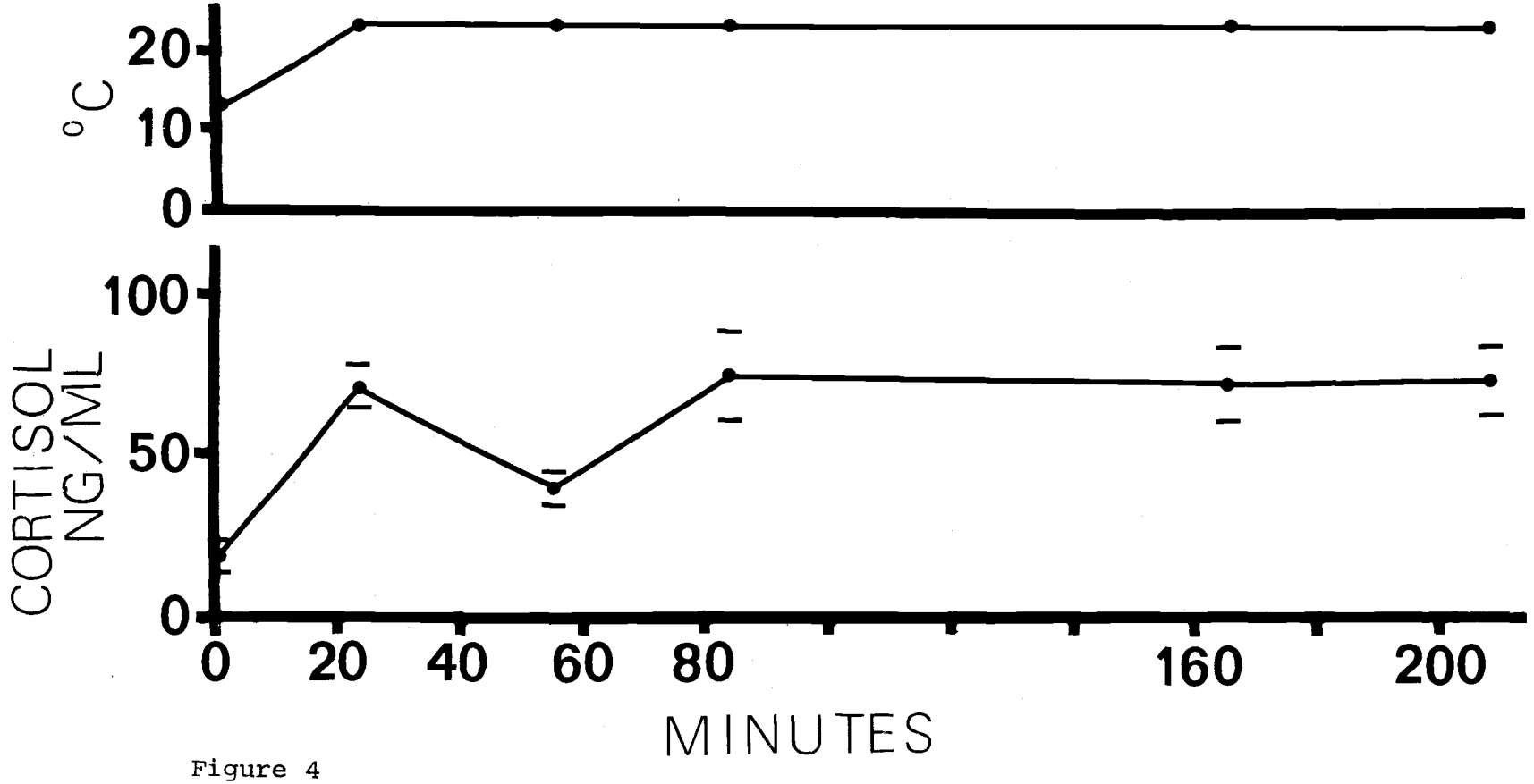

Figure 4

Figure 5. Plasma cortisol concentration (lower panel) over a 24-h period of juvenile cutthroat trout acclimated to different water temperature regimes: 13 C constant [open circles denote controls (C-1), solid squares denote different populations sampled each time C-2)] ; 13-23 C normal (N) fluctuating (solid circles); and 13-23 C reverse (R) fluctuating (open squares). Standard errors are indicated around means of six individual values. Water temperatures for the different groups are also shown.

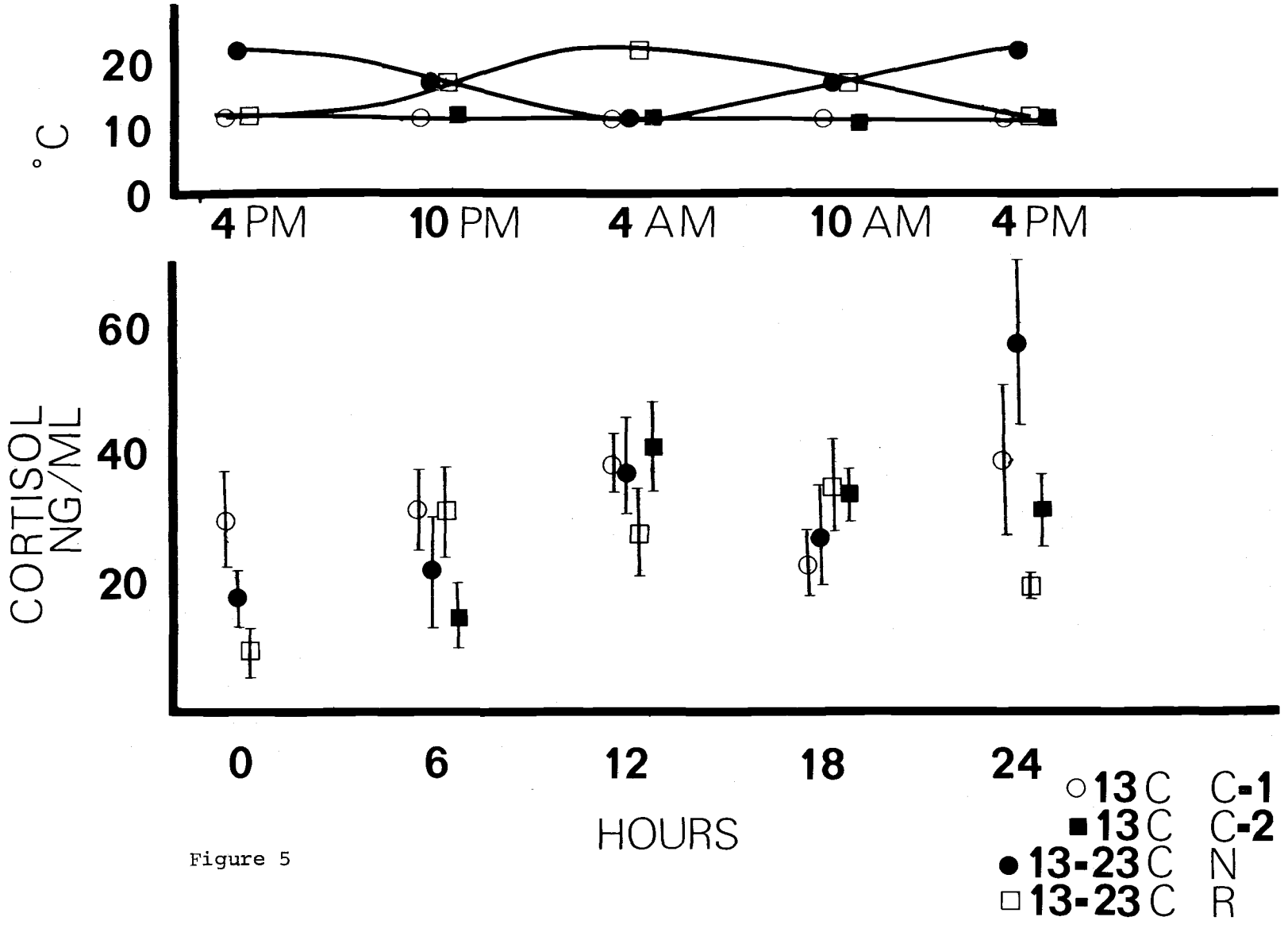
°C
20
10
0
4 PM
10 PM
4 AM
10 AM
4 PM
CORTISOL NG/ML
60
40
20
0
6
12
18
24
HOURS
○ 13 C C-1
■ 13 C C-2
● 13-23 C N
□ 13-23 C R

Figure 5

low concentrations throughout the day with no evidence of diurnal cycling. There was no difference in cortisol levels between control fish sampled repeatedly out of the same tank and fish sampled out of individual tanks.

Temperature: Effect on Response to Confinement

Two groups of cutthroat trout (9-10 cm) were acclimated to water temperatures of 9 C and 23 C. At time 0, or 0945 h, 23 February 1976, I sampled six fish from each temperature and dip-netted groups of six fish into a number of small 5.5-liter perforated buckets suspended in their respective tanks, severely confining the fish at their acclimation temperature. I sampled one different bucket of fish from each temperature at 10, 20, 40, and 70 min after confinement. Mean cortisol concentrations of juvenile cutthroat trout acclimated to different temperatures were the same (about 20 ng/ml) initially (Fig. 6). The warm and cool-water fish responded to handling similarly until the 70 min sample when fish in 23 C water failed to maintain an increasing cortisol concentration in response to continued confinement.

Discussion

After the onset of handling, there is an immediate, rapid rise in plasma cortisol from low basal levels to about 100 ng/ml (Fig. 2).

Figure 6. Plasma cortisol concentration over time of a confined juvenile cutthroat trout acclimated to 9 C (broken line) or 23 C (solid line). Standard errors are indicated around means of six individual values.

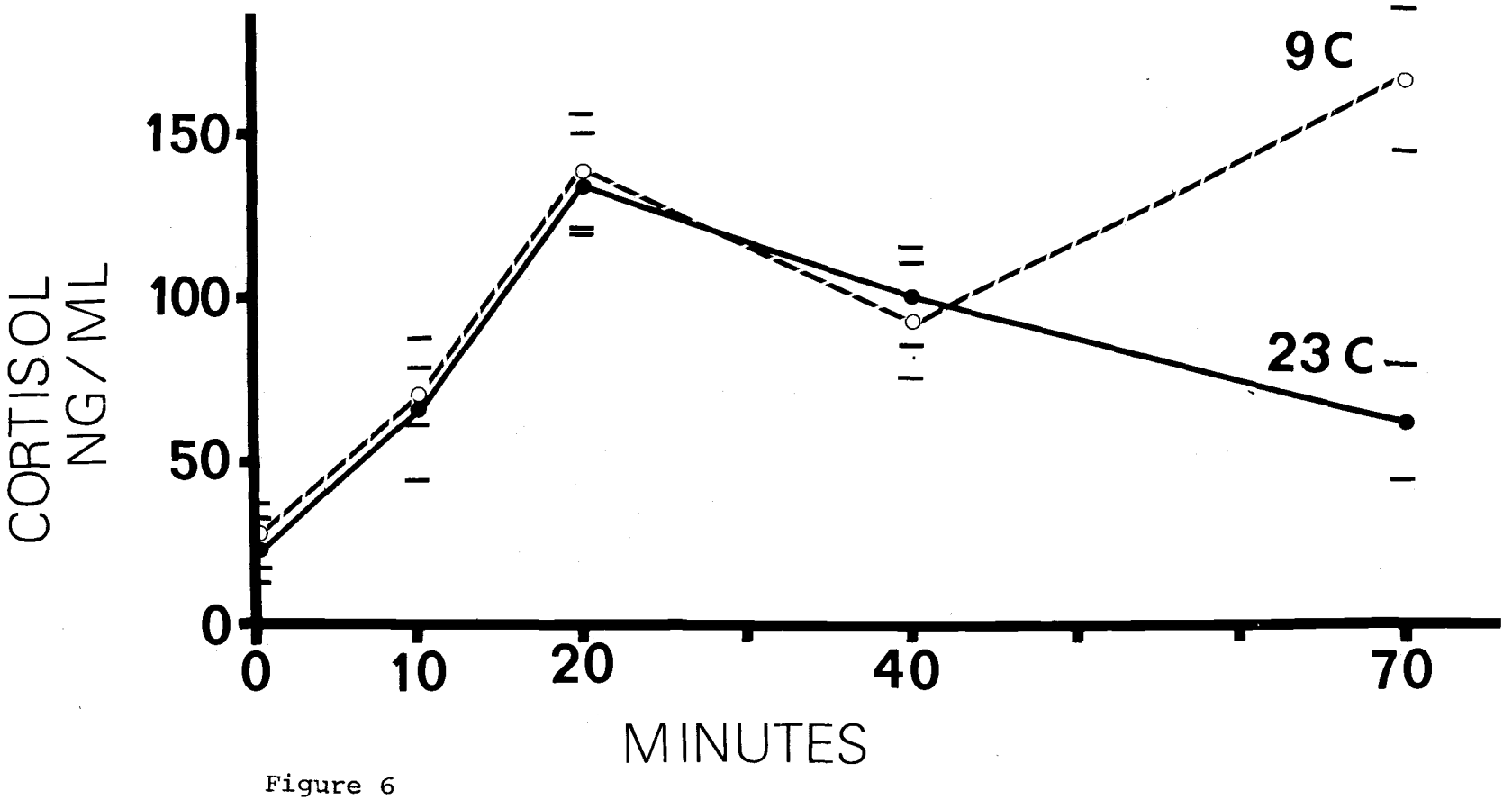

Figure 6

The scatter in Figure 2 indicates the considerable individual variability that is associated with the changes in cortisol during stress. The initial rapid rise is followed by a much slower but steady increase for at least 24 h (Fig. 3). I suspect the relatively elevated time 0 concentration in Figure 2 was due to about 1 h of prepatory activity that occurred around the tanks. In later trials, greater care was taken not to disturb the animals; however, it is impossible to know if one is measuring a true basal level of cortisol because some handling is necessary in order to obtain a blood sample. In some of the samples taken most rapidly, cortisol concentration was near or lower than our limit of detection (10 ng/ml), indicating that plasma concentrations of cortisol can be low in acclimated fish. However, "basal" levels can vary considerably. Pickford (1973) and Singley and Chavin (1975a) reported that cortisol becomes elevated by stress within seconds or minutes, similar to our findings. Others, however, suggested that handling or forced exercise caused a relatively slow (minutes to hours) interrenal response (Hill and Fromm 1968; Wedemeyer 1969; Spieler 1974). A few studies even indicate that netting and transporting (Wedemeyer 1972), transplanting and stocking (Gresswell 1973), and herbicides (Berry 1975) did not induce an interrenal secretion.

The response to a sudden increase in temperature in cutthroat trout (Fig. 4) seems similar to the immediate handling response in

chinook salmon (Fig. 2) in the initial rapid rise of cortisol. However, handling might be expected to generate a greater and continued increase than would temperature. A possible moderating effect of heat on the rise in cortisol concentration is also indicated in Figure 6, where fish in warm water failed to maintain an increasing cortisol concentration in response to continued handling, compared to fish in cool water.

Time 0 cortisol concentrations in fish acclimated to 9 C and 23 C were almost identical (Fig. 6) and fish acclimated to two opposed diurnal temperature cycles and to constant temperature had about the same concentrations over a 24-h period (Fig. 5), indicating that cortisol concentrations are similar in fish acclimated to different temperatures. However, fish acclimated to one temperature and subjected to a higher temperature (Fig. 4) displayed a cortisol response. Wedemeyer (1973) found that thermal shock depleted interrenals of coho salmon (Oncorhynchus kisutch) but not rainbow trout (Salmo gairdneri).

I found no evidence of a diurnal cycle in cortisol levels in either the moderate term handling study with chinook salmon or the diurnal temperature fluctuation study with cutthroat trout. Boehlke et al. (1966) reported that total glucocorticoids cycle over 24 h but that cortisol does not in channel catfish (Ictalurus punctatus). A daily rhythm in plasma cortisol was suggested for Gulf killifish (Fundulus

grandis) (Srivastava and Meier 1972; Garcia and Meier 1973; Meier and Srivastava 1975) and the goldfish (Singley and Chavin 1971, 1975b). Perhaps between-fish variation in cortisol of salmonids precludes detection of a rhythm, if indeed one is present. Pickford (1973) stated that the range in variation in cortisol was too great to allow detection of circadian cycles in mummichog (Fundulus heteroclitus). Data presented by Donaldson and Dye (1975) are not indicative of the presence of a diurnal cycle in cortisol of sockeye salmon (O. nerka).

Plasma concentration of cortisol is a function of secretion and clearance; changes in the balance between these two processes produced the alterations in concentration we observed; however, the exact mechanisms behind the cortisol dynamics of stressed fish await further elucidation. Additionally, changes in cortisol concentrations that occur between the acute stages of stress demonstrated in these trials and eventual acclimation or death need to be examined. However, plasma cortisol concentration does appear to be a sensitive reflection of the fish's response to environmental change and as such may have potential as an indicator of deviations from basal conditions.

III. CHRONIC CHANGES IN CORTISOL DURING CONFINEMENT AND EFFECTS ON GILL Na+K ATPase

Introduction

Increased plasma cortisol concentration in fishes after the onset of confinement or similar handling-fright type stress is well documented (Hane et al. 1966, Donaldson and MacBride 1967, Fagerlund and Donaldson 1970, Grant and Mehrle 1973, Fuller et al. 1974, Nemeth and Jurani 1974, Spieler 1974, Mazeaud et al. 1977). Much less is known, however, about the dynamics of cortisol concentration over time during a continuous stressor or after recovery from a short term stressor. Experimentation on cortisol concentration over time in stressed fishes has been limited to time intervals of less than 48 h, and the investigations have ended with plasma cortisol concentrations still elevated and only moderate, if any, mortality (Redgate 1974, Fryer 1975, Strange et al. 1977). This leaves open to question the nature of changes in cortisol concentration in fish that recover from a brief encounter with a severe stressor; that die from continuous exposure to a severe stressor; or that acclimate to continuous exposure to a moderate stressor. I conducted a series of experiments designed to elucidate changes in cortisol concentration from before the onset of a stressor until recovery, death, or acclimation. I felt that cortisol, a primary response to stress, would be

more sensitive and perhaps less complex than secondary responses (e.g., blood glucose, lactate). I measured gill Na+K ATPase, an enzyme necessary for saltwater adaptation, in juvenile salmon that had acclimated to a moderate stressor to determine a possibly deleterious effect of sublethal stress on the activity of this enzyme. A stress effect on ATPase is a real possibility, for as Epstein et al. (1971) have shown, gill enzyme activity may be under cortisol control.

Methods

Juvenile (0+ to 1+ yr) spring chinook salmon (Oncorhynchus tshawytscha) from the Rogue or Umpqua rivers were acclimated to circular tanks supplied with flowing 12 C well water for at least 2 weeks prior to experimentation. Plasma samples for cortisol assay were obtained from the fish, killed by a blow to the head, by severing the caudal peduncle and collecting the blood in heparinized capillary tubes. After centrifugation, samples were stored frozen. I determined plasma cortisol concentration by a competitive protein binding assay adopted from Murphy (1967) using a simplified method of preparing plasma for assay (Methods, Section V). Na+K ATPase was determined immediately on whole homogenate of gill filament tissue and related to protein content of the homogenate to yield a specific activity (Ewing and Johnson 1976).

Experimental Designs and Results

Severe Confinement: Short Term

At 0830 on 20 October 1976, 6 fish were sampled for plasma from a group of about 50 salmon (0+ yr, 10-15 cm) acclimated to a 90 cm diameter circular tank. The remaining fish were immediately netted into a small (10 cm x 30 cm, immersed to 15 cm) dip net suspended in the tank; this degree of confinement forced the fish into contact with each other and the net. After 0.5 h of confinement, 6 fish were sampled and the rest were released back into the tank. Six fish were then sampled at 1.5, 3.5, 6.5, 12.5, 24 and 48 h after confinement.

Plasma cortisol increased rapidly in response to severe confinement and remained elevated for over 6 h after release (Fig. 7). By 12 h after release, cortisol had returned to near basal levels. No mortalities occurred during the 0.5 h of confinement; however, moribund fish were present at 1.5 h (1 fish), 3.5 h (2 fish), and 6.5 h (2 fish).

Severe Confinement: Continuous

At 0930 on 25 May 1977, 4 fish were sampled for plasma from a group of about 45 salmon (1+ yr, 13-21 cm) acclimated to a 90 cm diameter circular tank. The remaining fish were immediately netted

Figure 7. Plasma cortisol concentration of juvenile chinook salmon subjected to 0.5-h of severe confinement in a dip net and then released. Shaded area indicates period of confinement. Standard errors are indicated around means of six.

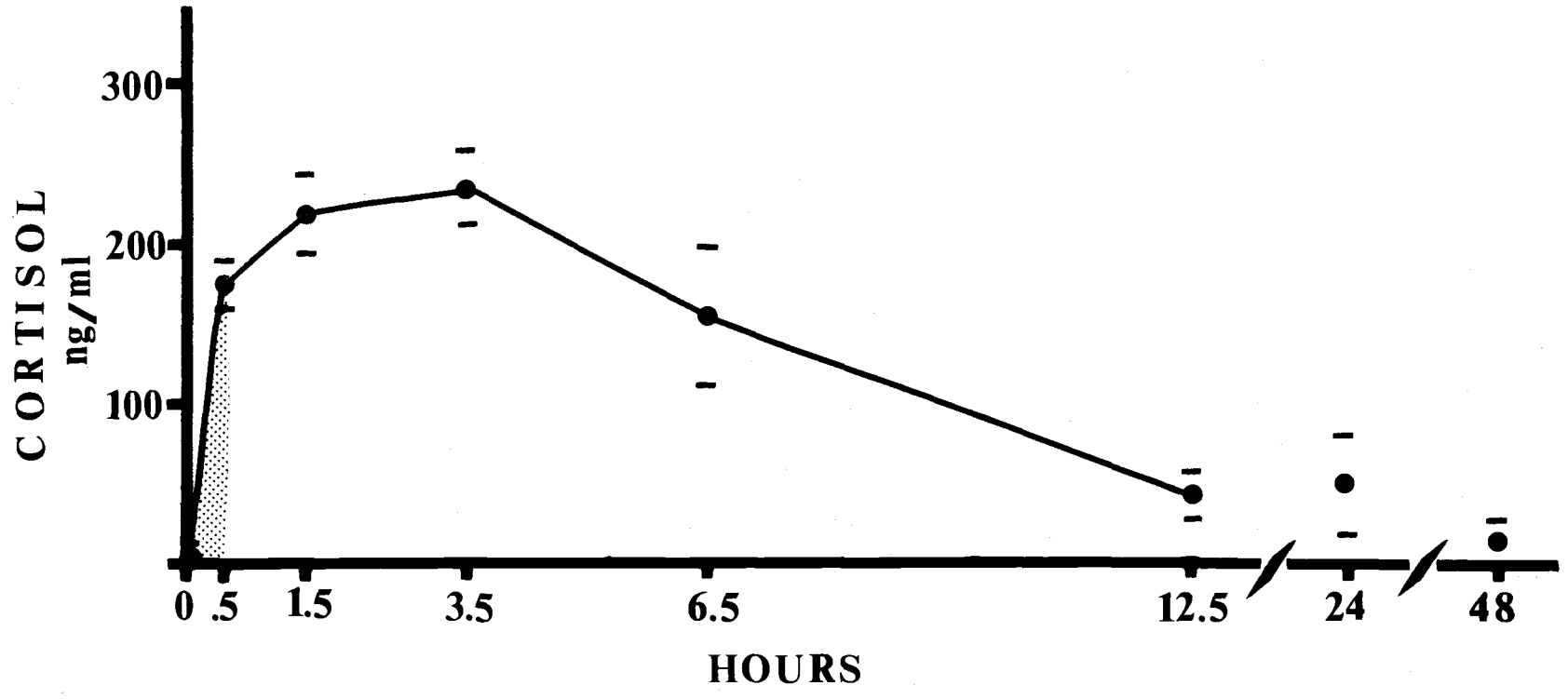

Figure 7

into a small (10 cm x 30 cm, immersed to 15 cm) dip net suspended in the tank forcing the fish into continuous contact. Six fish were sampled at 0.5, 1.5, 3.5, 6.5, and 12.5 h after confinement. Four unconfined fish from an adjacent 90 cm tank were sampled at 0, 3.5, and 12.5 h to serve as controls.

At first, plasma cortisol concentration increased rapidly in response to severe confinement; after 1.5 h, the rate of increase slowed and there was little change in plasma cortisol between the 6.5 and 12.5 h samples (Fig. 8). At 0.5 h after confinement, one fish with a spinal deformity was dead. Several fish were moribund at the 6.5 h sample, and by the 12.5 h sample--when the experiment was terminated--there were 8 alive, 4 dying, and 4 dead, approximating a median tolerance limit (TLm) of 12 h.

Moderate Confinement: Continuous

At 1330 on 21 July 1976, 6 fish were sampled from approximately 150 chinook salmon (0+yr. 10-13 cm) acclimated to a 150 cm diameter circular tank. This degree of confinement allowed each fish a small amount of space without necessarily touching the cage or another fish. Six fish from the live cage and 6 from an adjacent holding tank (controls) were sampled at about 1300-h each day for the next two weeks. Fish sampled from the live cage were replaced with fin-clipped individuals to maintain a consistent density. After

Figure 8. Plasma cortisol concentration of juvenile chinook salmon subjected to continuous severe confinement in a dip net (circles) and unconfined controls (squares). One-half of the confined fish were dead or dying at the 12.5 h sample. Standard errors are indicated around means of four (controls) or six (experimental).

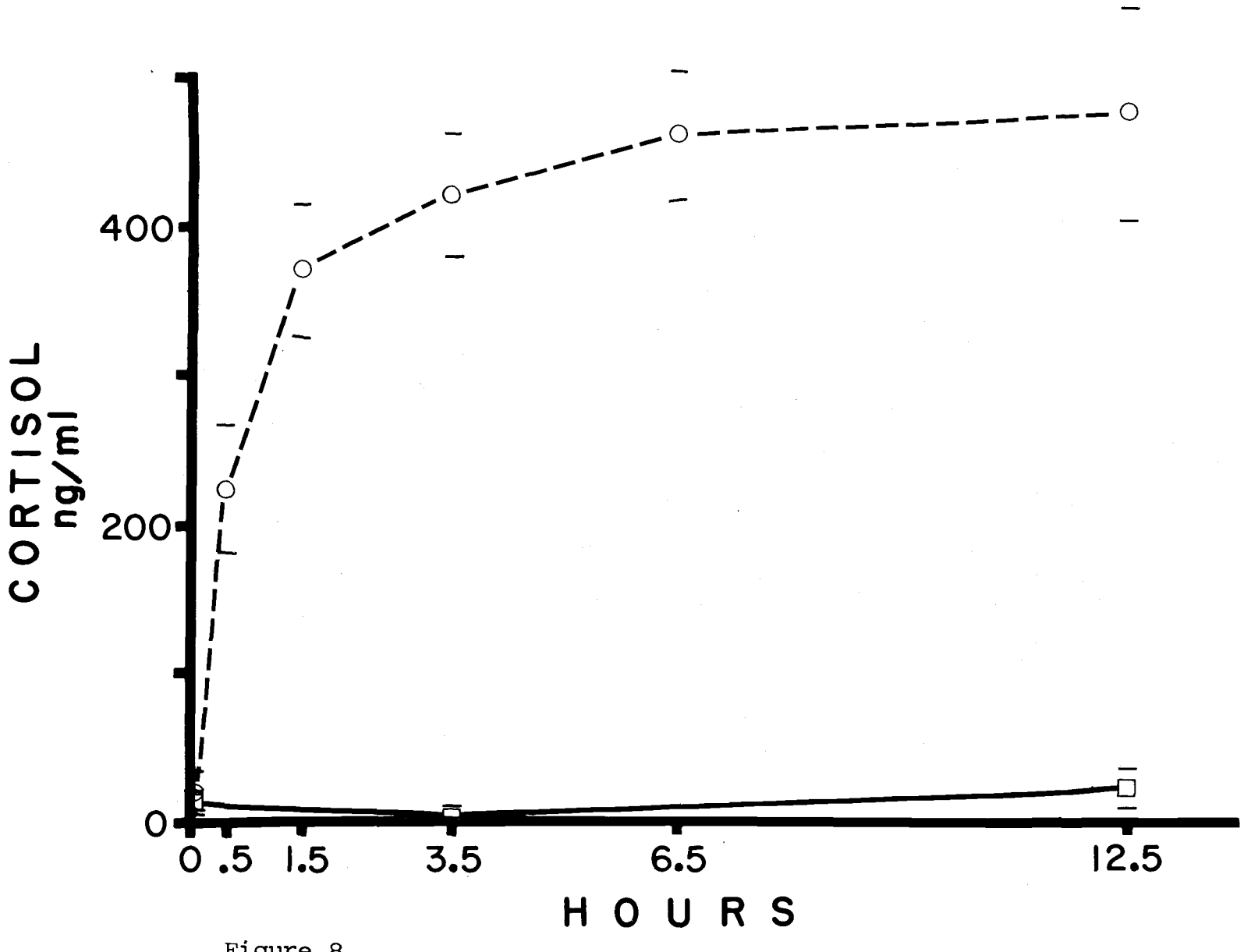

Figure 8

the day 14 sample, the experimental fish were released from the live cage and a sample was taken the next day (day 15) to assess the effect of release on fish that had been allowed to acclimate to a confined situation.

The experimental fish showed a definite but variable elevation in plasma cortisol concentration for several days after confinement with all confined fish having higher cortisol levels than all control fish on days 1 and 2 (Fig. 9). After day 4, most of the confined fish had cortisol concentrations comparable to control fish, all confined fish had cortisol levels near basal after day 8. During the period of elevated cortisol concentration in the confined fish, 1 dead and 3 moribund fish were noted. Two unexplained mortalities (one on day 3, one on day 7) occurred in the holding facility that was the source of controls during the experiment; however, there were over 1,000 fish in this tank and a small amount of mortality was not unusual. Release from the live-cage on day 14 caused no cortisol response evident on day 15.

Moderate Confinement: Effect on Gill Na+K ATPase

Experiment 1. On 9 September 1976, after determination of gill Na+K ATPase activity on a sample of 30 fish, 123 salmon (0+ yr, 8-11 cm) were counted into a bucket and then confined in a small live-cage (20 cm x 30 cm x 45 cm) suspended in their original 150 cm

Figure 9. Plasma cortisol concentration of a juvenile chinook salmon subjected to moderate confinement in a small live-cage (solid circles) or no confinement (open circles). Boxed circles indicate moribund fish. Solid line traces the means of confined fish and broken line the means of unconfined fish.

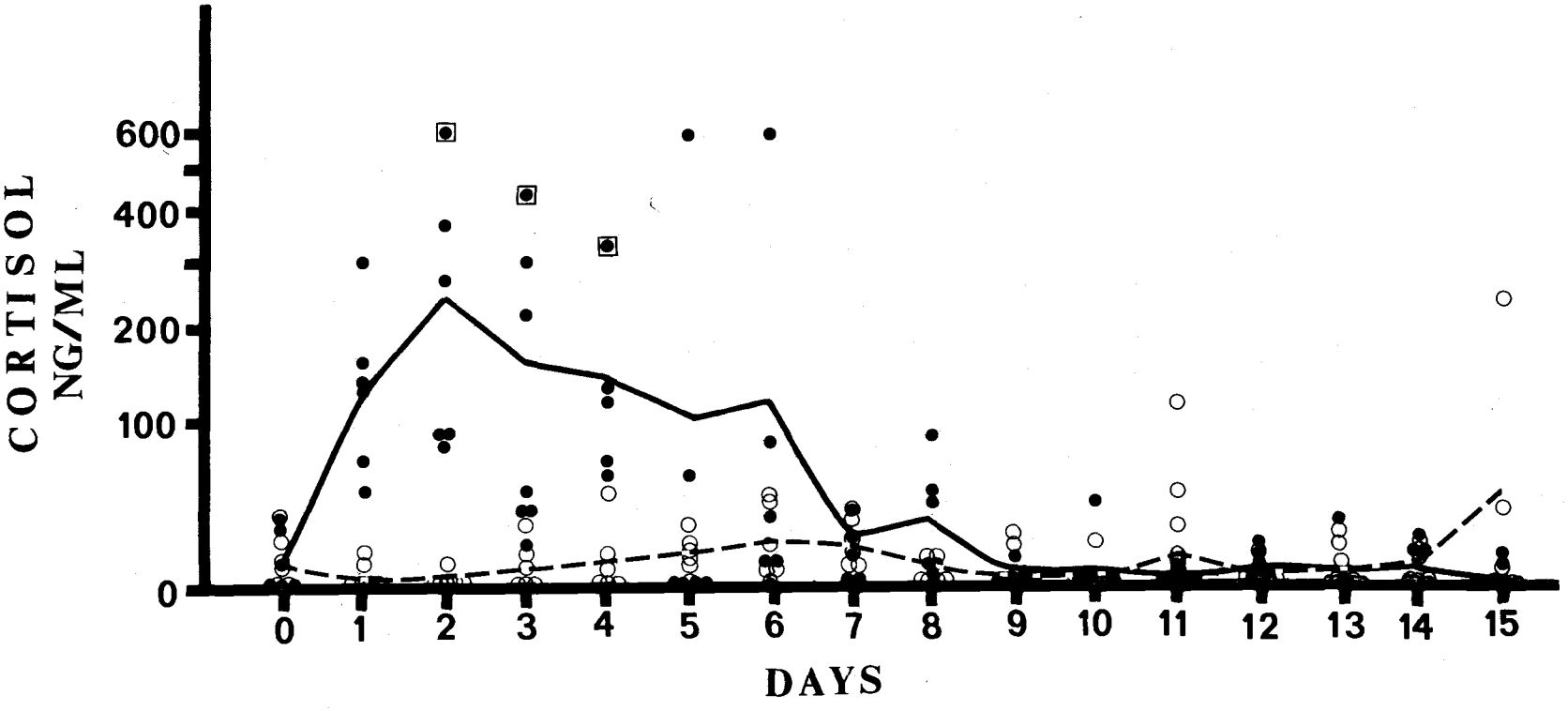
CORTISOL
NG/ML
600
400
200
100
0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
DAYS

Figure 9

holding tank. Fish maintained as usual in the holding tank were used as controls. Six fish were sampled for plasma from confined and control groups before confinement and 1, 3, 5, 7, 14, 21, and 26 days after confinement in order to document the changes in cortisol. On 5 October, 26 days after confinement, specific activity of gill Na+K ATPase was determined in a sample of 20 from the confined and control fish.

Plasma cortisol concentration was elevated in the confined fish during the first week returning to basal levels within 2 weeks; no change in cortisol occurred in the control fish. The mean of ATPase activity in the confined fish was substantially below the mean in the control fish, and there was no overlap of 95% confidence intervals (Fig. 10); however, the confined fish were smaller because they refused food during acclimation and gill Na+K ATPase is, to an extent, size dependent. An attempt to correct for the larger average size of the control fish was made by excluding fish over 11 cm in length from the control data. This produced similar average lengths for fish from both treatments, with ATPase activity in the stressed group still somewhat lower than the control group; however, the confidence intervals around the means of specific activity overlap.

Experiment 2. In an attempt to separate effects of reduced growth from stress effects on gill ATPase, starved as well as fed controls were used in this experiment. Starved controls and confined

Figure 10. Lower graph illustrates plasma cortisol concentration of juvenile chinook salmon subjected to moderate confinement in a small live cage (solid circles) and no confinement (open circles). Standard errors are indicated around means of six. Upper graph shows gill Na + K ATPase (wide bars) and length (narrow bars) of the fish before confinement (B), in fish stressed by confinement (S), in controls (C), and in controls less than 11 cm (C < 11). Post-confinement ATPase measurements were made on day 26. Standard errors are indicated around means of 20 or 30.

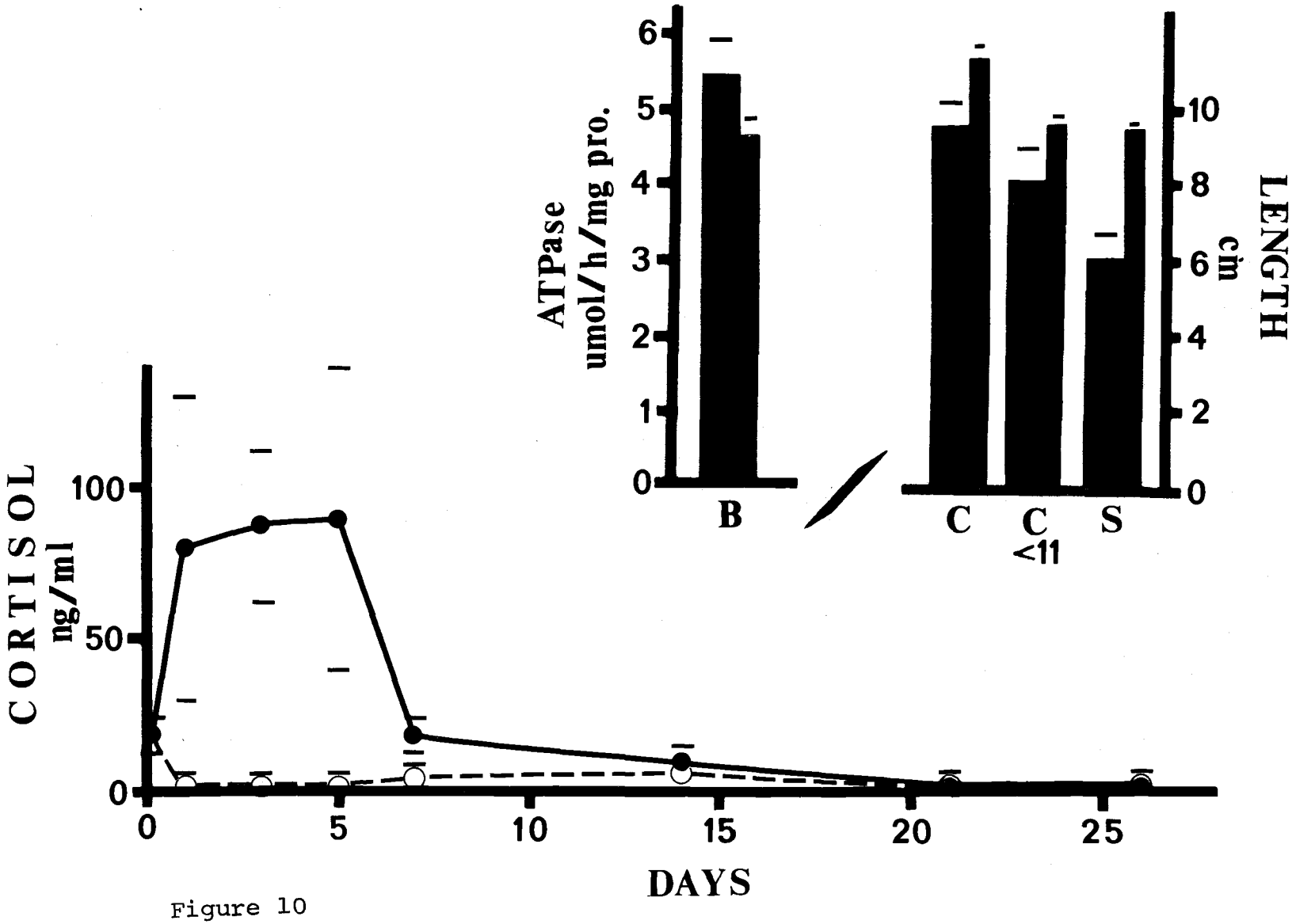
CORTISOL
ng/ml
100
50
0
0
5
10
15
20
25
DAYS
ATPase
umol/h/mg pro.
6
5
4
3
2
1
0
B
LENGTH
cm
10
8
6
4
2
0
C
C
<11
S

Figure 10

fish received no food while fed controls were fed daily. On the morning of 10 October 1976, 20 salmon (0+ yr, 9-13 cm) were sampled from each of two groups; one group served as the starved control and one as the fed control. Blood for cortisol determination was taken from the first six fish sampled from each group, and gill Na+K ATPase determined on all 20. After this sample, fish were taken from both these groups and a third group with an identical history and put into a small (20 cm x 30 cm x 45 cm) live-cage. On days 1, 2, 3, and 7 after confinement, six fish were sampled for plasma cortisol determination from the confined group and the starved controls. On day 16, 35, and 58, twenty fish from each group (confined, starved controls, fed controls) were sampled for cortisol and ATPase as on the first day except that on day 16 fifteen instead of twenty fish were sampled from the confined group.

Plasma cortisol concentration was elevated in the experimental fish during the first week, by day 16 cortisol had returned to basal levels (Fig. 11). The starved controls showed no cortisol response, and cortisol was always low in the fed controls. Specific activity of gill Na+K ATPase decreased in all groups during the experiment (Table 1). The greatest decline occurred in the confined fish and the least in the fed controls. The fed controls grew steadily and the stressed group did not grow. Unfortunately, sampling error

Figure 11. Plasma cortisol concentration of starved (open circles) and fed (squares) controls and moderately confined fish (solid circles) in experiment 2 on stress and ATPase.

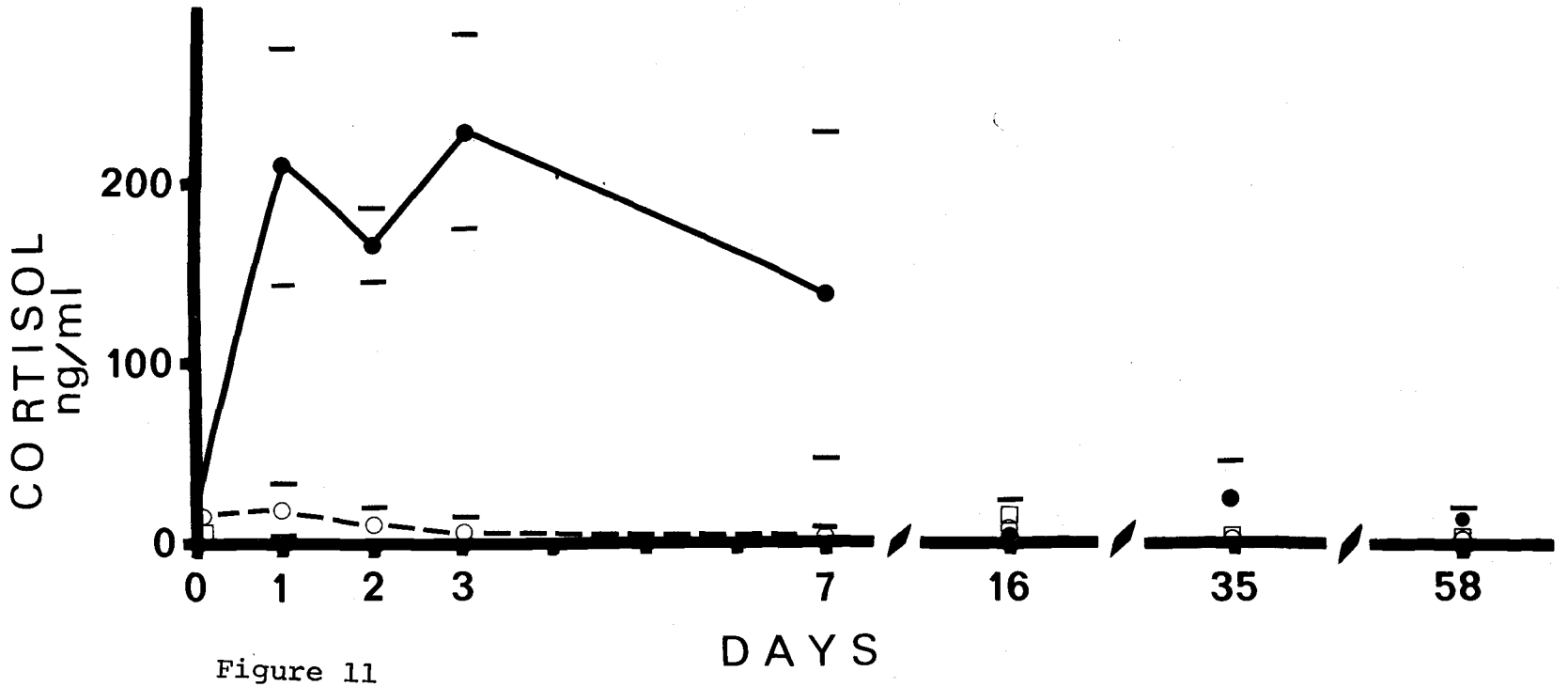

Figure 11

Table 1. Mean (±S. E.) gill Na+K ATPase specific activity (μmol/h/mg protein) and length (cm) in chinook salmon subjected to moderate confinement (Fig. 4, also). ATPase and length is presented for fed and starved unconfined fish as well.

	Days			
	0	16	35	58
fed control				
ATPase	4.3 ± .5	2.9 ± .4	3.3 ± .4	2.9 ± .3
length	11.2 ± .4	11.4 ± .4	12.8 ± .5	14.2 ± .5
starved control				
ATPase	4.4 ± .5	2.2 ± .4	1.7 ± .3	2.5 ± .3
length	11.0 ± .4	10.5 ± .4	10.8 ± .3	13.0 ± .3
confined				
ATPase	-	2.1 ± .2	0.9 ± .1	1.0 ± .1
length	-	10.7 ± .3	10.0 ± .3	10.6 ± .3

resulted in an apparent increase in the mean size of starved fish in the day 58 sample.

Discussion

Previous work on the dynamics of cortisol concentration over time in fishes subjected to handling-fright type stressors has involved mild to moderate (not rapidly lethal) stressors over relatively short periods. Redgate (1974) reported a steady increase in plasma cortisol concentration in carp sampled up to 25 min after the onset of continuous alarm produced by a struggling fish suspended in the experimental tank. Strange et al. (1977) found that moderate confinement elevated plasma cortisol in juvenile salmon for at least 48 h. In the investigations presented here, the continuous moderate confinement experiment (Fig. 9) as well as the cortisol data from the experiments on the effect of confinement on ATPase (Fig. 10 and 11) clearly demonstrate how plasma cortisol concentrations increased in response to a moderate stressor and then returned to prestress levels as the fish acclimated, the type of response classified as ideal adaptation by Precht (1958). Mortality associated with stressors always occurred while cortisol was elevated. Resumption of normal feeding activity coincided with the decline of cortisol to basal levels. The period of acclimation to a moderate confinement stressor, during which cortisol concentration was elevated and fish refused food, was

about one week. Acclimation to a similar new and noxious situation would be expected to take about the same time, while recovery from a brief stressor would take a much shorter time even if the brief stressor was more severe (Fig. 7). The change in cortisol during continuous moderate confinement differed from the change during continuous severe (rapidly lethal) confinement in the variability associated with the individual cortisol values. On day 3 in the continuous moderate confinement experiment cortisol concentrations range from almost basal to near the maximum level found in juvenile chinook salmon (note also the large standard errors for moderately confined fish in Figures 10 and 11). In the continuous severe confinement experiment there were no very low cortisol levels associated with the period of stress, all fish responding near their biological maximum (note small standard errors in Figure 8). This can be related to mortality in that most fish survive indefinitely under moderate confinement while severe confinement is rapidly lethal. Every fish we have sampled that was dying from a handling-fright type stressor has had substantially elevated plasma cortisol concentration, while well acclimated fish nearly always have levels of cortisol below 50 ng/ml and very often the levels are below our limit of detection (10 ng/ml).

The confinement stressors used in these investigations are probably comparable to most handling procedures used in fisheries management in the changes in cortisol they produce; however, other

stressors notably more covert pollution-type challenges such as cadmium or an antibiotic may induce different changes in cortisol or no changes at all (Schreck et al. unpublished data, MacBride et al. 1975). More overt pollution-type stressors that are recognized by the fish such as copper or kraft mill effluent produce a change in cortisol more similar to confinement (Schreck et al. unpublished data, Donaldson and Dye 1975, Dye and Donaldson 1974). Changes in cortisol during different stressors are variable, moreover, ideal acclimation (return of cortisol to basal levels) occurs in fish subjected to moderate confinement. These complications prevent cortisol or secondary responses related to cortisol (e. g., blood glucose, lactate) from being used as a "litmus test" for stress. Cortisol concentration, however, can be used in a controlled, experimental situation to assess stress and evaluate ways to reduce it (Strange and Schreck, in press).

In both experiment 1 and 2 on the effect of stress on gill Na+K ATPase, there was some decline in enzyme activity in the fed controls because late autumn is typically a period of normal decline in gill ATPase activity in Rogue stock spring chinook salmon. In experiment 2, ATPase activity in the starved controls and particularly in the stressed fish reached lower levels than in the fed controls. A mean specific activity of around 1.0 that occurred in the stressed group on days 35 and 58 is abnormally low for yearling chinook salmon, indicating a possible interference of sublethal confinement

stress with parr/smolt transformation. Sampling error that produced a substantially different mean length in the day 58 sample of starved fish complicates interpretation of these data since gill ATPase can be size dependent. It is likely that a more typical sample of starved fish on day 58 would have yielded a smaller mean size and a lower ATPase value making a less substantial difference in ATPase activity between the starved controls and confined fish. However, from day 35 data and from the size corrected data in experiment 1, I feel sub-lethal confinement stress probably depressed gill ATPase more than starvation alone. However, since fish do not normally feed during stress it may not be necessary to separate the two effects. The role of elevated cortisol concentrations during acclimation on the ultimate depression of gill ATPase is unclear. Epstein et al. (1971) found pharmacological doses of cortisol elevated gill Na+K ATPase in eels. The elevation of cortisol during the stress response does not result in increased gill ATPase in salmon--a more complicated control mechanism must exist.

IV. CHRONIC CHANGES IN CORTISOL DURING HEAT

Introduction

Short term experiments in Section II show that thermal shock results in an increase in plasma cortisol in juvenile salmonids and that acclimation temperature appears to affect the changes in cortisol during confinement. Long term experiments were conducted to elucidate complete cortisol dynamics to heat and to investigate the effect of water temperature on acclimation to confinement.

Methods

Spring chinook salmon (Oncorhynchus tshawytscha) or cutthroat trout (Salmo clarki) were acclimated for at least two weeks to tanks supplied with well water that could be temperature controlled without physical disturbance to the fish. Blood samples were obtained as rapidly as possible by severing the caudal penduncle and collecting the blood in heparinized capillary tubes; after centrifugation the samples were stored frozen. We determined plasma cortisol concentration by a competitive protein binding assay adapted from Murphy (1967) using a simplified method of plasma preparation (Methods, Section V).

Experimental Design and Results

Mild Thermal Shock: Continuous

At time 0, 1130 on 13 September 1976, 6 fish were sampled for plasma from each of two groups of about 60 Rogue stock juvenile (0+ yr, 9-14 cm) spring chinook salmon. The two groups were acclimated to flowing 12 C well water in two adjacent 90 cm temperature controlled tanks. Immediately after the time 0 sample, the water temperature in the experimental tank was raised to 20 ± 1 C in 20 min; the time was held at 12 C in the control tank. Six fish were sampled from both tanks on days 1, 2 , 3, 4, 6, 8, 10, 12, and 14 after day 0.

The experimental fish showed no substantial cortisol stress response to the heated water (Table 2). Although the average cortisol concentration in the experimental fish was often slightly higher than in the controls, only once was it greater than 50 ng/ml and it was never over 100 ng/ml; concentrations below these levels are too low to attribute to a specific cortisol response. Additionally, no mortalities occurred and the experimental fish resumed feeding on the second day after heating.

Temperature: Effect on Acclimation and Response to Confinement

At time 0, 1000 on 17 December 1976, 6 fish were sampled for

Table 2. Cortisol concentrations (mean = $\bar{X}$; standard error = SE) of juvenile chinook salmon acclimated to 12 C, subjected to a rapid heat rise to 20 ± 1 C and maintained at this temperature for two weeks. Controls were maintained at a constant 12 C.

		Cortisol (ng/ml			
		Subjected to heat (sample size = 6)		Control (sample size = 4)	
		$\bar{X}$	SE	$\bar{X}$	SE
Before heat		28	16	13	10
Days after heat:	1	12	9	0[a]	0
	2	28	12	5	5
	3	9	7	0[a]	0
	4	9	6	0[a]	0
	6	0[a]	0	3	3
	8	73	11	0[a]	0
	10	28	11	9	5
	12	38	22	3	3
	14	40	17	6	6

[a]All individuals below the limit of detection (< 10 ng/ml)

plasma from each of two groups of about 100 juvenile (0+ yr, 9-16 cm) cutthroat trout acclimated to adjacent temperature controlled 90 cm diameter tanks. The fish in one tank were living in flowing 12 C water and the fish in the other tank were living in flowing 22 C water. Immediately after the time 0, sample, all fish were dip netted into four perforated buckets (5.5 l) suspended in each tank, thus confining the fish at their respective acclimation temperatures. Six fish were sampled for plasma from both groups at 0.5, 1, and 2 h after confinement and then every 24 h for 8 days.

The fish acclimated to 12 C showed a moderate increase in plasma cortisol peaking at slightly over 100 ng/ml 1 h after confinement. Mean cortisol concentration was down to less than 50 ng/ml in 1 day, and very low basal concentrations were reached within 6 days (Table 3). The fish acclimated to 22 C showed an erratic increase in plasma cortisol peaking at 30 min after confinement. Warm-acclimated fish had a significantly higher cortisol level than the cool-acclimated fish at day 1; however, the warm-acclimated group returned to very low basal levels at least as rapidly as the cool-acclimated group.

Discussion

A rapid increase in water temperature from 12 C to 20 C elicited no definitive cortisol response in juvenile salmon. While the

Table 3. Plasma cortisol concentrations (mean = $\overline{X}$; standard error = SE) of juvenile cutthroat trout acclimated to two different temperatures and then subjected to confinement at their acclimation temperature. Sample size is six up to day 1 and four after day 1.

Sampling time	Acclimation temperature 12 C $\overline{X}$	12 C SE	22 C $\overline{X}$	22 C SE
Before confinement	3	3	8	5
After confinement: 0.5 h	99	20	173	10
1 h	122	20	64	19
2 h	108	29	83	29
1 day	23	4	113	22
2 day	69	30	12	6
3 day	20	9	3	3
4 day	10	6	8	4
5 day	36	15	3	3
6 day	4	4	0	0
7 day	4	4	0	0
8 day	6	6	3	3

short term thermal shock experiment in section II showed a definite, but not large, elevation in cortisol in response to heat in cutthroat trout the stressor was much more severe (13 C to 26 C with a median survival time of 6 h). Also, the salmon were from the Rogue River historically one of the warmest anadromous salmonid habitats in Oregon. In contrast to physical stressors (e. g. confinement) heat elicits only a small or no change in cortisol during exposure to potentially dangerous or even lethal temperatures severely limiting its use in the investigation of temperature stress.

Very warm acclimation temperatures apparently caused more erratic changes in cortisol during confinement than more normal acclimation temperatures with perhaps a more rapid return to very low basal levels, however, interpretation of this data is complicated by two factors. First, the use of two confinement buckets in each tank made individual container effects possible. Only one of the four buckets in each tank was sampled at each time period and fish in one bucket might have been more agitated than fish in another giving erratic changes in cortisol seen in the experimentals, though the controls, confined in the same way, had a fairly smooth stress response more typical of what is expected during acclimation (Section II). The second complicating factor is the relatively mild confinement used in this experiment which elicited a moderate and short lived elevation in cortisol and as discussed in Section III the milder the

confinement the more variable the elevation in cortisol. Despite these complications the data presented here does corroborate the finding in the similar, but shorter, experiment in Section II that warm acclimation temperature produces erratic changes in cortisol during confinement, however, the evidence is not conclusive.

V. ANESTHETIC AND CONFINEMENT ON SURVIVAL AND CORTISOL DYNAMICS

Introduction

Fish are subjected to extensive handling during procedures such as capture, tagging, and transport. Recommendations that could be used to decrease immediate mortality and increase future survival would be beneficial to fish culture and management. I investigated the relationship between anesthetic, handling, and stress to gain information on which to base recommendations for the reduction of handling stress; further, we compared anesthetic treatments to a mild saline solution suggested by McComas and Long (1976) for increasing resistance to handling stress.

Anesthetics, primarily tricaine methanesulfonate (MS-222), are used routinely in fisheries practice to immobilize fish during handling procedures. MS-222 is known to alter a number of physiological characteristics (Houston et al. 1971a, 1971b) and influence behavior after recovery (Goddard et al. 1974). However, the stress anesthetic places on fish in conjunction with mild handling, the comparative stress of different concentrations of anesthetic, and the effect of anesthetic on the future survival of fish is unknown. Elevation of plasma cortisol concentration has been correlated with a variety of physical and physiological stressors (e.g., Strange et al. 1977,

Mazeaud et al. 1977). I compared cortisol concentration in anesthetized and non-anesthetized fish that were transferred from one tank to another. Cortisol concentration was also measured in fish exposed continuously to different concentrations of MS-222. Finally, the value of treatment with buffered and unbuffered anesthetic and mild saline solution during handling was examined in terms of cortisol concentration and survival to a second stressor. Additionally, a simplified method of plasma preparation for cortisol assay was verified.

Methods

All fish used in the experiments were yearling spring chinook salmon (Oncorhynchus tshawytscha) 9-19 cm long from the Rogue River. Before experimentation, fish were acclimated under normal photoperiod for at least 1 week to 60 cm diameter circular tanks filled to 40 cm with flowing 12 C well water of circumneutral pH and hardness of about 100 mg/l as $CaCO_3$. An aqueous stock solution of 50 mg/ml MS-222 was used in the administration of the anesthetic. In the first experiment comparing anesthetization and no anesthetization in the presence of mild handling 0.2 ml 5 M imidozole per 50 mg MS-222, delivered concurrently, was used as a buffer. This concentration of buffer raised the pH of treated water slightly (0.3 pH unit) and in subsequent experiments 0.05 ml 5 M imidozole/50 mg MS-222 was used which resulted in a lowering of the pH 0.1 or 0.2

unit. In contrast, unbuffered MS-222 at 50 mg/l lowered the pH of the water 0.5 pH unit. All plasma samples were obtained as rapidly as possible by severing the caudal peduncle and collecting the blood in heparinized capillary tubes; after centrifugation, the samples were stored frozen.

The usual method of plasma preparation before determination of cortisol by competitive protein binding (CPB) assay is ethanol extraction requiring centrifugation to remove denatured protein from the ethanol fraction which is then assayed after evaporation. I characterized a simplified method in which the ethanol precipitated protein is left in the assay tube by splitting a series of salmon plasma samples with one aliquot assayed after usual extraction and the other after simply precipitating the protein, thus comparing cortisol values obtained on the same plasma following the two different methods of preparation. Also, different volumes of pooled plasma containing different levels of endogenous cortisol were processed in replicates of 12 by both procedures in order to compare the variablility of the two methods over a range of sample conditions.

Plasma samples were extracted by adding 0.5 ml of redistilled ethanol, stirring on a vortex mixer, centrifuging, and drawing off the ethanol supernatant. After extracting the protein pellet a second time, the combined ethanol fractions were evaporated to dryness. In the simplified procedure, the plasma sample was not extracted

but just precipitated with 0.5 ml ethanol, stirred, and evaporated to dryness. All samples were assayed by CPB adapted from Murphy (1967). First, 1.0 ml of 0.5% human male serum (aqueous solution) with cortisol binding sites "saturated" with tritiated cortisol was added to the dried samples in 15 x 85 mm culture tubes. The amount of tritiated cortisol used to saturate a 100 ml of 0.5% serum was 114 ng (29 Ci), although now we use about one-third that amount with good results. After allowing the samples to react for 5 min in a 45 C water bath, they were placed in an ice bath and the unbound corticoids removed by adding 80 mg Florisil^{R} (activated magnesium silicate, Baker) to each tube, shaking the tube for 30 s on a vortex mixer, and after 3 min removing a 0.5 ml aliquot of Florisil-free solution for scintillation counting in Insta-Gel^{R} (Packard). Duplicate cortisol standards (0, 0.25, 0.5, 1, 2, 3, 4, 6, 8 ng/tube) were processed along with the unknowns and cortisol concentration determined graphically.

Cortisol values obtained by extraction versus simple precipitation on split samples showed a 1:1 relationship ($y=1.00x+10.45$, $r^2=0.69$, where y equals cortisol measured in extracted samples and x equals cortisol measured in precipitated samples) over a range of 0-200 ng/ml (Fig. 12). Replicate determinations (n=12) on two pooled plasma samples (A and B) containing different levels of endogenous cortisol were as follows ($\overline{X} \pm$ SD, ng/ml): 10 µl subsamples plasma

Figure 12. Cortisol values determined after preparation by the extraction method versus cortisol values determined after preparation by the precipitation method in split plasma samples. Dashed line indicates regression: $y=1.00x+10.45$, $r^2=0.69$.

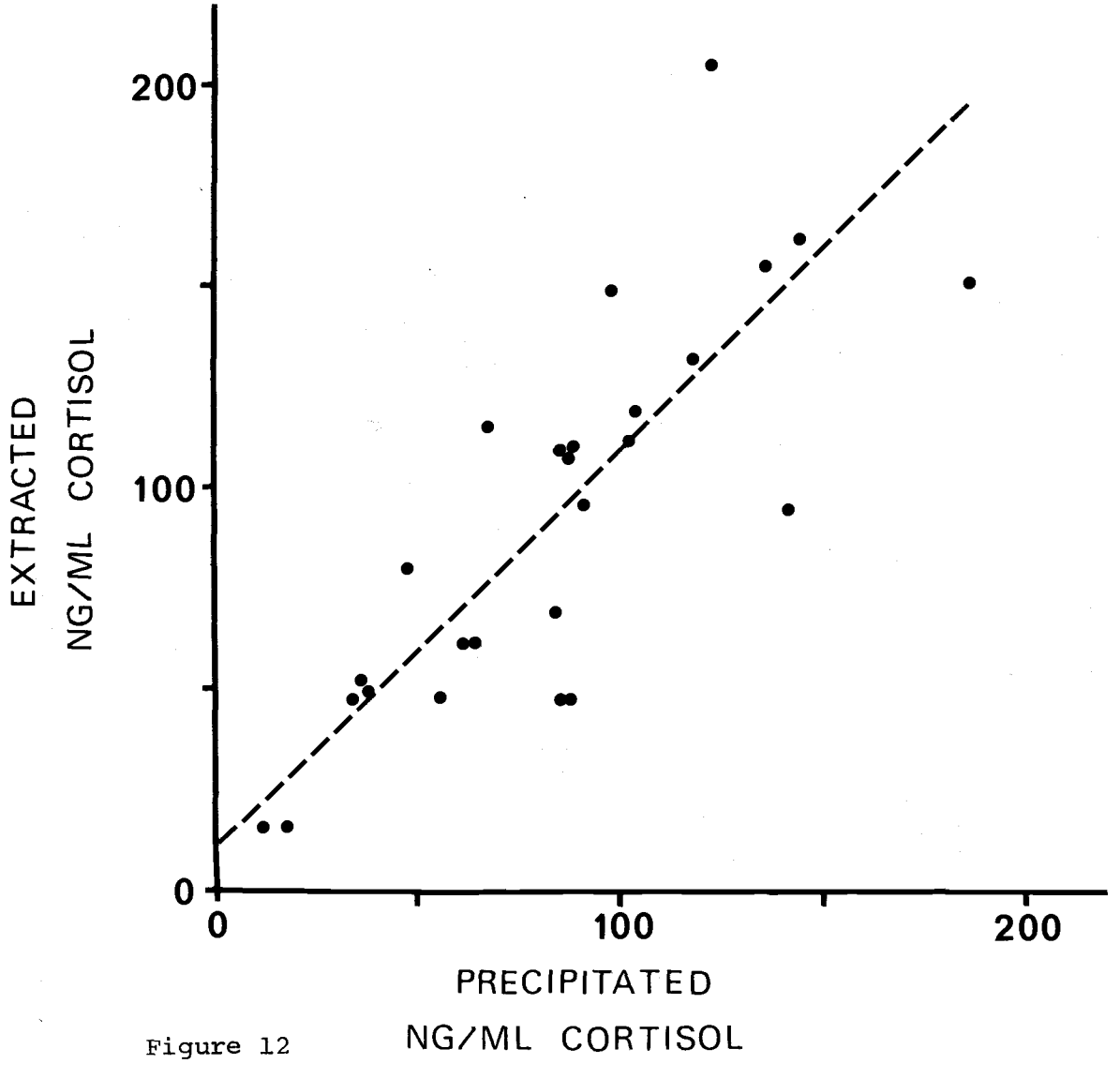
200
100
0
0
100
200
EXTRACTED
NG/ML CORTISOL
PRECIPITATED
NG/ML CORTISOL

Figure 12

A--extracted 243 ± 13, precipitated 190 ± 26; 25 µl subsamples plasma A--extracted 159 ± 20, precipitated 149 ± 30; 10 µl subsamples plasma B--extracted 502 ± 117, precipitated 456 ± 139. There are no significant differences in the variances in the mean cortisol concentrations between the two methods of sample preparation regardless of endogenous plasma concentration or subsample volume ($p=0.05$, F test).

Since the usual extraction procedure ("total corticosteroid" method used by Donaldson and Dye 1975) and the simplified precipitation methods of sample preparation yielded cortisol values that had a 1:1 relationship and no significant difference in variability, I felt confident in using the more rapid precipitation technique. I used thin layer chromatography and radiotracer methods to determine that consistently over 75% of the hormone activity measured by this assay is cortisol. Cortisone, the other important teleostean corticosteroid, contributes to the apparent concentration but is only about 10% as active as an equal amount of cortisol due to its lack of affinity for human serum.

Houston and Woods (1972) determined that blood concentrations of MS-222 reached a maximum of 56 mg/l in trout (Salvelinus fontinalis) exposed continuously to 100 mg/l of the anesthetic. We conducted an in vitro test that showed that his level of MS-222 did not affect the CPB assay. In the experiments with MS-222 10 µl

volumes of salmon plasma were assayed.

Experimental Design and Results

Anesthetic during Mild Handling

At 0915 on 11 November 1977, one group of about 50 yearling chinook salmon was immobilized by pouring 50 mg/l buffered MS-222 into the covered tank with a minimum of disturbance; six fish were sampled from another group which was not anesthetized to establish a pre-stress plasma cortisol level. Five minutes after anesthetization, the immobilized fish were transferred by dip net to an identical tank supplied with fresh, flowing water and blood samples were taken from six different fish at 5 min, 30 min, 1 h, 2 h, 7 h, and 24 h after transfer. The group of fish which was not anesthetized was similarly transferred and sampled at the same time.

The fish which had been anesthetized recovered within a few minutes after being placed in fresh water. The fish which were not anesthetized were considerably more agitated because they tried to avoid capture and struggled in the net while the anesthetized group showed no visible response to handling. Plasma cortisol concentration increased in both anesthetized and non-anesthetized fish after transfer with the anesthetized group having a consistently lower level during the first hour after transfer but taking longer to return to a

pre-transfer concentration (Fig. 13). The differences in cortisol concentration were slight, however, and the 95% confidence intervals overlapped at all time intervals.

Different Concentrations of Anesthetic

Beginning at 1215 on 15 March 1977, four groups of about 45 yearling chinook salmon each were exposed continuously to either 0, 25, 50, or 100 mg/l of buffered MS-222. After introduction of anesthetic, water flow was shut off, the tanks were supplied with aeration, and blood samples were taken at 5, 30, 60, 90, 120, and 180 min.

The group receiving the lowest concentration of MS-222 (25 mg/l) consistently had the highest cortisol concentration (Fig. 14). Most of the 25 mg/l group remained upright but unresponsive throughout the experiment. The fish receiving 50 mg/l had the next highest concentration of cortisol, were completely immobilized within five minutes of receiving the anesthetic, and displayed shallow respiration during the 3 h exposure; two of eight remaining fish in this group failed to recover after return to fresh water. The fish receiving no anesthetic were somewhat alarmed by the absence of the usual water flow in the tank and the periodic sampling, and displayed a moderate increase in cortisol concentration; however, there was no overlap of 95% confidence intervals between th 0 mg/l and the 25 mg/l or 50 mg/l groups at the last sampling time (180 min). The fish

Figure 13. Plasma cortisol concentration over time of anesthetized (50 mg/l buffered MS-222) and non-anesthetized yearling chinook salmon subjected to the mild handling stress of transfer to a new tank. Standard errors are indicated around means of six.

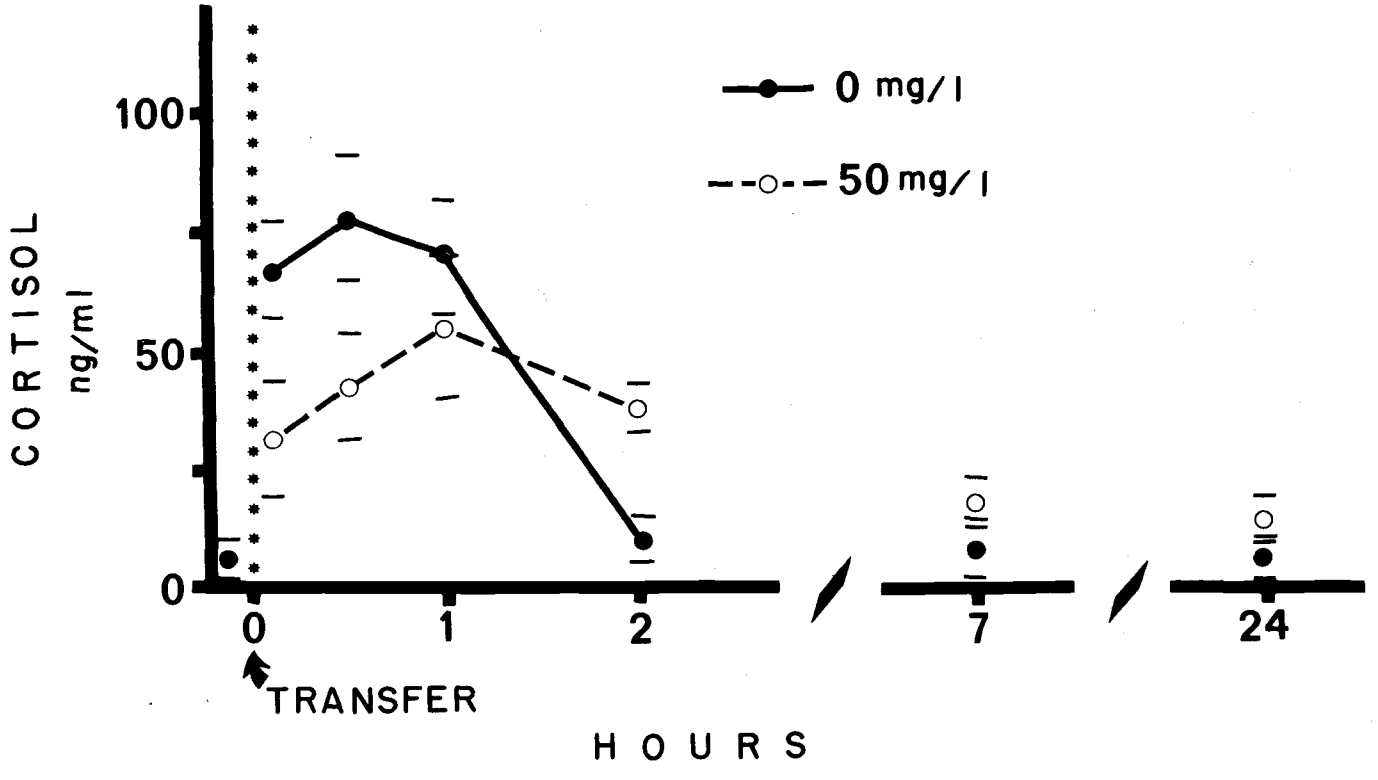

Figure 13

Figure 14. Plasma cortisol concentration over time of yearling chinook salmon exposed continuously to different levels of buffered MS-222. Standard errors are indicated around means of six. The fish exposed to 100 mg/l were dead by the first sample (30 min).

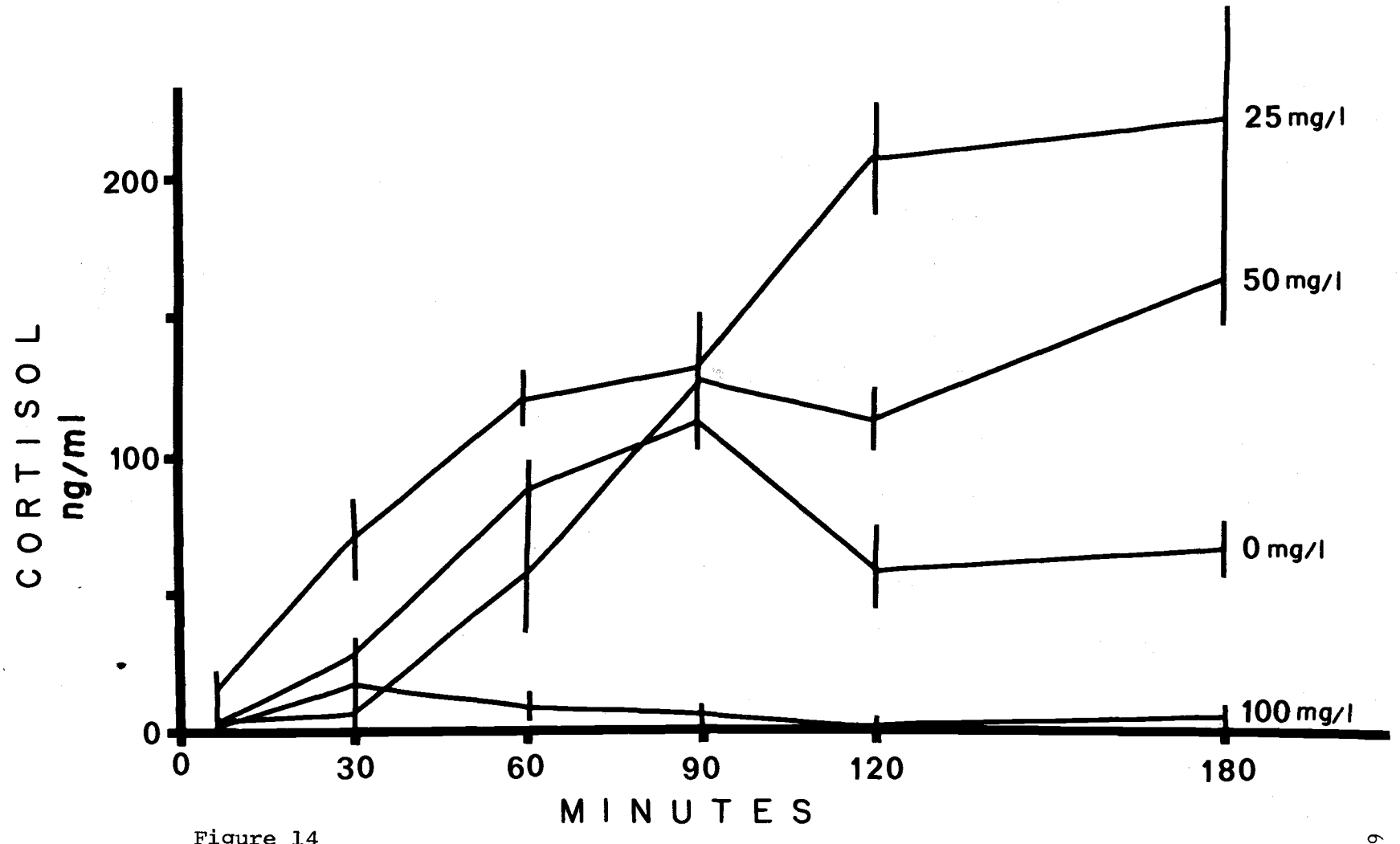
CORTISOL
ng/ml
200
100
0
0
30
60
90
120
180
MINUTES
25 mg/l
50 mg/l
0 mg/l
100 mg/l

Figure 14

receiving 100 mg/l exhibited no respiration and did not recover after return to fresh water at the first sampling period (30 min), although they bled freely and some heart beat was noted for the entire 3 h experimental period. No cortisol response was evident.

Anesthetization and Saline on Future Survival

The effect of anesthetization and saline treatment on yearling chinook salmon subjected to a handling stressor was evaluated in terms of plasma cortisol concentration and the ability to survive a second stressor. The first handling stressor consisted of dip netting groups of about 45 fish into a small net (30 cm x 10 cm immersed to 15 cm) suspended in their tank and keeping them confined for 30 min; during this time the water was shut off and aeration supplied. Anesthetic and saline treatments were administered during the first stressor. The three anesthetic treatments consisted of immobilizing the fish with buffered and unbuffered MS-222 (50 mg/l) prior to confinement and buffered MS-222 (same dosage) immediately after confinement (chasing and catching the fish as rapidly as possible in the dip net). The saline treatment was 0.5% NaCl supplied after confinement. Fish receiving no treatment except capture and confinement in a net served as controls. Plasma samples for evaluation of circulating cortisol were taken before confinement (6 fish per treatment) and after the first (30 min) stressor (12 fish per treatment). The second

stressor consisted of transferring the nets of fish to fresh, flowing water in adjacent tanks and keeping the salmon confined until half the controls (no anesthetic or saline supplied during first stressor) were dead. The remaining fish in all treatments were liberated into the tank and post-handling mortality noted. The treatment with buffered anesthetic before capture and a control were conducted on one day and the rest of the treatments and a control were conducted on another day.

The controls had the highest mortality when subjected to a second handling stressor and had a dramatic increase in cortisol concentration during the first (30 min) stressor (Table 4). Saline solution supplied only during the first stressor appeared to reduce mortality to the second stressor, but did not reduce the extent of cortisol elevation durng the first stressor. Anesthetic supplied before and during the first stressor substantially reduced mortality to the second stressor when the fish were no longer in the anesthetic; also, anesthetic suppressed the cortisol response during the first stressor. Buffered and unbuffered anesthetics supplied before capture reduced mortality the most, while buffered anesthetic supplied after initial netting was the anesthetic treatment that reduced mortality the least, although it was still substantially better than saline or no treatment.

Table 4. Plasma Cortisol concentration of yearling chinook salmon under different anesthetic and saline treatments before and after a severe 30 min handling stressor; controls received no treatment. Percent mortality to an immediately applied second handling stressor during which no treatment was given is also presented.

Date (1977) of experiment and treatment	Cortisol (ng/ml, X ± SE)		Mortality second stressor (%)
	Before capture (n = 6)	30-40 min After confinement (n = 12)	
21 April			
Control	32 ± 12	260 ± 20	85
Buffered anesthetic before capture	17 ± 8	13 ± 5	12
29 April			
Control	38 ± 13	202 ± 25	86
Unbuffered anesthetic before capture	68 ± 13	85 ± 15	19
Buffered anesthetic after capture	48 ± 12	74 ± 10	32
0.5% Na Cl after capture	69 ± 9	275 ± 22	62

Discussion

Brief anesthetization during mild handling resulted in plasma cortisol concentrations comparable to those in unanesthetized fish (Fig. 13). Fish exposed continuously to 25 mg/l MS-222 had an increase in cortisol concentration to over 100 ng/ml, a high level associated with stress, within 1 h (Fig. 14). Fifty mg/l produced little change in cortisol during the first 30 min; after 90 min, however, the 50 mg/l group was over 100 ng/ml. Wedemeyer (1970) also found no early (12 min) cortisol elevation in a salmonid (Salmo gairdneri) to an immobilizing dose of MS-222. The 0 mg/l group showed a mild stress response due to the termination of the usual water flow and the periodic sampling. The 0 ng/ml group's response does not act strictly as a control, because the activities that elevated cortisol in this group would not have affected the unresponsive, anesthetized individuals. Fish in the 100 mg/l group were dead, no opercle movement or recovery in fresh water, at the 30 min sample, but the fish bled freely and no rigor mortis occurred during the 3 h experiment. No elevation of cortisol occurred after death. Fuller et al. (1974) noted a decline in plasma cortisol concentration after death in a salmonid (Coregonus laveretus).

Anesthetization during severe handling spares the full cortisol increase experienced by the non-anesthetized fish (Table 4).

Corroborating this, Houston et al. (1971a) found less hyperglycemia, a secondary stress response probably dependent on cortisol, in anesthetized trout (Salvelinus fontinalis) than in non-anesthetized trout handled in the same manner. In our experiments, the anesthetized groups had substantially less mortality to a second stressor than control groups, indicating that the initial minutes of shock at the onset of handling, attenuated by the use of anesthetic, are particularly damaging to the organism in terms of future survival. Anesthetized groups also had lower levels of cortisol at the end of the first stressor. Lower cortisol concentration preceded slower mortality in a comparison of the two control groups as the April 29 control group with a lower cortisol concentration after the first stressor survived 2 h longer to the median survival time (11 h 25 min versus 9 h 25 min). Supplying buffered anesthetic after initial capture reduced mortality to the second stressor the least among the anesthetic treatments; moreover, some very early (< 1 h) deaths occurred during the second stressor which did not occur in any other group. Apparently, anesthetic supplied after the onset of handling places an additional burden on the animals initially, although over the long term this treatment reduced mortality more than saline or no treatment. Saline solution supplied during the first stressor appeared to reduce mortality to the second stressor without moderating the increase in cortisol. Perhaps saline has the indirect therapeutic value of reducing

osmoregulatory demands without reducing the stress of handling itself, as anesthetic apparently does. McComas and Long (1976) found mild saline reduced mortality substantially when the entire handling stress took place in salted water. The less dramatic reduction in mortality attributable to saline in our experiment was probably because salt was not supplied during the second, lethal stressor. Saline may be valuable primarily in reducing the initial mortality of handling while anesthetic appears to actually spare stress and, thus, increase chances for future survival.

The results of this study have potential for management application. The 50 mg/l dose of MS-222 immobilizes fish as rapidly as the quickly lethal 100 mg/l dose and is less stressful in terms of the cortisol response than 25 mg/l which merely depresses the fish. However, even at 50 mg/l anesthetization becomes increasingly stressful after 1 h. Anesthetization (50 mg/l, MS-222) increases the potential for future survival in fish subjected to rigorous handling through attenuation of stress by reduction of initial shock. Supplying the anesthetic before any handling yields the best results but is probably not often feasible; supplying anesthetic after netting results in better future survival than 0.5% salt solution which is better than no treatment. I evaluated the various treatments only in terms of survival to an immediately applied second handling stressor. Changes in normal behavior after anesthetization (Goddard et al. 1974) or

potential effects of such things as disease resistance might serve to qualify these statements.

My experiments also provide a firm link between an increase in plasma cortisol and reduced fitness in terms of increased mortality to a second stressor, strengthening the potential for the use of plasma cortisol concentration alone, as an indicator of sublethal stress of certain kinds.

VI. SEAWATER AND CONFINEMENT ON SURVIVAL AND CORTISOL DYNAMICS

Introduction

Cortisol is implicated in the osmoregulation necessary for seawater adaptation in teleosts. Cortisol stimulates the active absorbtion of water and salt from the lumen of the gut (Utida et al. 1972, Johnson 1973, Porthe-Nibelle and Lahlou 1975) a phenomenon necessary in maintaining hydration in seawater adapted fish. Pharmological doses of cortisol elevate Na+K ATPase in the gill and cause a silvering in freshwater eels (Anquilla rostrata) (Epstein et al. 1971), again suggesting a role in seawater adaptation. Moreover, increased activity of ACTH (adrenocorticotropin hormone) cells has been demonstrated in Atlantic salmon smolts (Olivereau 1975). We investigated possible interplay between the probable involvement of cortisol in osmoregulation and the demonstrated elevation of cortisol during stress in the following experiments with juvenile chinook salmon. Cortisol concentration was measured over time in salmon reared in freshwater and suddenly exposed to full strength seawater. Also, the effect of the sudden introduction of seawater on the cortisol stress response and survival to a severe handling stressor was explored.

Methods

Juvenile (0+ yr) spring chinook salmon 9-13 cm in length from the Rogue river were acclimated to circular tanks (60 cm diameter) supplied with flowing 12 C well water for 2 weeks prior to experimentation. Plasma samples for cortisol assay were obtained from the fish, killed by a blow to the head, by severing the caudal peduncle and collecting the blood in heparinized capillary tubes. After centrifugation, samples were stored frozen. We determined plasma cortisol concentration by a competitive protein binding assay adapted from Murphy (1967) using a simplified method of preparing plasma for assay (Methods, Section V).

Experimental Design and Results

At time 0, 800 on 9 September 1977, 6 fish were sampled from each of 2 tanks containing about 40 juvenile salmon. Water flow was then shut off and aeration introduced. A brine of synthetic sea salt (Marine Environment^{R}) was poured into one tank to yield seawater with 25 to 30 g/l dissolved solids and fresh water was poured into the control tank. Samples of 6 fish were taken from each of the tanks at 1, 6, 12, 28, and 52 h thereafter. Concurrently, another experiment was conducted in which both the salt and fresh water groups were severely confined in a small dip-net (10 cm x 30 cm

immersed to 15 cm) at the time of the introduction of the brine. Samples of 6 for cortisol determination were taken from both groups at 0.5 and 6 h after confinement. When 50% mortality was reached in the controls both groups were released and mortality during the next 2 days recorded.

In response to the introduction of seawater a slight increase in cortisol concentration from about 50 ng/ml to 100 ng/ml occurred after 1 h while cortisol concentration was at or below the time 0 level in subsequent samples (Table 5); fish in the control tank showed no such response. The large standard errors associated with the time 0 control and 52 h seawater sample was due to the presence of one outlyer in each sample. In the confinement experiment both salt and fresh water groups had an increase in plasma cortisol in response to capture and containment; however, the salt water group had a lesser increase which at the 6 h sample was half the concentration in the fresh water group. There was no overlap in 95% confidence intervals (Table 6). Mortality to severe confinement reached 50% in the fresh water group after 12 h 40 min at which time only 29% in seawater had succumbed. Ultimate mortality was correspondingly less in the seawater group (48% in salt versus 68% in fresh).

Table 5. Cortisol concentration in juvenile chinook salmon exposed to seawater (25 to 30 g/l dissolved solids) and unexposed controls. Seawater was introduced immediately after time 0 sample. Means and standard errors based on sample sizes of 6.

Time	Plasma Cortisol Concentration (ng/ml)			
	Seawater		Control	
	mean	standard error	mean	standard error
0	44	14	58	39
1 h	104	23	43	6
6 h	48	20	40	13
12 h	11	7	20	4
28 h	5	3	3	3
52 h	64	41	13	5

Table 6. Plasma cortisol concentration in juvenile chinook salmon severely confined in a small dip-net and exposed to seawater (25 to 30 g/l dissolved solids) and salmon similarly confined but maintained in fresh water. Confinement and seawater were initiated immediately after time 0 sample. Ultimate mortality to confinement also presented. Mean and standard errors based on sample sizes of 6.

Time	Plasma Cortisol Concentration (ng/ml)			
	Confined in Seawater		Confined in Fresh Water	
	mean	standard error	mean	standard error
0	31	13	43	15
0.5 h	130	33	177	20
6 h	188	23	381	22
	Mortality (%)			
	48		68	

Discussion

The increase in cortisol after introduction of full strength seawater (25 to 30 g/l dissolved solids) was slight and transient in the juvenile chinook salmon. In contrast, goldfish subjected to 9 g/l NaCl showed a greater increase that lasted for 9 days (Singley and Chavin 1975a). The difference may be that seawater is normally encountered in the life cycle of the salmon at approximately the size used in this experiment while goldfish do not usually encounter saltwater at all. A juvenile salmon is prepared to meet a hyperosmotic challenge and so seawater may not elicit a generalized stress response. In fact, the confinement experiment showed that the fish in seawater had less of an increase in cortisol during confinement than the fish in freshwater and survived slightly better as well. It may be that salmon at the particular size used in these experiments are more at ease in salt than in fresh water. The role of cortisol in seawater adaptation is not clear. The slight increase in cortisol after the introduction of salt may function to trigger osmoregulatory changes, but it is doubtful that a simple increase in plasma concentration of cortisol causes the complete reversal of osmoregulation needed for adaptation of seawater since a wide variety of relatively mild stressors result in similar changes in plasma cortisol concentration. The role of cortisol in seawater adaptation must be in concert with other regulatory mechanisms.

VII. SUMMARY AND CONCLUSIONS

Basal plasma cortisol concentrations of juvenile salmonids were very low in well acclimated fish. Cortisol concentration was nearly always below 50 ng/ml and often below 10 ng/ml, the limit of detection of the assay. Hundreds of plasma samples taken from controls or time 0 experimentals were assayed during the course of the experiments. Typically the mean cortisol concentration in these unstressed fish was between 10 and 30 ng/ml (Figs. 3, 4, 5, 6, 8, 9, 10, 11, 13, 14; Tables 2, 3; Appendix). These values were from both chinook salmon and cutthroat trout during all times of the day and year, the fish were all immature ranging in size from 9 to 16 cm and in age from 6 to 18 mo. Quite often there was no detectable cortisol (less than 10 ng/ml) in any fish out of a sample of 6 (Figs. 7, 8, 20, 22; Tables 2, 3). On two occasions cortisol concentrations in supposedly acclimated fish was near 50 ng/ml. In one instance the fish had been acclimated to new quarters only 1 week instead of the usual 2 or more weeks (Table 4). In the other case the fish were suffering from mild but widespread caudal fin erosion and had experienced high mortality during the 2 week acclimation (Tables 5, 6). On one occasion acclimated fish had a mean cortisol concentration of near 100 ng/ml (Fig. 2). This was the first experiment and in hindsight it seems likely that too much noisy preparatory activity

occurred around the experimental tanks during the hour before the first sample causing elevated time 0 concentrations. A useful generalization would be that in juvenile chinook salmon and cutthroat trout mean cortisol concentration would be less than 50 ng/ml regardless of time of day or season in acclimated fish and concentrations of over 100 ng/ml are associated with a stressor. Very low plasma cortisol (less than 10 ng/ml) may reflect an exceptionally comfortable situation for the fish, but this cannot be confirmed from the experiments conducted.

Immediate increases in plasma cortisol from low basal levels were seen after exposure to handling-confinement, heat, and seawater. The physical stressor of capture and confinement produced the fastest, largest, and most long lasting increase in plasma cortisol. Severe, rapidly lethal thermal shock (13 to 26 C) produced a mild (20 to 70 ng/ml) increase in plasma cortisol (Fig. 4) comparable to the gentle, physical disturbance of rapid dip-net transfer to an identical tank (Fig. 13). A non-lethal but substantial thermal shock (12 to 20 C) produced no definitive change in cortisol at all (Table 2) while any handling-confinement procedure resulted in an easily detectable elevation in cortisol. Introduction of seawater induced a slight, transient increase in plasma cortisol, again comparable to the mildest handling procedure. Acclimation to very warm (22 or 23 C) temperatures did not lessen the magnitude of the cortisol

increase during confinement, but apparently caused more erratic changes in concentration (Fig. 6; Table 3). An immobilizing dose (50 mg/l) of anesthetic (MS-222) induced a delayed increase in plasma cortisol concentration as compared to the immediate increase in cortisol produced by confinement, heat, or seawater. This dose of anesthetic did not result in elevated plasma cortisol at the 0.5 h sample and only after 1 h of exposure did a steady increase begin (Fig. 14). While anesthetic is physiologically traumatic (Houston et al. 1971a, 1971b) it presumably does not produce the fright and/or physical discomfort caused by the other stressors that are probably responsible for rapid elevation of cortisol.

After the immediate increase in plasma cortisol caused by confinement the cortisol concentration may return rapidly to basal, remain elevated until death, or return slowly to basal depending on the nature of the stressor. If the fish were liberated after 0.5 h of severe confinement cortisol remained elevated at about 200 ng/ml for several hours and then declined as the fish recovered until basal levels were reached about 12 h after release (Fig. 7). If the severe confinement was continuous, cortisol increased to near 400 ng/ml in 1.5 h with little further increase, and half the fish died within 12 h (Fig. 8). When the fish were subjected to a more moderate degree of continuous confinement to which they were able to acclimate without high mortality the cortisol stress repose lasted for 1 to 2 weeks

(Figs. 9, 10, 11). Typically, mean cortisol concentration increased to 100 to 200 ng/ml within the first day and remained at this level for 3 to 5 days, slowly declining to basal after about a week. The cortisol stress response to moderate confinement was less and more variable than the response to severe confinement because in moderate confinement a few individuals were stressed to the point of mortality but most were not, while under severe confinement all fish would eventually die. Fish acclimating to moderate confinement typically refused food during the period of elevated cortisol and resumed feeding as cortisol returned to basal during the second week of confinement. In contrast, during the moderate thermal shock (Table 2) that elicited little cortisol response the experimental fish showed reduced feeding for only one day. The elevation of plasma cortisol concentration and gradual return to basal levels during acclimation to continuous, moderate confinement in juvenile salmonids conforms to Precht's (1958) model of ideal compensation with the alarm (day 1), resistance (days 2-6), and adaptation (days 7-10) phases of Selye (1950) clearly evident (Figs. 9, 10, 11).

After acclimation to a non-lethal stressor sublethal effects of stress could be noted. Reduced growth during the period of stress is evident and due to lack of feeding and perhaps also because of additional energy expenditures (Fig. 10; Table 1). Further, a more subtle, but also potentially detrimental effect, reduction of gill Na+K

ATPase activity, appeared to occur several weeks after acclimation (Fig 10, Table 1). While a delayed reduction in Na+K ATPase might be expected to interfere with long term salt water adaptation, 9 to 13 cm chinook salmon performed better in terms of both survival and cortisol concentration when subjected to severe confinement immediately after introduction to seawater than when maintained in fresh water. It is clear that there are some and probably many more (e. g. possible increased susceptibility to disease) potentially detrimental effects of sublethal stress.

Several biological maximums associated with changes in cortisol during stress in juvenile salmonids are evident from this series of experiments. Consistently when non-anesthetized fish were captured and severely confined in fresh water there was a rate of increase in plasma cortisol concentration of 5 to 7 ng/ml/min during the first 30 min of confinement (Figs. 2, 7, 8; Tables 4, 6). This was the biological rate maximum for handling-confinement stressors and is probably the absolute rate maximum for any stressor since handling-confinement elicits the greatest cortisol response of any stressor yet examined. After this initial surge mean plasma cortisol concentration was 200 ng/ml and increased to about 400 ng/ml in the next hour after which there was little further increase (Fig. 8). In all cases where confinement was stringent enough to induce considerable mortality mean plasma cortisol concentration eventually reached a maximum

of 400 to 500 ng/ml (Figs. 3, 8; Table 6). Some individual values were in this range when fish were subjected to less severe confinement (Fig. 9). The biological maximum plasma cortisol concentration in response to stress in juvenile salmonids appears to be around 400 to 500 ng/ml with considerable individual variation.

As mentioned earlier an immobilizing dose (50 ng/ml) of anesthetic (MS-222) was peculiar in that it produced a cortisol response only after a delay of 0.5 to 1 h. Moreover, fish subjected to 0.5 h of severe confinement immediately after anesthetization showed no cortisol response and survived to a second stressor applied when they were no longer anesthetized much better than controls (Table 4). The smaller dose of anesthetic (25 ng/ml) produced a greater and less delayed increase in cortisol than the larger dose (50 ng/ml) (Fig. 14). Additionally an overdose of anesthetic (100 ng/ml) killed fish without eliciting an increase in cortisol, in contrast to mortality associated with confinement which was always accompanied by high levels of plasma cortisol.

In generalizing on this series of experiments a number of implications for the management of juvenile salmonids are evident. Use of a proper dose of anesthetic (50 mg/l MS-222) for a short time (less than an hour) could be employed to reduce stress and increase future survival during the intiiation of a handling procedure such as tagging or loading for transport. It is also useful to recognize that

feeding behavior is directly related to the cortisol stress response with the absence of feeding an indicator of possible stress. Further, physiological (adrenocortical) acclimation to new and noxious situations takes 1 to 2 weeks. It is also useful to realize that the onset of the generalized response to stress takes only minutes and these first moments of trauma are particularly damaging. Also, it is important to recognize that simple survival through a stressful situation does not mean the fish are back to normal; there are sublethal effects of stress that are potentially detrimental to future survival. Finally, the most important thing to remember while managing juvenile salmonids is that any human disruption of their daily routine of life is liable to trigger a generalized stress response with possible adverse effects.

Plasma cortisol concentration is not a "litmus test" for the presence of stress in juvenile salmonids. Changes in cortisol during stress are dynamic and sample time, relative to the onset of the stressor, is very important. Fish acclimate to noxious, potentially damaging situations with cortisol concentrations returning to basal; moreover, different stressors elicit different responses. These factors make it impossible to sample a fish from a stream or raceway and determine from cortisol concentration whether a stressor is present. If cortisol is found to be elevated, however, it can be assumed that the fish is stressed. The usefulness of the cortisol

assay to fisheries lies in its research application. In the carefully controlled situation cortisol concentration can be used to determine acclimation, compare relative stress, and assess methods for the reduction of stress.

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APPENDIX: MONTHLY MEASUREMENTS OF CORTISOL IN GROWING FISH

From July through November 1975, plasma samples were taken in conjunction with a gill $Na^+ + K^+$ ATPase experiment conducted at the Research Division Laboratory in which juvenile chinook were reared under different temperature and photoperiod regimes. During the period when plasma samples were taken, the chinook salmon grew from 7 cm to 11-17 cm, lost their parr marks, and displayed peaks in gill $Na^+ + K^+$ ATPase. No trends are apparent in plasma cortisol concentration that can be related to growth or parr-smolt transformation as indicated by morphology or gill $Na^+ + K^+$ ATPase. Even though the cortisol determinations were made on the first 4 fish sacrificed during each sampling period, there was enough difference in handling times (± 10 min) to introduce the variability seen in the cortisol levels and mask any subtle changes in basal levels of cortisol.

Plasma corticoid concentrations (mean ng/ml = $\overline{X}$, standard error = SE) in juvenile chinook salmon raised through parr/smolt transformation under different photoperiod and temperature regimes.

Tank # 9: Rogue River conditions.
Tank #10: 3 month advanced photoperiod, 8°C water temperature.
Tank #10: 3 month advanced photoperiod, 12°C water temperature.
Tank #12: normal photoperiod, 8°C water temperature.
Table #13: normal photoperiod, 12°C water temperature.

	$\overline{X}$	SE	No. of samples	Fish per sample
July				
# 9	23		1	4
#10	6		1	4
#11	15		1	3
#12	26		1	4
#13	27	10	4	1
August				
# 9	29		2	2
#10	37		2	2
#11	41	18	3	1
#12	36		2	2
#13	28	15	4	1
September				
# 9	33	11	4	1
#10	21	7	4	1
#11	0	0	4	1
#12	87	11	4	1
#13	29	10	4	1
October				
# 9	7	7	4	1
#10	0	0	4	1
#11	26	6	4	1
#12	19	18	4	1
#13	65	10	4	1
November				
# 9	28	14	4	1
#10	3	3	4	1
#11	71	8	4	1
#12	4	4	4	1
#13	40	21	4	1