METABOLIC CAPACITIES OF THE AFRICAN CICHLID *PSEUDOCRENILABRUS MULTICOLOR VICTORIAE*: DO DIFFERENT POPULATIONS DISPLAY THE SAME RESPONSE UNDER DIVERGENT DISSOLVED OXYGEN REGIMES?

by

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ABSTRACT

This thesis examines the metabolic capacity of an African cichlid, *Pseudocrenilabrus multicolor victoriae*, in divergent aquatic oxygen environments. In a field study, 20 *P. multicolor* from four different sites, in two different regions of Uganda (Mpanga River, Lake Nabugabo and satellite lakes) were analyzed to determine the metabolic capacity in individuals from habitats strongly divergent in dissolved oxygen (D.O.) levels (2.23 ± 1.15 to 7.53 ± 0.13). By analyzing enzyme activity of pyruvate kinase (PK), lactate dehydrogenase (LDH), citrate synthase (CS) and cytochrome C oxidase (CCO) in white skeletal muscle, heart, brain and liver, and malate dehydrogenase (MDH) and fructose 1,6-bisphosphotase (FBPase) in the liver, it was observed that the metabolic capacity differed between the two different geographic regions, Mpanga and Nabugabo, however sites within each region displayed little variation in enzyme activity. These results suggest that there may be a genetic component to metabolic capacity of *P. multicolor*.

Under laboratory conditions, the F_1 offspring from three different sites within the Mpanga region were reared under normoxia or hypoxia for one year. Analysis of six different enzymes from the carbohydrate metabolic pathway from four tissues (muscle, heart, brain and liver) indicated that the effect of dissolved oxygen was greater than the site of origin *within* this drainage.

Together these results highlight the importance of integrating field and lab studies to fully understand the interaction of phenotypic plasticity and long-term selection in physiological adaptation to long-term hypoxia exposure.

RÉSUMÉ

Cette thèse examine la capacité métabolique d'un cichlidé africain, *Pseudocrenilabrus multicolor victoriae,* dans des environnements aquatiques à régime en oxygène dissemblables. Dans cette étude, 20 *P. multicolor* provenant de quatre sites différents, dans deux régions différentes d'Ouganda (rivière Mpanga, Lac Nabugabo et lacs satellites), ont été analysés afin de déterminer la capacité métabolique des individus provenant des habitats fortement divergents dans leurs niveaux en oxygène dissous (D.O.) (2.23 ± 1.15 à 7.53 ± 0.13). En analysant l'activité des enzymes pyruvate kinase (PK), lactate déshydrogénase (LDH), citrate synthase (CS) et la cytochrome C oxydase (COX) dans le muscle squelettique blanc, le cœur, le cerveau et le foie, ainsi que la malate déshydrogénase (MDH) et la fructose 1,6-bisphophatase (FBPase) dans le foie, il a été observé que la capacité métabolique diffère entre les deux régions géographiques, Mpanga et Nabugobo, cependant, les sites dans chacune de ces régions présentent peu de variations dans l'activité enzymatique. Ces résultats suggèrent que la composition génétique des individus peut être entrain de déterminer la capacité métabolique de *P. multicolor.*

Dans des conditions de laboratoire, les rejetons F_1 provenant de trois sites différents de la région de Mpanga, ont été élevés dans des conditions de normoxie ou d'hypoxie pendant une année. L'analyse de six différentes enzymes de la voie métabolique des carbohydrates à partir de quatre tissus (muscle squelettique blanc, cœur, cerveau, foie) indique que l'effet de l'oxygène dissous était plus important que le site d'origine dans ce bassin de drainage.

Ces résultats soulignent l'importance d'intégrer les études de terrain et de laboratoire afin de mieux comprendre l'interaction entre la plasticité phénotypique et la sélection à long terme dans le contexte de l'adaptation physiologique à l'exposition à l'hypoxie à long terme.

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CHAPTER 1: GENERAL INTRODUCTION

The increasing frequency and intensity of aquatic hypoxia (low dissolved oxygen (D.O.)) induced by human activities has become an environmental stressor on a global scale (Diaz 2001; Chabot and Claireaux 2008). Thousands of square kilometres of water are affected by hypoxia worldwide, and this phenomenon will continue to worsen with increasing anthropogenic activities as well as the concomitant effects of global climatic change (Diaz 2001; Wu 2002; Chabot and Claireaux 2008; Pörtner and Farrell 2008; Somero 2010). Global climatic change is not only resulting in a rise in temperature (Carpenter *et al.* 1992; Dubravko *et al.* 1997), but also has direct effects on water salinity, pH, as well as D.O. Due to the physical properties of water, increases in temperature reduce oxygen solubility, making an organism's gas exchange more challenging. When dissolved oxygen levels in freshwater systems fall below 2.0 ml O_2 L^{-1}, many organisms are unable to survive. Also, under extreme conditions (D.O. < 0.5 ml O_2 L^{-1}), mass mortality can occur in some water-breathing organisms (Diaz 2001; Pollock *et al.* 2007; Diaz and Rosenberg 2008). Hypoxia is a natural environmental stressor in aquatic systems that are characterized by low light and low mixing (e.g., swamps) particularly in the tropics where high temperatures increase levels of organic decomposition, reduce oxygen tensions, and increase the oxygen consumption rates of ectothermic organisms (Chapman *et al.* 1996; Olowo and Chapman 1996; Martínez *et al.* 2004). In addition, since the early 1960's, industrialization and agricultural activities have increased water eutrophication, which directly affects dissolved oxygen concentration and has contributed to loss of habitat and biodiversity in some inland and coastal waters (Diaz 2001; Diaz and Rosenberg 2008).

Many fish species have developed a variety of strategies that allow them to survive hypoxia (Nikinmaa 2002; Nikinmaa and Rees 2005; Terova *et al.* 2008). Depending on the degree of D.O. variation, some organisms need to make minor adjustments, while others require greater changes (Wu 2002; Roessig *et al.* 2004). Under hypoxia stress, some strategies include behavioural, physiological and biochemical adaptation or a combination of these (Wannamaker and Rice 2000; Robb and Abrahams 2003; Martínez *et al.* 2006).

Behavioural Adaptations to Hypoxia

During periods of insufficient oxygen supply, organisms may be incapable of meeting the minimal energy demands to sustain life (Kramer 1987). One response by fishes is to relocate to areas with greater D.O concentrations. This may result in daily migrations, or seasonal migrations, depending on the water conditions and the environment (Magnuson *et al.* 1985; Robb and Abrahams 2002). However, when migration is not possible, fish must reduce their activity levels and oxygen consumption rates in order to reduce energy consumption (Zhou *et al.* 2000).

Fishes have evolved various respiratory strategies to increase the efficiency of oxygen uptake including the evolution of air-breathing organs or the use of aquatic surface respiration (ASR) (Kramer 1987; Chapman *et al.* 2002; Chippari-Gomes *et al.* 2005). Air breathing has evolved several times in fishes across many phylogenetic lineages and from a diversity of organs including lungs, modified buccal and pharyngeal cavities, swim bladder, and stomach (Jucá-Chagas 2004). Several water breathers increase oxygen uptake in hypoxic waters by using aquatic surface respiration (Kramer

and McClure 1982). During ASR, fish ventilate their gills with water from the air-water surface layer, where higher levels of oxygen are found due to diffusion (Kramer 1987; Chapman *et al.* 2002). However, both air breathing and ASR can incur great costs. In order to reach the water surface, organisms are forced to increase their activity level and therefore, must invest less time into other vital activities such as foraging for food (Chapman 1995). Furthermore, surfacing behaviour, whether air breathing or using ASR, can increase risk of predation from aerial or aquatic predators (Kramer 1983; Kramer 1987). Given these costs associated with surfacing (air breathing or ASR) fishes also use a variety of other physiological and metabolic adjustments in response to hypoxia that reduce the need for access to the surface.

Physiological and Metabolic Effects of Hypoxia Exposure

In general, fish size plays a major role in determining the physiological and metabolic requirements (Pelletier *et al.* 1993; Martínez *et al.* 2000; Nilsson and Östlund-Nilsson 2008). Depending on the size of the fish, its level of activity as well as its reproductive status, a balance between energy allocation and energy requirements is needed (Garvey and Marschall 2003). Larger fish require greater amounts of energy for daily body maintenance and growth (Rahel and Nutzman 1994). Furthermore, larger fish typically rely more on anaerobic metabolism compared to smaller individuals due to the high energy demands needed to transport oxygen across their larger bodies (Somero and Childress 1980; Goolish 1989).

Oxygen is an important basic molecule required to maintain life of aerobic respiration dependent organisms (Diaz and Breitburg 2009). As such, for fish and

aquatic invertebrates, dissolved oxygen is an important factor affecting not only their growth and reproduction, but also their survival (Karim *et al.* 2003). As previously stated, during hypoxia exposure, the level of activity of an organism needs to be reduced, which can affect its energy demands. One of the most important challenges under hypoxia stress is the maintenance of sufficient ATP levels. Cellular ATP production can be generated by aerobic or anaerobic pathways, the latter being less efficient than the former (Hochachka 1980; Boutilier 2001; Richards 2009). The amount of energy supplied to each cell and tissue in the body during hypoxia varies among and between organisms (Hochachka and Lutz 2001), and physiological, and metabolic adjustments in organisms when exposed to hypoxia are also highly variable (Sullivan and Somero 1980; West *et al.* 1999; Zhou *et al.* 2000; Martínez *et al.* 2006; Martínez *et al.* 2011).

In general, under hypoxia exposure, organisms tend to increase their anaerobic metabolism, and decrease the use of aerobic metabolism (Webster 2003). However, enzymatic levels have shown to differ across species, among individuals and also among different tissues (West *et al.* 1999; Cooper *et al.* 2002; Chippari-Gomes *et al.* 2005; Martínez *et al.* 2006; Farwell *et al.* 2007; Martínez *et al.* 2011). As an example, under prolonged exposure to hypoxia in the common carp (*Cyprinus carpio*), different responses were observed in the levels of muscle and liver lactate dehydrogenase (LDH). However, in the Gulf killifish (*Fundulus grandis*), Martínez *et al.* (2006) reported a different trend than the one reported on the common carp. Although a similar increase in the LDH activity levels were observed in the liver of *Fundulus grandis*, a decrease in LDH activity was found in the muscle tissue. Furthermore, significant differences at the tissue level have also been observed in two related Amazonian cichlids, *Astronotus*

crassipinnis and *Symphysodon aequifasciatus* (Chippari-Gomes *et al.* 2005). These results together along with many others, strongly suggest that tissues respond differently to hypoxia stress depending on their specific energetic demands, as well as the amount of oxygen supplied to each tissue. To better understand how the metabolic capacity of a fish can be affected by environmental dissolved oxygen, it is necessary to describe the different aerobic and anaerobic metabolic pathways involved in ATP production. I will first briefly describe the process of the oxidative pathway followed by anaerobic pathway that produces the least amount of ATP per carburant molecule, and finally the gluconeogenic pathway.

When oxygen is readily available, oxidative phosphorylation is the most important mechanism for ATP production (Salway 1999). This mechanism involves the breakdown of one glucose molecule, into two pyruvate molecules during a 10 step process, glycolysis. The pyruvate produced during glycolysis is further oxidized to H_2O and CO_2 in the Krebs cycle and electron transport chain. This breakdown of glucose through aerobic glycolysis, the Krebs cycle and the electron transport chain, produces a total of 38 ATP.

Under oxygen limitations however, glucose is broken down into lactate by the enzyme lactate dehydrogenase, producing two energy storing ATP. The lactate formed can then further be recycled in the liver, being converted back into glucose by a process referred to as gluconeogenesis. When organisms' glucose levels are exhausted, gluconeogenesis provides glucose for export to other tissues. Although seven of the reactions occurring in gluconeogenesis are similar to glycolysis, three reactions are unique and irreversible, preventing the simultaneous degradation of glucose by

glycolysis and resynthesis by gluconeogenesis. For the purpose of this thesis, I will focus on key enzymes from the aerobic, anaerobic and gluoconeogenic pathways.

Oxidative pathway

The main energy producing pathways for vertebrates, the Krebs cycle and the electron transport chain, occur in the mitochondria of the cell (Kramer 1987). The aerobic metabolic pathway converts organic substrates such as proteins, fats and carbohydrates into carbon dioxide to generate ATP. The final electron acceptor of this pathway is oxygen (Kramer 1987). The first enzyme that I focused on in this study, citrate synthase (**CS**), is an excellent indicator of aerobic metabolism (Yang and Somero 1993), and is responsible for catalyzing the first step in the citric acid cycle. As CS condenses acetyl coenzyme A with oxaloacetate, citrate is formed (Figure 1.1). The citric acid cycle enzymatically oxidizes acetyl groups to CO_2, while conserving energy in the reduced electron carriers NADH and $FADH_2$. The free electrons are then transferred to O_2, through the electron transfer chain. During the electron transfer, ATP is released by oxidative phosphorylation (Nelson and Cox 2000).

The second enzyme that I studied in my research was cytochrome c oxidase (**CCO**) or complex IV, which is a large enzyme of the inner mitochondrial membrane and is the last enzyme in the electron transfer chain (Figure 1.1) (Nelson and Cox 2000). The role of CCO is to carry electrons from cytochrome c with the aid of protons, to form water molecules. Although considered a good indicator of overall metabolic rate (Frick *et al.* 2008), CCO has shown to respond primarily to prolonged conditions of hypoxia (Zhou *et al.* 2000).

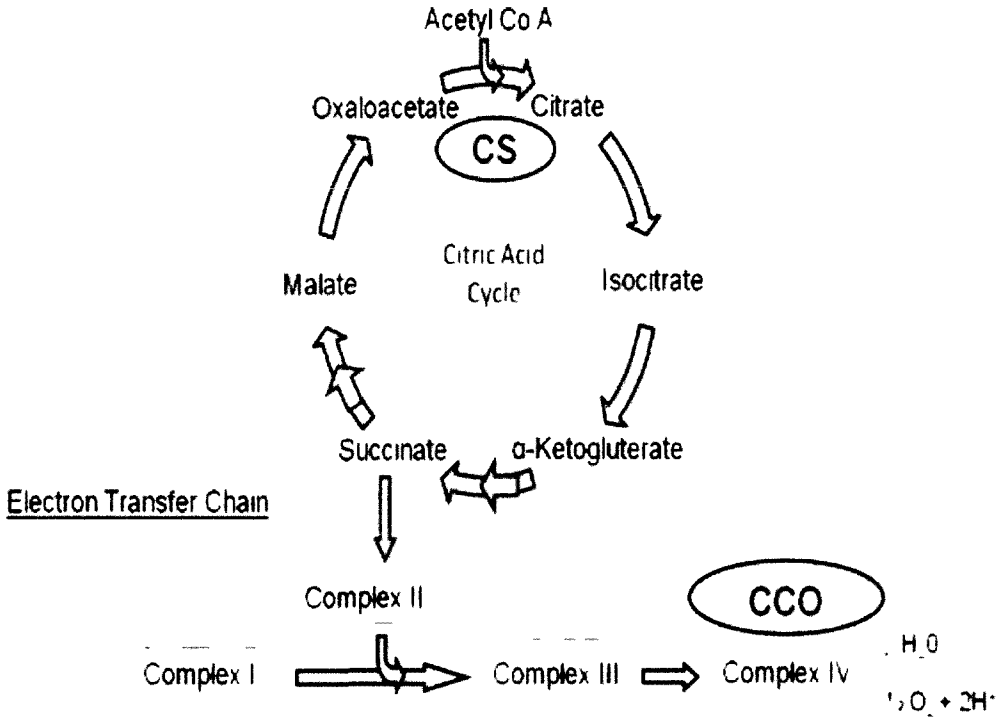

Figure 1.1. The process of acetyl CoA oxidation during the Krebs cycle. During the first reaction, the condensation of acetyl CoA with oxaloacetate is catalyzed by citrate synthase to form citrate. In the final step of the electron transfer chain, cytochrome c oxidase carries electrons from cytochrome c to oxygen and further reduces it to water (adapted from Nelson and Cox 2000).

Anaerobic pathway

When encountering hypoxia, fish experience a shortage of oxygen possibly inhibiting their ability to meet all metabolic processes by means of the aerobic pathway. Although a less efficient way of producing ATP, fish may readjust their metabolic machinery and rely more on the anaerobic metabolic pathway (Goolish 1991; Zhou *et al.* 2000; Martínez *et al.* 2004). The importance of anaerobic metabolism is primarily for the survival and fitness of a fish, as opposed to its contribution towards the total energy

budget (Goolish 1991). For example, anaerobic metabolism is often used in predator-prey interactions where the white muscle, having fast glycolytic fibres, provides the organism the ability to sprint swim while escaping predators (Almeida-Val *et al.* 2000; Gibb and Dickson 2002). The greater the anaerobic potential of a fish, the greater the survival chances are in oxygen poor environments (Hochachka 1980).

During glycolysis, glycogen is catalyzed to the level of pyruvate with the presence of NADH (Hochachka 1980; Goolish 1991; Rhoades and Pflanzer 2003). Pyruvate kinase (**PK**), the third enzyme measured in this study, catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate during the last step of glycolysis to create energy (Figure 1.2). Pyruvate can then be converted to acetyl coenzyme A, and then, it will be carboxylated to form oxaloacetate in the Krebs cycle. If oxygen is limited however, pyruvate is reduced to lactate (Horton *et al.* 2006). Lactate dehydrogenase (**LDH**), one of the most studied enzymes in fish (Coquelle *et al.* 2007), and the fourth enzyme I measured during this thesis, catalyzes pyruvate to lactate while regenerating NAD^+ from NADH (Figure 1.2) (Nelson and Cox 2000). The production of lactate is vital in a number of cells, especially where glucose is the main carbon source (Horton *et al.* 2006). However, the anaerobic glycolytic pathway assists in decreasing the chances of lactate build-up in the blood during hypoxia, and hence, preventing both blood and tissue pH levels from falling too low (Hochachka 1980). Overall, during severe hypoxia, adjustments made at the cellular level are highly correlated with the changes observed in the tissue metabolic capacity (Almeida-Val *et al.* 2000).

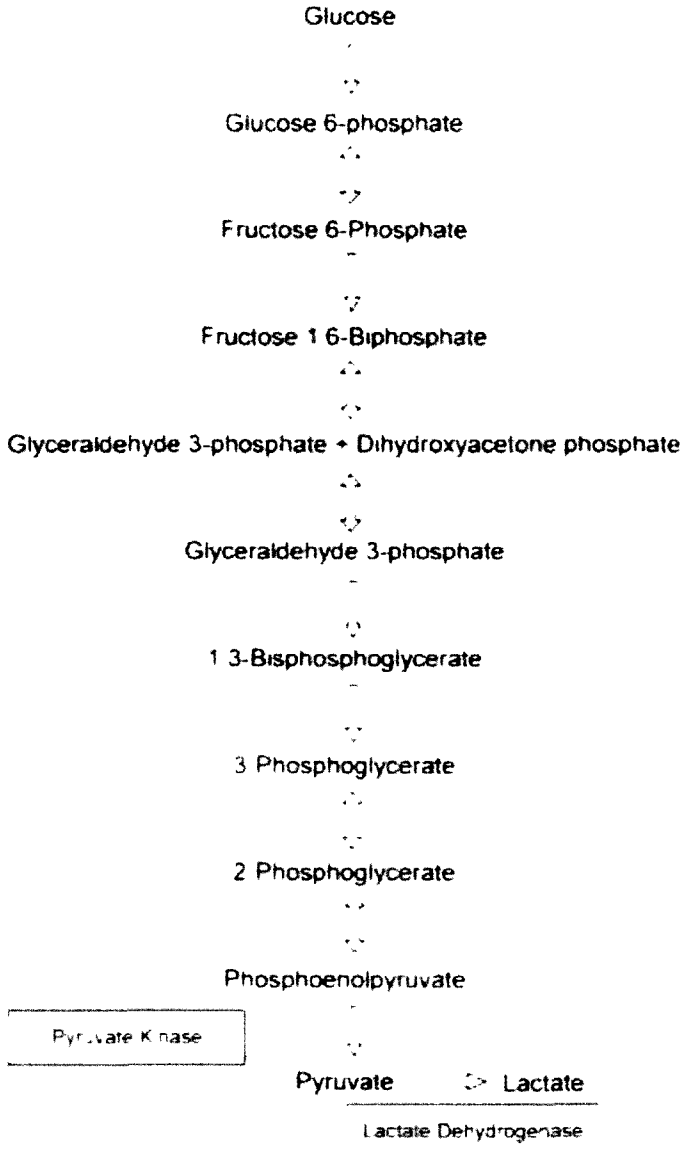

Figure 1.2. The process of glycolysis under anaerobic conditions. The phosphoryl group transferred from phosphoenolypyruvate, is catalyzed by the enzyme pyruvate kinase, to yield the product pyruvate. During anaerobic conditions, pyruvate is further reduced to lactate by the enzyme lactate dehydrogenase (adapted from Nelson and Cox 2000).

Gluconeogenic pathway

During hypoxia, sufficient levels of glucose may not be available from external sources or at an intercellular level (Horton *et al.* 2006). Also, glycogen levels, a major

energy reserve in the liver, are normally decreased under hypoxia (Zhou *et al.* 2000). However, in hypoxia tolerant organisms, higher levels of tissue glycogen have been observed when compared to organisms that are sensitive to hypoxia stress (Vornanen 1994; Richards *et al.* 2007). When glycogen sources become exhausted, and glucose must be supplied to tissues, gluconeogenesis may be activated to supply glucose required for anaerobic metabolism (Zhou *et al.* 2000). Gluconeogenesis, occurs primarily in the liver, and produces and supplies most of the body's glucose from substrates such as lactate, pyruvate and glycerol (Nelson and Cox 2000; Horton *et al.* 2006). Some enzymes such as malate dehydrogenase (**MDH**), the fifth enzyme I studied, are shared between the Krebs cycle and the gluconeogenic pathway. However, the other enzyme I measured, fructose 1,6-bisphosphatase (**FBPase**) is specific to gluconeogenesis. Malate dehydrogenase can be found in the cytoplasm and mitochondria (Almeida-Val *et al.* 2000). In the last step of the Krebs cycle, MDH catalyzes the oxidation of L-Malate to oxaloacetate, producing one NADH molecule (Figure 1.3) (Horton *et al.* 2006). Under gluconeogenesis, oxaloacetate is reduced to malate at the expense of NADH (Figure 1.3).

FBPase promotes the irreversible hydrolysis of C-1 phosphate. The enzyme catalyzes fructose 6-phosphate from fructose 1,6-bisphosphate (Figure 1.3) in the presence of metal cations such as magnesium, manganese, cobalt or zinc (Adamowicz *et al.* 2006). It has been reported that gluconeogenesis is increased under hypoxia, suggesting that there is an increased need in both the formation and exportation of glucose under stressful conditions (Martínez *et al.* 2006).

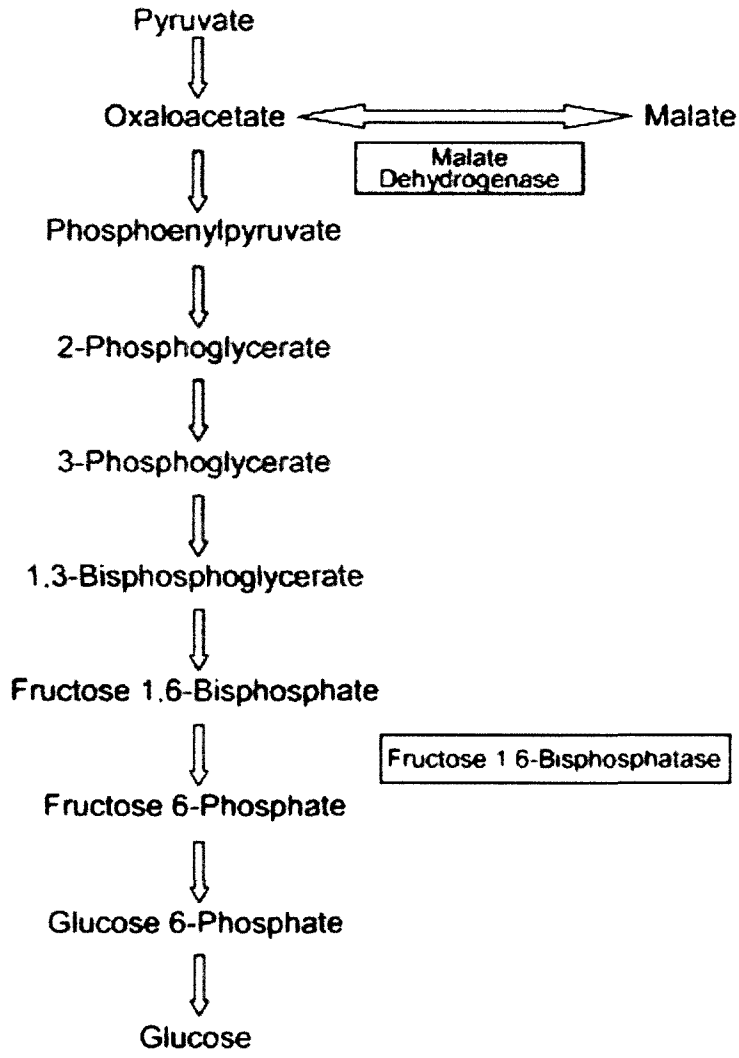

Figure 1.3. The process of gluconeogenesis. During this process, pyruvate is converted to glucose. Oxaloacetate, formed from pyruvate, is reduced to malate by malate dehydrogenase, which functions in both gluconeogenesis and the citric acid cycle. During an irreversible bypass, the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate is catalyzed by the enzyme fructose 1,6-bisphosphatase (adapted from Nelson and Cox 2000).

Study Problem

In the mouth brooding haplochromine cichlid fish, *Pseudocrenilabrus multicolor victoriae*, high levels of developmental plasticity have been observed between fish reared under different oxygen regimes (Chapman *et al.* 2002; Crispo and Chapman 2010). For example, Chapman *et al.* (2000) found that the F_1 generation of *P. multicolor* reared under hypoxia were characterized by gill surface area 18% larger than full sibs reared under normoxic conditions. The mass of the brain has also been reported to be smaller in *P. multicolor* when raised under hypoxia compared to when reared under normoxia (Chapman *et al.* 2008).

Recently, Crispo and Chapman (2008) studied the population structure of *P. multicolor* in the Mpanga River drainage of Western Uganda using neutral markers. They found low genetic divergence at microsatellite markers suggesting that either contemporary gene flow is high, or that colonization was recent, followed by insufficient time for significant neutral divergence to occur. High levels of plasticity in morphological traits such as gill size and brain size in *P. multicolor* (Chapman *et al.* 2008; Crispo and Chapman 2010) might have facilitated colonization of the hypoxic swamps, and/or current gene flow between environments might result in selection for developmental plasticity at the meta-population scale.

Despite the great amount of information on the ecology, behaviour, and morphology of this African cichlid, little information is available on the physiological adjustments used by this species to cope with hypoxia. Recently Martínez *et al.* (2009) found that when the F_1 generation of *P. multicolor* reared under hypoxia were held in common garden conditions for four weeks, both the haematocrit levels and LDH activity

levels were dependent upon the origin of the parental stock. However, with only one enzyme analyzed, it is difficult to get a broader view of the metabolic adaptations required by this species to survive under differing dissolved oxygen levels.

In this thesis, I explored the metabolic capacity of *P. multicolor* at a tissue level, in individuals from both natural conditions as well as in individuals reared under normoxic or hypoxic conditions in a laboratory setting. The metabolic capacity of *P. multicolor* was determined by measuring four different enzymes from three metabolic pathways in four tissues. In the white skeletal muscle, heart, brain and liver, the enzymes measured were pyruvate kinase, lactate dehydrogenase, citrate synthase and cytochrome C oxidase. An additional two gluconeogenic enzymes, malate dehydrogenase and fructose 1,6-bisphosphatase were measured only in the liver.

With the imminent threat of global warming on a great variety of freshwater and marine environments, it is very important to increase our understanding of the potential of organisms to respond to environmental change and the role of phenotypic plasticity in facilitating adaptive response. Physiological studies such as this one will help in predicting the effects of global warming and hypoxia by determining the link between local adaptation and plasticity on physiological patterns that facilitate animal tolerance and survival in stressful environments. As such, the main objective of my thesis was to determine if a variation in the metabolic capacities of different populations of *P. multicolor* exists across field sites that differ in dissolved oxygen regimes and when reared under divergent oxygen condition in a laboratory setting.

I first focused on the field fish, where I studied the metabolic capacity of natural populations of *P. multicolor* from four different sampling sites, within two different regions. For this chapter, based on earlier studies of *P. multicolor* demonstrating morphological and life-history divergence in traits between alternative oxygen environments (Chapman *et al.* 2008; Reardon and Chapman 2009), I compared variation in the metabolic capacity, within and between regions. I hypothesized that fish collected from sampling sites that differ in dissolved oxygen regimes would differ in their metabolic capacity. I also predicted that individuals inhabiting the fluctuating site would over-express their enzyme activity levels in order to cope with the seasonal changes in D.O. levels.

The purpose of the next chapter was to see as to what extent *P. multicolor* individuals show signs of physiological plasticity when reared under divergent oxygen regimes. To do this, I focused on the F_1 generation of *P. multicolor* from three different sampling sites within one region of Western Uganda, the Mpanga River system, where there is evidence for high levels of gene flow among sites (Crispo and Chapman 2008). Under lab conditions, the metabolic capacity was determined in the F_1 generation after a one year rearing experiment. The main objective of this chapter was to elucidate the role of site of origin and dissolved oxygen treatment on the metabolic capacity of *P. multicolor*. For this study I hypothesized that both population of origin as well as D.O. regime in which the offspring were reared would affect levels of the different enzyme activities. Since fish were under controlled lab conditions, and hypoxia was carefully monitored the following predictions were tested:

(1) Fish from normoxic origin will display greater anaerobic activity than fish from hypoxic origin when reared under hypoxia.

(2) Fish from the fluctuating D.O. site will display greater levels of enzyme activity when reared under hypoxia compared to individuals that inhabit more stable environments

The last chapter compiles the results from the lab and field studies to provide a more comprehensive view of the metabolic adjustments made by *P. multicolor* in response to divergent oxygen regimes. Hence, this chapter describes generalities and differences observed between the metabolic capacity of field fish and the F_1 generation reared under laboratory conditions. Together, these results will increase our knowledge on effects of divergent dissolved oxygen regimes on the metabolic capacity of hypoxia-tolerant organisms.

CHAPTER 2: NATURAL DISSOLVED OXYGEN REGIMES AND THE TISSUE METABOLIC CAPACITY OF THE AFRICAN CICHLID *PSEUDOCRENILABRUS MULTICOLOR VICTORIAE*

ABSTRACT

Previous work has focused on interdemic variation at the ecological, behavioural, and morphological levels in the African cichlid fish, *Pseudocrenilabrus multicolor victoriae*, inhabiting different dissolved oxygen (D.O) levels. However, little work has been done to determine effects of divergent D.O. levels on their metabolic capacity. *P. multicolor* was collected from four sites within two regions of Uganda: Bunoga (7.53 ± 0.13 mg O_2 l^{-1}), Kahunge (3.76 ± 1.70 mg O_2 l^{-1}), Lwamunda Swamp (2.23 ± 1.15 mg O_2 l^{-1}) and Lake Kayanja (7.17± 0.61 mg O_2 l^{-1}). Enzymes from the aerobic and anaerobic metabolism, pyruvate kinase (PK), lactate dehydrogenase (LDH), citrate synthase (CS) and cytochrome C oxidase (CCO) were examined in four different tissues, white skeletal muscle, heart, brain and liver. An additional two gluconeogenic enzymes, malate dehydrogenase (MDH) and fructose 1,6-bisphosphotase (FBPase) were examined only in the liver. Overall, the metabolic capacity of *P. multicolor* was tissue specific, possibly a strategy used to limit the energy expenditure during hypoxia exposure. Furthermore, despite regional differences between enzyme activities in most of the tissues, little variation was observed between the two sites within a region. The regional differences observed here suggest that the genetic composition may be playing an important role in determining the metabolic capacities of *P. multicolor* to a variable dissolved oxygen regime.

RÉSUMÉ

Des études précédentes se sont focalisées sur les variations interdémiques au niveau écologique, comportemental et morphologique chez le cichlidé africain, *Pseudocrenilabrus multicolor victoriae,* habitant des environnements présentant des niveaux en oxygène dissous (D.O.) différents. Cependant, peu d'études ont tenté de déterminer les effets de D.O. sur leur capacité métabolique. *P. multicolor* ont été échantillonné à partir de quatre populations dans deux régions de l'ouest de l'Ouganda : Bunoga (7.53 ± 0.13 mg O_2 l^{-1}), Kahunge (3.76 ± 1.70 mg O_2 l^{-1}), Lwamunda Swamp (2.23 ± 1.15 mg O_2 l^{-1}) and Lake Kayanja (7.17± 0.61 mg O_2 l^{-1}). Des enzymes du métabolisme anaérobie et aérobie, la pyruvate kinase (PK), la lactate déshydrogénase (LDH), la citrate synthase (CS) et la cytochrome C oxydase (COX) ont été examinées dans quatre tissus, le muscle squelettique blanc, le cœur, le cerveau et le foie. Deux enzymes gluconéogéniques, la malate déshydrogénase (MDH) et la fructose 1,6-bisphophatase (FBPase) ont été examinées pour le foie seulement. La capacité métabolique de *P. multicolor* variée selon les tissus, en indiquant une stratégie probable pour limiter l'utilisation d'énergie durant l'exposition à l'hypoxie. De plus, malgré les différences régionales entre les activités enzymatiques dans la plupart des tissus, peu de variations ont été observées entre les deux sites d'une même région. Les différences régionales observées ici et dans les études précédentes suggèrent fortement que la composition génétique semble jouer un rôle important dans la détermination des capacités métaboliques de *P. multicolor* en réponse à un régime variable en oxygène dissous.

INTRODUCTION

Several studies on East African freshwater fishes strongly suggest that environmental dissolved oxygen (D.O.) is an important variable contributing to phenotypic variation. In Uganda, East Africa, there are two rainy seasons that lead to biannual fluctuations in the levels of D.O. and other abiotic characteristics of aquatic systems (Chapman *et al.* 2008; Reardon and Chapman 2010). Some systems experience dramatic reductions in D.O. (hypoxia) during the dry season, while others experience hypoxia during the rainy season as a result of water inflow from hypoxic swamps (Chapman *et al.* 2002; Crispo and Chapman 2008). These oscillations in D.O., which have been found to occur not only in Africa, but worldwide, have forced a number of aquatic organisms to develop strategies to facilitate persistence under chronic and seasonal hypoxia (Chapman *et al.* 2002; Crampton *et al.* 2008; Chippari-Gomes *et al.* 2005; Mandic *et al.* 2009).

In general, under hypoxia, changes at the level of behaviour (Olowo and Chapman 1996; Wannamaker and Rice 2000), morphology (Chapman *et al.* 2008; Paterson *et al.* 2010), physiology (Martínez *et al.* 2006; Farwell *et al.* 2007), genetics (Crispo and Chapman 2008; Crispo and Chapman 2010), as well as life history traits (Blanck *et al.* 2007; Reardon and Chapman 2008) indicate that the capacity of some species to survive this stressor has played an important role on the evolutionary biology of hypoxia tolerant species. Recent studies suggest that the strategies used by different fish not only vary depending on the species, individuals, metabolic scope and tissues, but also depending on the intensity of hypoxia exposure and the duration of the exposure.

Physiologically, we observe different mechanisms acting under hypoxia exposure: a down-regulation of cellular energy turnover and an up-regulation of ATP

production (Hochachka *et al.* 1996; Jibb and Richards 2008). During hypoxia, mitochondria decrease the amount of ATP produced, reducing the capacity of the organism to maintain cellular energy balance (Richards 2009). When the severity of hypoxia increases, organisms must rely more heavily on the contribution of the anaerobic metabolic pathway to produce sufficient ATP, and sustain the reduced energy turnover (Hochachka *et al.* 1996; Pollock *et al.* 2007). Under prolonged exposure to hypoxia however, fish may find different ways to adjust metabolic needs and energy demands. A number of studies have focused on the metabolic strategies of fish species under laboratory conditions (Chippari-Gomes 2005; Farwell *et al.* 2007). Far fewer studies have examined the metabolic capacity of fish captured directly from their natural environmental conditions.

The small African haplochromine cichlid, *Pseudocrenilabrus multicolor victoriae* (Seegers), shows diverse suite of strategies in response to hypoxia in the field such as large gill surface area (Chapman *et al.* 2000) and small brain size (Chapman *et al.* 2008). Recently, it was suggested that the metabolic capacity of *P. multicolor* was dependent upon the site of origin of the parental stock, however this experiment was completed under laboratory conditions using only one enzyme, lactate dehydrogenase (LDH) (Martínez *et al.* 2009). Under natural conditions the metabolic capacity of *P. multicolor* is unknown. In this study I compared the metabolic responses of *P. multicolor* individuals captured from two different sites within each of the two regions in Uganda, East Africa. This is the first study to my knowledge to examine the metabolic capacity of *P. multicolor* from their natural normoxic, hypoxic, and fluctuating dissolved oxygen sites. In order to determine whether the metabolic capacity of this hypoxia

tolerant organism, varies under divergent dissolved oxygen environments, I measured citrate synthase (CS) and cytochrome C oxidase (CCO) (as a measure of the carbohydrate aerobic metabolism), pyruvate kinase (PK) and lactate dehydrogenase (as a measure of the carbohydrate anaerobic metabolism) in the muscle, heart, brain and liver tissues. The levels of malate dehydrogenase (MDH) and fructose 1,6-bisphosphatase (FBPase) were determined in the liver tissue only (as a measure of the carbohydrate gluconeogenic metabolism).

MATERIALS AND METHODS

Study sites and collection of fish

In June 2006, *P. multicolor* was collected from four different sites in Western Uganda, Africa. Two of the sites, Lake Kayanja and Lwamunda Swamp are located in the Nabugabo region close to Lake Victoria (Figure 2.1a), while the other two sites, Bunoga and Kahunge, are located in the Mpanga river basin of Western Uganda (Figure 2.1b).

The dissolved oxygen levels vary across the four sites within this study (Figure 2.2). Two of the four sites examined, Bunoga and Lake Kayanja remain normoxic year round with dry season values often over 7.0 ml O_2 L^{-1}, (Rosenberger and Chapman 2000; Crispo and Chapman 2008). The other site in the Nabugabo Region, Lwamunda Swamp, is hypoxic year round, although there is seasonal D.O. variation (Chapman *et al.* 2002). The Kahunge site, experiences both normoxic and hypoxic conditions during the year with low D.O levels during the rainy season (Figure 2.2) when the nearby Kantembwe Swamp overflows into Kahunge (Crispo and Chapman 2008).

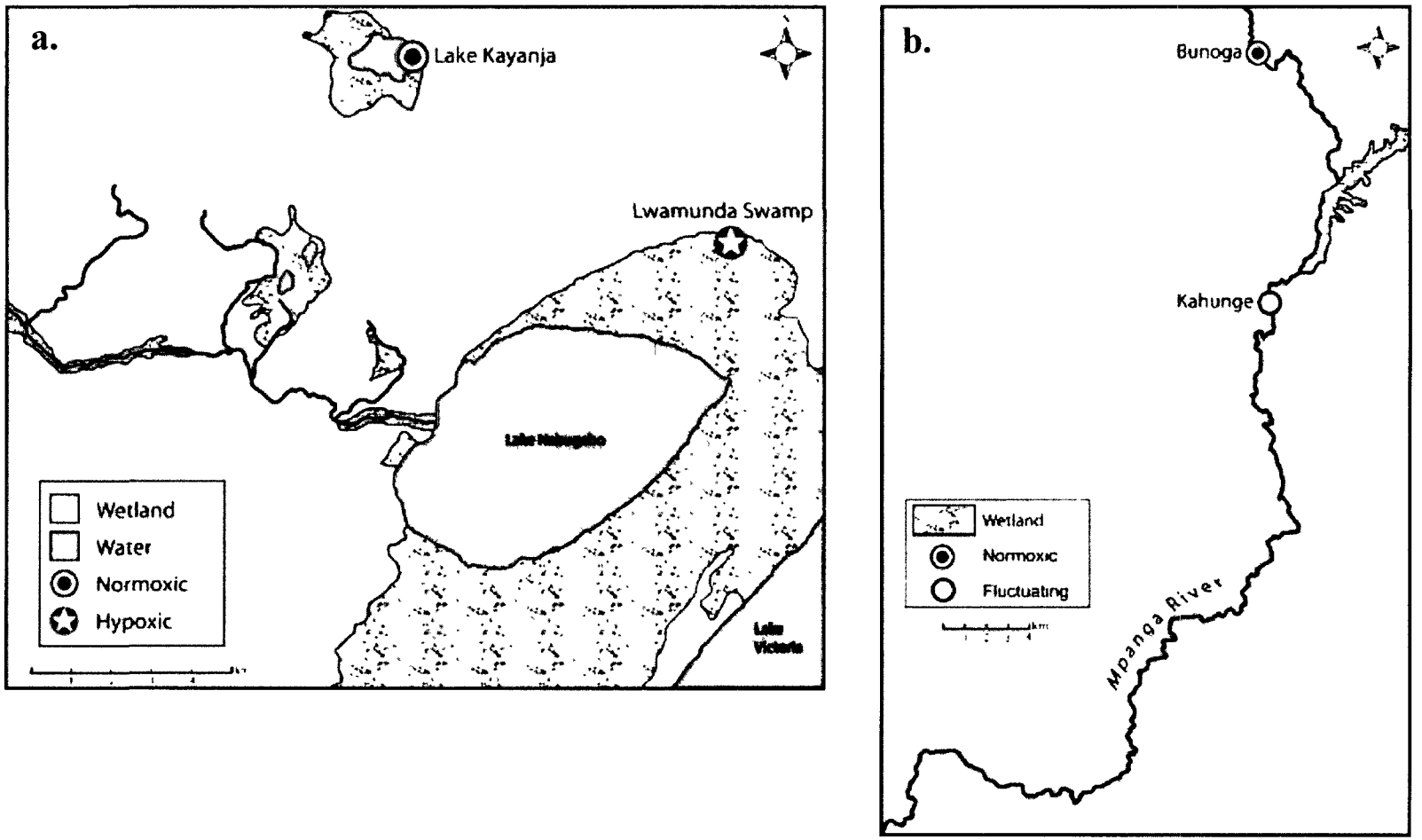

Figure 2.1. Sampling sites in Uganda, Africa. a., sites located within the Nabugabo region and b., the sites along the Mpanga river system (adapted from Crispo and Chapman 2008).

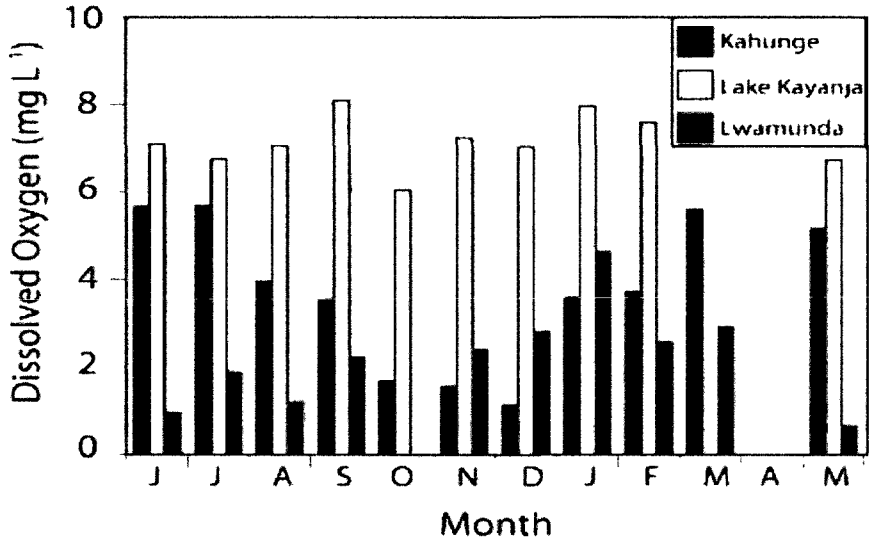

Figure 2.2. Monthly dissolved oxygen concentration (mg O_2 l^{-1}) of sampling sites in Western Uganda, Africa. Bunoga site is excluded from the graph due to logistical constraints in data acquisition. Yearly data recordings were taken from the following time periods: Kahunge, June 2006-May 2007; Lake Kayanja, June 1999-May 2000 and Lwamunda, June 2000-May 2001 (Data collected by Dr. Chapman's field assistants).

A total of 20 individuals were live captured from each site using minnow traps baited with bread. Fish were euthanized *in situ* with an overdose of buffered MS-222 (1g l^{-1} MS-222 and 4 g l^{-1} $NaHCO_3$). Individuals were sexed, and biometric traits were taken including mass (g ± 0.1), total length (cm ± 0.1) and standard length (cm ± 0.1). Fish were then individually wrapped, labelled and frozen in liquid nitrogen. Frozen samples were transported to McGill University in Montreal Québec, where they were stored at -80°C. Samples were finally transported to Laurentian University in Sudbury Ontario, on dry ice and immediately stored at -80°C, until tissues were ready to be assayed.

Enzyme Assays

Fish tissues (white skeletal muscle, heart, brain and liver) were quickly dissected on ice and immediately weighed (g ± 0.0001) and homogenized with a Polytron homogenizer (PT 1200) in an ice cold buffer consisting of 100mM of Hepes (pH 7.0), 0.1 mM of dithiotheritol (DTT), and 0.2% of Triton X-100 for three 20 s intervals. During and between homogenization, samples were kept on ice. Muscle and brain were homogenized in 10 or 50 volumes of buffer, while liver and heart were homogenized in 50 or 100 volumes depending on initial tissue mass. Homogenates were centrifuged using a Thermo IEC Micromax RF Refrigerated Microcentrifuge at 2400 *g* for 15 min at 4°C. Supernatants were kept on ice until enzyme activity was assayed.

Reactions were initiated by the addition of the enzyme specific substrate (substrate differentiated by *). Substrate concentrations were optimized to give maximal enzyme activity. The final protocols for pyruvate kinase, malate dehydrogenase and fructose 1,6-bisphosphatase were modified from Martínez *et al.* 2006 as follows:

<u>*Pyruvate Kinase*</u> (PYK; EC 2.7.1.40): 100 mM Hepes buffer (pH 7.4); 10mM KCl (heart, liver) 20mM KCl (brain), or 40mM KCl (muscle); 0.2 mM NADH; 2.5 mM ADP (heart) or 10 mM ADP (muscle, liver brain); 0.25 U ml^{-1} L-LDH (muscle, brain), 0.5 U ml^{-1} L-LDH (heart) or 1 U ml^{-1} L-LDH (liver); 5 mM $MgCl_2$ (muscle, liver, brain) or 10 mM $MgCl_2$ (heart); *2 mM PEP (muscle, liver, brain) or 4 mM PEP (heart).

<u>*Malate Dehydrogenase*</u> (MDH; EC 1.1.1.37): 100 mM Hepes buffer (pH 7.4); 0.2 mM NADH; *0.2 mM oxaloacetic acid.

Fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11): 100mM Hepes buffer (pH 7.4); 10 mM KCl; 0.4 mM NADP; 0.8 U ml^{-1} PGI; 0.72 U ml^{-1} Glucose-6-phosphate dehydrogenase; 6 mM $MgCl_2$; 0.5 mM EDTA; 0.05 mM Fructose 1,6-bisphosphate; * 10 µl of liver tissue.

The reaction conditions for citrate synthase, cytochrome C oxidase and lactate dehydrogenase were modified from standard protocols.

Citrate Synthase (CS; EC 2.3.3.1): 50 mM Trizma Base buffer (pH 8.0); 0.1 mM 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB); 0.1 mM Acetyl coenzyme A (muscle, heart) or 0.2 mM Acetyl coenzyme A (liver, brain); *0.75 mM oxaloacetic acid (muscle) or 1.5 mM oxaloacetic acid (liver, heart, brain) (Martínez *et al.* 1999).

Cytochrome C Oxidase (CCO; EC 1.9.3.1): 50 mM K_2HPO_4 and 50 mM KH_2PO_4 buffer (pH 7.0); 0.07 mM reduced cytochrome C. The reaction was compared against 0.07 mM of reduced cytochrome C and 0.33% K-ferricyanide. Cytochrome C was reduced by the addition of sodium hydrosulfite; *Tissue (Zhou *et al.* 2000).

Lactate Dehydrogenase (LDH; EC 1.1.1.27): 100 mM Hepes; 0.12 mM NADH; *1.0 mM pyruvate (Martínez *et al.* 2009).

Maximal enzyme activity was measured in a 96-well microplate reading spectrophotometer (Molecular Devices, Spectramax plus 384, Sunnyvale, CA, USA) at 25 °C. Rates from blank reactions (without substrates) were subtracted for determination of enzyme activities. For LDH, MDH, and PK, reactions were measured by the disappearance of NADH at 340 nm. The reduction of $NADP^+$ at 340 nm, was used to measure the reaction of FBPase. The CS reaction was measured at 412 nm to detect the

transfer of coenzyme A to DTNB, and CCO was measured at 550 nm to observe the oxidation of reduced cytochrome c. The value of 6.22, 13.6 and 19.1 were used as the millimolar extinction coefficients for NAD(P)H, DTNB and cytochrome C respectively. All enzyme activities were measured within four hours of tissue homogenization. Enzyme activity was measured in the following order, LDH, MDH, CS, CCO, PK and FBPase. Assays were run in quadruplicates and reactions were linear for $\geq$ 180 s.

Biochemicals and Chemicals

All chemicals and biochemicals were purchased from Sigma – Aldrich Co. (Oakville, ON, Canada), Roche Diagnostics Corporation (Mississauga, ON, Canada) and Thermo Scientific (Rockford, IL, U.S.A.)

Protein Analysis

Supernatants for protein analysis were kept at -80 °C until analyzed. The bicinchoninic acid (BCA) assay was used to determine the protein content in the supernatant fraction of tissue homogenates (Smith *et al.* 1985). The protocol was modified for use in a 96-well microplate reading spectrophotometer (Brown *et al.* 1989). Samples were diluted using distilled water to give a final dilution of 100x for muscle, heart and brain, and 200x for liver. A total of 10 µl of sample was added to quadruplicates of 200 µl BCA working reagent into individual wells of the 96-well microplate. Standards of 0 - 1 mg ml^{-1} of bovine serum albumin (BSA) were included as a standard curve with every plate. Plates were sealed, and incubated at 60°C for 30 min. After cooling for 10 min, plates were read at 562 nm at room temperature.

Calculations and Statistical Analysis

Enzyme activity was determined based on both the tissue mass (i.u. g^{-1} tissue) and the soluble protein content in tissue extracts (i.u. mg^{-1} protein). All variables were examined for normality using Shapiro-Wilks test, and tested for homogeneity of variance before analysis was completed. Tissue activity that did not meet the testing guidelines was $\log_{10}$ transformed. These included PK, CS and CCO activity in the muscle, all enzymes analyzed in heart and liver, and PK and CCO in the brain. As body size can affect the metabolic capacity, the contribution of mass and total length for each tissue enzyme was explored using one-way analysis of covariance (ANCOVA) when examining differences in activity levels between sampling sites. Fish body mass and length were significant covariates only in the case of brain CCO, heart LDH, and liver CS; and results were similar for mass and length. Therefore, mass was included in the final model as a covariate for the remaining of the analyses for these three tissues only. For each response variable, an ANOVA/ANCOVA was only used to test the effect of site of origin (Tukey *post hoc* analysis). As a difference may be present between the two regions, a nested ANOVA/ANCOVA was run where site of origin (random factor) was nested within regions (fixed factor). Statistical outliers detected in the ANOVA protocol (individuals where the value of the measured trait was more than two standard deviations from the mean), were removed from analyses. This resulted in the removal of one individual for muscle PK and CS; brain LDH and CS; liver MDH. Two individuals were removed from liver CS. Three individuals were removed from muscle CCO; liver LDH and FBPase; and finally, four individuals were removed from muscle LDH and brain CCO. All statistical analyses were completed using SPSS Statistics 17.0 (SPSS Inc.,

Chicago, Illinois, U.S.A.). All figures represented are shown using the raw untransformed data. Differences were considered significant when $P \leq 0.05$.

RESULTS

Overall, morphological traits: mass, length and Fulton's condition factor did not differ among sampling sites (Table 2.1). However, fish mass, length and Fulton's condition factor significantly differed between sexes ($F_{[1,78]} = 19.523$, $P < 0.001$; $F_{[1,79]} = 28.138$, $P < 0.001$; $F_{[1, 79]} = 5.021$, $P = 0.027$ respectively). In general, males were longer and heavier than females, while females had greater Fulton's condition factor than males. I also examined whether enzyme activity levels differed between sexes. Sex only affected the levels of muscle CS activity ($F_{[1,78]} = 6.128$, $P = 0.015$), where males showed greater activity levels than females.

Tissue Enzyme Levels

White skeletal muscle

In white skeletal muscle, nested ANOVA detected regional differences for PK enzyme activity (Figure 2.3), where individuals from the Mpanga River sites (Bunoga and Kahunge) had slightly greater PK activity compared to individuals from the Nabugabo region (Lake Kayanja and Lwamunda Swamp).

When looking for variation between sites of origin, the ANOVA results indicated significant differences for PK ($F_{[3,76]} = 4.725$, $P = 0.004$); LDH ($F_{[3,73]} = 3.366$, $P = 0.023$) and CS ($F_{[3,76]} = 6.095$, $P = 0.001$). No significant differences in CCO activity levels were observed between sites of origin ($F_{[3,74]} = 1.496$, $P = 0.223$).

Table 2.1. Morphological traits of *Pseudocrenilabrus multicolor victoriae* from two regions in Uganda, Africa. Mpanga region includes Bunoga (normoxic site) and Kahunge (fluctuating site) and Nabugabo region includes Lake Kayanja (normoxic site) and Lwamunda Swamp (hypoxic site). No significant difference was observed on the morphological traits between sites of origin.

	Mpanga Region		**Nabugabo Region**	
Morphological Traits	**Bunoga**	**Kahunge**	**Lake Kayanja**	**Lwamunda Swamp**
Mass (g)	4.29 ± 0.46	3.55 ± 0.39	3.07 ± 0.28	3.45 ± 0.43
Total length (cm)	6.33 ± 0.23	5.99 ± 0.20	5.75 ± 0.17	5.82 ± 0.23
Fulton condition factor (Mass / Length $_T$ $^{-3}$)*100	1.61 ± 0.05	1.56 ± 0.03	1.47 ± 0.08	1.62 ± 0.03
N	20	20	21	20

Note: Values are means ± S.E.

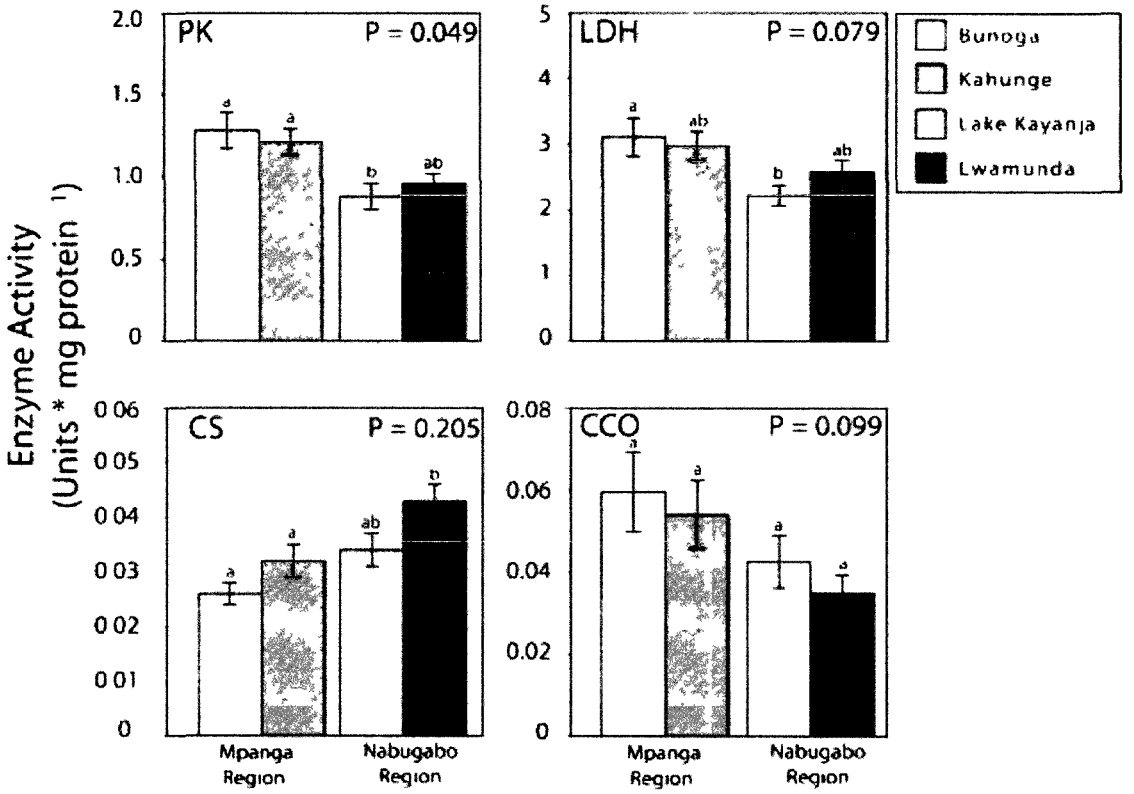

Figure 2.3. White skeletal muscle enzyme activity of *Pseudocrenilabrus multicolor victoriae* from Bunoga (normoxic), Kahunge (fluctuating), Lake Kayanja (normoxic), and Lwamunda Swamp (hypoxic). Enzymes measured were PK (pyruvate kinase), LDH (lactate dehydrogenase), CS (citrate synthase) and CCO (cytochrome C oxidase). *P values* indicate significant differences between regions, Mpanga Region and Nabugabo Region. Letters indicate significant differences among sites of origin. Bars with the same letter do not differ (Tukey *post-hoc* comparisons, $P > 0.05$). Data are shown as mean ± S.E.

Tukey *post-hoc* analysis was used in order to determine which sites of origin statistically differed in enzyme activity from one another. A significant difference was observed in the levels of PK activity between Kahunge (fluctuating) and Lake Kayanja (normoxic) $P = 0.018$, as well as between the two normoxic sites, Bunoga and Lake Kayanja ($P = 0.011$). No significant differences in PK enzyme activity were observed in fish from the other sites of origin ($P > 0.05$). Regarding LDH activity, significant differences were observed only between the two normoxic sites, Bunoga and Lake Kayanja ($P = 0.030$). No significant differences were observed between white skeletal muscle LDH activity measured in fish from the other sites of origin ($P > 0.05$). For CS activity, differences were found between Bunoga and. Lwamunda Swamp ($P < 0.0001$) and between Kahunge and Lwamunda Swamp ($P = 0.045$). However, no differences in the levels of CS enzyme activity between Bunoga and Lake Kayanja and between Kahunge and Lake Kayanja were observed ($P = 0.149$ and $P = 0.936$ respectively).

Heart Tissue

A nested ANOVA/ANCOVA detected that fish from the Mpanga region displayed significantly greater PK, LDH, and CS activity compared to fish from the Nabugabo region (Figure 2.4).

Results from ANOVA/ANCOVA indicated that between sites of origin, significant differences were observed for all enzymes analyzed, PK ($F_{[3,76]} = 6.061$, $P = 0.001$), LDH ($F_{[3,71]} = 7.806$, $P < 0.0001$), CS ($F_{[3,76]} = 9.867$, $P < 0.0001$) and CCO ($F_{[3,72]} = 3.606$, $P = 0.017$). The *post-hoc* analysis comparing sites of origin, showed significant differences on the levels of PK activity as follows: Bunoga and Lake Kayanja

(P = 0.017), Kahunge and Lake Kayanja (P = 0.005) and Kahunge and Lwamunda Swamp (P = 0.023). However, no statistical differences were observed between the other sites (P > 0.05).

Levels of heart LDH activity significantly differed between Bunoga and Lake Kayanja (P < 0.0001), Bunoga and Lwamunda Swamp (P = 0.004), Kahunge and Lake Kayanja (P < 0.0001) as well as between Kahunge and Lwamunda Swamp (P = 0.038). When examining the variation on the aerobic enzymes between the sites of origin, ANOVA results showed significant differences on the levels of CS activity between Bunoga and Lake Kayanja (P < 0.0001), Bunoga and Lwamunda Swamp (P = 0.038), Kahunge and Lake Kayanja (P = < 0.0001) and also between Kahunge and Lwamunda Swamp (P = 0.038). Finally, heart CCO activity differed only between Kahunge and Lwamunda Swamp (P = 0.023).

Brain tissue

Both anaerobic and aerobic enzyme activity were not significantly different at a regional level in the brain as detected using a nested ANOVA/ANCOVA (Figure 2.5).

Between sites of origin, ANOVA/ANCOVA results revealed that all four enzymes analyzed showed significant differences, PK ($F_{[3,77]}$ = 3.499, P = 0.019), LDH ($F_{[3,76]}$ = 6.724, P < 0.0001), CS ($F_{[3,76]}$ = 5.465, P = 0.002) and CCO ($F_{[3,71]}$ = 5.395, P = 0.002). The levels of PK activity were significantly different between Kahunge and Lake Kayanja (P = 0.019). However, no further differences between the other sites of origin were observed for this enzyme (P > 0.05).

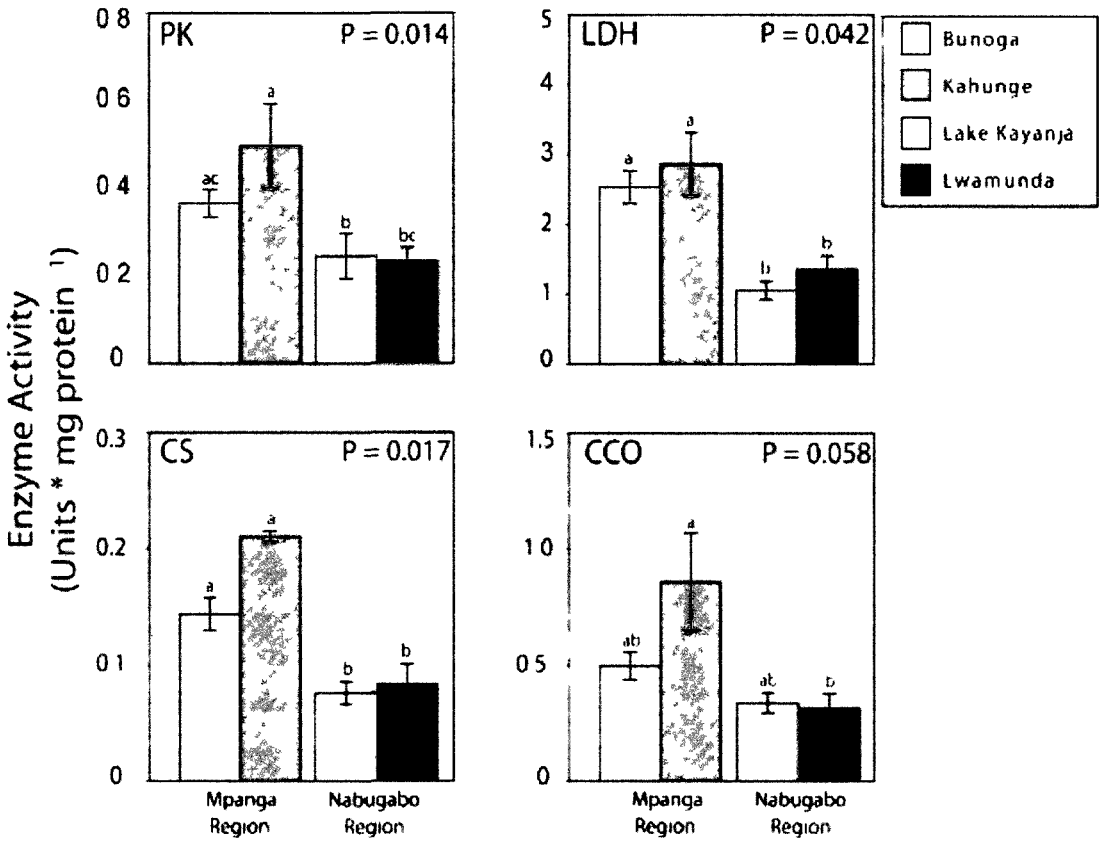

Figure 2.4. Heart enzyme activity of *Pseudocrenilabrus multicolor victoriae* from Bunoga (normoxic), Kahunge (fluctuating), Lake Kayanja (normoxic), and Lwamunda Swamp (hypoxic). Enzymes measured were PK (pyruvate kinase), LDH (lactate dehydrogenase), CS (citrate synthase) and CCO (cytochrome C oxidase). *P values* indicate significant differences between regions, Mpanga Region and Nabugabo Region. Letters indicate significant differences between sites of origin. Bars with the same letter do not differ (Tukey *post-hoc* comparisons, $P > 0.05$). Data are shown as mean ± S.E.

The *post-hoc* analysis results looking at the brain LDH activity variation between sites of origin showed significant differences between Bunoga and Lake Kayanja ($P = 0.009$) and Kahunge and Lake Kayanja ($P < 0.0001$). The level of LDH activity did not differ between the remaining sites analyzed ($P > 0.05$).

Levels of brain CS activity were only different between Kahunge and Lake Kayanja ($P = 0.001$). Lastly, minimal significant differences in CCO activity were observed between sites of origin. Although differences were observed in brain CCO activity between Kahunge and Lwamunda Swamp ($P = 0.002$), no significant differences in CCO activity were observed between the other study sites ($P > 0.05$).

Liver tissue

In this tissue, using a nested ANOVA, the only regional difference in enzyme activity was observed in the gluconeogenic enzyme, FBPase (Figure 2.6). Interestingly, FBPase activity was greater in the Nabugabo region compared to the Mpanga region, an opposite trend compared to the three other tissues examined during this study.

Between sites of origin, an ANOVA revealed significant differences, in PK activity ($F_{[3,76]} = 3.496$, $P = 0.020$), LDH activity ($F_{[3,73]} = 5.835$, $P = 0.001$), MDH activity ($F_{[3,75]} = 5.971$, $P = 0.001$) and FBPase activity ($F_{[3,74]} = 4.972$, $P = 0.003$) only.

When comparing the different sites of origin using a *post-hoc* analysis, differences in PK activity were only evident between Bunoga and Lwamunda Swamp ($P = 0.041$), as well as between two sites within the Mpanga region, Bunoga and Kahunge $P = 0.008$. The *post-hoc* analysis results showed differences in liver LDH activity between

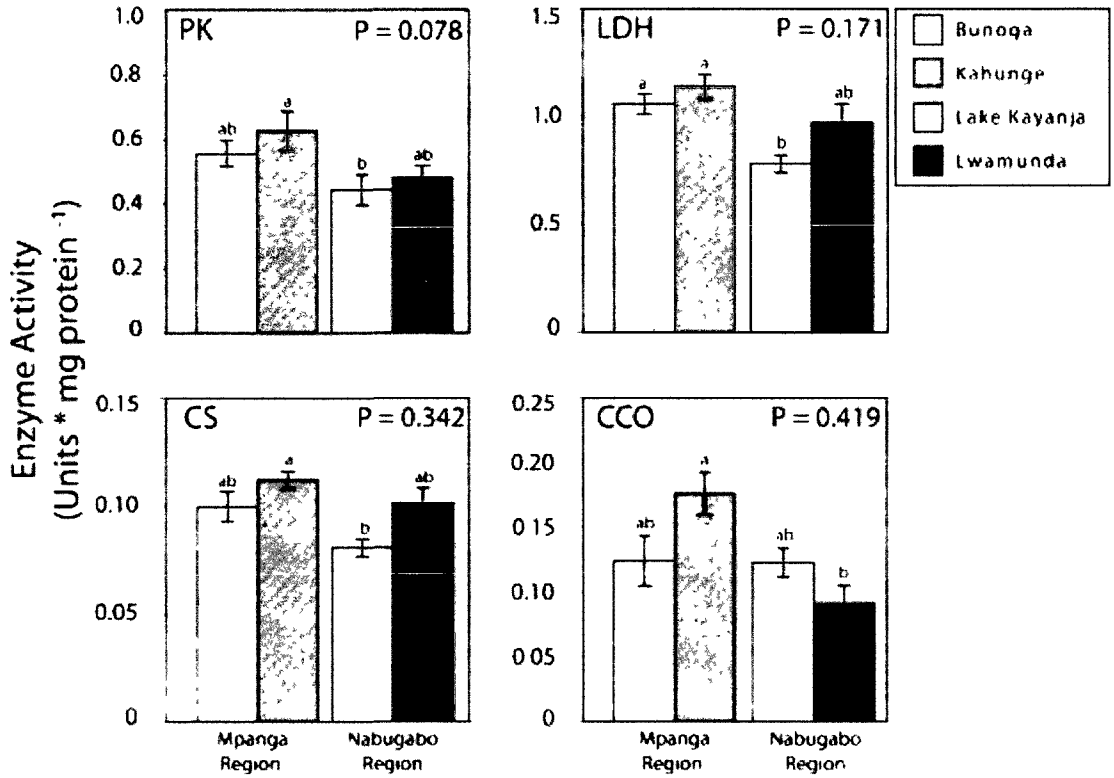

Figure 2.5. Brain enzyme activity of *Pseudocrenilabrus multicolor victoriae* from Bunoga (normoxic), Kahunge (fluctuating), Lake Kayanja (normoxic), and Lwamunda Swamp (hypoxic). Enzymes measured were PK (pyruvate kinase), LDH (lactate dehydrogenase), CS (citrate synthase) and CCO (cytochrome C oxidase). *P values* indicate significant differences between regions, Mpanga Region and Nabugabo Region. Letters indicate significant differences between sites of origin. Bars with the same letter do not differ (Tukey *post-hoc* comparisons, $P > 0.05$). Data are shown as mean ± S.E.

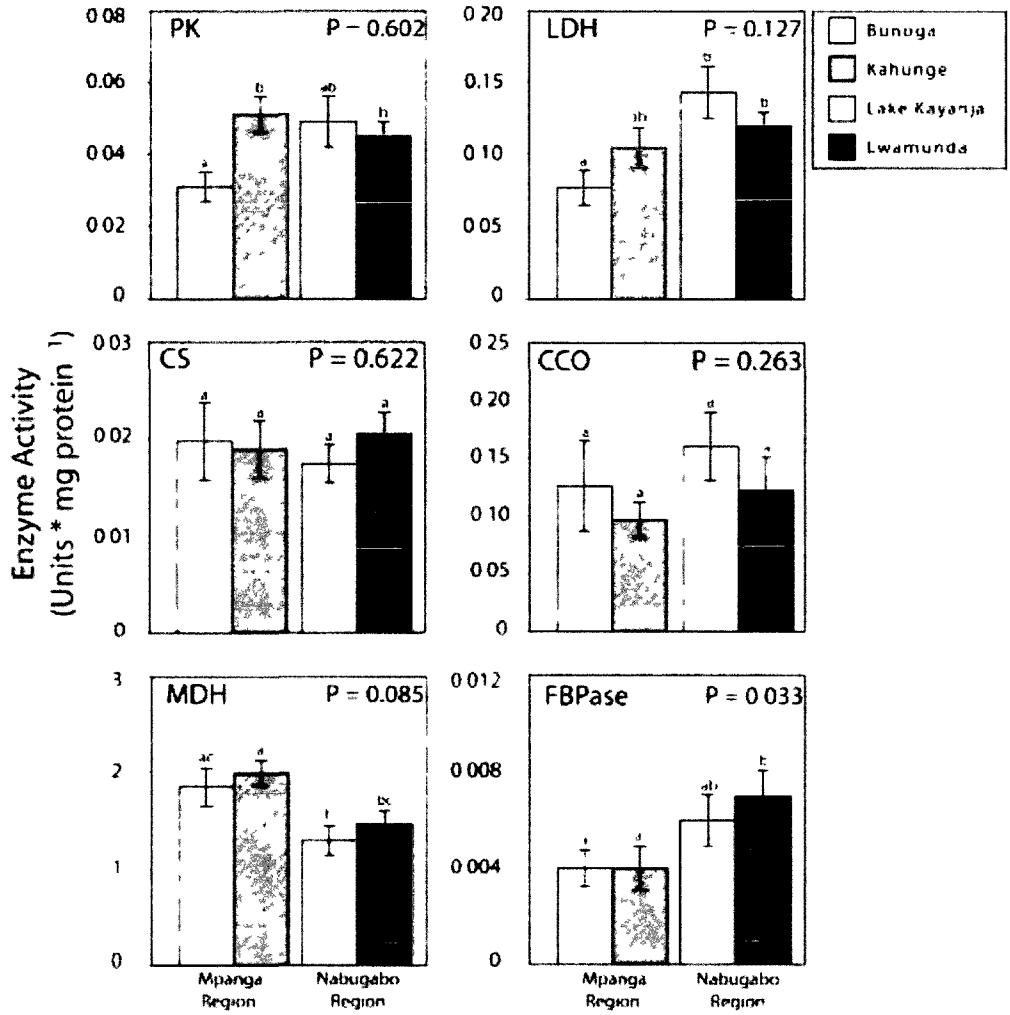

Figure 2.6. Liver enzyme activity of *Pseudocrenilabrus multicolor victoriae* from Bunoga (normoxic), Kahunge (fluctuating), Lake Kayanja (normoxic), and Lwamunda Swamp (hypoxic). Enzymes measured were PK (pyruvate kinase), LDH (lactate dehydrogenase), CS (citrate synthase) and CCO (cytochrome C oxidase). *P values* indicate significant differences between regions, Mpanga Region and Nabugabo Region. Letters indicate significant differences between sites of origin. Bars with the same letter do not differ (Tukey *post-hoc* comparisons, $P > 0.05$). Data are shown as mean ± S.E.

Bunoga and Lake Kayanja ($P < 0.0001$), and Bunoga and Lwamunda Swamp ($P = 0.004$), but not between the remaining studied sites ($P > 0.05$). When examining the gluconeogenic enzymes, MDH activity levels differed between Bunoga and Lake Kayanja ($P = 0.039$), Kahunge and Lake Kayanja ($P = 0.001$), and Kahunge and Lwamunda Swamp ($P = 0.045$), but not Bunoga and Lwamunda Swamp ($P = 0.452$). Finally, differences in liver FBPase activity were observed between Bunoga and Lwamunda Swamp ($P = 0.027$) and Kahunge and Lwamunda Swamp ($P = 0.008$).

Enzyme Activity Ratios

To determine whether there is a preferred metabolic pathway used by individuals from different sites of origin, enzyme activity ratios were calculated in all four tissues as follows: PK/CS (glycolysis/aerobic potential of the Krebs cycle), LDH/CS (glycolysis with pyruvate reduction/aerobic potential of the Krebs cycle), PK/LDH (glycolysis/glycolysis with pyruvate reduction), and MDH/LDH (gluconeogenesis and malate-aspartate shuttle during aerobic glycolysis/glycolysis with pyruvate reduction) calculated only in the liver (Table 2.2).

Overall, there was no preferred metabolic pathway used by fish from the two regions, or the four sites of origin. At a tissue level however, fish from the Mpanga region displayed greater anaerobic activity in the white skeletal muscle (LDH/CS ratio: $F_{[1,2]} = 19.204$, $P = 0.048$), and greater gluconeogenic activity in the liver (MDH/LDH ratio: $F_{[1,2]} = 41.504$, $P = 0.023$) than fish from the Nabugabo region.

While exploring whether sex had any effect on the ratios analyzed, results indicated a significant gender effect on LDH/CS and PK/CS ratios ($F_{[1,72]} = 11.368$, $P =$

0.001 and $F_{[1,75]}$ = 11.117, P = 0.001 respectively). Overall, females had greater anaerobic metabolism than males. No other differences were observed on the ratios between regions and sex.

Table 2.2. Enzyme activity ratio in white skeletal muscle, heart, brain and liver of *Pseudocrenilabrus multicolor victoriae*. Individuals were collected from Bunoga and Kahunge (Mpanga region) and Lake Kayanja and Lwamunda Swamp (Nabugabo region). Letters indicate statistically significant differences between sites of origin. Ratios with the same letter or no letter do not differ (Tukey *post-hoc*, P > 0.05). Data represent means ± S.E.

	Mpanga Region		**Nabugabo Region**	
	Bunoga	**Kahunge**	**Lake Kayanja**	**Lwamunda Swamp**
Muscle				
LDH/CS	127.18 ± 11.37^{a}	114.16 ± 16.68^{ac}	72.66 ± 6.81^{bc}	65.87 ± 7.92^{b}
PK/LDH	0.41 ± 0.02	0.42 ± 0.02	0.40 ± 0.04	0.39 ± 0.02
PK/CS	55.25 ± 6.12^{a}	45.59 ± 6.16^{a}	29.46 ± 3.74^{b}	24.53 ± 2.32^{b}
Heart				
LDH/CS	20.40 ± 2.80	17.71 ± 1.85	16.51 ± 2.01	22.87 ± 3.75
PK/LDH	0.19 ± 0.04	0.17 ± 0.02	0.33 ± 0.11	0.25 ± 0.08
PK/CS	3.03 ± 0.40	2.67 ± 0.37	4.57 ± 1.37	4.52 ± 0.83
Brain				
LDH/CS	14.67 ± 4.14	10.28 ± 0.58	10.05 ± 0.72	10.16 ± 0.97
PK/LDH	0.56 ± 0.06	0.57 ± 0.05	0.63 ± 0.08	0.54 ± 0.05
PK/CS	7.37 ± 1.65	5.89 ± 0.46	5.86 ± 0.73	4.89 ± 0.31
Liver				
LDH/CS	4.56 ± 0.51^{a}	6.45 ± 0.62^{b}	8.75 ± 0.87^{b}	6.54 ± 0.43^{b}
PK/LDH	0.46 ± 0.05^{ab}	0.50 ± 0.04^{a}	0.34 ± 0.02^{b}	0.35 ± 0.03^{ab}
PK/CS	2.26 ± 0.47^{a}	2.94 ± 0.18^{bc}	3.02 ± 0.38^{ac}	2.40 ± 0.25^{ac}
MDH/LDH	29.91 ± 4.96^{a}	24.01 ± 2.52^{a}	9.80 ± 0.68^{b}	11.65 ± 1.01^{b}

Interestingly, differences between sites of origin were evident only in the white skeletal muscle (LDH/CS $F_{[3,70]} = 8.002$, $P < 0.0001$; PK/CS $F_{[3,73]} = 12.321$, $P < 0.0001$) and liver tissue (LDH/CS $F_{[3,71]} = 10.651$, $P < 0.0001$; PK/LDH $F_{[3,73]} = 2.999$, $P = 0.036$; PK/CS $F_{[3,74]} = 2.947$, $P = 0.038$; MDH/LDH $F_{[3,72]} = 18.843$, $P < 0.0001$).

Between sites of origin, differences in muscle LDH/CS ratio were observed between Bunoga and Lake Kayanja ($P = 0.004$), Bunoga and Lwamunda Swamp ($P < 0.0001$) and Kahunge and Lwamunda Swamp ($P = 0.014$), however no differences were observed between the other study sites ($P > 0.05$). Differences in the PK/CS ratio in white skeletal muscle, were observed between, Bunoga and Lake Kayanja ($P < 0.0001$), Bunoga and Lwamunda Swamp ($P < 0.0001$), Kahunge and Lake Kayanja ($P = 0.039$) and Kahunge and Lwamunda Swamp ($P = 0.002$). Differences in PK/CS ratios were not observed between the other study sites examined in this study ($P > 0.05$).

In the liver tissue, LDH/CS ratios differed between Bunoga and Kahunge ($P = 0.018$), Bunoga and Lake Kayanja ($P < 0.0001$) and Bunoga and Lwamunda Swamp ($P = 0.006$). No differences LDH/CS were observed between the other sampling sites ($P > 0.05$). When examining whether differences exist between sites of origin in PK/LDH ratio, I only observed such differences between Kahunge and Lake Kayanja ($P = 0.033$). No difference in PK/LDH ratio was observed between the remaining sampling sites ($P > 0.05$). For PK/CS ratio, differences were observed between Bunoga and Kahunge ($P = 0.036$). Nevertheless, no differences were observed between the other sampling sites ($P > 0.05$). Lastly, significant differences were observed in liver MDH/LDH ratios between sampling sites for Bunoga and Lake Kayanja ($P < 0.0001$), Bunoga and Lwamunda Swamp ($P < 0.0001$), Kahunge and Lake Kayanja ($P < 0.0001$) and Kahunge and

Lwamunda Swamp (P = 0.001). The MDH/LDH ratio, did not differ between Bunoga and Kahunge (P = 0.700) or Lake Kayanja and Lwamunda Swamp (P = 0.643).

DISCUSSION

The present study was a further investigation into effects of divergent natural D.O. concentrations on the survival strategies of the African cichlid *P. multicolor*. Prior work on this species has suggested a certain degree of plasticity relative to the environment where they live (Chapman *et al.* 2002; Chapman *et al.* 2008; Martínez *et al.* 2009). In order to get a better understanding of whether the metabolic capacity of these fish varies with the dissolved oxygen concentration in their natural environment, a total of six enzymes from three pathways were analyzed. Overall, there was a high level of variation detected between regions, while fewer differences within regions were observed. Furthermore, fish from the Mpanga region (Bunoga and Kahunge sites) displayed greater anaerobic enzyme activity in white skeletal muscle, and greater gluconeogenic activity in the liver.

Previous studies have suggested that hypoxia will cause alterations of the metabolic pathways, resulting in increased anaerobic enzyme activity (Holeton and Randall 1967; Hochachka and Somero 2002; Webster 2003). In this study of *P. multicolor* under natural conditions, an overall up-regulation of the anaerobic pathway was not evident. Instead, this species displayed a great deal of variation among tissues and enzymes, similar to findings observed in both the common carp and killifish (Zhou *et al.* 2000; Martínez *et al.* 2006). Both, tissue and enzyme specificity has been suggested to result from adjustments made in order to meet tissue metabolic needs and oxygen requirements (Hochachka *et al.* 1996; Martínez *et al.* 2006). Prolonged or

reoccurring hypoxia exposure may select for varying tissue enzymatic activity. In populations of *Fundulus heteroclitus*, it was observed that differences in gene expression for specific metabolic pathways were expressed among populations, as well as among individuals within a population (Oleksiak *et al.* 2005). For *F. heteroclitus,* both aerobic and anaerobic metabolism responses, were individual specific, with some individuals expressing genes required for aerobic metabolism, while for other individuals, the expression of anaerobic enzymes was more important. It has been suggested that higher variation in gene expression is important for evolutionary change under varying conditions (Oleksiak *et al.* 2005; Garant *et al.* 2007). Changes in gene expression may allow an organism to conserve sufficient levels of ATP required for survival since it does not have to uniformly activate each of the enzymes that participate in the anaerobic metabolism.

Using neutral markers, Crispo and Chapman (2008) found that the genetic structure of *P. multicolor* individuals from different sites of origin similar to those studied here seemed to be influenced by the geographical distance between sites, but there was no evidence for a habitat effect (DO regime) within regions was observed. The differences observed on the genetic structure of individuals of *P. multicolor* are in accordance with my findings. In three out of the four tissues examined, white skeletal muscle, heart and liver, a regional difference between the Mpanga and Nabugabo region was observed. Greater activity of muscle PK activity as well as greater heart PK, LDH, CS, and closely significant CCO activity occurred in fish from the Mpanga region compared to those inhabiting the Nabugabo region. The opposite trend was observed in liver FBPase activity, with greater FBPase activity occurring in the Nabugabo region fish

compared to the Mpanga region fish. Furthermore, minimal differences between enzyme activity levels were observed between sites within a region, regardless of the oxygen regime. The only difference observed between sites of origin within a region was liver PK activity.

Different studies have suggested that increased genetic variability may allow an organism to increase its ability to adapt to environmental changes (Garant *et al.* 2007; Crispo and Chapman 2010; Somero 2010). Regardless of oxygen regime, genetic variability has been shown to be greater within the Nabugabo region, compared to populations inhabiting the Mpanga region (Crispo and Chapman 2008). The Nabugabo region fish may be better adapted to their environmental surroundings compared to the Mpanga region fish, by displaying lower metabolic capacity, regardless of oxygen regime. On the other hand, the higher levels of FBPase in fish from the Nabugabo region may be a result of the reduced glycogen levels in the liver of individuals from the hypoxic Lwamunda Swamp site. It has been suggested that gluconeogenesis becomes activated under long-term hypoxia. This activation could result from a decline in liver glycogen reserves due to food deprivation, lower food ingestion, as well as the need for liver glycogen to provide glucose to other organs within the organism (Hall *et al.* 2006; Hall *et al.* 2009). With insufficient glycogen reserves, the activation of gluconeogenesis will supply glucose for the anaerobic metabolism (Wright *et al.* 1989). Furthermore, the slightly higher levels of liver LDH in the Nabugabo region fish relative to fish from the Mpanga region indicates that Nabugabo fish may be utilizing the increased lactate levels to create greater levels of glucose through gluconeogenesis, decreasing the need to activate anaerobic activity in the extra-hepatic tissues, such as heart and brain as

observed in crucian carp (Nilsson and Lutz 2004). These regional differences and similarities among sites within a region suggest that a genetic variation is playing a role in determining the carbohydrate metabolic capacity of these organisms across the two regions studied.

The regional differences observed in the expression of the metabolic capacity of *P. multicolor* may also be a result of the environmental conditions in which these individuals live. It is possible that the specific habitat characteristics (river *vs.* lake), as well as the swimming energy demands depending on habitat (lotic *vs.* lentic) may be affecting the metabolic capacity. The greater anaerobic carbohydrate ratio observed in the white skeletal muscle of individuals from the Mpanga region (PK/CS and LDH/CS ratio) supports this. Fish inhabiting the river would require a greater anaerobic capacity in order to swim through the faster flowing river water while escaping from predators, capturing prey, or courtship behaviour during the reproduction period (Gibb and Dickson 2002). The ability for these fish to limit enzyme activity changes regardless of the level of oxygen, suggests that *P. multicolor* can withstand different environmental conditions without undergoing too much physiological stress.

Fluctuations in aquatic environments are not unusual under natural conditions, especially when examining the levels of dissolved oxygen. Environmental characteristics such as photosynthesis, light cycles, precipitation as well as river flow, can often change daily (Forsberg and Bergheim 1996); however, fish have developed a number of strategies to deal with constant fluctuations. Previous studies have reported changes in both behavioural as well as in parental care responses, while inhabiting fluctuating habitats (Breitburg *et* al. 1994; Takegaki and Nakazono, 1999). For example,

the male gobiid fish, *Valenciennea longipinnis*, adjusts parental care depending on fluctuations in oxygen. The male gobiid fish has shown to alter fanning frequency upon eggs depending on the level of oxygen during fluctuating times (Takegaki and Nakazono, 1999).

The present study is the first to my knowledge to observe the metabolic capacity of fish while under natural fluctuating D.O. concentrations. Fish from the Kahunge site, experience seasonal fluctuations in the level of D.O. throughout the season, ranging from 1.14 mg O_2 l^{-1} to 5.70 mg O_2 l^{-1} (Figure 2.1). Interestingly, in the more oxygen dependent organs examined, heart and brain, enzyme activity for both pathways, the anaerobic and aerobic, were overall greater in fish from the fluctuating Kahunge site. The over expression of enzyme activity may be necessary for Kahunge fish in order to protect vital organs required to maintain a homeostatic balance as suggested by Nilsson *et al.* (1993).

A major strategy for an organism to survive under hypoxia and fluctuating environments includes maintenance of sufficient levels of ATP or a level of ion homeostasis (Nilsson *et al.* 1993). The brain is one of the most sensitive organs of vertebrates and it consumes about 3% of the total body energy in ectotherms. As a center of control in all organisms, and in order to maintain neural processes, the brain requires high levels of ATP, which is mainly produced through glucose oxidation (Soengas and Aldegunde 2002). Furthermore, the brain is considered one of the most vulnerable organs to hypoxia due to its minimal glycogen and glucose reserves (Nilsson 2001). Vertebrates that are more sensitive to changing oxygen levels rapidly decrease the amount of available ATP in the brain tissue (Nilsson 2001). More hypoxia tolerant

organisms decrease the reduction of brain ATP levels while simultaneously decreasing lactate build up (Nilsson 2001). The lack of regional differences observed in the brain may suggest an important strategy by this organism to handle hypoxia. Unlike, hypoxia sensitive organisms that deplete ATP drastically due to the down regulation of the aerobic metabolism and utilization of anaerobic metabolism (Nilsson *et al.* 1993), *P. multicolor* seems to attain a level of homeostasis, supplying adequate levels of ATP to the brain, perhaps at the expense of other tissues like white skeletal muscle.

It is important to note, that for this study, the fish from the fluctuating Kahunge site were collected during the dry season, while oxygen levels are considered normoxic (5.5-6.0 mg O_2 l^{-1}); yet, individuals displayed higher enzymatic levels than individuals from normoxic sites. The observed higher levels of enzyme activity in fish from Kahunge site indicates that fish from this site have a greater plastic metabolic potential than fish from the other three study sites. The increased expression of enzyme activity observed in the Kahunge site, may allow these individuals to save energy in maintaining a larger pool of phenotypes, instead of changing them seasonally which may in return, be more energy expensive. This strategy of over expressing enzyme activity would have to be further investigated using populations of *P. multicolor* captured over the full year, in order to determine if similar trends are observed throughout the seasons. If little difference in metabolic capacity were observed over the year, it may suggest that individuals from the Kahunge site may be better adapted to the increasing hypoxic waters caused by climactic change and anthropogenic causes.

With the effects of global climactic change coupled with anthropogenic factors resulting from deleterious land uses, rising populations, pollution and increased fishing

industries, eutrophication is increasing, resulting in a number of hypoxic zones in both inland and coastal waters (Verschuren *et al.* 2002; Diaz and Brietburg 2009). Thus, it has become increasingly important to understand the response of fish to hypoxia across all levels from behaviour to metabolic capacity. It is possible that some aquatic organisms may be able to withstand the effects caused by human activity, and we may be able to extrapolate some of the findings in this study to other species. For example, it is likely that species occupying broad habitat ranges and/or characterized by high levels of phenotypic plasticity may be pre-adapted to handle environmental change relative to more stenotypic and less plastic species.

CHAPTER 3 – PHYSIOLOGICAL RESPONSES OF THE AFRICAN CICHLID *PSEUDOCRENILABRUS MULTICOLOR VICTORIAE* TO HYPOXIA REARING CONDITIONS: LIKE FATHER LIKE SON?

ABSTRACT

The African cichlid, *Pseudocrenilabrus multicolor victoriae* is a eurytopic species that exhibits high levels of developmental plasticity in response to oxygen availability in its environment. In this study, F_1 offspring from three sites within the Mpanga River drainage of Western Uganda characterized by different dissolved oxygen regimes (annual D.O. means: Bunoga 7.53 ± 0.13 mg $O_2 l^{-1}$, Kahunge 3.76 ± 1.70 mg O_2 l^{-1} and Kantembwe 0.21 ± 0.12 mg O_2 l^{-1}) were reared under either normoxia or hypoxia. After one year, enzymes from three different metabolic pathways (pyruvate kinase [PK], lactate dehydrogenase [LDH], citrate synthase [CS] and cytochrome C oxidase [CCO]) were measured in white skeletal muscle, heart, brain and liver. Two gluconeogenic enzymes, malate dehydrogenase (MDH) and fructose 1,6-bisphosphatase (FBPase), were examined in the liver only. Individuals reared under hypoxia displayed an up regulation in heart LDH, and CCO; a down regulation in brain CS, and liver CCO and MDH activity. This study demonstrates that the D.O. concentration plays a key role in determining the metabolic capacity of the F_1 generation of *P. multicolor.* Future studies, should examine the metabolic capacity of individuals reared under normoxia and hypoxia from different river systems that are more genetically distinct to explore more fully the interaction between oxygen and population effects.

RÉSUMÉ

Le cichlidé africain, *Pseudocrenilabrus multicolor victoriae,* est une espèce eurytope qui démontre de hauts niveaux de plasticité développementale en réponse à la disponibilité en oxygène dans son environnement. Dans cette étude, les rejetons F_1 provenant de trois sites du bassin de drainage de la rivière Mpanga dans l'ouest de l'Ouganda caractérisé par différentes régimes d'oxygène dissous (moyennes annuelles de D.O. : Bunoga 7.53 ± 0.13 mg O_2 l^{-1}, Kahunge 3.76 ± 1.70 mg O_2 l^{-1} et Kantembwe 0.21 ± 0.12 mg O_2 l^{-1}) ont été élevés dans des conditions normoxiques ou hypoxiques. Après une année, les enzymes de trois différentes voies métaboliques (pyruvate kinase [PK], lactate déshydrogénase [LDH], citrate synthase [CS] et cytochrome C oxydase [COX]) ont été mesurées dans le muscle squelettique blanc, le cœur, le cerveau et le foie. Deux enzymes gluconéogéniques, la malate déshydrogénase (MDH) et la fructose 1,6-bisphophatase (FBPase), ont été examinées dans le foie seulement. Les individus élevés sous hypoxie présentaient une régulation générale à la hausse dans le cœur de LDH et CCO; une régulation à la baisse dans le cerveau de CS, et dans le foie de l'activité de CCO, et MDH. Cette étude démontre que la concentration en D.O. joue un rôle clé dans la détermination de la capacité métabolique de la génération F_1 de *P. multicolor*. Les études futures devraient examiner la capacité métabolique des individus élevés sous conditions normoxiques et hypoxiques de différents systèmes de rivières qui ont démontré des variations génétiques.

INTRODUCTION

Low dissolved oxygen, hypoxia, has become a major stressor worldwide associated with eutrophication and pollution causing a dramatic increase in the extent of dead zones in coastal and inland waters (Dybas 2005; Diaz and Rosenberg 2008). Effects of hypoxia may be exacerbated by global warming particularly for ectothermic organisms (e.g., fish). Therefore it is becoming increasingly important to examine effects of hypoxia on the physiology of aquatic organisms under long-term exposure (Pörtner and Farrell 2008). Over the last few years, a number of laboratory experiments have examined behavioural, morphological and physiological adaptations of fish under different stressors such as temperature, pH and low dissolved oxygen (D.O.). However, when trying to establish a common hypoxia response pattern among fish under lab conditions, one needs to be cautious as even slight variations in acclimation duration (hours to weeks), the intensity of hypoxia exposure (species specific) as well as tank parameters that may affect the responses observed.

Organisms experience changes at different physiological levels under hypoxia exposure (Chippari-Gomes *et al.* 2005; Farwell *et al.* 2007). Some studies strongly suggest that for a given species, the capacity to survive under hypoxia highly depends on the length and intensity of the exposure (Greany *et al.* 1980). For example, when an organism is exposed to hypoxia for a short duration of time, a common response is to maintain oxygen delivery throughout the body by decreasing their energy expenditure (West and Boutilier 1998; Richards 2011). It has also been observed that under hypoxia conditions, glycolysis assists in ATP production without the need to reduce the overall ATP turnover (West and Boutilier 1998; Richards 2011). Under long-term exposure

however, there is an imbalance of ATP supply and demand leading to the depletion of cellular ATP (West and Boutilier 1998). In order to maintain the organisms' energetic needs, it is therefore necessary to balance both energy supply and demand under prolonged exposure to low D.O. (West and Boutilier 1998; Wu 2002; Richards 2011).

Depending on the developmental stage, individuals may be more vulnerable to a particular environmental stressor (Pollock *et al.* 2007). During the early developmental stages, the intensity, and duration of exposure to stressors such as hypoxia, may be extremely important for the future survival of the organisms (Spicer and Burggren 2003; Reardon and Chapman 2010). Studies have shown that immediately after hatching, fish experience a number of morphological, behavioural and physiological alterations, primarily due to the changes in their energy metabolism. For example, at the metabolic level, the activity of aerobic oxidative enzymes has been shown to be greater during earlier development, and observed to decrease as the fish grows. However, when examining anaerobic enzymes the opposite trend is observed, demonstrating that there are changes in metabolism and swimming modes as fish grow (Hinterleitner *et al.* 1987). The post-hatching larval stages are much more sensitive to environmental D.O. variation than the adult stage (Shang and Wu 2004). Therefore, exposure to hypoxia during the early developmental stages may promote adaptive changes that will be more suitable for survival under stressful conditions and that will enhance animal fitness (Nilsson *et al.* 2010).

Over the last 15 years, there has been an increase in the number of studies looking at different traits of a eurytopic African cichlid *Pseudocrenilabrus multicolor victoriae* (Seegers). This cichlid has been used as a model to study fish adaptation to

hypoxia at various levels because it naturally occurs across dramatic gradients of dissolved oxygen concentration from chronic extreme hypoxia to normoxia. This model fish displays a great level of developmental plasticity, as well as genetic diversity that relates not only to D.O., but also their geographical distribution. Phenotypic variation across populations has been observed in a number of traits including gill size, body shape, and brain mass (Chapman *et al.* 2000; Chapman *et al.* 2008; Crispo and Chapman 2010). In addition, some physiological responses of *P. multicolor* vary across field populations, including LDH (Martínez *et al.* 2009). These studies strongly suggest that local adaptation, genetic structure, and phenotypic plasticity are determining the capacity of *P. multicolor* to withstand hypoxia. There is evidence from studies of neutral markers that gene flow is high among *P. multicolor* population, but it is unclear as to whether phenotypic plasticity is driving gene flow, or if gene flow is selecting for an increased level of plasticity (Sterns 1989; Moore *et al.* 2006; Crispo and Chapman 2008).

Interestingly both the genetics of the organism as well as the environmental conditions can interact in such a way that the different genotypes may respond differently to the same environmental change (Moore *et al.* 2006). Variation in the genetic pool as well as the specific traits of the environment can play a key role in determining the metabolic capacities of individuals. As such, the aim of this study was to determine the role of plasticity on the metabolic capacity of the F_1 generation of *P. multicolor* from three different sites of origin reared under divergent D.O. regimes.

MATERIALS AND METHODS

Study Sites and Collection of Fish

Baited minnow traps were used to collect parental stock in June 2006, from three sites, along the Mpanga River in Western Uganda, Africa: Bunoga, Kantembwe and Kahunge (Figure 3.1). Bunoga site is situated along the river with open flowing waters, and remains normoxic year round (D.O. = 7.53 ± 0.13 mg O_2 l^{-1}). This site experiences subtle seasonal fluctuations in D.O. levels as a result of the surrounding anthropogenic land use (Crispo and Chapman 2010). Kantembwe is a vegetated swamp remaining hypoxic year round with a monthly D.O. variation of 0.21 ± 0.12 mg O_2 l^{-1}. The remaining site along the river, Kahunge, experiences a greater degree of seasonal D.O. fluctuation, with higher D.O. levels during the dry season (6.0 mg O_2 l^{-1}) and the lower values during the rainy season (1.5 mg O_2 l^{-1}) when the decomposing matter flows in from the adjacent swamp site (Chapman *et al.* 2002).

Rearing Laboratory Experiment

Live adult fish were transferred from Uganda, Africa to the Fish Respiratory Ecology Lab at McGill University in Montreal, Québec. Parental stocks were maintained under normoxic conditions (7.27 – 8.07 mg O_2 l^{-1}) until the broods were released. Fish were maintained in the same conditions as described in Crispo and Chapman (2010). To summarize, each family consisted of the brood from one male-female pair. Four full-sib families were raised in 2007-2008 from each sampling site. One week after broods were released from the female mouth, individuals from each family were divided into their experimental high and low dissolved oxygen treatment tanks, in which each combination of family and treatment were placed into a 14-ga. tank.

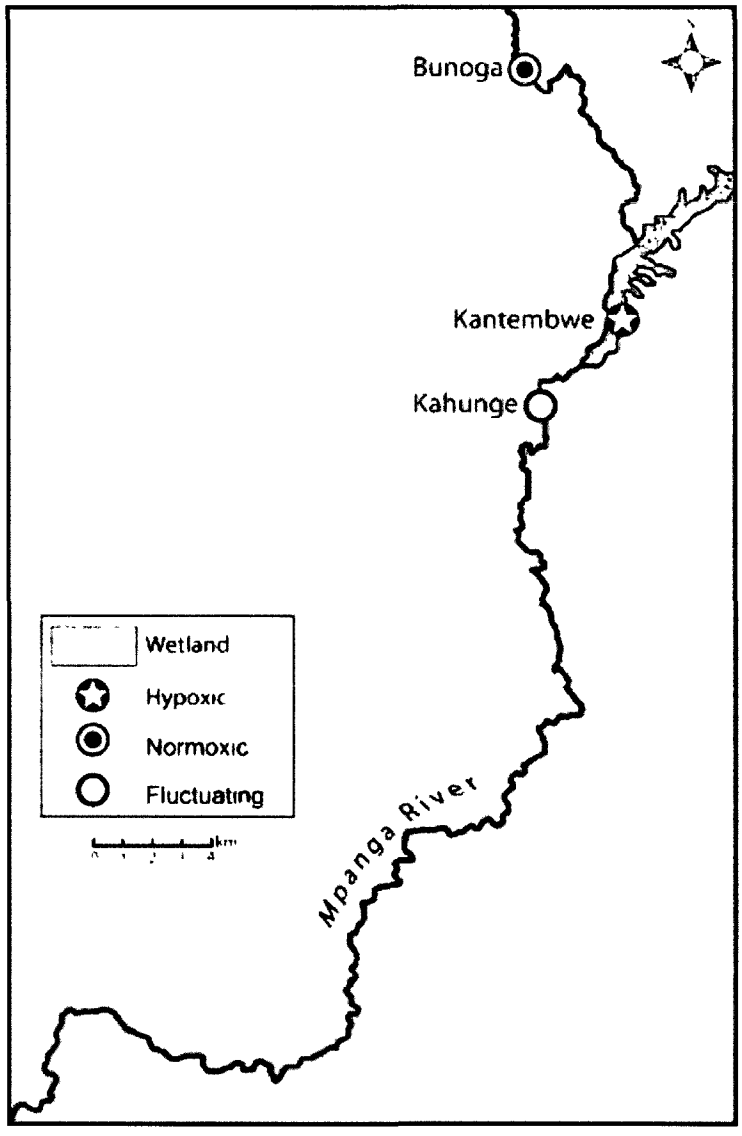

Figure 3.1. Location of sampling sites along the Mpanga River in Western Uganda, Africa. *Pseudocrenilabrus multicolor victoriae* were sampled at Bunoga (D.O. = 7.53 ± 0.13 mg O_2 l^{-1}) Kantembwe (D.O. = 0.21 ± 0.12 mg O_2 l^{-1}) and Kahunge (D.O. = 3.76 ± 1.70 mg O_2 l^{-1}). (Map adapted from Crispo and Chapman 2008).

After approximately 1 month, a ratio of 10 fish per tank was maintained until completion of the study to allow for growth that might be representative of what is observed in the field (no mortality was observed during the rearing period). During this study, tanks were maintained at a common temperature 25.5 ± 0.05°C, and exposed to a 12L:12D photoperiod. Temperature was recorded weekly using an OxyGuard Handy meter. Experimental tanks were equipped with Hagen Fluval® underwater filters with filter sponge. Normoxic treatment groups (7.27 – 8.07 mg O_2 l^{-1}) were aerated by constantly bubbling air through the water column. Low D.O. levels in the hypoxic treatment groups (0.54 – 1.29 mg O_2 l^{-1}) were monitored and controlled using a Point Four Systems Inc., PT4 Monitoring and Control System (Coquitlam, British Columbia) by releasing nitrogen gas into the water column when required. Fish were fed Hikari® First Bites™ fry bites once daily for the first three weeks after release from the females mouth, and a combination of fish bites and ground up TetraMin® Pro Tropical Crisps™ over the following two weeks. After one month of rearing, fish consumed ground up TetraMin® Pro Tropical Crisps™ daily for six days a week for the duration of the experiment. The F_1 generation was grown under experimental conditions for approximately one year before harvesting.

Enzyme Assays

Fish were euthanized using a buffered solution of tricaine methanesulfonate (MS222; pH 7.0), and measured for mass (g ± 0.1), total length (cm ± 0.1) and standard length (cm ± 0.1). Dissected tissues (white skeletal muscle, heart, brain and liver) were frozen in liquid nitrogen. Samples were transported to Laurentian University, Sudbury Ontario, on dry ice and immediately stored at -80 °C, until tissues were ready to be assayed.

Thawed tissues were weighed (g ± 0.0001) and homogenized using a Polytron homogenizer (PT 1200, Kinematica, Switzerland) in an ice cold buffer consisting of 100mM of Hepes (pH 7.0), 0.1 mM of dithiotheritol (DTT), and 0.2% of Triton X-100 for a total of three 20 s intervals. During and between homogenization, samples were kept on ice. Muscle and brain were homogenized in 10 or 50 volumes of buffer, while liver and heart were homogenized in 50 or 100 volumes depending on initial tissue mass. Homogenates were centrifuged using a Thermo IEC Micromax RF Refrigerated Microcentrifuge at 2400 *g* for 15 min at 4°C. Supernatants were kept on ice until enzyme activity was assayed.

Reactions were initiated by the addition of the enzyme specific substrate (substrate differentiated by *). Substrate concentrations were optimized to give maximal enzyme activity. The final protocols for pyruvate kinase, malate dehydrogenase and fructose 1,6-bisphosphatase were modified from Martínez *et al.* 2006 as follows:

Pyruvate Kinase (PYK; EC 2.7.1.40): 100 mM Hepes buffer (pH 7.4); 10mM KCl (heart, liver) 20mM KCl (brain), or 40mM KCl (muscle); 0.2 mM NADH; 2.5 mM ADP (heart) or 10 mM ADP (muscle, liver brain); 0.25 U ml^{-1} L-LDH (muscle, brain), 0.5 U ml^{-1} L-LDH (heart) or 1 U ml^{-1} L-LDH (liver); 5 mM $MgCl_2$ (muscle, liver, brain) or 10 mM $MgCl_2$ (heart); *2 mM PEP (muscle, liver, brain) or 4 mM PEP (heart).

Malate Dehydrogenase (MDH; EC 1.1.1.37): 100 mM Hepes buffer (pH 7.4); 0.2 mM NADH; *0.2 mM oxaloacetic acid.

Fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11): 100mM Hepes buffer (pH 7.4); 10 mM KCl; 0.4 mM NADP; 0.8 U ml^{-1} PGI; 0.72 U ml^{-1} Glucose-6-phosphate

dehydrogenase; 6 mM $MgCl_2$, 0.5 mM EDTA; 0.05 mM Fructose 1,6-bisphosphate; * 10 µl of liver tissue.

The reaction conditions for citrate synthase, cytochrome C oxidase and lactate dehydrogenase were modified from standard protocols.

Citrate Synthase (CS; EC 2.3.3.1): 50 mM Trizma Base buffer (pH 8.0); 0.1 mM 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB); 0.1 mM Acetyl coenzyme A (muscle, heart) or 0.2 mM Acetyl coenzyme A (liver, brain); *0.75 mM oxaloacetic acid (muscle) or 1.5 mM oxaloacetic acid (liver, heart, brain) (Martínez *et al.* 1999).

Cytochrome C Oxidase (CCO; EC 1.9.3.1): 50 mM K_2HPO_4 and 50 mM KH_2PO_4 buffer (pH 7.0); 0.07 mM reduced cytochrome C. The reaction was compared against 0.07 mM of reduced cytochrome C and 0.33% K-ferricyanide. Cytochrome C was reduced by the addition of sodium hydrosulfite; *Tissue (Zhou *et al.* 2000).

Lactate Dehydrogenase (LDH; EC 1.1.1.27): 100 mM Hepes; 0.12 mM NADH; *1.0 mM pyruvate (Martínez *et al.* 2009).

Maximal enzyme activity was measured in a 96-well microplate reading spectrophotometer (Molecular Devices, Spectramax plus 384, Sunnyvale, CA, USA) at 25 °C. Rates from blank reactions (without substrates) were subtracted for determination of enzyme activities. For LDH, MDH, and PK, reactions were measured by the disappearance of NADH at 340 nm. The reduction of $NADP^+$ at 340 nm, was used to measure the reaction of FBPase. The CS reaction was measured at 412 nm to detect the transfer of coenzyme A to DTNB and CCO was measured at 550 nm to observe the oxidation of reduced cytochrome C. The value of 6.22, 13.6 and 19.1 were used as the

millimolar extinction coefficients for NAD(P)H, DTNB and cytochrome C respectively. All enzyme activities were measured within four hours of tissue homogenization. Activity was measured in the following order, LDH, MDH, CS, CCO, PK and FBPase. Assays were run in quadruplicates and reactions were linear for $\geq$ 180 s.

Biochemicals and Chemicals

All chemicals and biochemicals were purchased from Sigma – Aldrich Co. (Oakville, ON, Canada), Roche Diagnostics Corporation (Mississauga, ON, Canada) and Thermo Scientific (Rockford, IL, U.S.A.)

Protein Analysis

Supernatants for protein analysis were kept at -80 °C until analyzed. The bicinchoninic acid (BCA) assay was used to determine the protein content in the supernatant fraction of tissue homogenates (Smith *et al.* 1985). The protocol was modified for use in a 96-well microplate reading spectrophotometer (Brown *et al.* 1989). Samples were diluted using distilled water to give a final 100 fold dilution for muscle, heart and brain, and 200 fold for liver. A total of 10 µl of sample was added to quadruplicates of 200 µl BCA working reagent into individual wells of the 96-well microplate. Standards of 0 - 1 mg ml^{-1} of bovine serum albumin (BSA) were included as a standard curve with every plate. Plates were sealed, and incubated at 60 °C for 30 min. After cooling for 10 min, plates were read at 562 nm at room temperature.

Statistical Analysis

All variables were examined for normality using Shapiro Wilks test, and tested for homogeneity of variance before analyses were completed. Tissue enzyme activities that did not meet the testing guidelines were $\log_{10}$ transformed. These included muscle CCO, heart CS, CCO and PK, brain CS, CCO, and PK, and all enzymes in the liver. Analysis of covariance (ANCOVA) was used to detect effects of sites of origin, D.O. treatment (normoxia vs. hypoxia), and the interaction between site of origin and treatment. Fish mass and length may have some effects on the levels of enzyme activity, and as such I explored their contribution in the model as covariates. Body mass and total length were both significant covariates for brain PK only (and the effects were similar for both covariates), therefore body mass was included as a covariate for this enzyme only. To consider the variation among families, family was nested within population, where family was the random factor and population was the fixed variable. Tukey *post hoc* analysis was used to determine differences between the levels of enzyme activity among sites. When the interaction between site of origin and oxygen treatment was significant, an ANOVA was used to assess the effect of oxygen treatment on sites of origin separately. Statistical outliers detected in the ANOVA protocol (individuals where the value of the measured trait was more than two standard deviations from the mean), were removed from analyses. This resulted in one individual being removed from brain CS; two from heart CCO and liver PK; three from muscle LDH, CS and CCO; three from brain PK, LDH and CCO; and three from liver PK, CS, MDH, and FBPase, and finally four from muscle PK; heart PK, LDH, CS; and liver LDH. All statistical analyses were completed using SPSS Statistics 17.0 (SPSS Inc., Chicago, Illinois, U.S.A.). All data

were considered statistically significant at $P < 0.05$. All figures presented are shown using the raw untransformed data.

RESULTS

In general, the F_1 fish from the three different study sites, Bunoga, Kantembwe and Kahunge differed in both body mass ($F_{[2,68]} = 3.785$, $P = 0.028$) and total length ($F_{[2,68]} = 4.258$, $P = 0.018$), but no difference was observed for Fulton's condition factor ($F_{[2,68]} = 1.237$, $P = 0.297$) (Table 3.1). Bunoga F_1 fish had greater body mass ($P = 0.027$) and total length ($P = 0.041$) compared to the Kantembwe fish. Kahunge F_1s were greater in total length compared to Kantembwe fish ($P = 0.033$). Significant differences were observed on body mass ($F_{[1,69]} = 4.072$, $P = 0.047$), total length ($F_{[1,69]} = 9.747$, $P = 0.003$), and condition factor ($F_{[1,69]} = 8.619$, $P = 0.005$) between D.O. rearing conditions. Individuals reared under hypoxia were smaller in body mass and shorter than individuals reared under normoxia. However, fish reared under hypoxia displayed greater Fulton's condition factor than individuals reared under normoxia (Table 3.1).

Dissolved oxygen had a significant effect on variation in enzyme activity of the heart, brain and liver. The tissues that showed the most variation in response to divergent D.O. environments during this study were the heart and liver, followed by the brain tissue (Table 3.2, 3.3, and 3.4). Interestingly, family nested within population had a significant effect on brain CCO activity (Table 3.3). Furthermore, in the liver tissue, population x treatment significantly affected the LDH levels (Table 3.4). Surprisingly, the rearing D.O. levels, population, and their interaction did not have any significant effect on the four enzymes examined in the white skeletal muscle (Table 3.5.).

Table 3.1. Morphological characteristics of *Pseudocrenilabrus multicolor victoriae* from Western Uganda, Africa. The F_1s were reared under normoxia or hypoxia conditions for approximately one year. Letters indicate significant differences between the F_1 generation reared under normoxia or hypoxia for each site of origin individually. Values with the same letter or no letter do not differ Tukey *post-hoc,* $P > 0.05$). Values are mean ± S.D.

	Bunoga		**Kantembwe**		**Kahunge**	
Parameter	**Normoxic**	**Hypoxic**	**Normoxic**	**Hypoxic**	**Normoxic**	**Hypoxic**
Body Mass (g)	1.93 ± 0.43^{a}	1.66 ± 0.44^{b}	1.54 ± 0.37	1.48 ± 0.33	1.83 ± 0.37^{A}	1.61 ± 0.33^{B}
Total Length (cm)	5.17 ± 0.45^{a}	4.80 ± 0.42^{b}	4.76 ± 0.33	4.65 ± 0.33	5.18 ± 0.29^{A}	4.80 ± 0.34^{B}
Fulton condition factor (Mass * Length $_T$ $^{-3}$)*100	1.39 ± 0.13	1.47 ± 0.13	1.41 ± 0.16	1.45 ± 0.07	1.32 ± 0.12^{A}	1.44 ± 0.12^{B}
N	12	12	12	12	12	11

In the heart tissue, fish displayed greater LDH and CCO enzyme activity when reared under hypoxia, nevertheless, the degree of variation was population dependent (Table 3.2, Figure 3.2). Comparing the specific enzymes changes due to oxygen regime within each site of origin, offspring from Bunoga and exposed to hypoxia displayed an up-regulation of LDH (21%) and CCO (21%) activity, while fish from Kantembwe origin displayed a 21% increase in LDH activity, and a 61% increase in CCO activity when reared under hypoxia. Lastly, Kahunge offspring, reared under hypoxia displayed an up-regulation of LDH (23%) and CCO (99%).

In the brain tissue, mass was positively correlated with brain PK activity. The rearing D.O. treatment, significantly affected the levels of CS activity, with offspring displaying lower brain CS activity when reared under hypoxia, compared to those fish reared under normoxia (Table 3.4). In general, offspring from Bunoga, down-regulated brain CS activity by 18% when reared under hypoxia (Figure 3.2). On the other hand, offspring from Kantembwe origin showed a 17% decrease in CS activity while reared under hypoxia relative to those reared under normoxia. Finally, fish from Kahunge origin displayed a 15% down-regulation in CS activity when reared under hypoxia conditions for one year (Figure 3.2).

In the liver tissue, rearing D.O. treatment significantly affected the levels of CCO and MDH activity, with hypoxia reared fish displaying lower levels of CCO and MDH activity compared to normoxia reared fish. Offspring from Bunoga origin displayed an 11% down-regulation in CCO activity and a 13% down-regulation in MDH activity (57% and 38% respectively) while reared under hypoxia relative to those reared under normoxia.

Table 3.2. Effects of rearing conditions, population of origin and family, on the levels of enzyme activity in heart of the African cichlid *Pseudocrenilabrus multicolor victoriae* offspring. Parental stocks were collected from Bunoga, Kantembwe and Kahunge sites from Western Uganda, Africa. F1s were reared under reciprocal D.O. levels (hypoxia and normoxia) for 12 months. *P* values are from ANOVA with rearing conditions, population of origin and family nested within population of origin as main effects.

Source of Variation	d.f.	MS	F	P
Pyruvate kinase				
Rearing D.O. Treatment	1	0.064	1.751	0.192
Population of Origin	2	0.079	3.069	0.096
Family (Population)	9	0.026	0.704	0.702
Population x Treatment	2	0.048	1.315	0.277
Error	52	0.036		
Lactate dehydrogenase				
Rearing D.O. Treatment	1	0.938	4.464	**0.039**
Population of Origin	2	0.027	0.106	0.900
Family (Population)	9	0.257	1.222	0.302
Population x Treatment	2	0.004	0.018	0.982
Error	52	0.210		
Citrate synthase				
Rearing D.O. Treatment	1	0.000	0.014	0.907
Population of Origin	2	0.057	0.729	0.509
Family (Population)	9	0.078	2.531	**0.017**
Population x Treatment	2	0.001	0.023	0.977
Error	52	0.031		
Cytochrome c oxidase				
Rearing D.O. Treatment	1	1.079	9.119	**0.004**
Population of Origin	2	0.086	0.568	0.586
Family (Population)	9	0.151	1.280	0.268
Population x Treatment	2	0.111	0.942	0.396
Error	56	0.118		

Table 3.3. Effects of rearing conditions, population of origin and family, on the levels of enzyme activity in liver of the African cichlid *Pseudocrenilabrus multicolor victoriae* offspring. Parental stocks were collected from Bunoga, Kantembwe and Kahunge sites from Western Uganda, Africa. F1s were reared under reciprocal D.O. levels (hypoxia and normoxia) for 12 months. *P* values are from ANOVA with rearing conditions, population of origin and family nested within population of origin as main effects.

Source of Variation	d.f.	MS	F	P
Pyruvate kinase				
Rearing D.O. Treatment	1	0.049	0.616	0.436
Population of Origin	2	0.027	0.205	0.818
Family (Population)	9	0.133	1.684	0.116
Population x Treatment	2	0.022	0.277	0.759
Error	53	0.079		
Lactate dehydrogenase				
Rearing D.O. Treatment	1	0.002	0.034	0.855
Population of Origin	2	0.135	1.331	0.311
Family (Population)	9	0.101	1.670	0.121
Population x Treatment	2	0.235	3.885	**0.027**
Error	51	0.061		
Citrate synthase				
Rearing D.O. Treatment	1	0.085	2.542	0.117
Population of Origin	2	0.113	2.035	0.185
Family (Population)	9	0.056	1.686	0.116
Population x Treatment	2	0.009	0.263	0.770
Error	52	0.033		
Cytochrome c oxidase				
Rearing D.O. Treatment	1	0.916	5.972	**0.018**
Population of Origin	2	0.304	1.573	0.259
Family (Population)	9	0.194	1.264	0.277
Population x Treatment	2	0.029	0.192	0.826
Error	55	0.153		
Malate dehydrogenase				
Rearing D.O. Treatment	1	0.252	12.178	**0.001**
Population of Origin	2	0.028	1.419	0.289
Family (Population)	9	0.019	0.935	0.503
Population x Treatment	2	0.057	2.729	0.075
Error	52	0.021		
Fructose 1,6-bisphosphatase				
Rearing D.O. Treatment	1	0.000	0.002	0.962
Population of Origin	2	0.016	0.207	0.817
Family (Population)	9	0.076	1.442	0.195
Population x Treatment	2	0.001	0.016	0.985
Error	52	0.053		

Table 3.4. Effects of rearing conditions, population of origin and family, on the levels of enzyme activity in brain of the African cichlid *Pseudocrenilabrus multicolor victoriae* offspring. Parental stocks were collected from Bunoga, Kantembwe and Kahunge sites from Western Uganda, Africa. F1s were reared under reciprocal D.O. levels (hypoxia and normoxia) for 12 months. *P* values are from ANOVA/ANCOVA with rearing conditions, population of origin and family nested within population of origin as main effects.

Source of Variation	d.f.	MS	F	P
Pyruvate kinase				
Mass	1	0.147	5.614	**0.022**
Rearing D.O. Treatment	1	0.035	1.353	0.250
Population of Origin	2	0.016	0.348	0.715
Family (Population)	9	0.046	1.768	0.097
Population x Treatment	2	0.070	2.676	0.078
Error	52	0.026		
Lactate dehydrogenase				
Rearing D.O. Treatment	1	0.013	0.441	0.510
Population of Origin	2	0.012	0.280	0.762
Family (Population)	9	0.043	1.429	0.200
Population x Treatment	2	0.042	1.396	0.257
Error	53	0.030		
Citrate synthase				
Rearing D.O. Treatment	1	0.076	4.757	**0.003**
Population of Origin	2	0.011	0.413	0.674
Family (Population)	9	0.026	1.625	0.131
Population x Treatment	2	0.003	0.207	0.813
Error	55	0.016		
Cytochrome c oxidase				
Rearing D.O. Treatment	1	0.114	2.481	0.121
Population of Origin	2	0.050	0.397	0.683
Family (Population)	9	0.127	2.760	**0.010**
Population x Treatment	2	0.111	2.414	0.099
Error	53	0.046		

Table 3.5. Effects of rearing conditions, population of origin and family, on the levels of enzyme activity in white skeletal muscle of the African cichlid *Pseudocrenilabrus multicolor victoriae* offspring. Parental stocks were collected from Bunoga, Kantembwe and Kahunge sites from Western Uganda, Africa. F1s were reared under reciprocal D.O. levels (hypoxia and normoxia) for 12 months. *P* values are from ANOVA with rearing conditions, population of origin and family nested within population of origin as main effects.

Source of Variation	d.f.	MS	F	P
Pyruvate kinase				
Rearing D.O. Treatment	1	0.239	2.129	0.151
Population of Origin	2	0.033	0.242	0.790
Family (Population)	9	0.137	1.223	0.302
Population x Treatment	2	0.149	1.330	0.273
Error	52	0.112		
Lactate dehydrogenase				
Rearing D.O. Treatment	1	0.646	0.779	0.382
Population of Origin	2	0.948	0.629	0.555
Family (Population)	9	1.516	1.827	0.085
Population x Treatment	2	0.128	0.154	0.857
Error	53	0.830		
Citrate synthase				
Rearing D.O. Treatment	1	0.000	2.807	0.100
Population of Origin	2	$3.54E^{-5}$	0.213	0.812
Family (Population)	9	0.000	1.162	0.338
Population x Treatment	2	$5.70E^{-5}$	0.397	0.674
Error	53	0.000		
Cytochrome c oxidase				
Rearing D.O. Treatment	1	0.005	0.069	0.794
Population of Origin	2	0.060	0.554	0.593
Family (Population)	9	0.108	1.434	0.198
Population x Treatment	2	0.087	1.161	0.321
Error	53	0.075		

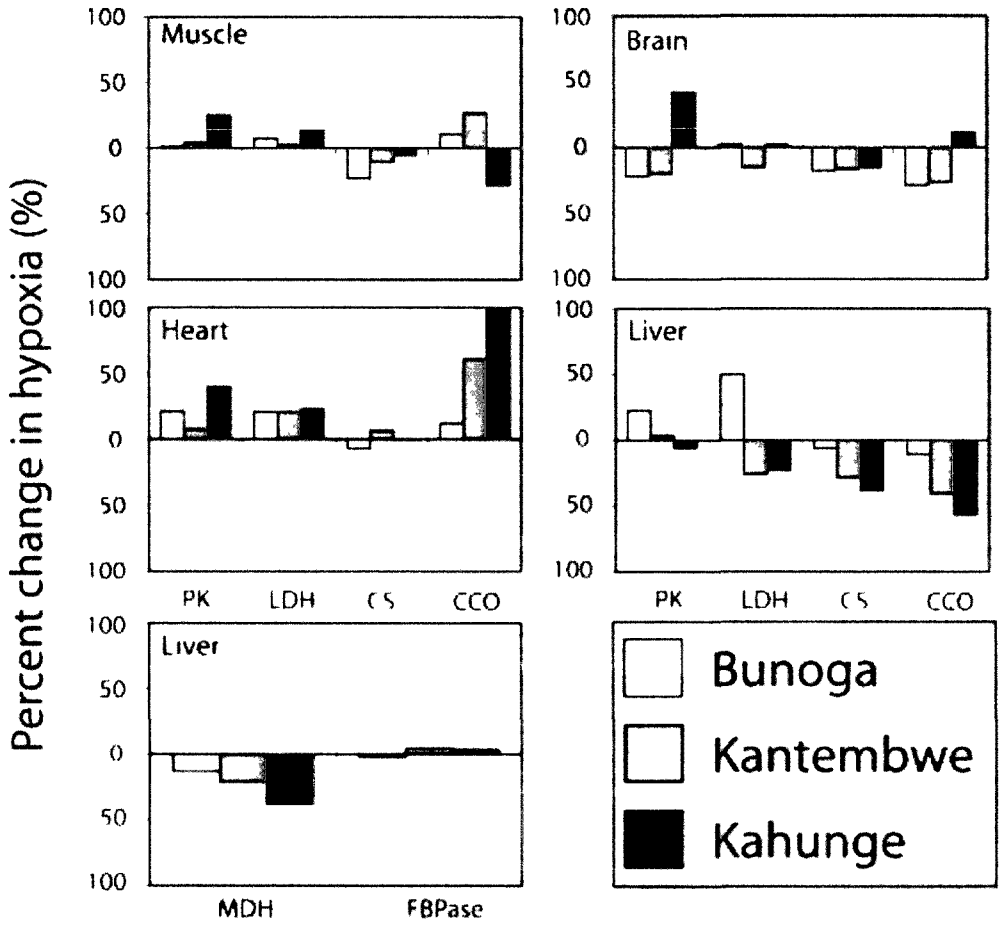

Figure 3.2. Percent change of enzyme activity of *Pseudocrenilabrus multicolor victoriae* in hypoxia relative to normoxia. The following enzymes, PK (pyruvate kinase), LDH (lactate dehydrogenase), CS (citrate synthase) and CCO (cytochrome C oxidase) were measured in muscle, heart brain and liver tissue, and MDH (malate dehydrogenase) and FBPase (fructose 1,6-bisphosphatase) in liver tissue of *Pseudocrenilabrus multicolor victoriae* reared under hypoxia compared to normoxia from Bunoga, Kantembwe and Kahunge.

Interestingly, in liver, the population x treatment interaction was significant for LDH (Table 3.3). When considering all three sites of origin, Bunoga offspring displayed an overall up-regulation under hypoxia, while Kantembwe and Kahunge offspring displayed a down-regulation in activity under hypoxia. When analyzing the three populations separately Bunoga F_1 were found to have higher LDH levels under hypoxia exposure ($F_{[1,20]} = 3.480$, $P = 0.017$), while Kantembwe and Kahunge offspring did not differ between normoxic and hypoxic reared fish ($F_{[1,22]} = 0.065$, $P = 0.801$ and $F_{[1,18]} = 2.136$, $P = 0.161$ respectively).

DISCUSSION

The present study demonstrated that populations of *Pseudocrenilabrus multicolor victoriae* reared under divergent oxygen regimes for approximately one year showed differences in enzyme activity associated with the D.O. environment in which they were reared. Interestingly, no significant effects of site of origin were observed. Crispo and Chapman (2010) quantified brain and gill size variation in response to rearing under high- and low-oxygen conditions for *P. multicolor* from 6 populations in the Mpanga River system and found high levels of developmental plasticity in these traits. Crispo and Chapman (2010) also found evidence for strong population effects on brain size and an interaction between population and rearing environment, suggesting genetic effects on brain size and an interaction between long-term selection and environmentally-induced plasticity. The results for enzyme activity are consistent with studies of gill and brain size under similar conditions, with a high level of developmental plasticity.

The lack of a population effect on enzyme activities may reflect high levels of gene flow in this system (Crispo and Chapman 2008). In addition, it is possible that the levels of oxygen used in my experiment were not sufficiently divergent to expose the population effect. For example, the Kantembwe site is characterized by extreme, chronic hypoxia averaging 0.21 mg O_2 l^{-1} over the year (Crispo and Chapman 2008). Thus, the F_1 offspring from this population may not have experienced sufficient hypoxia stress because the D.O. levels were not as low as they routinely experience in the field.

In fish, it has been observed that variation in tissue enzyme activity may be the result of fish size and/or lifestyle of the organism (Somero and Childress 1980). In this study, individuals were all submitted to the same D.O. levels, however, significant differences were observed in body mass and length at the end of the experiments; where individuals under hypoxia grew less and had lower mass than fish under normoxia. These results are in agreement with previous studies where fish living under hypoxic conditions have been shown to be typically lighter in mass and shorter in length due to the decreased amount of energy available for body maintenance activities (Kramer 1987; Karim *et al.* 2003). Changes in both body mass and length have been used as good indicators of the effects of hypoxia on fish (Cheung *et al.* 2007). As fish are exposed to hypoxia, their metabolic scope becomes reduced, limiting the amount of energy available for daily activity, growth as well as reproduction (Rijinsdorp *et al.* 2009; Nilsson *et al.* 2010). Interestingly, the condition factor (relative body mass) was higher in fish reared under hypoxia compared to fish reared under normoxia. However, this observation may not be a valid representation due to a level of sexual dimorphism. Females in general,

were in better condition than males, possibly a result of the larger gonads found in the females that was not taken into account in my measures of total mass.

In both white skeletal muscle and heart tissues there was evidence to suggest a general up-regulation of the anaerobic metabolism. Although this up-regulation was not always significant (tissue dependent), both PK and LDH activity were higher in fish reared under hypoxia, suggesting that in both tissues, the F_1s were heavily relying on anaerobic glycolysis for ATP production.

Surprisingly, a tissue metabolic variation amongst the three populations of *P. multicolor* was observed, suggesting that individuals display a level of intra-specific variation in tissue responses to hypoxia. In the gulf killifish, *Fundulus grandis*, variation in their tissue metabolic response to hypoxia has also been observed. After four weeks of hypoxia exposure, *F. grandis*, displayed a significant increase in the levels of some of enzymes in different tissues, while decreasing the same enzyme activity in other tissues (Martínez *et al.* 2006). It has been suggested that this level of intra-specific variation observed within tissue responses to hypoxia may be a result of the different tissue energetic needs and demands (Martínez *et al.* 2006; Martinez *et al.* 2011).

Interestingly, white skeletal muscle is the only tissue in which no statistical significant differences were observed between individuals reared under hypoxia or normoxia. In all other tissues, the rearing environment affected at least one of the enzymes. It has been suggested that the earliest stages of life can be very sensitive to different stressors (Pollock *et al.* 2007). It has previously been observed that fish development under hypoxia conditions may provide advantages for surviving life under hypoxia as an adult (Widmer *et al.* 2006). The exposure to hypoxia during the

developmental stages may assist in the selection for the optimal phenotypes needed during the adult stage (West-Eberhard 2003).

A degree of developmental plasticity in populations of *P. multicolor* may be an adaptive strategy selecting for multiple phenotypes, as opposed to selecting for one optimal phenotype (Marks *et al.* 2005; Widmer *et al.* 2006). This strategy would be especially beneficial in populations inhabiting the Kahunge site, which experiences a fluctuation of D.O. on a seasonal basis. Earlier studies have demonstrated significant plasticity in *P. multicolor* in several other traits including gill size, body shape, and brain size (Chapman *et al.* 2008, Crispo and Chapman 2008; Crispo and Chapman 2010). These results suggest that preserving a level of plasticity in different functional traits, including the enzyme activity, may be a beneficial strategy to use when coping with differing D.O. concentrations. In other fish species, it has been suggested that while under hypoxia stress, white skeletal muscle is more malleable than other tissues, due to its high glycolytic capacity, and therefore the need to up-regulate its metabolic capacity while under hypoxia is not a priority (Greaney *et al.* 1980; Somero and Childress 1980; Richards 2011). In order to maintain this high glycolytic capacity in the muscle, the more hypoxia tolerant organisms tend to reduce their metabolic rate in order to increase the longevity that glucose can support their metabolism (Richards 2011).

Despite the fact that little to no variation in the metabolic capacities were observed in the white skeletal muscle tissue, as mentioned above, the D.O. conditions in which *P. multicolor* were reared, may influence the observed trend. Unlike wild populations, the F_1 generation was fed daily which would decrease the need to search for food as they would under natural conditions. Also, the risk of predation by other species

is nonexistent under lab conditions. Combined, these two factors would decrease swimming activity, as it has been reported in other fish species under hypoxia acclimation (Wannamaker and Rice 2000; Martínez *et al.* 2006; Chabot and Claireaux 2008). It is possible however, that because individuals in this study were reared under such conditions and not acclimated like in other studies, the fact that no differences were observed in the levels of muscle enzyme activity may be a true representation of the life history changes needed for individuals from different sites of origin to adapt to the exposed conditions.

In two Amazonian cichlids, *Astronotus crassipinnis* and *Symphysodon aequifasciatus* (Chippari-Gomes *et al.* 2005), similar results to those reported here were reported. Heart LDH levels were greater than those of CS when exposed to hypoxic conditions, suggesting a greater anaerobic metabolism potential while under hypoxia. The heart of most vertebrates is very sensitive to declining oxygen levels (Nikinmaa 2002; Moore *et al.* 2006). It has been observed that under low oxygen levels, the heart will develop necrosis because of its inability to achieve equal levels of ATP supply and demand (Speers-Roesch *et al.* 2010). However, hearts in more hypoxia tolerant organisms tend to have elevated levels of glucose, increasing the rate of glycolysis and thus having a greater anaerobic capacity while under hypoxia (Treberg *et al.* 2007). Surprisingly, although, there is an activation of the anaerobic metabolism in *P. multicolor*, there is not a complete suppression of the oxidative metabolism. Levels of the aerobic enzyme, CCO, displayed up to 99% more activity in the heart of individuals under hypoxic conditions relative to those under normoxia.

Observed results from the heart tissue suggest the use of an independent modulation of specific enzymes on a given metabolic pathway that allows individual tissues to still utilize some aerobic enzymes while suppressing others, such as CS in this study. Similar independence of enzyme activity has also been observed in other species. In the common carp muscle, the same two aerobic enzymes (CS and CCO) showed an up-regulation and down-regulation respectively after exposure to hypoxia (Zhou *et al.* 2000). In *Leiostomus xanthuru*, gill CS levels were higher in fish submitted to hypoxia, while muscle CS level did not change (Cooper *et al.* 2002). In another African fish, *B. neumayeri,* the levels of CS and CCO were also tissue dependent and the degree of variation between hypoxia and normoxia acclimatized fish was not always in the same range or direction (Martínez *et al.* 2011). This independent modulation of the aerobic metabolic pathway may be the result of the duration of exposure to hypoxia, where some enzymes will respond only after long term exposure (Zhou *et al.* 2000). Furthermore, a change in the composition of the mitochondria membrane is also a way to modify the oxidative capacity of the mitochondria (Guderley and St. Pierre 2002). As the oxidative capacity of the mitochondria is set by their protein content, types of fatty acids and phospholipid head groups, it is possible that the independent enzyme modulation is the result of alterations made in the intrinsic properties of the mitochondria, such as changes in the lipid composition of the mitochondria membrane (reviewed in Guderley and St. Pierre 2002).

Although the rearing treatment did significantly affect the activity of some enzymes analyzed in the expected direction, the increase or decrease in activity was tissue specific. In a recent study examining the effects of hypoxia on the LDH levels of

the F_1 generation of *P. multicolor* from a normoxic and hypoxic origin, results showed that enzyme activity depended on both the population of origin as well as the acclimation history (Martínez *et al.* 2009). This study was completed after the F_1 offspring were reared under normoxia for at least 5 months prior to being acclimated for only four weeks under normoxia or hypoxia (Martínez *et al.* 2009). Although individuals used during this study were from different sites of origin, the fact that the sites in which parental stock were sampled from had only a minimal effect on the enzyme activity may be a result of the similar genetic structure as suggested by Crispo and Chapman (2008). On the other hand, the minimal effects of enzyme activity may also be due to the combination of the duration of time and development stage in which offspring were submitted to experimental conditions. During this study, offspring, after released from their mothers' mouth, were submitted to D.O. treatments after only week of normoxia exposure and were kept under experimental conditions for one year. Barrionuevo *et al.* (2010) found that when zebrafish were raised under hypoxia, they were able to better regulate aerobic and anaerobic activity. Also, it has been observed that *P. multicolor* displays a lower P_{crit} (critical partial pressure of oxygen) when reared under hypoxia compared to individuals under normoxia, suggesting that hypoxia reared fish have a greater tolerance to hypoxia (Barrionuevo *et al.* 2010; Reardon and Chapman 2010). Together, these studies suggest that site of origin played only a minimal role in determining enzyme activity. From all these studies it seems that a phenomenon known as "preconditioning" (prior exposure history) can greatly enhance fish capacity to withstand future hypoxia events (Gorgias *et al.* 1996; Mulvey and Renshaw 2000; Dowd *et al.* 2010). However, in this lab reared study, individuals did not have different prior

history exposure before they were placed under the experimental conditions and yet, they were able to better adapt to the hypoxic conditions regardless of the conditions in which parental stock originated from.

Populations of *P. multicolor* have inhabited the African waters for decades, and have shown a large number of traits enabling survival under hypoxic conditions. Strategies such as aquatic surface respiration (ASR), or increased gill size and external body shape under conditions of hypoxia (Chapman *et al* 2008), as well as an increased haematocrit levels (Martínez *et al.* 2009), may have increased the level of survivability and decreased the level of stress under hypoxia. Fish populations have shown to adjust rapidly under stressful conditions. The metabolic capacity of organisms exposed to hypoxia has shown to adjust depending on the duration under such conditions. When submitted to hypoxic conditions *Fundulus heteroclitus* was observed to turn back to its original enzyme levels after acclimation for 35 days (Greaney *et al.* 1980). It was reported that no change in enzyme activity would have been observed if not examined under acclimation conditions over a 35 day time period (Greaney *et al.* 1980). Also, changes in the common carp, regarding specific enzymes such as CCO were not observed until after 168 hours of exposure to hypoxia (Zhou *et al.* 2000). These previous results along with the current study, suggest that time of exposure is important when examining the metabolic capacity of fish. Therefore it may be necessary to complete more long-term hypoxia exposure experiments in order to increase the chances of achieving a valid representation of what may occur in the wild.

Since previous studies have indicated that the metabolic capacity will change over time (Greany *et al.* 1980), and with embryonic and larval stage being more sensitive

to stress compared to the adult stage (Shang and Wu 2004), it would be interesting to further examine the effects of hypoxia at an earlier stage through adulthood. As such, in order to facilitate the comparison of results amongst different developmental stages, species and time of exposure, in the future, it would be interesting to complete a series of studies in which eggs are placed in normoxia and hypoxia conditions and determine the metabolic capacity through time and determine the evolution of their metabolic needs. Although, studies on *P. multicolor* individuals have examined size and quantity of eggs produced, as well as the metabolic rate of the F_1 generation while under hypoxia and normoxia exposure (Reardon and Chapman 2008; Reardon and Chapman 2010), no research has been done on the metabolic capacity of individuals over a time course study from development to adult stage. This study may further explain the dependency of certain enzymes at different stages of life.

To conclude, this study demonstrates the need for more long-term rearing studies, to achieve more accurate results while looking at the metabolic capacity of different fish species. Previously the enzyme activity of *P. multicolor* was shown to be determined by both population of origin, and levels of D.O. in which the organism was acclimated to. In this study however, after being reared under similar conditions for one year, different results were obtained. Hence, time of exposure to specific conditions seems to play an important role when assessing the potential adjustment made by organisms to a specific stressor such hypoxia. Although over the last two decades we have seen a dramatic increase in the development of hypoxic zones with the concomitant decline of many non hypoxic tolerant species, this study shows that some fish, such as *P. multicolor* are able to adjust their metabolic capacity to compensate for alterations in environmental

stressors. This study has important implications for fish conservation. With the rising concerns of global warming and increasing dead zones due to anthropogenic stressors (Diaz and Rosenberg 2008), it is important to assess the physiological responses of organisms under longer-term exposure to the stressors. Although results in this study suggest subtle effects of hypoxia on the metabolic capacities of *P. multicolor,* it is evident that less hypoxia tolerant species are being and will continue to be affected by this phenomenon. More recent studies are providing further information about how hypoxia has been responsible for the declining of fish populations and how it greatly affects fish geographical distribution community assemblages (Diaz and Rosenberg 2008; Rijinsdorp *et al.* 2009; Nilsson *et al.* 2010; Martínez *et al.* 2011). With the specific range of tolerance differing between species (Rijinsdorp *et al.* 2009), it is important to increase the number of long-term laboratory studies to be able to better understand the ecological impacts that global warming will have at an individual, a population as well as a community level.

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSION

GENERAL DISCUSSION

This thesis blended results from a field survey and a laboratory rearing experiment to explore the metabolic response of the African cichlid, *Pseudocrenilabrus multicolor victoriae* to a widespread environmental stressor – hypoxia. The integration of the two studies provides evidence for a role of hypoxia in driving metabolic activities at the enzyme level, but also indicates the importance of population-specific and tissue-specific responses.

The aim of this research was to determine whether metabolic similarities exist at the enzyme level between individuals exposed to different dissolved oxygen regimes. This research also tried to determine if there was a metabolic pathway preference for individuals caught in the field from sites displaying different D.O. levels, as well as fish reared in the lab. Due to logistical constraints, fish from both studies were not collected exactly from all the same sites, limiting the capacity of comparison among sites of origin and regions. It was not possible to determine if individuals from the two regions, Mpanga and Nabugabo, respond differently to hypoxia conditions, as only Mpanga individuals were obtained for the laboratory study. Nevertheless, by synthesizing available data from field (Martínez *et al.* 2009 and this study) and lab studies, some inferences can be drawn.

The first approach taken was to plot data from the Bunoga and Kahunge sites, situated along the Mpanga River, in order to see whether the physiological responses at the level of enzyme activity in the four tissues (white skeletal muscle, heart, brain and liver) varied. Although some differences between field and lab enzyme levels were observed, in general, individuals that were reared in the lab, maintained similar enzyme

activity levels as those fish that were captured in the field from the same study site. These results suggest that hypoxia is one of the most influential factors affecting the metabolic capacity of wild populations of *P. multicolor*. On the other hand, the Kahunge populations displayed a greater number of changes between the field conditions and the normoxia reared laboratory fish, possibly as a result of the yearly fluctuations in D.O. levels that this particular population experienced in the wild.

When comparing the levels of enzyme activity in white skeletal muscle between field populations (Bunoga and Kahunge) and the F_1 generation of lab reared hypoxia and normoxia fish (Figure 4.1), it was observed that the lab reared fish displayed similar levels of activity to those that were captured in the field. The only difference observed using a Tukey *post-hoc* comparison was the lower white skeletal muscle CS activity of the Bunoga population in the field compared to that of fish reared in normoxic conditions ($P = 0.013$). On the other hand, heart tissue displayed slightly more variable results (Figure 4.2). For fish from the Bunoga site, field fish responded similarly to those reared under lab conditions for both CS and CCO activity ($P > 0.05$); however, heart PK levels were slightly greater in field fish compared to both normoxic reared ($P = 0.002$) and hypoxic reared ($P = 0.046$). Kahunge fish displayed greater heart PK and CCO activity in field fish compared to normoxic reared fish ($P = 0.035$; $P = 0.016$ respectively).

Brain enzyme activity levels in fish from the Bunoga population, were similar for PK, CCO and CS activity between both field and lab reared fish ($P > 0.05$). However, the Kahunge fish differed in both PK and CCO activity (Figure 4.3). The *post-hoc* analysis indicated that the field populations displayed greater PK and CCO activity compared to

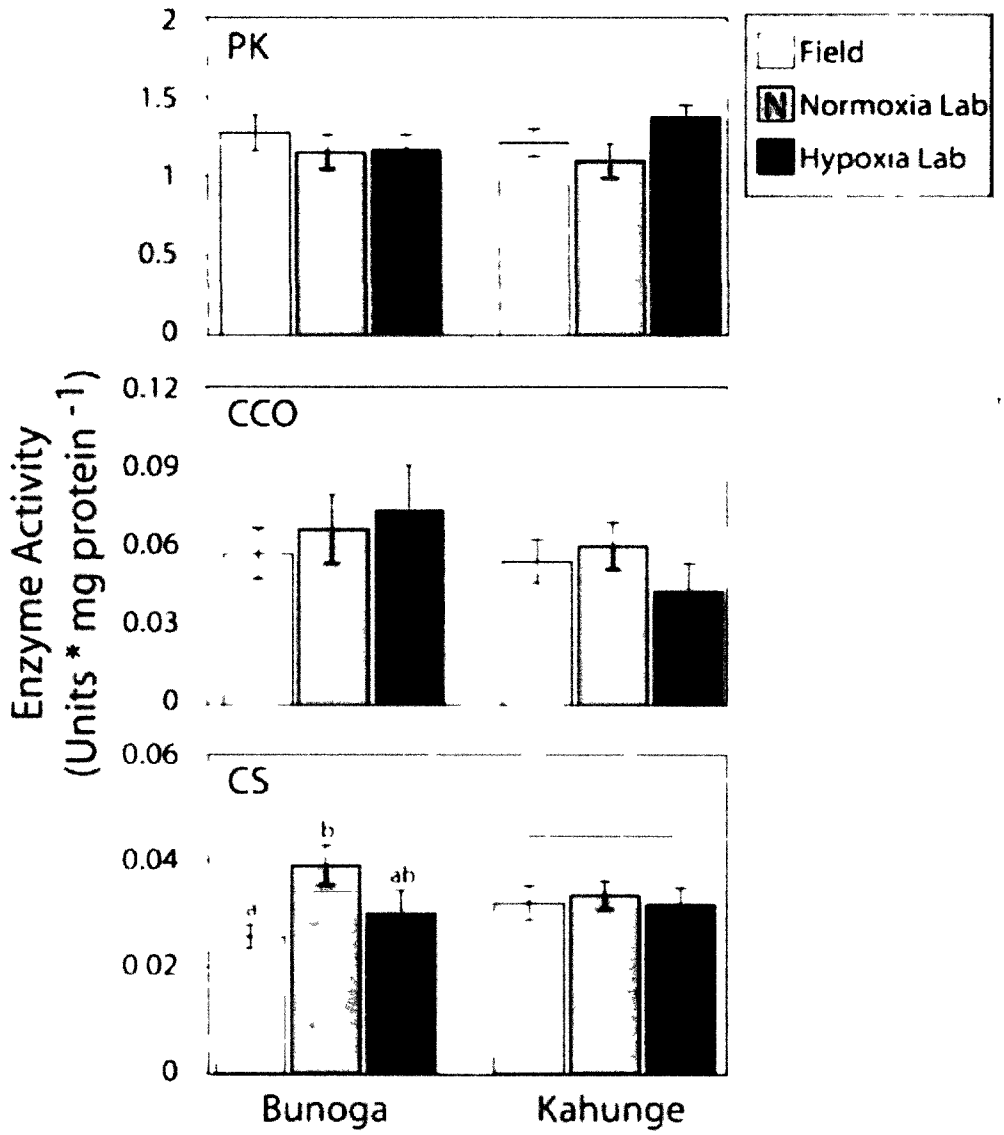

Figure 4.1. The enzyme activity levels in white skeletal muscle, of field and lab reared individuals of *Pseudocrenilabrus multicolor victoriae*. White bars represent field individuals from Bunoga and Kahunge sites, light grey bars represent F_1 lab individuals reared in normoxia, and dark grey bars represent F_1 lab individuals reared in hypoxia for approximately one year. Enzymes measured were pyruvate kinase (PK), cytochrome C oxidase (CCO) and citrate synthase (CS). Letters indicate significant differences between sites of origin. Bars with the same letter, and bars with line above, do not differ (Tukey *post-hoc* comparisons, $P > 0.05$). Data are shown as mean ± S.E.

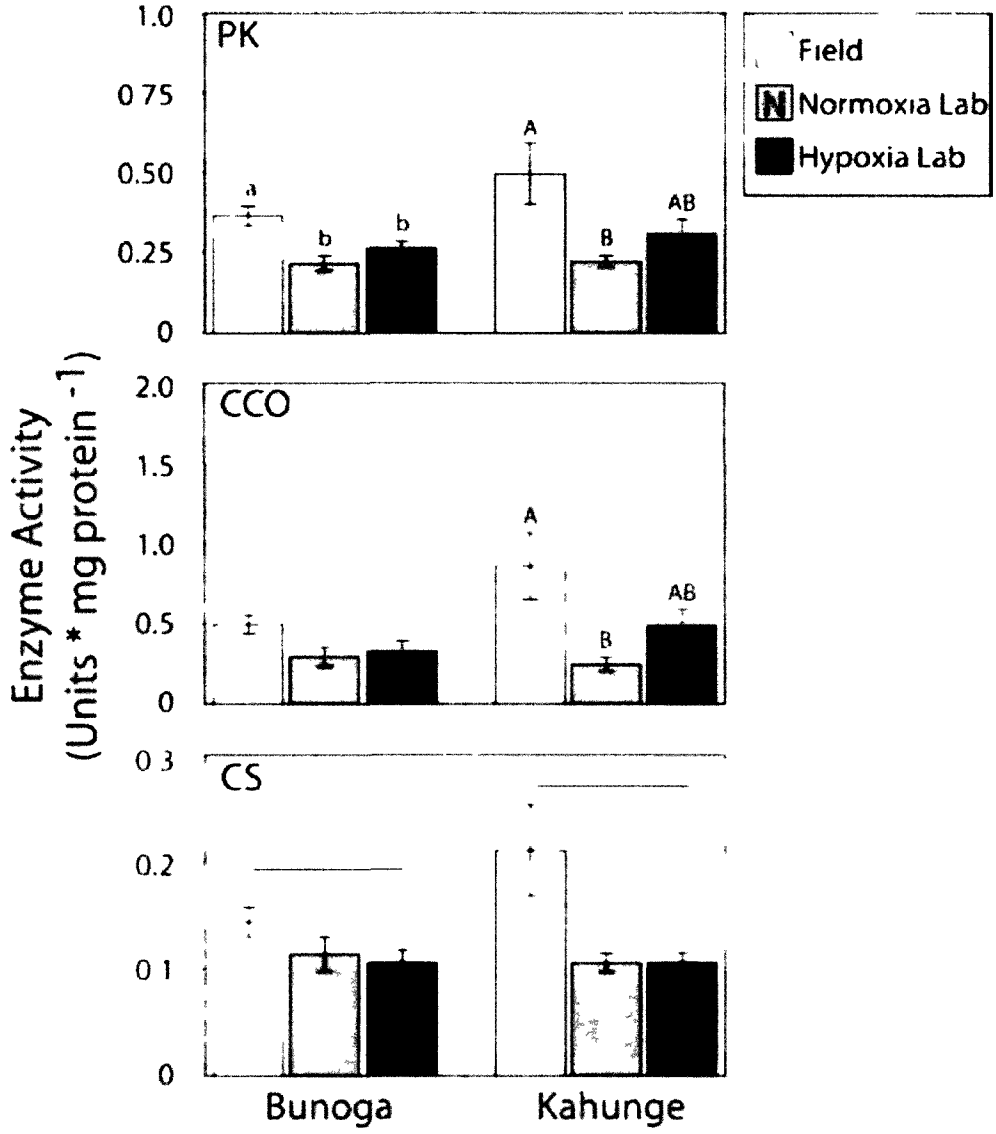

Figure 4.2. The enzyme activity levels in heart tissue, of field and lab reared individuals of *Pseudocrenilabrus multicolor victoriae*. White bars represent field individuals from Bunoga and Kahunge sites, light grey bars represent F_1 lab individuals reared in normoxia, and dark grey bars represent F_1 lab individuals reared in hypoxia for approximately one year. Enzymes measured were pyruvate kinase (PK), cytochrome C oxidase (CCO) and citrate synthase (CS). Letters indicate significant differences between sites of origin. Bars with the same letter, and bars with line above, do not differ (Tukey *post-hoc* comparisons, $P > 0.05$). Data are shown as mean ± S.E.

the normoxia reared fish ($P = 0.044$; $P = 0.031$ respectively), but similar activity levels to the F_1 generation reared in hypoxic conditions ($P > 0.05$).

The liver tissue, for PK, CCO and CS responded similarly between both field and lab reared fish ($P > 0.05$) from both Bunoga and Kahunge sites (Figure 4.4). Gluconeogenic enzyme activity however did differ between field fish and lab fish. Field fish from the Bunoga population displayed greater MDH activity than the F_1 generation reared in normoxia ($P = 0.001$) or hypoxia ($P < 0.0001$) lab conditions. The opposite was observed in the levels of liver FBPase, in which field fish displayed lower levels of liver FBPase activity than normoxia reared fish ($P = 0.002$) and hypoxia reared fish ($P = 0.003$). Similarly to the Bunoga population, the Kahunge field fish displayed greater MDH activity levels than the F_1 generation reared in normoxia ($P = 0.017$) or hypoxia ($P < 0.0001$). Liver FBPase activity was lower in the field populations of Kahunge compared to those of the F_1 generation reared in normoxia ($P < 0.0001$) or hypoxia ($P < 0.0001$).

Although data were not collected from both regions for the laboratory part of this study, a previous study was completed in which the F_1 generation from the Nabugabo Region, Lwamunda Swamp and Lake Kayanja populations, were raised under normoxic conditions for five months and then acclimated to normoxia or hypoxia conditions for approximately four weeks (Martínez *et al.* 2009). Despite that a number of challenges were evident while doing this type of comparison, such as year to year variation in the study, different individuals completing enzyme measurements, time of acclimation vs. rearing conditions, I thought it would be interesting to test whether differences between

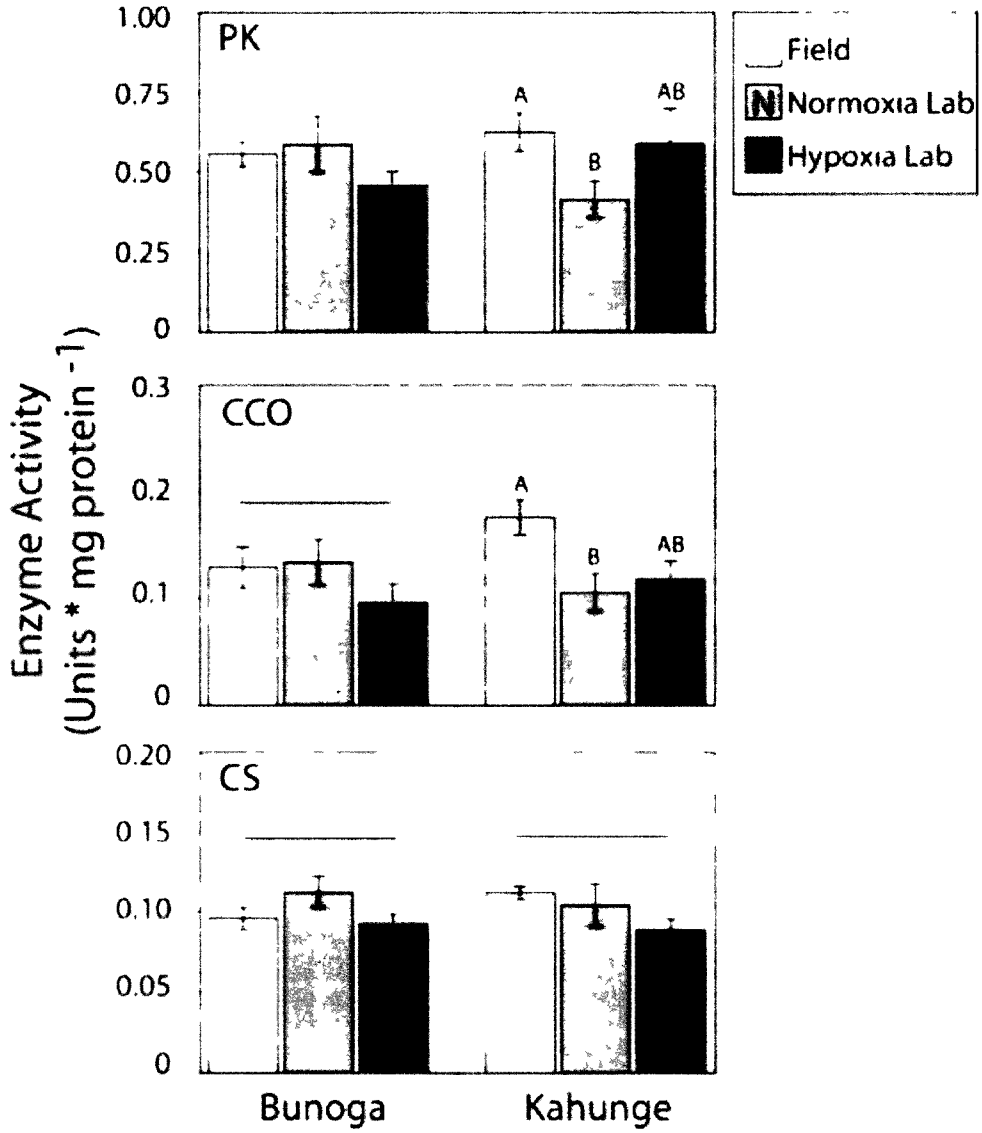

Figure 4.3. The enzyme activity levels in brain tissue, of field and lab reared individuals of *Pseudocrenilabrus multicolor victoriae*. White bars represent field individuals from Bunoga and Kahunge sites, light grey bars represent F_1 lab individuals reared in normoxia, and dark grey bars represent F_1 lab individuals reared in hypoxia for approximately one year. Enzymes measured were pyruvate kinase (PK), cytochrome C oxidase (CCO) and citrate synthase (CS). Letters indicate significant differences between sites of origin. Bars with the same letter, and bars with line above, do not differ (Tukey *post-hoc* comparisons, $P > 0.05$). Data are shown as mean ± S.E.

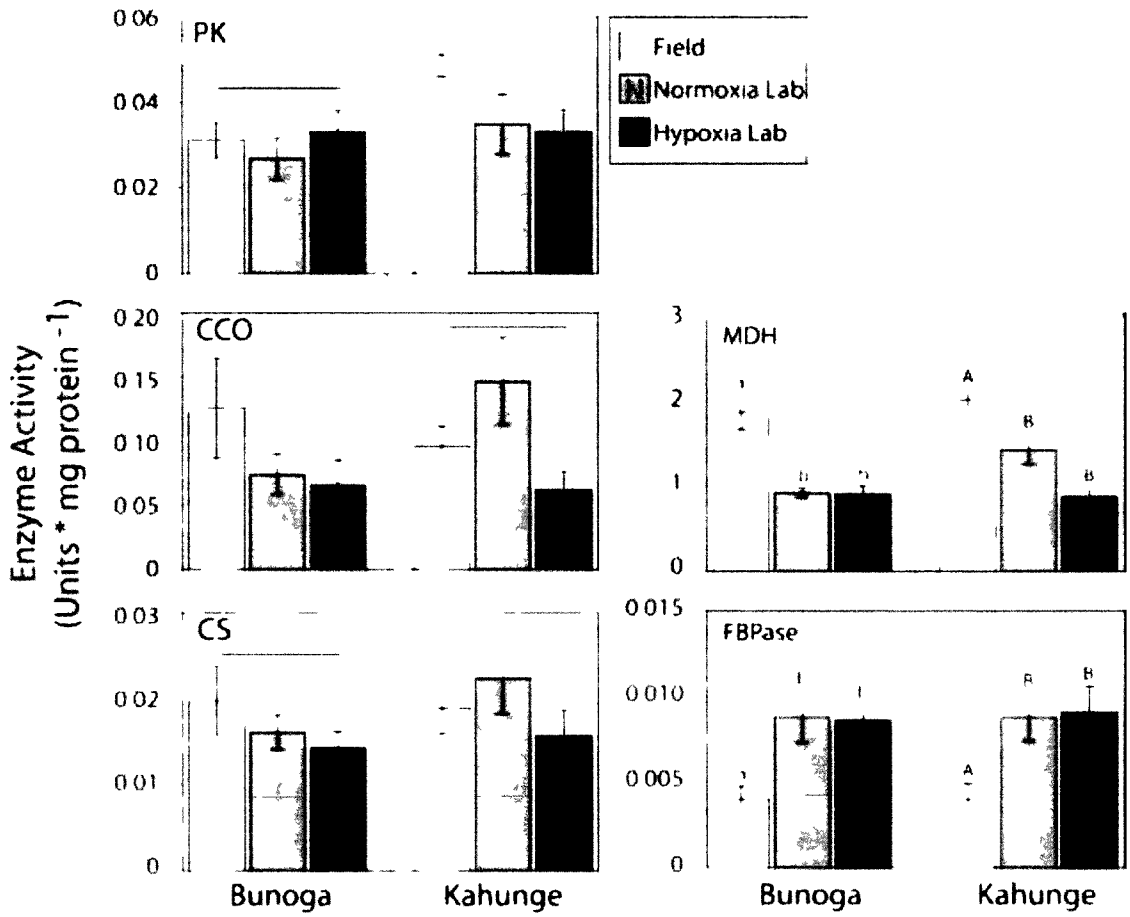

Figure 4.4. The enzyme activity levels in liver tissue, of field and lab reared individuals of *Pseudocrenilabrus multicolor victoriae*. White bars represent field individuals from Bunoga and Kahunge sites, light grey bars represent F_1 lab individuals reared in normoxia, and dark grey bars represent F_1 lab individuals reared in hypoxia for approximately one year. Enzymes measured were pyruvate kinase (PK), cytochrome C oxidase (CCO), citrate synthase (CS), malate dehydrogenase (MDH) and FBPase (fructose 1,6-bisphosphatase. Letters indicate significant differences between sites of origin. Bars with the same letter, and bars with line above, do not differ (Tukey *post-hoc* comparisons, $P > 0.05$). Data are shown as mean ± S.E.

regions were still evident, by using the LDH activity levels reported in Martínez *et al.* (2009) (Figure 4.5). For fish from the Mpanga region (Bunoga and Kahunge), both muscle and liver LDH activity was similar between the field population and the F_1 generation reared under both normoxia and hypoxia for one year ($P > 0.05$). The Bunoga fish displayed greater heart LDH activity compared to normoxia ($P = 0.001$) and hypoxia ($P = 0.015$) reared fish, as well as brain LDH activity compared to normoxia ($P = 0.002$) or hypoxia ($P = 0.003$). Similarly, Kahunge fish showed the same pattern, heart LDH activity was greater in field fish than the F_1 generation of normoxia reared ($P = 0.002$) and hypoxia reared fish ($P = 0.019$). Tukey *post-hoc* analysis indicated greater brain LDH activity in Kahunge field fish compared to normoxia reared ($P < 0.0001$) and hypoxia reared fish ($P < 0.0001$).

Nabugabo region fish (Lwamunda Swamp and Lake Kayanja) displayed differences in the level of LDH activity between field fish and the F_1 generation lab reared fish in all tissues, white skeletal muscle, heart, brain and liver. Lwamunda Swamp field fish displayed lower white skeletal muscle LDH activity than normoxia ($P< 0.0001$) and hypoxia ($P < 0.0001$) acclimated fish; however, normoxia acclimated fish had greater white skeletal muscle LDH activity than hypoxia acclimated fish ($P < 0.003$). Lower levels of LDH activity were also observed in the heart of field fish from Lwamunda Swamp compared to those acclimated to normoxia ($P < 0.0001$) and hypoxia ($P < 0.0001$). Similarly, brain LDH activity was significantly lower than normoxia acclimated fish ($P = 0.006$); but no difference was evident when comparing hypoxia acclimated fish ($P = 0.087$). Lastly, Lwamunda Swamp field populations displayed

lower liver LDH activities than normoxia acclimated fish ($P < 0.0001$), but similar levels to those of hypoxia acclimated fish ($P = 0.240$).

Lake Kayanja fish displayed a similar trend between field and lab fish as those from Lwamunda Swamp (Figure 4.5). The *post-hoc* analysis shows that Lake Kayanja field fish displayed lower white skeletal muscle LDH activity levels than normoxia acclimated ($P < 0.0001$) and hypoxia acclimated fish ($P < 0.0001$). While normoxia acclimated fish had lower white skeletal muscle LDH activity than hypoxia acclimated fish ($P = 0.001$). Field fish from Lake Kayanja also displayed lower heart, brain, and liver LDH activity than normoxia acclimated fish ($P < 0.0001$; $P = 0.008$; $P = 0.008$ respectively), as well as lower heart and brain LDH activity compared to hypoxia acclimated fish ($P < 0.0001$; $P = 0.0001$ respectively).

Interestingly, for individuals from Lwamunda Swamp and Lake Kayanja, the enzyme activity levels of field fish were overall lower than the laboratory fish enzyme activity levels. Bunoga and Kahunge individuals from the field, displayed similar muscle and liver LDH activity levels, compared to the normoxia and hypoxia reared fish. On the other hand, in the heart and brain tissues, field fish displayed overall greater activity than normoxia and hypoxia reared laboratory fish (Figure 4.5). These results suggest strong differences between the two different regions. In Chapter 2, results showed that there was a regional difference in the levels of most enzyme activities in the field fish however I was unable to show this in Chapter 3, due to the absence of data from the Nabugabo region. It should be noted that the discrepancy between results may be a result of how each study was conducted. It is clear in Figure 4.5 that there is a difference in the enzyme levels between the four weeks acclimated F_1 generation

compared to those that were reared under hypoxia or normoxia for one year. Time course changes in enzyme activities have been reported in previous studies (Greany *et al.* 1980; Zhou *et al.* 2000), showing that metabolic adjustments are made depending on the duration of exposure to hypoxia. In the study completed in this thesis, fish were reared for one year after only experiencing normoxia conditions for a maximum of one week, on the other hand, in Martínez *et al.* (2009), fish were acclimated for four weeks to experimental conditions after being held in normoxic conditions for five months. The duration of time before submission to experimental conditions could affect the activity levels of fish, as fish may have become pre-conditioned to the normoxic conditions, possibly making it more stressful for the fish when placed under hypoxic conditions.

Overall, the comparison between field and lab data suggests that the metabolic capacity is greatly affected by the level of dissolved oxygen in which *P. multicolor* is exposed to. However, it is evident that after comparing data with a previous study, Martínez *et al.* (2009), that the preconditioning of the organism to divergent dissolved oxygen regimes, is a major factor influencing the enzyme activity of *P. multicolor.* Combined, these results suggest that not one, but a combination of factors are responsible for the variation of the metabolic capacity.

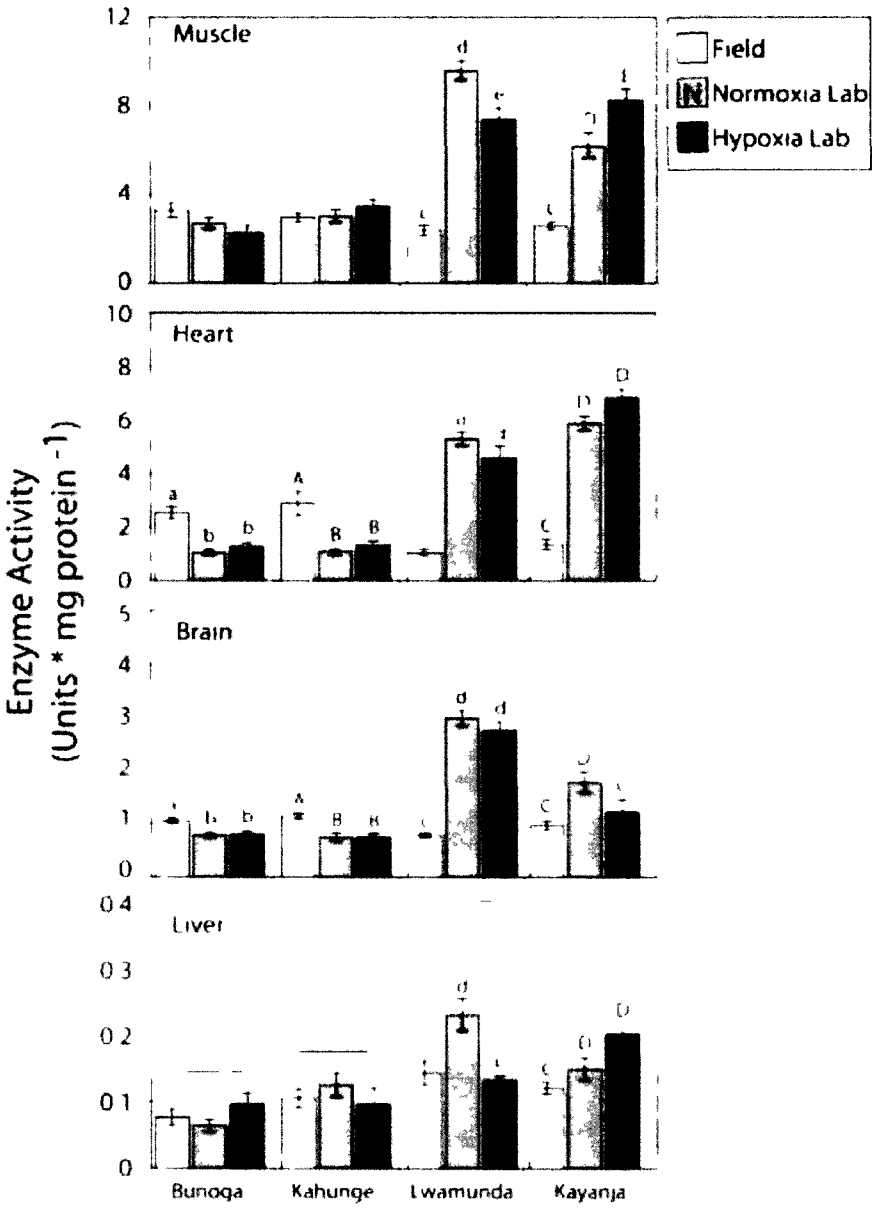

Figure 4.5. The lactate dehydrogenase (LDH) activity levels of *Pseudocrenilabrus multicolor victoriae* field populations from Bunoga and Kahunge (Mpanga Region) and Lwamunda Swamp and Lake Kayanja (Nabugabo Region) compared to F_1 lab individuals reared in normoxia or hypoxia for approximately one year (Bunoga and Kahunge) or acclimated for four weeks (Lwamunda Swamp and Lake Kayanja). Enzymes were measured in white skeletal muscle, heart, brain and liver. Letters indicate significant differences between sites of origin. Bars with the same letter, and bars with line above, do not differ (Tukey *post-hoc* comparisons, $P > 0.05$). Data are shown as mean ± S.E.

GENERAL CONCLUSION AND SUMMARY

Over the last few decades, a number of hypoxic dead zones around the world, as well as their duration and frequency has increased and has become of global concern (Dybas 2005; Pollock *et al.* 2007; Diaz and Rosenberg 2008). As the detrimental effects of global warming increase and human activities continue to affect the environment, there is a major need to determine patterns on the possible effects at an ecosystem scale. Studies on the fish physiological changes under stressed environments can assist in predicting ecological changes as well as future ecological trends (Pörtner and Farrell 2008). Therefore, using both a field study as well as a laboratory controlled study I tried to demonstrate the effects of hypoxia on the physiology of an African cichlid *Pseudocrenilabrus multicolor victoriae.*

My research has shown that the populations of *P. multicolor* display variation within populations as well as between populations in their metabolic capacity. I also showed that the enzyme activity is tissue specific for this species. Interestingly, for wild populations of *P. multicolor*, results strongly suggest a regional difference in the metabolic capacity between fish captured from the Nabugabo region and fish from the Mpanga region. Under laboratory conditions in a cross oxygen common garden experiment, results showed that the level of plasticity with regards to the metabolic capacity is quite high in this organism. This plasticity however, is tissue specific as well. This study also demonstrated that the level of dissolved oxygen plays a key role in determining the enzyme activity levels, compared to the discrete population of origin effect, with three out of the six enzymes depending on level of D.O. in which fish were in.

After comparison to a previous study in which fish were acclimated for a shorter duration, comparison has shown that the duration of time exposed to hypoxia can influence the metabolic capacity. Furthermore, the comparison also shows that the enzyme levels between the field and lab studies are quite similar, with Bunoga field populations resembling those of the F_1 lab generation more closely than the Kahunge populations.

As previously stated in Chapter 2 and Chapter 3, future work would have to determine the effects of the fluctuating D.O. levels on the Kahunge fish populations, by sampling and examining the metabolic capacity of fish throughout the year. Also, in the lab, it would be important to examine the effects of hypoxia at different intervals throughout all life stages, to determine how each life stage is affected by hypoxia over a generation.

This research demonstrates that although there is an increasing number of hypoxic dead zones occurring throughout the world, that some populations may, through a level of adaptive or developmental plasticity, be able to adjust at the physiological level as well as adjust their energy requirements to survive even when fluctuations are great, or there are decreasing levels of dissolved oxygen. I have shown that some populations respond more efficiently and effectively to changes in the ecosystem in which they inhabit. Fish have different life history stages, each of what might be affected differently by the effects of climate change (Rijinsdorp *et al.* 2009). It is therefore important to take into consideration all life history stages, while completing laboratory studies. Overall, the results that I have shown during this thesis may provide useful insight as to how other hypoxia-tolerant fish may be able to successfully respond

to increasing frequency of hypoxia events by a combination of a number of influential factors, such as pre-exposure to hypoxia, and variability in the expression of enzymes between different populations.

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