# Metabolic Effects Associated With Chronically Elevated Cortisol in Rainbow Trout (*Oncorhynchus mykiss*)

By

Donald E. Andersen

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UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

## ABSTRACT

The metabolic role of chronically elevated cortisol in otherwise unstressed rainbow trout, *Oncorhynchus mykiss*, was examined. Fish were fitted with mini-osmotic pumps which maintained plasma cortisol levels at approximately 100 or 200 $ng \cdot mL^{-1}$ for ten days. Plasma metabolites, liver enzyme activities, liver glycogen content, metabolic flux in isolated hepatocytes and alanine turnover were investigated. Plasma glucose, lactate and protein levels were unaffected by ten days of cortisol administration, despite a significant elevation in plasma cortisol. Plasma amino acids in cortisol treated fish ($1023.8 \pm 90.7\ \mu g \cdot mL^{-1}$) were significantly elevated compared to shams ($716.7 \pm 68.5\ \mu g \cdot mL^{-1}$) after nine days. Liver glycogen content was significantly reduced by cortisol treatment. The activities of the liver enzymes assayed were unchanged; likewise the fluxes of radioactive substrates to radiolabelled $CO_2$, glucose, and protein in isolated hepatocytes were unaffected in trout with chronically elevated cortisol compared to shams. Both the caloric and water contents of white muscle were unaffected by chronically elevated circulating cortisol levels. The cortisol treatment did not alter the turnover of alanine. These data do not support the purported role of cortisol as a glucocorticoid in rainbow trout. While chronically elevated cortisol may increase the supply of plasma amino acids, the hormone does not appear to alter the manner in which these potential gluconeogenic substrates are metabolized. The absence of other stressors may be partially responsible for the differences between this study and others in the literature.

## RESUMÉ

Les conséquence metaboliques du cortisol chroniquement élevé étaient examiner chez les truites arc-en-ciel, *Oncorhynchus mykiss*. Les poisons ont porté pendant 10 jours une mini-pompe osmotique maintenant le cortisol plasmatique à environs 100 ou 200 $ng \cdot mL^{-1}$. Les metabolites plasmatiques, l'activité des enzymes hépatiques, le glycogène hépatique, le flux metabolique de substrats radioactifs vers $CO_2$, glucose et protéines radiomarqués et le renouvellement du alanine sont examinés. Les teneurs plasmatiques en glucose, lactate, et protéines était inchangées après dix jours de traitement au cortisol, malgré une augmentation sensible du cortisol plasmatiques. Chez les poisons traiés, les concentrations d'acides aminés plasmatiques ($1023.8 \pm 90.7\ \mu g \cdot mL^{-1}$) était supérieures dans une mesure significative à celles mesurées chez les témoins ($716.7 \pm 68.5\ \mu g \cdot mL^{-1}$) après 9 jours. Le glycogène hépatique a sensiblement baissé avec le traitement au cortisol. L'activité des enzymes hépatiques évaluées est restée inchangée; de même, le flux de substrats radioactifs vers $CO_2$, glucose, et protéines radiomarqués dans des hépatocytes isolés n'a pas été modifié chez la truite à cortisol chroniquement élevé comparé aux temoins. Les contenues caloriques et hydrauliques du muscles blancs était inchangées par le traitement au cortisol. Le traitement au cortisol n'effect pas le renouvellement du alanine. Ces résultats ne confirment pas l'hypothèse seion lequelle le cortisol joue le rôle d'un glucocorticoide chez la truite arc-en-ciel. L'élévation chronique du cortisol peut faire augmenter l'apport d'acides aminés plasmatiques, mais il semble que le cortisol n'influe pas sur la façon dont ce substrat gluconéogène potentiel est métabolisé. L'absence d'autres factteurs de perturbation explique

peut-être en partie les différences qui est note. entre cette étude et celles dont on fait état dans la littérature du domaine.

## Acknowledgements

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# CHAPTER 1
# INTRODUCTION.

Growth rate is determined by the amount of energy directed to tissue synthesis after other metabolic processes have been satisfied (Brett and Groves, 1979). Fish are thought to sacrifice growth in response to the increased energetic demands of maintaining homeostasis under conditions of chronic stress. Therefore, fish require some type of "biochemical switch" to permit the shunting of energy from anabolic to catabolic processes when required. The plasma concentration of the steroid hormone cortisol is usually elevated when fish are subjected to chronic stress. The involvement of cortisol in promoting metabolic alterations in rainbow trout (*Oncorhynchus mykiss*) is the subject of this thesis.

## The Stress Response in Higher Vertebrates

Esch *et al.* (1975) defined stress as "the effect of any force which tends to extend any homeostatic or stabilizing process beyond its normal limit, at any level of biological organization". A stressor, either internal or external, is detected by sensory systems, resulting in a rapid rise in the plasma levels of cortisol and catecholamines. This is known as the primary effect of stress (Donaldson, 1981). These so-called stress hormones elicit physiological effects, such as the control of acid-base regulation, intermediate metabolism, and ionoregulation (Mazaud *et al.*, 1977; Mazaud and Mazaud, 1981). These physiological effects are said to be the secondary effects of the stress response. Finally, if the stress response is to be sustained over time then there will be a considerable energetic demand placed upon the animal, which may result in tertiary effects such as weight loss (Baker and

Wigham, 1979) and immunosuppression (Barton and Iwama, 1991). The stress response initially aids an animal in surviving a challenge, yet in time the stress response itself may become detrimental (Asterita, 1985).

The glucocorticoids are a group of 21-carbon steroids with many actions, the most important of which is to promote gluconeogenesis (Granner, 1990). Glucocorticoids also affect lipid and protein metabolism. In teleost fish, as in humans, cortisol is the predominant glucocorticoid (Butler, 1973).

The steroid hormone cortisol is generally considered to be an important mediator in maintaining homeostasis when an animal is subjected to a chronic stressor (Asterita, 1985). In mammals cortisol production by the adrenal cortex is regulated by pituitary adrenocorticotropin (ACTH) (Hadley, 1985). The release of ACTH is in turn regulated by corticotropin releasing hormone (CRH) which is synthesized and released from the hypothalamus. The mechanism of action for cortisol, as with all steroid hormones, involves its passage through the plasma membrane and subsequent binding to cytoplasmic receptors. The hormone-receptor complex is then transported into the nucleus where it binds to a specific segment of DNA, resulting in the production of complimentary messenger RNA. The mRNA enters the cytoplasm were they direct the synthesis of specific proteins. These proteins then function to alter the specific activities of the cell (Asterita, 1985).

While cortisol has many biological effects, such as reducing tissue inflammation through a stabilization of lysozyme membranes, the major site of action for glucocorticoids appears to be the liver (Thompson and Lippman, 1974). The effects of glucocorticoids are either direct or permissive. In a pioneer study, Ingle (1952) determined that the presence of

glucocorticoids allowed, or "permitted", an animal to respond to a graded stimulus with a graded response. Probably the most understood direct effect of glucocorticoids is the induction of liver enzymes (Voight *et al.*, 1978). In that study glucocorticoid treatment produced an increase in the level of the specific mRNA for tyrosine aminotransferase, thus establishing that alterations in transcription are an integral part of the mechanism of glucocorticoid action. Furthermore, increases in nuclear RNA polymerase activity indicate that RNA synthesis is involved in the hepatic response to glucocorticoids (Feigelson *et al.*, 1975).

Glucocorticoids appear to influence skeletal muscle metabolism in several ways. One of their extra-hepatic functions is to provide substrate for metabolic processes. Of major importance is the ability of corticosteroids to inhibit the synthesis of proteins in peripheral tissues (Shoji and Penington, 1977; Steele, 1975; Tomas *et al.*, 1979). This inhibition of peripheral protein synthesis is thought to be at the level of transcription (Rannels *et al.*, 1978). Amino acid transport into skeletal muscle is also inhibited by glucocorticoids (Krause-Friedmann, 1984). This inhibition of protein synthesis in conjunction with normal rates of protein degradation results in an elevation in plasma amino acid levels (Asterita, 1985). Therefore the overall effect of cortisol on intermediate metabolism appears to be an elevation in the level of circulating amino acids and an enhanced utilization of these potential gluconeogenic substrates through an increased induction of the enzymes responsible for hepatic gluconeogenesis.

**The importance of cortisol in the stress response of fish**

Teleost fish do not have distinct adrenal glands, instead cortisol is secreted by the interrenal tissue of the head kidney (Donaldson, 1981). The rate of cortisol secretion is directly regulated by pituitary ACTH (Donaldson, 1981; Sumpter *et al.*, 1986). As in mammals ACTH secretion is under the control of hypothalamic CRF which is secreted in response to stressors (Fryer and Peter, 1977; Donaldson, 1981).

As in mammals, increased cortisol secretion is clearly a component of the teleost stress response. While cortisol is often thought to act as a glucocorticoid, the metabolic effects of cortisol in teleost fish remain controversial. Chan and Woo (1978) demonstrated that in the Japanese eel, *Anguilla japonica*, a single intramuscular (IM) cortisol injection (1 $mg \cdot kg^{-1}$ body weight) caused a significant elevation in plasma glucose and amino acid levels. This protocol also stimulated liver glycogen deposition and increased the activity of the gluconeogenic enzyme, glutamic-oxaloacetic transaminase (GOT). Conversely, the activities of glutamic-pyruvic transaminase (GPT), fructose-1-6-bisphosphatase (FBPase) and glucose-6-phosphatase (G6Pase) were unaffected. In killifish (*Fundulus heteroclitus*), daily cortisol injections (20 $mg \cdot kg^{-1}$ body weight) for five days, resulted in hyperglycaemia, and in starved fish an increase in liver glycogen concentrations (Leach and Taylor 1982). Neither protein nor amino acid metabolism was affected in that study. Lidman *et al.* (1979) produced similar results in European eels, *A. anguilla*, using a slightly modified protocol (5 $mg \cdot kg^{-1}$ body weight) for ten days. More recently, Foster and Moon (1986) showed that in the American eel, *A. rostrata*, daily cortisol treatment (0.35 $mg \cdot kg^{-1}$ body weight) for ten days produced hypoglycaemia. This was a surprising finding which was certainly contrary to

the accepted dogma of glucocorticoid-induced hyperglycaemia. Unexpectedly, that study also reported a decline in the overall flux of $^{14}$C-labelled substrates to $^{14}$C-glucose in isolated hepatocytes, implying a decreased gluconeogenic activity, while activities of liver phosphoenolpyruvate carboxykinase (PEPCK) were elevated, suggesting an increased gluconeogenic activity. Daily intraperitoneal (IP) injections of cortisol (20 mg·kg$^{-1}$ body weight) for five days in rainbow trout, *Oncorhynchus mykiss*, increased the activities of the transaminase enzymes, GOT and GPT, implying an increase in the potential of amino acids as gluconeogenic substrates (Freeman and Idler, 1973). Interestingly, that study also reported that five daily injections of metopirone (SU4885) (50 mg·kg$^{-1}$ body weight) (Ciba Ltd.) increased GOT activity, yet this drug is known to impair glucocorticoid synthesis in rainbow trout by inhibiting the 11$\beta$-hydroxylation of adrenal steroids (Fagerlund *et al.*, 1968).

Given the discrepancies in the literature, there clearly is a need for further research on the metabolic effects of cortisol in teleost fish.

The mode of cortisol administration may be confounding the results of previous studies. If the direct metabolic effects of cortisol are to be isolated from those of other stress-induced changes (i.e., an elevation in circulating catecholamines), then it is essential that the hormone be administered in such a way as to minimize the disturbance to the animal. The majority of studies that have examined the metabolic effects of cortisol, however, have relied on frequent IP or IM injections to chronically elevate plasma cortisol levels. Foster and Moon (1986) demonstrated that this technique caused a massive post-injection elevation of plasma cortisol levels followed by its rapid clearance. After three days of treatment,

cortisol levels in treated fish were significantly below control levels. Owing to these large and continual oscillations in plasma cortisol concentrations, this mode of administration probably does not reflect the glucocorticoid status of teleost fish subjected to chronic stress. A recent study by Vijayan and Leatherland (1989) used IP implants of hydrogenated coconut oil containing cortisol to address this administration problem.

Reid and Perry (1991) recently demonstrated that chronically elevated plasma cortisol levels have a permissive role in the adrenergic response of rainbow trout to an acute stressor. That study showed that cortisol treatment resulted in significant increases in the number of erythrocyte "internalized", low affinity $\beta$-adrenoreceptors. When these cortisol "primed" trout were subjected to hypoxia the red cells exhibited an increased mobilization of the internalized receptors to the cell membrane surface. This finding is interesting because it suggests that fish subjected to chronic stress may have an enhanced response to acute stressors. Several studies have clearly shown that catecholamines have hyperglycaemic effects in fish (Murat *et al.*, 1981; Janssens and Lowrey, 1987; Wright *et al.*, 1989). Therefore it is possible that some of the studies which have concluded that cortisol has hyperglycaemic effects in teleosts (i.e., Chan and Woo, 1978; Lidman, 1979; Leach and Taylor, 1982) may simply be demonstrating an enhanced adrenergic response rather than a direct glucocorticoid response.

The aim of this thesis was to test the hypothesis that chronically elevated cortisol is responsible for an energetic repartitioning of metabolism, such that catabolic processes are favoured at the expense of anabolic processes. Specifically this thesis will investigate the

direct alterations in protein and carbohydrate metabolism which result from chronically elevated cortisol in the absence of other stress factors.

# CHAPTER 2

# MATERIALS AND METHODS

Rainbow trout were purchased from either Thistle Springs Trout Farm (Ashton, Ont.) or Linwood Acres Trout Farm (Campbellcroft, Ont.). Fish were held in 500 L fiberglass tanks supplied with flowing, dechlorinated, well aerated city of Ottawa tap water. The chemical composition of this water has been previously reported (Perry and Laurent, 1989). Photoperiod (12L:12D) and temperature (13 $\pm$ 1°C) were held constant throughout the experiment. Fish were fed daily to satiation with commercial trout pellets (Purina Trout Chow) prior to the experiments. Fish were permitted a minimum of 2 weeks to acclimate to laboratory conditions prior to each experiment. All experiments were performed between May and August. The fish used in experiments I, II, and III had a mean weight ($\pm$ SEM) of 178.1 $\pm$ 6.7 g. In all cases fish were sexually immature, thus size would not be expected to affect the manner in which they respond to cortisol treatment.

Mini-osmotic pumps (model 2001, Alza Corp., Palo Alto, CA) were used in all experiments as a means of chronically elevating circulating cortisol levels. The size of these pumps is approximately 2.5 x 0.6 cm. There design is rather simple, consisting of a small internal bladder, surrounded by a layer of osmolite and finally an outer semipermeable membrane. The inner bladders were filled with cortisol, as hydrocortisone 21-hemisuccinate (Sigma Chemical Co.), and then the pumps were surgically implanted into the peritoneal cavity of the fish. The surgical procedure involved anaesthetizing the fish using a 1:10 000 w/v solution of 3-aminobenzoic acid ethyl ester (MS-222, Sigma Chemical Co.) adjusted to pH 7.0 to 7.5 with sodium bicarbonate. An incision (1 - 1.5 cm) was made in the abdomen

and a cortisol-loaded pump was inserted. The incision was then closed using surgical silk. Water passes through the outer semipermeable membrane of the pump, resulting in a swelling of the osmolite. The layer of osmolite then presses on the inner cortisol containing bladder and ejects it's contents. According to the literature supplied by the manufacturer, at 13°C, these pumps will deliver their contents at a fixed rate of 0.264 $\mu L \cdot hr^{-1}$ for approximately 35 days.

The concentration of cortisol, which was loaded into the osmotic pump was determined by the average weight of the fish used in each experiment. Molecusol HPB 33% w/v (Pharmatec, Florida) was used as the carrier vehicle in all experiments owing to its high steroid solubility. The cortisol delivery rate was calibrated to maintain plasma cortisol concentrations of either 100 or 200 $ng \cdot mL^{-1}$. The sham treated fish received mini-osmotic pumps which delivered only the 30 % w/v molecusol vehicle.

Brown *et al.* (1986) determined the metabolic clearance rate (MCR) and degradation rate (DR) of cortisol in acid stressed (pH 5.0) rainbow trout. The acid treatment significantly increased the circulating cortisol levels of the fish to 48.4 $ng \cdot mL^{-1}$. The MCR of 15 $mL \cdot hr^{-1} \cdot 100\ g^{-1}$ b.w. for cortisol was not altered by the acid treatment, however the DR of cortisol was significantly increased to 2.1 $\mu g \cdot hr^{-1} \cdot 100\ g^{-1}$ b.w. Brown *et al.* (1986) explained that this increase in the DR in the acid-stressed fish was due to elevated levels of circulating cortisol. Since the metabolic clearance rate was unchanged, it was assumed that the endogenous cortisol production rate was equal to the cortisol degradation rate and if the two remained in equilibrium a constant plasma cortisol level would be established. Therefore, it was judged that delivering exogenous cortisol at the degradation rate of

2.1 $\mu g \cdot hr^{-1} \cdot 100\ g^{-1}$ b.w. would result in circulating levels of approximately 50 $ng \cdot mL^{-1}$ and if the delivery rate were doubled the circulating cortisol level would also double. The desired plasma cortisol levels for this study were 100 and 200 $ng \cdot mL^{-1}$, respectively. Therefore, exogenous cortisol needed to be delivered at a constant rate of 4.2 and 8.4 $\mu g \cdot hr^{-1} \cdot 100\ g^{-1}$ b.w.

**Experiment I. The effect of cortisol on plasma metabolites.**

Fish were surgically fitted with indwelling dorsal aortic cannulae (Soivio *et al.*, 1975) and cortisol-loaded mini-osmotic pumps. After the fish recovered from surgery they were placed in black Perspex boxes (volume 3 L) which were supplied with identical water as above. Food was not offered during this experiment as fish will not feed under these conditions. Two experimental groups of fish were used for this experiment. Group 1 received osmotic pumps which delivered cortisol at a continuous rate of 4.2 $\mu g \cdot h^{-1} \cdot 100\ g^{-1}$. This pumping rate maintained plasma cortisol levels near 100 $ng \cdot mL^{-1}$. Group 2 fish received pumps which administered cortisol at a rate of 8.4 $\mu g \cdot h^{-1} \cdot 100\ g^{-1}$ resulting in plasma cortisol levels near 200 $ng \cdot mL^{-1}$. Sham fish were fitted with pumps which delivered only the Molecusol HPB vehicle. Operations were performed in the morning (08:00-12:00 h) and a 500 $\mu$L blood sample was taken 4, 8 and 12 h following surgery through the dorsal aortic cannula. Thereafter, a 500 $\mu$L blood sample was taken at 11:00 h each day for the following ten days. Blood was centrifuged for 15 sec at 12 000 x g (Fisher Micro-centrifuge Model 235B) immediately after collection. The plasma was removed, frozen in liquid nitrogen, and stored at -70°C. Red blood cells were resuspended in heparinized (10

units $\cdot$ mL$^{-1}$) Cortland saline (Wolf, 1963) and re-injected into the animal through the cannula. Plasma cortisol concentrations were measured in all samples using a radioimmunoassay kit (ICN Biomedicals, Carson CA); the protein content of standards was adjusted to reflect values found in fish plasma, which increased assay sensitivity at low cortisol concentrations. Plasma glucose was measured enzymatically using hexokinase and glucose-6-P dehydrogenase as in Bergmeyer (1983).

Insulin may counteract the effects of elevated cortisol on plasma glucose (Rizza *et al.*, 1981). Therefore, a subsequent set of experiments was performed using fish which were deprived of food for 3 weeks prior to mini-osmotic pump implantation. This period of starvation was shown to significantly depress insulin levels in trout (Moon *et al.*, 1989). The experimental protocol was identical to that described above, except that the first blood sample was withdrawn 12 h after surgery, rather than 4 h post surgery. Plasma ninhydrin positive substances (amino acids) were measured using the phenol method of Troll and Cannan (1953) and plasma osmolarity was measured using a vapour pressure osmometer (Wescor, Inc.).

**Experiment II. The effect of cortisol on liver enzyme activities.**

Mini-osmotic pumps were implanted using the same protocol as above. The pumps delivered cortisol at a rate of 4.2 $\mu$g $\cdot$ h$^{-1}$ $\cdot$ 100 g$^{-1}$ resulting in plasma cortisol levels near 100 ng $\cdot$ mL$^{-1}$. Sham fish were fitted with pumps which delivered only the 30% Molecusol HPB vehicle. The fish were returned to a 500 L tank after surgery and resumed feeding 24 h later. Fish were fed to satiation once daily for eight days following surgery. Ten days after

pump implantation, a blood sample (2.0 mL) was withdrawn from the caudal vein/artery using a syringe rinsed with heparin (2500 units $\cdot$ $mL^{-1}$). Plasma was recovered and stored as above. Fish were killed by a sharp blow to the head and the livers were immediately excised and frozen between aluminum blocks cooled in liquid nitrogen. Livers were stored at -70°C until enzymes were analyzed.

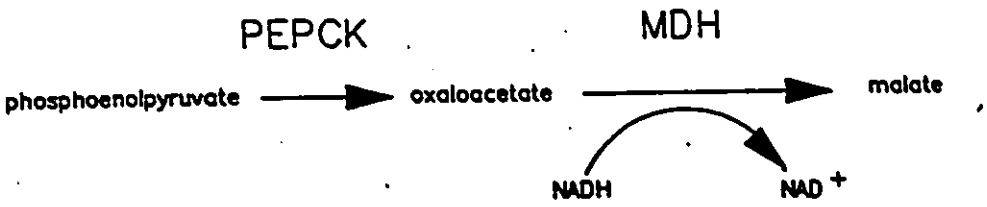
Plasma cortisol and glucose were analyzed as in Experiment I. Plasma lactate was measured enzymatically according to Bergmeyer (1983) and plasma protein using Folin's reagent (Lowry *et al.*, 1951).

To prevent the phosphorylation and/or dephosphorylation of liver enzymes, frozen liver pieces were homogenized with a Polytron PCU-2 (Brinkmann Instruments Co., Toronto, Ontario) in 4 vol of a "stopping buffer" containing 50 mM imidazole, 15 nM $\beta$-mercaptoethanol, 100 mM KF, 5 mM EDTA, 5 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The pH of this buffer was adjusted to 7.5. The liver homogenate was centrifuged for 2 min at 10 000 rpm and the resultant supernatant was desalted by passing it through a column of Sephadex G-25.

Liver enzyme assays were determined spectrophotometrically (Beckman DU-65, Fullerton, CA). This technique takes advantage of the fact that both NADH and NADPH absorb light of wavelength 340 nm. Neither $NAD^+$ or $NADP^+$ absorb light at that wavelength. Therefore, when $NAD^+$ is reduced to NADH (or $NADP^+$ to NADPH) the optical density at 340 nm will increase. Conversely, the oxidation of NADH to $NAD^+$ (or NADPH to $NADP^+$) results in a decreased optical density. The rate of change in optical density (i.e. the velocities), at 340 nm, is directly proportional to the activity of the $NAD^+$

(or $NADP^+$) dependant enzyme which is to be assayed. A standard curve of enzyme velocities versus enzyme activities was prepared and used to determine the activities of the liver supernatants.

If the enzyme to be assayed is not $NAD^+$ (or $NADP^+$) dependant, then the product of the reaction can be coupled to a reaction which is $NAD^+$ dependant. For example the activity of liver Phosphoenolpyruvate carboxykinase (PEPCK) can be assayed, even though it is not a $NAD^+$ dependant enzyme. This is done by adding NADH and excess malic dehydrogenase (MDH) to the preparation in which PEPCK is to be assayed. The oxaloacetate produced by PEPCK will be rapidly reduced to malate at the same time NADH will be oxidized to $NAD^+$ in the following manner.




The decrease in optical density can be monitored due to the oxidation of NADH and hence the activity of PEPCK can be determined.

All enzyme assays were performed at 10 °C as in Moon *et al.* (1989) and presented as activity units ($\mu mol \cdot min^{-1}$) per g liver wet weight. Unless noted, all activities reported are optimal under the conditions established.

Enzymes were assayed in a 50 mM imidazole buffer (pH 7.0 at 25 °C) unless otherwise noted. The following conditions from Mommsen *et al.* (1980) were used for individual enzyme assays:

Glycogen phosphorylase (GPase, EC 2.4.1.1): The two buffer system of Stalmans and Hers (1975) was used to differentiate GPase *a* from GPase total (i.e. *a*+*b*). Buffer A, which was used to determine the active form of the enzyme (GPase *a*) contained 50 mM potassium phosphate (pH 7.0), 10 mM EDTA and 10 mM caffeine. Buffer B, which is used to determine the activity of GPase *total*, contained 50 mM potassium phosphate (pH 7.0) and 0.25 mM EDTA, but no caffeine. The following were added to both buffers: 15 mM $MgSO_4$, 0.5 mM $NADP^+$, 10 $\mu$M glucose 1,6-bisphosphate, 2 mg dialyzed glycogen, and 2 units of phosphoglucomutase and 5 units of glucose-6- phosphate dehydrogenase. After establishing preliminary activity 2 mM of AMP was also added to each buffer. The percentage of GPase *total* which is in the active form GPase *a*, is determined by calculating the ratio the GPase activity using buffer A (i.e. with AMP and caffeine) to the activity of GPase using buffer B (with AMP, but no caffeine).

Pyruvate kinase (PK, EC 2.7.1.40): 100 mM KCl, 10 mM $MgCl_2$, 2.5 mM ADP, 0.15 mM NADH and 10 units PK-free LDH. PK was assayed using phosphoenolpyruvate concentrations of 0.1 and 5 mM (omitted for control). The activity ratio is equal to the PK activities at low [phosphoenolpyruvate]/ high [phosphoenolpyruvate]. Changes in the activity ratio is representative of changes in the degree of enzyme phosphorylation.

6-Phosphfructo-1-kinase (PFK, EC 2.7.1.11): A 50 mM Tris-HCl buffer (pH 7.8) was used to assay PFK. To this buffer was added 175 mM KCl, 17.5 mM $MgCl_2$, 0.25 mM NADH, and excesses of aldolase, triosephosphatase isomerase, and $\alpha$-glycerol phosphate dehydrogenase. The activity of PFK was determined using 6 mM fructose-6-P (omitted for control), and 2 mM ATP (Moon *et al.*, 1989).

Phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32): To the imidazole buffer was added 20 mM $NaHCO_3$, 0.5mM PEP, 1 mM $MnCl_2$, 0.1 mM NADH, excess malic dehydrogenase and, and 0.2 mM GDP (omitted for control).

Lactate dehydrogenase (LDH, EC 1.1.1.27): To the imidazole buffer was added 0.6 mM pyruvate (omitted for control) and 0.15 mM NADH were added to this buffer.

Glutamate-oxaloacetate transaminase (GOT, EC 2.6.1.1): To the imidazole buffer was added 10 mM α-ketogluterate (omitted for control), 25 mM aspartate, 0.05 mM pyridoxal-5'-phosphate, 0.32 mM NADH, and excess ammonium-free malate dehydrogenase.

Glutamate-pyruvate transaminase (GPT, EC 2.6.1.2): To the imidazole buffer was added 250 mM L-alanine, 10 mM α-ketogluterate (omitted for control), 0.15 mM NADH, and excess ammonium-free lactate dehydrogenase.

Glutamate dehydrogenase (GDH, EC 1.4.1.3): To the imidazole buffer was added 250 mM ammonium acetate, 0.1 mM EDTA, 0.1 mM NADH, 1 mM ADP, 14 mM α-ketogluterate (omitted for control).

Glucose-6-P dehydrogenase (G6PDH, EC 1.1.1.49): To the imidazole buffer was added 0.4 mM glucose-6-P (omitted for control), 0.4 mM $NADP^+$, and 7 mM $MgCl_2$

**Experiment III. The effect of cortisol on the flux of $^{14}C$-substrates in isolated hepatocytes.**

Experimental and sham fish were treated as in Experiment II. Isolated hepatocytes were prepared ten days after mini-osmotic pump implantation by the collagenase perfusion method of Moon *et al.* (1985).

This technique utilizes three different perfusion media. Medium A is Hanks medium (Hanks and Wallace, 1949) which consists of 176 mM NaCl; 5.4 mM KCl; 0.81 mM $MgSO_4$; 0.44 mM $KH_2PO_4$; 0.35 mM $Na_2HPO_4 \cdot L^{-1}$; 5.0 mM $NaHCO_3$; and 10 mM HEPES. The medium was gassed with 99.5% $O_2$, remainder $CO_2$ for 30 min, and the pH was adjusted to 7.6 using NaOH. Medium B is made up of Medium A to which was added 1.5% fatty acid free bovine serum albumin and 1 mM $CaCl_2$. Medium C is made up of Medium A to which was added 0.4 mg collagenase (*Clostridium histolyticum*) $\cdot mL^{-1}$ .

Animals were anesthetized using MS222 (1:2000 w/v) and a ventral incision was made to expose the liver. The hepatic portal vein was then cannulated allowing the liver to be perfused with well-oxygenated medium A by means of a peristaltic pump. The flow rate of the perfusate was kept close to 2 $mL \cdot min^{-1} \cdot g\ liver^{-1}$ so that aerobic conditions were maintained throughout the isolation process. This initial perfusion cleared the blood from the liver. The liver was then perfused with medium C containing collagenase to digest the liver. Following approximately 30 min of perfusion the liver was carefully excised from the animal and placed on a watch glass. Perfusion with medium C was continued until the liver showed visible signs of digestion. The liver was then placed in a petri dish and soaked with ice-cold medium A. Following the careful removal of the intact gall bladder, the liver was minced with a sharp razor blade and passed through a fine mesh. The cells were collected by centrifugation (Sorvall, model RC 28S) at 50 g for 2 min at 4°C. The supernatant from this process was decanted and the cells were resuspended in medium B. The cells were then washed three times using medium B, and each time they were collected using centrifugation. The cells were then resuspended in medium B to give a cell concentration of 25 mg wet

weight cells · $mL^{-1}$. The viability of the cells was tested by their ability to exclude trypan blue dye.

One mL aliquots of isolated cells, in resuspension medium B, were added to 20 mL glass vials containing either 5 mM alanine or 5 mM lactate, and 150 000 dpm of the equivalent [$^{14}$C]-labelled substrate (NEN Canada) was added to the appropriate vial (specific activities: alanine, 2627 MBq · $mmol^{-1}$; lactate, 2638.1 Mbq · $mmol^{-1}$). Cells were incubated in stoppered vials at 18°C for 2 h. The incubations were then terminated by the addition of 100 $\mu$L of 70% perchloric acid (PCA) according to Foster and Moon (1986).

The flux of $^{14}$C- substrates to radiolabelled metabolites were determined as follows: Radiolabelled protein was determined by centrifuging the acidified hepatocytes at 3 000 x g for 10 min. The resultant pellet was washed twice with 5% PCA, and then solubilized in 0.5 mL water and 1.0 mL NCS tissue solubilizer (Amersham Canada LTD). Scintillation counting was carried out the following day in 5.0 mL Aquasol-2 (NEN, Canada).

The flux of radioactive substrates to radiolabelled $CO_2$ was measured by means of a $CO_2$ trap (French *et al.*, 1981). Essentially, this trap is a small well, suspended over the cells during incubation. Inside the well was a piece of filter paper which, just prior to the addition of PCA to the vials was soaked with 0.3 mL hyamine hydroxide which "traps" $CO_2$. Following a two hour incubation, these filter papers were removed and added to 20 mL vials containing 5.0 mL Aquasol-2 for liquid scintillation counting.

Radiolabelled glucose was determined by passing the protein-free incubation supernatant through a pair of ion exchange columns. The first column contained Dowex AG-1 X 8 ($Cl^-$ form) which removed all negatively charged species from the supernatant. The

effluent from the first column was then passed through a second column containing Dowex 50 X 8 ($H^+$ form) to remove positively charged species. To ensure that [$^{14}$C]-labelled metabolites other than glucose were not contaminating the glucose fraction, plasma samples spiked with either [$^{14}$C]-labelled alanine or lactate were run through columns. In both cases these labelled metabolites were quantitatively recovered in the column effluent. An aliquot of the glucose containing supernatant was then added to a 20 ml vial containing 5.0 mL Aquasol-2 for liquid scintillation counting.

An LKB Rackbeta (model 1214) scintillation counter was used for all radioactive determinations and all counts were corrected for quenching using an external standard technique. Activities were then converted into $\mu$mol quantities of the appropriate metabolite, based upon the specific activity of the added radiolabel.

**Experiment IV. The effect of chronically elevated cortisol on the water and caloric content of white muscle.**

Fish were fitted with mini-osmotic pumps which maintained plasma cortisol levels near 100 ng$\cdot$mL$^{-1}$. The fish were then returned to a 500 L tank and were fed to satiation once daily for eight days after surgery. Fish were sacrificed by a sharp blow to the head ten days after pumps were implanted. A large sample (8-10 g) and a smaller sample (1-1.5 g) of white muscle was removed from each animal. Muscle water content was determined by weighing the small tissue samples before and after they were dried to a constant weight in an oven at 50°C.

The tissue caloric content was determined using an adiabatic oxygen bomb calorimeter (model 1241, Parr Instrument Co.) which was standardized using benzoic acid. The large muscle samples were used for calorimetric determination of white muscle. The muscle was dried and ground with a mortise and pestle. One gram aliquots of the ground tissue were pressed into pellets and placed in the stainless steel bomb. The bomb was then charged with 35 atm of oxygen and the pellet was ignited. The calculated heat of combustion, measured in calories, was corrected for both the heat of combustion of fuse wire and for the heat of formation of nitric acid, according to the procedures outlined for the equipment by the manufacturer.

**Experiment V. The effect of chronically elevated cortisol on alanine turnover in rainbow trout.**

Rainbow trout were fitted with mini-osmotic pumps which maintained plasma amino acid levels near 100 $ng \cdot ml^{-1}$. Ten days after pump implantation, fish were fitted with dorsal aortic canulae and were returned to black Perspex boxes (volume 3 L). Twelve $\mu$Ci of [$^3$H]-alanine (NEN Canada) in 100 $\mu$L of Cortland saline was injected through the cannulae. The cannulae were immediately flushed with an additional 250 $\mu$L of saline. Blood samples of 250 $\mu$L were taken 5, 15, 30, 60, 180, 570, and 1440 min after the labelled alanine injection. Plasma was prepared as in experiment I including the resuspension and re-injection of red blood cells after each blood sampling. Plasma alanine was quantified spectrophotometrically using alanine dehydrogenase (Williamson, 1985). Ion exchange resin chromatography was used to separate alanine from other labelled metabolites (Reilly, 1975).

Briefly, small (0.6 x 1.5 cm) Dowex 50x8 100-200 mesh W cation exchange resin columns were prepared. One hundred $\mu$L of plasma and 500 $\mu$L of 20 mg $\cdot$ mL$^{-1}$ (L+)$\alpha$-alanine (acidified to pH 2.75 with HCl) were added to the column simultaneously. The column was then rinsed twice with 2.0 mL of distilled water and the effluents were discarded. Four mL of 2.0 M triethylamine in 20% aqueous acetone were then run through the column and the eluent was collected into a clean scintillation vial. A 1.0 mL volume of the eluent was then added to 10.0 mL of scintillation fluor (Aquasol-2) and was counted using a liquid scintillation counter (LKB Rackbeta), with dpm correction capabilities. An alanine recovery efficiency of 84% for this protocol was determined by running a plasma sample spiked with a known amount of [$^{14}$C]-alanine through a column. To ensure that [$^{14}$C]-labelled metabolites other than alanine were not contaminating the alanine fraction, plasma samples spiked with either [$^{14}$C]-labelled glucose or lactate were run through columns. In both cases these labelled metabolites were quantitatively recovered in the column effluent. Alanine production and utilization rates were determined using the non steady-state substrate turnover equations of Bever *et al.* (1977).

**Statistical Analysis**

Statistical analyses for experiment I were performed using two-factor repeated measures ANOVA with analysis comparison of means utilizing Fisher's least significant difference (LSD) test at $p < 0.05$. All other statistical analyses were performed using either paired or unpaired Student's *t*-tests at a level of significance of $p < 0.05$.

# CHAPTER 3
# RESULTS

Experiment I. A surge in plasma cortisol levels occurred immediately after surgery in both experimental and sham trout (Fig. 1a). Plasma cortisol concentration was significantly elevated in a dose-dependent manner in the cortisol infused fish within 72 h of pump implantation and remained elevated until fish were killed at 240 h. Constant infusion using mini-osmotic pumps, therefore, was an effective method for chronically elevating cortisol titers in trout.

A post-surgery elevation of plasma glucose levels occurred in the initial 72 h in both sham and cortisol-infused fish (Fig. 1b). There was, however, no correlation between plasma glucose and cortisol concentrations at any time during the 240 h experimental period.

Repeating Experiment I using unfed fish demonstrated that nutritional status did not alter the effects of infused cortisol on plasma glucose levels (Fig. 2a). The extent of the post-operative cortisol and glucose surges observed in the fed fish, however, were less apparent.

Plasma amino acid levels in sham fish decreased after surgery (Table 1). This decrease in plasma amino acids was not observed in the cortisol-infused fish, where concentrations remained high throughout the experiment (Table 1). By 216 h, levels were significantly higher than shams, but were not different from the 24 h samples. Plasma amino acid concentrations were unrelated to level of exogenously administered cortisol. Plasma osmolarity was not affected by cortisol treatment (Table 2).

**Figure 1.** Time course of plasma cortisol and glucose in sham and cortisol treated fed rainbow trout. (closed circles, shams; open squares, cortisol nominally elevated to 100 $ng \cdot mL^{-1}$; open triangles, cortisol nominally elevated to 200 $ng \cdot mL^{-1}$). Vertical bars through each point represent 1 S.E.M.;* significantly different from sham treated fish; †significantly different from fish with cortisol nominally elevated to 100 $ng \cdot mL^{-1}$.

Fig. 1a

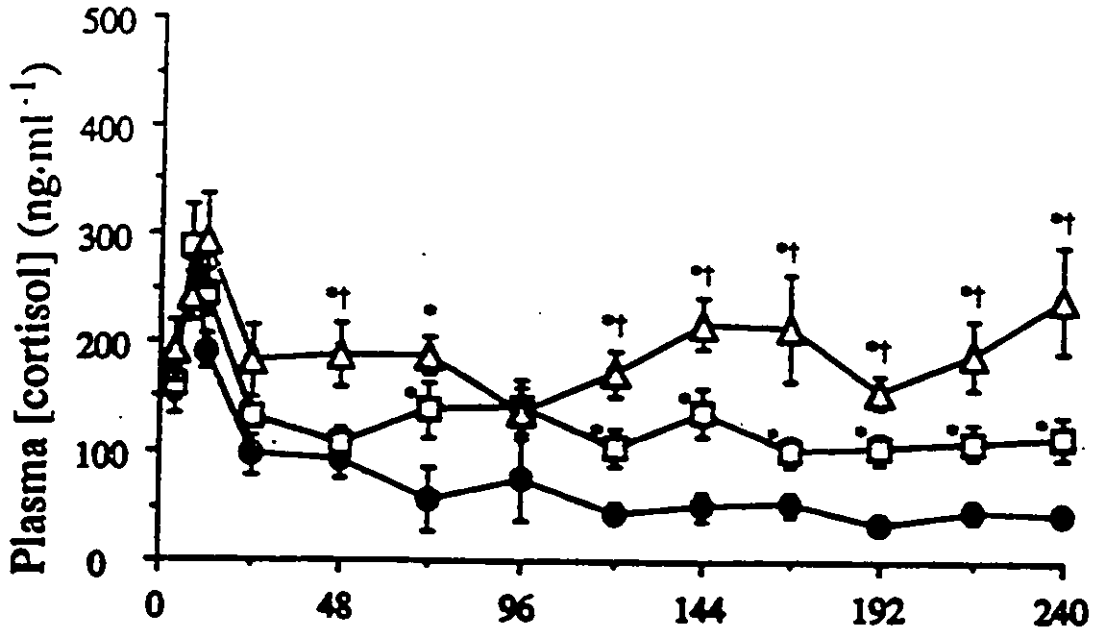

Plasma [cortisol] (ng·ml⁻¹)
500
400
300
200
100
0
0
48
96
144
192
240

Fig. 1b

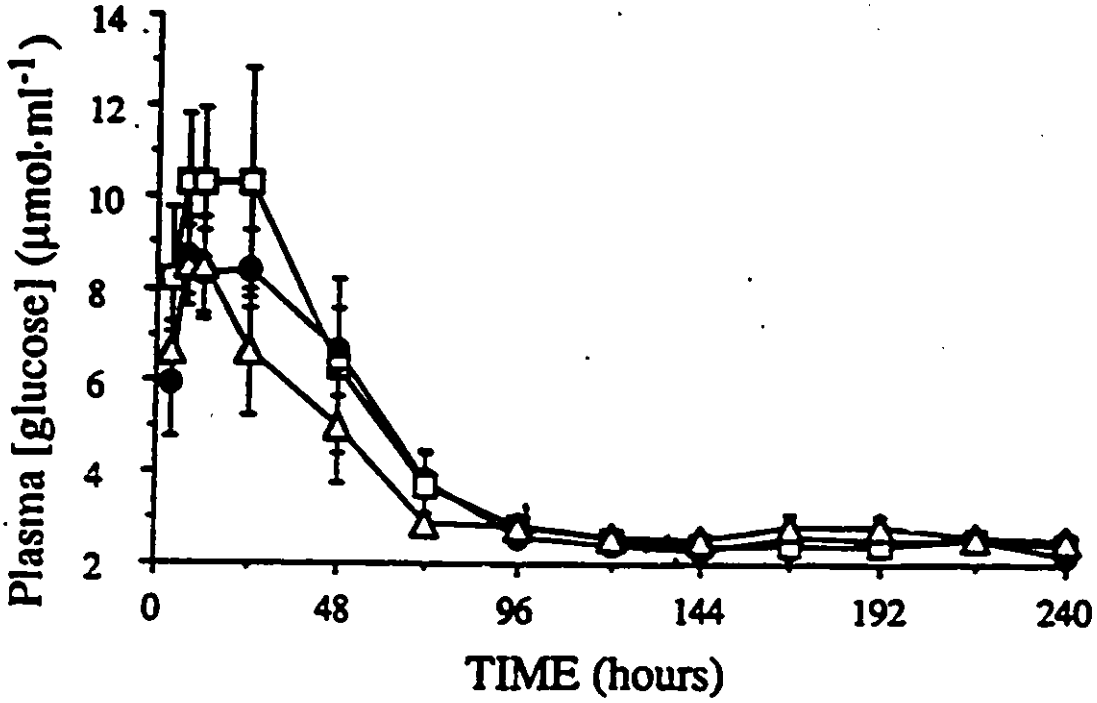
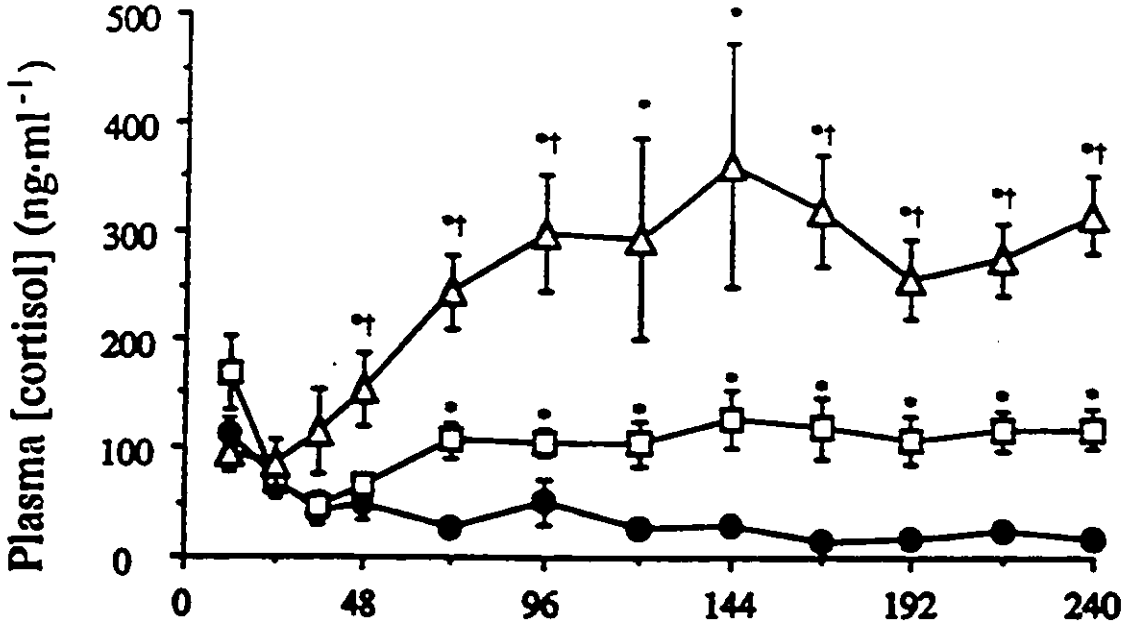

Plasma [glucose] (µmol·ml⁻¹)
14
12
10
8
6
4
2
0
48
96
144
192
240
TIME (hours)

**Figure 2.** Time course of plasma cortisol and glucose in sham and cortisol treated rainbow trout. Fish were deprived of food for three weeks prior to experiment. (closed circles, shams; open squares, cortisol nominally elevated to 100 $ng \cdot mL^{-1}$; open triangles, cortisol nominally elevated to 200 $ng \cdot mL^{-1}$). Vertical bars through each point represent 1 S.E.M.; * significantly different from sham treated fish; †significantly different from fish with cortisol nominally elevated to 100 $ng \cdot mL^{-1}$.

Fig. 2a


Plasma [cortisol] (ng·ml⁻¹)
500
400
300
200
100
0
0
48
96
144
192
240

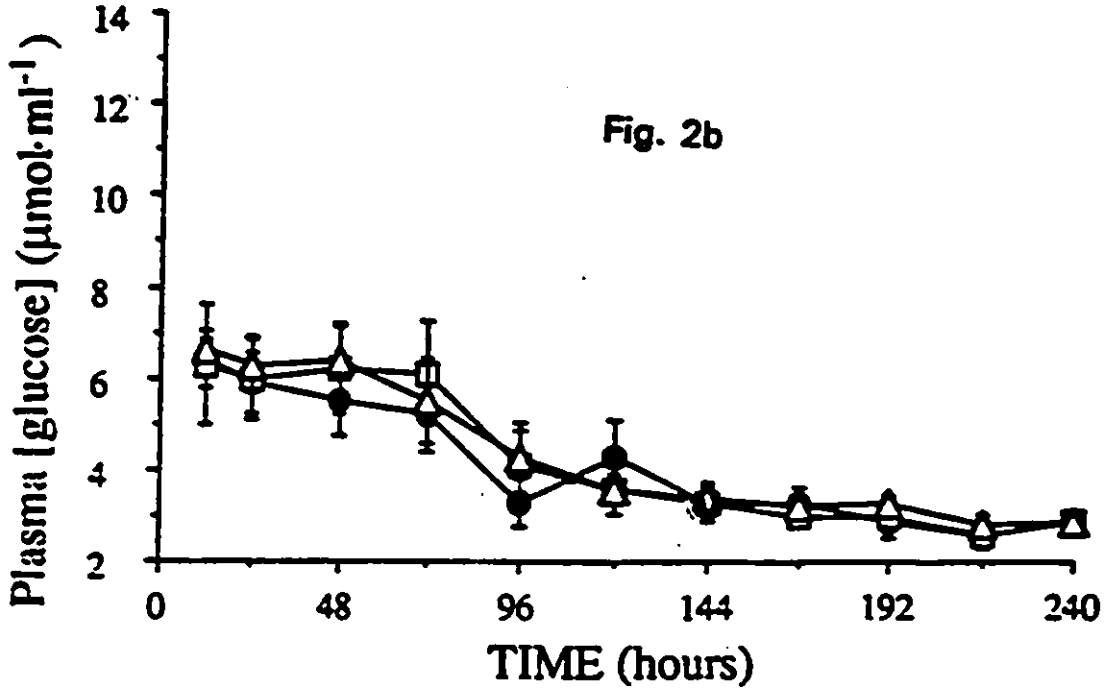

Plasma [glucose] (µmol·ml⁻¹)
14
12
10
8
6
4
2
0
48
96
144
192
240
TIME (hours)

Fig. 2b

**Table 1.** Total amino acid concentrations from unfed (3 weeks) rainbow trout fitted with mini-osmotic pumps which delivered either cortisol or vehicle (sham). Blood was sampled through a dorsal aortic catheter 24, 96 and 216 h after the pumps were implanted. Rates of cortisol administration were either 4.2 $\mu g \cdot h^{-1} \cdot 100\ g^{-1}$, resulting in plasma cortisol concentrations near 100 $ng \cdot mL^{-1}$ (Group I), or 8.4 $\mu g \cdot h^{-1} \cdot 100\ g^{-1}$ (group II), resulting in plasma cortisol concentrations near 200 $ng \cdot mL^{-1}$. (See Figure 2a.)

| Mean Plasma Amino Acids ($\mu g \cdot mL^{-1}$) $\pm$ 1 S.E.M. | | | |
|---|---|---|---|
| Time | Shams | Group I Cortisol 100 $ng \cdot mL^{-1}$ | Group II Cortisol 200 $ng \cdot mL^{-1}$ |
| 24 h | 1078.8±84.1 | 1044.5±155.3 | 953.1±88.8 |
| 96 h | 961.0±112.3 | 922.2±103.9 | 1025.4±111.3 |
| 216 h | 716.7±68.5[a] | 1023.8±90.7[b] | 942.3±63.7[b] |

[a] Significantly different from 24 h sample, 2-factor repeated measures ANOVA and Fisher's LSD, $p < 0.05$.

[b] Significantly different from sham treatment, 2-factor repeated measures ANOVA and Fisher's LSD, $p < 0.05$.

**Table 2.** Plasma osmolarity from unfed (3 weeks) rainbow trout fitted with mini-osmotic pumps which delivered either cortisol or vehicle (sham). Blood was sampled through a dorsal aortic catheter 24, 96 and 216 h after the pumps were implanted. Rates of cortisol administration were either 4.2 $\mu g \cdot h^{-1} \cdot 100\ g^{-1}$, resulting in plasma cortisol concentrations near 100 $ng \cdot mL^{-1}$ (Group I), or 8.4 $\mu g \cdot h^{-1} \cdot 100\ g^{-1}$ (group II), resulting in plasma cortisol concentrations near 200 $ng \cdot mL^{-1}$. (See Figure 2a.)

| Mean Plasma Osmolarity ($mOsmols \cdot kg^{-1}$) $\pm$ 1 S.E.M. | | | |
|---|---|---|---|
| Time | Shams | Group I Cortisol 100 $ng \cdot mL^{-1}$ | Group II Cortisol 200 $ng \cdot mL^{-1}$ |
| 24 h | 293.2 $\pm$ 5.6 | 280.3 $\pm$ 2.5 | 295.3 $\pm$ 2.5 |
| 96 h | 285.2 $\pm$ 3.0 | 284.3 $\pm$ 6.3 | 297.0 $\pm$ 4.1 |
| 216 h | 288.8 $\pm$ 6.3 | 294.2 $\pm$ 3.4 | 303.7 $\pm$ 4.9 |

**Experiment II.** Chronic cortisol elevation for 10 d did not significantly affect the optimal activity of a number of gluconeogenic and glycolytic enzymes (Table 3). The phosphorylation status of GPase and PK were also unaffected as indicated by the absence of any change in %GPase a and in the PK activity ratio. Furthermore, the plasma levels of glucose, lactate and total protein were unchanged by cortisol administration (Table 4).

**Experiment III.** The flux of lactate and alanine to protein, $CO_2$ and glucose in isolated hepatocytes was unaffected by the chronic administration of cortisol (Table 5). Initial hepatocyte glycogen concentration was significantly lowered by chronic cortisol administration, but neither glycogen depletion nor glucose production rates differed from shams.

It should be noted that the hepatocyte glycogen content of these particular fish was lower than those in other studies undertaken in this laboratory (e.g., Foster and Moon, 1988). To alleviate concerns about low initial glycogen levels, the experiment was subsequently repeated using fish from another supplier. These fish, however, had even lower hepatocyte glycogen contents. Except for differences in glycogen depletion and glucose production rates, no qualitative differences were noted between these two experiments . Such quantitative differences in glycogen depletion were expected based upon the differences in glycogen contents of the two groups of fish (Mommsen, 1986; Foster and Moon, 1990). The reason for hepatocyte glycogen levels below those previously reported remains unclear.

**Table 3.** Liver enzyme activities ($\mu$mol · min$^{-1}$ · g$^{-1}$ wet weight; except % GPase a and activity ratio) in sham and cortisol treated fed rainbow trout. Cortisol was elevated to about 100 ng · mL$^{-1}$ using mini-osmotic pumps. Fish were fed daily to satiation with commercial trout pellets, for eight days after surgery. Values are mean activity (units · g$^{-1}$) ± 1 SEM (n). Abbreviations: GPase, Glycogen phosphorylase; PK, Pyruvate kinase; Phosphofructokinase; PEPCK, phosphoenolpyruvate carboxykinase; LDH, lactate dehydrogenase; GOT, glutamate-oxaloacetic transaminase; GPT, glutamate-pyruvic transaminase; GDH, glutamate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; Activity ratio= Substrate activity at low [PEP]/activity at high [PEP]; % GPase a represents percentage of total glycogen phosphorylase (a+b) in the active form (a).

| Enzyme | Sham Treatment | Cortisol Treatment |
|---|---|---|
| GPase Total | 18.5± 1.7 (7) | 16.2 ± 1.7 (6) |
| %GPase a | 73.0 (5) | 70.0 (6) |
| PK | 70.5 ± 15.1 (8) | 71.5 ± 14.7 (6) |
| Activity ratio | 0.26 (8) | 0.18 (6) |
| PFK | 9.7 ± 2.2 (7) | 10.1 ± 3.7 (6) |
| PEPCK | 10.3 ± 1.8 (7) | 8.1 ± 1.2 (5) |
| LDH | 58.8 ± 8.7 (8) | 58.9 ± 6.7 (6) |
| GOT | 53.3 ± 5.5 (8) | 48.1 ± 2.9 (6) |
| GPT | 36.3 ± 6.3 (8) | 34.1 ± 6.8 (5) |
| GDH | 150.0 ± 24.3 (8) | 171.0 ± 26.1 (6) |
| G6PDH | 50.2 ± 3.8 (8) | 65.6 ± 8.4 (6) |

**Table 4.** Plasma metabolites in rainbow trout ten days after being fitted with mini-osmotic pumps which delivered either cortisol or vehicle (shams). Cortisol dosage rate was 4.2 $\mu g \cdot h^{-1} \cdot 100\ g^{-1}$. Values are means± 1 SEM (n).

| Parameter | Sham Treatment | Cortisol Treatment |
|---|---|---|
| Body Weight (g) | 173.6±8.7 (9) | 183.0±10.7 (8) |
| Plasma Cortisol ($ng \cdot mL^{-1}$) | 32.0±14.0 (9) | 91.5±21.1 (8)[a] |
| Plasma Glucose ($\mu mol \cdot mL^{-1}$) | 4.2±0.4 (9) | 3.7±0.3 (8) |
| Plasma Lactate ($\mu mol \cdot mL^{-1}$) | 0.5±0.07 (9) | 0.6±0.1 (8) |
| Plasma Protein ($mg \cdot 100 \cdot mL^{-1}$) | 25.4±0.8 (9) | 26.1±1.9 (8) |

[a]Significantly different from control value, Student's t-test $p<0.05$

**Table 5.** The rates of glucose and of $CO_2$ production from radioactive alanine and lactate and of substrate incorporation into protein by isolated rainbow trout hepatocytes. Hepatocytes were prepared from either sham or cortisol treated (approx. 100 $ng \cdot mL^{-1}$ plasma cortisol) fish. Fish were fed and had implants for ten days prior to sacrifice. Values are means$\pm$ 1 SEM (n).

| Parameter Measured | Sham Treatment | Cortisol Treatment |
|---|---|---|
| Initial Glycogen ($\mu mol \cdot g^{-1}$) | 42.6$\pm$8.9 (8) | 25.8$\pm$5.6 (8)[a] |
| **Lactate as Substrate** | | |
| Flux to Protein ($\mu mol \cdot g^{-1} \cdot h^{-1}$) | 0.64$\pm$0.13 (9) | 0.61$\pm$0.12 (8) |
| Flux to $CO_2$ ($\mu mol \cdot g^{-1} \cdot h^{-1}$) | 1.62$\pm$0.35 (9) | 1.54$\pm$0.22 (8) |
| Flux to Glucose ($\mu mol \cdot g^{-1} \cdot h^{-1}$) | 0.56$\pm$0.2 (9) | 0.79$\pm$0.3 (8) |
| Glycogen Depletion ($\mu mol \cdot g^{-1} \cdot h^{-1}$) | 9.7$\pm$2.6 (8) | 6.1$\pm$2.8 (8) |
| Total Glucose Production ($\mu mol \cdot g^{-1} \cdot h^{-1}$) | 3.0$\pm$0.6 (8) | 3.4$\pm$0.8 (8) |
| **Alanine as Substrate** | | |
| Flux to $CO_2$ ($\mu mol \cdot g^{-1} \cdot h^{-1}$) | 0.59$\pm$0.19 (9) | 0.87$\pm$0.14 (8) |
| Flux to Glucose ($\mu mol \cdot g^{-1} \cdot h^{-1}$) | 1.05$\pm$0.21 (9) | 1.67$\pm$0.53 (8) |
| Glucose Production ($\mu mol \cdot g^{-1} \cdot h^{-1}$) | 0.08$\pm$0.03 (9) | 0.10$\pm$0.03 (7) |
| Glycogen Depletion ($\mu mol \cdot g^{-1} \cdot h^{-1}$) | 8.6$\pm$3.0 (8) | 7.9$\pm$3.6 (8) |
| Total Glucose Production ($\mu mol \cdot g^{-1} \cdot h^{-1}$) | 2.3$\pm$0.5 (8) | 2.9$\pm$0.7 (8) |

[a] Significantly different from control value, Student's t-test $p<0.05$

**Experiment IV.** The alanine kinetic curves followed a simple exponential function, and alanine production and utilization rates were determined using the non steady state substrate turnover equations of Bever *et al.* (1977). In the cortisol treated fish the mean production and utilization rates were 3.95 $\pm$ 0.29 and 4.00 $\pm$ 0.35 $\mu mol \cdot min^{-1} \cdot ml^{-1}$, respectively. The production and utilization rates for the shams were 3.78 $\pm$ 0.32 and 3.97 $\pm$ 0.40 $\mu mol \cdot min^{-1} \cdot ml^{-1}$. There were no significant differences within treatments or between treatments.

**Experiment V.** The caloric content of white muscle was determined using a bomb calorimeter and was corrected for both the heat of combustion of fuse wire and for the heat of formation of nitric acid. The mean caloric content of the white muscle of cortisol treated fish was 5348.14 $\pm$ 42.78 $cal \cdot g^{-1}$ dry weight which was not significantly different from the measured value of 5273.28 $\pm$ 41.89 $cal \cdot g^{-1}$ dry weight for shams. The mean water content of cortisol treated fish was 76.3 $\pm$ 0.47% of wet weight, compared to 77.7 $\pm$ 0.61% for shams. These results indicate that the water and caloric content of white muscle was unaffected by chronically elevated plasma cortisol levels.

# CHAPTER 4
# DISCUSSION

In teleost fish, the stress-induced alterations in protein and carbohydrate metabolism that have typically been attributed to an activation of the pituitary-adrenal axis are highly variable (Storer, 1967; Butler, 1968; Chan and Woo, 1978; Lidman *et al.*, 1979; Leach and Taylor, 1982; Davis *et al.*, 1985; Foster and Moon, 1986; Barton *et al.*, 1987; Vijayan and Leatherland, 1989). Barton *et al.* (1987) suggested that these variable results may be due to the physiological status of the fish, dosages of cortisol used, or interactions of cortisol with other glucoregulatory hormones. Designing a well controlled experiment is important to all fields of science, but it is particularly critical when studying the physiology of stress. To gain an understanding of the direct metabolic effects of cortisol in teleost fish, the plasma levels of cortisol must be elevated and maintained at physiologically relevant concentrations in the absence of other stress factors. Obviously, this is difficult to achieve under laboratory conditions. Many of the early investigations of the metabolic effects of cortisol in teleosts relied on daily intramuscular (IM) or intraperitoneal (IP) injections as a means of elevating plasma cortisol levels (Butler, 1968; Chan and Woo, 1978; Lidman *et al.*, 1979). While these modes of drug delivery are common in physiological studies, they are probably not ideal techniques for investigating the role of cortisol in fish. Foster and Moon (1986) demonstrated that in American eels, ten daily IP injections of cortisol (0.35 $mg \cdot kg^{-1}$) resulted in large fluctuations in the plasma concentration of cortisol. Three hours after the last injection the plasma cortisol content of the treated eels was significantly elevated when compared to controls (400 $ng \cdot mL^{-1}$ for cortisol treated vs. 20 $ng \cdot mL^{-1}$ for controls).

However, 24 h after the injection, the treated fish had significantly lower cortisol levels (5.1 $ng \cdot mL^{-1}$ for cortisol treated vs. 37.3 $ng \cdot mL^{-1}$ for controls), indicating a very rapid clearance of the hormone. While cortisol may be secreted by the interrennals in a pulsatile fashion (Tam *et al.*, 1988), chronic stress typically results in the maintenance of elevated plasma cortisol levels (Donaldson, 1981). A further problem associated with daily injections is the stressful nature of the protocol itself. The netting and air exposure of the fish associated with the injection of cortisol obviously imparts a considerable amount of stress to the animal. Whiting and Wiggs (1977) demonstrated that in brook trout the sham treatment of daily saline injections resulted in elevated endogenous steroid secretions, indicating that the procedure was sufficiently stressful to activate the pituitary-interrenal axis.

Cortisol has also been administered by incorporating the hormone into the diet of fish (Pickering and Duston, 1983; Davis *et al.*, 1985; Barton *et al.*, 1987). While this protocol delivers cortisol in an unstressful manner, it still results in large post feeding plasma cortisol fluctuations (Barton *et al.*, 1987) which are very similar to those described by Foster and Moon (1986). Barton *et al.* (1987) also reported that after 10 weeks of such a protocol, plasma cortisol levels were significantly lower in the treated fish 24 h after their last meal. This indicates an increased activity of the steroid metabolizing enzymes of the liver, as suggested by Foster and Moon (1986). Several studies have used intraperitoneal implants of cortisol dissolved in cocoa butter (e.g. Pickering and Duston, 1983; Vijayan and Leatherland, 1989). These implants are an improved mode of administration since the cocoa butter or cholesterol will continually release cortisol, therefore avoiding the large oscillations in plasma hormone levels associated with injections or dietary supplementation. One problem

with this technique is that it is difficult to predict the plasma levels of cortisol for a given dose of steroid. The hormone release rate of these implants is proportional to their surface area. Unfortunately, the shape of the injected implant varies between animals and, therefore, so does the release rate. Furthermore it is impossible to determine if the coca butter releases the cortisol at a constant rate throughout a study.

In this study cortisol was administered by mini-osmotic pumps which were implanted into the peritoneal cavity of the fish. The advantages of this mode of administration are considerable. Mini-osmotic pumps deliver their contents at a fixed rate for up to 35 days at 13°C. Therefore, it is easy to deliver cortisol at a desired rate for the duration of an experiment, thus avoiding the fluctuations in plasma cortisol concentrations which are inherent in other protocols. Figures 1 and 2 indicate that the surgery required to implant the pumps was stressful to the fish. The mini-osmotic pumps require approximately 24 h at 13°C to attain their optimal pumping rate, therefore the post surgery rise in cortisol is due to endogenous production by the animal rather then the exogenous delivery from the pump. Further evidence of the source of this cortisol is the fact that sham treated fish demonstrated an identical post surgery elevation in cortisol. In the fed fish (Fig. 1a), plasma cortisol levels reached a maximum concentration of about 300 $ng \cdot mL^{-1}$ 12 h after surgery. However, in the fish which were deprived of food for 3 weeks prior to surgery, plasma cortisol levels peaked at about 150 $ng \cdot mL^{-1}$ 12 h after surgery. The food deprivation apparently altered the response of the fish to the stress of surgery. It is possible that 3 weeks of starvation was sufficiently stressful to reduce the ability of the trout to respond to further stressors. It should be noted that Barton *et al.* (1987) reported that a 3 week period of food

deprivation did not alter the magnitude of post stress cortisol levels associated with handling chinook salmon for 30 s. Fish routinely survive long periods of food scarcity in their natural habitats (Cunningham and Shuter, 1986), so 3 weeks of food deprivation should not be overly stressful. Within 72 h of surgery, plasma cortisol levels were significantly elevated in a dose dependent manner, when compared to sham treated fish, and remained elevated until the experiment was terminated at 240 h. After 72 h the plasma cortisol levels in the sham treated fish dropped to about 40 $ng \cdot mL^{-1}$ in the fish fed prior to the experiment and 25 $ng \cdot mL^{-1}$ in the fish deprived of food prior to the experiment. A previous study in which cortisol was measured for 16 days in dorsal aorta cannulated and confined rainbow trout showed a similar plasma cortisol profile (Brown *et al.*, 1986). That study demonstrated a post cannulation surge in cortisol to levels of about 70 $ng \cdot mL^{-1}$; 48 h after surgery circulating cortisol levels fell to 20 $ng \cdot mL^{-1}$ and remained close to this level for 16 days. Prior to cannulation these fish had plasma cortisol levels of about 10 $ng \cdot mL^{-1}$, indicating that the presence of the cannula was stressful. Wedemeyer *et al.* (1990) reported that in unstressed fish plasma corticosteroids are typically less than 30-40 $ng \cdot mL^{-1}$, which compares well to the measured cortisol levels in the sham treated fish from 72 to 240 h post surgery. On the other hand Pickering and Pottinger (1989) recently have suggested that unstressed salmonids should have plasma cortisol levels below 5 $ng \cdot mL^{-1}$. In that study fish subjected to a chronic stress had plasma cortisol levels of approximately 10 $ng \cdot mL^{-1}$, and were shown to have reduced disease resistance. While the goal of this study was to elevate cortisol in otherwise unstressed fish, the findings of Pickering and Pottinger (1989) suggest the possibility that both the cortisol and sham treated fish of this study may have been stressed.

If 5 $ng \cdot mL^{-1}$ is the true upper limit of circulating cortisol (Pickering and Pottinger, 1989) for unstressed fish, then a recent review of the literature (Barton and Iwama, 1991) would suggest that the control fish in most previous studies, in which circulating cortisol levels were examined, were stressed.

The use of mini-osmotic pumps in this study allowed for the selection of a desired plasma cortisol concentration. For most of the experiments in this study, plasma cortisol levels were raised to about 100 $ng \cdot mL^{-1}$. This concentration of circulating cortisol is indicative of the physiological levels seen in salmonids which have been subjected to a variety of moderate to severe stressors (Donaldson, 1981; Barton *et al.*, 1985; Patino *et al.*, 1987; Pickering and Pottinger, 1989). In coho salmon there may be a threshold level response to cortisol rather than a dose related response (Vijayan and Leatherland, 1989). To examine the possibility of a threshold effect in rainbow trout Experiment I, which examined the effect of cortisol on circulating metabolites, was repeated except that cortisol levels were raised to 200 $ng \cdot mL^{-1}$. As indicated by Figs. 1b and 2b, elevating plasma cortisol to 200 $ng \cdot mL^{-1}$ instead of 100 $ng \cdot mL^{-1}$ had no effect on the glycaemic status of the fish. Furthermore, Table 1 indicates that elevating the cortisol concentration beyond 100 $ng \cdot mL^{-1}$ did not indicate a threshold response when plasma amino acids and osmolarity were assayed.

The mini-osmotic pumps were successful at maintaining the circulating cortisol levels quite close to the desired levels for ten days (Figs. 1a and 2a). It has previously been reported that chronic cortisol treatment in American eels (Foster and Moon, 1986) and catfish (Davis *et al.*, 1985) resulted in an increased activity in the liver enzymes responsible for steroid metabolism. Both of these studies found that this increased metabolic clearance of

cortisol resulted in circulating cortisol levels that were actually lower in the treated fish than the controls when compared 24 h after the last treatment. Interestingly, there was no indication in the present study that continuous exogenous cortisol delivery resulted in an increased metabolic clearance of the steroid. The rate of drug delivery by the mini-osmotic pumps was constant. Therefore, if the cortisol treatment increased the metabolic clearance rate over the ten day treatment period, a gradual reduction in circulating cortisol levels would be expected. In the studies by Foster and Moon (1986) and Davis *et al.* (1985), the hepatic steroid metabolizing enzymes may have been stimulated by the pharmacological doses of cortisol which were administered, in addition, there may be species differences in the responses to chronically elevated cortisol levels.

**The role of cortisol in stress induced hyperglycaemia.**

Immediately following surgery there was a surge in plasma glucose concentrations in both cortisol and sham treated fish which lasted until about 72 h after surgery. This post-operative hyperglycaemia was present in both the fed fish (Fig. 1b) and the fish which were deprived of food for three weeks prior to surgery (Fig. 2b). The reason for repeating this experiment using food deprived fish was to ensure that insulin was not simply counteracting any effect of cortisol on plasma glucose levels (Rizza *et al.*, 1981). It has been demonstrated that 3 weeks of food deprivation reduces circulating insulin levels in trout (Moon *et al.*, 1989). The food deprived fish responded with a much smaller increase in plasma glucose concentration, yet it lasted for a longer period of time. The reason for this decreased response is not understood, since an increased glycaemic response would be expected if

insulin levels were actually reduced. For some unknown reason, the pre-operative period of food deprivation seems to have altered the carbohydrate metabolism of this group of fish.

An examination of Figs 1 and 2 indicates that the post-operative hyperglycaemia appears to co-vary with an elevation in circulating cortisol. It would be a mistake to attribute the hyperglycaemia solely to the elevation in cortisol. More likely it is indicative of the stressful nature of the surgical procedure and was due to elevated catecholamines (Mazeaud and Mazeaud, 1981). After approximately 72 h the plasma glucose levels in treated and sham fish decreased to about 2.75 $\mu mol \cdot mL^{-1}$ in the fed fish and remained close to this concentration until the experiment was terminated at 240 h. The plasma glucose levels in the food deprived group dropped to about 3.25 $\mu mol \cdot mL^{-1}$ by 96 h and were unchanged until 240 h. These plasma glucose concentrations are not different from the values reported in previous studies performed in this laboratory (Perry *et al.*, 1988). This indicates that the cortisol treatment did not result in hyperglycaemia. This finding is counter to the mammalian dogma that corticosteroids are glucocorticoids, and therefore, elevate plasma glucose levels (Kraus-Friedmann, 1984). In teleost fish there is considerable controversy surrounding the ability of exogenous cortisol to elevate plasma glucose levels. Several early studies have reported that cortisol induces hyperglycaemia in eels (Butler, 1968; Inui and Yokote, 1975; Chan and Woo, 1978; Lidman *et al.*, 1979). A conclusion common to these studies was that hepatic gluconeogenesis was stimulated by exogenous cortisol, and thus resulted in elevated circulating glucose concentrations. Hypophysectomizing an eel, as was done by Butler (1968) and Chan and Woo (1975), has many metabolic and hormonal effects in addition to the reduction in cortisol production of the interrenal tissue due to the absence

of ACTH. The removal of the pituitary will also eliminate the production and release of growth hormone, thyroid-stimulating hormone, and prolactin. Each of these hormones has a profound impact on the "normal" metabolism of an animal (Pilkis and El-Maghrabi, 1988). Interestingly, none of these early investigations directly examined the role of cortisol in liver metabolism, while arriving at the conclusion that cortisol was a glucocorticoid. All of these studies involved large pharmacological doses of cortisol (1 to 10 $mg \cdot kg^{-1}$ body weight) which would result in plasma cortisol levels exceeding the physiological range of a severely stressed fish. A more recent study of the metabolic role of glucocorticoids in eel metabolism indicated that 10 daily injections of cortisol actually resulted in hypoglycaemia (Foster and Moon, 1986). The daily dosage used in that study (0.35 $mg \cdot kg^{-1}$ body weight) was much smaller then the previous studies and yet the measured plasma cortisol level of 400 $ng \cdot mL^{-1}$ 3 h after day 10 of treatment was still considerably beyond the physiological range of a severely stressed fish (Donaldson, 1981; Barton and Iwama, 1991). Five daily injections (20 $mg \cdot kg^{-1}$) of cortisol resulted in hyperglycaemia in both fed and fasted killifish (Leach and Taylor, 1982). The measured circulating cortisol concentration in that study was 6560 $ng \cdot mL^{-1}$ 24 h after the fifth day of injections. There was no indication of increased skeletal muscle catabolism or enhanced liver gluconeogenesis associated with this elevation in cortisol. Therefore the hyperglycaemia was thought to be due to a cortisol stimulated inhibition of glucose utilization in peripheral tissue. This type of response to glucocorticoids was first described in mammals by Long *et al.* (1940). An initial decrease in glucose utilization in a marsupial was also reported by McDonald and Than (1976). However, if the glucocorticoid treatment was prolonged then an elevation in hepatic gluconeogenesis and

peripheral tissue catabolism would result. More recently Vijayan and Leatherland (1989) expressed support for a decreased glucose utilization in cortisol treated coho salmon. The findings of that study are particularly interesting when compared to this study since daily IP injections were avoided by using coco butter implants containing cortisol. Vijayan and Leatherland (1989) suggested that in coho salmon a threshold level of cortisol was necessary to stimulate gluconeogenesis, rather then a dose dependent response. It is possible that the cortisol levels in the fish of this study never reached this threshold, and therefore a hyperglycaemic response was never exhibited. It is more likely, however, that the contrasting results between these two studies was simply a reflection of inter-specific differences, since maintaining cortisol levels at 200 $ng \cdot mL^{-1}$ would almost certainly exceed any such physiological threshold. Tam *et al.* (1988) reported an increase in corticotrope secretory activity in brook trout maintained at pH 4.5 for 73 days. However, circulating cortisol concentrations were only elevated for 5 h after the commencement of acid exposure since a concomitant increase in the metabolic clearance rate eliminated any elevation in plasma cortisol levels. That study also reported hyperglycaemia beginning 4 days after the commencement of acidification and lasting for over a month. To examine whether this hyperglycaemia was a secondary stress response (Mazeaud *et al.*, 1977; Pickering, 1981; Wedemeyer and Mcleay, 1981) associated with the increased corticotrope secretion, Tam *et al.* (1988) injected rainbow trout with 0.5 mg cortisol and 0.1 mg adrenalin. This treatment did not result in hyperglycaemia, leading to the conclusion that cortisol was not responsible for the elevation in blood glucose levels in acid-stressed rainbow trout. While there are considerable differences between this study and that of Tam *et al.* (1988), both concluded

that elevated cortisol was not responsible for stress-induced hyperglycaemia. Conversely, Barton *et al.* (1987) fed juvenile rainbow trout cortisol laced food for 10 weeks. After 4 weeks plasma glucose levels were significantly elevated and after 10 weeks of treatment this hyperglycaemia was still apparent in the hormone treated fish.

The findings of this study contradict the conclusions of many previous studies. Close examination of these studies indicates there could be dose-dependent or dose-time-dependent trend to the results. Most early studies administered massive doses of cortisol to eels, but failed to actually measure the resulting plasma levels of the hormone. When Foster and Moon (1986) administered daily cortisol injections of 0.35 $mg \cdot kg^{-1}$ for 10 days, the plasma cortisol levels were 400 $ng \cdot mL^{-1}$ 3 h after the final injection. The circulating cortisol levels which would result from injections ranging from 1 to 10 $mg \cdot kg^{-1}$ (Butler, 1968; Inui and Yokote, 1975; Chan and Woo, 1978) can only be guessed, but they would most certainly not simulate the physiological conditions of even a severely stressed fish. Leach and Taylor (1982) measured circulating cortisol levels of 6560 $ng \cdot mL^{-1}$ in their study of *Fundulus heteroclitus*, which is an order of magnitude higher than the reported maximum cortisol levels in severely stressed *F. heteroclitus* (Leach and Taylor, 1980). It may be reasonable to question the physiological significance of the metabolic responses to such massive pharmacological doses of a drug. Vijayan and Leatherland (1989) demonstrated significantly elevated circulating cortisol levels in coho salmon receiving implants of 1, 5, and 10 mg/fish, yet only the highest dose resulted in hyperglycaemia. They suggested that in coho salmon a threshold level of hormone was necessary for gluconeogenic stimulation. Pickering and Duston (1983) using a similar protocol, gave cocoa butter of 5, 10, 25, and 50 mg to brown

trout in an investigation of the role of elevated cortisol in the susceptibility to bacterial and fungal infections. Over a one month period the mortality rate due to infection was 11% in the fish receiving a 5 mg implant compared to 6% for shams. The mortality rates in the fish given implants of 10, 25, and 50 mg was greater than 50% in each case. With this in mind it is possible that the hyperglycaemia which was reported by Vijayan and Leatherland (1989) was not due to a threshold level of cortisol, but instead was associated with a normal stress response due to an infection. There may also be a trend to the temporal resolution of the experiments which find hyperglycaemic responses to cortisol treatment. This study indicates that 10 days of cortisol treatment did not alter the glycaemic status of rainbow trout. Tam *et al.* (1988) found a similar effect after six days of cortisol treatment in brook trout. Conversely, chronic cortisol administration in juvenile brook trout resulted in hyperglycaemia after both 4 and 10 weeks of treatment (Barton *et al.*, 1987). Butler (1968) demonstrated that in hypophysectomized American eels, 10 days of cortisol treatment did not alter plasma glucose levels, but 21 days of treatment resulted in hyperglycaemia. Clearly, the presumed role of a purported stress hormone must be questioned if 21 days are required to elicit a response. It seems possible that the administration of exogenous cortisol itself may become stressful to teleosts, either through a compromised immune system, or some other unknown physiological mechanism.

**Plasma metabolites other than glucose.**

Ten days of cortisol treatment did not alter plasma lactate levels (Table 4). Elevated plasma lactate levels have been reported in stressed teleosts (Love, 1980; Pickering *et al.*,

1982), but this effect is probably due to an increase in excitement or exertion associated with the stress rather than a direct effect of cortisol (Wieser *et al.*, 1986). This is the first study to measure plasma lactate levels in salmonids with chronically elevated exogenous cortisol, so a close comparison of these results to others is not possible. Elevated blood lactate has been reported in eels after 14 daily injections of cortisol, yet after 1 and 4 days of this treatment there was no change in blood lactate levels (Lidman *et al.*, 1979). The conclusions of that study are somewhat confusing since the cortisol treatment was reported to stimulate gluconeogenesis, but the increased blood levels of lactate after 14 days may indicate a stimulation of glycolysis as well. The simultaneous elevation of plasma glucose and lactate is more characteristic of an adrenergic response rather than chronically elevated cortisol (Mazeaud and Mazeaud, 1981).

In mammals glucocorticoids are thought to increase hepatic protein synthesis. These proteins are then released into the circulation, increasing plasma protein levels (Kraus-Friedmann, 1984). Plasma proteins (Table 4) were unaltered by 10 days of cortisol treatment. This finding is in accord with most previous studies (Chan and Woo, 1978; Lidman *et al.*, 1979; Vijayan and Leatherland, 1989; Vijayan *et al.*, 1991). Conversely, Foster and Moon (1986) reported an increase in plasma proteins after 10 days of cortisol treatment.

Possibly the most interesting finding of this study was the alteration to circulating amino acid levels after 9 days of cortisol treatment. The results of this experiment (Table 1) seem to indicate that cortisol is capable of increasing the availability of amino acids to the liver, but the manner in which this potential gluconeogenic substrate is then metabolized is

not affected. Plasma amino acids were approximately 1000 $\mu g \cdot mL^{-1}$ 24 and 96 h after mini-osmotic pump implantation in both the sham and cortisol treated fish. By 216 h (9 days) after surgery the level of plasma amino acids was significantly lower in the sham treated fish (716 $\mu g \cdot mL^{-1}$ for shams versus 1023 $\mu g \cdot mL^{-1}$ for cortisol treated). While plasma amino acids were not measured prior to surgery, it appears that the stress response associated with the surgical procedure elevates plasma amino acids. The exogenous cortisol treatment is then capable of maintaining this elevation. Early studies by Idler and Clemens (1959) and Idler *et al.* (1959) suggested that cortisol was responsible for the increased muscle catabolism of migrating salmon. In higher vertebrates it is well documented that corticosteroids facilitate the mobilization of amino acids from skeletal muscle through a stimulation of tissue catabolism (Leung and Munck, 1975; Kraus-Friedmann, 1984). The resultant elevation in plasma amino acids is thought to be due to the inhibitory effect of glucocorticoids on the synthesis of peripheral proteins (Steele, 1975; Shoji and Pennington, 1977; Tomas *et al.*, 1979). This inhibition is believed to be at the level of translation (Kraus-Friedmann, 1984). Chan and Woo (1978) reported an increase in plasma amino acids 24 h after a cortisol injection in Japanese eels, but other studies have indicated exogenous cortisol administration does not elevate circulating amino acid levels in teleosts (Inui and Yokote, 1975; Leach and Taylor, 1980; Leach and Taylor, 1982; Foster and Moon, 1986). In a recent study by Tam *et al.* (1988), brook trout maintained at pH 4.5 had significantly elevated plasma amino acids. That study suggested that cortisol promoted protein catabolism, but that a stress induced reduction in the secretion of both growth hormone and thyroid hormone was responsible for a decreased incorporation of amino acids into protein. This latter stress

effect was thought to be responsible for the elevation in circulating amino acids since cortisol injections in non acid-stressed trout did not raise amino acid levels. Recently Hall-Angeras *et al.* (1991) administered a glucocorticoid receptor antagonist (RU 38486) to rats with sepsis to examine the role of corticosteroids in stress induced proteolysis. That study indicated that pretreatment of rats with RU 38486 significantly reduced the stress induced muscle protein breakdown. This finding suggests that in stressed rats, glucocorticoids have a necessary role to play in the increased mobilization of muscle protein.

In higher vertebrates aldosterone, which is produced by the adrenal glands, is the primary hormone responsible for electrolyte balance (Guyton, 1986). Salmonid interrenal tissue does not produce aldosterone in significant quantities, instead cortisol acts as the dominant mineralcorticoid hormone. The plasma osmolarity of the cortisol treated fish was not different from the shams (Table 2). This finding is somewhat surprising since cortisol is supposed to stimulate $Na^+/K^+$ ATPase activity (Jampol and Epstein, 1970). Furthermore Laurent and Perry (1990) has shown that treating rainbow trout with exogenous cortisol increases the number of chloride cells on the gill epithelia. Therefore cortisol might be expected to facilitate the uptake of $Na^+$ and $Cl^-$ and might be expected to increase the plasma osmolarity of a teleost. It is interesting to note that chronically treating coho salmon with cortisol had no effect on plasma osmolarity and actually lowered $Na^+/K^+$ ATPase activity (Redding *et al.*, 1986).

**The activity of key liver enzymes in cortisol treated fish.**

Glucocorticoids are thought to influence hepatic gluconeogenesis not only through an increased availability of gluconeogenic substrates but, by direct alteration to the activity of certain enzymes in the pathway (Kraus-Friedmann, 1984). A number of studies have demonstrated the induction of hepatic enzymes in mammals (Feigelson *et al.*, 1975; Voight *et al.*, 1978) and fish (Freeman and Idler, 1973; Inui and Yokote, 1975; Chan and Woo, 1978; Davis *et al.*, 1985; Vijayan *et al.*, 1991) after glucocorticoid treatment. Several tissues are dependent on a constant supply of glucose as a metabolic fuel. Because of the high protein diet of salmonids, most of this glucose requirement is met through hepatic gluconeogenesis (Moon, 1988). When an animal is stressed, hepatic gluconeogenesis is often reported to increase resulting in hyperglycaemia (Asterita, 1985). It is believed that this hyperglycaemia is mediated by cortisol and is maintained to ensure adequate substrate supply to glucose dependent tissues to enable the animal to combat the stressor.

This study examined the activities of several key regulatory enzymes in the glycolytic and gluconeogenic pathways of the liver of cortisol treated rainbow trout (Table 3).

Glycogen phosphorylase (GPase) is the hepatic enzyme responsible for regulating the entrance of the glucosyl units of glycogen into the glycolytic pathway (Lehninger, 1982). Alterations in the GPase %a, which is the active form of this enzyme, may be reflected in the glycogen content of the liver. It is interesting to note that the glycogen content of the hepatocytes isolated from cortisol treated fish (Table 5) was lower then the shams yet 10 days of cortisol treatment did not change the activity of total glycogen phosphorylase (GPase a+b) or GPase %a when compared to shams. The reported effect of cortisol on

liver glycogen metabolism in teleosts is controversial. Several studies have reported cortisol treatment increases liver glycogen (Butler, 1968; Inui and Yokote, 1975; Chan and Woo, 1978; Lidman *et al.*, 1979) while others have reported decreases (Storer, 1967; Davis *et al.*, 1985; Foster and Moon, 1986; Barton *et al.*, 1987; Vijayan and Leatherland, 1989; Vijayan *et al.*, 1991). This is the only study to examine the effect of exogenous cortisol administration on glycogen phosphorylase activity. Unfortunately, glycogen synthase activity has never been assayed in cortisol treated fish, so it's very difficult to explain the reported discrepancies concerning the effect of glucocorticoids on glycogen metabolism. Davis *et al.* (1985) has suggested that the response of liver glycogen to glucocorticoids may be species dependent.

Phosphofructokinase (PFK) catalyses the production of fructose-1,6-bisphosphate from fructose-6-phosphate by the transphosphorylation of ATP (Hers and Hue, 1983). In rainbow trout PFK is believed to be the rate limiting enzyme in liver glycolysis (Walton and Cowey, 1982). The activities of PFK and fructose-1,6-bisphosphatase (FBPase) are both mediated by the level of fructose-2,6-bisphosphate (Fru-2,6-P). When Fru-2,6-P levels are high PFK activity is inhibited and FBPase activity is stimulated. Conversely, when Fru-2,6-P are low the activity of PFK is stimulated and FBPase is inhibited. The level of Fru-2,6-P is itself controlled by a cAMP dependent phosphorylation of the bifunctional enzyme PFK2/FBPase2. When the enzyme is phosphorylated the synthesis of Fru-2,6-P is inhibited by the kinase and Fru-2,6-P hydrolysis is stimulated by the phosphatase (Pilkis *et al.*, 1988). Therefore any increase in the rate of hepatic gluconeogenesis, due to the corticosteroid treatment, should be associated with a decrease in PFK activity. But, the activity of PFK

(10.1±3.7 units·$g^{-1}$ for cortisol treatment versus 9.7±2.2 units·$g^{-1}$ for shams) was not altered by 10 days of cortisol treatment, indicating that the hormone did not inhibit glycolysis and therefore suggesting that gluconeogenesis was not stimulated by the hormone. Inui and Yokote (1975) reported that 10 days of cortisol administration stimulated gluconeogenesis in eels. FBPase was stimulated after 5 days of treatment, yet after 10 days of cortisol administration the activity of the enzyme was no longer significantly elevated, although the hyperglycaemia did not subside. That study also reported significantly increased PFK activity after 5 and 10 days of hormone treatment, indicative of an increase in glycolysis. When eels were given a single cortisol injection there was no change in FBPase activity in the 24 h following the treatment (Chan and Woo, 1978). Vijayan *et al.* (1990) reported that rainbow trout subjected to the stress of high stocking density had elevated PFK and FBPase activities when compared to fish kept at lower stocking density. The contradictory findings of that study and Inui and Yokote (1975) may indicate that measuring optimal PFK and FBPase activities is not an effective means of estimating intermediate metabolism. It is possible that any effect due to hormonal or stressor treatment is masked by variability in allosteric and substrate inhibitors and activators; unfortunately, none of these modulators have been estimated in this study.

Liver pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK) are key enzymes involved in the regulation of intermediate metabolism. PK catalyses the conversion of phosphoenolpyruvate to pyruvate. PEPCK is the enzyme which bypasses this step in the gluconeogenic pathway by converting oxaloacetate to PEP and is believed to be the rate limiting step in hepatic gluconeogenesis (Walton and Cowey, 1982). Regulation of PK

activity has also been proposed as a site of control for the rate of gluconeogenesis (Petersen *et al.*, 1987). In this study neither of these enzymes showed any change in their activities after 10 days of cortisol treatment. If cortisol was acting as a glucocorticoid in these fish then the activity of PEPCK would be expected to increase and that of PK would decrease. Thus the failure of the experimental treatment to alter the activity of these two enzymes strongly suggests that cortisol is not a glucocorticoid in rainbow trout. Foster and Moon (1986) reported an increase in the activity of the mitochondriai fraction of PEPCK, but not the cytosolic fraction of the enzyme, after 10 days of cortisol treatment; that study also reported no change in PK activity. Although the results of Foster and Moon (1986) differ from the results of this study, comparisons are difficult because rainbow trout only have mitochondrial PEPCK (Moon, 1988). When rainbow trout were subjected to the stress of high stocking density, gluconeogenesis from glycerol was stimulated, but the failure to find increased mobilization of proteins was supported by the unchanged activities of PK and PEPCK (Vijayan *et al.*, 1990).

In mammals it is accepted that glucocorticoids stimulate the utilization of amino acids as gluconeogenic substrates (Rosen *et al.*, 1959; Granner, 1990). To accomplish this the activities of several amino acid transaminases, glutamate dehydrogenase (GDH), and PEPCK are generally elevated (Krause-Friedmann, 1984). The $\alpha$-amino group of plasma amino acids are removed in the liver by transaminase enzymes. Most of the resultant keto acids, depending on their carbon-skeleton, then become potential gluconeogenic substrates. GDH is the enzyme responsible for the oxidative deamination of the glutamate, which along with the keto acids is a product of transaminase enzymes. As was previously discussed, PEPCK is

thought to be the rate limiting enzyme for the utilization of these substrates in the gluconeogenic pathway.

This study assayed glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT), and GDH in fish after 10 days of cortisol treatment. These enzymes are responsible for the entry of alanine and aspartate into the gluconeogenic pathway. There were no significant differences in the activities of these enzymes between the cortisol and sham treated fish. This finding is interesting because the cortisol treatment did increase the level of circulating amino acids (Table 1).

Several studies have examined GOT and GPT in cortisol treated fish. Freeman and Idler (1973) reported that in rainbow trout and brook trout both cortisone and cortisol stimulated liver GPT activity, but only cortisol stimulated GOT activity. These results have been cited frequently to support the role of corticosteroids as glucocorticoids in salmonids. However, it is worth noting that 5 daily cortisol injections of 20 $mg \cdot kg^{-1}$ were required to stimulate GOT activity in rainbow trout. When the cortisol dose used was reduced to 10 $mg \cdot kg^{-1}$ the activity of GOT was not significantly different from the sham treated fish. Unfortunately the circulating cortisol levels resulting from these treatments were not reported. One can safely speculate that had they been measured both experimental treatments would have resulted in plasma hormone levels far in excess of the physiological levels of severely stressed trout. Furthermore, Freeman and Idler (1973) reported that 5 daily injections of metopirone (50 $mg \cdot mL^{-1}$) also increased the activity of GOT, yet this drug is known to impair glucocorticoid synthesis in rainbow trout (Fagerland *et al.*, 1968). Thus, the conclusions of that study should probably be reviewed because a large pharmacological

dose of cortisol (10 $mg \cdot mL^{-1}$) did not stimulate GOT activity, but when glucocorticoid synthesis was blocked, the activity of the enzyme increased. Furthermore Fagerlund *et al.* (1968) reported that metopirone was leathal to rainbow trout when it was injected intramuscularly at a doses ranging from 35 to 45 $mg \cdot kg^{-1}$. Storer (1967) stated that cortisol elevated GPT activity in goldfish, but the physiological significance of the study is dubious owing to the massive doses (200 $mg \cdot kg^{-1}$) of cortisol which were injected. The studies by Chan and Woo (1978) and Inui and Yokote (1975) both reported significantly stimulated GOT and no change in GPT activities. While the differences in the results of these two investigations and the present study could simply be due to species differences, it is worth noting that Chan and Woo (1978) used hypophysectomized eels. Clearly, the surgical removal of an animal's pituitary will have wide ranging physiological and biochemical effects other than simply lowering cortisol titers. Thus it is difficult to make direct comparisons of that study and ones which administered cortisol to intact animals such as was done in this study. In support of the findings of this study, Foster and Moon (1986) reported that 10 days of cortisol treatment did not alter the activity of GOT or GPT. Likewise, GOT activity in both fed and fasted killifish was unchanged after 5 days of cortisol treatment, even though the dose was massive (Leach and Taylor, 1982). That study concluded that the hyperglycaemia that resulted from the experimental treatment was due to reduced peripheral glucose utilization rather than to increased gluconeogenesis from amino acids. In rat hepatocytes tyrosine aminotransferase (TAT) activity was shown to increase after glucocorticoid treatment (Iynedjian *et al.*, 1985). While it was not assayed in this study, several studies have examined the effect of corticosteroid administration on TAT activity in

teleosts. The administration of glucocorticoids to salmon (Fellman *et al.*, 1971) and the marine toadfish (Mommsen *et al.*, 1990) did not affect TAT activity. Conversely, when channel catfish (Davis *et al.*, 1985) and brook trout (Whiting and Wiggs, 1977) were treated with exogenous cortisol the activity of TAT increased significantly, leading both of these studies to conclude that, in teleosts, cortisol is a glucocorticoid. A recent investigation by Vijayan *et al.* (1991) reported that after 30 and 60 days of cortisol administration there was no change in metabolism of amino acids by brook trout. As in this study, Vijayan *et al.* (1991) found no change in the activity of GOT, GPT or PEPCK after chronic cortisol treatment. That study reported that rather than increasing the utilization of amino acids, administering cortisol to fed trout increased the mobilization and oxidation of lipids. The activity of several key enzymes associated with lipid metabolism were elevated by the cortisol treatment. Among these enzymes whose activity was elevated by cortisol was glucose-6-phosphate dehydrogenase (G6PDH), which generates reducing equivalents through the pentose phosphate pathway for fatty acid synthesis. The present study did not examine any lipid oxidative enzymes, but G6PDH was measured and it's activity was not altered by 10 days of cortisol treatment. This suggests that lipid turnover was probably not stimulated by cortisol treatment as it was in the study of Vijayan *et al.* (1991).

The activity of lactate dehydrogenase (LDH) was not altered by cortisol treatment (Table 3). Foster and Moon (1986) reported that LDH activity decreased in cortisol treated eels. This decrease was thought to be due to an overall decrease in liver protein content rather than a decrease in maximal enzyme activity. Unfortunately, liver protein content was not measured in this study.

**The effect of cortisol on substrate fluxes in isolated hepatocytes**

The use of hepatocytes isolated from teleost fish is a well established technique for analyzing hormonal regulation of liver metabolism (Moon *et al.*, 1985; Mommsen, 1986; Petersen *et al.*, 1987). Cortisol treatment did not result in significant differences in the activities of key enzymes of the gluconeogenic and glycolytic pathways, implying no hormonal alteration of liver metabolism. By examining the fluxes of radiolabelled substrates in isolated hepatocytes it was possible to directly analyze the role of chronically elevated cortisol on liver metabolism.

As previously discussed, cortisol acts as a glucocorticoid in mammals. If it had a similar function in rainbow trout then the rate of liver gluconeogenesis and thus the flux of $^{14}$C-labelled alanine and lactate to radiolabelled glucose would be expected to be elevated in hepatocytes isolated from cortisol treated fish. Actually, the flux of these two $^{14}$C-labelled substrates to radiolabelled glucose, $CO_2$, and protein was not affected by 10 days of hormone treatment (Table 5). These findings support the earlier results of this study, and further validate that cortisol does not function as a glucocorticoid in trout.

Only a single study (Foster and Moon, 1986) has previously investigated directly the metabolic role of cortisol in a teleost fish liver. That study examined the fluxes of radiolabelled substrates in isolated hepatocytes from cortisol treated eels. The findings of Foster and Moon (1986) suggested an overall decrease in the metabolic fluxes in hepatocytes isolated from eels following 10 days of cortisol injections. Not only were the fluxes of substrate to glucose decreased in the hormone treated eels, but the fish were also hypoglycaemic. In the present study the unchanged flux of substrates to glucose in the

cortisol treated trout is consistent with the unaltered glycaemic status of the animals. Renaud and Moon (1980) reported that the addition of 10 $\mu$M cortisol to the incubation medium stimulated gluconeogenesis in eel hepatocytes. An increased rate of gluconeogenesis was reported in the isolated hepatocytes within 1 h of the addition of cortisol. In mammals the effects of steroid hormones on enzyme induction are not apparent for several hours (Krause-Friedmann, 1984), therefore the results of Renaud and Moon (1980) are questionable. Longer term studies using cultured hepatocytes may be a more appropriate model for studies of this sort.

**The effect of cortisol on alanine turnover**

Alanine turnover has not been measured previously in teleost fish. In mammals elevated cortisol is associated with an increased utilization of amino acids as gluconeogenic substrates, therefore, alanine turnover increases as well. Alanine turnover was measured in this study as a means of further examining the importance of cortisol in amino acid metabolism. Earlier experiments in this study indicated that while the circulating levels of amino acids were elevated, the liver did not appear to increase it's utilization of this potential gluconeogenic substrate. The results of this experiment indicated that alanine turnover was not altered by 10 days of cortisol treatment. This supports the finding that 10 days of cortisol treatment did not alter the activities of the liver enzymes GDH and GPT, which are key enzymes in the utilization of amino acids. Conversely the increase in circulating plasma amino acids would seem to indicate an increase in the production of amino acids. At the present time it is not understood why the cortisol induced increase in plasma amino acid

levels did not alter the rate of alanine turnover. Especially given that Weber *et al.* (1986) showed that in tuna, the turnover of glucose and lactate were directly related to the concentration of plasma metabolites. It may be that uptake is a critical site for control of substrate flux in teleost fish hepatocytes.

**The effect of cortisol on the caloric and water content of muscle**

If cortisol stimulates the catabolism of white muscle in trout as reported by Butler (1973) and in mammals, one might expect a reduction in body mass. But it is also possible that body weight would be maintained and that the increased muscle catabolism simply results in an increase in muscle water content (Love, 1970) or a decrease in caloric content. To asses these possibilities, the water and caloric contents of white muscle were measured and found to be unchanged in cortisol treated rainbow trout.

The caloric content of white muscle in cortisol treated fish has not been measured prior to this investigation. However, the findings of the present study would suggest that, in the absence of other stress factors, the catabolic effect of cortisol is rather limited.

It has been reported that starvation results in the mobilization of muscle protein as an energy source (Love, 1980; Moon, 1983). The resultant changes in muscle structure are reflected in increased extracellular spaces within the tissue and thus an increased water content. It has been suggested that an increased muscle catabolism and resultant increase in tissue water content will also result from many chronic stressors such as lowered environmental pH (Cunningham and Shuter, 1986) or electroshock (Dehn and Schirf, 1986).

Whitings and Wiggs (1977) found that administering cortisol (IM injection 40 mg · $100g^{-1}$) to brook trout significantly increased muscle water content. While the results of that investigation conflict with this study, it is important to note that the dosages of cortisol used in these two studies were very different.

## Summary of findings

When compared to the findings of mammalian studies, the results of these experiments are unusual to say the least! Obviously, the findings of this study are not consistent with the accepted dogma that cortisol is a glucocorticoid in all vertebrates

Figures 1a and 2a demonstrate that mini-osmotic pumps can successfully maintain cortisol at a desired titre. While this treatment did not result in hyperglycaemia, it was not without effects. After 10 days of cortisol administration, the circulating level of amino acids was significantly elevated in the hormone treated fish. But, in the absence of other stress factors, this increase in plasma amino acids appears to be the only effect of the cortisol treatment. An examination of several key regulatory enzymes of the liver failed to show cortisol induced changes in their activities. When liver metabolism was directly examined using hepatocytes isolated from cortisol and sham treated fish, there was no change in the flux of radiolabelled substrates to $^{14}$C-labelled glucose, $CO_2$, and protein. The rate of alanine turnover was not affected by the cortisol treatment. Likewise, 10 days of hormone treatment did not alter the caloric or water contents of white muscle.

These results do not necessarily argue against the importance of cortisol as a component of the stress response in rainbow trout. Clearly, when salmonids are faced with

stressors such as acidification (Brown *et al.*, 1984;1986), confinement (Redding, 1984; Barton *et al.*, 1980), or disease (Donaldson, 1981) there is an associated elevation in plasma cortisol levels. The idea of a "permissive" role for glucocorticoids was first suggested by Ingle (1952). That study measured urea excretion as an indicator of the severity of surgical stress. It was demonstrated that a constant dose of glucocorticoids, which alone did not alter urea excretion, "permitted" a graded response to a graded stimulus. Therefore even though the effect of cortisol was indirect, it appeared to be an important component of the overall stress response. Since urea excretion is an indicator of the rate of amino acid metabolism, the findings of Ingle (1952) are an interesting comparison to the present study where the cortisol treatment alone, did not increase the utilization of amino acids as gluconeogenic substrates.

A recent study has suggested that permissive effects of cortisol are involved in the intermediate metabolism of rainbow trout (Reid and Perry, 1991). That study reported that 10 days of cortisol treatment increased the population of low affinity, internalized $\beta$-adrenergic receptors of erythrocytes but the number of high affinity receptors on the cell surface was not altered. When subjected to hypoxic conditions, there was a migration of receptors from the cell interior to the membrane surface, resulting in an increased adrenergic response in the erythrocytes from cortisol treated fish. In rainbow trout it is well established that catecholamines are elevated in response to stress (Perry *et al.*, 1989). Furthermore elevated catecholamines titers stimulate hepatic gluconeogenesis (Wright *et al.*, 1989) resulting in hyperglycaemia. Therefore it is possible that cortisol has limited direct effects

on the intermediate metabolism of an animal, but instead "primes" the animal such that it has an enhanced adrenergic response to stressors.

A further explanation of the results of this study could be that cortisol stimulates gluconeogenesis through increased substrate availability. The importance of the extrahepatic effect of cortisol (Krause-Friedmann, 1984) resulting in the mobilization of peripheral muscle and elevation of plasma amino acids should not be overlooked. Moon (1988) suggested that many carnivorous lower vertebrates, including rainbow trout, which have high dietary protein requirements maintain their glycaemic status primarily through gluconeogenesis. In these animals gluconeogenesis behaves as if it were permanently switched-on. Therefore the capacity, and possibly the need, to up regulate the pathway may be limited. While it was never investigated in this study, it is possible that, in rainbow trout, the stimulatory effect of cortisol on gluconeogenesis is limited to increasing the availability of potential substrates. The stress-induced metabolic alterations of omnivorous animals are considerably different than those of carnivores. They maintain their glucose status primarily through dietary consumption and glycogenolysis rather than gluconeogenesis. When subjected to stress, dietary consumption may decrease forcing omnivores to rely on gluconeogenesis to meet their glucose requirements. Therefore, omnivores must have some mechanism to stimulate both gluconeogenesis and the mobilization of lipids and proteins for use as gluconeogenic substrates. It is widely accepted that in mammals, glucocorticoids act in this manner. Conversely, during periods of anorexia, carnivorous animals, such as the rainbow trout, need not up regulate gluconeogenesis to maintain their glycaemic status. Instead, they may only need a mechanism to increase the mobilization of protein stores to supplement the protein

which is normally supplied through dietary consumption and an elevation in plasma cortisol would appear to function quite adequately in this capacity.

Therefore the model which I propose for rainbow trout is that, in the absence of other stress factors, chronically elevated cortisol will increase the mobilization of endogenous stores of protein and probably lipids (Vijayan *et al.*, 1991), but will not result in hyperglycaemia. The activities of the regulatory enzymes controlling the gluconeogenic pathway are unchanged by the hormone. While cortisol does not directly cause hyperglycaemia, it may "prime" an animal to respond with an enhanced adrenergic response if challenged with a stressor (Reid and Perry, 1991) resulting in hyperglycaemia.

This model is quite different from the widely held belief that cortisol behaves as a glucocorticoid in teleosts, but I can arrive at no other conclusion when examining the results of this study. Obviously, further research is needed to better understand the metabolic effects resulting uniquely from cortisol and those which result from permissive effects of cortisol on other stress hormones.

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# APPENDIX A.

**Experiment VI. The effect of chronically elevated cortisol on the rate of protein synthesis in specific tissues.**

As all steroid hormones, cortisol is thought to act upon the chromatin of a cell by altering the rate of DNA transcription of specific genes. The net effect of this is an alteration in the rate of synthesis of the proteins represented by these specific genes (Kraus-Friedmann, 1984). If hepatic gluconeogenesis is stimulated by cortisol then it is reasonable to assume that the production of gluconeogenic enzymes in the liver would be increased (Butler, 1973). The chloride cells of the gill are responsible for ion uptake (Perry and Laurent, 1979). The production of these cells might be predicted to increase if cortisol is able to stimulate ion uptake. In mammals glucocorticoids stimulate muscle catabolism and decrease muscle protein synthesis (Kraus-Friedmann, 1984). This experiment was, therefore, designed to investigate the role of cortisol in the rate of protein synthesis in the liver, gill, white muscle and red muscle of rainbow trout. Protein synthetic rates were measured using the method of Houlihan *et al.* (1986), which is outlined below.

This experiment differed from the others of this study in that soft water and acidified water were used. Soft water ($Na^+$ 0.05 mM, $K^+$ 0.015 mM, and $Cl^-$ 0.075 mM) was prepared by titrating deionized water with NaCl and KCl. Deionized water was continuously prepared by reverse osmosis treatment of city of Ottawa tap water. This system produced approximately 1.5 L of soft water per min, but varied depending upon the temperature of the incoming tap water. During winter months it was not possible to maintain adequate flow in

this system. Acidified water (pH 4.8) was prepared by titrating soft water with 0.01 N sulphuric acid.

Sixteen fish were acclimated to soft water and sixteen fish were acclimated to acidified soft water for three weeks prior to the experiment. Eight of the soft water fish were then randomly removed and fitted with a cortisol loaded mini-osmotic pump which maintained plasma cortisol levels near 100 $ng \cdot mL^{-1}$; the other eight fish were referred to as intact. Ten days later 8 fish from each treatment were individually removed and given a caudal injection of 150 mM L-phenylalanine and L-[$^{14}$C(U)] phenylalanine at 10 $\mu Ci \cdot mL^{-1}$ in Cortland saline. The injected dose was 0.50 $mL \cdot 100g^{-1}$ of body weight. Fish were then returned to a small (50 L) well aerated aquarium. Exactly 40 min after injection fish were killed by a blow to the head a blood sample was taken from the caudal vein/artery and liver, gill, white muscle, and red muscle samples were removed and frozen between aluminum blocks chilled in liquid nitrogen. This dissection was performed on ice and was completed in approximately 5 min.

Frozen tissue samples were weighed and homogenized in 3 mL of 2% PCA using a polytron PCU-2 (Brinkmann Instruments Co., Toronto, Ontario). The homogenate was then centrifuged (Sorvall, model RC 28S) at 2800 g for 15 min. The supernatant was removed and frozen at -20°C for later determination of homogenate free phenylalanine specific activity ($S_A$). The precipitate was washed 3 times with 5 mL of 2% PCA, with centrifugation at 2800 g for 15 min between each washing. These washings removed unincorporated labelled phenylalanine from the protein pellet. The supernatants from these washings were discarded. The resultant protein pellet was transferred to large screw top test tubes and subsequently

hydrolysed in 7.5 mL 6N HCl for 18 hours at 110 °C. The HCl was removed using a vacuum desiccator and the amino acids were resuspended in 5.0 mL of 0.5 M sodium citrate, pH 6.3.

The phenylalanine specific activity of the protein ($S_s$) and the homogenate free pool ($S_a$) were determined using the method of Garlick *et al.* (1980) in which phenylalanine is enzymatically converted to β-phenylethylamine (PEA). A 1.0 mL portion of supernatant or hydrolysate was incubated overnight at 50 °C with L-tyrosine decarboxylase suspended in 0.5 mL of 0.5 M sodium citrate, pH 6.3 (0.7 units·$mL^{-1}$ for supernatants and 1.4 units·$mL^{-1}$ for hydrolysates) containing 0.5 mg·$mL^{-1}$ pyridoxal phosphate. PEA was extracted by adding 1.0 mL of 3 M NaOH and vortexing with 10 mL of n-heptane. The organic layer was then removed and added to 5.0 mL of n-heptane and vortexed with 4.0 mL of 0.01 M $H_2SO_4$. A 1.0 mL sample of the aqueous phase of supernatants (2.0 mL for hydrolysates) was added to 10.0 mL of Aquasol-2 (NEN, Canada) for liquid scintillation counting. A further 1.0 mL of the supernatant and 50 μL of hydrolysate were assayed for PEA by the fluorometric method of Suzuki and Yagi (1976). A standard curve from 0 to 15 μM β-phenylethylamine (Sigma St. Louis, MO) in 0.01 M $H_2SO_4$ was used for comparison. To 1.0 mL of PEA was added 1.0 mL 50 mM ninhydrin (Sigma), 0.5 mL 2 mM leucylalanine (Sigma) and 2.5 mL of 1.0 M potassium phosphate, pH 8.0. Samples were incubated at 60 °C for 1 h, and then were allowed to cool to room temp. Fluorescence was measured at 495 nm emission and 390 nm excitation using a spectrofluorometer (G.K.Turner Associates, Palo Alto, CA; Model 430). All steps of this procedure subsequent to the incubation were carried out in darkness.

# RESULTS

The cortisol treatment did not significantly alter the rate of protein synthesis (Table 1) in any of the tissues examined. The rate of protein synthesis in the gills of the acidified soft water treated fish was significantly increased compared to soft water intact fish (fractional synthesis rate of 12.3% $\cdot$ day$^{-1}$ for acidified soft water vs. 6.8% $\cdot$ day$^{-1}$ for soft water intact fish). The acidified water did not affect the protein synthetic rate of the liver, white muscle, or red muscle. The elevated rate of protein synthesis in the gill is thought to be due to an increased population of chloride cells in the gill.

**Table 1.** Fractional rates of protein synthesis in % per day for sham, cortisol and acid treated fish. Fish were fed and cortisol treated fish received mini-osmotic pumps, loaded with cortisol 10 days prior to sacrifice. Mini-osmotic pumps were used to elevate cortisol to 100 $ng \cdot ml^{-1}$.

| Tissue Type | Shams n=3 | Cortisol n=6 | Acidified n=8 |
|---|---|---|---|
| Gill | 6.78±0.84 | 8.86±1.72 | 12.30±1.13[a] |
| Liver | 6.49±0.42 | 12.49±2.67 | 9.99±1.97 |
| White Muscle | 0.99±0.29 | 1.23±0.16 | 0.83 ±0.085 |
| Red Muscle | 0.89±0.05 | 1.06±0.11 | 1.17 ±0.29 |

[a]Significantly different from control value, Student's t-test, $p<0.05$

## DISCUSSION

The rates of tissue specific synthesis presented in Table 1 are quite close to previously published values. The study by Pocrnjic *et al.* (1983) was the first to use the method of phenylalanine "flooding" to examine protein synthetic rates in a teleost. That study reported protein synthetic rates in toadfish of 13.7% $\cdot$ day$^{-1}$ for liver, 7.7% $\cdot$ day$^{-1}$ for gill, and 0.23% $\cdot$ day$^{-1}$ for epaxial muscle. Houlihan *et al.* (1986) further refined the technique of phenylalanine "flooding" in rainbow trout and measured fractional rates of protein synthesis of 9.07% $\cdot$ day$^{-1}$ for gill, 1.25% $\cdot$ day$^{-1}$ for red muscle and 0.49% $\cdot$ day$^{-1}$ for white muscle.

Although the effect of glucocorticoids on protein synthesis has not been previously examined in teleosts, corticosterone has been shown to decrease the rate of protein synthesis in the muscle, but not the liver of rats (Garlick *et al.*, 1987).

The reliability of the results of this experiment are questionable for two reasons. The reverse osmosis system was not able to produce enough soft water to produce adequate flow through the system. Furthermore, it would be unreasonable to assume that these fish were able to acclimate to the experimental procedure and thus were unstressed. Fish mortality was significant amongst all three treatment groups, and there were signs of morbidity among the survivors. Therefore the failure to find differences in the tissue specific rates of protein synthesis between the cortisol treated fish and the shams may only be indicative of the stressful nature of the experimental procedures.

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