

Skeletal muscle composition in various breeds of domestic dogs (A comparative study)

Kathryn van Boom
VBMKAT001

Supervisor: Dr Tertius Kohn
Co-Supervisor: Prof Johan Schoeman

Thesis presented for the degree:
MSc (Med) in Exercise Science

University of Cape Town

May 2019

Division of Exercise Science and Sports Medicine,
Department of Human Biology,
University of Cape Town



The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

TABLE OF CONTENTS

DECLARATION.....	5
ACKNOWLEDGEMENTS	6
LIST OF KEY ABBREVIATIONS	7
ABSTRACT	8
CHAPTER ONE.....	9
1. LITERATURE REVIEW	9
1.1. What is the problem - an overview of the diabetes epidemic in humans and animals	9
1.2. Diabetes in humans.....	9
1.3. Type 2 Diabetes	10
1.4. The consequences and risk factors of diabetes in humans.....	11
1.5. Animal models of diabetes	12
1.6. Diabetes in dogs (<i>Canis lupus familiaris</i>)	14
1.7. Current diet of domestic dogs.....	15
1.8. The role of skeletal muscle	16
1.9. The skeletal muscle of dogs.....	20
1.10. Skeletal muscle fibre type as a risk factor for Type 2 Diabetes.....	22
1.11. Anticipated gain in knowledge	23
1.12. Aims and Hypothesis.....	24
CHAPTER TWO.....	25
2. DESCRIPTION OF DOG BREEDS	25
2.1. Introduction	25
2.2. Classification of the different breeds.....	26
2.3. Limitations of the study sample.....	31
CHAPTER THREE.....	32
3. SKELETAL MUSCLE MORPHOLOGY.....	32
3.1. Background.....	32
3.2. Methodology.....	32
3.3. Results	34
3.3. Discussion.....	39
CHAPTER FOUR	43
4. SKELETAL MUSCLE METABOLISM	43
4.1. Background.....	43
4.2. Methodology.....	43
4.3. Results	44

4.4. Discussion.....	47
CHAPTER FIVE.....	51
5. CONCLUSION	51
APPENDIX A	54
APPENDIX B.....	55
REFERENCES	61

DECLARATION

I, *Kathryn van Boom*, hereby declare that the work on which this dissertation/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 to be submitted for another degree in this or any other university.

I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate

Signature:

Date: 28/05/2019

ACKNOWLEDGEMENTS

There are many people I need to thank for their role in this research project. Firstly my Supervisor, Dr Tertius Kohn, for all his help, mentorship and encouragement throughout the past two years. His passion and enthusiasm for this work is evident in every conversation. With his guidance and support, Tertius allowed me to make this project my own. There were lots of ups and downs, but Tertius always remained positive and together we would plan the way forward. Under his mentorship, I have learnt so many new skills and have had countless opportunities to grow and develop in this field.

Thank you to my Co-Supervisor, Prof Johan Schoeman, for his initial assistance in writing the proposal and for taking the time to review and comment on this thesis. Thank you to Prof Amelia Goddard, Dr Malan van Zyl and Dr Chad Berman for their assistance in obtaining ethical approval for this project from the University of Cape Town and the University of Pretoria. Thank you to Paula Saner, from UCT, who assisted us in obtaining a DAFF Section 20 permit required to do research.

A special thank you to Dr David Grant and his team at Rondebosch Veterinary Hospital for their assistance in sample collection. Dr Grant was also instrumental in helping me draw up the sample collection procedure which allowed me to complete the animal ethics application. Thank you for your time and your enthusiasm for this study.

I cannot thank Dr Johan Steyl enough for coming onto this study, at the last minute, and collecting samples at the Onderstepoort Veterinary Hospital. Without Dr Steyl's involvement this study would not have been possible. I am so appreciative of the time you took to collect the samples and the amount you were able to collect in such a short time. Thank you again.

I would like to express my gratitude to the National Research Foundation (NRF) and the University of Cape Town for providing funding for this MSc degree.

A very big thank you to my Dad, who is my greatest fan and supporter. Thank you for constantly taking an interest in my work and always encouraging me to work hard and live out my dream. I am who I am because of everything that you are. Lastly, no acknowledgement section would be complete without thanking God who created this beautiful world for us to explore.

LIST OF KEY ABBREVIATIONS

ATP	adenosine triphosphate
BSA	bovine serum albumin
CK	creatine kinase
CS	citrate synthase
CSA	cross-sectional area
FCI	Fédération Cynologique Internationale (World Canine Organisation)
3-HAD	3-hydroxyacyl-CoA dehydrogenase
IHC	immunohistochemistry
IRS-1	insulin receptor substrate-1
LDH	lactate dehydrogenase
MHC	myosin heavy chain
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
PBS	phosphate buffer saline
TB	<i>Triceps brachii</i>
T2D	type 2 diabetes
μl	microlitre
μm ²	square micrometre
μmol	micromole
g/l	grams per litre
M	molar (mol per litre)
mg	milligrams
ml	millilitres
VL	<i>Vastus lateralis</i>

ABSTRACT

The rising rate of insulin resistance and type 2 diabetes in humans over the past two decades have been linked to increasing rates of obesity, aging and urbanisation. A similar pattern is occurring in domestic animals, specifically cats and dogs. Skeletal muscle is a vital organ in the regulation of blood glucose. Its composition in terms of muscle fibre type, metabolism and contractility can differ substantially between species, but is poorly studied in the domestic dog, in particular the various breeds. It was hypothesised that insulin resistance and type 2 diabetes may be associated with muscle fibre type, in particular, muscle with a low type I fibre content being a predisposing factor. Therefore, the aims of this study were to investigate the skeletal muscle fibre composition and metabolic profile in the *Triceps brachii* (TB) and *Vastus lateralis* (VL) of 16 breeds of domestic dogs (*Canis lupus familiaris*). A secondary aim was to correlate the skeletal muscle composition with breeds reported as having a high incidence of diabetes. Skeletal muscle samples were collected *post mortem* from the TB and VL of 38 dogs from different breeds, age and sex, and analysed for fibre type composition, fibre size, oxidative and glycolytic metabolic capacity (citrate synthase (CS), 3-hydroxyacetyl co A dehydrogenase (3-HAD), creatine kinase (CK) and lactate dehydrogenase (LDH) enzyme activities). There was no significant difference between the TB and VL in any of the measurements. Type IIA was the predominant fibre type for both muscle groups (TB: 43%; VL: 44%) followed by type I (TB: 33%; VL: 38%) and type IIX (TB: 24%; VL: 18%). The cross sectional area (CSA) of the fibres were all smaller compared to humans and other wild animals. Surprisingly, there was no difference in the CSA between the fibres types and muscle groups: Type I: TB: 1740 μm^2 ; VL: 1712 μm^2 , Type IIA: TB: 1690 μm^2 ; VL: 1720 μm^2 , Type IIX TB: 1726 μm^2 ; VL: 1791 μm^2). Metabolically, the muscle of the dog displayed a high oxidative capacity with high activities (all activities in $\mu\text{mol}/\text{min}/\text{g}$ protein) for CS (TB: 61; VL: 49) and 3-HAD (TB: 53; VL: 46). Lower CK (TB: 6115; VL: 6279) and higher LDH (TB: 1550; VL: 1478) activities than humans indicated a lower and higher flux through the high energy phosphate and glycolytic pathway, respectively. These results indicate that the dog has a predominance of type IIA fibres along with a higher oxidative capacity. There appears to be no pattern in fibre type profile that could be associated with a predisposition of a specific breed to insulin resistance and diabetes, although many of the breeds with a known risk did not form part of the study sample. This is the first study to characterise the skeletal muscle composition of a large population of dogs (16 breeds), but the association of breed to diabetes was not found. Future studies should include younger and more animals, as well as a diabetic population of dogs.

CHAPTER ONE

1. LITERATURE REVIEW

1.1. What is the problem - an overview of the diabetes epidemic in humans and animals

The rising rates of obesity, along with aging and urbanization have been linked to the increasing rates of diabetes in the human population (Wagner and Brath, 2012). According to the World Health Organisation (2016a), the prevalence of diabetes in adults have increased from 4.7% in 1980 to 8.5% in 2014. In 2015, there were 422 million people living with diabetes while 1.5 and 2.2 million deaths in 2012 were directly attributed to diabetes and high blood glucose, respectively. The International Diabetes Federation Atlas (IDF, 2017) predicted that the number of people living with diabetes globally will increase from 424.9 million in 2017 to a staggering 628.6 million in 2045.

A similar pattern was observed in the domestic animal populations, specifically in relation to the increasing rates of obesity (Osto and Lutz, 2015). In an Australian study investigating obesity in 2661 dogs, 34% of the dogs were overweight and 8% were obese as determined by veterinarians using the Hill's weight guide chart for dogs (McGreevy et al., 2005). In a 2015 survey looking at pet obesity in the United States, an estimated 20% of dogs and 28% of cats were obese (APOP, 2015). Both cats and dogs have varying rates of diabetes incidence and prevalence as well as risk factors for the disease, with diabetes being a relatively common endocrine disease in cats with a prevalence of 1 in 230 in a UK study (McCann et al., 2007). Diabetes in dogs is not as prevalent. From a cohort of 180 000 dogs investigated in Sweden, only 1 dog was diagnosed with diabetes for every 770 dogs, with different incident rates for different breeds (Fall et al., 2007).

Although the pancreas is responsible for insulin production and secretion in response to high blood glucose, it is the target of this hormone that is essential in the uptake of excess glucose. Skeletal muscle is a vital organ in the regulation of glucose in the body. Importantly, the slow twitch oxidative type I fibres have been attributed to higher levels of insulin sensitivity as opposed to the fast twitch type II fibres (Duan et al., 2017, Lefaucheur, 2010). Very few studies (with contrasting results) have investigated the fibre type and metabolic properties of skeletal muscle from various domestic dog breeds. Therefore, knowledge of these muscle characteristics would aid in our understanding of the onset of diabetes in dogs and humans.

1.2. Diabetes in humans

Diabetes Mellitus (or simply diabetes) is a non-communicable disease characterized by hyperglycemia and results from defects in insulin secretion or insulin sensitivity of target tissues,

such as skeletal muscle (Frayn, 2009, Gilor et al., 2016). There are two major forms of diabetes. Type 1 diabetes (T1D) is a condition that results in absolute insulin deficiency through the destruction of insulin secreting pancreatic β -cells (Daneman, 2006). This usually occurs through cell-mediated autoimmune attack on the β -cells. However, there is a form of T1D in which destruction of β -cells are not due to autoimmune attack, is less frequent, has no known cause and occurs primarily in people of African or Asian ancestry (Abiru et al., 2002, Daneman, 2006).

Type 2 Diabetes (T2D) is characterised by defects in insulin secretion due to β -cell dysfunction, as well as the concomitant presence of insulin resistance in tissue (Frayn, 2009, Stumvoll et al., 2005). Insulin resistance is the inability of insulin, at normal physiological concentrations, to exert its effect on insulin-sensitive tissue, such as skeletal muscle and liver (Frayn, 2009). The body compensates by increasing pancreatic β -cell function, resulting in increased insulin secretion. Over time, blood glucose concentration gradually increases as the pancreas fails to maintain the required increased secretion of insulin. Ultimately, glucose toxicity may lead to β -cell dysfunction (Stumvoll et al., 2003). Additionally, insulin resistance may progress to T2D if certain environmental and genetic risk factors, such as obesity, are present (American Diabetes, 2010, Stumvoll et al., 2005).

In humans, diabetes is defined as a fasting plasma glucose concentration above 7 mmol/L or a post-prandial concentration above 11.1 mmol/L, two hours after oral glucose administration (American Diabetes, 2010). Glycated haemoglobin (HbA1c) is a measure of average glucose concentrations over a period of three months and therefore provides a better representation of long term glucose concentrations (Frayn, 2009). An HbA1c above 6.5% is indicative of diabetes (American Diabetes, 2010).

1.3. Type 2 Diabetes

T2D is primarily a lifestyle disease characterised by high blood glucose concentrations and therefore it is important to understand the mechanisms of glucose regulation in the body. Insulin is a peptide hormone secreted from the β -cells of the pancreas in response to hyperglycemia. Hyperglycemia occurs after the ingestion of carbohydrates, which are then broken down into the various monosaccharides, predominantly glucose and fructose. Insulin promotes normal blood glucose concentration (between 3.9 and 5.5 mM) through cellular glucose uptake as well as by regulating carbohydrate, lipid and protein metabolism (Wilcox, 2005). Incidentally, glucagon secreted from the α -cells of the pancreas has the opposite effect, increasing blood glucose concentrations in response to hypoglycaemia.

Insulin mediates its action by binding to the extracellular component of insulin receptors, in response to glucose, which subsequently triggers a cascade of signalling pathways (Wilcox, 2005). This

includes the phosphorylation of the tyrosine residues on intracellular substrate proteins called insulin response substrate (IRS). There are four forms of IRS, with IRS-1 predominantly found in skeletal muscle. Phosphatidylinositol-3 kinase (PI3K) is activated by the phosphorylation of IRS-1. PI3K, in turn, promotes the translocation of glucose transporter 4 proteins (GLUT4) to the cell membrane via the phosphorylation of other proteins (such as Akt and PKC). Of these, GLUT4 is the major transporter protein in adipose tissue and skeletal muscle. The translocation of GLUT4 vesicles to the plasma membrane allows the uptake of glucose into cells. Importantly, skeletal muscle is the major tissue for glucose uptake, accounting for 60-70% of whole body insulin-mediated uptake (Smith, 2002). In addition, the liver and adipose tissue also contribute as sites of glucose uptake and storage.

Exercise, through muscle contraction, results in increased skeletal muscle glucose uptake (and can increase 16 fold). This occurs through various signalling pathways such as the increase in AMP, nitric oxide and calcium, which potentially converge at protein kinase C (PKC) phosphorylation (Ojuka and Goyaram, 2014). This leads to the translocation of GLUT4 to the muscle cell membrane and, therefore, the uptake of glucose into the cells. Once exercise stops, GLUT4 is returned to the cytosol through a process of endocytosis. Because of these two independent pathways (i.e. exercise and insulin secretion), frequent physical exercise is beneficial and prescribed for individuals with impaired glucose tolerance and insulin resistance (Dela et al., 2014, Ojuka and Goyaram, 2014). Low carbohydrate diets have also been shown to be effective in the management of diabetes and insulin resistance (Feinman et al., 2015). T2D is characterised by high blood glucose and, therefore, restriction of carbohydrate ingestion would lead to reduction in postprandial and overall glucose concentration and HbA1c (Accurso et al., 2008, Feinman et al., 2015). Ultimately, this would reduce the amount of insulin secreted by the pancreas in response to hyperglycemia and put less compensatory strain on the insulin-dependent signalling pathways.

As mentioned previously, insulin resistance is an inability of tissue (such as skeletal muscle and the liver) to respond normally to insulin and thus precedes T2D. In insulin resistant individuals, insulin signalling through IRS-1, PI3K and Akt is decreased, which ultimately leads to decreased GLUT4 translocation to the cell membrane and thus reduced insulin-mediated glucose uptake (DeFronzo and Tripathy, 2009). Consequently, the skeletal muscle protein content of IRS-1 and GLUT4 can act as markers of insulin sensitive tissue.

1.4. The consequences and risk factors of diabetes in humans

Diabetes increases the risk of cardiovascular morbidity and mortality (Resnick et al., 2001). There are both micro- and macrovascular complications associated with diabetes (Beckman et al., 2002). Some of the microvascular complications include nephropathy and retinopathy, while macrovascular

complications include diseases of the coronary arteries, peripheral arteries and carotid vessels. Importantly, hypertension, coronary artery disease and cerebrovascular disease occur more frequently in people with T2D compared to matched controls (Beckman et al., 2002). Visual impairment and blindness caused by diabetes occurs often in developed countries (Resnikoff et al., 2004).

There are many health implications of diabetes, which places a heavy economic burden on both patients and society. People with diabetes may require three times the health care resources compared to non-diabetics and diabetes care could make up 15% of a national healthcare budget (Wagner and Brath, 2012, Zhang et al., 2010). In 2017, an estimated \$726 billion was spent globally on diabetes-related healthcare for adults between the ages of 20 and 79 years (IDF, 2017). This diabetic epidemic has far-reaching consequences.

The rising rates of obesity, along with aging and urbanization have been linked to the diabetes epidemic with nearly 90% of T2D cases associated with obesity (Wagner and Brath, 2012). Obesity is defined as a body mass index of higher than 30kg/m² and in 2014, more than 600 million ($\pm 8.5\%$ of the total world population) adults worldwide were obese (WHO, 2016b). Genetic and environmental factors are important determinants of insulin resistance and β -cell dysfunction (Kahn et al., 2014). According to Kahn et al. (2014), the interaction between genes that affect adiposity and environmental factors (such as low energy expenditure, high caloric intake, nutrient composition, etc.) leads to obesity and associated insulin resistance. However, when genes for β -cell dysfunction are also present, the risk is greater that T2D will develop. In a study by Shai et al. (2006), it was found that the relative risk of diabetes is different between different ethnic groups with Asian, Hispanic and Black populations having a greater risk than white populations. Thus, different ethnicities are also a potential risk factor, but needs to be further investigated.

1.5. Animal models of diabetes

Historically, dogs have played a key role in our understanding of the pathophysiology and treatment of diabetes. In 1890, while studying the role of the pancreas in digestion, Von Mering found that the removal of the pancreas from a dog led to the presence of glucose in the urine (Mering and Minkowski, 1890). He concluded that some “factor” released from the pancreas (later called insulin) allows the body to utilise glucose. Later in the 1920's, a diabetic crossbreed by the name of Marjorie, was the first recipient of insulin treatment, which was the platform for future human treatments (Banting et al., 1922). Dogs are believed to have a form of diabetes similar to T1D, as dogs are dependent on insulin therapy to treat hyperglycemia (Kennedy et al., 2006, Nelson and Reusch, 2014). Secondly, the presence of autoantibodies against islet cells, insulin, pro-insulin, GAD5 and IA2 in dogs may indicate an immune-mediated component. Lastly, T1D in humans is strongly associated

with major histocompatibility complex class II polymorphisms, and associations have also been found between the canine dog leukocyte antigen and diabetes (Kennedy et al., 2006).

Cats are believed to develop a form of diabetes similar to human T2D, as they develop pancreatic islet amyloid deposits, which are a marker of T2D in humans (Hoenig, 2014, Hull et al., 2004). These depositions have yet to be discovered in dogs. In a study by Hoenig et al. (2000), they found that antibodies against β -cells and insulin were absent in healthy and diabetic cats. Therefore, cats do not experience autoimmune destruction, which is a characteristic of T1D.

Rats and mice are commonly used as animal models for T1D and T2D. In T2D rodent models, the characteristics of insulin resistance and β -cell failure are modelled (King, 2012). These include non-obese models such as hIAPP transgenic mice that express the human islet amyloid polypeptide (IAPP). Rodents do not naturally produce amyloid, which is an important aspect of T2D in humans (Matveyenko and Butler, 2006). However, there are many more rodent obese T2D models, which include monogenic models such as leptin deficient mice ($Lep^{ob/ob}$ mice) or polygenic models - the latter providing a better representation of the disease (King, 2012). Polygenic models include New Zealand obese (NZO) mice that are selectively bred to be leptin resistant and hyperinsulinemic, and the OLETF rat that is derived from a spontaneously diabetic rat – these rats have mild obesity and late onset hyperglycaemia.

There are many other rodent models for diabetes (transgenic or diet induced) as well as other non-rodent models to go along with the above mentioned cat and dog models (King, 2012, Osto and Lutz, 2015). Spontaneous T1D is relatively rare and unpredictable in large animal or non-rodent models. As a result, insulin resistance is induced through the surgical removal of the pancreas or the destruction of the pancreatic β -cells in these larger animal models (Osto and Lutz, 2015). T2D has been reported in several old world non-human primates which include macaque species, African green monkeys and baboons (Wagner et al., 2006). They develop similar clinical features and share similar risk factors (including obesity) as those observed in humans. Pigs have also been used as a model for T2D because of their phenotypic similarities to humans including cardiovascular anatomy and function, metabolism, lipoprotein profile, size, obesity tendency and omnivorous diets (Bellinger et al., 2006). Pig strains, such as the Yucatan minipig and Ossabaw pigs, have been developed to study mechanisms that lead to the development of diabetic complications (Bellinger et al., 2006).

Although rodent disease models are frequently used to study diabetes, the extrapolation of data obtained from these models to humans is relatively poor, primarily due to differences in environmental and physiological factors that promote obesity (Osto and Lutz, 2015). For example, human diets are more complex than the formulated rodent diets. Different rodent strains may also

lead to variability in metabolism (Lai et al., 2014). Rodents are also herbivorous. Due to the lack in understanding, there is a need to study alternative models such as dogs and cats, in which obesity occurs spontaneously, in order to broaden our knowledge of disease mechanisms. According to Osto and Lutz (2015), dogs and cats are good comparative models for human obesity and diabetes, because the clinical signs and genetic background of obesity, in terms of it being a polygenic disease with different genetic variations, are similar. They also share the same environment as humans, including the abundance of food, which may predispose them to similar environmental-based pathophysiology (Osto and Lutz, 2015).

It has already been proposed that the risk of acquiring T2D is different between the various human population groups (i.e. ethnicity) (Shai et al., 2006). The same has been observed for dogs, where the incidence of diabetes is higher in certain breeds (Fall et al., 2007). Therefore, studying obesity and T2D in cats and dogs can serve to (i) elucidate the mechanisms associated with acquiring T2D in these species and (ii) to gain a better understanding of the differences between different breeds, which may assist in elucidating the ethnic and, hence, genetic differences in humans. For the purpose of this thesis, only dogs will be discussed.

1.6. Diabetes in dogs (*Canis lupus familiaris*)

The diagnosis of diabetes in dogs is based on the presence of certain clinical signs and persistent hyperglycemia and glycosuria (Nelson and Reusch, 2014). The typical range of plasma glucose concentrations for diagnosis of diabetes is 180 - 220 mg/dL in dogs (Nelson and Reusch, 2014), which is equivalent to 10 - 12.2 mmol/L.

In a Swedish study that evaluated 180 000 insured dogs, 1 in every 770 dogs was diagnosed as being diabetic, but it appears the incident rates varied for the different breeds (Fall et al., 2007). The incidence was 1.33% in an Italian cohort of 186 dogs (Fracassi et al., 2004). There seems to be a disparity between European and American studies around which breeds are more predisposed to diabetes (Fall et al., 2007, Hoenig, 2014). It appears that Samoyeds, Swedish elkhound and lapphund, Fox terriers, Australian terriers, miniature and standard schnauzers, pugs and poodles are at a higher risk while American pitbull terriers, cocker spaniels, collies, golden retrievers, German shepherds and boxers have a lower risk for developing diabetes. Neutered, females and dogs with previous hyperadrenocorticism were also found to be at greater risk (Fall et al., 2007). Obesity has rarely been identified as a risk factor and there is presently no recognised association between obesity and diabetes in dogs (Rand et al., 2004).

Catchpole et al. (2008) proposed a diabetic classification system for dogs, independent of the human classification system, which focuses on the underlying causes of hyperglycemia. Primary insulin

deficiency diabetes (IDD) results from a lack of insulin production by the pancreas and is characterised by the progressive loss of β -cells. Primary insulin resistance diabetes (IRD) is a relative insulin deficiency that results from inadequate insulin function in insulin-sensitive tissues. IDD is the most common, with IRD usually progressing to IDD. Diabetes typically affects dogs between the ages of 5 to 12 years old (Davison et al., 2005) which is in contrast to human T1D, which commonly develop in childhood with a lower incidence in adulthood (Daneman, 2006). For convenience in this thesis, diabetes in dogs will either be referred to as diabetes or T2D.

1.7. Current diet of domestic dogs

1.7.1. Ancestral Diet of the wolf *Canis lupus*

In order to understand the current diet of domestic dogs, it is important to investigate the origins of these animals and the food preference of their ancestors. The dog (*Canis lupus familiaris*) belongs to the order Carnivora in which most animals rely on meat as their primary source of nutrition.

Dogs are believed to be the first species of animal to have been domesticated, but the exact details of this process are still unknown (Vilà and Leonard, 2001). Dogs possibly diverged from their ancestral species, the wolf *Canis lupus*, between 14 000 and 100 000 years ago. The ancestral wolf consumed a predominately meat diet although the dentition and habitats of the modern wolf are similar to jackals, and as such, more indicative of an omnivorous diet (Bradshaw, 2006). According to Bradshaw (2006), due to the long history of domestication and the wide diversity within the domestic dog species, it is challenging to generalise the feeding behaviour of these animals.

1.7.2. Modern Diet of the domestic dog *Canis lupis familiaris*

In a comprehensive study by Hewson-Hughes et al. (2013) on domestic adult dogs, the preference of dry and wet diets with varying protein, fat and carbohydrate composition was evaluated in different breeds. All these diets adhered to the minimum requirement of macronutrients, vitamins and minerals as stated in the US National Research Council guidelines (NRC., 2006). The study included five breeds of domestic dogs (papillon, miniature schnauzer, cocker spaniel, Labrador retriever and St Barnard). Interestingly, the target macronutrient composition was consistent between the breeds with 30%, 63% and 7% of the energy provided by protein, fat and carbohydrates, respectively. The high proportion of protein and fats may be associated with the hunting pattern of the ancestral wolf, which hunted in groups and therefore could kill larger prey with more protein and fat as opposed to the lone hunting style of the ancestral cat (Prothero, 1995). Another interesting finding by Hewson-Hughes et al. (2013) was the tendency of all five breeds to over-eat with 16 of the total 51 dogs being removed from the study as a result of reaching their maximum weight gain amount. Therefore, it is easy to understand the tendency of domestic dogs to become overweight. Wolves, for example, averaging

approximately 55 kg, can consume approximately 10 kg of meat in a single kill, amounting to 18% of their own body weight. Thus, the urge for dogs to overfeed may originate from its predecessor (Mech and Boitani, 2010).

Because of domestication, many dogs (as well as other domestic animals) are reliant on their owners for care and feeding. Therefore, the animal owner largely decides on the choice of diet for their pet. There are a wide variety of commercially available diets that owners could choose from with varying macronutrient content (protein, fat and carbohydrate). These diets should all adhere to a national regulatory body such as the National Research Council in the United States (NRC., 2006, Zicker, 2008). Generally, pet food is divided into three forms – dry, semi moist and moist or wet (Zicker, 2008). Dry food is usually the most convenient to store and to feed pets. It has a water content of less than 11% and a higher carbohydrate content than wet food, where cereals, legumes and other plant material form the major sources of the carbohydrate component (Hewson-Hughes et al., 2013, NRC., 2006, Zicker, 2008). Moist or wet foods are typically canned, have a higher water content of between 60 to 87%, and higher quantities of meat or protein than dry foods (Zicker, 2008). Semi-moist foods make up a smaller portion of the pet food market with a water content of 25 to 35% and a relatively high sugar content, which makes it highly palatable for dogs (Crane et al., 2010, Zicker, 2008).

Along with different forms of pet food, there are also a plethora of different brands that owners can select from. Examples of such brands include Whiskers and Catmor for cats, and Bobtail and Husky for dogs. Hill's manufactures food for both cats and dogs, with a variety of different diets targeting healthy animals or animals with specific diseases or conditions (www.hillspet.co.za). Diet trends are a common occurrence in human nutrition, but are also evident in animal diets. For example, the emergence of the natural pet food industry and a raw food diet has since seen the light in developed countries (Buff et al., 2014, Schlesinger and Joffe, 2011).

As a result of domestication, the diet is largely driven by the owners of cats and dogs. Although this thesis will not be focusing on diet or dietary interventions, future research should focus on its effects, especially with regards to skeletal muscle composition and diabetes.

1.8. The role of skeletal muscle

1.8.1. Overview and different fibre types

Skeletal muscle is a heterogeneous tissue consisting of a variety of muscle fibre types. A motor unit is defined as a number of muscle fibres innervated by a single neuron that, on activation, will stimulate all these fibres to contract at once (Schiaffino and Reggiani, 2011). Thus, motor units are recruited by the nervous system in order to fulfil a specific task - the more force required, the more motor units

activated. Thus, a direct relationship exists between exercise intensity and motor unit recruitment. The heterogeneity of muscle fibres allows skeletal muscle to perform a wide range of tasks, from maintaining body posture to locomotion (Schiaffino and Reggiani, 2011). Each muscle fibre consists of thousands of myofibrils, which in turn contains millions of myofilaments (Frontera and Ochala, 2015). The orderly arrangement of myofilaments forms sarcomeres, which are the smallest contractile unit of skeletal muscle. Actin and myosin are the two most abundant myofilament proteins.

Fibre types differ according to their structural, molecular, metabolic and contractile properties and these properties result in a variety of functional characteristics (Acevedo and Rivero, 2006). The components of skeletal muscle that brings about movement include the contractile proteins and metabolism, the latter system being responsible for providing adenosine triphosphate (ATP) to the contractile apparatus. The contractile properties of an individual muscle fibre are dependent on the myosin heavy chain (MHC) isoform that it expresses. There are three main MHC isoforms expressed in adult mammalian skeletal muscle, namely MHC I, IIA and IIX, giving rise to type I, type IIA and type IIX fibres, respectively (Bottinelli, 2001, Kohn et al., 2011).

Type I fibres have slow contraction speeds and rely on oxidative metabolism to supply the required ATP. The rate of ATP supply is sufficient to supply energy when the muscle is working at lower intensities over prolonged periods of time and allows these fibres to be highly resistant to fatigue. Type I fibres also have a dense capillary supply, small cross sectional area, high myoglobin concentration, low glycogen but abundant triglyceride stores (Lefaucheur, 2010, Kohn et al., 2011). They also have high numbers of mitochondria and GLUT4 content compared to type IIA and IIX fibres. Another property of this fibre type important to glucose homeostasis is that they appear to be more sensitive to insulin than the other fibre types.

Type IIX fibres display the opposite characteristics to type I fibres. Glycolytic metabolism is the main source of energy with higher glycogen content, low triglyceride and myoglobin concentrations, low capillary supply and large cross sectional areas (Kohn et al., 2011, Lefaucheur, 2010). These fibres have powerful and rapid contractions, but fatigue easily. They have low mitochondrial and GLUT4 content and, therefore, lower insulin sensitivity (Duan et al., 2017, Lefaucheur, 2010, Kohn et al., 2011).

Finally, type IIA fibres exhibit a mixture of type I and type IIX characteristics. Type IIB fibres have extremely fast contraction speeds and are mostly found in the muscles of smaller animals (e.g. rodents), and limited to specialised muscles in humans, such as the eye (Toniolo et al., 2007). More importantly, the relative proportion of any fibre type varies according to species, anatomical site and function (Schiaffino and Reggiani, 2011). Muscle fibres also exhibit plasticity, in terms of

composition, so they are able to change according to different physiological needs (Schiaffino and Reggiani, 2011).

An important characteristic of the muscle fibres that may be relevant to T2D is that the different types of fibres have varying degrees of insulin sensitivity and GLUT4 content (Daugaard and Richter, 2004). These are important characteristics, because skeletal muscles are the major site of glucose uptake. Indeed, Duan et al. (2017) proposed that targeting the reprogramming of muscle fibres to type I could help in the treatment of obesity and T2D. Therefore, it is important to study skeletal muscle fibre composition in relation to this disease.

1.8.2. Overview of metabolic pathways

The different muscle fibres types have varying oxidative and glycolytic capabilities (Figure 1). In order for muscle to perform its various functions, it relies on energy in the form of ATP. Only small quantities of ATP are stored, and is replenished *via* other fuel sources, which include phosphocreatine, blood glucose, glycogen and fat. All these fuels are metabolised to generate ATP via different metabolic pathways (Hocquette et al., 1998). Type I fibres are oxidative and, because of their high myoglobin content and capillary density, make use of substrates from blood such as blood glucose, free fatty acids and O₂ to yield energy (Frayn, 2009, Frontera and Ochala, 2015). In contrast, type IIX fibres have a much greater ATP consumption rate compared to type I, thus requiring ATP at a greater turnover rate. They rely on anaerobic glycolysis and make use of glucose-6-phosphate produced from glycogen breakdown within the muscle. In humans, most muscle groups comprise of type I, IIA and IIX fibres in varying proportions depending on the muscle group, training status and genetics of the individual (Frayn, 2009).

Following muscle contraction, ATP is immediately replenished by intramuscular pools of phosphocreatine. Creatine kinase (CK) facilitates the conversion of phosphocreatine to creatine in the cytoplasm of the muscle, producing ATP from adenosine diphosphate (ADP) (Frayn, 2009, Wallimann, 1994). CK has many isoforms within skeletal muscle and cardiac muscle - all of which are involved in ATP production in the cell (Wallimann, 1994). The metabolism of one phosphocreatine molecule produces one molecule of ATP and, thus, is a very limited source of ATP replenishment. Therefore, muscle is reliant on other sources to obtain more long term energy.

Blood glucose is brought into the cell through GLUT4 transporters. This action is mediated by insulin (after a meal) or muscle contraction (during exercise) (Wilcox, 2005). As in the liver, the glucose is either stored as glycogen or metabolised to pyruvate via the glycolytic pathway. Glucose-6-phosphate is produced from glycogen by phosphorylase and phosphoglucomutase, whereas blood glucose imported via the GLUT4 is phosphorylated to glucose-6-phosphate by hexokinase. There are many

different enzymes involved in glycolysis that ultimately produces two ATPs for every glucose molecule. Phosphofructokinase (PFK) is considered the pacemaker enzyme in glycolysis and as a result, plays an important regulatory role (Sola-Penna et al., 2010). PFK catalyses the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate and has three isoforms with only one, PFK-M, being expressed in skeletal muscle (Sola-Penna et al., 2010). Under anaerobic conditions, pyruvate is reduced to lactate via the lactate dehydrogenase (LDH) enzyme. Under aerobic conditions, pyruvate is converted into acetyl coenzyme A (acetyl CoA), which then enters the Krebs's cycle (or citric acid cycle) in the mitochondria.

The citric acid cycle is the most important pathway for energy production in the body and is the primary pathway for the oxidation of carbohydrates, proteins and lipids (Akram, 2014). Through β -oxidation, which consists of many steps and enzymes, fatty acids are converted to acetyl CoA and can therefore produce energy in the citric acid cycle (Houten et al., 2016). A marker of the flux capacity through this pathway is 3-hydroxyacyl-CoA dehydrogenase (3HAD), an enzyme involved in the later stages of β -oxidation (Houten et al., 2016).

Citrate synthase (CS) catalyses the joining of oxaloacetate with acetyl CoA to produce citrate, which is the initial step in the citric acid cycle (Akram, 2014). One ATP, one FADH_2 and three NADH molecules are produced for each cycle. The FADH_2 and NADH molecules then become oxidized transferring their electrons to the electron transport chain in the inner membrane of the mitochondria. As a result, 36 or 38 ATPs are produced from one exogenous or glycogen-derived glucose respectively, through oxidative phosphorylation. Depending on the length of the fatty acid chain, fatty acids can produce in excess of 100 ATPs after oxidative phosphorylation (Hocquette et al., 1998). However, the rate of fat metabolism is much slower than glucose and glycogen metabolism.

As described earlier, type I fibres are more reliant on oxidative metabolism, while type II fibres are more reliant on glycolytic metabolism to produce ATP. It is for this reason that type I fibres have a higher oxidative capacity while type II fibres have a higher glycolytic capacity – with type IIX having a higher glycolytic capacity than type IIA fibres (Schiaffino and Reggiani, 2011). Exercise training also plays a role in skeletal muscle fibre type distribution and metabolism. Endurance trained athletes tend to have a predominance of type I fibres and higher oxidative capacity compared to power/sprint athletes who tend to have a predominance of type II fibres and a higher glycolytic capacity (Kohn et al., 2007b, Schiaffino and Reggiani, 2011, Tesch et al., 1989).

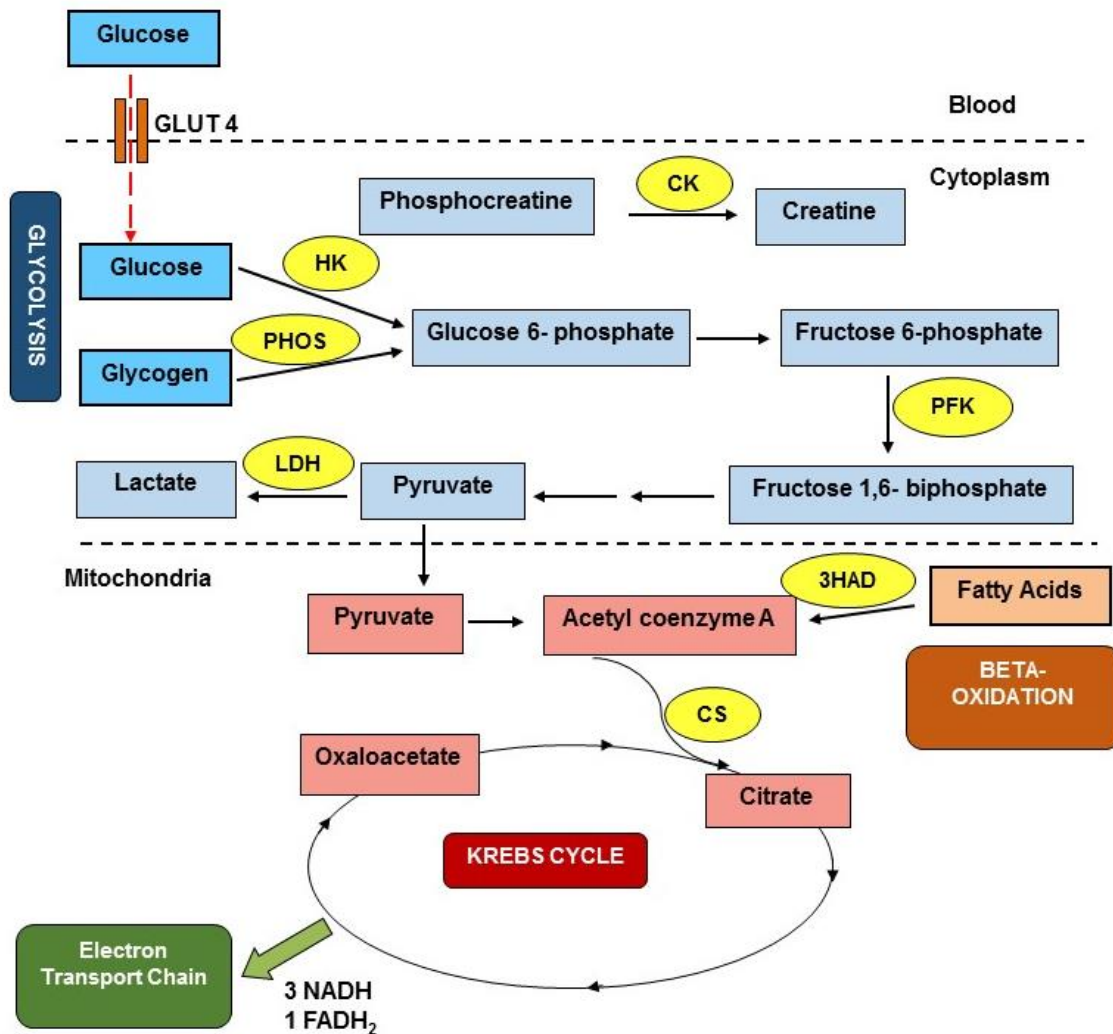


Figure 1. Schematic diagram of the skeletal muscle metabolic pathways that were investigated in this study. The yellow circles represent the enzymes used as flux markers of the respective pathways. HK, Hexokinase; PHOS, Phosphorylase; PFK, Phosphofructokinase; 3HAD, 3-hydroxyacyl-CoA dehydrogenase; CS, Citrate Synthase.

1.9. The skeletal muscle of dogs

Over the past 50 years, only seven studies have been published that have investigated the muscle composition and metabolic characteristics of dogs. In an early study by Gunn (1978), the histochemical properties of trained and untrained greyhounds were investigated. The technique that utilises the stability of the myosin ATPase enzyme at alkaline or acidic pH was used to differentiate between type I and II fibres. As there were no antibodies against the MHC isoforms at the time, this was the preferred technique used in many studies (Guy and Snow, 1981, Latorre et al., 1993, Snow et al., 1982). For type I fibres, the mATPase is unstable at a pH lower than 3.9 and greater than 10.4 while type IIA and IIX fibres can be differentiated at pH 4.6 where IIA fibres are unstable (Brooke and Kaiser, 1970). In the study by Gunn (1978), they separated type I and II fibres at pH 9.4 - fibres that have high mATPase activity (darker stains) are indicative of type II fibres and fibres with low mATPase activity (lighter stains) are indicative of type I fibres. There was no difference in type I

fibre composition between trained and untrained greyhounds in the *semitendinosus* muscle. Compared to other breeds, greyhounds (n=21) had 3% type I fibres compared to collies (n=5) with 23% (Gunn, 1978). This finding clearly illustrates a breed effect and that overall, dogs have a higher proportion of type II fibres compared to horses and humans (Kohn et al., 2011). However, a limitation of the comparative dog study of 1978 was that the researchers could not distinguish between type IIA, IIX and IIB fibres. In a similar study by Guy and Snow (1981) the fibre type of greyhounds (n=6), foxhounds (n=4) and crossbreeds (n=5) in different muscle groups were compared. Although this study consisted of a smaller sample size, their findings were similar. The average type II fibres in the *Vastus lateralis* and *Triceps brachii* were 97% and 94% for greyhounds, 81% and 65% for foxhounds, 61% and 77% for crossbreeds, respectively. The results show that along with various breeds, different muscle groups have varying proportions of type II fibres, as have been shown in rats (Delp and Duan, 1996).

Due to the difficulty in identifying the sub classes of type II fibres, there have been varying reports for the presence of type IIA and IIX fibres. Snow et al. (1982) used the mATPase stain and gel electrophoresis-derived enzyme-linked immunosorbent assay (GEDELISA) to identify the presence of type IIA fibres and another type II fibre with a similar oxidative-glycolytic capacity. This type II fibre did not correspond to type IIX fibres (called IIB in the paper) in MHC structure and was called IIDog fibres. In a study by Latorre et al. (1993) they found similar results to Snow et al. (1982), with the presence of type IIA, IIDog and I/II hybrid (referred to as IIC in the paper) fibres. They believed that the IIDog fibres did not correspond to type IIX fibres conventionally found in other species like rats. This type IIDog fibre was further investigated in several studies that aimed to achieve a definite, unambiguous identification of canine skeletal muscle fibre types and the MHC isoforms they express (Acevedo and Rivero, 2006, Strbenc et al., 2004, Toniolo et al., 2007). Toniolo et al. (2007) found that the limb and trunk muscles express MHC I, IIA and IIX and that the type IIDog fibres express the MHC IIX isoform. Therefore, type IIDog fibres are those expressing MHC IIX and could be type IIX and type IIAX hybrid fibres (Strbenc et al., 2004). The MHC IIA isoform was the most abundant with IIX commonly expressed as IIA/IIX hybrids. An oxidative type IIX fibre has also been found in antelope species such as the fallow deer and springbok, which may help these animals maintain high running speeds for an extended period of time (Curry et al., 2012). These oxidative IIX fibres may correspond to the type IIDog (IIX) fibre.

In an interesting study by Acevedo and Rivero (2006), they investigated a quantitative immunohistochemical approach to characterise hybrid canine skeletal muscle fibres. Their rationale was that many previous studies do not consider the hybrid phenotype - in terms of determining the dominance of one MHC isoform to another. Yet, hybrid fibres play an essential role in enabling a

muscle to fine tune its efficiency and in forming the bridge between “pure” fibres (Acevedo and Rivero, 2006, Stephenson, 2001). Acevedo and Rivero used microphotometric immunohistochemistry and SDS-PAGE to show a continuum of histological and immunohistochemical staining and semi-quantified the images obtained. The results varied slightly across muscle groups. Overall, one third of the fibres were hybrid type IA and IIX fibres. Interestingly, while there were very few “pure” type IIX fibres, most of the type IIX hybrids had a dominance of MHC IIX expression. In contrast, type IIA fibres made up 37% of the fibre composition followed by type I fibres with 27% and type IIX with 25%. This shows the high oxidative capacity of dog skeletal muscle which is probably linked to their high endurance ability (Acevedo and Rivero, 2006, Strbenc et al., 2004). However, a notable limitation of this study was the small sample size of four. A further limitation was that an average of 150 fibres were counted in total per muscle group, with only 118 fibres counted in the *Triceps brachii*.

Overall, the skeletal muscle fibre composition of dogs has been investigated over the past few decades with varying results. There seems to be a prevalence of type I and IIA fibres with a higher oxidative capacity compared to glycolytic capacity. However, all the studies mentioned above (Acevedo and Rivero, 2006, Gunn, 1978, Guy and Snow, 1981, Latorre et al., 1993, Snow et al., 1982, Strbenc et al., 2004, Toniolo et al., 2007) had very small sample sizes and did not always differentiate between breeds. It would be valuable to investigate these muscle characteristics in a wider variety of dog breeds.

1.10. Skeletal muscle fibre type as a risk factor for Type 2 Diabetes

The metabolism of various fibre types plays an important role in overall energy balance in muscle. Briefly, type I fibres rely on oxidative metabolism with higher mitochondrial numbers and a higher GLUT4 content compared to type IIA and IIX fibres (Lefaucheur, 2010). This would indicate that type I fibres are more sensitive to insulin. In a recent review by Duan et al. (2017), they provided evidence and hypothesised whether targeting the re-programming of fibres from type II to type I would be a potential therapeutic avenue for the treatment of obesity and T2D. Their hypothesis was based on the characteristic of muscle plasticity which indicates that, although the total number of muscle fibres in an individual is fixed from the time of birth, the fibre type composition can still be altered throughout an animal's life to allow for adaptation to different physiological requirements or stressors (Schiaffino and Reggiani, 2011). There are a number of signalling pathways responsible for specification of type I and type II fibres during embryonic development as well as muscle plasticity in adults, but falls beyond the scope of this thesis (Duan et al., 2017).

The clinical implications of skeletal muscle fibre composition and metabolism in relation to obesity and T2D have been investigated over the past 30 years. Lillioja et al. (1987) conducted a study looking at the insulin action (via glucose uptake) and muscle morphology of the *Vastus lateralis* in 23 Caucasian and 41 Pima Indian healthy and lean males. Glucose uptake was determined by euglycaemic clamp, while capillary density and fibre type were determined via periodic acid-Schiff and mATPase stains, respectively. Notably, there was a significant positive correlation between glucose uptake and capillary density (capillaries/mm²), which could be linked to the oxidative capacity of the muscle due to the increased capillaries. There was also correlations between glucose uptake and the percentage of type I fibres ($r = 0.29$, at maximal conditions) and the percentage of type IIX fibres ($r = -0.38$, at submaximal conditions). The authors proposed that these differences might be attributed to some biochemical differences between the fibre types, especially their oxidative capacity.

In a study conducted 10 years later by Simoneau and Kelley (1997), the oxidative (CS) and glycolytic (PFK, GAPDH, HK and PHOS) enzyme activities in the *Vastus lateralis* of 8 lean non-diabetic, 10 obese non-diabetic and 8 obese T2D human patients were investigated. The data was expressed as the ratio of glycolytic to oxidative activity (PFK/CS, GAPDH/CS, HK/CS, and PHOS/CS). T2D patients had a higher ratio of all glycolytic to oxidative enzyme capacity compared to their non-diabetic counterparts, indicating a higher oxidative capacity in healthy participants. In a similar study, Oberbach et al. (2006) investigated whether the changes in oxidative and glycolytic enzymes related to the specific muscle fibre types in healthy and T2D participants. SDH and LDH were used to measure oxidative and glycolytic activity respectively, while mATPase activity was used to determine fibre type. In the T2D group, there was lower SDH and higher LDH activity than the control group, indicating a higher glycolytic capacity. The T2D group also had a significantly higher percentage of type IIX (fast glycolytic) fibres and a significantly lower percentage of type I (slow oxidative) fibres compared to the control group. The authors hypothesised that the reduced oxidative capacity in the muscle of T2D patients was likely due to decreased percentage of oxidative fibres and not due to changes in individual muscle fibres. These abovementioned studies point to skeletal muscle fibre type being a risk factor for insulin resistance and T2D and warrant further investigation in both the human and animal population.

1.11. Anticipated gain in knowledge

Obesity and T2D has become an increasing concern in both the human and animal population. Dogs are a useful model to gain a clearer understanding of the physiology surrounding obesity and T2D

because (i) there are clinical similarities between humans and domestic dogs and (ii) domestic dogs share the same environment as humans and, therefore, develop obesity and T2D spontaneously.

Skeletal muscle is important in the uptake of glucose and plays a key role in glucose homeostasis in the body. Very little is known about skeletal muscle composition in dogs and whether certain fibre types may predispose animals to insulin resistance and diabetes. This study would help to characterise the skeletal muscle fibre type composition and metabolism of domestic dogs. This would form the basis for future studies in elucidating the mechanisms of acquiring diabetes in dogs. It may also create a model for further understanding of the disease in humans, especially surrounding the role of ethnicity in predisposition to diabetes. This may provide veterinarians and animal owners with the necessary knowledge to treat and prevent diabetes in domestic dogs.

1.12. Aims and Hypothesis

The primary aim of this study is to investigate the skeletal muscle fibre composition and metabolic profile in the *Vastus lateralis* and *Triceps brachii* skeletal muscle of 16 breeds (including mixed breed) of domestic dogs. The secondary aim is to determine whether the hypothesis, proposed by Duan et.al (2017), that type I fibres offer protection against insulin resistance and diabetes is valid when compared to predicted breed susceptibility.

This was achieved through fibre typing of the skeletal muscle and determining the muscle enzyme activities of specific marker enzymes in oxidative and glycolytic metabolism.

The hypothesis is that muscle fibre type and muscle metabolism will differ between the different breeds of dogs. Dog breeds with a predicted increased risk of diabetes would have a lower proportion of type I fibres.

CHAPTER TWO

2. DESCRIPTION OF DOG BREEDS

2.1. Introduction

This study comprised 38 canine muscle samples from the *Vastus lateralis* and *Triceps brachii*. The samples were acquired through opportunistic sampling from euthanased animals at Rondebosch Veterinary Hospital in Cape Town and the Onderstepoort Veterinary Hospital in Pretoria. The cohort of samples includes 16 dog breeds of varying ages, sex and weight. The aim of this chapter is to provide descriptive characteristics of these breeds, in relation to their physiological and physical traits.

The overall descriptive characteristics of the cohort of canines utilised in this study, are outlined in Table 1.

Table 1. Descriptive characteristic of 38 canines of various breeds. Typical parameters from the literature are included for lifespan and mean weight.

Breed	Typical Lifespan (years)	Typical Weight range (kg)	Quantity (n)	Sex (n)	Age (years)	Weight (kg)	Neutered (n)
Boerboel*	10 – 12	65 – 80	2	M	10	39	No
				M	0.4	15	No
Chihuahua*	14 – 18	1 – 3	2	F	13	5	No
				F	0.5	3	No
Chow Chow	10 – 12	23 – 34	1	F	10	22	No
Dachshund	12 – 14	7 – 14	4	F	8	9	Yes
				M	14	7	Yes
				M	9	6	No
				M	8	7	No
					10 ± 3	7 ± 1	
Dobermann	10 – 12	29 – 40	1	F	10	31	Yes
Fox terrier	10 – 13	8 – 10	3	F	13	5	Yes
				F	6	9	Yes
				M	1	--	Yes
					10 ± 5	7 ± 3	
German pointer	12 – 14	20 – 34	1	M	8	46	No
German shepherd*	10 – 12	34 – 43	2	F	1	18	No
				M	0.08	2	No
Greyhound	15 – 16	24 – 32	1	F	11	12	Yes
Jack Russell terrier	13 – 15	7 – 12	8	M	6	8	No
				M	10	13	Yes
				M	8	9	No
				M	6	8	No

Breed	Typical Lifespan (years)	Typical Weight range (kg)	Quantity (n)	Sex (n)	Age (years)	Weight (kg)	Neutered (n)
				M	10	6	No
				M	18	11	Yes
				M	18	7	No
				M	18	5	No
					12 ± 5	8 ± 2	
Labrador retriever	10 – 12	25 – 31	4	F	3	35	Yes
				F	7	30	Yes
				F	7	37	No
				F	10	21	No
					7 ± 3	31 ± 7	
Miniature pinscher	10 – 16	3 – 5	2	F	10	5	No
				M	9	3	Yes
					10 ± 1	4 ± 1	
Mixed breed**	--	--	3	M	4	10	No
				M	15	21	Yes
				M	10	28	Yes
					10 ± 6	20 ± 9	
Scottish terrier	12 – 13	8 – 10	2	M	6	8	No
				M	16	6	No
					11 ± 7	7 ± 1	
Pomeranian	12 – 16	2 – 3.5	1	F	9	4	No
Yorkshire terrier	14 – 16	1 – 4	1	F	10	3	Yes

Where applicable, data are expressed as mean \pm SD or expressed as the individual value. The typical characteristics of these animals were obtained from Hill's Pet Nutrition (Hill's, 2018), Pedigree (2014) and DogTime (2019). M, Male; F, Female. *Breeds that contain a puppy (below the age of 1). **Mixed breed contains two animals that are crossed with a German shepherd and one unknown cross.

2.2. Classification of the different breeds

Dogs were the first species of animal to become domesticated, although the exact dates are unknown with evidence ranging from 100 000 to 14 000 years ago (Bradshaw, 2006). As a result, the domestic dog is an extremely diverse species with over 300 breeds recognised by the Fédération Cynologique Internationale (FCI), which is the World Canine Organisation (Hedhammar and Indrebo, 2011). These animals have been bred over many years for a variety of reasons – from hunting to being companion dogs. As a result of the vast range of breeds, kennel clubs have developed all over the world with the general aim of advancing and protecting the welfare of dogs, particularly purebred dogs. In order to fully describe the recognised breeds, the FCI (and other kennel clubs) have breed standards, which describes the characteristics of the dogs within each breed. Dogs with similar traits, lineage, appearance or function are clustered into the same group – these groups are determined by the respective kennel clubs. However, only purebred dogs are recognised by kennel clubs. The FCI recognises 10 groups, each group encompassing many breeds (Figure 2) (FCI, 2019).

An important characteristic of skeletal muscle is its ability to adapt to physiological stress (Schiaffino and Reggiani, 2011). Fibre size and fibre type can change according to changes in nerve activity patterns, mechanical loading (e.g. exercise) and circulating factors such as hormones. Exercise training has been shown to cause changes in fibre type composition, such as endurance training that leads to an increase in type I fibres while resistance and sprint training leads to a shift to type II fibres (Andersen and Henriksson, 1977, Trappe et al., 2006). Due to genetics, there is also variability in fibre type composition between species that echo their physical ability. For example, the lion (*Panthera leo*) has significantly more type IIX fibres and higher glycolytic capacity in the *Vastus lateralis* compared to humans (Kohn et al., 2011), while antelope species such as the blesbok (*Damaliscus pygargus phillipsi*) have higher proportions of type IIX and IIA fibres with higher oxidative capacity, the latter to at least stand a chance to out run their predator (Kohn, 2014). The consensus therefore is that animals (including humans) that have speed and power have greater proportions of type II fibres, whereas those with endurance (especially humans) will harbour a greater number of type I fibres. This same pattern may be present in domestic dogs as they all exhibit different physical attributes – some have been bred for hunting while others bred for companionship. Therefore, the next section will briefly report on the various groupings of canines, their physical and lifestyle characteristics and their skeletal muscle fibre type, where available or proposed. Very little information with regards to

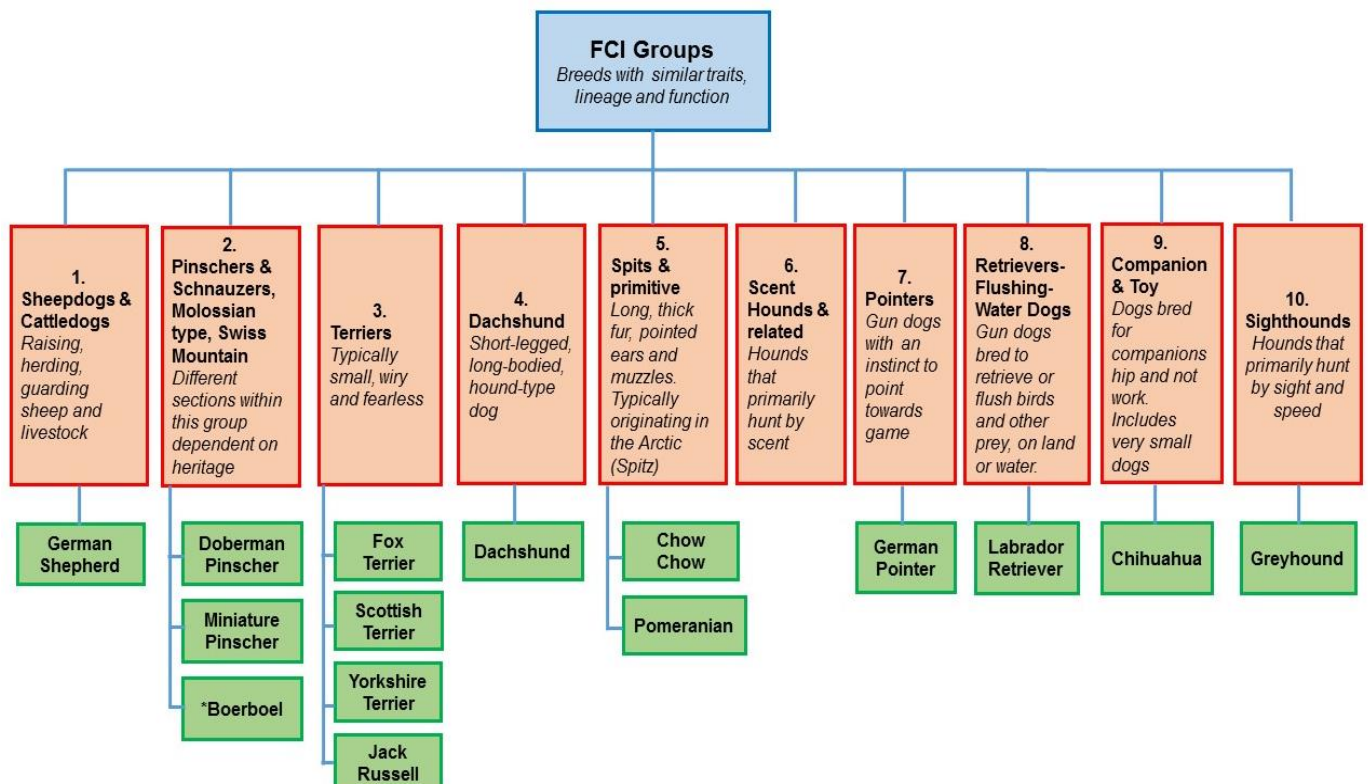


Figure 2. Grouping of different dog breeds according to the Fédération Cynologique Internationale (FCI) breed standard. Green rectangles indicate dog breeds included in the study sample. *The Boerboel is a South African breed that is not recognised by the FCI, however it is closely related to the Bulldog and Mastiff which fall in Group 2. Mixed Breed dogs are excluded from this figure.

actual physical strength, maximum sprinting speed and endurance are known as it has not yet been scientifically investigated. Thus, the content below are provided as predominantly observations.

Groups 1: Sheepdogs and Cattledogs

This group of dogs were bred for the purpose of raising, herding and guarding sheep and other livestock (KC, 2019). These dogs usually have thick waterproof coats to protect it from the elements.

One such breed is the German shepherd, which, according to the FCI, is a versatile working, herding and service dog. They were bred from two varieties of German herding dogs with the intention of creating a “working dog with high achievements”. This is evident from its worldwide recognition as a guide and tracking dog due to its good temperament and obedience. Physically, German shepherds are medium to large in size, very powerful and muscular. Based on these characteristics, they may have both high endurance and speed and hence, contain a high oxidative and glycolytic capacity with a high proportion of type IIA fibres.

Group 2: Pinschers and Schnauzers

In general, these dogs were bred to “work” on the farm. The group consists of 52 breeds ranging from Rottweilers to miniature schnauzers (FCI, 2019). The Dobermann (or Doberman pinscher) is a companion, protection or working dog (FCI, 2019). Dobermanns are physically large, powerful and muscular dogs, but not seen as the endurance type.

The miniature pinscher is considered a house and companion dog and actually forms part of the Toy Group in The UK Kennel Club (KC, 2019). They were bred with the purpose of hunting rats and other vermin. Miniature pinschers are smaller forms of the German pinscher from which it likely originates.

The boerboel is not recognised by the FCI, but is recognised by the Kennel Union of Southern Africa and the American Kennel Club (KUSA, 2016). Historically, the boerboel developed from the interbreeding of large bull and mastiff type dogs that were brought to South Africa by various settlers in the seventeenth century. They were used as farm dogs – defending the farm from predators, hunting game and protecting the family. Boerboels are large, strong, sturdy and very muscular dogs (KUSA, 2016).

Generally, the dogs in this group are strong and muscular dogs that were bred for protection. Therefore, their muscle characteristics would display strength and speed, a high type IIA and IIX fibre content, and be more glycolytic with poor oxidative capacity.

Group 3: Terriers

Dogs in this group tend to be small and energetic. Their ancestors were bred to hunt vermin. The fox terrier (smooth and wired hair) originated in Britain in the late 1800s and is considered a medium sized terrier. They are active and lively, with alleged high power-to-weight ratio (FCI, 2019). Initially bred to hunt foxes, they are considered fearless and would seek out foxes within burrows for the hunters (KC, 2019).

The hunt for rats, badgers and foxes on the Scottish Highlands were the primary reason for the breeding of the Scottish terrier. It is a small, short-legged and sturdy dog that is alert and agile despite its thick-set build (FCI, 2019).

The toy-sized Yorkshire terrier was bred in the 1850s to hunt rodents in textile mills and coal mines (FCI, 2019). Since the mid-1900s, the Yorkshire terrier has become famous as a companion dog. They are very small, alert dogs with a long hanging coat.

Therefore, the skeletal muscle of this group of canines may display a greater abundance of type I and IIA fibres compared to the powerful Pinschers and Schnauzers group. Their muscle may also be more oxidative.

Group 4: Dachshunds

Three varieties of dachshund – standard, miniature and rabbit dog make up this group. Dachshunds have been known since the Middle Ages with the first official kennel club established in 1888. Dachshunds were bred to track and hunt deer, badgers and rabbits above and below ground (FCI, 2019). Due to their low centre of gravity and short legs, they are very versatile hunting dogs specifically suited for hunting below ground. Based on these characteristics, one might expect their skeletal muscle to have similar oxidative and glycolytic capacity with a tendency for type I and IIA fibres.

Group 5: Spits and Primitive

This group consists of a 52 dog breeds ranging from the Siberian husky to the Pomeranian (FCI, 2019). Spitz dogs are characterised by long, thick fur, pointed ears and muzzles. Many of them originate from the Arctic regions of Siberia and were developed to hunt, herd and pull sleds. Primitive dogs are most likely classified as those who originated outside of Europe, many from South America and Asia.

The Chow Chow is possibly one of the world's oldest dog breeds with artefacts dating back to China's Han Dynasty in 206 BC. Chow Chows are related to the Nordic spitz and mastiff type dogs. They are

medium sized, powerful and muscular in stature and known for their lion-like manes (FCI, 2019). The Pomeranian is a miniature descendant of the German spitz that was utilised as a watch and companion dog. Pomeranians largely owe their popularity to Queen Victoria who became a breeder of Pomeranians in the mid-1800s and is credited for reducing them to toy size (KC, 2019). In other kennel clubs they form part of the Toy Group.

The animals in this group are very diverse and so would have different skeletal muscle characteristics. They would likely have similar oxidative and glycolytic capacity with a higher proportion of type IIA fibres.

Group 7: Pointers

Pointers and setters are a type of Gundog (or bird dogs) that instinctively point towards game with their muzzles, directing the hunter towards the game. German pointers (or German pointing dogs) can be divided into shorthaired and wirehaired. Although they are not directly related to one another, they are both versatile hunting dogs for land and water (FCI, 2019). Pointers are medium to large in size, with a high endurance enabling them to spend hours wading through water and bushes. Their skeletal muscle would likely reflect this endurance ability with higher oxidative capacity and a higher proportion of type I and IIA fibres.

Group 8: Retrievers – Flushing – Water Dogs

This group consists of Gundogs that primarily flush and retrieve birds and game from land or water. The Labrador retriever is believed to have originated on the coast of Newfoundland as a water dog that retrieved fish for the local fisherman (FCI, 2019). Labradors are preferred to act as guide dogs, service dogs and as companions. It is a large, strong, athletic dog with a water-resistant coat. It is capable of working long hours under difficult conditions as a Gundog. Therefore, the Labrador retriever would likely have similar skeletal muscle characteristics as the Pointers Group – higher oxidative capacity with more type I and IIA fibres.

Group 9: Companion and Toy

Dogs in this group were bred as small companions for humans. The Chihuahua originated in Mexico and is considered the smallest companion dog (FCI, 2019). Its ancestor, the Techichi, was an integral part of the Aztec culture in the 12th century. It has become famous in recent years as a “purse dog” due to its small, compact size. The Chihuahua has limited endurance and prefers short, slow walks. The skeletal muscle of the Chihuahua may have similar glycolytic and oxidative capacity with a higher proportion of type IIA and IIX fibres.

Group 10: Sighthounds

Sighthounds are hunting dogs that hunt by sight and speed as opposed to scent. The greyhound, which could be considered the flagship dog of this group, is believed to have originated in Ancient Egypt in 4000 BC. With its long, streamlined body, it is the fastest dog breed reaching speeds of 70 km/h (FCI, 2019). In an early study by Gunn (1978), they found that greyhounds had 97% type II fibres (using mATPase stain) in the *semitendinosus* muscle. Based on the high speed characteristics of the greyhound, higher proportions of type IIX fibres with high glycolytic capacity would be expected.

2.3. Limitations of the study sample

There are several limitations with this study sample. To start, the clinical reasons for euthanasia were not disclosed. The majority of the animals were older than 10 years, and as a result, “old age” was often recorded as the reason for euthanasia. This may have implications on the results, specifically the metabolism of the muscle and muscle fibre size. Secondly, as the sampling was opportunistic – thus, the breed, sex and age of the samples could not be controlled for or anticipated. Therefore, there are many breeds in this cohort with a sample size of only one or two.

CHAPTER THREE

3. SKELETAL MUSCLE MORPHOLOGY

3.1. Background

Previous studies have shown that the skeletal muscle of domestic dogs have a prevalence of Type I and IIA fibres with very few Type IIX fibres. However, most of these studies have contrasting results that may be due to their small sample size, limited dog breeds and, in some cases, minimal fibres counted and techniques not able to distinguish between the three fibre types (Acevedo and Rivero, 2006, Guy and Snow, 1981, Snow et al., 1982). Skeletal muscle plays a major role in maintaining glucose homeostasis with Type I fibres linked to increased insulin sensitivity and that potentially targeting these fibres may provide therapeutic treatment for obesity and T2D (Duan et al., 2017). This chapter aims to investigate the fibre type composition and cross sectional area of the VL and TB muscles in 16 different breeds of domestic dogs. The aims are:

- to support or provide new findings of previous studies (where data is available),
- to provide the fibre type profiles of the 16 breeds, and
- to assist in providing evidence for the proposed hypothesis that fibre type is associated with insulin resistance and T2D.

3.2. Methodology

3.2.1. Study Animals

The study sample included 38 dogs of various breeds, age, sex and weight (Table 1). These animals were euthanased for reasons not disclosed to the researchers. Sampling was opportunistic and collection took place at the Onderstepoort Veterinary Academic Hospital in Pretoria, as well as the Rondebosch Veterinary Hospital in Cape Town, with consent from the owner.

3.2.2. Ethics

Ethical approval for this study was obtained from the Faculty of Health Science Animal Ethics Committee, University of Cape Town (reference number: 017/023) and the Faculty of Veterinary Science Animal Ethics Committee, University of Pretoria (reference number: v026-18). A Department of Agriculture, Forestry's and Fisheries Section 20 permit was also granted for the research.

3.2.3. Tissue collection

Muscle samples were collected *post mortem* according to the procedure described by (Hennessey et al., 1997, Kohn et al., 2011). No animals were euthanased for the purposes of this study. Biopsies of approximately 50 to 100 mg were obtained from the VL and lateral head of the TB. After collection, the samples were cut into smaller pieces and rapidly frozen in liquid nitrogen. Samples were transported on dry ice to the Division of Exercise Science and Sports Medicine, Newlands and stored at -80°C until analyses.

3.2.4. Section preparation, fibre typing and fibre cross sectional area (CSA)

The fibre type of each VL and TB muscle sample was determined by fluorometric immunohistochemistry as described by Fry et al. (2014) with modifications. The frozen muscle samples were sectioned using a cryostat set to -25°C (Leica CM1100, Leica Biosystems) into 10µm thick serial cross sections and each section placed onto a microscope slides. Slides were stored at -20°C until processed.

Slides were removed from the -20°C and allowed to air dry. The sections were fixed in acetone at -20°C for 2 minutes, dried and hydrated in PBS. After blocking non-specific binding sites with 5% donkey serum (made up in PBS) for 1 hour at room temperature, the sections were incubated overnight at 4°C with primary antibodies against the MHC corresponding to type I (BA-D5) and type IIA (SC71) fibres. Type IIX fibres were lightly stained by SC71. All primary antibodies were purchased from Developmental Studies Hybridoma Bank (Iowa, USA). The following day, slides were washed in PBS and then incubated for one hour at room temperature with fluorescently-tagged secondary antibodies, namely AMCA goat anti-mouse IgG2b (catalogue number: 115-155-207) and AlexaFlour 488 goat anti-mouse IgG1 (catalogue number: 115-545-205) purchased from Jackson ImmunoResearch Laboratories. After washing in PBS, cover slips were mounted with a fluorescent mounting medium (Moviol) containing antifade and stored at 4°C until imaging.

Sections were visualised with a fluorescent microscope (Nikon Eclipse 80i, New York, USA) and photographs taken of a selected section at 10x magnification using a digital camera (Canon EOS 650D, Pretoria, South Africa). The number of fibres per fibre type was counted for a selected area of the section and expressed as a percentage of the total number of fibres counted. The fibres were classified as pure type I, IIA or IIX fibres (Figure 3A) with an average of 700 fibres counted and typed per section. Hybrid type IA and IIX fibres were identified, but made up less than 2.5% per hybrid type of the total fibre type composition. As a result, the percentage of hybrid fibres were divided and included in the type I, IIA or IIX category e.g. the percentage of type IA hybrids were halved and included in the type I and IIA groups. The CSA of a maximum of 50 fibres per fibre type

were determined per section using the computer programme ImageJ (version 1.50e, Maryland, USA). The image was broken up into quintiles (five areas) and the CSA of 10 fibres of each fibre type in each section was determined and averaged, this was to ensure that the entire muscle section was represented. A more detailed protocol described in Appendix A.

3.2.5. Statistical analyses

Statistical calculations were performed using GraphPad Prism 7.0e (GraphPad Prism, La Jolla, CA, USA). Values are expressed as mean \pm standard deviation and normality was checked with the Shapiro-Wilks normality test. Statistical analyses were not performed between different breeds as the sample sizes were too small. However, the fibre type and fibre CSAs from the VL and TB were compared to one another using the non-parametric Kruskal- Wallis one-way ANOVA multiple comparison test. The relationship between CSA and the weight of the animals were determined using a non-linear regression fit (two-phase decay for line of best fit). Significance was set at $P < 0.05$.

3.3. Results

3.3.1. Fibre type composition

In the TB and VL of all adult animals grouped (Figure 3B), type IIA fibres were the predominant fibre type with $43\% \pm 11$ and $44\% \pm 18$ respectively. This was followed by type I fibres with $33\% \pm 14$ and $38\% \pm 27\%$, whereas type IIX fibres represented $24\% \pm 16$ and $18\% \pm 18$, respectively. Using the Kruskal-Wallis ANOVA test, there was no significant difference between the TB and VL in type I, IIA or IIX fibres. All the fibre types had a large standard deviation, which is apparent when the spread of the individual animals is examined (Figure 3B).

Figure 3C and D shows the fibre type composition according to their breed, with each breed grouped according to the FCI breeding group standards. The German shepherd had a higher percentage of type IIA fibres (TB: 44%; VL: 49%) with an equal amount of type I and IIX fibres. The Dobermann had a higher percentage of type IIA fibres (TB: 50%; VL: 75%) followed by type I fibres (TB: 39%; VL: 21%). The VL of the miniature pinscher appears to have a distinct different fibre type profile than its TB. Type IIA dominated in the TB ($54\% \pm 3$) and type I dominated in the VL ($49\% \pm 59$). However, there was a large standard deviation in all the fibre types in the VL muscle group, and might be a sampling error. This was followed by $33\% \pm 3$ type I, $13\% \pm 0$ type IIX (TB) and $28\% \pm 27$ type IIA, $23\% \pm 33$ type IIX (VL). The boerboel also had contrasting TB and VL profiles with type IIX fibres having a higher percentage on average. The type I, IIA and IIX proportions were 29%, 29% and 42%

in the TB and 10%, 48% and 42% in the VL. Overall, the Pinschers and Schnauzers group had a fibre type composition of 33%, 47%, 20% and 32%, 45%, 23% for TB and VL, respectively.

The Terrier group had an average type I, IIA and IIX distribution of 30%, 43%, 27% and 42%, 41%, 17% for TB and VL, respectively. The fox terrier had predominantly type IIA fibres in the TB and VL ($44\% \pm 4$ and $48\% \pm 9$) followed by type I fibres ($39\% \pm 15$ and $23\% \pm 18$). The Scottish terrier had a similar percentage of type IIA fibres in the TB and VL ($44\% \pm 14$ and $47\% \pm 13$), but a higher percentage of type IIX fibres in the TB and VL than type I ($33\% \pm 29$ and $37\% \pm 27$). The Jack Russell had the largest sample size group, with 8 animals. Its fibre type composition were predominantly type IIA ($44\% \pm 11$) and type I ($30\% \pm 10$) in the TB. The VL had a predominance of type I fibres ($57\% \pm 24$) followed by type IIA fibres ($37\% \pm 20$). Unlike the other terriers, the Yorkshire terrier had a higher proportion of IIX fibres (48%) and IIA fibres (30%) in the TB. While each fibre type proportion was approximately 33% in the VL.

The fibre type distribution of the TB and VL of the dachshund were similar with a slightly higher percentage of type I fibres ($40\% \pm 19$ and $42\% \pm 31\%$). This was followed by type IIA ($35\% \pm 8$ and $36\% \pm 8$) and type IIX fibres ($25\% \pm 16$ and $22\% \pm 25$). There was a large standard deviation between the four samples, especially for type I and IIX fibres.

The Spits and Primitive group consisted of a Chow Chow and Pomeranian. The fibre type composition in the TB of these two breeds appeared the same (I: $20\% \pm 0.5$; IIA: $36\% \pm 0.3$; IIX: $44\% \pm 0.3$). On the other hand, the fibre type seems to be vastly different for the VL, with the Chow Chow having 70% type I and 30% type IIA and no type IIX fibres. In contrast, the Pomeranian had 49% type IIX, 40% type IIA and 11% type I fibres. This could potentially be due to sampling error.

The German pointer and Labrador retriever fall under the Pointer and Retriever-Flushing-Water dog groups, respectively, which are both largely classified as Gundogs. In the TB of the German pointer, the proportion of type I, IIA and IIX fibres were 47%, 50% and 3% while the proportions in the VL were 60%, 32% and 8%, respectively. There were four Labradors included in the sample group that appears to have a similar fibre type composition for the TB (I: $40\% \pm 22$; IIA: $43\% \pm 14$; IIX: $17\% \pm 20$), but more type IIA fibres in the VL (I: $36\% \pm 31$; IIA: $55\% \pm 29$; IIX: $9\% \pm 7$).

The Chihuahua, which falls under the Toy and Companion dog group, had a similar fibre type composition in both the TB and VL. Type IIA was the predominant fibre type (TB: 58%; VL: 46%) followed by type I (TB: 23%; VL: 25%) and type IIX fibres (TB: 19%; VL: 30%). The greyhound, considered the fastest of the canines, seems to have the lowest proportion of type I fibres of all the dogs (TB: 13%; VL: 2%). As a result, it had a high percentage of type IIA (TB: 50%; VL: 76%) and IIX fibres (TB: 37%; VL 22%).

The mixed breed category contained dogs with an incomplete breed history. On average, there was a higher proportion of type IIA fibres (TB: 57% \pm 4; VL: 48% \pm 18). The remainder comprised of type I (TB: 34% \pm 12; VL: 39% \pm 29) and type IIX fibres (TB: 10% \pm 9; VL: 14% \pm 12).

There were three puppies (dogs below the age of one) included in this cohort and they were grouped separately. The boerboel puppy had noticeably more type I fibres compared to the adult, with 43% and 56% for the TB and VL, respectively. Type IIA and IIX fibres represented 31% and 26% in the TB and 38% and 6% in the VL. The German shepherd appeared to have a higher proportion of type I (TB: 48%; VL: 35%) and IIA fibres (TB: 40%; VL: 56%) compared to the adult. In contrast, the Chihuahua puppy seemed to have a higher proportion of type IIX fibres (TB: 51%; VL: 27%) compared to the adult Chihuahua. The distribution of type I and IIA fibres were 20% and 30% in the TB and 9% and 65% in the VL.

3.3.2. CSA

The average CSA of type I, IIA and IIX fibres in the TB and VL is shown in Figure 4A. The average CSA of all the adult animals in the TB was 1740 $\mu\text{m}^2 \pm 586$ for type I fibres, 1690 $\mu\text{m}^2 \pm 606$ for type IIA fibres and 1726 $\mu\text{m}^2 \pm 674$ for type IIX fibres. The VL had similar CSA of 1712 $\mu\text{m}^2 \pm 613$ (type I), 1720 $\mu\text{m}^2 \pm 733$ (type IIA) and 1791 $\mu\text{m}^2 \pm 890$ (type IIX). There was no significant difference between type I, IIA and IIX fibres of the TB and VL using the Kruskal-Wallis ANOVA. Due the apparent similar CSA of type I, IIA and IIX fibres they were grouped together for each breed (excluding the puppies) and an average CSA determined (Figure 4B). The shaded area of Figure 4B represents the range of CSA from previous human studies conducted in the same laboratory (Kohn et al., 2011, Kohn et al., 2007a).

The range of average CSA in the TB was 1030 μm^2 to 3898 μm^2 . The German pointer had the largest fibres of 3898 μm^2 with no other breed having a CSA of greater than 3000 μm^2 . The mixed breed (2078 $\mu\text{m}^2 \pm 510$) and the Labrador (2006 $\mu\text{m}^2 \pm 497$) had the second and third largest CSA values, respectively. As expected, the Yorkshire terrier had the smallest CSA of 1030 μm^2 followed by the miniature pinscher at 1205 $\mu\text{m}^2 \pm 228$.

The range of average CSA in the VL was 770 μm^2 to 2758 μm^2 . The boerboel had the largest CSA followed by the Labrador (2188 $\mu\text{m}^2 \pm 1034$), mixed breed (2163 $\mu\text{m}^2 \pm 831$) and Chow Chow (2161 μm^2). The breeds with the smallest CSA were the Yorkshire terrier (770 μm^2), miniature pinscher (867 $\mu\text{m}^2 \pm 384$) and Chihuahua (1237 μm^2).

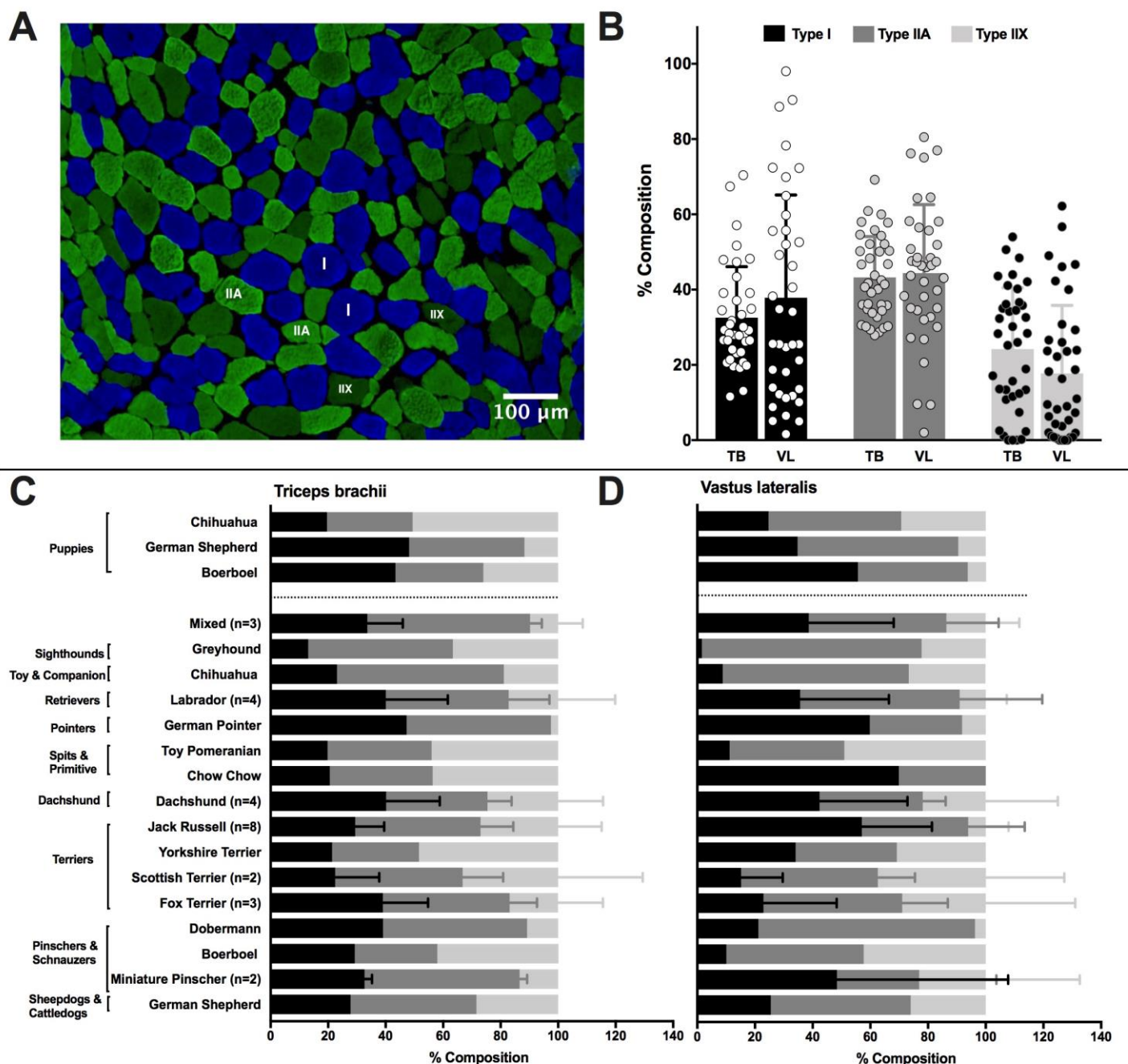
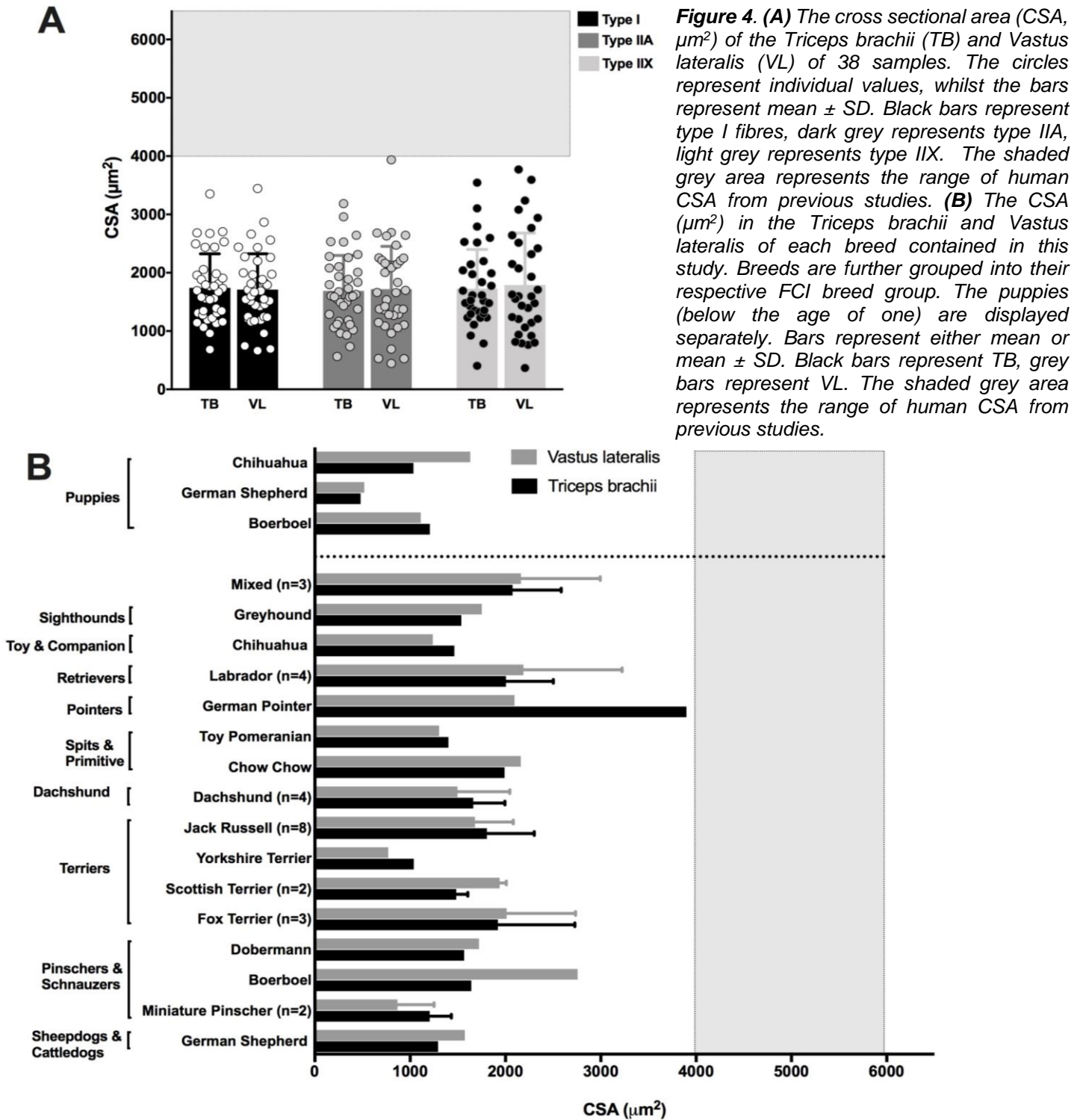


Figure 3. (A) Immunohistochemistry of a cross sectional cut of the Vastus lateralis in a dog sample. The blue fluorescence indicates type I fibres, the bright green fluorescence indicates type IIA and the lightly stained green indicates type IIX fibres. The image was captured at 10x magnification. (B) The fibre type composition (%) of the Triceps brachii (TB) and Vastus lateralis (VL) of 38 samples. The circles represent individual values, the bars represent mean \pm SD. Black bars represent type I fibres, dark grey represents type IIA, light grey represents type IIX. (C-D) The fibre type composition (%) in relation to overall total composition in the Triceps brachii and Vastus lateralis of each breed contained in this study, breeds are further grouped into their respective FCI breed group. The puppies (below the age of one) are displayed separately. Bars represent either mean or mean \pm SD. Black bars represent type I fibres, dark grey represents type IIA, light grey represents type IIX.

The three puppies included in the sample all had lower CSA compared to their respective adults. The Chihuahua had an average CSA of $1034 \mu\text{m}^2$ (TB) and $1630 \mu\text{m}^2$ (VL), respectively, while the boerboel had similar TB and VL values of $1207 \mu\text{m}^2$ and $1111 \mu\text{m}^2$ (VL). The German shepherd had the smallest CSA (TB: $479 \mu\text{m}^2$; VL: $519 \mu\text{m}^2$), but it was also the youngest animal (4 weeks).



3.3.3 Correlation between CSA and body weight

The correlation between the CSAs of the type I, IIA and IIX fibres and the weight of the animal was investigated using non-linear regression (Figure 5). The curve on the figure is representative of all the fibres grouped together. The TB has a stronger correlation ($r^2=0.40$) than the VL ($r^2=0.32$). Noticeably, the majority of the weight falls below 20 kg and, therefore, the data is clustered at the bottom of the graph.

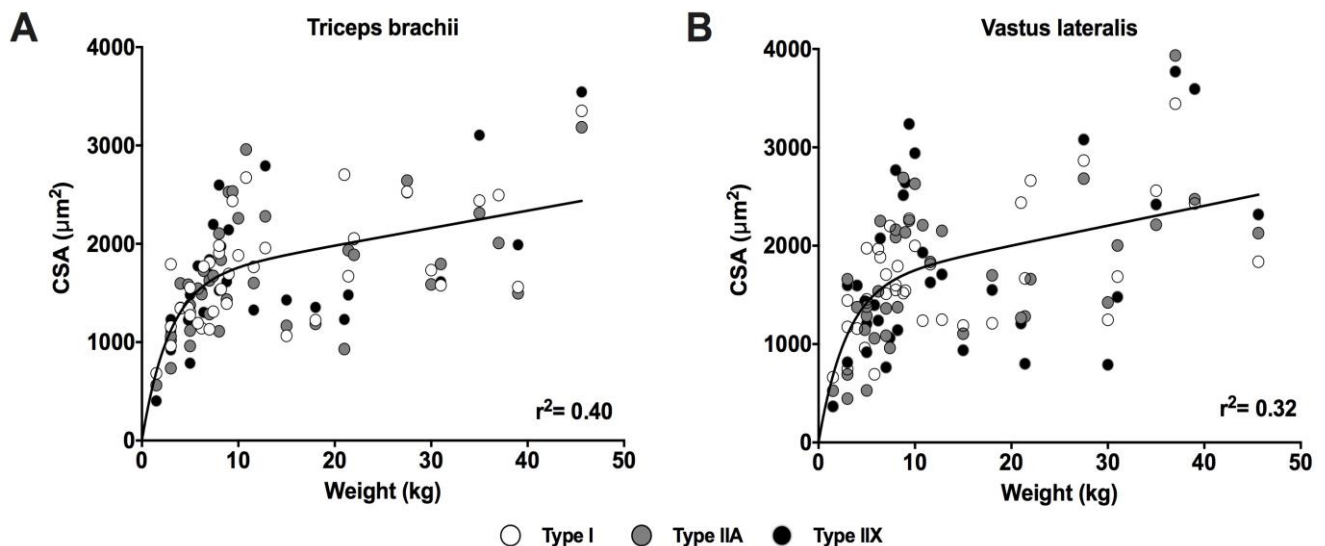


Figure 5. The correlations between CSA (μm^2) and animal weight in the (A) Triceps brachii and (B) Vastus lateralis. The circles represent type I (white), IIA (grey) and IIX (black) of each individual animal. The curve represents the non-linear regression of all fibre types grouped together.

3.3. Discussion

3.3.1. Fibre type composition

In this study, the predominant fibre type was the fast twitch oxidative type IIA fibre, followed by type I and IIX. This finding was evident in both the TB and VL muscle groups and is supported by previous research in dogs, including muscle groups such as the *Semitendinosus*, *Longissimus dorsi*, TB and VL (Acevedo and Rivero, 2006, Guy and Snow, 1981, Latorre et al., 1993, Strbenc et al., 2004, Toniolo et al., 2007). Acevedo and Rivero (2006) found that dog muscle fibres expressing MHC IIX were only found in the *Latissimus dorsi*, *Gluteus medius* and *Semitendinosus* (averaging 8% of the muscle fibres). Type IIX fibres were not found in the TB or *Vastus intermedius*. Acevedo and Rivero (2006) also found that hybrid IA and IIX comprised 28% of the total fibres across all muscle groups. Toniolo et al. (2007) used gel electrophoresis to determine the percentage of type IIX fibres in dog muscle. They found that type IIX fibres represented on average 22% of the five muscle groups investigated, but not all the muscle groups investigated expressed IIX fibres. The authors concluded that type IIX fibres are present in most, but not all limb muscles while type IIA fibres were the most abundant fibre type present. In the present study, 24% and 18% of the total fibres analysed were type IIX in the TB and VL, respectively (Figures 3B – D) and corresponds to the study by Toniolo et al. (2007). The major limitation of those studies was that very few fibres were studied in total across all muscle groups (Acevedo study: 1083 fibres; Toniolo study: 247 fibres) and that may account for the differences observed in the present study. A total of 52 000 fibres were counted for the TB and VL

across sixteen breeds of domestic dogs in the present study. There were limited number of hybrid fibres (IA and IIX) identified in the present study with only 5% identified in the TB and 6% in the VL. The low number of hybrid fibres may be due to the difficulty in identifying them through immunohistochemistry where type I, IIA and IIX fibres are clearly visible using the specific antibodies (Figure 3A). Alternatively, the high number of hybrid fibres may be due to inaccuracies in the Acevedo and Rivero (2006) study.

The proportions of type I, IIA and IIX fibres varied between the different FCI recognised groups as well as between the breeds. It is important to note that there were many breeds with a sample size of one (greyhound, Chihuahua, German pointer, Pomeranian, Chow Chow, Yorkshire terrier, Dobermann, boerboel and German shepherd) and therefore references to the results of these breeds are more indicative of a case study. From Figure 3C, the following breeds had higher proportions of type I and IIA fibres in the TB – Chihuahua, Labrador, German pointer, dachshund, fox terrier and Dobermann. While the breeds with a higher proportion of IIX fibres were the greyhound, Pomeranian, Chow Chow, Yorkshire terrier and boerboel.

However, this same pattern was not observed in the VL (Figure 3D), with Labrador, German pointer, Chow Chow, Jack Russell and miniature pinscher displaying on average a greater proportion of type I and IIA fibres. The Pomeranian, Scottish terrier and boerboel had higher proportions of type IIX fibres. Overall, the Labrador and German pointer had consistently higher numbers of type I and IIA fibres, which ties in well with their breed characteristic as Gundogs that have high endurance in order to retrieve game (FCI, 2019). The Pomeranian and boerboel seemed to have consistently higher proportions of type IIX fibres and matches the powerful characteristics of the latter breed, but is somewhat unexpected for the small Pomeranian (FCI, 2019). There were several dogs with thought-provoking fibre type profiles. In the VL, the greyhound only had 2% type I fibres, 76% type IIA and 22% type IIX fibres. This proportion is similar to previous reports on the VL of the greyhound of 96% type II fibres (IIA and IIX) – an expected composition due to their high athletic ability as race dogs (Guy and Snow, 1981). The Chow Chow had no type IIX fibres in the VL, but they were present in the TB. This was also seen in an individual dachshund, Jack Russell and miniature pinscher and the VL of a Labrador. The lack of type IIX fibres is likely due to the section of the skeletal muscle sample (Delp and Duan, 1996, Gunn, 1978) and is not necessarily indicative of a total lack of type IIX fibres in the muscle. Indeed, this hypothesis is confirmed in the analyses of the enzyme and MHC isoform content of a separate sample (Chapter 4). The MHC isoform content was investigated for each sample (data not shown) as an alternative method for fibre typing. There were high positive correlations between MHC isoform content and fibre type proportions for I, IIA and IIX respectively. This indicates that differences are not likely due to the method.

The Chihuahua, German shepherd and boerboel puppy had similar fibre type composition in the TB and VL. However, all three had different compositions to their respective adults – namely a higher percentage of type I fibres. This difference in fibre type, especially in the 4 week old German shepherd, may be due to their developing skeletal muscle (Schiaffino and Reggiani, 2011).

3.3.2. Fibre type and diabetes risk

In the comprehensive review by Hoenig (2014), the author used different epidemiological studies to identify which dog breeds were at a high or low risk for developing diabetes. According to Duan et al. (2017), fibre type composition is a potential risk factor in developing T2D. They proposed that the reprogramming of fibres to the more insulin sensitive type I fibres would be protective against the disease. Based on this concept, dogs with a higher proportion of type I fibres may be at a lower risk of developing T2D, while those with a lower proportion of type I fibres may be at a higher risk.

According to Hoenig (2014), the fox terrier (and potentially the Terrier group in general) have a higher incidence of T2D. In the present study, the fox terrier had a high proportion of type I fibres in the TB and a lower proportion in the VL (Figure 3C and D). This same pattern was observed for the Yorkshire terrier while the Jack Russell had a consistently higher percentage of type I fibres. Only the Scottish terrier had low type I fibres in both muscle groups. Based on these findings and the proposed hypothesis by Duan et al. (2017), it appears that only the Scottish terrier would be at an increased risk for developing T2D. The high risk category also included Samoyeds and Swedish elkhunds and lapphunds which form part of the Spits and Primitive Group (FCI, 2019, Hoenig, 2014). The present cohort did not contain those three breeds, but it did contain the Chow Chow and Pomeranian which fall into the same group. The Pomeranian had a lower proportion of type I fibres in the TB and VL, while the Chow Chow had a low proportion in the TB, but not in the VL. Interestingly, both dogs have approximately the same fibre type composition in the TB. This could indicate that dogs within this group are not as varied with their fibre type composition and as a result, fibre type composition may be a risk factor in their high T2D incidence.

Dogs at lower risk for developing T2D include the cocker spaniel and golden retriever (Retriever Group), and collie and German shepherd (Sheepdogs and Cattle dogs Group) (FCI, 2019, Hoenig, 2014). In this study, the Labrador forms part of the Retriever group and the German pointer forms part of the closely related Pointer Group. Both dog breeds had a high proportion of type I fibres and a very low proportion of IIX fibres. This finding suggests that the hypothesis proposed by Duan et al. (2017) may be true, in that a high proportion of type I fibres would lead to lower risk of T2D. However, the German shepherd had lower proportions of type I fibres compared to the Labrador and

German pointer. The dachshund and Jack Russell also had high proportions of type I fibres, however their risk for T2D is unknown.

There does seem to be trends surrounding fibre type and potentially correlating it to the incidence of diabetes, however, no definitive conclusion can be drawn from these findings. The results in this study are cross sectional in nature and the reasons why these animals were euthanased were also not disclosed. Ideally, the skeletal muscle composition should be investigated in a population of diabetic animals and healthy controls of the same age. Additionally, an intervention study to evaluate fibre type by inducing insulin resistance and T2D in an animal model would be better suited to prove or disprove the hypothesis that type I fibres decreases the risk of acquiring T2D. This may be an avenue for future studies.

3.3.3. Cross sectional area and correlations

The average fibre CSA determined in this study was $1729 \mu\text{m}^2$ across all fibre types in the TB and VL. There was no distinguishable difference in CSA between type I, IIA and IIX fibres across the 16 breeds. This value is slightly lower than what was reported by Acevedo and Rivero (2006) who investigated the CSA in different muscle groups in the dog. That study reported that the average CSA of type I and IIA fibres in the TB and *Vastus intermedius* was $2218 \mu\text{m}^2$ (I), $1446 \mu\text{m}^2$ (IIA) and $1925 \mu\text{m}^2$ (I), $2805 \mu\text{m}^2$ (IIA), respectively. The CSA of type IIX fibres was not determined in these muscle groups, but was $2357 \mu\text{m}^2$ in the *Latissimus dorsi*.

Comparatively, the CSA in the VL of humans are $5409 \mu\text{m}^2$ (I), $5174 \mu\text{m}^2$ (IIA) and $2968 \mu\text{m}^2$ (IIX) (Kohn et al., 2011). In that same study, the CSA of captive lions were $2014 \mu\text{m}^2$ (I), $2005 \mu\text{m}^2$ (IIA) and $3202 \mu\text{m}^2$ (IIX), while the caracal had similar CSAs as the lion, even though there is an approximately ten-fold difference in body weight between these two species. The CSA of the dog breeds in the present study were markedly smaller than humans and similar to that of captive lions and a caracal. As stated by Kohn et al. (2011), a smaller CSA does not necessarily mean that the muscle generates less power as this is dependent on other factors such as the number of muscle fibres recruited, fibre proportions and ATP supply. However, the present study did not investigate the skeletal muscle power production in the various breeds of the dog.

Based on Figure 5, it appears that there is a slight positive correlation between the weight of the dog and the CSA. Therefore, the larger the dog, the larger the CSA of the muscle fibres. The significance of fibre size in relation to the risk of developing insulin resistance is yet to be determined.

CHAPTER FOUR

4. SKELETAL MUSCLE METABOLISM

4.1. Background

The skeletal muscle of dogs have been shown to have a high oxidative capacity (Acevedo and Rivero, 2006, Gunn, 1978, Guy and Snow, 1981). Previous studies have used various enzymes as markers of oxidative and glycolytic capacity. In the present study, the markers of oxidative capacity include CS and 3HAD, which are marker enzymes of the Kreb's cycle and β -oxidation, respectively. CS is also a marker of mitochondrial content. CK and LDH act as markers of the high energy phosphate pathway and glycolytic capacity, respectively. This chapter aims to investigate the CS, 3-HAD, CK and LDH enzyme activities in the TB and VL of 16 breeds of domestic dogs.

4.2. Methodology

4.2.1. Sample

The maximum enzyme activities (CK, LDH, CS, 3HAD) were determined in the TB and VL muscle from 38 dogs.

4.2.2. Sample preparation for enzyme assays

Muscle samples were homogenised and prepared for enzyme analyses according Kohn et al. (2007b), with slight modifications. An amount of 10 to 20 mg wet weight muscle tissue was weighed. The samples were diluted to a ratio of 1:100 (1 mg muscle to 100 μ l buffer) with 0.1M potassium phosphate buffer, pH 7.3. The muscle piece was homogenised by hand using a glass homogeniser, followed by five times pulse sonication (Qsonica sonicators) for 10 seconds (one second pulse interval at 30% amplitude) on ice. See Appendix B for homogenising buffer preparation.

4.2.3. Homogenate protein concentration

The total protein concentration of each muscle homogenate was determined using the Bradford assay (Bradford, 1976). Bovine serum albumin (BSA) standards of 0 to 3 g/l (0.5 g/l increments) were used to construct a standard curve of known protein concentrations. Performing in duplicate, 5 μ l of each dog homogenate was pipetted into a clear 96-well microplate, followed by 250 μ l Bradford solution. The reaction was left to incubate for 5 minutes at room temperature, and the absorbance analysed at

595 nm using a microplate reader (BioTek Synergy HT). Using the BSA standard curve generated, the unknown protein concentration was calculated.

4.2.4. Fluorometric enzyme assays

The following enzymes were analysed fluorometrically to determine the maximum flux capacity through various metabolic pathways: CK, LDH, CS and 3HAD. The role of each of these enzymes have been described in chapter 1.

These enzyme assays were based on the conversion of NAD(P)H to NAD(P)⁺ (or vice versa) as the reaction progresses. Maximum enzyme activity was measured using fluorometry, in which the fluorescence of the reduced forms of NAD and NADP was determined at known time intervals. All reactions occurred at 25°C with an excitation and emission wavelength of 340 nm and 460 nm, respectively. The slope of fluorescence over time was calculated and converted to moles substrate using the NADH or NADPH standard curve. Maximum enzyme activity was expressed as $\mu\text{mol}/\text{min}/\text{g}$ protein. A detailed protocol for each assay is described in Appendix B.

4.2.5. Statistical analyses

Statistical calculations were performed using GraphPad Prism 7 (GraphPad Software Inc, USA). Values are expressed as mean \pm standard deviation and normality was checked with the Shapiro-Wilks normality test. The data were not normally distributed and a Kruskal- Wallis one-way ANOVA multiple comparison was used. The CK, LDH, CS, and 3HAD enzyme activities for all the animals combined were compared between the TB and VL. Statistical analysis was not performed between the different breeds as the sample sizes were too small. Significance was set at $P < 0.05$.

4.3. Results

4.3.1. Oxidative capacity

CS and 3-HAD activities are markers of oxidative capacity in the skeletal muscle of the various dog breeds (Figure 6A and B, Appendix B). In the TB and VL, the average CS activity across all the breeds was $61 \pm 16 \mu\text{mol}/\text{min g}$ protein and $49 \pm 13 \mu\text{mol}/\text{min}/\text{g}$ protein, respectively, while the average 3HAD activity was $53 \pm 14 \mu\text{mol}/\text{min}/\text{g}$ protein (TB) and $46 \pm 16 \mu\text{mol}/\text{min}/\text{g}$ protein (VL). There was no significant difference between the CS and 3HAD activities between the TB and VL. On the same figure, the average activities of recreationally active and endurance human athletes are shown for comparative purposes. This data was collected in the same laboratory and the average of six recreational and endurance athletes were used. These values are routinely used as reference points.

The CS activity of the recreationally active participants were 27 ± 7 $\mu\text{mol}/\text{min}/\text{g}$ protein, while the endurance athletes had more than double the amount at 57 ± 17 $\mu\text{mol}/\text{min}/\text{g}$ protein. The 3HAD activity of the same participants were 43 ± 12 $\mu\text{mol}/\text{min}/\text{g}$ protein (recreational) and 90 ± 15 $\mu\text{mol}/\text{min}/\text{g}$ protein (endurance).

The range of CS activities in the TB was 38 to 80 $\mu\text{mol}/\text{min}/\text{g}$ protein, while the range in the VL was similar at 33 to 66 $\mu\text{mol}/\text{min}/\text{g}$ protein. In the TB, the dachshund had the highest activity followed by the Dobermann (77 $\mu\text{mol}/\text{min}/\text{g}$ protein) and Scottish terrier (72 ± 21 $\mu\text{mol}/\text{min}/\text{g}$ protein), while the lowest activities were recorded for the Chow Chow, German pointer (43 $\mu\text{mol}/\text{min}/\text{g}$ protein) and Yorkshire terrier (44 $\mu\text{mol}/\text{min}/\text{g}$ protein). The breeds with the highest CS activities in the VL were the Dobermann, Chihuahua (64 $\mu\text{mol}/\text{min}/\text{g}$ protein) and greyhound (63 $\mu\text{mol}/\text{min}/\text{g}$ protein) and the breeds with the lowest activities were the German pointer, boerboel (36 $\mu\text{mol}/\text{min}/\text{g}$ protein) and miniature pinscher (37 ± 0.5 $\mu\text{mol}/\text{min}/\text{g}$ protein).

The range of 3HAD activities in the TB and VL were very similar at 29 to 78 $\mu\text{mol}/\text{min}/\text{g}$ protein and 28 to 73 $\mu\text{mol}/\text{min}/\text{g}$ protein, respectively. The Dobermann (TB: 78 $\mu\text{mol}/\text{min}/\text{g}$ protein; VL: 73 $\mu\text{mol}/\text{min}/\text{g}$ protein) and German shepherd (70 $\mu\text{mol}/\text{min}/\text{g}$ protein in both groups) had the highest activities in both muscle groups. The Chow Chow consistently had the lowest activities (TB: 29 $\mu\text{mol}/\text{min}/\text{g}$ protein; VL: 32 $\mu\text{mol}/\text{min}/\text{g}$ protein) along with the German pointer (TB: 43 $\mu\text{mol}/\text{min}/\text{g}$ protein) and boerboel (VL: 28 $\mu\text{mol}/\text{min}/\text{g}$ protein).

The boerboel, German shepherd and Chihuahua puppies had similar CS and 3-HAD activities compared to their corresponding adults. The Chihuahua (TB: 72 $\mu\text{mol}/\text{min}/\text{g}$ protein; VL: 45 $\mu\text{mol}/\text{min}/\text{g}$ protein) had a higher average CS activity than the German shepherd (TB: 44 $\mu\text{mol}/\text{min}/\text{g}$ protein; VL: 52 $\mu\text{mol}/\text{min}/\text{g}$ protein) and boerboel (TB: 50 $\mu\text{mol}/\text{min}/\text{g}$ protein; VL: 46 $\mu\text{mol}/\text{min}/\text{g}$ protein).

4.3.2. Glycolytic capacity

LDH and CK activities are markers of the glycolytic capacity as shown in Figure 6C and D and Appendix B. The average LDH activity across all dog breeds was 1550 $\mu\text{mol}/\text{min}/\text{g}$ protein ± 592 in the TB and 1478 $\mu\text{mol}/\text{min}/\text{g}$ protein ± 658 in the VL. In the TB and VL, the average CK activity was 6115 ± 1081 and 6279 ± 1529 $\mu\text{mol}/\text{min}/\text{g}$ protein, respectively. There was no significant difference between the LDH and CK activities between the TB and VL. Comparatively, the LDH activity of the recreationally active humans were 331 ± 165 $\mu\text{mol}/\text{min}/\text{g}$ protein and for the endurance athletes 557 ± 167 $\mu\text{mol}/\text{min}/\text{g}$ protein. These values are two to three times lower than the dog

activities. The CK activities of the recreationally active and endurance athletes were 5897 ± 1003 $\mu\text{mol}/\text{min}/\text{g}$ protein and $10\,573 \pm 1099$ $\mu\text{mol}/\text{min}/\text{g}$ protein, respectively.

The LDH activities of the different dog breeds had a range of 835 to 2514 $\mu\text{mol}/\text{min}/\text{g}$ protein and 891 to 3038 $\mu\text{mol}/\text{min}/\text{g}$ protein for the TB and VL, respectively. In the TB, the Pomeranian appears to have the highest activity followed by the Chihuahua (2296 $\mu\text{mol}/\text{min}/\text{g}$ protein) and Chow Chow (2289 $\mu\text{mol}/\text{min}/\text{g}$ protein), while the German pointer seems the lowest LDH activity followed by the fox terrier (1098 ± 278 $\mu\text{mol}/\text{min}/\text{g}$ protein) and miniature pinscher (1119 ± 115 $\mu\text{mol}/\text{min}/\text{g}$ protein). In the VL, the German shepherd had the highest activity (3038 $\mu\text{mol}/\text{min}/\text{g}$ protein) followed by the Yorkshire terrier (2469 $\mu\text{mol}/\text{min}/\text{g}$ protein) and the greyhound (2157 $\mu\text{mol}/\text{min}/\text{g}$ protein). The breeds with the lowest LDH activity were the German pointer (891 $\mu\text{mol}/\text{min}/\text{g}$ protein), Labrador (1095 ± 442 $\mu\text{mol}/\text{min}/\text{g}$ protein) and miniature pinscher (1101 ± 966 $\mu\text{mol}/\text{min}/\text{g}$ protein). The German pointer and miniature pinscher had consistently low LDH activities.

The activity range of CK was 4531 to 8134 $\mu\text{mol}/\text{min}/\text{g}$ protein (TB) and 4972 to 12 518 $\mu\text{mol}/\text{min}/\text{g}$ protein (VL). In the TB, the breeds with the highest activity were the Yorkshire terrier (8134 $\mu\text{mol}/\text{min}/\text{g}$ protein), dachshund (6684 ± 487 $\mu\text{mol}/\text{min}/\text{g}$ protein) and Chihuahua (6553 $\mu\text{mol}/\text{min}/\text{g}$ protein). The lowest CK activity belonged to the German pointer (4531 $\mu\text{mol}/\text{min}/\text{g}$ protein), Chow Chow (4817 $\mu\text{mol}/\text{min}/\text{g}$ protein) and German shepherd (5021 $\mu\text{mol}/\text{min}/\text{g}$ protein). Interestingly, the German shepherd had the highest activity in the VL of 12 518 $\mu\text{mol}/\text{min}/\text{g}$ protein, and was the fastest CK activity. This was followed by the Yorkshire terrier (7184 $\mu\text{mol}/\text{min}/\text{g}$ protein), Jack Russell (6977 ± 1518 $\mu\text{mol}/\text{min}/\text{g}$ protein) and Chihuahua (6976 $\mu\text{mol}/\text{min}/\text{g}$ protein). The breeds with the lowest activities were the German pointer (4972 $\mu\text{mol}/\text{min}/\text{g}$ protein), miniature pinscher (5151 ± 1618 $\mu\text{mol}/\text{min}/\text{g}$ protein) and fox terrier (5215 ± 869 $\mu\text{mol}/\text{min}/\text{g}$ protein). The Yorkshire terrier and Chihuahua had consistently higher CK activities, while the German pointer had consistently lower CK activity.

The puppies had lower LDH activity than their corresponding adults in the TB and VL. The Chihuahua (TB: 2390 $\mu\text{mol}/\text{min}/\text{g}$ protein; VL: 1569 $\mu\text{mol}/\text{min}/\text{g}$ protein) had the highest activity, followed by the German shepherd (TB: 1096 $\mu\text{mol}/\text{min}/\text{g}$ protein; VL: 1314 $\mu\text{mol}/\text{min}/\text{g}$ protein) and boerboel (TB: 1375 $\mu\text{mol}/\text{min}/\text{g}$ protein; VL: 896 $\mu\text{mol}/\text{min}/\text{g}$ protein). The CK activity of the puppies were higher than their corresponding adults in both muscle groups, with the TB having a higher activity than the VL. The Chihuahua (TB: 8706 $\mu\text{mol}/\text{min}/\text{g}$ protein; VL: 5736 $\mu\text{mol}/\text{min}/\text{g}$ protein), German shepherd (TB: 7712 $\mu\text{mol}/\text{min}/\text{g}$ protein; VL: 6896 $\mu\text{mol}/\text{min}/\text{g}$ protein) and boerboel (TB: 6999 $\mu\text{mol}/\text{min}/\text{g}$ protein; VL: 5889 $\mu\text{mol}/\text{min}/\text{g}$ protein).

■ Triceps brachii ■ Vastus lateralis ● Endurance athlete ● Recreationally active

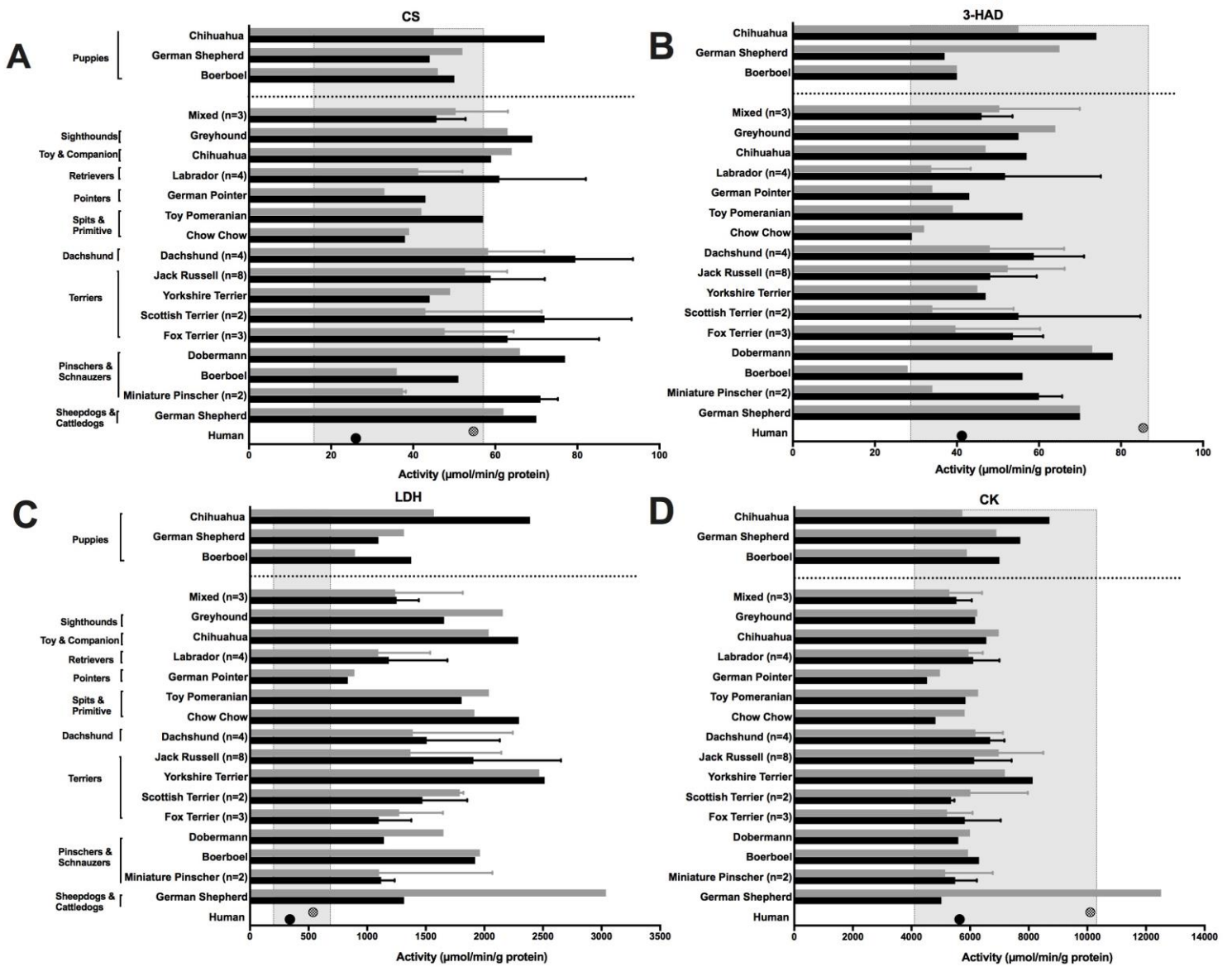


Figure 6. (A) CS, (B) 3-HAD, (C) LDH and (C) CK enzyme activities in the Triceps brachii (TB) and Vastus lateralis (VL) of each breed contained in this study. Breeds are further grouped into their respective FCI breed group. The puppies (below the age of one) are displayed separately. Bars represent either mean or mean \pm SD. Black bars represent TB, grey bars represent VL. The circles and shaded grey area represents the range of human enzyme activities from previous studies.

4.4. Discussion

4.4.1. Oxidative capacity

CS and 3-HAD act as markers of oxidative capacity with a higher activity indicating a higher flux through the respective pathway. The average activity in the TB and VL was very similar to one another, with no significant difference between the muscle groups. The range of recreationally active and endurance human athletes were included in the figures for comparative purposes. This unpublished data was collected in the same laboratory and used as reference guide. Endurance athletes

have nearly two times the CS and 3HAD activity of recreationally active humans, and conforms to published literature (Schiaffino and Reggiani, 2011). This indicates a higher oxidative capacity as there is an increased rate through the Krebs's cycle and β -oxidation, resulting in the muscle oxidising fat and carbohydrate as fuel in an efficient manner. The average CS activity across the dog breeds were similar to the endurance athletes, while the 3HAD activity was closer to that of the recreationally active humans. In comparison to wild animals, the dog has higher CS and 3HAD activity than the lion and caracal (Kohn et al., 2011), but lower activity than antelope species (Kohn, 2014). This finding indicates that dogs, overall, have a high oxidative capacity comparable to human endurance athletes, but not as high as antelope (such as the blesbok and springbok).

Across the different breeds, a wide range in CS and 3HAD activities exist. As mentioned in Chapter 3, it is important to note that there were many breeds with a sample size of one (greyhound, Chihuahua, German pointer, Pomeranian, Chow Chow, Yorkshire terrier, Dobermann, boerboel and German shepherd) and therefore references to the metabolic results of these breeds are more indicative of a case study. The Dobermann, German shepherd, greyhound, Scottish terrier and dachshund had the highest CS and 3HAD activities, on average, for the TB and VL. This would indicate that these breeds have a higher oxidative capacity and are able to more efficiently utilise the Krebs's cycle and β -oxidation to produce energy. For the German shepherd, Scottish terrier and dachshund, the higher oxidative capacity does match the function of these breeds (FCI, 2019). However, the Dobermann and greyhound are not considered endurance dogs but rather powerful and fast, respectively, thus a surprising find. The German pointer, Chow Chow and boerboel had overall low CS and 3HAD activities. This would indicate that these breeds have lower oxidative capacity that is closer to a recreationally active human. The boerboel is a powerful, muscular dog that would be expected to have a lower oxidative capacity (FCI, 2019). In contrast, the German pointer is considered a high endurance Gundog and would be expected to have a higher oxidative capacity as it also had one of the highest proportions of type I fibres in their muscles. In contrast, the results for CS and 3HAD were some of the lowest activities. The oxidative capacity of the Chow Chow matched the low proportion of type I fibres observed in Figure 3.

4.4.2. Glycolytic capacity

CK and LDH activity acts as a marker of the anaerobic capacity of the muscle, which is the ability to produce ATP rapidly without oxygen. The average LDH activity of the dog was three times higher than the endurance trained human, while the average CK activity was similar to recreationally active humans. In comparison to antelope species, the dog has a lower LDH activity and a similar CK activity (Kohn, 2014), but the felids (lion and caracal), have much higher LDH and CK activities

(Kohn et al., 2011). This would indicate that the dog has an increased ability to convert pyruvate to lactate under anaerobic conditions compared to endurance athletes, but a lower ability than antelope or felid species. This high glycolytic capacity is quite surprising considering the highly oxidative capacity and high proportions of type I and IIA fibres described in the previous chapter. However, the muscle fibre type does not necessarily define the oxidative or glycolytic capacity of the skeletal muscle, with other factors such as exercise training playing a role or merely genetics (Kohn et al., 2007a). The training and health status of the dogs included in this study were unknown at the time of euthanasia and could have potentially affected the results.

The different breeds had a variety of CK and LDH activities. Notably, the CK activity of the German shepherd in the VL was double the activity in the TB and almost double the average activity of the samples. The LDH activity in the VL was also double the activity of the TB. The explanation is not known and needs further investigation. Alternatively, this could indicate that German shepherds have a very high glycolytic capacity, which would link to the characteristics of this breed. It is considered a “high achievement working dog” with good endurance, speed and power (FCI, 2019). Other breeds with overall high CK and LDH activities include the greyhound, Chihuahua and Yorkshire terrier. This high glycolytic capacity is aligned with the bred for speed characteristic of the greyhound. The Yorkshire terrier and Chihuahua are very small dogs which are energetic, but have limited endurance (FCI, 2019), and may account for the higher glycolytic capacity in these breeds.

The breeds with generally lower CK and LDH activities were the German pointer, fox terrier and miniature pinscher. Interestingly, the German pointer had overall low oxidative and glycolytic capacity. This low glycolytic ability does align with the supposedly high endurance capabilities of this breed (FCI, 2019) as well as the low proportions of type IIX and IIA fibres found in this study. The original function of the Fox Terrier and Miniature Pinscher was to hunt vermin so they are not necessarily powerful dogs and would not need to produce rapid energy through glycolysis.

4.4.3. Muscle metabolism and T2D risk

Previous studies have shown that, along with fibre type composition being a potential risk factor, the metabolism of the muscle may be a risk factor for T2D (Oberbach et al., 2006, Simoneau and Kelley, 1997). These studies showed that human patients with T2D had a higher ratio of glycolytic to oxidative enzyme capacity compared to healthy controls. From the results of this study, it is clear that dogs have an inherently high oxidative capacity. Certain breeds (the Dobermann, German shepherd, greyhound, Scottish terrier and dachshund) had a greater capacity than other breeds. According to Hoenig (2014), the German shepherd is believed to have a lower risk of diabetes, which potentially

could be related to its high oxidative capacity because it did not have a particularly high proportion of type I fibres. The risk profile of the other breeds in relation to T2D is unfortunately unknown.

The German pointer, Chow Chow and boerboel had the lowest oxidative capacity of the various breeds. Interestingly, the German pointer had very high proportions of type I fibres, but some of the lowest oxidative capacity. However, this breed also had low glycolytic enzyme activity, which may balance the ratio of glycolytic to oxidative capacity. The Chow Chow falls under the Spits and Primitive Group, which have several breeds at high risk for diabetes (Samoyed, Swedish elkhund and lapphund) (FCI, 2019, Hoenig, 2014). Although the risk of the Chow Chow is not known, it does seem to have a low oxidative capacity and falls into a group with high risk breeds. The fox terrier is believed to have a high risk for diabetes. In this study, it had an average oxidative capacity and a higher proportion of type IIA fibres. The metabolism and fibre type of the fox terrier does not seem to align with its risk for diabetes. Although exercise is the preferred prescribed preventative measure to reduce the risk of acquiring insulin resistance and diabetes in humans, the dogs appear to all have a genetically moderate to high oxidative capacity. Thus, overall, it is difficult to establish whether the oxidative capacity associates with the risk for diabetes in dogs and requires data from diabetic dogs.

CHAPTER FIVE

5. CONCLUSION

Diabetes is becoming an increasing concern in the human population, with an estimated 422 million people living with the disease worldwide (WHO, 2016a). A similar pattern, although with a lower prevalence, has been observed in the domestic animal populations, primarily in cats and dogs (Fall et al., 2007, McCann et al., 2007). These species are believed to be excellent models to study obesity and diabetes in humans, since they share the same environment as humans and develop the disease spontaneously (Osto and Lutz, 2015). Interestingly, different dog breeds have been shown to be at a higher or lower risk for developing T2D (Hoenig, 2014). It appears that Samoyeds, Swedish elkhound and lapphund, fox terriers, Australian terriers, miniature and standard Schnauzers, pugs and poodles are at a higher risk, while American pitbull terriers, cocker spaniels, collies, golden retrievers, German shepherds and boxers are at lower risk of developing diabetes. With over 300 breeds recognised by the FCI and countless other breeds not recognised, the susceptibility of many breeds are unknown.

Due to the important role of skeletal muscle in the regulation of glucose in the body, it has been proposed that the re-programming of type II to type I muscle fibres may be a potential therapeutic avenue for the treatment of obesity and T2D (Duan et al., 2017). Therefore, this study aimed to characterise the skeletal muscle fibre composition and metabolism of different breeds of domestic dogs and to relate the fibre type composition with breeds that are reported to have a higher or lower risk of diabetes.

Type IIA fibres were shown to be the most prevalent fibre type in the breeds analysed, followed by type I and then type IIX. However, there was some variability across the different breeds, which may be attributed to their function and lifestyle. Breeds, such as the German pointer, dachshund, Labrador and miniature pinscher had much higher proportions of type I fibres compared to the greyhound, Pomeranian and boerboel. Additionally, the dogs analysed in this study seemed to have a higher oxidative capacity than glycolytic capacity, which links to their higher proportions of type I and IIA fibres, conforming to previous studies (Acevedo and Rivero, 2006, Snow et al., 1982, Strbenc et al., 2004, Toniolo et al., 2007). Breeds with a higher oxidative capacity includes the Dobermann, German shepherd, greyhound, Scottish terrier and dachshund. Notably, the specific breeds with a higher proportion of type I fibres do not necessarily have the highest oxidative capacity. Muscle fibre type does not solely dictate the metabolic properties of the muscle (Kohn et al., 2007a). In the study by Kohn et al (2007), Xhosa runners had a higher LDH activity in their type I and IIA fibres (LDH is

generally higher in type IIA) compared to their Caucasian counterparts. This could highlight the effect that training and lifestyle have on the metabolism of the muscle. Therefore, factors such as function and lifestyle of dogs may account for their high oxidative capacity, and not solely fibre type. Of course, genetics of the different breeds should not be ruled out, but would require a more in depth approach of analyses.

The fibre CSAs of the dogs were similar to that found in a previous study (Acevedo and Rivero, 2006). Notably, the fibres were markedly smaller than humans, but similar to lion and caracal fibre CSA (Kohn et al., 2011). There was a slight correlation between the weight of the dogs and their CSAs, with heavier dogs having a greater CSA. It would have been better to correlate muscle mass with CSA, as body weight itself includes too many factors (such as fat, organ size, etc) that can skew the results. The fibre CSA does not solely determine the power of the muscle, with the number of fibres recruited, fibre proportions and ATP supply playing a role (Kohn et al., 2011). However, the present study did not investigate power production in the skeletal muscle. Notably, oxidative fibres tended to be smaller than fibres that are more glycolytic, conforming to what was found in the present study (Kohn, 2014).

Unfortunately this study did not contain many breeds that have been classified as having a high or low risk of diabetes. The fox terrier (high risk) and German shepherd (low risk) were the only classified breeds in the current cohort. The fox terrier was the only dog that had a low proportion of type I fibres in the VL and overall, had an average oxidative and low glycolytic capacity. This does not conclusively align with the hypothesis proposed by Duan et al. (2017). The German shepherd, on the other hand, had a higher proportion of type I and IIA fibres with a high oxidative capacity, which potentially aligns with the hypothesis. No definitive conclusions can be drawn from the results although, theoretically, fibre type composition could potentially be a factor playing a role in the predisposition to diabetes mellitus in dogs. Due to the apparent genetically moderate to high oxidative capacity of the dog breeds, it is difficult to establish the association between oxidative capacity and the risk of diabetes in each breed.

In summary, this study was able to characterise the skeletal muscle of 16 breeds (including mixed breed) of the domestic dog. The strength of this study is that, for the first time, the muscle fibre type and metabolism of a large cohort of dog breeds were studied, providing for a solid foundation for future research on the health of these animals.

Limitations and Future directions

There were a few limitations to the study. First, muscle sampling was opportunistic – thus, the breed, sex and age of the samples could not be controlled for or anticipated. This resulted in many of the breeds having only one or two study animals (and therefore more indicative of a case study) with males and females being grouped together. Small sample sizes are not uncommon in this type of research (Acevedo and Rivero, 2006, Strbenc et al., 2004) with the novelty of this study being the diversity of breeds investigated. A second limitation was that the clinical reasons for euthanasia of the animals were not disclosed to the researcher. Many of the animals were older than 10 years, and as a result, “old age” was often recorded as the reason for euthanasia. This may have implications on the results, specifically the metabolism of the muscle and muscle fibre size. The results of this study are cross sectional in nature and include no known diabetic animals, therefore, it is difficult to draw definitive conclusions in relation to diabetes, fibre type and metabolism.

The present study has formed a foundation for future studies on the role of skeletal muscle fibre type composition and metabolism in dog diabetes. Going forward, skeletal muscle composition should be investigated in a population of diabetic animals and healthy controls of preferably the same age. Additionally, an intervention study to evaluate fibre type and metabolism by inducing insulin resistance and T2D in the dog would be better suited to prove or disprove the hypothesis that a predominance in type I fibres decreases the risk of acquiring T2D. Future studies should aim to include a greater sample size of each breed, so that statistical analyses can be conducted between the breeds. Finally, blood glucose regulation, mitochondrial function, insulin receptor integrity (e.g. IRS-1) and GLUT4 content should be investigated, because this will provide a clearer picture of oxidative capacity and insulin sensitivity, respectively, of the skeletal muscle. Due to the inherently high oxidative nature of the dog, it would also be beneficial to investigate the oxidative and glycolytic enzyme activity in each fibre type through single fibre techniques.

APPENDIX A

IMMUNOHISTOCHEMISTRY PROTOCOL

Reagents and antibodies

1. Acetone
2. Phosphate buffered Saline (PBS) 0.15M pH 7.4. In 1L PBS: 8g NaCl (0.14M), 1.26g Na₂HPO₄ (8.8mM), 0.2g KCl (2.7mM), 0.2g KH₂PO₄ (1.5mM), made up to 1L with distilled H₂O.
3. 5% Donkey serum made up in PBS.
4. Primary Antibodies: Monoclonal mouse BA-D5 IgG2b against MHC I, Monoclonal mouse SC-71 IgG1 against MHC IIA (lightly binds IIX in canine muscle). Antibodies purchased from Developmental Studies Hybridoma Bank (Iowa, USA)

Secondary antibodies: Polyclonal AMCA goat anti-mouse IgG2b (catalogue number: 115-155-207), Polyclonal AlexaFlour 488 goat anti-mouse IgG1 (catalogue number: 115-545-205). Antibodies purchased from Jackson ImmunoResearch Laboratories (Pennsylvania, USA).

Method

Slides were removed from -20°C storage and allowed to air dry before being fixed with acetone for 2 minutes. Slides were then hydrated in PBS for 2 minutes and allowed to incubate in 5% donkey serum (50 µL per section) for 1 hour at room temperature in a humidifying box. Following the blocking step, slides were incubated with a 1:50 dilution (in PBS) of BA-D5 and SC-71 primary antibody (50 µL per section) overnight at 4°C in a humidifying box. The next day, the slides were dunked and washed twice in PBS for 2 minutes. The following steps occurred in the dark. Slides were incubated with a 1:250 dilution (in PBS) of AMCA and AlexaFlour secondary antibody (50 µL per section) for 2 hour at room temperature in a humidifying box. After the slides were washed twice in PBS for 2 minutes, they were mounted with 30 µL and stored at 4°C until visualisation.

Sections were visualised with a fluorescent microscope (Nikon Eclipse 80i, New York, USA) and photographs taken of a selected section at 10x magnification using a digital camera (Canon EOS 650D, Pretoria, South Africa). Sections were small and all fit into the 10x magnification field of view. The number of each fibre type was counted for a selected section and expressed as a percentage of the total fibres. An average of 700 fibres were counted and typed per section. The cross sectional area (CSA) of a maximum of 50 fibres were determined for each fibre type per section using the computer programme ImageJ (version 1.50e, Maryland, USA). The image was broken up into quintiles (five areas) and the CSA of 10 fibres of each fibre type in each section was determined and averaged, this was to ensure that the entire muscle section was represented.

APPENDIX B

HOMOGENISING BUFFER

Stock solutions:

A. KH_2PO_4 0.1 M Mw 136.1

0.6804 g / 50 ml dH_2O

B. K_2HPO_4 0.1 M Mw 174.2

0.871 g / 50 ml dH_2O

Add 30 ml of buffer B to a small beaker and place pH probe into that solution. Use buffer A and adjust buffer to pH 7.30. This buffer can be stored at -20°C .

ENZYME ASSAY PROTOCOL AND DATA

All the enzyme assays described below use the principle of fluorometry. The measured fluorescence comes from reduced forms of NAD and NADP. The reaction can either in itself cause the increase or decrease (e.g. $\text{NADH} + \text{H}^+$), or be coupled to such a reaction. During analysis, the fluorescence is measured at known time intervals and difference per minute is calculated. Knowing the protein concentration, and its dilution, the enzyme activity, expressed as $\mu\text{mol}/\text{min}/\text{g}$ protein, is calculated. The fluorescence is measured at a sensitivity of 100.

NADH and NADPH Standard curve

Na_2CO_3 : 0.08 M; Mw 105.99; 0.848 g

NaHCO_3 : 0.02 M; Mw 84.01; 0.168 g

Make up to 100 ml dH_2O . This buffer is used to make up NADH and NADPH.

NADH: ± 5 mM; Mw 709.3

36 mg dissolve in 10 ml buffer

Heat NADH solution for 10 min in a water bath at 60°C to destroy NAD^+

NADPH: ± 5 mM; Mw 833.4

25 mg dissolve in 6 ml buffer

Heat NADPH solution for 10 min in a water bath at 60°C to destroy NADP^+

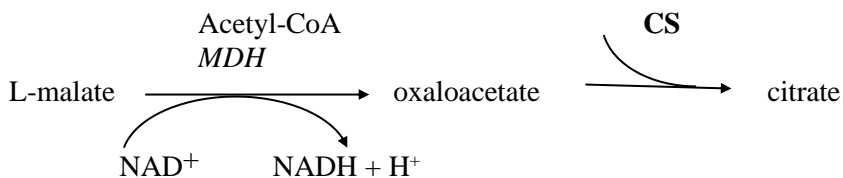
Generating NADH or NADPH standard curve:

1. Use black plates. Make sure the fluorometer is switched on for at least 20 minutes. Set the excitation wavelength to 340/11 nm and the emission wavelength to 460/40 nm.
2. Read the background fluorescence of each well.
3. Dilute the original NADH standard 11 times (1:10) with dH_2O . Dilute the original NADPH standard 3 times (1:2) with dH_2O .

4. Pipette 0, 2, 4, 6, 8, 10 μL volumes in duplicate into each well.
5. Add 250 μl Tris or Imidazole buffer to each well and read. The final concentration of the buffer is dependent on the concentration required in the respective assay.
6. Subtract each background value from each NADH or NADPH read.
7. Subtract the blank value from the rest of the measurements.
8. Draw a graph with the fluorescence values on the Y-axis and NADH or NADPH concentration (μM) on the X-axis.
9. Determine the slope, expressed as fluorescent units / μM

Citrate Synthase

Reaction:



Stock solutions:

Tris-buffer 1 M, pH 8.0 ; Mw 121.1 (Sigma T-1503)
12.11g / 100 ml dH_2O ; adjust pH

EDTA 0.1 M ; Mw 372.2 (Sigma E-5134)
3.722 g / 100 ml dH_2O

NAD^+ 0.1 M; Mw 663.4 (Roche 127 965)
69 mg / 1 ml dH_2O
Freeze at -80°C in aliquots

L-malate 0.1 M; Mw 156.1 (Sigma M-1125)
78 mg / 5 ml dH_2O
Freeze at -80°C in aliquots

Acetyl-CoA 3 mM; Mw 809.6 (Sigma A-2056)
Freeze at -80°C in aliquots

Dissolve 5 mg in 2 ml dH_2O , exact amount depending on analysis of each batch as indicated on vial. This will result in the [final] in 260 μl to be 60 μM .

Malate dehydrogenase (MDH) 5 mg/ml (Roche 127 256)

Reagent solution

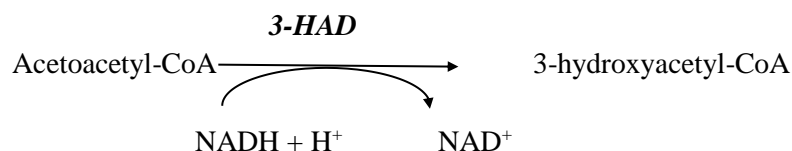
		25 ml:	50 ml:	120ml:	[Final]
Tris buffer	1 M, pH 8.0	2.5 ml	5.00 ml	12 ml	0.1 M
EDTA	0.1 M	625 μ l	1.25 ml	3.0 ml	2.5 mM
NAD ⁺	0.1 M	125 μ l	250 μ l	600 μ l	0.5 mM
L-malate	0.1 M	250 μ l	500 μ l	1.2 ml	1.0 mM
MDH	5 mg/ml	40 μ l	80 μ l	192 ml	8 μ g/ml

Fill up with dH₂O to desired volume. **Check pH 8.0**

Add 250 μ L of reagent solution, 10 μ L of dog homogenate, 5 μ L Acetyl-CoA to each well in duplicate. The assay runs for 3 minutes, use the maximum slope to determine Fl/min. Use NADH standard curve, as previously described, with 0.1M Tris.

3-Hydroxyacetyl CoA Dehydrogenase (3-HAD)

Reaction:



Stock solutions:

Imidazole buffer 1 M, pH 7.0; Mw 68.1 (Sigma I-0125)
6.8g / 100 ml dH₂O; adjust pH

EDTA 0.1 M ; Mw 372.2 (Sigma E-5134)
3.722 g / 100 ml dH₂O

NADH 0.1 M; Mw 709.4 (Roche 107 735)
17.7 mg / 250 μ l dH₂O
Freeze at -80°C wrapped in tin foil for not longer than 2 months.

Acetoacetyl-CoA 1 mM; Mw 971.6 (Sigma A-1625)

Dissolve 5 mg in approximately 1 ml dH₂O (4.9 mM). Dilute this to 1 mM aliquots and freeze at -80°C for later use. This will result in the [final] in 260 μ l to be 20 μ M.

Reagent solution

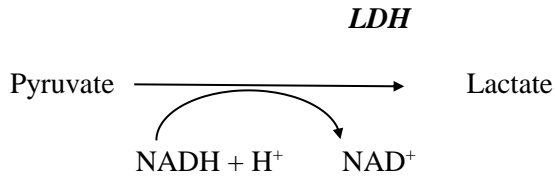
		25 ml:	50 ml:	120ml:	[Final]
Imidazole buffer	1 M	1.25 ml	2.5 ml	6 ml	50 mM
EDTA	0.1 M	1 ml	2 ml	4.8 ml	4 mM
NADH	0.1 M	7.5 μ l	15 μ l	36 μ l	30 μ M

Fill up with dH₂O to desired volume. **Check pH 7.0**

Add 250 μ L of reagent solution, 5 μ L of dog homogenate, 5 μ L Acetoacetyl-CoA to each well in duplicate. The assay runs for 5 minutes, use the maximum slope to determine Fl/min. Use NADH standard curve with 50 mM Imidazole.

Lactate Dehydrogenase (LDH)

Reaction:



Stock solutions:

Tris-buffer 1 M, pH 8.0 ; Mw 121.1 (Sigma T-1503)
12.11g / 100 ml dH₂O; adjust pH

EDTA 0.1 M ; Mw 372.2 (Sigma E-5134)
3.722 g / 100 ml dH₂O

NADH 0.1 M; Mw 709.4 (Roche 107 735)
17.7 mg / 250 μ l dH₂O
Freeze at -80°C wrapped in tin foil for not longer than 2 months.

Pyruvate 1.0 M; Mw 110.0 (Roche 128 147)
220 mg / 2 ml dH₂O
Freeze at -80°C in aliquots. [Final] will be 2 mM in 260 μ l

Reagent solution

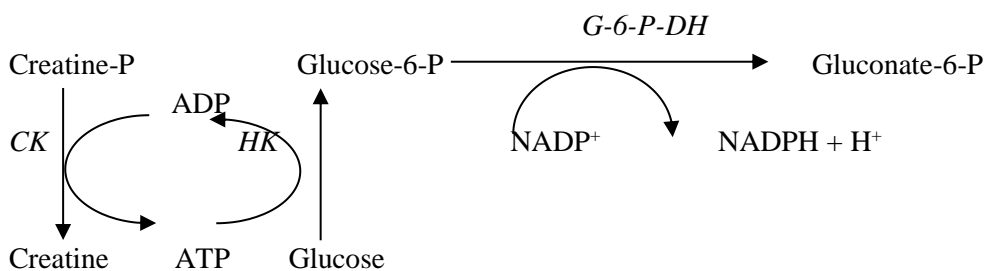
		25 ml:	50 ml:	120ml:	[Final]
Tris buffer	1.0 M	1.25 ml	2.5 ml	6 ml	50 mM
EDTA	0.1 M	1 ml	2 ml	4.8 ml	4 mM
Pyruvate	1.0 M	50 μ l	100 μ l	240 μ l	2mM
NADH	0.1 M	15 μ l	30 μ l	72 μ l	60 μ M

Fill up with dH₂O to desired volume. **Check pH 7.6**

Add 250 μ L of reagent solution, 2 μ L of dog homogenate to each well in duplicate. The assay runs for 2 minutes, use the maximum slope to determine FI/min. Use NADH standard curve with 50 mM Tris.

Creatine Kinase (CK)

Reaction:



Stock solutions:

Imidazole-EDTA-Mg-acetate buffer	EDTA 0.149 g; Imidazole 1.361 g; Magnesium acetate 0.429 g Dissolve in 150 ml dH ₂ O and pH to 6.7 with HCl.
Glucose	0.5 M; Mw 916.4 (Sigma G-7528) 915 mg/ 2 ml dH ₂ O
ADP	0.5 M; Mw 427.2 (Sigma A-2754) 213 mg/ 1 ml dH ₂ O
AMP	0.5 M; Mw 427.2 (Sigma A-2754) 213 mg/ 1 ml dH ₂ O
Creatine phosphate	0.5 M; Mw 327.2 (Roche 621722) 327 mg/ 2 ml dH ₂ O
Diadenosine Pentaphosphate (DAPP)	10 mg/ml Mw 916.4 (Sigma D4022)
NADP ⁺	0.1 M; Mw 787.2 (Roche 128058) 79 mg/ 1 ml dH ₂ O
N-Acetylcysteine (NAc)	0.5 M Mw 163.2 (Sigma A-7250) 82 mg/ 1 ml dH ₂ O
Hexokinase	1500 U/ml (Roche 1426362)
Glucose-6-phosphate dehydrogenase	1750 U/ml (Roche 127655)

Reagent solution

		25 ml:	50 ml:	120ml:	[Final]
Imidazole-EDTA-Mg buffer	pH 6.7	7.5 ml	15 ml	90 ml	
Glucose	0.5 M	80 µl	160 µl	960 µl	4 mM
ADP	0.5 M	40 µl	80 µl	480 µl	2 mM
AMP	0.5 M	100 µl	200µl	1.2 ml	5 mM
Creatine phosphate	0.5 M	600 µl	1.2 ml	7.2 ml	30 mM
DAPP	11 mM	9.15 µl	18.3 µl	110 µl	10 µM
NADP ⁺	0.1 M	80 µl	160 µl	960 µl	800 µM
NAc	0.5 M	400 µl	800 µl	4.8 ml	20 mM
QS to desired volume using dH ₂ O. This reagent can be frozen at -20 °C. Before use, add the following:					
Hexokinase	1500 U/ml	16.7 µl	33.3 µl	200 µl	2.5 U/ml
Glucose-6-phosphate DH	1750 U/ml	8.6 µl	17.1 µl	103 µl	1.5 U/ml

Fill up with dH₂O to desired volume. **Check pH 6.7**

Add 250 μ L of reagent solution, 0.5 μ L of dog homogenate to each well in duplicate. Alternatively samples can be diluted to 1:800. The assay runs for 6 minutes, use the maximum slope to determine FI/min. Use NADPH standard curve with 50 mM Imidazole.

Table 2. Mean CS, 3-HAD, LDH and CK activity for 38 dog samples and comparative human data

	Dog Enzyme Activity (μ mol/min/g protein)		Human Enzyme Activity (μ mol/min/g protein)	
	Triceps brachii (n=38)	Vastus lateralis (n=38)	Recreationally active (n=6)	Endurance athlete (n=6)
CS	61 \pm 16	49 \pm 13	27 \pm 7	57 \pm 17
3-HAD	53 \pm 14	46 \pm 16	43 \pm 12	90 \pm 15
LDH	1550 \pm 592	1478 \pm 658	331 \pm 165	557 \pm 167
CK	6115 \pm 1081	6279 \pm 1529	5897 \pm 1003	10573 \pm 1099

Data expressed as mean \pm SD

REFERENCES

1. ABIRU, N., KAWASAKI, E. & EGUCHI, K. 2002. Current knowledge of Japanese type 1 diabetic syndrome. *Diabetes/metabolism research and reviews*, 18, 357-366.
2. ACCURSO, A., BERNSTEIN, R. K., DAHLQVIST, A., DRAZNIN, B., FEINMAN, R. D., FINE, E. J., GLEED, A., JACOBS, D. B., LARSON, G. & LUSTIG, R. H. 2008. Dietary carbohydrate restriction in type 2 diabetes mellitus and metabolic syndrome: time for a critical appraisal. *Nutrition and Metabolism*, 5, 9.
3. ACEVEDO, L. M. & RIVERO, J. L. 2006. New insights into skeletal muscle fibre types in the dog with particular focus towards hybrid myosin phenotypes. *Cell and Tissue Research*, 323, 283-303.
4. AKRAM, M. 2014. Citric acid cycle and role of its intermediates in metabolism. *Cell Biochemistry and Biophysics*, 68, 475-8.
5. AMERICAN DIABETES, A. 2010. Diagnosis and classification of diabetes mellitus. *Diabetes Care*, 33 Suppl 1, S62-9.
6. ANDERSEN, P. & HENRIKSSON, J. 1977. Training induced changes in the subgroups of human type II skeletal muscle fibres. *Acta Physiologica Scandinavica*, 99, 123-125.
7. APOP. 2015. *Association for Pet Obesity Prevention. 2015 Obesity Risks and Facts* [Online]. Available: <http://petobesityprevention.org/pet-obesity-fact-risks> [Accessed 10 April 2017].
8. BANTING, F., BEST, C. H., COLLIP, J. B., CAMPBELL, W. R. & FLETCHER, A. A. 1922. Pancreatic extracts in the treatment of diabetes mellitus. *Canadian Medical Association Journal*, 12, 141.
9. BECKMAN, J. A., CREAGER, M. A. & LIBBY, P. 2002. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *Journal of American Medical Association*, 287, 2570-2581.
10. BELLINGER, D. A., MERRICKS, E. P. & NICHOLS, T. C. 2006. Swine models of type 2 diabetes mellitus: insulin resistance, glucose tolerance, and cardiovascular complications. *Institute for Laboratory Animal Research Journal*, 47, 243-258.
11. BOTTINELLI, R. 2001. Functional heterogeneity of mammalian single muscle fibres: do myosin isoforms tell the whole story? *Pflugers Archiv: European Journal of Physiology*, 443, 6-17.
12. BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Journal of Analytical Biochemistry*, 72, 248-254.
13. BRADSHAW, J. W. 2006. The evolutionary basis for the feeding behavior of domestic dogs (*Canis familiaris*) and cats (*Felis catus*). *The Journal of Nutrition*, 136, 1927S-1931S.
14. BROOKE, M. H. & KAISER, K. K. 1970. Three "myosin adenosine triphosphatase" systems: the nature of their pH lability and sulfhydryl dependence. *Journal of Histochemistry and Cytochemistry*, 18, 670-672.
15. BUFF, P., CARTER, R., BAUER, J. & KERSEY, J. 2014. Natural pet food: A review of natural diets and their impact on canine and feline physiology. *Journal of Animal Science*, 92, 3781-3791.
16. CATCHPOLE, B., KENNEDY, L. J., DAVISON, L. J. & OLLIER, W. E. 2008. Canine diabetes mellitus: from phenotype to genotype. *Journal of Small Animal Practice*, 49, 4-10.
17. CRANE, S., COWELL, C., STOUT, N., MOSER, E., MILLICAN, J., ROMANO, P. & CRANE, S. 2010. Commercial pet foods. *Small Animal Clinical Nutrition*, 157-190.
18. CURRY, J. W., HOHL, R., NOAKES, T. D. & KOHN, T. A. 2012. High oxidative capacity and type IIx fibre content in springbok and fallow deer skeletal muscle suggest fast sprinters with a resistance to fatigue. *Journal of Experimental Biology*, 215, 3997-4005.
19. DANEMAN, D. 2006. Type 1 diabetes. *The Lancet*, 367, 847-858.

20. DAUGAARD, J. R. & RICHTER, E. A. 2004. Muscle- and fibre type-specific expression of glucose transporter 4, glycogen synthase and glycogen phosphorylase proteins in human skeletal muscle. *Pflugers Archiv: European Journal of Physiology*, 447, 452-6.
21. DAVISON, L., HERRTAGE, M. & CATCHPOLE, B. 2005. Study of 253 dogs in the United Kingdom with diabetes mellitus. *Veterinary Record-English Edition*, 156, 467-471.
22. DEFRONZO, R. A. & TRIPATHY, D. 2009. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes Care*, 32 Suppl 2, S157-63.
23. DELA, F., PRATS, C. & HELGE, J. W. 2014. Exercise interventions to prevent and manage type 2 diabetes: physiological mechanisms. *Med Sport Sci*, 60, 36-47.
24. DELP, M. D. & DUAN, C. 1996. Composition and size of type I, IIA, IID/X, and IIB fibers and citrate synthase activity of rat muscle. *Journal of Applied Physiology*, 80, 261-70.
25. DOGTIME 2019. DogTime Media, Inc, California, USA. <http://www.dogbreedlist.info>.
26. DUAN, Y., LI, F., TAN, B., YAO, K. & YIN, Y. 2017. Metabolic control of myofibers: promising therapeutic target for obesity and type 2 diabetes. *Obesity Reviews*.
27. FALL, T., HAMLIN, H. H., HEDHAMMAR, Å., KÄMPE, O. & EGENVALL, A. 2007. Diabetes Mellitus in a Population of 180,000 Insured Dogs: Incidence, Survival, and Breed Distribution. *Journal of Veterinary Internal Medicine*, 21, 1209-1216.
28. FCI 2019. Fédération Cynologique Internationale, Thuin, Belgium. <http://www.fci.be>.
29. FEINMAN, R. D., POGOZELSKI, W. K., ASTRUP, A., BERNSTEIN, R. K., FINE, E. J., WESTMAN, E. C., ACCURSO, A., FRASSETTO, L., GOWER, B. A. & MCFARLANE, S. I. 2015. Dietary carbohydrate restriction as the first approach in diabetes management: critical review and evidence base. *Nutrition*, 31, 1-13.
30. FRACASSI, F., PIETRA, M., BOARI, A., ASTE, G., GIUNTI, M. & FAMIGLI-BERGAMINI, P. 2004. Breed distribution of canine diabetes mellitus in Italy. *Veterinary Research Communications*, 28, 339-342.
31. FRAYN, K. N. 2009. *Metabolic regulation: a human perspective*, John Wiley & Sons.
32. FRONTERA, W. R. & OCHALA, J. 2015. Skeletal muscle: a brief review of structure and function. *Calcified Tissue International*, 96, 183-95.
33. FRY, C. S., NOEHREN, B., MULA, J., UBELE, M. F., WESTGATE, P. M., KERN, P. A. & PETERSON, C. A. 2014. Fibre type-specific satellite cell response to aerobic training in sedentary adults. *Journal of Physiology*, 592, 2625-35.
34. GILOR, C., NIESSEN, S. J., FURROW, E. & DIBARTOLA, S. P. 2016. What's in a Name? Classification of Diabetes Mellitus in Veterinary Medicine and Why It Matters. *Journal of Veterinary Internal Medicine*, 30, 927-40.
35. GUNN, H. 1978. Differences in the histochemical properties of skeletal muscles of different breeds of horses and dogs. *Journal of Anatomy*, 127, 615.
36. GUY, P. & SNOW, D. 1981. Skeletal muscle fibre composition in the dog and its relationship to athletic ability. *Research in Veterinary Science*, 31, 244-248.
37. HEDHAMMAR, A. A. & INDREBO, A. 2011. Rules, regulations, strategies and activities within the Federation Cynologique Internationale (FCI) to promote canine genetic health. *Journal of Veterinary Science*, 189, 141-6.
38. HENNESSEY, J. V., CHROMIAK, J. A., DELLAVENTURA, S., GUERTIN, J. & MACLEAN, D. B. 1997. Increase in percutaneous muscle biopsy yield with a suction-enhancement technique. *Journal of Applied Physiology*, 82, 1739-1742.
39. HEWSON-HUGHES, A. K., HEWSON-HUGHES, V. L., COLYER, A., MILLER, A. T., MCGRANE, S. J., HALL, S. R., BUTTERWICK, R. F., SIMPSON, S. J. & RAUBENHEIMER, D. 2013. Geometric analysis of macronutrient selection in breeds of the domestic dog, *Canis lupus familiaris*. *Behavioral Ecology*, 24, 293-304.

40. HILL'S 2018. Hill's Pet Nutrition, Inc., Topeka, KS, USA. <https://www.hillspet.co.za>.
41. HOCQUETTE, J., ORTIGUES-MARTY, I., PETHICK, D., HERPIN, P. & FERNANDEZ, X. 1998. Nutritional and hormonal regulation of energy metabolism in skeletal muscles of meat-producing animals. *Livestock Production Science*, 56, 115-143.
42. HOENIG, M. 2014. Carbohydrate metabolism and pathogenesis of diabetes mellitus in dogs and cats. *Progress in Molecular Biology and Translational Science*, 121, 377-412.
43. HOENIG, M., REUSCH, C. & PETERSON, M. E. 2000. Beta cell and insulin antibodies in treated and untreated diabetic cats. *Veterinary Immunology and Immunopathology*, 77, 93-102.
44. HOUTEN, S. M., VIOLANTE, S., VENTURA, F. V. & WANDERS, R. J. 2016. The Biochemistry and Physiology of Mitochondrial Fatty Acid beta-Oxidation and Its Genetic Disorders. *Annual Review of Physiology*, 78, 23-44.
45. HULL, R. L., WESTERMARK, G. T., WESTERMARK, P. & KAHN, S. E. 2004. Islet amyloid: a critical entity in the pathogenesis of type 2 diabetes. *Journal of Clinical Endocrinology and Metabolism*, 89, 3629-43.
46. IDF 2017. *International Diabetes Federation. IDF Diabetes Atlas 8th edition. ISBN 978-2-930229-87-4*
47. KAHN, S. E., COOPER, M. E. & DEL PRATO, S. 2014. Pathophysiology and treatment of type 2 diabetes: perspectives on the past, present, and future. *The Lancet*, 383, 1068-1083.
48. KC 2019. The Kennel Club, The Kennel Club Limited, London, England. <https://www.thekennelclub.org.uk>.
49. KENNEDY, L. J., DAVISON, L. J., BARNES, A., SHORT, A. D., FRETWELL, N., JONES, C. A., LEE, A. C., OLLIER, W. E. & CATCHPOLE, B. 2006. Identification of susceptibility and protective major histocompatibility complex haplotypes in canine diabetes mellitus. *Tissue Antigens*, 68, 467-76.
50. KING, A. J. 2012. The use of animal models in diabetes research. *British Journal of Pharmacology*, 166, 877-94.
51. KOHN, T. A. 2014. Insights into the skeletal muscle characteristics of three southern African antelope species. *Biology Open*, 3, 1037-44.
52. KOHN, T. A., BURROUGHS, R., HARTMAN, M. J. & NOAKES, T. D. 2011. Fiber type and metabolic characteristics of lion (*Panthera leo*), caracal (*Caracal caracal*) and human skeletal muscle. *Comparative Biochemistry and Physiology Part A*, 159, 125-33.
53. KOHN, T. A., ESSEN-GUSTAVSSON, B. & MYBURGH, K. H. 2007a. Do skeletal muscle phenotypic characteristics of Xhosa and Caucasian endurance runners differ when matched for training and racing distances? *Journal of Applied Physiology*, 103, 932-40.
54. KOHN, T. A., HOFFMAN, L. C. & MYBURGH, K. H. 2007b. Identification of myosin heavy chain isoforms in skeletal muscle of four Southern African wild ruminants. *Comparative Biochemistry and Physiology Part A*, 148, 399-407.
55. KUSA 2016. Kennel Union of Southern Africa, Cape Town, South Africa. <https://www.kusa.co.za>.
56. LAI, M., CHANDRASEKERA, P. C. & BARNARD, N. D. 2014. You are what you eat, or are you? The challenges of translating high-fat-fed rodents to human obesity and diabetes. *Nutrition and Diabetes*, 4, e135.
57. LATORRE, R., GIL, F., VAZQUEZ, J., MORENO, F., MASCARELLO, F. & RAMIREZ, G. 1993. Skeletal muscle fibre types in the dog. *Journal of Anatomy*, 182, 329.
58. LEFAUCHEUR, L. 2010. A second look into fibre typing--relation to meat quality. *Meat Science*, 84, 257-70.
59. LILLIOJA, S., YOUNG, A. A., CULTER, C. L., IVY, J. L., ABBOTT, W., ZAWADZKI, J. K., YKI-JÄRVINEN, H., CHRISTIN, L., SECOMB, T. W. & BOGARDUS, C. 1987. Skeletal muscle capillary density and fiber type

- are possible determinants of in vivo insulin resistance in man. *The Journal of Clinical Investigation*, 80, 415-424.
60. MATVEYENKO, A. V. & BUTLER, P. C. J. I. J. 2006. Islet amyloid polypeptide (IAPP) transgenic rodents as models for type 2 diabetes. *ILAR Journal*, 47, 225-233.
 61. MCCANN, T. M., SIMPSON, K. E., SHAW, D. J., BUTT, J. A. & GUNN-MOORE, D. A. 2007. Feline diabetes mellitus in the UK: the prevalence within an insured cat population and a questionnaire-based putative risk factor analysis. *Journal of Feline Medicine and Surgery*, 9, 289-299.
 62. MCGREEVY, P., THOMSON, P., PRIDE, C., FAWCETT, A., GRASSI, T. & JONES, B. 2005. Prevalence of obesity in dogs examined by Australian veterinary practices and the risk factors involved. *Veterinary Record-English Edition*, 156, 695-701.
 63. MECH, L. D. & BOITANI, L. 2010. *Wolves: behavior, ecology, and conservation*, University of Chicago Press.
 64. MERING, J. V. & MINKOWSKI, O. 1890. Diabetes mellitus nach Pankreasexstirpation. *Archiv für experimentelle Pathologie und Pharmakologie*, 26, 371-387.
 65. NELSON, R. W. & REUSCH, C. E. 2014. Animal models of disease: classification and etiology of diabetes in dogs and cats. *Journal of Endocrinology*, 222, T1-9.
 66. NRC. 2006. *National Research Council. Nutrient requirements of dogs and cats*, National Academies Press.
 67. OBERBACH, A., BOSSENZ, Y., LEHMANN, S., NIEBAUER, J., ADAMS, V., PASCHKE, R., SCHÖN, M. R., BLÜHER, M. & PUNKT, K. 2006. Altered fiber distribution and fiber-specific glycolytic and oxidative enzyme activity in skeletal muscle of patients with type 2 diabetes. *Diabetes Care*, 29, 895-900.
 68. OJUKA, E. O. & GOYARAM, V. 2014. Mechanisms in exercise-induced increase in glucose disposal in skeletal muscle. *Diabetes and Physical Activity*. Karger Publishers.
 69. OSTO, M. & LUTZ, T. A. 2015. Translational value of animal models of obesity-Focus on dogs and cats. *European Journal of Pharmacology*, 759, 240-52.
 70. PEDIGREE 2014. Pedigree, Mars Australia Pty Ltd, Wodonga, Victoria, Australia <http://www.pedigree.com.au>.
 71. PROTHERO, J. 1995. Bone and fat as a function of body weight in adult mammals. *Comparative Biochemistry and Physiology Part A: Physiology*, 111, 633-639.
 72. RAND, J. S., FLEEMAN, L. M., FARROW, H. A., APPLETON, D. J. & LEDERER, R. 2004. Canine and Feline Diabetes Mellitus: Nature or Nurture? *The Journal of Nutrition*, 134, 2072S-2080S.
 73. RESNICK, H. E., SHORR, R. I., KULLER, L., FRANSE, L. & HARRIS, T. B. 2001. Prevalence and clinical implications of American Diabetes Association-defined diabetes and other categories of glucose dysregulation in older adults: the health, aging and body composition study. *Journal of Clinical Epidemiology*, 54, 869-876.
 74. RESNIKOFF, S., PASCOLINI, D., ETYA'ALE, D., KOCUR, I., PARARAJASEGARAM, R., POKHAREL, G. P. & MARIOTTI, S. P. 2004. Global data on visual impairment in the year 2002. *Bulletin of the World Health Organization*, 82, 844-851.
 75. SCHIAFFINO, S. & REGGIANI, C. 2011. Fiber types in mammalian skeletal muscles. *Physiological Reviews*, 91, 1447-531.
 76. SCHLESINGER, D. P. & JOFFE, D. J. 2011. Raw food diets in companion animals: a critical review. *The Canadian Veterinary Journal*, 52, 50.
 77. SHAI, I., JIANG, R., MANSON, J. E., STAMPFER, M. J., WILLETT, W. C., COLDITZ, G. A. & HU, F. B. 2006. Ethnicity, obesity, and risk of type 2 diabetes in women: a 20-year follow-up study. *Diabetes Care*, 29, 1585-90.
 78. SIMONEAU, J.-A. & KELLEY, D. E. 1997. Altered glycolytic and oxidative capacities of skeletal muscle contribute to insulin resistance in NIDDM. *Journal of Applied Physiology*, 83, 166-171.

79. SMITH, U. 2002. Impaired ('diabetic') insulin signaling and action occur in fat cells long before glucose intolerance--is insulin resistance initiated in the adipose tissue? *International Journal of Obesity and Related Metabolic Disorders*, 26, 897-904.
80. SNOW, D., BILLETTER, R., MASCARELLO, F., CARPENE, E., ROWLERSON, A. & JENNY, E. 1982. No classical type IIB fibres in dog skeletal muscle. *Histochemistry and Cell Biology*, 75, 53-65.
81. SOLA-PENNA, M., DA SILVA, D., COELHO, W. S., MARINHO-CARVALHO, M. M. & ZANCAN, P. 2010. Regulation of mammalian muscle type 6-phosphofructo-1-kinase and its implication for the control of the metabolism. *IUBMB Life*, 62, 791-6.
82. STEPHENSON, G. M. Hybrid skeletal muscle fibres: a rare or common phenomenon? Australian Physiological and Pharmacological Society, 2001. Citeseer, 69.
83. STRBENC, M., SMERDU, V., ZUPANC, M., TOZON, N. & FAZARINC, G. 2004. Pattern of myosin heavy chain isoforms in different fibre types of canine trunk and limb skeletal muscles. *Cells Tissues Organs*, 176, 178-86.
84. STUMVOLL, M., GOLDSTEIN, B. J. & VAN HAEFTEN, T. W. 2005. Type 2 diabetes: principles of pathogenesis and therapy. *The Lancet*, 365, 1333-1346.
85. STUMVOLL, M., TATARANNI, P. A., STEFAN, N., VOZAROVA, B. & BOGARDUS, C. 2003. Glucose allostasis. *Diabetes*, 52, 903-909.
86. TESCH, P., THORSSON, A. & ESSEN-GUSTAVSSON, B. 1989. Enzyme activities of FT and ST muscle fibers in heavy-resistance trained athletes. *Journal of Applied Physiology*, 67, 83-87.
87. TONIOLO, L., MACCATROZZO, L., PATRUNO, M., PAVAN, E., CALIARO, F., ROSSI, R., RINALDI, C., CANEPARI, M., REGGIANI, C. & MASCARELLO, F. 2007. Fiber types in canine muscles: myosin isoform expression and functional characterization. *American Journal of Physiology - Cell Physiology*, 292, C1915-26.
88. TRAPPE, S., HARBER, M., CREER, A., GALLAGHER, P., SLIVKA, D., MINCHEV, K. & WHITSETT, D. 2006. Single muscle fiber adaptations with marathon training. *Journal of Applied Physiology*, 101, 721-727.
89. VILÀ, C. & LEONARD, J. A. 2001. Canid phylogeny and origin of the domestic dog. *The Genetics of the Dog*, 1-11.
90. WAGNER, J. D., KAVANAGH, K., WARD, G. M., AUERBACH, B. J., HARWOOD JR, H. J. & KAPLAN, J. R. 2006. Old world nonhuman primate models of type 2 diabetes mellitus. *Institute for Laboratory Animal Research Journal*, 47, 259-271.
91. WAGNER, K. H. & BRATH, H. 2012. A global view on the development of non communicable diseases. *Preventive Medicine*, 54 Suppl, S38-41.
92. WALLIMANN, T. 1994. Bioenergetics: dissecting the role of creatine kinase. *Current Biology*, 4, 42-46.
93. WHO 2016a. Global report on diabetes WHO Geneva. WHO Press.
94. WHO 2016b. Obesity and overweight (Fact sheet N 311): WHO; 2011. h ttp.
95. WILCOX, G. 2005. Insulin and insulin resistance. *Clinical Biochemist Reviews*, 26, 19-39.
96. ZHANG, P., ZHANG, X., BROWN, J., VISTISEN, D., SICREE, R., SHAW, J. & NICHOLS, G. 2010. Global healthcare expenditure on diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice*, 87, 293-301.
97. ZICKER, S. C. 2008. Evaluating pet foods: how confident are you when you recommend a commercial pet food? *Topics in Companion Animal Medicine*, 23, 121-6.