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HEPATIC DISTRIBUTION OF α- AND β-ADRENOCEPTORS IN TROUT:

EFFECTS OF CORTISOL AND TEMPERATURE

by

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A thesis submitted to the School of Graduate Studies and Research in partial fulfillment of the degree of Masters of Science in Biology

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ABSTRACT

Exposure of an organism to a stressor results in a primary stress response. A part of this response involves the release of the catecholamines, epinephrine and norepinephrine, which exert their actions by binding to specific membrane binding sites or receptors termed adrenergic receptors or adrenoceptors. Two adrenoceptor types exist on hepatic membranes, and their occupation results in significant changes in the biochemistry of the hepatocyte. The distribution of α_1- and β_2-adrenoceptors on hepatic membranes is known to be species specific, and also seasonally dependent in cardiac tissue. This study tests the hypothesis that α- and β-adrenoceptor distribution can be modified by cortisol and long term temperature acclimation. Rainbow trout (*Oncorhynchus mykiss*) were injected with slow release hydrogenated coconut oil implants alone (sham) or containing cortisol for 10 - 14 days to elevate plasma cortisol levels. Hepatic membranes were purified on discontinuous sucrose gradients and α- and β-adrenoceptors were assayed from sham and cortisol injected trout as well as non-injected (naive) trout. No significant differences were found between any of the experimental groups for either the affinity (K_d) or receptor number (B_{max}) values of both receptor types. Cortisol treatment resulted in no change in either hepatic membrane phospholipid composition or plasma glucose, although plasma lactate decreased in cortisol injected fish. Adrenoceptor function was tested by examining glycogenolysis in isolated hepatocytes from sham and cortisol injected rainbow trout. Epinephrine significantly stimulated glucose release in sham injected trout only. This effect was blocked by both α-

and β-antagonists. In addition, studies on trout acclimated to 5 and 20°C did not demonstrate different receptor characteristics. These studies do not support the hypothesis that rainbow trout exposed to cortisol alter properties of hepatic adrenoceptors.

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INTRODUCTION

Epinephrine (EPI) and norepinephrine (NEPI), the two principal naturally occurring catecholamines, are important metabolic mediators in the short-term response to stressors (Fabbri and Moon, 1994). Both are released into the blood from chromaffin tissue primarily after sympathetic neural activation to produce a widespread variety of effects after binding to specific membrane bound receptors (Exton, 1988; Randall and Perry, 1992; Summers and McMartin, 1993; Fabbri and Moon, 1994). These receptors, termed adrenergic receptors or adrenoceptors possess specific affinities for catecholamines, both natural and synthetic which may be either agonists or selective antagonists, blocking agonist binding. The primary objective of this thesis is to examine how cortisol affects the distribution and function of the rainbow trout (*Oncorhynchus mykiss*) hepatic adrenoceptors. To better understand why and how stressors may affect these receptors, a brief description of their structures and functions is necessary.

I. ADRENOCEPTORS

Prior to 1948, the adrenoceptors were thought to consist of two different classes, one with mostly excitatory actions, the other with mostly inhibitory actions on the effector cells. However, this classification was not appropriate as the elicited response seen depended upon the tissue on which the receptor was located. Alquist (1948) working on various tissues from selected mammalian species, pharmacologically

classified these adrenoceptors as α and β, on the basis of the potency order of different adrenergic agonists and antagonists. These receptors were later classified into α_1, α_2, β_1, and β_2 based on the potency of specific new adrenergic ligands (Fabbri and Moon, 1994). Current molecular biological techniques have shown the need for further classification into numerous subtypes (Summers and McMartin, 1993). There are now recognized three types of α_1 (A, B, and C), four types of α_2 (A, B, C, and D), and a β_3 in addition to β_1 and β_2 (Bylund, 1992; Fabbri and Moon, 1994; Duman and Nestler, 1995). The probable functional significance of so many different subtypes is a more elaborate specificity of the physiological response by the cell (Bylund, 1992). On the other hand, there may be no functional significance as differences in affinities between the subtypes exist only for the synthetic agonists and antagonists. As well, there is no difference in the activation of the transduction pathways associated with these adrenoceptor subtypes (Graham *et al.*, 1996). So, the physiological significance of this multitude of receptor subtypes remains unresolved.

Regardless, all of the known adrenoceptors belong to the guanine nucleotide regulatory protein or G protein-linked superfamily of receptors. These receptors are 402-560 amino acids in length and contain seven hydrophobic transmembrane spanning regions of approximately 20 residues each (Summers and McMartin, 1993; Fabbri and Moon, 1994; Ruffolo and Hieble, 1994). Specific regions of the receptor are critical for proper functioning (Summers and McMartin, 1993). The amino-terminus, containing

sites for amino-linked glycosylation, is situated on the exterior of the cell while the carboxy-terminus, containing potential phosphorylation sites, is located in the cytoplasm (Benovic *et al.*, 1991).

Phosphorylation results in desensitisation of the receptor in order to maintain the cell's metabolic response within physiological limits (Sibley and Lefkowitz, 1985). There are two types of desensitisation, each associated with a different enzyme to phosphorylate the receptor. Homologous desensitisation refers to the process whereby after exposure to an agonist, the cell's response is reduced for that agonist only and not for any other ligands. The enzyme responsible for this in the β-adrenoceptors is β-adrenoceptor kinase (βARK). Heterologous desensitisation occurs after agonist exposure results in the decrease of the cell's response to all the agonists stimulating the identical pathway. Protein kinase A (PKA) which is activated by cyclic AMP (cAMP) is the enzyme responsible for this process (Lohse, 1993).

Receptor activation results in an exchange of GDP for GTP activating the G-protein complex, a trimeric protein. An interaction between the complex and a specific enzyme will regulate the level of the relevant intracellular second messenger. The cell membrane bound G-proteins are coupled to the third intracellular loop of the receptor and have been divided into separate classes based on their linkage to their respective effector systems (Summers and McMartin, 1993; Fabbri and Moon, 1994).

II. α-ADRENOCEPTORS

With the possible exception of the cyclostomes, α-adrenergic receptors occur in at least some tissues of all vertebrates studied to date (Johansson, 1984). In the livers of vertebrates, the α_1-adrenoceptors are believed to be of a more recent origin than the β-adrenoceptors. This was based on an apparent lack of hepatic α_1-adrenoceptors in the lower vertebrates whereas hepatic β-adrenoceptors were found in all classes of vertebrates (Sulakhe *et al.*, 1988). The significance of this as suggested by these authors was that the α_1-adrenoceptor coincidentally evolved with the sympathetic nervous system and the switch to neural catecholamine control from blood borne control of hepatic metabolism.

Anatomically, α_2-adrenoceptors may be found pre- and post-synaptically generally with inhibitory effects, while α_1-adrenoceptors seem to be exclusively post-synaptic with primarily stimulatory effects (Exton, 1985). However, the classification as α_1 and α_2 was based on pharmacological responses as opposed to site and function (Johansson, 1984). The α_1-adrenergic receptors are preferentially stimulated by NEPI and phenylephrine (PHE) and blocked by prazosin (PRZ) while the α_2-adrenergic receptors are more potently stimulated by EPI and clonidine and blocked by yohimbine (YOH) (Johansson, 1984; Ruffolo *et al.*, 1991). Currently, α_1 and α_2 are considered as two different receptors, no more related to each other than either is to the β-adrenoceptors (Bylund, 1992).

A. α_1-ADRENOCEPTORS

Activation of any of the α_1-adrenergic receptors results in the increase of intracellular free calcium concentration ($[Ca^{2+}]i$) although the underlying mechanisms vary between receptor subtypes (Exton, 1985; Summers and McMartin, 1993; Duman and Nestler, 1995). Ca^{2+} mobilization is accomplished through the coupling with phosphoinositide phospholipase C through the probable G_q-protein, initiating the hydrolysis of phosphatidylinositol-1,4-bisphosphate (PIP_2), a membrane phospholipid, producing 1,2-diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). DAG remains bound within the membrane layer while IP_3 diffuses into the cytosol (Berridge, 1993). These two second messengers activate protein kinase C (PKC) by reducing its requirement for Ca^{2+} and by releasing sequestered Ca^{2+} from intracellular stores through binding to a specific IP_3 receptor, respectively (Summers and McMartin, 1993; Fabbri and Moon, 1994).

The intracellular Ca^{2+} exerts its effects through binding to calmodulin, a ubiquitous calcium-dependent regulatory protein. The interaction between a variety of enzymes, as well as other cellular proteins with the Ca^{2+}-calmodulin complex produces the observed physiological responses which are tissue dependent. For example, the response is contraction in vascular smooth muscle, but in liver the response is glycogenolysis (Exton, 1985). The hepatic α_1-adrenoceptor activated glycogenolysis is mediated by allosteric activation of phoshorylase *b* kinase by changes in $[Ca^{2+}]i$ (Joseph *et al.*, 1984).

B. α_2-ADRENOCEPTORS

The α_2-adrenoceptors are coupled to a G_i or G-inhibiting protein which inhibit adenylate cyclase (ACase) activity thereby reducing the intracellular levels of cAMP (Exton. 1985; Summers and McMartin, 1993). α_2-Adrenoceptors can also operate by directly modifying ion channel activities such as the K^+ channel, the Ca^{2+} channel, and the Na^+/H^+ antiport exchanger (Summers and McMartin, 1993). Which transduction pathway the receptor is coupled to is tissue dependent and is most likely related to the cell's specific function (Duzic *et al.*, 1992). α_2-Adrenoceptors are found in the liver, although fewer in number than the α_1-adrenoceptors. A clear, significant role for α_2-adrenoceptor regulation of hepatic cAMP levels has yet to be elucidated (Exton, 1988).

III. β-ADRENOCEPTORS

The β-adrenoceptors are ubiquitously distributed throughout all the vertebrate classes (Fabbri and Moon, 1994). These receptors are coupled to the G_s or G-stimulating protein which activates ACase activity and operate by increasing intracellular levels of cAMP (Fabbri *et al.*, 1992; Summers and McMartin, 1993; Fabbri and Moon, 1994). This signal transduction pathway is the most studied and best understood G-protein coupled system (Levitzki *et al.*, 1993; Fabbri and Moon, 1994) and appears to be conserved in all vertebrates studied to date.

The binding of an agonist initiates the exchange or release of GDP for GTP at the G_α subunit leading to a destabilization. The activation of ACase is a result of the dissociation of the G_α subunit of the G_s protein as a consequence of the GTP binding and binding of G_α to ACase (Exton, 1985). The increase in production of cAMP is a result of ACase catalysing the hydrolysis of ATP. cAMP then activates protein kinase A (PKA) to modify several intracellular functions, including glycogenolysis (Exton, 1985; Fabbri and Moon, 1994).

The separation of the β-adrenoceptors into the β_1 and β_2 classes was based on the relative potencies of EPI, NEPI, and isoproterenol (ISO). Equal affinity for EPI and NEPI was demonstrated for the β_1-adrenoceptors while on the other hand, the β_2-adrenoceptors have a higher affinity for EPI than NEPI (Exton, 1985; Ruffolo *et al.*, 1991). As well, the β_1-adrenoceptors are selectively blocked by the antagonists practolol, metoprolol, and atenolol, whereas the β_2-adrenoceptors are selectively enhanced by the agonists salbutamol, metaproterenol, terbutaline, and soterenol (Exton, 1985). β-Adrenoceptors can be identified, in general, using either radiolabelled cyanopindolol or alprenolol.

IV. HEPATIC ADRENOCEPTORS IN TELEOSTS

The presence of β-adrenergic receptors in the hepatocytes of teleosts is undeniable and at least in rainbow trout (*O. mykiss*) they primarily appear to consist of the β_2

subtype (Reid *et al.*, 1992). The activation of these receptors by catecholamines increases cAMP synthesis (Fabbri and Moon, 1994) resulting in glucose mobilisation enhancing the metabolic potential of the organism (Perry and Reid, 1993). However, the control system in fish is less clear than in mammals where the adrenergic effects on carbohydrate metabolism are better defined (Sheridan and Muir, 1988).

The pathway for the elicited response may be species and/or possibly methodology related. For example, Morata *et al.* (1982) found that glucose release in rainbow trout liver slices was primarily by gluconeogenesis with no decrease or even sometimes an increase in glycogen content. While on the other hand, Moon *et al.* (1988) found that in the trout system, gluconeogenesis accounts for less than 10% of the total glucose released by the hepatocyte. Similarly, Mommsen *et al.* (1988) showed glycogenolysis was responsible for greater than 97% of the glucose produced by catecholamine treated and untreated rainbow trout hepatocytes. Birnbaum *et al.* (1976) also showed EPI induced an increased glucose release from isolated goldfish (*Carassius auratus*) hepatocytes via the glycogenolytic pathway.

After binding to β-adrenoceptors, EPI initiates glycogenolysis and gluconeogenesis by activating glycogen phosphorylase (GPase) and inhibiting pyruvate kinase (PyK) (Moon and Mommsen, 1990; Fabbri *et* al., 1992; Reid *et al.*, 1992). The activation of GPase results from a cascade of reactions initiated by the stimulation of ACase and the synthesis of cAMP (Brighenti *et al.*, 1987a). cAMP-dependent PKA phosphorylated inactive GPase b to the active GPase a form, thus activating glycogen mobilisation. PyK

is a regulatory enzyme in both gluconeogenesis and glycolysis and in its phosphorylated form (occurring by PKA) is less active allowing gluconeogenesis to be activated (Wright *et* al., 1989).

The use of adrenergic agonists and antagonists provides further evidence for a ß-adrenergic role in regulating hepatic glycogenolysis in many species of teleosts. Isoproterenol (ISO; β-agonist - Exton, 1985) has been found to be as effective as the naturally occurring catecholamines in stimulating glycogenolysis, while on the other hand, phentolamine (PHT: α-antagonist - Exton, 1985) has been found to be relatively ineffective in blocking this response. Propranolol (PROP; β-antagonist) has been found to abolish the adrenergic-stimulated glycogenolysis while PHT has been without much effect (for examples, see Birnbaum *et al.* (1976) - goldfish (*C. auratus*); Janssens and Lowrey (1987) - carp (*Cyprinus carpio*); Brighenti *et* al. (1987a,b) (*Ictalurus melas*) - catfish; Sheridan and Muir (1988) - chinook salmon (*Oncorhynchus tshawytscha*)).

No α-adrenoceptor pathway had been identified in the liver of fish or any other lower vertebrate prior to 1992 (Fabbri and Moon, 1994). Studies either found no (eg. Janssens and Lowrey (1987) - carp) or circumstantial (eg. Brighenti *et al.* (1987a,b) - catfish; Moon and Mommsen, (1990) - catfish, eel (*Anguilla rostrata* LeSueur)) evidence. Zhang *et al.* (1992a,b) provided the first evidence for the existence of and to link a physiological function to an α-adrenergic system in any teleost hepatic system using isolated hepatocytes of the American eel (*A. rostrata* LeSueur) and the brown bullhead (*I.*

nebulosus). These studies found that EPI could mobilise [Ca^{2+}]i in both eel and bullhead, and induce [Ca^{2+}]i oscillations in the eel hepatocytes qualitatively similar to α-agonist exposed rat hepatocytes, providing evidence for the presence of an α_1-adrenergic system; no changes in [Ca^{2+}]i to EPI were noted in trout hepatocytes. This evidence was supported by the fact that PROP was ineffective while PHT strongly inhibited [Ca^{2+}]i changes. Additional studies by Moon *et al.* (1993) using black bullhead (*I. melas*) hepatocytes found that the α-agonist phenylephrine (PHE) had a similar effect to EPI and the β-agonist ISO had little effect on [Ca^{2+}]i. The agonist potency ranking for the Ca^{2+} effect was reported to be EPI > PHE >>> ISO which is consistent with an α_1-adrenoceptor system of rats (Exton, 1988). Certainly changes in [Ca^{2+}]i are known in mammals to be controlled by the activation of the inositol polyphosphate pathway which is linked to an α_1-adrenergic system (Exton, 1985; 1988).

Fabbri *et al.* (1995a) found the first evidence for the presence of hepatic α-adrenoceptors in rainbow trout. Using the specific α_1-adrenoceptor antagonist ^{3}H-prazosin (^{3}H-PRZ), this group demonstrated a single class of receptors with specific binding to low affinity sites on purified hepatic membranes. However, EPI was found not to regulate intracellular levels of IP_3 and a physiological role for IP_3 in these hepatocytes has yet to be elucidated. However, Fabbri *et al.* (1995b) did find a dose-dependent EPI increase in IP_3 content in the hepatocytes of the American eel and the black bullhead, and specific IP_3-binding sites on hepatic membranes of both species.

Zhang *et al.* (1992a) found no EPI induced change in [Ca^{2+}]i in the hepatocytes of rainbow trout. Therefore, adrenergic control may show the species differences in fish that are demonstrated in mammals (Sulakhe *et al.*, 1988), possibly explaining the discrepancies found in the literature prior to 1993 (Fabbri *et al.*, 1994).

The effects of α-adrenergic agonists on hepatic carbohydrate metabolism have been equivocal. Janssens and Lowrey (1987) found PHT ineffective at inhibiting EPI-induced GPase activity in the carp (*C. carpio*) and concluded that hepatic α-adrenoceptors were not present in this species. Moon and Mommsen (1990) showed PHE-induced glycogenolysis and gluconeogenesis in the hepatocytes of three teleost species with significant species differences. These effects were blocked by PROP and decreased by PRZ and YOH. Therefore, these authors concluded that either a mixed α/β-adrenoceptor mechanism dominated by the β-receptors was regulating fish hepatocyte metabolism, or the mammalian defined α/β-agonist/antagonist scheme is not appropriate for fish preparations. A similar conclusion has been stated by Brighenti *et al.* (1987b) using PHE in catfish. While Moon *et al.* (1993) found evidence for the existence of at least an α_1-like adrenoceptor system in catfish, but the [Ca^{2+}]i changes observed could not be linked to the changes in GPase activities.

Fabbri *et al.* (1994) have demonstrated α-adrenoceptor involvement in the regulation of carbohydrate metabolism in the liver of catfish. The differences may lie in the methodological approach. This group used isolated hepatocytes suspended in a fine

BioGel P4 resin, which were continuously perifused with fresh medium to better mimic the *in vivo* situation. This could possibly avoid a negative feed-back effect by the removal of the products of cellular metabolism improving cell sensitivity and therefore increasing glucose release over statically incubated cells (Fabbri *et al.*, 1994). This is, however, the only study to show unequivocally that α-adrenoceptor binding is linked to carbohydrate metabolism in any fish species.

V. ADRENOCEPTOR DISTRIBUTION

There are several factors that are capable of influencing the distribution of adrenoceptors in a given tissue. Not surprisingly, one of these factors is species. Sulakhe *et al.* (1988) examined the distribution of α_1- and β_2-adrenoceptors in purified hepatic membranes from representatives of all vertebrate classes except fish. Differences in distribution of the adrenoceptors were found not only between classes, but also between species within a class. The authors found α_1-adrenoceptors to be barely detectable in both the frog (*Rana pipiens*) and the turtle (*Chrysemys sp.*). In birds, α_1- and β_2-adrenoceptors were found in about equal numbers in the liver of the budgerigar (*Melopsittacus undulatus*) and the white Leghorn chicken (*Gallus domesticus*). While in mammals, the β_1-adrenoceptors tended to dominate over the β_2-adrenoceptors (4 out of the 6 species examined), but varied from being approximately 1% of the total hepatic

adrenoceptor population in the guinea pig (*Cavia porcellus*) to almost 94% in the Swiss albino mouse (*Mus musculus*).

Seasonality has been found to effect the distribution of both the α- and β-adrenoceptors in cardiac tissue from various species. Peyraud-Waitzenegger *et al.* (1980) discovered in winter, the α-adrenoceptor mediated affects hypoventilation and bradycardia dominated, while in summer, the β-adrenoceptor mediated affects of hyperventilation and tachycardia were dominant in the European eel (*Anguilla anguilla* L.). A similar result was seen by Chiu and Chu (1989) with tiger frogs (*Rana tigrina*). Pleshka *et al.* (1996) compared the distribution of the β-adrenoceptors between hibernating and euthermic European hamsters. The β_1-adrenoceptors were found to be dominant in euthermic hamsters, while on the other hand, β_2-adrenoceptors dominated in hibernating hamsters.

In mammals, age has been found to affect hepatic adrenoceptor distribution. β-Adrenoceptors predominate in the fetus and neonates, while α-adrenoceptors dominate in adults (Huff *et al.*, 1991; Kajiyama and Ui, 1994; Slotkin *et al.*, 1994a; Kajiyama *et al.*, 1996). This change is believed to be related to the development of sympathetic innervation and the shift from circulating EPI to NEPI (Huff *et al.*, 1991). However, senescent rats augment their β-adrenergic responsiveness, unlike other animals (Eakes *et al.*, 1996), which probably is not related to sympathetic innervation.

Cortisol has been found to increase the number of surface β-adrenoceptors in the isolated hepatocytes of rainbow trout (Reid *et al.*, 1992), in the purified lung membranes of male Sprague-Dawley rats (Mano *et al.*, 1979) and the low-affinity internalized receptors in rainbow trout erythrocytes (Reid and Perry, 1991). The physiological significance of this observation in the trout hepatocytes was a more than doubling of the responsiveness to EPI. This led to a significant increase in the intracellular concentration of cAMP and an increased glucose production. In addition, the glucocorticoid, dexamethasone, was shown by Slotkin *et al.* (1994b) to increase ACase activity in the liver of fetal rats. As well, Huff *et al.* (1991) found daily injections of dexamethasone increased the rate of replacement of β-adrenoceptors with α_1-adrenoceptors in neonatal rat livers.

VI. CORTISOL

The exposure to a stressor, either physical or environmental, results in an endocrine or primary stress response (Vijayan and Moon, 1992; Gamperl *et al.*, 1994). Part of this response involves the release of the glucocorticoid, cortisol, which is the most active and abundant corticosteroid in teleost blood (van der Boon *et al.*, 1991). In teleost fish, release occurs from the interrenal tissue of the head kidney (comparable to the adrenal cortex of higher vertebrates) (Barton and Iwama, 1991; van der Boon *et al.*, 1991;

Gamperl *et al.*, 1994). This results in a secondary response by the organism involving metabolic and osmotic adaptations (Gamperl *et al.*, 1994).

Cortisol is considered to be a metabolic hormone, by effecting intermediary metabolism and thus re-establishing homeostasis (van der Boon *et al.*, 1991; Vijayan *et al.*, 1993a). However, in teleost fish, the metabolic effects of cortisol are controversial (see Andersen *et al.*, 1991 for references). For example, Chan and Woo (1978) found giving hypophysectomized Japanese eels (*Anguilla japonica*) a single intramuscular injection of cortisol (1 mg/kg) significantly increased the level of plasma glucose with a concurrent rising in levels of amino acids after 2 h. In the mummichug (*Fundulus heteroclitus*) it was shown by Leach and Taylor (1982), that a single cortisol injection (20 mg/kg body wt) raised blood glucose levels for at least 24 h. Conversely, Foster and Moon (1986) using a different protocol on American eels, showed daily intraperitoneal injections (0.35 mg/kg) for 10 days depressed plasma glucose 6 and 24 h after the final injection with no change in plasma amino acid levels. Andersen *et al.* (1991) implanted rainbow trout with mini-osmotic pumps to elevate plasma cortisol to 100 or 200 ng/ml for 10 days and found an increase in plasma amino acids with no change in plasma glucose, lactate or protein. The conflicting results seen in the literature may be due to such factors as physiological status of the fish, cortisol dosages used, or interaction with other glucoregulatory hormones (Barton *et al.*, 1987).

Nonetheless, in teleosts, the general consensus is cortisol stimulates metabolism (Reid and Perry, 1991) through a mobilisation of energy reserves to meet the increased

energy demands during the stressful period (Vijayan *et al.*, 1991; Vijayan and Moon, 1992; Gamperl *et al.*, 1994). The exact role cortisol has in the energy mobilisation is, however, poorly understood (Vijayan *et al.*, 1996). It is believed to be accomplished by cortisol having a catabolic effect on muscle protein resulting in an increase of amino acids for gluconeogenesis plus an induction of gluconeogenic enzymes (Andersen *et al.*, 1991; van der Boon *et al.*, 1991; Vijayan *et al.*, 1991; 1996). This process may be species dependent as Leach and Taylor (1982) found in *Fundulus* that protein catabolism was not the source of glucose, but gluconeogenesis from either lactate or glycerol, as well as, reduced glucose utilisation in peripheral tissues.

The severity and duration of the stress determines the extent of the release of cortisol (Barton and Iwama, 1991; Perry and Reid, 1993). A negative feedback system (reviewed by van der Boon *et al.*, 1991) controls the secretion of cortisol at the pituitary level. Briefly, there is an inhibition on the hypothalamus to release corticotropin-releasing factor (CRF) which, in turn, inhibits the release of adrenocorticotropic hormone (ACTH) from the pituitary (Barton *et al.*, 1987).

Cortisol enters the target cells, where it binds to a nuclear receptor which then binds to the DNA, and acts by regulating gene transcription (van der Boon *et al.*, 1991). As mentioned earlier, previous studies have found cortisol to affect β-adrenoceptors numbers in rainbow trout erythrocytes (Reid and Perry, 1991) and hepatocytes (Reid *et al.*, 1992). This is most likely the result of cortisol regulating the production of β-

adrenoceptor mRNA and therefore ultimately affecting β-adrenoceptors (Perry and Reid, 1993).

VII. PURPOSE OF STUDY AND HYPOTHESES

The adrenoceptors are involved in the stress response to aid in ameliorating the deleterious effects of stress thereby enabling an organism to better cope with the situation. However, there is surprisingly little information on how various stressors affect the distribution and function of the adrenoceptors. This study will test the hypothesis that cortisol will affect the distribution of the hepatic α_1- and β_2-adrenoceptors in a representative teleost, the rainbow trout. In addition, due to an opportunity to study temperature acclimated rainbow trout, I will test the hypothesis that temperature change can modify the adrenoceptor distribution of trout liver.

MATERIALS AND METHODS

I. Animals

Rainbow trout, *Oncorhynchus mykiss* Walbaum of either sex, weighing approximately 250 - 300 g were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) and transported to the University of Ottawa in the summer of 1995 and 1996. Fish were maintained in tanks of well aerated, dechloraminated City of Ottawa tap water at approximately 12°C with a constant 12L:12D photoperiod. Fish were fed 5 times per week with commercial trout pellets (Purina Trout Chow). All radioactive binding experiments were conducted between February and June. Glycogenolysis experiments were performed in November and December.

II. Cortisol Injections

Randomly selected fish were lightly anaesthetized with 3-aminobenzoic acid ethyl ester (125 mg MS-222/L H_2O), dried with a paper towel to remove excess moisture, weighed, and given a single intraperitoneal injection (0.2 ml/0.1 kg body weight) of hydrogenated coconut oil alone (SHAM) or containing 150 mg/kg cortisol (hydrocortisone, Sigma Chemical Co.) (TREATMENT). Mean weights for cortisol and sham injected fish were 324.2 ± 12.1 g and 329.8 ± 12.9 g (n = 20 for both), respectively. Fish were placed in tanks containing 115 L (± 2 L) of water at 12°C (± 0.5°C) and allowed to swim freely for 10 - 14 days. To determine general binding characteristics,

non-injected fish (NAIVE) were sampled directly from the main holding tank (mean weight 458.8 ± 12.5 g, n = 24). At the appropriate time, 2 fish were randomly selected, killed by a quick blow to the head and blood samples were withdrawn from the caudal peduncle into a heparinized syringe (22G 1 1/2" needle). Blood was centrifuged (Fisher microcentrifuge model 235B), plasma removed, frozen in liquid nitrogen, and stored at -80°C for future analysis. Livers, minus gallbladders, were then quickly excised and combined for membrane purification.

III. Membrane Purification

Membranes were purified using the method developed for fish liver by Fabbri *et al.* (1992) and modified by Gambarotta (1994). Briefly, once removed, livers were cleaned, weighed, and minced with scissors in ice cold homogenization buffer (Table 1). The combined livers were then homogenized on ice with a glass Dounce homogenizer followed by a tight-fitting, glass-teflon pestle, hand homogenizer. This homogenate was stirred on ice for 2 - 5 min, brought to 25 volume (w/v), filtered through a coarse nylon mesh (253 µm) and centrifuged at 3000 g (5750 rpm; Sorvall RC5B plus, SS-34 rotor) for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in a small volume of ice cold homogenization buffer, filtered through a fine nylon mesh (73 µm), brought to 10 vol (w/v) and re-centrifuged as before. Again the supernatant was

Table 1: Composition of the solutions for the purification of hepatic plasma membranes

SOLUTION	COMPOSITION	COMMENTS
HOMOGENIZATION BUFFER	1.0 $NaHCO_3$ 5.0 $CaCl_2$ 1.0 leupeptin	All concentrations are final and in mM. pH adjusted to 7.75 at room temp. Buffer without leupeptin stored at 4°C. Leupeptin aliquots (1.25 mg/50 µl) stored frozen at -20°C. One aliquot added to 500 ml buffer day of experiment.
INCUBATION BUFFER	1.0 $NaHCO_3$ 100.0 HEPES 120.0 NaCl 1.2 $MgSO_4$ 15.0 CH_3COONa 1.0 EDTA 2.5 KCl 10.0 glucose	All concentrations are final and in mM. Buffer without glucose stored at pH 5.5 at 4°C. pH adjusted to 7.75 after addition of glucose. Buffer contains glucose unless specified otherwise.

discarded and the pellet brought to 2 vol (w/v) by filtering through a fine nylon mesh. This was stirred on ice with 80% sucrose (w/v) to give a final sucrose concentration of approximately 31%. Two ml of this solution was then layered over a discontinuous sucrose gradient consisting of 45% (3.7 ml), 41% (4.4 ml), and 37% (1.9 ml) sucrose fractions (sucrose in homogenization buffer without leupeptin) and centrifuged in a Beckman L8-70M ultracentrifuge (SW 41 rotor) at 100 000 g (30 500 rpm) at 4°C for 2 h. The 37% fraction which contained the purified membranes (see Gambarotta, 1994) was recovered using an Eppendorf repeater pipette, diluted to 8% sucrose concentration with ice cold homogenization buffer and centrifuged at 4°C for 15 min at 25 000 g (16 500 rpm: Sorvall RC5B plus, SS-34 rotor). The supernatants were discarded and the pellets were resuspended in 8% sucrose and re-centrifuged as before. The final pellet was resuspended in 1.0 ml incubation buffer (Table 1), centrifuged for 1 min in a Fisher Model 235B microcentrifuge at room temperature. The supernatant was discarded and the walls were dried with lint free absorbent tissue before the pellet was frozen at -80°C for future analysis.

IV. α_1- and β_2-Adrenoceptor Binding Assay

The α_1- and β_2-adrenoceptors from the purified plasma membranes were characterized using saturation experiments according to the method of Gambarotta

(1994). Frozen pellets were resuspended in 1.0 ml incubation buffer. and an aliquot used to determine protein content by the method of Lowry *et al.* (1951) with bovine serum albumin (BSA) as standard. Samples were measured at 500 nm with a Spectronic 1001 Plus (Milton Roy). Suspensions were diluted to 300 μg protein per 50 μl with ice cold incubation buffer. The α_1-adrenoceptors were characterized using the specific α_1-adrenoceptor antagonist [7-methoxy-]^{3}H-prazosin (^{3}H-PRZ; Amersham. specific activity 76.0 Ci/mmol). The hydrophilic β-antagonist ((-)-4-(3-t-butylamino-2-hydroxypropoxy)-[5.7-^{3}H]benzimidazol-2-one) ^{3}H-CGP-12177 (^{3}H-CGP: Amersham. specific activity 48.0 Ci/mmol) was used to characterize the β_2-adrenoceptors. Initial aliquots of ^{3}H-PRZ and ^{3}H-CGP were diluted in a 1:1 95% ethanol:water mixture to give 225 nM and 75 nM stock solutions and final assay concentrations of 15 nM and 5 nM. respectively. When possible. these concentrations were verified by disintegrations per minute (dpm) on a liquid scintillation counter. Stock solutions were diluted with incubation buffer without glucose and all points were run in duplicate. Fifty μl of the purified membrane fraction were incubated for 15 min at room temperature (19 ± 1°C) in a final volume of 150 μl in the presence of either ^{3}H-PRZ or ^{3}H-CGP. Total and non-specific binding were determined in the absence or presence of either 10 μM non-radioactive PRZ or 10 μM alprenolol (ALP) in incubation buffer for the α_1- and β_2-adrenoceptors. respectively. Aliquots of the non-radioactive PRZ were prepared in pure dimethylsulfoxide (DMSO. final assay concentration = 0.33%) and stored at -20°C while ALP was prepared fresh

immediately before use. Assays were terminated by placing the tubes on ice, layering 300 µl of 3% ice cold BSA (RIA-grade) in incubation buffer, and centrifuging at 10°C for 3 min in a Fisher Model 235B microcentrifuge or an Eppendorf centrifuge 5415C. The supernatants were aspirated and the pellets were resuspended in 0.2% BSA (RIA-grade) and re-centrifuged as before. Supernatants were again aspirated and the tips containing the pellets were cut off into scintillation vials. Membranes were dissolved by shaking at 10°C for 24 h in 5 ml scintillation cocktail (ACS II; Amersham) and radioactivity was determined using a Packard 2500TR liquid scintillation counter. Specific binding was calculated by subtracting the average dpm of the non-specific from the corresponding average total dpm. Total, non-specific, and specific dpm were converted to fmol by dividing the dpm by the specific activity of the radioisotope. Saturation curves were obtained by plotting the fmol/mg protein versus the increasing radioisotope concentrations. Binding affinities (K_d) and maximal number of receptor sites (B_{max}) were determined by Scatchard analysis using the "EBDA" and "LIGAND" (Munson and Rodbard, 1980) computer programs (Biosoft-Elsevier).

V. Membrane phospholipid composition

The effect of cortisol on membrane phospholipid composition was performed by Dr. Georges Zwingelstein of the Institut Michel Pacha, Université de Lyon, France. Both membranes purified specifically for phospholipid composition and aliquots (100 µl) of the purified membranes from TREATMENT and SHAM fish were stored frozen at

-80°C. Phospholipid extraction and composition was performed using the methods of El Babili *et al.* (1996) and McClelland *et al.*(1995), respectively.

VI. Plasma Measurements

Plasma samples were assayed for cortisol, glucose, and lactate. Plasma cortisol was determined on 25 µl samples with a commercial radioimmunoassay kit (ICN Pharmaceuticals). Standards were diluted 2.5 fold to correct for the low protein content of fish plasma (Reid *et al.*, 1992), thereby increasing the sensitivity of the assay for low cortisol concentrations (Andersen *et al.*, 1991). Radioactivity was measured on either a Packard Cobra Auto-Gamma counter or a LKB WALLAC 1282 COMPUGAMMA universal gamma counter. Plasma concentrations were determined by comparing to a standard curve.

For glucose, plasma samples were diluted with double distilled water and measured using the glucose oxidase-peroxidase method (Bergmeyer and Bernt, 1963). Plasma lactate was measured enzymatically by measuring the change in absorbance of reduced nicotinamide adenine dinucleotide (NADH) after addition of lactate dehydrogenase (LDH) (1500 units/ml) (Juergensen, 1996). Both plasma glucose and plasma lactate were measured on a Packard SpectraCount microplate photometer.

VII. Temperature Acclimation

The effect of long term temperature acclimation on the distribution of hepatic α_1- and β_2-adrenoceptors was carried out in the Department of Zoology at Arizona State University. Tempe. Arizona. Immature rainbow trout of either sex were obtained from the Alchesay National Fish Hatchery (Whiteriver, AZ) and maintained in large. circular fibreglass tanks. Fish were acclimated to 5°C (mean final weight 209.2 ± 8.8 g) and 20°C (mean final weight 229.7 ± 15.2 g) for at least six months prior to use and were fed daily to satiation with Glencoe Mills (MN) trout food.

Fish randomly selected from either tank, were killed by a blow to the head. weighed. and livers minus the gallbladders were quickly excised. The livers from three to five fish were weighed and combined for plasma membrane purification using the method of Armstrong and Newman (1985) modified by Robertson and Hazel (1995). Briefly. chopped up livers were suspended in 9 vol (w/v) of homogenizing medium [250 mM sucrose. 20 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4 at 20°C]. 50 µl of 0.1 mg/ml DNase added, and homogenized on ice with a Potter-Elvehjem homogenizer with a teflon pestle. The homogenate was then filtered through nylon gauze (pore size 250 µm). The filtrate was then placed in 50 ml polycarbonate centrifuge tubes. 15 ml of 41% buffered sucrose (20 mM Tris. pH 7.4 at 20°C) added, and centrifuged for 30 min at 4°C at 35 000g (17 000 rpm) with a Beckman JA 20 rotor. The membrane containing fraction was recovered with a Pasteur pipette, diluted four-fold with homogenizing medium. and

centrifuged at 7 000g (10 000 rpm) for 15 min. The final pellet was resuspended in a small volume of homogenizing medium. One ml of this fraction was then layered over 20 ml of 18% Percoll (Sigma) solution and centrifuged at 33 000 g (19 000 rpm) with a 50.2 Ti rotor for 25 minutes. The membrane fraction was collected by forcing the bottom gradient upwards with a 66% sucrose solution and recovering the uppermost band. Approximately 5 to 6 ml of this fraction was diluted to a total of 40 ml with cold buffered saline (100 mM NaCl, 10 mM Tris, pH 7.4 at 20°C). This fraction was then centrifuged for 2 h at 30 000 rpm with the 50.2 Ti rotor. The Percoll bead was recovered, diluted in incubation buffer and the protein content measured using the bicinchoninic acid (BCA) protein assay (Smith *et al.*, 1985) with BSA as standard. The α- and β-adrenoceptors were characterized using saturation assays as previously described (specific activities of ^{3}H-PRZ and ^{3}H-CGP = 73.0 and 46.0 Ci/mmol, respectively), but with a few slight modifications. Protein content of the membrane suspensions varied from 169.5 to 306.5 μg/50 μl (mean warm = 197.8 ± 11.7 μg/50μl; mean cold = 263.8 ± 12.2 μg/50 μl), and assays with all points as singlets were run for 30 min at 15°C. Radioactivity was determined using a Beckman microprocessor controlled liquid scintillation counter. Data were analysed by Scatchard analysis as previously described.

VIII. Hepatocyte isolation

Fish were injected with coconut oil alone or coconut oil containing cortisol (either hydrocortisone or hydrocortisone 21-hemisuccinate (Sigma)) (112.5 mg/kg) as described previously. Mean weights for sham and cortisol injected fish were 270.8 ± 9.4 g and 242.7 ± 7.7 g (n = 19 and 16), respectively. Hepatocytes were isolated according to the method of Moon *et al.* (1985) 10 - 14 days post-injection. Solution compositions are listed in Table 2. Fish were killed by a blow to the head, blood samples taken as previously described, and a mid-ventral incision to expose the liver was made. The hepatic portal vein was cannulated and medium A was pumped through the liver at approximately 2 ml/min with a Buchler polystaltic pump for 5 to 10 min to clear the liver of blood. Medium B was then used for an additional 20 to 25 min to satisfactorily digest the liver. At this time, the perfusion was stopped and the liver minus the gallbladder was removed and placed in a petri dish containing a small volume of cold Medium A. The liver was finely chopped with a razor blade and filtered through two nylon screens (253 and 73 μm, respectively). Hepatocytes were then rinsed 4 times by centrifugation at 90 g (1 000 rpm) at 4°C for 2 min in a Sorvall RC5B plus centrifuge (SS34 rotor; Du Pont Canada). The first two rinses were using Medium A, the third with half Medium A and half Medium C, and the fourth time in Medium C. The final pellet was resuspended in Medium C and left on ice for approximately 1 h. At this time, the pellet was resuspended in fresh Medium C and the cells were counted and viability was determined via trypan blue exclusion. Cell concentration was determined by adding 100 μl of cell suspension to

Table 2: Media composition for the isolation of rainbow trout hepatocytes

MEDIUM	COMPOSITION	COMMENTS
A	0.8 $MgSO_4$ 0.33 NaH_2PO_4 0.44 KH_2PO_4 136.7 NaCl 5.4 KCl 5.0 HEPES 5.0 HEPES-Na	All concentrations are final and in mM. Stored as 5X at 4°C. 5.0 mM $NaHCO_3$ and 1.0 mM EGTA added. pH adjusted to 7.63 at room temp.
B	Medium A (without EGTA) + collagenase: 0.15 mg/ml	pH 7.63 at room temp.
C	Medium A (without EGTA) + 1.5 mM $CaCl_2$ 2% Bovine Serum Albumin (BSA)	pH 7.63 at room temp.

pre-weighed 1.5 ml polypropylene centrifuge tubes and centrifuged for 1 min at 13 000 g in a Fisher Model 235B microcentrifuge or Eppendorf Model 5415C at room temp. The tubes were re-weighed after the supernatant was decanted and the removal of excess fluid with lint-free absorbent tissue.

IX. Glycogenolysis

To test adrenoceptor function, static glycogenolysis experiments were performed. Approximately 50 mg of cells were incubated for 1 h at room temp with EPI (bi-tartrate salt) (final concentration = 1 μM) alone or with either the α_1-antagonist PRZ or the β-antagonist PROP (final antagonist concentrations = 1 mM). Preliminary experiments with pure DMSO (final assay concentration = 3.3%) showed no significant effects on the hepatocytes as glucose release was similar to controls. Cells were given a 15 min preincubation with the antagonists before the addition of EPI. Time 0 (placed on ice) and 1 h controls were run in the presence of Medium A (without Ethylene Glycol-bis(β-Aminoethylether) N.N.N'.N'-Tetraacetic acid (EGTA)) only or with either PROP or PRZ. Assays were terminated with the addition of perchloric acid (final concentration = 6%). Samples were then either frozen at -20°C for future analysis or assayed immediately. Samples were centrifuged 5 min at room temp at 13 000 g in a Fisher Model 235B microcentrifuge or Eppendorf centrifuge 5415C. The resulting supernatants were then analysed for total (glycogen + glucose) and free glucose content using the

glucose oxidase-peroxidase method as previously described. For the total glucose assay, 50 μl of the supernatant was incubated for 2 h at 37°C with 500 μl amyloglucosidase in sodium acetate buffer (pH 4.8) to hydrolyze glycogen. The assay was terminated with perchloric acid (final concentration 6%) and centrifuged as previously described. The resulting supernatant was then assayed for glucose content as per the free glucose. Glycogen content is determined as the difference between total glucose and free glucose after appropriate blank subtraction.

X. Chemicals

All nonradiolabelled adrenergic agonists and antagonists were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Chemicals for the solutions for the various procedures were purchased from Sigma Chemicals Co., BDH (Toronto, ON), ICN Biochemical Inc. (Mississagua, ON), Fisher Scientific Co. (Fair Lawn, NJ, USA) or from local suppliers and were of the highest purity available. For the glycogenolysis experiments, epinephrine and propranolol were made fresh immediately prior to use in Medium A (without EGTA). Prazosin was dissolved in DMSO and frozen aliquots were stored at -20°C.

XI. Statistical Analysis

Comparisons between groups were determined by either one-way ANOVA. Kruskal-Wallis one-way ANOVA on Ranks followed by a multiple comparison test (Tukey or Dunn, respectively) when required, unpaired t-test or Mann-Whitney Rank Sum Test with the computer software program Sigma Stat 2.0 (Jandel Scientific). F-test was used by the program LIGAND to determine the appropriateness of the one-site versus the two-site model for both adrenoceptor types. Level of significance was taken at $P < 0.05$.

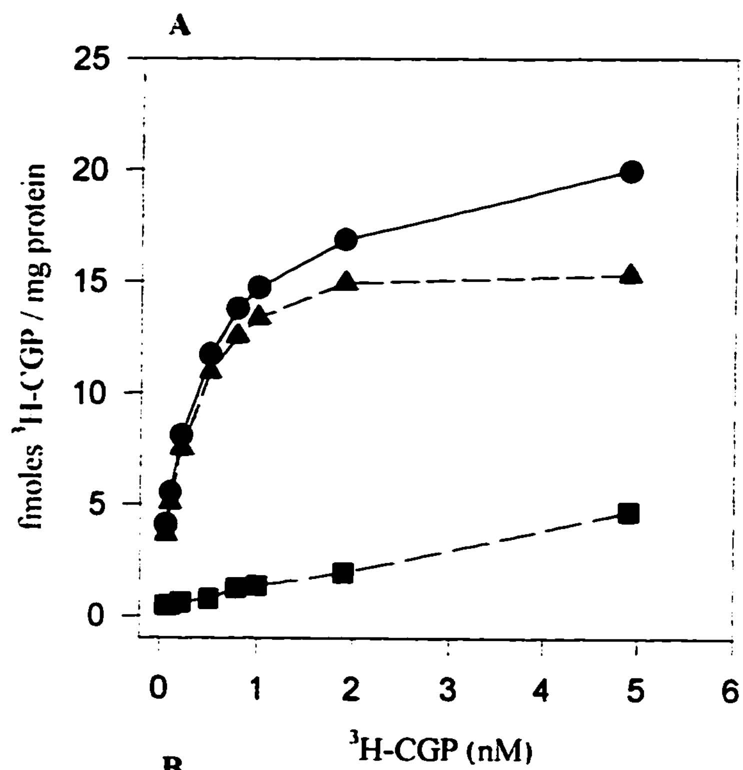
RESULTS

I. General Binding Characteristics

α_1- and β_2-Adrenoceptors were characterized on hepatic membranes purified by ultracentrifugation on discontinuous sucrose gradients. The 37% sucrose fraction which was recovered was previously shown by Fabbri *et al.* (1992) in bullheads to contain a greater than 2-fold enrichment of the plasma membrane marker 5'-nucleotidase (5'NTase) compared to the other fractions present in the gradient. Gambarotta (1994) found an almost 3-fold enrichment in 5'NTase in this fraction over the crude homogenate in purified eel and bullhead hepatic membranes. The incubation period of 15 min was based upon trials conducted by Fabbri *et al.* (1994; 1995b) examining purified hepatic bullhead membranes.

General binding characteristics were determined in non-injected (NAIVE) rainbow trout obtained directly from the main holding tank. Specific binding of ^{3}H-CGP to the β-adrenoceptors was saturable, representing approximately 66% (mean, n=6) of total binding at 5 nM (Figure 1A). Similarly, specific binding to the α-adrenoceptors of ^{3}H-PRZ was also saturable, representing 40% (mean, n=6) at 15 nM (Figure 2A). Data were analyzed by Scatchard analysis with the EBDA-LIGAND computer program (see Materials and Methods). Association curves and Scatchard plots of both receptor types representative of what was found in this group of fish are shown in Figures 1 and 2. A one-site model was found to significantly fit the data better than the two-site model for both the α- and β-adrenoceptors ($P < 0.05$) by the F-Test statistical evaluation using

Fig 1A, B: A representative association curve (A) and Scatchard plot (B) of β_2-adrenoceptors from purified rainbow trout hepatic membranes. (A) Membranes (300 μg protein/50 μl) were incubated for 15 min at room temp with increasing concentrations of tritiated CGP-12177 (^{3}H-CGP) in either the absence (Total binding, circle) or presence of 10 μM alprenolol (ALP) (NS binding, square). Specific binding (triangle) was determined by subtracting the NS binding from the Total. (B) Scatchard plot of data from (A) transformed with the program EBDA.


A
25
20
15
10
5
0
fmoles ^{3}H-CGP / mg protein
0
1
2
3
4
5
6
^{3}H-CGP (nM)

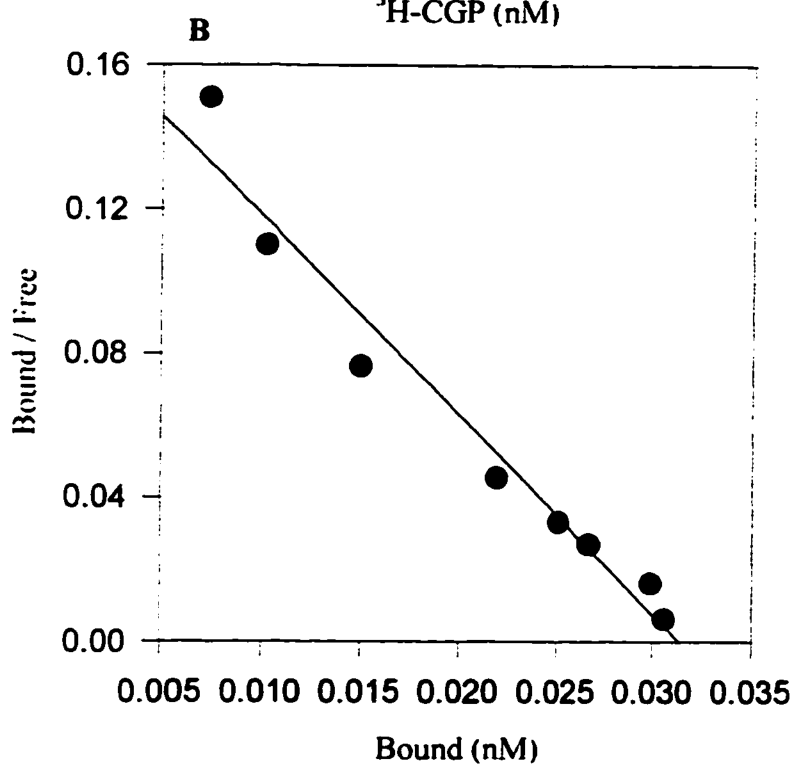
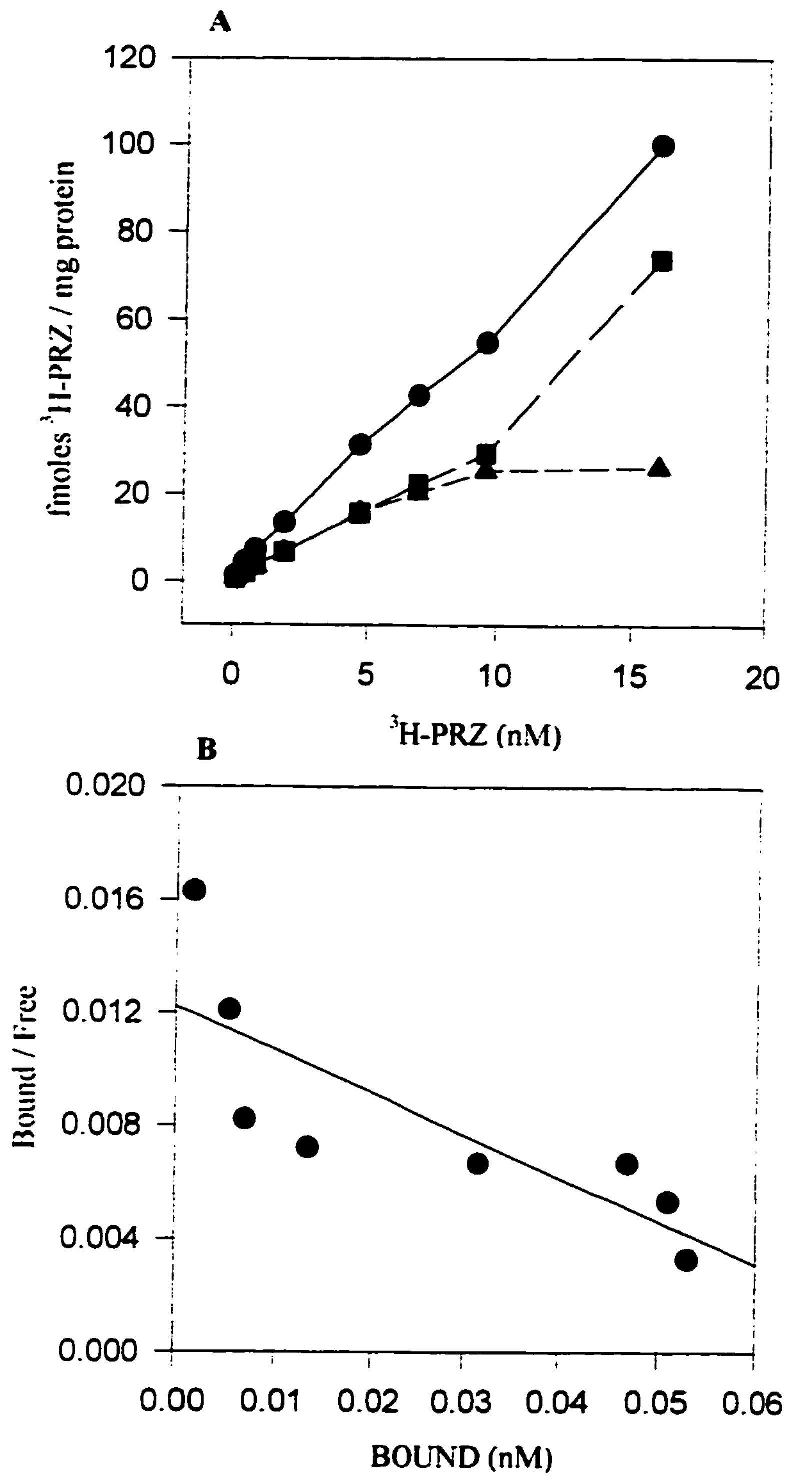

B
0.16
0.12
0.08
0.04
0.00
Bound / Free
0.005
0.010
0.015
0.020
0.025
0.030
0.035
Bound (nM)

Fig. 2A, B: A representative association curve (A) and Scatchard plot (B) of α_1-adrenoceptors from purified hepatic membranes of rainbow trout. (A) Membranes (300 µg protein/50 µl) were incubated for 15 min at room temp with increasing concentrations of tritiated prazosin (^{3}H-PRZ) in either the absence (Total binding, circle) or presence of 10 µM non-radioactive PRZ (non-specific (NS) binding, square). Specific binding (triangle) was calculated by subtracting NS from the total. (B) Scatchard plot of data from (A) transformed using the program EBDA.


A
120
100
80
60
40
20
0
fmoles ^{3}H-PRZ / mg protein
0
5
10
15
20
^{3}H-PRZ (nM)
B
0.020
0.016
0.012
0.008
0.004
0.000
Bound / Free
0.00
0.01
0.02
0.03
0.04
0.05
0.06
BOUND (nM)

LIGAND. The equilibrium binding constants (K_d) and maximal binding sites (B_{max}) are shown in Figures 3 and 4.

II. Cortisol

A. Plasma Concentration

Plasma samples from cortisol (TREATMENT), coconut oil only injected (SHAM), and NAIVE rainbow trout were assayed for cortisol, glucose and lactate. Values are summarized in Table 3 along with mass and hepatosomatic index (HSI) used in these experiments. Plasma cortisol values were significantly higher in the TREATMENT group compared to the SHAM and NAIVE groups ($P < 0.001$). There was no significant difference in plasma glucose between any of the three experimental groups ($P > 0.05$). Plasma lactate was significantly lower in the TREATMENT fish compared to the NAIVE group only ($P < 0.05$).

B. Effects on Binding

To determine any effect a 10 - 14 day cortisol exposure may have on the binding characteristics, saturation experiments as previously described were undertaken for TREATMENT and SHAM rainbow trout. As was seen with NAIVE fish, specific binding of ^{3}H-PRZ was saturable in both TREATMENT and SHAM fish, representing

Fig 3A, B: Dissociation constants (K_d) (A) and maximal number of binding sites (B_{max}) (B) of hepatic β_2-adrenoceptors from cortisol (TREATMENT), coconut oil only (SHAM), and non-injected (NAIVE) rainbow trout. Values are means ± SEM (n = 5 (TREATMENT, SHAM) or 6 (NAIVE)). No significant differences were found by one-way ANOVA ($P > 0.05$).

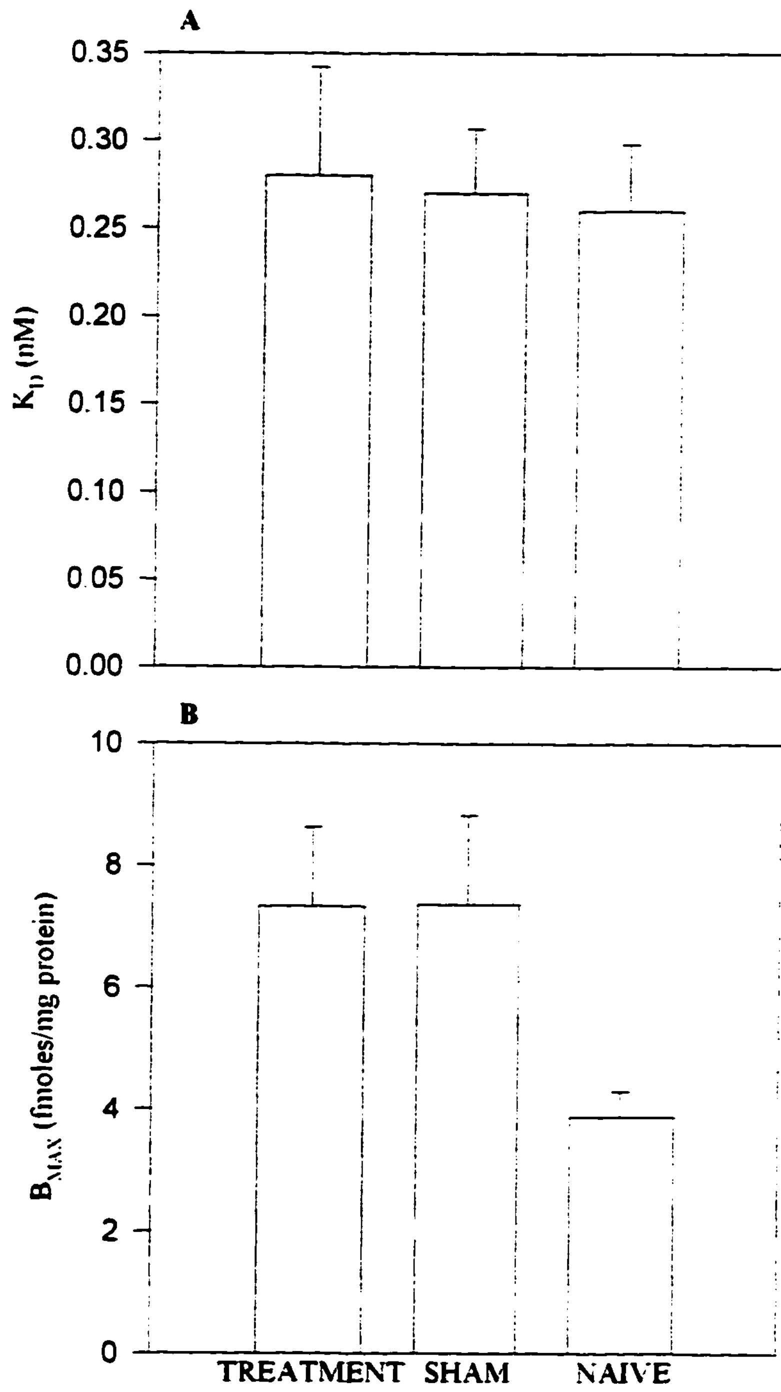

A
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
K_D (nM)
B
10
8
6
4
2
0
B_MAX (fmoles/mg protein)
TREATMENT
SHAM
NAIVE

Fig 4A, B: Dissociation constants (K_d) (A) and maximal number of binding sites (B_{max}) (B) for the hepatic α_1-adrenoceptors of cortisol (TREATMENT), coconut oil only (SHAM), and non-injected (NAIVE) rainbow trout. Values are means ± SEM (n = 5 (TREATMENT, SHAM) or 6 (NAIVE)). No significant differences were found by one-way ANOVA ($P > 0.05$).

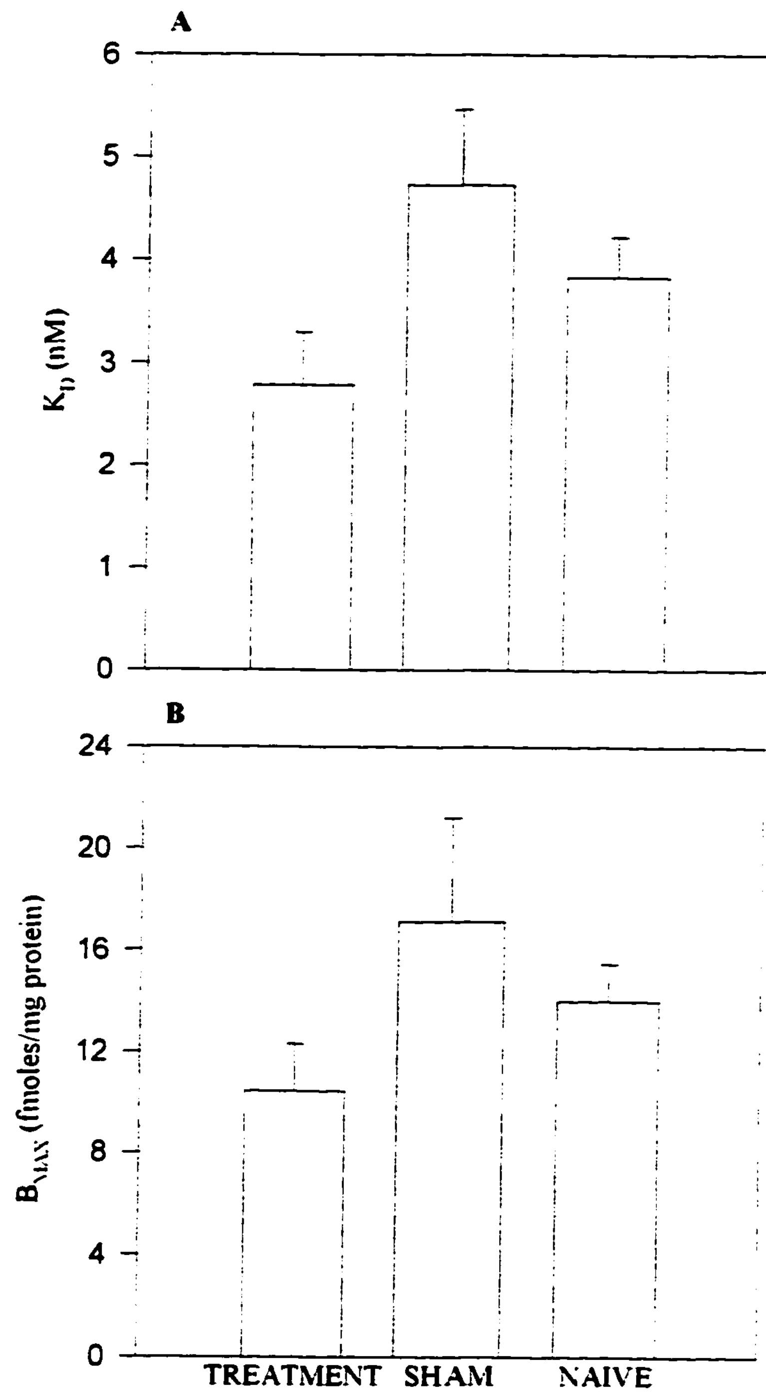

A
6
5
4
3
2
1
0
K_D (nM)
B
24
20
16
12
8
4
0
B_{MAX} (fmoles/mg protein)
TREATMENT
SHAM
NAIVE

Table 3: Body mass, hepatosomatic index (HSI), and plasma cortisol, glucose, and lactate values in rainbow trout injected with coconut oil containing 150 mg/kg cortisol (hydrocortisone) (TREATMENT), coconut oil alone (SHAM) and sampled 10 - 14 days later, or not injected (NAIVE). All values are means ± SEM (n = 20 - 24). HSI is liver mass/body mass x 100. * indicates significantly different from the other two groups (mass, one-way ANOVA followed by Tukey's test, $P < 0.05$; plasma cortisol, one-way ANOVA on Ranks followed by Dunn's test, $P < 0.05$). # indicates significantly different from TREATMENT (one-way ANOVA on Ranks followed by Dunn's test, $P < 0.05$).

	TREATMENT	SHAM	NAIVE
MASS (g)	324.2 ± 12.1	329.8 ± 12.9	458.8 ± 12.5 *
HSI	1.14 ± .07	1.10 ± .07	0.97 ± .04
PLASMA CORTISOL (ng/ml)	219.9 ± 25.5 *	35.0 ± 6.7	28.1 ± 4.3
PLASMA GLUCOSE (mM)	4.28 ± 0.49	4.35 ± 0.59	4.39 ± 0.72
PLASMA LACTATE (mM)	1.86 ± 0.16	2.25 ± 0.17	2.61 ± 0.15 #

approximately 40% (mean. n=5) of total binding at 15 nM. In the β–adrenoceptors. specific binding of ^{3}H-CGP was also saturable in both groups. representing approximately 75% (mean. n=5) of total binding at 5 nM. Scatchard analysis as previously described was used to assess the binding data (see Figures 1 and 2 for the β- and α-adrenoceptors. respectively). Again. F-test statistical analysis found a one-site model fit the data significantly better than the two-site model ($P < 0.05$) for both SHAM and TREATMENT fish for both the α- and β-adrenoceptors. Binding affinities (K_d) and sites (B_{max}) are shown in Figures 3 and 4. No significant differences were found between any of the experimental groups for either the K_d or B_{max} of both receptor types ($P > 0.05$).

C. Membrane Characteristics

The proportions of the various categories of membrane phospholipids for TREATMENT and SHAM injected fish are presented in Table 4. The 20:5n-3/20:4n-6 represents the ratio of those two polyunsaturated phospholipids which produce prostaglandins with antagonistic effects towards each other. No significant differences were found in the membrane phospholipid compositions between the two experimental groups either as individual phospholipids (data not shown). or as grouped data (Table 4. $P > 0.05$).

Table 4: Relative hepatic membrane phospholipid composition in rainbow trout injected with coconut oil containing 150mg /kg cortisol (hydrocortisone) (TREATMENT) or coconut oil alone (SHAM) and sampled 10 - 14 days later. All values are means ± SEM [(n = 9 (TREATMENT) or 11 (SHAM)]. Analysis was performed by Dr. Georges Zwingelstein of the Institut Michel Pacha, Université de Lyons, France. No significant differences were found between the two experimental groups (unpaired t-test or Mann-Whitney Rank Sum Test following ARCSIN transformation, $P > 0.05$).

	TREATMENT	SHAM
SATURATED	24.88 ±0.87	23.62 ± 1.06
MONOUNSATURATED	16.88 ± 0.94	17.85 ± 0.95
POLYUNSATURATED	53.02 ± 2.11	53.41 ± 1.35
20:5n-3/20:4n-6	3.63 ± 0.64	3.69 ± 0.95

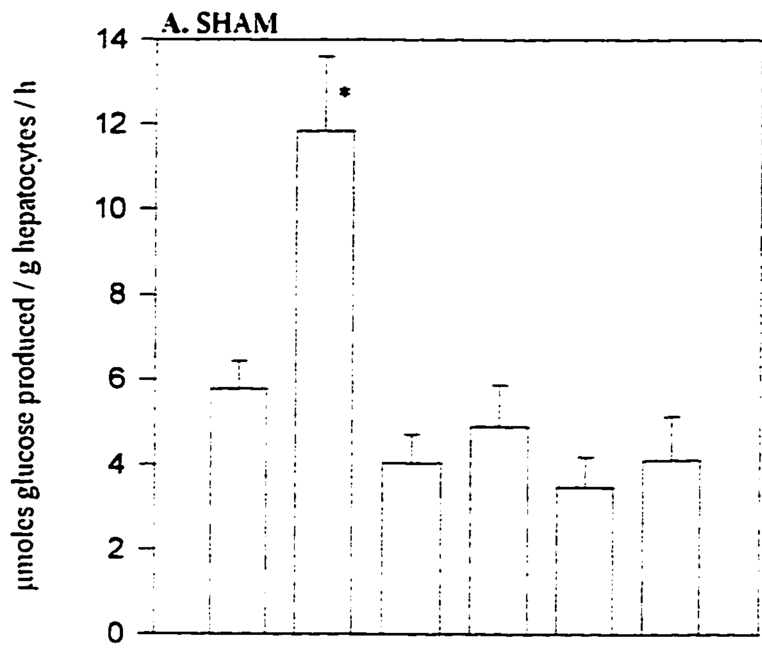
III. Glucose Production

To determine any effect cortisol may have on hepatic α_1- and β_2-adrenoceptor function, hepatocytes were isolated from rainbow trout injected with slow release coconut oil implants. Epinephrine significantly enhanced glucose release compared to control in the sham injected trout. In addition, this effect in the sham group was significantly blocked by both the α- and β-antagonists, PRZ and PROP (Figure 5A, $P < 0.001$). Any significant differences in the cortisol injected trout were masked by the large variabilities, but the same trends were seen (Figure 5B). No significant differences were found for any treatment between cortisol and sham injected trout (one-way ANOVA, $P > 0.05$). Mean initial glycogen contents between sham and cortisol injected rainbow trout were 41.2 ± 6.4 and 30.9 ± 9.6 µmol/g hepatocytes, respectively. No significant difference between the initial glycogen values was found (unpaired t-test, $P > 0.05$).

A. Plasma Values

Rainbow trout injected for the adrenoceptor function study were assayed for the same plasma constituents as for the adrenoceptor binding study. Values, along with body mass are shown in Table 5. As in the binding study, plasma cortisol was significantly elevated with the coconut oil implants in the TREATMENT fish compared to the SHAM fish ($P < 0.001$). Again, no significant difference was seen with either plasma glucose

Fig 5A, B: Hepatocyte glucose production from (A) coconut oil only (SHAM) and (B) cortisol injected (TREATMENT) rainbow trout. Isolated hepatocytes were incubated at room temperature for 1 h in the absence (CTL. PROP, PRZ) or presence of 1 μM epinephrine (EPI) alone or with either the β-antagonist propranolol (EPI + PROP) or α-antagonist prazosin (EPI + PRZ). Hepatocytes were pre-incubated with antagonists (final concentrations of both = 1 mM) for 15 min prior to addition of EPI. * indicates significant difference ($P < 0.001$) compared to the 5 other treatments by one-way ANOVA followed by Tukey's test. Values are means ± SEM, n = 10 (A) and 8 (B).


A. SHAM
14
12
10
8
6
4
2
0
μmoles glucose produced / g hepatocytes / h
*

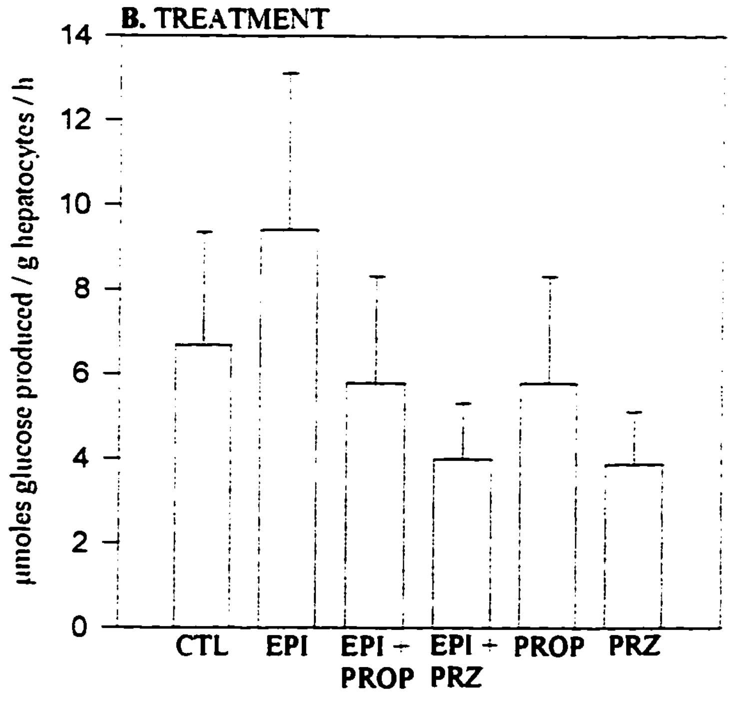

B. TREATMENT
14
12
10
8
6
4
2
0
μmoles glucose produced / g hepatocytes / h
CTL
EPI
EPI + PROP
EPI + PRZ
PROP
PRZ

Table 5: Body mass. plasma cortisol, glucose, and lactate in rainbow trout injected with coconut oil containing 112.5 mg/kg cortisol (hydrocortisone or hydrocortisone 21-hemisuccinate) (TREATMENT) or coconut oil alone (SHAM) and sampled 10 - 14 days later. All values are means ± SEM (n = 16 (TREATMENT) or 19 (SHAM)). * indicates significantly different from SHAM (mass. unpaired t-test. $P < 0.05$: cortisol. Mann-Whitney Rank Sum Test. $P < 0.001$).

	TREATMENT	SHAM
MASS (g)	242.7 ± 7.7 *	270.8 ± 9.4
PLASMA CORTISOL (ng/ml)	80.0 ± 11.4 *	24.5 ± 5.9
PLASMA GLUCOSE (mM)	5.96 ± 0.77	4.77 ± 0.47
PLASMA LACTATE (mM)	2.43 ± 0.20	2.45 ± 0.41

($P > 0.05$) or plasma lactate between cortisol and sham injected fish.

IV. Temperature

The effect of long term temperature acclimation on hepatic α_1- and β_2-adrenoceptor distribution was performed on rainbow trout acclimated to 5°C and 20°C. Hepatic membranes were purified by centrifugation on a Percoll self-generating density gradient solution. This method was shown by Robertson and Hazel (1995) to result in an almost 4-fold enrichment of 5'NTase in the liver of rainbow trout.

Specific binding of ^{3}H-CGP was saturable in both warm and cold acclimated rainbow trout, representing approximately 62% and 40% of total binding at 5 nM, respectively. However, specific binding of ^{3}H-PRZ was not saturable in either warm or cold acclimated trout. Data were analyzed by Scatchard analysis as previously described. The data for both receptor types in both warm and cold acclimated trout were found to fit the one-site model significantly better than the two-site model using LIGAND (F-test, $P < 0.05$). The K_d and B_{max} of both receptor types for warm and cold acclimated trout are summarized in Table 6. No significant differences were found between the two groups for either K_d or B_{max} for either the α_1- or β_2-adrenoceptors ($P > 0.05$).

Table 6: Dissociation constants (K_d) and maximal number of binding sites (B_{max}) for the α_1- and β_2-adrenoceptors in warm (20°C) and cold (5°C) acclimated rainbow trout purified hepatic membranes. All values are means ± SEM (n = 5 - 6). No significant differences were found for either receptor type (unpaired t-test or Mann-Whitney Rank Sum Test. P > 0.05).

	K_d (nM)	B_{max} (fmol/mg protein)
ALPHA: warm	119.7 ± 79.7	671.7 ± 424.7
cold	19.46 ± 9.26	64.72 ± 22.91
BETA: warm	0.56 ± 0.16	5.65 ± 0.73
cold	0.84 ± 0.27	6.25 ± 1.58

Obviously the highly variable results observed for the α-adrenoceptors mask any possible thermal effects on these receptors.

DISCUSSION

The rainbow trout is a common experimental model in fish studies. In addition, the adrenoceptors, in particular, the β-adrenoceptors, are among the most extensively studied of the G-protein linked receptors. These receptors play an important role in an animal's stress response. However, very little is known about how the distribution and function of these receptors are affected by various stressors, especially in the lower vertebrates. This study examined the effect of the corticosteroid, cortisol on hepatic α_1- and β_2-adrenoceptor distribution and function in the rainbow trout. In addition, the effect of long term temperature acclimation on distribution of the receptors was also examined. This discussion will place the results of my study into context with similar studies reported in the literature.

I. General Binding Characteristics

The distribution of the α- and β-adrenoceptors was determined on purified hepatic membranes with the use of saturation assays. Using the same technique, Fabbri *et al.* (1995a) characterized both α- and β-adrenoceptors in rainbow trout liver. The authors, using the β-antagonist ^{3}H-CGP-12177, found a single class of β-adrenoceptors with a K_d of 0.36 ± 0.032 nM and a B_{max} of 8.61 ± 1.1 fmol/mg protein. These results are in close agreement with the findings of my study. As seen in Figure 3A, a single class of β-adrenoceptors was found with K_d of 0.26 ± 0.04 nM and B_{max} of 3.9 ± 0.4 fmol/mg

protein for the NAIVE fish. This is in contrast to the findings of Fabbri *et al.* (1992) with the purified hepatic membranes of bullheads. Using the hydrophobic β-adrenergic antagonist (-)-[^{3}H]dihydroalprenolol ([^{3}H]DHA), Fabbri *et al.* (1992) found two classes of binding sites: a low affinity site with a K_d of 62.3 ± 19.5 nM and a B_{max} of 452.0 ± 109.9 fmol/mg protein, and a high affinity site with a K_d of 2.04 ± 0.46 nM and a B_{max} of 46.7 ± 13.7 fmol/mg protein.

For the α-adrenoceptors, using the α-antagonist, ^{3}H-PRZ, Fabbri *et al.* (1995a) found a single class of receptors with a K_d of 15.4 ± 3.5 nM and a B_{max} of 75.2 ± 13.5 fmol/mg protein. Despite the fact that I too report a single class of α_1-adrenoceptors, as can be seen from Figure 4, there is a difference in both affinity and receptor number. Binding affinity for ^{3}H-PRZ from this study was 3.8 ± 0.4 nM for the NAIVE group with a B_{max} of 14.0 ± 1.5 fmol/mg protein. Differences can be explained by the fact that while both studies used 300 μg protein/50 μl and determined NS-binding with 10 μM non-radioactive PRZ, Fabbri *et al.* (1995a) had a high ^{3}H-PRZ concentration of only 10 nM versus 15 nM from this study. Although, the authors state the receptors were saturated, the K_d must have been extrapolated given that their K_d was 15 nM and the maximum [^{3}H-PRZ] used was 10 nM, thus one must question the validity of these results by Fabbri *et al.* (1995a).

Fabbri *et al.* (1995b) found a single class of α_1-adrenoceptors using ^{3}H-PRZ in purified hepatic membranes of eel (*A. rostrata*) and bullhead (*I. melas*) with K_d and B_{max}

values of 3.0 ± 0.24 and 2.2 ± 0.18 nM and 108 ± 20.4 and 178 ± 13.4 fmol/mg protein, respectively. While receptor numbers vary considerably between the two species from Fabbri *et al.* (1995b) and the one species from my study, the K_ds are relatively similar. With all three species possessing only a single class of receptors and a similar affinity for the same ligand, it is probable that the same subtype exists in all three species. García-Sáinz *et al.* (1995) using $[^{125}I]$HEAT found a single class of high affinity α_1-adrenoceptors in hepatic membranes of the catfish (*Ictalurus punctatus*) with a K_d of 0.1 ± 0.023 nM and B_{max} of 33 ± 3 fmol/mg protein. Competition studies suggested the receptor belongs to the α_{1B} subtype. As only saturation experiments were performed in this study, it can not be positively concluded whether or not rainbow trout possess the same subtype.

In contrast, neither Janssens and Lowrey (1987) nor Janssens and Grigg (1988) were able to detect any α-adrenoceptors in hepatic plasma membrane preparations of carp (*C. carpio*) and Australian lungfish (*Neoceratodus forsteri*), respectively. No specific binding was found with either ^{3}H-PRZ or the α_2-adrenergic ligand ^{3}H-yohimbine (^{3}H-YOH). However, β-adrenoceptors were found in both species. Using $[^{125}I]$-iodocyanopindolol (^{125}I-ICP), a single class of receptors was found in the carp with a K_d of 0.083 nM and B_{max} of 45.8 fmol/mg protein. While ^{125}I-ICP found two classes of receptors in the lungfish, a high affinity site with a K_d of 0.020 nM and a low affinity site with a K_d of 0.50 nM.

The same species variability of adrenoceptor distribution seen in teleosts is also seen in the other vertebrate classes. For example, Janssens and Grigg (1988) were unable to find any evidence for the existence of hepatic α-adrenoceptors in either the axolotl (*Ambystoma mexicanum*) or the toad (*Xenopus laevis*). Using the same tritiated α-antagonists as in the carp and lungfish, the authors were unable to detect any specific binding on these hepatic membrane preparations. In contrast, ^{125}I-ICP bound with high affinity to one class of β-adrenoceptors in both species. Using ^{3}H-PRZ, Sulakhe *et al.* (1988) found hepatic α_1-adrenoceptors to be barely detectable in purified plasma membranes of the frog, *R. pipiens*. Hemmings and Storey (1994) found α_1-, α_2- and β_2-adrenoceptors to be present in hepatic plasma membranes of the wood frog, *Rana sylvatica*, and their distribution to be affected by freezing.

Janssens and Grigg (1992) reported similar findings in the long-necked turtle, *Chelodina longicollis*, as in the toad and axolotl. In contrast, Janssens and Giuliano (1989) found ^{3}H-PRZ, but not ^{3}H-YOH, bound specifically to hepatic membranes preparations of the western netted dragon, *Amphibolurus nuchalis*. Sulakhe *et al.* (1988) reported similar findings for the turtle, *Chrysemys sp.* as for the frog.

As stated in the Introduction, Sulakhe *et al.* (1988) reported significant species variations in hepatic adrenoceptor distribution in mammals. The only exception may be birds, but only two species were examined. Gutiérrez-Venegas and García-Sáinz (1993) found similar numbers of α_1-adrenoceptors in chicken hepatocytes as Sulakhe *et al.*

(1988) in purified chicken hepatic membranes. Thus, teleosts display the large species differences in adrenoceptor distribution that are reported in at least three of the other four classes of vertebrates.

II. Cortisol

A. Plasma concentrations

The use of coconut oil as a slow release implant has been previously shown to be an effective and practical method to continuously raise plasma cortisol levels in different species of fish (for examples, see Vijayan *et al.* (1991) - brook charr (*Salvelinus fontinalis*), Vijayan *et al.* (1994), Reid *et al.* (1996) - rainbow trout (O. *mykiss*), Vijayan *et al.* (1993b; 1996) - sea raven (*Hemitripterus americanus*)). As can be seen from Table 3, plasma cortisol in the TREATMENT fish was significantly increased compared to the SHAM and NAIVE fish. Similarly, plasma cortisol was significantly elevated in the TREATMENT fish versus the SHAM injected fish in the glucose production study (Table 5).

The effects of cortisol on plasma glucose in teleosts reported in the literature are extremely variable. No significant differences were found between any of the experimental groups in either the binding or glucose production studies (Tables 3 and 5, respectively) after 10 - 14 days of cortisol elevation. These results were also seen by Andersen *et al.* (1991) and Vijayan *et al.* (1994) in rainbow trout with similar levels of plasma glucose as in my study. In contrast, cortisol was found to increase plasma glucose

values in the Japanese eel (*A. japonica*) (Chan and Woo, 1978), and the sea raven (Vijayan *et al.*, 1996), but to decrease in brook charr (Vijayan *et al.*, 1991), and American eels (*A. rostrata*) (Foster and Moon, 1986).

Clearly, species differences probably exist in the role cortisol plays as a glucocorticoid in teleost fish. It should be noted, however, that these studies, apart from using different species, not only used different cortisol concentrations, but different methods to elevate plasma levels and different time periods. Therefore, a more consistent methodological approach may be necessary to elucidate the role of cortisol in carbohydrate metabolism in fish.

Cortisol effects on plasma lactate appear equally inconsistent. Cortisol elevation for 10 - 14 days resulted in a significant decrease in plasma lactate in TREATMENT fish compared to NAIVE fish and a trend towards a decrease (P = 0.070) compared to SHAMS (Table 3). However, in the glucose production study, no difference in plasma lactate was found between TREATMENT and SHAM injected fish (Table 5). This discrepancy is probably related to the different levels of plasma cortisol seen in the two TREATMENT injected groups of fish.

Bollard *et al.* (1993) reported a single injection (20 µg) of cortisol significantly elevated plasma lactate 1 h post-injection, followed by a gradual decline to control levels after 24 h in the snapper, *Pagrus auratus*. Similarly, Lidman *et al.* (1979) found 14 daily injections (5 mg/kg body weight) of cortisol significantly raised plasma lactate levels in the European eel (*A. Anguilla*). While, both Andersen *et al.* (1991) and Vijayan *et al.*

(1991) found no difference in plasma lactate in rainbow trout and brook charr, respectively, from constant cortisol elevation. These last two studies reported similar plasma cortisol concentrations to the values seen in my glucose production study thereby supporting a possible dose-dependent effect of cortisol on plasma lactate in rainbow trout.

B. Effects on Binding

Affinities and numbers of binding sites for TREATMENT and SHAM injected fish for β- and α-adrenoceptors are shown in Figures 3 and 4, respectively. As was seen with the NAIVE fish, a single class of both receptor types was found, supporting the findings of Fabbri *et al.* (1995a). However, the same inconsistencies with the α-adrenoceptors between my study and that of Fabbri *et al.* (1995a) were also seen with both TREATMENT and SHAM injected fish.

The effect of cortisol on β-adrenoceptor number and function in isolated rainbow trout hepatocytes was examined by Reid *et al.* (1992). Also using ^{3}H-CGP, as with my study, the authors found cortisol had no effect on affinity with K_ds of 55.1 ± 8.3 nM for shams and approximately 45 ± 10 nM (approximation taken from Figure 1B of Reid *et al.* (1992) as an actual value was not given) for cortisol treated trout. The large difference in affinities for ^{3}H-CGP between this study and both Fabbri *et al.* (1995a) and my studies, is probably related to the different protocols used. My study and that of Fabbri *et al.* (1995a) used purified membranes, a 15 min incubation period and determined NS-binding

with 10 μM ALP, a non-selective β-antagonist (Exton, 1985). In contrast, Reid *et al.* (1992) used isolated hepatocytes, a 1 h incubation period and determined NS-binding with 200 μM ISO, a non-selective β-agonist (Exton, 1985). In addition, Reid *et al.* (1992) had a high ^{3}H-CGP concentration of 40 nM and therefore, like Fabbri *et al.* (1995a) also had to extrapolate K_d values from concentrations below their reported K_d.

In terms of B_{max}, Reid *et al.* (1992) found an approximate 3.5 fold increase in the number of cell surface receptors in the cortisol treated trout compared to the shams, while no difference was found in my study between treatment fish and both sham and naive fish. Although both studies had similar treatment periods, Reid *et al.* (1992) used mini-osmotic pumps to elevate plasma cortisol to 132.1 ± 14.8 ng/ml whereas my study employed slow release coconut oil implants which elevated plasma cortisol levels to 219.9 ± 25.5 ng/ml. One possible explanation may be, for this time period, the level of cortisol in my study may have resulted in an increase in receptor number at an earlier period, and the continuous exposure at this level of cortisol has resulted in receptor sequestration and a return to control numbers. Receptor sequestration is a protective desensitisation mechanism an organism can use to prevent overstimulation by a hormone in a particular tissue (Heinsimer and Lefkowitz, 1982; Milligan *et al.*, 1994). In addition, there is also a large difference in plasma cortisol values between the two studies. Values for my sham and naive groups were 35.0 ± 6.7 and 28.1 ± 4.3 ng/ml versus 1.8 ± 0.9 ng/ml for Reid *et al.* (1992). Although, Gamperl *et al.* (1994) noted that plasma cortisol

values in non-stressed fish can vary from 2 - 42 ng/ml. it is possible that at the cortisol levels in my two control groups, receptor numbers were already at a maximum and no further increase in number was possible. In fact, Gamperl *et al.* (1994) in the same review, reported that cortisol levels in stressed fish vary from as little as 20 ng/ml up to 500 ng/ml although less than 300 ng/ml is characteristic. Possible support for this may be seen in a study by Reid and Perry (1991) examining the effect of cortisol on rainbow trout erythrocyte β-adrenoceptors. Using a similar protocol to Reid *et al.* (1992) where hepatocyte adrenoceptors were studied, plasma cortisol levels were raised with mini osmotic pumps to 118 ± 5.9 ng/ml in treated fish while values in shams were 23 ± 1.4 ng/ml for periods in excess of10 days. No significant difference between the two groups was found in either the cell surface receptor's affinity for ^{3}H-CGP or in the number. A significant difference was, however, found in the number of internalized low affinity receptors using ([^{3}H]DHA). The use of purified membranes did not permit this possibility to be determined in my study.

C. Membrane Characteristics

Cortisol was found not to affect the relative composition of phospholipids in purified rainbow trout hepatic membranes (Table 4). In contrast, glucocorticoids have been found to affect fatty acid composition in mammals. Kapitulnik *et al.* (1986) found the synthetic glucocorticoid, dexamethasone, significantly increased the content of the

polyunsaturated fatty acids, arachidonic (20:4) and linoleic acid (18:2), while decreasing the content of the monoenoic fatty acids, oleic (18:1) and palmitoleic acid (16:1), in hepatic microsomal membranes of fetal rats. Gonzales *et al.* (1990) found dexamethasone increased the short chain fatty acid, myristic (14:0) and unsaturated fatty acid (16:1), accompanied by a decrease in stearate (18:0) in human fetal lung explants. In addition, the latter authors also found dexamethasone stimulated both fatty acid synthesis and fatty acid synthetase activity. Recently, Gudbjarnason and Benediktsdóttir (1996) found daily administration of either EPI or NEPI for 7 or 15 days results in the replacement of both 18:2 and 20:4 with docosahexaenoic acid (22:6) and a synchronous decrease in the B_{max} of β-adrenoceptors in heart muscle membranes of rats. This study, however, did not determine if there was any change in the overall function of the receptors.

No other studies are available in teleost fishes regarding changes im membranes by cortisol. Given that no changes in adrenoceptor distribution were noted, the analysis of membrane changes was not particularly informative.

III. Glucose Production

One process in the liver that is regulated by the catecholamines is glycogenolysis (Janssens and Lowrey, 1987; Vijayan *et al.*, 1993a). This is accomplished by modifying

the activity of glycogen phosphorylase (GPase), the enzyme directly responsible for the breakdown of glycogen (Sheridan and Muir, 1988; Wright *et al.*, 1989). A β-adrenoceptor role in glycogenolysis in teleost livers has been demonstrated unequivocally (for examples, see Birnbaum *et al.* (1976) - goldfish (*C. auratus*) Brighenti *et al.* (1987a,b) - catfish (*I. melas*); Janssens and Lowrey (1987) - carp (*C. carpio*); Sheridan and Muir (1988) - chinook salmon (*O. tshawytscha*); and Wright *et al.* (1989), Michelsen and Sheridan (1990) - rainbow trout). Part of the supporting evidence was the observation that in fish hepatocytes the non-selective β-antagonist PROP effectively blocked catecholamine stimulated glycogenolysis while the non-selective α-antagonist PHT had little effect. As can be seen from Figure 5A, 1mM PROP significantly blocked 1μM EPI stimulated glucose release in the sham injected fish, supporting a functional role of the β-adrenoceptor in this system.

On the other hand, evidence for an α-adrenoceptor role in the hepatic glycogenolysis of teleosts has been, at best, circumstantial (Brighenti *et al.*, 1987a,b; Janssens and Lowrey, 1987; Michelsen and Sheridan, 1990; Moon and Mommsen, 1990). For example, Janssens and Lowrey (1987) found no evidence for α-adrenergic stimulated glycogenolysis in cultured liver pieces of carp, as EPI actions were blocked only by PROP and not by PHT. These authors also were unable to find any effect of Ca^{2+} as an intracellular messenger on glycogenolytic activity.

In contrast, Michelsen and Sheridan (1990) found evidence of mediation of hepatic glycogenolysis by Ca^{2+} in liver pieces of rainbow trout. Basal glucose release was more pronounced in calcium-containing medium than in calcium-free medium. As well, glucose release in the calcium-containing medium was enhanced with the calcium ionophore, A23187. It should be noted both the Janssens and Lowrey (1987) and the Michelsen and Sheridan (1990) studies used liver pieces as opposed to isolated hepatocytes. Liver slices contain damaged cells on the external surface and therefore represent a non-uniform system (Brighenti *et al.*, 1987b). Birnbaum *et al.* (1976) using isolated goldfish hepatocytes found EPI stimulated glycogenolysis to be unaffected by both calcium and A23187. However, basal glycogenolysis was enhanced by the addition of 1 - 4 mM calcium. Meanwhile, Moon and Mommsen (1990) found PRZ reduced PHE stimulated glycogenolysis in bullhead, eel, and trout hepatocytes. Although considered to be an α_1-agonist, the PHE effects were also blocked by PROP. This led to the conclusion by the authors of a mixed α/β-adrenergic receptor mechanism in these three teleost species. As can be seen from Figure 5A, 1 mM PRZ was just as effective as 1 mM PROP at blocking 1 μM EPI stimulated glucose release in hepatocytes isolated from the sham injected fish.

Similar results seen in teleosts in the regulation of hepatic glycogenolysis have also been reported in amphibians and reptiles. Janssens *et al.* (1983) were only able to detect a β-adrenergic role in the axolotl (*A. mexicanum*) as only PROP was effective at blocking

EPI-stimulated glucose release. Similar results were found by both Janssens and Grigg (1984) and Janssens and Grigg (1987) in the toad (*X. laevis*). In fact, in the latter study, contrary to both Michelsen and Sheridan (1990) in rainbow trout and Birnbaum *et al.* (1976) in goldfish, Ca^{2+} was found not to have any effect on the rate of glycogenolysis. This was also seen by Janssens *et al.* (1986) in the axolotl (*A. mexicanum*) and by Janssens and Grigg (1992) in both the long-necked turtle (*C. longicollis*) and the loggerhead turtle (*Caretta caretta*). However, Janssens and Giuliano (1989) could not exclude the possibility of a limited α-adrenergic role in hepatic glycogenolysis of the western netted dragon (*A. nuchalis*). Although similar results as the other studies were seen with respect to Ca^{2+}, PHT slightly blocked the actions of EPI.

Cortisol has been found to have possibly both a direct and indirect effect on glycogen metabolism (Vijayan and Leatherland, 1992; Vijayan and Moon, 1992). This is through a possible interactive and/or permissive effect with other glucoregulatory hormones such as the catecholamines (Vijayan *et al.*, 1993a). The lack of a significant increase in EPI stimulated glucose release in the cortisol injected fish (Figure 5B) contradicts the findings of Reid *et al.* (1992). These authors found cortisol treatment produced a 2.1-fold increase in rainbow trout hepatocyte responsiveness to EPI. A similar result was seen by Vijayan *et al.* (1993b) in sea raven hepatocytes. However, Vijayan *et al.* (1994) found no increase in hepatocyte responsiveness to epinephrine due to cortisol in rainbow trout with coconut oil implants. The metabolic potential of the

hepatocytes was, however, enhanced by a significant increase in alanine oxidation and gluconeogenesis.

In addition, the net rate of glycogenolysis is related to the initial hepatic glycogen content (Moon *et al.*, 1988; Foster and Moon, 1990; Mommsen and Moon, 1990). Also, liver glycogen metabolism is affected by the prior nutritional state of the animal (Sheridan and Mommsen, 1991; Vijayan and Moon, 1992) Initial hepatic glycogen content of the cortisol injected trout was quite variable at 30.9 ± 9.6 µmol/g hepatocytes. Although not significantly different, initial hepatic glycogen contents of the sham trout were less variable at 41.2 ± 6.4 µmol/g hepatocytes. The lack of a cortisol effect on hepatic glycogen content was also seen by Vijayan *et al.* (1993a) in coho salmon (*Oncorhynchus kisutch*), Vijayan *et al.* (1994) in rainbow trout and by Vijayan *et al.* (1996) in the sea raven. Andersen *et al.* (1991) found chronic cortisol treatment for 10 days significantly reduced hepatic glycogen content in rainbow trout by almost 50%. However, unlike in my study, the fish in this study were placed in black 3 L plexiglass boxes and were not fed for the duration of the experiment. While similar trends were seen between the two experimental groups, the relatively larger variabilities in the TREATMENT fish hide any possible significant effects that may be present.

IV. Temperature

As can be seen from Table 6, the results of this study are extremely variable and as such, few, if any, conclusions can be drawn. This experiment was conducted at Arizona

State University over a short period and, therefore, there was no time to evaluate the results of the earlier experiments to determine if any modifications were necessary.

Herman *et al.* (1996) examined the effect of long term temperature acclimation on the α_1-, α_2-, and β-adrenoceptors in American bullfrog (*Rana catesbeiana*) atrium, ventricle, and kidney membranes. Using ^{3}H-PRZ, ^{3}H-RX821002, and ^{125}I-ICP, for the α_1-, α_2-, and β-adrenoceptors, respectively, no significant temperature effects were found for either K_d or B_{max} in any receptor type for any tissues examined. It is important to note, however, that neither my study nor that of Herman *et al.* (1996) examined adrenoceptor function.

The physiologically functional adrenoceptors are found in the cell membrane. The function of the adrenoceptors has been found to be affected by the composition of the surrounding phospholipids (Williams *et al.*, 1995). It is well known that temperature acclimation is a factor in the phospholipid membrane composition of poikilotherms (Hazel, 1990; Hazel *et al.*, 1991). For example, cold temperatures promote the replacement of shorter chain monounsaturated fatty acids with longer chain polyunsaturated fatty acids (Hazel and Zerba, 1986) to maintain membrane fluidity by a process known as homeoviscous adaptation (reviewed by Hazel, 1995).

Receptor function was examined by Keen *et al.* (1993) who looked at β-adrenoceptor density and epinephrine sensitivity in cardiac tissue of rainbow trout acclimated to 8 and 18°C. Hearts from the fish acclimated to 8°C had an approximate 10-

fold increase in sensitivity to epinephrine when tested both *in situ* and *in vitro*. The β-adrenoceptors were characterized in the isolated sarcolemmal fractions of ventricles with ^{125}I-ICP. While no difference was found in the K_d, the B_{max} was significantly higher in the 8°C acclimated trout with an almost 3-fold increase in the number of receptor sites per square μm. The authors concluded that this increase was a possible explanation for the increased adrenergic sensitivity. Therefore, a future study examining hepatic receptor function and the signal transduction pathways in trout acclimated to different temperatures should be conducted.

V. Conclusions

This thesis examined the effect of cortisol and temperature acclimation on the distribution of the hepatic α_1- and β_2-adrenoceptors in rainbow trout. A few conclusions can be drawn from these studies.

First, both α- and β-adrenoceptors are present in hepatic tissue of rainbow trout. The characteristics of these receptors are similar to previous studies done on these receptors in trout hepatic membranes. It is not possible, however, to subtype these receptors from my study.

Second, use of cortisol containing coconut oil implants significantly altered plasma cortisol, occasionally plasma lactate, but not plasma glucose in rainbow trout.

Third, the elevation of plasma cortisol for 10 - 14 days did not modify the relative composition of the hepatic membrane phospholipids.

Fourth, distribution of both adrenoceptor types in purified hepatic membranes was not modified by either cortisol exposure or long term temperature acclimation.

Fifth, EPI stimulated glucose release was blocked by both PROP and PRZ indicating both adrenoceptor systems may be involved in hepatic glycogenolysis in rainbow trout hepatocytes. Unfortunately, my study could not separate which type predominated.

Clearly more studies need to be done to elaborate on the precise roles these receptors have on regulating hepatic metabolism in teleosts as well as on the factors that may affect their functioning. These studies should, but not exclusively, include cortisol elevation effects on the signal transduction pathways, as well as both a longer and a shorter time period of cortisol exposure. Other known stressors, such as hypoxia should also be tested. In addition, due to the species effects seen with the adrenoceptors, other species of teleosts should be examined. These studies, as well as others, should help to determine which, if either, receptor type, is more important in the regulation of hepatic metabolism in teleosts.

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