THE RESPONSE OF THE INTERRENAL GLAND OF RAINBOW TROUT (SALMO GAIRDNERI) TO STRESS.

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ABSTRACT

THE RESPONSE OF THE INTERRENAL GLAND OF RAINBOW TROUT (*SALMO GAIRDNERI*) TO STRESS.

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A study was conducted to determine the response of the interrenal gland of rainbow trout to chronic exposure to sub-lethal stress and to measure some metabolic effects of high circulating cortisol levels. Exposure of fish to 0.02 and 0.20 mg/l of environmental hexavalent chromium for one week significantly elevated plasma cortisol levels as determined by fluorescence in sulfuric acid. Fish exposed to these concentrations of Cr^{6+} for two and three weeks had plasma cortisol levels similar to those of control fish. Fish exposed to 20 or 30 mg/l of Cr^{6+} (levels approaching the 48 hr TLm for largemouth bass) for three days had elevated cortisol levels, but fish exposed for six, seven or ten days did not. Fish forced to swim for one-half hour twice daily at about 0.6 meters/sec for one and two weeks did not have elevated plasma cortisol levels when sampled 24 hours after the last exercise period. Fish forced to swim for two or four hours and sampled immediately did have plasma cortisol levels significantly above those of

control fish. Fish were treated with exogenous cortisol by implanting pellets in the body cavity, resulting in plasma cortisol levels of over 100 µg%. Fasted fish so treated for one week had higher liver glycogen stores and secreted more nitrogen than did control fish. Plasma glucose levels were not elevated in treated fish. Fish similarly treated but fed during the experimental period had liver glycogen stores and nitrogen excretion rates similar to control fish. A seasonal variation in plasma cortisol levels of untreated fish was found. Under laboratory conditions, untreated groups of fish sampled in summer and early fall showed average cortisol levels of 30-40 µg%; groups of fish sampled under similar circumstances in winter had average plasma cortisol levels ranging from 58 to 76 µg%. Treatment with exogenous ACTH, carried out at a time when untreated fish showed high cortisol levels, did not cause a significant increase in these levels. The effect of chronic low-level stress in circulating cortisol levels of rainbow trout is transitory and does not appear to be a useful indication of the existence of chronic stressful conditions. It is also unlikely that this response is a major causative factor in any untoward effects caused by chronic exposure to sub-lethal stress in rainbow trout.

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by

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INTRODUCTION

Human activities have resulted in many chemical and physical changes in natural bodies of water. Many of these changes are detrimental to fish and to other inhabitants of these waters. Often, alterations such as the addition of chemical substances, elevation of temperatures and lowering of dissolved oxygen concentration as well as alterations of stream volumes and stream bed characteristics occur simultaneously, making the assessment of the consequences extremely difficult (Brett, 1958). Many natural waters have shown gradual changes in species composition of fish population, resulting in a reduction or complete absence of more desirable species of fish (Hynes, 1960). Often these changes occur as a result of a complex combination of habitat and water quality changes which cannot be demonstrated to be detrimental by classical techniques used to measure acute toxicity. In order to plan amelioration of the effects of past habitat alterations, information is required on the physiological effects on fish and other organisms of these changes.

Considerable information has been obtained on the specific effects of the addition of chemical substances to water (Hynes, 1961; Jones, 1964). Most of this informa-

tion has been obtained by bio-assay techniques and is concerned with acutely toxic levels. Little is known about the effects of long-term exposure to sub-lethal levels of toxic chemical substances. Attempts have been made to extrapolate acute toxicity data to arrive at a so-called threshold concentration at which no detrimental effect is expected. Even for substances whose physiological toxic action is well established, such extrapolation is not very successful (Jones, 1964). With substances whose toxic action is not established, the determination of tolerable levels of chemical pollutants is even more difficult.

Chromium often occurs in wastes from plating and metal-treating operations. The physiological mechanism of its toxicity is poorly understood. Fromm and Schiffman (1958) exposed largemouth bass to solutions of hexavalent chromium and found a 48 hour median tolerance limit (TLm) of 195 mg chromium per liter. Pathological changes occurred in the digestive tract of fish exposed to chromium at 94 mg/l. Prolonged exposure to much lower levels are also toxic and a threshold level has not been established.

Two groups of rainbow trout exposed to water containing 2.5 mg/l of Cr^6 experienced about 50% mortality in 36 and 19 days (Fromm and Stokes, 1962). These authors found that rainbow trout took up hexavalent

chromium from water containing as little as 0.0013 mg/l. Fish exposed to 0.05, 0.10 and 0.15 mg/l took up chromium at a steady rate throughout a period of 28 days, at which time the experiment was terminated. There was no evidence of levelling off of the uptake at this time. Mortality in these experiments was light and the physiological consequences of the accumulation is unknown.

Many classes of habitat alterations are non-chemical and do not lend themselves to evaluation by bioassay techniques, but do impose a physiological stress on fish. The term "stress" is often used without adequate definition. Brett (1958) has proposed a definition as follows: "Stress is a state produced by any environmental factor which extends the normal adaptive responses of an animal, or which disturbs the normal functioning to an extent that the changes of survival are significantly reduced."

For example, alterations in the amount or quality of physical resting cover has been shown to profoundly affect stream-dwelling fish. The improvement of cover in streams by the addition of current-interrupting devices was shown to increase the carrying capacity of these streams to trout (Shetter, et al. 1946). Conversely, experimental destruction of cover in a stream by Boussu (1954) resulted in a reduction in fish populations. Miller (1958) created artificial overpopulations of cutthroat trout

in an Alberta stream by adding fish to a section already occupied by a normal native population. Fish were similarly added to a section from which the native population was previously removed. The fish in the overpopulated section suffered high mortality, which was determined by daily search for dead fish and by a census two months after the addition of the fish. Mortality was light in the previously unoccupied section. Dead and moribund fish were examined and no obvious cause of death was noted. The competition for resting cover by trout implied by the above studies has been documented by Newman (1956), who observed fish in a natural stream from a submerged observation chamber. The fish observed formed a social hierarchy in which each fish defended a resting place from subordinate fish. Temporary absence of a fish from the area resulted in movement of each subordinate to the usual resting place of its immediate superior in the hierarchy. Thus, all fish in the area observed by Newman were more or less continuously competing for resting cover. A condition of inadequate cover, either from destruction of cover-producing features of a stream or from overpopulation caused by introduction of superfluous fish, could result in physiological reactions which might be detrimental to the fish.

The imposition of stressful conditions of any sort might be expected to cause certain common physiological

responses which might be used to verify the existence of adverse environmental stimuli or to evaluate the magnitude or seriousness of known noxious conditions. One such physiological response which has been elicited in higher vertebrates by a wide variety of noxious stimuli is an increase in secretory activity of the adrenal cortex or interrenal gland.

The adrenal cortex or interrenal tissue of vertebrates secretes steroid hormones and is, in part, under the influence of the anterior pituitary gland. The steroids secreted by the adrenal cortex are commonly divided into two groups according to their principal physiological action (Selye, 1950). Mineralocorticoids, of which aldosterone is an example, have a regulating effect on the retention and secretion of monovalent cations. The secretion of mineralocorticoids is not influenced greatly by the pituitary tropic hormone, adrenocorticotropin (ACTH).

Glucocorticoids, so-called because of their promotion of gluconeogenesis, have been identified in all vertebrates which have been investigated (Pickford and Atz, 1957; Chester Jones, et al., 1959; Bern and Nandi, 1964). Three compounds universally occur as secretion products of incubated adrenal tissue and in venous adrenal blood. These compounds, cortisol, cortisone and corticosterone are C-21 steroids with the following charac-

teristics: ketone groups at carbon 3 and 20, a hydroxyl group at carbon 21, a double bond between carbons 4 and 5 and an oxygen atom (=O or -OH) at carbon 11.

The rate of secretion of the carbohydrate-active steroids is regulated by ACTH, secreted by the anterior pituitary gland. Secretion of ACTH is regulated, in turn, by a neurohormone secreted by the hypothalamus into the portal blood supply of the anterior pituitary gland.

The terms, mineral-regulating and carbohydrate-regulating are indicative of the principal effects of the steroids, but these effects are not strictly mutually exclusive. Mineralocorticoids show weak carbohydrate activity; glucocorticoids, particularly corticosterone, show weak mineral regulating activity.

Selye (1946) proposed that disease states in humans shared many common symptoms which are attributable principally to the response of the adrenal cortex to the stress of the disease-causing agent or trauma. These common symptoms were termed the "General Adaptation Syndrome". Selye and his co-workers were able to mimic the characteristics of a number of diseases by subjecting rats to non-specific stress such as surgical trauma, restraint and bone fracture (Selye, 1950). The significance of this syndrome to human medicine is apparently not as great as Selye first suggested and his generalizations have

been criticized by many others (Ingle, 1954; Engel and Frederichs, 1957; Rosch, 1958).

However, investigation of the adrenal cortex of mammals and the homologous tissue of other vertebrates has shown that this gland does respond to non-specific noxious stimuli by increasing its secretion of carbohydrate-active steroid hormones. These hormones apparently serve the function of altering metabolic processes to increase availability of glucose, largely at the expense of protein synthesis. Some of the effects of elevated levels of glucocorticoids, if maintained for an extended period of time, are detrimental to the animal. Among these effects are suppression of inflammation and granulation, suppression of antibody formation and resistance to infection, suppression of reproductive function, and suppression of growth (Christian, 1963).

Several physiological changes resulting from increased circulating levels of glucocorticoids are characteristic enough to be used as indicators of the levels of activity of the gland. Among these are elevation of plasma glucose levels, elevation of liver glycogen stores, involution of the thymus gland, reduction of lymphocytes and eosinophilic leucocytes.

The hypothesis that the pituitary-adrenal axis plays an important role in the response to environmental

stress by wild animal populations was first suggested by Christian (1950) to explain the regulation of population levels in mammals. Studies conducted on wild and laboratory populations of rodents and other mammals since 1950 have shown that increased population density and the resulting increase in social interaction results in decreased reproduction and maternal care as well as increased vulnerability to infectious diseases. Among the effects of crowding on reproduction are 1) the delay of puberty in both sexes, 2) suppression of spermatogenesis in the male 3) suppression of ovulation, 4) increased failure of implantation, 5) increased intrauterine mortality and 6) suppression of lactation and maternal care. These effects have been associated with and attributed to increased secretion of carbohydrate-active cortical steroids (Christian, 1959, 1963). Christian (1950) originally thought that an exhaustion of the adrenal gland might be responsible for the untoward effects associated with crowding. It has since become apparent that these effects are associated with elevated activity of the gland.

The adrenocortical tissue of most bony fishes is not contained in a discrete organ as in mammals, but is scattered along the cardinal veins in the anterior kidney (Chester Jones, et al. 1959). The identity of the tissue, first described on the basis of histological appearance has

been confirmed in many species by its reaction to hypophysectomy and to stimulation by exogenous pituitary extracts and mammalian ACTH (Pickford and Atz, 1957). Rasquin (1951) demonstrated that carp pituitary extracts or mammalian ACTH caused hypertrophy of the interrenal tissue. Rinfret and Hane (1955) injected extracts of salmon pituitary glands into rats and demonstrated ACTH activity as indicated by ascorbic acid depletion.

Secondary effects due to cortical hormone lack or excess have been studied by hypophysectomy and by ACTH administration, respectively. Hypophysectomy caused depletion of glycogen in the liver of starving eel (Hatey, 1951). Treatment of Astyanax mexicanus with ACTH for several days resulted in involution of lymphoid tissue (Rasquin and E. A. Atz, 1952).

Cortical steroids present in the blood of several species of fish have been identified. Cortisol is the principal glucocorticoid in all species studied (Chester Jones, et al. 1959, Bern and Nandi, 1964). Cortisone, corticosterone or both have also been identified in some species. Cortisone was present in amounts greater than cortisol in the plasma of spawning sockeye salmon (Schmidt and Idler, 1962). Robertson and his co-workers have studied the activity of the interrenal tissue of pacific salmon and of both searun rainbow trout (steelhead) and non-migration

rainbow trout (Hane and Robertson, 1959; Robertson and Wexler, 1959, 1960; Robertson et al., 1961, 1963). The universal post-spawning mortality in the pacific salmon is associated with hyperplasia of the interrenal gland and elevated levels of 17-OH corticosteroids (17-OHCS). Many tissues show degenerative changes similar to those found in Cushing's syndrome in man, a condition caused by excessive secretion of adrenal steroids. The higher post-spawning mortality which occurs in searun rainbow trout compared with that in non-migratory races is also associated with elevated interrenal activity during the pre-spawning period. These investigators have been able to produce degenerative changes similar to those occurring in salmon by administration of exogenous cortisol to immature non-migratory rainbow trout (Robertson, et al. 1963). Circulating levels in sockey salmon were found to increase about four-fold during the period of the upstream spawning migration (Idler, et al., 1959; Schmidt and Idler, 1962). Elevated levels were also found in spawning coho salmon and to a less marked extent, in Atlantic salmon. The latter, like the rainbow trout, spawns annually after reaching sexual maturity.

Responses of interrenal tissue of fish to experimentally induced stress has not been studied extensively. Hatey (1958) has demonstrated an elevation in plasma levels

of 17-OH Corticosteroids in carp subjected to short-term stress of forced swimming and of removal from the water for short periods of time. Rasquin (1951) subjected Astyanax mexicanus to cold shock, which resulted in the release of lymphocytes. Fish of this species reared in total darkness showed hypertrophy and hyperplasia of the interrenal tissue and an involution of lymphoid tissue (Rasquin and Rosenbloom, 1954). These authors suggested that this indicated that rearing in total darkness constituted a stress.

Erickson (1967) placed individually marked green sunfish in small aquaria in groups of four and observed the resulting social interaction. Individuals were rated according to the position which they assumed on the social hierarchy. Subsequently, the volume of interrenal tissue was estimated. Fish of lower social rank had significantly greater volumes of interrenal tissue. Fish of this species previously had been shown to grow more slowly when held in groups of four in four liters of water than when held alone in one liter of water (Allee, et al., 1948). Higher ranking fish in groups grew faster than lower ranking fish. Erickson's subsequent observations suggest that the interrenal gland played a role in the variations in growth observed by Allee and his associates.

McKim (1966) measured urine metabolites of

17-OH corticosteroids produced by rainbow trout subjected to low oxygen levels and to sub-lethal concentrations of synthetic detergent (alkyl benzene sulfonate). Both treatments resulted in an initial increase in output of 17-OHCS metabolites. The output diminished to a level similar to that of control fish in about one week, although the stressful stimulus was applied continuously during this period.

The present study was undertaken to determine whether chronic exposure to low levels of noxious stimuli will cause an increase in plasma levels of glucocorticoids in rainbow trout and to determine some metabolic effects of elevated levels of glucocorticoids.

A response by the interrenal gland, if elicited by sublethal noxious stimuli, singly or in combination, would be extremely useful as a diagnostic aid for evaluating a wide variety of disturbances ranging from low-level chemical pollution to highway construction along streams and other engineering works effecting the physical characteristics of streams. Physiological parameters which constitute valid evidence for harmful stimuli are urgently needed by resource management personnel for aiding in negotiations with individuals, business concerns and public agencies who have altered or seek to alter fish habitat in a manner detrimental to fish. Such physiological information is also needed for demonstrating, in legal actions, the existence of detrimental conditions.

MATERIALS AND METHODS

Fish. The fish used in the study were rainbow trout (Salmo gairdneri) obtained from the Michigan Conservation Department hatcheries at Harrietta, Michigan or Grayling, Michigan. Fish were about two years old, sexually immature and ranged in body weight from about 80 grams to 180 grams. Fish were transported to the laboratory in groups of 200 in a tank containing 300 l of water aerated with a motor-driven propellor and, when appropriate, kept cool by adding ice during the period of transportation (about 3 hours).

All fish were held in the laboratory for at least one week before use in experiments. Holding facilities consisted of a group of 300 l tanks in a controlled temperature room with a controlled light regime. Temperature was held at 13° C ±1° and the lights were on from 7 am to 9 pm (14 hours). Each tank held 25 kg or less of fish and received a continuous moderate (1 l/min) flow of dechlorinated tap water. Tanks were aerated with compressed air delivered through air stones.

Fish were fed commercial trout pellets at intervals of 2-4 days at a rate of approximately one percent of body weight per day. When excessive amounts of feces collected in the tanks, fish were moved to clean tanks.

Exposure to hexavalent chromium. Fish were exposed to environmental hexavalent chromium in tanks containing 100 l of aged tap water. Chromium was added in the form of potassium dichromate. The water was maintained at 13°C ± 1° and aerated through air stones. Daily light regime was as described above.

Eight fish (total body weight of about 1 kg) were held in each tank. At three-day intervals fish were fed and moved to clean tanks.

Exposure to chronic forced exercise. Fish were exercised for periods of one-half hour twice daily in an annular rotating tank similar to that described by Fry (1947). Dimensions of the tank were as follows: outside diameter - 100 cm, inside diameter - 80 cm, height - 15 cm. The tank was filled to a depth of 10 cm. Thus, the cross-sectional area of the water was 10 cm by 10 cm. The circumference at the center of this annular body of water was 283 cm.

Water in the tank was aerated with an air stone mounted in a stationary position. A pair of electrodes were placed upstream from the air stone. An a.c. potential of 3 volts was applied to discourage fish from drifting with the water. The tank was mounted on a circular platform which was driven by a variable-speed gearmotor.

The tank was located in an illuminated room whose temperature was maintained at 13° C ± 1°. The out-

side wall of the tank was made of clear plastic while the bottom and inside wall were made of opaque plastic. When placed in the rotating tank, the fish oriented visually on the surroundings and swam against the moving water to maintain a constant position with reference to these surroundings.

Fish were exercised in groups of eight. The dissolved oxygen content of the water was not materially lowered by this number of fish during the exercise period.

After fish were placed in the moving tank and all had oriented in the proper direction, the speed of rotation was increased until one or more fish appeared to have difficulty in maintaining position. The electrical stimulus was then applied to encourage these fish. The resulting behavior was as follows: most fish would maintain place very well, sometimes moving less than a few centimeters in 10 minutes. One or several fish would slowly lose ground against the moving water. When the caudal fin of one of these fish entered the field between the electrodes, the fish would dart foreward for about a half a meter and would then maintain place for a period of a few seconds to several minutes before again falling back toward the electrodes.

The tank speed was manipulated so that the slowest swimming fish appeared to be having considerable

difficulty in maintaining position, but were not swept through the shocker. This velocity ranged from 10 to 17 revolutions per minute. The velocity of the water was determined by observing the movement of saturated cotton balls placed in the tank and was found to be about 0.80 times the tank velocity at the speeds used. Thus, the fish were swimming at velocities ranging from 0.40 to 0.65 meters per second.

A group of fish from the same lot was moved from one tank to another each time the experimental fish were exercised. These fish served as controls.

Treatment with exogenous cortisol. For the purpose of determining some metabolic effects of high circulating levels of cortisol, levels were raised by administering exogenous cortisol by intraperitoneal implantation.

Pellets used in the experimental animals were made from cortisol (Nutritional Biochemicals Corp., Cleveland) diluted with two parts of cholesterol. The pellets were made in a manner similar to that used by Robertson et al., (1963). The cortisol and cholesterol were mixed in a dry state, wetted with diethyl ether to make a slurry and stirred constantly while the ether was evaporated by the heat of the hand. The resulting granular material was then weighed into 45 mg portions (containing 15 mg of

cortisol) and pressed into pellets 6.3 mm (0.25 inches) in diameter.

Initially, pellets were pressed with a force of 150 kg over the 6.3 mm diameter area. These pellets, when implanted in fish for periods of 1 and 2 weeks, failed to increase circulating cortisol levels. When removed from the fish, they appeared not to have been eroded by dissolution. Further trials were made with pellets made at various pressures. Pellets formed at forces of 50 kg or less crumbled within the body cavity, while those formed with forces of 70 kg or more did not release cortisol at a sufficient rate. Pellets formed at a force of 57 kg (125 lb) were found to resist crumbling and to release cortisol at a rate sufficient to increase circulating cortisol to the desired level.

Pellets of cholesterol only were made in a similar manner and were implanted in fish used as controls in these experiments. Cholesterol is not released into the circulation when implanted in this manner (Robertson, et al., 1963).

Pellets were placed in fish under MS 222 anesthesia. Two pellets were placed in each fish (body weight - 100 to 180 gm). An incision 12-15 mm long was made in the ventral body wall anterior to the spleen and caudal to the stomach. The incision was located to the left of a

medial position in fish receiving cortisol pellets and to the right of midline in control fish. After the pellets were placed within the body cavity, the incision was closed with a single suture.

Determinations of metabolic parameters were made 1 and 2 weeks after implantation. Fish were fasted during this period.

Treatment with exogenous ACTH. Mammalian ACTH (Depo* ACTH, Upjohn Co., Kalamazoo, Mich.) was diluted with 300 mOs Ringer's solution to a concentration of either 2 units per ml or 4 units per ml and was injected into the peritoneal cavity under MS 222 anesthesia.

Blood samples were taken from fish under MS 222 anesthesia. Blood was taken from the anterior end of the dorsal aorta (Schiffman, 1959) in syringes previously wetted with a minimal amount of heparin solution (1000 U.S.P. units per ml). Because of well established diurnal rhythms of circulating corticoid levels of man and other mammals, blood samples were consistently taken at the same time of day (9-11 am). Since this study was begun, a diurnal rhythm of plasma levels of adrenal steroids has been reported in carp (Boehlke, et al., 1966).

Blood was centrifuged at room temperature within 10 minutes after drawing and plasma was separated

by aspiration immediately thereafter. Plasma used for glucose determination was treated for precipitation of protein within 30 minutes; plasma used for cortisol determination was frozen and stored at -15°C.

Liver glycogen was determined colorimetrically by the phenol-sulfuric acid method of Montgomery (1957). Liver samples of 0.5 to 1.0 gram were taken from the fish immediately after removal of a blood sample. The tissue was weighed as rapidly as possible on a Roller-Smith torsion balance, placed in 30% KOH and immediately heated in a boiling water bath. After 20 minutes of digestion, the tissue was cooled and the glycogen was precipitated by adding sufficient 95% ethanol to produce a solution of 60% ethanol. The precipitate was centrifuged, washed with 60% ethanol, dissolved in distilled water and diluted with water to produce a concentration appropriate for the colorimetric method. An aliquot of 2.0 ml was treated with 0.1 ml 80% phenol and 5.0 ml conc. sulfuric acid and read at 490 mμ on a Bausch and Lomb Spectronic 20 colorimeter. The amount of glycogen present in the aliquot was determined from a curve made by treating known quantities of glycogen in a similar manner. Calculations were made to express glycogen content in grams per 100 gm of liver tissue (wet wt).

Plasma glucose was determined with commercial enzymatic reagent (Gluostat, Worthington Biochemicals, Inc. Freehold, N. J.). Protein was precipitated with barium hydroxide and zinc sulfate. A 10 minute reaction period was used. Absorption was read at 490 mμ; glucose concentration was determined from a standard curve.

Measurement of nitrogen excretion. Total nitrogen excretion was determined by placing fish in individual plastic tanks containing 4.0 l of water for a period of 24 hours. Aeration was provided, temperature was held at $13^{o}C \pm 1^{o}$ and light regime was maintained at 14 hrs light and 10 hrs darkness. Disturbance of the fish was avoided. An aliquot of the water was digested with sulfuric acid and steam distilled. The resulting solution was treated with Nessler's reagent and absorption was measured at 410 mμ (APHA, 1965). Nitrogen content of the water was determined from a standard curve, corrected for nitrogen present in the water supply and expressed as mg N excreted per kg of fish per day.

Plasma cortisol was measured by fluorescence in ethanolic sulfuric acid. This method is dependent on a fluorescent property exhibited by cortisol and corticosterone but only feebly by cortisone (Sweat, 1954). The principal steroid secreted by the interrenal gland of rainbow trout is cortisol

(Hane and Robertson, 1959; Nandi and Bern, 1965). Cortisone is also present in plasma and in interrenal extracts, but in lesser amounts. An alternative method for determination of plasma steroids, the colorimetric determination of products or reaction of steroids with phenylhydrazine (Silber and Porter, 1954) was considered and rejected. This method is dependent on a 17α, 21 dihydroxy-20 keto steroid configuration and measures cortisol and cortisone. The results of assays by this method are usually expressed as 17 hydroxy cortical steroids (17-OHCS). Robertson and co-workers used this method in their study of the histological effects of exogenous cortisol (Robertson, et al., 1963). The method has the disadvantage of requiring a blood sample several times larger than can be obtained from a fish of the size available for the present study.

The cortisol assay procedure used was similar to that of Guillemin et al. (1959). Plasma samples used were 0.2 ml and the quantities of reagents used were reduced accordingly. The fluorescence-inducing solvent used was 70% conc. H_2SO_4, (reagent, DuPont, Wilmington) 30% absolute ethanol (V/V), which produced optimal sensitivity to cortisol.

Steps in extraction and fluorescence measurement were:

1. 0.2 ml of plasma was placed in a 15 ml glass stoppered centrifuge tube and was diluted to 2.0 ml with distilled water. (2.0 ml of distilled water was used as a reagent blank).

2. 4.0 ml of spectro grade 2, 2, 4-trimethyl pentane (Eastman Kodak Co., Rochester, N. Y.) was added to each tube. Tubes were stoppered, shaken vigorously for 20 sec and centrifuged for 10 minutes.

3. The upper layer (2, 2, 4-trimethyl pentane) and the protein layer at the interface were carefully aspirated and discarded.

4. The water phase was diluted by addition of 2.0 ml of distilled water and 5.0 ml of chloroform (ACS Reagent, J. T. Baker Chemical Co., Phillipsburg, N. J.) was added. Shaking and centrifugation were repeated and the upper (water) phase was removed and discarded.

5. One-half ml of 0.1 N sodium hydroxide was added to the chloroform and the tubes were shaken briefly and centrifuged for 3 min. The NaOH layer was removed.

6. A 4.0 ml portion of the chloroform phase was transferred to a tube containing 1.0 ml of freshly made 70:30 ethanolic sulfuric acid. The tube was stoppered, shaken and centrifuged. The chloroform phase was removed.

7. A portion of the ethanolic sulfuric acid was transferred to a cuvette and the fluorescence was read 40

minutes after the chloroform and acid were shaken together.

Fluorescence was measured with a Turner model 110 filter fluorometer (G. K. Turner Assoc., Palo Alto, Calif.) equipped as follows:

1. cuvette holder: micro adapter with high sensitivity conversion kit.

2. light source: Turner #110-853 blue lamp.

3. primary filter: Baird-Atomic interference filter with peak transmittance at 468 mμ (Baird-Atomic, Inc., Cambridge, Mass.)

4. Secondary filter: Corning CS4-94 blue-green (Corning Glass Works, Corning, N. Y.) and Turner 110-818 sharp cut yellow. This combination of filters has a maximum transmittance at about 540 mμ.

A standard curve was prepared by treating known amounts of cortisol in the same manner as plasma samples.

RESULTS

Exposure to environmental chromium. Two groups of fish were held in water containing 0.02 and 0.20 mg/l of Cr^{6+}. At these levels, Cr^{51} was found by Fromm and Stokes (1962) to accumulate steadily in rainbow trout throughout a 28 day experimental period.

After a one week exposure, the fish exposed to 0.20 mg/l Cr^{6+} had plasma cortisol levels nearly twice those of control fish (54.3 µg% vs 30.5 µg%, $t = 2.46$, $p < 0.01$). Fish exposed to the lower level (0.02 mg/l) also had plasma cortisol levels significantly higher than control fish (44.6 µg%, $t = 1.80$, $p < 0.05$).

After exposures of two and three weeks, however, chromium treated fish had plasma cortisol levels essentially similar to those of control fish (Table 1).

Groups of fish were also exposed to environmental hexavalent chromium at levels approaching those found to be lethal to largemouth bass by Fromm and Schiffman (1958). Fish exposed to Cr^{6+} at 20 mg/l for 3 days showed plasma cortisol levels of 56.8 µg%, compared with 37.8 in control fish ($t = 2.60$, $p < 0.01$). Fish exposed for 6 days and 7 days showed no significant increase in cortisol levels over control fish; fish exposed for 10 days showed

Table 1. Plasma cortisol levels (µg%) in rainbow trout after exposure to environmental hexavalent chromium at low levels.

Length of Exposure	Level of Exposure		
	Control	0.02 ppm	0.20 ppm
1 wk	30.5±4.1[‡] (6)[+]	44.6±5.8* (6)	54.3±3.8** (6)
2 wks	39.2±7.6 (6)	41.0±5.2 (6)	40.6±2.4 (6)
3 wks	41.2±2.2 (6)	34.0±2.4 (6)	37.4±2.4 (6)

[‡]Mean ±s.e.
[+]Number in parentheses denotes sample size.
*Significantly different from control fish ($p < 0.05$; $t = 1.80$).
**Significantly different from control fish ($p < 0.01$; $t = 2.46$).

levels elevated above control levels, but lacking significance at the 0.05 level ($t = 1.61$, $0.10 > p > 0.05$ Table 2). Thus, such exposure to levels of hexavalent approaching 48 hr median tolerance level produced a response of a much lower magnitude than that in spawning pacific salmon to which Robertson and co-workers attributed detrimental degenerative secondary responses. Furthermore, this response was apparently transitory.

Exposure to exercise. Groups of fish were exposed to two daily half-hour periods of forced exercise for one week and two weeks. The speed of the tank was regulated so that the fish were swimming as rapidly as they were willing to do for the half-hour period. This speed was usually about 0.6 meters per second.

Blood samples were taken for cortisol determination after one and two weeks of this regime. Fish were rested for 24 hours before blood samples were taken. It was anticipated that the exercise regime might elevate plasma cortisol levels. Instead, the exercised fish exhibited lower cortisol levels than did the control fish (Table 3).

In order to evaluate the possibility that this apparent depression of cortisol levels was accompanied by elevated levels immediately after exercise, two groups of

Table 2. Plasma cortisol levels (µg%) in rainbow trout after exposure to environmental hexavalent chromium at near-lethal levels

Length of Exposure	Level of Exposure	
	Control	20 ppm
3 days	37.8±4.9 (5)	56.8±9.0** (6)
6 days	29.4±6.0 (5)	35.9±4.5 (5)
10 days	46.2±3.9 (6)	59.8±8.6 (6)
	Control	30 ppm
7 days	50.0±6.0 (6)	58.8±2.6 (6)

fish were forced to swim in the annular tank for 2 and 4 hours, respectively. Blood samples were taken immediately while the tank, containing remaining fish, continued to turn. Blood was also taken from undisturbed fish from the same lot. Plasma cortisol levels in the exercised fish were somewhat elevated above that of control fish; the elevation after 4 hours was statistically significant (Table 4).

Metabolic effects of treatment with exogenous cortisol. Some characteristic effects of elevated levels of adrenal cortex activity in mammals include an increase in liver glycogen levels, an elevation in plasma glucose levels, and an increase in the utilization of amino acids for energy metabolism and gluconeogenesis, with a resulting increase in nitrogen excretion. These metabolic parameters were measured in fish whose circulating levels of cortisol was elevated by administration of exogenous hormone.

Fish were treated by implanting pellets as described above. The fish were then held as a group in 300 l tanks for six days, at which time they were placed in individual tanks for 24 hours for the determination of nitrogen excretion. At the end of this time, the fish were anesthetized and samples of blood and liver were taken.

In fish which were fasted from two days before implanting of cortisol pellets to the time of the deter-

Table 3. Cortisol levels in fish exposed to forced exercise at about 0.6 m/sec for one-half hour twice daily. Blood sampled 24 hours after exercise.

duration	plasma cortisol (µg%)
Control (5)	55.4±2.8
1 wk (5)	48.0±2.4 ($t = 2.0$, $p < 0.05$)
2 wks (5)	46.6±2.1 ($t = 2.5$, $p < 0.05$)

Table 4. Cortisol levels in fish exposed to forced exercise for 2 or 4 hours. Blood samples taken immediately.

duration	plasma cortisol (µg%)
Control (5)	54.4±3.5
2 hrs (5)	61.0±2.3 ($t = 1.40$, $p > 0.05$)
4 hrs (5)	64.2±4.3 ($t = 1.96$, $p < 0.05$)

minations (a total of nine days), the glycogen content of the liver and nitrogen excretion were elevated over that found in control fish. Plasma glucose levels in treated fish did not differ from that of control fish (Table 5).

A similar experiment was conducted with fish which were fed at a rate of 2.0% of body weight per day during the period between implanting of pellets and sampling. Neither liver glycogen levels nor nitrogen excretion was elevated in the cortisol treated fish (Table 6).

Seasonal variations in circulating cortisol levels. Plasma cortisol levels in untreated fish were measured at various times of the year and were found to vary seasonally in sexually immature rainbow trout. Early experiments carried out in summer and early fall, resulted in plasma cortisol levels in untreated fish of between 30 and 40 µg%. These fish were held in small groups in 100 l tanks containing about 1 kg of fish.

In July, 1966, control levels in fish confined individually in 4.0 l of water for 24 hours exhibited plasma cortisol levels of 49 µg%. The latter level, although representing a control level for experiments designed to show effects of exogenous cortisol on metabolic parameters, may not be directly comparable to the earlier determinations, because of confinement required to determine nitrogen excretion levels.

Table 5. Effects of one week exposure to exogenous cortisol on nitrogen excretion, liver glycogen and plasma glucose of fasted rainbow trout.

parameter	control (6)	experimental (8)
plasma cortisol	48.7±4.5 ug%	125.7±16.0
liver glycogen	0.59±0.16 gm%	1.17±0.12*
nitrogen excretion	219±29 mgN/kg/day	306±61+
plasma glucose	86.3±11.5 mg%	74.1±11.0

*Significantly higher than control ($p < 0.05$, $t = 2.49$)

+($p > 0.05$, $t = 1.16$)

Table 6. Effects of one week exposure to exogenous cortisol on nitrogen excretion and liver glycogen of fed rainbow trout.

parameter	control (7)	experimental (6)
liver glycogen	3.63±0.69 gm%	3.55±0.56
nitrogen excretion	348±77 mgN/100/day	326±12

Cortisol levels in control fish from experiments conducted in October, 1965 were 30 to 40 µg%, similar to those found in July under similar laboratory circumstances.

Fish sampled in late November exhibited higher plasma cortisol levels (54.4±3.5 µg%) as did those taken in February (58.0±4.1 µg%). The nutritional condition of these fish was comparable to that of the fish mentioned above, excepting the July, 1966 group. The latter were fasted for seven days prior to measurement of nitrogen excretion.

After this seasonal variation was noted, it was intended that additional values for spring would be obtained. An unexpected epidemic among the fish in holding tanks made this determination impossible. Cortisol levels in various control groups and untreated fish are given in Table 7.

Response to ACTH. Experiments to determine the time course of response to ACTH injection were carried out at a time of year when plasma cortisol levels were quite high (December-January). Responses to ACTH were very slight and not statistically significant, suggesting that the interrenal tissue of untreated fish were producing cortisol at near-capacity levels.

Fish with body weights of 120-150 grams were treated uniformly with 0.5 units of ACTH. Blood samples

Table 7. Plasma cortisol levels in control and untreated fish at various times of the year.

month	circumstances	plasma cortisol
July	Control groups for exposure to Cr^{6+}	30.5±4.1 (6) 39.2±7.6 (6) 41.2±2.2 (6)
October	Control groups for exposure to Cr^{6+}	37.8±4.9 (5) 29.4±6.0 (5) 46.2±3.9 (6) 50.0±6.0 (6)
October	Control group for determination of metabolic effects of exogenous cortisol. (Cholesterol implanted, fasted for 9 days, confined for 1 day).	48.7±4.5 (6)
December	Control for acute exercise	54.4±3.5 (5)
January	Untreated	66.2±1.9 (5)
February	Untreated	61.6±5.4 (5)
February	Untreated group used to determine N excretion (fasted 8 days, confined 1 day).	58.4±4.5 (5)
March	Control for ACTH response (saline injected).	57.9±4.1 (6)
March	Control for ACTH response (saline injected).	53.8±2.5 (6)

were taken under MS222 anesthesia at 0.25, 0.5, 1, 2, 4, 8 and 24 hours. Levels at 0.5, 1 and 2 hours were moderately elevated, but the elevation was not statistically significant.

A similar experiment in which ACTH dose was adjusted to body weight produced a lesser response, even though control values were somewhat lower. Data from these experiments are given in Table 8.

Table 8. Plasma cortisol levels (ug%) in rainbow trout at various times after administration of exogenous mammalian ACTH.

Group A: fish treated with 0.5 units per fish.								
time (hrs.)	contr.	0.25	0.5	1	2	4	8	24
no.	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)
plasma cortisol	69	65	94	96	82	70	74	69
±s.e.	13.4	10.0	14.7	4.5	7.9	8.5	13.4	3.5

Group B: fish treated with 0.5 units per 100 gm body wt						
time (hrs.)	contr	0.5	1	2	4	10
no.	(8)	(4)	(4)	(3)	(4)	(3)
plasma cortisol	58	61	61	64	61	57
± s.e.	4.1	9.3	3.8	6.3	6.8	5.0

A group of fish was treated with doses of 0.3, 0.6 or 1.2 units of ACTH per 100 grams of body weight and blood was sampled two hours later. This experiment was performed in December, when control levels of plasma cortisol were elevated but when a moderate response was obtained with a dose of 0.5 units per fish (Table 8, part A). Plasma cortisol levels of treated fish were not significantly elevated above those of control fish (Table 9, part A).

Table 9. Plasma cortisol levels (µg% in rainbow trout after administration of various amounts of exogenous mammalian ACTH.

Group A: experiment conducted in December				
treatment (units/100 gm)	control	0.3	0.6	1.2
no.	(6)	(6)	(6)	(6)
plasma cortisol ± s.e.	76 5.5	87 8.8	93 4.0	87 7.7
Group B: experiment conducted in March				
treatment (units/100 gm)	control	0.5	1.0	2.0
no.	(6)	(6)	(6)	(6)
plasma cortisol ± s.e.	68 3.7	73 3.6	64 3.6	62 3.7

Because of the high levels of plasma cortisol found in control fish, the effect of handling, fin clipping and injection of Ringer's solution was investigated. None was found to cause an elevation of plasma cortisol levels. The undisturbed control fish showed an average cortisol level of 74 ug%, nearly identical to that of the control fish in the last experiment mentioned above.

A similar experiment to determine dose response was carried out in March when plasma cortisol levels in untreated fish were somewhat lower. Again, no significant differences were found. Results, based on samples taken two hours after ACTH injection, are given in Table 8, part B.

DISCUSSION

Exposure of rainbow trout to environmental hexavalent chromium, both at near-lethal levels (20-30 mg Cr^{6+}/l) and at much lower levels (0.02 and 0.2 mg Cr^{6+}/l) resulted in an elevation of cortisol levels significantly above those of control fish. However, the elevation was transitory. While fish exposed to the low concentrations of Cr^{6+} for one week showed these elevated levels, fish exposed to the same conditions for two or three weeks had circulating cortisol levels similar to control fish. Exposure to the higher levels produced a significant elevation only at three days and did not do so at six, seven or ten days.

Somewhat similar results were obtained by McKim (1966), who measured output of urinary 17-OHCS metabolites in rainbow trout exposed to a continuous stress of sub-lethal environmental levels (3, 5 and 7 mg/l) of the detergent alkyl benzene sulfonate. Excretion rates of 17-OHCS of experimental fish rose about two-fold in the first 24 hours of exposure but in all cases decreased to only slightly above (at 7 mg/l ABS) or equal to (at 3 mg/l and 5 mg/l) the rates of control fish at the end of seven days.

The transitory nature of interrenal response of rainbow trout to continuously applied stress suggests that the response of this gland does not play a major role in any adverse effects due to chronic exposure to low-level stress. Such a response would also be of little value for the detection of the estimation of magnitude of sub-lethal stresses in natural environments.

The slight depression of circulating cortisol found in fish subjected to intermittent forced exercise may have resulted from the occurence of a similar transitory response. The minimum period of experimental treatment used was one week. At the end of a similar period, the response of fish exposed to ABS by McKim had diminished completely or nearly so. The fish exposed to Cr^{6+} in the present study, however, showed a definite response at one week but not at two weeks.

It is possible that the twice-daily half-hour forced exercise did cause temporary responses which diminished between exercise periods. Such a response would not have been detected at the time of sampling (24 hours after the last exercise period). This delayed sampling time was chosen so that any chronic elevation of cortisol level could be detected without interference from a possible acute immediate effect.

The groups of fish subjected to forced exercise

and sampled immediately did show elevated plasma cortisol levels. Similar results caused by acute exercise have been reported in the past (Hatey, 1958).

The metabolic effects of treatment with exogenous cortisol were quite moderate. The circulating cortisol levels produced (over 100 µg/100 ml plasma) were similar to those which Robertson and his co-workers (1963) found nearly 100% lethal when maintained over a period of 8 weeks and were about twice the levels caused by exposure to near-lethal levels of environmental chromium.

Elevated circulating cortisol increased liver glycogen stores and nitrogen excretion rates over that of control fish only in fasted fish. These parameters in cortisol-treated fish fed at normal rates did not differ from those of control fish.

Plasma glucose levels were not elevated by exogenous cortisol in either fasted or fed fish. Elevated plasma glucose levels are usually associated with high circulating glucocorticoid levels in mammals (Turner, 1966). Some observations in fish suggest a similar effect. Nace (1955) found average plasma glucose levels of 113 mg% in toadfish treated with cortisol and 75 mg% in untreated fish. Values were variable, overlapped and no measure of deviation was given. Significant elevation of plasma glucose levels of goldfish resulted from ACTH injection

for one week (Oguri and Nace, 1966). Robertson et al. (1961) found plasma glucose levels of spawning steelhead trout about 50% higher than in immature non-migratory rainbow trout. This difference was associated with an elevation of circulating 17-OHCS in the spawning fish. A more useful comparison, spawning vs non-spawning adult steelhead trout was not made.

The apparent important role of the interrenal gland in bringing about the degenerative changes observed in spawning salmon has been given strong support by the successful reproduction of most of these changes by the administration of exogenous cortisol to immature rainbow trout (Robertson, et al., 1963). The high corticosteroid levels found in migrating pacific salmon may be an unusual phenomenon associated with a simultaneous demand for long-sustained exertion of migration and of maturation of a large biomass of gonadal tissue and gametes. The results of the present study and those of McKim (1966) suggest that the response of the interrenal gland to chronic sub-lethal stress does not play a major role in the genesis of adverse effects which may be caused by these stressors. Admittedly the number of stressors or stress situations thus far tested is limited.

There is some evidence that the responsiveness to ACTH of the adrenal cortex of rats (Langecher,

et al., 1957, cited by Mangili, et al., 1966) and humans Bierich, et al., 1959) is decreased by high circulating corticoid levels. A similar effect has been reported in Pacific salmon (Oncorhynchus tshawytscha) by Hane, et al. (1966). Injection of 0.5 units/kg of mammalian ACTH increased plasma 17-OHCS four-fold in non-spawning fish caught in the ocean. Increases nearly as marked were elicited in sexually undeveloped fish captured at the beginning of their spawning migration. Responsiveness diminished as the fish approached sexual maturity, coincident with increased plasma 17-OHCS levels. Little response to ACTH could be elicited in spawning fish, whose 17-OHCS levels were greatly elevated.

In the present experiments the elevation of plasma cortisol levels following intraperitoneal injection of mammalian ACTH was very low. This response may have been minimal because the experiments were carried out at a time of year when the plasma cortisol levels of untreated fish were high. Doses of mammalian ACTH used by the aforementioned workers as well as those used in the present study, when expressed in terms of body weight, are about 100 times that required for maximal stimulation of the adrenal cortex of dogs (Guillemin, 1963).

SUMMARY

1. A study was conducted to determine the response of the interrenal gland of rainbow trout to chronic exposure to sub-lethal stress and to determine some metabolic effects of high circulating cortisol levels.

2. Exposure of fish to 0.02 and 0.20 mg/l of environmental hexavalent chromium for one week significantly elevated plasma cortisol levels. Fish exposed to these concentrations of Cr^{6+} for two and three weeks had plasma cortisol levels similar to those of control fish.

3. Fish exposed to 20 or 30 mg/l of Cr^{6+} (levels approaching the 48 hr TLm for largemouth bass) for three days had elevated cortisol levels, but fish exposed for six, seven or ten days did not.

4. Fish forced to swim for one-half hour twice daily at about 0.6 meters/sec for one and two weeks did not have elevated plasma cortisol levels when sampled 24 hours after the last exercise period.

5. Fish forced to swim for two or four hours and sampled immediately did have plasma cortisol levels significantly above those of control fish.

6. Fish were treated with exogenous cortisol by implanting pellets in the body cavity, resulting in plasma cortisol levels of over 100 µg%. Fasted fish so treated for one week had higher liver glycogen stores and secreted more nitrogen than did control fish. Plasma glucose levels were not elevated in treated fish.

7. Fish similarly treated but fed during the experimental period had liver glycogen stores and nitrogen excretion rates similar to control fish.

8. A seasonal variation in plasma cortisol levels of untreated fish was found. Under laboratory conditions, untreated groups of fish sampled in summer and early fall showed average cortisol levels of 30-40 µg%; groups of fish sampled under similar circumstances in winter had average plasma cortisol levels ranging from 58 to 76 µg%.

9. Treatment with exogenous ACTH, carried out at a time when untreated fish showed high cortisol levels, did not cause a significant increase in these levels.

10. The effect of chronic low-level stress on circulating cortisol levels of rainbow trout is transitory and does not appear to be a useful indication of the existence of chronic stressful conditions. It is also

unlikely that this response is a major causative factor in any untoward effects caused by chronic exposure to sub-lethal stress in rainbow trout.

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