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FACULTÉ DES ÉTUDES SUPÉRIEURES  
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FACULTY OF GRADUATE AND  
POSTDOCTORAL STUDIES

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GRADE - DEGREE

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Rainbow Trout, *Oncorhynchus mykiss*

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AND POSTODORAL STUDIES

**THE LONG-TERM EFFECTS OF DIETARY CARBOHYDRATES ON  
GLUCOSE METABOLISM IN RAINBOW TROUT (*Oncorhynchus mykiss*)**

**Gavin J.S. Harman**

Thesis submitted to the  
School of Graduate Studies and Research  
University of Ottawa  
In partial fulfillment of the requirements for the  
Masters of Science in Biology

Ottawa-Carleton Institute of Biology  
L'Institute de biologie d'Ottawa-Carleton

Thèse soumise à  
l'École des études supérieures et de la recherche  
Université d'Ottawa  
En vue de l'obtention de la maîtrise ès sciences biologie

January 2004

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395 Wellington Street  
Ottawa ON K1A 0N4  
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395, rue Wellington  
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*Your file* *Votre référence*  
*ISBN: 0-494-01486-5*  
*Our file* *Notre référence*  
*ISBN: 0-494-01486-5*

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## ABSTRACT

Much remains unknown about the physiological effects of prolonged dietary carbohydrates on carnivorous fish such as rainbow trout, *Oncorhynchus mykiss*. In this thesis, the long-term effects of carbohydrates on trout glucose regulation are investigated by characterizing the metabolite profiles of fish fed with or without carbohydrates. Two groups of fish were maintained on either carbohydrate-rich (HC, 32.9% of diet by weight) or deficient (LC, 1% of diet by weight) diets for a six-month period. Plasma, liver and muscle tissue samples were assayed for various metabolites. The HC group showed elevated plasma glucose concentrations, increased abdominal fat, and higher levels of glycogen and lipid in muscle and liver, indicating a greater degree of energy storage.

Examination of a wide variety of enzymes revealed higher activities for the glycolytic enzymes HK, GK and PDH and lipogenic enzymes FAS and G6PDH. Conversely, the glucogenic enzymes ALT, AST and GDH were lower in the HC group, as was the lipolytic enzyme CPT. Indirect calorimetry was used to calculate respiratory exchange ratios ( $R_E$ ) of 0.84 in the HC and 0.61 in the LC groups. Together, these findings indicate a tendency toward the utilization of carbohydrates by the HC group as opposed to fats and proteins by the LC group.

Intravenous glucose tolerance tests were performed in each group and showed no significant differences in glucose tolerance. Continuous tracer infusion was used to measure glucose turnover in each group to determine whether changes in glucose concentration in response to a glucose load were due to changes in rates of glucose appearance ( $R_a$ ), disappearance ( $R_d$ ) or both. The load did not significantly alter either plasma glucose

concentrations or flux, but trout from the HC group were found to have higher glucose turnover rates, which were similar to those of mammalian species.

This research has demonstrated that rainbow trout can acclimate to high carbohydrate diets and modify several physiological parameters and biochemical pathways to both utilize and store newly available energy provided in the form of dietary glucose.

## RÉSUMÉ

Les effets d'une alimentation riche en glucose sur la physiologie des poissons carnivores tels que la truite arc en ciel, *Oncorhynchus mykiss*, sont encore très peu connus. Dans cette thèse, l'effet à long terme des glucides diète sur la régulation du glucose ont été étudiés en caractérisant le profil métabolique des truites nourries avec ou sans glucose. Deux groupes de poissons ont été nourris en utilisant soit une diète riche en glucose (HC, 32.9% de glucose dans la diète (g:g)), soit déficiente en glucose (LC, 1% de glucose dans la diète (g:g)), pendant six mois. Plusieurs métabolites ont été mesurés dans le plasma, le foie et le muscle. Une augmentation du taux de glucose dans le plasma, de la masse lipidique abdominale, ainsi que du glycogène musculaire et hépatique ont été retrouvés chez les truites HC, indiquant un plus grand stockage d'énergie.

L'activité des enzymes glycolytiques (tels que HK, GK et PDH) et adipogènes (FAS et G6PDH) était augmentée et l'activité des enzymes glucoformatrices (ALT, AST et GDH) diminuée dans chez les truites HC ainsi que l'enzyme adipogène CPT. La calorimétrie indirecte a été utilisée pour calculer le ratio d'échange respiratoire ( $R_E$ ) qui était de 0,84 dans chez les truites HC et 0,61 chez les truites LC. Ces données indiquent que les truites HC

utilisent préférentiellement les glucides par opposition au groupe LC utilisant plutôt les protéines et les acides gras comme substrats énergétiques.

Un test intraveineux de tolérance au glucose n'a révélé aucune différence entre les groupes. L'injection d'un marqueur a été utilisé pour évaluer le renouvellement du glucose dans chaque groupe. Les taux d'apparence ( $R_s$ ) et de disparition ( $R_d$ ) du glucose ont été mesurés. La dose de glucose injectée n'a pas affecté la concentration de glucose plasmatique ou son flux, mais les truites du groupes HC avait un renouvellement de glucose plus élevé que les truites LC et comparable a celui des mammifères.

Cette étude a démontré que les truites sont capables de s'adapter à une diète riche en glucides et modifient plusieurs paramètres physiologiques et biochimiques afin d'utiliser et de stocker l'énergie fournie par leur diète sous forme de glucose.

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## LIST OF ABBREVIATIONS

<b>Abbreviation</b>	<b>Meaning</b>
2Ira	Infusion rate of 20 $\mu\text{mol}/\text{min}/\text{kg}$
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
CPT	Carnitine Palmitoyl Transferase
CS	Citrate Synthase
FAS	Fatty Acid Synthase
G6PDH	Glucose-6-Phosphate Dehydrogenase
GK	Glucokinase or Hexokinase IV, high $K_m$ hexokinase
GLUT-1–12	Glucose Transporters- isoforms 1 through 12
GLUT-4	Facilitative Glucose Transporter of adipose and skeletal muscle tissues
GTT	Glucose Tolerance Test
HC	High Carbohydrate Diet
Ira	Infusion rate of 10 $\mu\text{mol}/\text{min}/\text{kg}$
IVGTT	Intravenous Glucose Tolerance Test
LC	Low Carbohydrate Diet
LDH	Lactate Dehydrogenase
NIDDM	Non-Insulin-Dependent Diabetes Mellitus
PDH	Pyruvate Dehydrogenase
PEPCK	Phosphoenol Pyruvate Carboxykinase
PK	Pyruvate Kinase
Ra, Rd	Rate of Appearance, Rate of Disappearance
RM	Red Muscle
RT	Rainbow Trout ( <i>Oncorhynchus mykiss</i> )
WM	White Muscle

## ACKNOWLEDGEMENTS

From the Mesopotamians to the Balts of Eastern Europe, the phenomenon of Moon worship has endured the rise and fall of an untold number of civilizations. Several centuries later, little has changed. I would like to extend my most heart-felt thanks to Dr. Thomas W. Moon, whose gravitational pull has repeatedly drawn me back towards his lab for three of the most illuminating years of my life.

Other heavenly bodies to be praised include all the members of my thesis committee: Dr. Steve F. Perry, Dr. Kathleen M. Gilmour and Dr. Jean-Michel Weber. These three individuals made my project possible by lending to me their valuable ideas, insight, and lab equipment. In particular, Steve Perry granted me unprecedented use of his laboratory setup for respiratory studies and offered training and technical support without hesitation or monetary reimbursement.

Additional thanks go to those many talented Masters students and PhDs who have extended their expertise to my cause. This includes Francois Haman, who guided me through the continuous tracer infusion method, Jeff Richards, who generously introduced me to PDH, and Jean-Marc Renaud who helped me with the statistical analysis of the glucose tolerance tests. I am grateful for the diligent work of summer students Carol-Ann Seymour and Thomas Arnason, for allowing me to earn a Masters degree without ever having to do any molecular biology for myself. No less deserving of my gratitude are old school department members Pat Desforges, for showing me how to cannulate fish, and Michel Lortie, for helping me unlock my full Moon potential.

Like so many others, I am truly grateful to have entrusted the care of my fish to Bill Fletcher of the Animal Care and Veterinary Services Facility. He is a man of the fish with a heart of gold.

Words fail to describe my appreciation for all the lab mates I have had the opportunity to work and play with during my Masters project. These are the people that make the difference between a tolerable and an enjoyable experience.

Most importantly, I would also like to thank my family for their love and support, and the countless numbers of brave rainbow trout who laid down their lives in the pursuit of this goal. To quote the great eastern philosopher, Eiji Kusuhara: “We salute your ultimate sacrifice at the altar of science!”

This research was funded by a research grant from NSERC to Dr. T.W. Moon.

# 1. INTRODUCTION

## 1.1. Rationale for the Study

The aquaculture industry is growing more rapidly than any other food-producing sector. Unlike capture fisheries whose growth has been halted by polluted and over-fished waters, fish farming is playing an increasingly important role in meeting the world's fish demand (FAO World Fisheries Department, 2002). With aquacultured fish production expanding so quickly, the importance of understanding fish physiology and nutrition has never been greater. Many aspects of fish growth and metabolism remain unresolved despite a growing interest and a relatively long history of fish research, especially in Canada. One major step towards achieving practical, economically and environmentally sound methods of fish rearing involves developing appropriate diets. In countries such as Canada and the United States, where the majority of commercial fish consumed are carnivorous (Reay, 1979), fish farmers rely on expensive, fat and protein-rich diets to produce fast growing fish. Such diets result in expensive feed costs, a high requirement for fishmeal and fish oils, and significant nitrogenous wastes leading to further pollution risks (Moon, 2001). Increasing the amount of dietary carbohydrates may provide an alternative to these costly feeds; however, the apparent inability of many fish species, and in particular carnivorous species, to effectively utilize dietary carbohydrates is an obstacle that must be addressed. This thesis examines the metabolic impact of dietary carbohydrates on a carnivorous teleost, the rainbow trout (*Oncorhynchus mykiss*).

## 1.2. Glucose Tolerance

For mammals, carbohydrates are the most abundant bulk nutrient and a major source of biological energy through their oxidation in the tissues. Glucose is one of the only circulating nutrients that passes the blood brain barrier, and is thus a key nutrient for the brain (Nelson and Cox, 2000). Dietary carbohydrates typically supply more than half of the total energy consumed by humans, however specialized feeding strategies developed in other vertebrates over the course of evolution may allow them to function with lower carbohydrate requirements. With this lowered intake of dietary carbohydrates comes a reduced ability to process excess glucose effectively, which is seen in animals such as the teleost fish when subjected to a glucose tolerance test.

### 1.2.1. Glucose tolerance tests

Glucose intolerance is a term used to describe the inability of a human to recover quickly from a glucose load. The main diagnostic tool for glucose tolerance is a glucose tolerance test (GTT). A bolus of glucose is delivered to the subject and periodic blood samples analyzed to assess the rate of glucose clearance from the plasma. The time required to recover from hyperglycemia depends on the form of the bolus, which may be delivered orally (O), intravenously (IV) or intraperitoneally (IP). Values for human fasting plasma glucose (FPG) concentration fall within a range of 5.5 - 7.0 mM. In healthy subjects, plasma glucose returns to FPG concentrations within 1-2 h following an oral glucose tolerance test (OGGT) using a standard dose of 750 mg glucose per kg body weight. Individuals whose plasma glucose concentrations remain above 11.1 mM after 2 h are considered glucose intolerant (Davidson *et al.*, 1999). Glucose tolerance tests are used to detect diabetes

mellitus, a disease of impaired pancreatic  $\beta$ -cell function and insulin sensitivity that compromises glucose utilization.

### 1.2.2. Glucose tolerance in fish

Glucose tolerance tests are the common method used to detect diabetes in humans, but are used to test fish glucose tolerance as well. Fish consistently show persistent hyperglycemia in a GTT, regardless of the route of glucose administration (Moon, 2001). Channel catfish (*Ictalurus punctata*) given a 250 mg IV (Ottolenghi *et al.*, 1995) or 1670 mg oral (Wilson and Poe, 1987) glucose dose per kg recovered from hyperglycemia 11 h or 6 h, respectively following this dose. Tilapia (*Tilapia mossambica*) showed elevated plasma glucose past 6 h with a 2000 mg/kg IP load (Wright *et al.*, 1998), whereas rainbow trout showed hyperglycemia to 18 h with a 300 mg/kg IP load (Harmon *et al.*, 1991). Meaningful comparisons between species can only be drawn when the dose and route of glucose administration are the same. A comparative study among three species demonstrated glucose tolerance in an OGTT to be greatest in the omnivorous carp (*Cyprinus carpio*), followed by the semi-carnivorous red seabream (*Pagrus major*), and poorest in the carnivorous yellowtail (*Seriola quinqueradiata*) (Furuichi and Yone, 1981). In an IVGTT comparison (250 mg glucose/kg) among carnivorous rainbow trout and American eel (*Anguilla rostrata*) and the relatively omnivorous black bullhead catfish (*Ameiurus melas*), decreasing levels of glucose tolerance were observed in the order catfish > trout > eel (Legate *et al.*, 2001). Table 1.1 summarizes the results of GTTs performed in several species of varying carnivorous character. Trophic levels for each species are presented; trophic levels increase with an organism's position in the food chain and generally correlate positively with the level of

carnivorous behaviour (Mittelbach *et al.*, 1988). A direct correlation between trophic level and glucose tolerance is difficult to quantify due to the lack of standardization in route and dosage of glucose administered between studies. In comparative experiments, however, the species with the greatest omnivorous or herbivorous character had the greatest degree of glucose tolerance, while the more carnivorous species had the lowest tolerance. Variations between species notwithstanding, in all cases fish may be considered, by the standards used for a warm-blooded omnivore (i.e. human), glucose intolerant. The mechanistic basis for glucose intolerance in fish is not fully understood, but may be related to a dysfunction in hormonal or metabolic glucose regulation.

Table 1.1. Observations of glucose tolerance tests performed in various teleost fish; adapted from Moon (2001).

Species	Route <sup>a</sup>	Dose (mg/kg)	Trophic level <sup>c</sup>	Remarks
<b>Palmer and Ryman, 1972</b> Rainbow trout	O	b	3.55 ± 0.58	Hyperglycemia at least to 7 h
<b>Harmon et al., 1991</b> Rainbow trout	IP	300	3.55 ± 0.58	Hyperglycemia at least to 18 h
<b>Blasco et al., 1996</b> Brown trout	IV	500	3.45 ± 1.09	Hyperglycemia corrected by 8 h fasting
<b>Legate et al., 2001</b> Rainbow trout	IV	250	3.55 ± 0.58	Hyperglycemia corrected by 24 h
American eel (6 mo fasted)			3.62 ± 0.54	Hyperglycemia at least to 24 h
Bullhead catfish			3.37 ± 0.49	Hyperglycemia corrected by 30 min
<b>Mazur et al., 1992</b> Chinook salmon	O	1670	3.93 ± 0.69	Hyperglycemia at least to 36 h

Species	Route <sup>a</sup>	Dose (mg/kg)	Trophic level <sup>c</sup>	Remarks
<b>Wilson and Poe, 1987</b> Channel catfish	O	670	3.25 ± 0.49	Hyperglycemia corrected by 6 h
<b>Ottolenghi et al., 1995</b> Channel catfish	IV	250	3.25 ± 0.49	Hyperglycemia corrected by 11 h
<b>Falkmer, 1961</b> Daddy sculpin	IM	500	3.84 ± 0.66	Hyperglycemia at least to 9 h
<b>Furuichi and Yone, 1981</b> Carp	O	167	3.03 ± 0.28	Hyperglycemia related to diet (approx. 5 h for carp; longer for other species) Hyperglycemia dependent upon prior feeding with dextrin
Red sea bream			3.63 ± 0.51	
Yellow tail			3.27 ± 0.50	
<b>Wright et al., 1998</b> Tilapia	IP	2000	2.71 ± 0.32	Hyperglycemia exceeded 6 h
<b>Ince and Thorpe, 1974</b> Silver European eels	IV	500	3.89 ± 0.65	IV-hyperglycemia corrected by 9 h O- minor hyperglycemia
	O			

<sup>a</sup> Route indicates method of glucose administration used: O, oral; IV, intravenous; IM, intramuscular; IP, intraperitoneal.

<sup>b</sup> A 1 g dose of glucose was given orally, but fish weights were not reported

<sup>c</sup> Trophic level estimated from a number of food items using a randomized re-sampling routine (<http://www.fishbase.org>).

### 1.3. Hormonal Control of Plasma Glucose

The endocrine system is an essential mediator of glucoregulation in mammals. Among the most important hormones involved are the hypoglycemic hormone insulin and the hyperglycemic hormone glucagon. In times of hyperglycemia, insulin acts directly on tissues to remove glucose from the plasma while also preventing peripheral glucose production or release from tissues. Glucagon promotes endogenous glucose production and release from the liver, elevating plasma glucose to correct hypoglycemia. A lack of these hormones or the secondary functions of insulin associated with intermediary metabolism may help explain differences in glucose tolerance between mammals and fish.

#### 1.3.1. Insulin

Insulin is the principal glucostatic hormone in mammals. Insulin is secreted by the pancreas, a mixed gland containing both endocrine and exocrine components (Norris, 1999). The endocrine portion is comprised of small masses called islets, which are spread throughout the acinar exocrine tissue. Islets are in turn made up of four distinct cell types: glucagon-secreting  $\alpha$  cells, pancreatic polypeptide or PP cells, somatostatin-secreting  $\delta$  cells, and insulin secreting  $\beta$ -cells (Youson and Al-Mahrouki, 1999). Insulin is stored within granules inside  $\beta$ -cells and released in times of hyperglycemia to re-establish normoglycemia. The secretion of insulin is mediated by a glucose sensing system involving the facultative glucose transporter GLUT-2 and the glycolytic enzyme glucokinase (GK), or hexokinase IV (HK IV) (Postic *et al.*, 2001). GLUT-2 is a bi-directional glucose transporter found predominantly on liver and  $\beta$ -cell membranes. It belongs to a much larger family of glucose transporters with up to eight well-characterized and as many as twelve total members in mammals (Khan and Pessin, 2002). GLUT-2 has a low affinity for glucose that ensures

glucose flux into and out of the cell is held at levels proportional to plasma glucose concentrations (Zierler, 1999; Voet *et al.*, 2002). Following a glucose load, this glucose sensing system initiates a chain of events that returns plasma glucose levels to their initial state. In a healthy mammal, glucose passively enters  $\beta$ -cells along its concentration gradient through GLUT-2. Inside the cell, the catabolic process of glycolysis metabolizes glucose. The first committed step of glycolysis is the phosphorylation of glucose by GK to glucose-6-phosphate. The low affinity of the GK ( $K_m = 5\text{-}12\text{ mM}$ ; Blin *et al.*, 1999) enzyme ensures that glucose phosphorylation varies linearly with cytosol glucose concentrations, in much the same manner that entry of glucose into the cell through GLUT-2 varies with plasma glucose concentration. The coupling of these two processes causes glucose phosphorylation to increase or decrease depending upon plasma glucose content. Because glycolysis is an energy-producing pathway, the intracellular adenosine triphosphate/adenosine diphosphate (ATP/ADP) ratio is consequently matched to plasma glucose. Increased ATP/ADP ratios reduce the activity of ATP-sensitive  $K^+$  channels ( $K_{ATP}$  channels). Under normoglycemic conditions, the outward rectifying  $K_{ATP}$  channels maintain the  $\beta$ -cell membrane in a hyperpolarized state by allowing the free flow of ions across the membrane. Inhibition of  $K_{ATP}$  channels by elevated ATP concentrations results in blocking ion flux and cell depolarization leading to the initiation of membrane action potentials. Insulin containing granules are stimulated to move towards the cell membrane where insulin is released by exocytosis into the blood stream (Aguilar-Bryan *et al.*, 2001). Circulating insulin binds primarily to receptors on skeletal muscle and adipose tissue, recruiting internalized glucose transporters (GLUT-4) that take up plasma glucose thereby restoring normoglycemia. This process is summarized in Figure 1.1.

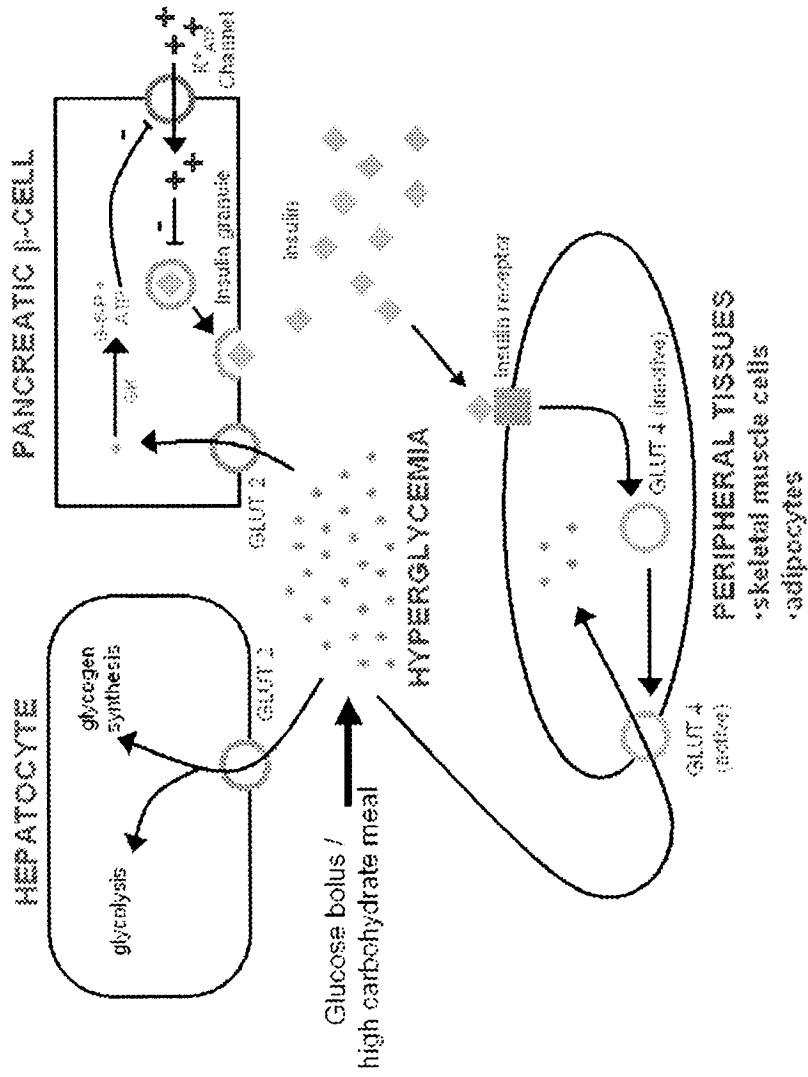


Fig 1.1: Cartoon depicting the mechanisms responsible for re-establishing normoglycemia after a glucose challenge in mammals.

In fish, the major role of insulin may not be the correction of plasma hyperglycemia. Unlike mammals, fish do not have a compact pancreas *per se*, but instead a grouping of endocrine cells, sometimes surrounded by exocrine tissue, which is collectively called the Brockmann body or bodies (Epple and Brinn, 1987). Four cell types have been found in fish Brockmann bodies: A, B, D and F cells. Insulin is secreted by B-, or  $\beta$ -cells, while A and D cells secrete glucagon and somatostatin, respectively (Youson and Al-Mahrouki, 1999). Insulin is highly conserved amongst the vertebrates, and is present in fish at concentrations slightly higher than those seen in mammals (Mommsen and Plisetskaya, 1991). This finding must be interpreted carefully, however as the radioimmunoassay used to quantify fish insulin cannot discern between insulin and pro-insulin, an insulin precursor with no established physiological action (Plisetskaya, 1998). Several studies report 2- to 3-fold increases in plasma insulin during GTTs in brown trout (Blasco *et al.*, 1996), Chinook salmon (Mazur *et al.*, 1992) and carp, yellow tail and red seabream (Furuichi and Yone, 1981), demonstrating that glucose administration can stimulate insulin release. Insulin concentrations were, however, seen to decrease in at least one IPGTT in rainbow trout (Harmon *et al.*, 1991). It should also be noted that while glucose is capable of eliciting insulin release, amino acids, and in particular arginine, are the most potent insulin secretagogues in fish (Mommsen and Plisetskaya, 1991). Despite these differences, it is generally agreed that fish possess insulin in quantities similar to those of mammals (Mommsen and Plisetskaya, 1991).

Insight into the capacity for the fish islet organ to produce insulin was gained through studies of Brockmann body xenotransplantation from fish to mammals. Tilapia are considered as possible donors for diabetics in need of islet transplants (Wright, 1992). Tilapia may provide a convenient alternative to the limited supply of human donors, and the ease of

harvesting anatomically distinct Brockmann bodies would reduce the need for expensive procedures of islet isolation (Wright, 1994; Wright and Schrezenmeir, 1995). The most successful transplantation experiments are carried out using islet encapsulation, the enclosure of tilapia islet grafts within semipermeable membranes with small pores that allow passive transport of insulin, glucose and oxygen, but prevent entry of larger immunoglobulins (Lanza and Chick, 1994, 1997; Kuhlreiber *et al.*, 1999). In mice, B cells of the pancreas can be destroyed by the drug streptozotocin in order to create a mammalian diabetic model. Transplantation of tilapia islets into streptozotocin-diabetic mice not only maintained long-term normoglycemia, but also mammalian-like glucose tolerance profiles (Wright *et al.*, 1992; Yang *et al.*, 1997). The fact that islets from a glucose intolerant species could impart glucose tolerance in a recipient species lends support to the notion that fish  $\beta$ -cells have the appropriate glucose-sensing system and can produce insulin but the fish may be resistant to its effects.

The structure and function of the fish  $\beta$ -cell might also become more clear through studies of  $K_{ATP}$  channel manipulation using drugs such as diazoxide and glibenclamide. As noted above, pancreatic  $\beta$ -cell  $K_{ATP}$  channels indirectly control insulin secretion by maintaining hyperpolarization of the cell (also see Fig. 1). The channels can be inhibited by high ATP/ADP ratios, causing action potentials that stimulate the exocytosis of insulin-containing granules.  $K_{ATP}$  channels are composed of two subunits: the sulfonylurea receptor (SUR) and the KIR6.x pore (Aguilar-Bryan and Bryan 1999). While SUR is generally associated with nucleotide sensitivity and pharmacology, KIR6.x is associated with inward rectification of  $K^+$  by forming the conductance pathway for ion flow (Babenko *et al.*, 1998). Glibenclamide is a sulfonylurea that promotes insulin secretion through interaction with the

SUR subunit and closing of the  $K_{ATP}$  channel (Khanna and Singh, 1983). Conversely, diazoxide is a hyperinsulinemia-attenuating drug that acts to keep the  $K_{ATP}$  channel open, thus inhibiting insulin secretion. Marked hypoglycemia observed in the freshwater fish, *Puntius conchoni* after glibenclamide administration and the lack of effect in streptozotocin-diabetic fish suggest that insulin secretion in fish is influenced in a manner similar to mammals (Loubatieres *et al.*, 1969; Bhatt and Gill, 1979). Continuing experimentation with diazoxide and other  $K_{ATP}$  channel modulating drugs may contribute to our understanding of  $\beta$ -cell function in fish.

The role of insulin in vertebrates is not strictly limited to carbohydrate metabolism. Insulin belongs to a larger family of peptide hormones that also includes insulin-like growth factors I and II (IGF-I and IGF-II). These peptides are involved in several physiological processes, including cell metabolism, differentiation and growth (Planas *et al.*, 2000a). In mammals, the primary function of insulin is to regulate carbohydrate metabolism, while IGF-I and -II are primarily involved in growth, cell differentiation, and fetal development (Planas *et al.*, 2000). In fish, the roles of these hormones are not well differentiated, as both IGF and insulin have strong growth promoting effects (Mommensen and Plisetskaya, 1991). The reason for this lack of specificity in fish becomes more obvious upon the examination of insulin and IGF receptors.

### 1.3.2. Insulin receptors and glucose transporters

If insulin content is not responsible for the sluggish response to hyperglycemia in fish, another possibility is that the “problem” lies at the level of peripheral insulin receptors and the GLUT proteins they activate (see Fig 1.). Binding studies have revealed that insulin

binds specifically to receptors on rainbow trout isolated skeletal muscle and hepatocyte membranes (Albett *et al.*, 1983; Gutiérrez and Plisetskaya, 1990; Plisetskaya *et al.*, 1993). Specific binding has also been reported in carp ovaries (Gutiérrez *et al.*, 1993), hepatocytes (Segner *et al.*, 1993) and red and white muscles (Banõs *et al.*, 1998). In a comparison of three species, insulin binding and tyrosine kinase activities (the functional properties of the insulin receptor) were highest in omnivorous carp and tilapia, and lower in the more carnivorous brown trout (*Salmo trutta*) and gilthead seabream (*Sparus aurata*) (Parrizas *et al.*, 1995). Generally, insulin receptor densities are highest in the most herbivorous, and hence most glucose tolerant fish species (Navarro *et al.*, 1999). Fish insulin receptors may be down-regulated by hyperinsulinemia and fasting, or specific binding enhanced by feeding a high carbohydrate diet (Gutiérrez *et al.*, 1991).

Complicating the issue of peripheral insulin receptors is the fact that in vertebrates, insulin receptors do not exclusively bind insulin. Insulin and IGF-I receptors are members of the protein tyrosine kinase superfamily, bearing two  $\alpha$  and two  $\beta$  subunits linked by disulphide bonds to form an  $\alpha\beta$ - $\alpha\beta$  heterotetramer (LeRoith *et al.*, 1995). The  $\alpha$  subunits are external to the membrane and include the ligand-binding site, while the  $\beta$  subunits penetrate the phospholipid bilayer and contain the tyrosine kinase activity within the cell cytosol; binding of a ligand activates tyrosine kinase that in turn activates secondary phosphorylation signals (Navarro *et al.*, 1999). IGF-II receptors are structurally unrelated, and lack tyrosine kinase activity due to a shortened cytosolic tail (Lane, 1981). Though insulin, IGF-I and -II receptors recognize and discriminate ligands if present in physiologically relevant ranges, some cross interactions exist (Navarro *et al.*, 1999). Notably, in mammals IGF-II binds to all three receptors; the IGF-II receptor acts to bind circulating IGF-II, clearing it from the

bloodstream. This allows control over the levels of IGF-II that reach target tissues, where their physiological action is carried out by binding to insulin and IGF-I receptors (Stewart and Rotwein, 1996). In fish, these receptors differ in relative distribution and ligand specificity from their mammalian counterparts (Parrizas *et al.*, 1995). In skeletal muscle, fish generally have lower insulin receptor densities than do mammals (Planas, 2000b). Another interesting point is that in all fish tissues, insulin receptors are vastly outnumbered by IGF-I receptors. The opposite is true for vertebrates such as mammals, suggesting a trend for increased specialization of insulin function in the evolution of vertebrates (Planas *et al.*, 2000b). Still, insulin binding and receptor numbers are immaterial unless fish possess responsive, functional glucose transporters.

Several distinct GLUT isoforms (GLUT-1–12) are identified in mammals. At present, the class I glucose transporters (GLUT-1–4) have the best-characterized kinetics, tissue distribution and substrate specificity (Khan and Pessin, 2002), while the roles of other GLUTs remain unclear. Following a glucose challenge, the timely return to normoglycemia relies on peripheral uptake of glucose into primarily skeletal muscle and adipose tissue by GLUTs (see Fig. 1). GLUT-1 and -4 are facilitative transporters that allow glucose to enter cells by following glucose concentration gradients. GLUT-1 transporters are ubiquitously distributed and are responsible for basal glucose uptake into most tissues. GLUT-4, the most thoroughly studied glucose transporter, also transports glucose from the blood to the tissues but is activated in an insulin-dependent manner. GLUT-4 is found primarily in skeletal muscle and adipose cells and is the chief transporter used to remove glucose from the blood during hyperglycemia (Zierler, 1999; Voet *et al.*, 2002). Translocation of GLUT-4 containing granules and their insertion into the cell membrane is initiated by the binding of insulin to the

insulin receptor; this activity accounts for up to 30% of the reduction in plasma glucose levels following an IVGTT (Hovorka *et al.*, 2002). GLUT-2 is the bi-directional glucose transporter that ensures glucose flux into and out of the liver is held at appropriate levels depending on blood glucose concentrations (Zierler, 1999; Voet *et al.*, 2002), and is essential to “glucose sensing” in  $\beta$ -cells discussed earlier (see section 1.3.1). These and other GLUTs work in concert to ensure a continuous supply of glucose to all cells and to re-establish plasma glucose homeostasis when disturbed from equilibrium.

Until recently, little was known regarding the presence of GLUTs in fish. With modern advances in cloning, this has begun to change. Three GLUT isoforms with high sequence homologies to mammalian GLUTs were identified in fish. The first of these was found in brown trout and was primarily expressed in skeletal muscle, gill, kidney and adipose tissue. This glucose transporter, designated as btGLUT, is homologous to GLUT-4 (Planas *et al.*, 2000b). Using arginine as a secretagogue, Capilla *et al.* (2002) raised insulin levels to examine their effect on btGLUT expression. Expression of btGLUT mRNA is predominant in white and red muscle tissues, but only in red muscle was expression up-regulated by insulin.

Cloning of GLUTs in rainbow trout and closely related salmonids confirmed the presence of a functional GLUT-1 type transporter, predominantly in trout cardiac muscle (Teerijoki *et al.*, 2000). Further investigation revealed a GLUT-1 homologue in rainbow trout muscle, denoted OnmyGLUT-1 (Teerijoki *et al.*, 2001). Expression of this GLUT does not appear to be under the influence of insulin, which suggests it may behave in the same constitutive manner as mammalian GLUT-1 (Capilla *et al.*, 2002). Hybridization of the

OnmyGLUT1 probe with genomic DNA from several species of the order Salmoniformes suggests GLUT genes are widespread in this group (Teerijoki *et al.*, 2000).

OnmyGLUT-2, a putative mammalian GLUT-2 homologue, was identified in rainbow trout liver by Krasnov *et al.* (2001). Cytochalasin B, phloretin and 2-deoxy-D-glucose inhibited the uptake of 3-O-methylglucose in isolated trout hepatocytes, suggesting that OnmyGLUT-2 is indeed a functional facilitative glucose transporter in these cells (Krasnov *et al.*, 2001).

Taken together, the currently identified fish GLUTs support the possibility that fish possess a mechanism for regulated glucose uptake into peripheral tissues, which at least in the case of btGLUT (a GLUT-4 homologue) is insulin-dependent in red muscle. The presence of the GLUT isoforms varies with fish species. Severe peripheral resistance to insulin in tilapia has been attributed to the absence of an insulin-sensitive glucose transporter; this study, however was undertaken using heterologous antibodies to mammalian GLUTs (Wright *et al.*, 1998). So even though tilapia islets transplanted into diabetic mice confer normal glucose tolerance profiles to the recipient, they may not do so for the donor tilapia. Further studies at the protein level must be conducted to determine whether sequence homology and insulin-controlled expression of mRNA indicate that fish glucose transporters play a significant physiological role in glucoregulation.

#### **1.4. Regulation of Cellular Glucose Metabolism**

Assuming that plasma insulin concentrations are adequate and peripheral tissues contain the appropriate GLUTs, the last possible weak link in glucoregulation is tissue glucose metabolism. In the mammalian model, glucose enters the cell and is phosphorylated

to glucose-6-phosphate (G-6-P) by a hexokinase. G-6-P is metabolized by the enzymes of glycolysis producing two moles of pyruvate that may enter the tricarboxylic acid (TCA) cycle. The TCA cycle in turn generates reducing equivalents used by the electron transport chain to produce energy for cellular work. The mechanisms that govern cellular respiration in fish, however, may not entirely conform to these mammalian paradigms.

#### 1.4.1. Glycolysis

Once inside the cell, the ultimate fate of glucose is determined by the action of enzymes. These enzymes ensure glucose storage and oxidation, while maintaining the steep concentration gradient across the membrane that allows glucose to enter the cell through appropriate GLUTs. Glucose oxidation is accomplished by glycolysis and the TCA cycle, collectively called intermediary metabolism. Glycolysis is comprised of a series of enzyme catalyzed reactions that generate ATP while converting one mole of glucose into two moles of pyruvate (Nelson and Cox, 2000). The major pathways involved in intermediary metabolism are outlined in Figure 1.2. The first committed step in glycolysis is glucose phosphorylation by hexokinase. A family of four hexokinases with varying kinetics and specific tissue distributions exist in mammals. Of these, hexokinase IV (HK-IV) or glucokinase (GK) has the highest  $K_m$  (therefore, lowest affinity) for glucose and is exclusively localized to liver and pancreatic  $\beta$ -cells in mammals (Blin *et al.*, 1999). Aside from enabling insulin release (see section 1.3.1 and Fig. 1.1), this enzyme is involved in controlling hepatic glucose utilization rates (Postic *et al.*, 2001). Mutations in the GK gene are linked to non-insulin-dependent diabetes mellitus (NIDDM) in mammals (Blin *et al.*, 1999), hence an absent or deficient GK could explain glucose intolerance in fish. However,

GK gene expression and enzyme activity were verified in rainbow trout and gilthead seabream (Panserat *et al.*, 2000). In fact, GK mRNA from trout and seabream are increased 4 h after feeding a high carbohydrate meal, consistent with increases in GK enzyme activity seen after 6 h in these species (Caseras *et al.*, 2000; Panserat *et al.*, 2000). The presence and functionality of other glycolytic enzymes, such as pyruvate kinase (PK) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase2) have also been evaluated. Expression of these enzymes in mammals is regulated by a variety of factors including dietary carbohydrates, hormones like glucagon, as well as intermediate products of glycolysis (Thorens, 1992; Pilkis *et al.*, 1995; Yamada and Noguchi, 1999). Deduced amino acid sequences of trout PFK-2/FBPase2 and PK are up to 80% similar to their mammalian counterparts. While rainbow trout fed high levels of carbohydrates had higher PFK-2/FBPase2 mRNA levels than fasted trout, gene expression of PK was always high, irrespective of nutritional status (Panserat *et al.*, 2001b). Due to the bifunctional nature of PFK-2/FBPase2, increased expression due to diet could lead to either increases or decreases in fructose-2,6-bisphosphate (F-2,6-P<sub>2</sub>), activating or inhibiting glycolysis. In fact, F-2,6-P<sub>2</sub> is the most potent regulator of glycolysis identified to date (Pilkis *et al.*, 1995). The phosphorylation status of the PFK-2/FBPase2 enzyme thus requires further investigation.

#### 1.4.2. The tricarboxylic acid cycle

In order to recover the electron carriers required to sustain glycolysis under anaerobic conditions, lactate dehydrogenase (LDH) converts pyruvate to lactate and produces NAD<sup>+</sup>. Under aerobic conditions, however, pyruvate is oxidized to CO<sub>2</sub>, ATP and water by the citric acid or tricarboxylic acid (TCA) cycle (Nelson and Cox, 2000). The TCA cycle yields larger

amounts of energy than glycolysis alone by providing the electron transport chain with reducing equivalents that facilitate the production of ATP. The multicomponent enzyme complex pyruvate dehydrogenase (PDH) converts pyruvate to Acetyl-CoA where it condenses with oxaloacetate to initiate the TCA cycle by forming citrate. Several enzymes are involved in the TCA cycle, which serve to generate reducing equivalents for the production of ATP in the electron transport chain, as well as reform oxaloacetate to perpetuate the cycle (Nelson and Cox, 2000). Hochachka (1969) and others (Saurez and Hochachka, 1987; Walton, 1985) have reviewed the status of the TCA cycle in fish mitochondria, the site of the TCA cycle reactions, and found rainbow trout mitochondria are capable of oxidizing all TCA cycle intermediates. All TCA cycle enzymes have been identified in trout liver mitochondria, and so it has been concluded that fish possess a complete, functional TCA cycle (Walton, 1985).

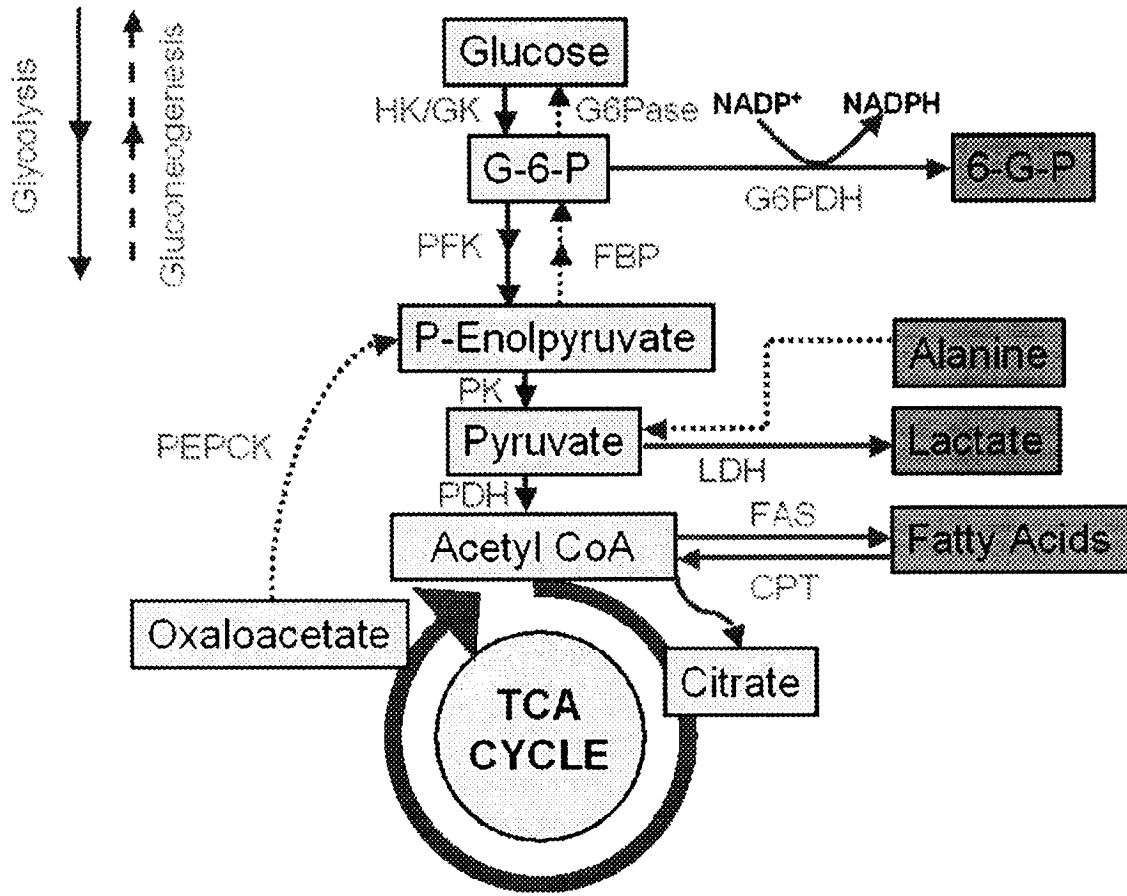


Fig 1.2: Cartoon illustrating the pathways of intermediary metabolism: glycolysis, the tricarboxylic acid (TCA) cycle and the gluconeogenic pathways. See text for abbreviations.

### 1.4.3. Gluconeogenesis

Mammals carefully balance glycolysis with gluconeogenesis in order to regulate plasma glucose concentrations. When the liver is presented with excess glucose, the glucose-sensing system activates glycolysis, while the formation of glucose by gluconeogenesis is blocked (Rencurel and Girard, 1998). Gluconeogenesis is generally reserved for times when dietary glucose is unavailable and must be formed from carbohydrate or non-carbohydrate precursors. Two molecules of phosphoenol pyruvate are converted back to glucose through the glycolytic reactions, which are all reversible with the exception of those catalyzed by HK,

PFK and PK. Reverse reactions to bypass these unidirectional enzymes are catalyzed by glucose-6-phosphatase (G6Pase), fructose biphosphatase (FBPase) and phosphoenol pyruvate carboxykinase (PEPCK). Pathways of both glycolysis and gluconeogenesis are shown in Figure 1.2. Lactate along with specific amino acids, which are readily available in a carnivorous fish diet, are important gluconeogenic substrates. Gluconeogenesis is an energetically expensive process, requiring four ATP and two GTP high-energy phosphates to produce one glucose molecule (Nelson and Cox, 2000).

Mammals keep gluconeogenesis under tight control, but in fish this does not appear to be the case (Panserat *et al.*, 2001). Dietary carbohydrates do not strongly depress gene expression of either PEPCK or G6Pase in rainbow trout (Panserat *et al.*, 2000). Furthermore, FBPase gene expression is not significantly reduced by dietary carbohydrate in rainbow trout hepatocytes (Panserat *et al.*, 2001). Lack of down-regulation of these genes suggests that trout may be unable to alter hepatic glucose production in response to hyperglycemia. Similar observations have been made in other carnivorous fish; FBPase activity is unaffected by dietary carbohydrate in both Atlantic salmon and perch (Tranulis *et al.*, 1996; Borrebaek and Christophersen, 2000). The continuous formation of glucose is a logical expectation for fish, which consume little dietary carbohydrates but rely on glucose to support cell function in key tissues such as the brain and kidney, where exogenous glucose is oxidized directly (Hemre *et al.*, 2001). Yet constitutive gluconeogenesis is not a property shared by all fish species; omnivorous carp show a depression in the activity of G6Pase and other gluconeogenic enzymes when placed on a diet supplemented with starch, glucose or fructose (Panserat *et al.*, 2000). European seabass and gilthead seabream likewise demonstrate repressible gluconeogenesis, as FBPase activity can be inhibited by diet (Meton *et al.*, 1999).

As with glucose tolerance and insulin receptors, glucose flux through the opposing pathways of glycolysis and gluconeogenesis is species specific, and may provide the strongest cause for glucose intolerance in fish (Panserat *et al.*, 2001).

If some teleost fish are truly unable to adjust gluconeogenic flux, it would be useful to measure glucose turnover rates during a glucose challenge. Practical methods of measuring accurate *in vivo* metabolite turnover rates have been available for many years and are used widely in mammalian metabolite studies (Hetenyi *et al.*, 1983). Adapting these methods to fish has been difficult, partly because of their small body size, but a reliable continuous tracer infusion method for measuring substrate turnover *in vivo* was developed and validated for rainbow trout by Haman *et al.* (1997). The application of this technique to measure glucose flux in a live fish exposed to a glucose load might help reveal underlying causes for glucose intolerance.

It is important to consider whether fish actually fit the glucose intolerant paradigm. While their slow performance during glucose tolerance tests is well documented (see Moon, 2001), these tests do not take into consideration the low metabolic rate of most fish. Fish are ectotherms, and their range of thermal tolerance is very wide. In desert lakes, species live in temperatures in excess of 50 °C, while in Antarctic waters, fish withstand temperatures as low as -1.86 °C (Hockachka and Somero, 2002). Accordingly, oxygen consumption rates are one-tenth that seen in similar sized mammals, and blood flow rates, nutrient uptake and nutrient demands are expected to correlate with these values (Moon, 2001). It should come as no surprise that glucose clearance is scaled down in fish, since glucose turnover is known to be much lower than in mammals (Weber and Zwingelstein, 1995).

## 1.5. Manipulation of Fish Diets

Although interest in fish carbohydrate has gained popularity over recent decades, human interest in the nutrition of fish is hardly a new concept. The discovery of confined fish depicted on ancient mosaics provide evidence that rudimentary aquaculture dates as far back as the Roman Empire (Halver, 1989). McCay and Dilley (1927) first attempted to identify the anti-anemic factors essential for maintaining captive trout for more than 3 months. It would be twenty years before "factor H" was found to be a vitamin B12 - folic acid combination. Much of the subsequent research in this area centered on determining similarities and differences between dietary requirements of fish and the already well documented dietary requirements of many mammals and birds. Salmonid fish such as the rainbow trout were the focus of these early studies, and have remained one of the most widely used experimental models. This is likely due in part to the fact that salmonids, and in particular rainbow trout, make up the majority of fish aquacultured in North America (Brown, 1977).

In the late fifties, salmonids were reported to have vitamin and amino acid requirements similar to those of mammalian and avian species (Halver, 1989). Proteins, however, accounted for up to 70% of dietary calories, a much higher level than that seen in most "warm blooded" animals, and reflected in the high protein content of fish diets at the time (Phillips, 1969). In the wild, fish have developed carnivorous lifestyles, with few exceptions (Cowey and Walton, 1989). It follows then, that fish must have also developed a metabolism adapted to deal with dietary proteins. Efforts to replace dietary protein with other sources of energy were usually met with limited success. While the use of fats or carbohydrates in protein-sparing diets allowed some reduction in protein without curtailing

weight gain, higher levels of carbohydrates in most fish diets resulted in lowered digestibility and growth (Tunison *et al.*, 1941; Phillips *et al.*, 1948; Inaba *et al.*, 1963).

The potential benefits of overcoming fish glucose intolerance appeal to scientists and aquaculturists alike. Not surprisingly, diet manipulation studies have been a key tool in our attempts to assess, explain and modify glucose metabolism and tolerance in fish (Hemre *et al.*, 2001). Early recommendations by Phillips (1948) were that carbohydrates should not exceed one-tenth of the dry dietary matter in the salmonid diet. Levels above this were seen to cause reduced growth, high liver glycogen levels and increased mortality. Despite confirmation by Hilton and Atkinson (1982), many have challenged the claim that carbohydrates are unsuitable for the average teleost diet. Buhler and Halver (1961) fed chinook salmon a series of diets in which the protein level was reduced from 71 down to 40% by increasing dextrin levels up to 43%. All diets produced similar growth, and the replacement of protein by dextrin increased the protein efficiency ratio (PER) of the remaining protein. Similar results were achieved in rainbow trout fed diets containing 30% and 50% raw cornstarch. The same growth and a better PER were seen than in diets with twice the protein content (Luquet, 1971). A wide miscellany of carbohydrate sources has been incorporated into fish diets including glucose, sucrose, pre-cooked and gelatinized starch (Tiews *et al.*, 1976; Pieper and Pfeffer, 1978). Digestibility of complex carbohydrates seems to be low, but at given levels, simple sugars such as glucose provide digestible energies near 100%. Bergot (1979a) devised an experimental diet containing 30% glucose and a protein level reduced from 60-30% to feed trout, but observed a negative effect on growth and feed conversion rate. However, the same diet with a protein level of 45% allowed trout to tolerate and efficiently utilize this high level of glucose (Bergot, 1979b). These

experiments provided the first promising examples of how fish can be made to utilize dietary carbohydrates. More recent studies have focused on the impact of carbohydrate diets on hormonal and biochemical aspects of glucose regulation.

The effect of dietary carbohydrates on key enzymes of metabolism has been studied in several fish species. The carnivorous gilthead seabream, one of the most extensively cultured fish in Mediterranean countries, demonstrates an ability to modify enzyme activities of liver glycolysis-gluconeogenesis after exposure to significant amounts of carbohydrate in the diet (Meton *et al.*, 1999). Dietary compositions ranging from 9.9 - 31.6 g carbohydrate per kg diet and correspondingly 58.5 - 37.4 g protein per kg diet were fed to seabream for 22 days. The enzymes PFK and PK were stimulated by the high carbohydrate diets, as were G6PDH and 6PGDH. These increases favour activation of the glycolytic and pentose-phosphate pathways, respectively. Activation of the pentose-phosphate pathway by the conversion of glucose-6-phosphate to 6-phospho-gluconolactone both utilizes glucose and provides NADPH reducing equivalents for the conversion of glucose to fatty acids (Nelson and Cox, 2000). Activity of the gluconeogenic enzyme FBPase-1 was reduced along with activities of alanine transaminase (ALT) and aspartate transaminase (AST). The latter two enzymes are involved in amino acid catabolism and act by converting their respective amino acids into a common product, glutamate, for oxidation and anabolism (Nelson and Cox, 2000). A comparative study between gilthead seabream and the relatively glucose tolerant common carp confirms the inhibition of gluconeogenesis by dietary carbohydrates in seabream, citing increased hepatic expression of genes for G6Pase and FBPase. Increased gluconeogenesis in carp was also observed, as was evidenced by up-regulation of PEPCK gene expression (Panserat *et al.*, 2002). An array of enzymes was also measured in the more

herbivorous tilapia (*Oreochromis niloticus* x *O. aureus*) fed both starch- and glucose-rich diets over 4 weeks (Lin and Shiau, 1995). Previous studies established starch as a better utilized substrate than glucose. Comparison of hepatic enzyme activity between starch- and glucose-fed fish revealed significantly higher G6PDH and phosphogluconate dehydrogenase (PGDH) activities in fish on the high starch diet, implicating a higher capacity for fatty acid biosynthesis. This implication is corroborated by the weight gain experienced by the starch-fed group which was nearly two-fold that of the glucose-fed group. Interestingly, there were no differences observed in either the glycolytic enzymes HK, PFK, or the gluconeogenic enzyme G6Pase between the two groups, even when fish on the starch diet were switched to glucose halfway through the experiment and vice versa. This study suggests the capability for tilapia to adjust hepatic control of fatty acid production depending on diet regime, but not necessarily the pathways of glycolysis and gluconeogenesis. Rainbow trout have been used in numerous feeding experiments, with conflicting reports. Abel *et al.* (1978) reported high hepatic GK activities in trout maintained on high starch or sucrose diets, yet several others (Shatton *et al.*, 1971; Nagayama and Ohshima, 1974; Cowey *et al.*, 1977; Nagayama *et al.*, 1980; Fideu *et al.*, 1983; Sundby *et al.*, 1991; Tranulis *et al.*, 1991) were unable to detect any effect of hexose phosphorylation activity due to high carbohydrate diets. The problem of the latter group of studies may stem from the lack of appropriate analytical methods to actually quantify GK activities in the presence of the more prevalent HK activities (Borrebaek *et al.*, 1993). Recently, Panserat *et al.* (2000) showed that both GK mRNA and enzyme activity are highly induced with a single meal containing 24% glucose. It was also shown that G6Pase activity remained high after the glucose meal, despite decreased G6Pase expression, implying poor control over gluconeogenesis in response to dietary carbohydrates.

## **1.6. Research Hypothesis and Study Objectives**

In western society, carnivorous teleost fish including the rainbow trout are popular among consumers, the fish farmer and the scientist. Improvement upon high protein diets through the use of higher levels of dietary carbohydrates would provide benefits for all, but to do so requires a better understanding of the role carbohydrates play in fish metabolism. It is the goal of this study to investigate the long-term effects of a high carbohydrate diet in rainbow trout using two experimental diets containing low and high levels of glucose. I will test the hypothesis that by altering flux through the pathways of intermediary metabolism and the TCA cycle, rainbow trout can increase their tolerance and utilization of glucose in response to changes in dietary carbohydrates. Changes in enzyme expression and activity, body composition and growth are determined by examination of the carcass and tissues. Differences in growth between the two groups will be used to determine the protein efficiency ratio of each diet. Glucose tolerance and turnover following glucose challenges are evaluated using IVGTTs and continuous tracer infusion methods. Indirect calorimetry is used to determine differences in substrate utilization between the two groups.

## **2. METHODS**

### **2.1. Experimental Animals**

Female rainbow trout (*Oncorhynchus mykiss*), weighing approximately 100-150 g, were transported from Linwood Acres Trout Farm (Campbellcroft, ON) to the University of Ottawa Aquatic Care Facility. Fish were maintained in 230 L fibreglass holding tanks supplied with well-aerated, dechloraminated City of Ottawa tap water at 13 °C, under a constant 12L:12D photoperiod. The trout were non-selectively divided into groups of 25 fish per tank and fed 1% body weight daily, with either a low carbohydrate (LC) or high carbohydrate (HC) diet, over a period of 6 months. The Animal Care Committee of the University of Ottawa approved (protocol number BL-160) the feeding and handling of all fish.

### **2.2. Diets**

Two diets, a low carbohydrate diet (<1% by weight) and a high carbohydrate diet (32.9% by weight), were formulated by and purchased from Zeigler Brothers, Inc. (Gardners, PA). Composition of the diets is presented in Table 2.1.

Table 2.1. Composition of experimental diets

<b>Percent by weight<sup>a</sup></b>	LC Diet	HC Diet
Carbohydrate	1.00	32.90
Protein	40.40	40.50
Fat	12.06	12.09
Crude Fiber	32.30	1.50
<b>Percent by energy<sup>b</sup></b>		
Carbohydrate	1.23	29.03
Protein	49.70	35.83
Fat	36.79	26.53
Total digestible energy (kJ/g)	12.80	17.80

<sup>a</sup> contributions to total mass and energy made by ash, vitamins and minerals are not shown.

<sup>b</sup> energy contributions were derived from energy yields of complete oxidation of glucose (15.71 kJ/g), palmitate (39.06 kJ/g), and average protein (15.75 kJ/g) (Halver, 1989).

Two groups of trout were used in these experiments. One group began feeding in November 2001 and continued through to May 2002. Blood and tissues were sampled on May 15, 2002 between 12:00 and 13:00 h. Respirometry studies were carried out in June on fish from this group. Metabolites and enzymes were measured in frozen tissue samples between June and August. A second group of trout was received in September 2002 and held until June 2003 under the same experimental conditions as the first group. Glucose tolerance tests were conducted between January and March, and glucose turnover studies were conducted in June.

### 2.3. Tissue Collection and Preparation

Four hours after the final meal, trout were netted from each tank and anaesthetized. The anaesthetic was prepared by dissolving 1 g benzocaine (Sigma Chemical Co., St. Louis, MO) in 10 ml of 95% ethanol. This solution was diluted 1:60,000 in 13 °C City of Ottawa tap water, in which the fish was then placed. Body weight and length were recorded and blood was sampled immediately by caudal puncture using a heparinized syringe. Blood was briefly stored on ice and subsequently centrifuged at 14,000 x g (Eppendorf 5414c) to separate plasma, which was aliquotted and frozen at -80 °C for later analysis. Fish were killed with a swift blow to the head and tissues were collected within five minutes. Red and white muscle tissues from anterior, middle and posterior sections of the epaxial region were collected and freeze-clamped between aluminum blocks cooled in liquid N<sub>2</sub>. Liver was excised, weighed and freeze-clamped. Liver and muscle tissue were stored at -80 °C until enzymes and metabolites were assayed. All visible adipose tissue was scraped from the gastrointestinal tract and weighed to estimate a visceral body fat index.

### 2.4. Calculations of Body Parameters

Recorded body weights (B<sub>w</sub>) and lengths (L) were used to calculate the condition factor (K) and specific growth rates (SGR<sub>exp</sub>) of each fish using the following equations:

$$K = 100 \cdot \frac{B_w}{L^3} \quad (\text{Anderson \& Neumann, 1996})$$

where weight is in g and length is in cm.

$$\text{SGR}_{\text{exp}} = 100 \cdot [\ln(B_{\text{w initial}}) - \ln(B_{\text{w final}})] \cdot t^{-1} \quad (\text{Ricker, 1979})$$

where t is time in days of the feeding trial (200 d).

Hepatosomatic index (HSI, % of BW) was calculated using the measured values for liver weight ( $W_{\text{Liver}}$  in g) and body weight (g):

$$\text{HSI} = 100 \cdot \frac{W_{\text{Liver}}}{B_{\text{w}}}$$

Protein efficiency ratio (PER) was calculated using the wet weight gain of the fish (g) and the amount of crude protein fed to the fish ( $W_{\text{PROTEIN}}$ ):

$$\text{PER} = \frac{B_{\text{w final}} - B_{\text{w initial}}}{W_{\text{PROTEIN}}} \quad (\text{Halver, 1989})$$

## 2.5. Metabolite Assays

All assays used a SPECTRAMax PLUS 384 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at room temperature and quantities calculated using SOFTmax PRO (Molecular Devices).

### 2.5.1. Glucose

Plasma glucose concentrations were measured using the coupled reaction of glucose phosphorylation by hexokinase and G6PDH production of NADH measured

spectrophotometrically at 340 nm. Assays were carried out using a reaction mixture consisting of 60 mM Trizma base, 40 mM Tris-HCl, 1 mM MgSO<sub>4</sub>, 2 mM NAD<sup>+</sup>, 1 mM ATP, 0.1 units/ml G6PDH (*Leuconostoc*) and 0.06 units HK. After a 30 min incubation period at room temperature, spectrophotometric readings were taken. Glucose concentrations were calculated based on a standard curve of D-glucose concentrations.

### 2.5.2. Protein

Measurements of protein content used frozen tissue homogenized in 50 mM imidazole buffer. The Bicinchonic acid (BCA) protein assay (Sigma, St. Louis, MO) was employed with bovine serum albumin (BSA) as a standard and measured spectrophotometrically at 540 nm.

### 2.5.3. Glycogen

Tissue glycogen was assayed by measuring the glucose produced by the breakdown of glycogen with amyloglucosidase. Frozen tissues were rapidly sonicated with a Kontes Micro Ultrasonic cell disrupter (Mandel Scientific) in ice-cold 7% perchloric acid (PCA) at a 4:1 (v/w) dilution, then centrifuged for 5 min at 10,000 x g. One hundred µl of the supernatant was incubated in a 12 x 75 mm test tube for 2 h with 50 µl NaHCO<sub>3</sub> (1 M), and 1 ml amyloglucosidase in acetate buffer (1 mg/ml in 17 mM glacial acetic acid, 70 mM NaCHOOH, pH 4.8). The reaction was terminated by the addition of 25 µl 70% PCA. The mixture was centrifuged for 1 min in a Sorvall GLC-2 clinical centrifuge (Dupont Industries) and the supernatant was assayed for glucose as described in section 2.5.1, except using glycogen as a standard. Varying concentrations of glycogen (type II from oyster, Sigma

Chemical Co., St. Louis, MO) were used as standards and were carried through the assay in an identical manner as the tissue samples.

#### 2.5.4. Amino acids

Amino acids were assayed according to the method of Troll and Cannan (1953). Dilutions of plasma (final volume 250  $\mu$ l) containing between 0.05-0.5  $\mu$ moles amino acids were incubated in a boiling water bath with 0.5 ml 0.4% phenol in ethanol and 0.5 ml of 50 x diluted 0.01 M KCN in  $\text{NH}_3$ -free pyridine. The assay mixture was allowed to reach water temperature at which time 0.1 ml of 280 mM ninhydrin in ethanol was added and boiled for an additional 5 min. The solution was cooled and brought to 5 ml with 60% ethanol. The solution was then read spectrophotometrically at 570 nm, using varying concentrations of leucine that had undergone the same treatment as the samples. This method does not detect proline.

#### 2.5.5. Triglycerides

Determination of total plasma triglycerides was performed using the GPO-TRINDER assay (Sigma) according to the manufacturer's instructions.

#### 2.5.6. Lipids

Lipids were measured gravimetrically using the method of total lipid extraction of Bligh and Dyer (1959), in which tissue is suspended in a final methanol-chloroform ratio of 1:1. This method was chosen over the earlier method of Lees and Stanley (1956), a rapid method of lipid extraction that uses a 1:2 methanol-chloroform ratio, but has the

disadvantage of employing large and inconvenient volumes of solvent (Bligh and Dyer, 1959). Tissue samples were crushed into powder with a mortar and pestle in liquid N<sub>2</sub>. One gram powder was suspended in 5 vol of a methanol-chloroform mixture in a 2:1 v/v ratio. The suspension was homogenized for 2 min using a Kinematica polytron (Brinkmann Industries) at low speed. One ml chloroform per g tissue was added to the mix and homogenized for a further 30 sec. One ml double distilled H<sub>2</sub>O per g tissue weight was added and the mixture was homogenized for a further 30 sec.

The mixture was passed through a piece of Whatman No. 1 filter paper on a Buchner funnel with suction into a vacuum flask. When dry, the residue on the paper was pressed to recover unfiltered solvent. The residue was kept for determination of dry weight and the filtrate was transferred to 15 ml polypropylene centrifuge tubes and centrifuged at 2400 rpm for 20 min. The upper methanol layer was removed by aspiration and the lower chloroform layer was washed in 1:4 volumes of a medium containing 50% methanol, 3% chloroform, and 0.2% CaCl<sub>2</sub>. After a further centrifugation at the same settings, the methanol layer was removed and the chloroform layer was evaporated in air in a pre-weighed aluminum pan. Once dry, the weight of the lipid left in the pan was recorded and used to calculate tissue lipid content.

## **2.6. Enzyme Assays**

### **2.6.1. Enzyme assays in Mommsen buffer**

The following enzymes were measured spectrophotometrically in 96-well microplates using the SPECTRAMax PLUS 384 microplate spectrophotometer and SOFTmax PRO.

Except where noted otherwise, tissues were homogenized in 5 vol Mommsen buffer (50% glycerol, 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM β-mercaptoethanol, 0.5 mM EDTA-Na<sub>2</sub>, pH 7.4), centrifuged for 10 min at 10,000 x g and dilutions of the supernatant were used for the assay. Reaction conditions and final substrate concentrations are given below for each enzyme. Activities were assayed by adding substrate last and any activity in the absence of substrate was subtracted from the total to give actual activities. All activities were optimized for substrate concentrations. Activities were calculated based upon an extinction coefficient for NAD(P)H at 340 nm of 6.22 cm<sup>2</sup>/μmole or dithionitrobenzoic acid (DTNB) at 412 nm of 12.2 cm<sup>2</sup>/μmole unless noted otherwise. Conditions noted are final concentrations in the microplate well; variable extract volumes were used depending upon tissue activities.

**LDH (EC 1.1.1.27)**

0.12 mM NADH  
1.0 mM Pyruvate  
50 mM imidazole buffer, pH 7.4

**G6PDH (EC 1.1.1.49)**

100 mM MgCl<sub>2</sub>  
5 mM NADP<sup>+</sup>  
10 mM G-6-P  
50 mM imidazole buffer, pH 7.4

**PK (EC 2.7.1.40)**

30 mM KCl  
10 mM MgCl<sub>2</sub>  
0.12 mM NADH  
0.5 mM PEP  
2.5 mM ADP  
20 units LDH  
50 mM imidazole buffer, pH 7.4

**PEPCK (EC 4.1.1.49)**

0.12 mM NADH  
0.5 mM PEP  
20 mM NaHCO<sub>3</sub>  
1 mM MnCl<sub>2</sub>

0.2 mM deoxyguanosine diphosphate

50 mM imidazole buffer, pH 7.4

note: malate dehydrogenase (MDH) is conventionally added to the assay mixture to initiate the reaction, but was excluded in my assays due to sufficient levels of MDH already present in the tissue homogenate

**Fatty Acid Synthase (FAS; EC 2.3.1.85)**

tissue homogenized in 1.5 vol phosphate-bicarbonate buffer (70 mM KHCO<sub>3</sub>, 85 mM K<sub>2</sub>HPO<sub>4</sub>, 9 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM dithiothreitol, pH 8.0)

0.12 mM malonyl CoA

1.2 mM EDTA

1.2 mM β-mercaptoethanol

38 μM acetyl CoA

1.2 mM NADPH

575 mM K-PO<sub>4</sub> buffer, pH 7.0

**Carnitine Palmitoyl Transferase (CPT; EC 2.3.1.21)**

0.1 mM DTNB

100 μM palmitoyl CoA

5 mM L-carnitine

50 mM Tris-HCl buffer, pH 8.0

**Citrate Synthase (CS; EC 2.3.3.1)**

0.1 mM DTNB

0.3 mM acetyl CoA

0.5 mM oxaloacetate

50 mM Tris-HCl buffer, pH 8.0

**Aspartate Aminotransferase (AST; EC 2.6.1.1)**

0.12 mM NADH

7 mM α-ketoglutarate

8 units malate dehydrogenase (MDH)

25 μM pyridoxal 5'-phosphate

40 mM L-aspartate

50 mM imidazole, pH 7.8

**Alanine Aminotransferase (ALT; EC 2.6.1.2)**

0.12 mM NADH

7 mM α-ketoglutarate

8 units MDH

25 μM pyridoxal 5'-phosphate

200 mM L-alanine

50 mM imidazole, pH 7.8

#### Glutamate Dehydrogenase (GDH; EC 1.4.1.3)

250 mM ammonium acetate  
0.1 mM EDTA  
0.12 mM NADH  
1 mM ADP  
14 mM  $\alpha$ -ketoglutarate  
50 mM imidazole, pH 7.4

#### 2.6.2. Hexokinase (EC 2.7.1.1) and glucokinase (EC 2.7.1.2)

Activity of hexokinase (HK) was assayed by coupling the production of glucose-6-phosphate by HK to the reduction of  $\text{NADP}^+$  to NADPH by G6PDH. The production of NADPH is measured spectrophotometrically at 340 nm. This assay is based upon recent studies by Panserat *et al.* (2000, 2001).

The homogenization buffer used consisted of 8 mM Trizma base, 4 mM EDTA, and 2 mM dithiothreitol (pH 7.6) with 100x diluted Sigma protease inhibitor cocktail (Sigma, P8340) added at 0.5  $\mu\text{l}/\text{ml}$  of buffer. Tissue was homogenized in a 1.5 ml plastic conical centrifuge tube using a 1:10 (w/v) dilution and a Wheaton overhead stirrer (Wheaton Industries) equipped with a Kontes pellet pestle; the resulting homogenate was centrifuged for 10 min at  $900 \times g$  (Eppendorf 5414c). The supernatant was kept and used immediately for the assay. The buffer and tissue were kept at 4 °C for the duration of the homogenization process and the tissue extracts kept on ice until the start of the assay.

The assay was performed in a buffered salt medium (8 mM  $\text{MgCl}_2$ , 1 mM KCl, 15 mM  $\text{KH}_2\text{PO}_4$ , 20 mM  $\text{NaHCO}_3$ , 80 mM Tris, 4 mM EDTA, 2.6 mM dithiothreitol, 2 mM NADP, pH 8.0) in a 200  $\mu\text{l}$  volume, to which was added 0.1 U/ml G6PDH and 6PGDH just prior to the assay. Twenty  $\mu\text{l}$  D-glucose was added to the assay mix in concentrations of 10 mM (final concentration = 1 mM) or 1.2 M (final concentration = 120 mM) to measure the

activities of the low Km HK and total HK, respectively. The reaction was initiated by adding 10 µl of tissue extract to the assay mix in a microplate well, and the assay allowed to proceed for 20 min at 37 °C. Activities of GIDH (glucose dehydrogenase) were also measured by performing the assay as described above, but without adding G6PDH or 6PGDH to the assay mixture. Glucokinase (GK) activity was calculated by subtracting low Km HK activity and 1/3 GIDH activity from the total HK activity according to Panserat *et al.* (2000).

### 2.6.3. Pyruvate dehydrogenase (EC 1.2.4.1)

The activity of pyruvate dehydrogenase (PDH) as both active and total was measured indirectly in a two-step radiometric assay according to the methods of Richards *et al.* (2002). The first part of the assay produced acetyl CoA from tissue samples incubated with pyruvate. The second part involved the use of radiolabeled oxaloacetate to produce labeled citrate using citrate synthase (CS) and the acetyl CoA from the first part of the assay.

#### 2.6.3.1. Acetyl CoA production

The production of acetyl CoA was performed according to the method of Constantin-Teodosiu *et al.* (1991). Active PDH activity was determined by homogenizing tissue samples in 1:15 w/v buffer containing 200 mM sucrose, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 5 mM EGTA, 50 mM Tris-HCl, 5 mM Cl<sub>2</sub>CHCOOH, 0.1% Triton-X 100 and 50 mM NaF (sodium fluoride), pH 7.8. The tissue was homogenized using a pestle attached to an overhead stirrer. The resulting homogenate was used directly for the assay.

In 12x75 plastic test tubes, 60 µl homogenate was added to 720 µl assay buffer (100 mM Trizma base, 0.5 mM EDTA, 100 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 3 mM NAD<sup>+</sup>, 1 mM CoASH, 1

mM thiamine pyrophosphate chloride, pH 7.8) and vortexed briefly. Thirty  $\mu$ l 26 mM pyruvate was added to initiate the reaction. The reaction was terminated after 1, 2 and 3 min of incubation at 15 °C by removing 200  $\mu$ l portions of the assay mixture and adding them to microtubes containing 40  $\mu$ l 3% PCA. The completed reaction mixtures were neutralized with 10  $\mu$ l  $K_2CO_3$  (1 M), frozen in liquid  $N_2$  and stored at -80 °C for later analysis of acetyl CoA.

Total PDH activity determination was made using the methods described above, except the homogenization buffer used contained 10 mM D-glucose, 10 mM  $CaCl_2$  and but no NaF.

#### 2.6.3.2. Acetyl CoA measurements

Samples from the PDH reaction above were assayed for acetyl CoA using the method of Cederblad *et al.* (1990). Samples were thawed on ice and centrifuged at 10,000 x g (Eppendorf 5414c) for 3 min. The supernatant was diluted 2x and used for the assay, which was conducted at room temperature, except where noted otherwise. To 200  $\mu$ l of either the sample or a standard acetyl CoA solution ranging from 0-1  $\mu$ M was added 20  $\mu$ l  $CuSO_4$ :K-acetate-DTT mix (1 mM  $CuSO_4 \cdot 5H_2O$ , 400 mM K-acetate, 100 mM dithiothreitol (DTT) in 0.5 M HEPES, pH 7.4) in 12x75 plastic test tubes. The tubes were incubated for 30 min during which time a  $^{14}C$ -oxaloacetate mixture was prepared in a separate test tube by combining 0.5 M HEPES, 11 mM EDTA, 8 mM  $\alpha$ -ketoglutarate, 18 units glutamic-oxaloacetate transaminase (GOT) and L-U- $^{14}C$  aspartate (Amersham; specific activity 10-30  $\mu$ Ci/mmol,  $62.2 \times 10^6$  DPM). The  $^{14}C$ -oxaloacetate producing reaction was terminated after 10 min with 6% PCA and neutralized 10 min later with 334  $\mu$ l KOH (0.6 mM) containing 11

mM EDTA. To each test tube was added 20  $\mu$ l EDTA (60 mM), 30  $\mu$ l N-ethylmaleimide (NEM) (30 mM), 20  $\mu$ l of the  $^{14}$ C-oxaloacetate mixture and 10  $\mu$ l of CS (133 units/ml). The tubes were incubated for 20 min followed by the addition of 30  $\mu$ l transamination mix (178 mM glutamate, 137 units GOT). After a final 20 min incubation, 1 ml of dowex resin (50WX8-100; prepared by suspending 3 g dowex per 5 mL distilled H<sub>2</sub>O) was added to each assay tube, the tube covered with a plastic plug and mixed by inversion for 2 min. As controls, the assay was performed on samples for which pyruvate was not added during the PDH assay to initiate the acetyl CoA producing reaction. The tubes were centrifuged at 3000 rpm for 2 min using a clinical centrifuge (Dupont Industries), and 500  $\mu$ l of the resulting supernatant was placed into a scintillation vials containing 5 ml aqueous scintillation fluid, and counted on a Beckman Coulter LS 6500 multi-purpose scintillation counter. Rates of production of acetyl CoA were deduced from citrate concentrations and used to calculate activity of the phosphorylated, active form of the PDH enzyme complex as well as the total (phosphorylated and dephosphorylated) PDH activity.

## **2.7. Liver GK mRNA Expression**

Total RNA was isolated from LC- and HC-fed trout using the Trizol (Invitrogen) extraction technique. RNA quality and quantity were verified by gel electrophoresis and spectrophotometry. Tissues were removed from the fish 4 h after the last meal as Panserat *et al.* (2000, 2001) determined this to be the point of highest GK mRNA expression in trout. Northern blots of trout liver RNA samples were prepared using Northern Max<sup>TM</sup> kit (Ambion, Austin, TX). Fifteen  $\mu$ g of total RNA were loaded on a 14 cm, 1.0% formaldehyde denaturing gel and run at 95V for 2.5 h in MOPS running buffer. Following electrophoresis

RNA was transferred overnight to a positively charged nylon membrane, Bright Star™ (Ambion). Membranes were prehybridized for approximately 2 h in 10 ml Ultrahyb™, hybridization solution (Ambion). Northern blots were run to semi-quantitatively assess the expression of GK using hybridization probes for GK (available on Genbank, accession number AF053331). A  $\beta$ -actin mRNA probe provided by C. Doyon (see Doyon *et al.*, 2003) was used for comparison. These experiments were undertaken during the summer of 2002 by Ms Carol Seymour, a NSERC Summer Scholarship student.

## **2.8. Insulin Assays**

Trout plasma insulin levels were assayed from blood treated with the protease inhibitor benzamidine (10 mM final concentration) by Dr. Isabel Navarro (Dèpartement de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Spain). This is a routine radioimmunoassay using bonito insulin as standard and rabbit anti-bonito insulin as antiserum (Gutiérrez *et al.*, 1984).

## **2.9. Flow-through Respirometry**

Rainbow trout ranging from 400-1000 g were fasted for 24 h while adapting to a 4000 ml black Perspex flow-through box (15 cm x 20 cm x 50 cm) continuously supplied with dechloraminated City of Ottawa tap water at 13 °C. Just prior to the experiment, the flow rate was adjusted to approximately 300 ml/min. Water was sampled using a peristaltic pump from either the inflow or outflow of the chamber and passed across PO<sub>2</sub> and PCO<sub>2</sub> recording electrodes (E101 O<sub>2</sub> and E201 CO<sub>2</sub>, Cameron Instrument Company). The electrodes were attached to a blood gas meter (Cameron Instrument Company) and data output was acquired

using Acqknowledge software (BIOPAC Systems, Inc.). Calibration of the PO<sub>2</sub> recording electrode was accomplished by sampling either a “zero oxygen solution” (158.7 mM Na<sub>2</sub>O<sub>3</sub>S) or a beaker of water bubbled with a 99.5% air, 0.05% CO<sub>2</sub> gas mixture to establish settings of 0 and 156 Torr, respectively. The latter solution was used to establish a 3.75 Torr setting for the PCO<sub>2</sub> electrode, while a beaker of water bubbled with 99% air, 1% CO<sub>2</sub> was used for a setting of 7.5 Torr. For all calibrations, solutions were sampled until a steady value was reached on the blood gas meter and for at least a 10 min period. During both the calibration and the experiments, all water sampled was kept chilled to 13 °C. PO<sub>2</sub> and PCO<sub>2</sub> measurements were taken from the inflow water first, and switched to outflow after steady readings were obtained. After measuring the outflow, the inflow water was re-sampled to ensure readings from the electrodes had not drifted from the original values.

Oxygen uptake (MO<sub>2</sub>) and carbon dioxide production (MCO<sub>2</sub>) were calculated using partial pressure differences between water entering and exiting the chamber. Water flow rate, fish mass and solubility coefficients were taken into account for these calculations:

$$MO_2 = \Delta PO_2 \cdot CWO_2 \cdot \frac{f}{W_B} \quad (\text{Gilmour } et al., 2001)$$

where  $\Delta PO_2$  is the partial pressure difference of O<sub>2</sub> (in Torr) between water flowing into and out of the chamber, CWO<sub>2</sub> is the solubility coefficient for O<sub>2</sub> in water (in mM/Torr), f is the water flow rate through the chamber (in l/h) and W<sub>B</sub> is the body weight of the fish (in kg).

$$MCO_2 = \Delta PCO_2 \cdot CWCO_2 \cdot \frac{f}{W_B} \quad (\text{Gilmour } et al., 2001)$$

where  $\Delta\text{PCO}_2$  is the partial pressure difference of  $\text{CO}_2$  (in Torr) between water flowing into and out of the chamber,  $\text{CWCO}_2$  is the capacitance coefficient for  $\text{CO}_2$  in water (in mM/Torr),  $f$  is the water flow rate through the chamber (in l/h) and  $W_B$  is the body weight of the fish (in kg).

The capacitance coefficient of  $\text{CO}_2$  in 13 °C dechloraminated City of Ottawa tap water was determined empirically from a  $\text{CO}_2$  combining curve. Concentrations of  $\text{CO}_2$  in water of varying  $\text{PCO}_2$  values were measured using a Capni-con 5 Total  $\text{CO}_2$  analyzer (Cameron Instrument Company). The concentration (in mM) was plotted against the partial pressure (in Torr) and the slope was taken as the capacitance coefficient (Fig. 2.1). The curve yielded the regression equation  $\text{CWCO}_2 = 0.34 + 0.095 \text{ PCO}_2$ ,  $r=0.98$ ,  $P<0.05$ .

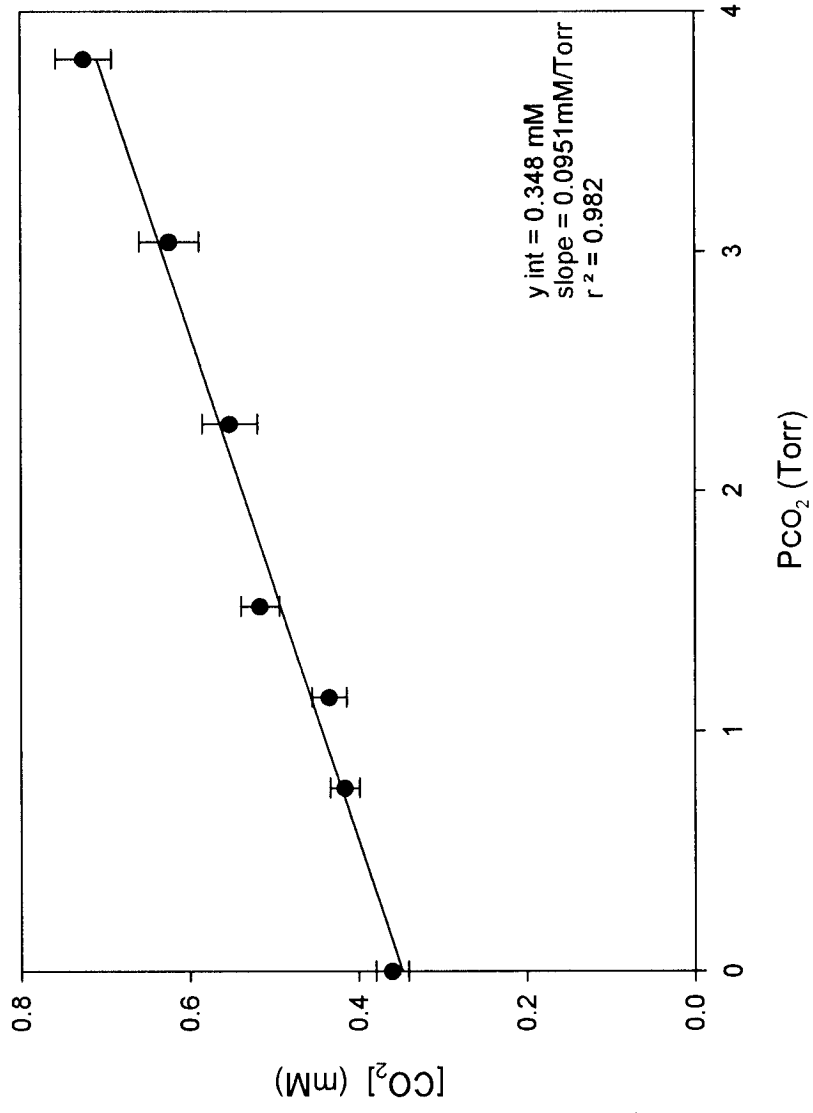


Figure 2.1:  $\text{CO}_2$  combining curve for dechloraminated City of Ottawa tap water at 13 °C. Values are means  $\pm$  SEM,  $n = 9-11$ .

## 2.10. Surgical Procedures

Cannulation of the dorsal aorta was performed using the method of Soivio *et al.* (1975). Trout were lightly anaesthetized in benzocaine solution as outlined above in section 2.3. A securing “grommet” was created from a small piece of PE 160 (Intramedic) tubing, heat-flared at one end. The grommet was threaded through a hole pierced through the snout of the trout with the flared end against the roof of the mouth. Fish were then placed ventral side up on an operating table, and the gills were supplied with flowing oxygenated, benzocaine water at 13 °C. A sharpened guide wire was inserted into a 20 cm piece of PE 50 tubing. The tip of the wire and cannula were used to penetrate the roof of the mouth at the level of the first gill arch and were advanced until blood moved up the cannula upon removal of the wire. The cannula was rinsed with heparinized Cortland saline (124 mM NaCl, 5.1 mM KCl, 1.6 mM CaCl<sub>2</sub>, 0.9 mM MgSO<sub>4</sub>, 11.9 mM NaHCO<sub>3</sub>, 3 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4) and fitted to a 60 cm extension made of PE 50 tubing with a section of a 23-gauge needle. The extension was passed through the grommet and the portion of tubing that remained in the mouth was stitched to the roof of the mouth with 2.0 surgical silk (Serag Weissner, Germany). Two simple knots were used to seal the open end of the extension after flushing the line with heparinized saline to prevent blockage of the cannula by clots. Fish were revived and placed in a 4000 ml flow-through black Perspex box with a continuous supply of aerated water at 13 °C. A 24 h period of recovery was allowed before any experimentation was carried out on the fish.

## **2.11. Glucose Tolerance Tests**

Fish used for glucose tolerance tests ranged in weight from 425-1000 g. After the 24 h recovery period, a 200  $\mu$ l blood sample was drawn to establish basal plasma glucose concentrations. Fish were then injected with a glucose bolus consisting of 250 mg glucose per kg body weight dissolved in Cortland saline as described by Legate *et al.* (2001), which was shown to be an adequate dose to achieve a state of hyperglycemia in trout, while still allowing them to return to normoglycemia by 24 h. Samples were taken 1, 5, 15, 30, 60, 120, 360 min and 24 h (1440 min) following the bolus injection. The blood samples were centrifuged and the plasma portion was frozen in liquid nitrogen and stored at -80 °C for later analysis.

## **2.12. Internal Urinary Bladder Cannula**

Cannulation of the urinary bladder was carried out according to the method described by Curtis and Wood (1990). Preparation of the trout and recovery from the surgery were the same as outlined above in section 2.9, and the bladder cannula was added just after cannulation of the dorsal aorta. A 45 cm section of PE 160 tubing was heat flared at one end and filled with 13 °C tap water. The flared end of the cannula was advanced dorsally 1.0-1.5 cm through the urogenital papilla into the urinary bladder. A seal was created by tying 2.0 cm through the urogenital papilla into the urinary bladder. A seal was created by tying 2.0 surgical silk around the papilla. Three purse-string ligatures (one just anterior to, and two to the side of the anal fin) were used to secure the cannula. Urine was allowed to exit the cannula by gravity, and was collected in plastic vials for storage at -80 °C.

## 2.13. Glucose Turnover

The continuous tracer infusion method developed by Haman and Weber (1996) was used to make measurements rates of glucose appearance (Ra) and disappearance (Rd), as well as glucose turnover.

### 2.13.1. Double cannulation of dorsal aorta

The continuous tracer infusion method requires the placement of two cannulae into the dorsal aorta. Cannulae are placed using the method of Soivo *et al.* (1975) (as detailed in section 2.8) with modifications presented by Haman and Weber (1996). Trout were prepared for insertion of cannulae using the methods described above for the implantation of a single cannula, with the exception that two grommets were pierced through the snout instead of one. The first cannula was implanted at the level of the third gill arch and advanced 8 - 10 cm caudally into the dorsal aorta. This cannula was later used as the infusion line. The second cannula entered the dorsal aorta at the level of the first gill arch and was used for blood sampling. Both cannulae were anchored to the roof of the mouth with surgical silk and threaded separately through one of the grommets. Trout used for the continuous tracer infusion study ranged in weight from 450-1200 g. Trout were allowed 24 h of recovery in a black Perspex box before experimentation.

### 2.13.2. Continuous tracer infusion

6-<sup>3</sup>H]-D-glucose (Amersham; specific activity 28 Ci/mmol) was infused into the dorsal aorta by one of two syringe pumps both connected to the same cannula by two PE-50 lines that converged into a single extension. The rate of infusion was always 1 ml/h. The

infusion was started by a priming dose of [<sup>3</sup>H]-D-glucose infusate equivalent to 90 min of infusion before starting the first pump. Infusate was prepared daily by drying 22.25 µl radiolabelled glucose (approx. 6.0×10<sup>7</sup> CPM) under nitrogen and then dissolving it in 5 ml Cortland saline. A portion of this infusate was held back for determining exact infusion activity.

At 40, 50 and 60 min of infusion, 200 µl blood samples were drawn from the sampling line. Following the 60 min blood sample, the second syringe pump was started that contained a cold glucose concentration necessary to produce an infusion rate of 10 µmol/kg/min. This infusion rate (IRa) was equal to typical values for normal rates of glucose appearance in rainbow trout (10 µmol/min/kg; Haman and Weber, 1996). Blood samples were taken 5, 15, 30, 60, 90 and 120 min after starting the IRa infusion. The IRa infusion was stopped after 120 min and the syringe was replaced by one containing a more concentrated glucose dose that gave an infusion rate twice IRa, and was hence denoted 2IRa. The pump was quickly restarted and blood was sampled at time intervals identical for those in the IRa infusion.

All blood samples were centrifuged and stored for glucose measurement as previously described (section 2.5.1.). Sixty µl aliquots of plasma were pipetted into plastic scintillation vials and made up to 4 ml with ASC II scintillation cocktail. Vials were counted in triplicate for 3 min with a Beckman Coulter LS 6500 multi-purpose scintillation counter. Glucose turnover, rates of appearance (Ra) and disappearance (Rd) for the 40, 50 and 60 min intervals were calculated using the steady state equation of Steele (1959):

$$Ra = \frac{F}{SA}$$

where  $F$  is the infusion rate of the isotope (DPM/kg/min) and  $SA$  is the plasma glucose specific activity (DPM/ $\mu$ mol).

$Ra$  and  $Rd$  for the IRa and 2IRa time intervals were calculated using the non-steady state equation of Wolfe (1992):

$$Ra = \frac{F \cdot pV [(c_1 + c_2)/2] [(SA_2 - SA_1)/(t_2 - t_1)]}{(SA_1 + SA_2)/2}$$

$$Rd = Ra - pV [(c_2 - c_1)/(t_2 - t_1)]$$

where  $pV$ , a measure of distribution of glucose in the animal, is the rapidly mixing pool volume (50 ml/kg),  $F$  is the infusion rate of the isotope (DPM/kg/min),  $SA$  is the plasma glucose specific activity (DPM/ $\mu$ mol),  $c$  is the plasma glucose concentration in  $\mu$ M and  $t$  is the sampling time at times 1 and 2.

## 2.14. Statistics

Glucose tolerance test data were analysed by the SAS statistical analysis program (University of Southern California), with the assistance of Dr. Jean-Marc Renaud (Department of Cellular and Molecular Medicine, University of Ottawa). All other data conversions used Microsoft Excel 2000, graphs were plotted using SigmaPlot 2001 (SPSS Inc., Chicago, IL) and statistical differences were evaluated using SigmaStat 2.0 (SPSS Inc.). A value of  $P < 0.05$  was accepted to indicate significant differences.

## **3. RESULTS**

### **3.1. Body Parameters**

After 6 months of feeding rainbow trout on the experimental low carbohydrate (LC) and high carbohydrate (HC) diets, several body parameters were measured (Table 3.1). Both final body weight and length were significantly higher in the HC group, as were weights of liver and visceral fat. Hepatosomatic index (HSI) and specific growth rate (SGR<sub>exp</sub>) were calculated using body weight and liver weight. Both were significantly higher in the HC group. The protein efficiency ratio (PER) was significantly elevated in the HC group. Condition factor (K) was not significantly different between the two groups.

### **3.2. Metabolite Profiles**

Several metabolites were estimated in plasma and tissues of LC and HC trout sampled 4 h after the final meal (Table 3.2). In plasma, a significant difference existed between groups for plasma glucose concentrations; LC concentrations were 5.39 mM compared to 8.59 mM for the HC group. Triglyceride concentrations were two-fold higher in the HC compared with the LC group, while amino acid concentrations were more than 2-fold lower in the HC group. No significant differences existed in plasma protein concentrations. Plasma insulin concentrations were not significantly different between the LC and HC groups at either 4 h or 8 h after feeding. Just prior to feeding, fish fasted for 24 h had plasma insulin concentrations of 10 and 17 ng/ml in the LC and HC groups, respectively, but the differences were not significant (Fig. 3.1).

Several differences were noted amongst stores of energy fuels in liver and muscle tissues. Liver protein was significantly higher in the LC group while the percentage of liver lipids and glycogen were approximately 2- and 5-fold higher, respectively, in the HC group.

White muscle (WM) demonstrated no significant differences in protein between groups, but lipid and glycogen contents were both significantly higher in HC-fed fish compared with the LC fish. Glycogen was also measured separately in epaxial WM sampled from the anterior (“front”), middle and posterior (“back”) regions. The only differences observed were in the front region, where glycogen was significantly higher in the HC group. Measurement of dry weight revealed no difference between WM water content. Red muscle (RM) lipid percentage was slightly higher in the HC group, while no differences were detected in either protein or water content between groups.

Table 3.1 Body Parameters for trout fed the LC and HC diets

	LC	HC
$W_i$ (g)	$98.3 \pm 5.6$	$100.1 \pm 5.3$
$W_f$ (g)	$307 \pm 14.1$	$390.6 \pm 16.1^*$
L (cm)	$28.1 \pm 0.26$	$30.97 \pm 0.54^*$
$W_{LIVER}$ (g)	$4.08 \pm 0.20$	$8.68 \pm 0.66^*$
$W_{FAT}$ (g)	$2.07 \pm 0.29$	$5.12 \pm 0.68^*$
HIS	$1.34 \pm 0.07$	$2.23 \pm 0.15^*$
SGRexp (% x day <sup>-1</sup> )	$1.02 \pm 0.045$	$1.24 \pm 0.041^*$
PER	$2.05 \pm 0.06$	$2.39 \pm 0.04^*$
K	$1.39 \pm 0.06$	$1.31 \pm 0.03$

Values are means  $\pm$  SEM (n = 10): initial and final body weights ( $W_i$  and  $W_f$ ); final lengths (L); liver weights ( $W_{LIVER}$ ); visceral fat weight ( $W_{FAT}$ ); hepatosomatic index (HSI); specific growth rate (SGRexp); protein efficiency ratio (PER); condition factor (K). Significant differences between LC and HC groups are represented by \* (for PER, Mann Whitney test; for all other parameters, t-test;  $P < 0.05$ ).

Table 3.2: Metabolite concentrations in plasma, liver and muscle tissue of rainbow trout fed the LC and HC diets

		LC	HC
Plasma	Protein (mg/ml)	313.9 ± 41.6	325.9 ± 35.0
	Amino Acids (mg/ml)	3.39 ± 0.319	1.65 ± 0.247*
	Glucose (mM)	5.39 ± 0.320	8.59 ± 0.800*
	Triglycerides (mM)	0.324 ± 0.069	0.662 ± 0.116*
Liver	Protein (mg/g)	984.8 ± 117.4	807.1 ± 188.4*
	Lipid (%)	1.18 ± 0.172	2.11 ± 0.203*
	Glycogen (mg/g)	23.9 ± 2.96	102.0 ± 6.36*
White Muscle	Protein (mg/g)	553.8 ± 92.3	592.2 ± 80.5
	Lipid (%)	1.716 ± 0.287	3.044 ± 0.370*
	Glycogen (total mean) (mg/g)	2.35 ± 0.110	3.52 ± 0.311*
	Front	2.35 ± 0.225	4.07 ± 0.572*
	Middle	2.05 ± 0.138	3.05 ± 0.529
	Back	2.63 ± 0.169	3.45 ± 0.516
	Water (%)	82.9 ± 0.67	82.0 ± 0.40
Red Muscle	Protein (mg/g)	432.2 ± 93.0	514.5 ± 88.5
	Lipid (%)	7.13 ± 0.98	10.66 ± 0.97
	Water (%)	85.9 ± 0.27	85.9 ± 0.24

Values are means ± SEM (n = 10). Significant differences between LC and HC groups are represented by \* (t-test, P<0.05).

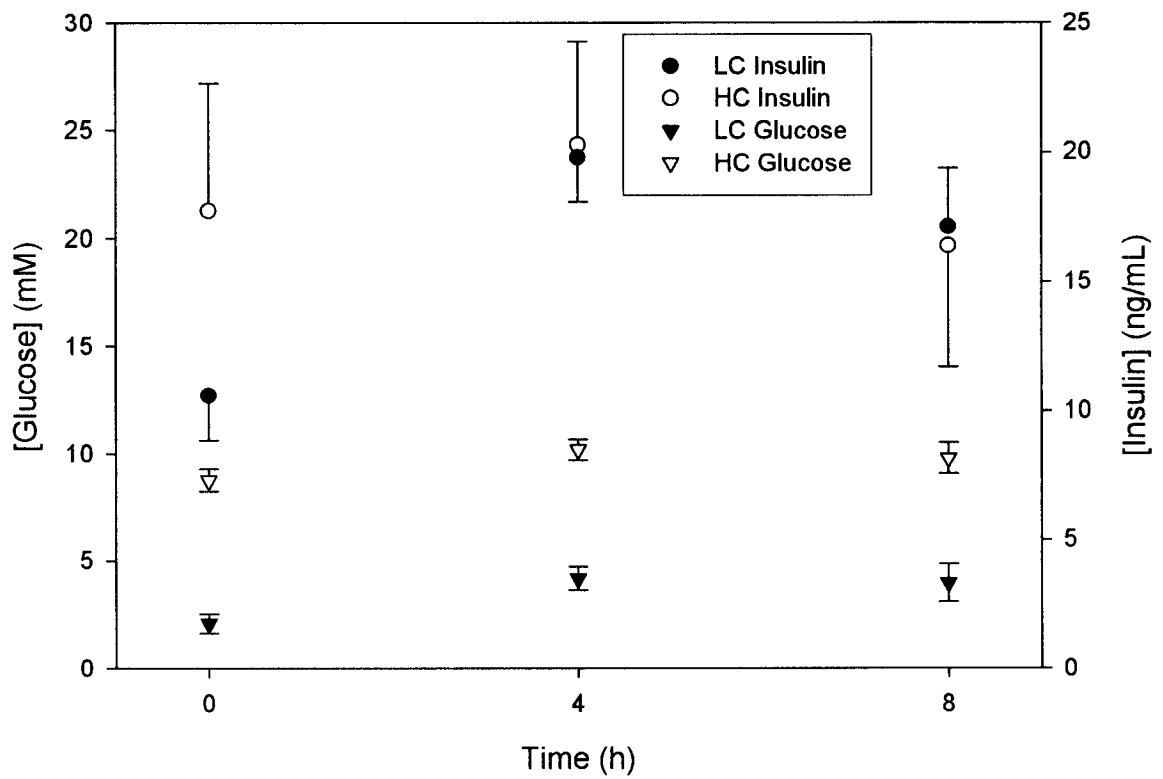


Fig 3.1: Plasma glucose (triangles) and insulin (circles) concentrations 0, 4 and 8 h after feeding trout with the low carbohydrate (LC, closed symbols) or high carbohydrate (HC, open symbols) diets. All values are means  $\pm$  SEM (n = 4); some upper and lower error bars removed for clarity. No significant differences were observed among glucose or insulin concentrations between LC and HC groups or over time (ANOVA,  $P < 0.05$ ). Note different axes.

### 3.3. Enzyme Profiles

Activities of all enzymes were measured in samples taken 4 h after the final feeding of trout fed either the LC or HC diets.

#### 3.3.1 Liver

Liver enzymes were categorized into three groups depending on their metabolic role: carbohydrate metabolism, lipid metabolism or amino acid metabolism.

The greatest difference between groups for the carbohydrate metabolism category was observed for the high  $K_m$  form of hexokinase, termed glucokinase (GK), which had activities over 3-fold higher in the HC compared with the LC group (Fig. 3.2A). The low  $K_m$  hexokinase (HK) activity was approximately 30% higher in the HC group. Phosphoenol pyruvate carboxykinase (PEPCK) was nearly 2-fold higher in the LC group. Also significantly different were pyruvate kinase (PK) and lactate dehydrogenase (LDH), which were higher in the LC-fed than the HC group. No significant difference was observed in citrate synthase (CS) activities between groups (Fig. 3.2A).

The lipid metabolism-related enzymes fatty acid synthase (FAS) and glucose-6-phosphate dehydrogenase were both significantly higher in the HC compared with the LC group by factors of 6 and 2, respectively (Fig. 3.2B). Carnitine palmitoyl-CoA transferase (CPT) activities were more than 5-fold higher in the livers isolated from LC- compared with HC-fed trout (Fig. 3.2B).

Activities were more than 2-fold greater in the LC compared with the HC group for all three amino acid metabolism enzymes estimated, including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glutamate dehydrogenase (GDH) (Fig. 3.2C).

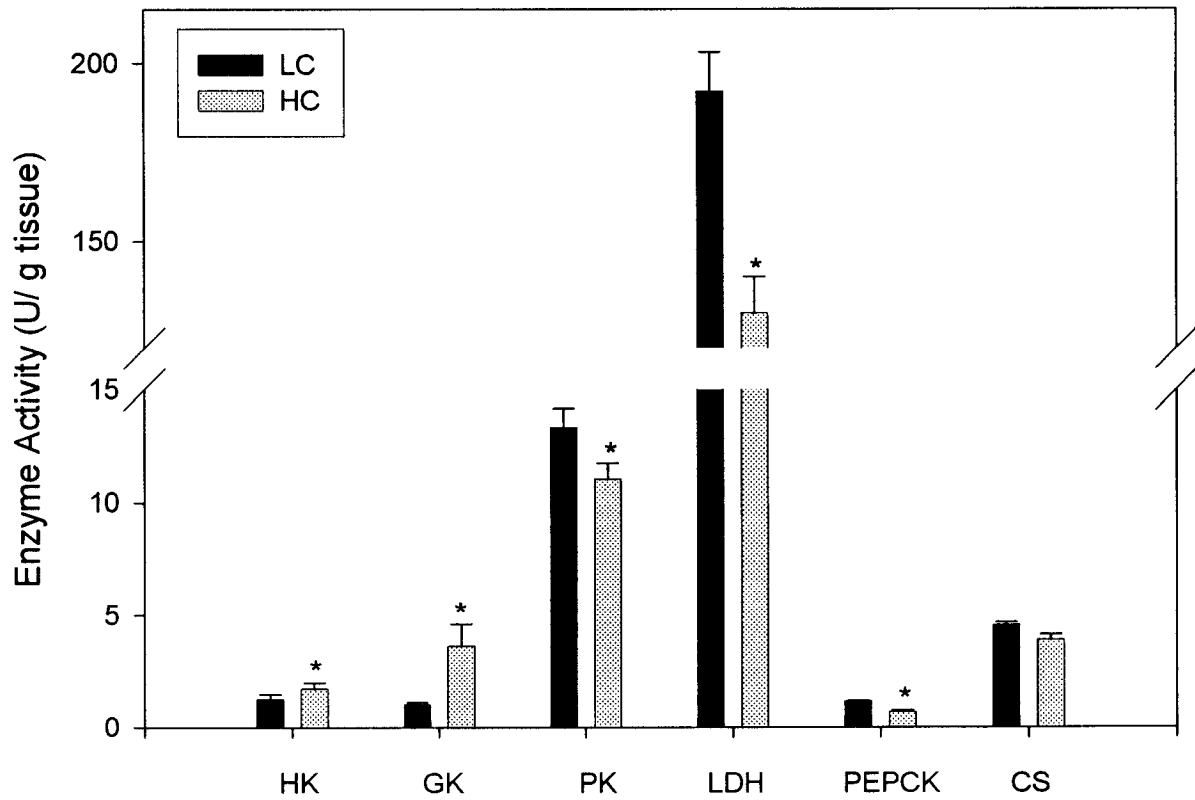


Fig 3.2: A) Activities of enzymes involved in carbohydrate metabolism in the livers of rainbow trout fed the LC (black bars) or HC (gray bars) diets. 1 U = 1  $\mu$ mol/min. All values are means  $\pm$  SEM, n = 10. Significant differences between LC and HC activities for each enzyme are represented by \* (t-test, P < 0.05). Abbreviations noted in text.

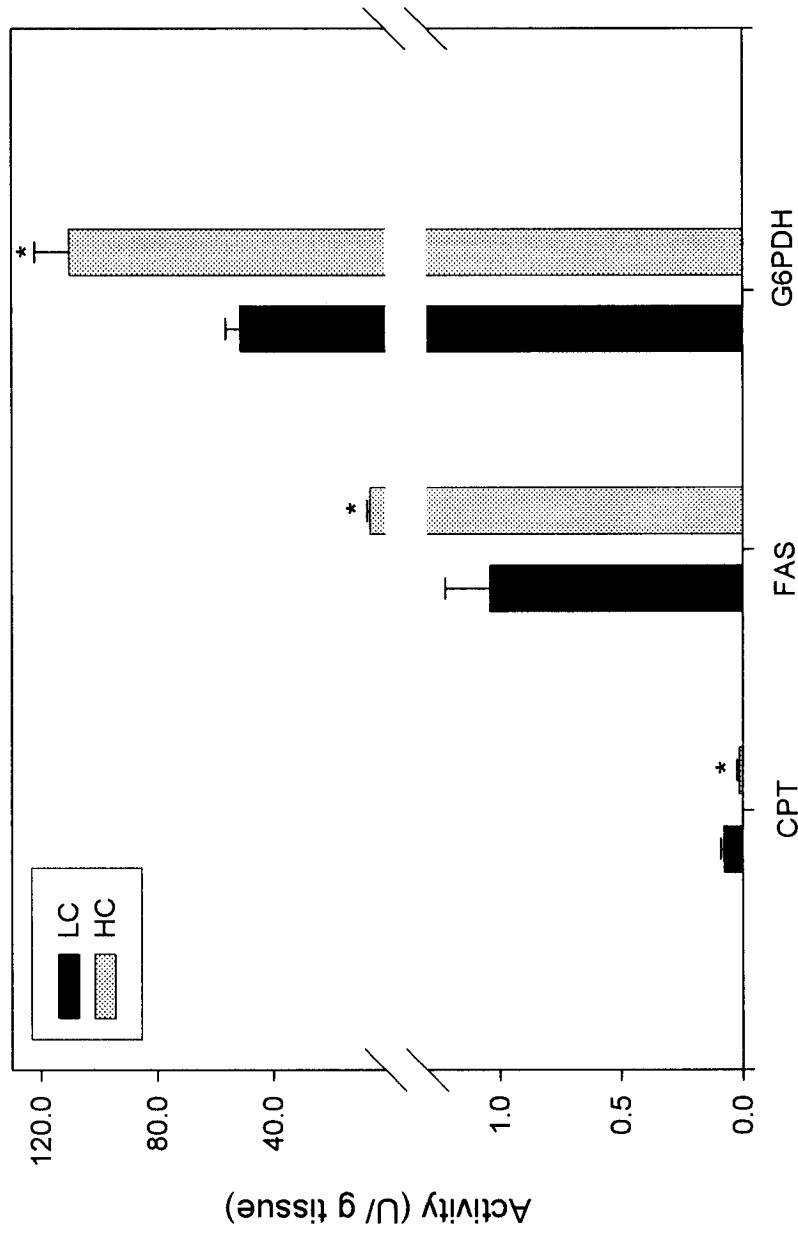


Fig 3.2: B) Activities of enzymes involved in lipid metabolism in the livers of rainbow trout fed the LC (black bars) or HC (gray bars) diets. 1 U = 1  $\mu\text{mol}/\text{min}$ . All values are means  $\pm$  SEM, n = 10. Significant differences between LC and HC activities for each enzyme are represented by \* (t-test,  $P < 0.05$ ). Abbreviations noted in text.

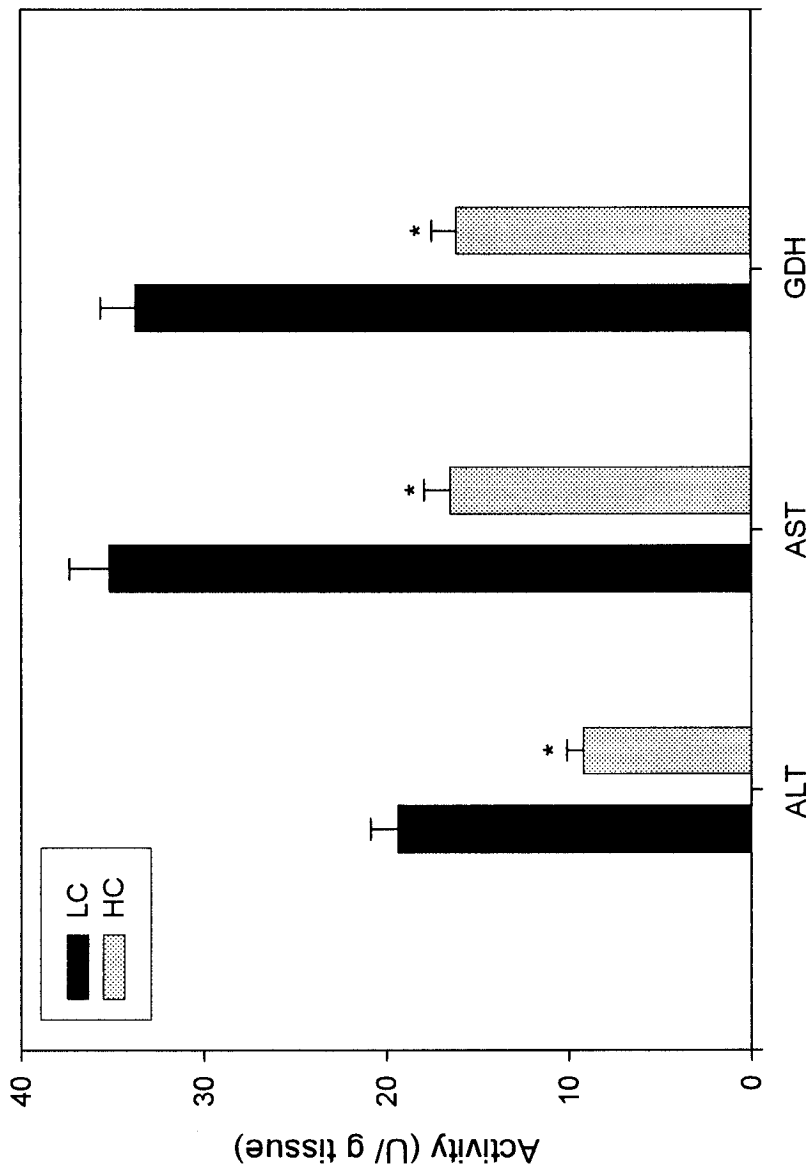


Fig 3.2: C) Activities of enzymes involved in amino acid metabolism in the livers of rainbow trout fed the LC (black bars) or HC (gray bars) diets. 1 U = 1  $\mu\text{mol}/\text{min}$ . All values are means  $\pm$  SEM, n = 10. Significant differences between LC and HC activities for each enzyme are represented by \* (t-test,  $P < 0.05$ ). Abbreviations noted in text.

### 3.3.2. White Muscle

Activities of selected enzymes were estimated in WM sampled from the front, middle and posterior sections of the epaxial region. G6PDH activities were more than 2-fold higher in HC- than LC-fed trout WM from the front region, while no differences were observed in middle and posterior sections (Fig. 3.3A). Conversely, LDH activity was significantly higher in WM sampled from the posterior of HC-fed trout, while front and middle regions showed no differences (Fig. 3.3B).

Activity of PK was significantly higher in posterior WM of HC-fed trout only, while PEPCK activity was significantly higher in both HC-front and HC-middle sections (Fig. 3.4). No significant differences were found between LC and HC groups for CS measured in WM from any region.

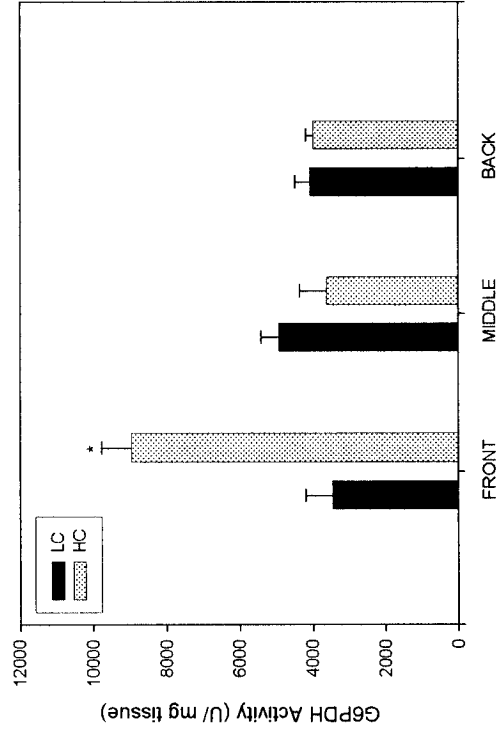
Active and total pyruvate dehydrogenase (PDH) activities were measured only in WM sampled from the posterior epaxial region (Fig. 3.5), a region where activities of most other glycolytic enzymes were seen to have their highest activities. Active PDH (PDHa) was approximately 7-fold higher in the HC compared with the LC group while total PDH (PDHt) activity was one-third lower in the HC group. PDHa and PDHt values were used to calculate the percentage of PDH activity that was active compared with total activity of PDH. The HC group exhibited a percent active PDH form that was 10-fold higher than in the LC group.

### 3.3.3. Red Muscle

Activities of PK, LDH and PDHa and PDHt (Fig. 3.6, 3.7) were also estimated in RM. In RM from HC-fed trout, PK activities were significantly higher than in RM of LC-fed trout, and LDH activities were more than 2-fold higher than that of LC RM (Fig. 3.6).

There were no significant differences between HC and LC fish for PDH activity in RM, although the trend observed was opposite that observed in WM; in the HC group, PDHa activity was slightly lower, and PDHt was slightly higher than the LC group. The percent of active PDH was lower in the HC group, but the difference was not statistically significant (Fig. 3.7).

A



B

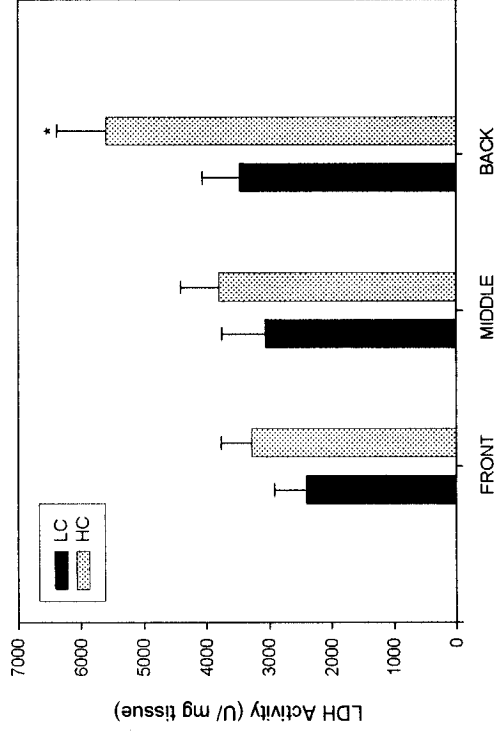


Figure 3.3: Activities of G6PDH (A) and LDH (B) in white muscle of rainbow trout fed the LC (black bars) or HC (gray bars) diets. Tissue was sampled from anterior, middle and posterior sections of epaxial muscle. Activities are U/mg tissue wet weight, where 1 U = 1  $\mu\text{mol}/\text{min}$ . All values are means  $\pm$  SEM,  $n = 10$ . Significant differences between LC and HC activities for each enzyme are represented by \* (ANOVA,  $P < 0.05$ ). Abbreviations noted in text.

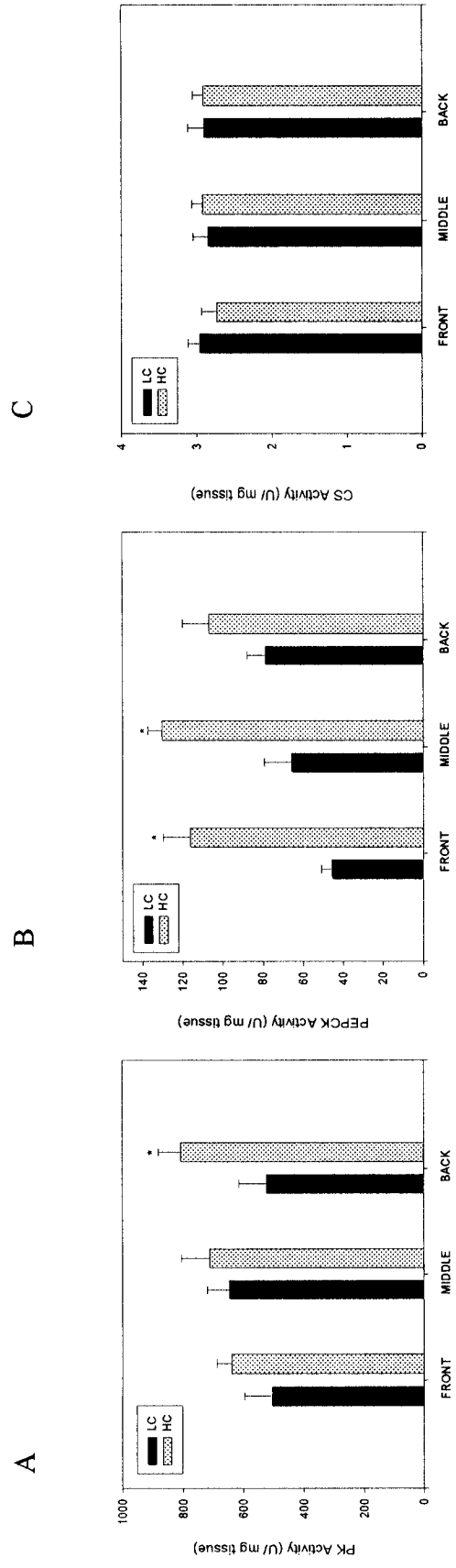


Figure 3.4: Activities of PK (A), PEPCK (B) and CS (C) in white muscle of rainbow trout fed the LC (black bars) or HC (gray bars) diets. Tissue was sampled from anterior, middle and posterior sections of epaxial muscle. Activities are U/mg tissue weight, where 1 U = 1  $\mu\text{mol}/\text{min}$ . All values are means  $\pm$  SEM, n = 10. Significant differences between LC and HC activities for each enzyme are represented by \* (ANOVA,  $P < 0.05$ ). Abbreviations noted in text.

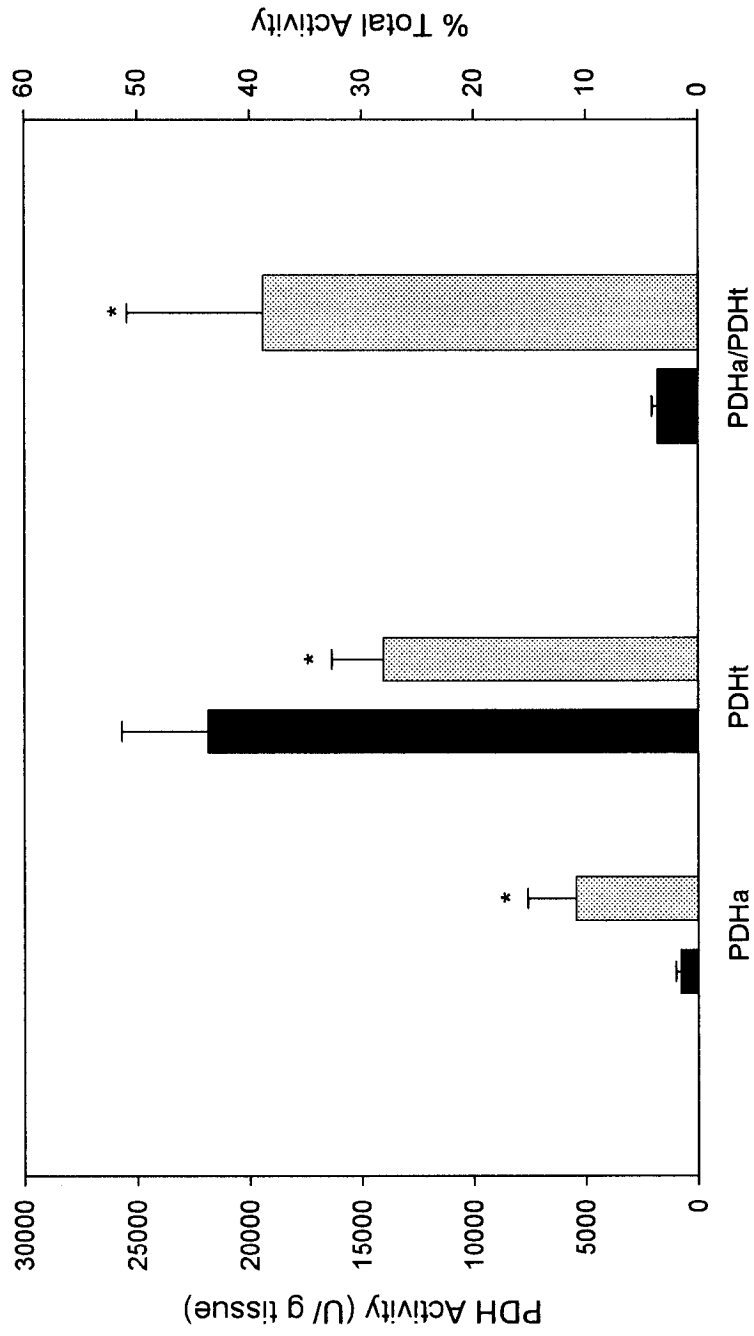


Figure 3.5: Activities of active (PDHa) and total (PDHt) pyruvate dehydrogenase and the activity ratio (PDHa/PDHt) in posterior epaxial white muscle of rainbow trout fed the LC (black bars) or HC (gray bars) diets. Activities are U/mg tissue weight, where 1 U = 1  $\mu\text{mol}/\text{min}$ . All values are means  $\pm$  SEM, n = 6. Significant differences between LC and HC activities for each enzyme are represented by \* (for activity ratio, Mann-Whitney test on arcsine transformed data, for all others, t-test;  $P < 0.05$ ). Note different axes

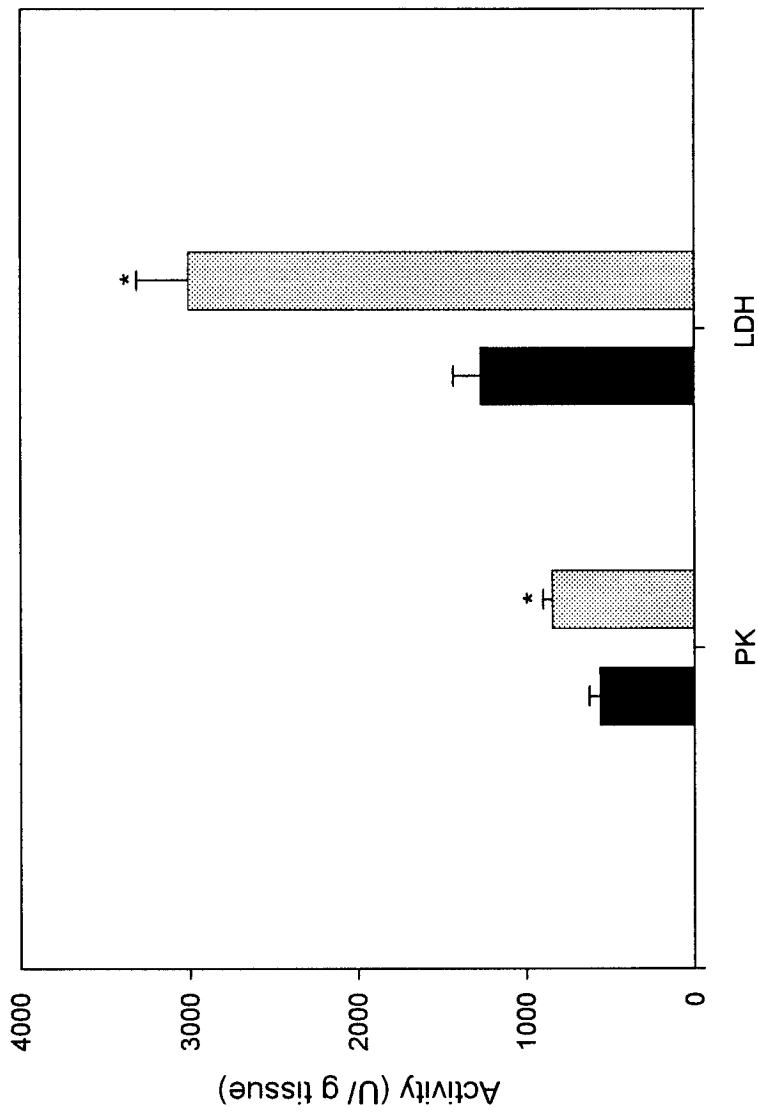


Figure 3.6: Activities of pyruvate kinase (PK) and lactate dehydrogenase (LDH) in the posterior section of red muscle of rainbow trout fed the LC (black) or HC (grey) diets. 1 U = 1  $\mu\text{mol}/\text{min}$ . All values are means  $\pm$  SEM, n = 10. Significant differences between LC and HC activities for each enzyme are represented by \* (t-test,  $P < 0.05$ ).

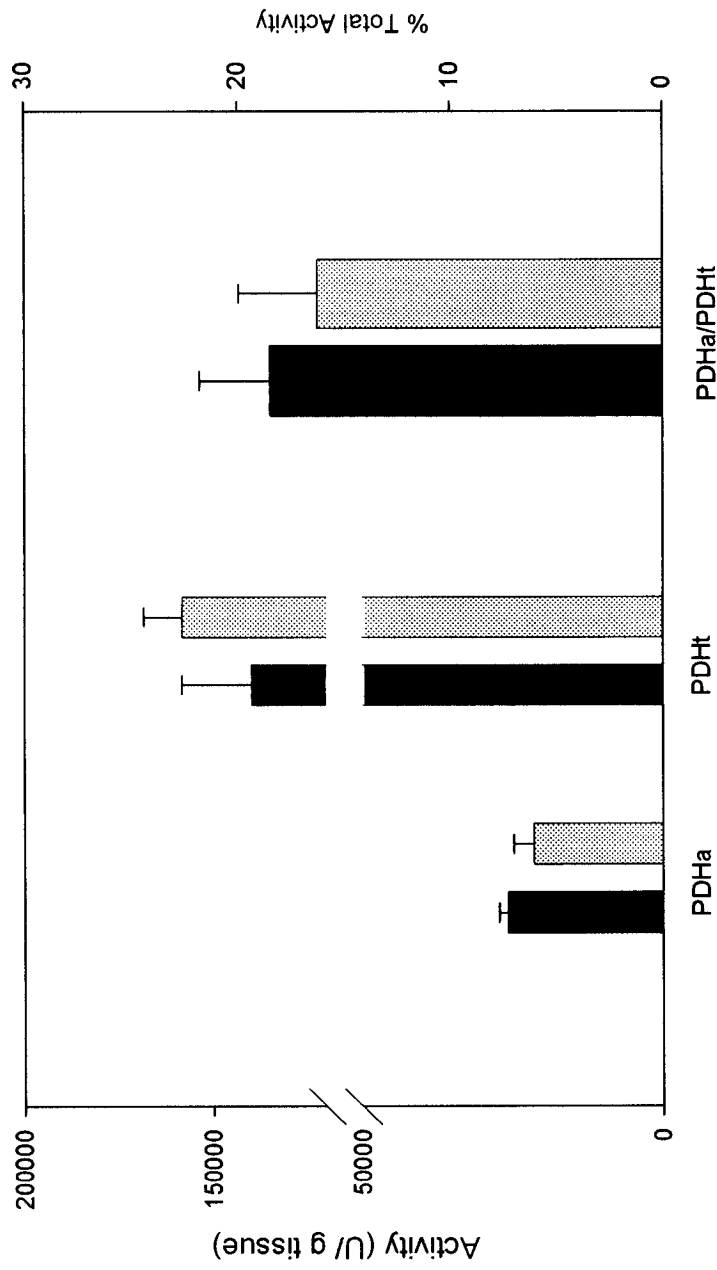


Figure 3.7: Activities of active (PDHa) and total (PDHt) pyruvate dehydrogenase and the activity ratio (PDHa/PDHt) in red muscle of rainbow trout fed the LC (black) or HC (grey) diets. 1 U = 1  $\mu\text{mol}/\text{min}$ . All values are means  $\pm$  SEM, n = 10. There are no significant differences between LC or HC groups in activity of either form of PDH.

### 3.3.4. Glucokinase mRNA expression

The relative levels of GK mRNA were measured in liver of LC- and HC-fed trout compared with levels of  $\beta$ -actin mRNA (Fig. 3.8A). GK mRNA was barely detectible in the LC group, while the GK mRNA levels were very prominent in the HC-fed trout. The ratio of GK to  $\beta$ -actin mRNA in the HC group was  $1.25 \pm 0.34$  or more than 300-fold greater than in the LC group, which had a ratio of  $0.0037 \pm 0.0017$  (Fig. 3.8B).

## 3.4. Flow-through Respirometry

Indirect calorimetry using a flow-through respirometer estimated  $O_2$  consumption ( $MO_2$ ) and  $CO_2$  production ( $MCO_2$ ) in resting trout from the LC- and HC-fed groups (Fig. 3.9). The respiratory exchange ratio ( $R_E$ ) was calculated from these values as  $\frac{MCO_2}{MO_2}$ . There were no significant differences between  $MCO_2$  values, which were  $2.82 \pm 0.23$  and  $2.73 \pm 0.34$  mmol/h/kg for the LC and HC groups, respectively.  $MO_2$  was significantly higher in trout fed the LC diet at  $4.70 \pm 0.40$  mmol/h/kg compared with  $3.23 \pm 0.32$  mmol/h/kg in the HC group. The resulting  $R_E$  value was  $0.84 \pm 0.03$  in the HC group, which was significantly higher than the  $R_E$  value of  $0.61 \pm 0.03$  in the LC group.

## 3.5. Intravenous Glucose Tolerance Tests

### 3.5.1. IVGTT plasma glucose concentrations

Trout from the LC- and HC-fed groups were challenged with an intravenous glucose tolerance test (Fig. 3.10). A bolus of 250 mg D-glucose/kg fish was injected through the indwelling dorsal aorta cannula and blood was sampled over the following 24 h. The sample

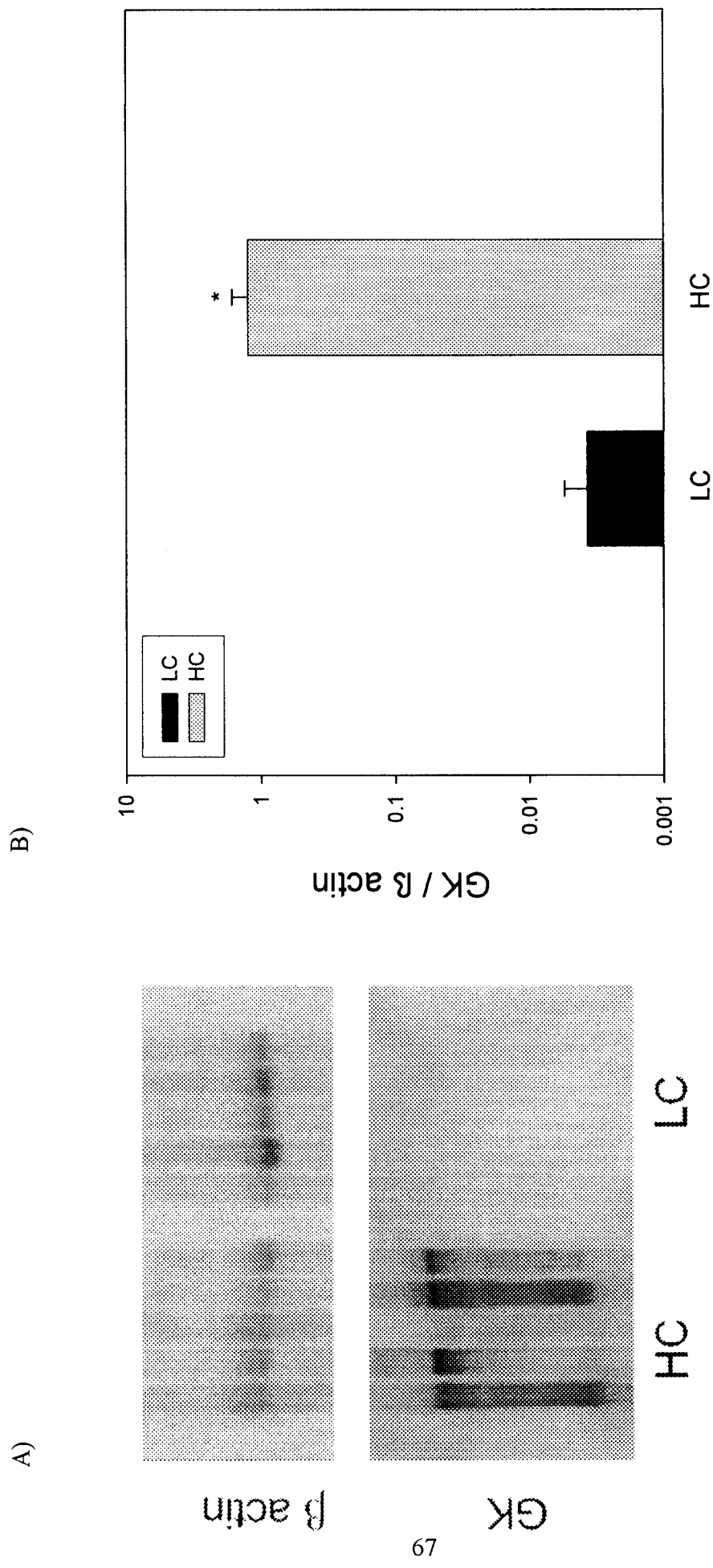


Figure 3.8 Northern blot (A) and GK/ $\beta$ -actin ratio (B) of rainbow trout mRNA from liver probed with rainbow trout GK cDNA for LC and HC diet groups. All values are means  $\pm$  SEM,  $n = 5$ . Significant difference between LC and HC group represented by \* (Mann-Whitney test on arcsine transformed ratios,  $P < 0.05$ ).

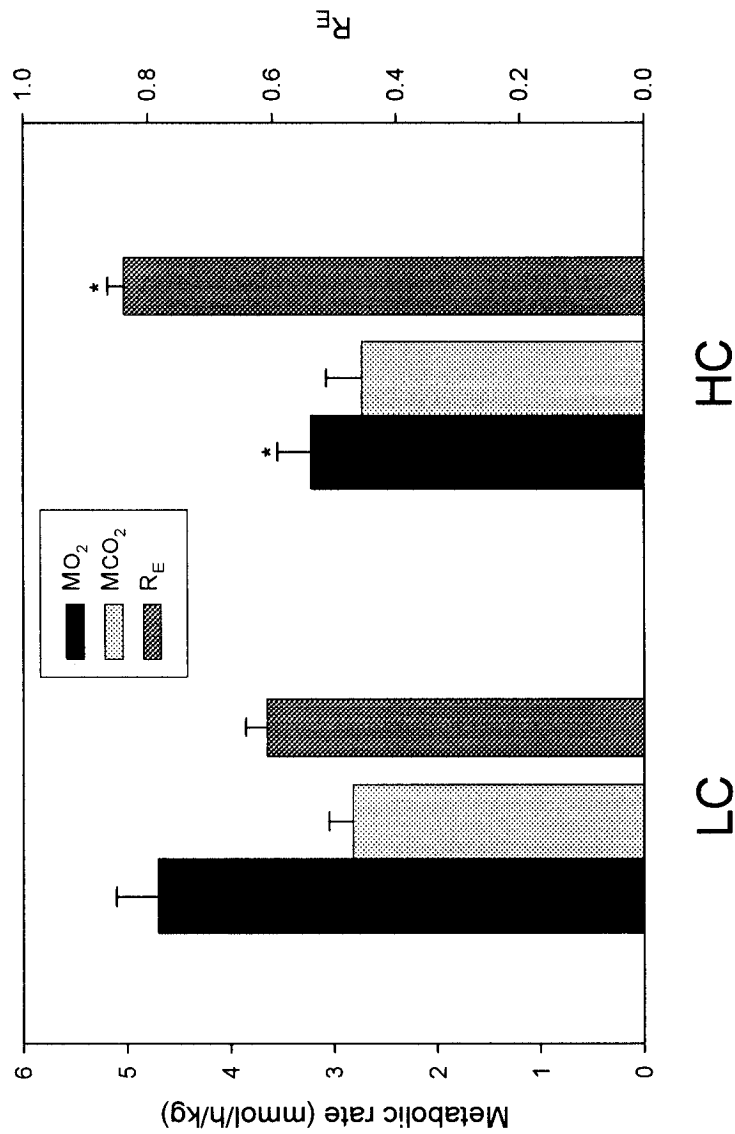


Figure 3.9: O<sub>2</sub> consumption (MO<sub>2</sub>, black bars), CO<sub>2</sub> production (MCO<sub>2</sub>, light gray bars) and respiratory exchange ratio (R<sub>E</sub>, dark gray bars) for rainbow trout fed the LC and HC diets. The values presented are means ± SEM, n = 6. Significant differences between LC and HC groups are represented by \* (MO<sub>2</sub> and MCO<sub>2</sub>: t-test, P<0.05; R<sub>E</sub>: Mann-Whitney Rank Sum test on arcsine transformed ratios, P<0.05). Note different axes.

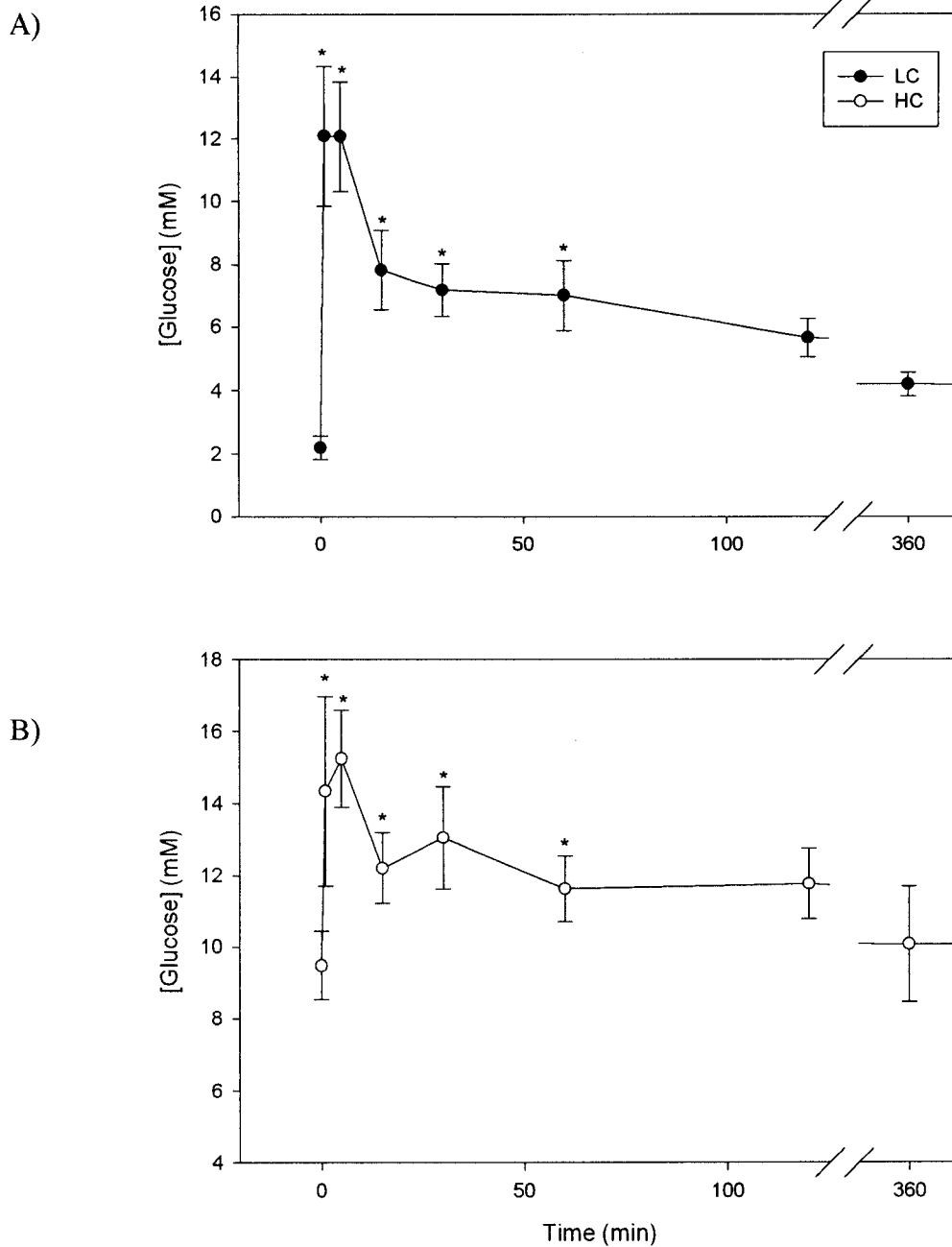


Figure 3.10: Plasma glucose concentrations in rainbow trout fed the LC (A) or HC (B) diets and injected with 250 mg D-glucose/kg body weight. Time 0 concentrations indicate resting (24 h after surgery) plasma glucose (pre-injection) concentrations. All values are means  $\pm$  SEM of  $n = 7$  independent experiments. Concentrations significantly higher than fasting values are indicated with \* (repeated measures ANOVA, Dunnetts method). Values of glucose concentration at 24 h (1440 min) were not statistically different from time 0 concentrations.

was taken just prior to the injection ( $t = 0$  min) was used to establish resting plasma glucose concentration (RPG).

Plasma glucose concentrations rose from 2.2 to 12.0 mM following the first minute of injection in the LC-fed group (Fig. 3.10A). Glucose levels quickly dropped to 7.8 mM but remained significantly elevated above RPG until 6 h after the bolus injection. The RPG value of the HC-fed group is higher than that of the LC group, while the initial increase following the bolus injection was less pronounced (Fig. 3.10B). Plasma glucose rose from 9.5 to 14.3 mM after one minute, and rapidly decreased. Plasma glucose concentration was no longer significantly higher than RPG by 6h.

### 3.5.2. Plasma insulin concentrations

Plasma insulin concentrations were estimated as noted in the Materials and Methods using a heterologous fish radioimmunoassay. Samples were taken from one LC and one HC fish during an IVGTT at several time intervals as described above in section 3.5.1. Insulin values rose from 4 to 14 ng/ml in the LC fish, and returned to baseline levels within 30 min following the glucose challenge (Fig. 3.11A). In the HC fish (Fig. 3.11B), the resting insulin value was lower than that observed in the LC fish, and the increase in concentration achieved a peak concentration of only 6 ng/ml from an initial value of 2.9 ng/ml.

### 3.5.3. Urinary glucose concentrations

Urine was collected from a bladder cannula in two individual trout from each diet group during an IVGTT (Fig. 3.12). Complications arising from the clogging of bladder cannulae with reproductive tissue prevented the continuation of the experiments, making

statistical comparisons impossible; however, interesting trends were observed. In the LC fish, initial urinary glucose concentrations were close to 0 mM and remained low for the first hour following the bolus injection. Glucose concentrations rose gradually at 2h and peaked after 6 h at 7 mM. After 24 h glucose remained elevated above the initial value at 1.6 mM. Initial glucose concentrations in the HC fish were 10 mM and peaked at 13 mM within the first 30 min following the injection. Urinary glucose dropped below initial values after 6 h to 5 mM where it remained at 24 h.

The volume of urine produced during each time interval was also recorded and used to calculate urine flow rate and mass of glucose excreted for one individual fish from each diet group (Table 3.3). Total urinary output was higher for the HC fish, and the amount of glucose excreted (expressed as a percentage of the glucose injected) was 7.25%, or three-times greater than the glucose excreted by the LC fish.

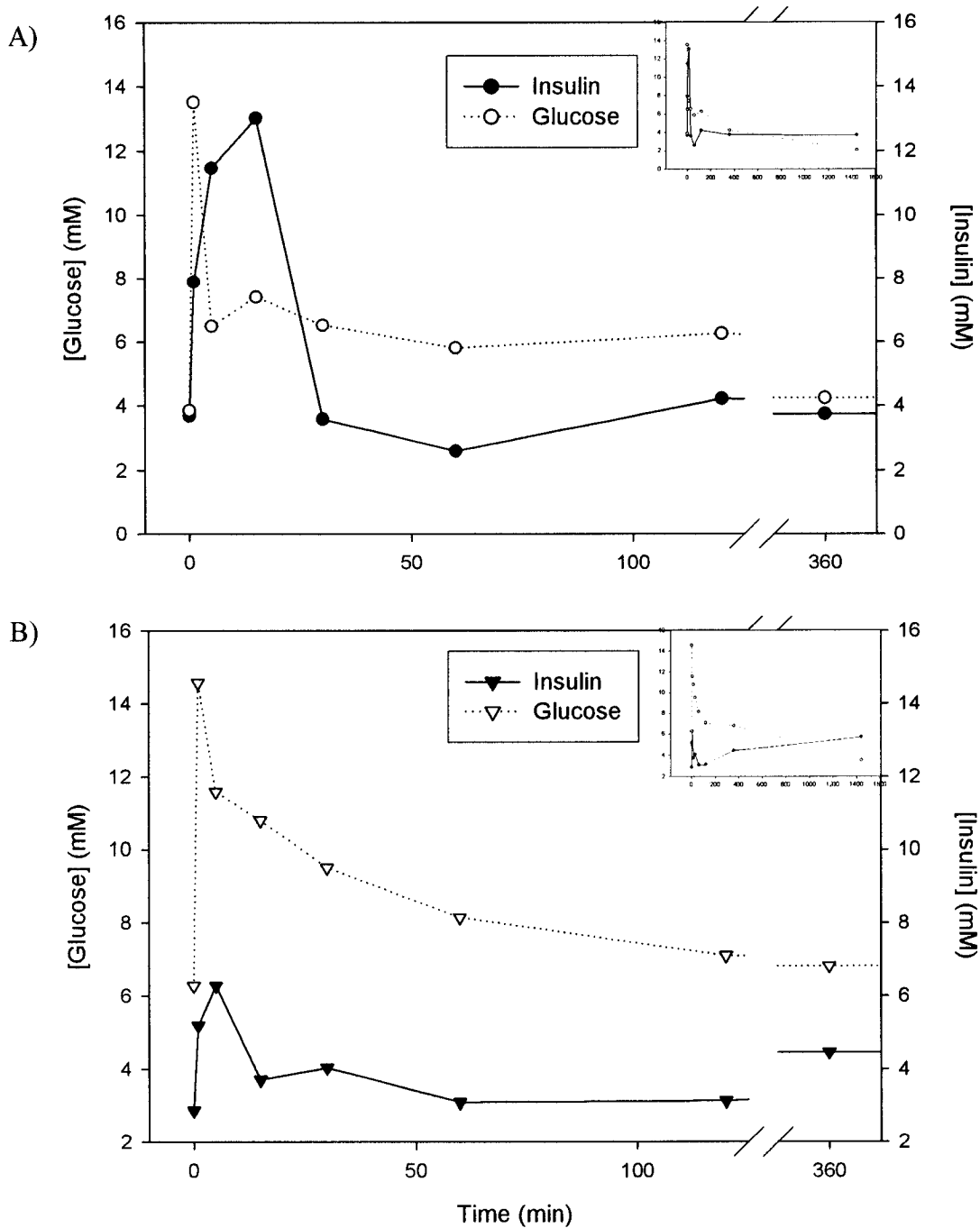


Figure 3.11: Plasma glucose (open symbols) and insulin (closed symbols) concentrations of individual rainbow trout fed the LC (A) or HC (B) diets and injected with 250 mg D-glucose/kg body weight, n = 1. Time 0 concentrations indicate fasting plasma glucose (pre-injection) and insulin concentrations. Insert shows entire 24 h sampling period. Note different axes.

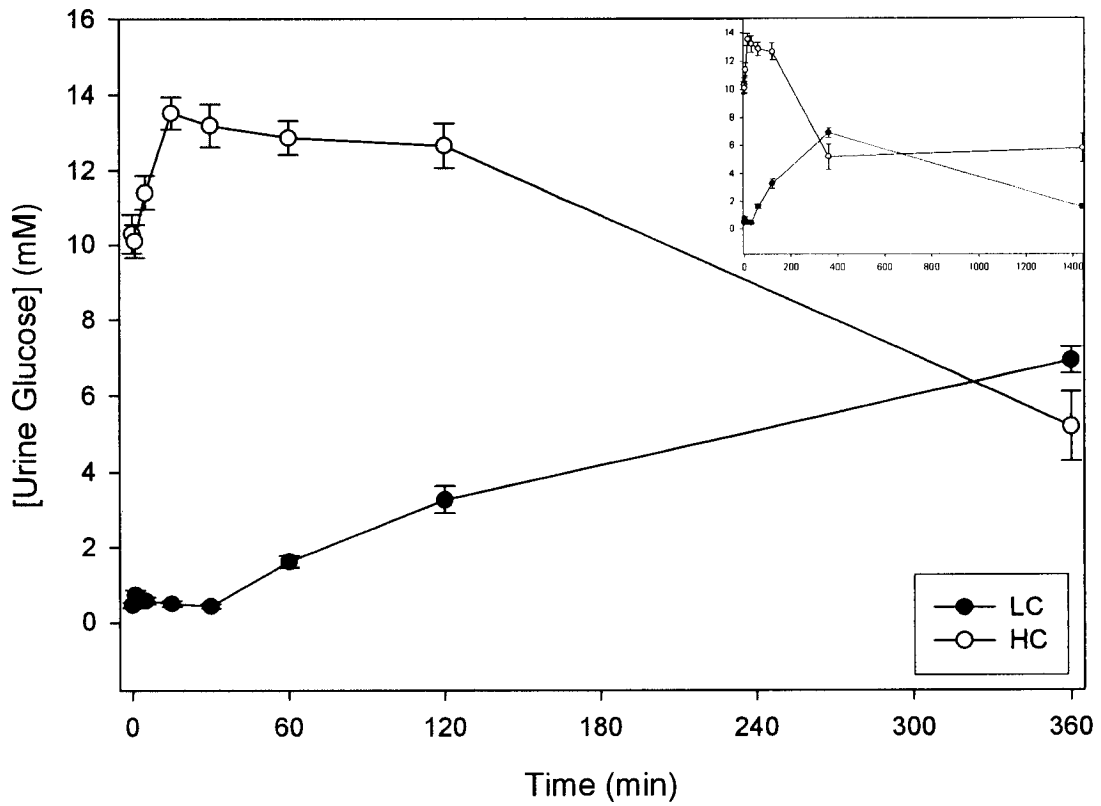


Figure 3.12: Urine glucose concentrations of rainbow trout fed the LC (open circles) or HC (closed circles) diets and injected with 250 mg D-glucose/kg body weight. Time 0 concentrations indicate fasting urine glucose (pre-injection) concentrations. All values are means  $\pm$  SD of  $n = 2$  independent experiments. Statistical analysis was not performed due to insufficient sample size.

### 3.6. Glucose Turnover

The rate of appearance (Ra) and the rate of disappearance (Rd) of glucose were estimated using the continuous tracer infusion method in LC- and HC-fed rainbow trout. Values for Ra and Rd were obtained prior to a 10  $\mu\text{mol/kg/min}$  infusion of D-Glucose (IRa dose), and at several time intervals after the IRa dose began. After 2 h, the dose was increased to 20  $\mu\text{mol/kg/min}$  and Ra and Rd measurements continued. The IRa and 2IRa doses were subtracted from the Ra values obtained to take into account the exogenous rate of appearance of glucose.

Glucose concentration and specific activity of the plasma samples taken during infusion were used to calculate glucose turnover and are shown in Figure 3.13. Plasma glucose concentrations in the HC group fluctuated widely between individuals, leading to high standard error of the mean (Fig. 3.13A). Six fish were used for the experiment, and of these, two had initial glucose concentrations of approximately 10 mM, one had a concentration of 4.8, and three had concentrations below 4 mM. Plasma glucose was higher in the HC than the LC group throughout the entirety of the infusion. Glucose concentration did not deviate significantly from initial values during the experiment in either the LC or HC group (Fig. 3.13A,B).

During the infusions, Ra and Rd decreased gradually in both the LC and HC groups (Fig. 3.14). In the LC group (Fig. 3.14A), glucose turnover was approximately 45  $\mu\text{mol/kg/min}$  before the infusion and dropped to 13  $\mu\text{mol/kg/min}$  after 2 min of the IRa infusion. Rd stabilized between 15-20  $\mu\text{mol/kg/min}$ , while Ra reached a plateau at approximately 9  $\mu\text{mol/kg/min}$  for the remainder of the IRa infusion. When the rate of

infusion was increased to 2IRa, Ra dropped to calculated values of approximately zero and as low as  $-4 \mu\text{mol/kg/min}$ . Rd remained steady at  $15 \mu\text{mol/kg/min}$ .

Glucose turnover rates were higher overall in the HC group (Fig. 3.14B), starting at  $137 \mu\text{mol/kg/min}$  and decreasing to  $80 \mu\text{mol/kg/min}$  with the IRa infusion. Rd decreased linearly as the infusion progressed, but leveled off at approximately  $50 \mu\text{mol/kg/min}$  when the 2IRa infusion began. Ra followed the same pattern, but fell from 70 to  $30 \mu\text{mol/kg/min}$ , where it remained for the 2IRa infusion.

During the infusion,  $\text{MO}_2$ ,  $\text{MCO}_2$  and  $\text{R}_E$  were determined as in section 3.4; values were taken prior to infusion and twice during both IRa and 2IRa infusions (Table 3.4).  $\text{R}_E$  values were consistently higher in the HC group than the LC group, however infusion of D-glucose did not cause significant changes in any variable measured in either group.

Table 3.3: Plasma and urine glucose concentration, urine flow rate, and mass of glucose lost to excretion during an IVGTT with 250 mg/kg body weight bolus in rainbow trout fed the LC and HC diets. Values presented are from one individual from each group.

	Time (min)	[Glucose] <sub>plasma</sub> (mM)	[Glucose] <sub>urine</sub> (mM)	Urine flow rate (ml/h)	Mass excreted (mg/kg body weight)	% bolus excreted
LC	0	1.68	0.04	1.20	$2.14 \times 10^{-4}$	
	1	10.66	0.03	0.25	$1.93 \times 10^{-4}$	
	5	5.80	0.03	0.33	$5.05 \times 10^{-4}$	
	15	4.60	0.04	0.35	$9.33 \times 10^{-4}$	
	30	3.92	0.08	0.42	$4.50 \times 10^{-3}$	
	60	4.11	2.53	0.34	0.24	
	120	3.13	5.24	0.33	1.91	
	360	3.01	4.98	0.06	0.08	
	1440	2.63	2.62	0.32	3.98	
					Total=6.21	2.49
HC	0	9.36	12.86	27.60	2.35	
	1	12.68	12.43	3.11	1.28	
	5	12.66	12.62	0.71	0.60	
	15	9.32	12.62	0.13	0.16	
	30	10.97	13.42	1.91	5.08	
	60	8.71	13.07	0.05	0.27	
	120	9.21	13.22	0.34	7.15	
	360	9.19	0.24	0.47	0.04	
	1440	9.18	0.20	0.89	1.21	
					Total=18.14	7.25

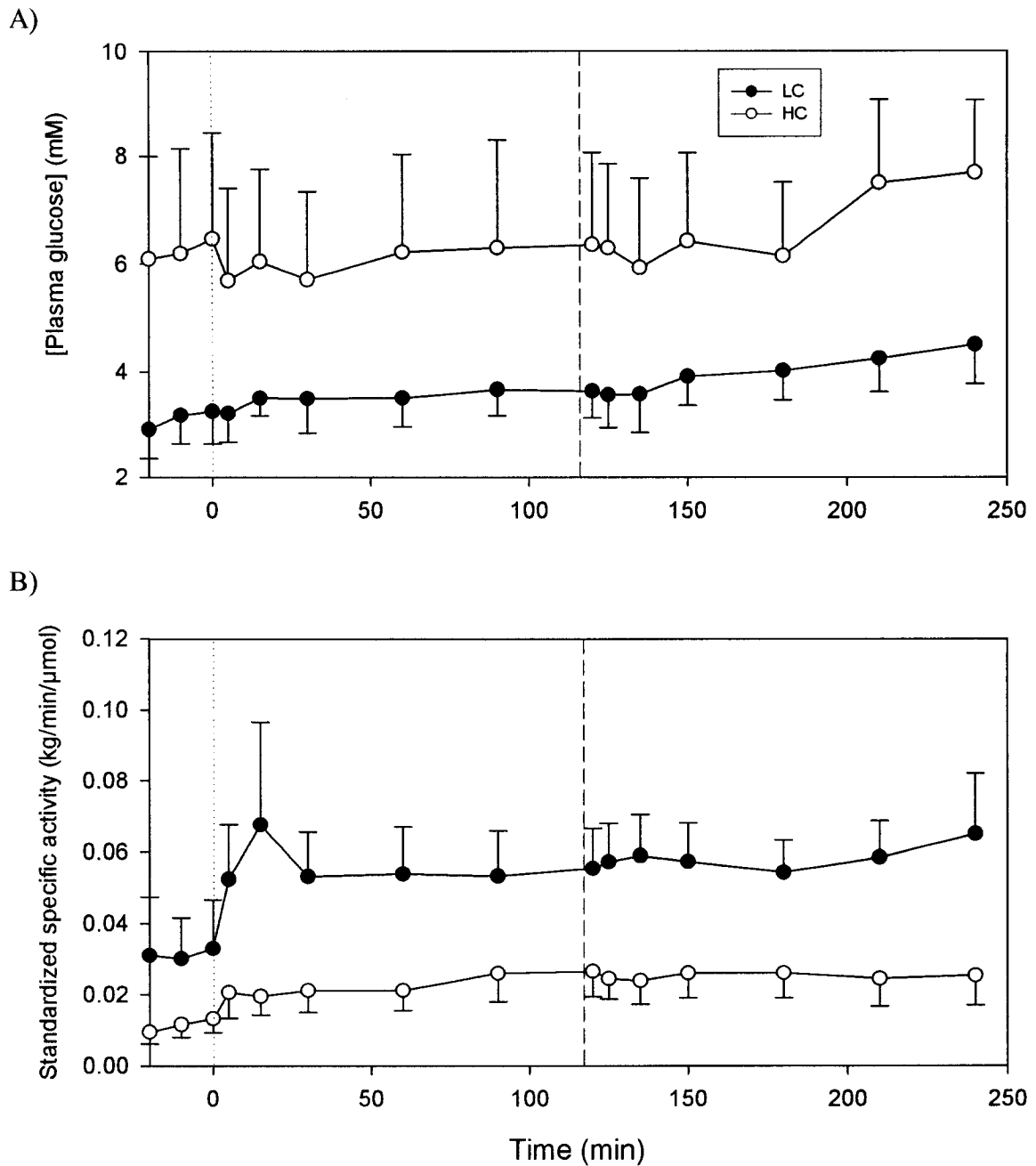


Figure 3.13: Plasma glucose concentration (A) and standardized glucose specific activity (= specific activity/infusion rate) (B) during primed continuous infusions of 6- $^3\text{H}$  glucose in rainbow trout fed the LC (black circles) and HC (white circles) diets. At time = 0, unlabeled D-glucose was infused at a rate of 10  $\mu\text{mol/kg/min}$  (IRa, dotted line). At time = 120 min, the rate of infusion of unlabeled D-glucose was doubled to 20  $\mu\text{mol/kg/min}$  (2IRa, dashed line). All values are means  $\pm$  SEM,  $n = 6$ .

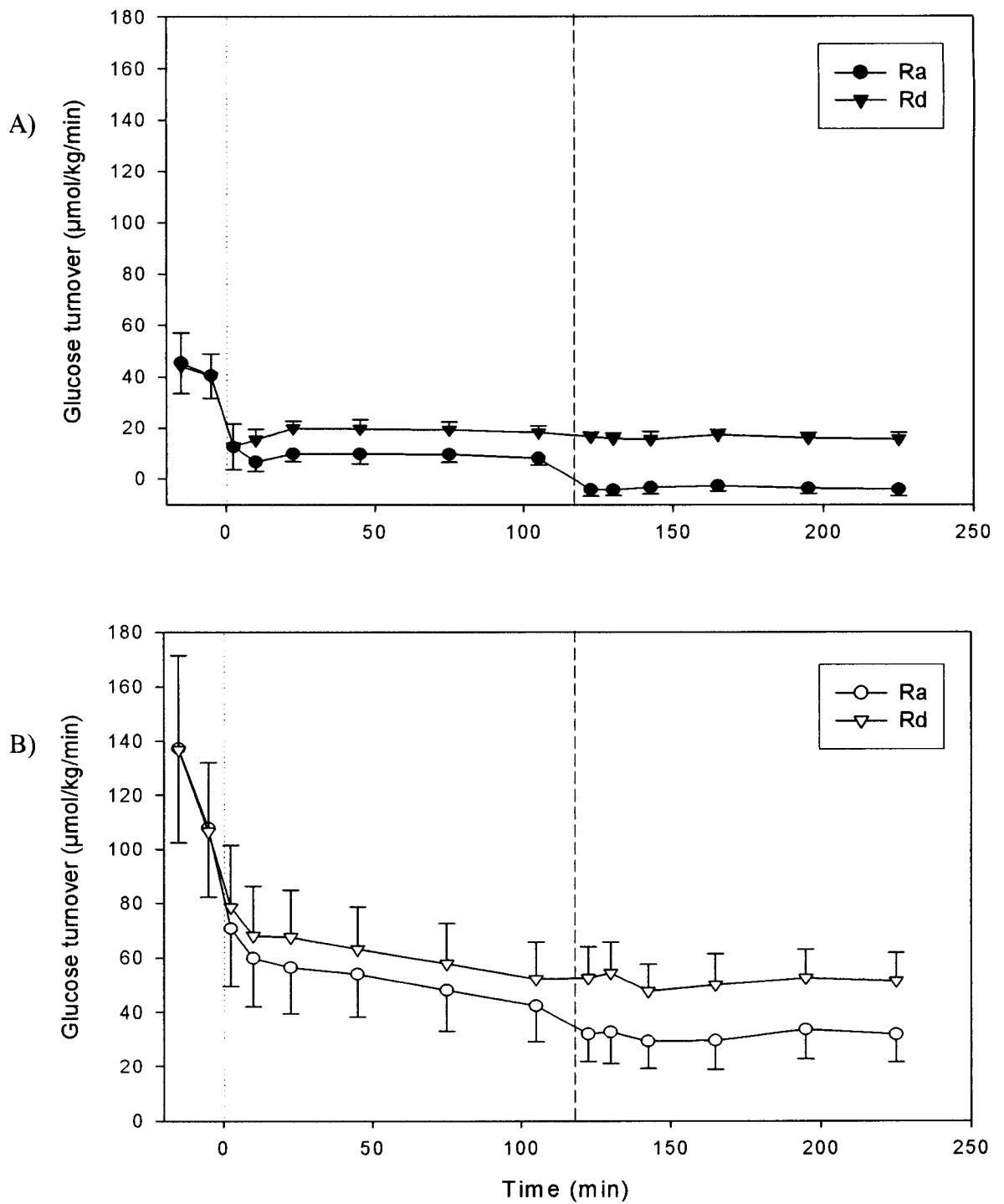


Figure 3.14: Glucose turnover rates in rainbow trout fed the LC (A) or HC (B) diets, during continuous tracer infusion. Rates of appearance and disappearance of glucose are represented by circles and triangles, respectively. At time = 0, unlabeled D-glucose was infused at a rate of 10 µmol/kg/min (IRa, dotted line). At time = 120 min, the rate of infusion of unlabeled D-glucose was doubled to 20 µmol/kg/min (2IRa, dashed line). All values are means ± SEM, n = 6.

Table 3.4: O<sub>2</sub> consumption (MO<sub>2</sub>), CO<sub>2</sub> production (MCO<sub>2</sub>) and respiratory exchange ratio (R<sub>E</sub>) during primed continuous infusions of 6-[<sup>3</sup>H] glucose in rainbow trout raised on LC and HC diets.

	Time (min)	MO <sub>2</sub> (mmol/h/kg)	MCO <sub>2</sub> (mmol/h/kg)	R <sub>E</sub>
LC	0	3.16 ± 0.70	1.86 ± 0.49	0.59 ± 0.04
	60	3.00 ± 0.57	1.66 ± 0.49	0.52 ± 0.05
	120	3.15 ± 0.53	1.81 ± 0.38	0.56 ± 0.04
	180	2.99 ± 0.47	1.74 ± 0.33	0.57 ± 0.04
	240	2.78 ± 0.38	1.65 ± 0.31	0.58 ± 0.05
HC	0	2.81 ± 0.27	2.15 ± 0.28	0.76 ± 0.03
	60	2.78 ± 0.33	2.06 ± 0.31	0.73 ± 0.03
	120	2.90 ± 0.27	2.08 ± 0.24	0.72 ± 0.04
	180	2.96 ± 0.24	2.14 ± 0.24	0.72 ± 0.04
	240	3.07 ± 0.27	2.16 ± 0.28	0.70 ± 0.05

All values are means ± SEM, n = 6. Infusion of IRa dose began at time 0, and infusion of the 2IRa dose at time 120 min. Neither metabolic rates nor respiratory exchange ratio were significantly different from initial values at any time after infusion began (repeated measures ANOVA).

## 4. DISCUSSION

The aim of this study was to assess the ability of the rainbow trout to modify its metabolism to better tolerate glucose in response to alterations in dietary carbohydrate. Previous studies involving manipulations of dietary carbohydrate demonstrated a wide range of effects on growth, feed utilization, hormone secretion and biochemical regulation depending on the fish species, diet composition and complexity of the carbohydrate. Typical diets previously used in our laboratory contain approximately 400 g protein, 100 g fat and 150 g carbohydrate per kg diet. In this study, trout were fed with diets containing similar amounts of fat and protein (approx. 120 and 400 g/kg diet respectively) but with higher carbohydrate content in the high carbohydrate diet (329 g/kg diet) compared to the low carbohydrate diet (10 g/kg diet; see Table 2.1). The carbohydrate sources of the HC diet were equal parts glucose and dextrin. Energy yields for the complete oxidation of glucose (15.71 kJ/g), palmitate (39.06 kJ/g), and an average amino acid (15.75 kJ/g) (Halver, 1989) were used to calculate energy contributions to the total digestible energy content of each diet. Due to the larger amount of carbohydrate, the HC diet had a total digestible energy of 17.8 compared with 12.80 kJ/g in the LC diet; thus these diets are non-isoenergetic (or isocaloric). Many diet manipulation experiments used diets with isoenergetic or near isoenergetic contents to ensure the observed effects in the fish were due to nutrient, not energy, differences (Bergot, 1979a; García-Gallego *et al.*, 1995; Panserat *et al.*, 2000). Unfortunately, to ensure isoenergetic conditions, either protein and/or fat levels must change if carbohydrate levels are manipulated. This means that conclusions regarding nutrient composition and physiological or biochemical changes are tenuous, as nutrients other than carbohydrates are changing. Given that protein and fat contents remained identical by mass in the two diets

used in my experiments, the results from my experiment can be interpreted as changes in either carbohydrate or total energy or both. Excess energy provided by carbohydrates in the HC diet was manifested as stored glycogen and fat, and allowed protein sparing to help drive muscle growth (Table 3.2).

#### **4.1 Body Parameters and Growth**

The early experiments of diet manipulation by Philips *et al.* (1948) stated that to best promote growth, 12% starch was the optimum carbohydrate content for trout feed. Many studies have since reached differing conclusions by using various proportions of nutrients in the diets. By maintaining a constant protein content and altering ratios of carbohydrate to fat, Brauge *et al.* (1995) showed that a 23:14 compared with a 60:14 carbohydrate to fat mass ratio yielded higher growth rates, fat deposition and protein retention, but only in fish held at 18 °C not at 8 °C. Garcia-Gallego *et al.* (1995) used diets with a constant fat content and increased maize starch from 200 to 500 g/kg digestible mass to substitute for protein (which decreased from 454 to 206 g/kg digestible mass). Growth was inversely proportional to carbohydrate content, and food intake decreased with decreasing protein content. The nutrient composition of the HC diet used in my experiments closely resembles that of the high carbohydrate diet used in the trout feeding experiment of Bergot (1979a). The authors noted that after 8 weeks of feeding, the high carbohydrate diet (composed of 30, 49, 14% by weight glucose, protein and lipid, respectively) yielded growth rates up to 18% higher than diets with identical lipid and protein but containing only 15 % glucose or using starch as an energy source.

The majority of growth in fish arises from buildup of muscle tissue, particularly white muscle (WM) fibres that comprise more than half of the total body mass. Although protein synthesis is higher in liver and gill than in WM, the low rate of protein degradation in WM compared with other tissues gives WM a higher growth rate efficiency (Houlihan *et al.*, 1989). In carnivorous species such as the rainbow trout, dietary protein provides the raw materials for protein synthesis and muscle growth. Part of this amino acid supply must be conserved for gluconeogenesis in order to produce glucose, which is the preferred oxidative substrate for nervous tissue and blood cells but is not a large portion of the natural trout diet (Cowey *et al.*, 1977). The goal of a high carbohydrate diet is to provide readily available glucose for these tissues so that a greater proportion of protein is reserved for growth. The increased protein efficiency ratio (PER) obtained by supplying other nutrients to replace it is referred to as protein sparing (Garcia-Gallego *et al.*, 1995). This can be accomplished by partial substitution of protein with carbohydrate or fat, or by simply increasing the amount of carbohydrate, fat or both. This method is limited by the assumption that all dietary protein is used for growth, and does not take into account use of protein for size maintenance. Furthermore, it does not take into account factors such as fish age, length or size although it remains the most widely used method for evaluating protein quality in fish (Halver, 1989). In the present study, protein and fat content by mass were kept constant between the diets, while carbohydrate was increased in the HC diet. PER was significantly higher in the HC group, which is an indication that a greater proportion of the available dietary protein is being used for growth. The significantly larger increases in length and weight of the HC group led to a higher specific growth rate (SGR) of this group compared with the LC group (Table 3.1). In addition to greater overall growth, internal inspection of the carcass and removal of

abdominal fat revealed more than twice the amount of peritoneal fat tissue in the HC group than the LC group. Liver mass was also disproportionately greater in the HC-fed group, leading to a HSI nearly twice than that of the LC group. It is also interesting that the plasma amino acid content of the LC-fed group was about double that of the HC group, implying these components were likely being mobilized from peripheral tissues for liver gluconeogenesis. Since the increased growth of the HC group was not constant between tissues, it is probable that specific physiological processes are altered in liver and adipose tissue.

## **4.2 Intracellular Glucose Metabolism**

### **4.2.1 Glycolysis**

Four hours after feeding, several tissues were collected from rainbow trout to measure enzymes and metabolites involved in intermediary metabolism. Plasma glucose concentrations 4 h after feeding were significantly higher in the HC group at 8.59 mM compared with 5.39 mM in the LC group (Table 3.2). The higher glucose levels of the HC group could be due to several factors. In addition to the greater glucose content of the HC diets, a poor ability to increase glycolysis, to suppress gluconeogenesis or glycogenolysis (glycogen breakdown), or to otherwise metabolize or excrete glucose would result in elevated plasma glucose concentrations. Values obtained in the HC group were close to the plasma glucose concentrations of 11 mM measured 6 h after feeding a similar high carbohydrate diet by Bergot *et al.* (1979a). Plasma insulin concentrations tended to be higher in the HC group 4 h after feeding compared to the LC group, but the differences were not statistically significant. In the HC group, insulin levels 4 h post-feeding were no different

from levels 8 h following or just prior to feeding. Plasma insulin in the LC group tended to rise 4 h post-feeding to levels similar to the HC group, where they remained up to 8 h post-feeding (Fig. 3.1). Values for the HC group were 17 ng/ml, similar to those measured in trout 6 h post-feeding a high carbohydrate (30% starch) diet (Capilla *et al.*, 2002), while the LC group had values of only 10 ng/ml 4 h post-feeding. The differences seen between the LC and HC groups in the enzymes and metabolites measured point to many biochemical pathways where these processes may have been modified by long-term exposure to dietary carbohydrates.

Once absorbed into the bloodstream, glucose has many fates. It can be excreted, oxidized in the cells, or stored in tissues as lipids or glycogen. Glycogen was measured in liver and WM. In both locations, glycogen was found to be higher in the HC group, with a 5-fold difference between groups in liver. Liver glycogen levels of the HC group were twice that measured by Bergot *et al.* (1979a) after feeding trout for 8 weeks with a high carbohydrate diet; trout fed with lower carbohydrate diets had liver glycogen concentrations of the LC group (Table 3.2).

The necessity for carbohydrate storage depends partly on how much glucose is in excess of that which can be utilized by cellular glycolysis. Several enzymes of glycolysis were measured to determine whether the increase in glucose storage in the HC group might be accompanied by increases in glucose utilization. Hexokinase, the first enzyme to interact with glucose inside the cell, initiates glycolysis by converting glucose into glucose-6-phosphate. HK activity was significantly higher in livers of HC-fed trout compared with LC-fed trout (Fig. 3.2A). GK, the high  $K_m$  hexokinase found exclusively in liver and pancreatic cells, was nearly 4-fold higher in liver of the HC group, as would be predicted for a tissue

with higher glucose uptake. Hepatic HK and GK activities have previously been shown to increase after the introduction of a high carbohydrate diet (Panserat *et al.*, 2000; Capilla *et al.*, 2003). After a 10-week feeding period of a diet composed of 40% protein, 16% lipid and 20% starch, Panserat *et al.* (2000) reported GK activities 10-fold higher than in trout fed a carbohydrate-free diet when measured 6 h after feeding. Capilla *et al.* (2003) also fed rainbow trout over a 10-week period with a virtually identical high carbohydrate diet, and observed a 3-fold increase in GK compared with trout fed with a diet devoid of carbohydrate. Expression of GK mRNA was also elevated in the high carbohydrate diet compared with the carbohydrate-free diet, which showed no detectible GK mRNA. Induction of GK mRNA expression by dietary carbohydrate was confirmed in the present study, which also demonstrated increased mRNA in the HC group 4 h after feeding, while GK mRNA in the LC group was undetectable (Fig. 3.7).

Values for the activity of PK in trout liver (Fig. 3.2A) were similar to those measured by Moon *et al.* (1988). PK, which catalyses the glycolytic conversion of PEP to pyruvate, had a significantly lower activity in the HC group, contrary to the prediction. This does not necessarily preclude the HC group from having higher liver glycolytic activities, since the conversion of PEP to pyruvate may not be the rate-limiting step in liver glycolysis. Panserat *et al.* (2001) examined mRNA expression of PK in the liver of rainbow trout fed either a low (-Cho) or high (+Cho) carbohydrate diet. Six and 24 h after feeding, expression of PK was not significantly different between the two groups, however, levels of PK in the +Cho group had a tendency to be lower than in the -Cho group at 6h, but higher than the -Cho group at 24 h post-feeding. The authors concluded that, as in mammals, there is no apparent nutritional effect on the regulation of PK; my study, however showed a statistically significant decrease

in hepatic PK activity in HC-fed trout consistent with the trend previously seen in PK mRNA expression by Panserat *et al.* (2000). The long duration of the present study may account for impacts not seen in the 10-week experiment of Panserat *et al.* (2000). In WM, PK activity was estimated in the anterior, middle and posterior regions to determine how location alters the status of muscle enzyme activities (Fig. 3.4A). Fish muscle is known to experience different strains depending on location, which influences both the type and quantity of proteins synthesized locally as well as mitochondrial proliferation (Moyes *et al.*, 1989). In the back (posterior) region, where activity of the muscle tends to be higher during swimming, PK was found to be significantly higher for the HC group than the LC group. In the front and middle sections, which tend to be less active, no significant differences in PK between LC and HC were seen, though PK still tended to be higher in the HC group. Based on the observations of most enzyme activities in WM, only the posterior region of red muscle (RM) was assayed for enzyme activities. Here, PK was also found to be higher in the HC group. LDH was also assayed in liver, WM and RM (Fig. 3.2A, 3.3 B, 3.6). Under conditions of limited oxygen supply, LDH catalyses the conversion of pyruvate to lactate, and generates  $\text{NAD}^+$  to allow the continuation of glycolysis. In mammals and fish, LDH has numerous isozyme forms with differing affinities for pyruvate,  $V_{\text{max}}$  values and tissue distributions. The significance of LDH activity increases is poorly understood and surrounded by controversy, as this enzyme is not thought to limit pathway flux. Activities of liver LDH were significantly lower in the HC group than the LC group; in this case, higher LDH activities would be significant to enhance lactate utilization as a gluconeogenic substrate (Nelson and Cox, 2000). WM LDH activity was consistently higher in the HC group, but showed statistical significance only in the back region. The HC group also showed significantly

higher LDH activity in RM. It is not known whether differences in LDH activity between the two experimental groups are related to their relative degrees of aerobic metabolism. Under aerobic conditions, pyruvate is converted to acetyl CoA that enters the TCA cycle in the mitochondria. The PDH enzyme complex catalyzes this reaction. Richards *et al.* (2002) measured PDH activity in rainbow trout RM and WM during graded aerobic exercise to determine substrate utilization at 30, 60 and 90% of the critical swimming speed, the maximum speed that can be sustained indefinitely by the fish. It was demonstrated that PDH activation increased with exercise and was maximally activated at exhaustion. The differential recruitment and substrate preferences of RM and WM during exercise have also been subjects of much research due to the convenient spatial separation of the two muscle types (Wilson and Egginton, 1994; Moyes and West, 1995; Burgetz *et al.*, 1998). In the present study, measurement of PDH activities in RM and WM of resting trout were used to assess possible changes in substrate preference, and especially the use of the excess glucose by mitochondrial oxidative phosphorylation (Fig. 3.5, 3.7). WM active PDH (PDHa) activity was 7-fold higher in the HC group than the LC group while the total PDH (PDHt) activity was lower; the PDHa/PDHt ratio was significantly higher in the HC group compared with the LC group, and had values comparable to those reported by Richards *et al.* (2002). These data provide evidence that trout fed the HC diet preferentially oxidize glucose through mitochondrial oxidative phosphorylation than trout fed the LC diet. In RM PDHa and PDHt values were higher, as were the PDHa/PDHt ratios compared to WM, however no significant differences were seen between the LC and HC groups (Fig. 3.7).

### 4.2.2 Lipid metabolism

Glucose that is not utilized or stored as glycogen may be converted into lipid. In addition to a greater proportion of body fat, the HC group had a 2-fold greater level of plasma triglycerides compared with the LC group (Table 3.2). Lipid percentages by weight in liver and muscle tissues of the HC group are also twice as high as in the LC group. Curiously, HC fed trout consistently demonstrate a higher fat content despite the fact that lipids make a lower contribution to the total digestible energy of the HC diet compared to the LC diet. This could be explained by a shift in substrate preference in the HC group to favour carbohydrate metabolism over lipid metabolism, and/or by an increase in lipogenic activity. Three enzymes (G6PDH, FAS and CPT) were measured to examine potential changes in lipid regulation. G6PDH catalyses the first reaction of the pentose phosphate pathway. In mammals and fish, the pentose phosphate pathway plays a major role in the production of NADPH, which provides reducing power for the biosynthesis of fatty acids in liver and adipose tissue (Nelson and Cox, 2000). Liver G6PDH was significantly higher in the HC group, supporting a greater reducing capacity for lipogenesis in this tissue (Fig. 3.2B). Although the pentose phosphate pathway is not highly active in skeletal muscle, G6PDH was found to have a significantly higher activity in the WM of the HC group sampled from the anterior epaxial region. FAS, also a lipogenic enzyme promoting the conversion of malonyl CoA to fatty acyl CoA for fatty acid synthesis, had more than 6-fold greater activity in the liver of the HC group, lending further support for the notion that lipogenesis is significantly activated by the HC diet (Fig. 3.2B). The estimation of CPT activity, which promotes  $\beta$ -oxidation by the conversion of fatty acids to Acetyl CoA, was used to determine whether decreased lipolysis could possibly account for the differences in fat between the two groups

(Fig. 3.2B). An 8-fold reduction of CPT in the HC group implies a much reduced capacity for  $\beta$ -oxidation, thus suggesting that both increased lipogenesis and decreased lipolysis are responsible for the higher degree of fat storage in the HC group.

#### 4.2.3 Gluconeogenesis

One hypothesis to explain the poor control of plasma glucose by carnivorous teleost fish is an inability to regulate gluconeogenesis (Moon, 2000). Several enzymes involved in the gluconeogenic pathway were measured to examine the impact of dietary carbohydrate on gluconeogenesis. The irreversible conversion of phosphoenol pyruvate (PEP) to pyruvate by PK is bypassed by PEPCK to produce PEP from the citric acid cycle intermediate oxaloacetate (see Fig. 1.2). Although dietary carbohydrates have been shown to decrease gluconeogenic enzyme expression in common carp and gilthead seabream (Panserat *et al.*, 2002), such has not been the case in trout. Panserat *et al.* (2001) concluded that hepatic PEPCK gene expression is not regulated by dietary carbohydrates in rainbow trout, after feeding fish for 10 weeks with either a low carbohydrate (-Chb) or high carbohydrate (+Chb) diet and detecting no differences in PEPCK mRNA. The authors suggested that the first step of the hepatic gluconeogenic pathway is functional and highly active irrespective of the dietary carbohydrate supply. Enzyme activities of PEPCK in the liver, however, showed significant differences between the two groups in the present study; PEPCK activity was nearly 2-fold higher in the LC group compared with the HC group (Fig. 3.2A). As with PK, differences in gene expression of this enzyme may not have been detected in previous studies due to their shorter durations. Changes in enzyme activities may also be observed without changes in gene expression due to post-transcriptional and post-translational modifications. Many amino acids are glucogenic, producing precursors for gluconeogenesis upon

deamination (Nelson and Cox, 2000). The production of pyruvate from alanine,  $\alpha$ -ketoglutarate from glutamate and oxaloacetate from aspartate are catalyzed by ALT, GDH and AST, respectively. In the carnivorous gilthead seabream, activities of ALT and AST are seen to be significantly lower in fish fed with high carbohydrate diets compared with low carbohydrate diets after 18 days of feeding (Metón *et al.*, 1999). In rainbow trout, fish fed the HC diet have significantly lower activities of AST, ALT and GDH compared with the LC group, which has nearly 2-fold greater activities for all three enzymes (Fig. 3.2C). These results suggest that like the seabream, trout exhibit a large degree of adaptation to changes in dietary carbohydrate by regulation of hepatic enzymes, and may have the ability to at least partially deactivate gluconeogenesis.

### 4.3 Glucose Tolerance

In the carnivorous rainbow trout, resting plasma glucose (RPG) values range from approximately 5 mM (Haman *et al.*, 1997) to 8.2 mM (Legate *et al.*, 2001). Resting plasma glucose values in this study were 2.2 and 9.5 mM for the LC and HC groups, respectively (Fig. 3.10). Intravenous glucose tolerance tests (IVGTTs) using 250 g glucose per kg fish performed by Legate *et al.* (2001) demonstrated a prolonged hyperglycemia that peaked at 22 mM and was corrected by 24 h in the rainbow trout, a result considered glucose intolerant in mammalian species. In the present study, the LC and HC groups showed similar plasma glucose concentration profiles throughout the IVGTT despite the initial difference in RPG (Fig. 3.10). In both, glucose levels peaked between 12 and 14 mM within the first five minutes following a 250 mg/kg body weight glucose injection, and returned to baseline values within 6 h. By the standards set for mammalian species, trout fed with either LC or

HC groups are considered glucose intolerant. Although there were no detectable differences in glucose tolerance between the groups, the difference between RPG and the peak glucose concentration were smaller for the HC compared with the LC group. The same increase was expected for both groups since each received the same bolus, however the peak values were similar in spite of drastically different baseline values. This discrepancy could be explained if RPG values in the HC group were closer to maximal plasma values, or by differences in glucose space (the total fluid space available to glucose) or by tubular transport maximum (the maximal rate of reabsorption of glucose in the kidney).

Studies on urinary glucose excretion in fish are rare and mostly qualitative, citing only the phenomenon of slight glucose presentation in the urine (Furuichi *et al.*, 1986; Lin, 1991). The renal threshold (the concentration beyond which glucose appears in the urine) for urinary glucose excretion was measured in tilapia orally administered carbohydrates and injected glucose and was found to be approximately 6 mM (Lin *et al.*, 2000). Two fish from either the LC or HC group were outfitted with a bladder cannula and urine was collected during an IVGTT to determine urinary glucose concentrations. The sporadic nature of urine flow and interference from reproductive processes in the trout made utilization of the bladder cannulation technique difficult. In the fish from which urine was successfully collected, intriguing differences were seen between the LC and HC groups (Fig. 3.12). In the LC group, urinary glucose was negligible prior to the glucose injection, and remained at concentrations close to 0 until the 30 min time point when concentrations rose steadily to a peak value of 7 mM at 6 h. By 24 h, urinary glucose concentration fell to 2 mM. In contrast, the HC fish had urinary glucose concentration above 10 mM even before administration of the bolus. Glucose concentrations rose more quickly than in the LC group, peaking at 14 mM within the first 30

min, and also fell to values below initial concentrations within 6 h. The calculated mass of glucose excreted over the 24 h period following the glucose load in the HC fish was 3-fold that of the LC fish (Table 3.3). The higher urinary glucose output of the HC fish implies that under resting conditions, plasma glucose concentrations are above the renal threshold for glucose in this group. The fact that the maximum plasma glucose concentration reached was about 14 mM, which is the value observed for the urinary concentration in the HC fish, is also intriguing. These results should be interpreted carefully however, due to both the small sample size and the fact that internal bladder catheterization underestimates the ionoregulatory effectiveness of the whole renal system, since functions of the urinary bladder itself are bypassed (Curtis and Wood, 1991).

In one individual fish from each group, insulin was measured in plasma sampled during an IVGTT (Fig. 3.11). In both fish, insulin concentrations rose from baseline, reaching maximum values within the first 15 min following the bolus injection. In the LC individual, the baseline levels were higher than the HC individual, and the increase was much greater as well, rising from 6 to 14 ng/ml compared to the smaller increase of only 3 to 6 ng/ml seen in the HC fish. Previous studies reported changes in plasma insulin during GTTs (see Table 1.1); red seabream, yellowtail and chinook salmon showed 2-fold increases during oral GTTs, while carp showed a 3-fold increase, and in an IVGTT brown trout showed a 2.5-fold increase (Furuichi and Yone, 1981; Mazur *et al.*, 1992; Blasco *et al.*, 1996). In rainbow trout, a transient increase was seen by Legate *et al.* (2001) during an IVGTT, but was not significantly different from baseline values at any point in time. Harmon *et al.* (1991) actually saw a decrease in plasma insulin following an intraperitoneal GTT in rainbow trout.

So whether insulin is responsible or even involved in the slow decrease in plasma glucose following a GTT remains unanswered.

#### 4.4 Respirometry

Indirect calorimetry was used to determine whether or not substrate utilization was modified in response to the experimental diets. It was predicted that the respiratory exchange ratio ( $R_E$ ) of the HC group would reflect an increased usage of carbohydrate due to the higher proportion of carbohydrate in the diet. The HC group showed a significantly higher  $R_E$  value of 0.84 compared with 0.61 in the LC group (Fig. 3.9). Estimation of substrate utilization derived from indirect calorimetry is founded in the stoichiometric relationships between  $O_2$  and  $CO_2$  in the complete oxidation of metabolic substrates (Frayn, 1983).  $R_E$  is actually an estimate of the true respiratory quotient ( $R_Q$ ), which represents the actual rate of  $CO_2$  production divided by the rate of  $O_2$  consumption. For glucose oxidation, 1 mole of  $CO_2$  is produced for each mole of  $O_2$  consumed, and thus the predicted  $R_Q$  for an organism exclusively utilizing glucose as a substrate is 1. For a typical fat such as palmitate, the stoichiometric  $O_2$  to  $CO_2$  ratio is 102:145, yielding an  $R_Q$  of 0.7. The  $R_E$  describes the instantaneous relationship between  $MO_2$  and  $MCO_2$  as determined from gas or water exiting a face mask or other respirometry apparatus (Warren *et al.*, 2002). The calculated  $R_E$  values indicate substrate preference in the LC group for fats and protein and in the HC group for carbohydrates, suggesting that dietary carbohydrates can alter substrate preference to favour dietary carbohydrates as an energy source. Strangely, the LC group value of 0.61 is below the lower limit of 0.7 for  $R_Q$ . However, processes that counter substrate oxidation (such as gluconeogenesis and lipolysis) interfere with the calculation of  $R_E$ . Furthermore, because gas

exchange is measured at the whole organism level and not the tissue level, estimates of  $R_E$  may be lower than actual  $R_Q$  values (Frayn, 1983).

#### 4.5 Glucose Turnover

The continuous tracer infusion method of Haman and Weber (1996) was used to detect differences in rates of glucose appearance ( $R_a$ ) and disappearance ( $R_d$ ) in trout raised on experimental diets and subjected to steady infusion of glucose. Legate *et al.* (unpublished data) previously used the continuous tracer infusion method to monitor changes in glucose turnover during a standard IVGTT.  $R_d$  increased transiently but non-significantly after the injection, while  $R_a$  was found to be significantly higher than pre-injection values at 2 and 4 h. These observations led the authors to believe that the liver does not reduce its output of glucose within the first 2 hours of exposure, possibly contributing to the high plasma glucose levels seen in rainbow trout following a glucose load. The introduction of a glucose bolus during the continuous tracer infusion method, however, makes calculations of  $R_a$  difficult because the system is pushed far out of steady state. It is for this reason that an alternate infusion line was used to slowly inject glucose at a controlled rate. Baseline  $R_a$  and  $R_d$  values in the LC and HC groups were 4- and 10-fold higher, respectively, than resting values obtained by Haman and Weber (1996) and were rapidly falling before a second infusion equivalent to  $10 \mu\text{mol/kg/min}$  ( $I_{Ra}$ ) was simultaneously administered (Fig. 3.14A,B). A possible explanation for these initial values is that the fish had not yet achieved steady state. Interestingly, the standardized specific activities of the plasma during the priming period (Fig. 3.13B) were nearly 4- and 10-fold lower in the LC and HC groups, respectively, compared to those of Haman and Weber (1996). These low values can partially account for

the extremely high values calculated for Ra and Rd, as glucose turnover is inversely proportional to the specific activity. Sixty minutes after an initial priming dose, the 6- $^{3}\text{H}$ -D-glucose plus a second infusion equivalent to 10  $\mu\text{mol/kg/min}$  (IRa) were simultaneously administered. By this time the LC group had a glucose turnover rate of 13  $\mu\text{mol/kg/min}$ , which is a typical resting value for rainbow trout (Haman and Weber, 1996; Haman *et al.*, 1997). The decrease in glucose turnover was accompanied by a proportionate increase in specific activity. By comparison, when the IRa infusion was initiated, the glucose turnover rate of the HC group was 7-fold higher at 70  $\mu\text{mol/kg/min}$ , a much higher glucose turnover rate than any reported even for mammals. No increase in specific activity of the HC group was observed during this period, which may explain why glucose turnover did not decrease as it did in the LC group. No explanation could be found for unusually low specific activity values, or for the increase in specific activity of the LC group at the time of the IRa infusion, but their patterns are reflected in the values of glucose turnover. Despite the marked fluctuations in glucose turnover, within each group Ra and Rd were the same prior to the IRa infusion, indicating no net production or removal of glucose from the circulation. When the IRa infusion was doubled to 20  $\mu\text{mol/kg/min}$  (2IRa), the Rd in the LC group remained constant at values near 16  $\mu\text{mol/kg/min}$ , while Ra dropped to levels near zero, which suggests that glucose production was halted completely. Ra reached a minimum of  $-4$   $\mu\text{mol/kg/min}$ , a theoretically impossible result. The values for Ra were determined by subtracting the exogenous glucose infusion rate from the values of Rd calculated from the plasma glucose concentration and specific activity. There were some similarities to the pattern of glucose turnover observed in the HC group, though values of Ra and Rd were generally higher. Rd decreased from an initial value of 78 to 52  $\mu\text{mol/kg/min}$  during the

course of the IRa infusion and held constant when the 2IRa infusion was initiated. Ra steadily decreased from 70 to 42  $\mu\text{mol/kg/min}$  during the IRa infusion and reached a steady value of around 32  $\mu\text{mol/kg/min}$  for the duration of the 2IRa infusion. As observed in the LC group, Ra decreased by magnitudes corresponding to the increases in infused glucose, implying a well-controlled reduction in glucose production in order to maintain a constant plasma glucose concentration. This ability to lower Ra may suggest that trout can halt glucose production by the liver in response to a glucose challenge, and is the first known demonstration in fish. Indirect calorimetry was used to estimate  $R_E$  during the glucose turnover experiment (Table 3.4), and  $R_E$  was not seen to change in either group throughout the continuous tracer infusion, indicating no acute effects of the exogenous glucose administration on changes in substrate preference.

## 5. CONCLUSIONS

While there is a very detailed understanding of carbohydrate metabolism in mammals, the question of whether carnivorous fish have the capacity to effectively utilize dietary carbohydrates has received little attention by comparison. This thesis investigated the use of dietary carbohydrates by a carnivorous teleost, the rainbow trout, over a longer period and measuring a wider array of enzyme and metabolite changes than previous studies.

Feeding fish with HC diets resulted in increased body mass and larger specific growth rates, when protein and fat composition of the diets were held constant. The HC diet also resulted in greater amounts of body fat and both lipid and glycogen deposition in liver and muscle tissue. The capacity for lipid storage was elevated in the HC group in part by

increased hepatic activities of the lipogenic enzymes FAS and G6PDH, and decreased activities of the lipolytic enzyme CPT.

The potential for flux through glycolysis was higher in the HC group, which had increased activities of GK, HK and PDHa. Activity of the gluconeogenic enzymes PEPCK, GDH, AST and ALT were lower in the HC group, providing strong evidence that increased dietary carbohydrate can reduce the potential for glucose synthesis by the gluconeogenic pathway. Elevated levels of plasma amino acids in the LC-fed trout imply increased substrate availability for hepatic gluconeogenesis in this group.

Glucose tolerance tests showed no significant difference in the ability of fish fed the HC diet to recover from a glucose challenge, although the HC group had a tendency to be more resistant to increases in plasma glucose, as seen by a smaller rise in plasma glucose concentration following the load.

Estimations of the respiratory exchange ratio established that long-term feeding of dietary carbohydrates leads to carbohydrates as a preferred substrate for energy production as do increased activities of PHDa.

When glucose was infused at a rate of 10  $\mu\text{mol/kg/min}$  into trout fed with either the LC or HC diets, glucose concentrations in both groups were unaffected, as were both  $R_a$  and  $R_d$  even after the infusion of glucose was doubled to 20  $\mu\text{mol/kg/min}$ . The HC group exhibited glucose turnover rates that more closely resembled those of humans, compared to the LC group, which had rates nearly 10-fold lower.

Over the past 50 years, scientists have increasingly challenged the conventional notion that carnivorous teleost fish such as the rainbow trout are glucose intolerant. This work monitored the long-term effects of dietary carbohydrates and is a significant addition to

the current body knowledge. The results shown here demonstrate that rainbow trout can tolerate a diet composed of 33% carbohydrate, 40% protein and 12% fat, and potentially utilize dietary carbohydrates to drive growth and produce energy. The protein efficiency ratio is higher in this diet compared to one devoid of carbohydrates. This study used diets with identical lipid and protein content while carbohydrate content was manipulated and as such has the inherent limitations of non-isocaloric diets. Accordingly, the findings should be interpreted cautiously, but nevertheless have promising implications for the potential sparing of protein by carbohydrate replacement. It is hoped that these results encourage future research that employs isoenergetic diets or pair-feeding methods to control caloric intake. If glucose can be used as a partial substitute for dietary protein, the benefits to the aquaculture industry could be substantial. With the formulation of high carbohydrate, low protein diets, decreased feed costs and reduced output of harmful nitrogenous wastes could be achieved, while still meeting the world's ever-increasing demand for aquacultured fish.

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