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The Cost of Ethanol Synthesis During Recovery from Exhaustive Exercise in Grass Carp (Ctenopharyngodon idella)

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

The Cost of Ethanol Synthesis During Recovery from Exhaustive Exercise in Grass Carp (*Ctenopharyngodon idella*)

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Abstract

Grass carp (*Ctenopharyngodon idella*) reduce white muscle glycogen (~14 μmol glucosyl units/g wet tissue) in response to exhaustive exercise. This reduction results in a small increase in muscle lactate (~9 μmol/g wet tissue) and a larger increase in muscle ethanol (~30 μmol/g wet tissue). Tissue-specific and whole-body measures of glycogen, ethanol and lactate confirm that ethanol is the major "anaerobic" glycolytic end-product. Additionally, while peak muscle and blood ethanol levels occur immediately post-exercise, the excretion of ethanol to the environment is delayed, occurring over a 30-minute period beginning ~105 minutes following exercise. As the total amount of ethanol synthesized in the white muscle does not account for that synthesized in the whole-body, it may be that the red muscle is also involved. The clearance and excretion of ethanol to the environment following exercise represents ~100% of the whole-body glycolytic pool used during exercise and therefore represents a significant carbon cost to the muscle's glycolytic pool.

Keywords: Grass Carp, Burst Exercise, Recovery, Anaerobic Metabolism, ADH, Ethanol.

Co-Authorship Statement

Simon Bradford – Lead researcher and author

Dr. Louise Milligan – Supervisory role, advised on appropriate methodology and metrics required for the completion of research goals. Also taught the required surgical techniques and served as primary thesis editor.

Dr. James Staples – Served as secondary editor

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Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

AAT	Amino acid transferase
ACC	Acetyl-CoA carboxylase
ADP	Adenosine diphosphate
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ATP	Adenosine triphosphate
BCAAs	Branched-chain amino acids
BC-KA	Branched-chain keto acid
BCAD	Branched-chain keto acid dehydrogenase
cAMP	Cyclic adenosine monophosphate
CK	Creatine kinase
EPOC	Excess post-exercise oxygen consumption
ETC	Electron transport chain
FA	Free fatty acids (non esterified)
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GP	Glycogen phosphorylase
HSL	Hormone-sensitive lipase
LDH	Lactate dehydrogenase

MS-222	Tricaine methane sulfonate
NAD^+	Oxidized nicontinamide adenine nucleotide
NADH	Reduced nicotinamide adenine nucleotide
PC	Pyruvate carboxylase
PCA	Perchloric acid
PCr	Phosphocreatine
PDC	Pyruvate decarboxylase subunit of pyruvate dehydrogenase
PDH	Pyruvate dehydrogenase
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
PK	Pyruvate kinase
PKA	Protein kinase A
SCOT	Succinyl-CoA Transferase
TCA	Tricarboxylic acid cycle
TG	Triacylglycerols
$\dot{V}\,O_2$	Rate of oxygen consumption
$\dot{V}$ EtOH	Rate of ethanol excretion

Chapter 1 - Introduction

An animal's capacity for movement has profound effects on its distribution, predator-prey interactions and reproductive behavior (Bennett, 1979). Some animals can achieve high speeds but show little stamina, while others move more slowly over long distances. The study of animal movement has led physiologists, behaviourists and ecologists to quantify both endurance and burst activity capacities of a number of taxa (Kieffer, 2000).

The study of exercise in fish was pioneered by Edgar Black and his colleagues (Black et al., 1962). This work defined an important avenue of research in describing the large anaerobic capacity of fish and their response to exhaustive exercise. Almost 60 years of continued research has extended our understanding of the response to exercise. This research has identified large inter-species variation with respect to capacities for sustained aerobic and burst-type anaerobic swimming (Kieffer et al., 1996; Kieffer, 2000), as well as potential mechanisms of recovery (Milligan and Wood, 1986; Wood, 1991; Milligan and Girard, 1993; Wang et al., 1994, Moyes and West, 1995) and the effects of muscle pH and hormones on these processes (Walsh and Milligan, 1989; Milligan, 1996).

In water, only 1/10,000th of the oxygen supply is available to aquatic organisms in comparison to terrestrial animals. From an evolutionary perspective, this likely supported the evolution of a larger scope of anaerobic activity compared to air breathers (Black, 1962; Bennett, 1978). The difference in respiratory media is further magnified when oxygen consumption is considered in relation to exercise. Although locomotor costs are relatively similar between fish and terrestrial ectotherms of the same mass, oxygen consumption increases exponentially with increasing speed in swimming fish but linearly in walking amphibians or reptiles (Gleeson, 1991). The exponential relationship in fish is due to both the increased metabolic cost of extracting and delivering oxygen to the working muscles (Gallaugher et al., 2001) as well as the increased drag as a result of the swimming media. Furthermore, the metabolic rate and therefore oxygen requirements of fish are relatively lower than those of air breathing ectotherms (Jackson and Prange,

1979). Which allows adjustments in gill ventilation and transport to successfully maintain much of a fish's daily swimming activity within its aerobic scope. However, circumstances such as spawning migrations, predator evasion and prey capture require a rapid increase in muscular power output (Beamish, 1978) that exceeds aerobic capacity and is therefore supported anaerobically. Burst-type exercise to exhaustion in fish can, therefore, be a particularly useful model system for identifying metabolic regulatory processes and allows identification of rate-limiting factors in exercise performance and recovery (Milligan, 1996; Kieffer 2000).

Exercise Metabolism in Fish

The swimming capacity of fish is commonly evaluated using critical swimming speed (U_{crit}), which reflects the maximum speed that can be sustained aerobically. This provides a general measure of the metabolic pathways (aerobic or anaerobic) that dominate during graded swim performance tests (Lowe, 1996) and has allowed standardization and comparison of swim performance across a variety of species. Based on the measurement of U_{crit}, swimming activity can be separated into three categories that reflect the type of metabolism employed. Sustained swimming at speeds below U_{crit} can be maintained for >240 minutes and yields respiratory and circulatory adjustments to meet energetic needs aerobically (Beamish, 1978). Prolonged swimming at speeds approaching U_{crit}, are also fuelled aerobically but can only be maintained for 20-200 minutes and results in fatigue (Beamish, 1978). Finally, burst-type swimming at speeds greater than U_{crit}, is fuelled anaerobically, and can only be maintained for <20 seconds (Brett, 1964).

The locomotory muscles of fish are also divided into categories and reflect the type of swimming for which they are recruited. The red, oxidative type I or slow twitch fibers have a well developed blood supply, numerous mitochondria, high myoglobin concentration as well as, high electron transport chain (ETC) and tricarboxylic acid cycle (TCA) enzyme activities (Johnston, 1977). The characteristics of the type I fibers correlate well with aerobic supply of energy for contraction and allow sustained or prolonged swimming. Conversely, the white, glycolytic or type II fibers constitute a much larger mass, have fewer mitochondria and lower myoglobin concentration

(Johnston, 1977). The characteristics of the type II fibers support rapid, anaerobic provision of energy supplying a large and therefore powerful muscle group and correlate well with their recruitment in achieving high speeds during burst-swimming.

In the natural environment, a single bout of burst-type swimming is not exhausting; however, multiple sprints, one shortly after another, are likely necessary to capture prey or avoid predators. Consequently, burst-swim performance depends on three endogenous fuels stores within the muscle: adenosine triphoshate (ATP), phosphocreatine (PCr), and glycogen (Beamish, 1978; Kieffer, 2000). During the early stages of burst activity (10-15 sec.), the energetic requirements of the type II muscle are maintained through re-phosphorylation of adenosine diphosphate (ADP) to ATP by transfer of inorganic phosphate from PCr (Dobson and Hochachka, 1987). As time spent swimming increases, the ratio of PCr/Cr declines and is no longer sufficient to maintain the concentration of ATP. As a consequence of repeated contraction, the myoplasmic ratio of ADP to ATP and the concentration of Ca^{2+} increases, signaling a cascade of events through cAMP and protein kinase A (PKA) that activates glycogen phosphorylase and increases glycolytic flux in order to maintain ATP supply to the muscle (Voet et al., 2001).

Activation of glycogen phosphorylase results in the breakdown of muscle glycogen to glucose-6-phosphate (G6P), which serves as the glycolytic substrate for continued ATP synthesis (Voet et al., 2001). Increased rates of glycogenolysis and glycolysis create increasing amounts of pyruvate and continual reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH in the cytoplasm (Dobson and Hochachka, 1987). Typically, pyruvate synthesized by glycolysis is transported into the mitochondria using a monocarboxylate transporter and converted to acetyl-CoA via pyruvate dehydrogenase (PDH) before entering the tricarboxylic acid cycle (TCA). The oxidation of acetyl-CoA within the TCA serves to replenish the glycolytic intermediate NAD^+ required for continued oxidative synthesis of ATP (Fig. 1) (Brooks et al., 1999). However, as the glycolytic flux is increased, the synthesis of pyruvate exceeds its rate of oxidation and creates a mismatch of NAD^+ supply and demand resulting in disequilibrium of redox potential (Dobson and Hochachka, 1987). In order to maintain

redox equilibrium, excess pyruvate is diverted to lactate dehydrogenase (LDH) in the cytosol. The conversion of pyruvate to lactate serves to replenish NAD^+ and allows the continued anaerobic synthesis of ATP (Wood, 1991) (Fig. 1). However, the accumulation and dissociation of lactic acid in the cytosol to lactate and metabolic protons (Milligan, 1996) cause disturbances of muscle and blood pH as well as ionic and osmotic balance that lead to exhaustion (Wang et al., 1994).

Overall, the physiological and metabolic response to exhaustive exercise in fish, for example rainbow trout (*Oncorhynchus mykiss)*, is characterized by an exponential increase in oxygen consumption and the depletion of muscular energy reserves (ATP, PCr and glycogen) causing a rapid decline in muscle and blood pH as a result of the accumulation and dissociation of lactic acid. The recovery time required for the clearance of metabolic end products and restoration of pH and ionic/osmotic balance may therefore represent a constraint on the potential for subsequent bouts of burst exercise. Furthermore, the duration of subsequent bouts is likely also dependent on the storage capacity of type II muscle energy substrates (glycogen, ATP or PCr).

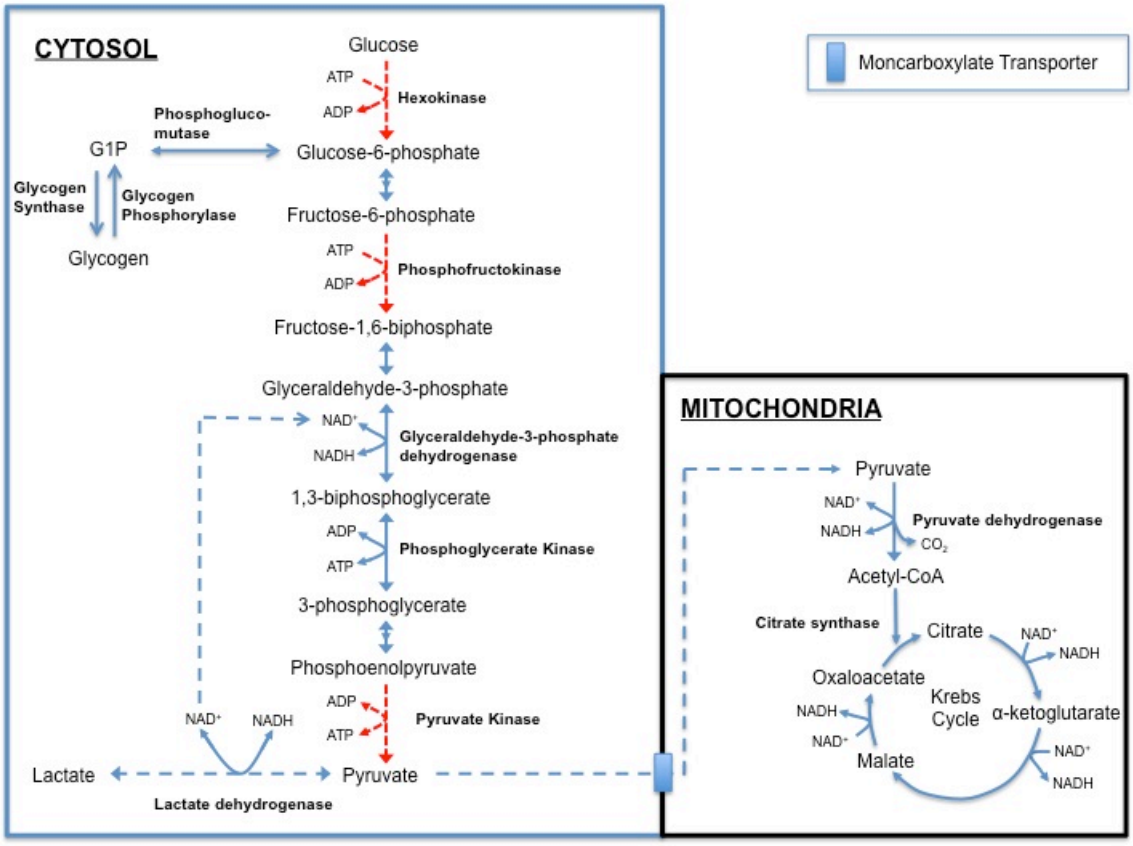

Figure 1: Schematic model of glycolysis and Krebs cycle including enzymes (shown in bold) and their locations in a muscle cell. Dashed red arrows indicate irreversible reactions, dashed blue arrows indicate alternate pathways used depending on cell redox state. ATP = adenosine triphosphate; ADP = adenosine diphosphate; NAD^+ = nicotinamide adenine dinucleotide; NADH = reduced nicotinamide adenine dinucleotide. Adapted from Berg et al. (2002)

Recovery from Exercise in Fish

During recovery from exhaustive exercise, elevated oxygen consumption is sustained for ~2h and is termed "excess post-exercise oxygen consumption (EPOC)". In adult sockeye (*Oncorhyncus nerka*) and coho (*O. kisutch*) salmon, Lee et al. (2003) determined that EPOC can be divided into fast and slow components linked to the cardioventilatory costs of the recovery process (Wood, 1991). The fast component (~20 min) reflects the oxidative restoration of PCr and ATP as well as tissue O_2 replenishment. However, the explanation for the contributions made by the slow component (~2 hours) remains somewhat incomplete. Lactate clearance and glycogen re-synthesis accounts for only ~25% of the slow component and takes 4-12h (Milligan and Wood, 1986; Milligan and Girard, 1993), so is therefore not the only contributor. The re-establishment of ion, acid-base and fluid homeostasis may significantly contribute to the slow component of EPOC (Wood, 1991).

Investigation of the patterns and strategies of post-exercise lactate clearance revealed that the possible fates of lactate vary among species (Gleeson, 1996). Post-exercise studies of mice indicate that blood and muscle lactate concentration are in equilibrium and that oxidization to carbon dioxide and water accounts for the majority of lactate removal (40%), while incorporation into tissue glycogen accounts for only a small fraction (~3%) during recovery (Hatta et al., 1994). However, while post-exercise muscle lactate accumulation is comparable in fish, blood lactate is often much lower (Milligan, 1996). This disequilibrium between the muscle and blood lactate pools reflects lactate retention for glyconeogenic use as ~80-85% of muscle-borne lactate in rainbow trout (*Oncorhynchus mykiss*), coho salmon (*Oncorhynchus kisutch*) and starry flounder (*Platichthys stellatus*) can be accounted for through *in situ* replacement of muscle glycogen (Turner et al., 1983; Milligan and McDonald, 1988).

Lactate supported muscle glycogenesis can occur by one of three biochemical pathways. These pathways are distinguished by the mechanism through which pyruvate is converted back to phosphoenolpyruvate (PEP). The carboxylation/decarboxylation of pyruvate though pyruvate carboxylase (PC) and PEP carboxykinase (PEPCK) is generally thought to be isolated to hepatic and renal tissue, as fish skeletal muscle lacks

PC activity (Crabtree et al., 1972). Of the two remaining possible pathways, reversal of pyruvate kinase (PK) and the pathway catalyzed by cytoplasmic malic enzyme and PEPCK, the reversal of PK has more support as (I) PEPCK is absent in all type II fish muscle studied except marlin (*Makaira nigricans*) (Moyes and West, 1995; Suarez et al., 1986), (II) the PK reaction is close to equilibrium (Shulte et al., 1992) and (III) that PK activity is sufficiently high in type II muscle to explain glycogenesis (Moyes and West, 1995). The retention of lactate and *in situ* glycogenesis observed in fish, as well as leopard frog (*Rana pipiens*), American toad (*Bufo americanus*), and tiger salamander (*Ambystoma tigrinum*), is beneficial as releasing lactate to the blood space exposes the lactate pool to oxidation and use by other tissues (Withers et al., 1988; Fournier and Guderley 1993; Wickler and Wagner 1995). By minimizing the loss of the carbon substrate to oxidation or use by other tissues, the fish may be more successful in complete muscle glycogen replenishment without having to forage or risk exposure to predation in a glycogen-depleted state (Gleeson and Dalessio, 1989; Fournier and Guderley, 1992).

In order for lactate to be used as a glyconeogenic substrate, the muscle requires an alternate oxidative substrate to form the ATP required. The substrates used to oxidatively generate ATP may be synthesized through three different mechanisms: (I) amino acid transamination, (II) hydrolysis of triacylglycerols or (III) ketone body synthesis. Milligan (1997) constructed a framework where cortisol-stimulated amino acid transamination and oxidation contributes the ATP required for replenishment of glycogen in rainbow trout. Briefly, in response to cortisol stimulation, hepatic BCAAs (leucine, isoleucine and valine) are released to the plasma. Once taken up by the muscle, transamination of BCAAs with α-ketoglutarate via transaminase creates glutamate and the respective branched-chain keto acid (BC-KA). The BC-KA can supply the TCA through branched-chain keto acid dehydrogenase (BCAD) while glutamate and excess pyruvate are converted to alanine via alanine aminotransferase and released back to the plasma. Once in the plasma, alanine may support hepatic gluconeogenesis or, perhaps more importantly, oxidation by cardiac or red muscle tissue contributing to the replenishment of whole-body ATP and glycogen stores to their pre-exercise levels (Milligan, 1997) (Fig.2).

The second alternative is that catecholaminergic stimulation in response to stress, such as exhaustive exercise, may activate protein kinase A (PKA) causing the hydrolysis of triacylglycerols and an increase in circulatory free fatty acids (FA) and glycerol (Tocher, 2003). Circulatory glycerol can be taken up by the liver in rainbow trout for re-synthesis of triacylglycerols (Bernard et al., 2003), while muscular uptake and β-oxidation of circulating FAs supplies the TCA intermediates required for oxidative ATP generation and supports muscular glycogen re-synthesis (Richards et al., 2002a, Fukao et al., 2004) (Fig. 3). An increase in mitochondrial acetyl-CoA concentration as a result of β-oxidation likely inhibits pyruvate dehydrogenase and, in the skeletal muscles, functions to direct both pyruvate and lactate away from an oxidative state and towards a glycogenic fate while also supplying the ATP required.

The third possible pathway for supplying the required ATP for muscular glycogenesis arises as a result of an increased concentration of FAs in the liver. The production of hepatic acetyl-CoA as a result of β-oxidation can exceed the hepatic cellular energy requirements and cause ketogenic formation of three inter-convertible, but distinct, compounds known as “ketone bodies”: Acetoacetate, 3-hydroxybutyrate and acetone (Fukao et al., 2004). In the event of ketogenesis, both free acetoacetate and 3-hydroxybutyrate are released to circulation as a potential oxidative substrate for ATP synthesis in extra-hepatic tissues (Fig. 4). Although the activity Succinyl-CoA-3-ketoacid coenzyme A transferase (SCOT) in the muscle is only 5% of that reported for the heart in humans (Williamson, 1991), the large proportion of total body mass represented by the skeletal muscles in fish would suggest that muscles may have an appreciable capacity for the generation of ATP through oxidation of ketone bodies that could aid in the energetic support of muscle glycogenesis. Furthermore, the increased concentration of acetyl-CoA would again direct pyruvate and lactate away from an oxidative fate by inhibiting the normal activity of PDH.

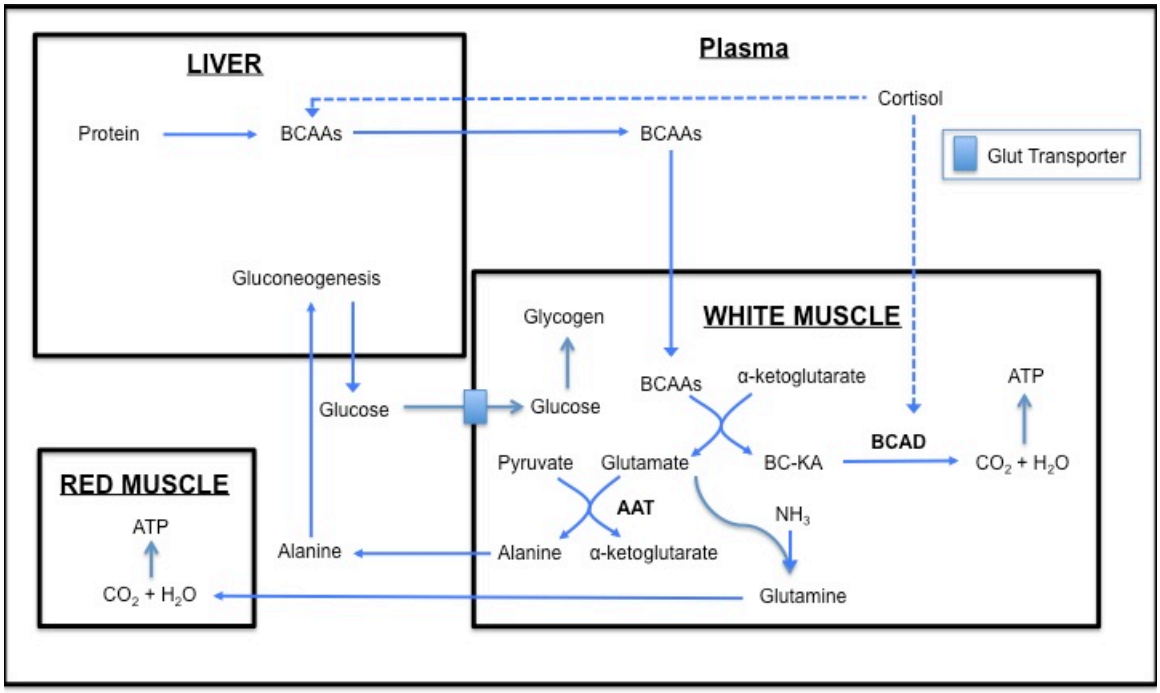

Figure 2: Schematic model for the use of amino acid transamination and oxidation in the supply of ATP and hepatic glycogen during recovery from exhaustive exercise in fish. Dashed arrows = hormonal targets; BCAA = branched-chain amino (leucine, isoleucine and valine); BC-KA = branched-chain keto acid; AAT = amino acid transferase BCAD = Branched-chain keto acid dehydrogenase. Adapted from Milligan (1996).

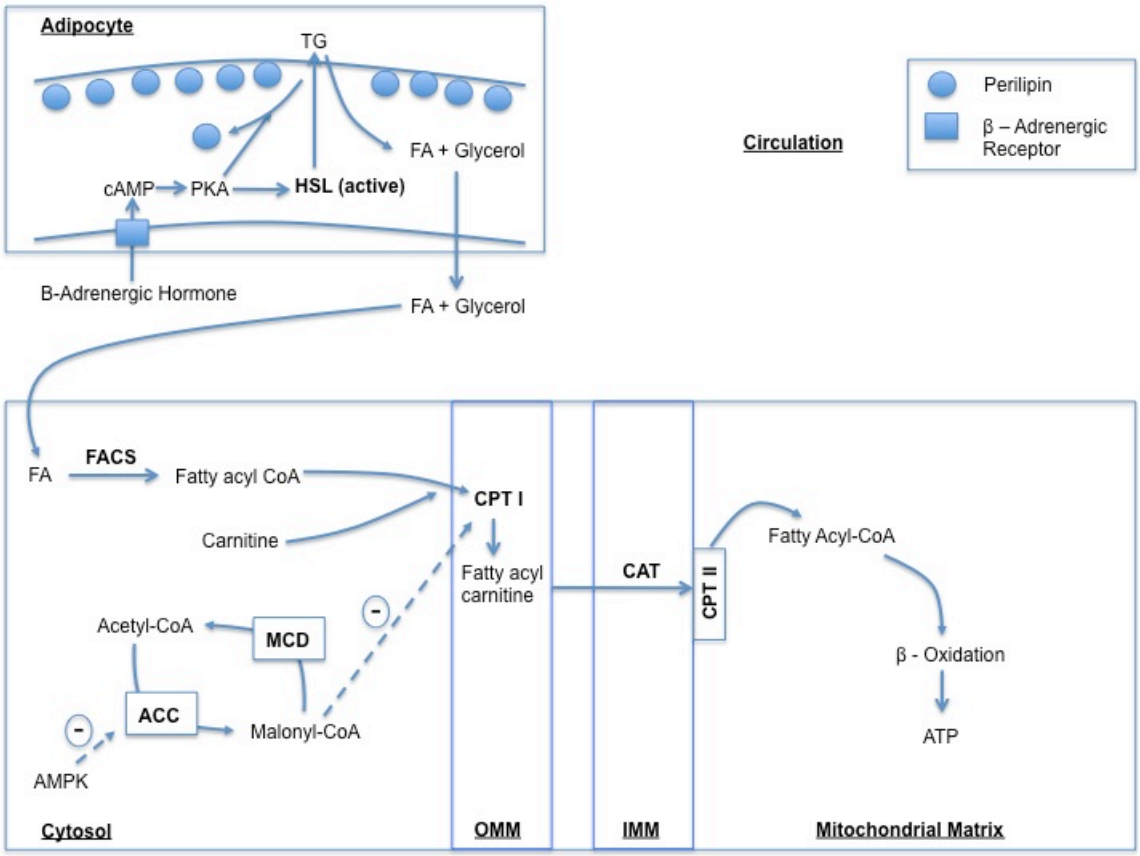

Figure 3: Schematic model for the increased mitochondrial β-oxidation of fatty acids through adrenergic hormone stimulation in response to exercise in fish. Dashed arrows = inhibition; Bolded text = enzymes; TG = triacyl glycerol; cAMP = cyclic adenosine monophosphate; PKA = Pyruvate Kinase; HSL = Hormone sensitive lipase; FA = Free fatty acid; FACS = Fatty Acyl-CoA synthetase; MCD = Malonyl-CoA decarboxylase; ACC = AcCoA carboxylase; AMPK = AMP activated protein kinase; CPTI = carnitine palmityl transferase I; CAT = Carnitine/Acyl-carnitine transferase; CPT II = Carnitine palmityl transferase II; ATP = adenosine triphosphate; OMM = Outer mitochondrial membrane; IMM = inner mitochondrial membrane. Adapted from Fukao et al. (2004).

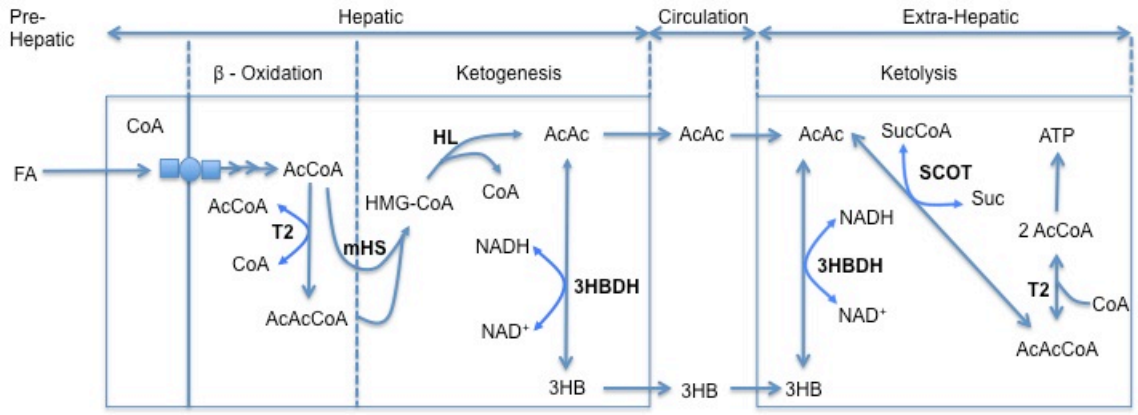

Figure 4: Schematic model of ketogenesis through fatty acid oxidation and transport of ketone bodies to extra-hepatic tissue for ketolytic ATP generation to support muscular glycogen replenishment after exercise. Bolded text = enzymes; FA = free fatty acid; CoA = Coenzyme A; AcCoA = Acetyl-CoA; AcAcCoA = Acetoacetyl-CoA; AcAc = Acetoacetate; T2 = AcAcCoA thiolase; mHS = Mitochondrial HMG-CoA synthase; 3HB = 3-hydroxybuturate; 3HBDH = 3-hydroxybuturate dehydrogenase; SucCoA = Succinyl-CoA; Suc = Succinyl; SCOT = Succinyl-CoA: 3-ketoacid coenzyme A transferase. Adapted from Fukao et al. (2004)

Alternative Glycolytic End-Product Metabolism

The goldfish (*Carassius auratus*) (Shoubridge and Hochachka, 1980), European bitterling (*Rhodeus amarus*) (Johnston and Bernard, 1982), crucian carp (*Carassius carassius*) (Wissing and Zebe, 1988) and grass carp (*Ctenopharyngodon idella*) (Bradford and Milligan, unpublished results) rely on the production of alternative, excretable glycolytic end-products (e.g. ethanol), to survive hypoxia. In order for these fish to remain active in an hypoxic environment, whole-body metabolism is depressed and those tissues that remain active operate anaerobically and are dependent on large stores of hepatic carbohydrates (Bickler and Buck, 2007). The use of anaerobic metabolism in maintaining activity results in the synthesis of lactate that is transported through the blood from extra-muscular tissues to the skeletal muscle for conversion to ethanol. Upon transport of lactate into the skeletal muscle, lactate is converted to pyruvate via lactate dehydrogenase (LDH) and enters the mitochondria through a monocarboxylate transporter. Covalent modification of PDH leads to the decoupling of the decarboxylase subunit of the PDH enzyme complex (Van Waversveld et al., 1989) and permits conversion of pyruvate to acetylaldehyde and CO_2. Acetylaldehyde then diffuses into the muscle cytoplasm where it is rapidly converted to ethanol by alcohol dehydrogenase (ADH) and along with CO_2 can be excreted through the gills (Mandic et al., 2008) (Fig 5). The conversion of lactate to ethanol removes the potential disruption of ionic/osmotic and pH balance associated with lactate accumulation, while the coupling of LDH and ADH maintain NAD^+/NADH redox potential allowing continued glycolytic ATP synthesis (Voet et al., 2001) (Fig. 5). However, although this strategy functions to maintain potential activity under hypoxic stress, conversion of lactate to ethanol and its excretion also means that lactate is no longer available as a glycogenic substrate upon re-oxygenation of the environment (Nilsson, 1988).

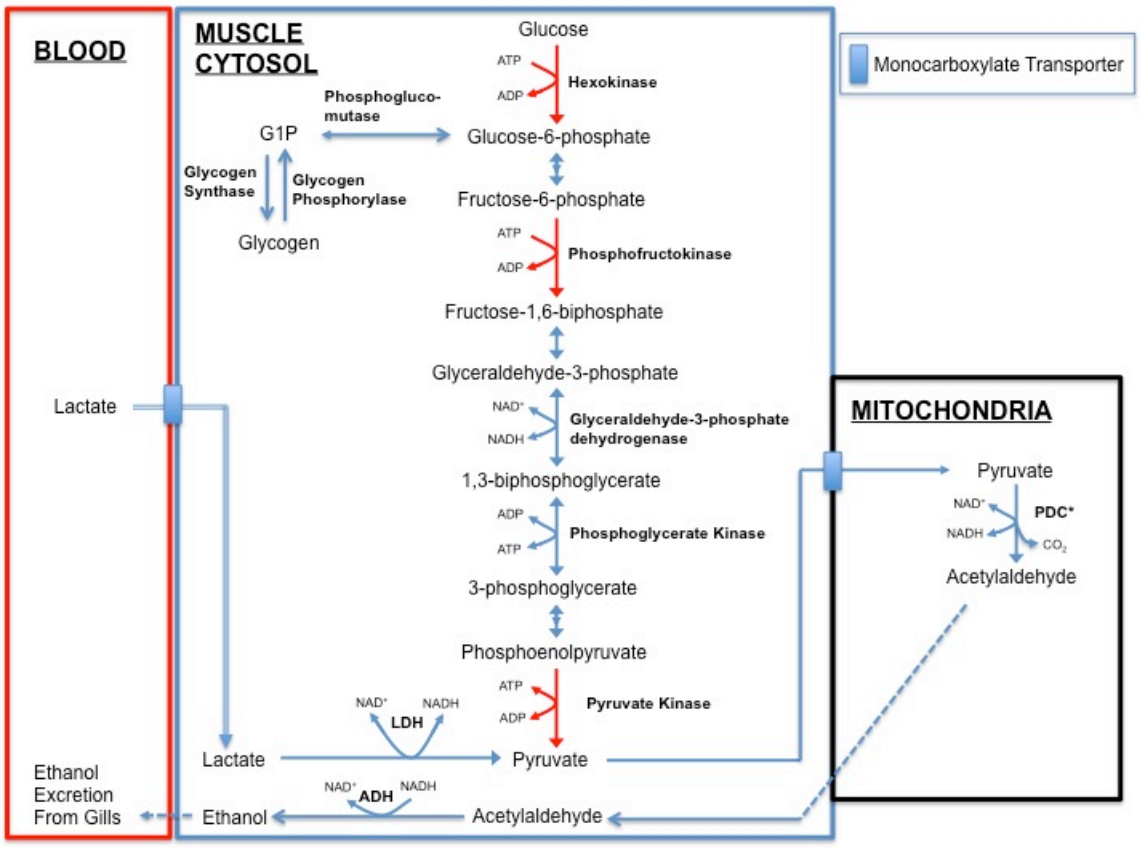

Figure 5: Schematic model of ethanol synthesis and excretion during hypoxia (double lines) or in response to exhaustive exercise in hypoxia-tolerant cyprinids. Bolded text = enzymes; Dashed lines = diffusion; LDH = lactate dehydrogenase; ADH = alcohol dehydrogenase; PDC* = pyruvate decarboxylase subunit of pyruvate dehydrogenase.

Unlike hypoxia, exhaustive exercise requires an increased whole-body glycolytic flux in order to sustain the energetic demands of muscle contraction. As the flux is increased, the rate of pyruvate synthesis from glycogen catabolism exceeds that of oxidation creating the potential for pyruvate accumulation. In hypoxia-intolerant species, the rate of glycolytic ATP production is maintained by shunting pyruvate to lactate through the use of LDH, restoring the NAD^+ required for continued anaerobic glycolysis. However, Davidson and Goldspink (1978) observed that, in goldfish, s lactate accumulated post-exercise was much lower than predicted from the corresponding change in glycogen if lactate was the major anaerobic end product. Mandic et al. (2008) suggested that, in goldfish, pyruvate may be shunted through a covalently modified PDH to synthesize acetylaldehyde and ultimately ethanol as observed during hypoxia. The conversion of acetylaldehyde to ethanol via alcohol dehydrogenase (ADH) in the cytosol serves the same function as LDH in replacing the NAD^+ required for continued glycolysis (Mandic et al., 2008). If in response to exercise, ethanol is produced and excreted because of a mismatch between pyruvate synthesis and oxidation then, the clearance of anaerobically formed ethanol suggests a substantial loss from the whole-body glycolytic pool and, therefore, a significant cost to the organism.

As grass carp have the ability to synthesize the alternative end product ethanol during hypoxic insult (Bradford and Milligan, unpublished results), it is possible that the synthesis of ethanol is constitutive whenever anaerobic metabolism occurs. Furthermore, the previous research on exhaustive exercise has focused on carnivorous species, such as the rainbow trout that have a high protein but relatively low carbohydrate intake in their natural diet. As herbivores, grass carp have much easier access to carbohydrates and therefore represents an interesting model to investigate the metabolic changes associated with recovery from exhaustive exercise as well as estimate the potential impacts of glycolytic carbon loss through ethanol synthesis and excretion.

Hypotheses and Objectives

The objectives of this thesis were 1) to determine if exhaustive exercise results in the synthesis and excretion of ethanol as a consequence of exercise, 2) to determine the location of ethanol synthesis and the potential substrates used to support end-product synthesis, and 3) if ethanol is excreted to the environment, whether the clearance and excretion of ethanol represents a significant loss from the glycolytic carbon pool. The following two hypotheses were tested: 1) Exhaustive exercise will result in the depletion of skeletal muscle energy metabolites (PCr, glycogen and glucose) resulting in the synthesis of ethanol as the major glycolytic end product, and 2) The synthesis and excretion of ethanol to the environment represents a significant loss from the grass carp's glycolytic carbon pool.

Chapter 2 - Materials and Methods

Experimental Animals

Triploid grass carp (*Ctenopharyngodon idella*) (n=75, 30.7-59.6 g) were certified and obtained from The Aquaculture Center for Excellence, Lethbridge Community College, Lethbridge, Alberta (License # 1058068, Appendix 4) and held at the University of Western Ontario (UWO) greenhouse in a 1500L concrete tank under flow-through conditions (4 mL/min) of aerated, de-chlorinated City of London water. All fish were acclimated to laboratory conditions for one month in the holding system maintained at 18-24 ^{o}C by an electronically controlled water heater. The fish were fed daily on a diet of sow thistle grown in the UWO greenhouse and supplemented with Koi pellets (Vandermeer Nursery, Ajax, Ontario). Twenty-four hours prior to experimentation, fish were isolated and fasted in a respirometer (Qubit Systems, D008, V = 2.25 L) or 4 L black plastic boxes (406 mmx83 mmx102 mm) covered with an opaque lid, under flow-through (8mL/min) conditions kept at a temperature of 18 ± 3 ^{o}C. The UWO Animal Use Subcommittee, in accordance with the Canada Council on Animal Care guidelines, approved all protocols for holding and handling of fish (Appendix 3).

Experimental Design

Preliminary experiments revealed that following 4 hours of recovery, oxygen consumption and ethanol excretion had returned to resting status. Therefore, three experimental series were carried out in order to investigate the physiological and metabolic changes that occur as a consequence of exercise and during a 4-hour recovery period. All experimental series compared the resting condition to the response after exhaustion or a designated recovery treatment.

Series 1 – As a consequence of exhaustive exercise, is ethanol excreted to the environment during recovery?

Using the intermittent respirometry setup (Loligo Systems), oxygen consumption ($\dot{V}$ O_2, μmol O_2/g*min) and ethanol excretion ($\dot{V}$ EtOH, μmol/g*min) was determined (N

= 16) (see Appendix. 1). After 24-hours acclimation, each respirometer was switched from a flow-through (5 mL/min) to a recirculation format (5mL/min) in which the respirometer was flushed for two minutes at fifteen-minute intervals from a temperature controlled (18-24 ^{o}C) oxygenated water reservoir in order to replace the volume of the respirometer with fresh, normoxic (minimum 6 mg/L O_2) water. A small plastic tube was mounted inline to allow water samples (1 mL) to be taken directly from the respirometer using a syringe (BD, Franklin Lakes, N.J.) immediately before and after each 2 minute flushing cycle for analysis of ethanol concentration and calculation of $\dot{V}$ EtOH (µmol EtOH/g*min). Resting $\dot{V}$ O_2 (µmol O_2/g*min) and $\dot{V}$ EtOH were measured for a period of 1 hour, after which each fish was individually released to a 20 L exercise tank and exercised to exhaustion by manual chasing until unresponsive to further stimulation (approx. 2-3 min). Once unresponsive, the fish were then transferred back to their respective respirometers where $\dot{V}$ O_2 and $\dot{V}$ EtOH were monitored throughout a 4-hour recovery period.

Series 2 – How does exhaustive exercise affect the grass carp's energy metabolites? Does ethanol synthesis in the tissues account for the timing and amount of ethanol excreted to the environment?

Fish were allowed at least seven days of recovery from the first experiment before being used in following experiments. Fish were then randomly assigned to one of nine treatments (Rest, Exhausted or Recovery for 0.25hr, 1hr, 1.5hr, 1.75hr, 2hr, 2.5 hourand 4hr, N = 4-6). Each fish was placed in a separate 4L plastic box with an opaque lid for 24 hours under flow-through conditions of aerated, de-chlorinated City of London water kept at a constant temperature (18 ± 3^{o}C) prior to experimentation. Resting fish were euthanized via an overdose of tricaine methane sulfonate (1.3g MS-222 and 2 g $NAHCO_3$; Syndel, Vancouver, B.C.) administered directly into the box. Immediately after death, blood was drawn (~100-500µL) via caudal puncture using a 1mL syringe (BD, Franklin Lakes, N.J.) that has been flushed with heparinized saline (50U/mL). The heart, liver and white muscle were excised, freeze-clamped and stored under liquid N_2 until transfer to a -80^{o}C freezer. White muscle was dissected dorsal to the lateral line and anterior to the dorsal fin. The entire dissection process from euthanasia to completed

dissection took approximately six minutes. The remaining eight fish were individually transferred to the exercise tank and manually chased to exhaustion. The exhausted fish were euthanized immediately after exercise and sampled as described above, while the recovery groups were returned to their respective boxes. Each recovery group was euthanized and sampled in the same manner as described above upon reaching the designated recovery period. All fish tissue samples were collected at the same time each day in an effort to reduce any possible differences between sampling days.

Series 3 – Does ethanol synthesis in the whole-body account for the difference observed between ethanol excretion to the environment and its clearance from individual tissues? If so, does ethanol excretion represent a significant loss from the whole-body glycolytic carbon pool?

A third experimental series identical to that described in "Series 2" was carried out to measure the changes in whole-fish metabolites, as the results of ethanol synthesis in the individual tissues (Series 2) did not account for the amount of ethanol observed to be excreted in Series 1. The changes in whole-body metabolites were further used to calculate the percentage of the whole-body glycolytic pool lost through ethanol clearance. As a significant correlation was found to exist between body weight and the metabolites measured (Appendix 3), all results were standardized to the weight of a 30g fish. The resting, exhausted, and 1 hour and 2 hour recovery treatment periods were selected as those that represented key time points to observe whole-fish metabolite changes. Fish were assigned to one of four treatments (N = 3) and placed in a separate 4 L plastic box with an opaque lid for 24 hours under flow-through conditions kept at 18 ± 3 ^{o}C prior to experimentation. Resting fish were immediately euthanized via administration of an overdose of tricaine methane sulfonate (1.3 g MS222 and 2 g $NaHCO_3$, Syndel, Vancouver, BC) directly into the box and were then frozen using liquid N_2. The treatment groups were individually transferred to the exercise tank and manually chased to exhaustion. The exhausted group was immediately euthanized as described above, while the recovery groups were returned to their respective flux boxes. Each recovery group was euthanized as described above and frozen under liquid N_2 upon completion of the designated recovery time. All fish were sampled at the same time each day in an effort to

reduce any possible differences between sampling days and placed in an -80 ^{o}C freezer until analysis.

Analytical Measurements

Oxygen Concentration

Oxygen concentration (μmol/L) was measured using the Loligo Systems (DAQ-PAW-G4S) intermittent respirometry system. This system permits a user to designate a time interval to switch between recirculation or flow-through formats allowing either the collection of oxygen concentration data or flushing of the respirometer (Appendix. 1). During recirculation, the system functions as a closed loop where water from the respirometer is pumped past an inline galvanic oxygen probe (±0.1% accuracy) and back to the respirometer. The data from the oxygen probe were collected using a USB connection to a laptop computer and analyzed using AutoResp 4 respirometry software (Loligo Systems #AR12610).

Metabolite Analysis

Frozen whole animal or individual muscle, liver, and heart samples were individually ground to a fine powder in liquid N_2 with an insulated mortar and pestle to ensure that the aliquots taken were representative of the individual tissues or whole-body. Aliquots of white muscle (~100 mg), liver (80-150 mg) and heart (15-40 mg) were homogenized by vortexing for 5 min in 1.0 mL ice-cold 8% perchloric acid (PCA). Whole blood (100 μL) was mixed 1:2 with 8% PCA and set on ice. Aliquots of whole-animal (~100 mg) were homogenized for 5 min in 1.0 mL ice-cold 8% PCA using a tissue homogenizer, and those intended for glycogen/glucose/G6P analysis were set aside while the remainder were neutralized with 2.5 M K_2CO_3 and centrifuged at 5000 *g* for 5 min. Supernatants were then withdrawn and stored at -80 ^{0}C until analysis. All metabolites measured were run in parallel with known standards and all NAD^+/NADH coupled reactions were measured at a wavelength of 340 nm and absorbance read using a SPECTRAmax 340PC spectrophotometer (Molecular Devices, Sunnyvale, California, USA) (See Appendix. 2 for details of enzymatic reactions). Whole-body metabolites were standardized to a 30 g body weight as a relationship between whole-body metabolites and body mass was observed (Appendix 3).

Glycogen, Glucose-6-Phosphate (G6P) and Glucose. Aliquots of whole animal or separated tissue homogenates (200 uL) were taken for glycogen digestion and the remainder of the homogenate was centrifuged at 5000 *g* for 5 min to determine glucose/G6P. The acidic and neutralized glycogen homogenates were compared and acidic homogenates yielded increased recovery values. Acidic glycogen homogenates were incubated at 38^0C and digested to glucose using excess glucoamylase (2 mL, ≥10 kUL^{-1}, Sigma-Aldrich) in 0.2 M acetate-buffer (pH = 4.8) with shaking for two hours. The digestion reaction was terminated by addition of 1 mL 0.6 M PCA and centrifuged at 5000 *g* for 15 minutes. The supernatant was then neutralized with 1 M $KHCO_3$ and centrifuged again for 5 minutes at 5000 *g*. Both glycogen and glucose supernatants (50 uL) were added to a 2 mL cuvette and the first absorbance was recorded. 1 mL of reaction buffer (1 mM ATP, 0.9 mM βNAD^+, >700 U/L G6PDH) was then added. The increase in absorbance was followed until constant and the second absorbance was recorded. 5 uL of hexokinase (280 kU/L, Sigma-Aldrich) was then added and absorbance followed until stable when a final absorbance was recorded. The difference in absorbance after the addition of reaction buffer and after the addition of hexokinase will be proportional to the concentration of G6P and glucose, respectively (Bergemeyer 1965).

ATP/PCr. Separated tissue supernatants were neutralized with 3 M K_2CO_3 and centrifuged for 5 minutes at 5000 *g*. Neutralized supernatant was incubated in 2 mL buffer (G6PDH ≥2 kU/L Sigma-Aldrich, 0.01 M βNAD^+, pH= 8) for 10 minutes and the absorbance was read. 5 µL of hexokinase (HK) (1.5 $kUmL^{-1}$, Sigma-Aldrich) was then added and allowed to incubate for 20 minutes before a second absorbance reading was taken. 20 µL of 0.1 M adenosine diphosphate (ADP) was added and allowed to incubate for 10 minutes and a third absorbance reading was taken. 10 µL of creatine kinase (≥700 kUL^{-1}, Sigma-Aldrich) was added and allowed to incubate for 25 minutes and a final absorbance reading was taken. The difference in absorbance between incubation with G6PDH/ βNAD^+ buffer and addition of HK and between the addition of ADP and creatine kinase will be proportional to the concentration of ATP and CP, respectively (Bergemeyer, 1965).

Lactate. Supernatants and standards were assayed in glycine buffer (0.6 M glycine, 0.5 M hydrazine buffer, pH= 9.4), 2.5 mM NAD^+, and excess lactate dehydrogenase (LDH, ≥14 kUL^{-1}) (Bergemeyer, 1965). Cuvettes were incubated for 30 minutes at 37 ^oC and absorbance read at 340 nm.

Ethanol. Supernatant and standards were assayed in a clear 96-well plate using a pre-made kit (DIET-500, QuantiChrom^{TM}). The kit is based on the reduction of dichromate by ethanol and results in a bluish chromic product (Cr^{3+}). The intensity of color was measured by absorbance at 680nm and is proportional to concentration of ethanol (mM) (Jetter, 1950). Ethanol excretion was calculated by the following equations:

1. EtOH (umol) =
   (Concentration of EtOH (mM)*Relative Volume of Respirometer (L))/1000)
2. $\dot{V}$ EtOH (µmol EtOH/g*min) = (EtOH (umol)/body weight (g))/15 min

Statistical Analysis

Data are presented as means (±SEM) where N represents the number of fish used in each treatment group. Statistical analyses were performed using SPSS software. A repeated measures ANOVA followed by a post-hoc Tukey's HSD was used to determine statistical differences between the resting and recovery treatments in series 1. Based on the results of an F-test, a single factor one-way ANOVA assuming unequal variance followed by Dunnett's C test was used to determine statistical differences between the resting and recovery treatments for series 2 and 3. All graphical representations of data were constructed using R modeling software (version 2.14.2).

Chapter 3 - Results

Excretion of ethanol to the environment was not significantly different from rest until 1.75 to 2 hours of recovery when peak excretion was observed (Fig. 6, $p=0.04$). Oxygen consumption increased 2.5-fold immediately post-exercise compared to resting values and remained significantly elevated until 90 minutes of recovery ($p=0.052$) (Fig. 7).

The breakdown of glycogen resulted in the accumulation of the end products lactate and ethanol; however, the recovery and distribution patterns observed for these metabolites were different from one another. Even at rest, the muscle contained ethanol (31.19 ± 5.53 μmol/g tissue) and a similar concentration was observed in the blood (35.59 ± 2.06 μmol/mL) (Fig. 8a,c). Immediately post-exercise, white muscle and blood ethanol were significantly elevated by ~2-fold compared to resting values (Fig. 8a,c, $p=0.002$). Heart and liver ethanol were not significantly different from rest and remained relatively stable throughout 4 hours of recovery (Fig. 8b, d).

At exhaustion, white muscle, liver, heart and blood lactate were all significantly elevated post-exercise (Fig. 9). Muscle lactate (Fig. 9a) remained elevated, while liver (Fig. 9b) and blood (Fig. 9c) lactate peaked at 1.5 hours of recovery. Blood lactate remained significantly elevated throughout the 4-hour recovery period while muscle, liver and heart lactate (Fig. 9d) were no longer significantly different from rest by 2.5 hours of recovery.

At exhaustion, muscle ATP (Fig. 10a) and PCr (Fig. 10b) were both significantly decreased, by ~3 μmol/g tissue and ~5 μmol/g tissue, respectively, compared to resting values. Muscle ATP was no longer significantly different from resting values by 1 hour of recovery and remained relatively stable throughout the remainder of the recovery period. Muscle PCr was no longer significantly different from resting values after 15 minutes of recovery but continued to increase and by 1.75 hours of recovery, peak PCr content was significantly greater than resting values (Fig. 10b, $p=0.03$). PCr content was no longer significantly different from rest after 2 hours of recovery and remained stable throughout the remainder of the 4 hours of recovery observed.

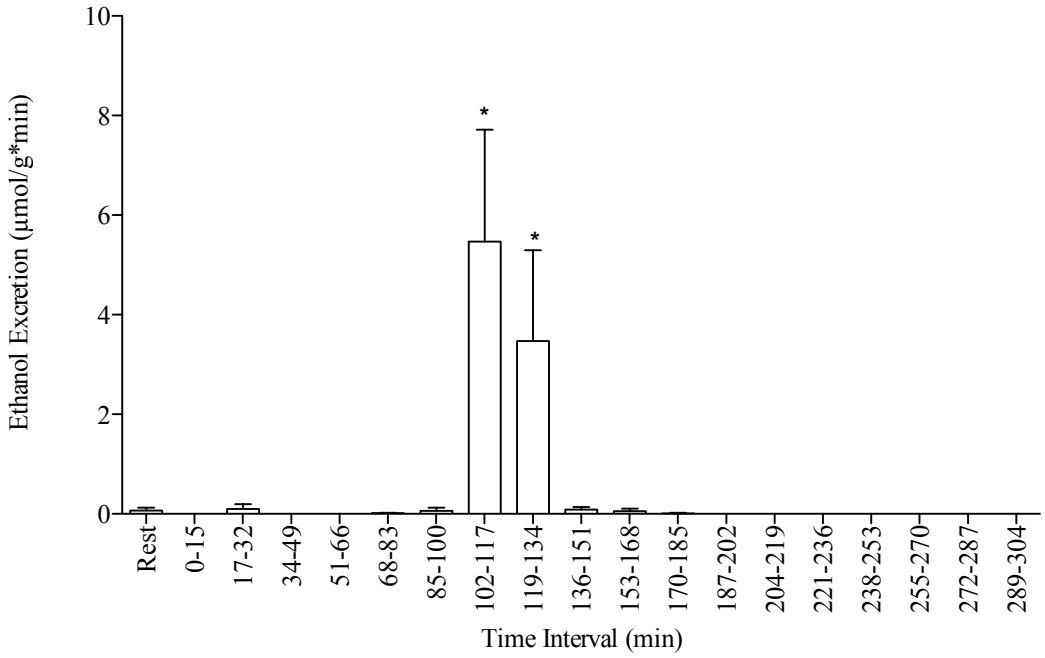

Figure 6: Ethanol excretion at rest (R) and throughout 4 hours of recovery from exhaustive exercise in grass carp. Asterisks (*) above data points indicate significant differences from resting condition (R) ($p < 0.05$). All values are presented as means ± S.E.M (n=6). Note: The difference of two minutes between each 15-minute interval displayed is as a result of the flushing cycle.

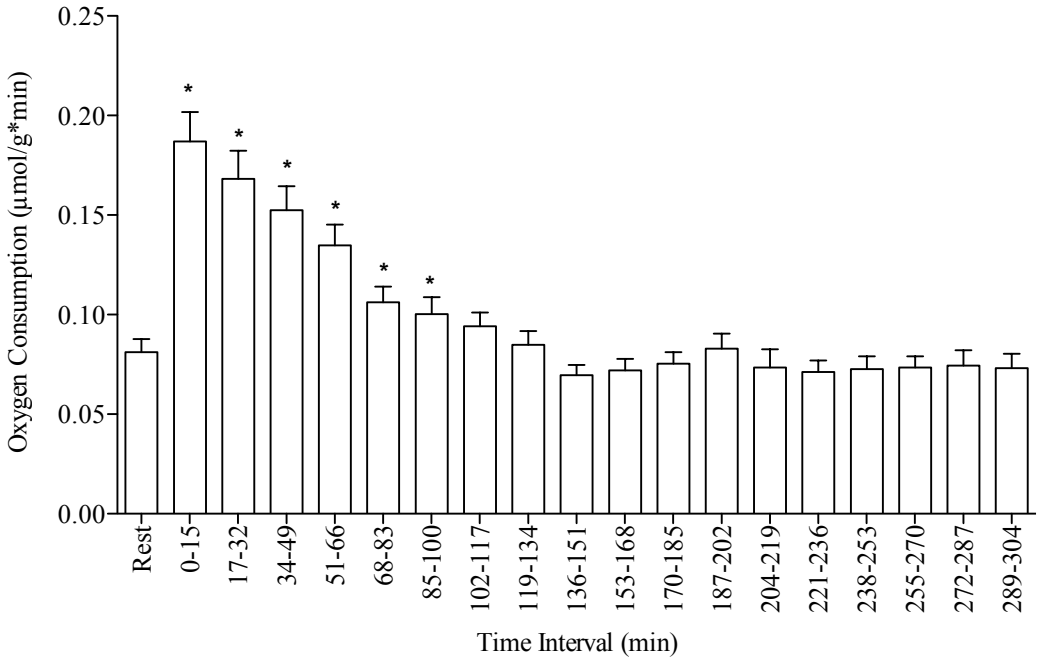

Figure 7: Oxygen consumption at rest (R) and throughout 4 hours of recovery from exhaustive exercise in grass carp. Asterisks (*) above data points indicate significant differences from resting condition (R) ($p < 0.05$). All values are presented as means ± S.E.M (n=6). Note: The difference of two minutes between each 15-minute interval displayed is as a result of the flushing cycle.

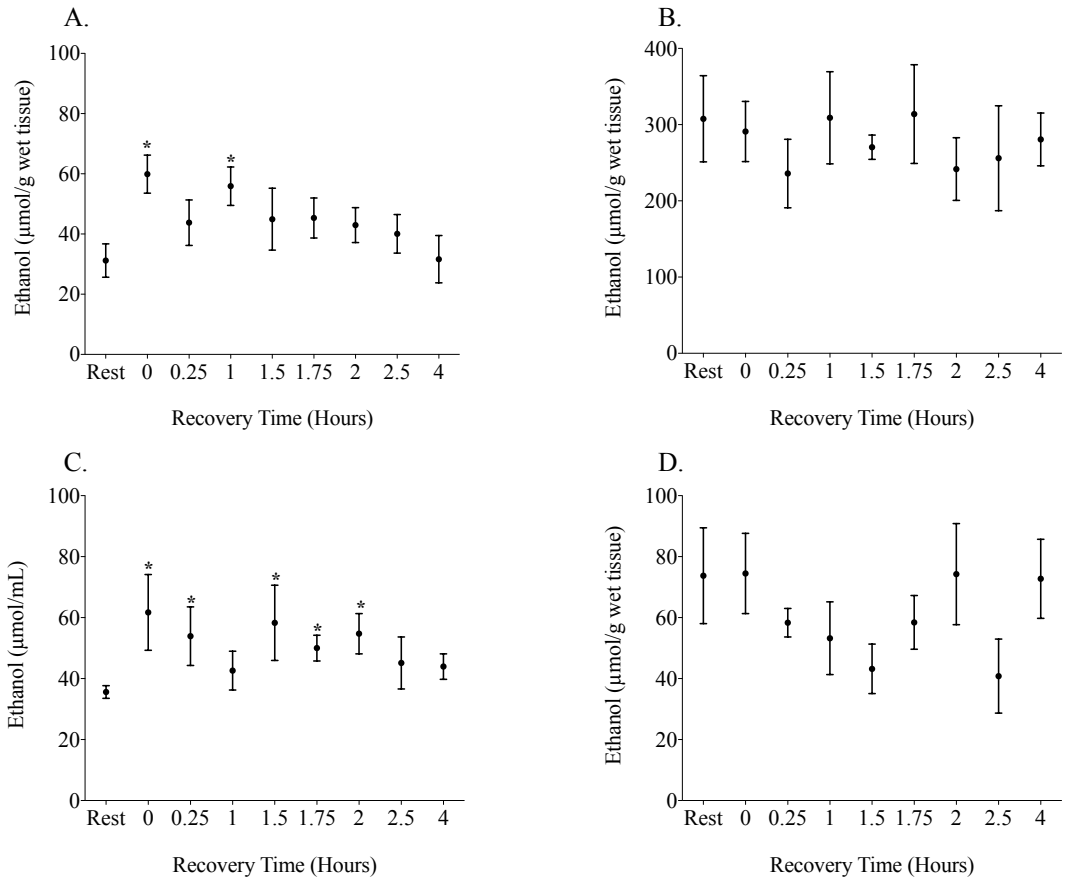

Figure 8: White muscle (A), liver (B), blood (C) and heart (D) ethanol at rest (R) and throughout 4 hours of recovery from exhaustive exercise in grass carp. Asterisks (*) associated with data points within a panel indicate significant differences from rest condition ($p < 0.05$). All values are presented as means ± S.E.M (n=6).

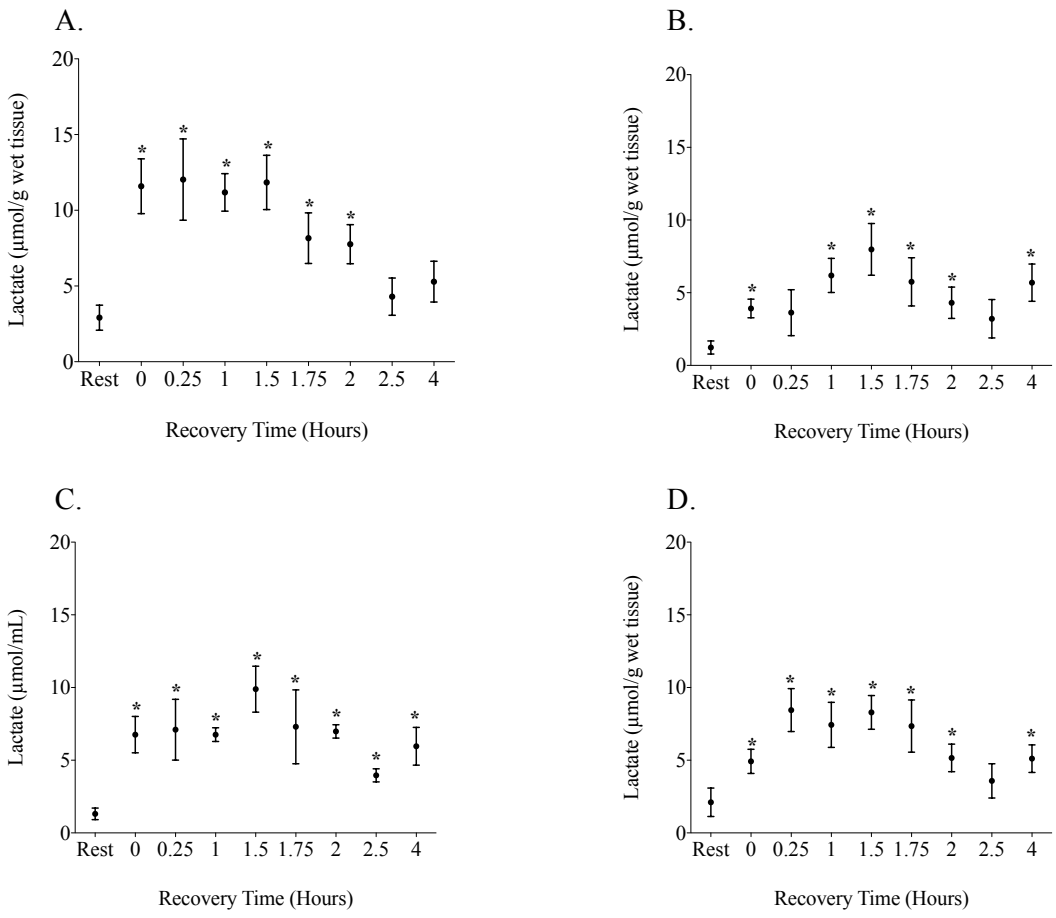

Figure 9: White muscle (A), liver (B), blood (C) and heart (D) lactate at rest (R) and throughout 4 hours of recovery from exhaustive exercise in grass carp. Asterisks (*) associated with data points within a panel indicate significant differences from rest condition ($p < 0.05$). All values are presented as means ± S.E.M (n=6).

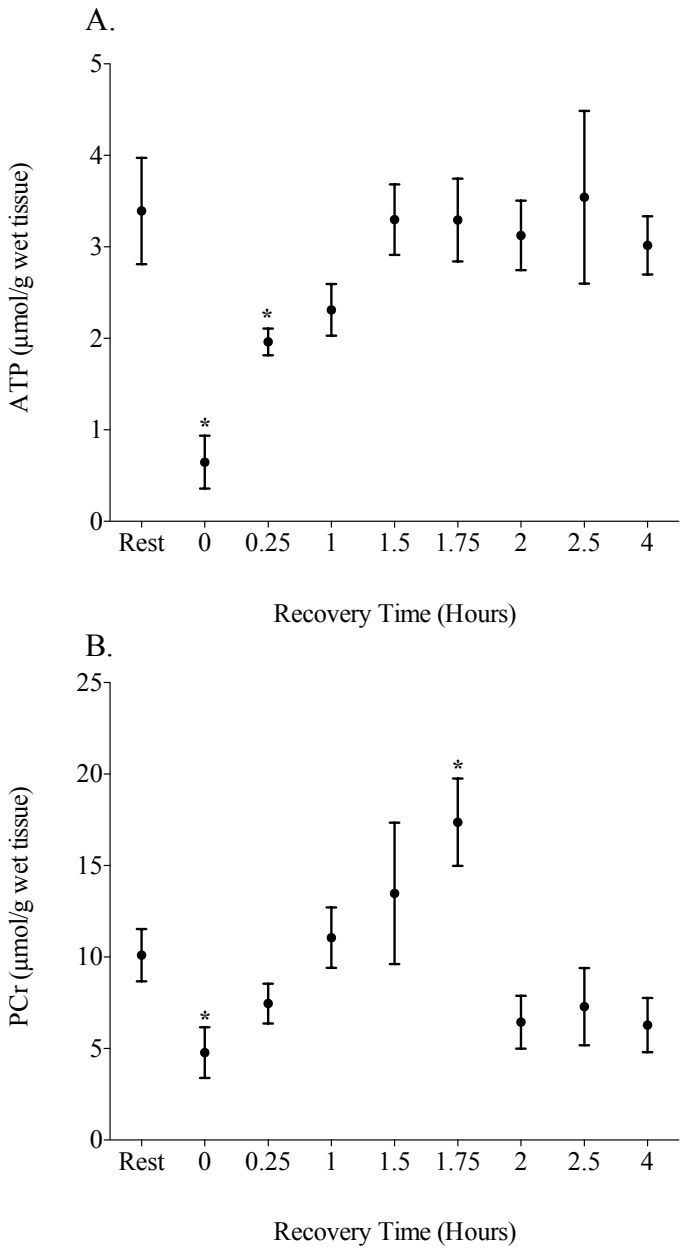

Figure 10: White muscle ATP (A) and PCr (B) at rest (R) and throughout 4 hours of recovery from exhaustive exercise in grass carp. Asterisks (*) associated with data points within a panel indicate significant differences from rest condition ($p < 0.05$). All values are presented as means ± S.E.M (n=6).

An ~80 % reduction in white muscle glycogen ($p=0.006$, Fig. 11a) immediately post-exercise compared to rest confirms that glycogen is the predominant substrate used to power exercise. Liver glycogen fluctuated throughout the recovery period; however, none of the observed changes during the 4-hour recovery period were statistically significant (Fig. 11b). White muscle, blood, and liver glucose were significantly increased immediately post-exercise compared to the resting value (Fig. 12). White muscle glucose (Fig. 12a) remained significantly elevated throughout 4 hours of recovery. Liver glucose returned to resting values within 15 minutes after exercise and remained relatively stable throughout the 4-hour recovery period (Fig. 12b), the decline of liver glucose can be followed by a concomitant rise in blood and heart glucose, while both blood and heart glucose returned to resting values by 2.5 hours (Fig. 12c & d).

My study indicated that the amounts of whole-body glycogen and glucose at exhaustion were significantly correlated with body weight (Appendix 3). All whole-body measurements were therefore standardized to 30 g body weight. The measurements of whole-body lactate (Fig. 13a) paralleled the pattern observed in separated tissues (Fig. 10) and remained significantly elevated through 2 hours of recovery. Whole-body ethanol synthesis (Fig. 13b) accounts for those changes observed in the white muscle tissue (Fig. 8a) but were significantly higher than the sum of the separated tissues selected for analysis; however, the total loss of ethanol from the whole-body (~3000 μmol) was still less than the total ethanol excreted (~3600 μmol) based on calculations for a 30 g fish. The measurement of both whole-body and tissue specific lactate and ethanol content confirm that ethanol was the major anaerobic end product. Whole-body measurements of glycogen (Fig. 14a) and glucose (Fig. 14b) were similar to those trends observed in

individual tissues, and, when the changes in whole-body metabolites are converted to glucosyl unit equivalents, the clearance of ethanol represents ~100% of the decrease in whole-body glycogen and glucose (Table 1).

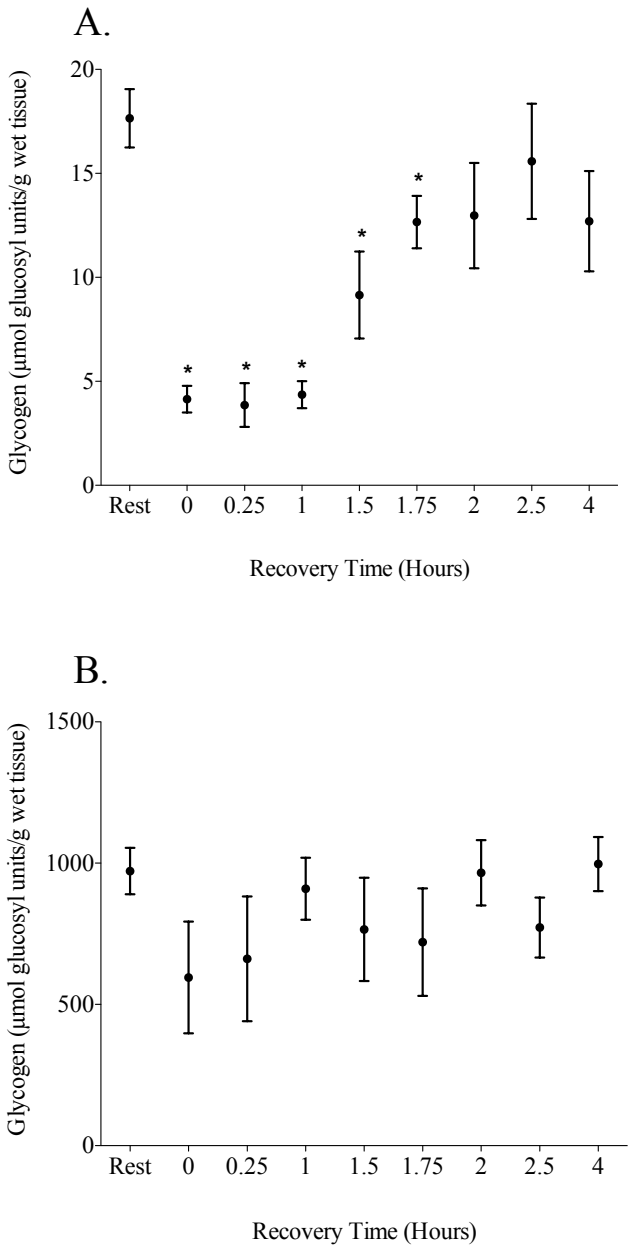

Figure 11: White muscle (A) and liver (B) glycogen at rest (R) and throughout 4 hours of recovery from exhaustive exercise in grass carp. Asterisks (*) associated with data points within a panel indicate significant differences from resting condition ($p < 0.05$). All values are presented as means ± S.E.M (n=6).

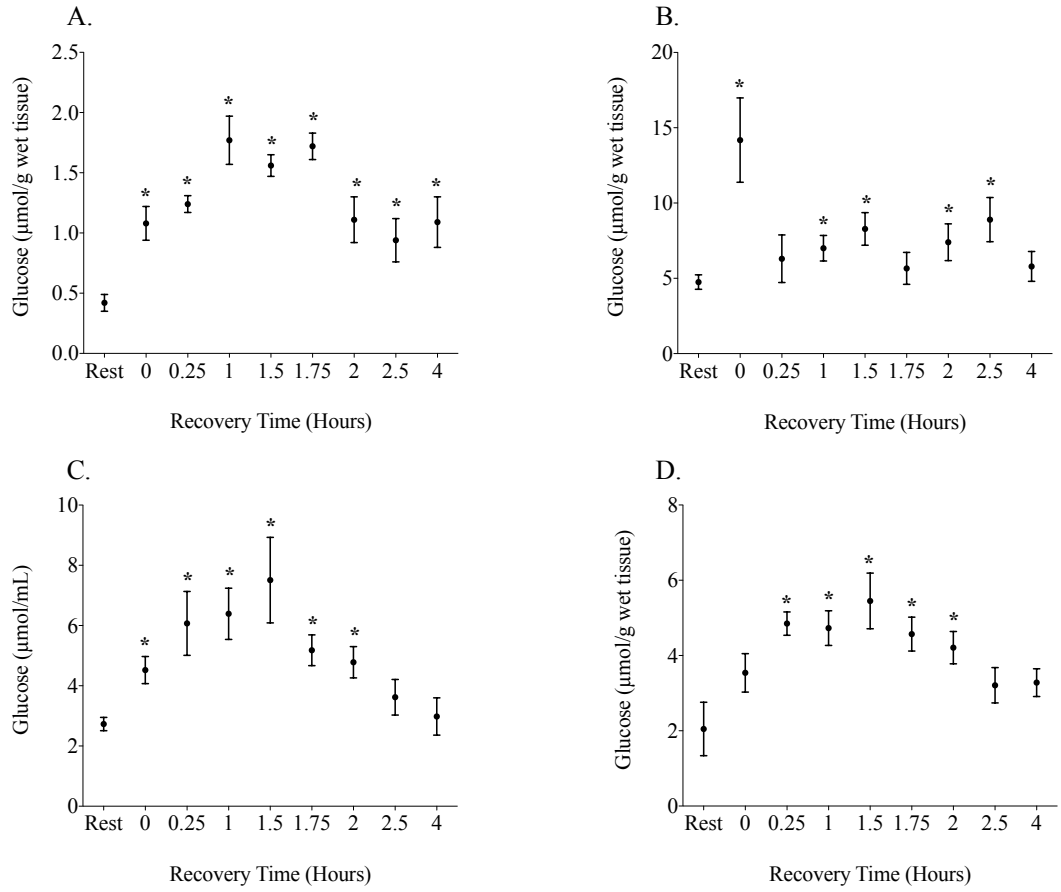

Figure 12: White muscle (A), liver (B), blood (C) and heart (D) glucose at rest (R) and throughout 4 hours of recovery from exhaustive exercise in grass carp. Asterisks (*) associated with data points within a panel indicate significant differences from rest condition ($p < 0.05$). All values are presented as means ± S.E.M (n=6).

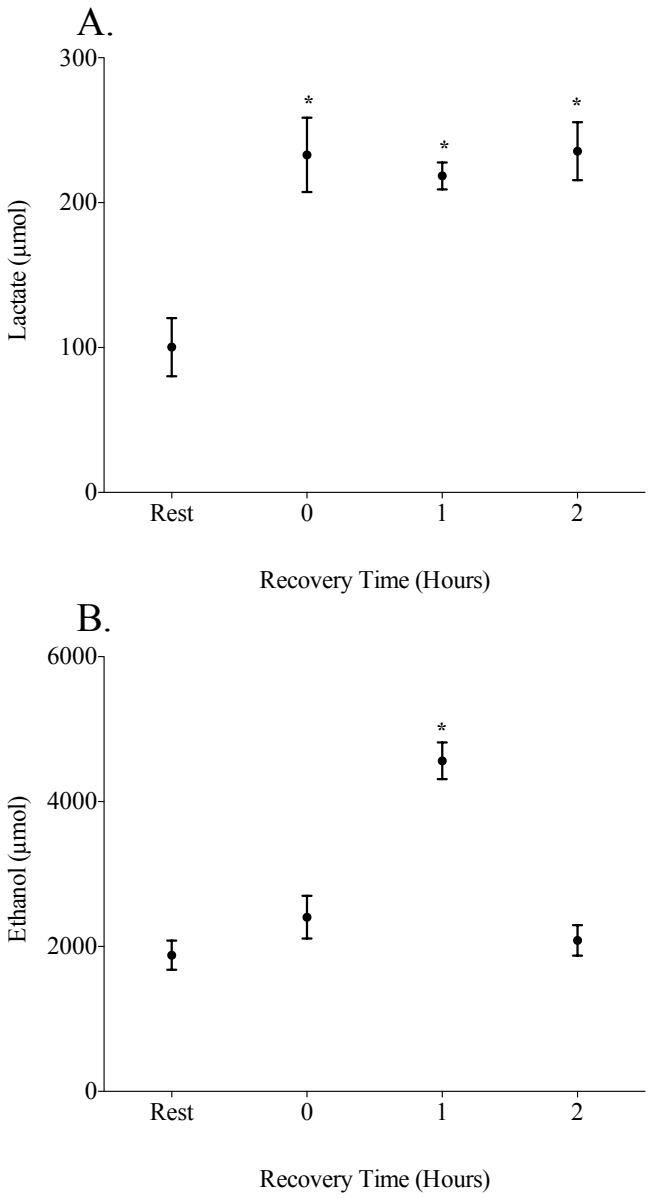

Figure 13: Whole-body lactate (A) and ethanol (B) at rest (R) and throughout 2 hours of recovery from exhaustive exercise in grass carp. Values are standardized to 30g body weight. Asterisks (*) associated with data points within a panel indicate significant differences from rest condition ($p < 0.05$). All value presented as means ± S.E.M (n=3)

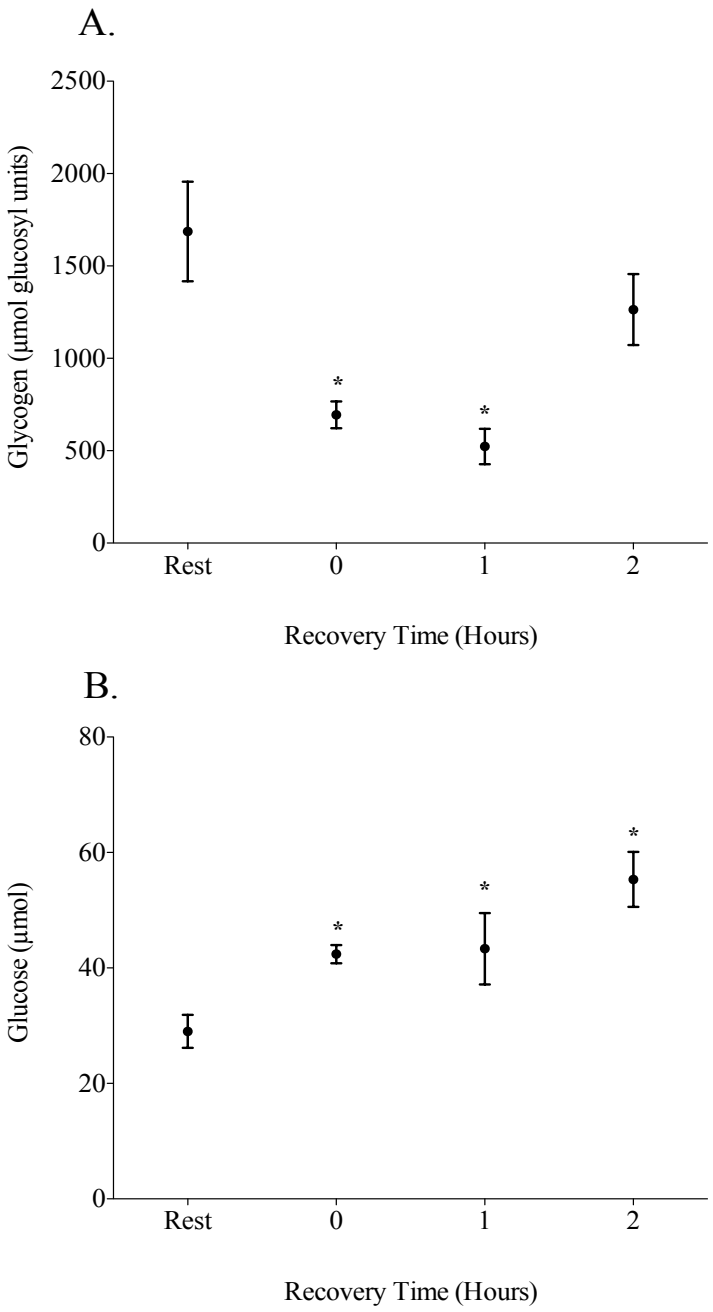

Figure 14: Whole-body glycogen (A) and glucose (B) at rest (R) and throughout 2 hours of recovery from exhaustive exercise in grass carp. Values are standardized to 30g body weight. Asterisks (*) associated with data points within a panel indicate significant differences from rest condition ($p < 0.05$). All values are presented as means ± S.E.M (n=3).

Table 1: Percentage of the whole body glycolytic pool lost as ethanol as a consequence of exercise in grass carp. All metabolites are standardized to the weight of a 30g fish as a significant correlation between body mass and metabolites was found. All metabolites are represented as glucose equivalents.

For a 30g Fish	**Glycogen (µmol)**	**Glucose (µmol)**	**Ethanol (µmol)**	**Lactate (µmol)**
Rest	1686.49	29.02	940.06	50.13
Time 0	694.32	42.39	1202.55	116.48
1 Hour Recovery	522.77	43.33	2281.58	109.24
Total Change	-1164.49	14.31	1341.58	66.35
% of glycolytic pool			113.81	5.63
1 Hour Recovery			2281.58	109.24
2 Hour Recovery			1042.42	117.76
Total Change			-1239.16	8.52
% Glycolytic Pool Lost as Ethanol			106.41	

Chapter 4 - Discussion

Despite the occurrence of peak ethanol concentration immediately post-exercise in the muscle and blood (Fig. 8a, Fig. 8c), ethanol excretion to the environment was not seen until 1.5-2 hours into recovery (Fig. 6). This disconnect between synthesis and excretion is further magnified as increased oxygen consumption (Fig. 7), and associated increases in gill perfusion, lamellar recruitment, stroke volume and heart rate as a result of exhaustive exercise (Evans et al., 2005) would be expected to facilitate ethanol efflux from the gill upon synthesis and diffusion from the muscle to the plasma. Although the timing of ethanol clearance from the muscle (Fig. 8a) to the blood (Fig. 8c) was similar to that observed in excretion (Fig. 6), the amount excreted per 30g fish (3600 μmol) was significantly more than the sum of ethanol loss from the individual tissues (~1180 μmol ethanol, Fig. 8). Further measurements using the whole animal revealed that a total of ~ 3000 μmol of ethanol were lost from the whole-body (Fig. 13b) and therefore accounts for the majority of observed excretion to the environment (Fig. 6). Furthermore, when calculated as a percentage of the whole-body glycolytic pool (Table 1), the clearance of ethanol accounts for ~100% of glycolytic substrates used as a consequence of exercise, and therefore represents a significant glycolytic cost to the grass carp. The temporal difference between ethanol excretion and its synthesis in the tissues measured may reflect the possibility of ethanol retention and or storage. Retention and pulsatile ethanol excretion has also been observed in goldfish at similar recovery time points in response to exhaustive exercise (Mandic et al., 2008); however, the tissues responsible for retention and/or trigger for release have yet to be identified.

Immediately post-exercise, the glycolytic end products ethanol and lactate were synthesized (Fig. 8, 9). However, as the increase in white muscle ethanol (Fig. 9a) was ~3.5-fold that of muscle lactate (Fig. 9a), ethanol is confirmed as the major glycolytic end product of exhaustive exercise. A smaller (~2-fold) difference in the post-exercise accumulation of ethanol compared to lactate has been observed in goldfish (*Carassius auratus),* and is supported by the difference in white muscle ADH and LDH activity, 14 μmol/g/min and 7.47 μmol/g/min at 25 ^{o}C, respectively (Johnston et al., 1975; Nilsson, 1988). Therefore, it may be that a difference in ADH and LDH activity is also

responsible for the ~3.5-fold difference in white muscle ethanol and lactate accumulation observed in grass carp.

Following exercise, a reduction of whole-body glycogen by ~1000 μmol glucosyl units (Fig. 14a), would be expected to yield ~2000 μmol of lactate and/or ethanol equivalents. However, at exhaustion, only ~1/3 of that amount is observed to accumulate in the whole-body (~130 μmol lactate, ~525 μmol ethanol, Fig. 13a, b). Using the assumption that a 30 g fish possess' approximately 18 g of muscle, 1.4 g of liver, 0.02 g of heart and 1.5 mL of blood, this discrepancy between glycogen breakdown and end product accumulation is reduced to ~1/2 of expected or ~3.71 μmol glucosyl units/g tissue (ethanol ~540 μmol, Fig. 8; lactate ~168 μmol, Fig. 9). At exhaustion, the cytoplasmic concentration of Ca^{2+} remains elevated as a result of muscle contraction and potentially supports an increased availability of fish muscle GLUT transporters (Ihleman et al., 1999). An increased availability of muscle GLUT transport could support the observed increase in muscle glucose (Fig. 12a) supplied by the breakdown of glycogen to glucose in the liver (Fig. 11b, Fig. 12b) during recovery. Furthermore as muscle ATP was depleted at exhaustion (Fig. 8), the increase in muscle AMP would act to promote phosphofructokinase and direct any glucose taken up by the muscle towards the glycolytic end products lactate or ethanol. Therefore the changes in whole-body glycogen and glucose collectively account for the appearance of the end products lactate and ethanol.

Interestingly, the patterns of ethanol synthesis and accumulation were also different when whole-body and tissue measurements are compared. Peak ethanol accumulation occurred immediately post-exercise in the white muscle (Fig. 9a) and blood (Fig. 9c). However, ethanol content for the whole-body was not significantly different from rest until 1 hour of recovery (Fig. 13b). Upon closer inspection, while the changes in white muscle and blood ethanol content were significant within the respective tissues, the total change in ethanol for all the tissues measured between rest and exhausted is only ~530 μmol (Fig. 9). In comparison, the change in whole-body ethanol between rest and exhausted are actually quite similar, ~525 μmol, although not significant with respect to whole-body ethanol content (Fig. 13b). Furthermore, the substantial increase in whole-

body ethanol after 1 hour of recovery (~2500 μmol) would suggest that an unmeasured tissue is of significant importance to whole-body ethanol synthesis. In goldfish, red muscle fibers occupy approximately 10% of the myotome (Johnston and Lucking, 1978) and have an ADH activity (31.7 μmol/g/min) approximately 3.5-fold greater than that of the white muscle fibers. If this estimation of myotome composition holds true in grass carp, then red muscle would account for approximately 1.8 g of a 30 g fish. The red muscle would therefore have the potential to synthesize ~ 3500 μmol of ethanol over the period of one hour and is of potential interest in identifying the source of the observed increase in whole-body ethanol synthesis during recovery.

By using the existing knowledge of exercise recovery in salmonid models and incorporating the results of this study, it is suggested that the release of stress hormones (catecholamines and cortisol) act to supply FAs and BCAAs that may in part be used to support the observed muscle and whole-body glycogen re-synthesis (Fig. 11a, Fig. 14a). The release of catecholamines stimulates the release of FAs and glycerol through activation of PKA and subsequent stimulation of hormone sensitive lipase (HSL) (Fukao et al., 2004; Fig. 3). Extracellular glycerol has the potential to act as a glycogenic substrate, although its contribution to the skeletal muscle glycogen pool in rainbow trout is limited in preference of lactate (Kam and Milligan, 1996). If exhaustive exercise resulted in similar cytosolic glycerol accumulation as in rainbow trout (0.1μmol/mL; Richards et al., 2002b) then glycerol has the potential to contribute only ~6% of the observed glycogen replenishment. However, this may be an underestimation of the contribution of glycerol as the post-exercise accumulation of muscle lactate in rainbow trout (~25 μmol/g wet tissue, Richards et al., 2002a) is more than twice that observed in grass carp (~9 μmol/g wet tissue, Fig. 10a). This difference in lactate accumulation between grass carp and rainbow trout may therefore alter the contribution of glycerol as a potential glycogenic substrate and in part explain the glycogen replenishment observed during recovery (Fig. 11a, Fig. 14a). Furthermore, the oxidation of FAs in the liver in the presence of elevated glucose (Fig. 12b) creates the potential for hepatic acetyl-CoA concentration to exceed the oxidative capacity of the TCA (Fukao et al., 2004). The accumulation of hepatic acetyl-CoA would promote ketogenic release of acetoacetate and/or 3-hydroxybutarate to circulation. Once in circulation, these ketone bodies may be

used as a source of muscular acetyl-CoA and therefore oxidative ATP synthesis. As a result of FA oxidation, the increased concentration of acetyl-CoA within the muscle mitochondria likely inhibits PDH and therefore the oxidation of any pyruvate formed as a result of increased concentration of glycerol. Therefore, the oxidation of FAs may, in part, support both the substrate and energetic needs required for the observed muscular glycogen re-synthesis (Fig. 11a).

In rainbow trout (*Oncorhynchus mykiss*), the release of cortisol as a result of inactivity during recovery from exercise stimulates hepatic proteolysis causing the release of BCAAs to circulation (Milligan, 1997), and has been identified as a primary stress response hormone in grass carp (Yavuzcan-Yildiz and Kirkagac-Uzbilek, 2001). Once taken up by the muscle, BCAAs supply glutamate and the respective branched-chain keto acid that may act as an additional source of oxidative ATP, while glutamate and excess pyruvate are converted to alanine. Once in the plasma, alanine supports hepatic gluconeogenesis and release into circulation (Milligan, 1997) supporting the observed elevation of blood (Fig. 10c), liver (Fig. 10b), and whole-body glucose (Fig. 14b). However, the contribution of blood glucose to muscle glycogenesis may be limited as relatively little evidence exists for the use of specific GLUT transporters in fish. The only evidence of potential increase and availability of GLUT transporters is a consequence of the increased cytoplasmic concentration of Ca^{2+} as a result of muscular contraction (Ihleman et al., 1999). If the appropriate GLUT transporters were present in grass carp white muscle, circulatory glucose upon uptake to the muscle could be available as a glycogenic substrate via phosphorylation to G6P by HK (Milligan, 1997) (Fig.2). Furthermore, rainbow trout and grass carp have a similar profile of muscular glycogen replenishment at ~2 hours of recovery (Fig. 9a) when the levels of circulatory cortisol are reduced (Milligan 1996). If cortisol plays a similar role in stimulating BCAA release and inhibiting muscular glycogen replenishment in grass carp during recovery then it potentially explains the pattern of observed glycogen replenishment observed at ~2 hours of recovery.

Overall, it is clear that the grass carp white muscle is functionally different from that of other fish species, like rainbow trout. The synthesis of ethanol as the predominant

end product occurs in both hypoxia and exhaustive exercise as a result of anaerobic metabolism. The metabolic depression associated with hypoxia tolerance allows glycogen stores to be conserved, while ethanol synthesis permits the clearance of anaerobic extra-muscular lactate. The excretion of ethanol under hypoxic conditions therefore represents a relatively small loss of glycolytic substrate in exchange for maintaining activity levels. However, during burst exercise, where the rate of metabolism and therefore glycogen depletion is greatly increased, the synthesis and excretion of ethanol represents a significant loss from whole-body glycolytic pool to the environment. The oxidation of FAs, and BCAAs in combination with liver glycogen stores could supply the ATP requirements and substrates required for the observed replenishment of the muscular glycogen stores during recovery. However, after repeated bouts of exercise the liver's capacity to support muscle glycogen replenishment would likely be dependent on replenishing its own glycogen stores through feeding.

Chapter 5 - Conclusions and Future Work

The results of the present study indicate that in response to exhaustive exercise, grass carp (*Ctenopharyngodon idella*) use glycogen as the primary energetic substrate for anaerobic glycolysis resulting in ethanol as the predominant glycolytic end product. As the synthesis of ethanol exceeds that of glycogen depletion, glucose may also contribute to post-exercise ethanol synthesis as a result of increased GLUT transporter availability and phosphofructokinase activation in response to the cytosolic ATP/AMP ratio. The differences observed in the amounts of ethanol synthesized between tissue specific and whole-body measures may be linked to a difference in ADH activity between red and white muscle fibers.

Interestingly, all of the ethanol synthesized is excreted to the water over a 30-minute period, after approximately 1.75 hours of recovery from exhaustive exercise. The clearance of ethanol from the whole-body accounts for ~100% of glycolytic substrates used as a consequence of exercise, and therefore represents a significant cost in terms of lost carbon from the grass carp's glycolytic pool. Future experiments involving the dissection and *in vitro* perfusion of grass carp gills with ethanol may yield some explanations as to the retention of ethanol and/or the trigger for its release during recovery. Furthermore, dividing the recovery tank to separate ethanol excretion from the gills compared to that lost through urination may also be of benefit in further isolating the site responsible for ethanol retention and/or excretion.

It is suggested that stress hormones and their stimulation of hepatic proteolysis and BCAA/FA/ketone body oxidation may play a role in supporting the use of liver supplied glycolytic carbon for the observed replenishment of the muscle glycogen stores. It is assumed that multiple bouts of exercise would deplete the grass carp's liver glycogen stores and therefore require feeding to replenish whole-body glycolytic pools. The results of my study provide insight into the distinct differences between the herbivorous grass carp and the carnivorous, salmonid models of exercise physiology. The grass carp's access to a carbohydrate rich diet may offset the requirement to retain glycolytic end products as substrates for muscle glycogen replenishment as observed in the rainbow

trout. This research provides the initial calculations and findings for generating a comprehensive model of recovery from exhaustive exercise in these hypoxia-tolerant cyprinids.

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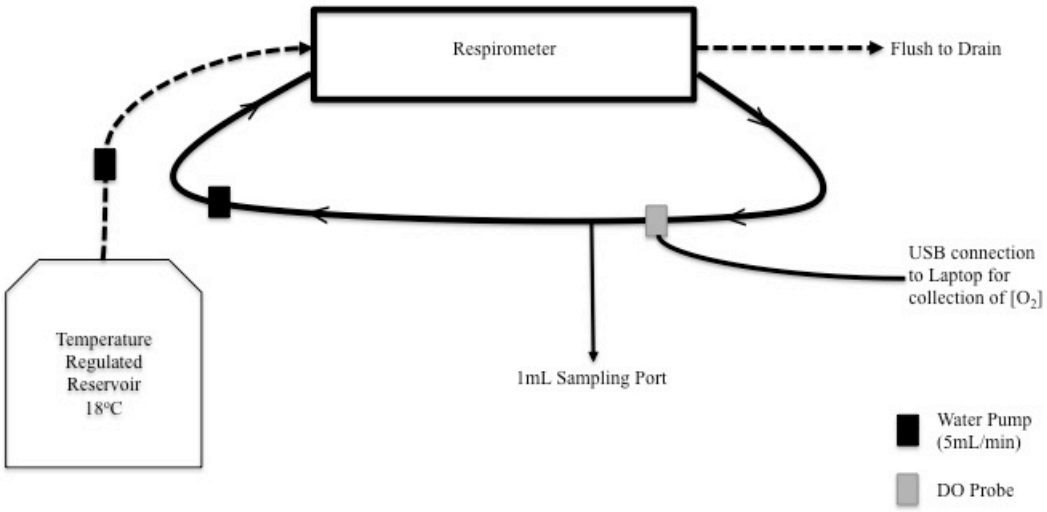
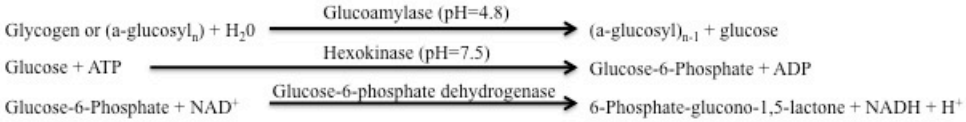
Appendices

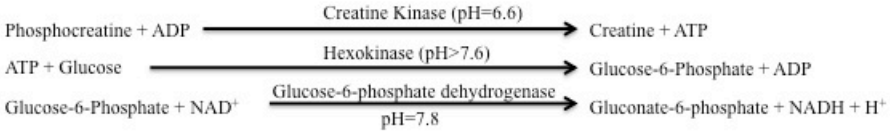


Appendix 1: Schematic of Qubit Systems intermittent respirometry apparatus with sampling port and USB connection allowing collection water samples and O_2 concentration data.

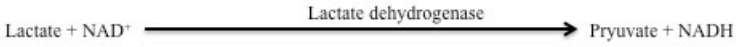
A. Glycogen/Glucose/G6P



B. ATP/PCr

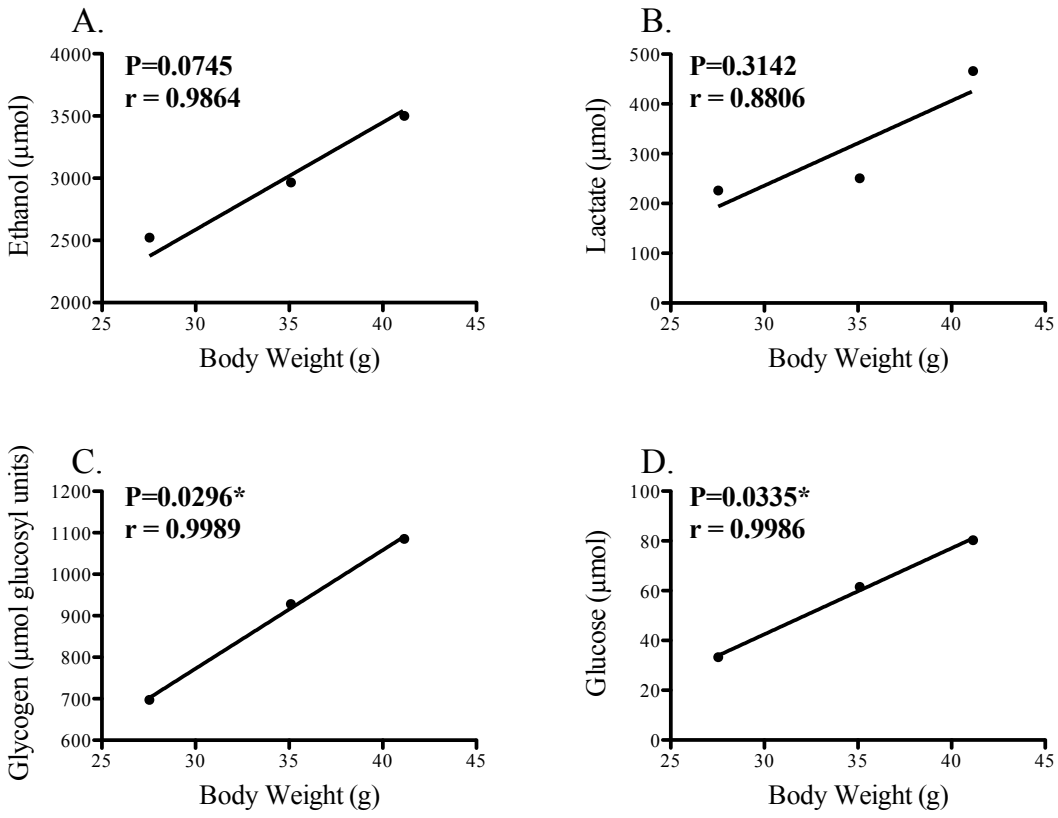


C. Lactate

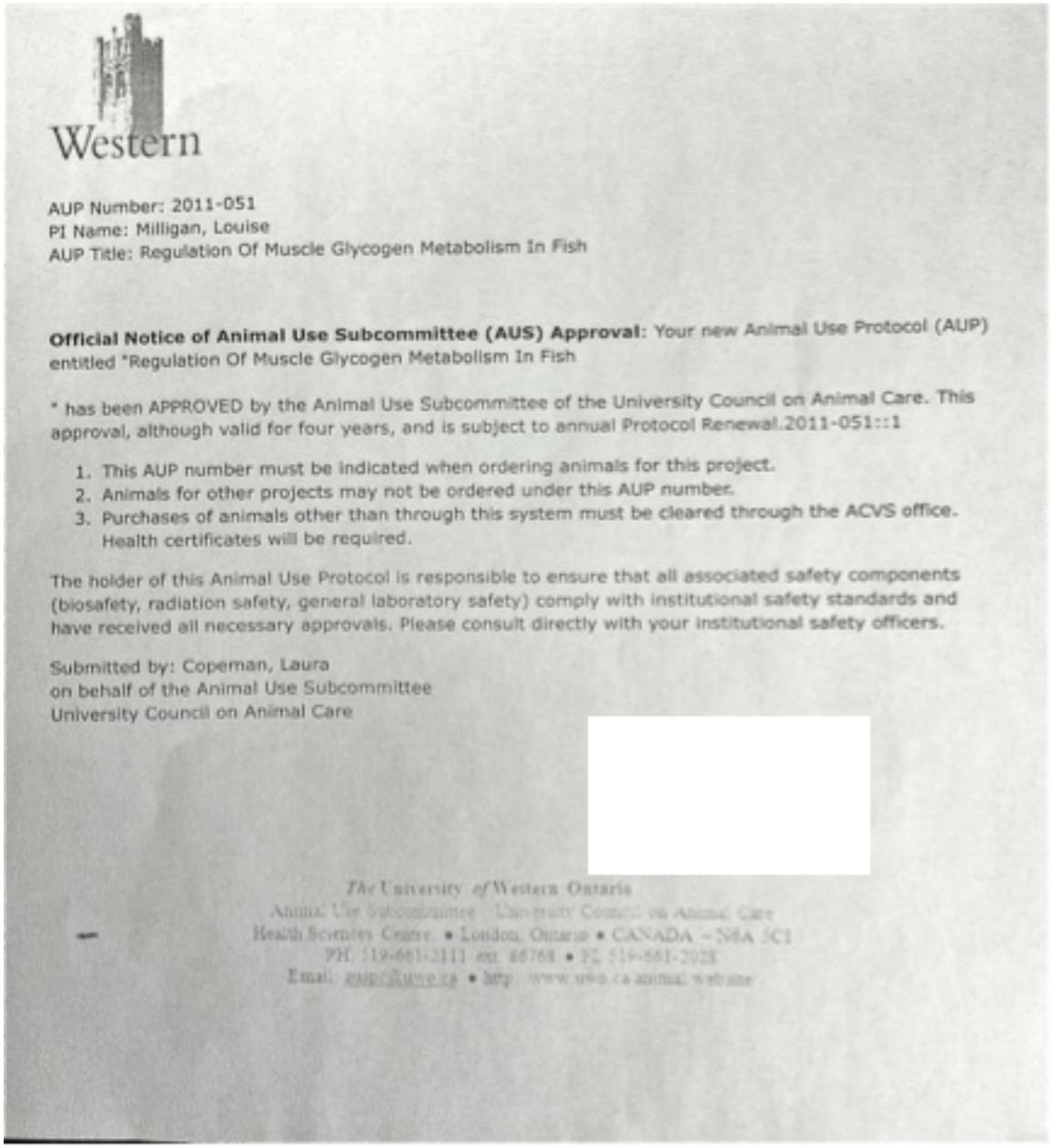


D. Ethanol

Appendix 2: Details of chemical reactions used for the basis of metabolite analysis.



Appendix 3: The relationship of grass carp body weight (g) with post-exercise metabolites (ethanol (A), lactate (B), glycogen (C) and glucose (D)). Asterisks associated with P values within a panel denote significant correlation (P<0.05).



Western

AUP Number: 2011-051
PI Name: Milligan, Louise
AUP Title: Regulation Of Muscle Glycogen Metabolism In Fish

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Regulation Of Muscle Glycogen Metabolism In Fish

* has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2011-051::1

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

The University of Western Ontario
Animal Use Subcommittee / University Council on Animal Care
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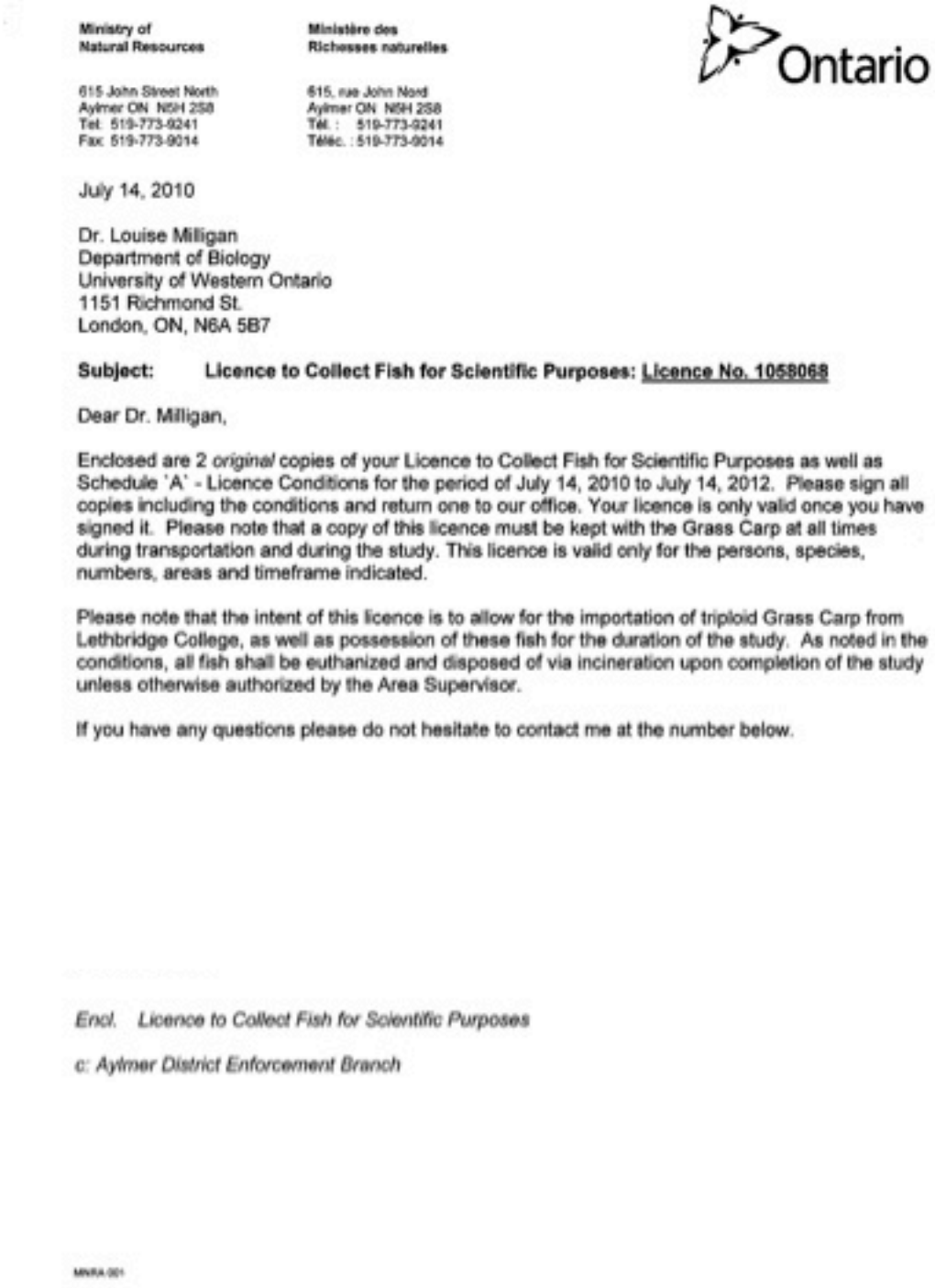
Email: auspc@uwo.ca • http: www.uwo.ca/animal/website

Appendix 4: Animal husbandry protocol for care and use of grass carp. Granted to Dr. C.L. Milligan, UWO London.



Ministry of Natural Resources

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July 14, 2010

Dr. Louise Milligan
Department of Biology
University of Western Ontario
1151 Richmond St.
London, ON, N6A 5B7

Subject: Licence to Collect Fish for Scientific Purposes: Licence No. 1058068

Dear Dr. Milligan,

Enclosed are 2 *original* copies of your Licence to Collect Fish for Scientific Purposes as well as Schedule 'A' - Licence Conditions for the period of July 14, 2010 to July 14, 2012. Please sign all copies including the conditions and return one to our office. Your licence is only valid once you have signed it. Please note that a copy of this licence must be kept with the Grass Carp at all times during transportation and during the study. This licence is valid only for the persons, species, numbers, areas and timeframe indicated.

Please note that the intent of this licence is to allow for the importation of triploid Grass Carp from Lethbridge College, as well as possession of these fish for the duration of the study. As noted in the conditions, all fish shall be euthanized and disposed of via incineration upon completion of the study unless otherwise authorized by the Area Supervisor.

If you have any questions please do not hesitate to contact me at the number below.

Encl. Licence to Collect Fish for Scientific Purposes

c: Aylmer District Enforcement Branch

MNRA 001

Appendix 5: License to collect a restricted fish (Grass carp, *Ctenopharyngodon idella)* for the purpose of scientific research. Granted to C.L. Milligan, UWO London.

Curriculum Vitae

Mr. Simon Bradford

A. Date Curriculum Vitae Prepared: December 16, 2012

1. EDUCATION

Sep. 2010 – Dec 2012	M.Sc. Biology – Specialization in Animal Physiology Faculty of Biology, University of Western Ontario London, Ontario, Canada. Supervisor: Dr. Louise Milligan. Project Title: "Exhaustive exercise and recovery in grass carp (*Ctenopharyngodon idella*)".
Sep. 2004 – Apr. 2010	H.BSc. - Specialization in Biology Faculty of Biology, University of Western Ontario London, Ontario, Canada.

2. RESEARCH, TEACHING AND EMPLOYMENT

Sep. 2010 – Dec. 2012	Teaching Assistant Department of Biology, University of Western Ontario Courses: Biology, Animal Physiology, Genetics and Advanced Genetics
Apr. 2010 – Sep. 2010	Summer Research Assistant Supervisor: Dr. Louise Milligan
Sep. 2009 – Apr. 2010	Honours Research Thesis Department of Biology, University of Western Ontario Supervisor: Dr. Louise Milligan Project Title: "The Ability of Grass Carp (*Ctenopharyngodon idella*) to Alleviate Winter Driven Hypoxic Stress Through Anaerobic Ethanol Production"
Apr. 2009 – Sep. 2009	Work Study Student Department of Biology, University of Western Ontario Supervisor: Dr. Sashko Damjanovski

3. PRESENTATIONS

Oct. 2012	Presentation: 3rd Annual Biology Graduate Research Forum. London, Ontario Title: "Exhaustive Exercise and Recovery in Grass Carp (*Ctenopharyngodon idella*)".

Feb. 2012	Presentation: Annual Comparative Physiology and Biochemistry Workshop. Peterborough, Ontario Title: "Exhaustive Exercise and Recovery in Grass Carp (*Ctenopharyngodon idella*)".
Apr. 2010	Presentation: Undergraduate Biology Day London, Ontario. Title: "The Ability of Grass Carp (*Ctenopharyngodon idella*) to Alleviate Winter-Driven Hypoxic Stress Through Anaerobic Ethanol Production".

4. EXTRACURRICULAR ACTIVITIES

Nov 2009 – Present	Volunteer: Children's Wish Foundation Car Rally. Raise funds for the Children's Wish Foundation, silent auction organization and on-site fundraising.

5. TRAINING AND CERTIFICATIONS

Safe Campus Community Training
Accessibility for Ontarians with Disabilities (AODA) Training
Comprehensive WHMIS Certification
Nuclear Radiation Safety Certification
Lab Safety and Hazardous Waste Disposal Certification