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Glucose Tolerance in

3 Teleost Species

By

© Nicola J. Legate

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ABREVIATIONS

GT	Glucose tolerance
GTT	Glucose toleranace test
IGTT	Intravenous glucose tolerance test
OGTT	Oral glucose tolerance test
Ra	Rate of appearance
Rd	Rate of disappearance
IDDM	Insulin dependant diabetes mellitus
NIDDM	Non-insulin-dependant diabetes mellitus
CB	Cytochalasin B
HK	Hexokinase
P/N	Percoll/Nycodenz

ABSTRACT

Fish are generally considered to be glucose intolerant in relation to omnivorous mammals. Glucose intolerance may be an expected consequence of the carnivorous lifestyle of many fish. Comparatively, however, some omnivorous fish have higher glucose tolerance than carnivorous fish. This suggests differences in the mechanisms of regulation of glucose clearance of carnivorous and omnivorous fish. This study tests the hypothesis that glucose tolerance in fish is related to nutrient preference and that this tolerance is correlated with hexokinase activity and tissue glucose transporters.

Differences in glucose clearance abilities of three fish species, the carnivorous rainbow trout (*Oncorhynchus mykiss*) and American eel (*Anguilla rostrata*) and the omnivorous bullhead catfish (*Ameiurus melas*) were investigated in this study. Glucose tolerance was compared between the three species and between feeding and 6 month fasted eels. Glucose tolerance was assessed by administering an intravenous glucose tolerance test (IGTT) , injecting 250 mg glucose/kg body weight and taking blood samples over 24 h. Feeding eel and catfish were found to be more glucose tolerant than fasting eel and rainbow trout. Glucose turnover was investigated in the rainbow trout during the glucose tolerance test and the rate of appearance of glucose did not decrease until 2 h after a glucose load which may contribute to the glucose intolerance seen.

To test what factors might contribute to differences in glucose tolerance, two steps within the glucose uptake pathway were examined in white muscle. Hexokinase activities were investigated but no differences in HK activities were detected between species. These studies established that HK was not the rate limiting step of glucose uptake into the white muscles.

In mammals GLUT-1 and GLUT-4 are the glucose transporters present in skeletal muscle and GLUT-4 is the insulin-responsive glucose transporter that regulates much of a glucose load. The white muscle of all three species were probed for mammalian GLUT-1 and GLUT-4 transporters and no mammalian GLUT-1 or GLUT-4 protein or mRNA were detected.

Glucose transport studies were performed on white muscle membrane vesicles prepared using a Percoll/Nycodenz technique from all three species. Radioactively labeled glucose uptake provided evidence for the presence of a stereospecific, saturable glucose transporter in the white muscle of all three species. This transporter, however, did not have inhibition kinetics indicative

of a mammalian GLUT indicating that the three fish studied have a glucose transport system, but it is different from the one found in mammals.

This study provides evidence for differences in glucose tolerance between the three fish species examined based on feeding type (omnivorous and carnivorous). The precise bases for these differences appear not to be associated with hexokinase activities. Hepatic glucose output, in the rainbow trout, does not decrease with a glucose load which may hinder swift glucose clearance from the blood. The mammalian GLUT-1 and GLUT-4 glucose transporters were not detected in the white muscle of any of the fish species. However, a stereospecific, saturable glucose transporter was found in white muscle using kinetic analyses, but the activities of this transporter did not differ between species. The differences in glucose tolerance in these three species may result from a combination of these factors or may reside in features other than those studied here.

Résumer

Comparativement aux mammifères omnivores, les poissons sont considérés intolérant au glucose. L'intolérance au glucose peut-être une conséquence attendue du style de vie carnivore de plusieurs poissons. Par contre, certains poissons omnivores ont démontrés une tolérance au glucose plus élevé que des poissons carnivores. Ceci suggère des différences dans les mechanismes du système de régulation du glucose des poissons carnivores et omnivores. Cette étude á pour but de vérifier l'hypopthèse suivante : La tolérance au glucose chez les poissons est reliée à la préférence des nutriments et que la tolérance au glucose est corrélé á l'activité de l'hexokinase (HK) et les transporteurs membranaires de glucose.

La question suggérant des differences chez les méchanismes de la régulation du glucose sanguin fut investiguée chez trois espèces de poissons, la truite arc-en-ciels (*Oncorhynchus mykiss*) et l'anguille américaine (*Anguilla rostrata*), soient deux poissons carnivores, ainsi que la barbotte noir (*Ameiurus melas*), une espèce omnivore. De plus, la tolérance au glucose fut comparées entre les trois espèces ainsi qu'entre des anguilles nourrits et jeûnées pendant une période de six mois. Les anguilles de cette étude étaient de différentes classe d'âges, donc, les observations ne peuvent pas être seulement préscrites à des différences dûe á l'état nourrit des anguilles. Le test de la tolérance du glucose fut effectué par administration d' IGTT, par injection de 250 mg de glucose/ kg et par collection d'échantillons de sang sur une période de 24 heures. Les résultats démontrent que les anguilles nourrit et les barbottes ont une tolérance plus élever que les anguilles jeûnées et les truites arc-en-ciel. Le renversement du glucose a été mesurer chez la truite arc-en-ciel et nous a permis de déterminer que même si le taux d'élémination du glucose sanguin a augmenté, le taux d'apparition est resté inchanger, ce qui en sorte pourrait contribuer à l'observation de l'intolérance chez cette espèce après l'injection de glucose.

Deux étapes responsable de l'influx du glucose furent examinées dans le muscle blanc pour déterminer quels facteurs seraient à l'origine des différences dans la régulation du glucose sanguin. Ceux-ci sont l'activité de l'hexokinase et la présence des transporteurs membranaires GLUT-1 et GLUT-4. Aucune différences furent observées entre les espèces concernant l'activité de l'hexokinase, mais l'étude a établie qu'il y avait sufisamment d'hexokinase dans le muscle blanc et que cette étape n'est pas une étape limitante dans l'influx du glucose dans le muscle.

Deuxièment, les mammifères possèdent un système de transport de glucose incluant les transporteurs membranaires GLUT-1 à GLUT–7. GLUT-1 et GLUT-4 sont présent dans les muscles squelettique et Glut-4 est le transporteur membranaire répondant à l'insuline qui régularise presque entièrement une élévation de glucose suite à une injection de glucose ou un repas. Aucun des transporteurs « mammaliens », soient proteines et ARN méssager fuent détectés dans les muscles blancs des espèces étudiées.

Une préparation de vésicules membranaires de muscles blancs fut utilisée pour éffectuer une étude sur le transport du glucose. L'influx de glucose radioactif donne de l'évidence pour la présence d'un transporteur membranaires de glucose stéréospécifique et saturable dans le muscle blanc des trois espèces. Par contre, ce transporteur n'avait aucune inhibition cinétiques indicatif d'un Glut mammalien. Les trois poissons étudier ont en effet un système de transport de glucose, mais il est différent de celui des mammifères.

Les résultats de cette étude suggèrent qu'il y a des différences dans la tolérance du glucose entre les trois espèces étudier et que ces différences sont basées sur les types carnivores ou omnivores. La base précise ne semble pas être associé à l'activiter de l'hexokinase. Le flux du glucose hépatique chez la truite arc-en-ciel ne décroit pas avec une accroissement de glucose (ex. par injection), ce qui pourrait empêcher l'élimination du glucose du sang. Les transporteurs membranaires GLUT-1 et GLUT–4 mammalien ne furent détecter chez aucune des espèces étudiées. Par contre, un transporteur stéréospécifique et saturable fut détecter dans les muscles blancs utilisant des analyses cinétiques, mais l'activité de ce transporteur ne diffère pas entre les trois espèces. Les différences de la tolérance de glucose observées peuvent-être le résultat d'une combination de ces facteurs ou peut-être le résultat d'autre méchanismes.

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INTRODUCTION

1. INTRODUCTION

Fish are generally thought to have a limited ability to utilize carbohydrate, especially compared with mammals. Although the precise explanation for this is not fully known, mammalian skeletal muscle and adipose tissue do have glucose transporters that take glucose from the circulating blood in the basal state and an insulin stimulated glucose transporter which enhances glucose clearance in the postprandial state. The clearance of glucose from the plasma is much more sluggish in fish leading a number of authors to state that fish are glucose intolerant (Falkmer 1961; Palmer and Ryman 1972; Furuichi and Yone 1980; Mommsen and Plisetskaya 1991; reviewed by Garcia Gallego 1994; Wilson 1994). Investigation of glucose clearance in fish is important to identify how it differs from the well regulated and studied mammalian system and whether it can be modified in order to reduce costs associated with fish rearing. Past studies will be reviewed that look at glucose tolerance in fish. In addition the mammalian system of glucose uptake will be reviewed in order to compare the two systems.

1.1. Glucose Intolerance in Fish

Classically fish have been considered to have a limited ability to utilize carbohydrate and have been classified as glucose intolerant. Carnivory is a predominant feeding strategy of many fish and these species have metabolisms that evolved primarily to deal with high protein diets (Halver 1989). There are, however, fish that have evolved omnivorous and herbivorous lifestyles and these species would have a higher exposure to carbohydrate and potentially a greater capacity to deal with carbohydrate (Halver 1989).

Glucose intolerance is a clinical term used in the diagnosis of insulin-dependent diabetes mellitus (IDDM) which is diagnosed by the use of a glucose tolerance test (GTT). A GTT involves administering a bolus of glucose either orally or intravenously, and if plasma glucose values do not return to baseline within 1-2 h, the subject is considered to have impaired glucose tolerance (National Diabetes Data Group 1979). This same procedure has been administered to fish as an index of glucose tolerance.

Several studies have examined glucose tolerance in a variety of fish species. An early, in-depth study was reported by Falkmer (1961) who examined blood sugar regulation in the daddy sculpin (*Cottus scorpius*) and performed a GTT by injecting 500 mg glucose/kg body weight intramuscularly. A marked hyperglycemia was detected 5 min after glucose injection, blood glucose peaked 30-45 min post-injection and levels approached a normal range 9-24 h post-injection. Removal of the pancreatic tissues (iselectomy) resulted in most sculplins studied becoming hyperglycemic even in the absence of a glucose challenge. Falkmer concluded from these and other studies that glucose levels were maintained by processes similar to, but more sluggish then, those found in mammals. Palmer and Ryman (1972) performed oral glucose tolerance tests (OGTT) in which 1 g of glucose was administered to rainbow trout (*Oncorhynchus mykiss*) through a stomach tube. These fish showed both pronounced and persistent hyperglycemia with blood glucose values still elevated 7 h after glucose ingestion. Intravenous glucose tolerance tests (IGGT) performed on channel catfish (*Ictalurus punctata*) (250 mg glucose/kg body weight) showed that blood glucose increased sharply within 1 min after injection, but hyperglycemia was reduced dramatically over the first hour with concentrations close to starting values 24 h after glucose injection. Furuichi and Yone (1981) performed GTTs

on three fish species with widely different feeding patterns; carp, an omnivore, red sea bream, a semi-carnivorous fish and yellowtail also known as the Japanese amberjack, a carnivore. These fish were administered a gelatin capsule containing 167 mg glucose/100 g body weight orally. Five hours later the omnivorous carp was normoglycemic, whilst the red sea bream and yellowtail were hyperglycemic with the latter species showing the highest values. Although all three fish were glucose intolerant compared with mammalian omnivores, the omnivorous carp had a higher glucose tolerance than either the semi-carnivorous red sea bream or the carnivorous yellowtail. This implies that even though fish are glucose intolerant by mammalian standards, there are differences among fish species that relate to dietary intake of carbohydrate.

Garcia-Gallego *et al.* (1995) compared carbohydrate utilization by European eel (*Anguilla anguilla*) and rainbow trout fed 4 isoenergetic diets with increasing carbohydrate and decreasing protein contents. The results supported a greater ability to use carbohydrate by the eel than the trout. The authors suggested that this may be a combination of the trout requiring a greater quantity of protein for growth and a reduced ability to use dietary carbohydrates. Furthermore, they proposed that carbohydrate utilization in the carnivorous eel is more comparable with either omnivores or herbivores than to that of the carnivorous trout. At the termination of this 3 month feeding study, a number of parameters of carbohydrate utilization were examined. Blood glucose values in eel fed all 4 diets were not significantly different regardless of the carbohydrate content, while in trout a direct relationship existed between increasing blood glucose and increasing carbohydrate content of the diets. This difference in ability to regulate blood glucose with changes to dietary carbohydrate content correlated with pyruvate kinase (PK) activities, which were used as an index of glycolytic metabolism. PK

activities did not change in trout, while there was an increase in PK activities with small increases in carbohydrate and an increase in enzyme substrate affinity for higher carbohydrate dietary contents in the eel (Suarez *et al.* In Press). These two studies do support different abilities of apparently carnivorous fish species to use carbohydrate in their diet, a key issue within the aquaculture industry. In addition, they support the idea that the ability to utilize the excess carbohydrate was key to glucose tolerance.

A limited number of experiments have been done on *in vivo* glucose utilization in fish. These studies calculate glucose turnover and the rate of appearance of glucose (Ra) from the liver and kidney and the rate of disappearance of glucose (Rd) into the tissues. Evidence supporting an enhanced ability to utilize carbohydrate by the American eel (*Anguilla rostrata*) was reported by Cornish and Moon (1985). American eels exhibited a consistently high plasma glucose concentration but in addition high turnover and clearance rates compared to other fish species including the kelp bass (*Paralabrax clathratus*; Bever *et al.* 1977), coho salmon (*Oncorhynchus kisutch*; Lin *et al.* 1978) and rainbow trout (Haman *et al.* 1997). They also reported that eels fasted for 6 months showed higher glucose turnover and clearance rates than eel fasted for 15 months. Weber *et al.* (1986) reported higher glucose turnover in the skipjack tuna (*Katsuwonus pelamis*) than in other teleosts which was probably a consequence of their high metabolic rates. Glucose turnover rates in the skipjack tuna were at least as high as those reported for mammalian species. West *et al.* (1992) reported white and red muscle glucose utilization in resting and exercising rainbow trout. These experiments showed a significant increase in glucose utilization by red muscle but not white muscles with exercise. Garin *et al.* (*Dicentrarchus labrax*) (1987) examined glucose turnover in sea bass and made comparisons

with both mammal and bird values from the literature and concluded that glucose metabolism is slower in fish. Haman *et al.* (1997), using a continuous tracer infusion method, measured glucose turnover rate in rainbow trout exposed to hypoxia. They reported that hepatic glucose production was briefly stimulated at the onset of hypoxic stress, but that this increase was not accompanied by an increase in glucose clearance resulting in hyperglycemia. Shanghavi (1998 personal communication) found that during submaximal exercise (1.5 body lengths/sec), rainbow trout rate of appearance (Ra) and rate of disappearance (Rd) of glucose both decrease. None of these studies, however, address glucose utilization after a glucose load.

1.2. Insulin

Insulin is a key regulatory hormone in mammalian glucose homeostasis (reviewed by White and Kahn 1994; Saltiel 1996; Cortright and Dohm 1997). Plasma insulin suppress glucose output by the liver and stimulates glucose uptake by peripheral tissues (Holness *et al.* 1996). The major secretagogue of insulin secretion in mammals is plasma glucose concentrations. With ingestion of a meal, absorption of amino acids and certain gut factors augment insulin secretion (Holness *et al.* 1996). This postprandial secretion of insulin allows for the clearance of a glucose load. Glucose clearance is accomplished by insulin binding to insulin-specific tissue receptors that are members of the receptor tyrosine kinase family (Tavare and Siddle 1993). A complex signal transduction pathway ultimately moves intracellular vesicles containing the glucose transporter GLUT-4 (a sodium-independent facilitative transporter), to the plasma membrane significantly increasing glucose transport into the cell (Klip *et al.* 1996). A lack of insulin or defects within the insulin signaling pathway disrupts glucose uptake in mammals as is

seen in either insulin-dependent diabetes mellitus (IDDM) or non-insulin-dependent diabetes mellitus (NIDDM) in humans. When examining glucose intolerance in teleosts, a reasonable place to begin would be with circulating plasma insulin levels and insulin binding to its receptors.

1.3. Insulin in Fish

The teleost endocrine pancreas has an unique structure. It is present as an accumulation of endocrine cells adjacent to the gall bladder termed the principle islet or when this islet is surrounded by a rim of exocrine tissue it is known as a Brockmann body (Epple and Brinn 1987). The cell types found in fish endocrine pancreatic tissue are A, B, D and F cells (Epple and Brinn 1987). Functionally A, B, and D cells each secrete a specific hormone namely glucagon, insulin and somatostatin, respectively. Originally it was assumed that because fish were glucose intolerant that they were deficient in insulin, but with the development of homologous radioimmunoassays (RIAs) for a number of species, high levels of circulating insulin have been detected in many fish species including the American eel (1.1 ± 0.1ng/ml) and the rainbow trout (2.2 ± 0.1 ng/ml) (reviewed by Mommsen and Plisetskaya 1991). Insulin has been found in all fish examined to date, from hagfish and lampreys to tuna and species in between (Mommsen and Plisetskaya 1991). There is some debate, however, whether the high plasma insulin concentrations reported in most fish represent bioactive insulin, as some may be the inactive C-peptide that may be immunoreactive in the RIA (Plisetskaya 1997). It has been shown that amino acids, particularly lysine and arginine, are secretagogues of insulin and in fact are more potent secretagogues than glucose in eel (Ince and Thorpe 1977), trout (Mommsen and Plisetskaya 1991) and even the omnivorous catfish (Ronner and Scarpa, 1987).

Even though amino acids are more effective in promoting insulin release, insulin injected into fish is hypoglycemic. Leibson and Plisetskaya (1967) produced hypoglycemia in a number of cyclostomes and bony fish, including scorpion fish (*Scorpaena porcus* L.), sea bass (*Spicara smaris*), horse mackerel (*Trachurus mediterraneus ponticus*), goby (*Gobiidae sp.*) and carp (*Cyprinus carpio*) by injecting pig or ox insulin (30-80 IU/kg). In another study, Plisetskaya *et al.* (1985) reported fish insulin injected into coho salmon produced significant hypoglycemia. Lewander *et al.* (1976) injected European eels with insulin (100 IU/kg) that resulted in hypoglycemia 10 h after injection with a maximum hypoglycemia 96 h after administration. One hundred and sixty eight hours after injection, blood glucose levels were only slightly higher than controls. Liver glycogen was almost completely depleted between 24 and 96 h after insulin administration but showed some recovery by 168 and 336 h. Muscle glycogen fluctuated, showing a significant but transient increase by 168 h. Carneiro and Amaral (1982) injected bovine insulin (350 IU/kg) intraperitoneally into *Pimelodus maculatus*, a South American teleost, and reported maximal reduction of plasma glucose between 12 and 24 h after injection with restoration of plasma glucose levels by 72 h. In addition they found a marked depletion in liver glycogen. Ottolenghi *et al.* (1981) injected both fed and fasted (20 days) catfish (*Ictalurus melas*) with bovine insulin (60 IU/kg) and reported that there was a decrease in blood glucose levels in both groups although the hormone effect was more pronounced in the fed animals. In both fed and fasted animals a significant decrease in liver glycogen was detected while in red and white muscles insulin increased the glycogen levels of both fed and fasted animals. Ablett *et al.* (1981) injected rainbow trout with bovine insulin every 48 h for 56 days, at either a low (0.05-0.5 IU/ kg) or high dose (0.5-5.0 IU/kg). At day 57 plasma glucose values were 6.5% lower

than controls in the low dose group and 24.6% lower in the high dose group; however, in contrast to the previously mentioned studies, no significant change was found in either muscle or liver glycogen content. These data show glucose clearance from the plasma in response to insulin although the studies presented demonstrate that this response appears to be slow acting even with injection of a wide range of insulin doses (0.05-350 IU/ kg body weight). However, a definite hypoglycemia is seen with injection of insulin which supports a possible role for this hormone in the glucose uptake pathway in fish.

Islectomy or pancreactomy, the removal of islet tissue that secretes insulin, has been performed in a number of studies. Kelly (1993) islectomized the goby (*Gillichthys mirabilis*) in an attempt to induce IDDM. Sixteen days after islectomy, plasma glucose levels were 2.5-fold and 1.5-fold higher than intact or sham islectomized animals and 31 days after the experiment plasma glucose values were 3-fold higher than sham iselectomized animals. Islectomized gobies were injected with bovine insulin in three doses (0.01, 0.1, 1.0 U/kg) which reduced plasma glucose in a dose-dependent manner, reaching control plasma glucose values with the highest dose. Spontaneous diabetes in carp is called Sekoke disease and it has been reported that the β cells of the pancreatic islets of these fish are degranulated with more clear islet tissues (Nakamura *et al.* 1970). In contrast to these examples, pancreatectomy performed on eel (Lewis and Epple 1972) and river lamprey (*Lampetra fluviatilis*) (Hardisty 1975) did not show significant hyperglycemia from control or sham animals.

Binding studies have shown that insulin binds specifically to a receptor on both skeletal muscle and hepatocyte membranes of rainbow trout (Albett *et al.* 1983; Gutierrez and Plisetskaya 1990; Plisetskaya *et al.* 1993) and that the overall specific binding of insulin is

enhanced when trout were fed a high carbohydrate diet (Gutierrez *et al.* 1991). Segner *et al.* (1993) reported specific insulin binding in primary culture carp hepatocytes, as well Gutierrez *et al.* (1993) found specific receptors for insulin in carp ovaries. Banos *et al.* (1997) found that specific binding of insulin to red muscle of carp and trout were higher than reported values for white muscle. Parrizas *et al.* (1994) compared insulin binding and tyrosine kinase activity in three fish species with different nutrient preferences; the brown trout (*Salmo trutta fario*) and gilthead sea bream (*Sparus aurata*) which are more carnivorous than the carp or tilapia (*Tilapia mossambica*). They reported that the omnivorous carp and tilapia had more numerous insulin receptors with higher tyrosine kinase activity. These data agree with the findings of Furichi and Yone (1981) mentioned earlier that found carp had a higher glucose tolerance than either the carnivorous sea bream or yellowtail.

These studies illustrate that fish produce sufficient insulin, that insulin binds specifically to insulin receptors in a variety of tissues, and that insulin does produce hypoglycemia in at least some fish species. All of these facts seem to support a similar role of insulin in fish as in mammals.

1.4. Growth Promoting Functions of Insulin

Much of the research examining the effects of insulin in fish have focused on the possible metabolic role of this hormone. This may reveal an unconscious bias of researchers in which insulin's important and dramatic glycemic affects in humans are extrapolated to fish. In the human disease diabetes, lack of insulin in IDDM or a breakdown in the insulin/glucose transport signaling pathway, results in mild to severe health complications. This traditional view of insulin

in mammals has lead many researchers, including this one, to focus on the glucose uptake and metabolic functions of insulin when it may be that the primary role of insulin in fish is amino acid uptake and growth. Fish have a large protein demand which may represent a quantitative difference between fish and omnivorous mammals (Christiansen and Klungsoyr 1987). King and Kahn (1981) studied the relative potencies of several insulins on growth and metabolic activities in mammalian bioassay systems. They established that the bonito insulin tested was more potent as a growth factor than as a metabolic agent. In addition their data indicated that the growth promoting and metabolic activities of insulin are distinct and that the growth function is less affected by amino acid substitutions. Plisetskaya *et al.* (1991) reported that amino acids, and in particular arginine, have a strong insulinotropic effect in coho and chinook salmon and in rainbow trout. In addition, Ince and Thorpe (1977) reported that the initial rise in insulin was greater after the administration of the amino acids lysine and arginine than glucose at the same dose. Ronner and Scarpa (1987) report that glucose stimulated insulin release in channel catfish but that L-arginine did so to a larger extent. This indicates that even in omnivorous catfish amino acids are potent secretagogues. In a study on rainbow trout, Albett *et al.* (1981) reported that insulin has a growth promoting effect. ^{14}C-glucose and ^{14}C -glycine were used to look at *in vivo* metabolism in the northern pike (*Esox lucius*) (Ince and Thorpe 1976). They reported that the highest specific activity was found in liver glycogen, however, insulin injection caused a significant reduction in liver glycogen and protein while no significant changes were recorded in muscle glycogen. Insulin significantly stimulated the incorporation of ^{14}C-glucose carbon into both liver and muscle lipid and protein. In addition insulin significantly increased the incorporation of ^{14}C -glycine into muscle protein. Mommsen and Plisetskaya (1991) state that

increases in uptake of amino acids in the liver and skeletal muscle are "*much more pronounced and experimentally reproducible than insulin effects on hepatic glucose uptake or carbohydrate metabolism in general*" (p.239). Insulin's role in glucose metabolism in fish is interesting and a valid pursuit, but it may be that insulin in fish plays a more important role in amino acid uptake and growth.

1.5. Membrane Glucose Transporters

All animal tissues have the potential to metabolize glucose given the presence of the glycolytic pathway in all tissues, even if they utilize a non-carbohydrate substrate preferentially. Glucose is a hydrophilic molecule and so does not pass readily through the essentially lipid hydrophobic plasma membrane. To ensure glucose entry, most cells retain an energy-independent facilitated diffusion transport system, transporting glucose into and out of most animal tissues simply following concentration gradients (reviewed by Thomas *et al.* 1992; Ismail-Beigi 1993; Mueckler 1994; Thorens 1996).

Glucose transport in mammalian cells is facilitated by a family of membrane glucose transport proteins referred to as GLUTs (1-7). GLUTs 1-4 and 7 are facilitative diffusion glucose transporters while GLUT-5 is a fructose transporter expressed in the small intestine (Handberg 1996). GLUT-6 is a pseudogene that is not expressed at the protein level (Kayano *et al.* 1990). These glucose transporter isotypes are 50-60 kDa glycoproteins that contain 12 transmembrane-spanning domains, a large cytoplasmic loop, a long extracellular loop with a glycosylation site and the amino- and carboxy- terminals oriented toward the cytoplasm (Handberg 1996). Sequence identity between isotypes ranges from 40-65%, the greatest

divergence being at the extracellular loop and carboxy- and amino-tails (Handberg 1996). It has been established that at least two glucose transporters are present in muscle and in adipose tissues, the two insulin sensitive tissues (reviewed by Thomas *et al.* 1992; Muekler 1994; Klip *et al.* 1996; Ebeling *et al.* 1998). GLUT-1 has a wide tissue distribution in many fetal and adult tissues, including muscle and fat, and is responsible for providing basal glucose uptake (Muekler 1994). GLUT-4 is expressed in adipocytes, cardiac and skeletal muscles and is the insulin-responsive transporter (Birnbaum 1989, Fukumoto *et al.* 1989; James *et al.* 1989).

The mechanical activity and, therefore, energy requirements of muscles vary greatly. It follows that these muscles would need a glucose transport system that can respond to large variations in need for glucose (Elbrink *et al.* 1975). Intracellular levels of glucose are kept low in muscle through the phosphorylation of glucose by the enzyme hexokinase producing glucose-6-phosphate. Glucose is metabolized quickly in mammalian muscle suggesting that the transport of glucose is the rate limiting step in glucose utilization (Elbrink *et al.* 1975). Skeletal muscle is the primary tissue responsible for insulin-dependent glucose uptake in the fed state (approximately 75-95%). In the postprandial state, skeletal muscle is responsible for only about 20% of whole body glucose uptake, and insulin-mediated glucose uptake (IMGU) and non-insulin-mediated glucose uptake (NIMGU) each represent about half of this uptake (Baron *et al.* 1990). As concentrations of glucose in the blood rise after a glucose load, skeletal muscle becomes the organ that determines overall glucose uptake (Baron *et al.* 1990).

GLUT-1 is present on the plasma membrane of mammalian muscle cells where it provides glucose uptake required for basal cellular activity (Marette *et al.* 1992). As mentioned previously, it is the insulin-responsive transporter, GLUT-4 that is primarily responsible for

disposal of a glucose load. In the basal state (no large insulin release as seen after a meal) GLUT-4 is present primarily in a pool of intracellular vesicles that are recruited to the membrane surface in response to insulin (Phillips *et al.* 1996). The translocation of GLUT-4 from the intracellular to the cell membrane has been established as the main way that insulin stimulates glucose transport in both muscle and fat cells. Henriksen *et al.* (1990) reported that maximal insulin-stimulated transport was greatest in rat soleus muscle composed of >80% type I fibres (slow twitch oxidative) consistent with a higher content of GLUT-4 protein content than the epitrochlearis that consists of 65% type IIb fibres (fast twitch glycolytic) and a lower content of GLUT-4 protein.

The mechanism of insulin signal transduction which results in sequestering the intracellular pool of glucose transporters to the plasma membrane is complicated and pieces of this pathway are still unclear (reviewed by Thomas *et al.* 1992; Mueckler 1994; Stephens and Pilch 1995; Klip *et al.* 1996; Saltiel 1996). Upon insulin binding to its receptor, the receptor autophosphorylates critical tyrosine amino acids on the cytosolic beta subunit of the insulin receptor (Saltiel 1996). The binding of insulin to its receptor and subsequent autophosphorylation increases the activity of the receptor for a group of adapter molecules leading to increased phosphorylation of substrates including IRS-1 (insulin receptor substrate-1) (White *et al.* 1993). Upon tyrosine phosphorylation, IRS-1 associates with multiple intracellular signaling molecules including the phosphatidylinositol-3 kinase, Grb2 and SH2 domain containing tyrosine specific kinases (Waters *et al.* 1993). The initial tyrosine kinase activity of the insulin receptor is translated into downstream phosphorylations and dephosphorylations of specific proteins on serine and threonine residues. Eventually this signal is relayed to intracellular

membrane compartments containing GLUT-4 glucose transporters. When this signal is received, the vesicles move to the plasma membrane surface by a yet to be understood mechanism where the transporters are inserted into the plasma membrane by membrane-membrane fusion (Klip *et al.* 1996). Once in position, these additional GLUT-4s allow greater glucose flux across the membrane and glucose availability to the cellular metabolic machinery. In addition to insulin stimulation of GLUT-4 it was reported that contraction stimulates GLUT-4 translocation in skeletal muscle (reviewed by Cortright and Dohm 1997). Douen *et al.* (1990) reported that not only does contraction stimulate GLUT-4 translocation, but muscle contraction increased the number of GLUT-4 transporters in the plasma membrane without depleting the insulin-sensitive intracellular pool of transporters. These results suggest the existence of distinct insulin- and exercise-recruitable intracellular pools of GLUT-4.

1.6. Fish Glucose Transporters

Limited studies have investigated the presence of glucose transporters in various fish tissues and most of these studies examined glucose transport in the erythrocyte. Young *et al.* (1994) reported that glucose transport in the red blood cells of Pacific hagfish (*Eptatretus stouti*) was rapid and mediated by a saturable, stereospecific mechanism that is sensitive to cytochalasin B (CB) inhibition. Soengas and Moon (1994) examined glucose uptake in the American eel and reported a saturable, sodium- independent, stereospecific glucose transporter that was inhibited by CB. Tihonen and Nikinmaa (1991) looked at glucose transport in the erythrocyte of the river lamprey (*Lamptera fluviatilis*) and the carp (*Cyprinus carpio*) and reported saturation kinetics for the lamprey but not for the carp. A later study by Tihonen *et al.* (1995), however, reported

that glucose transport in the carp erythrocyte was inhibited by CB and phloretin and concluded that if a glucose transporter does exist it has an extremely low affinity for glucose as it was not saturated over physiological concentrations of glucose. Tse and Young (1990) reported a CB-sensitive saturable transporter in the common eel (Anguilla japonica) but not in the rainbow trout or the paddyfield eel (*Monopterus albus*).

Wright *et al.* (1997) performed western blot analysis on tilapia heart, brain, liver, and red and white muscle protein probing with mammalian GLUT-1 and GLUT-4 antibodies. They failed to report the presence of GLUT-4 protein in any of the tissues assayed, while GLUT-1 was detectable only in brain and heart. Northern blot analysis was performed on the above tissues using a GLUT-4 mammalian cDNA probe and again none was detected. Thus, from the limited studies undertaken to date, few fish tissues demonstrate classic mammalian facilitated glucose transporters.

1.7. GLUT-4 Overexpression and Ablation in Transgenic Mice

Recent studies looking at the overexpression of the GLUT-4 transporter have lent further support to the functional role of this transporter in mammalian skeletal muscle. Leturque *et al.* (1996) examined glucose tolerance and insulin action in transgenic mice overexpressing GLUT-4 protein by two-fold in skeletal muscle. They reported that plasma glucose concentrations were lower and plasma insulin concentrations were slightly lower in transgenic compared with age-matched control mice. Glucose disposal during an oral GTT was enhanced in transgenic mice overexpressing the GLUT-4 protein, while these mice secreted two-fold less insulin than controls. These mice were rendered diabetic through the injection of streptozotocin

(STZ) which selectively destroys pancreatic β-cells. Fed transgenic STZ-induced diabetic mice had significantly lower blood glucose levels than fed STZ-induced diabetic control mice. In addition insulin action was investigated by performing an insulin tolerance test (1.6 U/ kg body weight) and the effect of insulin was two-fold greater in transgenic STZ-induced diabetic rats than in control STZ-induced diabetic rats. These data show that physiological overexpression of GLUT-4 in skeletal muscle improves glucose tolerance and overall insulin action. Tsao *et al.* (1996) examined mice that overexpressed GLUT-4 exclusively in fast twitch muscle. Their conclusions complement those of Leturque *et al.* (1996) as transgenic mice showed a 3-4-fold increase in GLUT-4 protein in fast twitch muscle and insulin-stimulated glucose uptake increased 2.5-fold over control mice. In addition Tsao *et al.* (1996) found that insulin increased glucose uptake which resulted in increased glycogen content exclusively in fast twitch muscles. These studies emphasize the importance of the GLUT-4 glucose transporter in insulin-stimulated glucose disposal in mammals and its importance in regulating a glucose load.

Results from the overexpression of the GLUT-4 glucose transporter in skeletal muscle in mice have demonstrated the importance of GLUT-4 in clearing a glucose load and underscores its role in insulin-responsive glucose uptake. In a study reported by Katz. *et al.* (1995), the opposite situation was investigated as they disrupted the GLUT-4 gene in order to produce GLUT-4 null mice. No GLUT-4 mRNA or protein were detected in the heart or skeletal muscles of these mice, and there was no upregulation of GLUT-1 in skeletal muscles to compensate for the absence of GLUT-4. In GLUT-4 null mice, males showed a 34% decrease in fasting and a 20% increase in fed glycemia when compared with normal controls while blood glucose levels in females were not significantly different from controls in either the fed or fasted state. Fasting

insulin levels were unaltered in GLUT-4 null mice but fed insulin levels were 5-6 times higher than in control mice. Oral GTTs were administered and both male and female GLUT-4 null mice cleared glucose as efficiently as the normal controls, but demonstrated decreased sensitivity to insulin during an insulin tolerance test. Glucose homeostasis without the GLUT-4 glucose transporter was a surprising result and suggests that other mechanisms are compensating for the loss of the GLUT-4 transporter. As mentioned GLUT-1 was not upregulated in these muscles, but it was upregulated by 1.5-fold in heart, although this increase is thought unlikely to contribute to normal postprandial glycemia as the heart constitutes a small percentage of the insulin responsive tissue of the animal. GLUT-5 and GLUT-3 levels were assessed in skeletal muscle and were not seen to be higher than controls. Finally GLUT-2 was found to be overexpressed 1.7-fold in the liver, but the role of GLUT-2 overexpression in maintaining normoglycemia remains undetermined. It may be that under normoglycemic conditions, insulin reduces hepatic glucose production and that the increase in GLUT-2 seen in the liver contributes to hepatic glucose uptake and maintenance of normoglycemia in these GLUT-4 null mice. It was also proposed by the authors that a possible explanation is that another insulin-responsive glucose transport system had been activated in the muscle. A follow-up study by Stenbit *et al.* (1996) looked at the ability of muscles lacking GLUT-4 to take up deoxyglucose in the presence and absence of insulin in GLUT-4 null mice. This study examined glucose uptake and glycogen synthesis in soleus muscle (84% slow oxidative fibers) and extensor digitorum longus (EDL) fast twitch glycolytic-oxidative fibers. As a slight sexual dimorphism was seen in the study by Katz *et al.* (1995), males and females were examined separately. Glucose transport and glycogen synthesis were decreased in the EDL muscles of GLUT-4 null mice under both basal and insulin-

stimulated conditions. In the male soleus muscle basal glucose uptake was twice that observed in control males and glycogen accumulation was higher than in control, however there was no response to insulin. Glucose uptake in GLUT-4 null female mice soleus muscle was normal as compared to control mice. GLUT-4 null female soleus muscle did respond to insulin although this response was blunted in comparison with controls; however, uptake was 2 fold higher than basal and glycogen synthesis showed a 20% increase over basal levels. These results demonstrate that GLUT-4 is necessary in the EDL of mice for insulin-stimulated glucose uptake. This does not seem to be the case in the soleus muscle as basal uptake levels in the GLUT-4 null male are elevated but not responsive to insulin while the uptake levels in the female are similar to controls and are stimulated by insulin. The authors suggest two possible explanations . One, even though the expression of GLUT-1 in the muscles is not increased, there may be an increase in the 'efficiency' or the intrinsic ability of GLUT-1 to transport glucose across the plasma membrane. Two, there is an unknown transporter expressed exclusively in the red muscle of GLUT-4 null mice that is translocated to the membrane in response to insulin in female soleus muscle but does not respond to insulin in males. It may be that glucose homeostasis in the GLUT-4 null mice is maintained by a combination of these factors. These studies all point to the fact that the presence of GLUTs in mammalian skeletal muscle confers insulin-sensitive glucose transport, but the absence of GLUT-4 does not preclude insulin sensitivity of muscle transport.

1.8 Hexokinase

Hexokinases (HK) are a family of enzymes that have been designated hexokinase I-IV or A-D in mammals (Iynedjian 1993). Hexokinase IV is a low affinity hexokinase and has been

designated a glucokinase; this enzyme is usually restricted to the liver of mammals. Hexokinases all catalyze the reaction

$$\text{D-glucose} + \text{ATP} \Rightarrow \text{D-glucose-6-phosphate} + \text{ADP}$$

This enzyme is an important step in glucose transport, as it maintains a steep glucose concentration gradient across the membrane by keeping intracellular glucose concentrations low (Osawa *et al.* 1996). This enzyme is the first step in dealing with glucose once it has entered the cell, and one form of this enzyme (HK-IV or glucokinase) is thought to be a glucose sensor and key to some forms of diabetes in mammals (Grupe *et al.* 1995).

Tranulis *et al.* (1991) found HK activity in the liver of rainbow trout and reported that activities were unchanged after a 25 day fast. Borrebaek *et al.* (1993) reported the presence of a low affinity HK or glucokinase in salmon (*Salmo salar*) liver, the activity of which increased with high carbohydrate feeding. Trannulis *et al.* (1996) also report the presence of a gluocokinase in the liver of the Atlantic salmon; other labs, however, have failed to report a 'glucokinase' in any other fish liver. Fideau *et al.* (1983) reported that HK activities in the liver of rainbow trout was higher in trout fed a high carbohydrate diet than a high protein diet. Moon and Johnston (1981) examined hexokinase activities in the liver of plaice (*Pleuronectes platessa*) starved for 4 months and reported a decrease in HK activities. However, Sundby *et al.* (1991) reported that after starvation there was an increase in HK activities in salmon liver but a reduction in cod (*Gadus morhua*) liver. In addition they found that injection of human insulin in salmon enhanced HK activities.

Hexokinase has been reported to be present in many tissues of fish, including both red and white skeletal muscles of rainbow trout, cod and plaice (Knox *et al.* 1980). Hexokinase

activity was detected in lake whitefish (*Coregonus clupeaformis*) red and white muscles and an increase in activity was seen in white muscle of cold acclimated fish (Blier and Guderley 1988). In the white muscle of plaice starved for 4 months, Moon and Johnston (1981) reported a decline in HK activities while HK activities in the red muscle remained constant. At this step in the glucose uptake pathway in muscles, there does not appear to be a major difference between fish and mammals.

1.9. Non-Insulin-Dependent Diabetes Mellitus

A number of authors have drawn a parallel between carbohydrate metabolism in fish and the human pathological state of diabetes (NIDDM) (Falkmer 1961; Palmer and Ryman 1972; Kelly 1993). Patients with NIDDM are characterized by persistent fasting hyperglycemia in the presence of normal levels of insulin, mild insulinopenia or above normal levels of insulin associated with insulin resistance (National Diabetes Data Group, 1979). As mentioned previously, fish are generally considered to be glucose intolerant and show persistent hyperglycemia after a glucose load. Also as noted above, fish have sufficient to high levels of circulating insulin. The etiology of NIDDM is unclear, although it may lie somewhere in the insulin signaling pathway that eventually stimulates the translocation of glucose transporters to the cell surface (Cortright and Dohm 1997). Whether fish are truely a good NIDDM model requires greater knowledge of the basis of glucose intolerance in these organisms.

1.10. Hypothesis and Purpose of Study

Fish are considered to be glucose intolerant relative to mammalian omnivores. Early studies, however have looked primarily at carnivorous fish species (Falkmer 1961; Palmer and Ryman 1972; Mommsen and Plisetskaya 1991). As noted above, there are indications of differences in the extent of glucose intolerance in different species, including within carnivores (Furuichi and Yone 1981; Garcia-Gallego *et al.* 1995; Suarez *et al.* 1995). This study examines glucose tolerance and uptake in three species, the rainbow trout (*Oncorhynchus mykiss)*, American eel (*Anguilla rostrata)*, and black bullhead catfish (*Ameiurus melas)*. This allows comparison between species with different nutritional backgrounds (carnivorous vs. omnivorous). This study tests the hypothesis that the ability of fish to clear a glucose load is related to the nutrient preference of the fish. The prediction, therefore, is that the omnivorous catfish will have a higher glucose tolerance than the carnivorous trout, and the eel will have a glucose tolerance profile more similar to the catfish than the trout. In addition, skeletal muscle hexokinase activity and the expression of skeletal muscle glucose transporters will be higher in the omnivore (catfish) than in the carnivore (trout). White muscle in fish may constitute as much as 90% by weight of the fish (Christiansen and Klungsoyr 1987). This being the case glucose uptake and hexokinase activity were examined in this tissue as quantitatively it should play a large role in whole body glucose homeostasis. Elucidating this system and establishing possible contributing factors to glucose intolerance in fish may have repercussions for the study of glucose intolerance in NIDDM.

CHAPTER 2:
MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Experimental Animals

Rainbow trout, *Oncorhynkiss mykiss*, of either sex and weighing between 250-350 g were obtained from Linwood Acres Trout Farm (Cambellcraft, ON). Trout were maintained at 13°C and were fed five times a week with Floating Trout Feed Grower Pellets (Martin Mills). Immature American eels, *Anguilla rostrata*, weighing 300-500 g were obtained from the St. Lawrence River at Bainsville, Ontario. Eels were maintained at 13°C and were not fed for 6 months at the time of experiments. Experiments on feeding eels were performed on immature American eels weighing 250-300 g obtained from the South Shore Trading Co., Port Elgin, N.B. The New Brunswick eels were maintained at 22°C and were fed 5 times a week with Royal Lifetime extruded eel feed obtained from South Shore. Black bullhead catfish, *Ameiurus melas*, of either sex and weighing 150-200 g, were obtained from Willow Pond Aqua Farm, Inc. (Canadaigua, NY). Catfish were maintained at 13°C and were fed five times a week with AquaMax fish diet for omnivorous fish (Purina Mills). Crude protein content of the fish food was 40% for trout, 36% for catfish and 46% for eel. An estimate of crude carbohydrate content of the fish food is 27% for trout, 35% for catfish and 20% (ash and carbohydrate) for eel. All animals were transported to the University of Ottawa aquatic care facility and were maintained in tanks of well aerated, dechloraminated City of Ottawa tap water at a constant 12L:12D photoperiod.

2.2. Surgical Procedures

2.2.1. Rainbow Trout

Trout were cannulated through the dorsal aorta using the methods of Soivo *et al.* (1975). Fish were lightly anaesthetized using 0.1% 3-aminobenzoic acid ethyl ester (MS-222) neutralized with an equal weight of sodium bicarbonate. A hole was pierced through the snout and a small piece of PE 160 (Clay Adams) tubing, heat-flared at the end, was threaded through the hole with the flared end against the roof of the mouth. Fish were then placed on an operating table ventral side up where their gills were continuously irrigated with 0.1% MS-222, oxygenated water. A sharpened guide wire was inserted into a 20 cm piece of PE-50 (Clay Adams; ID = 1.14mm ; OD = 0.965mm) tubing. The tip of the wire and cannula was inserted through the roof of the mouth at the level of the first gill arch until blood moved up the cannula. The cannula was then rinsed with heparinized (1mg/ml) Cortland saline (in mM: 124 NaCl, 5.1 KCl, 1.6 $CaCl_2$, 0.9 $MgSO_4$, 11.9 $NaHCO_3$, 3 NaH_2PO_4) and fitted to an 80 cm piece of PE 50 tubing using a 23 gauge needle. The PE 50 tubing was then threaded through the PE 160 tubing in the snout and the tubing remaining in the mouth was stitched to the roof of the mouth with 2-0 surgical silk. This cannula was used for blood sampling and injection of glucose. It remained patent for at least 2 days and was frequently rinsed with heparinized saline to ensure clots did not block the cannula opening. Fish were allowed to recover for at least 24 h before experimentation.

2.2.2. Bullhead Catfish

Catfish were cannulated through both the caudal vein and artery using the technique of Axelsson and Fritsche (1994). Fish were lightly anaesthetized with 0.01 % MS-222 neutralized with sodium bicarbonate. Fish were placed on an operating table while fully oxygenated, anesthetic water was pumped over the gills. An incision was made in the caudal region along the lateral line. The muscle and connective tissue were cleared from the incision with a Q-tip or blunt probe until 2-3 vertebral spines were visible. The caudal vein could be seen at this point, while the artery lay behind the vein and was cannulated blindly. A small hole was made in the vessel using a 23 gauge needle and an 80 cm piece of PE 50 tubing was quickly inserted into the hole and advanced into the vessel as far as possible (approximately 10 cm or 1/3 of the fish length). After both cannulae were in place, the incision was stitched closed with 2-0 surgical silk. The caudal vein cannula was used for injection of glucose while that in the caudal artery was used for blood sampling. Catfish were allowed to recover for 24 h before experimentation.

2.2.3. American Eel

Eels were cannulated through the caudal artery and vein using the techniques described above for the catfish. Eels were lightly anaesthetized with 0.1% MS-222 neutralized with sodium bicarbonate. Eels were placed on an operating table exposed to air and an incision was made in the caudal region along the lateral line about 10 cm from the tip of the tail. Muscle and connective tissue were cleared with a Q-tip or blunt probe until a minimum of 2 vertebral spines were visible. These spines were broken and removed and the caudal vein and artery were exposed. The vessel to be cannulated was cleared and freed from all adhering tissue and isolated

by slipping a pair of small bent forceps under it. A small hole was cut into the vessel using micro scissors and an 80 cm piece of PE 50 tubing was inserted into both the artery and the vein and was threaded along 1/3 of the fish length and secured with 2-0 surgical silk. Both vessels were tied posteriorly to the placement of the cannulae and the incision was closed with surgical silk. The cannulae were secured by stitching them to the skin. The caudal vein cannula was used for injection of glucose while the caudal artery cannula was used for blood sampling. Eels were allowed to recover for 24 h before experimentation.

2.3. Glucose Tolerance Test

Glucose tolerance tests were performed on trout, catfish, fasting St. Lawrence River eels and feeding New Brunswick eels.

All fish were allowed to recover from surgery for at least 24 h in a black Perspex box. At the beginning of an experiment, a 200 µl blood sample was taken to establish baseline glucose and hematocrit levels and in some cases, insulin concentrations. From this sample, 100 µl of whole blood was stored with benzamidine (10 mM final concentration) for insulin analysis and 20 µl was used to measure hematocrit. Hematocrit was measured using heparinized 20 µl hematocrit tubes centrifuged in an International Clinical centrifuge (Needham, MA) at 5000 x g; percent packed cells was determined using a Micro-hematocrit capillary tube reader. The remaining 80 µl was centrifuged for 1 min in an Eppendorf microfuge (5415 C) at 15000 x g and the plasma was removed from the red blood cells to measure glucose concentration. The red blood cells were gently resuspended in 80 µl of Cortland saline and reinjected into the animal. The samples for insulin and glucose analysis where snap frozen in liquid nitrogen and stored at -80°C until

assayed. Fish were then each given a bolus injection of glucose, 250 mg glucose per kg fish. Blood samples were taken at 1, 5, 10, 15, 30, 60, 120, 360 min and 24 h (1400min) after injection to measure plasma glucose and insulin concentrations in eel and trout and plasma glucose in catfish. Hematocrit values were also checked at 360 min.

Controls for all three species were run using the above protocol but replacing the bolus injection of glucose with an equivalent injection of Cortland saline. Blood samples were taken to measure plasma glucose only over a 6 h period.

2.4. Glucose and Insulin Assays

Plasma glucose was assessed using the glucose oxidase assay available as a commercial kit (GOD PAP, Bohringer Mannheim). Briefly, plasma was diluted 10 times with double distilled water. Twenty µl samples placed in individual wells of a 96 well plate and 200 µl of GOD PAP reagent was added. Samples were run in triplicate. Each plate was run with a D-glucose standard curve and plates were read on a Packard Spectracount™ at a wavelength of 510 nm. Data were analyzed using the Microplate reader 2.01 I-Smart Software.

Plasma insulin samples from trout were assayed by Dr. Erika Plisetskaya (University of Washington, Seattle) using a homologous salmonid radioimmunoassay (Plisetskaya *et al.* 1986). Eel plasma insulin was measured by a heterologous RIA by Dr. Isabel Navarro (University of Barcelona, Spain) (Gutierrez *et al.* 1984).

2.5. Hexokinase assay

Randomly selected trout and catfish were killed with a swift blow to the head while New Brunswick feeding eel were killed by decapitation. The skin was removed, and a 5 g piece of white muscle was removed from the anterior epaxial region. White muscle was homogenized in a buffer consisting of 50 mM imidazole, 15 mM β-mercaptoethanol, 5 mM EDTA and 1-2 crystals of phenylmethylsulphonyl fluoride (PMSF) at a 1:1 (w/v) dilution using a Kinematica (Kriens-Luzern, Switzerland) homogenizer. Homogenization was carried out on ice at a setting of 5 for 10 sec, twice. The homogenate was centrifuged in an Eppendorf microfuge (5415 C) for 15 min at 15000 xg and the supernatant was used directly for assay.

Hexokinase was assayed using a buffered salt medium (50 mM imidazole, 7.5 mM $MgCl_2$, 2.0 mM KCl) to which was added an ATP regenerating system (10 mM creatine phosphate, 0.9 IU/ml creatine phosphokinase), 0.5 mM $NADP^+$, 2.5 mM ATP, and 0.5 U glucose-6-phosphate dehydrogenase in a 1.0 ml total volume. Homogenate (10 µl) was added to this mixture and cuvettes were inverted several times and allowed to stand for 5 minutes to remove endogenous glucose. Glucose was then added in concentrations between 0 and 100 mM. Increases in NADPH production were followed at a wavelength of 340 nm using the kinetics program on a Beckman DU-65. Background activity (a sample was measured adding only homogenate without the addition of glucose) was subtracted from activities in the presence of glucose. Activities were converted to µmoles NADPH/min by using the extinction coefficient for NADPH (6.22 cm^2/µmole) .

2.6. Northern and Western Blots

2.6.1. RNA extraction

Randomly selected trout and catfish were killed with a blow to the head. Feeding eels from New Brunswick were killed by decapitation. A piece of epaxial white muscle was removed from just below the dorsal fin. Total RNA was isolated from 1.5 g of white muscle using TriPure™ Isolation Reagent (Boehringer Mannheim) and the RNA was resuspended in diethylpyrocarbonate (DEPC)-treated RNase free water. Total RNA was isolated from 5 eels, 5 trout and 5 catfish. A 10 μg sample of RNA from each fish was then centrifuged in a Savant Speed Vac (Sc110) until completely dry. RNA was boiled to denature and stored on ice.

Northern (mRNA) and western (protein) blots were performed at the University of Waterloo in the lab of Dr. Arend Bonen with the assistance of Xiao Xia Han and Yoga Arumugan. Both northern and western blots were run using the techniques routinely used in the Bonen laboratory as outlined in Wright (1997). These techniques are briefly outlined below.

2.6.2 Northern Blot

Ten μg total RNA was loaded onto an agarose gel and subjected to electrophoresis (100 V for 3 h) to separate the RNA for northern blot analysis. The gel was loaded with marker RNA, tRNA, heart mix (rat RNA) and soleus muscle (rat RNA). The RNA in the agarose gel was then transferred to a nitrocellulose membrane through capillary action, by laying the membrane on the gel and layering filter paper and paper towels on top. Salt of sodium citrate (SSC, 10x) buffer moves through the gel and the membrane to the filter paper and transfers the RNA from the gel

to the membrane. RNA on the membrane was stained with methylene blue to visualize 28S and 18S ribosomal bands. The nitrocellulose membrane was incubated with a ^{32}P-2`-deoxy-cytidine-5`-triphosphate cDNA GLUT-4 or GLUT 1 probe (American Type Tissue Collection) at 42 °C for 12 h. The membrane was washed with 10x SSC and placed in an autoradiographic cassette with Kodak autoradiographic film at -80°C for 12 h. The film was processed per the manufactures recipes.

2.6.3. Western Blot

A piece of white muscle taken from the epaxial region below the dorsal fin from 3 individual trout, 3 eels and 3 catfish was 'snap' frozen in liquid nitrogen and stored at -80°C. Sixty mg of this white muscle was used for western blot analysis. Samples were prepared by homogenization using a Kinematica (Kriens-Luzern, Switzerland) homogenizer on ice at a setting of 5 for 10 sec, twice in buffer (in mM: 30 Hepes, pH 7.4, 210 sucrose, 2 EGTA, 40 NaCl, and 2 phenylmethylsulphonyl fluoride) followed by mixing with 3 ml of 1.167 M KCl and 58.3 mM pyrophosphate. The total membrane fraction was recovered by centrifugation at 256,000 xg in a Beckman L8-70M ultracentrifuge (SW 41 rotor) for 75 min at 4°C. The supernatant was discarded and the pellet was homogenized a second time with buffer and centrifuged in an Eppendorf microfuge (5415 C) at 3000 RPM and the supernatant was used for western blot analysis. Ten µl of supernatant was used for determination of protein content by the Bicinchoninic Acid (BCA) method (Pierce Chemical Co., IL).

Fifty µg of protein was loaded onto a 10% Sodium Dodecyl Sulfate-polyacrylamide gel and was electrophoresed for 1 h at 150 V. The protein was transferred from the gel to a nitrocellulose membrane by laying the membrane on the gel in transfer buffer (methyl glycine in

double distilled water) for 1 h at 110 V. This membrane was coated with 10% milk powder and incubated overnight. The milk powder was removed and the membrane was incubated with GLUT-4 or GLUT-1 commercially available antibodies (East Acres Biological) in 10% milk powder. The membrane was washed with 3x TBST (Tris-NaCl and tween 20) and was incubated with horseradish peroxidase-labelled anti-rabbit IgG (Amersham, Oakville, ON). An enhanced chemiluminescense detection system (Amersham, Oakville, ON) was used to view GLUT-4 and GLUT-1 proteins. The membrane was then exposed at - 80°C in an autoradiograph cassette with Kodak film for 12 h and developed as above.

2.7. Muscle Glucose Transport

Glucose transport was determined in membrane vesicles isolated from skeletal muscle of the 3 fish species using radioactive glucose.

2.7.1. Membrane vesicle isolation

Vesicles were isolated from the white muscle of trout, New Brunswick feeding eels and catfish. Trout and catfish were randomly selected and killed with a swift blow to the head. Eels were randomly selected and decapitated. Vesicles were isolated from white muscle using a technique based upon that previously published by Standen *et al.* (1984), Burton *et al.* (1988), Juel (1991), Ploug *et al.* (1993), Bonen *et al.* (1998). Forty to fifty g of white muscle was removed from the anterior epaxial region and immersed in a buffer consisting of 140 mM KCl and 5 mM MOPS adjusted to pH 7.4. A razor blade was used to make cuts parallel to the myosepta. The pieces of muscle were transferred to a flask containing 40 ml of a collagenase medium, consisting of 400 U/ml collagenase (Type IV hepatocyte collagenase, Sigma, St. Louis,

MO) and 0.025 mM $CaCl_2$. Type IV collagenase was chosen after testing the effectiveness of other types (Type II and Type IA collagenase from Sigma and Type 4 from Worthington). The flask was shaken gently in a water bath at 34°C for 1 h. The collagenase solution was pipetted into a beaker and the muscle pieces were washed with 20 ml of 10 mM EDTA in 140 mM KCl and 5 mM MOPS pH, 7.4 and again this solution was pipetted into the beaker. This suspension was centrifuged at 100 xg for 10 min in a Sorvall GLC-2 centrifuge at room temperature and the supernatant was removed and combined with Percoll (Sigma, St. Louis, MO) to give a final Percoll concentration of 24%. A three layer density gradient was created in a 15 ml conical plastic tube using 8 ml Percoll/vesicle suspension, 3 mls 4% Nycodenz (Sigma, St. Louis, MO) in 140 mM KCl and 5 mM MOPS buffer pH 7.4, and finally topped with 1 ml of 140 mM KCl and 5 mM MOPS. The density gradient was centrifuged at 1000 xg for 45 min in a Sorvall GLC-2 centrifuge at room temperature. Vesicles floated through the Percoll and Nycodenz layers and accumulated in a cloudy layer at the Nycodenz- KCl/MOPS (top layer) interface. The fat globules that collected on top of the buffer layer were removed by aspiration. The cloudy layer containing the vesicles was carefully removed using a Pasteur pipette and centrifuged at 4500 xg for 30 min in a Sorvall RC SB Plus (SS 34 rotor) at 4°C. The supernatant was carefully removed by aspiration and the pellet was resuspended in 300-400 µl of the KCl/MOPS buffer. Twenty µl of this vesicle suspension was removed for BCA (bicinchoninic acid) protein analysis (Pierce). Protein samples were assayed with a standard curve of bovine serum albumen and were read at a wavelength of 562 nm on a Beckman DU-65. The vesicle suspension was diluted to 1 µg protein/µl with 140 mM KCl/5 mM MOPS, pH 7.4.

Muscle vesicles from all three species were viewed using an Axiophot photomicoscope (Zeiss, West Germany) and were photographed using a Hamamatsu chilled CCD camera (Hamamatsu photonics, Japan). The trout and eel vesicles were photographed using phase contrast microscopy while catfish vesicles were photographed using bright field microscopy. Image analysis was performed using the Metamorph imaging system version 3.0 (Universal Imaging Corp.) and images were printed from Corel Draw (version 8.0).

2.7.2. Glucose Transport

Fifty µl of vesicle suspension containing 50 µg of protein was placed in a plastic 1.5 ml centrifuge tube. Increasing amounts of D-glucose were added so that the final concentration varied from 0 to 18.75 mM D-glucose. Mannitol was used in a reciprocal manner so that the total concentration of sugar was always 18.75 mM. This was mixed with 0.5 µCi D-[6-^{3}H] glucose specific activity 28 Ci/mmol , (Amersham, Oakville, ON) and 0.1 µCi L-[1-^{14}C] glucose specific activity 58 mCi/mmol (Amersham, Oakville, ON) The specific activities were equalized between ^{3}H and ^{14}C-glucose by adding 1.92×10^{-6} mM cold D-glucose. This mixture was vortexed and was incubated for 60 sec at which time 1 ml of ice cold 'stop' buffer (200 µM phloretin in KCl/MOPS buffer, pH 7.4, 1% BSA) was added to the tube and the tube was immediately centrifuged at 15000 xg in an Eppendorf microfuge (5415 C) for 2 min at room temperature. The supernatant was removed by aspiration and 1 ml of stop buffer was again added to rinse the pellet and immediately removed by aspiration. The sides of the tube were dried using an extra low lint Kimwipe™ and the tip of the centrifuge tube containing the pellet was cut into a 10 ml plastic scintillation vial. Scintillation cocktail (2.5 mls, ASC II; Amersham) was added and these

tubes were shaken in the dark for 12 h and then counted on a Beckman LS 6500 scintillation counter using the double label counting program for 1 h.

In some experiments the ability of cytochalasin B (20 μM final concentration) and phloretin (200 μM final concentration) to inhibit D-glucose uptake was assessed. Experiments were performed under the exact conditions of the glucose transport experiments except vesicles were incubated with inhibitor for 10 min before transport experiments were initiated.

Counting efficiencies were assessed by counting samples, comparable in cpm to amounts being counted in the experimental vials, of pure D-[^{3}H]glucose and L-[^{14}C]glucose separately and then together to check for spill-over correction. These results indicated that the automatic correction feature on the L56500 provided accurate measurements so the automatic counting feature was routinely employed.

2.8. Rate of Glucose Turnover in Trout

The rate of disappearance of glucose (Rd), rate of appearance of glucose (Ra) and glucose turnover were calculated for rainbow trout employing the continuous tracer infusion method routinely used in J-M. Webers laboratory (see Haman and Weber, 1996).

2.8.1. Double Dorsal Aortic Cannulation

Using the continuous infusion tracer method requires the placement of two cannulae. Cannulae are placed using the methods of Soivo *et al.* (1975) (as described above for single dorsal aortic cannulation) with modifications presented by Haman and Weber (1996). Trout were prepared for insertion of both cannulae as in the surgical procedures for implantation of a

single cannula described above, with the exception that two holes are pierced through the snout. Briefly the first cannula is implanted between the third gill arch and is advanced 8-10 cm caudally into the dorsal aorta. This cannula is used for infusion of radioactive glucose. The second cannula is implanted between the first gill arch and is only advanced a few cm. This cannula is used for blood sampling. Both cannulae are stitched to the roof of the mouth and threaded separately through the snout openings. Trout were then allowed to recover for 24 h in a black Perspex box before experimentation.

2.8.2. Continuous tracer infusion

[^{3}H]-D-glucose (specific activity 28 Ci/mmol) (Amersham) was infused into the first cannulae with a calibrated syringe pump at 1 ml/h. The infusion was started with a priming dose of [^{3}H]-D-glucose infusate equivalent to 90 min of infusion before starting the pump. Infusate was prepared daily by drying radiolabelled glucose under nitrogen and then dissolving it in Cortland saline. A portion of this infusate was counted in order to determine exact infusion activity rate.

One hundred and fifty µl blood samples were taken after 40, 50 and 60 min of infusion. After the 60 min blood sample was taken, a bolus of 250 mg glucose/ kg fish was injected through the sampling cannulae. Blood samples were then taken after 61, 65, 70, 75, 90, 120, 180, 420 min after infusion. Each sample was centrifuged in an Eppendorf microfuge (5415 C) and the plasma was removed. Twenty µl of plasma was used to measure glucose concentration using the commercial GOD PAP kit as mentioned above. Glucose activity was measured in 10 µl of plasma. The plasma was pipetted into a plastic scintillation vial, dried under N_2 and 3 ml of

ASC II scintillation cocktail was added. Vials were counted for 3 min in the Tri-Carb 2500 scintillation counter (Packard) with external quench correction. Glucose turnover, Ra and Rd for times 40, 50 and 60 were calculated using the steady state equation while Ra and Rd for times 61, 65, 70, 75, 90, 120, 180, 420 min were calculated using the Steele (1959) non-steady-state equation.

2.9. Statistics

Graphs were constructed using Sigma Plot 3.0 software (Jandel Corp.). Data are expressed as means ± SEM of (n) independent experiments. Statistical analyses were undertaken using SigmaStat 2.1 software (Jandel Corp.).

For the glucose tolerance experiments, baseline (control) glucose concentrations were compared to each plasma glucose concentration after injection of glucose to test for significant differences (repeated measures one way analysis of variance, ANOVA, Dunnetts method). Insulin plasma concentrations were also analyzed in this manner.

Hexokinase activities were plotted as Eadie Hofstee plots (v vs. v/s) in order to determine Km and Vmax by linear regression. Glucose transport activities were plotted using a Lineweaver-Burke plot (1/v vs. 1/s) in order to calculate Km and Vmax by linear regression analysis for each individual fish. Average Km and Vmax values were calculated from these individual values, and compared with one way ANOVA

CHAPTER 3:
RESULTS

3. RESULTS

3.1. Glucose Tolerance Tests

Trout, catfish, feeding and fasting eel were each challenged to a glucose tolerance test. A bolus of 250 mg D-glucose/kg fish was injected and blood samples were taken over time. The blood sample taken at time 0 before glucose injection was used to establish baseline plasma glucose and insulin values.

Rainbow trout given a bolus glucose injection increase plasma glucose values from 6.2 to 21.9 μmoles/ml by 1 min after injection (Fig. 3-1 A). Values slowly decrease over time although remain significantly above baseline 360 min after injection ($p<0.05$). Plasma glucose values are no longer significantly different by 24 h (1440 min). Plasma insulin concentrations also peak by 1 min after injection of glucose and then decrease over time (Fig 3-1 A). However, plasma insulin values are not significantly different from baseline at any time after glucose injection

Plasma glucose and insulin concentrations for the 6 month fasting American eel before and after a bolus injection of glucose are shown in Figure 3-1 B. After a bolus injection, plasma glucose levels increase from 13.3 to 34 0 μmoles/ml by 1 min and then decrease slowly over time. Values remain significantly elevated ($p<0.05$) from 1 to 360 min after injection compared with pre-injection values. At 24 h after injection, plasma glucose values are significantly below baseline. Plasma insulin levels follow glucose changes, peaking 1 min after injection of the glucose bolus and decreasing over time. Again these values were not significantly different from baseline.

The response of fed American eels to a bolus glucose injection is shown in Figure 3-1 C. Again plasma glucose values peak 1 min after injection, increasing from 9.2 to 32.6 μmoles/ml.

Figure 3-1 A: Plasma glucose (closed circles) and insulin (closed squares) concentrations in rainbow trout injected with 250 mg D-glucose/kg body weight. Time 0 concentrations indicate baseline (pre-injection) values. All values are means ± SEM of n = 6 independent experiments. All plasma glucose concentrations were significantly different from baseline ($p<0.05$), except at 24 h (1440 min) after injection of glucose (repeated measures ANOVA, Dunnetts method). The increase in plasma insulin after injection was not significantly different from baseline (repeated measures ANOVA).

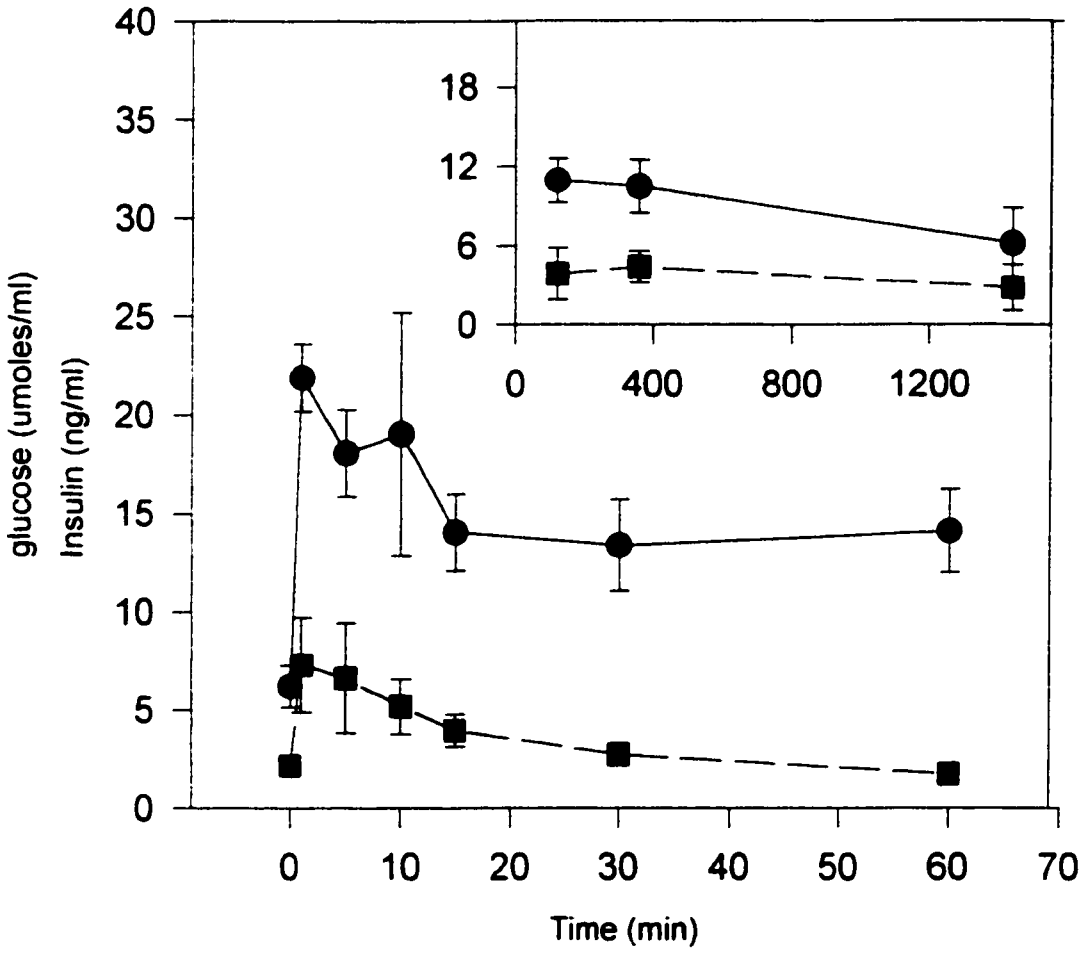

glucose (umoles/ml)
Insulin (ng/ml)
Time (min)
0
5
10
15
20
25
30
35
40
0
10
20
30
40
50
60
70
0
6
12
18
0
400
800
1200

Figure 3-1 B: Plasma glucose (closed circles) and insulin (closed squares) concentrations in 6 month fasted American eels injected with 250 mg D-glucose/kg body weight. Time 0 concentrations indicate baseline (pre-injection) values. All values are means ± SEM of n = 5 independent experiments. All plasma glucose concentrations were significantly different from baseline (repeated measures ANOVA, Dunnetts method, $p<0.05$). The increase in plasma insulin after injection was not significantly different from baseline (repeated measures ANOVA).

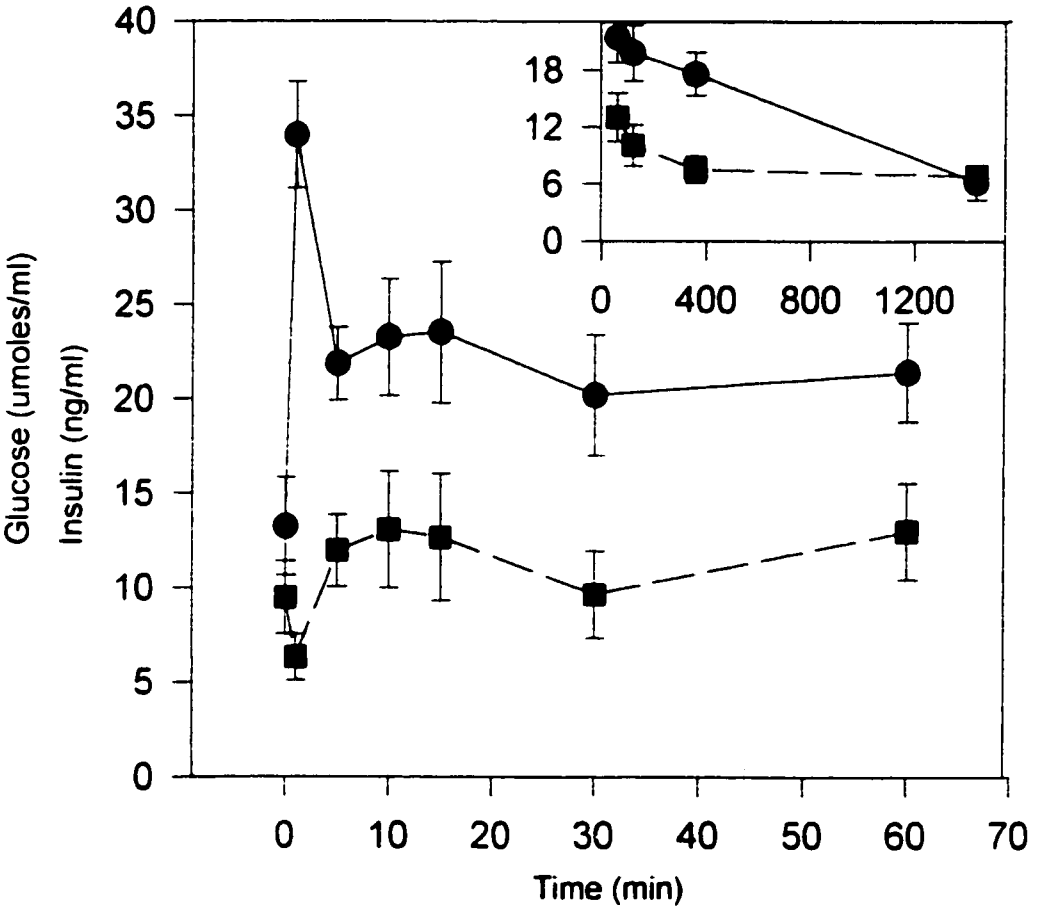

Glucose (umoles/ml)
Insulin (ng/ml)
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
Time (min)
18
12
6
0
0
400
800
1200

Figure 3-1 C: Plasma glucose (closed circles) concentrations in fed American eels injected with 250 mg D-glucose/kg body weight. Time 0 concentrations indicate baseline (pre-injection) values. All values are means ± SEM of n = 6 independent experiments. Plasma glucose concentrations were not significantly different from baseline 60 min after injection of glucose (repeated measures ANOVA, Dunnetts method).

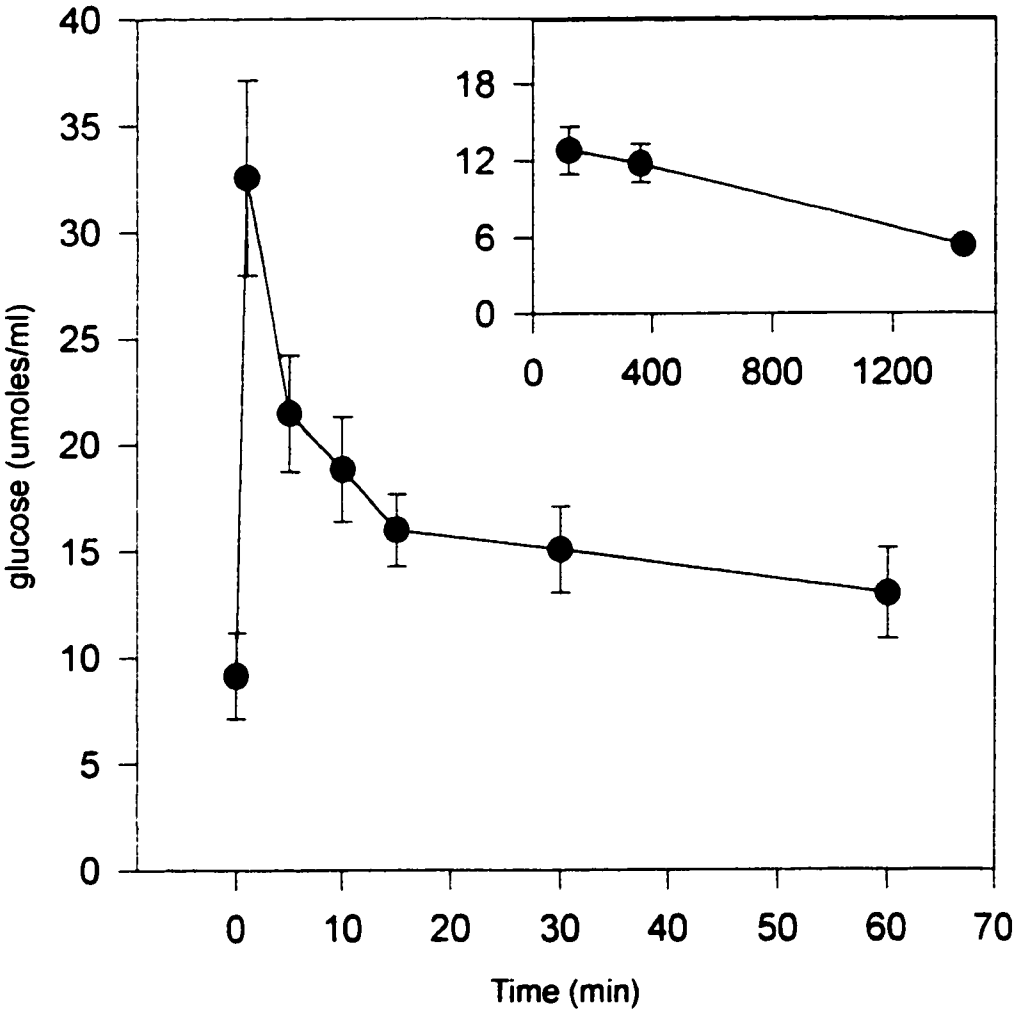
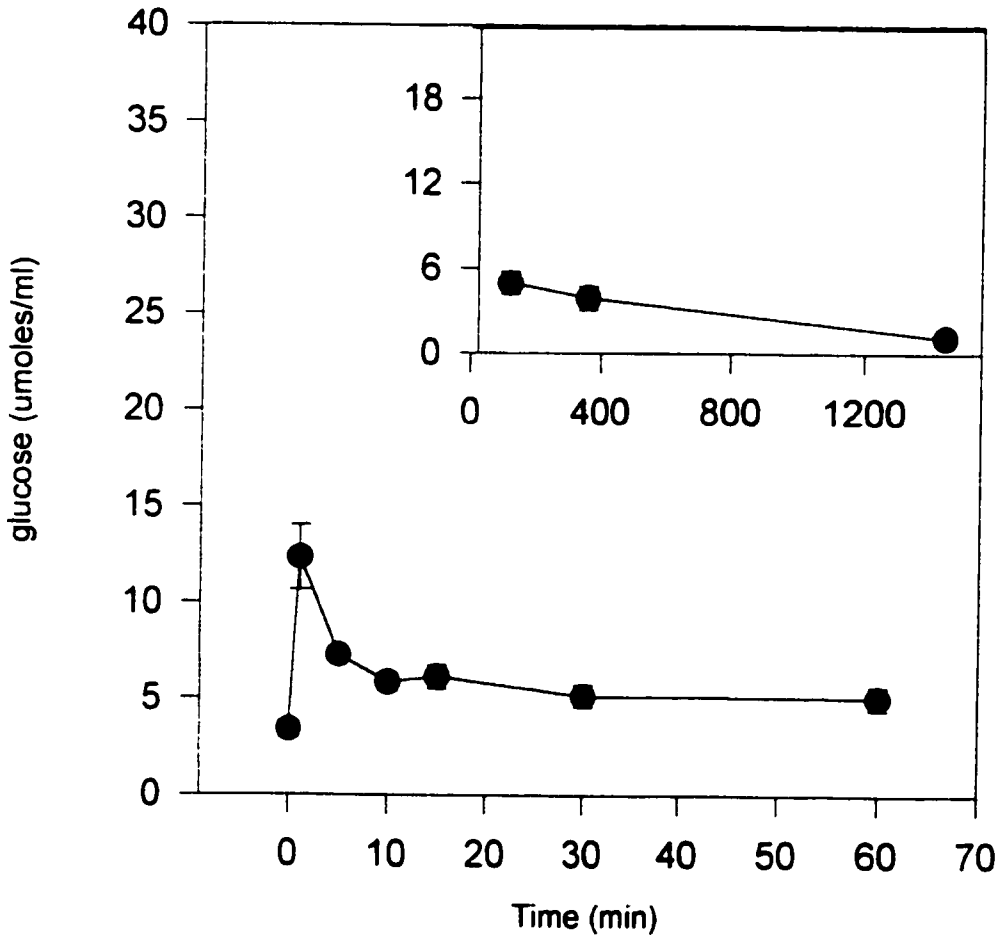

40
35
30
25
20
15
10
5
0
glucose (umoles/ml)
0
10
20
30
40
50
60
70
Time (min)
18
12
6
0
0
400
800
1200

Figure 3-1 D: Plasma glucose (closed circles) bullhead catfish injected with 250 mg D-glucose/kg body weight. Time 0 concentrations indicate baseline (pre-injection) values. All values are means ± SEM of n = 6 independent experiments. Plasma glucose concentrations were not significantly different from baseline 30 min after injection of glucose (repeated measures ANOVA, Dunnetts method).


glucose (umoles/ml)
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
Time (min)
18
12
6
0
0
400
800
1200

Values remain significantly elevated ($p<0.05$) 30 min after injection; however, by 60 min post-injection, plasma glucose values are not significantly different from baseline. Insulin concentrations were, unfortunately not evaluated in this group of eels.

Plasma glucose concentrations for bullhead catfish before and after injection of a bolus of glucose are reported in Figure 3-1 D. After injection plasma glucose values peak by 1 min, increasing from 3.4 to 12.3 μmoles/ml. After injection, values remain significantly elevated for 10 min. Plasma glucose values are not significantly different from baseline at 30 min after injection. Catfish cleared a bolus of glucose from the plasma much faster (10-30 min) than trout (6-24 h) and fasting eel (6-24 h) and slightly faster than feeding eel (30-60 min).

Plasma glucose concentrations for trout, catfish and fasted American eel after injection of a bolus of saline without glucose are shown in Figure 3-1 E. Experiments followed the procedures of the glucose tolerance tests exactly except the bolus injection did not contain glucose. Plasma glucose values are not significantly elevated after saline injection. Eel plasma glucose values are over 3 times higher than trout and 5 times higher than catfish plasma glucose values. Hematocrit levels did not change for any of the species throughout the experiments.

3.2. Glucose Turnover in Rainbow Trout

The rate of appearance of glucose (Ra) and the rate of disappearance of glucose (Rd) were estimated using the continuous infusion tracer method in the rainbow trout. Values for Ra and Rd were obtained before and after injection of a bolus (250 mg/kg) of cold D-glucose. Table 3-2 lists Ra, Rd, and plasma glucose concentration at time 40, 50 and 60 min of infusion, after measurement of Ra and Rd at 60 min the bolus of glucose was administered. After injection of the bolus of glucose, Rd values increase dramatically followed by a general decrease. This

Fig 3-1 E: Plasma glucose concentrations for rainbow trout (closed circles), bullhead catfish (closed triangles) and fasted American eel (closed squares) injected with Cortland saline after time 0. Experiments followed the identical protocol as the glucose tolerance experiments except glucose was absent from the saline. All values are means ± SEM of n = 3 independent experiments. There were no significant differences in plasma glucose concentration after injection of saline (repeated measures ANOVA, Dunnetts method).

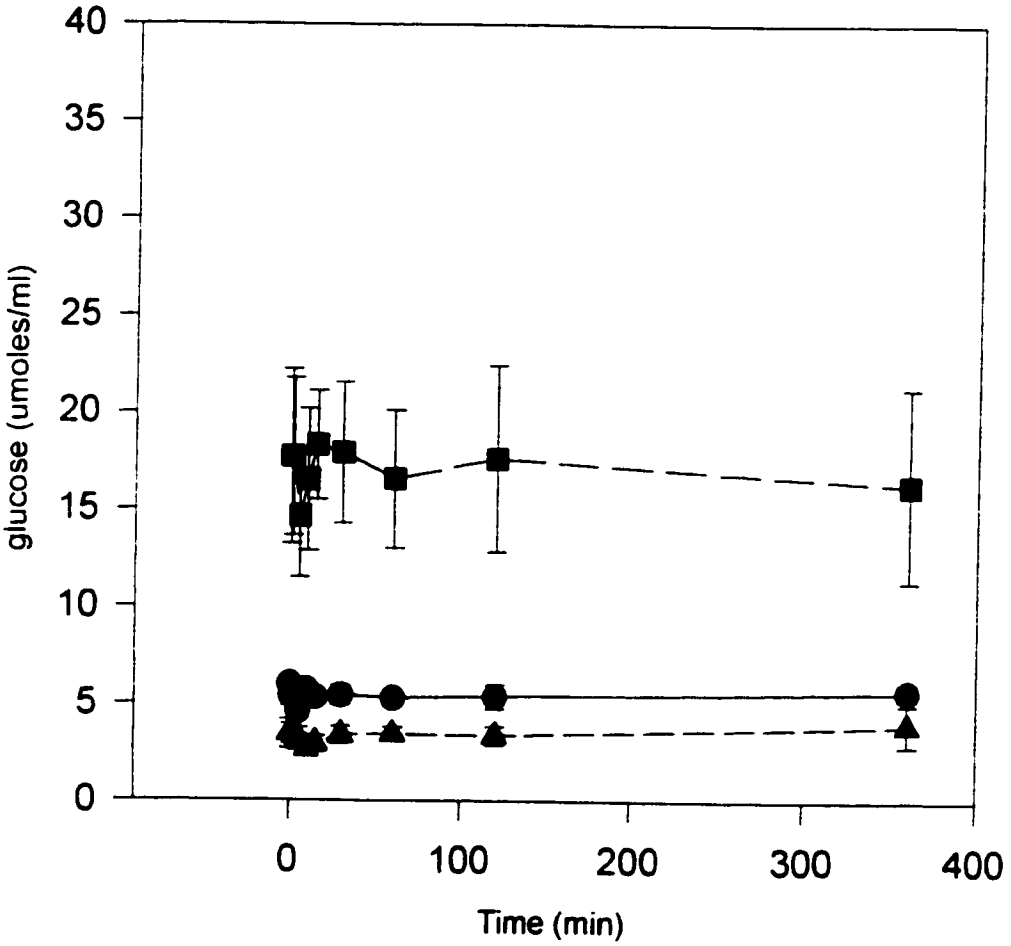

40
35
30
25
20
15
10
5
0
glucose (umoles/ml)
0
100
200
300
400
Time (min)

Table 3-2: Mean plasma glucose values, rates of disappearance (Rd) and rates of appearance (Ra) of glucose in rainbow trout infused with [^{3}H-D] glucose. The time represents time of infusion and a bolus of 250 mg cold glucose/kg body weight is injected immediately after 60 min. Forty, 50 and 60 min represent post-glucose injection values. All values are means ± SEM, n = 5, independent experiments. The Ra of glucose was significantly lower than time 40 min at 180 and 300 min after injection of the bolus of glucose (repeated measures ANOVA, Dunnetts method). The Rd of glucose is not significantly higher than baseline at any point (repeated measures ANOVA, Dunnetts method).

Time (min)	Glucose (mM)	Rd (μmoles/kg/min)	Ra (μmoles/kg/min)
40	9.42 ± 0.80	16.08 ± 2.82	16.08 ± 2.82
50	8.74 ± 0.89	14.40 ± 2.02	14.40 ± 2.02
60	9.11 ± 0.57	15.94 ± 2.98	15.94 ± 2.98
63	15.40 ± 1.14	50.61 ± 9.97	12.39 ± 3.19
67.5	13.87 ± 0.97	29.63 ± 3.36	14.23 ± 2.60
72.5	13.46 ± 1.25	18.64 ± 8.41	14.6 ± 2.37
82.5	12.48 ± 1.06	17.67 ± 3.98	14.4 ± 2.24
105	11.18 ± 0.99	15.82 ± 2.09	13.66 ± 1.98
150	10.63 ± 1.17	12.58 ± 1.71	12.12 ± 1.59
300	8.26 ± 1.05	9.90 ± 0.72	9.41 ± 0.79

increase, however, was not found to be significantly higher than baseline. Ra values remain relatively constant. The injection of a bolus of glucose probably placed the system so far out of steady state that early values after injection are not valid for calculating Ra. However, 2 h after injection of the bolus of glucose, Ra values are significantly lower than pre-injection values implying that it takes this long for the liver to decrease glucose output.

3.3. Skeletal Muscle Hexokinase activities

Hexokinase (HK) activities in the white muscle of trout, eel and catfish demonstrate normal hyperbolic kinetics over a range of glucose concentrations from 0-1 mM (Fig. 3-3 A,B,C). Concentrations from 0-10 mM glucose were originally investigated but activity plateaued by 1 mM glucose. Kinetic constants (Km and Vmax) for HK for each fish were calculated from individual Eadie Hofstee plots where activity (v) is plotted against v/s (s, substrate concentration). The y intercept of the plot is Vmax and the negative slope is the Km. No significant differences exist between Km or Vmax values for trout, eel or catfish HK using a one way ANOVA (Table 3-3).

3.4. Glucose Transport Northern and Western Blots

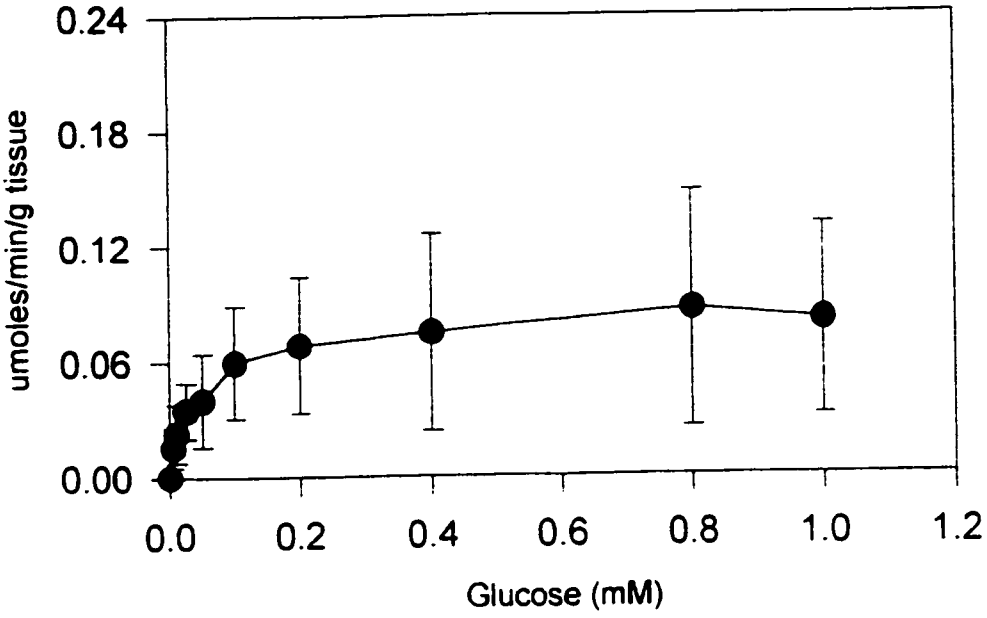
Northern blot analysis was performed on white muscles from rainbow trout, fed American eels and bullhead catfish using rat heart and red muscle as positive controls. Blots were probed with a mammalian GLUT-4 cDNA probe. No hybridization was detected for trout, eel or catfish mRNAs (Fig. 3-4). There was, however, clear hybridization with the rat red muscle and rat heart mRNAs. Blots were also probed with a mammalian GLUT-1 cDNA probe and again no hybridization was detected with the fish mRNAs, while there was with both rat red muscle and heart mRNAs.

Figure 3-3 A: Activity of hexokinase in the white muscle of American eel (6 month fasted) incubated with concentrations of glucose between 0-1 mM. Activities are μmoles/min/g tissue and are means ± SEM, n = 6 independent experiments.

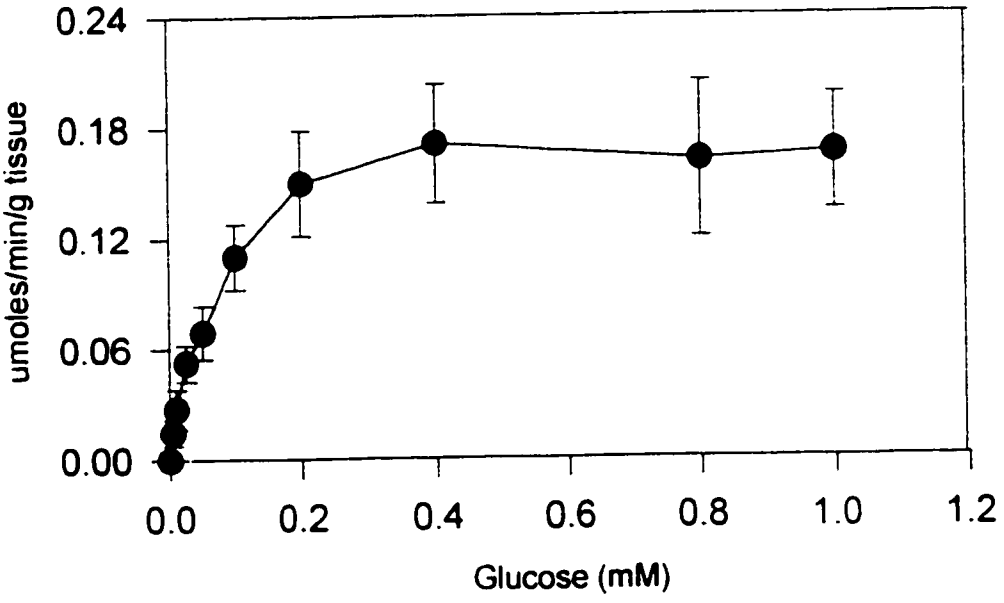
Figure 3-3 B: Activity of hexokinase in the white muscle of bullhead catfish incubated with concentrations of glucose between 0-1 mM. Activities are μmoles/min/g tissue and are means ± SEM, n = 6 independent experiments.

Figure 3-3 C: Activity of hexokinase in the white muscle of rainbow trout incubated with concentrations of glucose between 0-1 mM. Activities are μmoles/min/g tissue and are means ± SEM, n = 6 independent experiments.

A


0.24
0.18
0.12
0.06
0.00
umoles/min/g tissue
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Glucose (mM)

B


0.24
0.18
0.12
0.06
0.00
umoles/min/g tissue
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Glucose (mM)

C

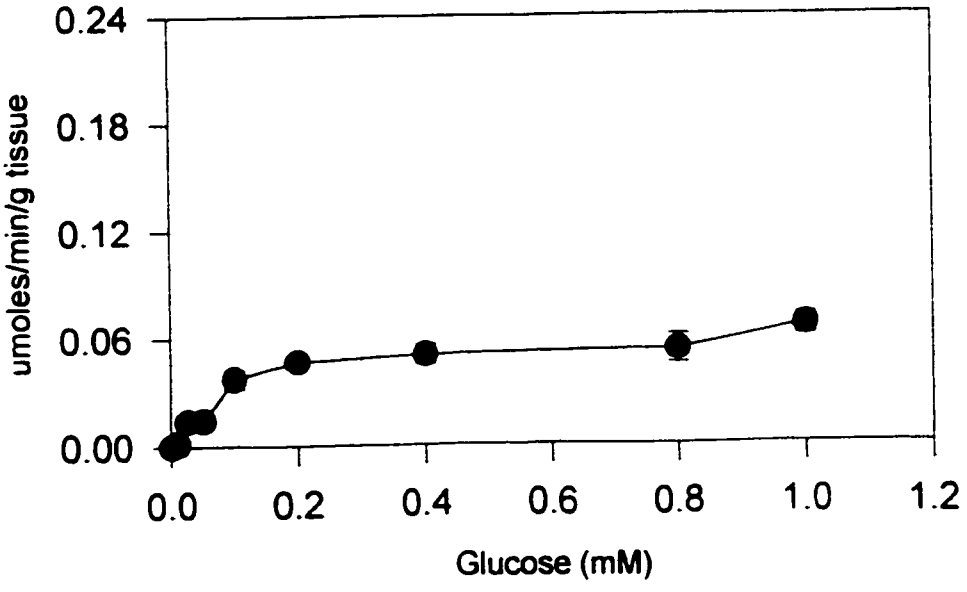

0.24
0.18
0.12
0.06
0.00
umoles/min/g tissue
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Glucose (mM)

Table 3-3 : Hexokinase activities in the white muscles of rainbow trout, fasted American eel, and bullhead catfish. All values are means ± SEM, n = 5-6, independent experiments. No significant differences exist between Km or Vmax values of any fish (one way ANOVA).

	Km (mM)	Vmax (μmoles/min/g)
Trout	0.10 ± 0.046	0.07 ± 0.008
Bullhead	0.05 ± 0.018	0.19 ± 0.044
Eel	0.16 ± 0.089	0.10 ± 0.029

Western blot analysis was also run on white muscle of the three species of fish with rat red muscle as a positive control. Blots were probed with mammalian anti-GLUT-1 and GLUT-4. Again, no cross reactivity was detected with either the GLUT-4 or GLUT-1 probes in the fish while there was with the rat red muscle.

3.5. Skeletal Muscle Glucose Transport

Membrane vesicles were isolated from the white muscle of trout, fed eels and catfish (Fig. 3-5 E) using a standard Percoll-Nycodenz gradient centrifuge method. Vesicles were incubated with increasing concentrations of D-glucose and kinetic uptake curves were constructed. L-[^{14}C]glucose was used as an indicator of diffusional uptake and values were subtracted from D-[^{3}H]glucose values to provide values plotted on each figure. Glucose transport (pmoles/mg protein/sec) was assessed in vesicles incubated with increasing concentrations of D-glucose between 0 and 18.75 mM. Transport is shown for each species (Fig. 3-5 A-C) and in each species, transport does plateau. Variation at each point is large and not atypical of experiments of this type (A. Bonen, personal communication). There was a reproducible drop in transport rate in the trout vesicles at 4 mM D-glucose that can not be explained by technical difficulties (Fig. 3-5 A). In contrast the eel transport curve (Fig. 3-5 B) showed a consistent increase at 2.5 mM and an inhibition at a D-glucose concentration greater than 12.5 mM. Catfish (Fig. 3-5 C) showed relatively low activities, but little inhibition. Double reciprocal plots (Lineweaver-Burke plots) are presented as means for the three species (Fig. 3-5 D). Linear regression analysis was performed on each individual double reciprocal plot and Km and Vmax values were determined. Mean Km and Vmax values of glucose transport are presented in Table 3-5. No significant

Figure 3-4. Northern blot of rainbow trout, American eel and bullhead catfish mRNA probed with mammalian GLUT-4 cDNA. No hybridization can be detected with trout, eel or catfish mRNA. The two far right lanes show rat mRNA that has hybridized with the GLUT-4 probe.

Fig 3-5 A: D-glucose transport (pmoles glucose/mg protein/sec) in membrane vesicles isolated from rainbow trout white muscle incubated with increasing concentrations of D-glucose. All values are means ± SEM, n = 7 independent experiments.

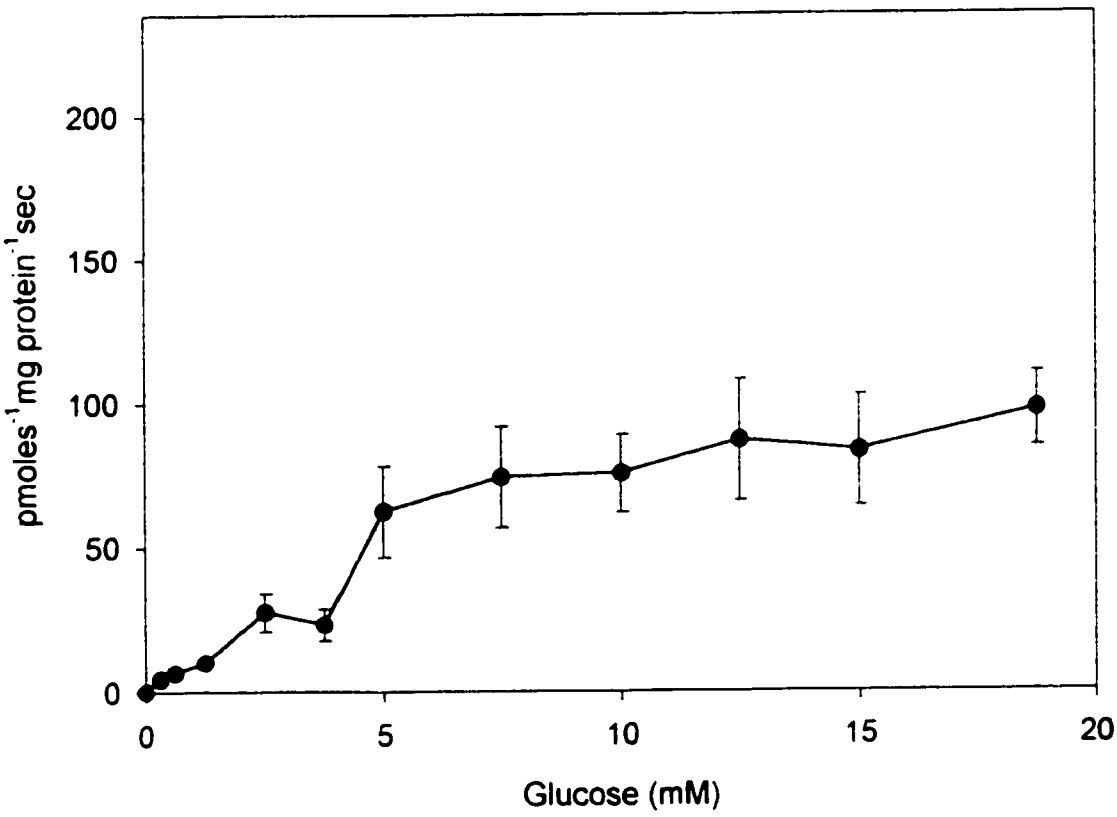

200
150
100
50
0
pmoles-1mg protein-1sec
0
5
10
15
20
Glucose (mM)

Fig 3-5 B: D-glucose transport (pmoles glucose/mg protein/sec) in membrane vesicles isolated from fed American eel white muscle incubated with increasing concentrations of D-glucose. All values are means ± SEM, n = 7 independent experiments.

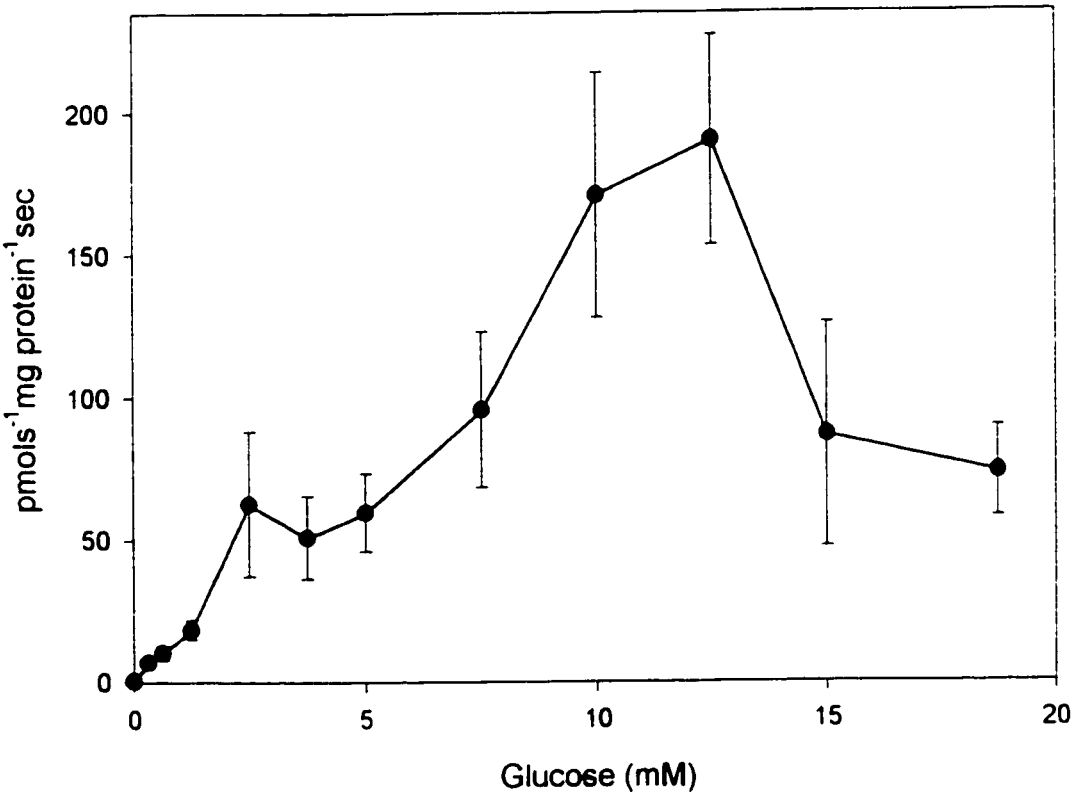
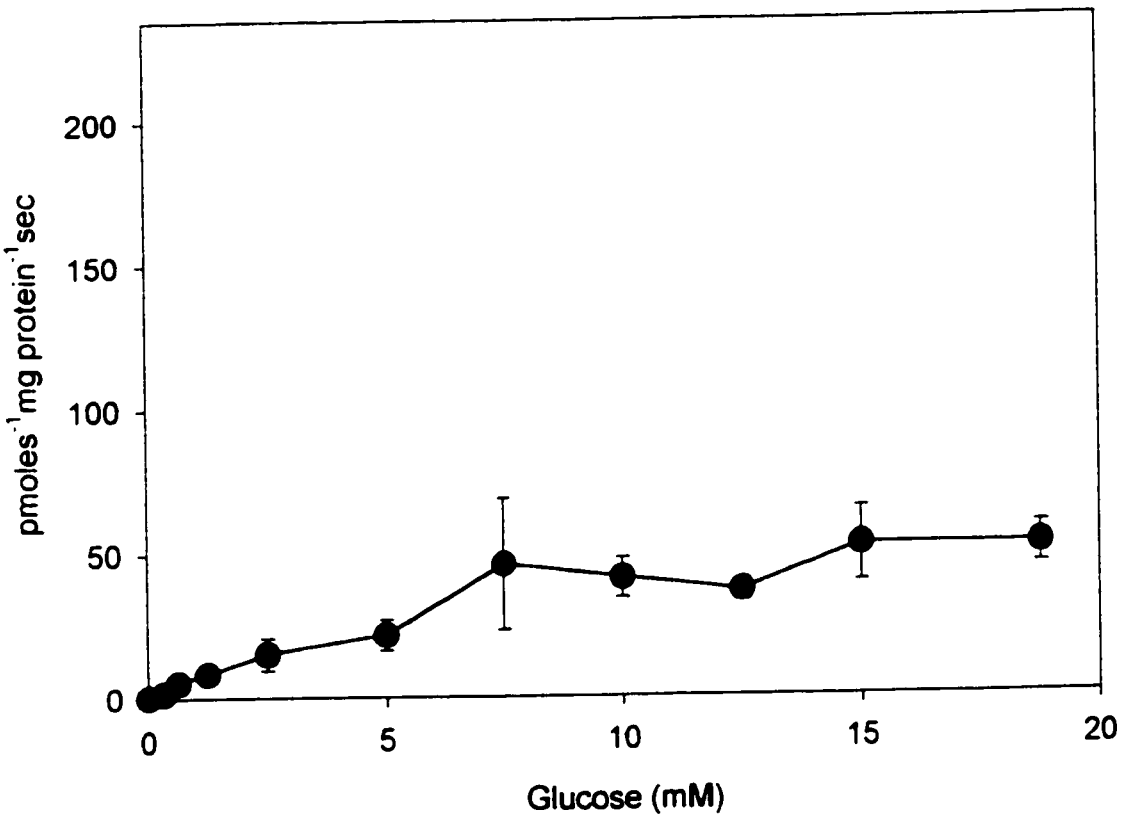

200
150
100
50
0
pmols-1mg protein-1sec
0
5
10
15
20
Glucose (mM)

Fig 3-5 C: D-glucose transport (pmoles glucose/mg protein/sec) in membrane vesicles isolated from bullhead catfish white muscle incubated with increasing concentrations of D-glucose. All values are means ± SEM, n = 5 independent experiments.

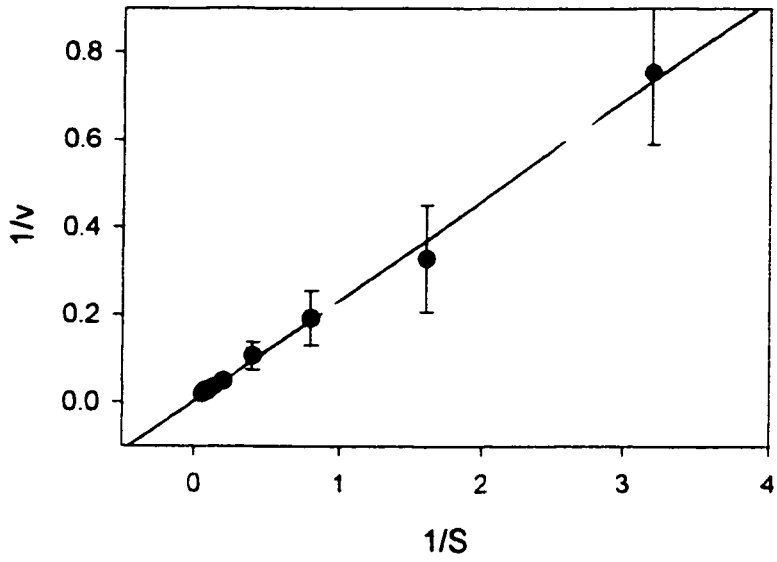

200
150
100
50
0
pmoles-1mg protein-1sec
0
5
10
15
20
Glucose (mM)

differences exist between Km or Vmax values from trout, eel or catfish (one way ANOVA), probably as a result of the significant individual variations between values.

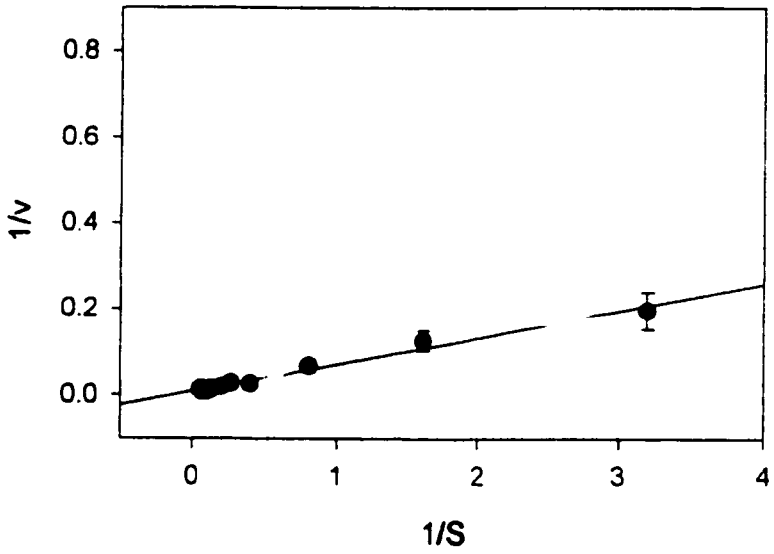
In some of the glucose uptake experiments the ability of 20 μM cytochalasin B (CB) and 200 μM phloretin to inhibit uptake of D-glucose was tested (Table 3-6) in white muscle membrane vesicles of all three species. Experiments followed the identical protocol as the kinetic uptake experiments, except vesicles were incubated with either CB or phloretin for 10 min before the experiment. Mean glucose uptake values for the vesicles incubated with the inhibitors were compared to control values not exposed to inhibitors (t-test) and no significant difference in uptake of D-glucose was detected.

Fig 3-5 D: Mean Lineweaver-Burke (double reciprocal) plots for D-glucose transport in solute muscle membrane vesicles prepared from (A) rainbow trout, (B) fed American eels and (C) bullhead catfish. Values are means ± SEM, n = 5-7 independent experiments. The regression line through the average values is shown as a solid line.

A


0.8
0.6
0.4
0.2
0.0
1/v
0
1
2
3
4
1/S

B


0.8
0.6
0.4
0.2
0.0
1/v
0
1
2
3
4
1/S

C

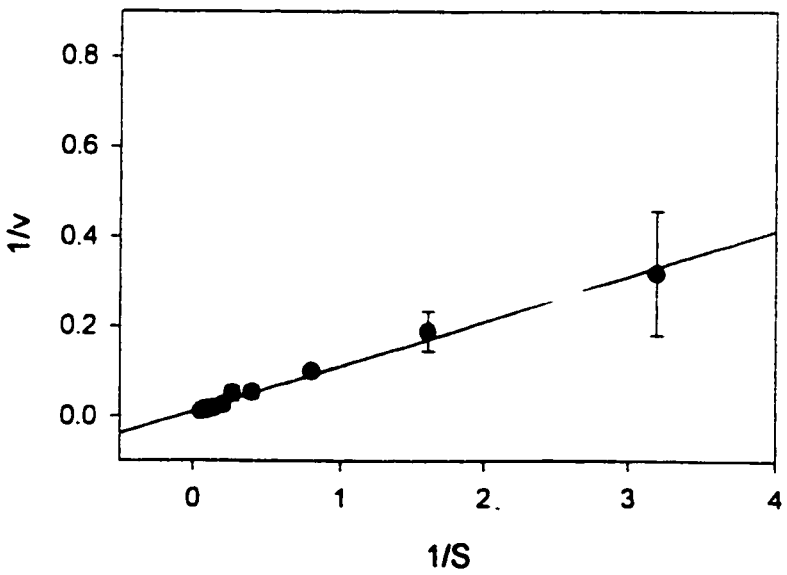

0.8
0.6
0.4
0.2
0.0
1/v
0
1
2
3
4
1/S

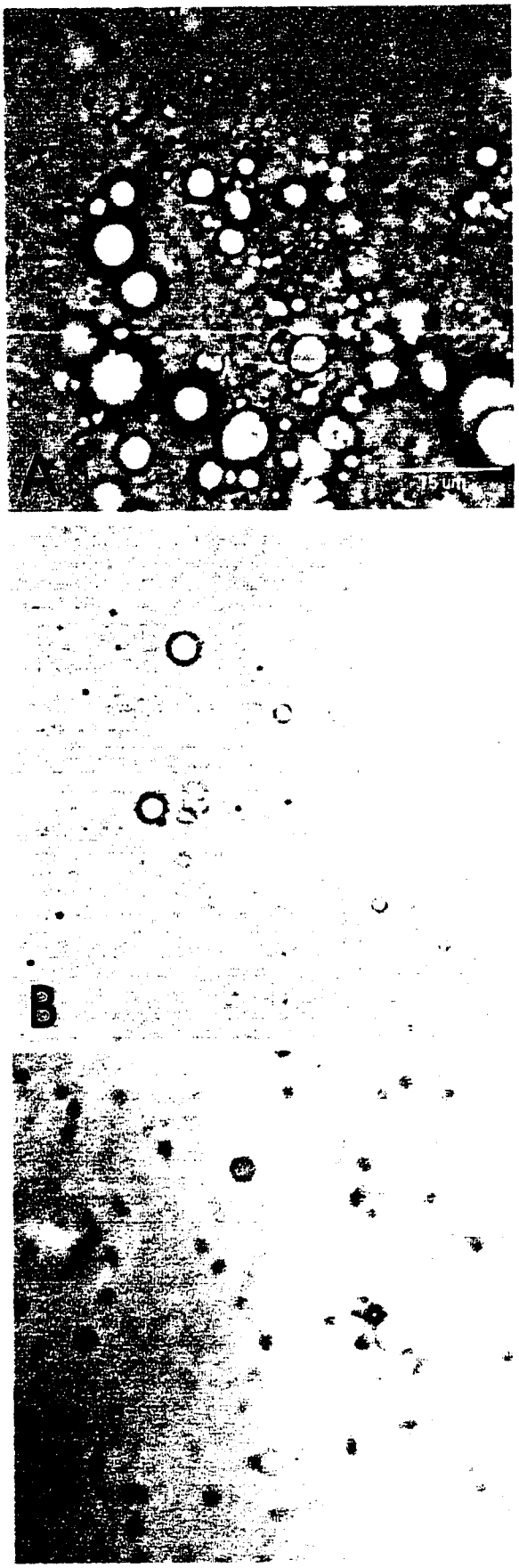
Table 3-5: Km and Vmax values obtained from individual double reciprocal plots for glucose transport in membrane vesicles of rainbow trout, feeding American eel, and bullhead catfish (see Fig. 3-5 D). All values are means ± SEM, n = 5-7 independent experiments. No significant differences exist between the Km or Vmax values of any species (one way ANOVA).

	Km (mM)	Vmax (pmoles/mg/sec)
Trout	9.3 ± 2.5	98.7 ± 14.6
Eel	8.1 ± 1.5	153.6 ± 36.0
Catfish	14.2 ± 4.3	75.6 ± 10.7

Table 3-6: Glucose transport (pmoles/mg/sec) in white muscle membrane vesicles from rainbow trout, American eel and bullhead catfish incubated with 20 μM cytochalasin B (CB) or 200 μM phloretin for 10 min before glucose uptake experiments were performed at two concentrations of D-glucose (2.5 mM and 7.5 mM). All values are means ± SEM of n = 3-6 independent experiments.

		2.5 mM Glucose	7.5 mM Glucose
Trout	Cyto B	33.16 ± 11.63	79.53 ± 28.90
	Phloretin	33.55 ± 7.26	150.27 ± 73.57
	Control	23.70 ± 15.56	75.40 ± 20.67
Eel	Cyto B	38.49 ± 13.65	89.61 ± 29.55
	Phloretin	22.13 ± 5.17	92.43 ± 50.22
	Control	40.21 ± 8.61	91.88 ±38.51
Catfish	Cyto B	20.35 ± 5.85	209.1 ± 133.15
	Control	10.12 ± 2.92	46.03 ± 22.83

Figure 3-5 E. Photomicrograph of white muscle vesicles isolated using the Percoll/Nycodenz technique from (A) rainbow trout (B) American eel (C) bullhead catfish


A
B

CHAPTER 4:
DISCUSSION

4. DISCUSSION

4.1. Glucose tolerance

Several groups performed oral or intravenous (IV) glucose tolerance tests (GTTs) on a variety of species to determine glucose tolerance (Palmer and Ryman 1972; Furuichi and Yone 1981; Machado *et al.* 1989; Ottolenghi *et al.* 1995). In the present study glucose tolerance tests were performed by IV injection on all fish (Figs. 3-1 A-D). These GTTs were performed to test i) for species differences in glucose tolerance, ii) whether improved glucose tolerance was associated with normal high carbohydrate diets, iii) whether the American eel has a glucose tolerance profile similar to the carnivorous trout or a selected omnivore, the bullhead catfish. Prior to the IV glucose tolerance tests (IGTT), baseline plasma glucose values differed between species with eel>trout>bullhead (Figs. 3-1 A-D). The values obtained are consistant with those reported by Haman *et al.* (1997) for trout (8.2 μmoles/ml), Cornish and Moon (1985) for American eel (11.0 μmoles/ml), and Ottolenghi *et al.* (1995) for channel catfish (2.3 μmoles/ml). The injection of 250 mg glucose/ kg body weight rapidly increased plasma glucose values in all 4 groups and peaked within 1 min after injection (Figs. 3-1 A-D). The peak values followed the same pattern as the baseline values with eel>trout>bullhead. This quick and dramatic peak in plasma glucose is typical of an IGTT in which glucose is injected directly into the blood stream. Similar results were seen after 1 min in channel catfish (Ottolenghi *et al.* 1995) also injected with 250 mg glucose/kg body weight. These peak plasma glucose values 1 min after glucose injection indicate insufficient time for mixing of glucose in the blood. Increases in plasma glucose 5 min after glucose injection may be a better indication of change and these values were (in μmoles/ml) 11.9 in trout, 8.6 and 12.32 in fasting and fed American eels, and 3.9 in bullhead catfish. At

either 1 or 5 min after injection, it is clear that the initial change in plasma glucose in catfish is lower than trout or eel. The bases for such differences is not known. Assuming complete mixing, the change in concentration after glucose injection should have been the same, unless the glucose space differs between species. Glucose space (the total fluid space available to glucose) values have been reported to range from 106 ml/kg in sea bass (*Dicentrarchus labrax*) to approximately 400 ml/kg in the toadfish (*Opsanus*) with values in between for other fish species (Garin *et al.* 1987). I can only conclude that at least part of the explanation for differences in plasma glucose concentrations is related to differences in glucose space between species.

Plasma glucose values in trout and fasted eels remained significantly elevated above baseline at 6 h post-glucose injection. Palmer and Ryman (1972) performed an oral GTT on rainbow trout by administering 1 g of glucose through a stomach tube and reported that mild hyperglycemia persisted for 24 h. In addition Ince and Thorpe (1974) performed an intra-arterial glucose loading using 500 mg glucose per kg body weight on European silver eels fasted for at least 4 weeks, and reported that blood glucose values recovered to normal levels at 24 h. In contrast to these plasma glucose values in trout and fasted eels, fed eel plasma glucose values were no longer significantly different from baseline 1 h after glucose injection while catfish values were not significantly different at 30 min (Figs. 3-1 A-D). Even though values are not significantly different from baseline, fed eel and catfish plasma glucose values remained slightly elevated between 2-6 h after glucose injection. These results indicate that rainbow trout and fasting American eel are glucose intolerant using the criteria established for mammals (National Diabetes Data Group 1979), however differences in metabolic rates and size may make this comparison invalid. A more appropriate comparison can be made between the 4 groups of fish

tested and there are differences in glucose tolerance among these 4 groups. Both the catfish and fed American eel are relatively more glucose tolerant than the rainbow trout and the fasted American eels. These data support my hypothesis that glucose tolerance is greater in omnivorous fish than in carnivorous fish. Catfish are categorized as omnivorous while eel and trout are thought to be primarily carnivorous. Garcia-Gallego *et al.* (1995), however, reported that the European eel did utilize carbohydrate in a manner consistent with the more omnivorous species. Machado *et al.* (1989) showed that starvation induced a progressive reduction in the rate of glucose clearance in carnivorous fish which may explain the fact that glucose tolerance in the fasted American eel was poorer than when it was fed. This supports the conclusion of Garcia-Gallego *et al.* (1995) that feeding European eels do utilize carbohydrate more effectively than the carnivorous rainbow trout. The two groups of eels examined, fed and fasted, were probably from two different age groups- the fasted eels were older than fed eels (no precise age determination was made). A better comparison would have been fed versus fasted young eels, but this was not possible due to time restrictions. My results are also similar to those of Furichi and Yone (1981) who performed oral GTTs, administering 1.67 g of glucose/kg body weight in carp (omnivorous), red sea bream (semi-carnivorous), and yellowtail (carnivorous). They found that the omnivorous carp returned plasma glucose concentrations to baseline faster (5 h) than either the red sea bream or yellowtail where plasma glucose remained elevated at 5 h after administration. The Furichi and Yone (1981) study showed that carp plasma glucose levels remained elevated longer than the omnivorous example in my study (i.e., bullhead catfish). A partial explanation for this difference may be that in an oral GTT, glucose must first pass through the stomach before entering the blood and, therefore, glucose takes longer to reach the blood

than in an intravenous GTT. In addition, the high dose of glucose (>6-times) may also partially explain why plasma glucose values in the omnivorous carp remained elevated for 5 h as opposed to 30 min in the omnivorous catfish. Finally it may be that within different omnivorous species vary in glucose tolerance, possibly as a result of the amount of carbohydrate typically ingested in the diet.

In a study by Blasco *et al.* (1996) ^{3}H-deoxyglucose uptake rates in brown trout were examined under basal conditions and under the influence of an intra-aortic glucose load (500 mg/kg body weight). In the basal state, rates of tissue glucose uptake per g tissue were in the order intestine>> kidney>gills>liver>red muscle>white muscle. Red muscle took up glucose at a 2-4-fold higher rate than white muscle. After injection of the glucose load which resulted in a strong and persistent hyperglycemia that lasted for >8 h, glucose uptake rates in the red and white muscle increased 4- and 3-fold, respectively, while glucose uptake in the other tissues did not change significantly. Since white muscle represents at least 50% of body weight, it is potentially the major site of glucose uptake and in respect to other tissues, took up 64% of glucose after the glucose load while red muscle took up 14% even though flux per mg tissue is higher in red muscle. This study confirms that fish white muscle is the major site of glucose disposal after a glucose load and supports the focus on white muscle glucose uptake in my study. Blasco *et al.* (1996) also measured plasma insulin and reported that the intra-aortic glucose load provoked a 2-3-fold increase in plasma insulin compared to pre-load values in both fed and fasted animals. From their results they concluded that skeletal muscle is the only tissue which may be clearly modulating a glucose load.

4.2. Glucose Turnover in Rainbow trout

A continuous tracer infusion method was used to determine the rate of disappearance (Rd) and rate of appearance (Ra) of glucose in trout before and after injection of a bolus of glucose. Haman *et al.* (1997) verified the use of this technique in establishing turnover rates in the rainbow trout. Baseline Ra and Rd values (Table 3-2) are comparable to resting values obtained by Haman *et al.* (1997) of approximately 17 μmol/kg/min. Cornish and Moon (1985) examined glucose turnover in the American eel and reported that Ra and Rd were 38 and 77 μmol/kg/min in fed and 6 month fasted eels, respectively. Garin *et al.* (1987) examined glucose turnover in the sea bass and reported that Ra and Rd values were 0.55-0.65 μmol/kg/min. Machado *et al.* (1989) reported glucose turnover values of 3.7-4.3 μmoles/kg/min in fed *Hoplias malabaricus* while Bever *et al.* (1977) reported a value of 1.94 μmoles/kg/min in the fed kelp bass (*Paralabrax clathratus*). These data demonstrate that there are wide variations in turnover rates between species. The data of Cornish and Moon (1985) show high glucose turnover rates in the eel, comparable with those reported for mammals. Furthermore, Weber *et al.* (1986) reported glucose turnover rates for skipjack tuna (*Katsuwonus pelamis*) at least as high as those reported for mammalian species.

The Rd of glucose in the trout increased after injection of glucose but this increase was short lived and was not found to be significantly higher than pre-injection values. Ra is difficult to calculate as the introduction of the glucose bolus moves the system so far out of steady state; however, at 2 and 4 h after injection of glucose, Ra was significantly higher than pre-injection values. These high Ra values imply that the liver does not reduce its output of glucose after a

glucose load until 2 hours after exposure and may contribute to the high plasma glucose levels seen in the rainbow trout after a glucose load. It is possible that differences in the regulation of glucose output by the liver in the different species in my study may contribute to glucose tolerance.

4.3. Insulin in Fish

Baseline plasma insulin concentrations were more than 4 times higher in American eel than in rainbow trout (Figs. 3-1 A-B) and were comparable to those reported by Mommsen and Plisetskaya (1991) for both the European eel (1.1 ng/ml) and rainbow trout (2.2 ng/ml). Moon *et al.* (1989) reported insulin values of 12.1 ng/ml in fed, and 2.0 ng/ml in 6 week fasted rainbow trout. Plasma insulin concentrations peaked at 1 and 10 min after glucose injection in the trout and the eel, respectively; however, no concentrations were significantly different from baseline at any time measured over the 24 h period. Ince and Thorpe (1977) examined plasma glucose and insulin variations in trout kept under constant benzocaine anesthesia. A significant hyperglycemia was reported, reaching a maximum after 30 min of anesthesia and returning to control values by 2 h. Plasma insulin levels were elevated after 5 min, but declined to lower, relatively stable values thereafter. Furuichi and Yone (1982) measured plasma insulin during an oral GTT (1.67 mg glucose/ kg body weight) in carp, red sea bream and yellowtail. Baseline plasma insulin values for all three species were similar (approximately 20 μU/ml) and values peaked 2 h after glucose administration, reaching approximately 60 μU/ml in the omnivorous carp, 52 μU/ml in the semi-carnivorous red sea bream, and 44 μU/ml in the yellowtail. It is interesting to note that in this study after a glucose load, plasma insulin concentrations were highest in the omnivorous

species. The slower insulin release reported in the Furuichi and Yone (1982) study may be in part due to the oral ingestion of glucose (see above). Blasco *et al.* (1996) reported that after an intra-aortic glucose load of 500 mg/kg, brown trout plasma insulin peaked 30-60 min at approximately 9 ng/ml in trout fasted 3-5 days and at about 14 ng/ml in trout fasted 9-15 days. Pre-injection plasma insulin values were around 2.5 ng/ml in trout fasted 3-5 days and 5 ng/ml in trout fasted 9-15 days. Fasting is shown here to increase baseline plasma insulin concentrations and increase the insulin response to a glucose load; this may somewhat explain the higher plasma insulin concentrations seen in the eel used in my study that had been fasted for 6 months. However, Moon *et al.* (1989) reported the more consistent observation of a major decrease in rainbow trout plasma insulin after 6 weeks of fasting (12.1 to 2.0 ng/ml).

The large doses of glucose used in the GTTs of these previous experiments (Furiuchi and Yone 1982; Blasco *et al.* 1996) may be partially responsible for the larger insulin release. In my study insulin release after glucose injection was not significantly different from baseline values, but obviously insulin release was detectable 1 min after glucose injection (Figs. 3-1 A-B).

It still remains unclear whether insulin is playing a role in clearing glucose from the blood in the fish I studied. In the other fish glucose tolerance studies mentioned above, there was an increase in plasma insulin concentration after injection or oral administration of a glucose load. In a normal human, plasma insulin values are 10-20 μU/ml in the postprandial state which is similar to values reported in fish, but these values increase to a maximum of 80-130 μU/ml after a glucose load which is higher than maximal plasma insulin values (approximately 50-70 μU/ml) reached in the fish examined by Furuichi and Yone (1981). Glucose is a secretagogue of insulin but it is not as potent as amino acids in fish (Mommsen and Plisetskaya 1991). In addition, in

studies injecting insulin into fish, hypoglycemia is seen but doses of insulin administered to illicit this response are usually supra-physiological (Leibson and Plisetskaya 1967; Kewander 1976; Plisetskaya *et al.* 1985). Mommsen and Plisetskaya (1991) do state that insulin does stimulate glucose uptake into the liver and skeletal muscles in some fish species. If in fact this is the case, insulin in fish is stimulating glucose uptake to a lesser extent than in mammals and possibly through different mechanisms.

4.4. Hexokinase

After glucose enters the cell, the first enzyme to interact with it is hexokinase. Hexokinase phosphorylates glucose in the presence of ATP and ions to glucose-6-phosphate in order to maintain a glucose concentration gradient from outside to inside the cell. This is a prerequisite for glucose entry into the glycolytic pathway or its conversion to glycogen. White muscle hexokinase from rainbow trout, bullhead catfish and fed American eels could not be differentiated based upon Km or Vmax values (Fig 3-3A-C; Table 3-3). These results demonstrate the presence of HK in the white muscle of all three species, but fail to establish a correlation between enzyme activity and the nutritional background of the fish. Knox *et al.* (1980) investigated HK activities in a number of tissues including white muscle. They reported activities similar to the values reported here of (in μmoles/min/g tissues or units/g) 0.06 for rainbow trout, 0.02 for cod and 0.07 for plaice. Blier and Guderley (1988) reported HK activities in white muscle of 0.09 (units/g tissue) in lake whitefish.

The Km values were below 0.02 mM for each species assayed (Table 3-3). These values define muscle HK as a low Km, high affinity enzyme which means that any glucose entering the

cell will be phosphorylated by this enzyme and that this is probably not a site of deficiency in fish glucose tolerance. Blasco *et al.* (1996) reported that all 2-deoxyglucose taken up into the skeletal muscle of the brown trout was phosphorylated which suggests that phosphorylation is not a limiting step. In my study HK activities (Table 3-3) are much higher than glucose transport rates (Table 3-5) in all three species, again implying that HK activity is not rate limiting to glucose deposition in white muscle.

4.5. GLUT-4 and GLUT-1 in Fish White Muscle

Mammalian skeletal muscle glucose transport occurs by two facilitative glucose transport isoforms, GLUT-1 and GLUT-4 (see section 1.5). Immunoblotting and immunofluorescence indicate that both transporters are present in the plasma membranes of red and white muscles, although there is a greater abundance of transporters in red than white muscles in the rat (Marette *et al.* 1992). GLUT-1 has been reported to reside constitutively in perineural sheaths and endoneural vessels with relatively low levels being detected in the actual muscle plasma membrane. The contribution of GLUT-1 in insulin sensitive tissues is reported to be minimal in relation to GLUT-4 (Korani *et al.* 1991; Leturque *et al.* 1996).

The presence of a GLUT-1 and GLUT-4 glucose transporter in the white muscle of rainbow trout, American eel and bullhead catfish was investigated using northern (mRNA) and western (protein) blot analyses. The mRNA of white muscle of all three species was probed with a mammalian GLUT-1 cDNA probe and no hybridization was detected. The mRNA was then probed with a mammalian GLUT-4 cDNA probe (see Fig. 3-4) and again no hybridization was detected. Samples were compared with rat soleus and heart mRNA which did react with the

probe indicating that the northern blot procedure itself was working. Western blot analysis was performed on protein extracts of all three species and blots probed with a mammalian antibody for both GLUT-1 and GLUT-4. Again no cross reactivity was detected with either probe. Rat red muscle protein was run as a control and did cross react with both probes. These results suggest that there is no glucose transporter homologous to mammalian GLUT-4 or GLUT-1 present in the white muscle of rainbow trout, American eel or bullhead catfish. These results support a study by Wright *et al.* (1997) that could not detect the presence of GLUT-1 or GLUT-4 in either red or white skeletal muscles of tilapia (*Oreochromis nilotica*) using western and northern blot analyses. This group used the same GLUT-1 and GLUT-4 mammalian probes used in my study and in fact the same laboratory (Dr. A. Bonen, University of Waterloo). It may be argued that these probes are specific for mammalian transporters and that species specificity could be the reason for no cross reactivity. However, Wright *et al.* (1997) also looked for GLUT-1 and GLUT-4 in heart, liver and brain, and GLUT-1 was detected in the heart and brain of tilapia. GLUT-1 levels in heart were ten-fold higher than those in rat and in addition GLUT-1 levels in brain are many-fold higher than in rat. This indicates that GLUT-1 is detectable in at least one species of fish with the probes used in my study.

GLUT-4 is the insulin-regulated transporter in mammalian skeletal muscle and adipose tissue the two tissues primarily responsible to clear a glucose load (see section 1.5). As stated earlier, fish are relatively glucose intolerant which is thought to be due to an inability of peripheral tissues to clear glucose from the blood. Lack of a homologous GLUT-4-like transporter may begin to explain this inability to regulate a glucose load and the relative dissociation of insulin from glucose uptake in fish (Mommsen and Plisetskaya, 1991). However,

as indicated by experiments with GLUT-4 null mice (Katz *et al.* 1995; see section 1.7), other transporters may be present. Also, Blasco *et al.* (1996) clearly demonstrated that white muscle is the major site for glucose uptake in the brown trout.

4.6. Glucose Transport in Fish White Muscle

Plasma membrane transport of various types and the properties of ionic channels have been examined by many researchers using muscle vesicle preparations (Standen *et al.* 1984; Burton *et al.* 1988; Juel 1990; McDermott and Bonen 1993; Ploug *et al.* 1993). Most authors have isolated muscle vesicles from mammalian muscles, but Standen *et al.* (1984) reported a successful preparation from frog muscle. Juel (1990) used the collagenase digestion, Percoll/Nycodenz (P/N) vesicle isolation method on rat muscle to determine if this preparation could be used to study lactate transport. He reported the formation of large vesicles that he used to study lactate transport and which he suggested could be used to examine other transport systems. Ploug *et al.* (1993) used the P/N isolation technique on rat muscle to examine glucose transport. Recently K. Labaree and L. Milligan (personal communication) used the P/N technique on white muscle of rainbow trout and were able to isolate vesicles and characterize a lactate transporter in this tissue. In my study the P/N isolation was performed on the white muscle of rainbow trout, bullhead catfish and feeding American eels. Muscle vesicles from all three fish were examined using a confocal microscope and vesicles ranged in size to a maximum of approximately 3 μm in diameter (Fig 3-5 E). Vesicles incubated with radiolabled glucose indicated a stereospecific glucose transporter is present in the white muscle of all three species (Fig. 3-5 A-C). Kinetic analyses, reported on Table 3-5 , demonstrate differences between

species with respect to both Km and Vmax values. Km values ranged from 9-14 mM and Vmax values were lower for trout than either catfish or eel; unfortunately the high error on this assay precluded identification of significant differences in these kinetic values. The Km for glucose transport in eel and trout white muscle is close to baseline plasma glucose values for these animals (see Figs. 3-1,3-4), but the Km in catfish vesicles is much higher than baseline plasma glucose. Thomas *et al.* (1992) reported Km values of human glucose transporters transfected into *Xenopus* oocytes as 20.9 mM for GLUT-1 and 1.8 mM for GLUT-4. Soengas and Moon (1995) reported a glucose transporter on the red blood cell of the American eel with a Km of 10.4 mM that is close to the values obtained for the rainbow trout and American eel muscle in my study. Evidence in the eel red cell supports the transporter being of the GLUT-1 variety (Soengas and Moon 1995). A glucose transporter has been characterized on the red blood cells of Pacific hagfish (Young *et al.* 1994) and of Japanese eels (Tse and Young 1990) again consistent with a GLUT-1-like system. Interestingly, the rainbow trout and most other fish species do not have a similar red blood cell glucose transporter (Tse and Young 1990). Ploug *et al.* (1993) reported stereospecific glucose uptake in rat skeletal muscle vesicles with a Km of 16 mM and a Vmax of 272 pmoles/mg vesicle protein/sec. They reported the presence of a GLUT-4 transporter but not a GLUT-1 transporter in the vesicles used in their study. King *et al.* (1989) used a small vesicle rather than a large vesicle preparation to examine glucose transport in rat skeletal muscle, and reported a Km of 20 mM and a Vmax of 58 pmoles/mg/sec. Thus, even in the same muscle, at least Vmax values can be very different depending upon the procedure to isolate the membrane vesicles.

Cytochalasin B (CB) and phloretin inhibit carrier-facilitated glucose transport as shown in a number of studies (King *et al.* 1989; Ploug *et al.* 1993; Young *et al.* 1994; Soengas and Moon 1995). Muscle vesicles were incubated with 20 μM CB or 200 μM phloretin for 10 min prior to beginning transport studies. No inhibition of D-glucose uptake could be detected in these experiments (Table 3-6). I can only conclude that either this particular white muscle transporter is insensitive to CB and phloretin, or that the specific concentrations used were inadequate to elicit a response. Further studies are needed to characterize this vesicle transporter.

My study with white muscle in rainbow trout, American eel and bullhead catfish detected no mammalian GLUT-4 or GLUT-1. However, according to my glucose uptake studies in white muscle vesicles from all three species, there is a glucose transporter present that demonstrates saturable, stereospecific kinetics. It may be suggested that the glucose transporter observed in my study is similar to the yet to be identified transporter potentially present in GLUT-4 null mice that maintained glucose homeostasis without a GLUT-4 (Stenbit *et al.* 1996) (see section 1.7). In normal mice, the effects of this transporter may be masked by the more active/abundant GLUT-4 and in mice with a functioning GLUT-4 transporter the contribution of this transporter may be small but performs a compensatory role in the absence of GLUT-4. This fish transporter may be a primitive glucose transporter that secondarily acquired insulin sensitivity in vertebrates where precise glucose regulation was needed. Comparative studies within vertebrates other than fish and mammals of tissue Na^+-independent glucose transporters (presumably GLUT-like transporters) do not exist.

4.7. Does Insulin Play a Role in Glucose Clearance in Fish?

In my study it remains unclear whether insulin performs a role in clearing glucose from the plasma of eel and trout. In both fish, after injection of glucose there was an almost immediate increase in plasma insulin. In the rainbow trout, I measured the rate of disappearance of glucose (Rd) and found that it increased after injection of a bolus of glucose. The only evidence from my study that insulin and glucose clearance are related is that a slight increase in plasma insulin coincides with an increase in Rd for the rainbow trout, which is obviously not conclusive. In the studies mentioned above and in the introduction, a similar increase in insulin concentration is reported after injection of a bolus of glucose and in the study by Blasco *et al.* (1996) there is uptake of glucose into skeletal muscle. In addition injection of insulin into a variety of fish is associated with a hypoglycemic response. The doses of insulin injected are usually pharmacological, however, these may be the experimental doses needed for a researcher to detect a response. The problem with such doses is that they may mask the more subtle effects of insulin while activating a non-specific process.

In mammals insulin regulates a glucose load by translocating the GLUT-4 transporter. In this study and the study by Wright *et al.* (1997) no GLUT-4 protein or mRNA was detected in the skeletal muscle which Blasco *et al.* (1996) identified as THE major site of glucose uptake in brown trout. These results indicate that the highly regulated mammalian role of insulin in translocating GLUT-4 to the plasma membrane may be absent in fish.

The correlation between insulin release in response to glucose and a hypoglycemic response to insulin can still not be ignored. A glucose transporter present in fish may be moderately insulin-responsive or insulin may work through a separate pathway in fish. In

mammals it has been established that GLUT-4 is translocated by at least two different mechanisms: one in response to insulin and the other to contraction, and these two responses are found to be additive (Douen *et al.* 1990; Cortright and Dohm 1997). The fish glucose transporter may be regulated by contraction in skeletal muscle or by some yet to be investigated pathway. Another option is that the fish glucose transporter may only be present in the plasma membrane (comparable to GLUT-1) and is not translocated by insulin or any other mechanism. Moreover the fish transporter (or transporters) may have varied distribution and expression in different fish species, possibly based on exposure to carbohydrate in the diet.

The increase in insulin reported in response to a glucose load may be a response that eventually evolved into the metabolic functions of insulin classically seen in mammals. Relatively high circulating levels of insulin may be attributable to its growth promotion function which would be much more relevant to the lifestyles of most fish which consume high levels of protein and are indeterminate growers. Glucose intolerance in fish is a good indicator that whatever the glucose clearance system is in fish, it is not a very efficient or highly regulated one, but is sufficient for the lifestyle of the fish.

4.8. Non-Insulin-Dependent Diabetes Mellitus

Non insulin dependent diabetes mellitus (NIDDM) is usually associated with insulin resistance in the peripheral tissues (adipose and skeletal) and/or a defect of insulin production and secretion from the pancreatic β cells. As mentioned in the introduction, a number of authors examining glucose tolerance in fish have drawn a parallel between this system in fish and the human disease diabetes (Falkmer 1961; Palmer and Ryman 1972; Furiuchi and Yone 1982;

Mommsen and Plisetskaya 1991). NIDDM patients may demonstrate hyperinsulinemia and a blunted response to insulin manifested in poor glucose clearance. This situation may seem on the surface to present a similar profile to fish which also have high levels of circulating insulin and a sluggish glucose clearing response.

It has been suggested that a defect in GLUT-4 expression in skeletal muscles and adipocytes of NIDDM patients could be responsible for insulin resistance in these individuals. Garvey *et al.* (1991) reported that pre-translational events specifically suppress expression of the GLUT-4 transport protein in the adipocytes of NIDDM patients. The major part of glucose uptake, however, occurs in skeletal muscle (adipocytes contribute less than 5%). This led Eriksson *et al.* (1992) to measure GLUT-4 mRNA and protein in muscle biopsies from NIDDM patients and their first degree relatives and compare them with GLUT-4 levels in insulin-sensitive control subjects. They did not find reduced expression of GLUT-4 mRNA or protein in skeletal muscle of these subjects. This being the case they concluded that there may be a defect in the signaling or in the functioning of GLUT-4.

In my study and the study performed by Wright *et al.* (1997), no GLUT-4 or GLUT-1 mRNA or protein were detected in the white muscle of the fish studied. In the NIDDM patients studied no reduction in GLUT-4 was seen in the skeletal muscle. It may be that GLUT-4 signaling or functioning is disabled in the skeletal muscle of NIDDM patients. If this is the case then fish and NIDDM patients may have similar compensatory mechanisms to try to maintain glucose homeostasis without a GLUT-4, even though only one of them have had much success.

4.9 Conclusions

This thesis has examined glucose tolerance and two of the steps in the glucose uptake pathway in three species of teleosts, the rainbow trout, the bullhead catfish and the fed and fasted American eel. A few conclusions can be drawn from this work.

First, the rainbow trout and fasting American eel are glucose intolerant when compared with the clinical criteria established for this disease condition in humans.

Second, there are differences in glucose tolerance between the groups, with catfish and fed American eels having higher glucose tolerance than the carnivorous trout or fasted American eel.

Third, Ra does not decrease until two hours after injection of a bolus of glucose implying that some contribution to glucose intolerance in the trout at least, is associated with liver function.

Fourth, the white muscle has sufficient hexokinase to deal with glucose entering the cell and that this enzyme does not contribute to glucose intolerance in these fish. Properties of this enzyme were not found to be significantly different between the three species of fish and so cannot be related to nutrient preference of the fish.

Fifth, no homologous GLUT-1 or GLUT-4 glucose transporters could be detected in the white muscle of the three species examined using mammalian generated probes for mRNA and protein.

Sixth, that a saturable, stereospecific glucose transporter is present in the white muscle of all three species, the properties of which are not consistent with mammalian GLUT-1 or GLUT-

4. In addition the transporters properties were not significantly different in the three species studied and so cannot be related to nutrient preference.

Further work must be done to elucidate the glucose uptake system in fish more clearly. GLUT-1 has been detected in specific tissues of one fish and it must be examined in others. The glucose transporter that I have detected in the white muscle of these fish must be characterized and further comparative studies using red muscle from these species and red and white muscle of other fish examined. As well the question of whether insulin plays a role in regulation of this transporter still needs to be addressed. The absence of rapid, reliable, simple homologous RIAs for fish insulin has been and continues to be a problem in the studies of fish glucose homeostasis. These studies, as well as others, should help tease out the glucoregulatory system in fish and may have consequences to patients with NIDDM who may be attempting to regulate glucose without a functional GLUT-4.

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