

Inactivity and overfeeding related changes in skeletal muscle composition and metabolism in captive felids – are they at risk of developing metabolic syndrome?

By:

Daneil Feldmann

Student number: FLDDAN002

Supervisor: Dr Tertius Kohn

Co-Supervisor: Dr Adrian Tordiffe

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Division of Exercise Science and Sports Medicine
University of Cape Town
Sports Science Institute of South Africa
Boundary Road, Newlands
Cape Town
South Africa



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DECLARATION

I, Daneil Feldmann, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is submitted for another degree in this or any other university.

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Date: 31st October 2016

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BSA	bovine serum albumin
CS	citrate synthase
CSA	cross-sectional area
3-HAD	3-hydroxyacyl-CoA dehydrogenase
IHC	immunohistochemistry
IR	insulin receptor
IRS-1	insulin receptor substrate-1
LDH	lactate dehydrogenase
MHC	myosin heavy chain
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NCD	non-communicable diseases
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PDK	phosphatidylinositol-dependent kinase
PFK	phosphofructokinase
PI3K	phosphatidylinositol kinase
TAG	triacylglycerol
TIID	type II diabetes
μl	microliter
μm^2	square micrometre
g/l	grams per litre
M	molar (mol per litre)
mg	milligrams
ml	millilitres
nm	nanometres

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Table 1

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ABSTRACT

Obesity and physical inactivity are established key risk factors in the development of skeletal muscle insulin-resistance and type II diabetes in human and domestic animal populations. Wild animals are similarly exposed to these risk factors as a result of captivity. This study aimed at describing the skeletal muscle properties of captive lions (*Panthera leo*) who present with obesity and are physically inactive, with a group of free roaming wild lions. Skeletal muscle biopsies were collected from the *Triceps brachii* and analysed for fibre type composition, fibre size, and maximum oxidative (citrate synthase (CS), 3-hydroxyacyl CoA dehydrogenase (3HAD) and glycolytic (phosphofructokinase (PFK) and lactate dehydrogenase (LDH) enzyme capacities. The type IIA fibres were the predominant fibre type in both the wild (48%) and captive (44%) lions. No significant differences were evident in the overall fibre type composition between groups, although a trend towards less type I (28% vs 36%) and more type IIX (28% vs 16%) fibres in the captive group were observed. The captive lions displayed significantly larger cross-sectional areas (CSA) of the type I ($5847\mu\text{m}^2$ vs $3318\mu\text{m}^2$) and IIA fibres ($8344\mu\text{m}^2$ vs $4360\mu\text{m}^2$) with no difference evident in the CSA of the IIX fibres. Relationships were observed between body mass and the CSAs of type I ($r = 0.68$, $P = 0.0002$) and IIA ($r = 0.44$; $P = 0.03$) fibres. Metabolically, the captive lions displayed higher flux through the glycolytic pathway as represented by their higher PFK activities (551 ± 287 vs. $338 \pm 123\mu\text{mol}/\text{min}/\text{g}$ protein, $P < 0.05$). In contrast, their CS activities (11 ± 3 vs. $14 \pm 2\mu\text{mol}/\text{min}/\text{g}$ protein) were lower than free roaming lions, suggesting lower oxidative capacity. No differences in LDH and 3HAD activities were observed between the two groups. Relationships were observed between 3HAD activity and MHC IIX fibre content ($r = -0.43$, $P = 0.001$), 3HAD activity and MHC I content ($r = 0.55$, $P = 0.001$). A positive correlation was observed between CS activity and MHC I content ($r = 0.35$, $P = 0.009$). In light of previous research, the data collected from this project follows that observed in exercise training models, where a sedentary lifestyle decreases oxidative capacity of muscle and increases type II fibre content. However, due to the low sample size in the free roaming wild lion group, interpretation of the data is difficult. Therefore, future research must strive to increase the sample number of the free roaming group.

CHAPTER 1

1. LITERATURE REVIEW

1.1 BURDEN OF DISEASE

Humans live in an ever-changing environment where continuous improvements in technology alongside increased urbanization, have encouraged a lifestyle of decreased levels of daily energy expenditure, and increased sedentary behaviour in leisure, work and transport activities (Oggioni et al., 2014; Wagner and Brath, 2012). Furthermore, due to growing populations and increasing job demands the need for larger amounts of easily accessible, affordable food sources has increased (Oggioni et al., 2014). This need has resulted in an influx of large supermarket chains that provide an abundance of readily available, inexpensive food choices which are predominantly highly processed, and contain high amounts of fat, sugar and sodium (Poutanen, 2012). Overeating on these nutritionally poor foods in combination with reduced activity levels have resulted in a higher prevalence of overweight and obese individuals the world over (Cecchini et al., 2010; Poutanen, 2012).

Physical inactivity and obesity are established lifestyle risk factors for non-communicable diseases (NCD) such as cardiovascular disease, cancer, respiratory disorders and type II diabetes (TIID) (Shaw et al., 2010). NCDs account for 60% of mortality rates worldwide and have reached epidemic proportions across both developed and developing societies (Cecchini et al., 2010; Warren et al., 2014). Furthermore, projected data suggest that NCDs shall surpass all other diseases, as the leading cause of death by the year 2030 (Wagner and Brath, 2012). TIID is the most prevalent NCD, with current estimates of 347 million people worldwide living with the disease (World Health Organisation – WHO). Furthermore, projected data provided by WHO suggest that a doubling of diabetes-associated deaths will occur from the year 2005 to 2030.

TIID is associated with debilitating health implications such as damage to the eyes, kidneys, nerves, heart and blood vessels (American Diabetes Association, 2010). If not controlled, TIID can cause heart disease, high blood pressure, stroke, kidney failure, amputations and blindness (Huang et al., 2012). It is thus clear how this global epidemic has placed great economic burden on both patient and society, in terms of life expectancy and health-care costs (Danaei et al., 2011).

1.2 GLUCOSE CONTROL AND TYPE II DIABETES

T2DM is a disease of lifestyle and is characterised by chronically high blood glucose levels. In a healthy non-diabetic state, the regulation of blood glucose is tightly controlled by the pancreatic hormones, glucagon and insulin. Glucagon, secreted by the pancreatic α -cells, acts to increase blood glucose when levels are too low (hypoglycaemia), as is the case during exercise or fasting (Koeslag et al., 2003). Insulin, secreted by the pancreatic β -cells, is responsible for the lowering of blood glucose in response to increased levels (hyperglycaemia) after the ingestion of a meal (Rosenthal et al., 2001). Once glucose enters the blood stream, it is distributed to various organs of the body for either adenosine triphosphate (ATP) synthesis or storage. Fat tissue, liver and skeletal muscle are sites for glucose storage, with skeletal muscle being the predominant location. A group of glucose proteins termed GLUTs transport glucose from the blood across the muscle plasma membrane and into the cells. The two isoforms mainly involved in the uptake of glucose at the muscle cell membrane, are GLUT1 and GLUT4 (Ojuka and Goyaram, 2014).

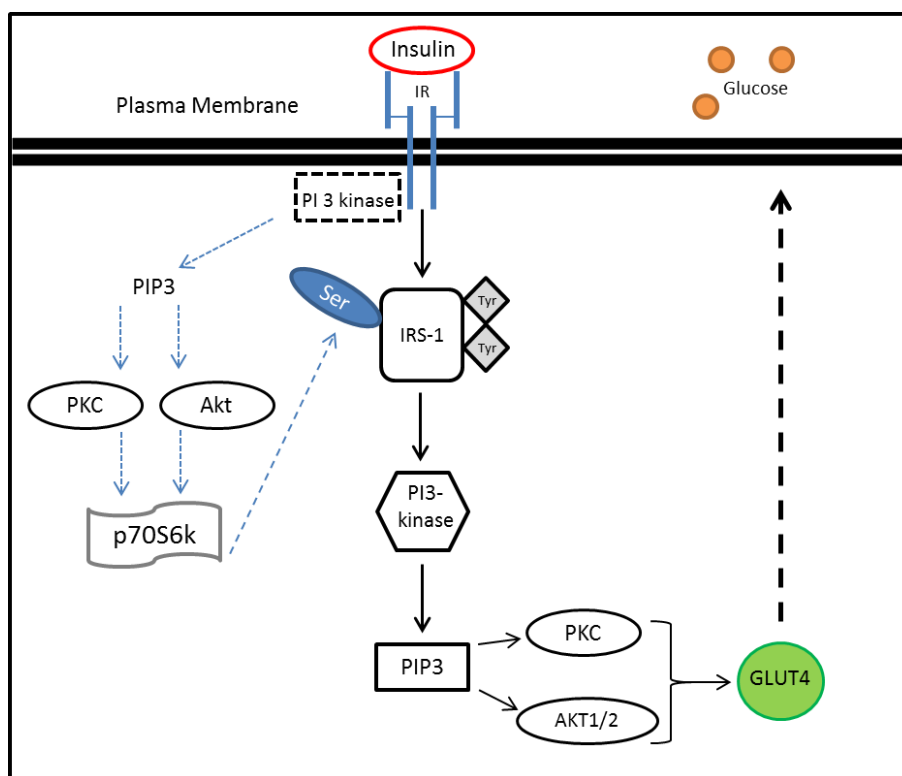


Figure 1: Signalling pathways responsible for the uptake of glucose in healthy skeletal muscle. Insulin secreted from the pancreatic β -cells binds to the insulin receptor protein on the outer cell membrane. Tyrosine residues on IRS-1 are phosphorylated which signals a set of numerous activation processes which ultimately leads to the translocation of GLUT4 vesicles to the cell membrane for glucose uptake. Schematic adapted from Shao *et al.*, 2002.

The process of glucose uptake into a muscle cell is initiated by insulin. Raised insulin levels, post glucose absorption, results in the binding of glucose to the insulin receptor (IR) located on the plasma membrane (Figure 1). This triggers a cascade of signalling pathways, which includes the phosphorylation of tyrosine residues on the insulin receptor substrate 1 (IRS-1). Subsequently, phosphatidylinositol kinase (PI3K) is activated, which in turn catalyses phosphatidylinositol 4, 5 diphosphate into phosphatidylinositol 3, 4, 5 triphosphate. Downstream molecules such as phosphatidylinositol-dependent kinase (PDK) and protein kinase B (PKB [Akt]) have a pleckstrin homology domain that enables these molecules to migrate toward the plasma membrane. In skeletal muscle, this PI3-kinase–Akt activation is an essential step, which brings about the translocation of the GLUT4 vesicles to the outer cell membrane to enhance glucose uptake (Blanco et al., 2010). In an insulin resistant state however, an alternative pathway is stimulated. Instead of the tyrosine residues, serine 307 on the IRS-1 are phosphorylated through PIP2 and Akt signalling (Figure 1). This results in a decrease in IRS-1 tyrosine phosphorylation, and hence PI3-kinase activation which ultimately reduces the translocation of GLUT4 vesicles to the cell membrane and thus the uptake of glucose into the cell (Simon-Szabo et al., 2016).

TIID is a progressive disease and preceded by an insulin-resistant state. Insulin-resistance is recognized as the inability of insulin to effectively promote glucose uptake by the skeletal muscle. This occurs as a result of the body's attempt to mitigate the lack of muscle cell sensitivity to glucose. A counter compensation response results in a further increase in insulin secretion by the pancreatic β -cells to maintain normal levels of blood glucose – euglycemia (Lazar, 2005). Hyperinsulinemia develops as a result of elevated insulin levels, and if no remedial action is taken, and the insulin-resistant state persists long-term, the β -cells are no longer able to secrete the necessary amounts of insulin to compensate for cell insensitivity, and the blood reaches a state of chronic hyperglycaemia, i.e. complete TIID. The insensitivity of the muscle cell membrane to insulin is due to the inactivation of the IR as a result of the degradation of the IRS-1 protein – Figure 1 (Cheng, 2005). Insulin-resistant skeletal muscle can thus be distinguished from insulin sensitive muscle primarily from reduced IRS-1 content, as well as a reduced GLUT4 protein content (Chavez et al., 2008).

Of noteworthy mention is the existence of an insulin-independent pathway through which muscle cells can take-up glucose by means of muscle contraction (i.e. physical activity). Although the exact mechanisms underlying contraction-induced glucose uptake are less defined in comparison to those brought about by insulin, it has been shown that several intracellular signals, which result in an increase in GLUT4 content and subsequent glucose transport, are involved (Ojuka and

Goyaram, 2014). Physical activity has thus been advocated as a remedy to improve insulin sensitivity, in those individuals presenting with insulin-resistance (Dela et al., 2014).

1.3 OBESITY AND PHYSICAL INACTIVITY AS KEY RISK FACTORS

Obesity and physical inactivity have long been identified as key risk factors in the onset of insulin-resistance and T1DM (Abubakari and Bhopal, 2008; Unger, 1997; Wang et al., 2015). Although a strong association exists between obesity and T1DM, and inactivity and T1DM, the mechanisms on exactly how these risk factors lead to the disease remain unclear (Codoner-Franch and Alonso-Iglesias, 2015).

It has been proposed that adipose tissue dysfunction plays a vital role in the pathogenesis of obesity-related insulin-resistance and T1DM. Adipose tissue is the main storage organ for fat deposits in the body, and is instrumental in buffering the daily ingestion of dietary fat. Buffering action is accomplished through the suppression of non-esterified fatty acids into circulation, and by increasing the clearance of triacylglycerol (TAG). In obese individuals, overloaded fat cells result in a reduced capacity to buffer fatty acids and TAG, thus leading to their storage in non-adipose tissue, such as the pancreas, liver and skeletal muscle. The accumulation of free fatty acids in skeletal muscle is proposed to interfere with the insulin signalling pathway as the lipotoxicity enhances IRS-1 serine 307 phosphorylation, and thus propagates the inactivation of the IRS-1 protein (Morino et al., 2006; Simon-Szabo et al., 2016). Furthermore, Koves *et al* (2005) suggest that excess fat tissue in obese individual's increases incomplete fat oxidation and the accumulation of β -oxidative intermediates, which may in addition to the free fatty acids, interfere with insulin-signalling (Koves et al., 2005).

With regards to physical inactivity, there is no doubt that there is an association between a sedentary lifestyle and the risk of developing insulin-resistance. The pathways involved however, are poorly understood. A large amount of research has investigated the effect of bed rest on metabolic responses and insulin sensitivity, however this is not relevant to the current research scope of the present investigation. This project will focus on the effect of reduced physical activity, and not complete immobile behaviour. A reduction in physical activity is associated with a decrease in insulin sensitivity, and changes to the morphological structure and composition of skeletal muscle tissue (Hamilton et al., 2014).

1.4 EFFECTS OF DETRAINING ON SKELETAL MUSCLE

Skeletal muscle is a highly adaptable and malleable tissue that responds to environmental and physiological changes, by alterations in muscle fibre size, and fibre type composition. Detraining refers to a period of insufficient or reduced training stimulus, which results in a reversal of exercise adaptations. The magnitude or extent to which muscle adaptations reverse, are dependent on the degree and duration of physical inactivity (Bogdanis, 2012).

Skeletal muscle, in addition to being responsible for movement, plays a large role in the disposal of glucose to regulate blood glucose concentrations. The contractile properties of skeletal muscle are classified according to the myosin heavy chain (MHC) isoform expressed. Three such isoforms exist in adult mammalian skeletal muscle, namely type I, type IIA and type IIX. A fourth isoform type IIB exists in small mammals such as the rat, and in specialised muscles such as those found in the larynx or human eye. Type I muscle fibres are characterised by a slow contraction speed, small cross-sectional area (CSA) large amounts of mitochondria, and depend predominantly on aerobic metabolism for ATP. Type IIX muscle fibres possess a faster speed of contraction, a larger CSA, and depend primarily on anaerobic metabolic pathways for the delivery of ATP. Type IIX fibres contain very little mitochondria, and fatigue rapidly in comparison to the fatigue-resistance type I fibres. Type IIA fibres display characteristics similar to type IIX in terms of contraction speed, however are comparable to type I fibres in that they are fatigue resistant (Kohn et al., 2011; Schiaffino et al., 2007).

The key alterations which occur in mammalian skeletal muscle as a result of reduced physical activity, include the transition in muscle fibre phenotype towards an increased expression of the type IIX isoform (Bruton, 2002). Furthermore, in terms of the morphology of the fibres, detraining is accompanied with an increase in the size of the type I and IIA fibres, with a reduction in type IIX fibre CSA (Bogdanis, 2012).

Numerous studies have examined these effects in both human and animal models. Detraining of domestic cats results in a reduction of type I fibres and a simultaneous increase in the proportion of type II fibres of the gastrocnemius muscle (Hernandez et al., 1997). Horses exposed to a period of endurance training displayed increased capillary density, aerobic enzyme activity and type I fibres in skeletal muscle. These adaptations were shown to completely reverse to pre training levels, following a detraining period of three months (Serrano et al., 2000). Human work carried out by Larsson and Ansved (1985) has shown as much as a 16% decrease in the proportion of quadriceps type I fibres, after a detraining period (Larsson and Ansved, 1985). It is thus evident

that changes in muscle fibre type occur in humans and domesticated animals. It is unclear however if similar alterations occur in large wild animals, such as wild felids, which also express all three muscle fibre types in skeletal muscle (Kohn et al., 2011).

In addition to changes in muscle morphology and composition, reduced physical activity has further effects on the metabolic pathways of skeletal muscle. More specifically, detraining and obesity are associated with alterations in the maximal activities of key enzymes involved in fuel metabolism. Citrate synthase (CS) and 3-hydroxyacyl-CoA dehydrogenase (3HAD) are oxidative enzymes involved in aerobic carbohydrate and fat metabolism. CS is responsible for catalysing the first reaction of the citric acid cycle, and thus serves as vital indicator of the citric acid cycle oxidative capacity. 3HAD is a key role player in β -oxidation, and therefore is a useful indicator of fat metabolism (Serrano et al., 2000). On the anaerobic side, phosphofructokinase (PFK) is one of the most important enzymes regulating glycolysis, as it functions as a rate limiting step in catalysing the ATP-dependent phosphorylation to convert fructose-6-phosphate into fructose 1,6-bisphosphate and ADP. Lactate dehydrogenase (LDH) catalyses the conversion of pyruvic acid to lactic acid in the absence of, or when oxygen is in short supply, and therefore is a useful indicator of anaerobic capacity (Mujika and Padilla, 2001).

The activities of CS and 3HAD are observed to decrease with detraining, as evidenced in both animal (Sertie et al., 2013) and human studies (Linossier et al., 1997). Conversely, detraining is associated with an upregulation in the maximal activities of anaerobic enzymes PFK and LDH (Mujika and Padilla, 2001). The undesired increase in the activity of PFK and LDH indicates a loss of oxidative capacity, and a greater reliance on the glycolytic pathway (Mujika and Padilla, 2001). This signals a reduced capacity for fuel oxidation (Chi et al., 1983) and thus poses a negative consequence on metabolic health, by contributing to the risk for metabolic aberrations.

1.5 OBESITY AND DIABETES IN ANIMALS

As in humans, animals are also at risk of developing metabolic disease as a result of obesity and sedentary behaviours (D'Eath et al., 2009; Osto and Lutz, 2015). Furthermore, obesity in domestic pets is associated with a higher risk of health problems such as type II diabetes, cardiorespiratory disease, urinary disorders, and reproduction complications (Muranaka et al., 2011). These animals become overweight as a result of overfeeding, as in addition to routine daily pet food portions, they are often supplemented with more “palatable” human foods as a reward, sign of affection or merely to dispose of excess meals (Rohlf et al., 2010).

Extensive research has been carried out on the prevalence of TIID in domestic cats and dogs, but very little data exists on the incidence and occurrence of this disease in wild animals. As traditionally wild animals - now housed in captivity - are also becoming overweight or obese, largely as a result of frequent feeding and the removal of behavioural activities of foraging, hunting or chasing prey (Hartstone-Rose et al., 2014) it is of importance that their potential risk for developing metabolic disease (insulin-resistance and type II diabetes) be investigated.

1.6 DIABETES IN DOMESTIC CATS

Diabetes in domestic house cats closely resembles that of TIID in humans, as it too is characterised by insulin-resistance of the liver, adipose tissue and skeletal muscle, in addition to the destruction of the pancreatic β -cells through amyloid deposition (Lutz and Rand, 1995). Although there is some evidence for a genetic predisposition to TIID, obesity remains the major risk factor for the development of the disease. The exact mechanisms of insulin-resistance development in cats are not yet known, but research to date has shown reduced GLUT4 (glucose transporter) expression and lower levels of IRS-2 protein in obese vs. lean cats (Nelson and Reusch, 2014). What is interesting about domestic feline diabetes is the ability for complete diabetic remission. Diabetic remission is characterised by the ability to maintain euglycemia without exogenous insulin or oral hypoglycaemic agents, and occurs when the pancreatic β -cells recover from a state of destruction, and are able to produce sufficient insulin again (Gottlieb et al., 2015). The reversal of diabetes in cats has largely been linked to changes in diet, and more specifically a transition from foods higher in carbohydrates, to those containing less carbohydrate. A study comprising 63 cats diagnosed with diabetes were fed either a moderate carbohydrate-high fibre diet (MC-HF) or a low carbohydrate-low fibre diet (LC-LF). Although both groups were able to revert to a healthy non-diabetic state, the LC-LF diet was deemed the preferred option, as 68% of cats in comparison to 41% on the MC-HF diet, no longer required artificial insulin (Bennett et al., 2006).

Further evidence of this diabetic remission phenomenon is provided by a case selection study carried out by Lien and Huang (2012). The consolidation of medical records (1999 to 2001) from an animal clinic in Taiwan, comprising of 29 cats diagnosed with diabetes, showed that a low carbohydrate diet was the only factor associated with clinical diabetic remission (Lien and Huang, 2012). As feline diabetes mellitus closely resembles that of the human form (Osto and Lutz, 2015) felines may serve as a useful model for further human diabetes research.

1.7 LOW CARBOHYDRATE, HIGH FAT DIET (LCHF)

An investigation into the long-term effects of a diet low in carbohydrates, can be observed through populations which have adopted this way of eating for centuries. One such population is the indigenous people of the Arctic - the Inuit. The traditional diet of indigenous arctic populations is low in carbohydrates and very high in animal fat (marine mammals and fish) specifically omega-3 fatty acids (Sefidbakht et al., 2016). Historically, the prevalence of T1DM was rare among these populations, however with more recent exposure to a Westernised lifestyle where the traditional diet has been replaced with increased refined carbohydrates (i.e. table sugar) and a reduction in physical activity (Munch-Anderson et al., 2012) there has been an increase in the prevalence of chronic diseases of lifestyle, such as T1DM and cardiovascular disease (Jeppesen et al., 2014). Animals too have been shown to benefit from high fat feeding, for which a good example are Alaskan sled-dogs. These animals perform incredible feats of endurance running in extreme environmental conditions, fuelled by a diet high in fat (> 50% of energy content) and low in carbohydrate (< 20%) (McKenzie et al., 2005). To date as far as our research group is aware, there is no documented evidence in the literature that indicates that these animals are at risk for the metabolic syndrome. Based on these observations, a diet low in carbohydrate may have some merit in glycaemic control, and therefore the control of T1DM.

1.8 WILD FELIDS IN CAPTIVITY

Due to shrinking natural habitats, a decrease in natural prey availability, poaching, and the illegal trade industry, wild felids such as lions, cheetahs and leopards are being housed and bred in captivity in facilities such as zoos and game reserves (Shepherd et al., 2014). Although providing a safe environment, a captive setting is not without challenges. Physical activity levels are lower as a result of smaller spaces and the absence of hunting and chasing prey (Mason et al., 2013; Slingerland et al., 2009). Furthermore, living environments create unnatural social densities, which affect animal behaviours (Mason et al., 2013). These factors, in addition to overfeeding practices has led to weight gain and lethargic behaviours which are now observable in the South African setting. Communications with Dr Adrian Tordiffe (South African wildlife veterinarian) has elucidated on the current situation of lions in captivity. Dr Tordiffe has observed captive lions being almost 50% heavier, and possessing a far greater percentage body fat than their wild counterparts. Based on the current literature and knowledge on obesity and physical inactivity in human and domestic pet populations, this study is therefore of imperative research interest, to

determine if the current captive scenario is placing these animals at risk for metabolic disorder and disease

1.9 ANTICIPATED GAIN IN KNOWLEDGE

The development of insulin-resistance resulting from an unhealthy lifestyle has affected the human and domestic animal population similarly. Although obesity and physical inactivity risk factors have been unquestionably linked to the onset and progression of this disease, the exact mechanisms are yet to be clarified.

Further understanding of the mechanisms involved in the metabolic pathway, and insulin-signalling which lead to insulin resistance, may offer a window into the pathophysiology of this disease in the human. In addition, the effect of a diet low in carbohydrate on skeletal muscle metabolism and glucose pathways, may provide a platform for further research into a LCHF diet as a tool for diabetic control.

Lastly, the effects of a captive lifestyle on traditionally wild animal skeletal muscle metabolism and structure will be examined. This insight may provide veterinarians and conservationists with further knowledge on whether wild animals (specifically felids in this study) like humans and domestic pets are also at risk for developing metabolic disturbances, and thus aid in the promotion of their health and well-being.

CHAPTER 2

2. COMPARISON OF SKELETAL MUSCLE MORPHOLOGY OF WILD AND CAPTIVE LIONS

2.1 INTRODUCTION

A sedentary lifestyle has known effects on the composition and structural components of skeletal muscle. These changes include a shift towards more type II fibres and a reduced fibre cross-sectional area (Bruton, 2002) and have been well documented in humans and laboratory animals (Hernandez et al., 1997; Larsson and Ansved, 1985). Assuming that free-roaming wild lions are more active than captive lions, the hypothesis that the latter will have detraining related adaptations in fibre type composition and muscle fibre area, was investigated.

2.2 METHODS

2.2.1 Study Animals

The study sample comprised of two groups, namely wild free roaming lions, and captive lions. The muscle samples for the wild group were sourced from wild lions located in Phinda Private Game Reserve, where typical wild animal behavioural activities of roaming and hunting occur. The captive lion muscle samples were collected from lions housed in enclosures at Buisfontein Safari Lodge, Ukutula Lodge & Lion Park, and the National Zoological Gardens (Figure 2).

At Buisfontein and Ukutula, enclosures varied in size from 300m² to 2500m², and diets ranged from whole chickens six days a week (Buisfontein), to horse or cattle carcasses once a week (Ukutula). The lions at the National Zoological Gardens were housed in small hospital enclosures (3m x 6m) or in zoo enclosures (100m x 30m), and fed supplemented beef (Predator Power, Health tech, South Africa) on alternate days.

Muscle biopsies (*Triceps brachii*) from the captive lions were collected while animals were anaesthetised for management purposes, treatment or other registered research projects. The biopsies from the wild lions were collected from animals that were culled due to overpopulation. After collection, the muscle samples were divided into smaller sections, rapidly frozen in liquid nitrogen on site, and transported on dry ice to the laboratory, and stored at -87 °C until analysis.

The two sample populations consisted of 7 wild lions (3 females, and 4 males) and 48 captive lions (31 females, and 17 males). Due to inadequate muscle sample size, histology could not be performed on all 48 captive animals.



Figure 2: Geographical locations of the study population. A Google Maps image shows the position of the study lions in the context of Southern Africa (Map data ©2016 AfriGIS (Pty) Ltd, Google).

2.2.2 Ethics

Ethical approval for this project was obtained from the Faculty of Health Sciences (FHS) Animal Ethics Committee, reference number 15/028 at the University of Cape Town, and the NZG Research Ethics and Scientific Committee, reference number P14/12.

2.2.3 Tissue Collection

Muscle biopsies were performed by a qualified veterinarian skilled in the collection of muscle tissue (Dr Adrian Tordiffe). Briefly, the skin over the *Triceps brachii* was disinfected with F10 SC sterilant (Health & Hygiene, Roodepoort, South Africa). A 2 cm skin incision was made with a sterile scalpel blade and the incision extended down into the *Triceps brachii*. The exposed muscle was grasped with sterile forceps and a small piece (approximately 3 mm x 5 mm) removed, placed in a cryovial, and kept on ice. Following the biopsy, a sterile gauze swab was applied to the wound until the bleeding had subsided. The skin was sutured with 2/0 catgut (Ethicon, Johnson & Johnson, Midrand, South Africa) and the wound sprayed with an antiseptic wound spray (Necrospray, Bayer Animal Health, Johannesburg, South Africa). For the euthanized wild lions,

the same procedure was followed, except the skin was not disinfected, nor were any sutures applied after sample collection.

2.2.4 Fibre Typing and Cross-Sectional Area (CSA)

Fibre size and type was determined using fluorometric immunohistochemistry (IHC) as described by Fry et al. (2014) with modifications. Serial cross-sections were cut using a cryostat, after which slides were blocked with 5% bovine serum albumin for 1 hour at room temperature, followed by incubation with primary antibodies overnight at 4°C. All primary antibodies were from Developmental Studies Hybridoma Bank (DSHB – Iowa, USA) and validated for their specificity to MHC I (BA-D5) and MHC IIA (N2.261) fibres in lions (Kohn et al., 2011). The following day, the slides were washed and incubated with fluorescent-tagged secondary antibodies for two hours at room temperature (Jackson Immunoresearch Laboratories: AMCA goat anti-mouse IgG2b (catalogue no. 115-155-207) and AlexaFluor 488 goat anti-mouse IgG1 (catalogue no. 115-545-205). Slides were then thoroughly washed and mounted with a fluorescent mounting media (moviol), and stored at room temperature in the dark overnight to cure (Fry et al., 2014). All sections were visualised using a fluorescent microscope (Nikon Eclipse i80, Japan) and the image captured at 10x magnification using a digital camera (Canon EOS 650D). Fibres were classified as either pure type I, IIA, or IIX (Figure 3), hybrid fibres were excluded from the results, as they represent less than 1% of the total fibre pool. All fibres in view were included in the analysis, and approximately 200 fibres per muscle section for each animal were typed. The CSA of each fibre was determined using pre-calibrated software (Image J version 1.48) and grouped according to their fibre type. For a detailed explanation of the protocol, refer to Appendix A: Section 1.

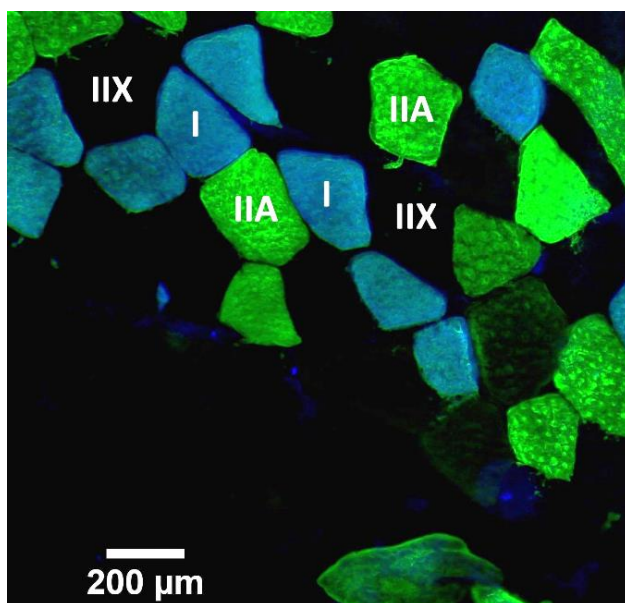


Figure 3: Example of immunofluorescent secondary antibody tagging to determine muscle fibre type. The green (excitation 450-490) indicates the type I and IIA fibres, and the blue (excitation 365/10) indicates only the type I fibres. Type IIX fibres were identified as those that remained unstained.

2.2.5 Statistics

All values are expressed as mean \pm standard deviation (SD). All the data was assessed and statistically analysed using Graphpad Prism version 6.05. The Shapiro-Wilk test for normality indicated that non-parametric statistics be used for all analyses. The data did not meet the assumptions of normality and homogeneity of regression, which are required to run an ANCOVA. Therefore, as there is no non-parametric alternative to an ANCOVA, to determine an age-effect (account for age as a confounder) scatterplots and regression models for each outcome variable against age was generated (Appendix A: Section 2). Furthermore, Mann-Whitney U tests were performed to identify differences in outcome variables between groups, and the Spearman's rank correlation coefficient was used to determine relationships between specific outcome variables. Significance was defined as $p < 0.05$ for the outcome variables.

2.3 RESULTS

2.3.1 Sample Characteristics

The captive lions were found to be significantly older than the wild lions, with a large age range of 2-16 years compared to the wild group (1-8 years). Furthermore, the captive lions were significantly heavier than the wild lions with a mean body mass of 188 ± 50 kg, in comparison to 136 ± 27 kg observed in the wild lions (Table 1).

2.3.2 Fibre Type Composition

For both the captive and wild lions, type IIA fibres were the most predominant fibre type with $44\% \pm 16$ in the captive lions, and $48\% \pm 15$ observed in the wild lions (Figure 4). The proportions of type I and IIX fibres, however, differed between groups. In the captive lions, there were equalled proportions of type I ($28\% \pm 11$) and IIX fibres ($28\% \pm 19$), while in the wild lions the type IIX fibres contributed much less ($16\% \pm 19$) to the overall fibre count in comparison to the type I fibres ($36\% \pm 13$). There was no statistical significant differences in the proportions of type I, IIA or IIX fibres between the captive and wild lions. There was however a trend towards less type I fibres, and more type IIX fibres in the captive lions, with similar proportions of the type IIA isoform between groups (Figure 4). Furthermore, there was no difference in the fibre type composition between the male and female lions within the captive and wild groups (Table 3, Appendix A: Section 2). However, for both the captive and wild lion groups, the male lions tended to possess more type I and IIA fibres, with little to no type IIX muscle fibres in comparison to the female lions in both groups (Figure 4).

From the standard deviations provided above, and through visual inspection of the data in Figure 4, there was a large range in the percentage contribution of each fibre type within groups. Animals within the captive and wild groups showed large differences in the percentage contribution of each fibre type. Furthermore, there was evidence of clustering within groups for example in the wild group, all the male lions had considerably more type I fibres than the female lions. Similarly, the majority of the male lions in both the captive and wild groups had very few, or no type IIX fibres in the *Triceps brachii* muscle sample (Figure 4).

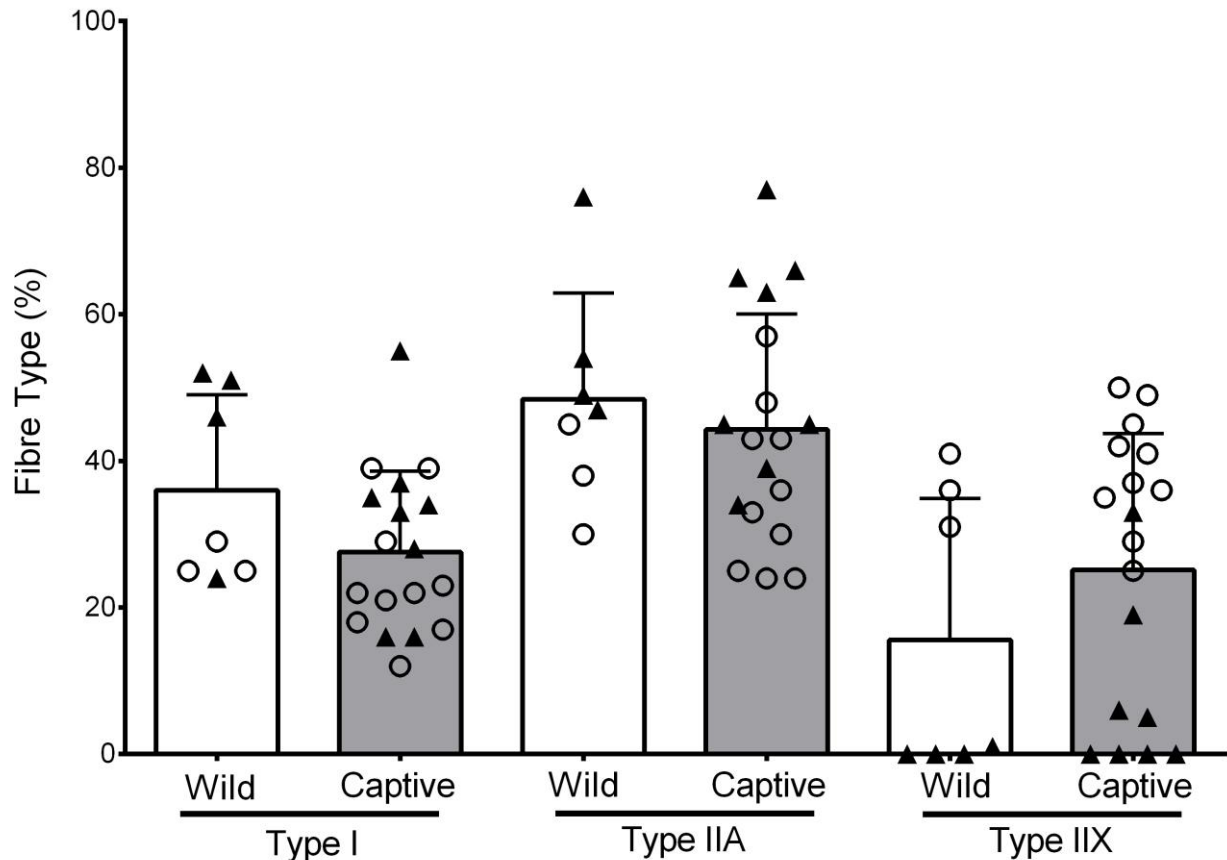


Figure 4: Fibre type composition (%) of the *Triceps brachii* in the captive and wild lions. The male and female lions are represented by open circles (females) and closed triangles (males). The bars represent means \pm SD. For exact values and ranges refer to Table 3, Appendix A: Section 2.

2.3.3 CSA

Within both groups, the cross-sectional areas of the type IIX fibres were the largest, and the type I fibres the smallest. Statistical analyses (Table 1) between groups found type I fibres to be significantly larger in the captive lions ($5847\mu\text{m}^2$ vs. $3318\mu\text{m}^2$). Additionally, the captive group had significantly larger type IIA fibres ($8344\mu\text{m}^2$) in comparison to the wild lions ($4360\mu\text{m}^2$). However, no difference was noted in type IIX fibre size, despite the mean fibre sizes being considerably different ($10122\mu\text{m}^2$ in the captive versus $6658\mu\text{m}^2$ in the wild lions). Furthermore, there was no difference in the fibre size of any of the fibre types between male and female captive and wild lions (Table 1).

Table 1 – Sample population characteristics, and CSA of the *Triceps brachii* in the wild and captive lions. Values are expressed as mean \pm standard deviation. The range for each group is in parentheses.

	Captive (n=18)			Wild (n=7)		
	Male (n = 8)	Female (n = 10)	Pooled	Male (n = 4)	Female (n = 3)	Pooled
Age (yrs)	5.1 \pm 1.4	5.4 \pm 3.9	5.2 \pm 3.0 (2-16)	2.5 \pm 1.7	3.7 \pm 3.8	3.0 \pm 2.6 * (1-8)
Weight (kg)	230 \pm 36	153 \pm 29	188 \pm 50 (102-292)	144 \pm 27	125 \pm 32	136 \pm 27 * (133-184)
CSA (μm^2)						
Type I	6594 \pm 2633	5248 \pm 1358	5847 \pm 2075 (2644 – 10009)	3385 \pm 1449	3228 \pm 726	3318 \pm 1110 * (2308 – 5499)
Type IIA	8263 \pm 3512	8409 \pm 2935	8344 \pm 3106 (3521 – 14817)	3844 \pm 1610	5048 \pm 1971	4360 \pm 1733 * (2418 – 6837)
Type IIX	10564 \pm 5028	9990 \pm 2952	10122 \pm 3288 (5153 – 16342)	N/A	6658 \pm 3428	6658 \pm 3428 (4383 – 10601)

* Different between wild and captive lions ($P < 0.05$)

2.3.4 Correlations

There was no association found between body mass and the proportion of type I, IIA or IIX fibres. However, there was a relationship (Figure 5) between body mass and the CSA of type I ($r = 0.68$, $P = 0.0002$) and type IIA fibres ($r = 0.44$; $P = 0.03$). No relationship was evident between type IIX fibre size and body mass.

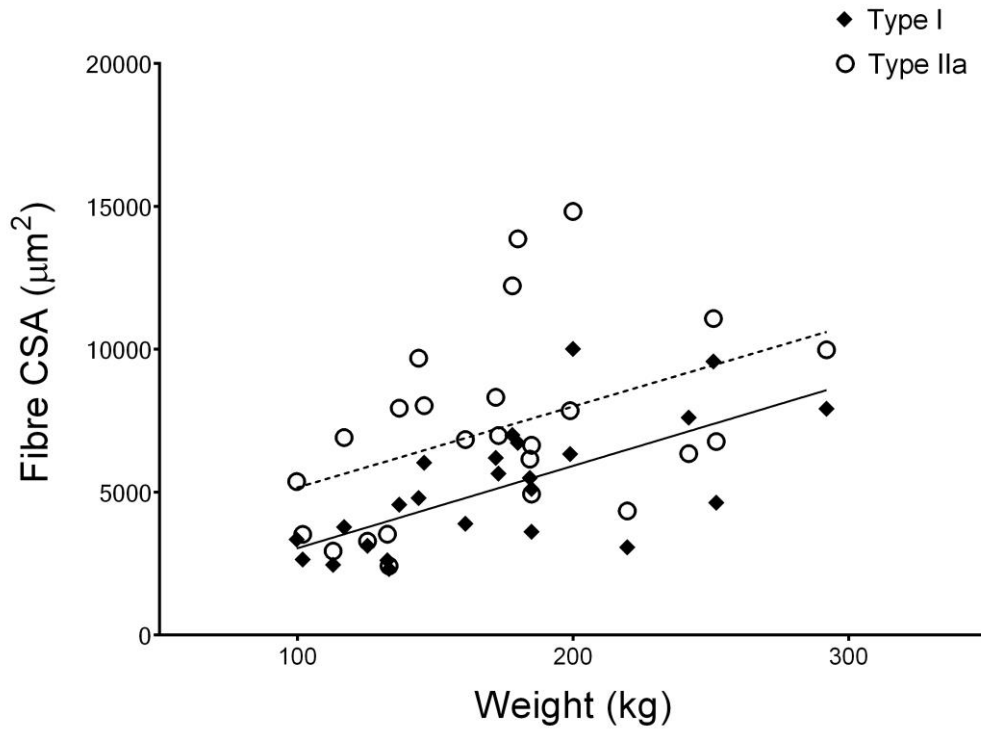


Figure 5: Correlations between CSA (μm^2) and body mass. Data for the wild and captive groups were pooled to assess relationships. The regression line for the type I fibres is depicted by the solid line, with the dotted line representing the regression line for the type IIA fibres. $r = 0.68$ and $r = 0.44$ for the type I and type IIA fibre areas, respectively. Correlations were significant with a $P < 0.05$.

2.4 DISCUSSION

Reduced physical activity is associated with various alterations in the skeletal muscle fibre composition and morphology. These alterations have negative consequences on muscle metabolism, and have been linked to chronic diseases of lifestyle, such as type II diabetes (Oggioni et al., 2014). Due to the nature of captivity, where animals live in enclosed spaces and the need to hunt or forage for food has been removed, sedentary behaviours and obesity are factors of important consideration. The captive lions in this study were heavier than the wild lions and thus it was of interest to identify and determine the skeletal muscle composition and morphology in the captive lions, in order to ascertain if these lions are not only overweight, but possibly displaying the muscle characteristics indicative of sedentary behaviours.

One of the key muscle morphological features of detraining in humans, is a shift in muscle fibre composition from a predominance in type I towards more type IIX fibres (Bruton, 2002; Hernandez et al., 1997). This adaptation has also been observed in domesticated animals (Hernandez et al., 1997; Serrano et al., 2000; Sertie et al., 2013), but it is unknown whether this same adaptability is prevalent in the skeletal muscle of wild animals, such as felids. Kohn et al. (2011) compared the fibre type and skeletal muscle metabolism of lions, caracal and humans, but the study was limited in that only 2 lionesses made up the lion sample group (Kohn et al., 2011). The present research is therefore the first study that has had such a large sample size to investigate, and describe the skeletal muscle physiology of wild and captive lions.

2.4.1. Fibre Type Composition

In contrast to findings by Kohn et al. (2011), where type IIX fibres were identified as the predominant fibre type in two captive female lionesses, the current study showed a predominance of type IIA fibres in the *Triceps brachii* in both the captive and wild lion sample groups. This inconsistency may be the result of biopsy location, as the study by Kohn et al. (2011) examined muscle samples collected from the *Vastus lateralis*. This observation therefore not only illustrates the importance of identifying and comparing the same muscle within and between species, when investigating skeletal muscle physiological characteristics (Bruton, 2002), but also may indicate potential differences in the fibre type profile of the muscle groups comprising the anterior and posterior limbs of the lion.

On the other hand, this discrepancy may purely be as result of the sample selection. From the data in the current study (Figure 4) it was observed that the majority of the male lions possess more

type I and type IIA fibres than the female lions, and have little to no type IIX fibres. Therefore if the male lions were excluded from this study, the results may be more in line with those observed in the Kohn et al. (2011) study.

It must be noted that the difference in muscle fibre composition observed between male and female wild felids is a novel finding, as to date there has been no evidence suggesting they differ in their muscle physiological makeup. Muscle function is greatly influenced by fibre type composition (Cornachione et al., 2011; Delp and Duan, 1996) and in humans, certain muscle groups show predominance of one fibre type, which strongly relates to the function of that particular muscle (Gollnick et al., 1974). The difference in fibre composition observed in the male and female lions, may indicate a potential functional difference of the muscle. Whether this is genetically determined, or an adaptation is unclear and warrants further investigation in follow-on studies. This finding highlights the importance of the homogeneity of a sample group, however as the lack of type IIX fibres observed in the male lions was an unexpected observation in the current study, it wasn't a consideration when collecting the muscle samples.

In terms of the overall muscle fibre profile, there was no statistical difference observed in the fibre type composition between the captive and wild lions. However, there was a tendency towards less type I (28% vs 36%) and more type IIX (28% vs 16%) fibres in the captive lions, which may indicate a potential adaptation towards a more glycolytic fibre type profile and thus be in support of detraining associated maladaptation's observed in human and animal models (Bruton, 2002; Hernandez et al., 1997). However, due to the large variation (standard deviations) in the data, in addition to the lack of statistical significance, it cannot be conclusively stated that this is indeed the case.

2.4.2. Cross-Sectional Area

The literature on the effects of endurance training cessation on muscle cross-sectional area in humans, appears to show conflicting results amongst studies. Some studies report an increase in the size of type I (Dahlstrom et al., 1987; Houston et al., 1979; Larsson and Ansved, 1985) and IIA fibres (Houston et al., 1979). Other studies however, found a decrease in the cross-sectional areas of type I, IIA (Amigo et al., 1998; Klausen et al., 1981) and IIX fibres (Bogdanis, 2012), or no change at all in the fibre cross-sectional areas (Houmard et al., 1992). Overall, the majority of the literature appears to support the general trend towards a decrease in the fibre cross-sectional areas with the cessation of strength training, and a general increase in the size of the fibres with detraining from endurance type activities (Mujika and Padilla, 2001).

Research on the effects of detraining on skeletal muscle fibre cross-sectional areas in animals is limited, however based on the available data, the observations seem more consistent than those seen in humans. Hernandez et al. (1997) investigated the effect of varying activity levels on the fibre cross-sectional area in domestic cats. The authors reported no difference in the fibre cross-sectional areas of the animals which were caged for over 20 months, when compared to those that were exercised daily (Hernandez et al., 1997). Furthermore, long-term detraining in horses was also found to illicit no changes in the mean cross-sectional areas of type I, IIA and IIX fibres (Serrano et al., 2000).

In addition to hunting and chasing prey activities, which require great strength and speed. In their natural habitat, wild lions also engage in physical activities of low intensity, such as patrolling territories and periods of extended walking to seek out new hunting grounds (Mosser and Packer, 2009). Thus an endurance capacity, in addition to a largely favoured strength physiological profile would be anticipated. The significantly larger type I and IIA fibres, and the trend towards larger type IIX fibres observed in the captive lions, are comparable to detraining observations in human endurance athletes (Mujika and Padilla, 2001). Thus in comparison to the wild lions, there may be some indication that lower levels of physical activity in the captive lions, are resulting in changes in their skeletal muscle morphology.

On the other hand, other factors such as hydration and body mass (correlations described below) may also be influencing the muscle fibre morphology results. Water accounts for approximately 75 % of muscle mass in human individuals, and with dehydration there is a considerable decline in muscle volume (Hackney et al., 2012). Based on the assumption that free-roaming wild lions have less access to freely available drinking water in comparison to lions in captivity, in theory, the wild lions may have been less hydrated than the captive lions during the biopsy collection. Although a potential factor of relevance, this was deemed an unlikely event as thirst is very well regulated in lions, and they are able to concentrate their urine dramatically to retain body fluid. Moreover, as the muscle biopsies were collected in the winter season, and lions do not have significant sweat glands, moisture loss would have been minimal (communication with Dr. Tordiffe). The present study was unable to control for this factor, and although unlikely, it cannot be ruled out as a mitigating factor. In future studies, this variable should be incorporated through the measurement of water loss, from the muscle samples pre and post freeze-drying.

2.4.3 Correlations

The average weight of a healthy adult male lion is approximately 190 kg and that of a lioness approximately 128 kg (Skinner and Chimimba, 2005). The captive lions in the present study are not only significantly heavier than the wild lions, but they are also overweight (mean of 230 kg and 156 kg for the male and female lions, respectively). Obesity in humans is associated with an increase in skeletal muscle fibre area as a result of ectopic lipid deposition. More specifically, this lipid deposition occurs primarily in the oxidative type I and IIA fibres (Pisto et al., 2012). The association observed between type I and IIA fibre areas and body mass in the wild and captive lions is an interesting finding, as it may indicate that the larger type I and IIA fibres observed in the captive lions are potentially linked to their greater body mass, and not necessarily simply to their lower activity levels. To investigate this theory, future research should measure the quantity of lipid stored in the various muscle fibre types, in order to determine if the captive lions harbour a higher lipid content in the type I and IIA fibres, in comparison to wild lions.

2.5 CONCLUSION

From the skeletal muscle composition and morphology results, there is insufficient evidence to suggest that a shift towards a more glycolytic fibre type profile is apparent in the captive lions. Although there was indication of a difference in the cross-sectional areas, due to the potential influence of confounding variables, it cannot be conclusively stated that the captive lions are showing skeletal muscle morphological signs of being detrained, in comparison to the wild lions.

To gain a more comprehensive representation of the potential effects of captivity, to either support or refute the above notion, it was necessary to investigate vital enzymatic activities involved in skeletal muscle metabolism.

CHAPTER 3

3. METABOLISM

3.1 INTRODUCTION

Reduced physical activity is associated with a downregulation in the enzymes of aerobic metabolism, and an upregulation in the enzymes of the glycolytic pathway (Chi et al., 1983; Linossier et al., 1997). As these adaptations increase the risk for metabolic disturbances, such as insulin-resistance, it is paramount that their activities are investigated within the captive lion. The glycolytic enzymes examined were lactate dehydrogenase and phosphofruktokinase, as lactate dehydrogenase (LDH) serves as an indicator of anaerobic capacity, and phosphofruktokinase (PFK) of glycolytic flux (Serrano et al., 2000). Furthermore, the oxidative enzyme citrate synthase (CS) is the first enzyme involved in the citric acid cycle, and thus a key indicator of oxidative flux. Lastly, 3-hydroxyacyl-CoA dehydrogenase (3HAD) activity was examined as it serves as a key marker of β -oxidation, and thus a useful indicator of the capacity to metabolise fat (Linossier et al., 1997; Mujika and Padilla, 2001).

3.2 METHODOLOGY

3.2.1 Sample Population

The maximum enzyme activities of CS, 3HAD, PFK and LDH were determined in forty eight captive, and seven wild lion muscle samples.

3.2.2 Preparation of muscle homogenates for Enzyme Assays and SDS-PAGE

All muscle PAGE sample homogenates prepared for enzyme analyses and SDS-PAGE were carried out according to Kohn *et al.* 2007, with modifications. An amount of ± 40 mg wet weight muscle tissue was freeze-dried overnight under vacuum and at -50°C . A small portion of the freeze-dried muscle was then weighed, and 0.1 M potassium phosphate homogenising buffer (pH 7.3) added to obtain a ratio of 1:400 (i.e. 400 μl buffer for every 1mg tissue). Samples were then pulse-sonicated (Qsonica sonicators) three times for ten seconds (pulse interval set to one second and an amplitude of 30%) on ice. The homogenising buffer preparation is reported in Appendix B.

3.2.3 Protein quantitation

The total protein concentration of each homogenate was determined using the assay developed by Bradford (1976). A bovine serum albumin (BSA) stock solution of 0.5 g/l was utilised to conduct a series of standard BSA protein solutions (concentration range 0.0 – 0.5 g/l). A volume of 10 µl of each unknown lion homogenate sample was then pipetted in duplicate into separate wells of a clear 96-well microplate and 250 µl Bradford reagent added to each well. The reaction was left to incubate for 5 min at room temperature, after which the absorbance was measured at 595 nm. A standard protein curve was constructed from known BSA concentrations, fitted with a linear equation and the protein content of the lion samples quantified (Bradford, 1976). For a detailed protocol of the Bradford protein assay, refer to Appendix B.

3.2.4 MHC isoform content

Muscle homogenate samples (prepared as outlined above) were separated into their distinct isoforms according to methods described by Kohn et al. (2007). Briefly, a volume of sample containing approximately 0.5µg total protein was loaded on 7% polyacrylamide gels containing 30% glycerol (Hoefer SE600, Holliston, MA, USA). Gels were packed in ice and run in the cold at constant 70V for 4h, followed at 275V for 20h. After silver staining, the gels were scanned and the density of the separated bands quantified using the Un-Scan-It software package (Silk Scientific Corporation, Orem, UT, USA). Three bands were identified (Figure 6) and each band was expressed as a percentage of the total densitometric profile of the three bands

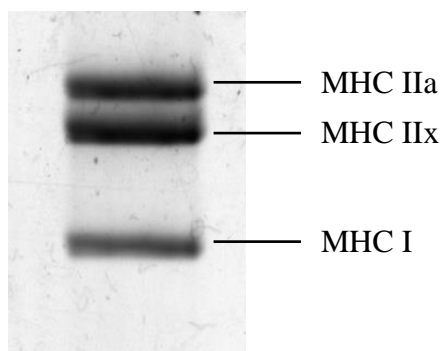


Figure 6: Myosin heavy chain (MHC) isoform separation and content of *Triceps brachii* homogenate samples using gel electrophoresis.

3.2.5 Enzyme Activities

Maximal activities of enzymes 3-HAD, LDH, PFK and CS were determined using fluorometric methods described by Kohn et al. (2007) with slight modifications. The technique relies on the principle of fluorescence, where enzyme activity is indirectly measured via the disappearance or appearance of NADH over time. The excitation and emission wavelengths for all assays was 340

nm and 460 nm, respectively, and all the reactions were performed at 25 °C. For each assay, the maximum linear slope was determined and expressed relative to the amount of protein calculated from the Bradford assay ($\mu\text{mol}/\text{min}/\text{g}$ protein) (Essen-Gustavsson and Henriksson, 1984). Please refer to Appendix B: Section 1, for complete reagent buffer preparations and detailed protocols.

3.2.6 Statistics

The Shapiro-Wilk normality test was carried out on the population characteristics and each outcome variable. Based on the results, non-parametric statistics were implemented to analyse differences between groups (Mann Whitney U test) and relationships between measured variables (Spearman's correlation coefficient).

3.3 RESULTS

3.3.1 MHC content

MHC isoform content quantified by gel electrophoresis, was compared to the fibre type content identified by IHC techniques performed in Chapter 2. The results in Figure 7 display the inconsistencies between the two methodologies. There were significantly more ($P = 0.001$) type IIA fibres identified by the IHC technique (45%) in comparison to the gel electrophoresis method (32%). Furthermore, significantly ($P = 0.002$) less type IIX fibres were identified by the IHC technique (24 %) in contrast to the gel electrophoresis method, where 41% of the overall fibre composition comprised of the MHC IIX isoform. No differences were observed with regards to the MHC I content, with a proportion of 30% identified by the IHC technique, and 27% via the gel electrophoresis method.

However, as was similarly observed for the IHC results (Chapter 2), no significant differences in the MHC isoform composition was observed between the captive and wild lion homogenate samples (Figure 11, Appendix B, Section B).

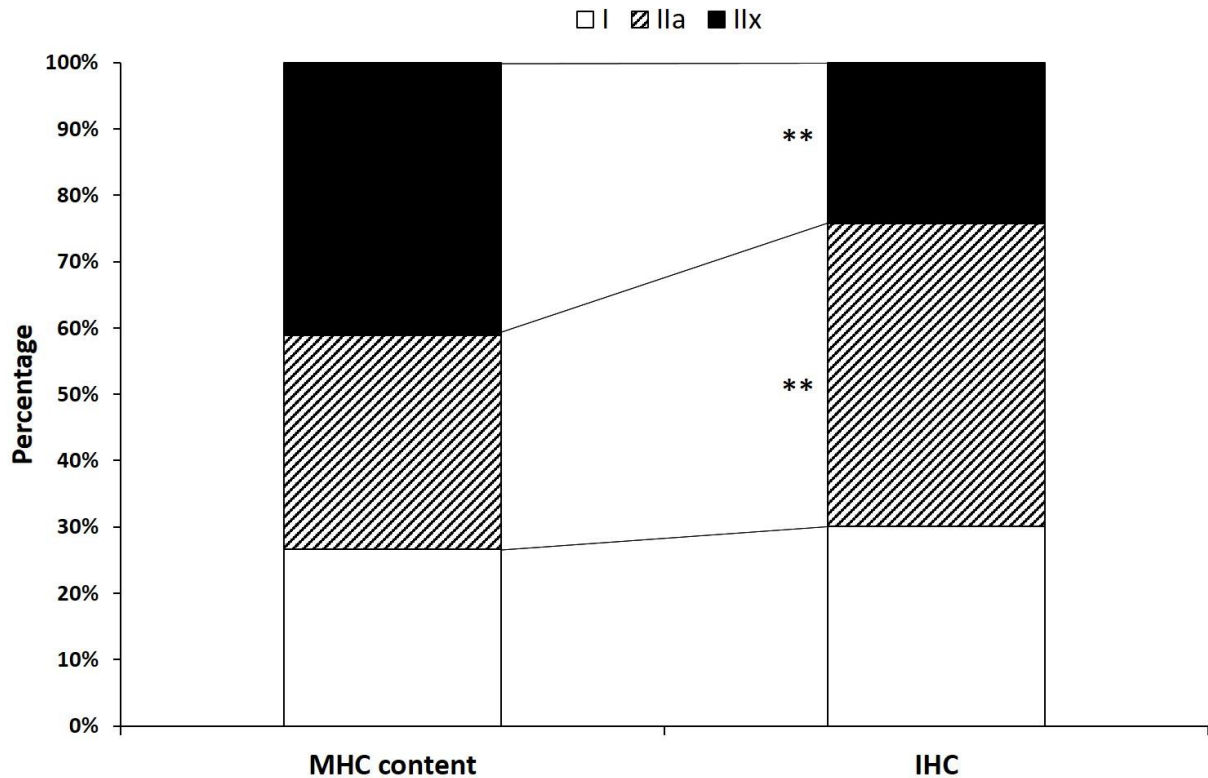


Figure 7: Comparison of muscle fibre type determined from immunohistochemistry (IHC) vs. the MHC isoform content determined from gel electrophoresis. Data for the wild and captive lions were pooled for this analysis. Different: ** $P < 0.01$.

3.3.2 Enzyme Activities

Very similar LDH activities were observed between the captive ($1833 \pm 433 \mu\text{mol}/\text{min}/\text{g}$ protein) and the wild lions ($1816 \pm 368 \mu\text{mol}/\text{min}/\text{g}$ protein). Maximal PFK activities however were 1.6 x higher in the captive lions (551 ± 287 vs. $338 \pm 123 \mu\text{mol}/\text{min}/\text{g}$ protein, $P = 0.03$) including the two female outliers (Figure 8a). This statistical difference ($P = 0.04$) in PFK activities between groups, was still evident when the two female outliers were removed from the analysis.

Furthermore, no differences in LDH or PFK activities were evident between the male and female lions within the captive and wild lion groups. Refer to Appendix B, Table 5 for enzyme activity means and standard deviations.

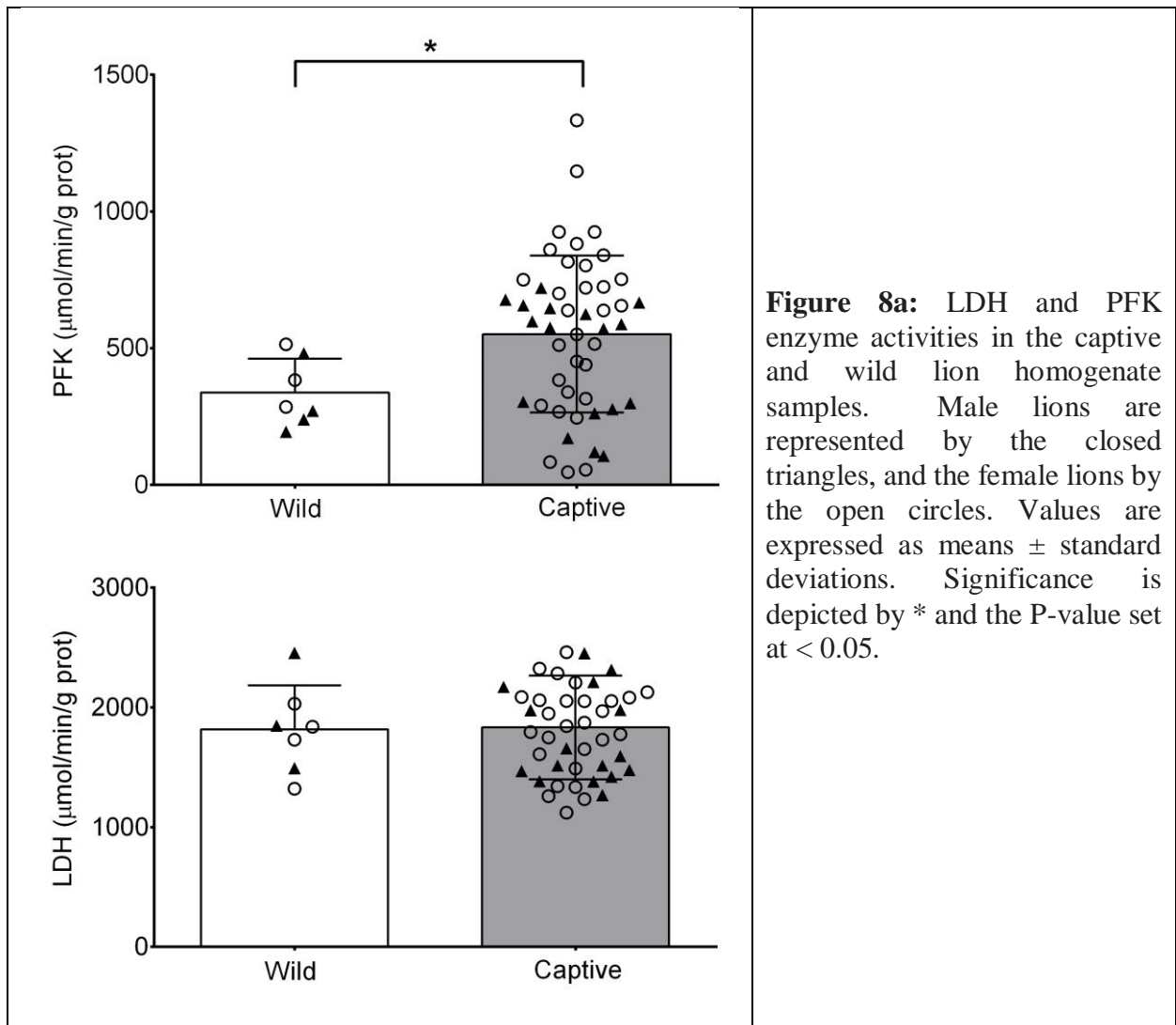
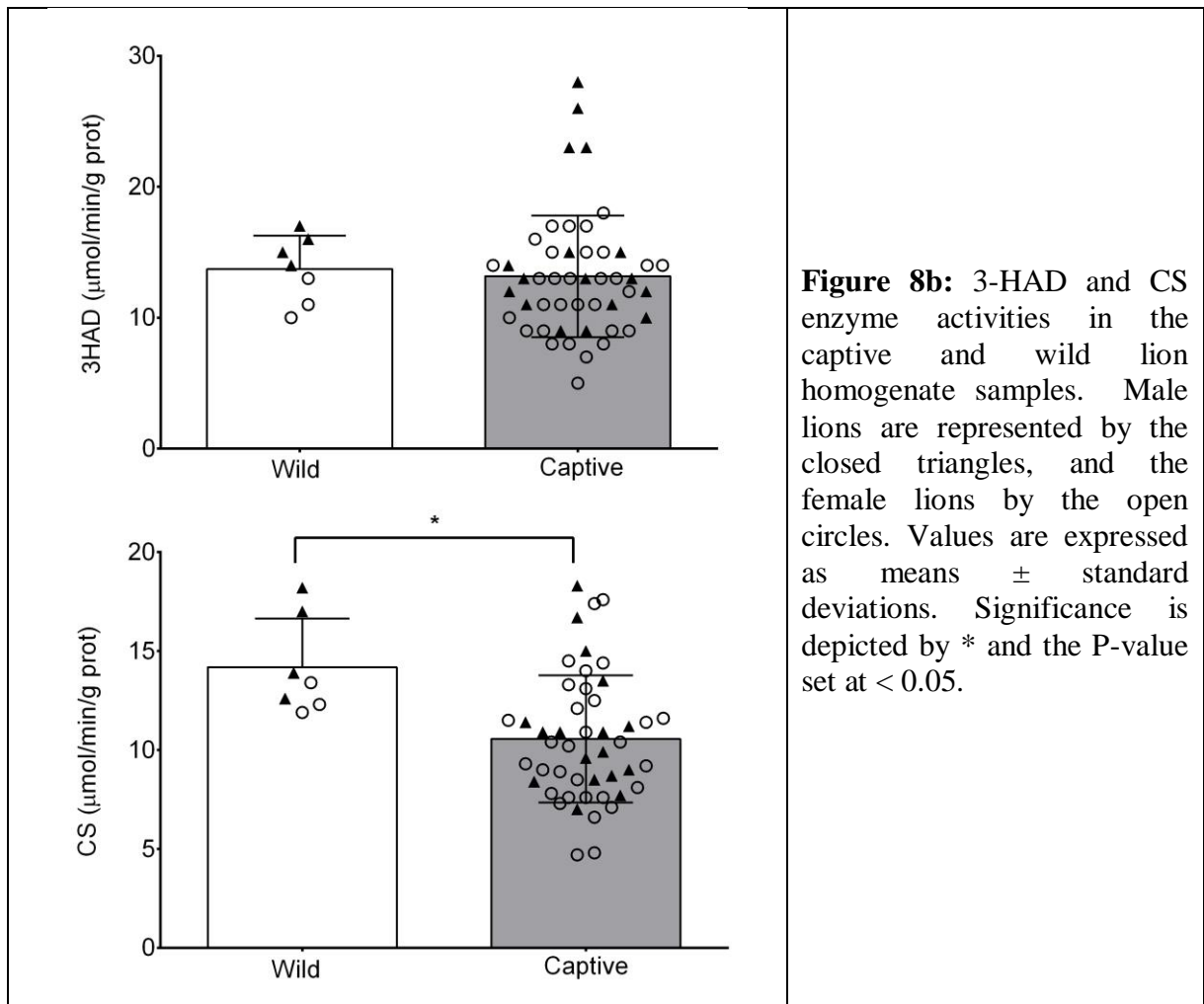


Figure 8a: LDH and PFK enzyme activities in the captive and wild lion homogenate samples. Male lions are represented by the closed triangles, and the female lions by the open circles. Values are expressed as means \pm standard deviations. Significance is depicted by * and the P-value set at < 0.05 .

In terms of oxidative capacity, there was no difference in the maximal 3HAD enzyme activities between the wild ($14 \pm 3 \mu\text{mol}/\text{min}/\text{g}$ protein) and captive lions ($13 \pm 5 \mu\text{mol}/\text{min}/\text{g}$ protein). However, maximal CS activities were found to be significantly higher (1.3 x) in the wild lions (14 ± 2 vs. $11 \pm 3 \mu\text{mol}/\text{min}/\text{g}$ protein, $P = 0.003$) (Figure 8b). Again, no difference was observed between male and female lions (Appendix B, Table 5).



3.3.3 Correlations

Although a positive relationship was observed between 3HAD activities and MHC I content of the homogenate samples ($r = 0.55$; $P = 0.001$), this relationship was only evident when the captive and wild lion groups were pooled. When analysed individually, there was only an association evident between the captive group and MHC I content. Similarly, the negative relationship observed between 3HAD activity and MHC IIx content was only evident when both the wild and captive groups were pooled. However, when analysed as separate groups, there was only an association between 3HAD activity, and MHC IIx content in the captive group (Figure 9).

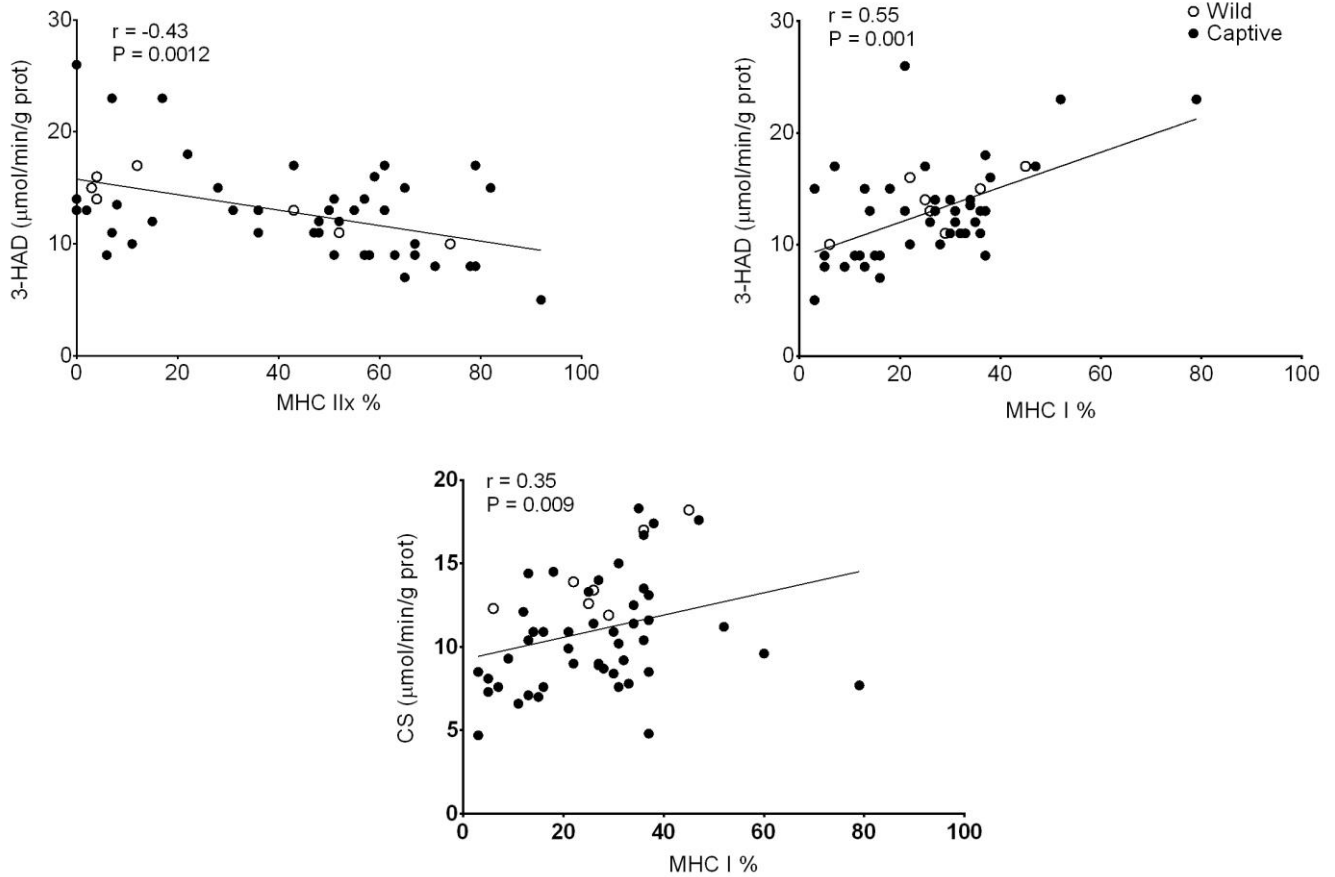


Figure 9: Relationship between enzyme activities and fibre type proportion. Data for the captive and wild lions were pooled for correlation analyses. Significance was set at $P < 0.05$.

Furthermore, a significant positive association (Figure 9) was found between CS activity and MHC I content ($r = 0.35$, $P = 0.009$). Again this was only the case when the groups were pooled, and individually there was only an association within the captive group. No relationship was found between PFK or LDH activities, and any of the MHC isoform contents. Furthermore, no associations were found between body mass and any of the enzyme activities measured.

3.4 DISCUSSION

3.4.1 MHC content

There are various methods of identifying the fibre type profile of muscle samples, e.g. ATPase histochemistry, antibodies directed against the MHC isoforms, homogenate and single fibre sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). Each method has limitations and advantages. In this study, immunohistochemistry and homogenate gel electrophoresis were selected to determine the muscle fibre type (type I, IIA and IIX) and MHC isoform content, respectively. Each technique made use of a separate piece of muscle, divided from the same biopsy muscle section. The incomparable results not only highlight the variability in muscle fibre type composition within a muscle biopsy, but also questions whether either method can be used as a stand-alone technique to determine the fibre type profile of a particular muscle. It is important to consider that the smaller sample size used for IHC (18 captive and 7 wild lions) may have been a limitation of the technique, and thus if a larger sample size were used, the results may better reflect those observed from the homogenate gel electrophoresis technique. Overall, gel electrophoresis and IHC are complimentary techniques, and both provide important information on muscle physiology. To improve the validity of results, future work should consider incorporating both methods when gathering quantitative, and qualitative information on muscle fibre composition and structure.

Despite the inconsistencies revealed between methods, the gel electrophoresis method was in support of the findings observed by the IHC technique in Chapter 2, as there was no difference in MHC I, IIA or IIX content of the *Triceps brachii* between the wild and captive lions.

3.4.2 Enzyme Activities

As detraining is associated with a downregulation in oxidative enzyme flux (CS and 3HAD), and a concomitant upregulation in the enzymes of anaerobic (PFK and LDH) metabolism (Chi et al., 1983; Serrano et al., 2000; Wibom et al., 1992), the significantly lower CS and higher PFK activities measured in the captive lions, provides some evidence to suggest that the captive lions may be showing signs of detraining.

Previously, it was generally accepted that muscle fibre type dictated the activities of oxidative and glycolytic enzymes (Essen et al., 1975; Lowry et al., 1978). However, more recent work has elucidated to there being a wide range, and much overlap in the activities of glycolytic and oxidative enzymes within each muscle fibre isoform (Kohn and Myburgh, 2007; Nakatani et al., 2000). Thus muscle fibre types are not defined by metabolic enzyme activities and vice versa, but are rather dependant on a stimulus (i.e. training)(Kohn and Myburgh, 2007). Although no difference in the muscle fibre composition (Chapter 2) was evident between the captive and wild lions. The lower CS and higher PFK activities observed in the captive lions, are thus potentially indicative of a lower oxidative capacity, and a higher glycolytic flux as a result of a sedentary lifestyle.

On the other hand, despite the observed differences in CS and PFK activities, there was no difference in 3HAD (capacity to metabolise fat) or LDH activities between the wild and captive lions. LDH catalyses the conversion of lactate to pyruvate, and vice versa in the oxygen-independent metabolic pathway (Valvona et al., 2016), and bearing in mind that lions are sprinters by nature (Kohn et al., 2011), it was anticipated that current sedentary behaviours in the captive lions would have resulted in them displaying lower LDH activities.

Interestingly, detraining studies in human individuals have observed a significant reduction in muscle oxidative potential, while the glycolytic potential (PFK and LDH activities) remained unchanged after 7-12 weeks of detraining (Linossier et al., 1997; Simoneau et al., 1987). Although a difference in PFK activities was observed between the wild and captive lions, perhaps detraining in lions (captive group) does not affect the anaerobic pathway (LDH activity) in the same manner as the glycolytic pathway (PFK activity).

3.4.3 Correlations

As associations were only evident between enzyme activities and MHC content within the captive group, this questions whether a true relationship exists between these variables. Furthermore, if there was indeed an association between fibre type and enzyme activity, then it would presumably be evident with all enzyme activities. However, this was not the case as no relationship was evident between anaerobic enzymes PFK and MHC I or MHC IIx content, or LDH and MHC I or MHC IIx content. Furthermore, as previous research has failed to show true correlations between muscle fibre type, and enzyme activities in animal models (Curry et al., 2012; Kohn and Myburgh, 2007; Smerdu et al., 2009) the relationships observed in the current research may be an artefact of pooling the data sets, or merely a co-incidence.

3.5 *CONCLUSION*

Based on the analyses of the key enzymes involved in the metabolic pathway, there is currently insufficient data to suggest that there is any meaningful difference in the efficiency and capacity of these enzymes between the captive and wild lions. Therefore, it cannot be concluded that the captive lions in this study are displaying any enzymatic metabolic disturbances as a result of greater body weight, and lower physical activity levels.

CHAPTER 4

4. CONCLUDING REMARKS

The current global population is approximately 7 billion people, which according to the Global Footprint Network (Accessed July 2016) relates to three times the sustainable level. With this continual growth of the human population, there is an ever-increasing need for more space and basic resources. Various issues have arisen as a result of overpopulation, which include, but not limited to the depletion of natural resources, degradation of the environment, conflict and wars, unemployment and the destruction of wild animal habitats (Mason et al., 2013). Due to the latter, many wild animals have been forced into a captive existence to prevent their extinction. However, despite this seemingly protective practice, animals in captivity are faced with other challenges, such as unnatural social and habitual behaviours, close proximity to humans, altered climates (Mason et al., 2013) and sub-optimal nutrition (Hartstone-Rose et al., 2014).

The concerns and challenges faced by wild animals in captivity can largely be paralleled with those experienced by human populations as a result of rural-urban migration (Mason et al., 2013). Human urbanisation is associated with an improvement in overall living standards, yet it is also accompanied by less favourable changes in lifestyle. Improved technology leads to increasing sedentary behaviours during activities of leisure, in the workplace, and due to better modes of transport (Oggioni et al., 2014; Wagner and Brath, 2012). Furthermore, populations living in urban settings tend to consume more processed foods, which are less nutritious and contain more calories (Poutanen, 2012).

From an objective perspective, it seems apparent that both human and animal populations are similarly exposed to, and at risk of health deteriorations as a result of lifestyle factors. The characteristics of a captive scenario in which animals become overweight or obese due to reduced physical activity, and unaccustomed feeding practices mimic obesity and sedentary behaviours experienced by human populations, exposed to urban lifestyles.

Based on this premise, the current research project aimed to determine if, like humans and domestic cats, reduced physical activity and obesity in captive lions places these animals at risk for metabolic disturbance, and potential pre-indicators for insulin-resistance (changes in muscle composition, morphology and metabolism). Although there are numerous reports of TIID presenting in domestic cats (Gostelow et al., 2014; Gottlieb et al., 2015; Lutz and Rand, 1995) to

date there have been no reported cases of insulin-resistance or TIID in captive lions. The evidence from this research study is insufficient to suggest or refute any indication of potential metabolic disturbance within the captive group. Although there appears to be some evidence which points toward a more glycolytic profile in comparison to the wild lions, this cannot be conclusively stated without further research.

Alternatively, although the captive lions present with obesity and considerably lower levels of physical activity, they may be presenting with what's termed "healthy obese". In humans, the phenomenon of long term obesity and even morbid obesity, with no signs of ill health or metabolic syndrome symptoms has been termed metabolically healthy obese (Hwang et al., 2012; Munoz-Garach et al., 2016). The aetiology of healthy obesity is unclear, however genetics (Berezina et al., 2015) and lifestyle factors such as diet and physical activity (Stelmach-Mardas and Walkowiak, 2016) have been speculated as key role players (Navarro et al., 2015). Currently, there is no research that has looked at genetic factors between wild and domestic felines. However, there is evidence for a considerable difference in their diets. Lions in captivity are primarily fed chicken, beef and horse carcasses, with an extremely low percentage contribution of carbohydrate to their total energy intake (Lorenzo et al., 2014; Nistor et al., 2013). Domestic cats, on the other hand, are fed pelleted diets which comprise up to 50% carbohydrate (Slingerland et al., 2009). Given that diabetic domestic cats are able to revert to a non-diabetic state, when their diets are altered to contain a very low carbohydrate content (Bennett et al., 2006; Lien and Huang, 2012) it could be possible that the captive lion diet may be playing a critical role in maintaining their metabolic health. Exploration into the effect of diet, and more specifically, a low carbohydrate diet, on the metabolic health of these lions, and within other species, deserves further investigation.

In summary, this study has provided an important stepping-stone in wild and captive felid research, and laid the foundation for subsequent studies to explore the health of various animal species living in captivity. Furthermore, this research has provided valuable insight into possible key factors, which may be fundamental in the prevention and/or control of chronic metabolic disease, such as insulin-resistance and type II diabetes in the human population.

CHAPTER 5

5. LIMITATIONS AND FUTURE DIRECTION

One limitation of this study is the relatively small sample size of the wild lion group. Lions are listed as vulnerable animals on the IUCN Red List of Threatened Species for 2014, and therefore muscle samples are entirely opportunistic. A greater sample size, particularly for the wild lions, would have strengthened the analysis, and any potential differences which may exist between the wild and captive lions. Furthermore, increasing the sample size would increase the statistical power of the analyses, and thus reduce the risk of potential type I and II errors.

Another limitation of the study was the large variation within each group which was evident in the majority of the outcome measures. This may have been as a result of using both male and female lions in the study, and thus highlights the importance of controlling for large standard deviations through a more homogenous sampling group.

An unforeseen limitation of the study was made evident through the difference in fibre composition observed between male and female lions. As only the female lions appear to express type IIX fibres, only three animals from the wild group could be compared with the captive group in terms of type IIX fibre composition and cross-sectional area. The restricted study population may have therefore under-powered the comparison between the lion groups, and potentially reduced the generalizability of the results to the broader captive lion population.

Ideally, the analysis of blood parameters such as plasma glucose and insulin levels would have been useful in providing a better picture of the metabolic status of the captive lions. However, as a result of a stress response when the animal is anaesthetised, these parameters fluctuate considerably, and therefore cannot be used to accurately indicate potential metabolic disturbance and/or possible insulin-resistance. As an alternative to blood analysis, a follow-on study should investigate the insulin signalling pathway at the level of the muscle, as this is not affected by changes in the immediate stress response, and thus fluctuations in blood parameters. This could be accomplished through the expression of the IRS-1 protein, to determine if the metabolic health of the captive lions is compromised in comparison to the wild lions. Furthermore, future work should examine mitochondrial function in the captive lions, through high resolution respirometry, in order to assess if any irregularities are present in the mitochondrial pathways.

APPENDIX A

SECTION 1

IMMUNOHISTOCHEMISTRY - METHODOLOGY

Immunohistochemistry techniques were adapted from Fry *et al.*, 2015 with modifications.

Protocol

Serial cross-sections (8µm) were cut on a Leica portable cryostat at -22°C. Sections were then fixed in acetone for 2min, followed by air drying. Sections were hydrated in 0.15M phosphate buffer saline (PBS) and incubated (50µl per section) with 5% BSA in a humidifying box, for 1 hour at room temperature. Succeeding the blocking step, sections were incubated with 1:50 primary antibodies (BA-D5 and 2F7) in a humidifying box overnight at 4°C. The following day the slides were washed twice in PBS buffer and incubated with 1:250 secondary antibodies (AMCA and Alexa Fluor) in a humidifying box at room temperature for 2 hours. For all primary and secondary antibodies, a volume of 50µl was pipetted for each section. All slides were mounted in moviol medium, and stored at 4°C until viewing.

All slides were viewed using a Nikon Eclipse i80 fluorescent microscope and images captured at 10x magnification using a digital camera (Canon EOS 650D). Fibre types were assessed by colour differentiation according to the fluorescent-tagged secondary antibody, and Image J pre-calibrated software was used to measure the cross-sectional area of each fibre. A minimum of 20 fibres was set as the threshold for fibre-type identification and cross-sectional area measurements.

0.15M Phosphate Buffer Saline (PBS) solution - pH 7.4.

Chemical	Amount	Adjust the pH with HCl or NaOH to 7.4 and make-up to 1L
NaCl	8 g	
Na ₂ HPO ₄ anhydrous	1.15 g	
KCl anhydrous	0.2 g	
KH ₂ PO ₄ anhydrous	0.2 g	

SECTION 2

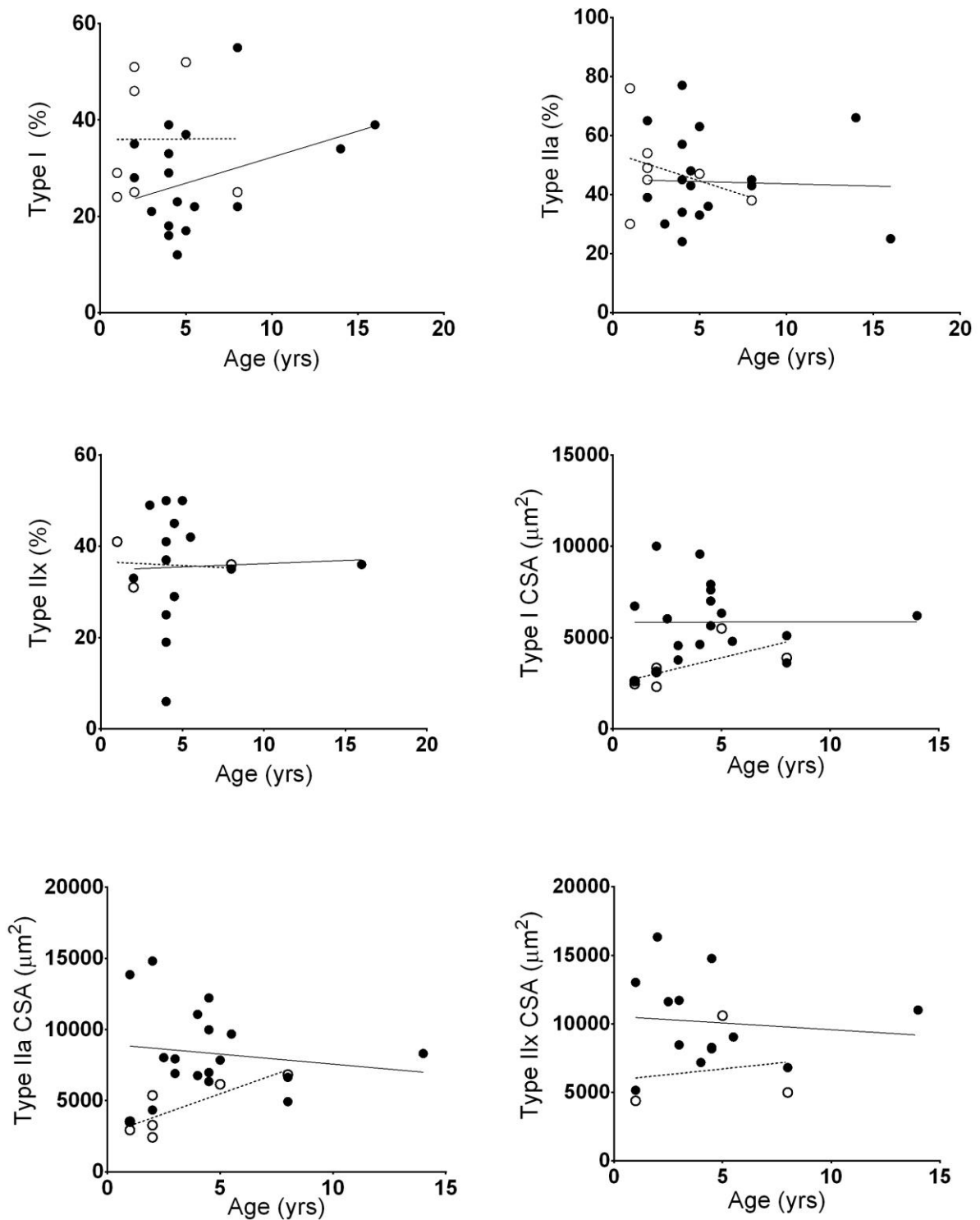


Figure 10: Scatter plots of age and each outcome variable. Wild lions are depicted as open circles and the dotted regression line, with the captive lions as closed circles and the solid regression line.

Table 2 - Linear regression for age as a confounder. Each outcome variable was plotted against age, and the p value computed to determine if the slope was significantly non-zero. Significance was set at $p < 0.05$. P values in bold were identified as significant.

	Wild Lions	Captive Lions
Type I %	p = 0.99	p = 0.13
Type IIA %	p = 0.46	p = 0.89
Type IIX %	p = 0.92	p = 0.90
Type I CSA	p = 0.10	p = 0.99
Type IIA CSA	p = 0.02	p = 0.58
Type IIX CSA	p = 0.89	p = 0.74

When groups were analysed separately, there was only an effect of age in the wild group with regards to type IIA CSA. Furthermore, when the groups were pooled, age was not found to effect any of the outcome variables.

Table 3 - Fibre type composition (%) of the *Triceps brachii* in the captive and wild lions. Values are expressed as mean \pm standard deviation. The range for each group is in parentheses.

Fibre Type (%)	Captive (n=18)			Wild (n=7)		
	Male (n = 8)	Female (n = 10)	Pooled	Male (n = 4)	Female (n = 3)	Pooled
Type I	32 \pm 12.4	24 \pm 8.9	28 \pm 11.0 (12-55)	43 \pm 16.2	26 \pm 2.2	36 \pm 13.0 (24-52)
Type IIA	54 \pm 15.5	36 \pm 11.3	44 \pm 15.8 (24-77)	57 \pm 16.5	38 \pm 7.1	48 \pm 14.5 (30-76)
Type IIX	8 \pm 11.9	39 \pm 8.2	28 \pm 18.8 (0-50)	0 \pm 0.6	36 \pm 5.1	16 \pm 19.3 (0-41)

There was no significant differences ($p > 0.05$) in the fibre type composition, between the male and female lions in the captive and wild groups.

APPENDIX B

SECTION 1

Homogenising Buffer Solution - pH 7.30.

Stocks	[Stocks]	Mr
A - KH ₂ PO ₄ anhydrous	0.1 M	Dissolve 0.68 g in 50 ml dH ₂ O
B - K ₂ HPO ₄ anhydrous	0.1 M	Dissolve 0.87g in 50 ml dH ₂ O

Add 30 ml of Buffer B (homogenising buffer) to a beaker and use buffer A to adjust the pH to 7.30. Store the buffer at 4°C short term, and -20°C for long term.

Bradford Protein Assay

The assay method used was developed by Bradford 1987. The principle of the method relies on Coomassie G250 dye which binds protein and results in a colour change. This change in colour alters the absorbance from 465 nm to 595 nm. By generating a standard curve using concentrations of a known substance, in this case BSA. The protein concentration of an unknown sample can be quantified. The microplate-based method was utilised for this study.

Protocol:

Bradford reagent composition and protocol.

Note - The reagent is a concentrate and thus needs to be diluted 1:4 before use.

Chemical	Volume	Add the ethanol to the Coomassie powder. Add the phosphoric acid slowly and stir with a glass rod. Fill up to 500 ml with dH ₂ O. Filter the reagent through Whatmann no. 1 paper.
Coomassie G250 powder	0.5 g	
95% ethanol	125 ml	
H ₃ PO ₄	250 ml	
dH ₂ O	500 ml	

Standard Curve

Make-up a 0.5M BSA solution in an Eppendorf, and pipette the following volumes into each well of the microplate.

Well	0.5 M (μ l)	dH ₂ O (μ l)	Bradford Reagent	[Final] g/l
Blank	-	10 μ l	250 μ l	0.0
Standard 1	2 μ l	8 μ l	250 μ l	0.1
Standard 2	4 μ l	6 μ l	250 μ l	0.2
Standard 3	6 μ l	4 μ l	250 μ l	0.3
Standard 4	8 μ l	2 μ l	250 μ l	0.4
Standard 5	10 μ l	-	250 μ l	0.5

SECTION 2

MHC Content – Gel electrophoresis technique:

Gel electrophoresis was used to determine MHC isoform content in the homogenate samples of the wild and captive lions. No difference was found between the proportion of MHC I, IIa or IIx fibres between groups.

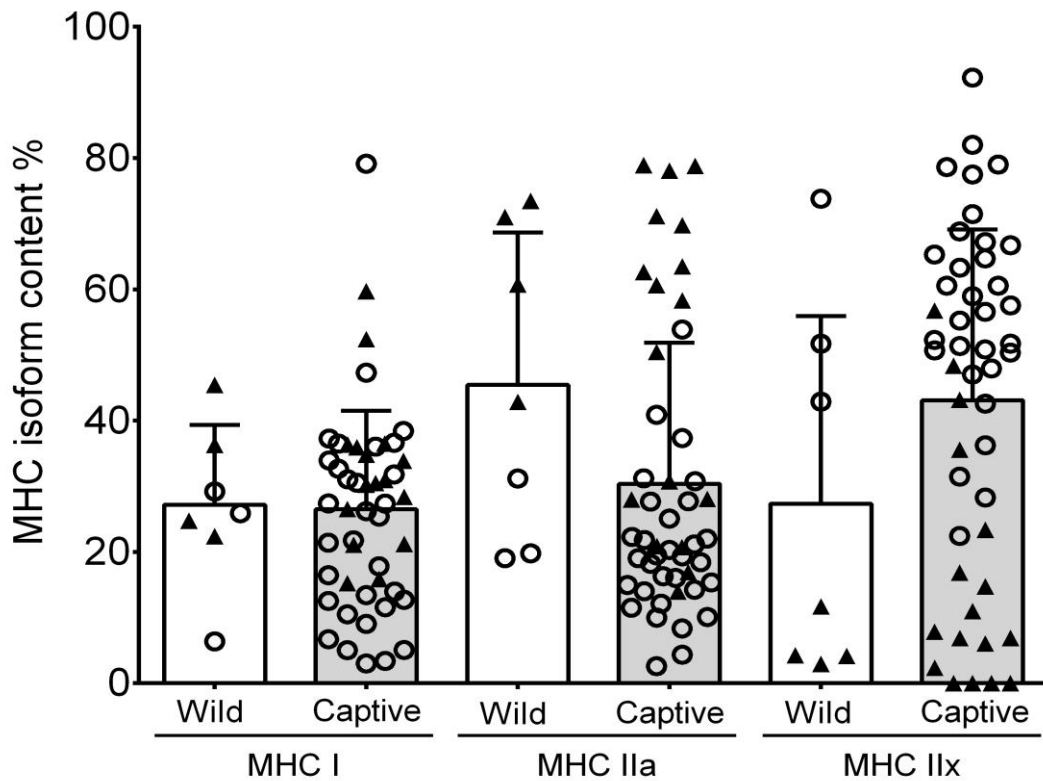


Figure 11: MHC isoform content of the wild and captive lions using gel electrophoresis methods. Male lions are represented by the closed triangles, and the female lions by the open circles. The bars represent means and standard deviations.

Enzyme Assays:

Table 5 - Enzyme activities ($\mu\text{mol}/\text{min}/\text{g}$ protein) for the captive and wild lions. Values are provided in mean and standard deviations.

	Captive (n=48)			Wild (n=7)		
	Male (n=17)	Female (n= 31)	Pooled	Male (n=4)	Female (n=3)	Pooled
PFK	462 \pm 219	599 \pm 311	551 \pm 287	296 \pm 127	394 \pm 115	338 \pm 123 *
LDH	1736 \pm 384	1889 \pm 457	1833 \pm 433	1730 \pm 299	1931 \pm 487	1816 \pm 368
3-HAD	15 \pm 6	12 \pm 3	13 \pm 5	16 \pm 3	11 \pm 2	14 \pm 3
CS	11 \pm 3	10 \pm 3	11 \pm 3	15 \pm 3	13 \pm 1	14 \pm 2 **

* Different between groups, $p < 0.05$, ** different between groups, $p < 0.005$

Nicotinamide adenine dinucleotide (NADH) Standard

With each of the enzyme activity assays, an NADH standard curve is constructed using a standardised NADH dilution series. In order for this curve to be accurate, it is critical that the exact concentration of the standard NADH solution is known. The NADH solution is therefore standardised by measuring the absorbance of the solution in a spectrophotometer and applying the Beer-Lambert law ($A = \epsilon cl$). With the measured absorbance, known molar absorptivity of NADH, and known path length of 1 cm, one can determine the exact concentration in the standard solution. A quartz cuvette is used in the standardisation procedure as it allows for transmission of the ultra violet light (340 nm) which is used to excite the NADH molecules.

NADH concentration determination:

Reagent	Molecular Weight	Required Amount	Volume dH ₂ O	Concentration
Na ₂ CO ₃	105.99	0.848 g	100 ml	0.08 M
NaHCO ₃	84.01	0.168 g		0.02 M
Add both reagents into one beaker and make-up to 100 ml with dH ₂ O				

Procedure:

1. Dissolve approximately 36 mg of NADH (1 vial) in 10 ml of above buffer.
2. Heat NADH solution for 10 minutes in a water bath set at 60 °C to destroy any NAD⁺.
3. Add 1 ml 0.1 M Tris buffer pH 8.0 to at least 3 quartz cuvettes.
4. Set the wavelength to 340 nm on the spectrophotometer. Place a cuvette into the spectrophotometer and blank it.
5. Pipette 20 µl NADH solution into the cuvette
6. Using parafilm, cover the top of the cuvette, invert a few times and then read absorbance.
7. Repeat with remaining cuvettes.

Calculation:

$$\frac{\text{NADH abs}}{6270} \times \frac{1020 \times 10^{-6}}{20 \times 10^{-6}} = \text{mol/l NADH}$$

Generating NADH standard curve:

The excitation wavelength was set to 340 nm and emission wavelength to 460 nm on the fluorometer. The background fluorescence of each well was read and the original NADH standard was diluted 11 times with dH₂O. The volumes in the below table were then pipetted in duplicate into each well of a micro-plate.

Well Row	NADH (μl)
A	0
B	2
C	4
D	6
E	8
F	10
G	15

300 μl of 0.1M Tris buffer was added to each well and the fluorescence read. The background fluorescence was then subtracted from the respective NADH fluorescence, and the blank value (row A) subtracted from the rest of the measurements.

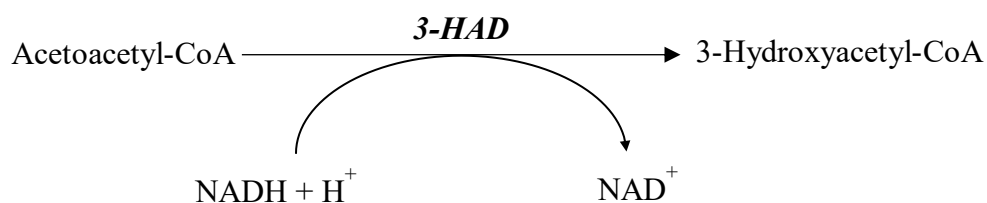
The NADH concentrations in each well was calculated as follows:

- Divide original NADH concentration by 11 to account for dilution.
- Use $C_1V_1 = C_2V_2$ to calculate final concentration in each well.

Plot a standard curve of fluorescence vs. NADH concentration, and determine the slope. The slope gives fluorescence/μM NADH and is used in calculation of enzyme activity. Note: curve should be linear.

3-Hydroxy-Acetyl-CoA Dehydrogenase (3-HAD) Methodology

Reaction:



Principle:

With fluorometry one measures fluorescence, which comes from reduced forms of NAD and NADP. The reaction can either in itself cause the increase or decrease in e.g. $\text{NADH} + \text{H}^+$, or be coupled to such a reaction, as in this case. During analysis, the fluorescence is measured at known time intervals and difference per minute (Δ) is calculated. Knowing the weight of the sample and its dilution, the enzyme activity, expressed as mmol/min/kg substrate turnover, is calculated. * starts the reaction.

Chemicals:

Trizma-Base	Sigma T-1503
Ethylenediaminetetra-acetic acid (EDTA)	Sigma ED 2 SS
Acetoacetyl-CoA	Sigma A-1625
NADH	Roche 107 735

Stock solutions (store at $-40\text{ }^{\circ}\text{C}$):

Chemical	Concentration	Molecular Weight
Tris – Buffer	1 mol/l, pH 8.0 - 12.11g / 100 ml dH ₂ O; adjust pH	121.1
EDTA	0.1 mol/l - 3.722g / 100 ml dH ₂ O	372.2
NADH	0.1 mol/l - 0.0709 g / 1ml dH ₂ O	709.4
Acetoacetyl-CoA	1 mmol/l	971.6

Reagent solution

		25 ml	50 ml	120ml	[Final]
Tris buffer	1.0 mol/l	1.25 ml	2.5 ml	6 ml	50 mM
EDTA	0.1 mol/l	1 ml	2 ml	4.8 ml	4 mM
NADH	0.1 mol/l	7.5 μ l	15 μ l	36 μ l	30 μ M

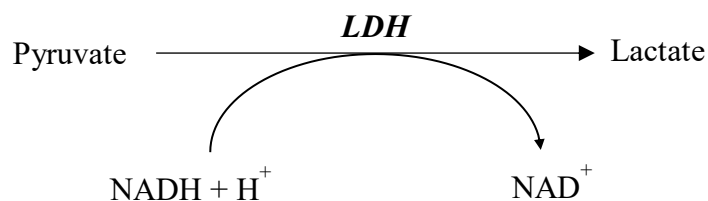
Fill up with dH₂O to required volume, and check pH 8.0

Calculation:

$$\frac{\Delta}{\text{min}} \times \frac{\text{Standard NADH} \times 10^{-7} \text{ mol/l} \times 500 \mu\text{l} \times 0.260 \times 10^{-3} \text{ l}}{1.234 \times 10^{-6} \text{ kg} \times 5 \mu\text{l}}$$
$$= 10^{-4} \text{ mol/kg/min} = 10^{-1} \text{ mmol/kg/min.}$$

Lactate Dehydrogenase Methodology

Reaction:



Principle:

With fluorometry one measures fluorescence which comes from reduced forms of NAD and NADP. The reaction can either in itself cause the increase or decrease in e.g. $\text{NADH} + \text{H}^+$, or be coupled to such a reaction, as in this case. During analysis, the fluorescence is measured at known time intervals and difference per minute (Δ) is calculated. Knowing the weight of the sample and its dilution, the enzyme activity, expressed as mmol/min/kg substrate turnover, is calculated.

Chemicals:

Trizma-Base	Sigma T-1503
Ethylenediaminetetra acetic acid (EDTA)	Sigma ED 2 SS
NADH	Roche 107 735
Pyruvate	Roche 128 147

Stock solutions: (Store at -40°C):

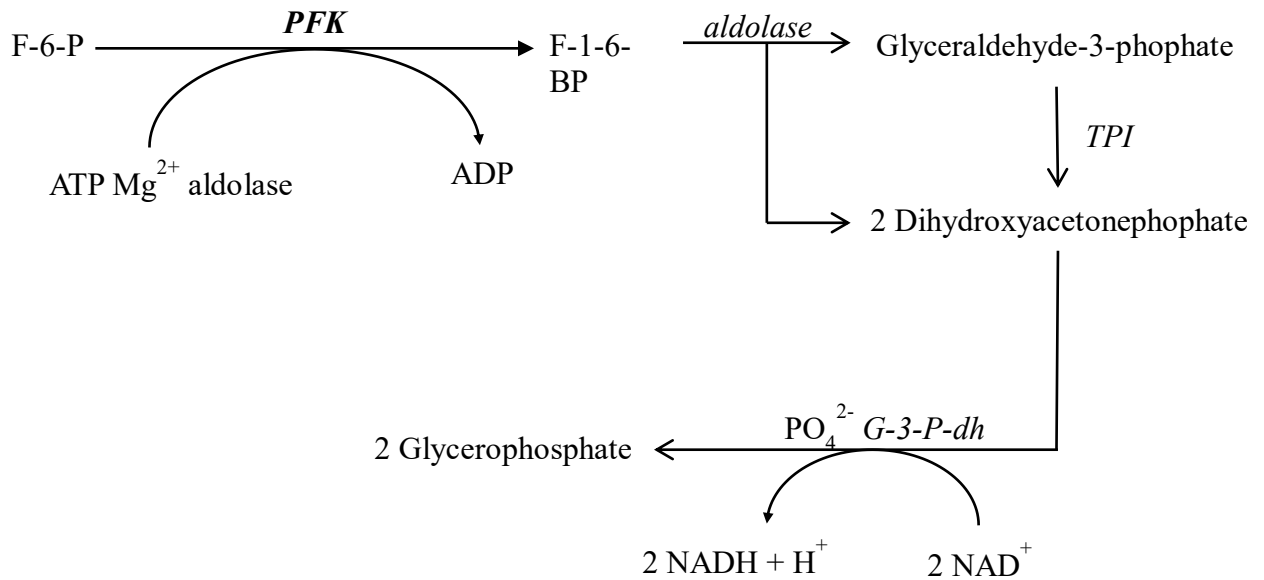
Chemical	Concentration	Molecular Weight
Tris – Buffer	1 mol/l, pH 8.0 - 12.11 g / 100 ml dH ₂ O; adjust pH	121.1
EDTA	0.1 mol/l - 3.722g / 100 ml dH ₂ O	372.2
NADH	0.1 mol/l - 0.0709 g / 1ml dH ₂ O	709.4
Pyruvate	0.1 mol/l - 22 mg/ 2ml dH ₂ O	110
TEA	0.05 mol/l - 1.857 g / 200 ml dH ₂ O	185.7

Reagent solution

		25 ml	50 ml	120 ml	[Final]
Tris buffer	1.0 mol/l	1.25 ml	2.5 ml	6 ml	50 mM
EDTA	0.1 mol/l	1 ml	2 ml	4.8 ml	4 mM
NADH	0.1 mol/l	7.5 μ l	15 μ l	36 μ l	30 μ M
Fill up with dH ₂ O to required volume, and adjust pH to 8.00					

Phosphofructokinase Methodology

Reaction:



Principle:

With fluorometry one measures fluorescence which comes from reduced forms of NAD and NADP. The reaction can either in itself cause the increase or decrease in e.g. NADH + H⁺, or be coupled to such a reaction, as in this case. During analysis, the fluorescence is measured at known time intervals and difference per minute (Δ) is calculated. Knowing the weight of the sample and its dilution, the enzyme activity, expressed as mmol/min/kg substrate turnover, is calculated. Please note that 2 molecules of NAD⁺ are formed and that you must divide the activity by 2. The sample will start the reaction.

Chemicals:

TRIS-Base	Sigma T-1503
ATP	Sigma A-5394
5'AMP	Sigma A-1877
MgCl ₂	Merck 5833
Na ₂ HPO ₄	Merck 6580
Fructose-6-phosphate – Na ₂	Sigma F-3627
NADH	Roche 107 735
β-Mercaptoethanol	Sigma M-6250
Aldolase	Roche 102 644
Triosphosphate-isomeras (TPI)	Roche 109 754
Glyceraldehyde-3-phosphate-dehydrogenase	Roche 127 752
Bovine serum albumin (BSA)	Sigma A-2153

Stock solutions: (Store at -20° C):

Chemical	Concentration	Molecular Weight
Tris – Buffer	1 mol/l, pH 8.0 - 12.11g / 100 ml dH ₂ O; adjust pH	121.1
ATP	0.1 mol/l - 0.1102 g / 2 ml dH ₂ O	551.1
NADH	0.1 mol/l - 0.0709 g / 1ml dH ₂ O	709.4
AMP	0.1 mol/l - 0.0694 g / 2 ml dH ₂ O	347.2
MgCl ₂	1 mol/l - 2.0331 g / 10 ml dH ₂ O	203.31
Na ₂ HPO ₄	0.25 mol/l - 2.225 g / 50 ml dH ₂ O	177.99
F-6-P	0.1 mol/l - 0.068 g / 2 ml dH ₂ O	340.1
BSA	10% - 1g/10 ml dH ₂ O	

Reagent solution

		25 ml	50 ml	120ml	[Final]
TRIS	1 mol/l; pH 8.0	1.25ml	2.5 ml	6 ml	0.05 M
ATP	0.1 mol/l	250 μ l	500 μ l	1200 μ l	1 mM
AMP	0.1 mol/l	250 μ l	500 μ l	1200 μ l	1 mM
MgCl ₂	1 mol/l	50 μ l	100 μ l	240 μ l	2 mM
Na ₂ HPO ₄	0.25 mol/l	125 μ l	250 μ l	600 μ l	1.25 mM
F-6-P	0.1 mol/l	250 μ l	500 μ l	1200 μ l	1 mM
NADH	0.1 mol/l	2.5 μ l	5 μ l	12 μ l	10 μ M
BSA	10%	125 μ l	250 μ l	600 μ l	0.05 %
β -Mercaptoethanol	14.2 M	2 μ l	4 μ l	9.6 μ l	1 mM
Aldolase	20 mg/2ml	30 μ l	60 μ l	144 μ l	12 μ g/ml
TPI	2 mg/ml	38 μ l	75 μ l	180 μ l	3 μ g/ml
G-3-P-dh	10 mg/ml	20 μ l	40 μ l	96 μ l	10 μ g/ml
Fill up with dH ₂ O to		25 ml	50 ml	120 ml	
Check pH 7.60					

Calculation:

Eg: 1.234 mg sample homogenized in 500 μ l buffer.

$$\frac{\Delta}{\text{min}} \times \frac{\text{Standard NADH} \times 10^{-7} \text{ mol/l} \times 500 \mu\text{l} \times 0.260 \times 10^{-3} \text{ l}}{1.234 \times 10^{-6} \text{ kg} \times 5 \mu\text{l}}$$

$$= 10^{-4} \text{ mol/kg/min} = 10^{-1} \text{ mmol/kg/min.}$$

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