ACUTE RESPONSES OF THE THYROID OF THE STARVED RAINBOW TROUT (*SALMO GAIRDNERI*) TO CARBOHYDRATE

by

Beverly Ann Himick

A thesis
presented to the University of Manitoba
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
The Department of Zoology

Winnipeg, Manitoba

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BEVERLY ANN HIMICK

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

The acute effects of refeeding a single meal on plasma T_3 (3,5,3'-triiodo-L-thyronine) and plasma T_4 (L-thyroxine) were examined in previously-starved rainbow trout (*Salmo gairdneri*). An increase was observed in plasma T_4 within 2 h post- feeding, with a peak at 4 h. Feeding-induced plasma T_4 elevations occurred only in trout weighing less than 250 g and when refed a ration exceeding 0.37% of body weight. The feeding-mediated elevation in plasma T_4 is believed to represent an increase in thyroidal T_4 secretion. Plasma T_3 was not altered consistently within 8 h post- feeding.

The mechanism whereby food intake causes an acute increase in pT_4 was investigated. Sight, smell and taste of food did not elevate plasma T_4 as demonstrated by exposure of fish to either a food extract or to simulated food pellets. Postprandial changes in pT_4 were not dependent on energy intake. However dietary composition, and in particular the amount of carbohydrate consumed, dictated the degree to which plasma T_4 was elevated, regardless of the carbohydrate type (dextrin or cerelose). Intraperitoneal injections of D-glucose (0.2 to 2.0 g/kg) into groups of trout demonstrated a dose dependent and causal relationship between glucose and plasma T_4. In starved trout cannulated

in the dorsal aorta to permit serial blood removal, injected D-glucose (0.85 g/kg) revealed an increase in plasma T_4 within 1 h of plasma glucose elevation. Glucose did not cause short-term alterations in plasma T_3.

Glucagon (4.0 μg/g) caused an elevation in plasma T_4 within 2 to 3 h post-injection into starved, cannulated trout. An increase in pT_4 may occur through glucagon-mediated elevations in plasma glucose which occur within one hour. Insulin (10 IU/kg) may also stimulate thyroidal T_4 secretion in starved trout. Plasma T_3 remained unaltered by both pancreatic hormones.

Synthetic thyrotropin-releasing hormone (TRH) caused an increase in plasma T_4 within 1 h of injection (1.0 μg/g) into starved, cannulated trout. TRH did not act to alter plasma glucose or T_3.

It is concluded that acute postprandial plasma T_4 elevations in trout likely depend on carbohydrate availability in the diet and the degree to which plasma glucose becomes elevated following ingestion. Increased secretion of insulin and glucagon after feeding may participate to increase plasma T_4. A rapid response of plasma T_4 to TRH suggests that TRH acts as a thyrotropin-releasing factor and that the potential exists for glucose to stimulate thyroidal T_4 secretion through enhanced activity at the higher levels of the hypothalamo-pituitary thyroid axis.

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Glossary of Abbreviations

T_4 = L-thyroxine

T_3 = 3,5,3'-triiodo-L-thyronine

rT_3 = reverse T_3

pT_4 = plasma T_4

pT_3 = plasma T_3

FT_4 = free T_4

FT_3 = free T_3

*I T_4 = $[^{125}I]$ T_4

*I T_3 = $[^{125}I]$ T_3

$*I^-$ = $^{125}I^-$

T_4Ab = T_4 antibody

T_3Ab = T_3 antibody

TH = thyroid hormones

pTH = plasma TH

TSH = thyroid-stimulating hormone

TRH = thyrotropin-releasing hormone

HPT-axis = hypothalamo-pituitary thyroid axis

MS222 = tricaine methane sulfonate

ip = intraperitoneal

bw = body weight

IU = international unit

NSB = non-specific binding

TCR = total counts reference

cpm = counts per minute

GSS = glucose standard solution

PGO = peroxidase/glucose oxidase

d/d = distilled and deionized

OD = optical density

CV = coefficient of variation

SEM = standard error of measurement

PE = polyethylene

PCV = packed cell volume

Chapter I

INTRODUCTION

In many respects, the thyroid of the rainbow trout, *Salmo gairdneri*, resembles that of higher vertebrates (Eales, 1979; 1985). Thyroid follicular epithelial cells trap inorganic iodide and, through a process of tyrosine iodination and coupling, thyroid hormones (TH) are synthesized and stored in the colloid of the follicle lumen. Upon stimulation, TH are released by colloidal pinocytosis and endocytosis of the follicle cells. L-thyroxine (T_4) is the predominant hormone secreted by the fish thyroid (Chan and Eales, 1976; Grau *et al*., 1986). Following entry into the circulation, T_4 binds to several classes of plasma proteins (Eales, 1987). However, an extremely small percentage ($< 1.0\%$) of total T_4 remains "free" or unbound in the circulation (FT_4). This fraction represents the probable physiologically active pool of T_4 in the plasma. Thyroidal T_4 release is stimulated by TSH (thyroid-stimulating hormone) from the pituitary, which is in turn regulated by neuroendocrine pathways from the hypothalamus. There is some evidence to suggest that, as in the mammal, a negative-feedback system system exists in fish whereby homeostatic adjustment of FT_4 is achieved.

Peripherally, plasma FT_4 is in a steady state with protein-bound T_4 in the tissues. Free T_4 leaves the circulation and enters the cells where it may become enzymatically converted to 3,5,3'-triiodo-L-thyronine (T_3) by the loss of a single phenolic iodine. T_3, which is considered the metabolically active TH, then enters the plasma free T_3 (FT_3) hormone pool. Upon encountering a target cell, T_3 binds to the cellular nuclear receptor and initiates metabolic changes, presumably through increased messenger RNA and protein synthesis (Eales, 1985).

Many factors act to influence thyroid function in fish. Amongst them are alterations in food availability. For example, diets deficient in protein or low in ration can suppress peripheral T_3 production and plasma T_4 (pT_4) clearance in trout when fed over an extended period (Higgs and Eales, 1977; 1979). The inability of fish to cope with high dietary carbohydrate (CHO) is similarly accompanied by a reduction in the plasma T_4 to T_3 ratio suggesting decreased T_4 monodeiodination (Leatherland, 1982; Leatherland _et al_., 1984). Dietary lipid levels of 15% or greater also lower TH metabolism (Leatherland _et al_., 1984).

Total deprivation of food causes severe suppression of teleost thyroid metabolism. In trout, 3 days starvation is sufficient to eliminate the daily cycles of plasma T_3 (pT_3) and T_4 (Brown _et al_., 1978; Eales _et al_., 1981). In addition, basal T_4 levels are decreased (Flood and Eales,

1983). Fasting also reduces the number of hepatic T_3 receptors (Van Der Kraak and Eales, 1980; Bres, 1988). Extended periods of starvation decrease peripheral T_4 to T_3 conversion <u>in vitro</u> in the liver of the trout (Shields and Eales, 1986).

Two studies conducted in salmonid fish reveal that feeding rapidly alleviates fasting-induced thyroid suppression. Flood and Eales (1983) demonstrated elevated pT_4 4 h after refeeding previously-starved rainbow trout, resulting in the re-establishment of the daily cycles of pTH. Feeding also enhanced pT_4 clearance in brook trout (Higgs and Eales, 1977), suggesting that pT_4 elevations with food intake probably occur through enhanced thyroidal secretion. T_4 monodeiodination may also increase with refeeding in both rainbow trout and brook trout. However, the means by which a meal rapidly "normalizes" thyroidal status remains undefined.

The underlying theme of this thesis has been to determine the mechanism(s) whereby food ingestion stimulates T_4 release from the thyroid of the rainbow trout. Initial experiments were conducted to confirm previous findings that refeeding starved trout a single meal elevates pT_4 (Flood and Eales, 1983). Once this was established, an attempt was made to determine more precisely the time course of pT_4 changes after food intake. Based on the peak T_4 response, a standard experimental protocol was developed for use in all subsequent experiments.

To determine the feeding stimuli responsible for elevations in pT_4, experiments were conducted to test the effects of sight, smell and taste of food. However, pT_4 changed mainly in response to both ration and specific dietary composition. An interaction between CHO and T_4 formed the basis of further studies which led to the investigation of a possible causal relationship between glucose and pT_4.

The short-term effects of insulin and glucagon on pT_4 were also examined because of their immediate release during feeding, as well as their involvement in CHO metabolism. Both of these pancreatic hormones alter T_4 secretion and peripheral TH metabolism in mammals (Gavin and Moeller, 1983; Glade and Reimers, 1985; Attali _et al_., 1986). However, little information exists with respect to insulin and glucagon actions on the teleost thyroid.

Finally, experiments were conducted to examine the acute response of pT_4 to thyrotropin-releasing hormone (TRH). In the mammal, feeding-mediated pT_4 elevations occur, in part, through increased hypothalamic TRH secretion (Spira and Gordon, 1986). As of yet, a role of TRH in the regulation of the fish thyroid has not been established. Evidence of short-term pT_4 stimulation by TRH in trout would reinforce the possibility that feeding-induced T_4 secretion could occur as a result of changes at the higher levels of the hypothalamo-pituitary thyroid (HPT)-axis.

Chapter II

SELECTED LITERATURE REVIEW

2.1 ACUTE STARVATION AND THYROIDAL STATUS IN THE HOMEOTHERM

In the bird and mammal, short-term fasting produces a marked depression in thyroidal status. With starvation, serum T_3 levels are decreased (Decuypere and Kuhn, 1984; Hugues et al., 1986; Nowak and Slebodzinski, 1986), and serum rT_3 levels are elevated (Gavin and Moeller, 1983; Slebodzinski et al., 1982; Komaki et al., 1986). Circulating T_4 levels either increase (Pethes et al., 1984; Klandorf and Harvey, 1985; Houpt et al., 1986), decrease (Naito et al., 1981; Kinlaw et al., 1985) or remain unchanged (Mueller et al., 1982; Jaedig and Faber, 1982; Komaki et al., 1986). These changes are the direct result of alterations at the level of the HPT-axis.

During starvation, both basal TSH levels and the response of TSH to TRH stimulation from the hypothalamus are suppressed (Naito et al., 1981; Hugues et al., 1983, 1987; St. Germain and Galton, 1985). In man, fasting for 30 h effectively lowers TSH and eliminates the nocturnal peak in TSH diurnal cycling (Hugues et al., 1984). Starvation continued to 96 h blunts the response of TSH to TRH at the

level of the pituitary (Burger et al., 1980). Such a response occurs within 24 h of fasting in domestic fowl (Mitchell and Raza, 1986).

Modifications of the HPT-axis during starvation result in overall changes in TH peripheral metabolism. Hormonal secretion from the thyroid gland is suppressed (Wartofsky and Burman, 1982), as are the turnover rates of both T_4 and T_3 (Ingbar and Galton, 1975; Decuypere and Kuhn, 1984; Kinlaw et al., 1985). Activity of the 5'-deiodinase enzyme, which converts T_4 to T_3, is also decreased (Gavin and Moeller, 1983; Decuypere and Kuhn, 1984; Pethes et al., 1984; Nowak and Slebodzinski, 1986). This results in a decrease in the levels of circulating T_3. Inhibition of T_4 transmembrane transport into cells also contributes to suppressed T_3 levels (Van Der Heyden et al., 1986). With less T_4 substrate available during periods of reduced dietary intake, a decrease in T_3 production occurs.

Several factors may contribute to fasting-induced decreases in the responsiveness of TSH to TRH. The effects of injected T_3 into starved rats suggest that the pituitary acquires an increased sensitivity to circulating TH levels (Burger et al., 1980). TSH suppression has also been linked to a decrease in glucocorticoids, which occurs during periods of reduced food intake (Brown and Hedge, 1973; Pamenter and Hedge, 1980). The number of TRH-binding sites in the rat pituitary does not appear to be altered with fasting (Hugues et al., 1987).

High hypothalamic somatostatin levels during starvation have also been shown to inhibit both the release of TSH and the response of thyrotropes to TRH (Spira and Gordon, 1986). Similarly, fasting-induced increases in dopamine may also inhibit TSH release (Foord *et al*., 1986). Recently, FT_4 levels have been implicated in the regulation of the HPT-axis during fasting. An increase in FT_4 results in a decrease in TSH secretion (Komaki *et al*., 1986; Van Der Heyden *et al*., 1986). During starvation free fatty acids (FFA) become elevated in plasma. FFA compete with pT_4 in binding to plasma proteins and cause an increase in FT_4 levels.

2.2 REFEEDING AND THYROIDAL STATUS IN THE HOMEOTHERM

Feeding a single meal after short- or long-term starvation results in a partial to full reversal of fasting-induced TH alterations (Wartofsky and Burman, 1982; Danforth, 1986). Serum T_3 levels are increased while rT_3 and T_4 levels are decreased to pre-fasting conditions (Jaedig and Faber, 1982; Pasquali *et al*., 1982; Dauncey and Ingram, 1986; Glade and Reimers, 1985; Klandorf and Harvey, 1985). Several studies suggest that CHO and/or caloric content are important dietary components in mediating such TH changes.

In the piglet, isocaloric diets varying in dietary composition result in a drastic change in TH only when CHO

as opposed to protein is refed (Dauncey *et al.*, 1983). Similarly, in man it has been demonstrated that hypocaloric diets are capable of re-establishing altered TH levels only if CHO is present (Koppeschaar *et al.*, 1985). In fact, the level of CHO present in the diet may dictate the degree to which normalization of thyroidal status occurs (Pasquali *et al.*, 1982; Koppeschaar *et al.*, 1985). It has been suggested that a threshold with respect to dietary CHO may exist above which refeeding effects on TH are exerted (Pasquali *et al.*, 1982).

In the horse, the magnitude of TH change appears to depend on the energy level in the diet, regardless of its dietary source (protein or CHO) (Glade and Reimers, 1985). This study also demonstrated that consumption of excess energy results in the most notable thyroid metabolism changes. O'Brian *et al.* (1980) have similarly shown that caloric content of a diet is more important than CHO in causing thyroid normalization following starvation in the rat. Because of the difficulty in separating the effects of CHO and energy, it has generally been accepted that both contribute to altering TH metabolism in the mammal.

Refeeding stimulates the HPT-axis in the mammal (Wartofsky and Burman, 1982; Danforth, 1986). Serum TSH levels increase within the first hour of food intake (Hugues *et al.*, 1984; 1986; Komaki *et al.*, 1986). Glucose may act directly on the hypothalamus and/or pituitary to cause this

reversal. Rojdmark and Nygren (1983) have demonstrated that oral administration of glucose corrects the blunted TSH response to TRH in fasted rats. Somatostatin does not appear to act in the re-activation of TSH responsiveness during refeeding, despite its involvment during starvation (Hugues et al., 1986).

Food intake also induces changes in the peripheral metabolism of T_4 and T_3. Glucose increases the activity of the 5'-deiodinase enzyme, which results in an increased conversion of T_4 to T_3 (Ingbar and Galton, 1975; Gavin et al., 1981; Sato et al., 1983; St. Germain and Galton, 1985). In the bird, it has been demonstrated that a shift in feeding time causes a parallel shift in plasma glucose, T_3, and maximal 5'-deiodinase activity (Decuypere and Kuhn, 1984). According to Saito and Ishikawa (1981), a deficiency in CHO seriously alters T_4 metabolism by decreasing T_3 production and increasing T_4 to rT_3 conversion. This latter effect occurs through stimulation of the 5-deiodinase enzyme (Wartofsky and Burman, 1982).

2.3 STARVATION AND THYROIDAL STATUS IN FISH

Most studies on fish indicate that with chronic starvation thyroid function is suppressed. Early histological studies demonstrated a decrease in thyroid follicular cell height with prolonged fasting in salmon (McBride, 1967). Rainbow trout deprived of food for 40-65

days similarly exhibited depressed pT_4 (Milne et al., 1979; Leatherland et al., 1977). Fasting for 4 weeks has been shown to suppress daily cycling in pT_3 and pT_4 in rainbow trout (Cook and Eales, 1987). Shields and Eales (1986) have demonstrated that in vitro hepatic 5'-monodeiodinase activity also decreases in trout starved for 14 days. Fasting for extended periods in trout decreases $*T_4$ ($[^{125}I]$ T_4) biliary excretion (Eales and Sinclair, 1974). Higgs and Eales (1978) have demonstrated that $*T_4$-injected brook trout exhibit lower $*T_4$ biliary-fecal excretion rate, T_4 MCR and $*T_4$ deiodination rate when starved for 12-37 days.

Acute starvation also decreases thyroid metabolism in fish. In the rainbow trout, food deprivation for 72 h results in the elimination of pT_4 and pT_3 daily cycling (Brown et al., 1978; Eales et al., 1981). Similarly, in vivo and in vitro studies in the trout both suggest that three days of fasting results in a decrease in hepatic T_3 nuclear receptor capacity, as measured relative to hepatocyte DNA, with no apparent change in binding affinity (Van Der Kraak and Eales, 1980; Bres, 1988). Starvation for 6 to 8 days suppresses basal levels of both pT_4 and pT_3 (Flood and Eales, 1983).

2.4 REFEEDING AND THYROIDAL STATUS IN FISH

There are few studies which have examined the effects of refeeding on TH recovery in starved fish. Flood and Eales (1983) demonstrated that food intake in rainbow trout starved for 6 days increased both pT_3 and pT_4 within 4 h. T_4 monodeiodination to T_3 increased within one hour after refeeding in chronically starved brook trout (Higgs and Eales, 1977). An increase in $*T_4$ biliary-fecal excretion occurred within 24 h of refeeding in trout starved for two weeks (Eales and Sinclair, 1974).

Dietary composition alters fish thyroid hormone metabolism in some instances. Higgs and Eales (1979) chronically fed brook trout a diet low in protein (15%) and caloric content (2.4 kcal/g). In relation to fish fed a high-protein/high-caloric diet, they observed a decrease in pT_4, $*T_4$ conversion to $*T_3$ ($[^{125}I]\ T_3$) and $*T_4$ biliary-fecal excretion. On the contrary, Leatherland _et al_. (1980) reported no effects on thyroid metabolism following the feeding of varied dietary protein amounts to rainbow trout acclimated at different temperatures. When fed over one month, dietary CHO has been shown to have varied effects on pT_3 and pT_4 in trout (Leatherland _et al_., 1984). Dietary lipid also influences thyroid metabolism. An increase in unsaturated fat causes an elevation in pT_4 when fed to trout at low temperatures (Leatherland _et al_., 1977).

2.5 GLUCOREGULATORY HORMONES AND TH METABOLISM IN THE HOMEOTHERM

Associated with fasting and refeeding are changes in the levels of the main glucoregulatory hormones, insulin and glucagon. While starvation inhibits pancreatic insulin release, at the same time increased plasma glucagon levels act to mobilize glucose from hepatic glycogen stores. Current studies suggest that these pancreatic hormones also regulate TH metabolism during dietary manipulation (Wartofsky and Burman, 1982; Gavin and Moeller, 1983).

In man, hyperglycemia induced by intravenous glucagon administration is accompanied by a rapid decrease in pT_3 within one hour, followed by an increase in rT_3 at 4 hours (Kabadi and Premachandra, 1985). Similar findings in glucagon-treated hypothyroid individuals injected with T_4 suggest that glucagon acts peripherally to alter TH metabolism (Kabadi and Premachandra, 1987). *In vivo* studies have demonstrated a negative correlation between 5'-deiodinase activity and glucagon in hepatocytes of the fasted rat (Gavin and Moeller, 1983).

The acute effects of glucagon have also been examined at the level of the thyroid gland. Attali *et al*. (1984) have demonstrated a rapid elevation in TH concentration after exposing the perifusate of rat thyroid minces to glucagon. More recent studies, however, have shown no effect on TH release when intact thyroid follicles of the dog are

perfused with both pharmacological and physiological doses of glucagon (Laurberg and Iversen, 1987).

Feeding-induced increases in insulin have been positively correlated with 5'-deiodinase activity in horses (Glade and Reimers, 1985). Studies of cultured rat hepatocytes demonstrate a direct stimulation of 5'-deiodinase activity by insulin (Sato and Robbins, 1981). Hepatocytes from fasted rats exposed to insulin for 5 h display an increase in previously suppressed 5'-deiodinase activity. Gavin _et al_. (1987) have shown that both glucose and insulin reverse the effects of 48 h fasting in the rat by increasing _in vitro_ T_3 production in hepatocytes.

The effects of insulin on thyroidal function of streptozotocin-induced diabetes mellitus in the rat have also been well documented. Whereas diabetes mimics the effects of starvation on TH metabolism, insulin treatment causes an increased activity at the levels of both the hypothalamus and pituitary, as well as peripherally by increasing T_4 to T_3 deiodination in the liver and kidney (Wartofsky and Burman, 1982; Ferguson _et al_., 1985).

The mechanism of insulin-mediated elevations in 5'-deiodinase activity remain unclear. Increased glucose uptake into hepatocytes in response to insulin may stimulate new 5'-deiodinase synthesis or re-activate the inactive enzyme. However, 5'-deiodinase activity was not found to be

stimulated by glucose in rat hepatocytes (Sato and Robbins, 1981). Brisson-Lougarre and Blum (1981) have identified the presence of insulin-binding sites in the plasma membranes and nuclei of isolated bovine thyroid cells suggesting a possible direct stimulation by insulin on thyroid tissue.

Insulin also influences TH concentrations through alterations at the level of the HPT-axis. Insulin injections decrease both serum glucose and TSH in the rat (Brown _et al_., 1976). This may result from decreased release of hypothalamic TRH which has been shown to occur with insulin treatment in the rat (Leung _et al_., 1975). Rojdmark and Nygren (1983) have also demonstrated an inhibition of pituitary thyrotrope function by low glucose levels. Glucagon appears to have no effect on TSH (Komaki and Premachandra, 1985). However, in the bird, increased pT_4 3 h after refeeding (when glucagon levels are high) suggests that alterations at the level of the HPT-axis are possible (Mitchell and Raza, 1986).

Thyroidal status has been shown to regulate pancreatic function in the homeotherm. When made hypothyroid, rats exhibit decreased growth of pancreatic tissue (Kenny _et al_., 1987). In addition, blood glucose levels are elevated with decreased T_4 (Brown _et al_., 1976). In the hyperthyroid mammal, the actions of insulin on glucose metabolism are inhibited (Mueller _et al_., 1987). Furthermore, chronic administration of T_4 in the rat causes a decrease in insulin

binding to target tissue receptors (Mueller *et al*., 1986). Isolated rat pancreatic islets perfused with T_4 have shown a delayed effect of insulin secretion (Yamameto *et al*., 1987).

TRH within the gastrointestinal tract also acts to influence pancreatic function in the mammal. Cholecystokinin release of the pancreas is controlled by TRH (Blanco *et al*., 1987). Food intake and the secretion of gastric acid also increase TRH secretion in the mouse stomach (Uberti *et al*., 1986). TRH release during food intake may regulate TH metabolism both peripherally and centrally through TSH.

2.6 GLUCOSE, GLUCOREGULATORY HORMONES AND TH METABOLISM IN THE FISH

Few studies have examined the interactions between TH, glucoregulatory hormones and glucose in fish. Early studies in the carp, *Cyprinus carpio*, demonstrated suppressed plasma glucose levels 48 h after injection of high doses of T_3 and T_4, with an elevation in heart and muscle glycogen content (Murat and Serfaty, 1970; 1971). In the Pacific hagfish and lamprey, T_3 and T_4 injections at possibly pharmacological doses caused a decrease in plasma glucose within 24 h (Plisetskaya *et al*., 1983; Plisetskaya and Gorbman, 1983). TH may participate in the regulation of glucose levels in lower vertebrates, acting possibly to decrease plasma glucose. However, use of such high doses of TH presents the

difficulty of establishing whether or not these effects are exerted through catabolic actions of TH. Recent studies have revealed no effect of oral T_3 administration on plasma insulin levels or on dietary CHO utilization in rainbow trout (Hilton et al., 1987).

Data concerning the interactions of glucoregulatory hormones and TH are scarce. This may be due, in part, to the fact that while glucagon and insulin act antagonistically in the regulation of CHO metabolism in the mammal, their role in the lower vertebrates remains unclear. Glucagon is released in response to a lack of circulating glucose. On the other hand, glucose overloading in the teleost results in a sustained hyperglycemia over several hours, suggesting little homeostatic regulation of plasma glucose by insulin. In the perfused Brockmann bodies of the catfish, D-glucose does stimulate insulin release. However, certain amino acids such as arginine appear to be much more potent secretagogues (Ronner and Scarpa, 1987). Porcine insulin prevents glycogen depletion in isolated rainbow trout hepatocytes, but does not alter glucose levels (Foster and Moon, 1987).

Plisetskaya et al. (1983) have demonstrated that decreased insulin levels in the Pacific hagfish are accompanied by a significant decrease in pT_3 within 24 h and an increase in plasma glucose within 72 h. Plasma T_4 levels appear unaltered. Porcine insulin injections into carp

increase pT_3 with no change in pT_4 (Kaminska *et al*., 1985). Recent *in vivo* studies in the turtle, *Chrysemys dorbigini*, have shown an uptake of [^{125}I]-insulin by the thyroid gland 30 min after radiolabel injection (Marques *et al*., 1985). This suggests that, as in the mammal, a direct effect of insulin on the thyroid may occur in the lower vertebrates.

2.7 TRH IN POIKILOTHERMS WITH SPECIAL REFERENCE TO FISH

Alterations of the HPT-axis during starvation and refeeding are well established in the mammal (see 2.1 and 2.2). However, little information exists regarding the effects of dietary manipulation on TRH and TSH in the poikilotherm. There are several possible reasons for this. Firstly, a suitable TSH assay for fish (and certain other poikilotherms) does not exist and as such, circulating levels of TSH cannot be measured accurately. Secondly, much controversy surrounds the role of TRH in the poikilotherm brain. Only in the last few years has an attempt been made to clarify hypothalamo-pituitary interactions regarding thyroidal control in the teleost and other lower vertebrates.

For example, earlier studies have shown that intravenous injections of synthetic TRH have no effect on thyroidal function in the turtle, *Chrysemys picta* (Sawin *et al*., 1981). However, more recent studies demonstrate that TRH does stimulate TSH release in the reptile and that both T_4

and T_3 have clear negative feedback effects on TRH-mediated TSH release (Denver and Licht, 1987). Similarly, the apparent stimulation of the thyroid by TRH acting on the pituitary in the frog, *Rana ridibunda* (Darras and Kuhn, 1982), contradicts earlier studies demonstrating the lack of TRH effects on the amphibian thyroid (Vandesande and Aspeslagh, 1974).

In fish, acute responses of pituitary TSH cells to TRH have been demonstrated (Tsuneki and Fernholm, 1975). However, other *in vivo* and *in vitro* studies fail to show a thyrotropic response to TRH (Peter and McKeown, 1975; Crim *et al*., 1978). In some cases TRH has even been shown to inhibit TSH release from the pituitary (Peter, 1973; Crim *et al*., 1978; Ball, 1981). Although the role of TRH in the HPT-axis has not yet been resolved, the recent discovery of TRH receptors in teleost pituitary homogenates (Burt and Ajah, 1984) supports the theory that TRH is involved in pituitary regulation.

Chapter III

MATERIALS AND METHODS

3.1 FISH MAINTENANCE AND HOLDING CONDITIONS

Rainbow trout of the Mt. Lasson strain were obtained from the Rockwood Experimental Hatchery in Balmoral, Manitoba. Stock fish were held in 2.3-kL tanks receiving a constant flow of aerated and dechlorinated Winnipeg city water at 12 $\pm$ 1°C. Photoperiod was held constant at 12L:12D and, at approximately 1300 - 1330 h each day, fish were fed a 1% ration of 4P trout pellets (1/8"; 40.0% protein, 10.0% fat, 15.0% CHO 3.0% fibre, 7500 IU (International Units)/kg vitamin A, 3000 IU/kg vitamin D_3, 100 IU/vitamin E, 800 mg/kg ascorbic acid; Martin Feed Mills Ltd., Elmira, Ontario). Rations have been expressed throughout the thesis as dry food weight.wet body weight^{-1}.10^2(%).

Prior to an experiment, trout (mean wt. range 36.6 - 414.4 g) were anesthetized in 18 L of aerated water at 12 $\pm$ 1°C containing 1.2 g of tricaine methane sulfonate (MS222; Syndel Laboratories, Vancouver, B.C.). They were weighed to the nearest 0.1 g and then randomly assigned to 205-L tanks receiving running water at 12 $\pm$ 1°C. Photoperiod remained unaltered at 12L:12D. At 0930 h daily, prescribed rations

of trout pellets were dropped through a small hole in the translucent lid of each tank. Fish were acclimated under these conditions for 7 to 21 days, after which any food particles and feces were removed from the bottom of each tank. Trout were then starved until day 4, when experimental treatments began.

3.2 EXPERIMENTAL TREATMENTS

3.2.1 Feeding

To examine the effects of quality and other food properties on pTH, experimental diets were fed once at 0815 h or 0830 h. Tanks of trout received food in a staggered sequence (20 to 30-min intervals) in the order in which each tank of fish would be bled. This permitted groups of fish to be sampled as closely as possible at the same interval after feeding. Trout were fed by removing a rubber stopper from the hole in the tank cover and dropping pellets through the opening. Control fish did not receive food. However, the stopper on the tank lid was removed to initiate the swimming and thrashing movements characteristic of trout anticipating a meal.

Experimental diets consisted of either simulated (plastic) trout "pellets" or custom-formulated pellets of known nutrient content. Plastic pellets were created by cutting brown plastic electrical wire insulation into pieces approximately 3.0 x 5.0 mm. Custom-formulated diets varied

in both CHO type (dextrin = partially hydrolyzed corn starch or cerelose = D-glucose) and the CHO/lipid content (high CHO/low lipid, medium CHO/medium lipid and low CHO/high lipid). In trout, different digestibilities exist for both dextrin (77%) and cerelose (99%) (Cho *et al.*, 1982). The specific composition of each diet type is presented in Appendix 1. These diets were prepared by Dr. D.A. Higgs, Department of Fisheries and Oceans, West Vancouver, B.C..

3.2.2 Food extract

An extract of fish food was prepared by boiling 54.1 or 62.2 g (representing a ration of 3 or 5% bw) of Martin 4P trout pellets in 1 L of distilled and deionized (d/d) water for approximately 3 h. The solution was then cooled to room temperature and centrifuged at 2,000 G (Sorvel) for 20 min to remove suspended food and lipid. The final extracted product was filtered and chilled to 12°C.

At 0930 h, the food extract was administered to trout by pouring the entire solution through a funnel near the incoming flow of water. Controls were treated in a similar manner except that d/d water (12°C) was introduced into the tank at 0950 h.

3.2.3 Injection

Groups of trout were injected intraperitoneally (ip) with either D-glucose (anhydrous; Fisher Scientific Co.), bovine insulin (25 IU/g, crystalline; Sigma), a mixture of bovine and porcine glucagon (crystalline; Sigma) or synthetic TRH (Sigma). Injections commenced at 0815 h or 0830 h with 20- to 30-min intervals between each treatment. Because of the insolubility of insulin and glucagon in physiological saline, a 0.1M acetate buffer (463 mL/L 0.2 M acetic acid, 37 mL/L of 0.2 M sodium acetate; pH 3.27) and a 0.05M Tris/HCl buffer (250 mL/L 0.2 M Tris; pH 9.00) were tested as suitable vehicles for hormone injection.

Prior to injection, trout were anesthetized within their tank following methods outlined previously (see 3.1). When ventral side up and displaying no detectable fin movement, each fish was removed and ip injected (20 - 500 μL) on the right ventral surface just anterior to the pelvic fins by use of a 26-gauge, 5/8-inch needle and a 1-ml tuberculin syringe. In some experiments, a Hamilton repeating dispenser was used. After injection, each fish was returned to its tank which had during the interim been flushed with water to remove anesthetic.

3.3 BLOOD SAMPLING

At 2, 4, 6 or 8 h after being treated, groups of fish were netted and anesthetized (1.2 g MS222 in 18 L water). When body and fin movements ceased, each trout was removed and a 1-mL blood sample was taken from the caudal vessel with a heparinized 21-gauge, 1-1/2 inch needle and a 1- or 3-ml syringe. Fish were then sacrificed by concussion. Each group was sampled within approximately 10 to 15 min. Blood was placed directly into 1.5-ml disposable polystyrene centrifuge tubes and centrifuged for 2 min at 15,000 G (International Centrifuge model MB or IEC Centra-M centrifuge). The supernatant plasma was collected with a Pasteur pipette and stored at -70°C in 2-mL disposable plastic sample cups sealed with Parafilm. Within one week of collection, samples were analysed for pT_4, pT_3, and plasma glucose. In several experiments samples were analysed immediately after collection.

3.4 ESTIMATION OF FOOD INGESTION

After sacrifice, each fish was weighed to the nearest 0.1 g and the stomach and intestine were examined for food contents. In experiments involving food quantity, the amount of food ingested by each trout was measured by use of a technique modified from that of Brett and Higgs (1970). The stomach and intestine of each fish were excised and their contents rinsed with 95% ethanol into an aluminum

weighing pan. The mouth was also examined, and if any food was present, it was added to the pan. The effects of ethanolic rinsing on the weight of food was tested by parallel extraction of 1.0 g of finely-ground 4P trout pellets. After 24 h evaporation, all pans were placed into an oven and dried at 105°C for 24 h. When cooled to room temperature, the weight of food present in each pan was determined after correction of weight loss through extraction (0.032 g).

3.5 CANNULATION TECHNIQUE

Aortic catheterization was performed following modified procedures of both Swift (1981) and Brown _et al_. (1986). Each trout to be cannulated (407.4 - 877.5 g) was taken from stock, anesthetized, weighed to the nearest 0.1 g, and placed ventral side up on an operating chamber. Its lower jaw was gently lifted and hooked on a thread to keep the mouth open during the course of the operation. To prevent drying of the skin, the fish was placed on two sponges soaked with water. A flexible rubber tube, the end of which was inserted into a pipette bulb with a hole at the tip, was placed inside each operculum. This allowed the gills to be constantly perfused with aerated, dechlorinated water at 12 $\pm$ 1°C containing MS222 (1.2 g in 18 L) and NaCl (150 Mm) (during the first three quarters of the operation), or dechlorinated water at 12 $\pm$ 1°C (during the last quarter of the operation) (Fig. 1 a).

Three sutures were placed on the inside of the mouth by use of fine-pointed hemostats, 1/2-inch circle suture needles (cutting edge, Stevens and Sons Inc., Northlands Inc.) and 00-silk braided suture thread (Ethicon Sutures Ltd., Peterborough, Ontario). Sutures number one and two were on the midline palate, while suture number three was on the right side of the mouth (Fig. 1 b). A fourth suture was carefully placed on the edge of the right operculum (Fig. 1 c).

An indwelling catheter (sheath gauge 18, needle gauge 20; Sovereign), previously flushed with ice-cold heparinized Cortland saline (50 heparin units mL^{-1}; Allen and Hanbury's, Glaxo Canada Ltd.; Cortland saline (Wolf, 1963): 4.25 g NaCl, 0.215 g $CaCl_2.2H_2O$, 0.38 g KCl, 0.41 g NaH_2PO_4, 0.12 g $MgSO_4$, 1.00 g $NaHCO_3$ / 1 L d/d water), was then inserted through the dorsal midline tissue between the second and third gill arch until the needle had just penetrated the dorsal aorta (Fig. 1 b). The catheter was held in place while the needle was gently removed. A cannula (Clay-Adams PE 50), flushed with heparinized Cortland saline was then inserted via the catheter into the dorsal aorta. Attached to the cannula at the distal end was a 25-gauge needle (5/8-inch) which was in turn attached to a 3-way stopcock, which could be adjusted to permit the flow of ice-cold heparinized Cortland saline from either a 1- or 3-ml syringe. To prevent blood clotting at the implanted end of

the cannula the polyethylene tubing was pre-soaked, in some instances, overnight at room temperature in a 2% TD-Mac heparin complex (Polysciences, Inc.). After implantation, the pulsing of blood within the cannula was checked and the tubing was sealed off at the distal end with Plasticine. The catheter was then removed and the cannula secured with the sutures in the mouth. The cannula was directed out through the right operculum and tied to the fourth suture (Fig. 1 c). In total, the operation usually lasted 10 to 15 min.

After the operation, each trout was placed into a 57-L glass aquarium, shielded with white material and a translucent lid and receiving a constant flow of 12 $\pm$ 1°C water. The cannula was allowed to float freely on the surface of the water. Trout recovered from the operation within approximately 20 to 30 min.

Every 24 h, at 0930 - 1000 h, the cannula dead volume was removed and replaced with fresh heparinized Cortland saline (250 heparin units mL^{-1}). This prevented blood clotting in the cannula. Occasionally, blood did not flow. Fish were anesthetized within their tank, and the cannula was gently tugged to loosen it slightly from the suture knots. Fish which did not completely recover from the operation were not used and were sacrificed by concussion. Trout were not fed following cannulation.

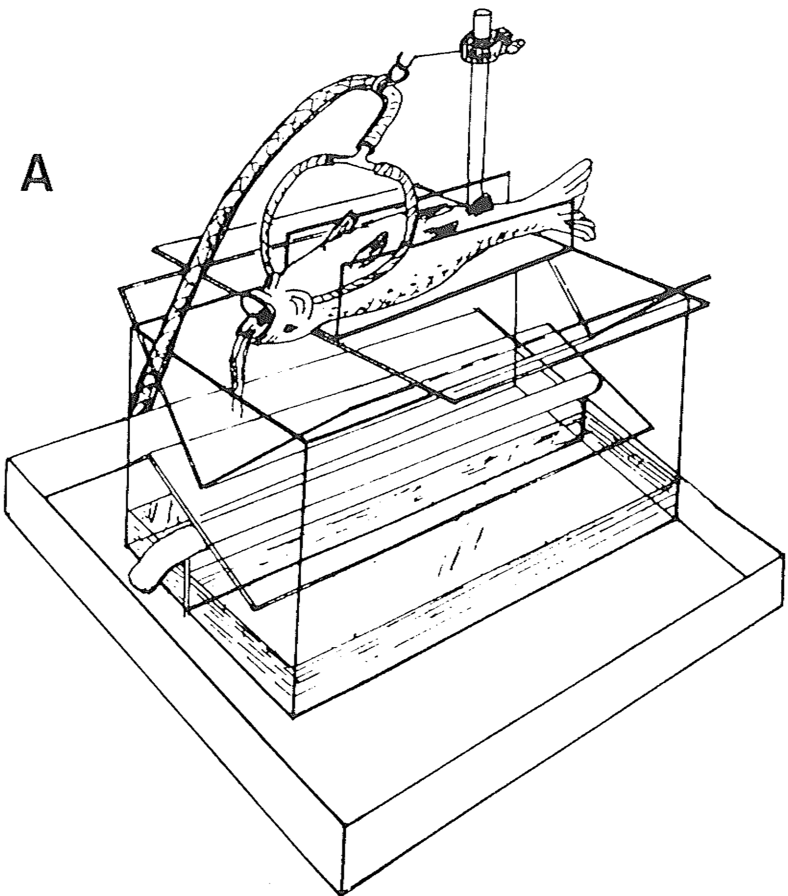
Figure 1: Aspects of aortic cannulation in trout.

A. Diagram of apparatus used during catheterization.

B. Positions of the first three sutures. Sutures 1 and 2 were placed on the midline of the dorsal palate; suture 3 was placed to the right of the midline.

C. Suture 4 was placed on the outer edge of the operculum.

The catheter was inserted between the second and third gill arches and the cannula was secured with all four sutures.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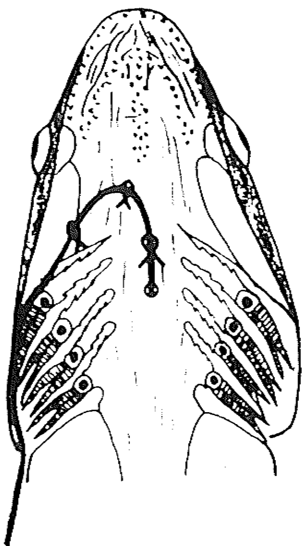
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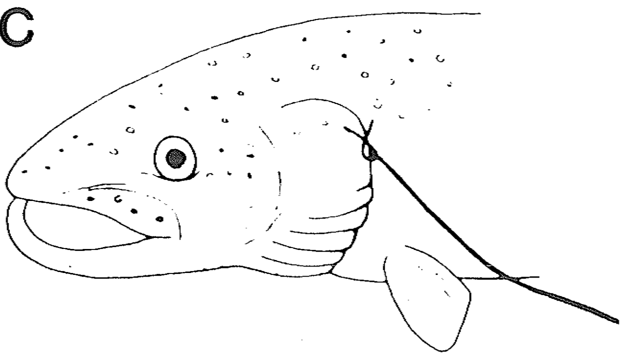

C

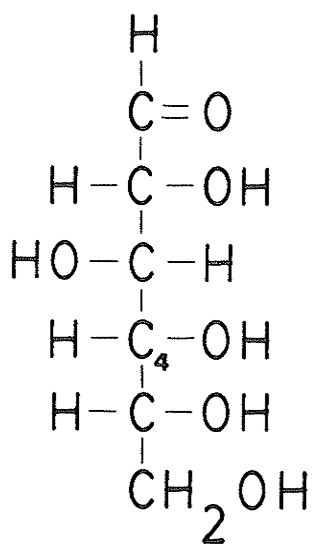
3.6 INJECTION AND SAMPLING OF CANNULATED FISH

At 0830 - 0900 h, the heparinized saline within the cannula was withdrawn with a 25-gauge needle and a 3-ml syringe. A 500-µL blood sample was collected and replaced with approximately 250-µL of fresh heparinized Cortland saline (50 heparin units ml^{-1}). Each fish was then anesthetized within its tank by the addition, through a funnel, of a concentrated solution of MS222 (3.8 g MS222 in 250 mL of water). Care was taken not to startle or disturb the fish in any way during this step. When the fish was ventral side up and devoid of any body movements, the lid was removed and the fish was raised to the water surface. With the gills and body still immersed, the belly was raised out of the water and an ip injection was administered.

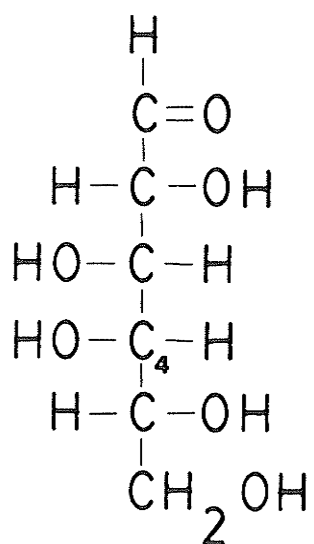
Cannulated trout were injected with 0.85 mL/kg of either D-glucose, D-galactose, a stereoisomer of D-glucose (Fig. 2), bovine insulin, a mixture of bovine and porcine glucagon or synthetic TRH. Saline-injected (0.7% NaCl) trout served as controls and were run throughout the course of all monosaccharide and hormone injections.

After injection, each trout was gently lowered to the bottom of the tank. While the fish was still motionless, half the water in the tank was bailed out to remove anesthetic. Fresh water was poured into the tank at the end opposite the fish until the water line was similar to

Figure 2: Structures of D-glucose and D-galactose. D-galactose differs from D-glucose by the rotation of one hydroxyl group at chiral carbon 4.



D-Glucose



D-Galactose

pre-treatment conditions. Each trout began to regain equilibrium within 30 min of being injected.

At hourly intervals, a 500 μL blood sample was removed (0 h to 7 h). Each sample was placed directly into a 1.5-mL polystyrene centrifuge tube for plasma separation. Some blood was withdrawn from the centrifuge tube into a heparinized micro-hematocrit capillary tube for determination of hematocrit or packed cell volume (PCV). The capillary tube was centrifuged at 15,000 G for 2 min and PCV was determined using a Criticap microhematocrit tube reader (Sherwood Medical Industries, Montana). Samples were analysed within one week for pT_4, pT_3 and glucose. Once sampling had ended, the fish was sacrificed.

3.7 PLASMA ANALYSES

3.7.1 Thyroid hormones

Plasma T_4 and T_3 were analysed in duplicate by use of either a separate T_4 or T_3 radioimmunoassay (RIA) (Brown and Eales, 1977) or a combined T_4 / T_3 RIA which measures both T_4 and T_3 in the same plasma sample (Omeljaniuk *et al.*, 1984). In both assay types, Isolab Quik-Sep columns (8.0 cm x 1.0 cm) containing G-25 Sephadex (0.3 g per column) were used.

Working standards (20 ng T_4 or T_3/mL) were created by the evaporation of 0.5 mL of a stock solution (200.0 ng T_4 or

T_3/mL ethanol:ammonia, 99:1 (v/v)) followed by reconstitution with 5.0 mL of bovine serum albumin (BSA) buffer (KH_2PO_4 1.1998 g, $Na_2HPO_4 7H_2O$ 8.144 g, NaN_3 0.065 g, albumin fraction V 1.00 g / 1 L d/d water; pH 7.4). For the combined RIA, 0.5 mL of a stock solution containing 200 ng T_4/mL and 200 ng T_3/mL was used. Serial dilutions were then made with BSA buffer to yield 10.0, 5.0, 2.5, 1.25, 0.62 and 0.31 ng/mL standards. BSA buffer alone constituted 0 ng/mL standard. Volumes of 400 μL of each standard were pipetted into sample vials, sealed with Parafilm and frozen at -70°C until needed.

$[^{125}I]$ T_4 ($*T_4$; sp.ac. > 1200 μCi/μg) and $[^{125}I]$ T_3 ($*T_3$; sp.ac. > 1200 μCi/μg) were obtained from Amersham Searle (Oakville, Ontario).

Antibodies generated against T_4 and T_3 were obtained from Calchemical Lab. Supplies Ltd. (Calgary, Alberta).

Separate RIA analysis of pT_4 or pT_3

The separate T_4 and T_3 RIA is based on the ability of unlabeled hormone to displace labeled hormone from specific antibody binding sites. The assay is conducted on miniature G-25 Sephadex columns which, at the appropriate pH, bind T_4 and T_3 weakly but still permit interaction between hormone and the antibody sites. The free and antibody-bound hormone can be separated following incubation with antibody, since elution with buffer causes all antibody-bound hormone to be

eluted from the column, leaving the free hormone still weakly bound to the Sephadex. By counting the radioactivity eluted with the antibody, one can construct a standard curve and determine the hormone levels in plasma samples through interpolation.

In the assay, corrections are applied for the inorganic $^{125}I^-$ contamination of the radiolabeled T_4 and T_3 and for the non-specific (non-saturable) binding (NSB) of the labeled hormones in the antibody fraction. The advantage of this solid-phase procedure for thyroid hormones is that, prior to addition of antibody, both inorganic iodide and plasma proteins that bind T_4 and T_3 (which would interfere with T_4 or T_3 binding to the antibody) can be eluted.

<u>Separate RIA procedure</u>

1. The column sequence was randomized and the columns were drained of stored 0.1 N NaOH and recapped at the bottom.

2. Into each column, 100 µL of either $^*T_4$ ($5 - 7 \times 10^3$ cpm; T_4 RIA) or $^*T_3$ ($5 - 7 \times 10^3$ cpm; T_3 RIA) was pipetted. Similar volumes were added to 3 tubes (16 x 125 mm) containing 2.4 mL of either barbital buffer for the T_4 RIA (15.6 g barbital sodium C-IV, Fisher Scientific Co., in 1 L d/d water; pH 8.6) or phosphate buffer for the T_3 RIA (26.8 g sodium phosphate dibasic heptahydrate, 11.2 g disodium ethylenediamine tetraacetate in 1 L d/d water; pH 7.4). These tubes represented the total counts reference (TCR) of

the radioactivity used in each assay. The TCR comprises the actual level of labeled hormone used, together with contamination from inorganic iodide ($*I^-$).

3. Into each column, 100 µL of either standard (0 - 20.0 ng/mL) or plasma sample was added. Each standard was run in triplicate whereas each sample was run in duplicate.

4. Three additional columns designated for measurement of T_4 or T_3 radioiodide ($*I^-$) contamination, as well as three columns measuring T_4 or T_3 NSB received 100 µL of 0 ng/mL standard. The total volume added to each column was 200 µL.

5. The contents of each column were mixed by swirling and the bottoms were uncapped and drained to waste.

6. $*I^-$ was eluted from each column with 2.0 mL of barbital buffer (T_4 RIA) or phosphate buffer (T_3 RIA). All eluates were run to waste except for fractions from iodide columns which measured $*I^-$ contamination. These were collected in tubes and set aside for counting.

7. Tubes were placed under each column, followed by the addition of 0.5 mL of T_4 antibody (T_4Ab, 1:5500 in barbital buffer; T_4 RIA) or T_3 antibody (T_3Ab, 1:22500 in phosphate buffer; T_3 RIA). NSB columns received 0.5 mL of barbital (T_4 RIA) or phosphate (T_3 RIA) buffer only.

8. Columns were covered and incubated at room temperature for a minimum of 1.5 h (T_4 RIA) or 5 h (T_3 RIA) to allow interaction between the hormone and antibody.

9. The hormone/Ab complex was eluted with 2.0 mL of barbital (T_4 RIA) or phosphate (T_3 RIA) buffer, leaving free T_4 or T_3 bound to the Sephadex.

10. All tubes were sealed with Parafilm and counted to 2% error in a Beckman 5000 Gamma counter set for ^{125}I detection.

11. Columns were regenerated by washing off residual T_4 or T_3 with 8.0 mL of diluted human plasma (1:10 dilution with barbital (T_4 RIA) or phosphate (T_3 RIA) buffer) followed by 16.0 mL of d/d water and 4.0 mL of 0.1 N NaOH. Precautions were taken when using the human plasma. Columns were capped and stored in 0.1 N NaOH.

12. The eluant counted in the gamma counter represented the bound (B) fraction of thyroid hormone. The free (F) hormone remaining bound to the column Sephadex was calculated as the difference between the corrected TCR (inorganic iodide subtracted from TCR placed on each column) and the B fractions collected. After subtracting NSB from B for each standard or sample value, the B/F values were logit transformed, where logit B/F =

$$\ln\left[\frac{B/F}{[B/F]_0} \bigg/ 1-\frac{B/F}{[B/F]_0}\right]$$

and plotted against the logarithm of the hormone concentration (Fig. 3). From this standard regression, pT_4 and

pT_3 levels were determined by interpolation. All calculations were made with an RIA data processing system (Moncayo, 1984) run on an Apple IIc computer.

Combined RIA analyses of pT_4 and pT_3

For simultaneous measurement of T_4 and T_3 within one plasma sample, miniature Sephadex columns can be adapted to be used in a combination T_4/T_3 RIA. In this procedure, both labeled hormones are added onto the column along with the plasma samples or standards. Following elution of plasma proteins and inorganic iodide, T_4 antibody is added and allowed to incubate. Once the T_4/T_4Ab complex is eluted, T_3Ab is added and the T_3 RIA is conducted.

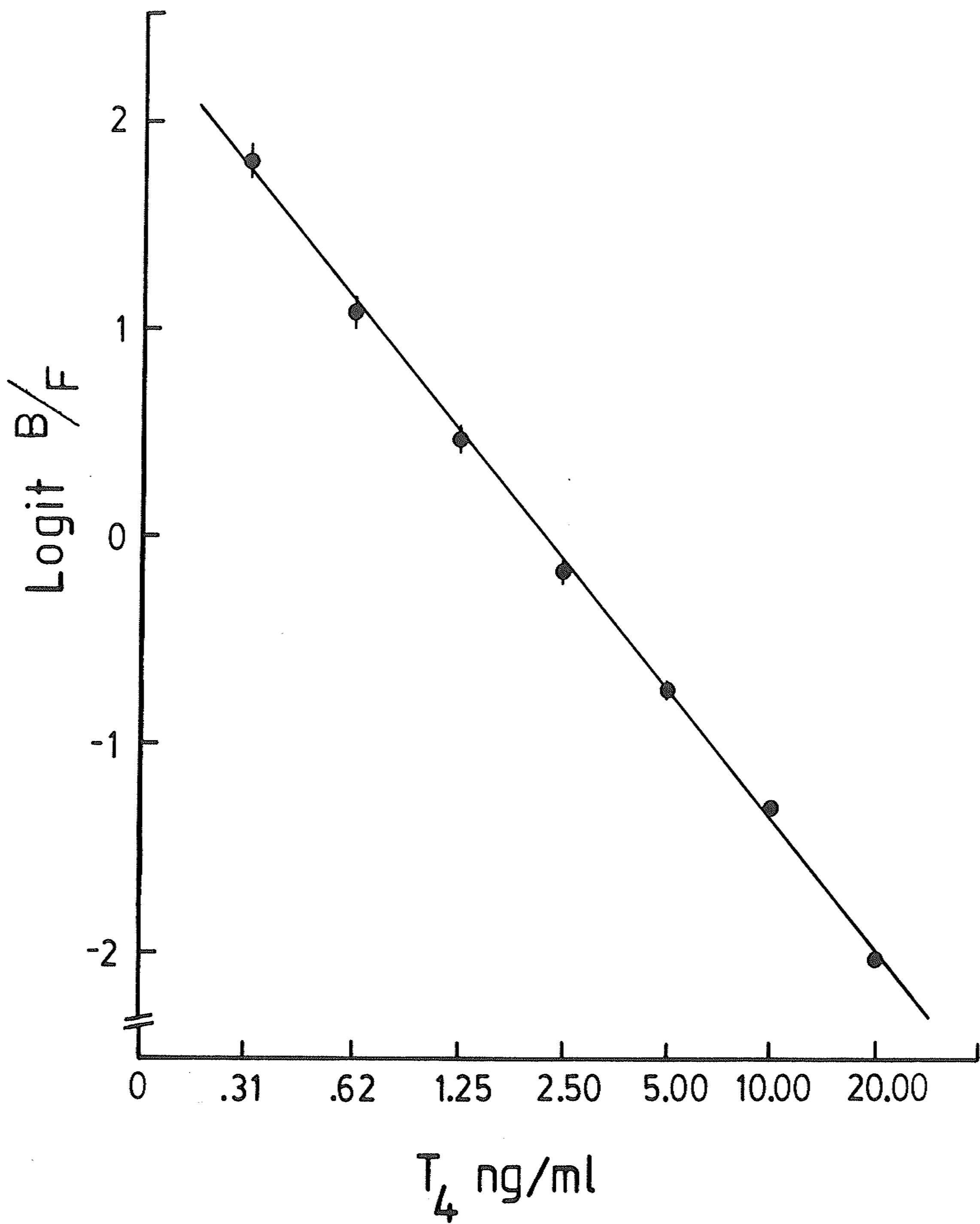
Combined RIA procedure

1. The column sequence was randomized and the columns were drained of stored 0.1 N NaOH and recapped at the bottom.

2. Into each column, 50 µL of $*T_4$ (5 - 7 x 10^3 cpm) and 50 µL $*T_3$ (5 - 7 x 10^3 cpm) were added with the exception of T_4 NSB and $*I^-$ columns (no $*T_3$ added) and T_3 NSB and $*I^-$ columns (no $*T_4$ added). To these columns 50 µL of 0 ng/mL standard was added.

3. Into each column, 100 µL of either standard (0 - 20.0 ng/mL) or sample was added. To all T_4 and T_3 NSB and $*I^-$ columns, 100 µL of 0 ng/mL standard was added. All

Figure 3: An example of an RIA regression for T_4 standard values. B/F values (logit transformed) were plotted against the logarithm of the T_4 concentration. Plasma T_4 was determined by interpolation. Vertical bars represent mean ± 1 SEM.



2
1
0
-1
-2
Logit B/F
0
.31
.62
1.25
2.50
5.00
10.00
20.00
T4 ng/ml

standards were measured in triplicate whereas all samples were measured in duplicate. The total volume added to each column was 200 μL.

4. The contents of each column were swirled and the bottoms were uncapped and drained to waste.

5. $*I^-$ was eluted from all columns with 2.0 mL barbital buffer. Fractions were collected in tubes from both T_4 and T_3 iodide columns and were set aside to be counted.

6. Tubes were placed under each column and 0.5 mL T_4Ab was pipetted onto each column with the exception of T_4 NSB columns which received 0.5 mL of barbital buffer only.

7. All columns were covered and incubated at room temperature for a minimum of 1.5 h to allow interaction between T_4 and antibody.

8. The T_4/T_4Ab complex was eluted with 2.0 mL barbital buffer, leaving free T_4 bound to the Sephadex. Fractions from all columns were collected in tubes and sealed with Parafilm. T_4 NSB columns were set aside. Tubes were counted at 2% error in a Beckman 5000 Gamma counter set for ^{125}I detection.

9. Tubes were placed under all columns and 0.5 mL T_3Ab was pipetted onto each column. T_3 NSB columns received 0.5 mL phosphate buffer only.

10. All columns were covered and incubated at room temperature for a minimum of 5 h to allow interaction between T_3 and antibody.

11. The T_3/T_3Ab complex was eluted with 2.0 mL phosphate buffer, leaving only free T_3 bound to the Sephadex. Fractions from all columns were collected in tubes, sealed with Parafilm and counted at 2% error in a Beckman 5000 Gamma counter set for ^{125}I detection.

12. Columns were regenerated in a manner similar to step 11 of the separate RIA procedure with the exception that only phosphate buffer was used.

13. Calculations were conducted in a similar manner to that for the separate RIA analysis.

To measure interassay variation, samples from a stock of pooled rainbow trout plasma were run in duplicate with each RIA performed. For 12 T_4 and T_3 assays, the coefficient of variation (CV%) was 8.47% and 4.48% respectively.

To measure intraassay variation, samples from a stock of pooled rainbow trout plasma were run on 20 randomly-selected columns. The CV% was 2.76% (T_4) and 1.69% (T_3).

3.7.2 **Glucose**

Plasma glucose concentration was measured in duplicate by use of Sigma test kit no. 510. To prevent possible glucose breakdown during the assay, all samples and testtubes were kept on ice.

Reagents

To prepare the peroxidase/glucose oxidase (PGO) enzyme solution, one capsule of PGO enzyme powder (Sigma) was added to 100 mL d/d water and placed in an amber bottle. To prepare reagent A, 1.6 mL of 0-dianisidine dihydrochloride (Sigma) was added to the 100 mL of enzyme solution. This reagent remained stable for approximately 3-4 weeks (or until a change in color occurred) and was stored in the dark at 0 - 5°C.

Assay Procedure

1. To polystyrene centrifuge tubes containing 270 µL of d/d water, a 30-µL aliquot of plasma was added.

2. After mixing, 100 µL of each diluted sample was pipetted into a tube containing 100 µL of d/d water.

3. Working standards, ranging from 0 to 400 mg/100 mL, were prepared in duplicate by serial dilution of a glucose

standard solution (GSS; 100 mg/dL, Sigma) with d/d water (see Appendix 2 for dilution procedure). These tubes were randomly distributed amongst the tubes containing diluted plasma samples from step 2.

4. To the first 20 tubes, 2.3 mL of reagent A was added followed by 5 sec of vortexing.

5. All 20 tubes were then incubated in a Metabolyte water bath (New Brunswick Sci. Co.) set at 37 ± 1°C and shaker speed 2.5 (80 rpm).

6. Every 5 min, for a total of 30 min, 20 additional tubes containing reagent A were added to the incubator.

7. At the end of a 30 min incubation, each set of 20 tubes was removed and the optical density (OD) of each sample was determined in disposable cuvets (10.0 x 10.0 x 45.0 mm) by use of a Spectronic 601 spectrophotometer (Milton Roy Co.) set at 450 nm.

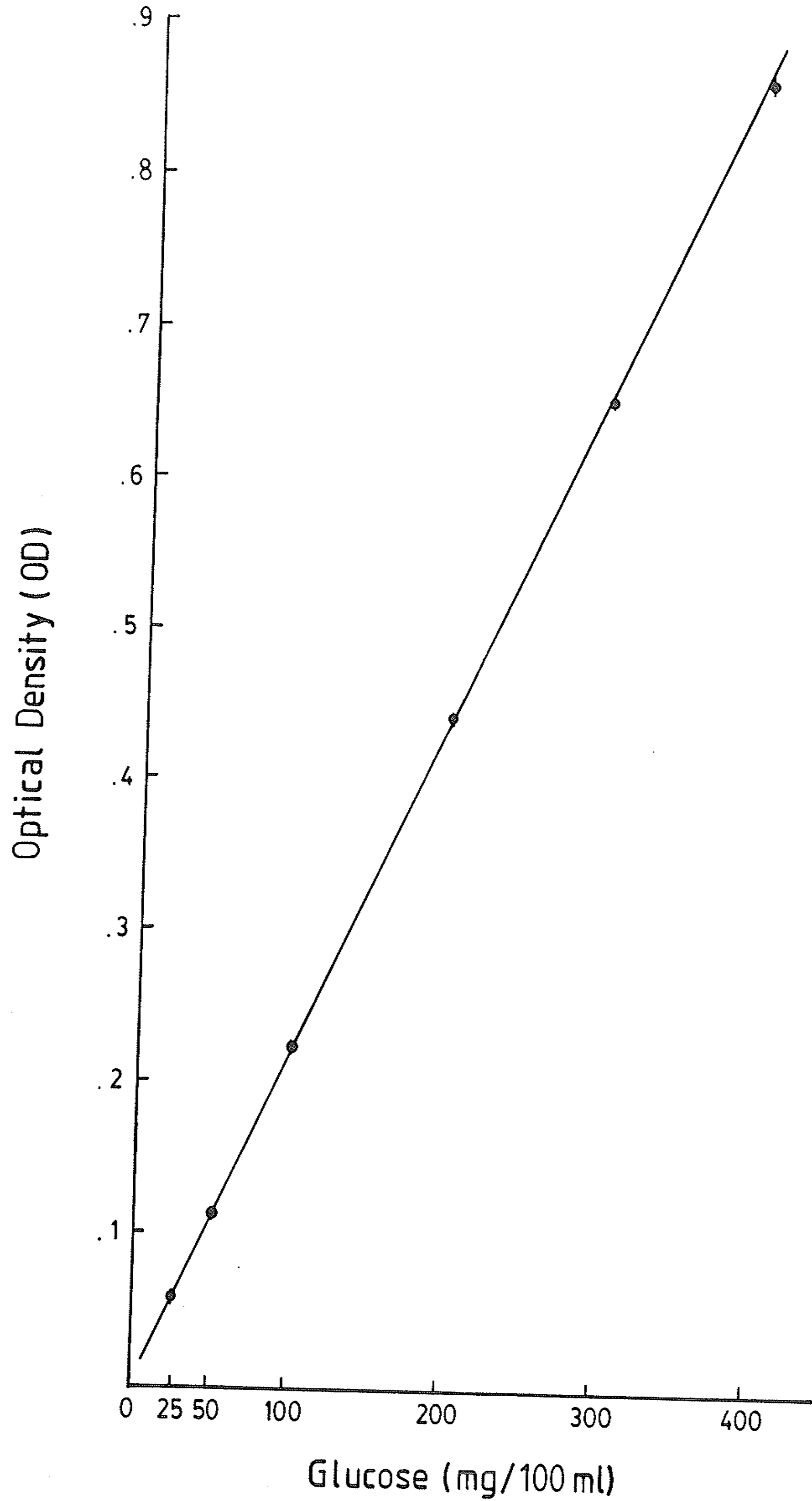
8. A linear regression of the mean OD against the glucose concentration (mg%) of each standard was run, where OD (y) represents the linear function of glucose concentration (x) (Fig. 4). The slope of the line was as follows:

$$y = Bo + B_1x,$$

where Bo represents the y intercept (value of y when x = 0) and B_1 represents the slope of the line. The glucose concentration in each plasma sample was determined by interpolation.

To monitor interassay variation, samples from a stock of pooled rainbow trout plasma were run in duplicate with each assay performed. For 12 assays, the CV% was 2.98%.

Figure 4: An example of a glucose standard regression. Optical density (OD) was plotted against glucose concentration. Plasma glucose was determined by interpolation. Vertical bars represent mean ± 1 SEM.



.9
.8
.7
.6
.5
.4
.3
.2
.1
Optical Density (OD)
0
25
50
100
200
300
400
Glucose (mg/100 ml)

3.8 STATISTICAL ANALYSES

Statistical analyses were applied using the Statistical Analysis System (SAS; SAS Inst. Inc., 1982) or a statistical data processing system (Moncayo, 1984). Differences within experimental groups of fish were measured by use of a one-way analysis of variance (ANOVA) or a two-way ANOVA with interaction. If a difference between means existed, a two-tailed Student's-t test or Tukey's standardized range test was applied. The statistical level for significance was considered as $p = 0.05$.

Because of variable dependence in samples obtained from cannulated trout, data were subjected to multivariate analysis of variance (MANOVA) with repeated measures and univariate (ANOVA) repeated measure tests. MANOVA tests were applied to test if an overall effect over time and within treatments existed with combined experimental and control data. Univariate repeated measures tests were employed to establish (i) differences over time in combined experimental and control data, and (ii) the difference over time due to treatment with experimental and control data analysed separately. Multiple comparisons were employed to establish the difference between each time interval and the initial time ($T = 0$) within both the control and experimental groups separately, and to examine the magnitude of difference between $T = 0$ and $T = n$ within control and experimental data.

Chapter IV

EXPERIMENTAL PROTOCOL AND RESULTS

4.1 ACUTE EFFECTS OF REFEEDING ON PLASMA T_4, T_3 AND GLUCOSE

Experiment 1

To establish the effects of acute starvation and refeeding on pT_4 and pT_3, 2 groups of 15 trout (mean wt. 43.0 g) were fed a 3% ration for 16 days and then starved (May). Mortality was 0%. On day 4 of starvation, one group of fish was refed a 3% ration. Both groups were sampled 4 h later.

Refeeding caused a significant two- to threefold increase in pT_4, as well as an elevation in pT_3 (Fig. 5 a).

In a second trial, 2 groups of 15 trout (mean wt. 106.0 g) were fed a 3% ration for 16 days and then starved (September). Mortality was 6.5%. On day 4 of starvation, one group of fish was refed a 3% ration. Both groups were sampled 4 h later.

As in the first trial, pT_4 was significantly increased with refeeding (Fig. 5 b). However, pT_3 was significantly decreased with food intake (Fig. 5 b).

It is concluded that refeeding starved trout causes a marked increase in pT_4 at 4 h. Food intake may cause an increment or decrement in pT_3.

Experiment 2

To establish a time profile of the acute effects of refeeding on pT_4, pT_3 and plasma glucose, 8 groups of 15 trout (mean wt. 46.8 g) were fed a 2% ration for 7 days and then starved (February). Mortality was 0%. On day 4 of starvation, 4 groups of fish were refed a 2.5% ration. Four other groups remained starved. Both starved and fed fish were sampled 2, 4, 6 or 8 h later.

A two-way ANOVA revealed a significant interaction between food intake and time only with respect to pT_4 ($p < 0.001$). PT_4 was elevated in fed trout at all time intervals relative to starved fish ($p < 0.001$) (Fig. 6 a). Multiple comparisons analysis (least-squared means) revealed that in fed trout, pT_4 was greater at 2 and 4 h as compared to 6 and 8 h ($p < 0.05$). In starved fish, pT_4 remained unchanged over all time intervals.

A significant difference in plasma glucose between fed and starved trout was present over all time intervals ($p < 0.001$) (Fig. 6 c). No interaction between treatment (fed or starved) and time on plasma glucose was present. PT_3 remained unchanged in both fed and starved fish over all time intervals.

Figure 5: The acute effects of refeeding on pT_4 and pT_3. Trout were starved for 3 days and refed a single meal (3%). Fish were sampled 4 h later. Plasma T_4 and pT_3 increased in trial A, whereas pT_4 increased and pT_3 decreased in trial B.

Vertical bars represent mean ± 1 SEM.

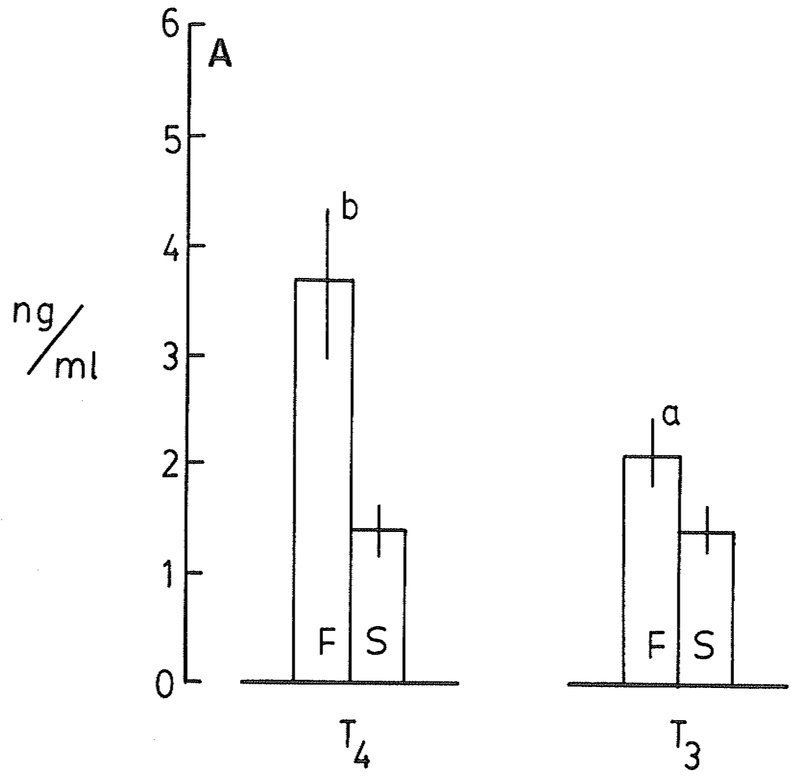
F = refed

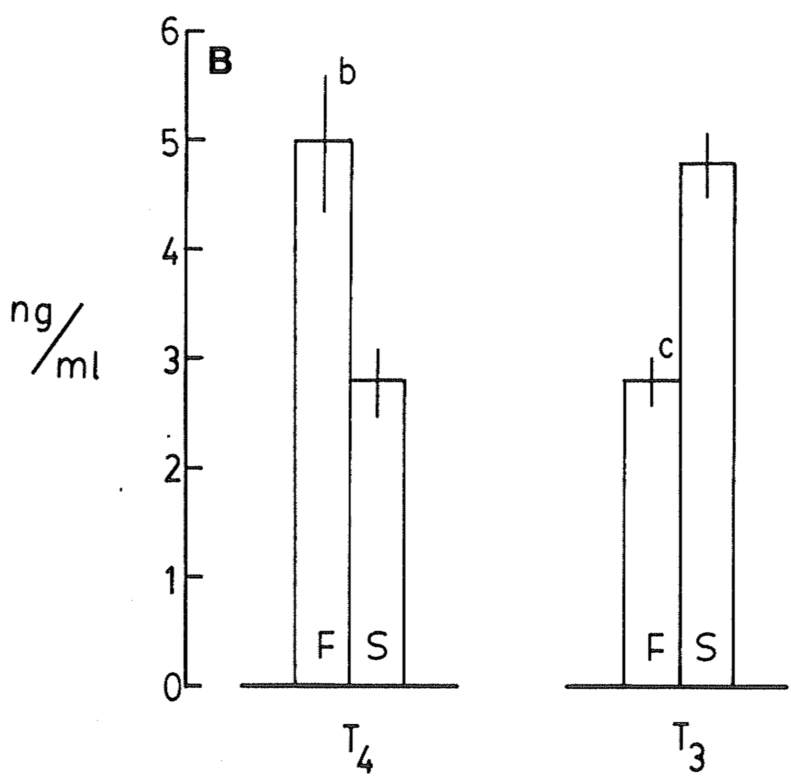
S = starved

a = $p < 0.05$ (relative to controls)

b = $p < 0.01$

c = $p < 0.001$


A
ng/ml
6
5
4
3
2
1
0
b
a
F
S
F
S
T_4
T_3


B
ng/ml
6
5
4
3
2
1
0
b
c
F
S
F
S
T_4
T_3

It is apparent that refeeding mediates an increase in plasma glucose and pT_4 within 2 h. At 4h pT_4 is maximal, after which levels decline. Such results establish the basis of the protocol employed in subsequent experiments where trout were starved for 3 days and pT_4 was examined 4 h following experimental manipulation.

Plasma glucose increased within 2 h of refeeding and remained elevated to 8 h. Plasma T_3 was not significantly altered over time with food intake (Fig. 6 b).

Figure 6: Time profile of the acute effects of refeeding on pT_4, pT_3 and plasma glucose. Trout were starved for 3 days and refed a single meal (2.5%). Fish were sampled 2, 4, 6 and 8 h later.

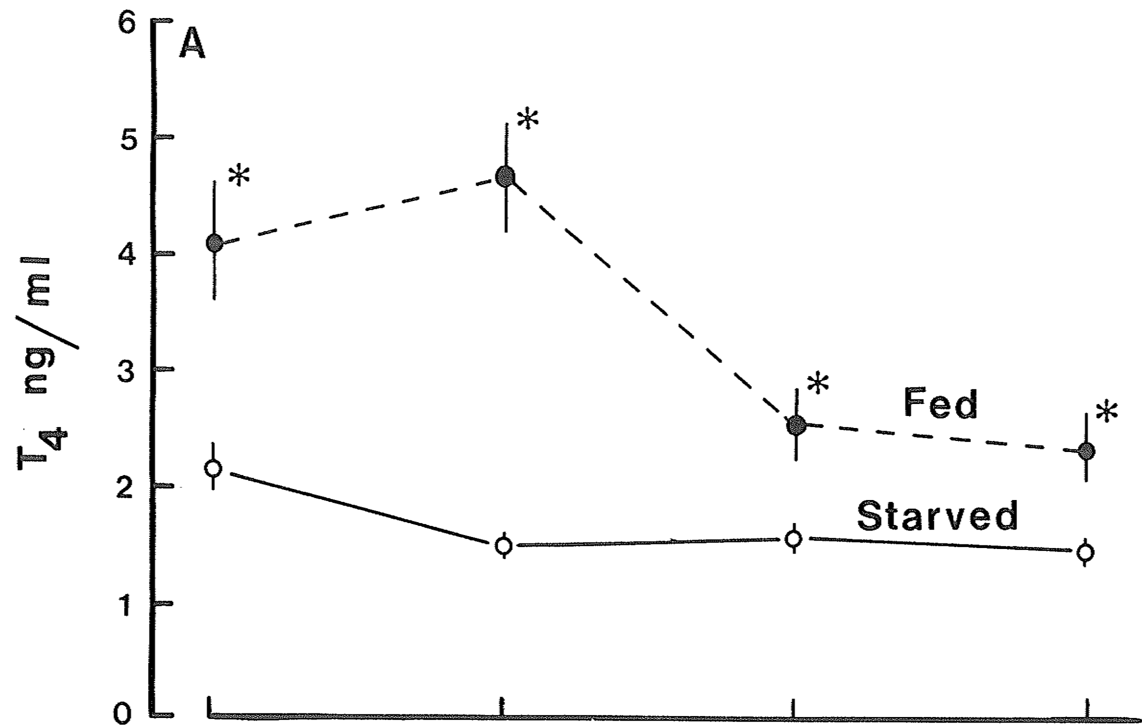
A. Plasma T_4 increased within 2 h in fed fish (- - ● - -) and peaked at 4 h, as compared to starved controls (——0——).

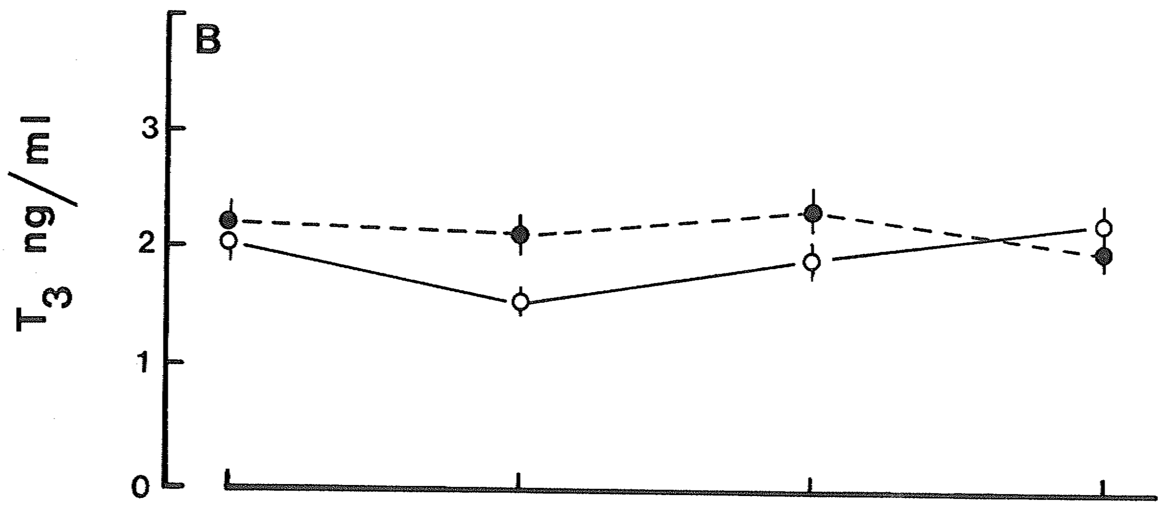
B. Plasma T_3 remained unaltered in fed and starved trout.

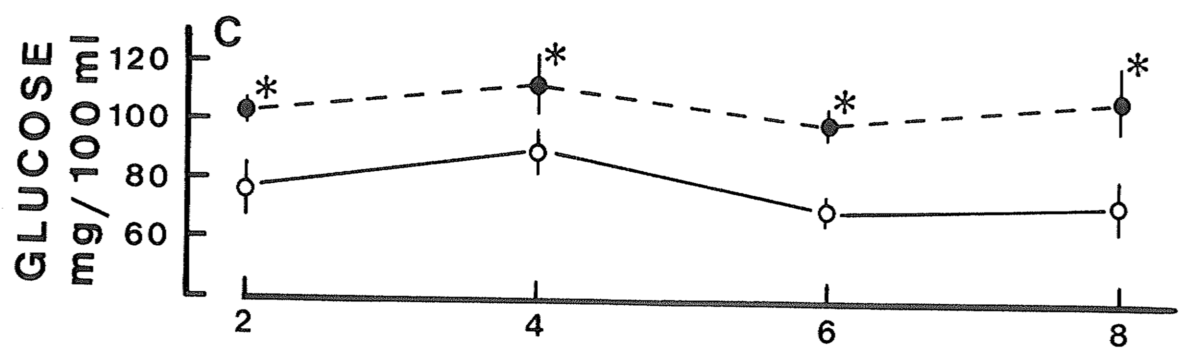
C. Plasma glucose increased within 2 h of refeeding and remained elevated to 8 h.

Vertical bars represent mean ± 1 SEM.

* = $p < 0.001$ (relative to controls)


A
T_4 ng/ml
6
5
4
3
2
1
0
Fed
Starved
*


B
T_3 ng/ml
3
2
1
0


C
GLUCOSE mg/100 ml
120
100
80
60
*
2
4
6
8
HOURS POST-REFEEDING

4.2 THE EFFECTS OF FOOD RATION ON PLASMA T_4 AND T_3

Experiment 3

To examine the effects of food quantity on pT_4, 4 groups of 15 trout (mean wt. 91.6 g) were fed a 3% ration for 8 days and then starved (January) (Trial 1). Mortality was 1.7%. On day 4 of starvation, 3 groups of fish were refed a quantitatively different ration (0.37, 0.71 and 3.57%). The fourth group served as starved controls. Fish were sampled 4 h later.

All rations caused a significant increase in pT_4 relative to starved controls ($F = 2.906$) (Table 1). Plasma T_3 was not altered with refeeding ($F = 2.310$), although with the greatest ration administered, trout exhibited the highest pT_3.

In trial 2, 4 groups of 15 trout (mean wt. 93.7 g) were fed a 3% ration for 9 days and then starved (February). Mortality was 3.3%. On day 4 of starvation, 3 groups of fish were refed a 0.11, 0.29 or 2.94% ration. The fourth group remained starved. Blood was collected 4 h after presentation of food.

Refeeding raised pT_4 only in trout fed the 2.94% ration ($p < 0.001$) (Table 1). Plasma T_3 was significantly increased only in fish fed 0.11% (Table 1). Plasma glucose was not significantly different between groups of trout (Table 1).

However, plasma glucose was considerably higher than normal levels of 50 to 70 mg/100 mL documented in trout by Thorpe and Ince (1976) and of trout refed in Experiment 2.

In a third trial, 4 groups of 15 large trout (mean wt. 268.1 g) were fed a 1% ration for 11 days and then starved (March). Prior to fasting, trout were occassionally observed to be gasping for air at the water surface. Mortality was 1.7%. On day 4 of starvation, 3 groups of trout were refed a 0.23, 1.33 or 1.92% ration. The fourth group served as starved controls. Blood was collected 4 h later.

Refeeding did not alter pT_4 or plasma glucose (Table 1). However, food intake did elevate pT_3 above control levels in trout fed the 0.23% ($p < 0.01$) and 1.33% ($p < 0.02$) rations (Table 1). Plasma glucose levels were unchanged in fed trout as compared to starved controls (Table 1).

In trial 4, 4 groups of 15 large trout (mean wt. 414.1 g) were fed a 1% ration for 10 days and then starved (May). As in trial 3, fish were observed to be gasping for air prior to experimental manipulation. Mortality was 10.0%. On day 4 of starvation, three groups of trout were refed a 0.45, 1.08 or 1.81% ration. A fourth group remained starved. Fish were sampled 4 h later.

Relative to starved controls, pT_4 was depressed in trout fed the 1.08% ration ($p < 0.001$), while pT_3 was decreased in trout fed the 0.45% ration ($p < 0.001$) (Table 1).

It is concluded that smaller trout (approximately 100 g) consistently exhibit an elevation in pT_4 when rations exceeding 0.37% bw are refed. In larger fish (>250 g), amounts of food up to 1.92% bw do not stimulate pT_4. Gasping for air by trout in trials 3 and 4 was probably not due to suspected excessive total chlorine in the city water supply, since analysis of chlorine during these periods revealed similar levels throughout all 4 feeding trials.

Inconsistent changes in pT_3 with refeeding suggest periperal alterations in TH metabolism which may be the result of food intake.

Table 1. Plasma T_4, T_3 and glucose levels in starved trout 4 h after refeeding varied food rations.

Trial	Mean Weight (g)	Food Eaten (% bw)	N	T_4 (ng/mL)	T_3 (ng/mL)	plasma glucose (mg/100 ml)
1	90.6	0	15	$1.4 \pm .23$	$2.3 \pm .17$	-----
	90.2	0.37	15	$2.4 \pm .24^c$	$2.3 \pm .15$	-----
	91.5	0.71	15	$2.1 \pm .26^a$	$2.4 \pm .22$	-----
	94.0	3.57	14	$2.3 \pm .31^a$	$3.0 \pm .35$	-----
2	92.6	0	15	$2.0 \pm .24$	$2.2 \pm .13$	147 ± 22
	95.6	0.11	15	$2.5 \pm .28$	$3.0 \pm .29^a$	135 ± 15
	96.3	0.29	15	$2.4 \pm .27$	$2.0 \pm .21$	173 ± 24
	90.1	2.94	13	$4.2 \pm .58^d$	$2.6 \pm .27$	176 ± 27
3	263.3	0	15	$1.4 \pm .26$	$2.3 \pm .23$	107 ± 17
	270.4	0.23	14	$1.6 \pm .24$	$3.8 \pm .49^c$	125 ± 11
	274.2	1.33	15	$2.0 \pm .32$	$3.6 \pm .43^b$	106 ± 4
	264.6	1.92	15	$1.4 \pm .32$	$3.1 \pm .29$	110 ± 13
4	420.1	0	15	$2.2 \pm .13$	$3.2 \pm .30$	-----
	409.7	0.45	12	$2.2 \pm .24$	$1.8 \pm .18^d$	-----
	416.9	1.08	13	$1.3 \pm .17^d$	$2.4 \pm .29$	-----
	409.7	1.81	14	$1.8 \pm .23$	$3.7 \pm .32$	-----

* Chlorine levels in the city water ranged from 250 to 340 ppb during all 4 feeding trials.

a = $p < 0.05$ (relative to controls)   c = $p < 0.01$

b = $p < 0.02$ d = $p < 0.001$

4.3 THE EFFECTS OF FOOD PERCEPTION ON PLASMA T_4 AND T_3

4.3.1 Food extract

Experiment 4

To establish the effects of a food extract on pT_4, 2 groups of trout (mean wt. 81.4 g) were fed a 3% ration for 14 days and then starved (August) (Trial 1). Mortality was 3.3%. On day 4 of starvation, either food extract (12°C) or a similar volume of water (12°C) was introduced into the inflow of one of two tanks. Both groups of trout were sampled 4 h later.

Despite feeding behaviour exhibited in trout receiving the food extract, both pT_4 and pT_3 remained unaltered relative to control fish (Table 2).

In a second trial, 3 groups of 15 trout (mean wt. 119.7 g) were fed a 3% ration for 9 days and then starved (January). Mortality was 2.2%. On day 4 of starvation, a similar volume of either food extract (12°C) or water (12°C) was added to the inflow of one of two tanks. Fish in the third tank remained undisturbed. Groups of fish were sampled 4 h later.

As in trial 1, no significant changes occurred in pT_4 after administration of food extract or water to fish

despite feeding behaviour exhibited in trout exposed to the extract (Table 2). Because of a problem with the T_4 RIA standards, pT_4 is represented as the counts per minute (cpm) in the antibody fraction.

It appears that the odor and taste of an extract from trout pellets do not mediate changes in either pT_4 or pT_3.

Table 2. Acute influence of fish food extract on pT_4 and pT_3 in starved trout.

Trial	Treatment	N	T_4 (ng/mL)	T_3 (ng/mL)
1	Water	15	$7.6 \pm .34$	$6.0 \pm .62$
	Extract	14	$7.3 \pm .82$	$4.7 \pm .57$
2	No Addition	14	15879 ± 88 (cpm)[1]	----
	Water	15	17921 ± 72	----
	Extract	15	15701 ± 66	----

[1] measured in counts per minute (cpm) as a result of a problem with RIA standards in the antibody fraction.

4.3.2 Sight of food

Experiment 5

To examine the acute effects of the sight of food on pT_4, 2 groups of 15 trout (mean wt. 189.2 g) were fed a 2% ration for 14 days and then starved (November). Mortality was 0%. On day 4 of starvation, plastic pellets equivalent by volume to a 2% ration were presented to one of the two groups of trout. Trout were sampled 4 h later.

Presentation of plastic pellets initiated feeding behavior, but did not cause a significant alteration in pT_4 or pT_3 (Table 3). Examination of the stomach and intestine revealed the presence of no plastic pellets suggesting that they were not ingested.

Unchanged pT_4 after refeeding plastic pellets suggests that sight is not involved in the elevation of pT_4 during food intake.

Table 3. Plasma T_4 and T_3 in starved trout exposed to simulated trout pellets made of plastic.

Treatment	N	T_4 (ng/mL)	T_3 (ng/mL)
Starved	15	$1.8 \pm .42$	$2.9 \pm .33$
Plastic pellets	14	$2.3 \pm .51$	$3.7 \pm .31$

4.4 THE EFFECTS OF DIETARY COMPOSITION ON PLASMA T_4, T_3 AND GLUCOSE

Experiment 6

To examine the effects of dietary CHO on pT_4, pT_3 and plasma glucose, 8 groups of 14 trout (mean wt. 69.4 g) were fed a 2.5% ration of trout pellets for 18 days and then starved (August). Mortality was 0%. On day 4 of starvation, 6 tanks of fish were refed a 2.5% isocaloric diet varying in both the type of CHO (cerelose or dextrin) and the CHO to lipid ratio. The six diets presented to trout were: low cerelose/high lipid, medium cerelose/medium lipid, high cerelose/low lipid, low dextrin/high lipid, medium dextrin/medium lipid and high dextrin/low lipid. Fish in two tanks remained starved and served as controls. Blood sampling occurred 4 h post-refeeding.

A one-way ANOVA revealed no significant differences between the two control groups with respect to pT_4, pT_3 and plasma glucose. These groups were therefore combined and treated as one group.

A significant difference between groups of fish was present with respect to pT_4 ($p < 0.001$), pT_3 ($p < 0.01$) and plasma glucose ($p < 0.001$). Multiple comparisons analysis (Tukey's studentized range test) of the means of each variable revealed that relative to starved controls (i) a significant difference in pT_4 of trout refed high and medium

dextrin and high cerelose existed ($p < 0.05$) (Fig. 7 a), (ii) plasma glucose was higher in fish refed both types of high and medium CHO diets ($p < 0.05$) (Fig. 7 b) and (iii) a significant elevation in pT_3 occurred only when trout were refed the high cerelose diet ($p < 0.05$) (Fig. 7 c).

A two-way ANOVA revealed no effect of CHO source on pT_4 or pT_3. However, a significant effect of CHO type was present on plasma glucose in fish ($p < 0.001$). The level of CHO refed influenced pT_4 ($p < 0.001$) and plasma glucose ($p < 0.001$) in trout but did not alter pT_3. No interaction existed between CHO source and level with respect to pT_4, pT_3 and plasma glucose.

It is evident that regardless of the CHO type refed to trout, the level at which dietary CHO is present dictates the degree to which pT_4 is altered. Similar isocaloric content within all diets refed also suggests that CHO is more important than energy intake in mediating a reversal of fasting-induced suppression of T_4.

Plasma T_3 is elevated only when a diet high in cerelose is refed.

Figure 7: Plasma T_4, T_3 and glucose of trout refed diets varying in CHO type and level. Trout were starved for 3 days and refed a single isocaloric meal (2.5%) of trout pellets varying in CHO type (cerelose or dextrin) and the CHO/lipid ratio. Fish were sampled 4 h later.

A. The degree to which pT_4 was elevated depended on the level of dietary CHO regardless of the type of CHO refed.

B. Plasma glucose levels were elevated according to the level of dietary CHO present.

C. Plasma T_3 was significantly elevated in trout refed HC only.

Vertical bars represent mean ± 1 SEM.

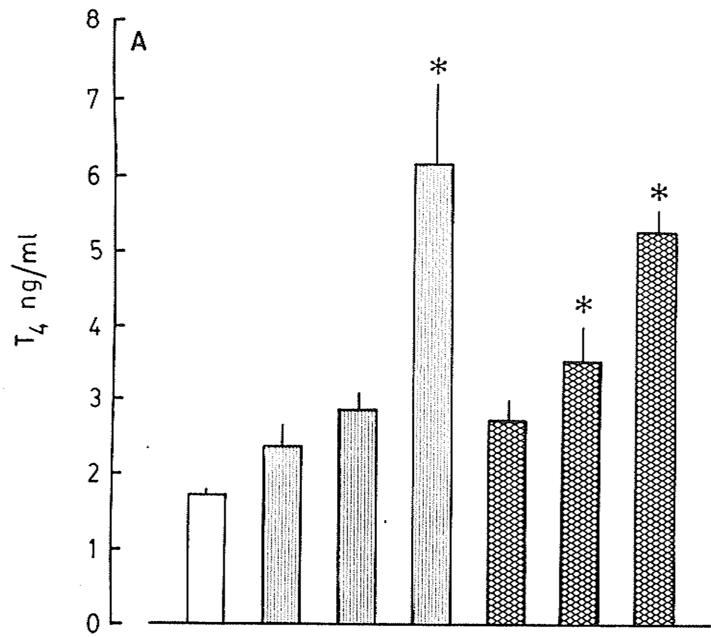
* = $p < 0.05$ (relative to controls)

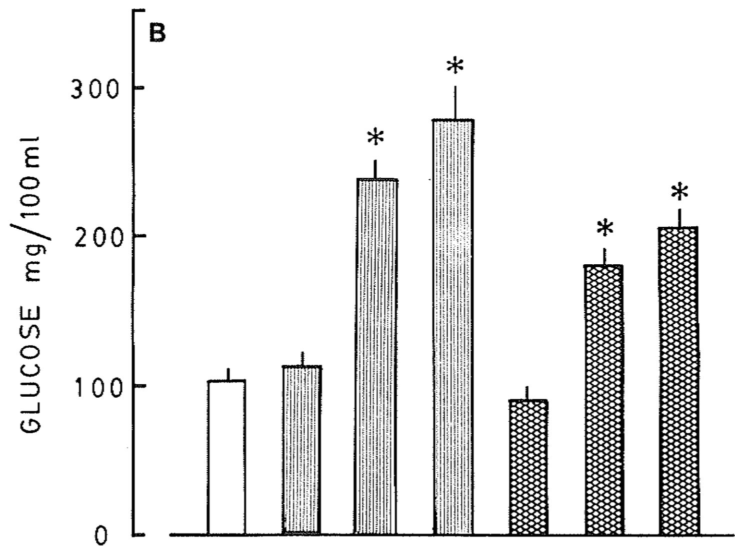
L = low

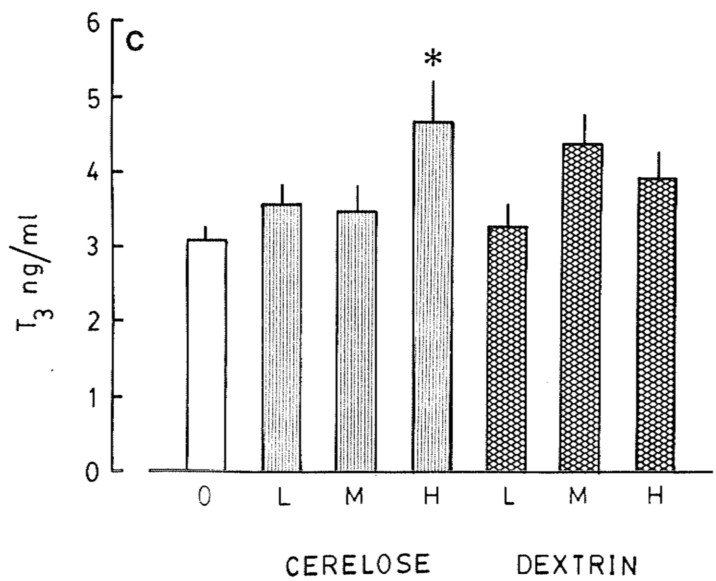
M = medium

H = high

(See Appendix 1 for proximate analysis of diets)


A
T_4 ng/ml
8
7
6
5
4
3
2
1
0
*
*
*


B
GLUCOSE mg/100ml
300
200
100
0
*
*
*
*


C
T_3 ng/ml
6
5
4
3
2
1
0
*
0
L
M
H
L
M
H
CERELOSE
DEXTRIN

4.5 RELATIONSHIP BETWEEN GLUCOSE AND PLASMA TH

Experiment 7

To examine the possible causal relationship between plasma glucose and pT_4, 3 groups of 15 trout (mean wt. 48.9 g) were fed a 3% ration for 7 days and then starved (July). Mortality was 0%. On day 4 of starvation, groups of trout were injected with 500 µL of either 0.7% NaCl or 0.7% NaCl containing D-glucose (0.2, 2.0 g/kg). Fish were sampled 4 h following injection.

Plasma T_4 appeared to increase with an increasing dose of glucose. However, neither pT_4 elevations nor pT_3 changes were statistically significant relative to saline-injected controls (Fig. 8 a).

In a second trial, 4 groups of 15 trout (mean wt. 46.7 g) were fed a 3% ration for 12 days and then starved (October). Mortality was 3.3%. On day 4 of starvation, groups of trout were injected with 100 µL of either D-glucose (0.2, 2.0 g/kg) in 0.7% NaCl or 0.7% NaCl alone. One group of fish remained undisturbed. Trout were sampled 4 h later.

Glucose caused an elevation in pT_4 with all doses administered (Fig. 8 b). However, pT_4 was significantly increased only in fish injected with 2.0 g glucose/kg ($p < 0.01$). As compared to saline-injected controls, pT_4 was significantly lower in undisturbed trout ($p < 0.05$). Such

observations support findings by Brown _et al_. (1978) that handling increases pT_4. However, such changes do not occur to the extent that a masking of further pT_4 elevation results. Glucose and handling did not appear to cause short-term alterations in pT_3 (Fig. 8 b).

It is concluded that graded doses of glucose (0.2 to 2.0 g/kg) result in an increase in pT_4. Glucose does not appear to cause acute alterations in pT_3.

Experiment 8

To examine the acute effects of glucose on pT_4, pT_3, plasma glucose and PCV over time, 15 trout (528.1 - 800.0 g) were cannulated in the dorsal aorta (June - July) and starved. On day 4 of starvation, a blood sample was withdrawn from each trout at 0h (0900 h). While the fish was under anesthetic, a volume of 0.7% saline (0.85 mL/kg) or 0.7% saline containing D-glucose (0.85 g/kg) was injected. Fish regained equilibrium within 20 min. Samples were collected hourly for 8 h (1600 h).

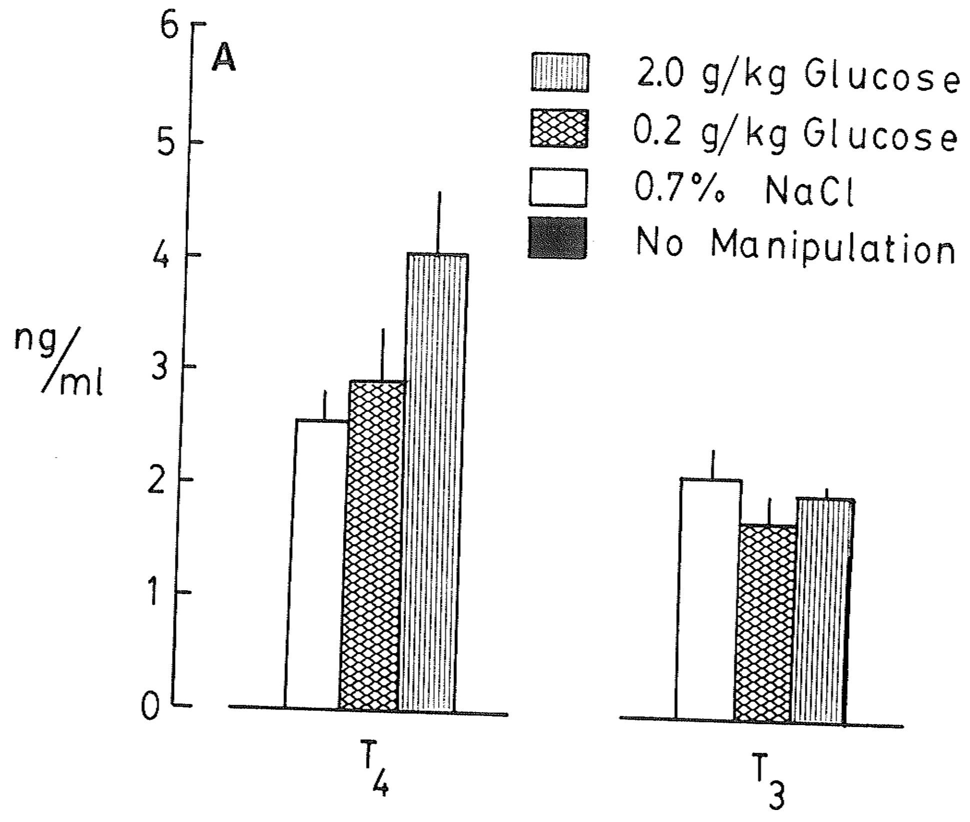
An overall difference between saline- and glucose-injected fish was present only with respect to pT_4 ($p < 0.002$) and plasma glucose ($p < 0.040$) (Fig. 9 a, c). Glucose administration caused a significant increase in plasma glucose at 1 h (156 mg/100 mL) as compared to 0 h (46 mg/100 mL), and remained elevated to 7 h (159 mg/100 mL).

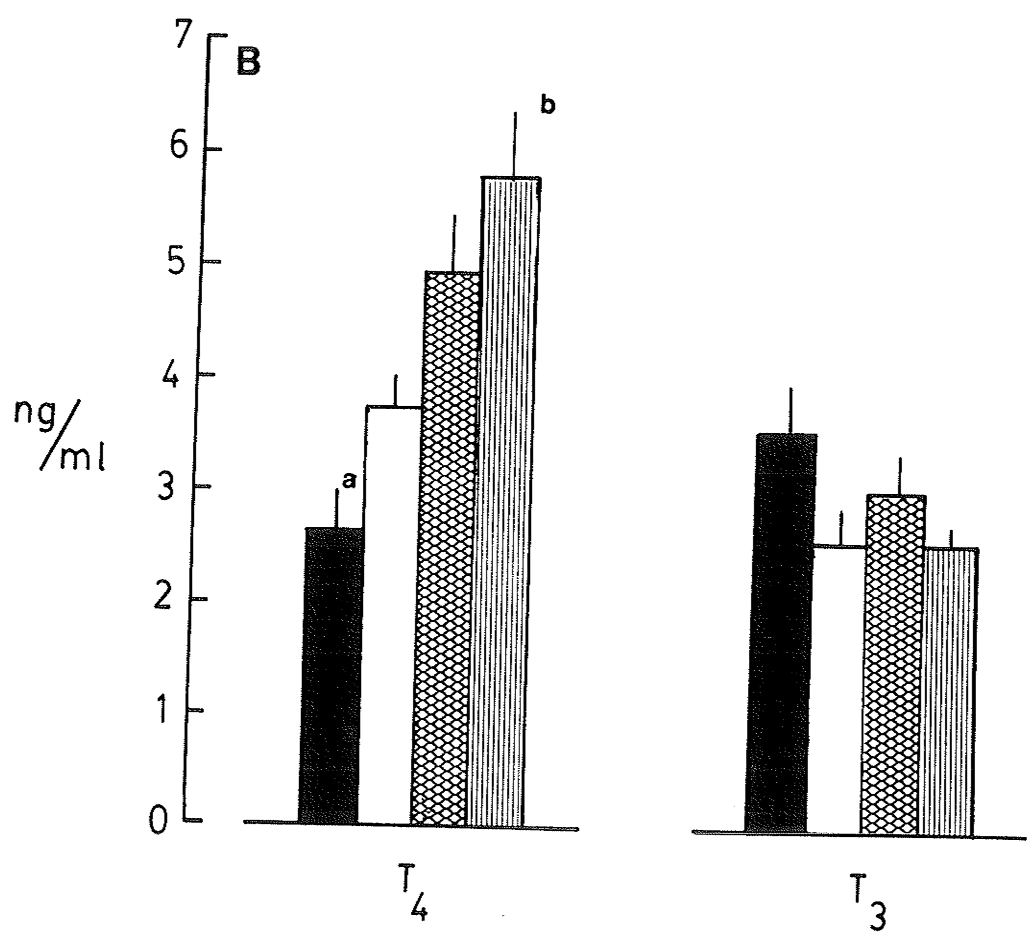
Figure 8: Plasma T_4 and T_3 at 4 h after D-glucose injection. Trout were starved for 3 days and ip injected with either 0.2 or 2.0 g/kg of glucose in 0.7% NaCl or 0.7% NaCl alone. Plasma T_4 was elevated in trial A and trial B 4 h after glucose administration. Plasma T_4 was significantly elevated in trout receiving saline injection as compared to undisturbed fish (trial B). Plasma T_3 remained unchanged in both trials.

Vertical bars represent mean ± 1 SEM.

a = $p < 0.05$ (relative to controls)

b = $p < 0.01$


A
2.0 g/kg Glucose
0.2 g/kg Glucose
0.7% NaCl
No Manipulation
ng/ml
0
1
2
3
4
5
6
T4
T3


B
ng/ml
0
1
2
3
4
5
6
7
a
b
T4
T3

Computed as the mean difference from time zero, pT_4 of glucose-injected fish exhibited a significant increase at 2 h (2.2 ng/mL) (or 1 h after plasma glucose levels had increased). At 3 h, pT_4 peaked (2.5 ng/mL), after which levels remained significantly elevated to 6 h (83 ng/mL).

Glucose injection did not significantly alter pT_3 and PCV (Fig. 9 b, d). Saline-injected fish showed no alterations over time in pT_4, plasma glucose, pT_3 or PCV.

Two cannulated control fish, which on day 4 recovered poorly from anesthetization and/or handling, were also sampled.

Recovering poorly from anesthetization, fish 1 (654.7 g) had approximately 500 μL of heparinized saline injected into its cannula at 1 h in an attempt to prevent blood clotting in the cannula. At 2 h, approximately 2.0 mL of blood was lost after hemostats punctured the cannula. As plasma glucose levels increased from 69 mg/100 mL (0 h) to 150 mg/100 mL (7 h), pT_4 increased from 1.8 ng/mL (0 h) to 5.2 ng/mL (7 h) (Fig. 10 a). Plasma T_3 remained unchanged throughout sampling (data not presented).

Fish 2 (605.5 g) also recovered poorly from experimental procedures, and was hyperactive during the period of sampling. It was also necessary to remove the tank lid several times in order to untangle the cannula which kept twisting and knotting. As with fish 1, an elevation in

Figure 9: Time profile of the acute effects of D-glucose on pT_4, pT_3, plasma glucose and PCV. Trout were cannulated in the dorsal aorta, starved for 3 days and ip injected with 0.85 mL/kg of 0.7% NaCl (n = 8)(- —0— -) or 0.7% NaCl containing glucose (0.85 g/kg) (n = 7) (——●——).

A. Plasma T_4 peaked within 2 to 4 h of glucose injection, after which levels began to decline.

B. Plasma T_3 remained unaltered in both saline- and glucose-injected trout.

C. Plasma glucose increased within 1 h after glucose injection and remained significantly elevated to 8 h.

D. PCV remained unchanged by both saline and glucose administration.

Vertical bars represent mean ± 1 SEM.

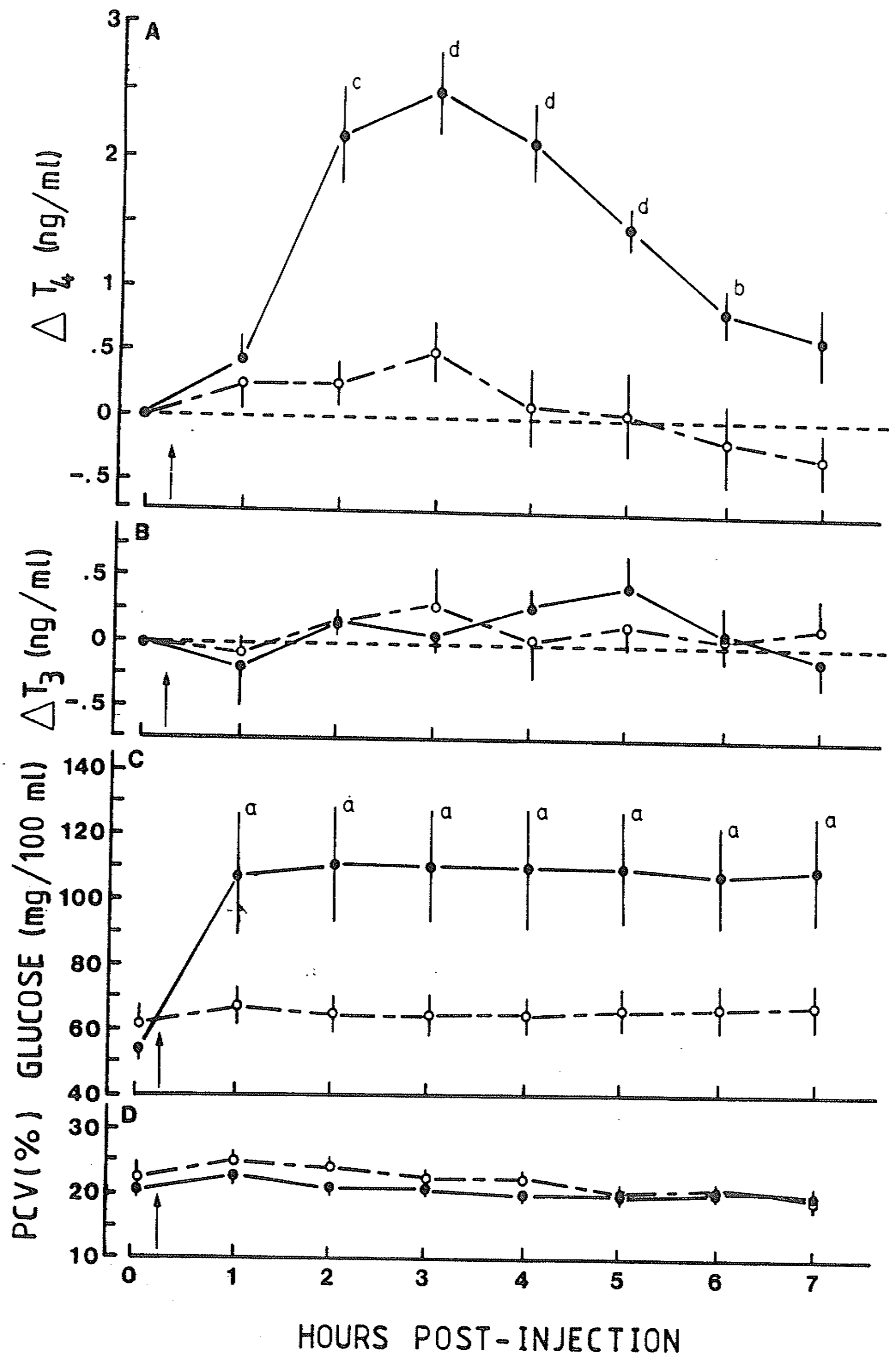
Arrows represent time of saline or D-glucose injection.

a = $p < 0.05$ (relative to 0 h)

b = $p < 0.02$

c = $p < 0.01$

d = $p < 0.001$


A
B
C
D
ΔT_4 (ng/ml)
ΔT_3 (ng/ml)
GLUCOSE (mg/100 ml)
PCV(%)
HOURS POST-INJECTION
a
b
c
d

plasma glucose from 147.6 mg/100 mL (0 h) to 192.0 mg/100 mL (4 h) was correlated with an increase in pT_4 from 2.61 ng/mL (0 h) to a maximum of 4.76 ng/mL (4 h) (Fig. 10 b). Plasma T_3 remained unchanged throughout sampling (data not presented).

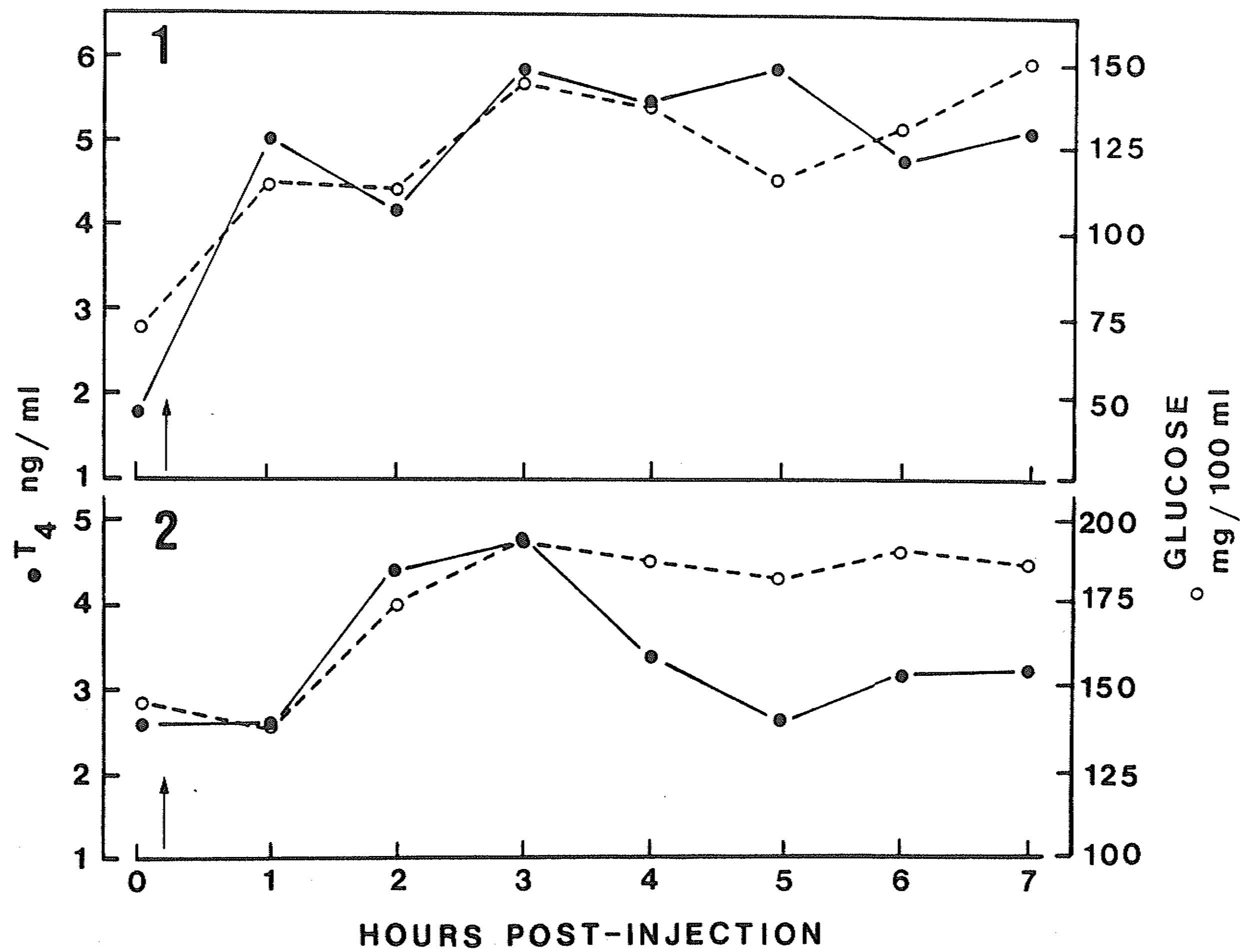
The relationship between endogenous plasma glucose and pT_4 was further examined by sampling two trout which were deliberately disturbed. Fish were cannulated (August) and starved. On day 4 of starvation, each trout was sampled at 0 h (0900 h) and then injected with 0.7% NaCl (0.85 mL/kg) while under anesthetic. Fish regained equilibrium within 20 min, after which blood samples were collected hourly for 8 h (1600 h). Fish were netted out of the water several times and disturbed within the tank during sampling.

Fish 1 (506.2 g) displayed a gradual increase in plasma glucose from 78 mg/100 mL (0 h) to 120 mg/100 mL (7 h) which was accompanied by an overall increase in pT_4 from 3.3 ng/mL (0 h) to 5.1 ng/mL (7 h) (Fig. 11 a).

Fish 2 (584.9 g) exhibited an elevation in plasma glucose at 0 h (60 mg/100 mL) to 7 h (93 mg/100 mL), with the greatest increase occurring between 2 h (74 mg/100 mL) and 4 h (93 mg/100 mL). Plasma T_4 rose during sampling, with the greatest increase also occurring between 2 h (2.5 ng/mL) to 4 h (5.0 ng/mL) (Fig. 11 b).

Figure 10: Plasma glucose and pT_4 in poorly-recovered cannulated trout. Saline-injected fish (1 and 2) which recovered poorly from anesthetization, handling and/or sampling showed rapid and parallel changes in plasma glucose (- - 0 - -) and T_4 (——●——).

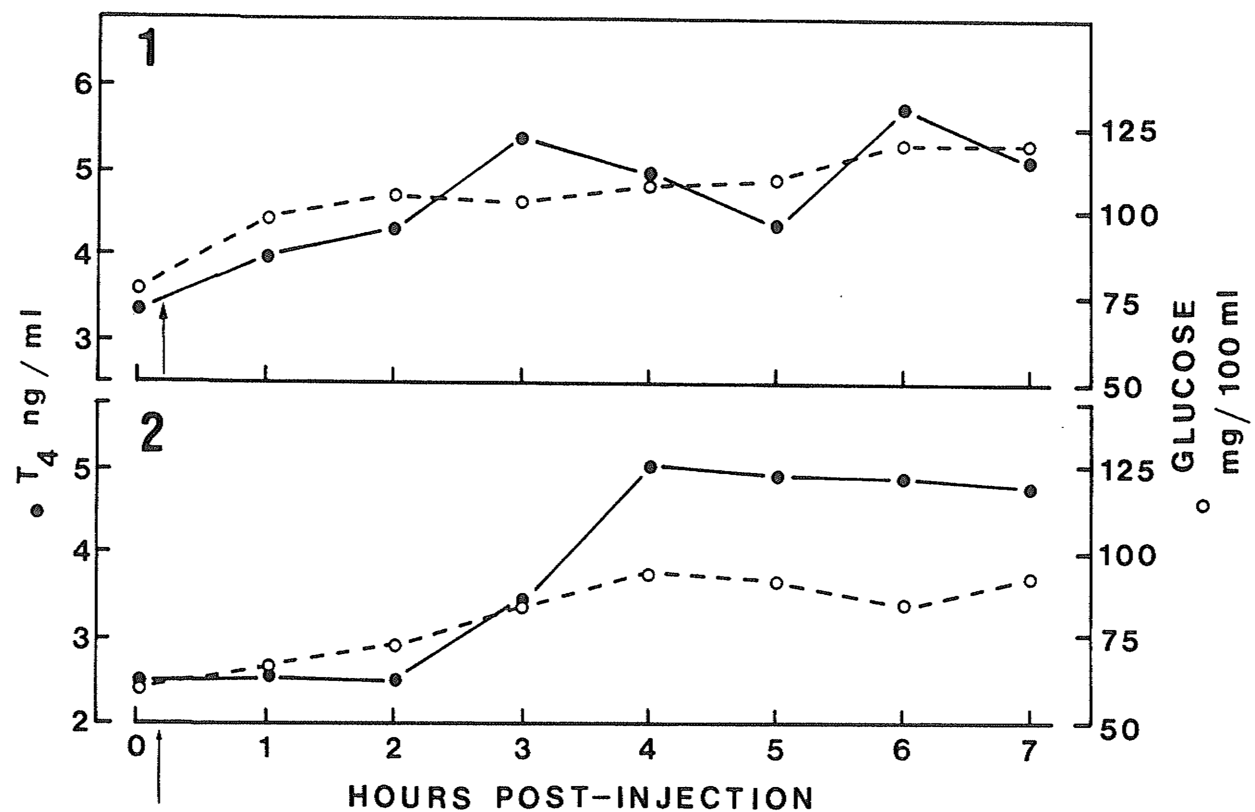
Arrows indicate time of saline injection.


1
2
6
5
4
3
2
1
5
4
3
2
1
150
125
100
75
50
200
175
150
125
100
0
1
2
3
4
5
6
7
HOURS POST-INJECTION
• T_4 ng / ml
○ GLUCOSE mg / 100 ml

It is evident that elevations in plasma glucose are accompanied by an increase in pT_4. Such a relationship appears to be causal, since an increase in pT_4 is preceeded by an elevation in plasma glucose. Glucose does not act to alter pT_3. Also apparent is that trout do not rapidly readjust plasma glucose levels and may remain hyperglycemic several hours after glucose-loading.

Figure 11: Plasma glucose and T_4 in deliberately disturbed cannulated trout. Increased plasma glucose levels (- - 0 - -) through netting and disturbance in saline-injected fish (1 and 2) are accompanied by an elevation in pT_4 (——●——).

Arrows indicate time of saline injection.


1
2
6
5
4
3
2
125
100
75
50
0
1
2
3
4
5
6
7
HOURS POST-INJECTION
• T_4 ng / ml
○ GLUCOSE mg / 100 ml

4.6 THE ACUTE EFFECTS OF D-GALACTOSE ON PLASMA T_4, T_3 AND GLUCOSE

Experiment 9

To examine the effects of a glucose stereoisomer on pT_4, pT_3, plasma glucose and PCV, trout (484.4 - 800.0 g) were cannulated (September - October) and then starved. On day 4 of starvation, fish were injected, while under anesthetic, with 0.85 mL/kg of 0.7% NaCl or 0.7% NaCl containing D-galactose (0.85 g/kg). Fish regained equilibrium within 20 min, after which blood samples were collected hourly for 8 h (1600 h).

An overall difference between saline- and galactose-injected trout existed only with respect to pT_4 ($p < 0.002$) (Fig. 12 a). When analysed as the mean difference from time zero, galactose caused a significant increase in pT_4 at 4 h (3.7 ng/mL), at which time pT_4 was also maximal. Plasma T_4 remained significantly elevated at 5 h (3.6 ng/mL) and 6 h (3.3 ng/mL).

Plasma glucose of fish 3 (October) was extremely high and as a result could not be measured accurately. High glucose levels may be due to the increased amount of dissolved chlorine (350 - 500 ppb) present in the city water at the time this particular trout was sampled. Glucose levels from fish 3 were therefore omitted from the calculated mean. In

the other two galactose-injected trout, as well as in control fish, plasma glucose showed no significant change over time (Fig. 12 c).

Plasma T_3 (Fig. 12 b) and PCV (Figure 12 d) were unaltered with time in both saline- and galactose-injected trout.

Galactose appears to cause an increase in pT_4 at 3 to 4 h post-injection. However, galactose does not alter plasma glucose, pT_3 and PCV following administration.

Figure 12: Time profile of the acute effects of D-galactose on pT_4, pT_3, plasma glucose and PCV. Trout were cannulated in the dorsal aorta, starved for 3 days and ip injected with 0.85 mL/kg of 0.7% NaCl (n = 8) (- --●-- -) or 0.7% NaCl containing D-galactose (0.85 g/kg) (n = 3) (——●——).

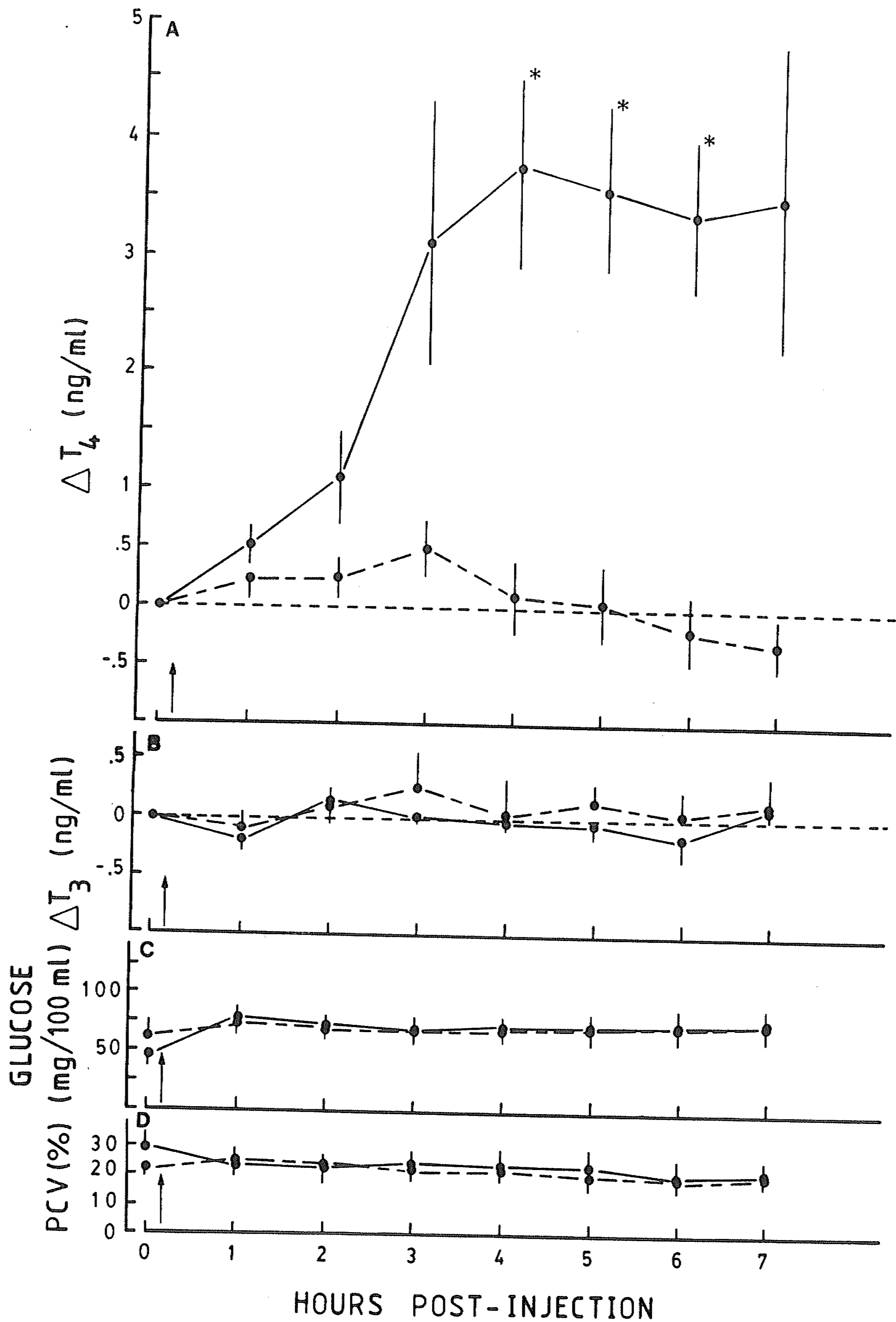
A. Plasma T_4 was elevated at 3 to 4 h and remained significantly increased until 6 h with galactose administration.

B,C and D. Plasma T_3, plasma glucose and PCV remained unaltered in both galactose- and saline-injected trout (n = 2 for glucose analysis).

Vertical bars represent mean ± 1 SEM.

Arrows represent time of saline or D-galactose injection.

* = $p < 0.05$ (in relation in 0 h)


A
B
C
D
ΔT_4 (ng/ml)
ΔT_3 (ng/ml)
GLUCOSE (mg/100 ml)
PCV (%)
HOURS POST-INJECTION
*
*
*

4.7 RELATIONSHIP BETWEEN GLUCOREGULATORY HORMONES, PLASMA T_4, T_3 AND GLUCOSE

Because of the insolubility of insulin and glucagon in physiological saline, the effects of an acetate buffer and a Tris/HCl buffer were examined on pT_4 before use as a vehicle for hormone injection.

Experiment 10

Three groups of 15 trout (mean wt. 47.4 g) were fed a 3% ration for 6 days and then starved (July). Mortality was 17.8%. On day 4 of starvation, trout were injected with 20 µL of either a 0.1 M acetate buffer (pH 3.27) or 0.05 M Tris/HCl buffer (pH 9.00). The third group of fish was not injected and remained undisturbed. Trout were sampled 4 h post-injection.

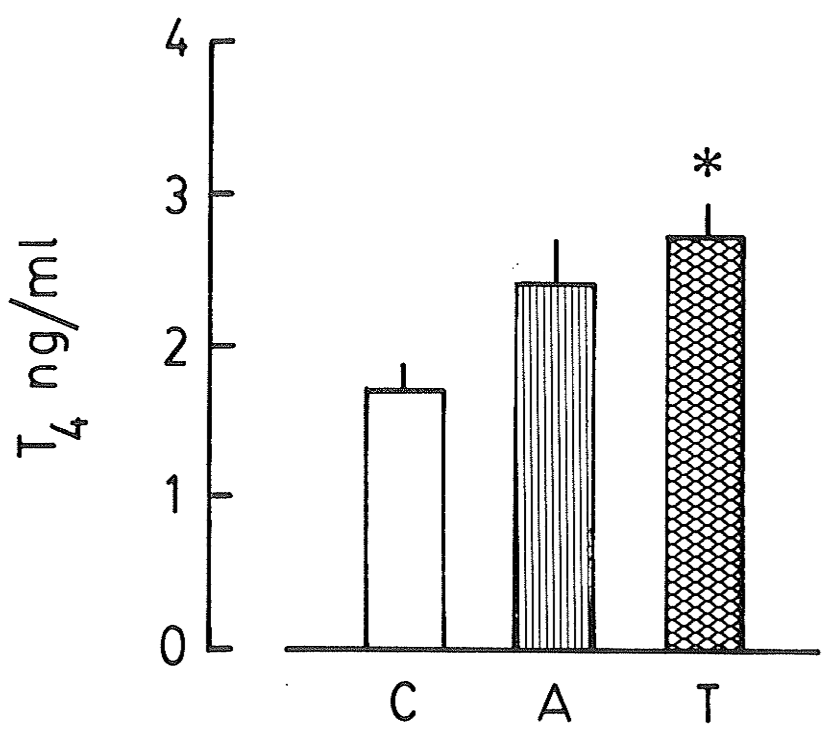
When compared to unhandled fish, pT_4 was significantly elevated in trout which were injected only with the Tris/HCl buffer ($p < 0.01$). Trout receiving the acetate buffer did not exhibit a significant increase in pT_4 (Fig. 13).

It is concluded that a 0.1 M acetate buffer (pH 3.27) is suitable for use as a vehicle for hormone injection into small fish where a small volume is required (Experiments 11 and 13). Acetate was not used to administer glucagon and insulin to cannulated trout in Experiments 12 and 14. Instead, a saline vehicle was employed due to a larger injection volume which was required (0.85 mL/kg).

Figure 13: The effects of an acetate and Tris/HCl buffer on pT_4. Trout starved for 3 days were ip injected with 20 µL of either 0.1 M acetate buffer (pH 3.27) (A) or 0.05 M Tris/HCl buffer (pH 9.00) (T). A third group of fish remained undisturbed (C). Only Tris/HCl caused a significant increase in pT_4 4 h after injection.

Vertical bars represent mean ± 1 SEM.

* = $p < 0.01$ (relative to controls)



T_4 ng/ml
4
3
2
1
0
*
C
A
T

4.7.1 Insulin

Experiment 11

To examine the effects of insulin on pT_4 and pT_3, 2 groups of 15 trout (mean wt. 47.2 g) were fed a 3% ration for 11 days and then starved (July) (Trial 1). Mortality was 0%. On day 4 of starvation, trout were injected with 20 µL of a 0.1 M acetate buffer (pH 3.27) or 0.1 M acetate buffer containing 10.0 IU bovine insulin/kg. Fish were sampled 4 h post-injection.

Insulin did not significantly alter pT_4. However, a decrease in pT_3 occurred ($p < 0.01$) (Table 4).

In trial 2, 3 groups of 15 trout (mean wt 113.6 g) were fed a 3% ration for 10 days and then starved (January). Mortality was 0%. On day 4 of starvation, trout were injected with 40 µL of a 0.1 M acetate buffer (pH 3.27) or 0.1 M acetate buffer containing 10.0 or 100.0 IU bovine insulin/kg. Trout were sampled 4 h later.

Trout injected with 10.0 IU/kg insulin exhibited a decrease in pT_4. An increase in pT_4 occurred with 100.0 IU insulin/kg injected, although levels were not significantly different from control fish. Plasma T_3 remained unaltered by both doses of insulin administered (Table 4).

In a third trial, 4 groups of 16 trout (mean wt. 41.0 g) were fed a 3% ration for 10 days and then starved (May).

Mortality was 1.6%. On day 4 of starvation, trout were injected with 20 μL of a 0.1 M acetate buffer (pH 3.27) or 0.1 M acetate buffer containing 10.0, 55.0 or 100.0 IU bovine insulin/kg. Sampling occurred 4 h post-injection.

An increase in pT_4 occurred in trout injected with both 55.0 ($p < 0.01$) and 100.0 ($p < 0.01$) IU insulin/kg (Table 4). Relative to acetate-injected controls, pT_4 did not change in fish receiving 10.0 IU/kg insulin (Table 4). Plasma T_3 remained unchanged with all doses of insulin (Table 4). Plasma glucose decreased with all insulin doses injected (10.0 IU/kg, $p < 0.001$; 55.0 IU/kg, $p < 0.01$; 100.0 IU/kg, $p < 0.05$) (Table 4).

Insulin may act in a biphasic fashion to alter pT_4. At 10.0 IU/kg, pT_4 is decreased in trout (trials 1 and 2), whereas at a higher dose pT_4 becomes elevated. Plasma T_3 is not altered in a consistent manner with insulin and usually does not change. Insulin was most effective in suppressing plasma glucose with the lowest dose (10.0 IU/kg) administered. At elevated doses of insulin, other factors (such as glucagon) may interact to regulate physiological actions of the hormone.

Experiment 12

To examine the acute effects of insulin on pT_4, pT_3, plasma glucose and PCV over time, trout (426.9 - 800.0 g)

Table 4. Plasma levels of T_4, T_3, and glucose in starved trout 4 h after bovine insulin injection.

Trial	Mean Weight (g)	Dose (IU/kg)	N	T_4 (ng/mL)	T_3 (ng/mL)	plasma glucose (mg/100 mL)
1	49.5	acetate	15	2.9 $\pm$ .45	3.1 $\pm$ .24	-----
	44.8	10.0	15	2.0 $\pm$ .19	1.9 $\pm$ .30^{b}	-----
2	111.3	acetate	15	4.6 $\pm$ .47	3.5 $\pm$ .49	-----
	111.1	10.0	15	3.4 $\pm$ .31^{a}	2.9 $\pm$ .43	-----
	118.3	100.0	15	5.9 $\pm$ .48	2.5 $\pm$ .13	-----
3	42.1	acetate	16	2.9 $\pm$ .30	2.2 $\pm$ .26	109 $\pm$ 12
	38.8	10.0	15	3.3 $\pm$ .36	2.8 $\pm$ .45	56 $\pm$ 3^{c}
	39.7	55.0	16	4.4 $\pm$ .36^{b}	2.4 $\pm$ .23	73 $\pm$ 2^{b}
	41.2	100.0	16	4.8 $\pm$ .44^{b}	2.3 $\pm$ .22	82 $\pm$ 5^{a}

a = $p < 0.05$ (relative to controls)

b = $p < 0.01$

c = $p < 0.001$

were cannulated and then starved (January). On day 4 of starvation, each trout was sampled at 0 h (0900 h) and injected (0.85 mL/kg), while under anesthetic, with 0.7% NaCl or 0.7% NaCl containing 10.0 IU bovine insulin/kg (in suspension). Fish regained equilibrium after 20 min. Samples were collected hourly for 8 h.

Univariate analysis (with repeated measures) revealed a significant overall difference between saline- and insulin-injected trout with respect to pT_4 and plasma glucose only (Fig. 14 a,c). High basal plasma glucose in insulin-treated trout may have contributed to significant differences between the control and insulin-treated trout.

Over time, trout injected with insulin exhibited an increase in pT_4 ($p < 0.020$). When analysed as the mean difference from time zero, pT_4 increased significantly at 2 h (1.7 ng/mL) and peaked at 4 h (2.6 ng/mL) to 5 h (2.6 ng/mL). At 7 h (1.6 ng/mL) pT_4 remained significantly elevated as compared to 0 h.

PCV was altered over time ($p < 0.004$) with insulin administration; however, a significant decrease occurred only at 7 h (Fig. 14 d).

Plasma glucose was altered over time within both insulin- ($p < 0.009$) and saline- ($p < 0.030$) injected fish. Insulin caused a significant decrease in plasma glucose from 4 h (175 mg/100 mL) to 7 h (149 mg/100 mL) relative to 0 h (197

mg/100 mL). Glucose was significantly elevated in control fish through 1 h (75 mg/100 mL) to 7 h (75 mg/100 mL) as compared to 0 h (63 mg/100 mL).

Plasma T_3 remained unaltered within both experimental and control fish (Fig. 14 b).

Bovine insulin may cause an acute elevation in pT_4 within 2 to 3 h. However, high basal glucose levels in several experimental trout may have influenced insulin actions. A delay in plasma glucose suppression occurred with insulin administration. Insulin did not alter pT_3 and decreased PCV at 7 h post-injection only.

Figure 14: Time profile of the acute effects of insulin on pT_4, pT_3, plasma glucose and PCV. Trout were cannulated in the dorsal aorta, starved for 3 days and then ip injected with 0.85 mL/kg of 0.7% NaCl (n = 9) (- —0— -) or 0.7% NaCl containing 10.0 IU bovine insulin/kg (n = 7) (——●——).

A. Relative to T = 0 h, insulin caused an increase in pT_4 at 2 h which peaked within 3 to 5 h.

B. Plasma T_3 remained unaltered in both insulin- and saline-injected trout.

C. Plasma glucose was significantly decreased with insulin at 4 to 7 h, whereas in control fish glucose became elevated.

D. Insulin injected trout exhibited a decrease in PCV at 7 h only.

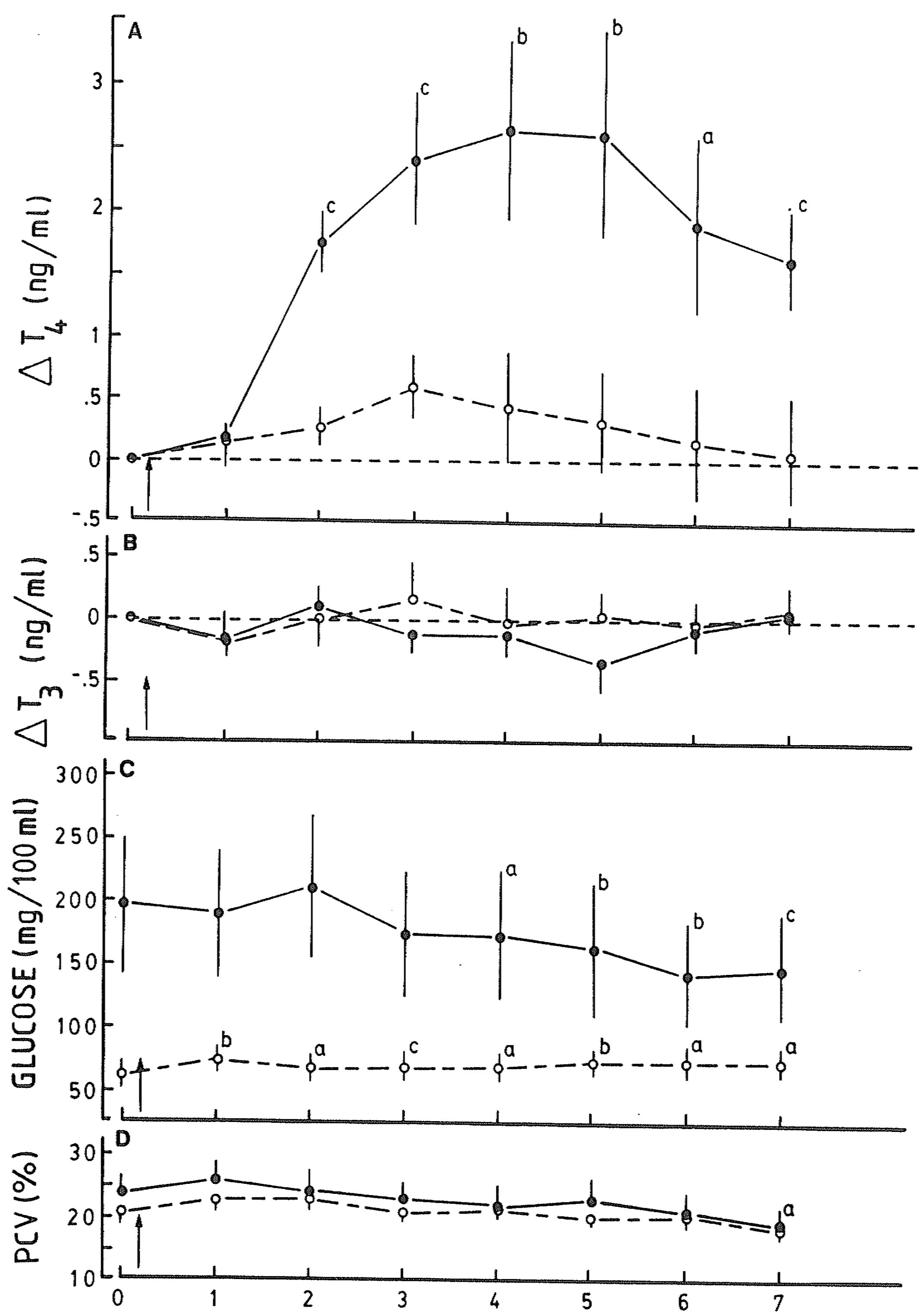
Vertical bars represent mean ± 1 SEM.

Arrows represent time of saline or insulin injection.

a = $p < 0.05$ (relative to 0 h)

b = $p < 0.02$

c = $p < 0.01$


A
B
C
D
ΔT_4 (ng/ml)
ΔT_3 (ng/ml)
GLUCOSE (mg/100 ml)
PCV (%)
HOURS POST-INJECTION

4.7.2 Glucagon

Experiment 13

To examine the acute effects of glucagon on pT_4 and pT_3, 2 groups of 15 trout (mean wt. 69.7 g) were fed a 3% ration for 7 days and then starved (May) (Trial 1). Mortality was 0%. On day 4 of starvation, fish were injected with 20 µL of a 0.1 M acetate buffer (pH 3.27) or a 0.1 M acetate buffer containing a mixture of 0.69 µg bovine and porcine glucagon/g. Trout were sampled 4 h post-injection.

Glucagon did not significantly alter pT_4 or pT_3 as compared to acetate-injected controls (Table 5).

In trial 2, 3 groups of 15 trout (mean wt. 61.9 g) were fed a 3% ration for 10 days and then starved (July). Mortality was 2.2%. On day 4 of starvation, fish were injected with 20 µL of a 0.1 M acetate buffer (pH 3.27) or a 0.1 M acetate buffer containing a mixture of 0.081 or 0.333 µg bovine and porcine glucagon/g. Trout were sampled 4 h post-injection.

As in trial 1, no significant changes occurred in pT_4 or pT_3 relative to acetate-injected controls (Table 5).

In a third trial, 3 groups of 15 trout (mean wt. 63.4 g) were fed a 1% ration for 17 days and then starved (March). Mortality was 2.2%. On day 4 of starvation, fish were injected with 20 µL of a 0.1 M acetate buffer (pH 3.27) or a

0.1 M acetate buffer containing a mixture of 0.143 µg bovine and porcine glucagon/g. A third group of fish remained undisturbed. Blood was collected 4 h post-injection.

Relative to acetate-injected trout, pT_4 was not altered with glucagon (Table 5). However, pT_4 was significantly lower in unhandled fish as compared to acetate controls ($p < 0.001$). No alterations occurred between groups of fish with respect to pT_3 or plasma glucose (Table 5).

Glucagon at doses of 0.08 to 0.69 µg/g does not appear to alter plasma glucose (trial 3), pT_4 or pT_3 at 4 h.

Experiment 14

To examine the acute effects of glucagon over time on pT_4, pT_3, plasma glucose and PCV, trout (458.2 - 800.0 g) were cannulated and then starved (January - Febuary). On day 4 of starvation, each trout was sampled at 0 h (0900 h) and injected (0.85 mL/kg), while under anesthetic, with 0.7% NaCl or 0.7% NaCl containing a suspended mixture of 4.0 µg bovine and porcine glucagon/g. Fish regained equilibrium after 20 min. Blood samples were collected hourly for 8 h (1600 h).

Univariate analysis (with repeated measures) revealed a significant difference between saline and glucagon-injected trout only with respect to pT_4 ($p < 0.030$) (Fig. 15 a).

Table 5. The effects of bovine and porcine glucagon on plasma T_4, T_3 and glucose in starved trout 4 h post-injection.

Trial	Mean Weight (g)	Dose (µg/g)	N	T_4 (ng/mL)	T_3 (ng/mL)	plasma glucose (mg/100 mL)
1	40.3	acetate	14	$5.3 \pm .62$	$3.1 \pm .20$	-----
	41.6	0.69	14	$4.9 \pm .70$	$3.6 \pm .39$	-----
2	63.9	acetate	15	$2.9 \pm .50$	$2.7 \pm .28$	-----
	61.9	0.08	15	$2.5 \pm .36$	$2.4 \pm .20$	-----
	60.0	0.33	14	$2.2 \pm .56$	$2.5 \pm .21$	-----
3	64.6	unhandled	15	$0.8 \pm .08^a$	$4.1 \pm .30$	105 ± 21
	62.0	acetate	14	$3.0 \pm .42$	$4.5 \pm .46$	147 ± 25
	67.3	0.14	15	$2.0 \pm .36$	$3.7 \pm .39$	157 ± 25

a = $p < 0.001$ (relative to controls)

Over time, glucagon-treated fish exhibited a significant elevation in pT_4 ($p < 0.040$). Plasma T_4 was increased at 3 h (2.5 ng/mL) and remained elevated until 5 h (2.1 ng/mL).

Plasma glucose was altered over time in both saline- ($p < 0.020$) and glucagon- ($p < 0.020$) injected trout (Fig. 15 c). Glucagon caused a significant glucose elevation at 1 h (111 mg/100 mL) as compared to 0 h (82 mg/100 mL) (Fig. 15 c). Levels remained elevated over time until 7 h (147 mg/100 mL). Relative to values at 0 h (61 mg/ 100 mL), plasma glucose levels were increased with saline administration at 1 h (73 mg/100 mL) and remained elevated to 7 h (73 mg/100 mL) (Fig. 15 c).

Glucagon also caused a significant decrease in PCV over time ($p < 0.002$) (Fig. 15 d). However, PCV values were not significantly different from those of control fish.

Plasma T_3 remained unaltered over time in both glucagon- and saline-treated fish (Fig. 15 b).

It is concluded that glucagon at a dose of 4.0 μg/g causes a elevation in plasma glucose at 1 h followed by a significant increase in pT_4 at 2 h. Glucagon does not alter pT_3. However, a decrease in PCV occurs over time.

Figure 15: Time profile of the acute effects of glucagon on pT_4, pT_3, plasma glucose and PCV. Trout were cannulated in the dorsal aorta, starved for 3 days and then ip injected with 0.85 mL/kg of 0.7% NaCl (n = 10) (- —0— -) or 0.7% NaCl containing 4.0 µg bovine and porcine glucagon/g (n = 7) (——●——).

A. Plasma T_4 increased at 2 h and peaked at 3 h after glucagon administration.

B. Plasma T_3 remained unaltered in both saline- and glucagon-injected trout.

C. Glucose levels were significantly increased over time with both glucagon and saline injection.

D. PCV was altered over all time intervals with glucagon administration.

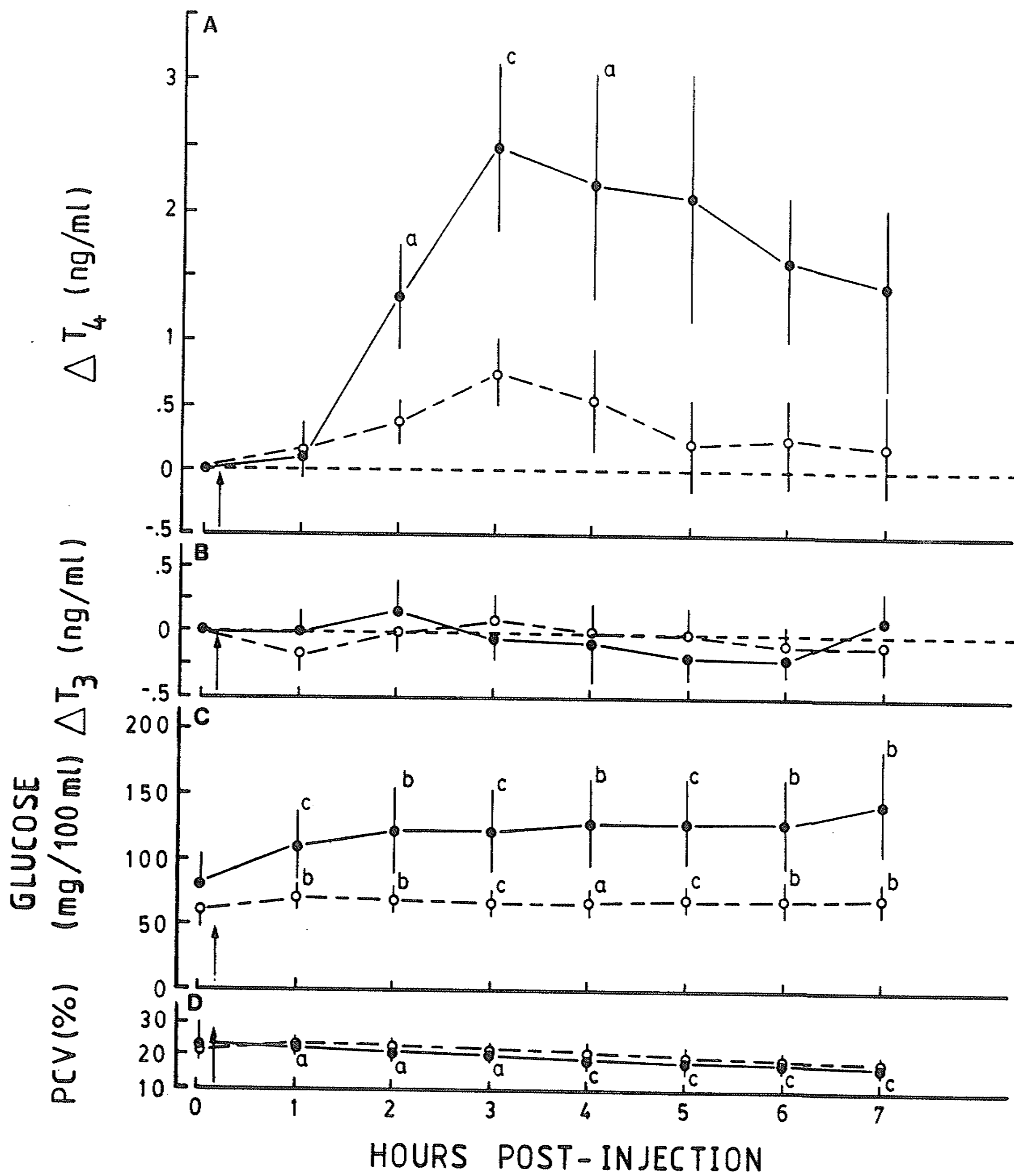
Vertical bars represent mean ± 1 SEM.

Arrows represent time of saline or glucagon injection.

a = $p < 0.05$ (relative to 0 h)

b = $p < 0.02$

c = $p < 0.01$


A
B
C
D
ΔT_4 (ng/ml)
ΔT_3 (ng/ml)
GLUCOSE (mg/100 ml)
PCV (%)
HOURS POST-INJECTION
0
1
2
3
4
5
6
7
3
2
1
.5
0
-.5
.5
0
-.5
200
150
100
50
0
30
20
10

4.8 THE ACUTE EFFECTS OF TRH ON PLASMA T_4 AND T_3

Experiment 15

To examine the acute effects of TRH on pT_4, 4 groups of 15 fish (mean wt. 36.6 g) were fed a 2% ration for 20 days and then starved (June). Mortality was 8.3%. On day 4 of starvation, trout were injected with 20 µL of either synthetic TRH (0.01, 0.10, 1.0 µg/g) in 0.7% NaCl or 0.7% NaCl alone. Fish were sampled 4 h post-injection.

One-way ANOVA revealed a significant difference between pT_4 of injected fish. Multiple comparisons revealed a significant difference between fish injected with 0.01 (2.4 ng/mL) and 1.0 (4.5 ng/mL) µg/g TRH ($F = 3.350$) (Fig. 16). When compared to saline-injected controls, TRH did not cause a significant increase in pT_4.

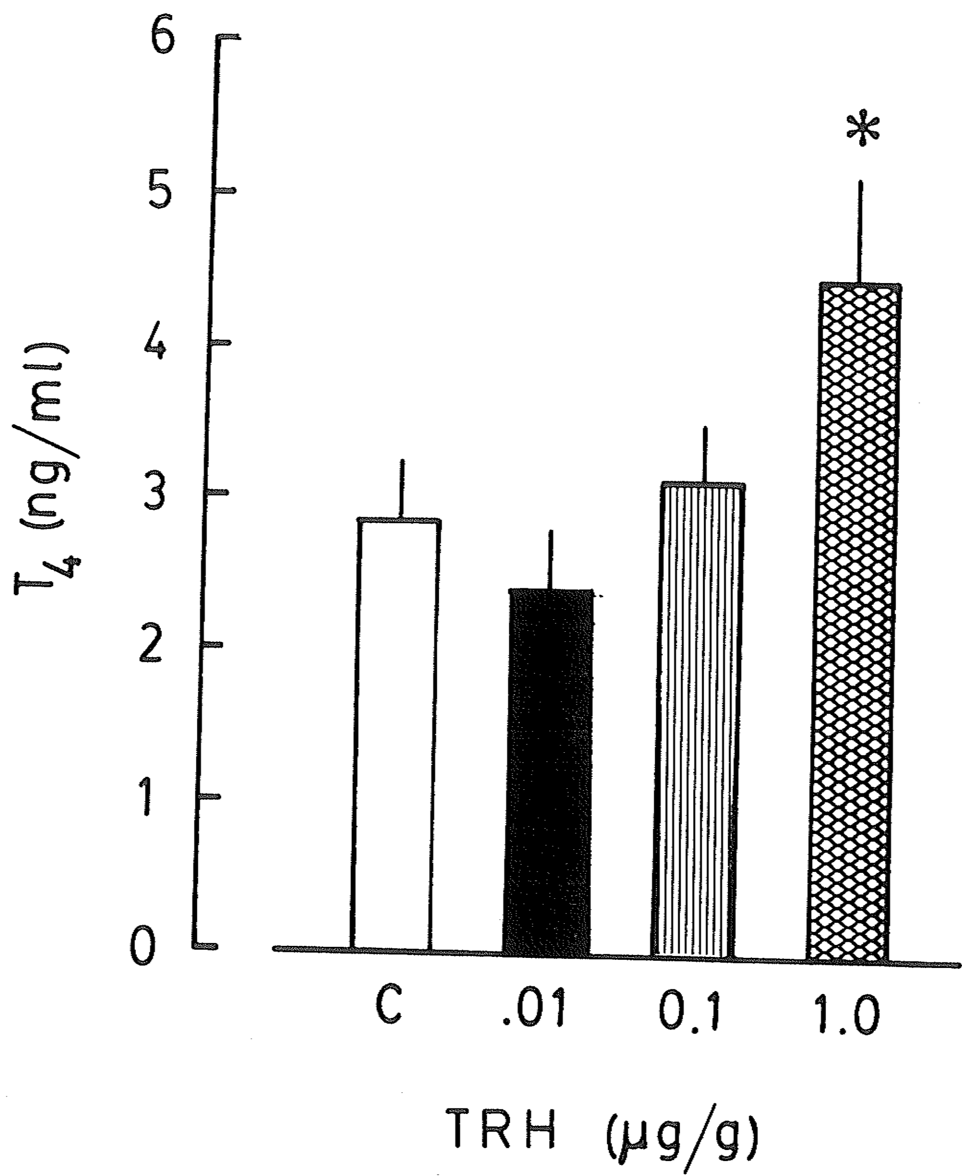
Experiment 16

To examine the acute effects of TRH over time on pT_4, pT_3, plasma glucose and PCV, trout (407.4 - 877.5 g) were cannulated and then starved (July - August). On day 4 of starvation, each trout was sampled at 0 h (0900 h), and injected (0.85 mL/kg) with 0.7% NaCl or 0.7% NaCl containing 1.0 µg synthetic TRH/g. After fish regained equilibrium, samples were collected hourly for 8 h (1600 h).

Figure 16: Acute influence of synthetic TRH on pT_4 in trout. Trout were starved for 3 days and ip injected with 20 µL of 0.7% NaCl or 0.7% NaCl containing 0.01, 0.10 or 1.0 µg/g synthetic TRH. A significant difference in pT_4 existed only between fish injected with 0.01 and 1.0 µg TRH/g.

Vertical bars represent mean ± 1 SEM.

* = $p = 0.05$ (relative to .01 µg/g injected)



6
5
4
3
2
1
0
T4 (ng/ml)
*
C
.01
0.1
1.0
TRH (µg/g)

A significant difference between saline and TRH-injected trout existed only with respect to pT_4 ($p < 0.002$) (Fig. 17 a). Over time, trout injected with TRH exhibited a significant elevation in pT_4 ($p < 0.031$). When computed as the mean difference from time zero, a significant increase in pT_4 occurred at 1 h (1.1 ng/mL). Plasma T_4 peaked at 2 h (2.5 ng/mL) to 3 h (2.6 ng/mL) after which levels declined. However, at 7 h (1.8 ng/mL) pT_4 remained significantly elevated relative to 0 h. Plasma T_4 remained unchanged in saline-injected trout.

Saline-injected trout exhibited a decrease in PCV only at 7 h ($p < 0.025$) (Fig. 17 d).

Plasma T_3 (Fig. 17 b) and glucose (Fig. 17 c) remained unchanged over time in both saline- and TRH-injected trout.

It is concluded that TRH at a high dose (1.0 µg/g) acts to elevate pT_4 within 1 h. Maximal T_4 levels are attained at 2 to 3 h post-injection. TRH does not cause alterations in plasma glucose, pT_3 or PCV over time.

Figure 17: Time profile of the acute effects of TRH on pT_4, pT_3, plasma glucose and PCV. Trout were cannulated in the dorsal aorta, starved for 3 days and then ip injected with 0.85 mL/kg of either 0.7% NaCl (n = 7) (- —0— -) or 0.7% NaCl containing 1.0 μg synthetic TRH/g (n = 7) (——●——).

A. TRH caused a significant elevation in pT_4 at 1h which reached maximal levels at 2h to 3 h.

B,C. PT_3 and plasma glucose remained unaltered in both saline- and TRH- injected trout.

D. PCV was decreased at 7 h only with saline administration.

Vertical bars represent mean ± 1 SEM.

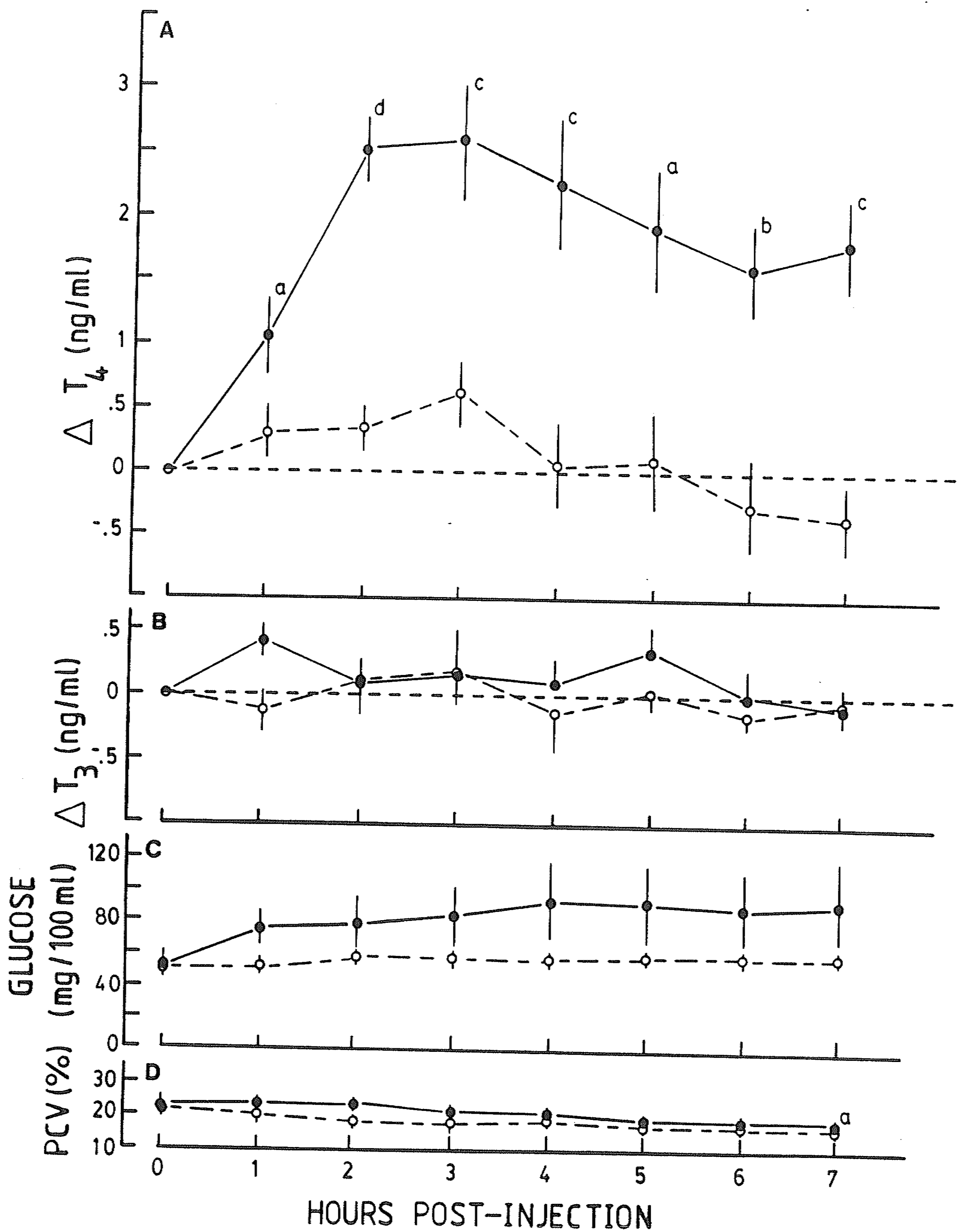
Arrows represent time of saline or TRH injection.

a = $p < 0.05$ (relative to 0 h)

b = $p < 0.02$

c = $p < 0.01$

d = $p < 0.001$


A
B
C
D
Δ T_4 (ng/ml)
Δ T_3 (ng/ml)
GLUCOSE (mg/100 ml)
PCV (%)
HOURS POST-INJECTION
a
d
c
c
a
b
c
a

Chapter V

DISCUSSION

5.1 REFEEDING AND PLASMA TH: A PROTOCOL FOR FURTHER INVESTIGATION

Initial experiments conducted in this thesis confirm previous findings by Flood and Eales (1983) of a twofold increase in pT_4 within 4 h of refeeding starved trout (Exp. 1 and 2; Fig. 5 and 6). Furthermore, postprandial pT_4 elevations are time-related (Exp. 2; Fig. 6). Food intake stimulates an increase in pT_4 within 2 h followed by a peak at 4 h. Increased pT_4 likely reflects rapid T_4 secretion from the thyroid rather than a decrease in T_4 clearance from plasma, since feeding has been shown to enhance T_4 clearance rate in trout (Higgs and Eales, 1977). Declining pT_4 6 to 8 h after refeeding may represent the combined effects of decreased thyroidal T_4 release and enhanced T_4 removal from the circulation.

Elevations in pT_4 with refeeding depend on fish size. After food intake, a rise in pT_4 occurs only in small fish (mean weight range 91.6 to 93.7 g) as opposed to larger trout (mean weight range 261.1 to 414.1 g) (Exp. 3; Table 1). Such observations are consistent with findings of feeding-induced pT_4 elevations in rainbow trout weighing

less than 200 g documented by Flood and Eales (1983). Other studies have revealed "blunted" responses in thyroid function to various stimuli in larger salmonids. Bres (1988) demonstrated a decrease in hepatic T_3 receptor number after rainbow trout reached approximately 150 g. Similarly, reduced T_4 monodeiodination to T_3 has been observed in mature trout (C.A. Shields and J.G. Eales, data unpublished). Decreased thyroidal output observed in large fish in response to feeding may therefore be the result of a low T_4 demand due to decreased peripheral T_3 utilization. Variable basal T_4 levels of control fish between experiments may be due to pT_4 seasonal differences which have been demonstrated in trout (Laidley and Leatherland, 1988).

A rise in pT_4 also depends on the quantity of food refed. In small trout, a minimum of 0.37% bw is required to elicit a response in pT_4 (Exp. 3; Table 1). Increasing rations in excess of this amount cause graded responses in T_4 release. This agrees with studies conducted by Flood and Eales (1983). They demonstrated an elevation in previously suppressed pTH after refeeding a meal of 1.1% bw or more to starved trout. The amount of food consumed has also been shown to dictate the degree to which pTH are altered in domestic fowl (Klandorf and Harvey, 1985).

Refeeding starved trout caused marked elevations only in pT_4 amongst the variables measured. Such findings are in accordance with other studies conducted in fish demon-

strating major changes in T_4 following acute thyroid stimulation. Brown _et al_. (1978) showed an elevation in pT_4, with no change in pT_3, 2 to 6 h after handling and injection in rainbow trout. Chan and Eales (1976) observed a rapid increase only in pT_4 after TSH injection into brook trout. Grau _et al_. (1986) demonstrated acute release of T_4, but no secretion of T_3, following perifusion of the parrotfish thyroid _in vitro_ with TSH.

The slight fluctuations in pT_3 after a meal (Exp. 1, Fig. 5) suggest little T_3 release from the fish thyroid. In contrast, feeding causes the release of both T_4 and T_3 from the thyroid gland of the mammal (Wartofsky and Burman, 1982). It cannot be established with certainty whether such small and inconsistent changes in pT_3 result from alterations in peripheral T_4 to T_3 conversion. On the basis of studies utilizing $*T_4$, increased T_4 monodeiodination has been demonstrated in both the brook trout and rainbow trout after refeeding (Higgs and Eales, 1977; Flood and Eales, 1983). Increased pT_3 4 h after food intake (Exp. 1, Fig. 5) may therefore be the result of enhanced T_3 production. Decreased pT_4 at 6 to 8 h post-refeeding in Experiment 2 may also represent pT_4 deiodination (in addition to increased uptake of T_4 from the plasma). However, during this time interval T_3 may remain within the cell instead of entering the pT_3 pool.

Based on presented findings, a standard protocol was developed whereby short-term pT_4 responses to various stimuli could be investigated. To eliminate the effects of daily pT_4 cycling during experimental treatment trout were subjected to 3 days of starvation, since 3 days fasting has been shown to suppress pT_4 diurnal rhythms in salmonids (Exp. 2; Brown et al., 1978; Flood and Eales, 1983). The pT_4 response to experimental manipulation was examined at 4 h post-treatment, since at this time postprandial pT_4 is maximal.

5.2 MECHANISM OF ELEVATED PT_4 DURING REFEEDING

Several factors may stimulate thyroidal T_4 release after food intake. However, it is unlikely that olfactory and gustatory responses of trout to food are involved. Prepared extracts of fish food did not cause an increase in pT_4 4 h after presentation to fish (Exp. 4; Table 2). Similarly, administration of simulated food in the form of plastic pellets did not elicit a rise in pT_4 (Exp. 5; Table 3). These findings suggest little participation of sight, smell and taste in stimulating pT_4 secretion following a meal.

Graded pT_4 elevations with increasing rations refed (Exp. 3; Table 1) suggest that trout respond either to total caloric intake or to amounts of particular ingredients. Refeeding isocaloric diets to fish (Exp. 6; Fig. 7) demonstrates that dietary composition is an important influencing

factor of acute pT_4 changes. In particular, the level of CHO refed can modify the extent to which pT_4 rises, regardless of the CHO type. High levels of either dextrin or cerelose produced a marked elevation in plasma glucose and pT_4 whereas low CHO diets did not. A diet containing medium dextrin also caused a modest increase in plasma glucose and pT_4.

Unaltered glucose levels may explain the small change in pT_4 witnessed in trout refed low CHO diets in Experiment 6 (Fig. 7). It is possible that an elevation in plasma glucose is required to stimulate T_4 release from the thyroid. In support of this are concomitant increases in plasma glucose and pT_4 observed within 2 h of refeeding (Exp. 2; Fig. 6). Unchanged postprandial pT_4 in large trout may also be the result of unaltered plasma glucose (Exp. 3; Table 1).

Lipid may interact with CHO to modify the response of pT_4, since experimental diets administered in Experiment 6 varied reciprocally in CHO and lipid content in order to maintain an isocaloric state. Sufficient levels of dietary lipid may act to suppress T_4 secretion from the thyroid. This would explain unaltered pT_4 in trout refed a medium cerelose/medium lipid diet despite a marked elevation in plasma glucose. In the rat, fat intake causes only slight changes in pTH and TSH as opposed to CHO feeding, suggesting little stimulation or possibly inhibition of the HPT-axis by lipid (Burger *et al*., 1980; Ullrich *et al*., 1985).

Graded pT_4 elevations in response to diets containing increasing amounts of CHO in Experiment 6 demonstrate the lack of dependence on energy intake in altering pT_4, since all diets tested were isocaloric. The apparent greater importance of CHO as opposed to caloric intake in stimulating the thyroid system agrees with much mammalian literature (Pasquali *et al.*, 1982; Koppeschaar, *et al.*, 1985; Danforth, 1986) but contradicts other recent studies (Dauncey, *et al.*, 1983; Glade and Reimers, 1985).

While refed CHO modifies teleost thyroid secretion, it appears that dietary CHO levels must be sufficiently high to provide stimulation ($\leq$14.9%). Although fish food is composed primarily of protein (~40%), CHO is present at a level of approximately 15% in most diets (Martin Feed Mills, pers. comm.). CHO uptake by the gut probably elevates plasma glucose, the degree of glucose increase dictating the magnitude of the rise in pT_4. Alteration of pT_4 after a meal may also depend on other interacting nutrients, such as lipid. Varied pT_4 responses to meal size (Exp. 3; Table 1) may reflect the amount of food required to elevate plasma glucose to a critical level.

5.3 RELATIONSHIP BETWEEN PLASMA GLUCOSE AND PT_4

Several experiments within this thesis present evidence of a causal relationship between plasma glucose and pT_4. Doses of 0.2 and 2.0 g/kg D-glucose injected ip into trout result in an acute increase in pT_4 within 4 h (Exp. 7; Fig. 8). In glucose-injected (0.85 g/kg) cannulated trout, an elevation in plasma glucose is followed within one hour by a significant rise in pT_4 (Exp. 8; Fig. 9). These data also reveal that it is the initial surge in plasma glucose which is associated with an increase in pT_4, since a decline in pT_4 occurs after rapid elevation despite sustained glucose levels. The decline in pT_4 may be attributed to increased T_4 utilization by the tissues or a decrease in thyroidal T_4 secretion. Prolonged elevation of plasma glucose, witnessed in trout after glucose-loading (Exp. 8; Fig. 9) has also been demonstrated in the silver eel, *Anguilla anguilla*. Intra-arterial injection of glucose at doses of 10 to 500 mg/kg caused a sustained hyperglycemia over several hours (Ince and Thorpe, 1974; 1977). Such results illustrate the "diabetic-like" nature of fish, as compared to the mammal, following a glucose load.

Trout which responded poorly to handling and anesthetization (Fig. 10) provide further evidence of a relationship between plasma glucose and pT_4. An increase in glucose was always accompanied by an elevation in pT_4. After deliberate disturbance (Fig. 11), trout also exhibited

concomitant elevations in pT_4 and plasma glucose. However, despite similar increases in pT_4 under both conditions (poorly recovered or deliberately disturbed), the degree of plasma glucose change appeared to be not as great in trout which were netted and disturbed. This suggests that either modest glucose alterations are sufficient to cause acute elevations in pT_4 or that factors other than glucose contribute to increase pT_4 during handling and/or stress.

Glucose-mediated pT_4 elevations in trout support findings of thyroid activity altered by glucose in homeotherms. In domestic fowl, a feeding-induced shift in plasma glucose produces a parallel shift in peak T_3 and maximal 5'-deiodinase activity (Decuypere and Kuhn, 1984). In the rat, increased plasma glucose 3 h after food intake is accompanied by a concomitant elevation in pT_4 and a slight increase in TSH (Burger et al., 1980). Klandorf and Harvey (1985) demonstrated that glucose-injected fowl (2.0 g/kg) exhibit enhanced T_4 to T_3 conversion within 3 h, preceded by an increase in thyroidal T_4 secretion.

5.4 MECHANISM OF GLUCOSE-MEDIATED ELEVATIONS IN PT_4 AND POSSIBLE ROLES OF THE THYROID IN GLUCOSE METABOLISM

Glucose likely acts to increase pT_4 through direct stimulation of the HPT-axis. In trout, pT_4 elevation within one hour of glucose injection (Exp. 8; Fig. 9) indicates the potential for glucose action on the thyroid tissue itself.

Glucose may also act at the pituitary to stimulate TSH release which would, in turn, stimulate thyroidal T_4 secretion. Alternatively, T_4 elevations in response to glucose may occur as a result of hypothalamic TRH release. Experiment 16 (Fig. 17) demonstrates that TRH is capable of causing an increase in pT_4 within 1 h in trout. Stimulation of the HPT-axis by glucose has been demonstrated in the rat. Rojdmark and Nygren (1983) observed an acute hormonal release from the thyroid gland _in vitro_ after exposure of tissue to glucose. CHO has also been shown to increase thyrotropic TSH within one hour (Hugues _et al_., 1983).

The possibility that glucose acts through altered glucoregulatory hormones to enhance thyroidal T_4 secretion in the trout also exists. Such indirect actions cannot be ruled out and are discussed in further detail in section 5.6.

Increased plasma glucose may trigger thyroidal secretion in order to counteract hyperglycemia. In the Pacific hagfish and lamprey, plasma glucose levels are decreased 24 to 48 h following T_4 or T_3 administration (Plisetskaya _et al_., 1983; Plisetskaya and Gorbman, 1983). T_4 injection into carp results in hypoglycemia within 48 h concommitant with an increase in the deposition of muscle and heart glycogen (Murat and Serfaty, 1970; 1971). Implantation of capsules containing 6-propylthiouracil (6-PTU), a goitrogen which inhibits TH synthesis, causes a gradual elevation in

plasma glucose within one week in the Pacific hagfish (Plisetskaya *et al.*, 1983). Kaminska *et al.* (1985) have demonstrated a clear negative correlation between pT_3 and blood glucose levels in the carp.

Elevations in pT_4 following glucose stimulation may act to re-establish glucose homeostasis by increasing oxidation of excess circulating glucose. Activation of the pentose-phosphate pathway by T_4 has been demonstrated in the liver of the brook trout, *Salvelinus fontinalis* (Hochachka, 1962). In the rat, elevated TH levels cause increased oxidative metabolism of glucose in the brain (Sabell *et al.*, 1985). Such an event may also occur in trout.

It cannot be established from data obtained in Experiment 9 (Fig. 12) whether glucose actions are specific in mediating acute T_4 secretion from the thyroid of the trout. Galactose, a stereoisomer of glucose, causes rapid elevation in pT_4. However, a difference exists between galactose- and glucose-mediated pT_4 changes over time despite injection of equimolar concentrations of each carbohydrate. Galactose-induced pT_4 elevation is delayed 1 to 2 h as compared to glucose injection (Exp. 8; Fig. 9) and is followed by a sustained plateau.

Unchanged plasma glucose following galactose injection suggests no involvement of galactose-induced gluconeogenesis (or galactose conversion to glucose) in stimulating pT_4.

The slower pT_4 response to galactose suggests that either (i) an extra step in the chain of events leading to T_4 secretion exists, (ii) galactose is converted to some other substrate, which causes more prolonged T_4 release or (iii) galactose uptake from the coelom following injection is much slower than glucose uptake. This later event may have resulted, since galactose is far less soluble in saline than is glucose.

Unlike glucose, galactose does not cause release of insulin from the Brockmann bodies of the catfish, *Ictalurus punctatus* (Ronner and Scarpa, 1987) and suggests that fish can distinguish between closely related hexoses. This is in accordance with studies conducted in the toadfish, *Opsanus tau*, where the islets of Langerhans can distinguish between the D- and L- isomers of glucose (Cooperstein and Lazarow, 1969).

In summary, an acute increase in plasma glucose causes a rise in pT_4 within 1 h. Glucose-mediated elevations in pT_4 likely result from excess thyroidal T_4 secretion due to direct stimulation of the HPT-axis. An increase in pT_4 may serve to counteract hyperglycemia by enhancing oxidation of excess circulating glucose. Glucose action may be specific, since pT_4 is altered differently by glucose and galactose.

5.5 RELATIONSHIP BETWEEN INSULIN, GLUCAGON AND PT_4

Both insulin and glucagon levels have been shown to increase shortly after feeding in salmonids and other teleosts (Emdin, 1982; Hilton *et al*., 1987; Plisetskaya *et al*., 1987; Suarez and Mommsen, 1987), raising the possibility that these pancreatic hormones also play a role in elevating pT_4. In support of this mechanism, Plisetskaya *et al*. (1983) demonstrated that injection of antibodies generated against human insulin caused a decline in pT_3 within 24 h in the Pacific hagfish. This was followed by an increase in plasma glucose at 48 h. Kaminska *et al*. (1985) showed that a bolus of insulin injected into carp increased pT_3 over time. Data presented here indicate the possibility of acute stimulation of pT_4 by both insulin and glucagon following administration to trout.

In Experiment 12 (Fig. 14), cannulated trout injected with 10 IU/kg bovine insulin exhibited an elevation in pT_4 within 2 h followed by a suppression of plasma glucose at 4 h. Ludwig *et al*. (1977) showed a similar decrease in plasma glucose within 4 h of bovine insulin (10 IU/kg) injection into rainbow trout, with glucose recovery at 24 h. Other studies have also shown an effect of insulin on glucose levels in fish. Inui and Gorbman (1977) demonstrated depressed plasma glucose within 24 h of injecting 1.0 to 1000.0 IU/kg bovine insulin in the Pacific hagfish. Ince and Thorpe (1974) revealed that while 2.0 IU/kg codfish

insulin produces a significant decrease in blood glucose at 0.5 h in the silver eel, bovine insulin at this dose does not alter glucose levels. In the fish, the decrease in plasma glucose due to insulin likely results from oxidative clearance rather than from glycogen deposition of glucose (Ince, 1983; Suarez and Mommsen, 1987).

Plasma T_4 elevations in large cannulated trout by 10 IU/kg insulin (Exp. 12; Fig. 14) are at variance with results of Experiment 11 (Table 4) where 55 to 100 IU/kg insulin was required to stimulate pT_4 in smaller non-cannulated fish. Discrepancies between results from these two experiments may be attributed to several factors.

First, insulin suspended in saline (Exp. 12; Fig. 14) may have been taken up more effectively from the coelom than insulin dissolved in acetate. Rapid clearance of insulin in acetate may alter the amount of hormone necessary to be injected. Despite a small injection volume (20 uL), the acidity of the acetate buffer used may also influence pT_4 responsiveness to insulin. While acetate does not alter pT_4 (Exp. 10; Fig. 13), low pH (3.27) may act to release catecholamines which could inhibit insulin action in fish (Ince, 1980).

Secondly, differences may be due to fish size and/or seasonal variation in the effects of bovine insulin. In Experiment 11 (Table 4), fish weighed between 41.0 and 113.6

g, whereas cannulated trout (Exp. 12; Fig. 14) averaged between 426.9 and 800.0 g. The thyroid of younger fish may be less sensitive to the effects of mammalian insulin than older fish. Alternatively, seasonal sensitivity of trout hepatocytes to insulin has been documented (Petersen *et al*., 1987). This, in addition to seasonal variations in the levels of fish plasma insulin (Ince, 1983; Gutierrez *et al*., 1988), may contribute to the differences observed in pT_4 response, since Experiment 12 was conducted in January whereas Experiment 11 was run in May, July and January.

Finally, differences in basal glucose levels between the fish used in Experiments 11 and 12 may have been a factor. Instances of elevated glucose in Experiment 12 may be due to incomplete recovery from cannulation or to seasonal differences in plasma glucose between trout in Experiments 11 and 12. In the carp, Kaminska *et al*. (1985) demonstrated lower glucose levels in summer as opposed to winter months.

Insulin may act at different levels of the HPT-axis to elevate pT_4. Stimulation may occur at the thyroid, since in the turtle, *Chrysemys dorbigini*, acute [^{125}I]-insulin uptake by the thyroid (suggesting the presence of insulin-binding sites) has been demonstrated (Marques *et al*., 1985). Insulin may also stimulate higher levels of the fish HPT-axis. Insulin receptors, capable of binding mammalian insulin, have been isolated in the brain of the salmon (Leibush, 1984). In the rat, treatment with insulin

following induced diabetes mellitus (a condition which exerts effects similar to that of starvation on thyroidal status) results in enhanced hypothalamic and pituitary secretion (Wartofsky and Burman, 1982).

It is unlikely that elevated postprandial insulin levels stimulate the thyroid system by changing plasma glucose. Glucose levels decline 4 h following insulin injection, or 2 h after pT_4 has risen.

Elevated postprandial plasma glucose may cause an increase in thyroidal T_4 release through enhanced insulin secretion. In contrast to the response of insulin to elevated glucose in the mammal however, insulin responds less readily to glucose fluctuations in the lower vertebrates. Emdin (1982) showed only a twofold increase in insulin when hagfish were loaded with D-glucose (0.5 g/kg). Similarly, both _in vivo_ (Ince and Thorpe, 1976) and _in vitro_ (Patent and Foa, 1971) studies in fish demonstrate little insulin participation in glucose homeostasis. In fact, in fish certain amino acids (arginine and lysine) appear to be more potent secretagogues of insulin release than glucose (Ince and Thorpe, 1977; Ince, 1979; Plisetskaya _et al_., 1987; Ronner and Scarpa, 1987).

As with insulin, mammalian glucagon (4.0 µg/g) injected into trout caused an acute elevation in pT_4 within 2 to 3 h (Exp. 14; Fig. 15). Glucagon also caused an elevation in

plasma glucose within 1 h which remained sustained over 8 h. Increased glucose by glucagon probably results through enhanced hepatic glycogenolysis (Ince, 1983) or elevated gluconeogenesis (Murat _et al_., 1978; Suarez and Mommsen, 1987). The effect of a dose of 4.0 μg/g glucagon injected into trout is in accordance with studies conducted in carp by Murat _et al_. (1978) in which 2.0 μg/g glucagon resulted in hyperglycemia within 10 h. Preliminary experiments with 2.0 μg/g glucagon injected into trout revealed no changes in plasma glucose; therefore a higher dose (4.0 μg/g) was employed.

Unaltered pT_4 by glucagon in Experiment 13 (Table 5) likely reflects an insufficient amount of hormone injected (0.08 to 0.69 μg/g) to elicit a physiological response. This is suggested by little change in plasma glucose after glucagon administration in trial 3 of Experiment 13.

Glucagon may act directly on the thyroid to stimulate T_4 release. Stimulation of thyroidal T_4 release by glucagon has been demonstrated in the rat _in vitro_ (Attali _et al_., 1984). Glucagon may also act to increase pT_4 through alteration of plasma glucose. Elevated glucose levels within one hour of glucagon injection could serve as a stimulus to enhance thyroidal T_4 release as is revealed in Experiment 8 (Fig. 9).

In summary, mammalian glucagon (4.0 µg/g) causes an acute elevation in pT_4 within 2 to 3 h post injection in trout. Bovine insulin may also act to cause a rapid elevation in pT_4. Both glucoregulatory hormones may act directly at the level of the HPT-axis to enhance thyroidal T_4 secretion. Glucagon-mediated increases in plasma glucose which occur within 1 h could also act to elevate pT_4. Release of insulin and glucagon during food ingestion suggests their possible involvement in elevating pT_4 after refeeding in the fish.

5.6 TRH AND PLASMA T_4

Acute pT_4 elevations by TRH support previous findings of TRH stimulatory actions in certain poikilotherms (Darras and Kuhn, 1982; Denver and Licht, 1987), but contradict other studies (Peter, 1973; Ball; 1981; Sawin *et al*., 1981). Experiments 15 and 16 (Fig. 16 and 17) both demonstrate that synthetic TRH at a dose of 1.0 µg/g elevates pT_4. Furthermore, Experiment 16 (Fig. 17) reveals an increase in pT_4 by TRH within 1 h, reaching maximal levels 2 to 3 h later. Similar time-dependent pT_4 elevations by TRH have been demonstrated in other poikilotherms. In the cannulated frog, *Rana ridibunda*, intravenous injection of 0.1 µg/g TRH stimulates an increase in pT_4 within 2 to 4 h (Darras and Kuhn, 1982). Jacobs *et al*. (1988) have shown that TRH (0.1 to 1.0 µg/g) elicits a rapid elevation in pT_4 within 2 h of

injection into the metamorphosed axolotl, *Ambystoma mexicanum*.

Acute pT_4 responses to TRH in trout suggest that TRH functions as a thyrotropin-releasing factor. TRH-binding sites have been isolated in the pituitaries of several marine teleosts and the goldfish (Burt and Ajah, 1984). This presents the possibility of direct stimulatory action of TRH on the fish pituitary. Rapid release of TSH has been demonstrated in the turtle in response to TRH (Preece and Licht, 1987). Similar control of TSH release in the fish would result in rapid thyroidal T_4 secretion (Grau *et al.*, 1986).

TRH may also act indirectly to stimulate T_4 through glucagon release. Control of pancreatic activity by TRH has been demonstrated in the mammal (Blanco *et al.*, 1986). Similarly, TRH injection into the CNS of the rat causes acute release of glucagon (Alwmark and Greeley, 1987). However, unchanged plasma glucose, as well as the extremely rapid pT_4 response to TRH, suggests otherwise.

In the trout, refeeding-mediated thyroidal T_4 secretion may result through stimulation of hypothalamic TRH release. Postprandial changes in plasma glucose, insulin and/or glucagon may act directly at the brain to cause TRH secretion. A similar mechanism of enhanced activity at the HPT-axis has been documented in mammals following CHO refeeding.

Chapter VI

CONCLUSIONS

Feeding tends to restore pT_4 previously depressed through fasting in the rainbow trout. A single meal refed to trout following acute (3 days) starvation results in a two- to threefold increase in pT_4 within 2 h. These changes appear to be related to both fish size and the ration size refed to small fish. The increase in pT_4 is probably due to an increase in thyroidal T_4 secretion rather than a decrease in pT_4 clearance.

Several mechanisms may be involved in stimulating T_4 secretion during feeding. However, it is unlikely that the sight, smell and taste of food participate. Rather, specific dietary composition, as opposed to the CHO type or caloric content, appears to be important in mediating the increase in pT_4. High CHO diets provide maximal stimulation of thyroidal T_4 release while a meal low in CHO results in unchanged pT_4.

A causal relationship between glucose and pT_4 exists in trout. An increase in pT_4 occurs in association with, or one hour after, an elevation in plasma glucose. Glucose action may be specific, since a different pT_4 response profile occurs with the glucose stereoisomer galactose. However, further investigation into this area is required.

Glucose probably acts to increase pT_4 through direct stimulation of the HPT-axis. Glucose may act at the thyroid tissue itself, or at the pituitary and/or hypothalamus. Elevations in pT_4 in response to increased plasma glucose may serve to stimulate oxidation of excess circulating glucose in the liver and brain and to re-establish normal glucose homeostasis.

Increased insulin and glucagon secretion during food intake may also participate to increase T_4. Glucagon (4.0 µg/g) caused a rapid increase in pT_4 within 2 to 3 h, or 1 h after an elevation in plasma glucose occurred. Insulin (10 IU/kg) may also act to acutely elevate pT_4.

An increase in pT_4 within 1 h of TRH administration presents the possibility that postprandial changes in glucose, insulin and/or glucagon act to stimulate the hypothalamus and hence increase thyroidal T_4 release.

In conclusion, increased postprandial pT_4 elevations in small trout depend on the amount of dietary CHO and the degree to which plasma glucose becomes elevated. A rise in pT_4 may serve to regulate altered glycemic levels and hence prevent further hyperglycemia. Sustained plasma glucose, with little fluctuation after a meal, may reflect a new set-point established by the thyroid to maintain glucose homeostasis.

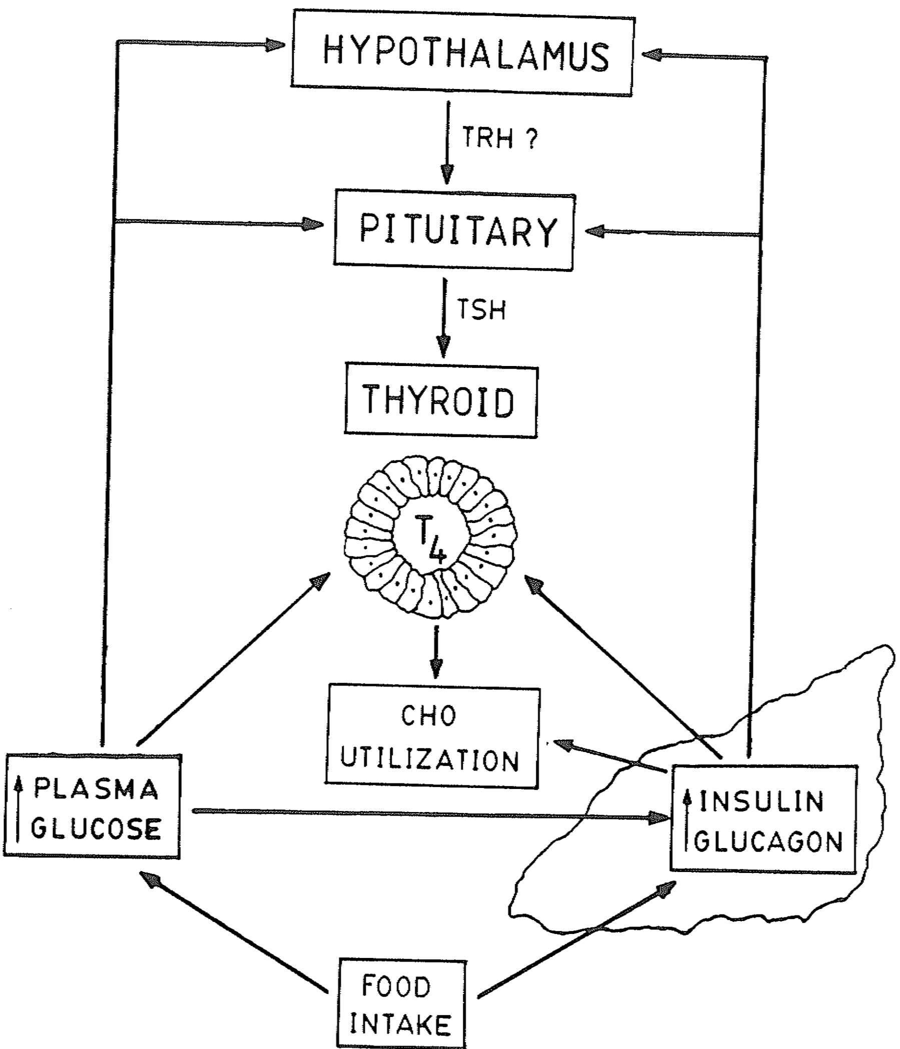
In the fish, a complex interrelationship may exist between the thyroid and the pancreas in regulating plasma glucose (Fig. 18). Besides controlling altered circulating glucose, insulin and glucagon may elevate pT_4 to reinforce regulatory action over glycemic levels. Of interest would be to examine the acute actions of glucose in fish rendered hypo- or hyperthyroid. Such experiments may confirm the regulatory role of the fish thyroid in glucose metabolism.

Figure 18: Interrelationship between the thyroid and pancreas in the regulation of plasma glucose in the trout.

Food intake causes an elevation in plasma glucose. Plasma glucose acts to enhance thyroidal T_4 release which may then increase CHO utilization.

Food intake and/or elevated plasma glucose cause the release of insulin and glucagon from the pancreas. Both pancreatic hormones act to increase T_4 release from the thyroid. Plasma T_4 may then contribute to the regulation of altered plasma glucose.

Glucose, insulin and glucagon may increase thyroidal T_4 secretion through stimulation at the higher levels of the HPT-axis.


HYPOTHALAMUS
TRH ?
PITUITARY
TSH
THYROID
T_4
CHO
UTILIZATION
PLASMA
GLUCOSE
INSULIN
GLUCAGON
FOOD
INTAKE

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APPENDICES

Appendix 1. Composition of experimental diets varying in dietary CHO type and content.

Ingredients (g/kg dry diet)	HC	MC	LC	HD	MD	LD
whole herring meal (steam-dried)	372.5	372.5	372.5	372.5	372.5	372.5
Euphausids (freeze-dried)	50.0	50.0	50.0	50.0	50.0	50.0
Soybean meal	150.0	150.0	150.0	150.0	150.0	150.0
Wheat middlings	70.0	70.0	70.0	70.0	70.0	70.0
Cellulose	----	74.0	148.2	----	74.0	148.2
Cerelose (D-glucose)	268.2	134.2	0.0	----	----	----
Dextrin (cornstarch)	----	----	----	268.2	134.2	0.0
Salmon-oil (stabilized)	17.3	77.3	137.3	17.3	77.3	137.3
Vitamin supplement	30.0	30.0	30.0	30.0	30.0	30.0
Mineral supplement	20.0	20.0	20.0	20.0	20.0	20.0
Permapoll (binder)	15.0	15.0	15.0	15.0	15.0	15.0
Choline chloride (60%)	5.0	5.0	5.0	5.0	5.0	5.0
Ascorbic acid	2.0	2.0	2.0	2.0	2.0	2.0
Estimated level (%) of:						
protein	39.0	39.0	39.0	39.0	39.0	39.0
lipid	7.0	13.0	19.0	7.0	13.0	19.0
carbohydrate	28.3	14.9	1.5	28.3	14.9	1.5
digestible energy (kcal/kg)	3905	3908	3905	3905	3908	3905
metabolizable energy (kcal/kg)	3425	3426	3426	3425	3426	3426

Appendix 2. Contents of tubes used to generate a glucose standard regression.

Standard (mg/100 mL)	d/d water (μL)	GSS (μL)	Total Volume (μL)
400	160	40	200
300	170	30	200
200	180	20	200
100	190	10	200
50	190	10 μL from "A"	200
25	190	10 μL from "B"	200
0 (Blank)	200	---	200

where "A" = 30 μL GSS and 30 μL d/d water
"B" = 30 μL "A" and 30 μL d/d water

d/d = distilled and deionized
GSS = glucose standard solution

Appendix 3

Aortic cannulation as a technique to study acute plasma TH changes in trout

Experiments conducted throughout this study provide evidence that aortic catheterization can be effectively employed to examine acute TH alterations in trout. Despite a reported decrease in PCV due to serial sampling (Lane, 1979), no changes occurred in PCV over time in control trout with the exception of 7 h post saline injection in Experiment 17. Similarly, blood glucose has been recognized as a sensitive indicator of acute stress in fish (Wedemeyer, 1972; Mazeaud and Mazeaud, , 1981; Brown *et al*., 1986). However, altered plasma glucose occurred only in Experiments 13 and 15. Ince and Thorpe (1977) reported that repeated blood removal of 2.25 mL h^{-1} in the cannulated eel (300 - 510 g) caused no significant alterations in plasma glucose.

Total blood removal was approximated at 20 to 49%. While this seems high, slight PCV alterations suggest small influence by the volume of blood collected. In addition, pT_4 alterations occurred within the first 4 h of experimental manipulation, a time when only 10 to 25% total blood was removed.

Sampling on day 4 allows for sufficient recovery time from surgery, since Brown *et al*. (1986) demonstrated stabilization in plasma glucose, PCV and cortisol 2 days after

trout are cannulated. This is in agreement with decreased plasma glucose witnessed 2 days following aortic cannulation in the silver eel (Ince and Thorpe, 1974; Thorpe and Ince, 1976).