Epidemiological, pathological and metabolomic characterisation of an acquired myopathy of dogs in New Zealand

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Abstract

'Go Slow' myopathy (GSM) is an idiopathic myopathy in dogs in New Zealand, characterised by an acute onset of trembling, weakness and collapse, followed by a prolonged period of exercise intolerance. In the first part of this thesis, the epidemiology of the disease was investigated using a telephone survey to obtain information regarding the diet, exercise, and health of affected dogs. Eighty-six confirmed cases were included in this study, and ingestion of wild pig in the week prior to the onset of clinical signs was a consistent finding (76/86 dogs; 88%; 95% confidence interval = 82 – 95%). Cases occurred most commonly in the upper North Island, particularly in Northland.

The aim of the second part of this thesis was to characterise the pathology of GSM in the same 86 dogs included in the epidemiological study, using serum biochemistry (78 dogs), histology (20 dogs), and electron microscopy (4 dogs). Acutely, affected dogs had increased serum creatine kinase and aspartate aminotransferase activities, corresponding with the histological finding of skeletal muscle degeneration in the absence of inflammation. Ultrastructural changes in skeletal muscle included mitochondrial hypertrophy, intramitochondrial inclusions and increased sarcoplasmic glycogen. Similar lesions were observed in the skeletal muscle of wild pigs from areas where GSM occurred in dogs. Affected dogs also had increased serum alanine aminotransferase activities due to accumulation of lipid and glycogen in hepatocytes. Overall, the microscopic findings were consistent with a toxic myopathy.

To further investigate the pathogenesis of the disease, liver samples were collected from 15 affected dogs and 24 clinically normal dogs for untargeted metabolic profiling using liquid chromatography-mass spectrometry. Comparison of spectra between affected and normal dogs revealed a widespread decrease in phospholipids, and increases in selected dicarboxylic acids and N-acetylated branch chain amino acids in affected dogs. No causative compounds were identified although several candidate mass spectrometric features were identified for future investigation.

Taken together, the results of these studies suggest that ‘Go Slow’ myopathy is a toxic mitochondrial myopathy in dogs that is associated with the ingestion of wild pork. The findings reported aid in the prevention, diagnosis, and management of cases, with the primary suggestion being that owners avoid feeding wild pork in areas where the myopathy occurs. Further work is required to elucidate the cause of this disease.

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Abbreviations

AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
ChE	Cholesteryl ester
Cer	Ceramide
CK	Creatine kinase
cf-DNA	Cell-free DNA
DG	Diglyceride
EM	Electron microscopy
EMG	Electromyography
FC	Fold change
FDR	False discovery rate
GRMD	Golden Retriever muscular dystrophy
GPx	Glutathione peroxidase
HE	Haematoxylin and eosin stain
LC	Liquid chromatography
miRNA	Micro-RNA
MPI	Ministry for Primary Indurstries
MRI	Magnetic resonance imaging
neg	Negative ionisation mode
NMR	Nuclear magnetic resonance spectroscopy
MS	Mass spectrometry
PA	Phosphatidic acid
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
pos	Positive ionisation mode
PS	Phosphatidylserine
SM	Sphingomyelin
TG	Triglyceride
m/z	Mass to charge ratio
rt	Retention time

Chapter 1

Background and review of the literature

1.1 Background

In 2004, the Ministry for Primary Industries (MPI, formerly the Ministry of Agriculture and Forestry) investigated a 'mystery disease' that was first recognised in the late 1990s in pig dogs and working dogs in Northland, New Zealand.[1, 2] The case definition in this investigation included dogs with an acute onset of tiring rapidly with exercise, trembling/shivering, weakness, collapse and an inability to jump. Clinical signs developed within minutes to hours of exercise and resolved with rest and supportive care, although in some cases there was a prolonged return to fitness for hunting or work. Mortality was very low. Consistent clinical pathology findings in affected dogs included increased serum creatine kinase (CK), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, indicative of muscle damage.

At the time of these initial investigations, the overall prevalence of the disease was low, and most veterinary practices in Northland reported seeing only 2 – 4 cases of this 'mystery disease' per year. However, the prevalence on a single property could be high, with multiple dogs affected simultaneously. The MPI investigation included 46 affected dogs on 6 different properties, 5 of which were in Northland and one in Taupo. Dogs used for pig hunting were most commonly affected (30 cases), but working farm dogs were also at risk (16 cases). No age or sex predilections were reported, and most of the dogs were crosses of two or more breeds. Owners were questioned about the dogs' diet, hunting activity, concurrent diseases and any animal health products used, but this did not reveal any apparent risk factors for the development of the disease. Most cases occurred over the winter months, particularly June and July.

It was concluded that the disease was consistent with a myopathy induced or exacerbated by exercise, but a concurrent peripheral neuropathy or other neuromuscular disease could not be excluded. A list of differential diagnoses was compiled, and a toxic aetiology was considered most likely. The clinical findings were not consistent with any diseases exotic to New Zealand, and as a result no further testing or research was pursued at this time.

Since then, there have been sporadic reports of this myopathy,[3] but the disease has received very little attention from the veterinary profession in New Zealand. Anecdotal information from pig hunters and dog owners suggests that the prevalence and geographic spread of this disease might have increased since the 2004 investigation, but owners do not always seek veterinary care for affected dogs as there is no specific treatment. Within the veterinary and pig hunting communities, the disease has become commonly known as 'Go Slow', named for the exercise intolerance that is a characteristic feature in both acute and chronic cases. Throughout this thesis, the disease is referred to as 'Go Slow myopathy' (GSM) as the name 'Go Slow,' although colloquial, is widely recognised by veterinarians and pig hunters in New Zealand, and myopathy describes the characteristic pathological feature of the disease. Without a greater understanding of the disease there is no good, descriptive alternative, but it is hoped that in future, it can be renamed using terms that better reflect the epidemiology and pathology the disease.

In 2014, a cluster of cases in Okaihau, a small town in Northland, New Zealand, was reported by a veterinarian, which prompted renewed research interest in the myopathy. The following chapters detail epidemiological, pathological and metabolomic investigations of 'Go Slow' myopathy that took place between 2014 and 2017. The aim of these studies was to characterise the pathogenesis of the disease and investigate possible causes in order to address the paucity of knowledge surrounding this myopathy. This chapter will summarise the structure, function and metabolism of skeletal muscle in health, which is integral to understanding how it responds to injury and disease. Diagnostic tests used in the investigation of myopathies will be discussed, followed by an overview of general causes of myopathies in dogs.

1.2 Skeletal muscle in health

1.2.1 Structure of skeletal muscle

Skeletal muscle is a highly organised tissue that plays important roles in posture and locomotion, as well as amino acid and whole body metabolism.[4] Individual skeletal muscle cells are referred to as myofibres or myocytes, and each is surrounded by a cell membrane or sarcolemma, with the cytoplasm referred to as the sarcoplasm.[5] During development, each myofibre forms from the fusion of multiple myoblast cells, resulting in each myofibre having multiple nuclei located peripherally in the cell.[6] Although myofibres are post-mitotic and growth of skeletal muscle is adults is by hypertrophy only,[7] skeletal muscle is able to regenerate following injury due to the presence of satellite cells, which are the adult stem cells of skeletal muscle[5] and are able to proliferate and differentiate into new muscle fibres.[8] Following muscle injury or disease, satellite cells are activated by myogenic factors such as myoD, a protein that binds to regulatory regions of some muscle specific genes.[9]

In each individual myofibre, there are repeating contractile units called sarcomeres. At each end, the sarcomere is bounded by a Z line, from which thin actin filaments extend longitudinally in both directions (Figure 1.1). Each thin filament is composed of strands of actin arranged in a double stranded helix, wound together with strands of tropomyosin.[10] Complexes of a third protein, troponin, are also associated with the thin filaments and play a vital role in muscle contraction through their calcium binding activities.[11] Between the actin filaments in the centre of the sarcomere, there are thick myosin filaments. Small projections, named myosin heads, extend from the filaments and are able to bind ATP and actin in the process of muscle contraction. Where actin and myosin filaments overlap within a sarcomere, the muscle appears more electron dense, forming the A band. Near the Z lines, there are only actin filaments so the muscle appears lighter on electron microscopy, giving rise to the I band. In the centre of the sarcomere is the M line, and it is in this region that proteins such as creatine kinase are found (discussed later in this chapter).[12] Two other proteins, titan and nebulin, are also important in aspects of muscle function such as passive tension, stiffness and cell signalling.[5] Titan attaches to myosin and the Z disk to help stabilise thick filaments, and may also play a role in the generation of force during muscle

contraction.[13] Nebulin binds to actin filaments and aids in the regulation of thick-thin filament overlap and muscle contraction.[14]

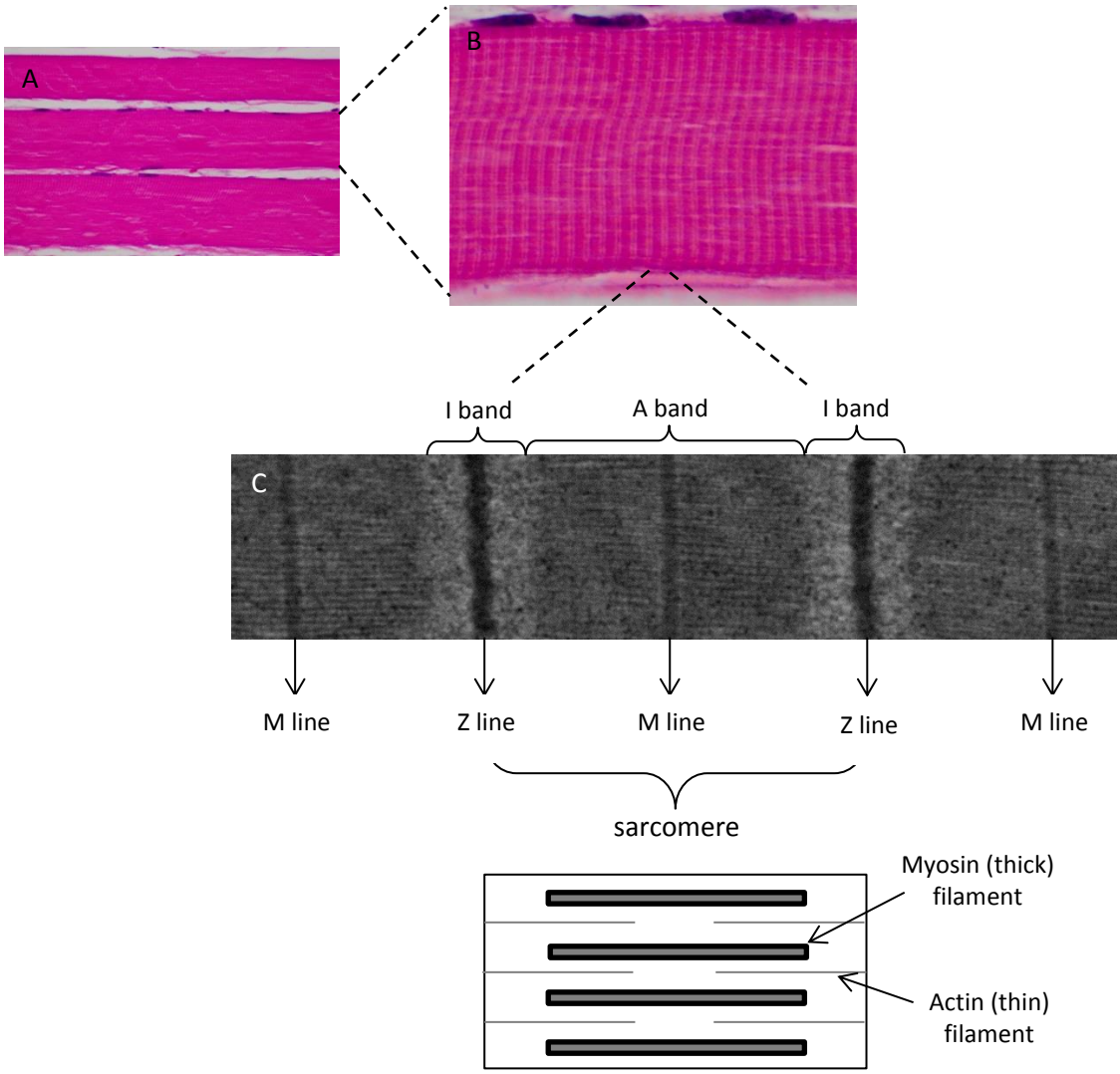

Figure 1.1: Basic structure of skeletal muscle. **A)** Three myofibres in longitudinal section, HE 200x. **B)** Individual myofibre, HE 200x. Note striations are visible at this magnification, and three peripherally located nuclei at the top of the image **C)** Individual myofibril, electron microscopy.

Other specialised features of skeletal muscle include the sarcoplasmic reticulum and transverse tubular system (T tubules). The sarcoplasmic reticulum lies beneath the sarcolemma and plays an important role in the storage, release and reuptake of calcium ions needed for muscle contraction.[5] The T tubules are invaginations of the sarcolemma that form a complex and dense network within a myofibre. They function in the propagation of action potentials, ensuring that all sarcomeres within a myofibre are stimulated to contract at the same time.[15]

Within a muscle, not all myofibres are the same. Skeletal muscle fibres can be categorised into specific fibre types based on their major myosin heavy chain component.[16] Type I ("slow twitch") fibres have slow rates of contraction, a high number of mitochondria and rely heavily on oxidative phosphorylation for energy production.[17, 18] These fibres are common in postural muscles and are relatively resistant to fatigue. This is in contrast to Type II ("fast twitch") fibres that are common in muscles involved in locomotion or directional movement. Type II fibres contract rapidly and have a high capacity for glycolysis. In many species, type II fibres can be further subdivided into two or three subtypes that differ in their oxidative capacity and resistance to fatigue,[16, 18] but these subtypes are not identifiable in dogs.[19] In general, the speed of contraction of a myofibre is related to the extent of sarcoplasmic reticulum development, while the oxidative capacity and fatigability is a function of myofibre mitochondrial content.[5]

In many aspects, skeletal muscle is similar to cardiac muscle, as both are types of striated muscle and there are enzymes, proteins and structures common to both. However, skeletal and cardiac muscle arise from different mesodermal progenitor cells in the embryo[20] and myopathies affecting the skeletal muscle of dogs are frequently not associated with any lesions in cardiac muscle.[21-24] Salient features of cardiac muscle that differ from skeletal muscle include the number of mitochondria (higher in cardiac muscle as it must function continuously without rest periods), [25] development of transverse tubular systems (which are wider and have an additional longitudinal component in cardiac muscle)[26] and the presence of intercalated discs (which function in the transmission of action potentials and are unique to cardiac muscle).[27] From a clinical perspective when investigating myopathies, it is important to note that cardiac myocytes are considered to be terminally-differentiated, post-mitotic cells and damage to the myocardium is irreversible,[28] in contrast to skeletal muscle which has capacity for regeneration.

1.2.2 Contraction of skeletal muscle

The translation of neuronal impulses into muscle contraction requires sequential activation of a number of voltage-gated ion channels that control the propagation of action potentials.[4] Muscle contraction is initiated when an action potential reaches the neuromuscular junction.[11] Activation of acetylcholine receptors causes depolarisation and opening of sodium ion channels in the junctional sarcolemma, generating an action potential in the muscle fibre which spreads along the length of the fibre via the T tubule system. The action potential stimulates the release of stored calcium ions from the sarcolemma into the cytosol, and calcium is then able to bind with the troponin complex on the thin filament. Binding of calcium to troponin causes a conformational change which results in the movement of tropomyosin and exposure of the myosin-binding sites on the actin filament.[12] The actin filaments are pulled in towards the centre of the sarcomere via repeated binding and detachment with the myosin heads (facilitated by ATP and ATPase), resulting in shortening of the sarcomere and contraction of the muscle.[5] Calcium ions are moved back into the sarcoplasmic reticulum by an ATP-dependent pump, and as calcium is removed from the cytosol, the myosin-binding sites of the actin filament become blocked again and muscle relaxation occurs.[11, 29] Abnormalities in any of the channels or components involved in this process can result in clinical signs of neuromuscular disease.[4]

1.2.3 Muscle metabolism in dogs

Skeletal muscle in dogs is optimised for endurance exercise, with a predominance of oxidative, rather than glycolytic fibre types.[30] At rest and during low to moderate intensity exercise, there is adequate oxygen availability and blood flow to support fatty acid oxidation as the primary energy source in muscle. When energy demands exceed the capabilities of oxidative metabolism, anaerobic pathways of glycogenolysis are utilised, which results in the accumulation of metabolites that contribute to the development of muscle fatigue. The balance of fatty acid oxidation and glycogen usage in muscle can also be affected by factors such as physical training and diet; high fat diets are recommended for canine endurance athletes as they promote the use of fat

as an energy source and spare muscle glycogen.[31] An overview of selected metabolic pathways in muscle is shown in Figure 1.2, and these pathways are discussed below.

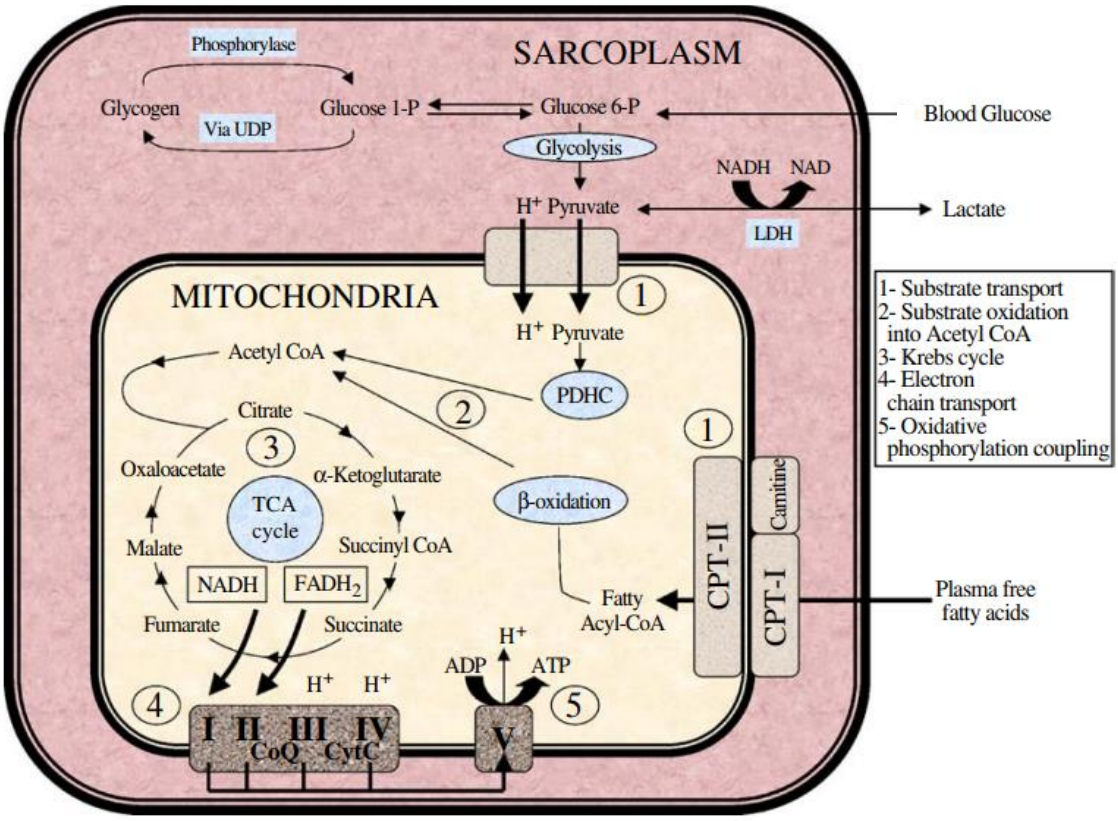

Figure 1.2: An overview of selected metabolic pathways within myocytes.
CoA, coenzyme A; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CoQ, coenzyme Q; CPT, carnitine palmitoyltransferase; CytC, cytochrome c oxidase; FADH2, reduced FAD; LDH, lactate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; PDHC, pyruvate dehydrogenase complex; TCA, tricarboxylic acid; UDP, uridine diphosphoglucose pyrophosphorylase (glycogen synthase). Figure reproduced with permission from Platt SR and Garosi LS. Neuromuscular weakness and collapse. *Veterinary Clinics of North America: Small Animal Practice* 2004; 34: 1281-1305.

1.2.3.1 Lipid metabolism and fatty acid oxidation in skeletal muscle

Fatty acids used as an energy substrate by muscle include non-esterified fatty acids released into circulation by lipolysis of adipocytes, and fatty acids produced within the muscle from lipolysis of intramuscular triglycerides or low-density lipoproteins.[32] The uptake of fatty acids from circulation into muscle can occur by passive diffusion and protein-mediated transport, and once inside the cell, fatty acids are bound to coenzyme A (CoA) to form fatty acid CoA esters.[32] The main oxidative pathway for fatty acids is β-oxidation, which occurs in mitochondria (Figure 1.2). Short and medium

chain fatty acids with less than 12 carbon atoms are able to cross directly into mitochondria, but the more abundant long chain fatty acids require carnitine for transport into mitochondria.[33] Carnitine can be provided in the diet (in meat and milk) or synthesised endogenously from lysine, and the largest stores of carnitine in the body are found in muscle and liver.[34] Deficiencies in carnitine in people can cause adult-onset myopathies characterised by myalgia (muscle pain) and muscle fatigue.[35] Carnitine-dependent transport of fatty acids is a three step process, starting with the action of carnitine palmitoyltransferase I (CPT I), which is located on the outer mitochondrial membrane and converts fatty acid CoA esters into their acylcarnitine equivalents.[36] This enzyme is the rate-limiting step of the "carnitine shuttle," and therefore plays an important role in the overall regulation of fatty acid oxidation. In the second step, the acylcarnitines are transported across the mitochondrial inner membrane by carnitine/acylcarnitine translocase (CACT) in exchange for free carnitine. Once inside the mitochondria, the acylcarnitines are converted back into their acyl-CoA esters by the third enzyme, carnitine palmitoyltransferase II (CPT II), and fatty acid oxidation can proceed.

The process of β-oxidation removes two carbon units from the fatty acid at a time, and fatty acids go through multiple cycles of β-oxidation to generate successively shorter fatty acids. The process is catalysed by various acyl-CoA dehydrogenase enzymes depending on the length of the carbon chain of the substrate, including very long chain acyl-CoA dehydrogenase (VLCAD, which acts on C14 – C20 substrates), long-chain acyl-CoA dehydrogenase (LCAD, most active with C14 substrates), medium chain acyl-CoA dehydrogenase (MCAD, C6 – C12 substrates) and short chain acyl-CoA dehydrogenase (SCAD, C4 – C6 substrates).[36] Deficiencies in these enzymes are responsible for some inherited fatty acid oxidation disorders, which usually present with clinical signs attributable to skeletal muscle myopathy, cardiomyopathy and hepatopathy.[37] Each cycle of β-oxidation generates acetyl CoA, as well as the reduced forms of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2), which contribute electrons to the respiratory chain and electron-transfer flavoprotein. The acetyl CoA produced enters the tricarboxylic acid cycle (TCA or Krebs cycle). As shown in Step 3 Figure 1.2, the TCA cycle generates further NADH and FADH2, which

carry electrons to complex I and II of the electron transport (respiratory) chain. The electrons are then passed sequentially to coenzyme Q10 (CoQ), complex III, cytochrome C (CytC), complex IV and O_2 to generate H_2O.[38] The energy generated is used to pump protons across the inner mitochondrial membrane, creating an electrochemical gradient. This gradient is used by ATP synthase (labelled as Complex V in Figure 1.2) to form adenosine triphosphate (ATP) via oxidative phosphorylation, and in this way the electron transport chain is coupled to oxidative phosphorylation. Experimentally, compounds that uncouple these processes can result in the development of skeletal muscle myopathies in animals.[39]

1.2.3.2 Carbohydrate metabolism in skeletal muscle

During high intensity exercise, oxidation of carbohydrates is required to meet the energy demands of the muscle.[40] The main carbohydrate in muscle is glycogen, and during exercise, muscle fatigue develops when glycogen stores are depleted. Uptake of glucose from the blood into muscle is stimulated by insulin, and insulin also activates glycogen synthase (UDP-glucose-glycogen glucosyltransferase, labelled as UDP in Figure 1.2), resulting in glycogen production in muscle when glucose is in excess.[41] During anaerobic exercise or under the action of adrenaline, glycogen is broken down to glucose-1-phosphate by glycogen phosphorylase. A second pathway of glycogen breakdown occurs in lysosomes, where glucose is produced under the action of the enzyme acid maltase.[40] In the sarcoplasm, glucose-1-phosphate is subsequently converted into glucose-6-phosphate, which is in turn converted into pyruvate through a multi-step glycolytic pathway (not shown). Pyruvate is transported across the inner mitochondrial membrane and through the action of the pyruvate dehydrogenase complex (PDHC) it is converted to acetyl CoA. From here, the TCA cycle and electron transport chain proceed as described in the discussion of fatty acid oxidation. If pyruvate cannot enter the TCA cycle due to a metabolic defect such as PDHC deficiency, pyruvate is instead converted into lactate (via lactate dehydrogenase) and alanine. Clinically, this leads to lactic acidosis, reduced energy production and a range of muscular and neurological effects.[42, 43]

1.2.3.3 Protein metabolism in skeletal muscle

Skeletal muscle is the major site of protein storage in the body, and during times of negative energy or protein balance, muscle is degraded to provide amino acids for gluconeogenesis.[44] The balance between protein synthesis and breakdown in skeletal muscle is regulated by a number of cytokines and hormones, including insulin and cortisol, and is influenced by dietary protein intake and resistance exercise. After protein or amino acid ingestion and with acute resistance exercise, muscle protein synthesis rates increase.[45, 46] Repeated resistance training can induce protein accumulation in muscle and lead to physiological hypertrophy. In addition to being a structural component of proteins, the branched chain amino acid leucine also plays a role in how cells sense amino acids in circulation to increase muscle protein anabolism,[47] and defects in leucine pathways have been associated with the development of mitochondrial myopathies.[48]

1.3 Diagnosis of muscle injury and disease

1.3.1 History and clinical signs

Diseases primarily affecting skeletal muscle (myopathies) can be difficult to differentiate clinically from neurological disease, and these are often considered together under the heading of 'neuromuscular disease'. A thorough clinical history, including age of onset, progression of signs, effect of exercise, diet, recent trauma and possible exposure to drugs or toxins, can aid in the differentiation of metabolic, infectious, traumatic and toxic myopathies.[49] Weakness is the most common clinical sign associated with myopathies, and may be constant or episodic.[50, 51] Peripherial neuropathies may also result in weakness and collapse, but are commonly associated with changes in spinal reflexes and sensation that are not seen in myopathies.[30] Other common signs of myopathies include exercise intolerance, fatigue and muscle pain (myalgia), but these are considered non-specific and may also occur with orthopaedic, rheumatologic and systemic diseases. Muscle cramps, contractures and impaired muscle relaxation (myotonia) are recognised in humans with myopathies, but may be harder to identify in animals. In cases of severe, acute muscle necrosis (rhabdomyolysis), myoglobin released from damaged myofibres is filtered by the

kidneys and may turn the urine brown (myoglobinuria).[52] If respiratory muscles are affected, hypoventilation may be noted.[49]

1.3.2 Biomarkers of muscle injury and disease in serum

Biomarkers are objective, quantifiable characteristics that can be evaluated as indicators of normal biological processes, pathological processes or response to therapy.[53] Biomarkers of muscle injury and disease in serum and plasma vary in their ability to differentiate cardiac and skeletal muscle involvement, and there are also important species differences. The field of biomarker discovery is rapidly expanding and this is by no means a complete list, but focuses on the enzymes and markers most relevant to this research.

1.3.2.1 Creatine Kinase

Creatine kinase (CK), also known as creatine phosphokinase, is an 84kDa enzyme that catalyses the reversible transfer of a high-energy phosphate bond from creatine phosphate to ADP, thus regenerating ATP.[54, 55] It is one of the most frequently used serum biomarkers of skeletal muscle damage, but is also present in cardiac muscle, intestine and brain. There are two genetically distinct subunits of CK, termed M-type for muscle and B-type for brain. Mitochondrial CK has also been detected in some species. Each CK enzyme is a dimer, which can be composed of the same or different subunits. In both cardiac and skeletal muscle, CK-MM is the predominant isoform. CK-MB mainly originates from the myocardium and has been used in human medicine in the diagnosis of myocardial infarction, but the proportion of CK-MB activity relative to total CK varies greatly between species[56] and immunological assays designed for human CK-MB measurement are unreliable in animals.[57] A third isoenzyme, CK-BB, is present predominantly in the brain, and also found at low levels in kidney, spermatozoa and skin.[58]

In skeletal muscle, CK is located along the M-line of the sarcomere and acts to buffer cellular ATP and ADP.[59] Energy is stored in resting muscle as creatine phosphate, and during muscle contraction CK catalyses the rapid conversion of ADP to ATP required for

muscle contraction. CK is also thought to be involved in translocating ATP from the mitochondrial matrix to the cytoplasm.[60]

Physical damage to myocytes disrupts the integrity of the cell membrane and allows part or all of the intracellular contents, including creatine kinase, to leak out into the extracellular space.[61] From there, creatine kinase and other muscle enzymes are transported by lymphatics into the intravascular space,[62] and increases in the activities of these enzymes can be detected in a venous blood sample. Detectable increases in serum and plasma CK activity can also occur with mechanical muscle damage from unaccustomed exercise or overtraining, or with metabolic disturbance of muscle.[7] In these situations, it is thought that the primary abnormality is a deficiency of ATP, leading to dysfunction of Ca^{2+}-ATPase and NA-K-ATPase membrane pumps. Calcium from the extracellular space moves into myocytes and acts to increase cell permeability and intracellular proteolytic enzyme activity, leading to the leakage of some cell contents into circulation.[7] Increased oxidative stress, inflammatory and immune responses may also play a role in muscle damage and CK release following mechanical or metabolic insults.

Clearance of creatine kinase from circulation is thought to occur primarily through the reticuloendothelial system[63] and liver,[64] with no renal elimination. With repeated episodes of muscle damage, the clearance of CK may be accelerated, resulting in a reduction in the magnitude of increases in CK activity in plasma.[65] The terminal half-life of elimination of CK in dogs is 2.5 hours following intravenous injection of supernatants of skeletal muscle homogenates and 6.5 hours after intramuscular injection, owing to limited absorption and incomplete bioavailability following intramuscular injection.[66] Due to the relatively short half-life of CK in plasma, it has a low sensitivity (0.32) for the diagnosis of muscle diseases, although specificity is high (0.82).[55] Clinically, CK activity in circulation usually peaks 6-12 hours after acute muscle injury.[67] Necrotising, dystrophic and inflammatory myopathies are usually associated with significantly increased serum CK activity, while CK may be normal or mildly increased in non-inflammatory muscle diseases.[68]

It is important to note that there is also significant variation in serum and plasma CK activities between individuals and depending on the test methodology used. CK activity is higher in serum than in plasma of dogs as platelets have some CK activity, but this difference is small in magnitude when compared to the increases seen with muscle diseases.[69] Artefactual increases in serum CK activity can occur with traumatic venepuncture due to needle-induced damage of surrounding muscles,[70] and increases may also be seen with severe hyperbilirubinaemia or haemolysis, but lipaemia has no effect.[55] In general, CK activity is lower in large dogs than in small dogs and decreases with age, but there are no significant differences between males and females.[69] Serum CK activity in an individual dog can vary markedly as a result of physical exertion, with significant increases seen in dogs during endurance exercise events such as the Iditarod Trail Sled Dog Race.[71] Short periods of maximal exertion, such as greyhound 400 metre sprint races, do not usually result in significant increases in serum CK activity,[72] although increases can be seen with cumulative sub-clinical muscle damage sustained over a racing season.[73]

1.3.2.2 Aspartate aminotransferase

Determination of aspartate aminotransferase (AST) activity in plasma or serum is often performed alongside measurement of CK activity to aid in the diagnosis of muscle damage or disease. Aspartate aminotransferase (also known as glutamic-oxaloacetic transaminase or GOT) catalyses the conversion of L-aspartate and α-ketoglutarate to oxaloacetate and L-glutamate, which are important metabolites in nitrogen metabolism[74] and the citric acid cycle.[75] Within cells, there are two fractions of AST: a mitochondrial or particulate isoenzyme that represents one-third of the total cellular AST activity, and a soluble or cytoplasmic isoenzyme that accounts for the remainder of cellular activity.[76] AST is found in many animal tissues including heart, skeletal muscle, liver, kidney, brain and mammary gland, and the proportion of the two isoenzymes can vary between tissues, with a high ratio of cytoplasmic to mitochondrial activity in heart, skeletal muscle and mammary gland.[77] The relative contributions of different tissues to serum aminotransferase activities is poorly understood, but clinically significant increases in both AST and alanine aminotransferase (see below)

are often associated with hepatocyte or myocyte injury or necrosis.[78] However, due to the lack of specificity of this enzyme,[79] increases in AST activity in circulation must be interpreted in light of the clinical signs and results of other diagnostic tests, including CK activity and tests of liver function.

Serum AST activity has a longer half-life than CK (12 hours in dogs, 50 hours in horses) and peaks 24 to 36 hours after acute muscle injury.[67] AST is primarily cleared from circulation by the liver, with studies in rats showing uptake of the enzyme by liver sinusoidal cells.[80] Similar to CK, intensive muscular exercise can significantly increase plasma AST activity,[81] and in humans, this effect can last for seven days or more.[82] In dogs, serum and plasma AST activities are similar in males and females[79] and remain consistent with age,[83] although higher activities have been reported in 12 year old beagles compared to younger beagles.[84] Haemolysis of samples results in artefactual increases in serum/plasma AST activities,[85] as AST in present in erythrocytes.[86]

1.3.2.3 Alanine aminotransferase

A second aminotransferase enzyme, alanine aminotransferase (ALT), also exists as mitochondrial and cytosolic (soluble) isoenzymes, but the mitochondrial fraction represents a small and variable part of total cellular ALT activity.[76] In glycolytic tissues such as cardiac and skeletal muscle, the cytosolic form predominates, but in gluconeogenic tissues like the liver and kidney, the concentrations of the cytosolic form are much more variable.[87] Like AST, ALT catalyses the transfer of an α-amino group (alanine) to the α-keto group of ketoglutaric acid, subsequently generating pyruvate (hence the former name of this enzyme was glutamate-pyruvate transaminase).[75]

The tissue distribution of ALT varies between species, and in dogs (as well as cats, rats, mice and ferrets) it is generally considered to be a liver-specific enzyme.[79, 83] In dogs, the relative tissue activities of ALT in cardiac and skeletal muscle are reported to be approximately 25% and 6%, respectively, of ALT activity in liver.[88] However, as the total muscle mass of an animal is much larger than the liver mass, large increases in serum

ALT can also be seen in association with some myopathies where there is no microscopic evidence of liver damage.[89]

In dogs, the reported half-life of ALT in blood ranges from 3 – 60 hours, with carbon tetrachloride exposure studies suggesting a half-life of 45 – 60 hours.[90] As with other transaminases, ALT is cleared from circulation by hepatic sinusoidal cells.[80] Increases in ALT following exercise have been reported in dogs used for hunting[81] and humans participating in intense muscular exercise such as weightlifting,[82] but no significant increases were observed in Thoroughbred horses immediately after race training.[91] ALT may gradually increase with age and in Beagle dogs, peak ALT concentrations were observed at 12 years of age, but declined by 14 years.[84] Unlike AST, even severe haemolysis does not affect the measured activity of ALT in serum.[92]

1.3.2.4 Myoglobin

Myoglobin is a small protein (153 amino acids), found in the sarcoplasm of cardiac and skeletal muscle cells. Each myoglobin molecule is able to bind one oxygen molecule, and oxygen bound to myoglobin can be used in mitochondrial respiration when blood-borne oxygen is inadequate.[68] Myoglobin is released into blood from damaged or dying muscle cells, and is rapidly filtered by the glomerulus and excreted in urine (myoglobinuria).[67, 93] Consequently, myoglobin has a short half-life in serum (reported to be less than 10 minutes in dogs) and normalises rapidly after muscle injury ceases compared to CK.[94] In cases of rhabdomyolysis (widespread breakdown of skeletal muscle), renal filtration of the large amounts of myoglobin released into circulation can result in proximal tubular necrosis, tubular obstruction and renal vasoconstriction, leading to acute renal failure.[95]

The amino acid sequence of myoglobin is highly conserved between species, but species differences in tertiary structure and antigenic features mean that human myoglobin assays are not suitable in most animal species, as the antibodies used fail to cross-react.[56] For this reason, myoglobin is not routinely used in the diagnosis of muscle diseases in dogs or other animals, and will not be further discussed here.

1.3.2.5 Cell-free DNA

Cell-free DNA (cfDNA) in plasma has been proposed as a novel indicator of exercise-induced muscle injury and over-training.[96, 97] Cell-free DNA consists of double-stranded fragments of DNA in plasma that are not bound to cells. In the past, cfDNA was thought to originate largely from cells undergoing apoptosis or necrosis, but in recent years it has been suggested that DNA may also be released from living cells as they proliferate.[98] Plasma concentrations of cfDNA are increased in various disease states in dogs and are associated with disease severity and prognosis,[99] and thus could be useful in determining the likelihood of recovery in myopathies such as 'Go Slow' myopathy (GSM) that can have a chronic clinical course. Similar to the other biomarkers discussed, plasma cfDNA concentrations in dogs are reported to increase with prolonged strenuous exercise,[100] but validated reference ranges have not been established in working and hunting dogs.

1.3.2.6 Muscle specific microRNAs

MicroRNAs (miRNAs) are non-coding sequences of RNA approximately 22 nucleotides long that have important roles in post-transcriptional regulation of gene expression.[101] miRNAs can be detected in blood and numerous miRNAs that are specific to or highly enriched in skeletal muscle have been described.[102, 103] The miRNAs show promise as biomarkers to aid in the diagnosis and understanding of various muscle diseases, including canine models of muscular dystrophy.[101, 104] As very little was known about GSM at the outset of the studies presented in this thesis, the identification of possible cases relied upon widely used, commercially available diagnostic tests (such as serum CK and AST activities) and miRNAs were not investigated. However, they are mentioned here as they provide a potential avenue to further characterise the disease in future.

1.3.3 Other tests used in the diagnosis of myopathies

1.3.3.1 Electromyography

Myopathies may cause alterations in the electrical activity of muscle, which can be detected on electrophysiological evaluation. Electromyography (EMG) is the recording

and interpretation of insertional, spontaneous and voluntary electric activity of skeletal muscle.[105] In animals, EMG is usually performed under sedation or general anaesthesia to enable the placement of needle electrodes within muscle, so recording of voluntary activity is limited. Myopathies may be associated with prolonged or decreased insertional potentials if there is instability of the muscle membrane, and abnormal spontaneous activity may be recorded.[106] Although EMG has limited value in differentiating between neuropathies and myopathies, it can help to determine which muscles and nerves are affected and therefore suitable for muscle biopsy.

1.3.3.2 Muscle biopsy

Muscle biopsy is an essential tool in the diagnosis of skeletal muscle diseases, and allows visual examination of myofibres, intramuscular nerve fibres and surrounding connective and vascular tissue.[4] An affected, but functional, muscle is chosen for biopsy based on clinical findings, ease of biopsy and EMG results. The area of EMG needle insertion should be avoided as this may cause localised muscle necrosis.[107] Usually, biopsy samples are harvested from the body of the muscle using an open surgical approach under general anaesthesia, although percutaneous needle muscle biopsies are common in large animals and have been successfully performed in dogs.[108] Formalin fixation and routine histologic processing of muscle biopsies may give a general overview of the disease process and enable identification of myofibre degeneration, regeneration, atrophy, inflammation and fibrosis. However, detailed histochemical analysis is not possible on formalin-fixed specimens, and for this reason, frozen biopsy samples are preferred. To freeze biopsy samples, the muscle is orientated within tissue-embedding media such as OCT (Sakura Finetek, Torrance, CA) and rapidly frozen in isopentane cooled in liquid nitrogen to prevent the formation of ice crystals. Sections are cut on a cryotome at -20°C, and a range of stains and histochemical reactions can be used to identify muscle fibre types, mitochondria, lipid, glycogen, lysosomes and macrophages.[107] Biopsy samples can also be fixed in glutaraldehyde for electron microscopy, which allows visualisation of individual sarcomeres and cellular organelles. With all methods of fixation, hypercontraction of muscle fibres can result in the formation of contraction bands, which can mimic acute

necrosis of myofibres.[109] Careful handling of biopsy specimens during collection and fixation can help to minimise artefactual contraction, and the presence of other features of degeneration (discussed below) can help to differentiate true muscle necrosis from artefactual contraction bands.

Muscle degeneration and necrosis are common microscopic finding in myopathies, and can affect whole muscle fibres or be segmental. As in other tissues, necrosis of myofibres is characterised by fragmentation of the sarcoplasm (with loss of cross striations), and hypereosinophilia with pyknotic or absent nuclei.[110] Based on the time course and distribution of lesions, muscle injury can be classified into four categories, which may aid in the identification of possible causes.[111] These categories include:

- *Focal monophasic reactions* due to a single, isolated traumatic event. In monophasic reactions, all lesions are in the same phase of injury or repair, consistent with a single episode of muscle damage.
- *Multifocal monophasic reactions* arise from a single event or insult that is capable of causing widespread muscle damage. This can include exposure to toxins, drugs or chemicals, or some metabolic disorders.
- *Focal polyphasic reactions* are due to repeated injury of the same site
- *Multifocal polyphasic reactions* result from continued muscle injury over a long period. Necrosis, inflammation and regeneration are seen concurrently and lesions are widespread. Potential causes include nutritional deficiencies, inflammatory myopathies and genetic diseases.

Experimental models of muscle injury in mice show that following direct injury to muscle or contractile overload, there is degeneration/necrosis of myofibres and widening of the interstitial space, indicative of oedema.[112] In these mice, infiltration of inflammatory cells peaked 3 days after injury and regenerating fibres, characterised by centrally located nuclei and sarcoplasmic basophilia, were first seen on days 3 – 7. Over time, new myofibres formed through the process of regeneration increase in size and nuclei move to periphery of the cells, and they become morphologically and functionally indistinguishable from uninjured myofibres.[113]

1.3.3.3 Diagnostic imaging

Radiography, ultrasonography and magnetic resonance imaging (MRI) may be useful in the diagnosis of some myopathies. Traumatic myopathies can occur with fractures and swelling, which may be appreciable on plain radiographs. If there is cardiac muscle involvement, changes in heart size may be seen on radiographs, but echocardiography is preferred for assessment of the heart. In humans, MRI has been used to characterise the severity and distribution of muscle involvement in some myopathies,[114] but this imaging modality is not routinely used in veterinary medicine.

1.3.3.4 Ancillary tests

A wide range of metabolic and molecular tests are available to aid in the diagnosis of myopathies in animals. Determination of lactate and pyruvate in blood, and carnitine profiling in plasma, urine and muscle using mass spectrometry may be useful in the diagnosis of mitochondrial myopathies.[68] Nuclear magnetic resonance (NMR) spectroscopy can be used to investigate changes in metabolites during exercise to characterise defects in muscle glycolytic and oxidative pathways.[4] Vitamin E and selenium deficiency can cause a nutritional myopathy,[115] and concentrations of these nutrients can be measured in blood and liver.

Genetic tests have been developed for some inherited myopathies and muscular dystrophies in animals, such as exercise-induced collapse in Labradors and dystrophin-deficient muscular dystrophy in Golden Retrievers.[68] Autoantibodies may be detected in some immune-mediated myopathies, like masticatory muscle myositis.[116] A number of infectious agents can be associated with myopathies, including *Toxoplasma gondii,*[117] *Neospora caninum*[118] and *Trichinella spiralis*,[119] so specific tests to identify the presence of these agents (including ELISA, PCR and immunohistochemistry) are appropriate in some cases.

1.4 Myopathies in dogs

1.4.1 Classification of myopathies in animals

Like diseases in other body systems, myopathies can be classified according to the time course of disease and microscopic appearance of lesions. Both of these features are important in the characterisation of a novel myopathy such as GSM, but for the purposes of this review, myopathies are classified based on the underlying cause. The following discussion briefly outlines the types of myopathies most relevant to this study, with specific examples given where appropriate. However, it is not a comprehensive list of myopathies in dogs and does not include diseases targeting the neuromuscular junction (such as myasthenia gravis) or endocrine or electrolyte diseases, which can also result in myopathies.

1.4.2 Degenerative (non-inflammatory) myopathies

Degenerative myopathies are characterised by myofibre necrosis. Inflammatory cells may be seen in response to necrosis, but are not responsible for initiating muscle damage as they are in the inflammatory myopathies.[120] Causes of degenerative myopathies include trauma, overexertion, nutritional deficiencies and toxicities.

1.4.2.1 Traumatic myopathies

Trauma to skeletal muscle can occur with impact injuries, lacerations, crush injuries, penetrating wounds and excessive stretching.[121] Muscle damage and the development of a fibrotic myopathy in the iliopsoas muscle of a dog has also been reported in association with the migration and surgical removal of a grass awn.[122] In dogs used for pig hunting, traumatic wounds are particularly common,[123] and penetrating wounds caused by the tusks of wild boars can be associated with significant muscle damage. Traumatic myopathies frequently cause a severe but transient elevation in serum creatine kinase,[50] and a slower elevation in aspartate aminotransferase.[67] If large regions of muscle are traumatised, as can occur with excessive pressure in crush syndrome, release of muscle contents into circulation can result in electrolyte imbalances and myoglobinaemia, leading to acute renal failure.[124]

1.4.2.2 Exertional myopathies

Muscle necrosis due to overexertion or excessive exercise has been reported in a wide range of species, and is referred to as an exertional myopathy or exertional rhabdomyolysis.[125] Altered blood flow to muscle and exhaustion of aerobic energy during overexertion leads to increased production of lactic acid and decreased delivery of oxygen and removal of cellular waste.[126] This damages myofibres, and when severe, can cause widespread muscle necrosis and rhabdomyolysis. The histological appearance of muscle in exertional myopathies and rhabdomyolysis depends on the time since the injury occurred. Within a few hours, myofibres show changes typical of acute necrosis, including hypereosinophilia and fragmentation of the sarcoplasm, with loss of cross striations, haemorrhage, and pyknotic or absent nuclei.[125] Brown granular casts of myoglobin may be seen in renal tubules and are associated with tubular necrosis. In lesions of a longer duration, necrotic myofibres can be mineralised, and there is infiltration of leukocytes and proliferation of myoblasts and sarcolemmal cells.

Exertional rhabdomyolysis is most commonly reported in dogs that participate in high energy exercise, such as sled racing.[127] In humans, genetic polymorphisms that may predispose to the development of exertional rhabdomyolysis have been identified,[128] and in horses it has been shown that underlying muscle disorders (such as polysaccharide storage myopathy) can play a role.[129] Specific risk factors for the development of exertional rhabdomyolysis have not been identified in dogs, and vitamin E and antioxidant status do not have any effect.[127]

1.4.2.3 Nutritional myopathies

Contraction of striated muscle generates large numbers of free radicals.[127] Antioxidant defence mechanisms, such as vitamin E and the selenium-dependent glutathione peroxidase system, are therefore important in protecting muscle from injury induced by free radicals. If animals (particularly livestock) consume a diet deficient in selenium and/or vitamin E, or if neonatal animals are born to a deficient dam, muscle degeneration and nutritional myopathy may result.[120] Affected muscles often appear grossly pale, leading to the common name of ‘white muscle disease’. In neonatal

animals with congenital nutritional myopathy, the most severe lesions are usually seen in the heart,[130, 131] whereas skeletal muscle lesions generally predominate in animals affected after three to four weeks of age.[115] A myopathy resembling white muscle disease has been reported in an adult sheep dog in New Zealand, which was characterised histologically by widespread skeletal muscle necrosis with loss of striations, swelling and fragmentation of the sarcoplasm and rare calcified fibres, with minimal interstitial cellular response.[131] Selenium deficiency was not confirmed in this case, but overseas there are case reports of selenium/vitamin E responsive myocardial degeneration in dogs,[132] and experimental feeding of selenium and vitamin E deficient diets to puppies causes a skeletal myopathy.[133]

1.4.2.4 Toxic myopathies

A wide range of plant toxins, fungal toxins and synthetic chemicals and drugs can be associated with the development of skeletal and cardiac myopathies in animals. Typically, toxic myopathies are characterised by myofibre necrosis, but some toxins also cause inflammation or interfere with mitochondrial, lysosomal or microtubular function.[134] Plant-related myopathies are most commonly seen in grazing animals, although some plants, such as *Ageratina altissima* (white snakeroot), are capable of causing a secondary toxicity in animals or humans that eat meat or milk from poisoned animals.[135] Determining the individual plant species implicated in a toxic myopathy may be difficult, and the toxic principles of many plants are unknown. A recent example of this is seasonal pasture myopathy/atypical myopathy in horses, where outbreaks were first recognised in the 1980s, but the underlying cause (hypoglycin A in box elder seeds) was only found in 2013 after extensive epidemiological and biochemical investigations.[136]

Certain drugs, chemicals and medications, either on their own or in combination, can cause muscle damage.[134] One of the most frequently recognised drug-induced myopathies in animals is ionophore toxicity. Ionophore antibiotics may be added to ruminant feed to promote growth and reduce the incidence of bloat, and ruminants are able to tolerate high ionophore concentrations. However, other animals

(particularly horses and dogs) are more sensitive to these drugs,[120] and can develop a cardiac and skeletal muscle myopathy if they are exposed to ionophores. In one case report, accidental contamination of dry dog food with monensin caused anorexia, dyspnoea, severe weakness, myoglobinuria and marked increases in serum CK, AST and lactate dehydrogenase activities in a 2-year-old sheltie dog.[137] Histological lesions of ionophore toxicity are characterised by multifocal monophasic muscle necrosis, and necrotic fibres are infiltrated with macrophages over time.[111] Ultrastructurally, there is marked swelling and disintegration of mitochondria. Both type I and type II muscle fibres are affected, but satellite cells can survive acute toxicity and aid in muscle regeneration. Confirmation of toxicity is through laboratory analysis for specific ionophores.[138]

In humans, there are numerous drugs that can cause skeletal muscle myopathies, including anticholesterolaemic drugs (statins), anti-inflammatory/immunosuppressive drugs, antinucleoside analogues, dietary agents and recreational drugs (cocaine, heroin, amphetamines and alcohol).[134] These myopathies are most commonly necrotising (degenerative), but can also be inflammatory or can target mitochondria, myofibrils or microtubules of muscle. Dogs are sometimes used in drug toxicity testing or as experimental models of drug-induced myopathies,[139,140] but drug-induced myopathies in dogs are otherwise rare.

1.4.3 Inflammatory myopathies

In inflammatory myopathies, also referred to as myositis, myofibre injury is directly caused by the actions of inflammatory cells. Inflammatory myopathies can occur with bacterial, parasitic, fungal and viral infections, or be immune-mediated.[120].

1.4.3.1 Bacterial myositis

Bacteria can be introduced into muscle by direct penetration (wounds and surgery), haematogenous spread, or extension from a local infection such as arthritis. Pyogenic bacteria such as streptococci and staphylococci are frequently involved,[141] and cause a focal necrotising myositis. In acute focal bacterial myositis, histology of affected

muscle shows necrosis of myofibres with infiltrates of inflammatory cells and the presence of bacteria, but serum CK and AST activities often remain normal.[141] In livestock, particularly cattle, sheep and horses, various clostridial species (including *Clostridium perfringens, C. septicum, C. novyi* and *C. chauvoei*)[142] can cause fatal haemorrhagic myonecrosis through the production of toxins under anaerobic conditions. Clostridial myositis is rare in dogs but has been reported, and in one case was attributed to the use of a contaminated needle for intramuscular injection.[143, 144]

Myositis in dogs has also been reported in cases of leptospirosis,[145, 146] but the disease typically presents with more severe systemic signs attributable to renal and liver injury.[146] Cases of leptospirosis in dogs in New Zealand are most commonly associated with *Leptospira interrogans* serovars *copenhageni* and *pomona*, and in the past, most clinical cases were reported in the Auckland and Waikato regions.[147] More recently however, antibodies to these serovars, as well as *L. borgpetersenii* serovars *hardjo* and *ballum,* have been detected in dogs throughout New Zealand, with breeds of working farm dogs shown to be at greater risk of exposure to *L. borgpetersenii* serovar *hardjo.*

1.4.3.2 Parasitic myositis

Protozoal organisms and nematodes may be found incidentally within muscle, or may cause significant clinical disease. In herbivores, rodents, pigs, birds and reptiles, protozoal cysts of *Sarcocystis* spp. are a common incidental finding[120] and do not typically incite an inflammatory response unless they rupture. In dogs, *Neospora caninum* or *Toxoplasma gondii* protozoal organisms are able to infect skeletal and cardiac myocytes and cause an inflammatory myopathy, although neurological signs often predominate.[118, 148] Dogs are intermediate hosts for *T. gondii* and definitive hosts for *N. caninum*, and can be infected by these protozoal organisms through the ingestion of bradyzoites in tissue cysts of intermediate hosts, or ingestion of sporulated oocysts in cat faeces (*Toxoplasma*).[149] Bradyzoites develop into tachyzoites in the gastrointestinal tract, and tachyzoites then spread throughout the body in the lymphatic and vascular systems before forming tissue cysts is tissues such as skeletal and cardiac muscle.[150] Histologically, polymyositis caused by *Neospora* in dogs is

characterised by multifocal infiltrates of lymphocytes, plasma cells and macrophages,[151] and in chronic cases there may also be muscle fibre atrophy and fibrosis.[118] Organisms may not be visible within myofibres, and definitive diagnosis can require the use of immunohistochemistry (IHC), serology and PCR. Overseas, other protozoal organisms including *Leishmania infantum* and *Hepatozoon americanum*, as well as the rickettsial organism *Ehrlichia canis*, can cause myositis or muscle atrophy in dogs, often in addition to more generalised clinical signs.[152] These organisms, as well as the tick vectors of *H. canis* and *E. canis*, are currently exotic to New Zealand.[153]

Trichinella spiralis is a nematode parasite that can cause muscle damage in animals and humans. Infection in people is usually associated with under-cooked pork products, and although the organism is considered endemic in New Zealand, the reported prevalence in wild pigs is very low and clinical cases are rarely reported in humans.[154-156] The last confirmed finding of *T. spiralis* in pigs in New Zealand was in a single pig in 2007,[154] and extensive monitoring programs since 1969 (testing both commercially farmed and wild pig meat) have not detected any infected pigs.[156]

Animals are infected with *Trichinella* after eating larvae encysted in muscle, and digestive enzymes release the larvae within the small intestine. Adult worms develop within the intestinal mucosa and mate to produce larvae, which encyst within striated muscle. In people, invasion of muscle causes myalgia and weakness, with a peripheral eosinophilia.[141] Dogs are considered relatively resistant to infection and experimental dosing of dogs with large numbers of larvae (500 – 5,000) resulted in only mild vomiting and diarrhoea, with no significant increases in serum creatine kinase activity.[157] In New Zealand, a case of myostitis with peripheral eosinophilia in a working farm dog fed raw wild pig meat and horse meat was investigated in 2013, but *Trichinella* was ruled out as a cause using serum ELISA and western blot tests.[158] Overseas, there has been a case report of *Trichinella* causing clinical signs of neuromuscular disease, primarily muscle weakness, in a dog, and in this case larvae were observed histologically in muscle, surrounded by a thick hyaline capsule.[119] This was accompanied by increased fat in the endomysium, focal infiltrates of lymphocytes

and macrophages, variability in myofibre size, and evidence of muscle regeneration and atrophy. Hunting dogs might be more likely than other dogs to be exposed to *Trichinella* spp. due to opportunities they might have to consume wild pig tissues, and hunting dogs have been used as sentinels for monitoring *Trichinella* spp. in wildlife.[159]

1.4.3.3 Fungal and viral myositis in dogs

In humans, fungal causes of myositis (including *Aspergillus* spp. *Blastomyces dermatitidis, Candida spp*., *Coccidioides spp., Cryptococcus neoformans, Fusarium spp., Histoplasma capsulatum* and *Pneumocystis jiroveci*) have been documented but are considered rare.[141] A study of 200 cases of inflammatory cases in dogs did not include any cases of fungal myositis, and serology for various fungal organisms in seven of these dogs was negative.[160] The author of this thesis has personally diagnosed a case of systemic cryptococcosis in a pig hunting dog in New Zealand that presented with progressive lethargy, weight loss and a poor hair coat. In this case, yeast organisms were visible in multiple organs, including the liver and skeletal muscle (Figure 1.3), but were not associated with any significant muscle degeneration or inflammation. There are no reports of viral-induced myopathies or viral myositis in dogs in New Zealand.[152]

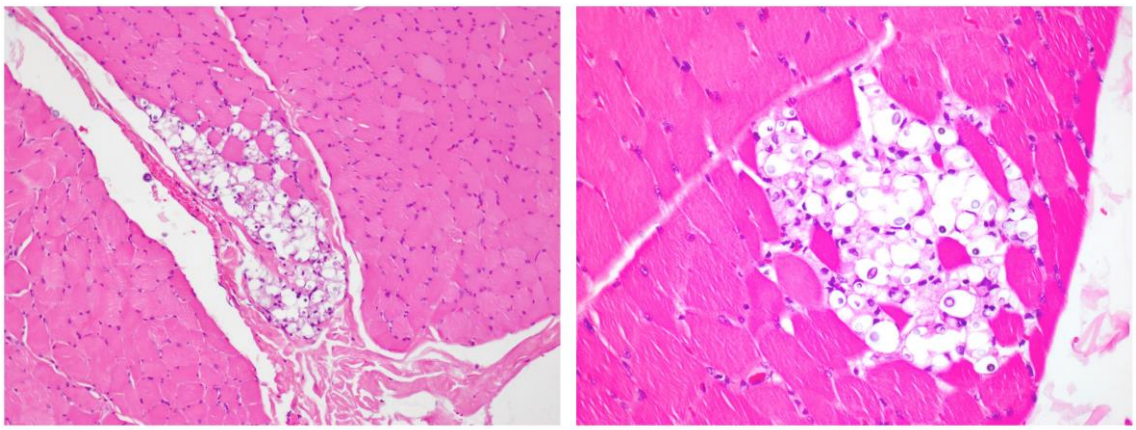

Figure 1.3: Skeletal muscle histology of a dog with systemic cryptococcosis (HE) at 200x (L) and 400x magnification (R). Basophilic, round 5 - 10µm diameter yeasts are surrounded by thick, non-staining capsules. Adjacent skeletal muscle fibres are normal and there is no inflammation.

1.4.3.4 Immune mediated myositis in dogs

Immune-mediated myositis has been reported in dogs, cats and horses and is usually characterised histologically by lymphocytic invasion of myofibres and perivascular lymphocytic inflammation.[120] Immune-mediated myopathies in dogs may be generalised or only affect focal muscle groups, such as masticatory muscle myositis and extraocular myositis. Polymyositis is a generalised immune-mediated muscle disease characterised clinically by muscle pain, weakness, a stilted gait and pyrexia.[161] Affected dogs have high serum muscle enzyme activities, electromyographic abnormalities, lymphocytic infiltrates in skeletal muscle, and are negative on serology for infectious diseases known to cause muscle damage. In masticatory muscle myositis, lesions are restricted to muscles innervated by the mandibular branch of the trigeminal nerve and result in jaw pain, inability to open the mouth and masticatory muscle atrophy.[160] Immune complex deposition in this disease is limited to type 2M muscle fibres, which are only found in masticatory muscles and are distinct from the fibre types in limb muscles.[162] Extraocular myositis results in bilateral exophthalmos without protrusion of the third eyelid.[163] The disease is rare and poorly characterised, but is most commonly reported in young, entire female Golden Retrievers and cases respond well to systemic corticosteroids at anti-inflammatory doses.

1.4.4 Inherited myopathies and muscular dystrophies

Inherited myopathies and muscular dystrophies have been reported in a number of dog breeds, and usually present with progressive muscle weakness and atrophy early in life.[164] Inheritance of some of these disorders is X-linked and only males are clinically affected, while other disorders have autosomal dominant or recessive inheritance. Inherited myopathies may primarily target myofibres (as in muscular dystrophy), ion channels (myotonia and channelopathies) or muscle metabolism (metabolic and mitochondrial myopathies).[120] One of the best researched inherited muscle diseases in dogs is Golden Retriever muscular dystrophy (GRMD), which is used as a model for Duchenne muscular dystrophy in people.[165] GRMD is an X-linked disease caused by mutations in the *DMD* gene that results in loss of the dystrophin protein. Dystrophin stabilises the muscle membrane by connecting the myofibre cytoskeleton to the

extracellular matrix,[164] so lack of this protein results in membrane fragility, leading to repeated cycles of myofibre necrosis, regeneration and eventually muscle atrophy and fibrosis.[166] Death can result from involvement of respiratory muscles or the heart. Other breeds in which inherited muscle diseases are reported include Labrador Retrievers,[167] Irish terriers,[168] Scottish terriers,[169] Cavalier King Charles spaniels,[170] Japanese Spitz,[171] Great Danes,[172] Weimaraners[173] and miniature Schnauzers.[174] An inherited mitochondrial myopathy has been reported in a German Shepherd dog, characterised ultrastructurally by subsarcolemmal accumulations of mitochondria and morphologically atypical mitochondria.[22] Similar mitochondrial abnormalities have also been reported in Old English Sheepdogs with episodic weakness associated with exertional myopathy and lactic acidosis.[175, 176]

1.4.5 Idiopathic myopathies

In addition to the degenerative and inflammatory myopathies of known causes, there are several myopathies reported in dogs for which the pathogenesis and aetiology are poorly understood. These include a number of acquired fibrotic myopathies, an idiopathic inflammatory polymyopathy in Hungarian Vizslas, and a poorly characterised syndrome of chronic fatigue in dogs. Fibrotic myopathies and muscle contractures usually affect a single muscle group, and present with lameness, pain and weakness, and the affected muscle is firm to touch.[177] Muscles reported to be affected include the semitendinosus, iliopsoas, sartorius, teres minor and gracilis.[177-179] Trauma, either acute or repetitive, is suspected to play a role in the development of these myopathies, and working dogs and young male German Shepherds are over-represented in cases of semitendinosus myopathy.[180, 181]

Vizsla idiopathic inflammatory myositis (VIP) is characterised clinically by dysphagia, regurgitation, sialorrhoea and masticatory muscle atrophy, and histologically by myofibre degeneration and endomysial, interstitial and perivascular mononuclear cell infiltrates in a range of skeletal muscles.[182, 183] No infectious agents have been associated with this disease and affected dogs had negative serological tests for masticatory muscle myositis and myasthenia gravis, but recent work has found an

association with specific dog leukocyte antigen class II haplotypes, suggesting that VIP is an immune-mediated disease.[184]

In humans, chronic fatigue syndrome (also known as myalgic encephalomyelitis) is a syndrome associated with post-exertional exhaustion, neurocognitive impairments, and impairments in energy production.[185] The aetiology of the syndrome is poorly understood, and although not considered to be a primary myopathy, functional and structural mitochondrial abnormalities have been observed in skeletal muscle of people with chronic fatigue syndrome.[186, 187] There are reports of apparent cases of chronic fatigue syndrome in dogs[188, 189] but the criteria used for diagnosis in these studies included various non-specific clinical signs such as lethargy, anorexia and weight loss, so there is the potential that these cases represent more than one disease process.

1.5 Conclusion

Myopathies are a diverse group of clinically important diseases in dogs. Investigation of possible myopathy cases relies on a combination of case history, clinical examination findings, measurement of biomarkers of muscle damage in blood, examination of muscle biopsies and ancillary testing where appropriate. Based on the findings of these investigations, myopathies can be broadly classified into degenerative, inflammatory or inherited diseases, which can aid in the refinement of likely aetiologies. This framework of investigation, combined with knowledge of normal skeletal muscle structure, function and metabolism, will be used to further characterise 'Go Slow' myopathy (GSM), an idiopathic myopathy in dogs in New Zealand.

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Chapter 2

Epidemiological features of 'Go Slow' myopathy

2.1 Introduction

As discussed in Chapter 1, 'Go Slow' myopathy (GSM) is a myopathy of unknown cause which has been reported to primarily affect dogs used for pig hunting (pig dogs).[1] In order to understand how this myopathy develops and investigate possible causes of the disease, it is first necessary to understand recreational hunting of wild pigs (*Sus scrofa*) and the role that dogs play in this. Hunting for wild game is a popular pastime in New Zealand and in 2014, it was estimated that 167,000 New Zealanders (5.0% of the population) participated in some form of hunting over the previous 12 months.[2] Animal species hunted in New Zealand include deer, pigs, goats, chamois and thar (collectively referred to as large or big game); rabbits, hares, possums and wallabies (small game) and ducks, swans, geese, pheasant and quail (game birds).[3] In a 2012 online survey of 1251 big game (deer, pigs, thar and chamois) hunters in New Zealand, 812 (65%) of respondents targeted pigs, often in conjunction with other game species.[4] The peak period of big game hunting activity, based on the days spent hunting each month and the average number of kills per day, was highest in April with a secondary peak in June. In general, pig hunters were reported to undertake more hunting trips of a shorter duration than hunters of other animals.

The use of dogs in pig hunting is common in New Zealand, but published data regarding the national pig dog population is lacking. One of the main motivations reported for pig hunting in New Zealand, in contrast to other types of game hunting, is the opportunity to work with dogs.[5] In a 1989 survey, it was reported that 23% of successful pig hunters used their own dogs for pig hunting, and a further 39% used dogs belonging to others.[6] Together, these hunters took 87% of the reported pig harvest, while the 38% of hunters not using dogs took only 13% of the total kill. This supports overseas studies that report high hunting success rates when targeting feral pigs with dogs,[7] and improved harvest rates when dogs are used in hunting, relative to

hunting without dogs.[8] Hunting pigs using dogs is not unique to New Zealand; in southern Australia, feral pigs are reported to be the most common species that are hunted using dogs,[9] and in California, USA, hunting with dogs was used as part of a successful programme to eradicate feral pigs from Santa Cruz island.[10]

Dogs used in pig hunting may be bred specifically for this purpose, or may have other primary functions, particularly as working farm dogs.[6] A large variety of breeds and cross-breeds of dogs are used in pig hunting in New Zealand, ranging in size from Fox Terriers to Great Danes.[5] When hunting pigs, dogs work as a pack and individual dogs may have different roles. Pig dogs can be categorised as finders, bailers or holders, or a combination of these. Dogs usually detect the presence of prey using smell,[8] and a dog that locates pigs is designated as a 'finder'. Dogs that are 'bailers' keep a pig stationary by barking at it, while a 'holder' bites part of the pig, such as the ears, tail or leg to prevent it from escaping before the hunter arrives.[11] There is considerable overlap between these roles, and dogs are often classified as 'finder-bailers' or 'finder-holders'. Upon catching up with the dogs, the hunter usually kills the pig by exsanguination using a knife in the neck or chest (referred to as 'sticking' a pig), or by gunshot.

There are no recent research publications regarding dogs used for pig hunting in New Zealand, and as outlined in Chapter 1, 'Go Slow' myopathy (GSM) was initially reported to primarily affect pig hunting dogs, with cases also observed in working farm dogs.[1] The aim of this study was to investigate the geographic distribution of cases of GSM, and the diet, hunting activities and general health of affected dogs. To appreciate the role and husbandry of dogs used in recreational hunting of wild pigs, a further aim of the present study was to gather data about the hunting activities, diet and health of pig hunting dogs in New Zealand.

2.2 Methods

Two separate surveys were conducted: a telephone survey with owners of dogs diagnosed with GSM (Appendix A), and an online survey regarding the hunting activities, diet and health of pig hunting dogs in New Zealand (Appendix B). The content, methodologies and time frames of the two surveys differed as the myopathy case survey was also part of a separate study investigating the pathology of the disease (Chapter 3). Due to the different selection methods for participation in each survey and different questions asked, it was not appropriate to directly compare the results of these surveys through the construction of logistic regression models or calculations of odds ratios and p-values. Rather, each set of data was analysed individually, and results are presented alongside each other, then further compared and contrasted in the discussion.

2.2.1 Myopathy case definition

For the purposes of this study, cases of GSM were defined as dogs that presented with a sudden onset of trembling, exercise intolerance and/or weakness and collapse. Less commonly, affected dogs also had vomiting and diarrhoea in the acute stages, but clinical examination (performed by a veterinarian) was otherwise normal. Muscle damage was confirmed on serum biochemistry or skeletal muscle histology (or both), and characteristic clinical pathology and microscopic findings are outlined in Chapter 3. Results of additional diagnostic tests, conducted at the discretion of the veterinarian, were not consistent with any known causes of myopathies in dogs.

2.2.2 Survey of myopathy cases

Putative cases of the myopathy were recruited at the time of diagnosis through notifications from veterinarians, veterinary pathologists and pig hunters from June 2014 to June 2017. The study was publicised through various presentations and short articles printed in several veterinary newsletters, pig hunting magazines and newspaper articles, as well as discussions about the disease on pig hunting interest pages on Facebook. A telephone questionnaire was completed with the owners of all affected dogs, with the exception of seven cases in which the survey was completed by

the primary veterinarian as the owners were not contactable. A copy of the survey is included in Appendix A. Locations of myopathy cases were defined as the GPS co-ordinates of where dogs had hunted immediately prior to developing clinical signs, or if this was not known or not applicable (for working farm dogs or pet dogs), the home address of the dog was used. Blood and/or tissue samples collected from dogs in order to fulfil the case definition were part of a separate study investigating the pathology of this myopathy (Chapter 3), which was approved by the Massey University Animal Ethics Committee (approval number 14/65).

2.2.3 Survey of hunting activities, diet, and health of pig hunting dogs

An online survey ("online pig hunting dog survey," Appendix B) was designed using commercially available software (SurveyGizmo, Boulder, CO, USA) to collect data regarding healthy pig hunting dogs. A risk assessment determined that the survey was low risk according to the criteria of the Massey University Human Ethics Committee, and full ethical approval was not required. Any responses that indicated the dog had experienced trembling, lethargy and/or collapse in the previous 12 months were excluded, due to the potential for these dogs to be undiagnosed cases of GSM. Partial responses that did not include dietary information and duplicates (identified as responses from the same latitude and longitude with the same number and names of dogs) were also excluded. A link to the survey was posted in a closed Facebook group called 'Pig Hunting Gear and Dogs For Sale NZ' on August 13, 2015 and respondents self-selected from this group. This group was chosen as it was the largest New Zealand pig hunting interest group on Facebook at the time, with approximately 9,000 members (although members are not required to be active hunters to join).

In the survey, respondents were asked to enter the names of up to three of their dogs, and the second dog entered was selected as the subject of subsequent questions regarding dog diet and health. Respondents were also asked to characterise the role that this dog played in pig hunting, with permitted answers being any combination of 'finder', 'bailer' and/or 'holder,' or open text entry.

2.2.4 Statistical analysis

Answers to survey questions with numerical or open text answers were summarised using simple descriptive statistics. The proportions of each of the multiple choice answers chosen were calculated, along with the approximate 95% confidence interval (CI) of each proportion, using the general formulas described by Dohoo *et al*.[12]

2.3 Results

2.3.1 Signalment of dogs

Of 112 possible cases of GSM, a total of 86 fulfilled the case definition and were recruited into the study from June 2014 to June 2017. Of these, 58 cases (67%; 95% CI = 58 – 77%) occurred in pig hunting dogs, 16 (19%; 95% CI = 10-27%) in pet dogs and the remaining 12 cases (14%; 95% CI = 7 – 21%) were in working farm dogs. The number of dogs belonging to each owner, the dog age, and the sex ratio are shown in Table 2.1 and are similar to the findings of the online pig hunting dog survey. Overall, 60 of the cases occurred in crossbred dogs (70%; 95% CI = 60 – 79%) and common breeds or crosses included heading dogs (13 dogs; 15%; 95% CI = 8 – 23%), cattle dogs (12 dogs, 14%, 95% CI = 7 – 21%), greyhounds (11 dogs, 13%; 95% CI = 6 – 20%) and Labradors (7 dogs, 8%, 95% CI = 2 – 14%). The affected purebred dogs were most commonly working farm dogs or pet dogs, (17 of 26 purebred dogs; 65%; 95% CI = 47 – 84%), rather than pig hunting dogs.

In the online pig hunting dog survey, 203 eligible responses were received between 13th and 20th August 2015. In 191 of the 203 responses, dogs were classified according to their role when hunting pigs, and 79 of these (41%; 95% CI = 34 – 48%) were reported to find, bail, and hold pigs. A further 49 dogs (26%; 95% CI = 19 – 32%) were characterised as both finders and bailers, and 35 dogs (18%; 95% CI = 13 – 24%) were both finders and holders. The remaining 28 dogs (15%; 95% CI = 10-20%) had a single role (finding, bailing or holding pigs) while hunting. Age, sex and breed characteristics of dogs in this survey are shown in Table 2.1. A total of 81 different breeds or crossbreeds of dog were described by 196 of the respondents, and the breeds that featured prominently in crosses included cattle dogs (66 dogs, 34%; 95% CI = 27 – 40%), greyhounds (62 dogs, 32%; 95% CI = 25 – 38%), bull terriers (37 dogs, 19%; 95%

CI = 13 – 24%), Labradors (26 dogs, 13%; 95% CI = 9 – 18%) and whippets (21 dogs, 11%; 95% CI = 6 – 15%).

Table 2.1: Age, sex and breed characteristics of dogs included in the 'Go Slow' myopathy (GSM) case survey conducted between June 2014 and June 2017, and an online pig hunting dog survey conducted in August 2015.

	GSM case survey	**Pig hunting dog survey**
Number of responses	86	203
Median no. of dogs per owner (range); *95% CI*	3 (1 – 16); *3 - 4*	3 (1 – 8); *3 - 4*
Median dog age in years (range); *95% CI*	*4 (0.5 – 14); 3- 5*	3 (0.3 – 15); *3 - 4*
Sex (% male/ % female); *95% CI*	56/44; *45-66/34-55*	55/45; *48-62/39-53*
Breed (% purebred / % crossbred); *95% CI*	30/70; *21-40/60-79*	9/91; *5-13/87-95*

2.3.2 Geographic distribution of myopathy cases and overall pig hunting activity

Locations of GSM cases are shown in Figure 2.1, and it can be seen that cases predominantly occurred in the upper half of the North Island. The number of cases in Northland was disproportionately higher than in other regions, with 65 of the cases (76%; 95% CI = 67 – 85%) occurring here. However, one particular veterinarian in Okaihau, Northland, was responsible for recruiting 43 of the cases (50%) into the study as she had a particular interest in the disease. The Waikato and Bay of Plenty each had three cases (4%; 95% CI = 0 – 7%) included in the study. In 2016, three cases occurred in the lower North Island, all of which were in the Wairarapa region, but prior to that, no cases had been reported in this area. Throughout the three-year study period, only two cases were recruited from the South Island. Both of these cases were in working farm dogs from the same property in Canterbury (represented by a single red dot in Figure 2.1). A common feature amongst cases was a history of having been on or around sheep and beef farms in the week prior to the initial onset of clinical signs (56 cases, 65%; 55 – 75%).

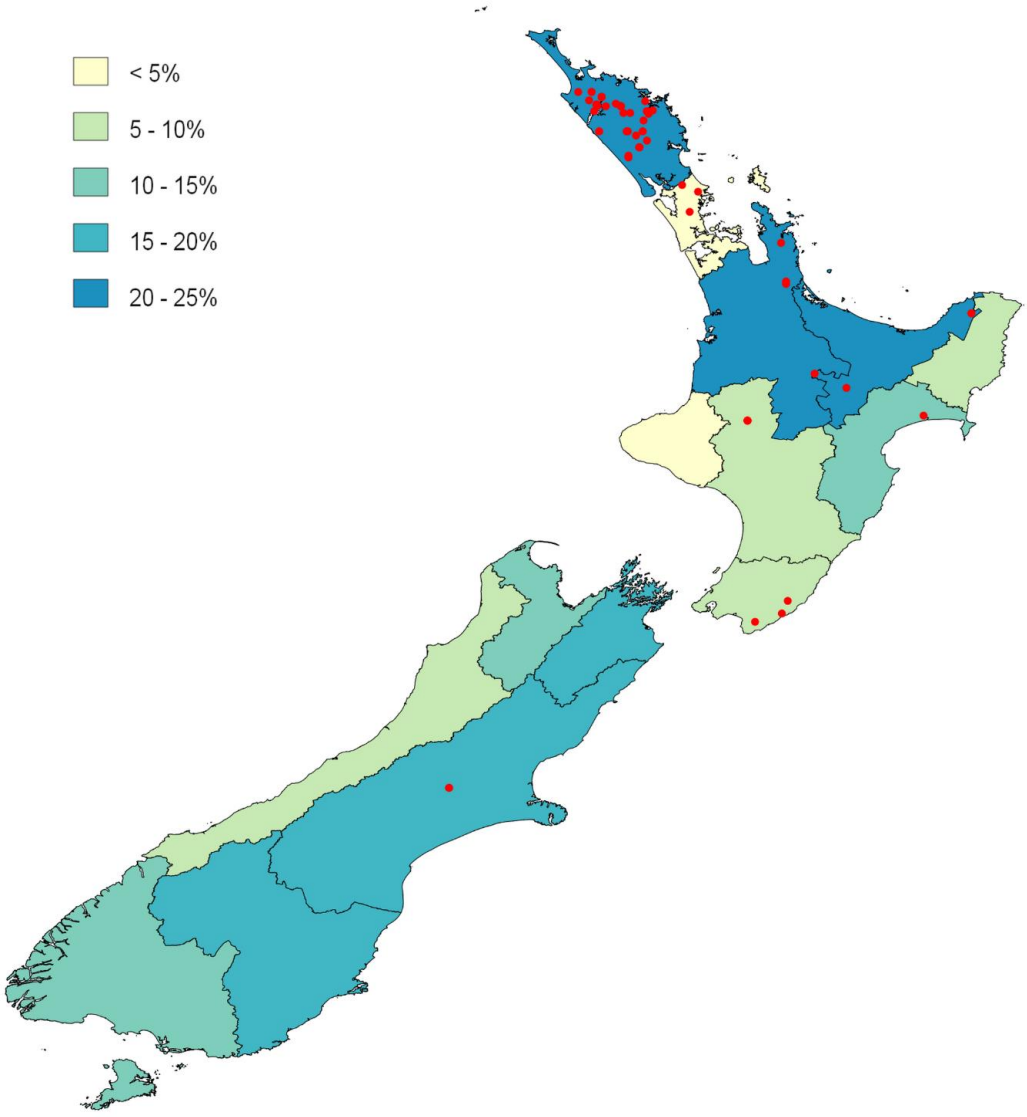

Figure 2.1: Map of New Zealand depicting the locations of 'Go Slow' myopathy cases in dogs between June 2014 and June 2017 (●). Case locations frequently represent more than one dog as some owners had multiple dogs affected, and in some popular hunting locations several independent cases occurred at different times. Shading of regions on the map denotes the percentages of pig dog hunting survey respondents which hunted for pigs in each region from August 2014 to August 2015. Respondents were able to select multiple regions, as applicable.

The proportions of online pig hunting dog survey respondents that hunted in each region of New Zealand over the preceding 12 months are also depicted in Figure 2.1, and shown with their 95% confidence intervals in Table 2.1. Of the 203 survey respondents, 100 (49%; 95% CI = 42 – 56%) reported that they had hunted in more than one region, and hunting of wild pigs using dogs was most common in the Bay of Plenty, Waikato and Northland (all in the North Island), followed by Otago, Canterbury

and Marlborough (South Island locations). A variety of land types were popular for pig hunting, with 167 respondents (82%; 95% CI = 77 – 88%) indicating that they regularly hunted on more than one land type. Similar proportions of respondents hunted in native bush (73%; 95% CI = 67% - 80%), on or around sheep and beef farms (71%; 95% CI = 65 – 78%) and in pine forestry plantations (71%; 95% CI = 65 – 77%). Less common hunting sites included dairy farms (32%; 95% CI = 26 - 38%) and tussock land (5%; 95% CI = 2 – 9%).

Table 2.2: Number and percentage of respondents who reported hunting for pigs in each region of New Zealand from August 2014 - August 2015, based on data from 203 respondents to the online pig hunting dog survey. Respondents were able to select multiple regions, as applicable.

Hunting region	*n*	% of respondents; *95% CI*
Northland	41	20.2; *15-25*
Auckland	10	4.9; *2-8*
Waikato	45	22.2; *17-28*
Bay of Plenty	47	23.2; *17-29*
Gisborne	17	8.4; *5-12*
Hawke's Bay	23	11.3; *7-16*
Taranaki	10	4.9; *2-8*
Manawatu	11	5.4; *2-9*
Wellington	14	6.9; *3-10*
Nelson/Tasman	26	12.8; *8-17*
Marlborough	34	16.7; *12-22*
West Coast	11	5.4; *2-9*
Canterbury	38	18.7; *13-24*
Otago	39	19.2; *14-25*
Southland	27	13.3; *9-18*

2.3.3 Seasonal occurrence of myopathy cases and hunting frequency

Cases of GSM occurred throughout the year, with a peak incidence in June (Figure 2.2). The percentage of cases that occurred in June was higher than the percentage of cases that occurred monthly from November to March (late spring, summer and early autumn), as indicated by the error bars in Figure 2 that represent the 95% confidence intervals of each percentage. The lowest percentage of cases occurred in December and March, and the 95% confidence intervals for these months do not overlap with the 95% confidence intervals for April, May, June, July, August and October.

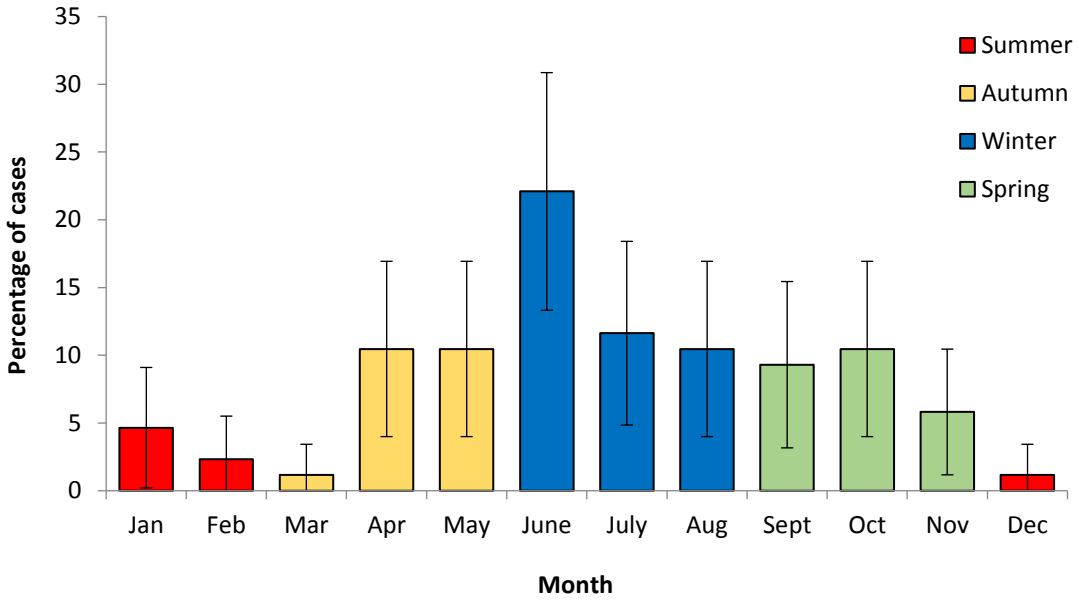

Figure 2.2: Bar graph showing the temporal distribution of 'Go Slow' myopathy cases in dogs, based on a series of 86 cases between June 2014 and June 2017. Cases are plotted according to the month in which clinical signs of the myopathy were first reported. Error bars show the 95% confidence interval for each percentage

In the pig hunting dog survey, hunting frequency was a categorical variable. Across all seasons, the most commonly selected hunting frequency was 'every week'. In winter, spring and autumn the second most common frequency was '2 to 3 times per week,' whereas in summer it was 'every 1 to 2 weeks,' suggesting that overall pig hunting might be less frequent in summer.

2.3.4 Diet of myopathy cases and healthy dogs used for pig hunting

Owners were questioned about what dogs affected by GSM had eaten in the week prior to first developing clinical signs. The results are presented in Table 2.3 and show that processed commercial dog food, which includes dry food (biscuits), dog roll and canned dog food, was the most common type of food fed, followed by the feeding of meat, offal and/or bones from wild animals. Tissues from wild animals were fed to 79 of the 86 affected dogs (92%; 95% CI = 86 – 98%), particularly tissues from wild pigs (76 dogs, 88%; 95% CI = 82 – 95%). All of the working farm dogs (including the two affected dogs from the South Island), and all of the pet dogs except two, had a history of eating wild pig. Of the 10 dogs not fed wild pig, eight dogs (9%; 95% CI = 3 - 15%)

could have had access to tissues from wild pigs, but were not directly observed to eat any (dogs that went missing temporarily in the bush or that might have had the opportunity to scavenge pig meat). This includes the two pet dogs that were reportedly not fed wild pig, and means that only 2 cases (2%; 95% CI = -1 – 1%) had no known possible access to wild pig. Of the 76 dogs confirmed to have eaten meat, offal and/or bones from wild pigs, 51 (67%; 95% CI = 57 – 78%) ate raw wild pig. In the remaining 25 cases (33%; 95% CI = 22 – 44%), the pig had been frozen and/or cooked prior to being fed to the dogs. In addition to wild pig, meat, offal and bones from a variety of farmed animals (obtained from a supermarket, butcher or homekill) were commonly fed to dogs diagnosed with the myopathy, while table scraps and milk were less frequently fed.

For reference, the diet of healthy pig hunting dogs in the week prior to the online survey (August 2015) is also included in Table 2.3. These results are not directly comparable to the myopathy case data, but give an overview of what may be commonly fed to dogs used for pig hunting. Commercial dog food was the most common type of food, followed by meat, offal and bones from farmed animals. Similar numbers of dogs were fed meat, offal and bones from wild animals (predominantly pigs) and table scraps (32% each, 95% CI = 25 – 38%). Respondents to this survey were also asked if their dogs were ever fed wild pig, not just in the preceding week (Q12, Appendix B). Of 194 valid responses to this question, 55 (28%; 95% CI = 22 – 35%) never allowed their dogs to eat meat, offal or bones from wild pigs, while a further 23 (12%; 95% CI = 7 – 16%) did not intentionally feed their dogs wild pig, but dogs might sometimes have the opportunity to scavenge meat or offal. Forty-four respondents (23%; 95% CI = 17 – 29%) usually gave their dogs offal/trimmings on a hunt or while butchering the pig. The remaining 72 respondents (37%; 95% CI = 30 – 44%) sometimes fed their dog wild pig as a meal (either raw, previously frozen or cooked).

Table 2.3: Diet of 86 dogs diagnosed with 'Go Slow' myopathy (GSM) in the week prior to the initial onset of clinical signs. For reference, the diet of 203 healthy pig hunting dogs in New Zealand over a 1-week period in August 2015 is also presented (pig hunting dog survey).

Dog diet over the preceding 7 days[a]	**GSM case survey %;** *95% CI*	**Pig hunting dog survey %;** *95% CI*
Commercial dog food	93; *88 – 98*	89; *85 – 93*
Table scraps[b]	16; *9 – 24**	32; *25 – 38*
Milk	3; 0 – 7	9; *5 – 13*
Meat, offal or bones from farmed animals	**70; *60 – 79***	**67; *61 – 74***
- Cattle	55; *44 – 65*	51; *44 – 58*
- Sheep	49; *38 – 59*	41; *35 – 48*
- Chickens	6; *1 – 11**	21; *16 – 27*
- Pigs	12; *5 – 18*	8; *5 – 12*
- Deer	1; *-1 – 3*	5; *2 – 9*
- Horse	5; *0 – 9*	0.5; *0 – 1*
Meat, offal or bones from wild animals	**92; *86 – 98***	**32; *25 – 38***
- Pigs	88; *82 – 95*	27; *20 – 33*
- Deer	6; *1 – 11*	16; *11 – 21*
- Possums	5; *0 – 9*	11; *7 – 16*
- Goats	3; *0 – 7*	8; *5 – 12*
- Rabbits	1; *-1 – 3*	1; *0 – 2*

[a] Survey questions are presented in Appendix A and B. In both surveys, respondents were able to select as many options as applicable

[b] Table scraps include food, other than meat, primarily prepared for human consumption.

2.3.5 Myopathy case presentation and overall pig dog health

All dogs diagnosed with GSM had been seen by a veterinarian for clinical signs related to the myopathy, as clinical examination performed by a veterinarian was part of the case definition for inclusion in the study. The most common clinical sign reported was lethargy/tiring easily with exercise (79% of cases; 95% CI =70 – 88%), followed by generalised muscle fasciculations or shaking (62%; 95% CI = 51 – 72%). Other clinical signs included collapse or weakness during exercise (34%; 95% CI = 24 – 44%), stiffness when walking (26%; 95% CI = 16 – 35%), vomiting (17%; 95% CI = 9 – 25%), and diarrhoea (15%; 95% CI 8 – 23%). The myopathy occurred in dogs that were rested and confined to kennels as well as dogs with a history of recent exercise, but in all cases, clinical signs were worsened by exercise. Fifty-two of the dogs (61%; 95% CI = 50 –

71%) initially developed clinical signs while exercising, or had a history of strenuous exercise (hunting, farm work or a greater than 30-minute run) in the 24 hours prior to the onset of signs. The clinical and pathological features of cases included in this study are discussed in further detail in Chapter 3.

In the online pig hunting dog survey, 194 valid responses were received to the questions related to dog health (Q13 and 14, Appendix B). In the 12 months prior to the survey, 114 of the dogs (59%; 95% CI = 52 – 66%) had been seen by a veterinarian. Wounds were the most common health problem reported in this sample of dogs, with 58 dogs (30%; 95% CI = 23 – 36%) wounded during the year. Of these 58 dogs, 17 (29%; 95% CI = 18 – 41%) had not been seen by a veterinarian in the previous 12 months, which might suggest that these wounds were either not serious enough to require veterinary treatment, that owners had managed the dog's wounds themselves, or that the wounds were fatal. Other common health issues identified included weight gain (8%; 95% CI = 4 – 11%), weight loss (7%; 95% CI = 4 – 11%), joint stiffness (6%; 95% CI = 3 – 10%), diarrhoea and skin problems (6% each; 95% CI = 2 – 9%).

2.4 Discussion

Cases of 'Go Slow' myopathy (GSM) were most commonly reported in pig hunting dogs, but working farm dogs and pet dogs were also affected. There were no apparent breed predilections or genetic predispositions for the disease as the majority of affected dogs (particularly the pig dogs) were crossbreeds and a wide variety of breeds were represented. Cattle dogs, greyhounds, Labradors featured commonly, as they do in the wider pig dog population in New Zealand, and all of the working farm dogs affected were heading dogs or heading dog crosses, which is a common breed of working dog in New Zealand[13, 14]. A high proportion of cases had spent time on or around sheep and beef farms in the week prior to developing clinical signs (either as working farm dogs, hunting dogs or pet dogs), but equally, a similar proportion of unaffected pig hunting dogs frequently hunted on or around sheep and beef farms. The median age, age range and sex distribution of dogs with the myopathy reflect that of the healthy pig hunting dogs included in the online survey, suggesting there are no age or sex predilections for this disease.

The most notable finding was that a high proportion of affected dogs ate meat, offal or bones from wild animals, particularly wild pigs, in the week prior to developing clinical signs of the GSM. Of the cases with a confirmed history of eating wild pig, approximately two-thirds had eaten raw meat, offal or bones, while in the remainder of cases, the pig tissues had been previously frozen and/or cooked (including cooked meat leftover from barbecues and roasts for human consumption). Aside from wild pig, the dietary intakes of dogs affected by the myopathy were similar to the healthy dogs in the online survey. In both surveys there is the potential for inaccuracies and omissions in owner recall, particularly in regards to the dietary intake of myopathy cases. Surveys were conducted with owners of affected dogs as soon as practicable following diagnosis, in order to maximise the accuracy of the owner's recollections. However, in some cases, several days or weeks had elapsed between the dog developing clinical signs and the diagnosis being confirmed. In addition, owners of affected dogs may be more likely to scrutinise their dog's diet in detail, compared to owners of healthy dogs.

Cases of GSM in the present study predominantly occurred within the upper half of the North Island of New Zealand, particularly in the Northland region. Fewer cases were recognised in the Waikato and Bay of Plenty regions, which were common hunting locations in the online pig hunting dog survey. The high number of cases reported in Northland could represent a spatial trend, or be a result of over-reporting in this region. The myopathy was first identified in Northland and the disease has received publicity in local newspapers and hunting newsletters, which could increase awareness of the disease and therefore recognition and recruitment of cases. Additionally, the cases were not selected at random and a veterinarian in Okaihau, Northland had a special interest in the disease and actively encouraged owners to seek veterinary care for suspected cases. As a result, this veterinarian recruited a high number (43) of dogs for the present study, which could contribute to selection bias. However, attempts were made to publicise the study nationwide and recruit cases from throughout New Zealand, and pathologists working in commercial diagnostic laboratories were aware of the disease and listed it as a differential diagnosis in any cases with consistent clinical pathology findings, regardless of geographic location. GSM is widely discussed

on pig hunting forums and Facebook pages and hunters throughout the country know of the disease, which could minimise geographic bias, but may contribute to the high proportion of cases in pig hunting dogs relative to pet dogs and working farm dogs, as the owners of hunting dogs could be more familiar with the disease. Selection bias is also possible in the online pig dog hunting survey, as 203 eligible responses were received from a group with approximately 9,000 members, and the survey was only advertised through Facebook. Furthermore, survey responses were only collected over a week in August and dog diets may change over the year to reflect the availability of different sources of meat.

Even taking into account possible sources of bias, ingestion of wild pig was a consistent feature of myopathy cases. There are no reports of myopathies in dogs associated with the ingestion of wild pig, but there are several organisms that could be transmitted from pigs to dogs and cause muscle damage. Infectious agents present in New Zealand that can infect both pigs and dogs and cause muscle damage include *Trichinella spiralis*[15], *Toxoplasma gondii,*[16, 17] *Neospora caninum*[18] and *Leptospira interrogans.*[19,20] However, these diseases usually present with clinical signs attributable to organ systems other than skeletal muscle, or no clinical signs (trichinosis in dogs[21]), and the presence of inflammatory cells or infectious agents would be expected on muscle histology, which are not recognised features in this myopathy (discussed in Chapter 3).

In addition to infectious organisms, dogs could potentially be exposed to toxic compounds through the ingestion of wild pigs, including insecticides, vertebrate pesticides or naturally occurring plant or fungal toxins. Historically, insecticides such as organochlorines and organophosphates have been widely used in the agricultural sector, and organochlorines in particular may persist in the environment for extended periods. Focal muscle necrosis has been reported with both organochlorines[22] and organophosphates.[23] and residues of organochlorines have been found in tissues of wild boars.[24] Vertebrate pesticides used in New Zealand include sodium monofluoroacetate (1080), sodium nitrite, cyanide, anticoagulants (pindone, diphacinonone, brodifacoum), cholecalciferol (Vitamin D_3) and elemental phosphorus.[25-27] The elimination half-lives of 1080, sodium nitrite and cyanide are

short,[26, 28] and the risk of secondary poisoning of dogs from pigs killed by hunters, rather than lethal doses of these pesticides, is likely to be small. Phosphorus and anticoagulant compounds, particularly brodifacoum, have longer elimination half-lives[26, 29] and secondary and tertiary poisonings of dogs with brodifacoum are possible,[30] but the clinical signs of exposure to these poisons are not consistent with a myopathy.[31]

Plant species present in New Zealand that have been reported to cause skeletal muscle lesions, or contain toxins known to damage muscle, include staggerweed (*Stachys arvensis*),[32] sycamore and box elder trees (*Acer* spp.), which contain the toxin hypoglycin that causes seasonal pasture myopathy in horses,[33, 34] and the native karaka (*Corynocarpus laevigatus*)[35] and kowhai trees *(Sophora* spp*)*[36]. Edible wild mushrooms present in New Zealand have also been reported to cause increases in serum muscle enzyme activities in people and mice, including *Tricholoma* spp.,[37, 38] *Lentinus edodes,*[39] *Amanita phalloides,* [40] *Boletus edulis* and others.[41] Overseas, the white snakeroot plant (*Ageratina altissima*, previously known as *Eupatorium rugosum*) has been reported to cause skeletal muscle damage in various animals,[42-44] as well as a secondary myopathy ('Milk Sickness') in people that ingest milk, meat, butter or cheese made from cattle which eat the plant.[45, 46] *Ageratina altissima* is not naturalised in New Zealand,[47] but two closely related species are.[48] Studies to investigate the pathology of GSM in dogs could provide further insight into how the disease develops and progresses, and therefore possible causes (Chapter 3).

The present study has shown that 'Go Slow' myopathy, an idiopathic myopathy previously reported in hunting and working dogs, can also occur in pet dogs. In affected dogs, clinical signs were exacerbated by exercise, but the disease also occurred in dogs that had been rested and confined to a kennel. Consumption of raw, previously frozen or cooked tissues from wild animals, particularly wild pigs, was a notable feature in cases of the myopathy. In this particular study, cases were most commonly reported in regions in the upper North Island of New Zealand, and in light of these findings, it would seem prudent to avoid feeding any tissues from wild pigs to dogs in these areas.

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Chapter 3

Clinical and microscopic pathology of 'Go Slow' myopathy

3.1 Introduction

As outlined in Chapter 1, the first cases of an idiopathic myopathy ('Go Slow' myopathy, GSM) in dogs in New Zealand were recognised in the late 1990's to early 2000's.[1] Preliminary investigations by the Ministry of Primary Industries (formerly known as the Ministry of Agriculture and Forestry) in 2004 reported that affected dogs presented with an acute onset of trembling, weakness, and collapse.[2] Clinical signs were induced or exacerbated by exercise and mortality was low, although some dogs had a prolonged return to full fitness and relapses were reported. Serum biochemistry findings in affected dogs included increased serum creatinine kinase (CK), aspartate aminotransferase and alanine aminotransferase (ALT) activities, which were interpreted to be indicative of muscle damage. Electromyography was performed in two cases, and no specific myopathic or spontaneous muscle activity was reported. These two dogs also had muscle biopsies taken, which showed scattered necrotic myofibres, with evidence of muscle regeneration in one dog and muscle atrophy in the other.

As part of the 2004 study, serological evaluation for exposure to *Neospora caninium, Toxoplasma gondii* and/or *Leptospira interrogans* was performed in eight cases, and all dogs tested had negative or low antibody titres to these organisms. Blood selenium concentrations were analysed in four dogs and were found to be within the reference range. The clinical signs and pathological features of the disease were considered consistent with a myopathy, but the possibility of a concurrent peripheral neuropathy or neuromuscular junction disorder (junctionopathy) could not be excluded. The preliminary investigations did not establish any underlying cause, but an exotic infectious disease was considered unlikely and research interest in the disease waned.

Over the following 10 years, there were sporadic reports of cases of the myopathy in Northland,[3] but no diagnostic testing was performed beyond routine serum biochemistry. In mid-2014, a veterinarian in Northland made contact with researchers at Massey University after seeing multiple suspected cases. Anecdotally, several of her clients also reported that similar clinical signs had occurred in their dogs in the past, but most had not sought treatment for their dogs as they perceived that veterinarians had limited knowledge of the disease and could do little to help in suspected cases.

The present study was designed to investigate the clinical course of GSM and to characterise the underlying pathology of the disease using a variety of techniques, including serum biochemistry, histology and electron microscopy.

3.2 Methods

3.2.1 Case recruitment

The present study was performed in conjunction with the study of epidemiological features of the disease in dogs, and the methods of case recruitment are the same as outlined in Chapter 2. Briefly, articles about GSM were written for several veterinary and laboratory newsletters, pig hunting magazines, newspapers and pig hunting interest groups on Facebook, along with presentations at various conferences and meetings to raise awareness of the present study. Cases were recruited through notifications from veterinarians, veterinary pathologists, pig hunters and dog owners over a three-year period from June 2014 to June 2017. The study protocol and sample collection procedures were approved by the Massey University Animal Ethics Committee (approval number 14/65).

The cases included in this study are the same as those in the epidemiological study, and the same case definition was applied. The case definition was devised based on the findings of the 2004 MAF study,[2] with input from veterinarians in Northland who had seen cases previously. Cases of GSM were defined as dogs that presented with a sudden onset of trembling, exercise intolerance and/or weakness and collapse. Less commonly, affected dogs also had vomiting and diarrhoea in the acute stages, but

clinical examination (performed by a veterinarian) was otherwise normal. Muscle damage was confirmed using serum biochemistry, which showed increases in serum creatine kinase and aspartate aminotrasferase, or skeletal muscle histology (or both). Results of additional diagnostic tests, conducted at the discretion of the veterinarian, were not consistent with any known causes of myopathies in dogs.

In suspected cases, relevant clinical histories and laboratory results were obtained from the consulting veterinarian. As soon as practical, owners of affected dogs were contacted by telephone to discuss the case history, the dog's recent diet and exercise/hunting activity. Owners were also asked to describe the onset and progression of clinical signs, as well as anything that appeared to improve or worsen the dog's condition. A copy of the survey form is included as Appendix A of this thesis. Where possible, owners were contacted again 2 to 4 weeks after the onset of clinical signs to provide follow up information regarding the progression and/or resolution of the disease. In cases that had not recovered and returned to full fitness after 4 weeks, further follow up phone calls were conducted at 3-4 weekly intervals for at least 3 months.

3.2.2 Haematology, serum biochemistry and serology

Blood samples from affected dogs were collected by the referring veterinarian in each case and samples were sent to commercial veterinary diagnostic laboratories for analysis (regional laboratories of Idexx Laboratories New Zealand Ltd. or of Gribbles Veterinary Pathology Ltd.). In cases recruited early in the study, a full serum biochemistry panel was performed where possible, along with a complete blood count (CBC). As a consistent clinical pathology picture of the myopathy emerged, a more cost-effective 'Go Slow' biochemistry panel was developed with Gribbles Veterinary Pathology, which included serum creatine kinase (CK), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities and bilirubin concentration. Serology to detect antibodies against *Leptospira* sp. was performed by Gribbles Veterinary Pathology (Palmerston North, NZ) using the microscopic agglutination test (MAT). In cases where the clinical history or laboratory results were atypical for the

myopathy, further tests were performed as required by the referring veterinarian as part of their diagnostic approach to the case.

3.2.3 Cell-free DNA (cfDNA) in plasma

Plasma samples were collected from eight pig hunting dogs belonging to the same owner, with the same history of recent exercise and hunting activity. Four of these were dogs that first developed clinical signs and biochemical changes consistent with GSM 4 days prior to sample collection, while the remaining four dogs were clinically normal, with normal serum biochemistry results. Venous blood samples were collected into tubes containing EDTA and centrifuged within 30 minutes to separate plasma, which was frozen and transported to Massey University. A total of 20µL of each plasma sample was used for cfDNA quantification with the Qubit® dsDNA HS Assay Kit and Qubit 2.0 (ThermoFisher Scientific, Waltham, MA, USA). The Qubit assay uses a fluorescent dye selective for double stranded DNA (dsDNA), and the amount of fluorescence recorded is proportional to the amount of dsDNA in the sample. All plasma samples were run as a single batch and the Qubit 2.0 was calibrated with the standards provided prior to use. The concentration of cfDNA in each sample was calculated using the manufacturer's dilution algorithm.

3.2.4 Post mortem examination, toxicology, histology and electron microscopy

Tissue samples for histology were collected from affected dogs that were euthanised as a result of the disease, or due to an inability to perform their primary function (working farm dog or hunting dog) to a level satisfactory to the owner. Post mortem examinations were performed by the referring veterinarian in each case, and 1-2 cm^3 samples from the middle of the triceps muscle in the forelimb and the biceps femoris in the hindlimb were collected into 10% neutral buffered formalin, along with a sample of liver (any lobe). In some cases, additional tissue samples were also collected at the discretion of the referring veterinarian. In four cases, 2 x 2 x 5mm sections of triceps, biceps femoris and liver were also collected immediately after euthanasia and fixed in 2% glutaraldehyde for electron microscopy. Muscle samples for electron microscopy were taken adjacent to the samples collected for histology, in the centre of the muscle

(away from tendinous insertions). Samples for electron microscopy were also collected in the same manner from one clinically normal dog to enable detection of any artefactual or post mortem changes that might arise during tissue sampling and processing. Fresh liver samples were collected from two dogs and frozen at -20^{o}C, then shipped to a commercial toxicology laboratory (Landcare Research, Lincoln, NZ) for analysis of brodifacoum residues. Formalin-fixed samples were routinely processed for histology at Massey University, and stained with haematoxylin and eosin (HE). Samples fixed in glutaraldehyde for electron microscopy were post-fixed in osmium tetroxide and embedded into epoxy resin. Thin sections from resin blocks were stained using lead citrate/uranyl acetate, and viewed using a FEI Tecnai G2 Biotwin Transmission Electron Microscope at the Manawatu Microscopy and Imaging Centre (Massey University, Palmerston North, NZ).

3.2.5 Statistical Analysis

For analysis of serum biochemistry results, cases were grouped according to the time of sampling relative to the onset of clinical signs (Day 1, Day 2, Days 3 – 10, Days 11 – 30 and >30 days). Serum CK, AST and ALT activities were log transformed to achieve a normal distribution, and the groups based on time of sampling were compared to each other using a one-way ANOVA in R Studio.[4] If the *p* value obtained from the ANOVA was significant ($p < 0.05$), pairwise comparisons were performed with a Bonferroni correction applied to identify the sampling groups that differed from each other.

3.3 Results

3.3.1 Clinical signs and disease progression

A total of 86 eligible cases were recruited during the study period. The most common clinical sign of GSM in dogs was lethargy and tiring easily with exercise, reported in 68 dogs (79.1% of cases). Many of these dogs also had generalised muscle fasciculations, trembling or shaking (53 dogs, 61.6% of cases), which were usually most severe in the forelimbs. Twenty-nine dogs (33.7%) presented with collapse during exercise, weakness or difficulty standing, and 22 dogs (25.6%) were stiff when walking or were judged to be painful by the referring veterinarian. Vomiting (15 cases, 17.4%) and

diarrhoea (13 cases, 15.1%) were also reported, particularly in dogs with no history of recent exercise. In four of these 13 dogs, the diarrhoea was described as haemorrhagic. Less common clinical signs noted by veterinarians or owners included bilaterally dilated pupils with normal pupillary light reflexes (six dogs, 7.0%), polydipsia/polyuria and tachypnoea (four dogs each, 4.7%) and dark brown or red discolouration of the urine (three dogs, 3.5%). Clinical signs were exacerbated by exercise or exertion in all cases. Dogs that first presented more than one week after the onset of signs (24 cases) were normal on physical examination at rest, and clinical signs were appreciable only after exercise. In 52 cases (60.5%), clinical signs were first observed during or within 24 hours of strenuous exercise, including hunting, farm work or a run of greater than 30 minutes duration.

Clinical improvement with rest (including resolution of trembling, weakness, vomiting and diarrhoea) was seen in most cases within 1 to 10 days, with no apparent associations between recovery and any treatment or supportive care given. However, recurrence of clinical signs was commonly reported. Of the 86 dogs included in the study, 48 (55.8%) were reported by their owners to recover fully within 3 months, defined as a return to their prior fitness/normal exercise tolerance with no residual effects of the myopathy. Two cases (2.3%) were lost to follow-up after the initial presentation, leaving 36 cases (41.9%) which did not recover during the standard follow-up period of 3 months. It should be noted that many of the dogs that did not fully recover improved enough to tolerate a low level of exercise, but could no longer perform their primary duties (usually hunting or stock work) to the satisfaction of the owner. As a result, 14 of these dogs (16.3% of all cases) were euthanised at the owner's request. Another two dogs (2.3%) were euthanased on welfare grounds during the acute phase of the disease, as their clinical condition deteriorated despite intensive supportive care. Four affected dogs (4.6%) were euthanased for reasons unrelated to GSM (including unrelated injuries or pre-existing behavioural problems).

3.3.2 Haematology

A CBC was performed at the time of initial presentation in 13 cases (15.1%). Erythrocyte parameters (including red blood cell count, haemoglobin concentration and haematocrit) were normal in 9 dogs (69.2%), while 3 dogs (23.1%) exhibited mild haemoconcentration, consistent with dehydration. The remaining dog had a mild anaemia (HCT 0.34, reference range 0.37 – 0.55 L/L), with no apparent clinical cause such as recent trauma or haemorrhagic diarrhoea. This anaemia was shown to be regenerative on repeat haematology one week later. Total and differential leukocyte counts were normal in six out of the 13 dogs (46.2%), while five dogs (38.5%) had a mild neutrophilia, consistent with either stress or inflammation. The remaining two dogs (15.4%) had eosinophilia, accompanied by mild monocytosis in one case and lymphopenia in the other. Platelet counts were normal in all dogs .

3.3.3 Serum CK, AST and ALT activities

Analysis of serum CK and AST ± ALT activities was performed in 78 of the 86 cases. The remaining eight cases were part of working or hunting dog packs where multiple dogs presented in an identical manner, but serum samples were not taken from all dogs at the time of initial presentation. As seen in Table 3.1 and Figures 3.1 – 3.2, there was marked variation of serum CK activities in dogs with GSM, with results ranging from 146 to 276,830 IU/L (reference range 0 to 385[a] or 609[b] IU/L, laboratory dependent). Twenty-four dogs had serum CK activities greater than 25,000 IU/L at the time of initial presentation; in 16 of these blood samples were collected on day 1, a further four cases were sampled on day 2, another on day 5, and three on day 10. Seven of these dogs were excluded from plots and statistical analysis as they were reported by the laboratory[b] as >25,000 IU/L rather than an absolute value. A one-way ANOVA of log transformed serum CK activities showed a significant difference between groups with different durations of clinical signs at the time of sampling ($p<0.001$). Pairwise comparisons with t-tests revealed that log transformed CK activities from dogs sampled on day 1 were significantly different to dogs sampled between day 11 and 30 ($p = 0.001$) and over 30 days ($p = 0.002$).

[a] Gribbles Veterinary Pathology, New Zealand

[b] New Zealand Veterinary Pathology / Idexx Laboratories, New Zealand

Serum AST activity was increased above the reference range in 73 of the 78 dogs (93.6%), with a range of 23 – 8,800 IU/L (reference range 0 – 51[a] or 79[b] IU/L, laboratory dependent). Mean serum AST activities were highest in dogs sampled within 10 days of the onset of clinical signs, but there was marked variation in results between dogs with a similar duration of clinical signs, as shown in Table 3.1 and Figures 3.3 – 3.4. A one-way ANOVA showed a significant difference in log transformed AST activities between groups sampled at different time points (p <0.001), and pairwise t-tests revealed that AST activities in dogs sampled on day 1 were significantly different to dogs sampled on days 11 – 30 (p = 0.0036), or at >30 days (p = 0.0199).

The serum ALT activity (Figure 3.5) was measured in 74 dogs at the time of initial presentation, and was increased above the reference range (0 – 88[a] or 75[b] IU/L, laboratory dependent) in 63 of these (85.1%). In contrast to CK and AST, log transformed ALT activities (Figure 3.6) did not differ significantly between sampling groups (p = 0.6872). There was a strong correlation between the log transformed CK and AST, as shown by the linear regression line (R^2 = 0.831) in Figure 3.7, but log transformed serum ALT activities were poorly correlated with both log transformed AST (R^2 = 0.322) and CK (R^2 = 0.160).

Table 3.1: Mean ± SD (range) activities of CK, AST and ALT (IU/L) in serum of dogs with 'Go Slow' myopathy at the time of initial presentation, grouped according to the time elapsed since the onset of clinical signs.

Variable	Day 1 (n = 33)^	Day 2 (n = 15)ǂ	Day 3 -10 (n = 11)	Day 11 – 30 (n = 8)	>30 days (n = 11)
CK	36,243 ± 55,411 (1,072 - 276,830)	29,314 ± 45,131 (169 - 117,850)	35,152 ± 54,578 (146 - 174,810)	1,506* ± 1,129 (332 - 3,063)	2,212* ± 1,577 (357 - 4,500)
AST	1,760 ± 1,882 (125 - 8,267)	1,586 ± 1,740 (32 - 5,515)	1,800 ± 2,731 (23 - 8,800)	221* ± 189 (39 - 486)	350* ± 285 (57 - 900)
ALT	691 ± 1,589 (70 - 8,800)	399 ± 321 (50 - 926)	501 ± 465 (27 - 1,541)	431 ± 169 (192 - 584)	394 ± 511 (37 - 1,791)

^ n = 27 for CK

ǂ n = 14 for CK

* p < 0.05 compared with dogs sampled on day 1 (log transformed values)

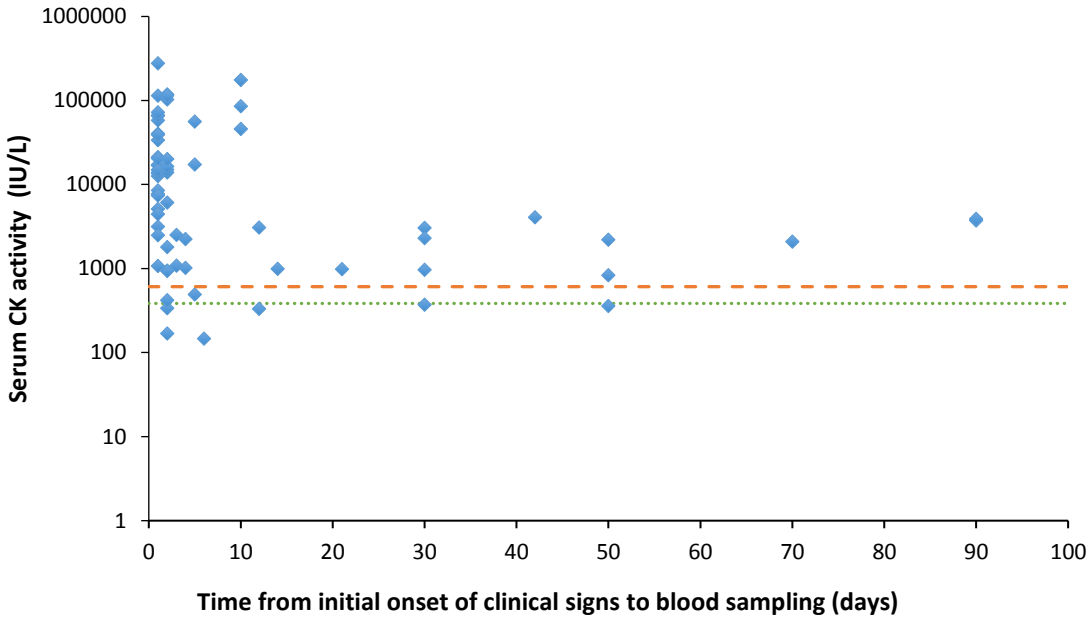

Figure 3.1: Scatterplot of serum creatine kinase (CK) activities in 67 dogs with 'Go Slow' myopathy at time since initial onset of clinical signs. Cases that were sampled >100 days *(n=4)* after the initial onset of clinical signs, and results reported as >25,000 IU/L *(n=7),* rather than an absolute value, are not shown. The dashed orange line represents the upper limit of the IDEXX Laboratories reference range (609 IU/L), while the Gribbles Veterinary Pathology upper limit is 385 IU/L (dotted green line)

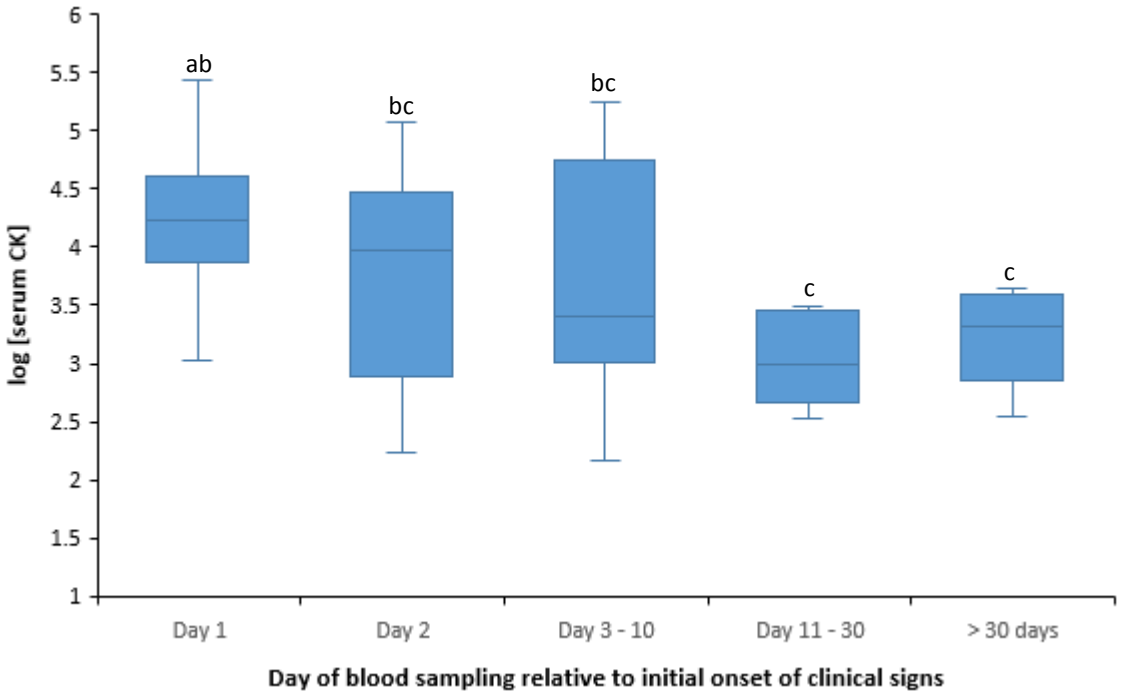

Figure 3.2: Boxplot of log transformed serum creatine kinase (CK) activities in 71 dogs with 'Go Slow' myopathy, grouped according to the time since initial onset of clinical signs. Results reported as >25,000 rather than an absolute value *(n=7)* are not shown. Different letters denote groups that are significantly different to each other ($p < 0.05$)

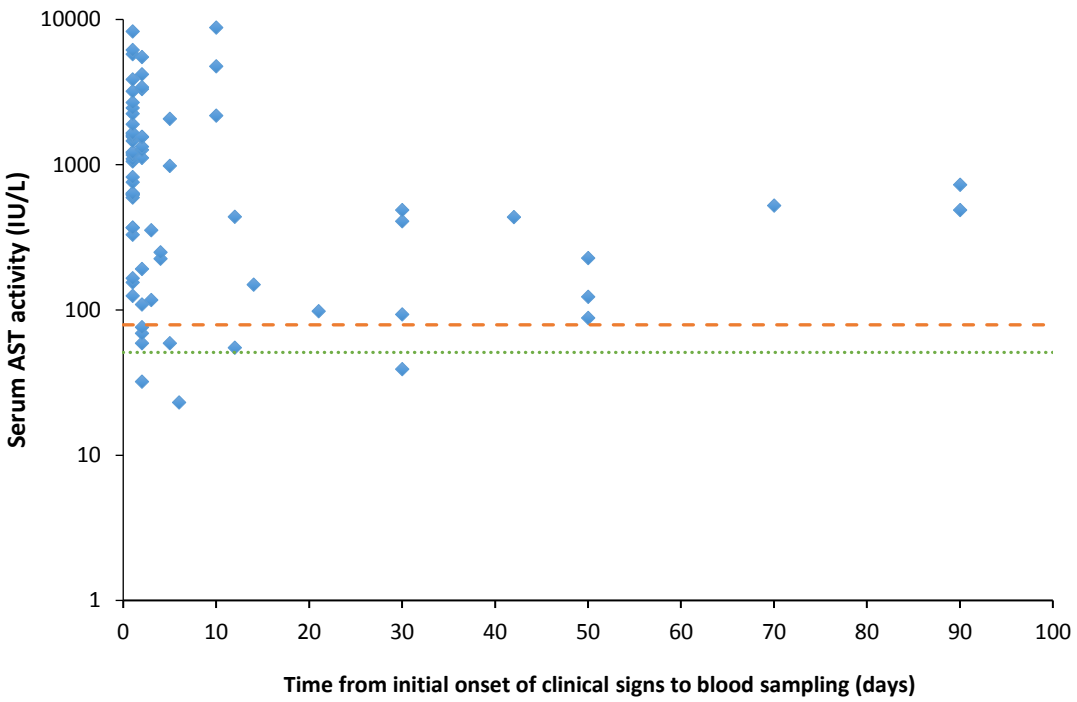

Figure 3.3: Scatterplot of serum aspartate aminotransferase (AST) activities in 74 dogs with 'Go Slow' myopathy at time since initial onset of clinical signs. Cases that were sampled >100 days (*n=4)* after the initial onset of clinical signs are not shown. The dashed orange line represents the upper limit of the IDEXX Laboratories reference range (79 IU/L), while Gribbles Veterinary Pathology has an upper limit of 51 IU/L (green dotted line)

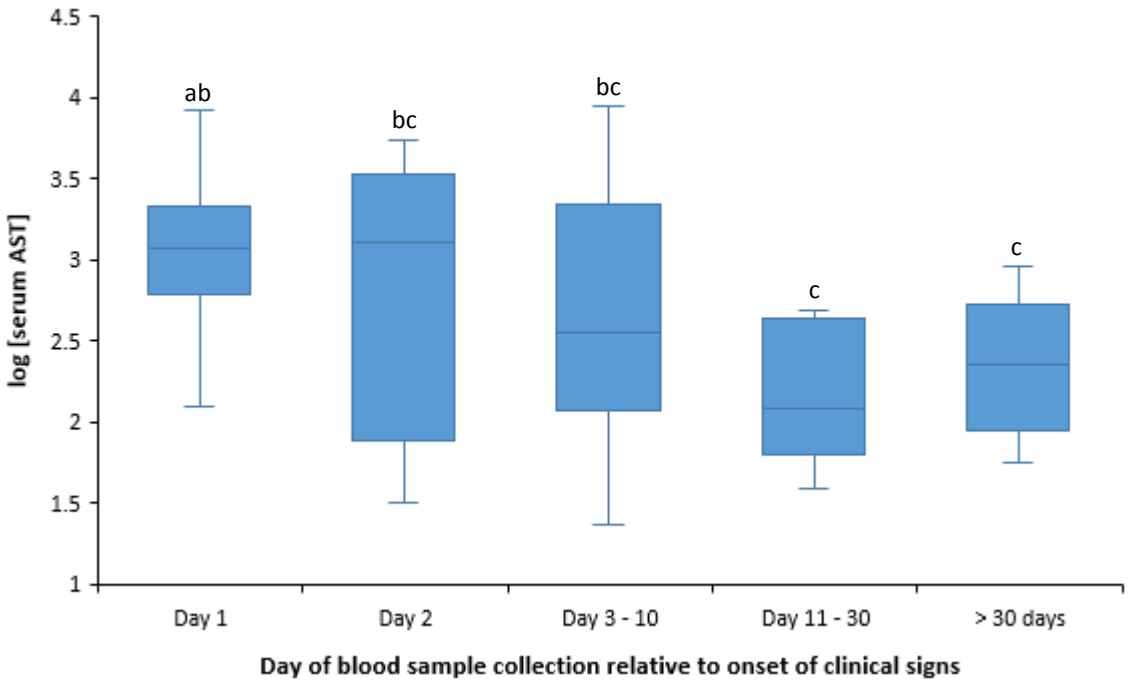

Figure 3.4: Boxplot of log transformed serum AST activities in 78 dogs with 'Go Slow' myopathy at the time of initial presentation, grouped according to the time since the first onset of clinical signs. Different letters denote groups that are significantly different to each other ($p < 0.05$)

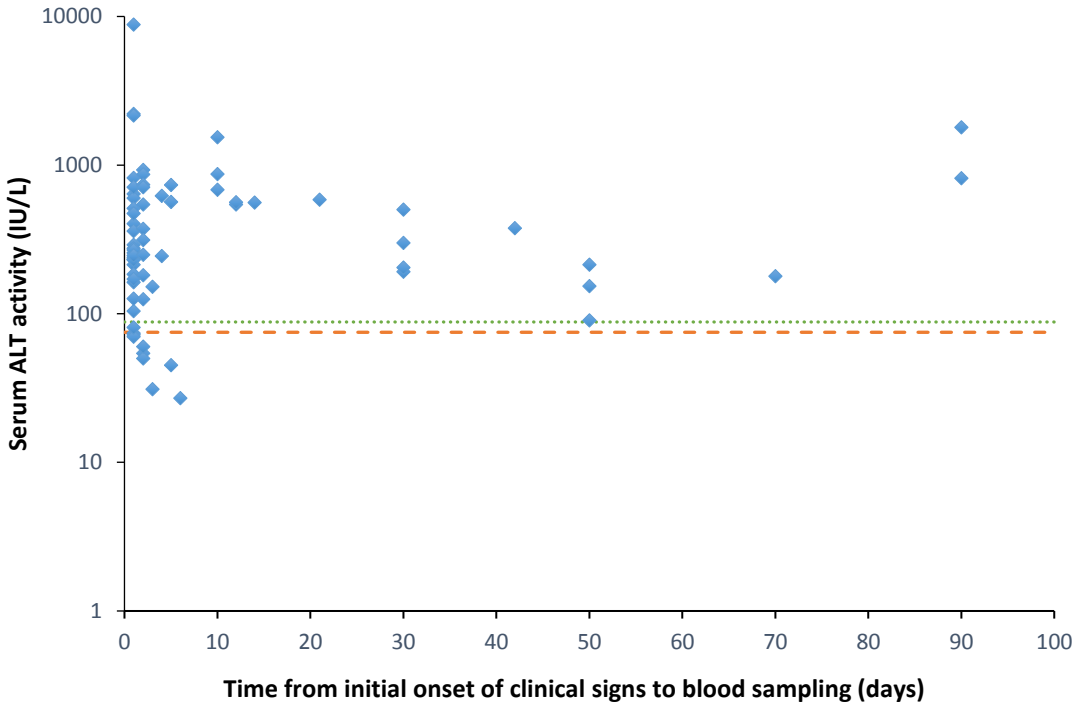

Figure 3.5: Scatterplot of serum alanine aminotransferase (ALT) activities in 70 dogs with 'Go Slow' myopathy at time since initial onset of clinical signs. Cases which were sampled >100 days (*n=4)* after the initial onset of clinical signs are not shown. The dashed orange line represents the upper limit of the IDEXX Laboratories reference range (75 IU/L), while Gribbles Veterinary Pathology has an upper limit of 88 IU/L (green dotted line)

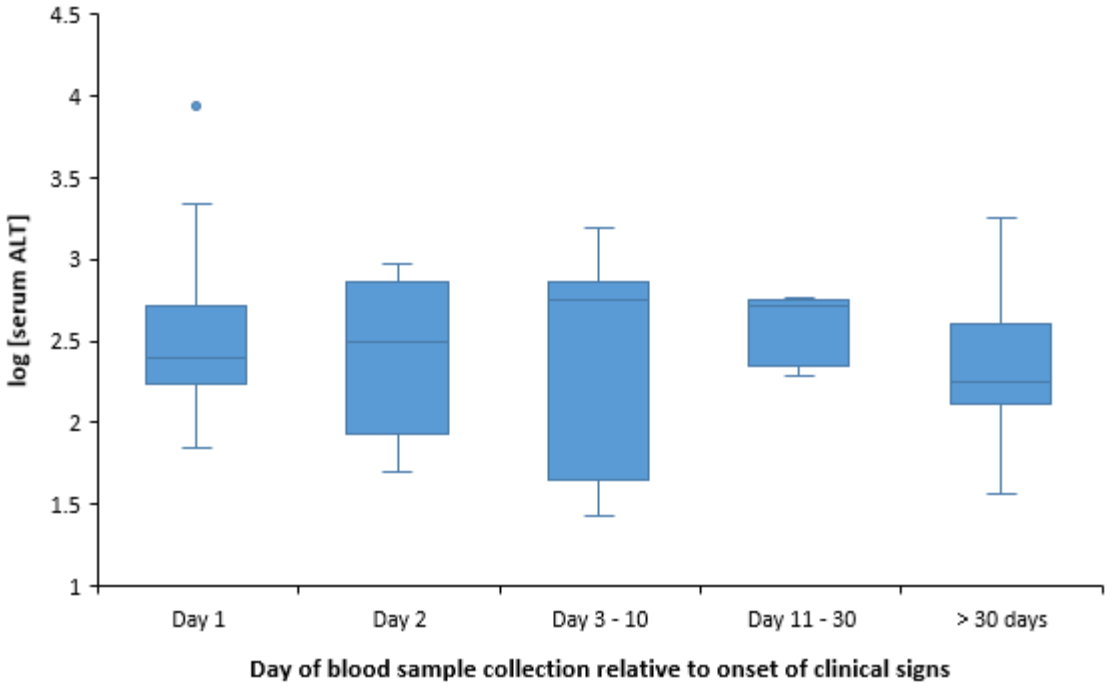

Figure 3.6: Boxplot of log transformed serum alanine aminotransferase (ALT) activities in 70 dogs with 'Go Slow' myopathy at the time of initial presentation, grouped according to the time since the first onset of clinical signs. There were no significant differences between the groups.

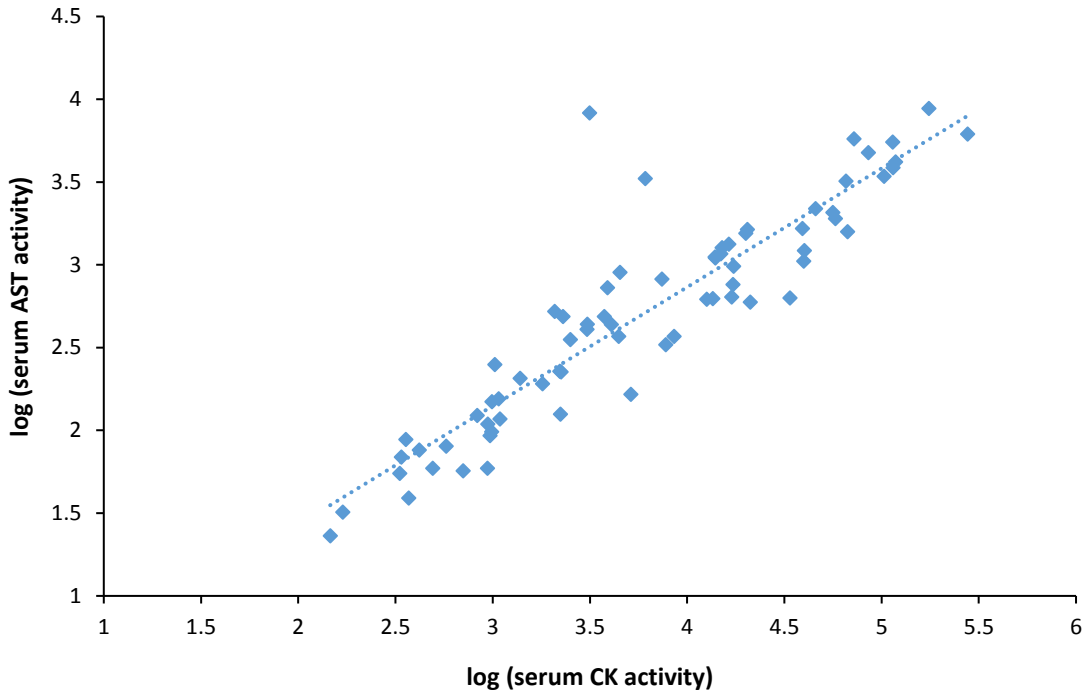

Figure 3.7: Scatterplot showing the relationship between logarithmically transformed creatine kinase (CK) and aspartate aminotransferase (AST) activities in the serum of 71 dogs with 'Go Slow' myopathy, with a linear regression line fitted ($y = 0.7175x - 0.0052$, $R^2 = 0.8311$)

In 13 cases, blood sampling for analysis of serum CK, AST and ALT activities was repeated during the recovery period (4 – 50 days after initial presentation). Five of these dogs had complete resolution of clinical signs at the time of subseq uent sampling, while the remaining eight dogs were normal at rest, but were reported to tire easily with exercise. As can be seen in Figure 3.8, the activities of all 3 enzymes in serum were decreased in the repeated samples, and two-tailed paired t-tests revealed significant differences between the initial and repeated sample means of log transformed CK ($p = 0.0017$), AST ($p = 0.0005$) and ALT ($p = 0.0497$) serum activities. Three cases were excluded from Figure 3.8 and statistical analysis as they were chronic at the time of initial presentation (21 – 365 days' duration of clinical signs), and the CK activities from a further two cases were omitted as they were reported as >25,000 IU/L rather than an absolute value.

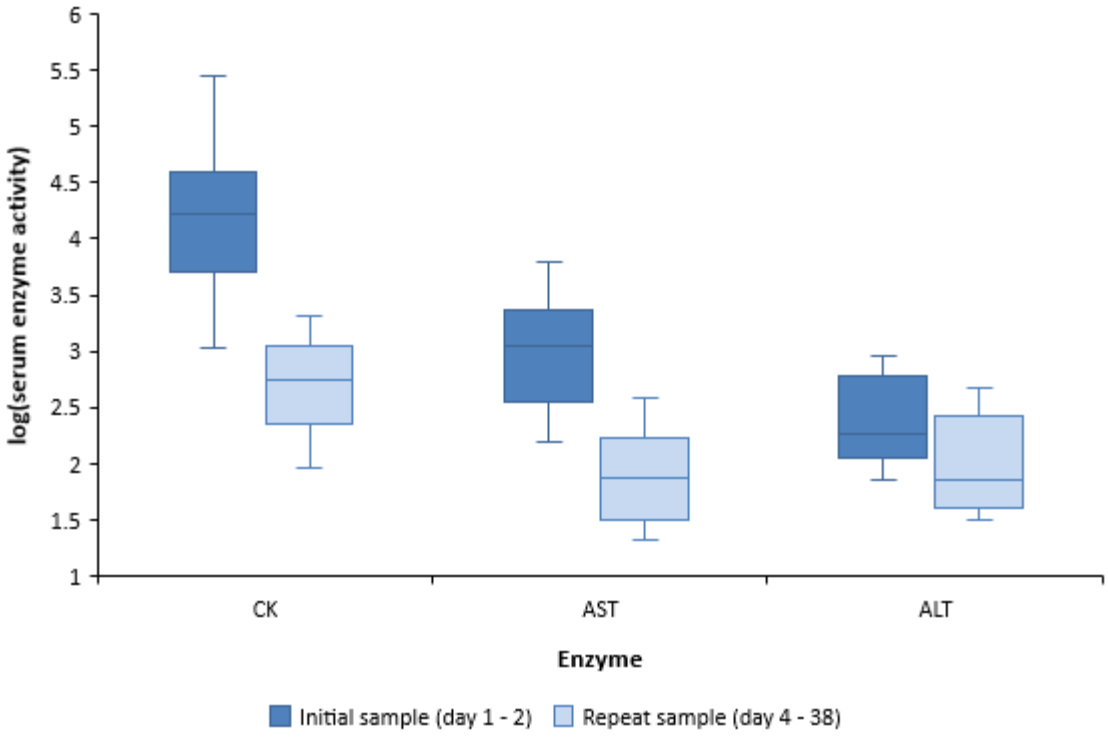

Figure 3.8: Boxplot of log transformed serum creatine kinase (CK) activities in eight dogs, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in 10 dogs with 'Go Slow' myopathy at the time of initial presentation, and in subsequent serum samples taken 4 – 38 days later.

3.3.4 Other serum biochemistry and toxicology results

The serum bilirubin concentration was included on a 'Go Slow panel' offered by commercial veterinary diagnostic laboratories, and was analysed in a total of 54 dogs (Table 3.2). Of these, bilirubin was within the reference range in 38 dogs (70.4%), low in 13 dogs (24.1%) and high in 3 dogs (5.5%). The three dogs with high serum bilirubin concentrations (values of 8.3, 13 and 17 µmol/L) were clinically dehydrated, with haemoconcentration evident on CBC and increases in urea, creatinine and phosphate on serum biochemistry. Two of these dogs also had severe elevations in ALT (2,159 and 8,800 IU/L) and mild increases in ALP (176 and 154IU/L respectively), suggesting that the hyperbilirubinaemia was hepatic in origin.

A full serum biochemistry panel was performed in 23 acute cases (12 samples analysed by Gribbles Veterinary Pathology Ltd., 11 by IDEXX/New Zealand Veterinary Pathology Ltd.). Results are present in Table 3.2, and the most common findings included low

serum chloride concentrations (below the applicable reference range in 13 dogs) and high serum phosphate concentrations (11 dogs). Four dogs had both high serum urea and creatinine concentrations (azotaemia) at the time of initial presentation. One of these dogs was euthanised due to the severity of clinical signs of the myopathy, while the other three dogs were monitored and showed no clinical evidence of renal dysfunction on follow-up, suggesting the initial azotaemia was pre-renal in origin or that the renal insult was mild. In these cases, urinalysis was either not performed, or urine samples were taken following intravenous fluid therapy, making interpretation of urine specific gravity difficult. Serum chloride concentrations were mildly decreased in 16 out of 22 dogs. The degree of hypochloraemia in these dogs is not considered clinically significant, but low serum chloride concentrations could be attributable to vomiting and diarrhoea in some cases, or might be indicative of an undiagnosed metabolic alkalosis[5] as blood gas and acid-base analyses were not performed.

In addition to routine biochemistry panels, serum cholinesterase (ChE) activity was measured in five dogs at the time of initial presentation to investigate the possibility of organophosphate poisoning (Table 3.2). There are no established reference ranges for serum cholinesterase activity in dogs, so samples from two normal dogs (provided by the diagnostic laboratory) were analysed as controls and returned activities of 4,691 and 8,798 delta pH units/hr. A cholinesterase activity of <50% of a normal animal is consistent with organophosphate exposure, although there is a large degree of variation amongst individuals and plasma or serum ChE activities may not accurately reflect brain ChE activity.[6] In three dogs, glutathione peroxidase (GPx) concentrations in plasma were analysed. Validated reference ranges for serum GpX concentrations in dogs were not provided by the veterinary diagnostic laboratory that performed the test, but the pathologists interpreted these concentrations as adequate to high based on information available in published scientific literature.

Table 3.2: Additional serum biochemistry results from dogs with 'Go Slow' myopathy at the time of initial diagnosis.

Variable	*n*	Mean ± SD	Range	Units	Reference range [a] ([b])
Bilirubin	54	2.0 ± 2.8	0.3 – 17.0	umol/L	1.0 – 3.0 (0 - 6)
ALP	25	74 ± 77	18 – 337	IU/L	0 – 87 (0 – 185)
Sodium	23	148 ± 4	137 – 159	mmol/L	141 – 153 (139 – 153)
Chloride	23	104 ± 5	95 – 119	mmol/L	106 – 117 (105 – 121)
Potassium	23	5.0 ± 0.5	4.0 – 6.3	mmol/L	4.0 – 5.4 (3.5 – 5.6)
Urea	25	9.6 ± 7.5	2.6 – 36.8	mmol/L	2.5 – 9.0 (3.6 – 11.4)
Creatinine	25	102 ± 117	41 – 569	umol/L	48 – 109 (53 – 123)
Phosphate	23	2.07 ± 0.99	1.00 – 4.17	mmol/L	0.92 – 1.82 (1.00 – 3.00)
Calcium	23	2.33 ± 0.28	1.73 – 2.77	mmol/L	2.08 – 2.82 (2.20 - 3.00)
Cholesterol	23	5.2 ± 1.3	2.7 – 9.2	mmol/L	3.27 – 9.82 (3.0 – 9.0)
Total protein	23	66 ± 7	55 – 81	g/L	54 – 74 (52 – 75)
Albumin	23	38 ± 4	28 – 44	g/L	33 – 44 (26 – 44)
Globulin	23	29 ± 6	18 – 42	g/L	19 – 35 (17 – 39)
Amylase	23	601 ± 221	243 – 1,232	IU/L	0 – 1074 (30 – 1,020)
Lipase	11	55 ± 32	15 – 121	IU/L	N/A (13 – 200)
Cholinesterase	5	4,703 ± 1,461	2,737 – 6,444	pH/hr	Controls: 4,691; 8,789
GpX [#]	3	23.6; 27.0; 32.2		KU/L	N/A

[a] Gribbles Veterinary Pathology Ltd. reference range

[b] IDEXX/New Zealand Veterinary Pathology Ltd. reference range

[#] Whole blood glutathione peroxidase, actual results listed instead of mean and SD

ALP = alkaline phosphatase

Blood clotting times (activated partial thromboplastin time, prothrombin time and thrombin time) were tested 2 acute cases and were normal in both. Brodifacoum concentrations were analysed in the liver of two chronic cases, with one negative result and the other at the limit of detection (0.005µg/g), consistent with previous exposure but not clinical toxicity.

3.3.5 Leptospirosis serology

Serology for *Leptospira interrogans* serovars Pomona and Cophenhageni and *Leptospira borgpetersenii* serovar hardjo was performed in three acute cases (day 1 – 2 of clinical signs) and four chronic cases (42 – 365 days). One of the acute cases returned a 1/400 titre for *L. borgpetersenii* serovar hardjo, while the remainder of cases tested were negative for all serovars. Serum from two of the dogs was also tested for antibodies to *L. borgpetersenii* serovars Ballum and Tarassovi, with negative results.

3.3.6 Cell-free DNA (cfDNA) concentrations in plasma

Plasma samples for determination of cfDNA were taken from eight dogs belonging to a single owner. Four of these dogs were clinically normal, and four were confirmed cases of GSM, with an initial onset of clinical signs four days prior to sampling. The plasma cfDNA concentrations of the normal dogs ranged from 413 – 632ng/mL, while concentrations in the affected dogs ranged from 556 – 850ng/mL, with two dogs above the upper limit (654ng/mL) of the suggested normal range. As shown in Figure 3.9, cfDNA was increased in plasma of affected dogs compared to unaffected dogs from the same hunting pack, but a two-tailed t-test revealed that the difference between the means of the 2 groups was not significant (p = 0.1556). There are no validated reference intervals for plasma cfDNA in dogs, but a suggested range of 512 - 654ng/mL (mean ± 2 standard deviations) was calculated using data from 24 healthy dogs presented in a paper by Burnett *et al,*[7] which used the same methods.

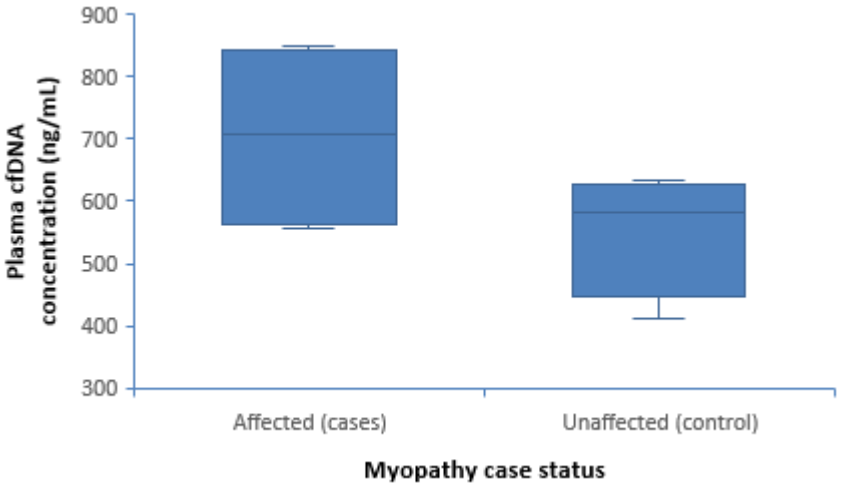

Figure 3.9: Boxplot of plasma cfDNA concentrations in four dogs with 'Go Slow' myopathy (4 days after the initial onset of clinical signs) and four clinically normal dogs.

3.3.7 Histology

Histology of skeletal muscle and liver samples was performed in 20 dogs. In all cases, the muscles sampled and the liver appeared grossly normal. Additional tissues were collected and examined histologically where possible, including heart (15 dogs), gastrointestinal tract (9), lung (9), kidney (7), spleen (6), brain (4), lymph node (3) and spinal cord (1). Samples for histology were collected at varying time points in relation

to the onset of clinical signs, with the earliest samples collected on day 2 (four cases). Other cases were sampled on day 3 (three dogs), day 5 - 6 (three dogs), day 10 - 20 (four dogs) and day 45 – 430 (six dogs). The most significant lesions were present within sections of skeletal muscle, with similar histological changes seen in the triceps, biceps femoris and other muscles sampled. In severe acute cases (7 days or fewer duration), there was scattered segmental degeneration of individual myofibres, characterised by fragmentation, vacuolation or hyalinisation of the sarcoplasm and loss of cross-striations (Figure 3.10A-B). Within the sarcoplasm of some degenerate myofibres there were small numbers of mixed inflammatory cells, predominantly macrophages, with rare neutrophils, lymphocytes and plasma cells (Figure 3.10C-D), but in most cases inflammation was minimal to absent. In muscle samples collected within 2 days of the initial onset of clinical signs, there was no evidence of muscle regeneration. In dogs sampled from day 3 onwards, regeneration of myofibres was a consistent feature, and acute myofibre degeneration and regeneration were seen within the same muscle bundles (Figure 3.10E-G). Regenerative fibres were smaller than surrounding normal myofibres, with lightly basophilic sarcoplasm and no clearly visible cross striations. These fibres had multiple, centrally located large nuclei, each with 1 -2 large nucleoli. In cases with mild clinical signs and small increases in serum creatine kinase activities, no overt myodegeneration or necrosis was seen. Instead, the predominant finding in these cases was the presence of scattered small (10 – 50% of the diameter of normal myofibres), hypereosinophilic triangular fibres in transverse sections (Figure 3.10H). There were also occasional thin bands of connective tissue running through the middle of larger myofibres in cross section, dividing them into 2 smaller fibres (fibre splitting). PAS staining of skeletal muscle sections was performed in four of the cases recruited early in the study (duration of clinical signs ranging from 2 days to 4 weeks), as well as one control dog. There were no appreciable differences in the pattern of fibre staining with PAS in these dogs.

In chronic cases (greater than 7 days' duration), there were scattered small triangular myofibres and fibre splitting as described in acute cases, resulting in variation in myofibre diameter in transverse sections. There were also occasional individual hypereosinophilic myofibres, separated from surrounding normal fibres by a thin rim

of clear space (Figure 3.11A) These myofibres had small, dark peripheral nuclei (pyknosis) and a homogenous appearance to the sarcoplasm, with a loss of cross striations. Variable numbers of degenerate and regenerative myofibres were also seen in chronic cases, with no significant inflammatory response, and in some cases the lesions were very subtle. In three dogs, there were multifocal areas of mildly increased fibrous connective tissue around myofibres in the triceps and biceps femoris, and some fibres in these areas had increased numbers of peripheral satellite nuclei. Scattered adipocytes were present in the centre of muscle bundles in two cases, alongside myofibre regeneration (Figure 3.11B), suggestive of myofibre loss and replacement with adipose tissue. In all cases, perimysial connective tissue, blood vessels and intramuscular sections of peripheral nerves were histologically normal. There was no evidence of *Trichinella spiralis* or other parasites in any of the muscle sections examined. No microscopic abnormalities were observed in the cardiac muscle samples from affected dogs (*n* = 15).

In the livers of 18 out of the 20 dogs sampled, the cytoplasm of centrilobular and mid-zonal hepatocytes was expanded by numerous indistinct, small, clear vacuoles (vacuolar hepatopathy) without peripheral displacement of the nucleus (Figure 3.11C-D). This is most consistent with microvesicular steatosis (accumulation of lipid in hepatocytes), although accumulation of glycogen within hepatocytes can also be associated with a similar vacuolar change. Special stains for lipid were not performed as all samples were formalin-fixed and paraffin embedded, and any lipid present is usually dissolved during processing. In three cases, the Periodic acid–Schiff (PAS) stain, which detects glycogen, was applied to liver sections and the clear material did not take up the PAS stain. In addition to the microvesicular change, there were also scattered foci of large, discrete clear vacuoles that displaced and compressed hepatocyte nuclei (macrovesicular steatosis) in one acute case (2 days' duration). In the two cases where hepatocytes were not vacuolated, there was marked congestion of sinusoids in centrilobular areas resulting in attenuation of hepatic cords. Periportal lymphatic vessels were occasionally dilated in affected dogs, but the portal areas were otherwise normal. There was no significant inflammation or hepatocyte necrosis.

Histologic changes were seen in the kidneys of two dogs that were euthanised due to clinical deterioration despite intensive symptomatic and supportive care. In these two dogs, numerous large, variably sized, brightly eosinophilic round droplets (interpreted as myoglobin) were present within the lumen of both proximal and distal convoluted tubules (Figure 3.11E-F). There was no degeneration of renal tubular epithelial cells associated with this, and glomeruli were normal. One of these dogs had increased serum urea, creatinine and phosphate concentrations on serum biochemistry, but urine specific gravity was not measured to allow differentiation between pre-renal and renal azotaemia. The kidneys were normal in the remaining five cases where samples were collected, and none of these dogs showed clinical signs or had biochemical changes suggestive of renal dysfunction.

Of the nine cases where fixed samples of the gastrointestinal tract were collected, six were histologically normal. In the remaining three dogs, there were multifocal areas of mild to moderate haemorrhage in the lamina propria and along the mucosal surface of the ileum and colon (Figure 3.11G-H). This was associated with mild blunting and fusion of villi, but there was no erosion of the mucosal surface or accompanying inflammation. The tunica muscularis was normal. All three of these dogs were reported to have haemorrhagic diarrhoea, and samples were collected within 5 days of the initial onset of clinical signs. Other tissues examined from affected dogs were within normal limits, including the brain, spinal cord and peripheral nerves.

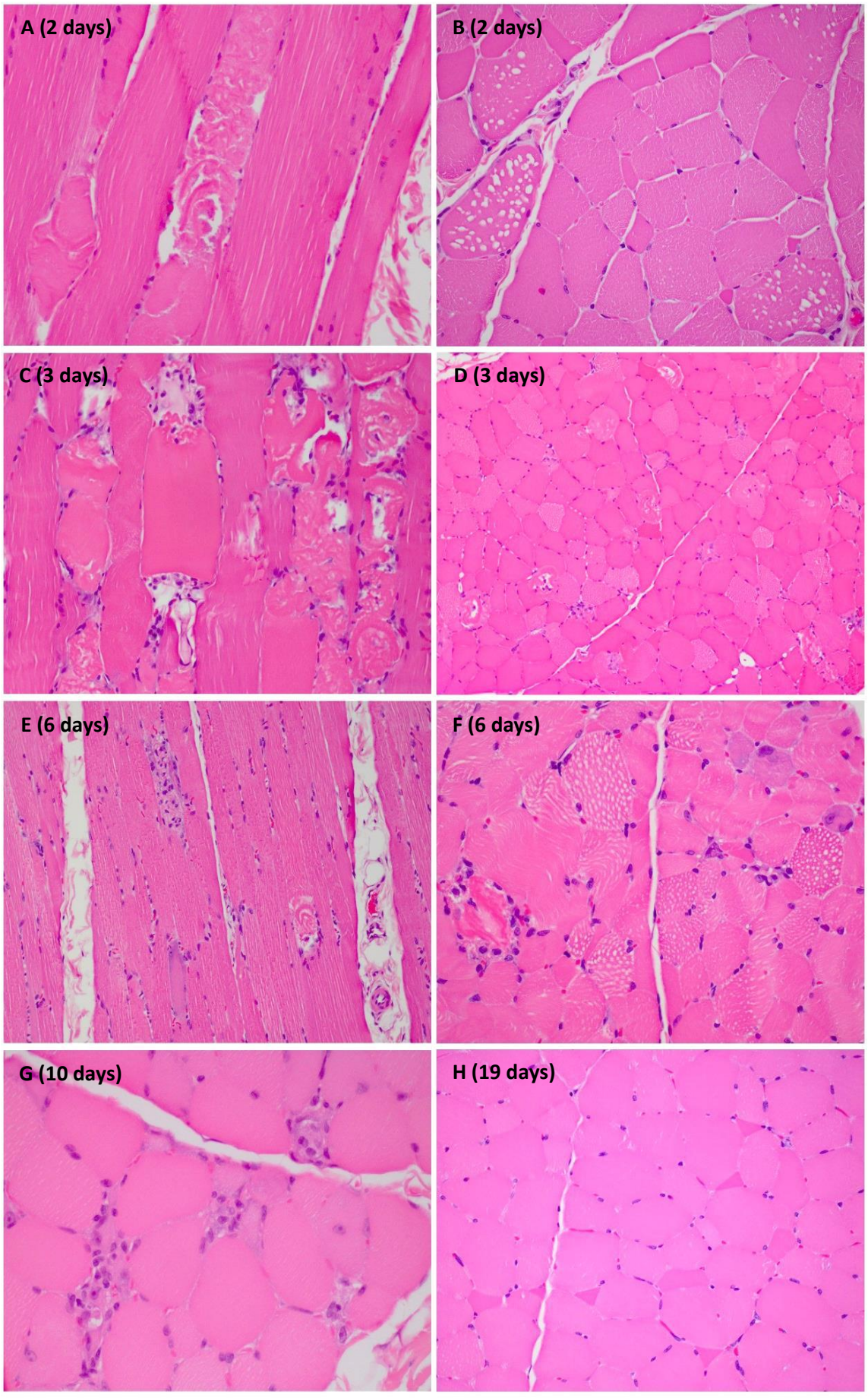
A (2 days)
B (2 days)
C (3 days)
D (3 days)
E (6 days)
F (6 days)
G (10 days)
H (19 days)

Figure 3.10 (opposite page): Photomicrographs of longitudinal and transverse sections of skeletal muscle from dogs with 'Go Slow' myopathy. The number of days refers to the time between the initial onset of clinical signs and sample collection. **A-B (2 days):** Scattered degenerate myofibres, characterised by disruption of the sarcoplasm and loss of cross striations. There are also several shrunken, hypereosinophilic fibres in the transverse section (B). **C-D (3 days):** Myofibre degeneration as in A, with small numbers of mononuclear inflammatory cells present within the sarcoplasm of degenerate fibres. Small numbers of regenerative fibres, characterised by basophilia of the sarcoplasm, are also seen in D. **E-F (6 days):** Acute degeneration of myofibres, degeneration with inflammation, and myofibre regeneration are seen within the same section. Regenerative fibres have sarcoplasmic basophilia and multiple, large nuclei that can be located centrally within the cell. **G (10 days):** Regeneration of myofibres; no degeneration is seen in this section. **H (19 days):** Small, angular, hypereosinophilic myofibres are scattered amongst normal myofibres.

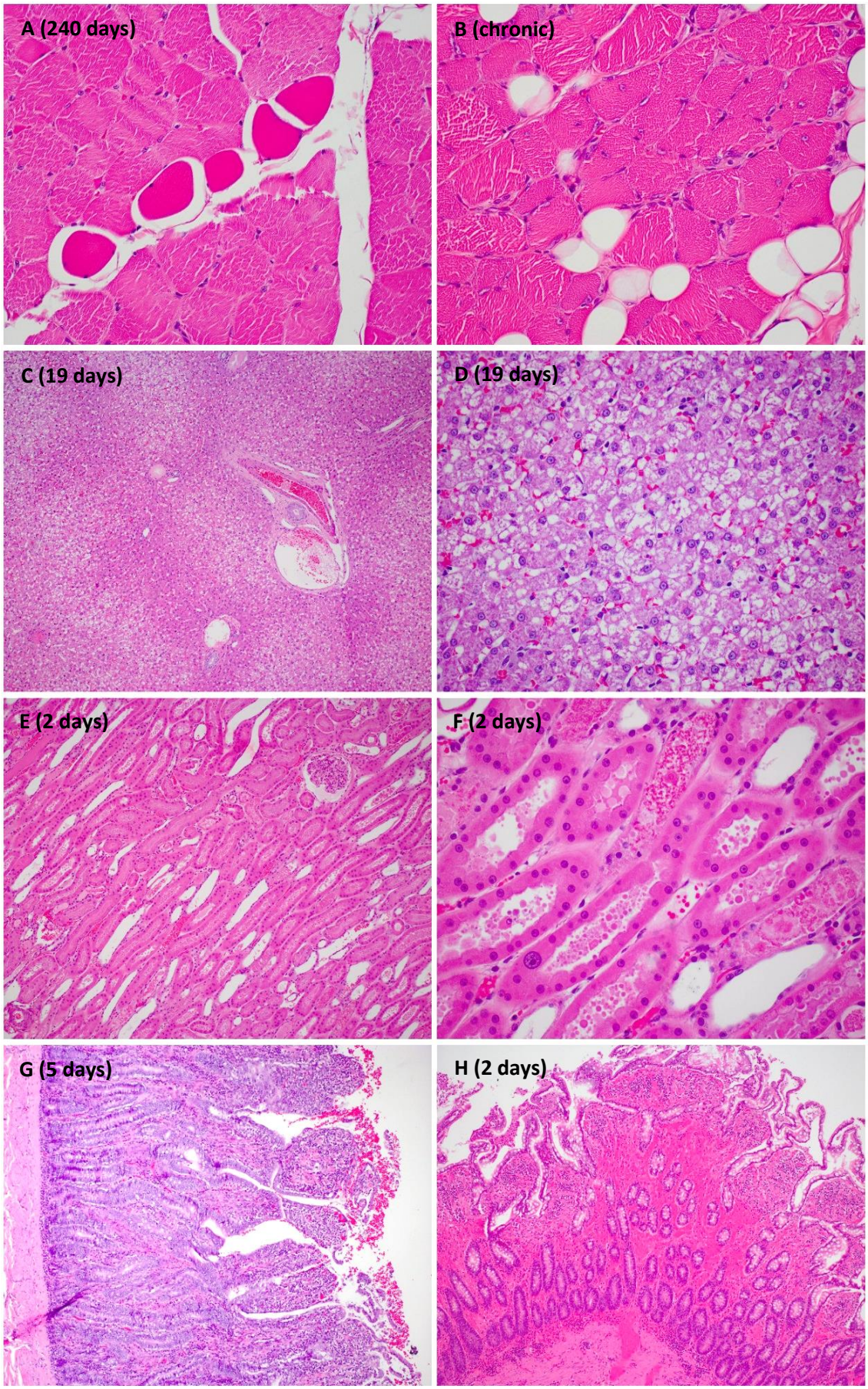
A (240 days)
B (chronic)
C (19 days)
D (19 days)
E (2 days)
F (2 days)
G (5 days)
H (2 days)

Figure 3.11 (opposite page): Photomicrographs of sections of skeletal muscle, liver, kidneys and intestine from dogs with 'Go Slow' myopathy. The number of days refers to the time between the initial onset of clinical signs and sample collection. **A (240 days):** Transverse section of skeletal muscle hypereosinophilic myofibres separated from surrounding normal fibres by a thin rim of clear space; nuclei are pyknotic. **B (chronic case, unknown duration):** Transverse section of skeletal muscle showing numerous fibres with centrally located nuclei, indicative of myocyte regeneration. Multifocally, myofibres are replaced by adipocytes with a large, clear cytoplasm. **C-D (19 days):** Section of liver at 100X (C) and 400X magnification. In centrilobular to mid-zonal areas there is vacuolation of hepatocyte cytoplasm. Periportal hepatocytes are normal. Lymphatic vessels adjacent to the portal area are dilated. **E-F (2 days):** Sections of kidney at 100x and 400x showing the presence of variably sized, brightly eosinophilic droplets within the lumen of renal tubules. Glomeruli are normal and there is no evidence of tubular degeneration. **G (5 days):** Section of ileum showing mild haemorrhage along the mucosal surface. **H (2 days):** Section of ileum showing haemorrhage within the lamina propria, with mild blunting and fusion of villi.

3.3.8 Electron microscopy

Electron microscopy was performed on samples of the triceps, biceps femoris and liver from four dogs with GSM (ranging from 2 – 50 days since the initial onset of clinical signs) and one clinically normal dog (Figure 3.12A /3.13A). Similar changes were observed in both the triceps and biceps femoris in all four affected dogs. The ultrastructure of the myofibrils themselves was normal, with clearly delineated Z lines, I bands and M lines, and the most significant changes were present adjacent to and within the mitochondria. In the most acutely affected dog (2 days' duration of clinical signs), there were numerous large, spherical to irregular, non-membrane bound globules of highly electron-dense material (lipid) between mitochondria (Figure 3.12B). These lipid globules appeared to compress adjacent myofibrils and mitochondria, but the mitochondria themselves appeared structurally normal.

This was in contrast to the remaining three cases (duration 6 – 50 days), where there were marked structural alterations in skeletal muscle mitochondria (Figure 3.12C-F). In these cases, there was widespread mitochondrial hypertrophy, with mitochondria frequently longer than a myofibril sarcomere in length. The outer membranes of the mitochondria were intact, but inner cristae were partially disrupted by the presence of mitochondrial inclusions. These inclusions ranged from thin filamentous/lamellar structures (Figure 3.12C & E) to round or irregular accumulations of small, dense granules (most consistent with glycogen), some of which were bounded by a single or

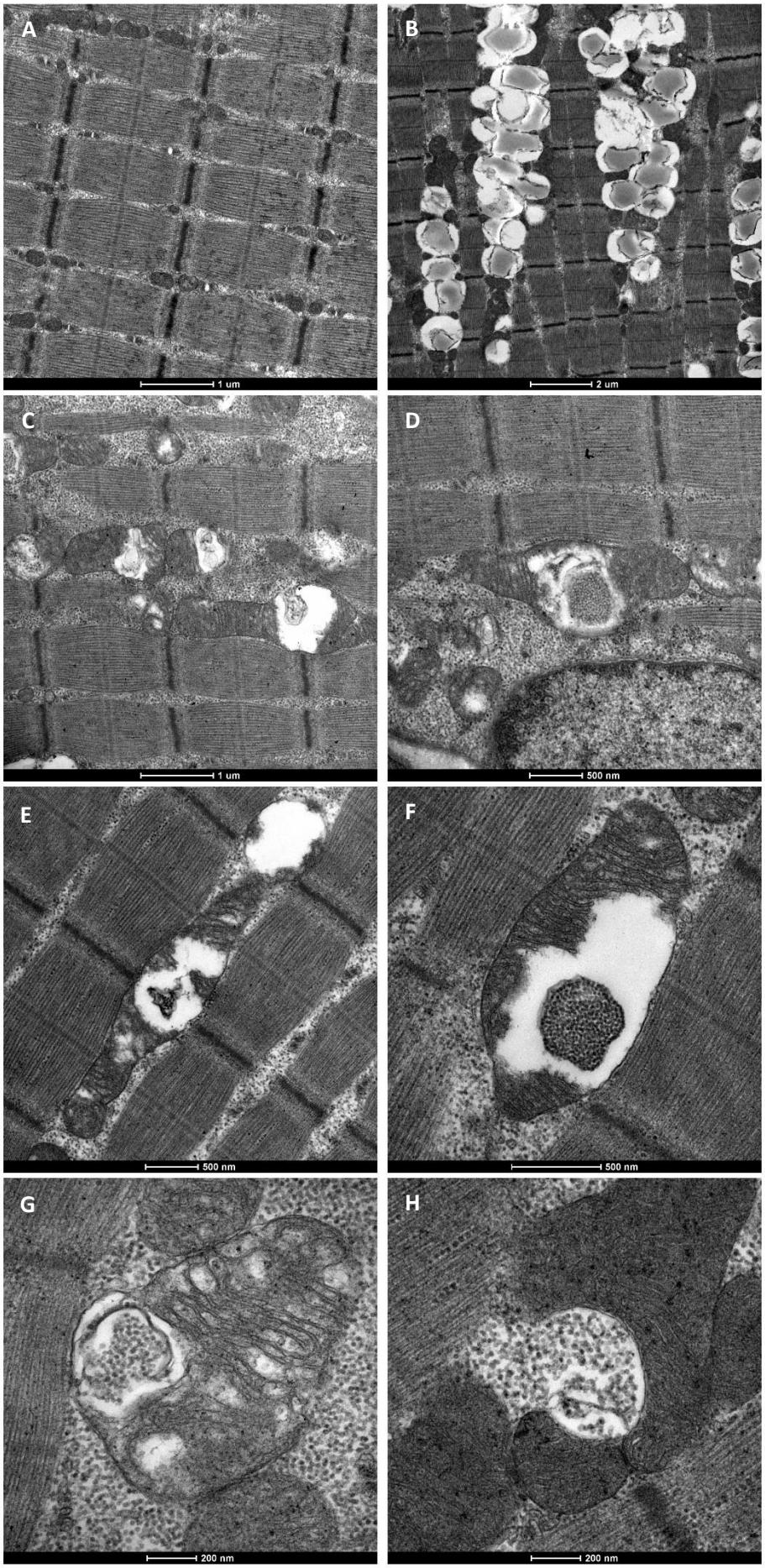
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Figure 3.12 (opposite page): Electron micrographs of skeletal muscle in dogs with 'Go Slow' myopathy (except A). **A:** Normal (unaffected) dog. **B:** Dog with initial onset of clinical signs of GSM 2 days previously. Large, circular to irregular globules of highly electron dense material (lipid droplets) are present in the sarcoplasm between mitochondria. **C-D:** Dog with initial onset of clinical signs 30 days previously. Mitochondria are markedly enlarged (hypertrophy) and compress adjacent myofibrils. Within mitochondria, cristae are partially disrupted by fibrillar granular inclusions. Note also the increased quantities of glycogen (small dense granules) in the sarcoplasm between myofibres compared to the normal dog (A). The nucleus is seen at the bottom right of image D and is normal. **E-G:** Dog with initial onset of clinical signs 50 days previously. Mitochondrial hypertrophy and inclusions. Granules within the inclusions have the same appearance as glycogen in the sarcoplasm, and are bound by a single or double membrane. **H:** Dog with initial onset of clinical signs 6 days previously. Concave appearance of mitochondria, surrounding glycogen granules in the cytoplasm.

double-layered membrane (Figure 3.12F-G). The mitochondrial cristae surrounding these inclusions were sometimes loosely arranged, but were otherwise normal. In one dog, occasional mitochondria had a concave shape at one end and appeared to be surrounding or engulfing glycogen in the adjacent sarcoplasm (Figure 3.12H). None of these ultrastructural changes were observed in the clinically normal dog (Figure 3.12A). In affected dogs, there were increased amounts of glycogen present within the sarcoplasm compared to the normal dog. The nuclei, nuclear membranes and cell membranes of myocytes were normal in all dogs.

In the liver in the most acute case (2 days' duration), there were variably-sized, round droplets of electron dense material (lipid globules) with the cytoplasm of hepatocytes (Figure 3.13B). Similar, more numerous globules were present within Kupffer cells (Figure 3.13C). In the remaining three cases, small amounts of lipid were seen within hepatocytes, but increased amounts of glycogen were also present in some cells and the glycogen compressed and displaced mitochondria and other organelles towards the periphery of the cell (Figure 3.13D). No structural abnormalities were observed in hepatic mitochondria in these cases. Occasional binucleate hepatocytes were seen, but nuclei were ultrastructurally normal.

In addition to skeletal muscle and liver, samples of cardiac muscle were examined from the normal dog (Figure 3.13E) and one GSM case (Figure 3.13F). There were no ultrastructural abnormalities in the cardiac sections examined and mitochondrial morphology was within normal limits.

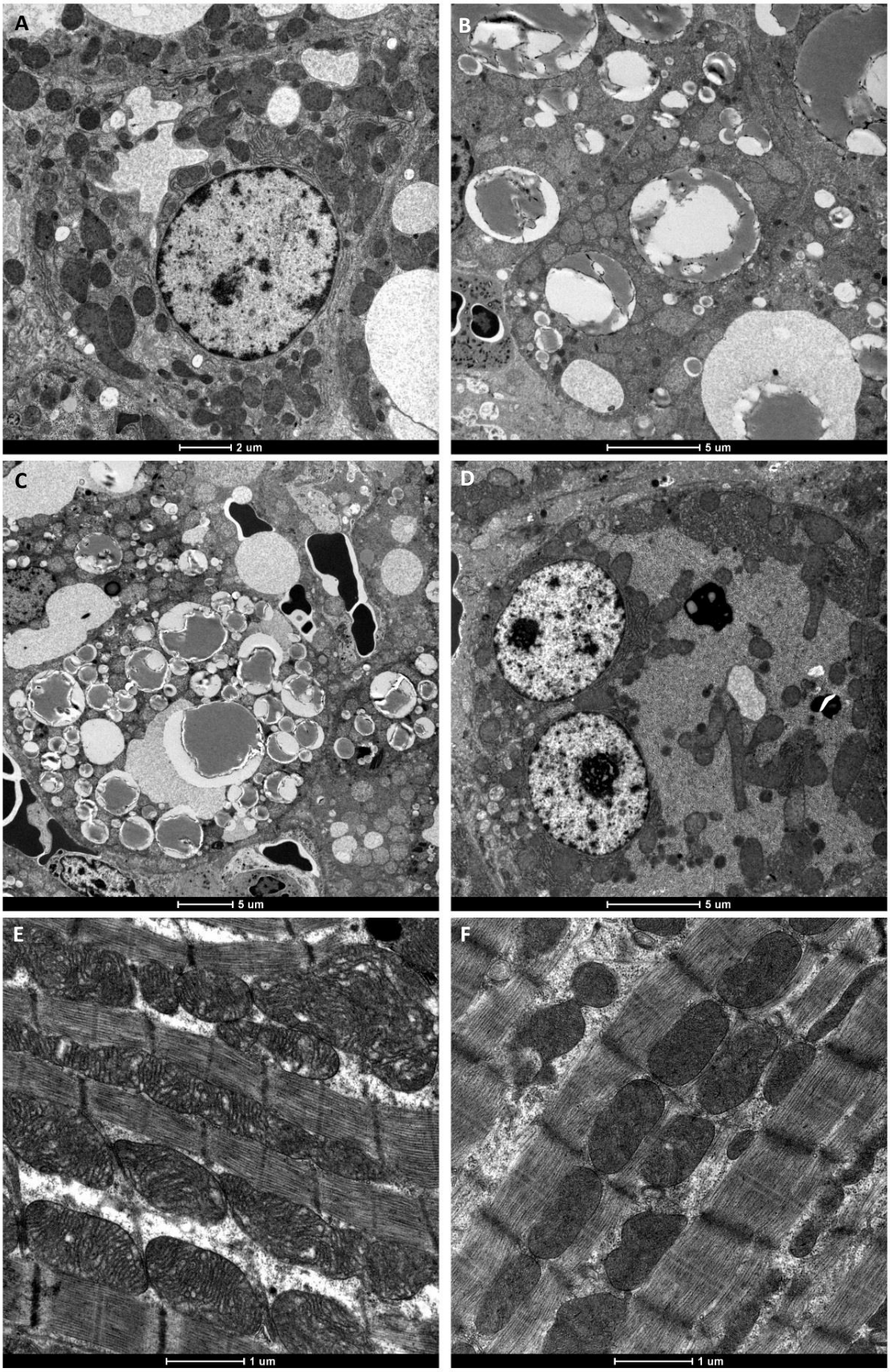
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Figure 3.13 (opposite page): Electron micrographs of sections of liver and heart in dogs with 'Go Slow' myopathy (except A and E). **A:** Normal (unaffected) dog liver. **B:** Dog with initial onset of clinical signs of GSM 2 days previously, showing large, spherical globules of electron dense material (lipid droplets) within the cytoplasm of hepatocytes. **C:** Same dog as B, showing variably sized lipid droplets within the cytoplasm of a Kupffer cell in a hepatic sinusoid. **D:** Dog with initial onset of clinical signs 6 days previously. Accumulation of fine, dense granules (glycogen) within the cytoplasm of a binucleate hepatocyte. Granules displace mitochondria, endoplasmic reticulum and other organelles towards the periphery of the cell. **E:** Cardiac muscle from a normal (unaffected) dog. **F:** Cardiac muscle from the same dog as B (6 days' duration of clinical signs). Mitochondria and myofibrils are ultrastructurally normal.

3.4 Discussion

Preliminary investigations in 2004 showed that 'Go Slow' myopathy (GSM) was a myopathy exacerbated by exercise, with clinical pathology findings of increased serum CK, AST and ALT activities.[2] The present study confirms and expands on these findings, and describes the histological and ultrastructural changes associated with the disease.

Acutely, affected dogs commonly presented with a sudden onset of muscle fasciculation, weakness, or collapse. Although non-specific, these clinical signs are recognised in association with myopathies,[8] where they are attributable to muscle degeneration and impaired muscle function. Variable degrees of muscle degeneration were observed histologically in dogs, with the most severe lesions seen in dogs sampled early in the course of the disease. Clinically, muscle degeneration was detectable on serum biochemistry, with consistent increases in CK and AST activities, which were most severe within 10 days of the initial onset of clinical signs. The finding of increased creatine kinase activity in circulation is usually associated with loss of muscle cell integrity (degeneration and necrosis), but CK can also be released from intact myofibres with altered membrane permeability due to depletion of ATP.[9] Serum CK activity is reported to have a moderate sensitivity (0.772) and specificity (0.791) for the diagnosis of primary muscle diseases in dogs,[10] and has a short half-life of 2-3 hours.[11] False negatives are attributed to the short half-life of CK in plasma, while false positives may be seen in secondary muscle disorders or with traumatic venepuncture. In the present study, acute cases had marked increases in serum CK activity, but in chronic cases and repeated samples, CK activities were often normal, corresponding with myofibre regeneration with minimal degeneration on histology. However,

degeneration and regeneration of myofibres were seen concurrently in some cases, suggesting that there is a degree of ongoing muscle damage (polyphasic necrosis), rather than just a single insult. This could be either be due to continued exposure of muscle to the agent or compound that causes the disease (through repeated ingestion or persistence in the body), or a single insult to muscle that has lasting effects on cell function, leading to ongoing myocyte degeneration that could be exaccerabated by physical exertion.

AST is present in the cytoplasm of muscle cells and hepatocytes and leaks into circulation with damage to these cells, but has a longer half-life than CK (12 hours).[5] In affected dogs, there was a strong correlation (0.83) between log transformed serum CK and AST activities, suggesting that AST was primarily of muscle origin rather than hepatic. Both CK and AST are also present in cardiac myocytes and serum activities of these enzymes can increase in some cardiomyopathies,[5] but histology and electron microscopy did not reveal any lesions in cardiac muscle in affected dogs. Specific tests of cardiac function were not performed in cases of GSM, as there was no clinical evidence of cardiac dysfunction on physical examination or post mortem of affected dogs.

Increases in serum ALT were also seen in GSM cases. The liver has the highest ALT activity of all tissues, but increases in ALT are not specific for liver disease as the enzyme is also found in cardiac and skeletal muscle.[12] Histologically, affected dogs had lesions within both the liver and muscle, but ALT in these cases appears to originate primarily from the liver, as there was no correlation between serum ALT and AST or CK activities. Furthermore, vacuolar change in hepatocytes was seen to a similar degree in both acute and chronic cases, and increases in ALT were similar in dogs sampled at different time points, whereas the magnitude of muscle damage and increases in CK and AST activity reduced over time.

Unlike CK, AST and ALT activities, increases in plasma cfDNA concentrations do not have tissue specificity. Any type of nucleated cell undergoing apoptosis or necrosis can release free double-stranded DNA into circulation (cell-free DNA),[13] although DNA

might also be released from living cells as they proliferate.[14] Cell-free DNA can be increased with prolonged strenuous exercise in dogs[15] and is increased in a range of disease states,[6] including trauma, sepsis, sarcomas,[16] mammary neoplasia[17, 18] and pulmonary thromboembolism.[19] As expected in animals with damage to tissues (skeletal muscle and liver), plasma cfDNA concentrations were increased above the suggested reference range in two of the four dogs with GSM. The range of cfDNA concentrations in affected dogs was similar to the findings of Burnett *et al.*, who reported a median concentration of 733 µg/L and an interquartile range of 622 – 818 µg/L in dogs with a range of diseases.[7] However, there was not a significant difference between affected and unaffected dogs in the present study. It is interesting to note that the four affected dogs used for cfDNA analysis had markedly increased serum CK activities ranging from 15,100 – 113,700 IU/L (reference range 0 – 609 IU/L), and yet increases in plasma cfDNA were not as remarkable. This could suggest that in myocytes undergoing degeneration, the damage to the sarcoplasm (and therefore release of CK into circulation) is greater than the damage to the nuclei (which could lead to release of cfDNA). As cfDNA is a relatively novel biomarker, its use and interpretation in specific diseases in dogs is limited, and further work to understand the release of cfDNA from different tissues (including muscle) is warranted.

Inflammation was not a prominent feature of the GSM cases in this study. Affected dogs did not consistently have inflammatory leukograms on haematology, and minimal inflammation was observed in sections of skeletal muscle. Numerous degenerate myocytes were seen without any accompanying inflammation. Where inflammation was observed, it was in response to muscle degeneration, rather than the inflammation preceding and causing degeneration, which shows that GSM is not an inflammatory myopathy (myositis). Bacterial, parasitic, fungal, viral and immune-mediated aetiologies are usually associated with myositis in animals,[20] so the lack of inflammation in GSM makes these causes less likely. In addition, there were no inflammatory lesions in other tissues, as might be expected with some inflammatory and infectious diseases that can cause muscle damage, such as leptospirosis[21] and neosporosis.[22]

In addition to skeletal muscle lesions, 18 of the 20 dogs (90%) in which liver sections were examined had a mild to moderate vacuolar hepatopathy affecting centrilobular to mid-zonal hepatocytes. In most cases, the appearance of the vacuolated hepatocytes was most consistent with accumulation of lipid within the cytoplasm (microvesicular steatosis), and this was confirmed ultrastructurally, although increased amounts of glycogen were also seen in some hepatocytes on electron microscopy. Excessive accumulations of both lipid[23] and glycogen[24] within hepatocytes can result in increases in serum ALT activity, which was a consistent serum biochemical finding in dogs with GSM. Hepatic accumulation of lipid occurs when uptake of lipids is greater than oxidation or secretion, such as with increased mobilisation of triglycerides during periods of high energy demand or with injury to hepatocytes that impedes fatty acid oxidation.[23] In particular, the microvesicular steatosis seen in affected dogs is often associated with toxic hepatopathies that cause mitochondrial injury and interfere with β-oxidation of fatty acids, as partially oxidised fatty acids have a lower surface tension than triglycerides, resulting in the formation of smaller vesicles.[25] Accumulation of glycogen in hepatocytes is frequently associated with steroidogenic hormone excess due to hyperadrenocorticism, exogenous glucocorticoids or illness-induced physiologic stress.[26] None of the cases had a history of recent glucocorticoid administration or any evidence of adrenal dysfunction, so glycogen accumulation is considered to most likely be secondary to physiologic stress or abnormalities in carbohydrate metabolism associated with the disease.

Accumulations of lipid and glycogen were also seen in the sarcoplasm of skeletal muscle myocytes in affected dogs on electron microscopy, but were not severe enough to be appreciable histologically. Lipid droplet accumulation in myocytes is reported with various defects in mitochondrial oxidative metabolism in both dogs and people,[27-30] and glycogen accumulation is associated with defects in carbohydrate and polysaccharide metabolism.[31, 32] Abundant glycogen and lipid droplets are also reported in skeletal muscle of human patients with chronic fatigue syndrome.[33] The clinical features of chronic fatigue in humans have some similarities to GSM in dogs, and mitochondrial dysfunction is suspected to play a role in chronic fatigue.[34]

Structural abnormalities in mitochondria, including mitochondrial hypertrophy and inclusions, were widespread in the sections of skeletal muscle from affected dogs examined ultrastructurally. These mitochondrial changes were observed in myocytes where myofibrils were structurally normal, suggesting that mitochondrial damage precedes myofibre degeneration. Increased mitochondrial size (hypertrophy) results from the fusion of individual mitochondria,[35] and is associated with metabolic defects leading to the accumulation of enzymes or substrates in the mitochondrial matrix.[36]

In addition to hypertrophy, skeletal muscle mitochondria in dogs with the myopathy frequently contained inclusions, ranging from small filamentous structures to aggregates of dense granules surrounded by a membrane. Filamentous inclusions (intramitochondrial fibrils) can occur in skeletal muscle and are commonly seen in patients with metabolic disease, uraemia or severe terminal illness.[36] However, the granular inclusions are more unusual, and electron microscopy images from one of the affected dogs suggest that these inclusions may form as a result of mitochondrial invagination around glycogen present in the sarcoplasm. Mitochondria with C-shaped morphology that appear to have engulfed cytosolic components have been reported in mouse fibroblasts treated with an uncoupling agent that disrupts the link between electron transport and generation of ATP.[37] In the mouse fibroblasts, the engulfed cytoplasmic contents were bounded by a double layered membrane (formed from the inner and outer mitochondrial membranes), similar to the inclusions seen in the affected dogs. Similar glycogen-containing mitochondrial inclusions or compartments have been sporadically reported in skeletal muscle in some inherited mitochondrial myopathies in people,[38-41] particularly myopathies where there are abnormalities in the electron transport chain and ATP synthesis. However, in most of these diseases crystalline or rod-like mitochondrial inclusions are also a feature, whereas crystalline inclusions were not seen in dogs in this study. Mitochondrial glycogen inclusions have also been reported in skeletal muscle of vitamin B12 deficient sheep[42] and hepatocytes of vitamin B2 (riboflavin) deficient mice.[43] In these deficiencies, it has been suggested that alterations in oxidative phosphorylation secondary to the accumulation of intermediates such as methylmalonic acid, or changes in mitochondrial phospholipids, could result in the morphological changes seen in mitochondria.

Mitochondrial myopathies are usually due to genetic[44] or toxic causes.[45] As discussed in Chapter 2, there was no breed, sex or age predisposition for GSM in dogs, and combined with the sudden onset of clinical signs, a toxic aetiology is more plausible than a genetic cause. In humans, toxic mitochondrial myopathies have been reported with several classes of drugs, particularly statins[46, 47] and reverse transcriptase inhibitors,[48-50] but no recent drug, supplement, or animal health product administration was reported in dog myopathy cases. Serum cholinesterase activities and liver brodifacoum concentrations, which were each tested in a small number of cases, were not consistent with organophosphate or anticoagulant toxicity.

The epidemiological studies described in Chapter 2 found that ingestion of raw or cooked wild pig was very common in cases of GSM, and cases were predominantly reported in the upper half of the North Island of New Zealand. Dogs could potentially be exposed to an environmental toxin through pig meat, and a plant or fungal toxin is considered most likely given the limited geographic distribution of cases. Plant species reported to cause muscle mitochondrial damage or dysfunction in animals include coffee senna (*Senna occidentals*);[51-53] sycamore and box elder trees (*Acer* spp.);[54, 55] and white snakeroot (*Ageratina altissima*, formerly *Eupatorium urticaefolium*).[56] Poisonings in animals with these plants have some similarities to GSM in dogs, but there are also some differences in the associated pathology. Coffee senna is reported to cause multifocal monophasic or polyphasic degeneration of skeletal muscle in cattle, rats, rabbits and chickens, and affected animals can also have centrilobular hepatic necrosis, skeletal muscle atrophy, cardiac muscle degeneration and renal tubular degeneration.[52, 57-61] Ingestion of sycamore and box elder trees containing hypoglycin A by horses can result in the development of atypical/seasonal pasture myopathy, a sudden-onset myopathy not related to exercise. In this disease, there is multifocal monophasic degeneration of striated muscle, with the postural, respiratory and cardiac muscles most severely affected.[62] The underlying pathological mechanism of this disease is a multiple acyl-CoA dehydrogenase deficiency (MADD), which leads to defects in the β-oxidation of short-, medium-, and long-chain fatty acids in mitochondria.[63] The white snakeroot plant can cause a primary skeletal and cardiac myopathy in grazing animals,[64-66] or a secondary myopathy ('milk sickness') in people

who consume meat or milk from animals that have eaten the plant,[67] and in this regard the disease is similar to the myopathy in dogs, which appears to be associated with the consumption of wild pigs. It is interesting to note that in all three of these plant toxicities (coffee senna, atypical myopathy and milk sickness), vomiting and/or diarrhoea is reported in a low proportion of cases,[59, 67, 68] which was also a feature of some GSM cases. The pathogenesis of these clinical signs and the haemorrhagic intestinal lesions seen in two cases is uncertain, although a number of poisonous plants are known to induce diarrhoea when consumed.[69]

It is also important to note that in many mitochondrial myopathies in people and animals, skeletal muscle lesions are accompanied by similar lesions in cardiac muscle,[70-72] whereas in the GSM cases, no histological or ultrastructural abnormalities were observed in the heart, and no cardiac arrhythmias were detected on physical examination of affected dogs. In cardiac muscle, mitochondria comprise approximately 35% of tissue volume, whereas in skeletal muscle, mitochondria represent approximately 3 – 8% of the tissue volume,[73] so it is somewhat surprising that a tissue so rich in mitochondria was not affected. There are differences between cardiac and skeletal muscle in the isoforms of some receptors expressed (such as the ryanodine receptor)[74] as well as differences in myocyte gene expression,[75] which could play a role in the apparent specificity of the myopathy for skeletal muscle, depending on the underlying mechanism of the disease.

In humans, statin drugs (also known as 3-hydroxy-3-methylglutaryl coenzyme A reductase or HMG-CoA reductase inhibitor) are used to impair cholesterol synthesis and protect against cardiovascular mortality, but can cause a skeletal myopathy.[76] These drugs have opposite effects on skeletal and cardiac mitochondria, which are thought to be mediated by the differential responses of each muscle type to reactive oxygen species (ROS) production.[77] In cardiac muscle, initial ROS production due to statin drugs induces mitochondrial biogenesis and activates antioxidant defence mechanisms through induction of peroxisome proliferator-activated receptor gamma co-activator (PGC-1) family pathways. Conversely, in skeletal muscle, initial ROS production overwhelms ROS-detoxifying capabilities, and excessive oxidative stress

leads to mitochondrial dysfunction and a decrease in PGC-1 expression, ultimately resulting in the development of a myopathy. This mechanism of differential responses in cardiac and skeletal muscle mitochondria is termed 'mitohormesis' and could explain the lack of lesions seen in cardiac muscle, particularly if the generation of ROS plays a role in the development of the disease. Synthetic statin drugs are not commonly used in dogs, but the mushroom species *Pleurotus ostreatus*, which is present in New Zealand, contains a HMG-CoA reductase inhibitor.[78]

Limitations of the present study include the relatively small number of dogs sampled for haematology, histology, electron microscopy and some additional tests such as plasma cfDNA concentrations. Haematology was performed in selected cases, mostly those recruited early in the study, but as there were no consistent changes observed in the haemogram of these dogs, haematology was not included in the eligibility criteria/case definition for this study. As the dogs included in the study were clinical cases seen by practicing veterinarians rather than experimentally-induced cases, sampling for histology was not standardised in terms of the timing of sample collection, site of muscle sampling within the triceps and biceps femoris and samples collected from other organs. All histological samples were fixed in formalin to ensure adequate tissue preservation prior to transport to Massey University, but this limited the use of special stains such as those for lipid, and precluded muscle fibre typing, which requires fresh muscle samples that are snap frozen in liquid nitrogen.

The lack of fibre typing means it is not possible to determine if the muscle lesions seen in affected dogs are restricted to a particular fibre type, which could have implications for the relative importance of different metabolic pathways (oxidative vs. glycolytic) in the pathogenesis of the disease. During the study, attempts were made to perform frozen histology and histochemical stains on muscle samples from affected dogs to better appreciate changes in muscle composition and function. However, as most cases were seen in rural regions of New Zealand and sample collection was performed on an opportunistic basis from affected dogs that were euthanised, deterioration and autolysis of samples before and during transit precluded the diagnostic use of fresh/frozen tissue samples.

In conclusion, the present study has confirmed that 'Go Slow' myopathy (GSM) primarily affects skeletal muscle, without concurrent cardiac muscle damage or nervous system involvement. Clinical signs include muscle tremors, weakness, collapse and lethargy in acute cases, and signs are exacerbated by exercise. Less commonly, affected dogs present with diarrhoea and vomiting (which may be haemorrhagic), particularly in cases with no recent history of exercise. Clinical improvement is usually seen within 10 days of the onset of disease, but many dogs that appear normal at rest have a prolonged period of exercise intolerance and fatigue, and some dogs never return to full fitness. Skeletal muscle degeneration in the absence of inflammation is the primary histological finding, and is detectable clinically by prolonged increases in serum CK and AST activities in affected dogs. Vacuolar change in centrilobular hepatocytes is also a consistent feature, and results in increased ALT activity in serum. The underlying ultrastructural lesions in muscle include mitochondrial hypertrophy and mitochondrial inclusions, indicative of a metabolic myopathy. Taken with the results of the epidemiologic studies presented in Chapter 2, a toxic aetiology (particularly a plant or fungal toxin) is suggested as the most likely cause of GSM. In order to further investigate possible causes of the disease and potential metabolic changes induced by mitochondrial dysfunction, techniques such as liquid chromatography-mass spectrometry could be used in untargeted metabolic profiling of animal tissue samples.[79] This approach is described in Chapters 5 and 6.

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Chapter 4

Muscle pathology of wild pigs (Sus scrofa) in New Zealand

4.1 Introduction

The epidemiologic study detailed in Chapter 2 established that ingestion of wild pig *(Sus scrofa)* tissues is very common in cases of 'Go Slow' myopathy (GSM), an idiopathic myopathy in dogs. Histologic and ultrastructural lesions in the skeletal muscle and liver of dogs with GSM are consistent with a mitochondrial myopathy and vacuolar hepatopathy (Chapter 3), and these findings are most consistent with a toxic aetiology. If affected dogs are exposed to a toxic compound or agent through eating tissues from wild pigs, this raises the question of whether pigs are similarly affected.

Wild pigs are an invasive pest that were first introduced into New Zealand in the 17th and 18th centuries through the deliberate release of domestic pigs descended from European and Asian animals.[1] Negative impacts of wild pigs include environmental destruction,[2] endangerment of native plants and animals,[3, 4] and spread of infectious disease.[5, 6] However, wild pigs can also be considered as a resource, as hunted wild pigs can be an important source of food for people and their pets.[7, 8] This is particularly true in some Maori and Polynesian communities, and there are implications for management of wild pigs under the Treaty of Waitangi.[9, 10] The last reported survey of the geographic range of wild pigs in New Zealand took place between 1993 and 1996, and at this time wild pigs occupied more than 93,000 km^2, or approximately 35% of the total land area (Figure 4.1).[11] Of this area, 5585 km^2 (6%) was in the Northland region (which comprises 5% of New Zealand's total land area[12, 13]), where GSM cases in dogs are most commonly recognised (Chapter 2). The Northland region has a total area of 13,286km^2, meaning that wild pigs occupy 42% of the land area in Northland.

Figure 4.1: Geographical distribution of the established range of feral pigs in New Zealand in 1995 – 1996, with point locations for new populations of feral pigs between 1993 and 1996. Reproduced with permission from Fraser KW, Cone JM, and Whitford EJ. A revision of the established ranges and new populations of 11 introduced ungulate species in New Zealand. *Journal of the Royal Society of New Zealand* 2000; 30: 419-437.

The total number of wild pigs in New Zealand is unknown, but previous estimates suggest a population size of 110,000 at an average density of 1.2 pigs per square kilometre[14] However, the density of pigs found in a given area can vary widely based on food availability and hunting pressure.[15] Farmland is the most common habitat for wild pigs, and was the most common land type used for pig hunting in a recent survey

(Chapter 2), but pigs are also found scrubland and in native and pine forests.[16] Wild pigs in New Zealand have been reported to occupy home ranges of 28 – 209ha, and travel an average of 0.8 – 1.7km per 24 hours, with a maximum 3.6km travelled per day.[15]

The diet of wild pigs is comprised of approximately two-thirds plant material and one-third animal tissues.[17, 18] Pigs obtain food mainly by foraging on the ground, with lesser amounts of food obtained by rooting, browsing and grazing. Wild pigs have been reported to eat a wide range of plant types (both native and introduced), including woody plants, herbs, monocotyledons and ferns.[18] Some fungal species also form part of the diet of wild pigs. Animal species eaten by pigs are predominantly invertebrates, with annelids (earthworms) being most common,[17, 18] although the carcasses of possums,[17] frogs[3] and various seabirds including albatross,[19] petrels,[20] shearwaters[21] and penguins[22] have been found in the stomachs of wild pigs. Pig predation on kiwi and takahe has been identified by wildlife pathologists in New Zealand (unpublished data) reinforcing their significance to the conservation of New Zealand native fauna.

If wild pigs ingest or are exposed to a toxin that can subsequently cause muscle and liver damage in dogs, it might be expected that the pigs would also show similar lesions. It is difficult to detect clinical signs of myopathies in wild pigs caught using dogs, as hunters might not be in close enough proximity to observe the pigs as they are being chased and cornered by dogs. Anecdotally, hunters report that pigs fed to dogs prior to the onset of GSM are in good body condition with no obvious gross abnormalities in tissues. However, tissue samples for histology are not usually available from these pigs, as by the time the meat is fed to dogs the rest of the carcass has often been frozen, cooked or discarded. Furthermore, wild pigs are often caught in remote locations with the viscera removed before leaving the site, so samples of organs such as liver are rarely available for examination.

The primary aim of the present study was to investigate whether wild pigs have microscopic muscle and liver lesions similar to those found in dogs with GSM. Due to the inherent difficulties in collecting samples from wild pigs, sampling in the present

study was largely opportunistic in nature. Where possible, samples were collected from wild pigs where parts of the carcass had been eaten by dogs that subsequently developed GSM. Samples from other wild and semi-wild pigs (see 4.2.1 below) that were not fed to dogs, but were caught in areas where GSM was reported to occur, were also collected. An additional aim of the present study was to check for the presence of nematode parasites on histological examination of muscle sections collected from wild pigs, as initial investigations into the myopathy in dogs in 2004 listed the nematode parasite *Trichinella spiralis* as a possible cause of the disease.[23]

4.2 Methods

4.2.1 Classification of wild pig samples

Samples collected from pigs were classified according to the following criteria:

- **Group A (5 pigs):** Wild pigs, from any region of New Zealand, that were eaten by dogs that subsequently developed GSM. The diagnosis of GSM in dogs was based on clinical examination, serum biochemistry and/or muscle histology findings (as outlined in the case definition in Chapter 3). Samples from these pigs were collected by hunters, and included skeletal muscle and liver samples for histology.
- **Group B (40 pigs):** Wild pigs caught in Northland, New Zealand, that were not fed to dogs and so were not linked to any known cases of GSM in dogs. Samples in this group were further categorised according to the method of sample collection:
  - Group B1 (13 pigs): Wild pigs caught during the Kaiwaka Three Furlongs Tavern Pig Hunt 2014. Samples of sternohyoideus muscle for histology were collected from these pigs by a local veterinarian as an addition to standard head and neck lymph node sampling for tuberculosis surveillance. The sternohyoideus muscle was chosen as it was easily accessible and is a site where *Trichinella* sp. has been found in wild boars.[24]
  - Group B2 (27 pigs): Wild pigs caught in Northland during 2014 and 2015 for human consumption. Samples of skeletal muscle (16 pigs) and liver (23 pigs) for histology were collected opportunistically by hunters.
- **Group C (7 pigs):** Semi-wild pigs from two neighbouring properties (Farm 1 and Farm 2) in Northland. Both these properties had a large population of wild pigs and

on Farm 1 the owner fed the pigs daily, resulting in formerly wild pigs becoming partially tame and living primarily on pasture. None of these pigs were linked to cases of GSM in dogs, but a dog on Farm 1 had been diagnosed with GSM in the past and commonly ate meat from cattle and pigs on the farm. Samples of skeletal muscle and liver for histology were collected by the farmer or a local veterinarian from a total of seven pigs on these farms that were killed due to poor health or for human consumption. Samples of cardiac muscle were also collected from five pigs.

4.2.2 Sample collection, fixation and processing

All samples for histology and electron microscopy were collected post mortem. The hunters, farmers and veterinarians who collected skeletal muscle samples were instructed to remove 2cm long, by 1cm wide, by 1cm deep samples from large, laterally located muscle bodies in the fore or hindlimbs (Group A and C) or the sternohyoideus muscle (Group B) using a knife. These samples were placed in 50mL plastic pottles containing 10% neutral buffered formalin as soon as practical. Samples of liver (approximately $2cm^3$), taken from any lobe, were collected into a separate 50mL pottle of formalin where possible. A high proportion of the muscle and liver samples collected by hunters were larger than specified (up to $5cm^3$), resulting in poor fixation of the centre of the sample.

All samples collected from pigs in Groups B and C were placed in formalin within 24 hours of the pig being killed, while samples from pigs in Group A were stored at room temperature and then fixed in formalin between 1 and 5 days after the pig died (after dogs who ate the pigs developed signs of GSM). Once in formalin, samples were fixed for between 2 to 21 days, prior to trimming longitudinal and transverse sections of muscle and sections of liver from well-fixed areas of the samples. Trimmed samples were then processed routinely for histology, embedded in paraffin, sectioned at 4µm and stained with haematoxylin and eosin (HE).

In addition to samples for histological examination, samples from electron microscopy were collected from two pigs in Group C. One of these pigs was a thin, semi-wild boar

from Farm 1 killed on humane grounds, while the other was a healthy pig from Farm 2 killed for human consumption. A local veterinarian collected multiple 2mm^3 samples of the triceps and biceps femoris muscles from these pigs immediately after death, and placed them in 2mL vials containing 2% glutaraldehyde. The fixed samples were kept refrigerated for approximately one month prior to being post-fixed in osmium tetroxide and embedded into epoxy resin. Thin sections from resin blocks were stained using lead citrate/uranyl acetate, and viewed using a FEI Tecnai G2 Biotwin Transmission Electron Microscope at the Manawatu Microscopy and Imaging Centre (Massey University, Palmerston North, NZ).

4.3 Results

4.3.1 Histology of skeletal muscle and liver from pigs eaten by GSM dogs (Group A)

Samples of skeletal muscle were examined from five pigs that dogs ate prior to developing GSM. In three of these pigs (60%), there were rare degenerate myofibres with disruption and hypereosinophilia of the sarcoplasm and loss of cross striations (Figure 4.2). No inflammation was observed in association with the degeneration. Skeletal muscle samples from the remaining two pigs (40%) were histologically normal. Samples of liver were also examined from these five pigs and no abnormalities were observed, apart from mild to moderate autolysis (consistent with the reported intervals between death and sample fixation).

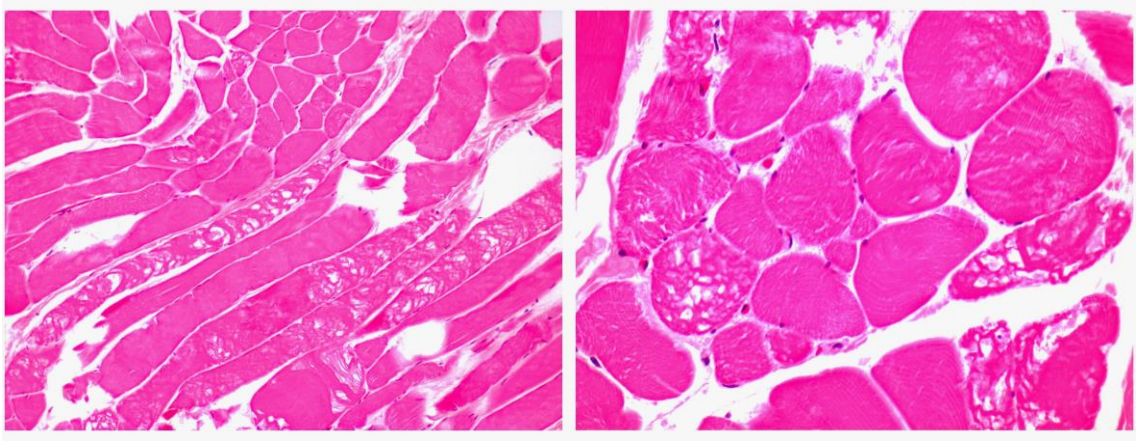

Figure 4.2: Longitudinal (left; 200x magnification) and transverse (right; 400x magnification) muscle sections from a pig eaten by a dog that subsequently developed 'Go Slow' myopathy. There is disruption of the sarcoplasm of myofibres with loss of cross striations, and swelling of individual myocytes

4.3.2 Histology of skeletal muscle and liver of wild pigs caught in Northland (Group B)

Sternohyoideus muscle samples were taken from 13 pigs that were caught during the 2014 Kaiwaka Three Furlongs Tavern Pig Hunt (Group B1). These 13 pigs were all estimated to be between two and four years of age, and had a median weight of 45kg (range 37 – 71.5kg). Two of the pigs (15%), both caught in the Dome Valley Forest between Warkworth and Wellsford, had scattered myofibres with disruption of the sarcoplasm and loss of cross striations (Figure 4.3A – B). No inflammation was associated with these fibres, and sections of sternohyoideus muscle from the remaining 11 pigs (85%) were normal.

Skeletal muscle samples were also collected from 16 of the 27 wild pigs (59%) sampled opportunistically by hunters (Group B2). In five of these 16 muscle samples (31%), there were rare degenerate myocytes with little to no inflammation (Figure 4.3C – D). There was no evidence of previous episodes of muscle degeneration in these sections, such as fibre splitting or myofibre regeneration. Muscle samples collected from the remaining eleven pigs (69%) were histologically normal.

Samples of liver were collected from 23 of the 27 wild pigs in Group B2. In the livers of two of these pigs (9%), there was periportal fibrosis and small to moderate numbers of inflammatory cells, predominantly lymphocytes and eosinophils. A further two pigs (9%) had high numbers of binucleate hepatocytes with normal liver architecture. A single pig (4%) had a single large granuloma in the liver, composed of macrophages, lymphocytes and eosinophils surrounding a central cystic cavity filled with eosinophilic material. Liver samples from the remaining 18 pigs (78%) were histologically normal, and none of the pigs had vacuolation of hepatocytes.

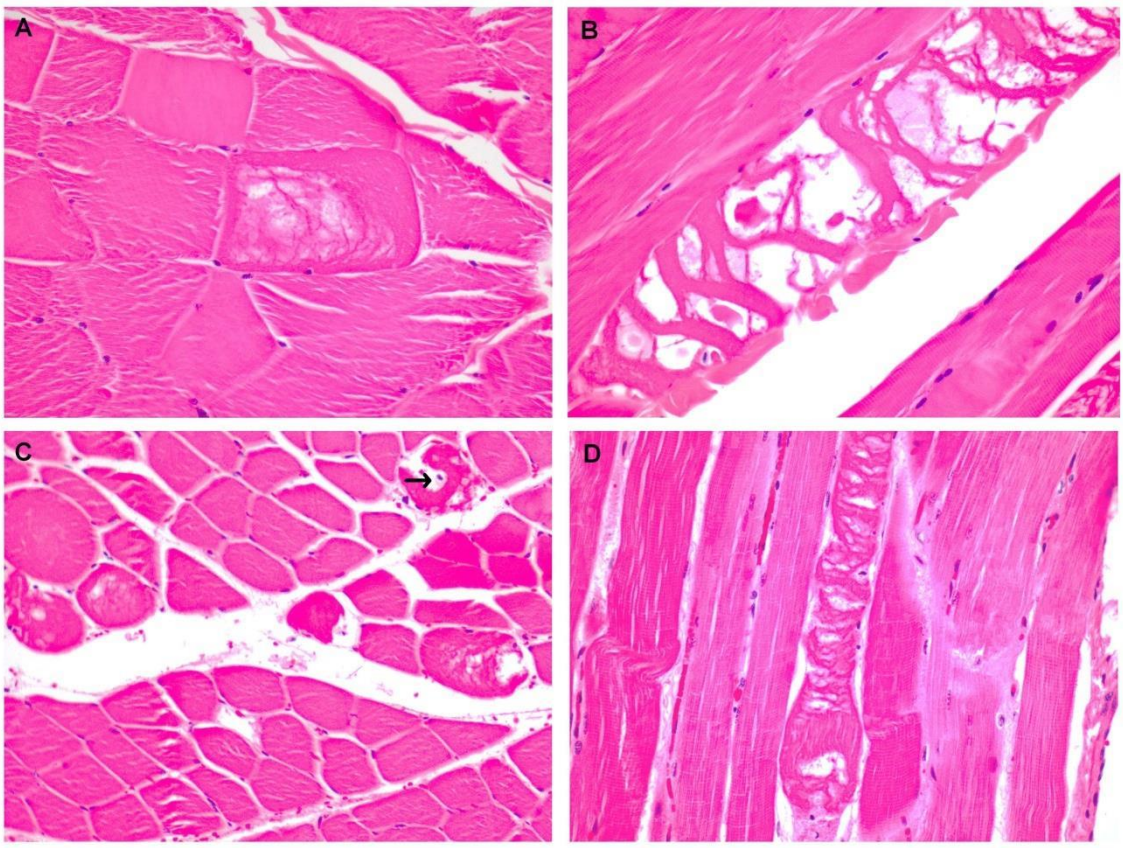

Figure 4.3: Histology of skeletal muscle samples from wild pigs in Northland, showing acute degeneration of scattered myocytes, with disruption of the sarcoplasm and loss of cross striations. **A-B:** Sternohyoideus muscle from a wild pig caught in the Dome Valley, Northland, 400x magnification. **C-D:** Unidentified skeletal muscle from a wild pig caught on farmland in an area where 'Go Slow' myopathy was reported to occur in dogs, 400x magnification. A single neutrophil is present in the sarcoplasm of a degenerate myocyte in image C (arrow).

4.3.3 Histology of skeletal muscle, heart and liver, and electron microscopy of muscle, from semi-wild pigs in Northland (Group C)

Skeletal muscle and liver samples for histology were collected from five pigs on Farm 1 (Pigs 1 – 5), where a dog had been previously diagnosed with GSM. Four of these pigs (Pigs 1 – 4) were in poor body condition and lagged behind other pigs in the group when moving, but clinical examination of these animals was not possible given their semi-wild state. The remaining pig from Farm 1 (Pig 5) was in good body condition and appeared healthy, and was killed for human consumption. Muscle and liver samples were also collected from two pigs (Pigs 6 – 7) from Farm 2 (a neighbouring farm with no history GSM cases in dogs) for histology. In addition, electron microscopy was performed on skeletal muscle samples from Pigs 4 and 7. Skeletal muscle histology and electron microscopic findings are summarized in Table 4.1.

Table 4.1: Muscle histology and electron microscopic findings in seven semi-wild pigs from two neighbouring farms in Northland.A dog on Farm 1 had been diagnosed with 'Go Slow' myopathy in the past, but none of the meat from these pigs was fed to dogs. Thin pigs were in poor body condition and appeared to lag behind other pigs in the group when moving.

Pig	Farm of origin	Clinical status	Skeletal muscle histology	Presence of sarcocysts	Electron microscopy
Pig 1	Farm 1	Thin	Normal	++	Not performed
Pig 2	Farm 1	Thin	Degeneration, inflammation, regeneration, mineralisation	++	Not performed
Pig 3	Farm 1	Thin	Single degenerate myofibre with inflammation	+	Not performed
Pig 4	Farm 1	Thin	Degeneration, inflammation, regeneration	+++	Mitochondrial hypertrophy and membranous inclusions
Pig 5	Farm 1	Healthy	Normal	++	Not performed
Pig 6	Farm 2	Healthy	Normal	0	Not performed
Pig 7	Farm 2	Healthy	Normal	0	Normal myofibrils and mitochondria

Sarcocysts key: 0 = absent; + = 1 – 2 per section; ++ = 3 – 5 per section; +++ = greater than 5 per section

The most significant histological lesions in skeletal muscle were seen in Pigs 2 and 4 (both thin pigs from Farm 1). Lesions were similar in both pigs, but were more severe in Pig 2, and included acute degeneration of scattered myofibres, characterised by hypereosinophilia and disruption of the sarcoplasm with loss of cross striations (Figure 4.4A). Small to moderate numbers of mononuclear inflammatory cells, predominantly macrophages with fewer lymphocytes and rare eosinophils, were present within the sarcoplasm of a small number of degenerate fibres (Figure 4.4B). In both pigs, there were rare regenerating myofibres with central nuclei and basophilia of the sarcoplasm (Figure 4.4C). There was also mineralisation of the sarcoplasm in scattered myocytes in Pig 2 (Figure 4.4D). Pig 3 had a single degenerate myocyte in longitudinal section that was infiltrated by neutrophils, macrophages, lymphocytes and eosinophils, but there were no other changes consistent with a myopathy in skeletal muscle samples from this pig.

Cardiac muscle samples were examined from Pigs 1 – 4 and 7 in Group C. Sections of cardiac muscle from Pig 2 (a thin pig from Farm 1) exhibited similar changes to those seen in the skeletal muscle of this pig, including scattered foci of mononuclear inflammation and mineralisation (Figure 4.4E), but no acute degeneration of cardiac myocytes was observed. There were no significant histological changes in cardiac muscle samples from the remaining pigs. However, an incidental finding in both cardiac and skeletal muscle samples from all pigs from Farm 1 (Pigs 1 – 5) was the presence of *Sarcocystis* sp. organisms within myocytes (Table 4.1). Sarcocysts were round to ellipsoidal with a thick, eosinophilic cyst wall, and contained numerous, curved basophilic bradyzoites (Figure 4.4F). No muscle degeneration or inflammation was seen in association with the sarcocysts. Muscle samples from pigs from Farm 2 (Pigs 6 and 7) did not contain any sarcocysts.

Sections of liver were examined from all seven pigs in Group C. Pigs 1 and 3 (thin pigs from Farm 1) had small numbers of lymphocytes, plasma cells, macrophages and eosinophils in periportal areas (Pig 1) or randomly distributed within lobules (Pig 3). Sections of liver from the remaining pigs had no histological lesions. None of the pigs exhibited any vacuolar change in hepatocytes.

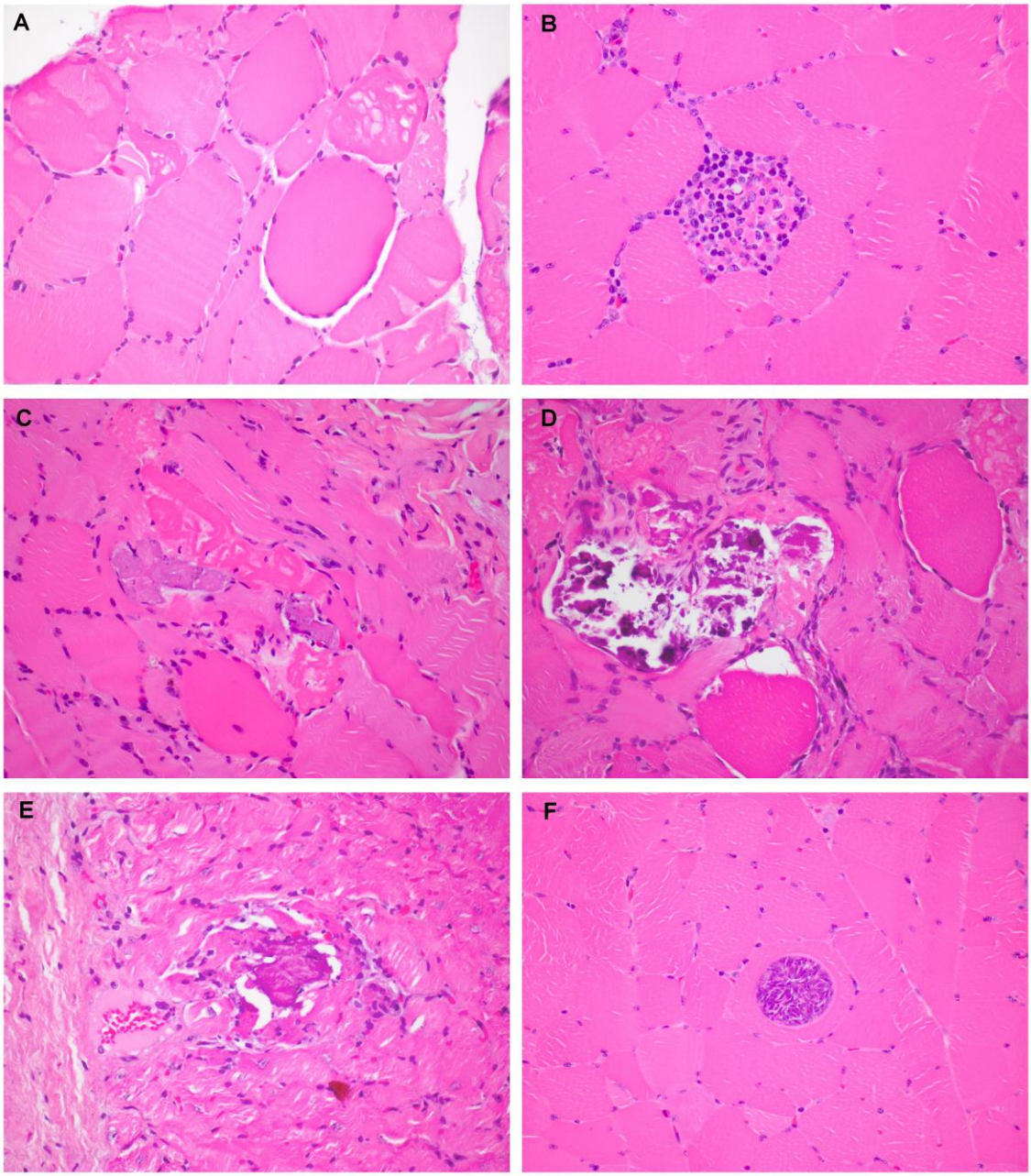

Figure 4.4: Histology of skeletal and cardiac muscle samples (400x magnification) from Pig 2, a semi-wild lethargic pig from a property with a history of 'Go Slow' myopathy in dogs. Similar lesions were seen in Pig 6. **A:** Acute degeneration of skeletal muscle myocytes, with hypereosinophilia and disruption of the sarcoplasm, and loss of cross striations**. B:** Mixed inflammatory cells within the sarcoplasm of a single degenerate skeletal muscle myocyte**. C:** Acute myodegeneration and concurrent regeneration in skeletal muscle. Regenerative myocytes have sarcoplasmic basophilia and centrally located nuclei. **D:** Focal mineralisation of skeletal muscle, seen as deeply basophilic granular material replacing a myocyte. **E:** Focal mineralisation of cardiac muscle**. F:** Incidental finding of a sarcocyst located within the sarcoplasm of a skeletal muscle myocyte. Note the absence of degeneration or inflammation associated with the organism.

Electron microscopy was performed on skeletal muscle samples from Pig 4 (a thin pig from Farm 1) and from Pig 7 (a clinically normal pig from Farm 2). No ultrastructural abnormalities were observed in Pig 7 (Figure 4.5A - B). In contrast, Pig 4 had widespread mitochondrial changes in skeletal muscle, with preservation of normal myofibrillar structure. Mitochondria were frequently enlarged and there was focal disruption and expansion of cristae by electron-dense filamentous and membranous inclusions (Figure 4.5C - D). In the sarcoplasm of myofibres, there were large quantities of uniform, dense granules consistent with glycogen, and occasionally small aggregates of these granules formed a circular structure bounded by a thin membrane (Figure 4.5E). For comparison, ultrastructural changes in the skeletal muscle of a dog with GSM are pictured alongside the pig electron microscopy images (Figure 4.5F).

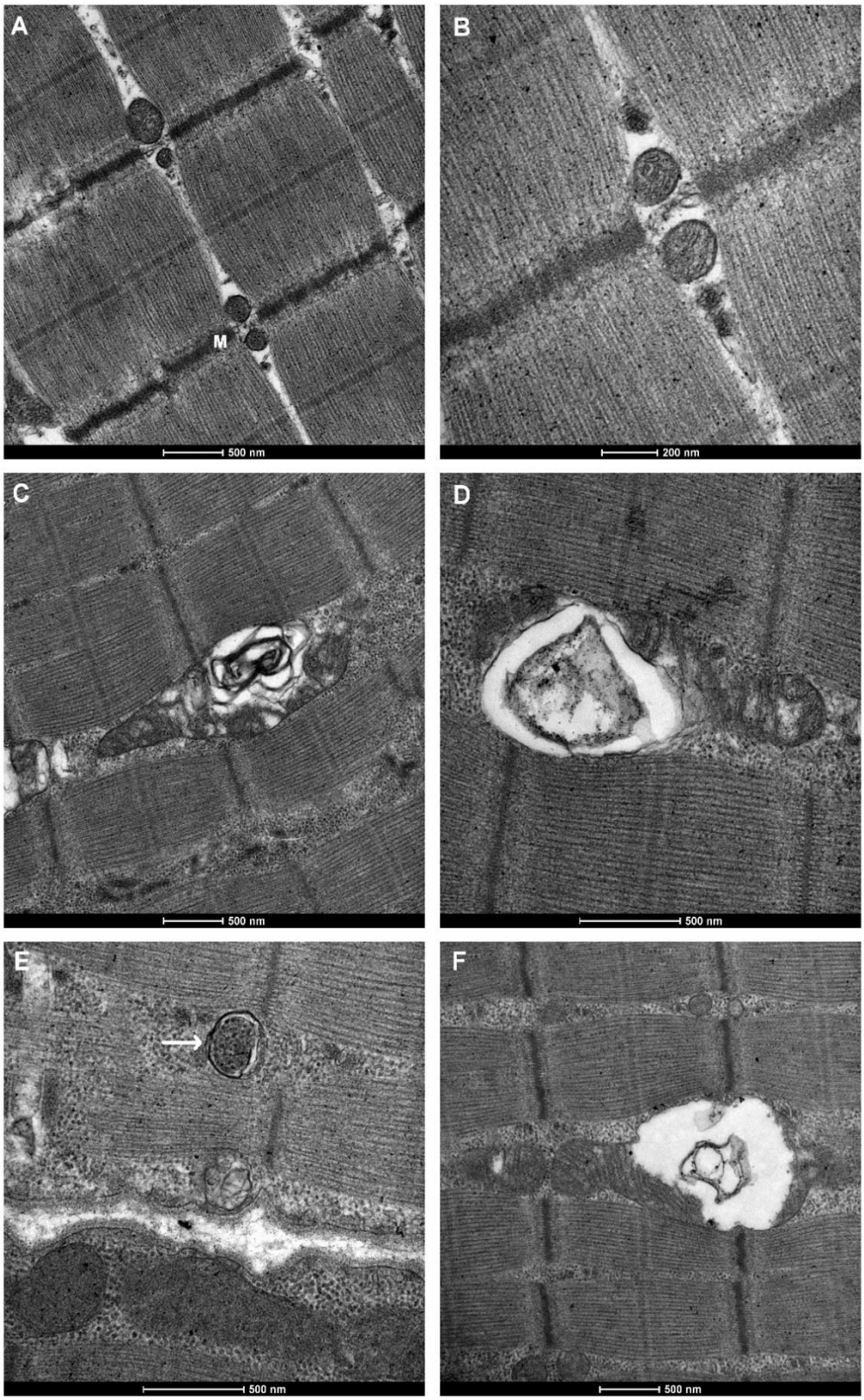

Figure 4.5: Electron microscopy of skeletal muscle from semi-wild pigs.**A – B:** Normal myofibres and paired mitochondria (M) from a clinically normal pig (Pig 7). **C – D:** Enlarged mitochondria with membranous whorls and inclusions disrupting and expanding mitochondrial cristae in a lethargic semi-wild pig (Pig 4) **E:** Increased glycogen is present within the myocyte sarcoplasm of Pig 4 compared to pig 7 (A). A focal accumulation of glycogen is bounded by a thin, electron dense membrane (arrow). **F:** Comparable mitochondrial lesions in the skeletal muscle of a dog with 'Go Slow' myopathy (Chapter 3).

4.4 Discussion

Histologically, degeneration of skeletal muscle with minimal to no inflammation is the primary lesion in dogs with 'Go Slow' myopathy (GSM, see Chapter 3), and similar changes were seen in three out of five (60%) pigs that had been fed to dogs who subsequently developed GSM. Muscle samples from the remaining two pigs (40%) that were fed to dogs who developed GSM were histologically normal, suggesting that muscle histology is not a useful indicator of which pigs might be associated with the myopathy in dogs.

Scattered degenerate myofibres were also present in 15% of sternohyoideus muscle samples from wild pigs in Northland, and 31% skeletal muscle samples from the fore and hind limbs of other wild pigs hunted in Northland (none of which were fed to dogs). In addition, skeletal muscle degeneration and regeneration were seen histologically in two thin semi-wild pigs out five pigs (40%) sampled on a farm where GSM had been previously diagnosed in a dog. Electron microscopy on skeletal muscle of one of these semi-wild pigs showed mitochondrial hypertrophy, membranous intramitochondrial inclusions and increased glycogen in the sarcoplasm, with preservation of normal myofibrillar structure. These ultrastructural changes are similar to those seen in dogs with GSM (Chapter 3), and are most commonly described in association with mitochondrial myopathies. Similar intramitochondrial filamentous inclusions in muscle have also been described with ischaemia and uraemic myopathy,[25] but these are not considered likely in the pig as additional ultrastructural changes associated with these conditions[26, 27] were not observed in the samples examined.

In people, mitochondrial myopathies have been described in association with various genetic [28] and toxic causes.[29] [25] Genetic myopathies reported in pigs include a familial myopathy in pietrain pigs[30] and malignant hyperthermia (porcine stress syndrome).[31] In pigs with malignant hyperthermia, muscle contraction and hypermetabolism can be triggered by stress or inhalational anaesthetics. The wild and semi-wild pigs included in this study could have experienced stress while being hunted, resulting in muscle damage if they had malignant hyperthermia. However, this would not explain the presence of more chronic muscle lesions such as regeneration and mineralization seen

in some of the Group C pigs. Furthermore, malignant hyperthermia causes rupture of mitochondria and a loss of glycogen granules ultrastructurally,[32, 33] whereas the pig skeletal muscle examined by electron microscopy in this study exhibited mitochondrial hypertrophy and increased glycogen. While genetic causes of myopathies are impossible to rule out in pigs in this study, the histological and electron microscopic changes are more consistent with a toxic aetiology, especially given the similarity to skeletal muscle lesions of GSM in dogs. Possible causes of toxic myopathies are discussed in Chapter 3, including plant and fungal toxins that feral pigs could be exposed to through their varied diet.

In addition to muscle lesions, most dogs with GSM had a vacuolar hepatopathy (microvesicular steatosis) on histology of the liver (Chapter 3). Liver lesions were not observed in pigs with muscle lesions in this study, suggesting that either these pigs did not have GSM, or there are species differences in tissue sensitivity or selectivity to the causative agent/compound. Some toxins which affect the liver in dogs (such as acetaminophen[34]) do not cause hepatocyte damage in pigs,[35] and there are species differences in expression of hepatic biotransformative enzymes, particularly the cytochrome P450 enzymes,[36] which could affect the hepatic metabolism and lesions caused by toxic compounds. The occurrence of microvesicular steatosis could also be influenced by species differences in hepatic capacity for fatty acid oxidation and ketogenesis,[37] as microvesicular steatosis can be associated with decreased mitochondrial oxidation of fatty acids.[38] As clinical examinations, serum biochemistry, and histology of other organs were not able to be performed on the pigs in this study, it is not possible to definitively determine whether the muscle lesions are attributable to GSM, with species differences in liver lesions, or if the lack of liver lesions indicates a different underlying disease process is occurring.

In earlier studies of the myopathy in dogs, the nematode *Trichinella spiralis* was considered as a possible causative agent.[23] There was no evidence of *Trichinella* or other nematode species in muscle sections examined from any of the pigs in the current study, including the pigs eaten by dogs that developed GSM. Furthermore, the clinical signs of trichinosis in dogs are not consistent with GSM, as *Trichinella* infections

in dogs are usually asymptomatic,[39, 40] and surveillance for *Trichinella spiralis* in New Zealand suggests a low prevalence in the wild pig population.[41, 42] Human cases of trichinosis are also rare in New Zealand,[43] and are usually associated with the ingestion consumption of raw or undercooked pork,[44] whereas cases of GSM have been reported in dogs fed well-cooked wild pig (Chapter 2). Due to these inconsistencies and the absence of any *Trichinella* larvae in wild pig samples examined, *T. spiralis* is considered unlikely to be implicated in the development of GSM, although direct examination of muscle for the presence of *Trichinella* larvae is insensitive.[45] The presence of *Sarcocystis* sp. organisms in muscle was common in pigs in this study, but was not associated with any muscle pathology and was considered an incidental finding.

Limitations of the present study include the opportunistic nature of sample collection, the small number of animals sampled, and the variation in sampling procedure and sample preservation, especially in wild pig samples collected by hunters. Delayed fixation of samples can result in autolysis, leading to disruption, contraction and clumping of myocyte sarcoplasm histologically,[46] which may appear similar to acute degeneration. However, autolysis was excluded as a cause of the muscle lesions in the present study as only scattered myocytes were affected and there were no other changes consistent with autolysis. Tissue preservation is also of particular importance for electron microscopy samples, as organelles like mitochondria degenerate rapidly following death of the animal or sample collection. No specific controls were included in the present study, but skeletal muscle samples for electron microscopy were collected from a thin pig from Farm 1 and a healthy pig from Farm 2 in the same manner to enable detection of any artefactual or autolytic changes resulting from sampling and fixation. No changes were seen in the healthy pig from Farm 2, while the mitochondrial lesions in the pig from Farm 1 are consistent with pathological rather than autolytic changes.

A further limitation of the present study is that it was not possible to perform clinical examinations or serum biochemistry on the pigs, as they were wild animals that were often caught in remote locations. In dogs with GSM, histologic and ultrastructural

changes in muscle can be interpreted in light of the clinical history and serum muscle enzyme activities (Chapter 3) in order to confirm the diagnosis, but in wild pigs the lesions have to be interpreted in isolation. Furthermore, although the lesions seen in pigs are similar to those in dogs with GSM, suggesting a common aetiology, this does not prove a causal link. In order to show a causal association between the ingestion of wild pig and the myopathy in dogs, it would be necessary to feed meat from pigs with skeletal muscle lesions to dogs. This was not ethically justifiable as currently there is no proven treatment for GSM in dogs and intentionally attempting to create cases of the disease would result in dogs suffering. The retrospective analysis of tissues from pigs known to have been fed to dogs that developed GSM showed that 3/5 (60%) of these cases had histological lesions consistent with GSM, providing further supportive evidence for this link, but as more is learnt about the disease in dogs, a prospective case-control study could become more feasible.

In conclusion, microscopic myocyte degeneration with minimal inflammation was seen in a total of 13 out of 41 (32%) skeletal muscle samples from wild and semi-wild pigs in Northland. Electron microscopy of muscle from a thin, semi-wild pig showed mitochondrial hypertrophy, intramitochondrial inclusions and increased sarcoplasmic glycogen. These changes are similar to those described in the skeletal muscle of dogs with GSM (Chapter 3), and ingestion of wild pork is associated with cases of the myopathy in dogs (Chapter 2). Taken together, these findings suggest that wild pigs could be implicated in the pathogenesis of GSM and may be similarly affected by the disease. However, not all wild pigs that were eaten by dogs subsequently diagnosed with GSM had histological evidence of muscle degeneration. Histology of skeletal muscle is therefore unlikely to be a useful screening tool to detect pigs that might be associated with the development of the myopathy if fed to dogs.

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Chapter 5

Untargeted metabolic profiling using liquid chromatography-mass spectrometry: Part A (aqueous liver extracts)

5.1 Introduction

The epidemiological and pathological features of an idiopathic myopathy in dogs, known as 'Go Slow' myopathy (GSM), are most consistent with a toxic myopathy associated with ingestion of tissues from wild pigs. The disease primarily affects mitochondria in skeletal muscle and increased amounts of lipid and glycogen are seen in the muscle and liver of affected dogs, suggesting that the disease may be associated with systemic or tissue specific alterations in metabolism.

As outlined in Chapter 3, a plant or fungal toxin is considered to be the most likely cause. There are numerous similarities between GSM in dogs and white snakeroot (*Ageratina altissima*) toxicity,[1-4] as the white snakeroot plant can cause a secondary toxic myopathy ('milk sickness') in people that eat meat or drink milk from animals who have eaten the plant. Extracts of the white snakeroot plant are reported to affect mitochondrial metabolism,[5] and the primary toxic compound is known as tremetone,[6] which has been detected in liver samples of poisoned cattle using gas chromatography-mass spectrometry.[7] *Ageratina altissima* is not naturalised in New Zealand, but two closely related species are. *Ageratina adenophora* has been associated with respiratory disease in horses,[3] and experimentally, leaf extracts can cause hepatotoxicity in mice, although specific toxic compounds have not been definitively identified.[8] The second related species*, Ageratina riparia,* is the subject of a biological control programme in New Zealand as it is considered an invasive weed,[9] but there are no published reports of toxicity in animals associated with this species.

Other secondary toxic myopathies recognised in people include coturnism, associated with consumption of migratory quail (*Coturnix* spp) in Europe,[10-12] and Haff Disease, which develops following ingestion of fish or crustaceans in the Baltic Sea region, USA

and China.[13, 14] Coturnism was thought to be due to quail eating seeds of *Galeopsis ladanum*, but recent studies do not support this,[15] and it been suggested that Haff Disease results from fish eating toxic blue-green algae.[16] However, the mechanisms of toxicity and causative compounds in both of these diseases remain unknown.

Laboratory tests are available to detect a large number of pesticides,[17, 18] insecticides[19] and drugs[20, 21] in biological samples, but testing for natural toxins can be more challenging.[22] GSM in dogs is hypothesised to be due to a plant or fungal toxin, but as with coturnism and Haff Disease, the causative species and toxic compounds are unknown. Characterisation of the pathology of the disease (Chapter 3) has provided clues as to the underlying pathogenesis, but using metabolomics to understand the cellular pathways involved could allow further refinement of possible causes and inform treatment decisions in clinical cases. Metabolomics, or metabolic profiling, enables detection of a wide range of analytes in biological samples, including amino acids, oligopeptides, fatty acids, sugars and biochemical pathway intermediates (such as the glycolytic and tricarboxylic acid cycle pathways). Principal methods used include ^{1}H nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS).[23] Both of these have advantages and disadvantages, but MS is considered to have higher sensitivity and can provide fragmentation patterns of compounds that aid in determining the identity of unknown molecules.[24]

Mass spectrometry is usually preceded by liquid chromatography (LC) or gas chromatography (GC). For GC-MS, non-volatile and polar compounds need derivatization prior to analysis, so sample preparation is often more complex than for LC-MS.[23] In LC-MS, liquid extracts from samples are injected onto a chromatographic column (e.g. C18) to separate the mixture into individual components with different retention times determined by their polarity.[25] The compounds are eluted from the column and ionized in the source of the mass spectrometer and accelerated in an electromagnetic field. The mass to charge ratio (*m/z*) of the ionised compound is detected and the resulting mass spectrum and retention time recorded. Untargeted extraction and LC-MS procedures can give insights into the pathogenesis, underlying

mechanisms and biochemistry of a disease,[23] and could aid in the identification of previously unrecognised toxins.

The aim of the present study was to perform untargeted metabolic profiling on liver samples collected from clinically normal dogs and those affected by GSM. The mass spectra of aqueous extracts of liver samples collected from healthy dogs and GSM cases were compared in order to detect significant differences between normal and affected dogs, with the aims of identifying cellular pathways altered in the disease and possible causative compounds.

5.2 Methods

5.2.1 Liver sample collection

Liver samples were collected post mortem from a total of 39 dogs, including 24 clinically normal dogs and 15 dogs affected by GSM. The clinically normal dogs were all euthanised for reasons unrelated to the study, including pound dogs that were not able to be rehomed and dogs that were aggressive or chased livestock. Dogs with the myopathy were part of the pathology study detailed in Chapter 3 (diagnosed on the basis of case history, clinical signs and muscle histology findings) and were euthanised for deterioration in their clinical condition despite supportive treatment, or due to an inability to perform their required role as a hunting or working dog as a consequence of the myopathy. The duration of clinical signs at the time of euthanasia ranged from 3 days to one year; four dogs were sampled at 3 days after the initial onset, six dogs were sampled between 7 and 19 days, and the remaining 5 dogs were sampled at greater than 50 days duration. As samples were collect post mortem, and dogs were not specifically euthanised for this study, ethical approval was not required.

From each dog, a liver sample at least $2cm^3$ in size was collected from any liver lobe by a veterinarian immediately following euthanasia. Samples were either refrigerated and shipped to Massey University, Palmerston North, where they were frozen at -20°C, or were frozen by the referring veterinarian prior to shipping on ice if a delay in sample transport was anticipated (e.g. samples collected late on a Friday or over a holiday

period). Samples were stored at -20°C for up to 18 months prior to extraction and analysis.

5.2.2 Preparation of aqueous extracts from liver samples

Sample extraction was performed using the procedure described by Want *et al*[23] for global metabolic profiling of animal tissues using LC-MS (Figure 5.1). This protocol generates aqueous and lipid extracts from tissue samples for the detection of polar and nonpolar compounds. Lipid phase extracts are discussed in Chapter 6. To prepare aqueous extracts, a 50mg section was taken from each liver sample and homogenised with 1.5mL methanol/water (1:1) using a bead beater (Precellys, Bertin Instruments, France) at 6,500Hz speed and 1.0mm diameter zirconia/silica beads (dnature diagnostics & research, Gisborne, NZ). The mixture was centrifuged at 10,000*g* for 10 minutes at 4°C, before collecting the supernatant and drying it in a Savant vacuum concentrator (Thermo Scientific, Waltham, MA, USA) for 180 minutes at 45°C. Dried extracts were frozen at -20°C for 5 days before being thawed and reconstituted in 120µL of 50:50 acetonitrile:water with 0.1% formic acid, with an internal standard of dicholorofluoroscein at 1µg/mL concentration. Blank samples were prepared in the same manner with no liver added, and control samples for quality control (QC) purposes were prepared by pooling 20µL from each of the 39 samples following extraction and resuspension. For each analysis, blanks were run first, followed by QCs, then randomised samples. A QC sample was also injected after every 10 samples.

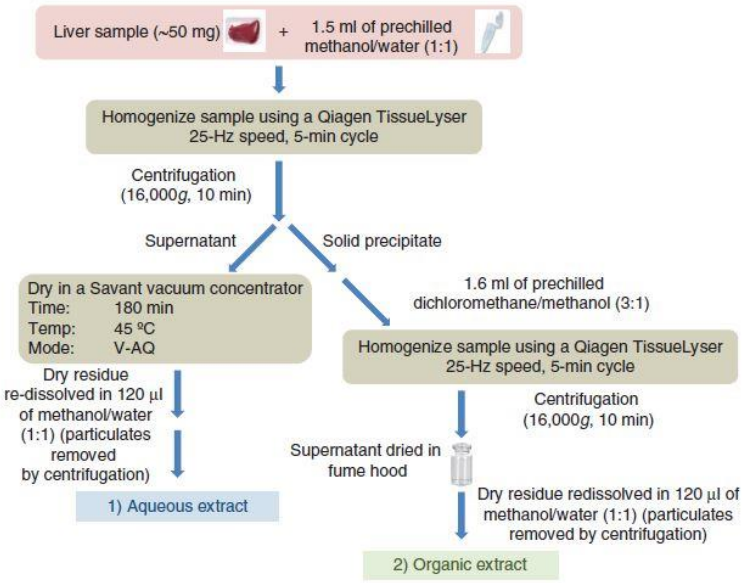

Figure 5.1: Two-step solvent extraction of liver samples to yield aqueous (present chapter) and organic (Chapter 6) extracts for liquid chromatography/mass spectrometry. Figure reproduced with permission from Want EJ, Masson P, Michopoulos F*, et al*. Global metabolic profiling of animal and human tissues via UPLC-MS*. Nat. Protocols* 2013; 8: 17-32.

5.2.3 Liquid chromatography – mass spectrometry (LC-MS) procedure

Aqueous (polar) liver extracts were analysed in both positive and negative ionisation modes using equipment and conditions as described by Fraser *et al.*[26] A Thermo LC–MS system (Thermo Fisher Scientific, Waltham, MA, USA) was used, consisting of an Accela 1250 quaternary UHPLC pump, a PAL auto-sampler fitted with a 15,000 psi injection valve (CTC Analytics AG., Zwingen, Switzerland), which cooled samples to 4^{o}C, a 20 µL injection loop, and an Exactive Orbitrap mass spectrometer with electrospray ionisation. A 2 µL aliquot of each samples was resolved on an Agilent RRHD SB-C18 column (150 mm×2.1 mm, 1.8 µm) at 25 °C, with a flow rate of 400 µL/min. A gradient elution programme using solvent A (water–formic acid (99.9:0.1, v/v)) and solvent B (acetonitrile–formic acid (99.9:0.1, v/v)) was applied as follows: held at 5% B (0–0.5 min), 5–99% B (0.5–13 min), held at 99% B (13–15 min), returned to 5% B (15–16 min) and allowed to equilibrate for a further 4 min prior to the next injection. The first 2.5 min and the last 6 min of the chromatogram were diverted to waste. Mass spectral data were collected in profile mode over a range of m/z 60–1200, at a mass resolution setting of 25,000 with a maximum trap fill time of 100 ms using the manufacturer's Xcalibur software package. Samples were run in positive and negative ionisation

modes separately. Positive ion mode parameters included: spray voltage, 3.5 kV; capillary temperature, 325 °C; capillary voltage, 50 V, tube lens 120 V. Negative ion mode parameters were as follows: spray voltage,−3.5 kV; capillary temperature, 325 °C; capillary voltage, −90 V, tube lens −80 V. For both modes, the settings for the nitrogen source gas used to nebulise the sample were the same: sheath gas, 40; auxiliary gas, 10; sweep gas, 5 arbitrary units.

5.2.4 Data processing and analysis

LC-MS data was processed as described by Fraser *et al.*[26] and Dalziel *et al.*[27] Compounds eluting between 2.5 and 14 minutes were extracted from the LC-MS data and converted to mzXML files using ProteoWizard (ProteoWizard Software Foundation, San Diego, CA). Peak detection, alignment and grouping were performed using XCMS software[28] and the peak intensity table generated was run through in-house R-based linear run-order correction normalisation and isotope annotation, with low-intensity signals filtered to remove background noise.

Data analysis was performed using the online tool Metaboanalyst, version 3.0 (McGill University, Montreal, Quebec, Canada).[29] For all analyses, dogs were separated into clinically normal (control) dogs and dogs affected by GSM. The data were log transformed to improve normality and autoscaled to allow comparison of compounds based on differences in their relative abundance in normal and affected dogs, rather than absolute concentrations.[30] Principal component analysis was used to provide an overview of the datasets and identify samples that could be outliers, then *t* test and fold change analyses were performed to identify differences between clinically normal (control) dogs and myopathy cases. In the t-test, a false discovery rate (FDR) correction was applied to reduce the risk of false positive results (Type I errors). Mass spectral features were considered significant if FDR < 0.05.

5.2.5 Compound identification

Data from the two ionisation modes were combined for compound identification. Significant LC-MS features were searched against the public domain databases HMDB (http://www.hmdb.ca) and Metlin (http://metlin.scripps.edu/), with an *m/z* tolerance of 10ppm allowed. Chromatograms of significant mass spectrometric features were viewed using MZmine 2[31] to assess whether features that shared the same retention time represented the same compound (adducts or fragments). Very low intensity unresolved peaks were removed. For features with no database matches, possible molecular formulae and isotope patterns were explored using the MZedDB MF generator (http://maltese.dbs.aber.ac.uk:8888/hrmet/index.html). Compounds designated as 'unknown' in the results are unknown either because there were no plausible database matches at this mass, or there were multiple biologically plausible matches that could not be differentiated on the basis of the MS spectra available.

5.3 Results

A total of 1253 features in positive mode and 621 features in negative mode were identified for statistical analysis. No run-order effects were evident on principal components analysis (PCA) and there was overlap between normal and affected dogs on PCA score plots (Appendix C). The *t* tests yielded 29 features that differed significantly (FDR <0.05) between groups in positive mode and 26 features in negative mode. Following identification of fragment ions and isotope peaks, a total of 38 significant features remained (annotated in Table 5.1). Of these, 19 matched biologically plausible compounds in the public domain databases, including various phospholipids, dicarboxylic acids and N-acetylated amino acids. A further seven mass spectrometric features were possible matches for one or more known compounds, including some plant-derived alkaloids, such as convoline, supinine, ambiline, phytocassane B, anastospemine and other tropane alkaloids. The remaining 12 significant features were not able to be identified, although possible molecular formulae were generated for five of those. Further characterisation based on those molecular formulae was not possible, as known compounds with those formulae were synthetic laboratory compounds that neither the dogs, nor the wild pigs they ate, were likely to have been exposed to.

Table 5.1: Mass spectrometric features from the aqueous analysis of liver samples that differ significantly (FDR >0.05) between dogs with 'Go Slow' myopathy (M) and normal (N) dogs

#	m/z	Mode	r.t (min)	Ions detected	Possible identification *[significant fragments]*	Formula	FDR	log2FC (M/N)	Level (M/N)
1	305.094	Pos	4.47	Unknown	Unknown	-	0.000	5.19	M>N
2	188.035	Neg	3.99	[M-H]-	Kynurenic acid	C10H7NO3	0.000	5.61	M>N
	144.045	Neg		[M-H-CO2]-			0.000	4.82	
3	263.084	Pos	3.29	?[M+H]+	Unknown	Possible: C16H10N2O2	0.001	3.70	M>N
4	172.097	Neg	4.75	[M-H]-	N-acetylleucine *[-130.087; +132.102; +128.107; +86.097]*	C8H15NO3	0.001	1.75	M>N
5	524.299	Neg	11.55	[M-H]-	PS(18:0)	C24H48NO9P	0.001	-1.08	N>M
6	452.280	Neg	10.89	[M-H]-	PE(16:0)	C21H44NO7P	0.001	-1.12	N>M
	454.294	Pos		[M+H]+	*[+255.234]*		0.008	-1.08	
	436.282	Pos		[M-H-H2O]-			0.008	-1.14	
7	346.221	Pos	5.41	[2M+NH4]+	Possible: kynuramine	C9H12N2O	0.001	2.42	M>N
8	316.211	Pos	5.24	Unknown	Unknown	-	0.003	1.21	M>N
9	331.249	Neg	8.75	[M-H]-	9,10,13-Trihydroxystearic acid	C18H36O5	0.006	0.80	M>N
10	480.312	Neg	13.04	[M-H]-	LysoPE(18:0)	C23H48NO7P	0.014	-1.11	N>M
	482.325	Pos		[M+H]+			0.032	-1.02	
11	452.280	Neg	10.68	[M-H]-	LysoPE(16:0)	C21H44NO7P	0.014	-0.74	N>M
12	371.227	Pos	4.1	? [M+H]+	Unknown	Possible: C16H34O9	0.015	-1.12	N>M
13	476.307	Pos	4.42	? [M+H]+	Unknown	Possible: C21H41N5O7	0.015	-0.58	N>M
14	610.373	Pos	12.32	[M+H]+	1-palmitoyl-2-glutaryl phosphatidylcholine (PGPC)	C29H56NO10P	0.015	-1.68	N>M
15	415.254	Pos	4.27	[M+H]+	Unknown	Possible: C18H38O10	0.015	-0.56	N>M
	432.281			[M+NH4]+			0.015	-0.55	
16	308.152	Pos	4.35	[M+H]+	Possible: convoline	C16H21NO5	0.015	1.30	M>N
	306.140	Neg		[M-H]-			0.028	1.27	
17	215.128	Neg	6.56	[M-H]-	Undecanedioic acid	C11H20O4	0.015	1.15	M>N
18	480.312	Neg	12.17	[M-H]-	LysoPE(18:0) *isomer of compound #10*	C23H48NO7P	0.016	-0.87	N>M
	540.331	Neg		[M+CH3COO]-			0.044	-0.68	

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Table 5.1 continued

	m/z	Mode	r.t (min)	Ions detected	Possible ID *[significant fragments]*	Formula	FDR	log2FC	Level
19	614.331 616.345	Neg Pos	10.15	[M-H]- [M+H]+	Unknown	Possible: C32H41N9O4	0.017 0.031	-0.97 -0.97	N>M
20	188.092	Neg	3.97	?[M-H]-	Possible: astanospermine OR N-methylcalystegines OR australine OR alexine	Possible: C8H15NO4	0.017	0.62	M>N
21	520.330	Pos	4.55	Unknown	Unknown	-	0.017	-0.61	N>M
22	315.254	Neg	10.33	[M-H]-	Dihydroxystearic acid	C18H36O4	0.019	0.84	M>N
23	125.097 169.087	Neg Neg	7.96	Fragment [M-H-H2O]-	Nonanedioic (azelaic) acid	C9H16O4	0.019 0.057	-0.78 -0.68	N>M
24	561.042	Pos	10.93	Unknown	Unknown	-	0.022	-1.53	N>M
25	575.713	Pos	10.88	Unknown	Unknown	-	0.022	-1.49	N>M
26	300.289 282.279	Pos Pos	12.61	[M+H-H2O]+ [M+H-2H2O]+	Phytosphingosine [*-299.258*]	C18H39NO3	0.022 0.043	-1.49	N>M
27	590.390	Pos	10.83	Unknown	Unknown	-	0.023	-1.49	N>M
28	231.123	Neg	5.91	[M-H]-	2- or 3-hydroxy undecanedioic acid	C11H20O5	0.023	1.05	M>N
29	229.109	Neg	5.28	[M-H]-	Possible: Oxo-undecanedioic acid *[-167.107; -139.113]*	C11H18O5	0.023	0.87	M>N
30	201.112	Neg	5.19	[M-H]-	Sebacic acid (decanedioic acid)	C10H18O4	0.026	0.98	M>N
31	599.320	Neg	10.28	[M-H]-	PI(18:0)	C27H53O12P	0.028	-0.75	N>M
32	335.222	Pos	6.56	?[M+H]+	Possible: phytocassane B OR crispanone OR dehydropinifolic acid prostaglandin A2/B2/C2/J2	Possible: C20H30O4	0.030	0.96	M>N
33	284.185	Pos	4.62	?[M+H]+	Possible: supinine OR amabiline	Possible: C15H25NO4	0.031	1.43	M>N
34	299.223	Neg	11.38	[M-H]-	Heptadecanedioic acid	C17H32O4	0.034	-0.70	N>M
35	197.117	Pos	8.08	?[M+H]+	Unknown	Possible: C11H16O3	0.043	0.83	M>N
36	213.149	Pos	6.81	[M+H-H2O]+	Dodecanedioic acid	C12H22O4	0.048	0.65	M>N
37	564.356	Pos	4.66	Unknown	Unknown	-	0.048	-0.53	N>M
38	158.081	Neg	3.63	[M-H]-	N-Acetylvaline	C7H13NO3	0.048	0.56	M>N

r.t. retention time; *FC* fold change; *FDR* false discovery rate
PE phosphatidylethanolamine; *PI* phosphatidylinositol; *PS* phosphatidylserine

5.4 Discussion

The significant mass spectrometric features identified in aqueous extracts of dog liver give an insight into metabolic pathways affected in an idiopathic myopathy of dogs ('Go Slow' myopathy, GSM), and also aid in the identification of compounds that could be implicated in the pathogenesis of the disease.

The most significant feature in terms of both FDR and fold change is a compound with an m/z of 305.094 and retention time of 4.42 minutes in positive mode (compound 1 in Table 5.1). The calculated fold change for this compound is 36.4, and examination of total ion chromatograms shows a clear peak at this mass and retention time in 14 out of 15 dogs affected by the myopathy (93%). In contrast, a defined peak in this region is only seen in two out of 24 normal dogs (8%), and the peaks present in these 2 normal dogs are of a low base peak intensity (maximum 3.0×10^4). At this mass, there are no matches to known compounds in the HMDB database, but in the METLIN database there are 119 matches (including 117 toxicants) for [M+H]+ adducts. However, MS/MS spectra are only available for two of these compounds, making further characterisation difficult. Possible molecular formulae of compounds with [M+H]+ adducts at this mass include $C_{23}H_{12}O$, $C_{16}H_{17}O_4P$, $C_{18}H_{12}N_2O_3$, $C_{15}H_{16}N_2O_3S$ or $C_{17}H_{17}ClO_3$, and of these, the isotope pattern of the peak is most consistent with either $C_{18}H_{12}N_2O_3$ or $C_{15}H_{16}N_2O_3S$. Most known compounds with these formulae are synthetic drugs or industrial chemicals unlikely to be present in extracts of dog liver, so further studies are required to identify this compound.

In addition to compound 1, there are three other unidentified significant mass spectrometric features that were higher in samples from affected dogs relative to normal dogs (compounds 3, 8 and 35 in Table 5.1). Searching for the masses of these compounds on public domain databases yielded varying numbers of matching compounds, but MS/MS spectral data was lacking for most, precluding definitive identification. Furthermore, the majority of the matching compounds were synthetic chemicals or flavouring ingredients unlikely to be present in aqueous extracts of liver samples from dogs, with the exception of one of the possible results for compound 3 (m/z 263.084, positive mode). At this mass is the [M+H]+ adduct of

3-methoxysampangine, a copyrine alkaloid with antifungal properties from the plant *Cleistopholis patens*.[32] This plant is native to Africa and is not found in New Zealand, but other species of plant belonging to the same family (*Annonaceae*) are present in New Zealand and similar bioactive substituted natural compounds have been found in ascidians (sea squirts) along the New Zealand coast.[33]

Four other significant mass spectrometric features that were increased in affected dogs could be consistent with plant-derived alkaloids. Compound 16 in Table 5.1 (m/z 308.152 in positive mode) has the same mass as the [M+H]+ adduct of convoline, a toxic tropane alkaloid from *Convululus* sp. plants. *Convolvulus arvensis* (field bindweed) is present in New Zealand and classified as an environmental weed.[34] Extracts of this plant can cause gastritis and hepatitis in mice,[35] and intestinal fibrosis in horses,[36] but there are no reports of muscle damage associated with ingestion of *Convolvulus* sp. Compound 20 (mz/188.092, negative mode) is consistent with the [M+H]+ adduct of compounds with a molecular formula of $C_8H_{15}NO_4$, which encompasses several tropane alkaloids derived from plants (including astanospermine, N-methylcalystegines, australine and alexine). These alkaloids are inhibitors of glucosidase[37-39] and galactosidase[40] enzymes, and are produced by several unrelated plant species, including *Castanospermum austr*ale[41] (Moreton Bay Chestnut) and *Lycium chinense*[40] (boxthorn), which are present in New Zealand. In people, inherited deficiencies in glucosidase enzyme activity result in lysosomal storage diseases that are associated with skeletal muscle lesions, such as Pompe disease. The skeletal muscle lesions of lysosomal storage diseases include accumulation of glycogen in myocytes, mitochondrial abnormalities and fragmentation of myocytes,[42, 43] and similar lesions occur in the muscle of dogs with GSM (Chapter 3). However, as the name suggests, glycogen accumulates within lysosomes in lysosomal storage diseases, whereas the glycogen in myocytes of affected dogs appeared to be extra-lysosomal, suggesting the diseases have a different underlying pathogenesis.

The remaining two mass spectrometric features with plant alkaloids as possible identities are compounds 32 (m/z 335.222, positive mode) and 33 (m/z 284.185, positive mode) in Table 5.1. At a mass of 335.222 are [M+H]+ adducts of compounds

with a molecular formula of $C_{20}H_{30}O_4$, including the plant-derived compounds phytocassane B (isolated from rice, *Oryza sativa*),[44] crispanone (from parsley, *Petroselinum crispum*)[45] and dehydropinifolic acid (from conifer trees, *Pinus silvestris*).[46] While those plants are present in New Zealand, the compounds are not reported to be toxic to mammals and at that mass there are also [M+H]+ adducts of various prostaglandins, so definitive identification of this peak is not possible. At a mass of 284.185 (compound 33) are [M+H]+ adducts of two pyrrolizidine alkaloids, supinine and amabiline, which accumulate in various plants in the borage family, Boraginaceae.[47-49] Chronic ingestion of pyrrolizidine alkaloids can result in hepatotoxicity,[48] but a single injection of supinine in mice resulted in no hepatic lesions,[47] suggesting that acute toxicity is unlikely.

Aside from highlighting possible compounds that could be implicated in the development GSM in dogs, the significant mass spectrometric features from the aqueous liver extracts aid in the identification of metabolic pathways affected in the disease. Kynurenic acid was more abundant in affected dogs relative to normal dogs, with a log2FC of 5.6, which corresponds to a fold change of 48.7. Kynurenic acid is one of three possible end products of the kynurenine pathway of tryptophan metabolism, with the other two products being picolinic acid and nicotinamide adenosine dinucleotide (NAD), as shown in Figure 5.1.[50] Kynurenic acid is generated through the action of kynurenine aminotransferase (KAT) enzymes, located in the inner membrane of mitochondria,[51] so changes in mitochondrial function could alter kynurenic acid production. Conversely, accumulation of kynurenic acid can impair the function of mitochondria in the heart and lower ATP production.[52] In the brain, kynurenic acid has neuroprotective properties and antagonises N-methyl-d-aspartate (NMDA) and α7 nicotinic acetylcholine receptors,[53] but kynurenic acid crosses the blood-brain barrier poorly.[54] In people, increased levels of kynurenic acid in tissues have been reported in a variety of diseases, including type II diabetes, vitamin B6 deficiency, inflammatory bowel disease, schizophrenia and Alzheimer's disease.[55, 56] Additionally, expression of KAT enzymes in skeletal muscle can be increased with endurance exercise through activation the PGC-1α1-PPARα/δ pathway, resulting in increased production of kynurenic acid. However, all of the affected dogs in this study had been rested for a

minimum of two days prior to sample collection, so the increases in kynurenic acid detected are unlikely to result from exercise alone.

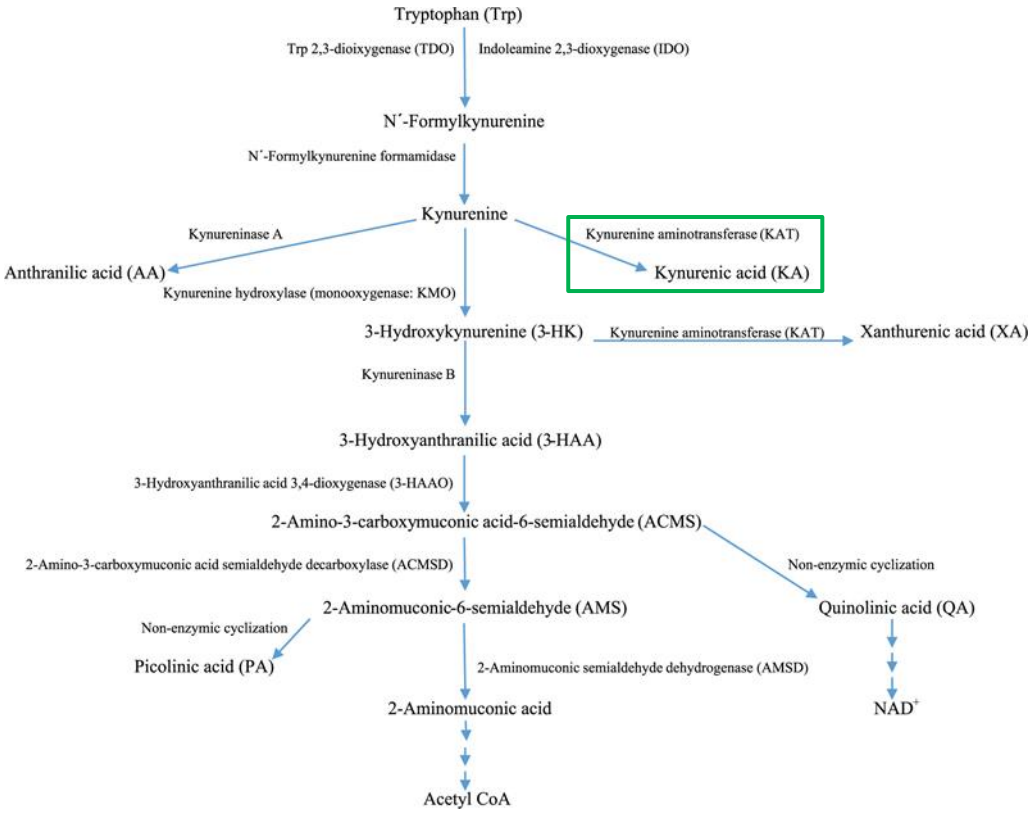

Figure 5.2: The kynurenine pathway of tryptophan degradation. Kynurenic acid is shown in the green box. Figure reproduced with permission from Badawy AA-B. Kynurenine Pathway of Tryptophan Metabolism: Regulatory and Functional Aspects. *International Journal of Tryptophan Research* 2017; 10: 1178646917691938.

The other metabolic disturbances detected on mass spectrometry in dogs with the myopathy included changes in the abundance of several medium dicarboxylic acids, a decrease in the abundance of seven different phospholipids, and an increase in the N-acetylated conjugates of leucine and valine. This specific combination of metabolic changes has not been previously reported, but could result from a defect in mitochondrial fatty acid oxidation or generalised mitochondrial dysfunction. Increased production and urinary excretion of dicarboxylic acids occurs in disorders of mitochondrial fatty acid oxidation,[57-60] and in the present study, dogs with the myopathy had increased liver levels of medium chain saturated dicarboxylic acids including sebacic (decanedioic) acid, undecanedioic acid and dodecanedioic acid. Affected dogs also had increased levels of hydroxylated and oxygenated carboxylic

acids, including 2- or 3-hydroxy undecanedioic acid, oxo-undecanedioic acid, dihydroxystearic acid and 9,10,13-trihydroxystearic acid. Hydroxylation of dicarboxylic acids occurs in ω-oxidation of fatty acids in liver microsomes,[61] and this pathway can be upregulated in response to defects in mitochrondrial β-oxidation.[62] Two saturated dicarboxylic acids with odd numbers of carbon atoms (nonanedioic and heptadecanedioic acid) were decreased in affected dogs relative to normal dogs, suggesting that oxidation of odd numbered carbon chains may not be impaired, although the increases in undecanedioic acid are not consistent with this.

Affected dogs had a relative reduction in the abundance of various phospholipids and their degradation products, particularly those incorporating saturated fatty acids. These include phosphatidylserine (18:0), phosphatidylethanolamine (16:0) lysosphosphatidylethanolamines (18:0 and 16:0), 1-palmitoyl-2-glutaryl phosphatidylcholine (PGPC), phytosphingosine and phosphatidylinositol (18:0). These changes could be due to decreased phospholipid production as a result of generalised mitochondrial dysfunction, as mitochondria are central to the production of phospholipid precursors and phosphatidylethanolamine,[63] and alterations in phospholipids can be seen in some mitochondrial diseases.[64] In some disorders of fatty acid oxidation, it has been shown that the intermediates of incomplete fatty acid oxidation produced are not incorporated into phospholipids,[65] which could influence the levels of different phospholipids present in tissue. Phospholipid hydrolysis can increase with fasting in tissues such as myocardium,[66] and in the present study the timing of the last meal relative to sampling was not recorded or controlled for in either the affected or normal dogs. However, fasting of up to four days in rats has no significant effect on liver phospholipid content,[67] and other metabolic changes that occur with fasting, such as increases in bile acids,[68] were not seen in either the aqueous or lipid extracts of liver (presented in Chapter 6). This suggests that the decreases seen in phospholipids in dogs affected by the myopathy are unlikely to be a reflection of any possible differences in recent feeding or fasting between the groups.

The other metabolic change noted in dogs with GSM was an increase in N-acetylated conjugates of the branch chain amino acids leucine and valine. These are unusual

amino acid conjugates, and have been detected in the urine of patients with Maple Syrup Urine Disease, a disorder of branch chain amino acid (BCAA) metabolism.[69, 70] Other mass spectrometric findings and clinical features of the myopathy in dogs are not consistent with a diagnosis of Maple Syrup Urine Disease,[71] but the presence of N-acetyl leucine and valine in the liver of affected dogs suggests there may be a defect in the metabolism of BCAA in these dogs. BCAA metabolism occurs entirely within mitochondria[72] and long chain fatty acids are thought to play a regulatory role in the process,[73] so mitochondrial disease or defects in fatty acid oxidation could influence BCAA levels in tissue. The oxidation of BCAA occurs predominantly in skeletal muscle, with a lesser amount in occurring in liver,[72] so the microscopic damage to these organs seen in dogs with the myopathy (Chapter 3) could also contribute to changes in BCAA and their conjugates.

If affected dogs do have a defect in mitochondrial function, specifically β-oxidation, this could explain some of the clinical signs and microscopic lesions seen in affected dogs. Clinical signs of the myopathy are exacerbated by moderate-intensity exercise, which is known to increase utilisation and oxidation of fatty acids within skeletal muscle.[74, 75] Microscopic lesions in dogs with GSM are confined to skeletal muscle and the liver (Chapter 3), and patients with disorders of mitochondrial fatty acid oxidation usually present with hepatopathy, skeletal myopathy and/or cardiac myopathy as fatty acid oxidation is most active in these tissues.[60] Defects in fatty acid oxidation can cause hepatic steatosis,[76-78] which was a common finding in affected dogs. Ultrastructural changes in the muscle of affected dogs include increased amounts of lipid and glycogen and mitochondrial hypertrophy, which can occur with the accumulation of substrates in metabolic disorders.[79] The morphological changes seen in mitochondria (Chapter 5) could be associated with mitochondrial dysfunction, as also suggested by some of the compounds identified on mass spectrometry.

Fatty acid oxidation disorders are most commonly inherited, but can also be acquired as various drugs and compounds can inhibit mitochondrial β-oxidation enzymes and/or sequester coenzyme A.[78] For example, seasonal pasture myopathy/atypical myopathy in horses is a toxic myopathy due to ingestion of box elder tree seeds containing

hypoglycin A, and is characterised by deficiency of multiple acyl-CoA dehydrogenases, resulting in defective mitochondrial β-oxidation.[80] In this disease, there are significant alterations in serum and urine acyl carnitines,[81] which were not detected during statistical analysis of dog liver mass spectrometric data. However, liver acyl carnitine levels content may not be reflective of plasma concentrations as different tissues (including liver and skeletal muscle) can contribute to plasma acyl carnitine pools and may respond to changes in lipid metabolism in different manners.[82, 83] Furthermore, extraction and analysis procedures in the present study were not specifically targeted towards carnitines and a range of acute and chronic cases were included, which could mask the detection of causative compounds or changes in metabolites that are only present in the acute phase of the disease. None of the plant alkaloids suggested as possible identities of significant mass spectrometric features are known to specifically inhibit mitochondrial fatty acid oxidation, but the toxic potential of these alkaloids in dogs and mechanisms of toxicity are largely uncharacterised. In vitro tests of mitochondrial function[84] and fatty acid oxidation could help to further investigate the pathways implicated in the disease, and analysis of acyl carnitines in plasma in acute cases could also be beneficial in exploring similarities to other toxic myopathies affecting mitochondria, such as seasonal pasture myopathy.

A limitation of the present study is the opportunistic nature of sample collection, leading to a lack of standardisation in sampling, and variation in the duration and severity of disease in affected dogs, and the timing of the last meal in relation to sampling. The liver mass spectrometric profile of dogs with the myopathy could change between the acute and chronic phases of the disease, but these differences would not be detectable with the sampling and analysis methods used. While analysis could be performed to compare acutely affected dogs (less than 7 days' duration of clinical signs) and chronically affected dogs (7 days or more), the statistical validity of such analysis would be highly questionable as only four dogs would be in the acute group and there can be a large degree of individual variation in metabolomic profiles.[85]

The mass spectra of liver samples may not accurately reflect changes in metabolism in skeletal muscle, but liver was chosen for this study as it allows better evaluation of

changes in whole body metabolism, and affected dogs also had liver lesions (Chapter 3). Although not standardised, liver samples are likely to be more metabolically uniform than skeletal muscle samples taken from different muscles.[86] The length of time that both normal and affected dog liver samples were stored for prior to analysis was variable, which could cause artefactual changes in compounds detected on mass spectrometry if there is sample degradation over time. However, the number of freeze thaw cycles samples are subjected to and the time between sample collection and freezing can have a greater impact on metabolomic profiles,[87, 88] and all liver samples in this study were frozen immediately at -20°C and only thawed prior to extraction.

The other main limitation is the lack of definitive identification of significant mass spectral peaks. Fragmentation patterns and retention times enabled tentative characterisation of some compounds, but these identities were not confirmed and some remained unknown. Peak identification is particularly difficult when dealing with possible plant-derived compounds, as the plant metabolome is more complex than mammalian systems and is estimated to include over 200,000 different compounds,[89] most of which have not yet been characterised. Further studies using more targeted extraction protocols, and the introduction of reference standards for compounds suggested as possible identities of unknown peaks could aid in the refinement of the data and identification of causative compounds. In addition, plant species known to produce the alkaloids identified as possible significant compounds could be harvested from regions where the disease is known to occur, and extracts or fractions of extracts of these plants could be tested in an *in vitro* model of mitochondrial function to refine possible causative species and compounds.

In conclusion, the significant mass spectrometric features identified in aqueous liver extracts from dogs with 'Go Slow' myopathy include changes in the abundance of various dicarboxylic acids, phospholipids and N-acetylated branch chain amino acids. These changes are suggestive of mitochondrial dysfunction, particularly involving pathways of mitochondrial of fatty acids. In addition, there were several compounds present at increased levels in the liver of affected dogs relative to normal dogs, and possible identities for these compounds include various plant-derived alkaloids that

could play a role in the development of the disease. Analysis of lipid extracts of the same liver samples from normal and affected dogs was performed to provide further insights into the pathogenesis of the myopathy (Chapter 6).

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Chapter 6

Untargeted metabolic profiling using liquid chromatography-mass spectrometry: Part B (lipid liver extracts)

6.1 Introduction

As described in previous chapters, 'Go Slow' myopathy (GSM) is a myopathy in dogs that is associated with the ingestion of wild pig, fed raw or after freezing and cooking (Chapter 2). Pathological features of the disease are most consistent with a toxic mitochondrial myopathy (Chapter 3), and structural changes in mitochondria have been observed in the skeletal muscle of affected dogs, as well as a wild pig from an area where the disease is known to occur (Chapter 4). Untargeted metabolic profiling of aqueous extracts of liver from affected dogs has shown changes in the abundance of various dicarboxylic acids, phospholipids and N-acetylated branch chain amino acids (Chapter 5). These findings are suggestive of mitochondrial dysfunction, particularly a defect in mitochondrial oxidation of fatty acids. In addition, several significant mass spectrometric features from aqueous liver extracts were possible matches with various alkaloids derived from plants, and overall, the epidemiological and pathological features of the disease are consistent with a plant or fungal toxin that dogs are exposed to through the ingestion of wild pigs.

The aim of the present study was to further investigate the metabolic changes associated with GSM in dogs by analysing lipid (organic) extracts of liver samples from affected dogs using liquid chromatography – mass spectrometry. Resultant mass spectra were compared to the mass spectra of lipid extracts of liver from clinically normal dogs to identify compounds, pathways and possible causative compounds implicated in the development of the myopathy.

6.2 Methods

6.2.1 Liver sample collection

The liver samples used for analysis of lipid (organic) extracts were the same samples as used for aqueous extracts (Chapter 5), so sample collection and storage procedures were identical to those previously described. Briefly, samples at least $2cm^3$ in size were collected from any lobe of the liver immediately post mortem. Liver samples were collected from a total of 24 clinically normal dogs and 15 dogs diagnosed with GSM on the basis of case history, clinical exam findings and muscle histology. Clinically normal dogs were euthanised for reasons unrelated to the study, while affected dogs were euthanised at the owner's request due to deterioration in their clinical recovery or an inability to perform their primary roles as hunting or working dogs as a result of the myopathy. The duration of clinical signs at the time of euthanasia ranged from 3 days to one year; four dogs were sampled at 3 days after the initial onset, six dogs were sampled between 7 and 19 days, and the remaining five dogs were sampled at greater than 50 days duration. As samples were collect post mortem, and dogs were not euthanised for reasons other than this study, ethical approval was not required. All liver samples were frozen at -20^{o}C for a maximum of 18 months prior to sample preparation and analysis.

6.2.2 Preparation of lipid extracts from liver samples

Lipid (organic) extracts were prepared using the procedure described by Want *et al.*[1] for untargeted metabolic profiling of tissue samples. The first step of tissue homogenisation was shared with the preparation of aqueous extracts (Chapter 5), and involved 50mg of tissue being homogenised with 1.5mL methanol/water (1:1) using 1.0mm diameter zirconia/silica beads (dnature diagnostics & research, Gisborne, NZ) and a bead beater at 6,500Hz (Precellys, Bertin Instruments, France). The mixture was then centrifuged at 10,000*g* for 10 minutes at 4°C, and the supernatant was used to prepare aqueous extracts while the tissue pellet was used for lipid extracts. 1.6mL of chilled dichloromethane/methanol (3:1) solution was added to the tissue pellet and the mixture homogenised using a bead beater as outlined in the initial step. The resulting mixture was then homogenised at 10,000*g* for 10 minutes at 4°C, with the

supernatant collected into glass vials. A 250µL aliquot of the supernatant of each sample was taken for LC-MS analysis and dried in a fume cupboard overnight. Dried samples were frozen at -20°C for 5 days before being thawed and reconstituted in 120µL of Folch solution (66.6% chloroform, 32.3% methanol, 1% water) containing a d31-PE internal standard at 10µg/mL concentration. Blank samples were prepared in the same manner with no liver added, and quality control (QC) samples were prepared by pooling 20µL from each of the 39 samples following extraction and resuspension. The samples were randomised before the run, and the run order in both positive and negative modes comprised blank samples, followed by QC samples, followed by dog liver samples. QC samples were also injected after every tenth sample, and retention time, mass error and signal intensity were monitored throughout the analysis to enable detection of any shifts in retention time or instrument variability.

6.2.3 Liquid chromatography – mass spectrometry (LC-MS) procedure

Lipid extracts of liver were analysed in both positive and negative modes using the procedure described by Dalziel *et al.*[2] An Acquity CSH C_{18} column (100 x 2.1 mm, 1.7µm inner diameter; Waters, Milford, MA) was used to separate the lipid extracts at 65°C with a gradient elution program at a flow rate of 600µL/min. The mobile phase consisted of two solvents: *solvent A* (acetonitrile-water-formic acid at a ratio of 59.95:39.95:0.1 vol/vol + 10 mM ammonium formate) and *solvent B* (isopropyl alcohol-acetonitrile-formic acid at a ratio of 89.95:9.95:0.1 vol/vol + 10 mM ammonium formate). The gradient elution program was as follows: 85–70% A (0–2 min), 70–52% A (2–2.5 min), 52–18% A (2.5–11 min), 18–1% A (11–11.5 min), held at 1% A (11.5–12 min), returned to 85% A (12–12.1 min), and held at 85% A (12.1–15 min). Column effluent was connected to the electrospray source of a Q-Exactive Orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Mass spectral data was collection over a mass range of *m/z* 200 – 2,000 in profile data acquisition mode with a mass resolution setting of 35,000 and a maximum trap fill time of 250ms using the manufacturer's Xcalibur software package. Parameters used in positive ion mode included a spray voltage of 4 kV, capillary temperature of 275°C and S-lens of 50 V. Parameters in negative ion mode included a spray voltage of

-4.0 kV, capillary temperature of 275°C and S-lens of -100 V. The settings for the nitrogen source gas used to nebulise the sample were identical for both modes. Five samples (the QC sample, one clinically normal dog and three of the dogs with GSM, incorporating both acute and chronic cases) were selected for data-dependent MS2 (ddMS2) fragment analysis, whereby ions produced in the initial MS step were further fragmented to aid in metabolite identification. ddMS2 analysis used the same settings as the primary MS analysis, with an isolation window of 1.5 m/z and normalised collision energy of 30.

6.2.4 Data processing and analysis

LC-MS data processing was performed as described for the aqueous extracts of liver (Chapter 5.2.4). Compounds eluting between 2.5 and 14 minutes were extracted, aligned and processed as previously described. The online tool Metaboanalyst, version 3.0 (McGill University, Montreal, Quebec, Canada)[3] was used for data analysis to detect differences between clinically normal dogs and those affected by GSM. Data were log transformed and autoscaled, and principal component analysis was used to give an overview of the data and highlight any outliers. P values were adjusted to give false discovery rates (FDRs) using the Benjamini and Hochiberg method.[4] T tests and fold change analysis was used to detect differences between the healthy and affected dogs, with FDRs of <0.05 considered significant.

6.2.5 Metabolite identification

Lipid annotations were performed by matching the processed data matrix against lipids identified in the samples subjected to ddMS2 analysis, using LipidSearch software (Thermo Scientific). Compounds not identified using LipidSearch were searched against online databases, namely HMDB (http://www.hmdb.ca/), METLIN (http://metlin.scripps.edu/) and LIPID MAPS (http://www.lipidmaps.org/) using accurate mass in combination with MS2 data. Where possible, organic phase compounds were putatively characterised, but not definitively identified, and the positions of fatty acyl groups and double bonds were not determined.

6.3 Results

For statistical analysis, a total of 2096 features were identified in positive mode and 1248 in negative mode. In negative ionisation mode, four samples (including three from clinically normal dogs and one GSM case) were not correctly injected by the autosampler and no valid spectra were obtained, so these samples were excluded from analysis. There was no clear separation between the affected and clinically normal dogs on principal component analysis (Appendix D) and no run order effects were evident. A total of 475 features were significantly different (FDR<0.05) between the groups in positive mode, and 39 features in negative mode. Adducts and isotopes were identified by visual examination of total ion chromatograms of these features, enabling refinement of the number of significant features to approximately 316 different compounds. Using MS2 data, 40 features were able to be identified in LipidSearch, including various phospholipids, sphingomyelins, ceramides and cholesteryl esters as listed in Table 6.1. All of these identified features were decreased in affected dogs relative to the normal dogs. Of the remaining significant features, the 20 with the lowest FDR are shown in Table 6.2. Ten of these features are large compounds or adducts (*m/z* > 1,000) that are higher in the dogs with GSM relative to normal dogs, and do not match with any known compounds on the databases searched. A further five of these twenty features are consistent with phospholipids and are decreased in affected dogs.

The remaining 256 significant features are shown in Appendix E. Possible identifications were made for 120 of these, while the rest did not match any biologically plausible compounds in the online databases. Possible identifications of compounds that were increased in dogs with GSM relative to normal dogs included anhydroretinol and retinal (vitamin A2), dolichol-20, and several diglycerides (42:1, 46:0), triglycerides (50:0, 58:2, 63:3, 68:4) and a monoglyceride (18:1). Compounds decreased in affected dogs included 61 different phospholipids (encompassing phosphatidic acids, phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines, phosphatidylinositols, phosphatidylglycerols, plasmenyl-phosphoethanolamines and plasmenyl-phosphatidylcholines), with the length and saturation of fatty acids in these phospholipids ranging from 34:0 to 44:12 (Appendix

E). In addition, three cardiolipin compounds (72:6, 72:8 and 74:8) were found to be possible matches for compounds significantly decreased in affected dogs. Various sphingolipids (sphingosine, dihydrosphingosine, and six sphingomyelins ranging from 19:1 to 44:1), sixteen ceramides (with fatty acid chains 30 – 44 carbons in total and one or two double bonds) and four cholesteryl esters are also listed in Appendix E as compounds decreased in affected dogs. Other significant mass spectral features that were tentatively identified using database matches and were decreased in affected dogs relative to normal dogs included hexadecenal, heptadecenal, heptadecadienal, octadecadienal, nonadecanedione, oxo-nonadecanoic acid, oleamide, palmitoleamide, spermic acid, 3-hydroxypristanic acid and N-hexadecanoyl pyrrolidine (Appendix E).

Table 6.1: Lipids identified using MS2 data in LipidSearch that were significantly lower in liver lipid extracts from dogs with 'Go Slow' myopathy (M) compared to clinically normal (N) dogs.

Lipid class/ID	Ion	m/z	Mode	r.t (min)	p value	FDR	log2FC (M/N)	Level (M/N)
Phosphatidylcholines (PC)								
PC(38:6)	[M+H]+	806.568	Pos	6.08	0.0001	0.003	-2.08	N>M
PC(40:7)	[M+H]+	832.580	Pos	6.12	0.0004	0.006	-1.29	N>M
PC(34:2)	[M+H]+	758.569	Pos	6.40	0.0004	0.006	-1.09	N>M
	[M+Na]+	780.547	Pos					
PC(34:3)	[M+H]+	756.552	Pos	6.01	0.0006	0.007	-1.36	N>M
PC(36:2)	[M+H]+	786.600	Pos	7.18	0.0007	0.008	-0.94	N>M
	[M+Na]+	808.577	Pos					
PC(40:6)	[M+H]+	834.597	Pos	6.83	0.0011	0.011	-1.74	N>M
PC(39:6)	[M+H]+	820.583	Pos	6.44	0.0016	0.013	-1.07	N>M
PC(33:2)	[M+H]+	744.553	Pos	6.03	0.0041	0.025	-1.02	N>M
PC(36:4)	[M+H]+	782.567	Pos	6.28	0.0053	0.029	-0.82	N>M
PC(32:0)	[M+H]+	734.567	Pos	6.95	0.0058	0.031	-0.97	N>M
PC(38:3)	[M+H]+	812.614	Pos	7.36	0.0059	0.031	-0.71	N>M
PC(40:5)	[M+H]+	836.611	Pos	7.33	0.0063	0.033	-0.10	N>M
PC(36:3)	[M+H]+	784.584	Pos	6.59	0.0089	0.042	-0.85	N>M
PC(32:0e)	[M+H]+	720.588	Pos	7.41	0.0092	0.043	-0.79	N>M
PC(34:1)	[M+H]+	760.584	Pos	7.01	0.0106	0.048	-0.56	N>M
Phosphatidylethanolamines (PE)								
PE(16:0/22:6)	[M+H]+	764.520	Pos	6.27	0.0000	0.002	-1.99	N>M
	[M+Na]+	786.502	Pos		0.0011	0.010		
	[M-H]-	762.510	Neg		0.0002	0.021		
PE(18:0/22:6)	[M+H]+	792.551	Pos	7.04	0.0000	0.003	-1.59	N>M
	[M-H]-	790.539	Neg	6.97	0.0015	0.046		
	[2M+H]+	1584.094	Pos		0.0001	0.003		
PE(16:0/18:2)	[M+H]+	716.521	Pos	6.62	0.0003	0.005	-1.17	N>M
	[M-H]-	714.51	Neg		0.0006	0.037		
	[2M-H]-	1430.03	Neg		0.0001	0.018		
PE(18:0/20:5)	[M+H]+	766.537	Pos	6.77	0.0016	0.013	-1.24	N>M
PE(16:0/20:4)	[M+H]+	740.521	Pos	6.49	0.0023	0.017	-0.80	N>M
	[M-H]-	738.51	Neg		0.0007	0.037		
PE(18:0/18:2)	[M+H]+	744.552	Pos	7.39	0.0067	0.034	-0.39	N>M
PE(18:1/22:6)	[M-H]-	788.524	Neg	6.25	0.0007	0.037	-1.42	N>M
PE(18:0p/20:4)	[M+H]+	752.557	Pos	7.62	0.0085	0.041	-0.88	N>M
PE(16:0/18:1)	[M+H]+	718.536	Pos	7.23	0.0100	0.045	-0.80	N>M
PE(18:0p/18:2)	[M+H]+	728.557	Pos	7.77	0.0100	0.045	-1.06	N>M

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Lipid class/ID	Ion	m/z	Mode	r.t (min)	p value	FDR	log2FC (M/N)	Level (M/N)
Phosophoinositols (PI)								
PI(18:0/22:6)	[M+NH4]+	928.588	Pos	6.08	0.0000	0.002	-1.63	N>M
	[M+H]+	911.56	Pos		0.0000	0.002		
	[M-H]-	909.553	Neg		0.0000	0.017		
PI(18:0/20:3)	[M+NH4]+	906.602	Pos	6.8	0.0039	0.024	-1.74	N>M
PI(18:0/22:5)	[M-H]-	911.56	Neg	5.97	0.0006	0.037	-0.75	N>M
PI(16:0/22:6)	[M+NH4]+	900.556	Pos	5.36	0.0001	0.003	-1.49	N>M
	[M-H]-	881.522	Neg	5.28	0.0008	0.037	-1.06	
Phosphatidylserines (PS)								
PS(18:0/22:6)	[M+H]+	836.542	Pos	6.17	0.0009	0.009	-1.48	N>M
PS(16:0/22:6)	[M-H]-	806.501	Neg	5.37	0.0001	0.018	-1.17	N>M
	[M+H]+	808.512	Pos	5.47	0.0003	0.005	-1.46	
PS(40:3)	[M-H]-	840.579	Neg	7.34	0.0005	0.037	-1.17	N>M
Sphingomyelins (SM)								
SM(d38:1)	[M+H]+	759.635	Pos	7.76	0.0021	0.016	-0.81	N>M
SM(d42:2)	[M+H]+	813.683	Pos	8.44	0.0026	0.018	-0.73	N>M
SM(d36:1)	[M+H]+	731.605	Pos	6.97	0.0066	0.034	-0.88	N>M
Ceramides (Cer)								
Cer (d18:1/24:1)	[M+H]+	648.628	Pos	9.28	0.0009	0.009	-0.87	N>M
	[M+Na]+	670.610	Pos		0.0000	0.003		
Cer(d18:1/22:0)	[M+H]+	622.613	Pos	9.34	0.0050	0.028	-0.86	N>M
Cer(d18:1/24:0)	[M+H]+	650.642	Pos	9.98	0.0088	0.042	-0.66	N>M
Cholesteryl esters (ChE)								
ChE(18:1)	[M+NH4]+	668.632	Pos	12.26	0.0002	0.004	-1.08	N>M
ChE(18:2)	[M+NH4]+	666.616	Pos	11.97	0.0064	0.033	-0.96	N>M

r.t. retention time; *FC* fold change; *FDR* false discovery rate

Table 6.2: Mass spectrometric features from the analysis of lipid extracts of dog liver that differed significantly between dogs with 'Go Slow' myopathy (M) and clinically normal (N) dogs. Only the top 20 features (based on FDR) are shown here, with the remainder included in Appendix E.

#	m/z	Mode	r.t (min)	Ion	Possible ID	Formula	FDR	log2FC (M/N)	Level (M/N)
1	750.506	Pos	5.89	[M+H]+	PC(34:6) or PE(37:6)	C42H72NO8P	0.002	-1.49	N>M
2	1693.605	Pos	13.9	Unknown	Unknown		0.002	1.28	M>N
3	1552.128 1550.115	Pos Neg	7.03 6.94	?[M+H]+ ?[M-H]-	Unknown		0.002 0.034	-1.42	N>M
4	396.382	Pos	3.37	?[M+NH4]+	Unknown	?C25H46O2	0.002	-1.73	N>M
5	1557.477	Pos	13.66	Unknown	Unknown		0.002	1.11	M>N
6	1943.819	Pos	12.26	Unknown	Unknown		0.002	-1.33	N>M
7	1625.538	Pos	13.78	Unknown	Unknown		0.003	1.25	M>N
8	1301.199	Pos	12.9	Unknown	Unknown		0.003	1.49	M>N
9	951.813	Pos	12.58	Unknown	Unknown		0.003	1.32	M>N
10	1617.472	Pos	13.51	Unknown	Unknown		0.003	1.54	M>N
11	1685.536	Pos	13.62	Unknown	Unknown		0.003	1.56	M>N
12	981.861	Pos	12.79	Unknown	Unknown		0.003	1.51	M>N
13	1520.138	Pos	6.40	Unknown	Unknown		0.003	-1.84	N>M
14	1415.299	Pos	13.2	Unknown	Unknown		0.003	1.00	M>N
15	1315.204	Pos	11.97	Unknown	Unknown		0.003	-1.53	N>M
16	862.627	Pos	7.28	[M+H]+	PC(42:6) or PE(45:6)	C50H88NO8P	0.003	-2.90	N>M
17	910.550	Pos	6.62	[M+NH4]+	PI(39:8)	C48H77O13P	0.003	-1.25	N>M
18	791.539	Pos	6.32	[M+NH4]+	PC(36:8) or PE(39:8)	C44H72NO8P	0.003	-1.78	N>M
19	1502.415	Pos	13.56	Unknown	Unknown		0.003	0.80	M>N
20	778.535	Pos	6.65	[M+H]+	PC(36:6) or PE(39:6)	C44H76NO8P	0.003	-1.19	N>M

r.t. retention time; *FC* fold change; *FDR* false discovery rate

6.4 Discussion

Mass spectrometric analysis of lipid (organic) extracts of the dogs' livers revealed several hundred compounds that differed significantly between dogs affected by 'Go Slow' myopathy (GSM) and clinically normal dogs. The results expand upon those obtained from analysis of aqueous extracts of the same liver samples (Chapter 5) and enable further identification of metabolic pathways that are altered in affected dogs.

From the compounds able to be identified (either tentatively or using MS2 data), several key metabolic changes and trends were apparent. Approximately 98 different phospholipid compounds were significantly decreased in affected dogs relative to normal dogs, including phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines, phosphatidylinositols and cardiolipins. Phospholipids are important components of cellular and subcellular membranes,[5] and in general, decreased levels of these compounds can be due to either reduced production or accelerated degradation. In addition, dogs with GSM in this study commonly had hepatic steatosis (Chapter 3), which results in structural changes in hepatocytes that could alter the relative proportions of different lipid classes in the liver. In people with hepatic steatosis and animal models of non-alcoholic fatty liver disease, different classes of phospholipids show distinct changes, with some phospholipids elevated and others decreased,[6, 7] in contrast to the dogs in the current study where all identified phospholipids were decreased. This suggests that the relative decrease in liver phospholipids in dogs affected by the myopathy is not solely attributable to hepatic lipid accumulation (steatosis).

Induction of phospholipase A2 has been reported in fatty liver disease in people, leading to increased breakdown of phospholipids.[8] However, accelerated phospholipid degradation, which can occur as a result of oxidative stress or ischaemia, usually results in accumulation of lysophospholipids,[9-11] whereas in the affected dogs in the present study, lysophospholipids were also decreased (Chapter 5 and Appendix E). Therefore, the alterations in phospholipids in affected dogs could instead be due to reduced production of these compounds, which is also supported by the decrease seen in some phosphatidic acid compounds (Appendix E), as phosphatidic acid is a precursor

for the synthesis of other phospholipids.[12] Synthesis of phospholipids occurs primarily in the endoplasmic reticulum, Golgi apparatus and mitochondria of cells,[12, 13] and studies regarding the pathology of GSM in dogs (Chapter 3) have shown that there are structural changes in muscle mitochondria, which raises the possibility that the disturbances in phospholipid metabolism could result from mitochondrial dysfunction.[14] In particular, cardiolipin and phosphatidylglycerol, which were decreased in affected dogs, are almost exclusively localised in mitochondria.[15] Cardiolipin also plays an important role in mitochondrial energy metabolism as it is a stimulatory cofactor for some mitochondrial enzymes[16] and is required for Complexes I and III of the mitochondrial respiratory chain to function.[17] As discussed in Chapter 5, the decrease in phospholipids in affected dogs is unlikely to be due to any potential differences between groups in the timing of the dogs' last meal, as liver phospholipids do not significantly change with fasting and other metabolic changes associated with fasting were not detected on statistical analysis.[18, 19]

In addition to phospholipids, various sphingolipids were decreased in liver samples of GSM dogs, including nine sphingomyelin compounds (Table 6.1 and Appendix E). The decreased levels of sphingomyelins could be secondary to the alterations seen in phosphatidylcholines, as sphingomyelins are generated through the transfer of a phosphocholine head group from phosphatidylcholine to ceramide.[20] Sphingosine, dihydrosphingosine and several ceramide compounds were also decreased in affected dogs (Appendix E), as well as aldehydes and amides of long chain fatty acids with 16 to 19 carbons. Long chain aldehydes such as hexadecanal (C16) can be produced by several different pathways, one of which is the oxidation and degradation of sphingolipids such as sphingosine.[20-22] The combination of decreased levels of sphingolipids and decreased fatty acid aldehydes suggest that the alterations in sphingolipids are more likely to result from a decrease in the production of these compounds, rather than increased degradation. Sphingolipids are primarily produced in the endoplasmic reticulum,[20] and while morphological changes were not appreciated in the endoplasmic reticulum of hepatocytes and myocytes of dogs with GSM on electron microscopy (Chapter 3), functional changes cannot be excluded. Interestingly, sphingolipids (particularly ceramides) are thought to act as important

signalling molecules and mediators of mitochondrial function[23, 24] and have been implicated in mitochondrial dysfunction in diabetic cardiomyopathy,[25] meaning that the alterations seen in sphingolipids in affected dogs could potentially contribute to the development of mitochondrial abnormalities instead of being a consequence of such changes.

A total of 72 compounds were increased in the lipid liver extracts of affected dogs relative to normal dogs. Fifty-one of these were large compounds or adducts (*m/z* greater than 900) that eluted late (retention times of 12 to 14 minutes) and did not match with any known compounds on the databases searched. It is possible that one or more of these mass spectrometric features could represent a toxic compound that causes or is central to the pathogenesis of GSM in dogs, but further characterisation or identification of these compounds was not possible as part of the present study. Of the compounds increased in affected dogs that were able to be tentatively identified using database matches, none are consistent with possible causative toxins.

Other possible compounds increased in dogs with GSM relative to normal dogs in the present study included anhydroretinol, retinal (vitamin A2), and dolichol-20. Both anhydroretinol and retinal are metabolites of retinol (vitamin A), and anhydroretinol has been shown to generate reactive oxygen species and precipitate cell death.[26, 27] The liver is central to vitamin A storage and metabolism, and excessive accumulation of fat in the liver (steatosis) can lead to disturbances in vitamin A metabolism and decreased retinol export to the periphery, with development of a functional vitamin A deficiency.[28, 29] Dogs with GSM commonly exhibited lipid accumulation within hepatocytes (microvesicular steatosis, Chapter 3), and on mass spectrometry, these dogs had increased liver levels of the anhydroretinol and retinal relative to normal dogs. Alterations in vitamin A metabolites could also be attributed to administration of a nutritional supplement to some of the affected dogs. This supplement contained high levels of certain vitamins and minerals, including vitamin A, and a single large dose of vitamin A has been shown to increase the concentrations of these vitamin A metabolites.[26]

The other known metabolite of interest that was increased in affected dogs was dolichol-20, a polyisoprenoid alchohol involved in the biosynthesis of N-linked glycoproteins.[30] Dolichol is metabolised slowly and accumulates in tissues in some lipid storage diseases and with aging, so the increased levels in GSM dogs could be due to the disease process or a reflection of the age of the dogs. Accurate aging of dogs in this study, particularly the normal dogs, was not possible as many of these dogs were pound dogs with no owners to provide relevant history. However, 17 out of 24 (71%) of the normal dogs were young dogs (less than 2 years of age), while only 3 out of 15 (8%) of the myopathy cases were known to be less than 2 years old, and the oldest case included was 14 years. Therefore, despite incomplete information regarding the age of dogs, it is likely that the mean age of the normal dogs was less than those with the myopathy, which could explain the difference in dolichol levels detected in liver samples.

Dogs with GSM also had significantly increased levels of several triglycerides, diglycerides and a monoglyceride relative to normal dogs (Appendix E). This is consistent with the microscopic finding of lipid accumulation in the hepatocytes of affected dogs (Chapter 3), and could reflect an imbalance between hepatic fatty acid uptake, lipogenesis, β-oxidation or lipid export from hepatocytes.[31] Results of mass spectrometric analysis of aqueous extracts of the same dog liver samples were suggestive of a defect in mitochondrial fatty acid oxidation (Chapter 5), and when fatty acid oxidation is inhibited, fatty acids are shifted towards the esterification pathway, resulting in increased tissue levels of triglycerides.[32, 33]

No potential causative toxic compounds were found in lipid extracts of liver from dogs affected by the idiopathic myopathy, and it is interesting to note that none of the significant mass spectrometric features are consistent with tremetone or dehydrotremetone, the toxic compounds in lipophilic extracts of *Ageratina altissima* (white snakeroot),[34-36] which can cause a similar clinical syndrome to GSM (as discussed in Chapter 5). Dehydrotremetone has been detected in the liver of a cow that died from white snakeroot poisoning using gas chromatography-mass spectrometry (GC-MS), and the compound was characterised by a peak with a mass

spectrum of m/z (relative intensity) 185 (100), 200 (44), 157 (38).[37] Analysis of extracts of snakeroot plants have revealed that tremetone has a molecular formula of $C_{13}H_{14}O_2$ and a mass spectrum of m/z (relative intensity) 202 (57), 187 (100), 169 (10), 159 (51), 144 (35).[38] In addition to the statistical analysis performed, visual inspection of total ion chromatograms of both aqueous and lipid liver extracts from affected dogs did not show any peaks consistent with the molecular ions or common adducts of either tremetone or dehydrotremetone, although the LC-MS protocol used is not optimised for the detection of these compounds so they cannot be completely excluded as a cause of the myopathy.

The limitations of the present study are similar to those outlined for the mass spectrometric analysis of aqueous liver extracts (Chapter 5), and include the inherent variation in dogs sampled, particularly the GSM cases. As liver sample collection was opportunistic, the severity and duration of disease, signalment of the dogs, diet, fed/fasted state, treatments administered (if any) and site of liver sampling were not standardised, and these factors could influence the metabolomic profile of the tissue. Cases and controls were not matched, so analysis was limited to group-level comparisons. A further limitation of this study is the lack of definitive identification of many significant mass spectrometric features. MS2 data enabled the identification of 40 lipid compounds that differed significantly between affected and normal animals, but other possible identifications were made on the basis of *m/z* and retention time, and were not confirmed. Furthermore, a large number of the significant features did not match any compounds listed in publically available databases, particularly the features with large *m/z* values that were increased in affected dogs. To further investigate the identity of these compounds, targeted extraction methods and MS2 analysis techniques such as precursor ion scanning could be useful.[39] However, efforts to identify unknown compounds in aqueous extracts might be more rewarding in the search for a possible causative compound, as some of these compounds are consistent with alkaloids from plants (Chapter 5). In order to determine the mechanisms responsible for the alterations in lipids seen in the present study, including the relative decrease in phospholipids in the liver of affected dogs, studies to assess specific aspects of hepatocyte metabolism could be undertaken, including mRNA

transcriptome profiling of genes involved in lipid metabolism[40] and functional assessments of isolated mitochondria *in vitro.*[41]

In conclusion, the results of the present study show that dogs with GSM have reduced hepatic phospholipid and sphingolipid levels relative to normal dogs. Mass spectrometric features consistent with several diglyceride and triglyceride compounds were increased in affected dogs, which is plausible in light of the findings outlined in other chapters of this thesis, including the presence of hepatic steatosis and mitochondrial abnormalities in dogs with the myopathy (Chapter 3). No potential causative toxic compounds were identified on analysis of lipid liver extracts, although there were 51 compounds or adducts present at higher levels in affected dogs that had a large *m/z*, eluted late and did not match any known compounds. Further investigations of lipid metabolism and mitochondrial function in dogs with GSM are warranted.

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Chapter 7

General discussion

7.1 Summary of findings

When this idiopathic myopathy was first described in the early 2000's, it was referred to as a "mystery disease in Northland pig dogs and working dogs," with clinical signs and serum biochemistry findings consistent with a myopathy, although a concurrent peripheral neuropathy or neuromuscular disorder could not be excluded.[1] The initial cases investigated in 2004 were confined to dogs on a small number of properties that had a high incidence of the disease, and no husbandry or dietary risk factors were able to be identified. At that point, and at the start of my research, it was unknown whether the myopathy was exertional, genetic, infectious or toxic in nature, although the disease was not considered to be consistent with any exotic infectious agents.

In order to investigate the pathogenesis and possible causes of the disease, the epidemiological (Chapter 2) and pathological studies (Chapter 3) were planned and undertaken concurrently, while the other studies evolved as the research progressed. The key findings of each study are summarised at the end of each chapter so will not be described individually here. Instead, a revised case description/definition that combines the findings of all studies is presented below, followed by a discussion of possible causes, areas for future research, treatments, and public health implications of this work.

7.1.1 Revised case description

The term 'Go Slow myopathy' (GSM) refers to a toxic myopathy in dogs that appears to primarily occur in the North Island of New Zealand. Affected dogs usually have a history of consuming wild pigs (including previously frozen and/or cooked wild pig) prior to the onset of clinical signs. There are no apparent breed, age or sex predilections for the myopathy, and although pig hunting dogs are the most commonly affected, case have also been seen in working farm dogs and pet dogs. Clinically, the

disease is characterised by a sudden onset of trembling, lethargy, exercise intolerance and/or weakness and collapse. Vomiting and diarrhoea also occur in a low proportion of cases. Clinical signs are exacerbated by exercise, and chronically affected dogs often appear normal at rest but signs recur with exertion. Some affected dogs never return to full fitness. Serum biochemistry shows moderate to severe increases in creatinine kinase, aspartate aminotransferase and alanine aminotransferase activities, which reflect histological findings of skeletal myocyte degeneration and vacuolar change in hepatocytes. Cardiac muscle is not affected, and the disease does not induce any significant inflammatory response on haematology or histology.

GSM causes structural changes in skeletal muscle mitochondria, including mitochondrial hypertrophy and membrane-bound intramitochondrial granular inclusions. There is also increased lipid and glycogen storage in liver and muscle, depending on the stage of the disease. In the liver, the disease is typified by an increase in the concentrations of kynurenic acid, specific medium to long chain dicarboxylic acids and N-acetylated amino acids, with a widespread decrease in phospholipids.

7.1.2 Possible causes

The definitive cause of the myopathy remains unknown, but taken together, the results of all studies in this thesis suggest that GSM in dogs is a toxic myopathy associated with the consumption of wild animals, particularly wild pigs. The epidemiological studies (Chapter 2) revealed a very high proportion of affected dogs ate wild pig prior to the onset of clinical signs, and studies of muscle pathology in wild pigs in areas where the disease is known to occur showed similar histological and ultrastructural lesions (Chapter 4) as those seen in affected dogs (Chapter 3). Despite this, it is important to recognise that many dogs in New Zealand are fed wild pork with no ill consequences, and causality cannot be proven without experimental feeding of wild pig to dogs, which would present significant challenges from both practical (as only certain pigs in certain areas are associated with the disease) and ethical

viewpoints (as the disease causes suffering in dogs and there is currently no proven treatment).

I propose that the mitochondrial toxicity in GSM is caused by a plant or fungal toxin that dogs are exposed to through the ingestion of wild pig. The disease has clinical,[2-5] histological[6, 7] and biochemical[8] similarities to poisoning in people and animals with tremetone and other toxic benzofuran ketones from the plants *Ageratina altissima* (white snakeroot) and *Isocoma pluriflora* (rayless goldenrod).[9, 10] White snakeroot poisoning in people is variably referred to as 'milk sickness,' 'the slows', or 'trembles', and develops following the consumption of meat or milk from animals that have eaten the plants.[4, 11] Despite these similarities, there is currently no evidence to suggest that tremetone is the causative compound in GSM in dogs, as *A. altissima* and *I. pluriflora* are not reported to be naturalised in New Zealand (although other *Ageratina* species are)[12, 13] and although the mass spectrometry analysis was not optimised for this compound, there were no significant mass spectral features consistent with tremetone in samples of dog liver analysed (Chapters 5 and 6.

Alkaloids derived from several plant species, some of which have known toxicity to animals, were amongst the possible identities of compounds increased in the livers of affected dogs on mass spectrometry. These include convoline from *Convolvulus aravensis,* which is present in both the North and South Islands of New Zealand,[14] and tropane alkaloids from various plant species that act as galactosidase and glucosidase enzyme inhibitors.[15-18] The toxic effects of these compounds differ in some respects from what is seen in GSM, but toxic effects can be species specific and the action of these compounds in dogs has not been described. In addition, it is possible that a causative toxic compound was amongst the significant features found on mass spectrometry (e.g. metabolite 1 in Chapter 5, with an *m/z* of 305.094) but was not identified as it has not been previously characterised or listed in online databases. Alternatively, if the toxin was present only in the acute phase of the disease, and ongoing muscle damage was due to lasting effects of the initial exposure rather than continued presence of the toxin in the body, it may not have reached the threshold for

significance in the mass spectrometric studies that included both acute and chronic cases.

To further investigate the cause of GSM, reference standards of plant alkaloids identified as possible compounds of interest could be introduced into extracts of dog liver samples for mass spectrometry. This would aid in confirming whether the significant mass spectrometric features discussed in Chapter 5 do in fact represent these alkaloids. Another avenue of investigation could be to take extracts of the plants of interest (such as *Convolvulus arvensis*) and examine the effect they have on the tissues of dogs, either by using an in vitro model such as isolated hepatocytes, myocytes or mitochondrial fractions of these tissues, or by dosing dogs with these extracts. For any studies such as this, it would be important that plant specimens were collected from properties where confirmed cases the myopathy have occurred, at a similar time of year to when cases where seen, as plant toxicity can vary with geographic location, season and different plant chemotypes.[2]

7.2 Limitations

The limitations of the individual studies have been discussed in each chapter, but overall, most of the limitations stem from the opportunistic nature of sample collection. Ideally, the epidemiological study (Chapter 2) and the serum biochemistry part of the pathology study (Chapter 3) would have been conducted as a case control study, where each affected dog that was presented to a veterinarian was matched to a healthy pig hunting dog, working farm dog or pet that also presented to the veterinarian, as appropriate. The histories and serum biochemical results from the healthy and affected dogs could then have been directly compared, allowing the calculation of odds ratios and definitive identification of risk factors and diagnostic criteria for the disease. Initial attempts were made to recruit control dogs, but unfortunately, the number of healthy hunting dogs presented to veterinarians involved in this study was very low, and the only time most of the participating veterinarians saw hunting dogs was when they were severely injured or unwell. Even if there had been sufficient numbers of control dogs for a case control study, matching on the basis

of factors such as breed, type of hunting dog or recent hunting activity would not have been possible due to the inherent diversity in this population of dogs.

It was also initially intended that muscle biopsy samples from affected dogs would be collected under anaesthesia at specified time intervals following the onset of clinical signs, to allow characterisation of the time course of the disease and the use of histochemical staining techniques on fresh/frozen tissue samples. However, it became apparent early on that affected dogs at various stages of the disease were being euthanised by veterinarians or their owners for clinical signs related to the myopathy, and it was decided to utilise this case material for the pathology and mass spectrometry chapters (with the same dogs included in all studies where possible), rather than performing surgical procedures on living dogs.

7.3 Future directions

The series of studies included in this thesis should be considered as an advance towards the full characterisation of this novel myopathy in dogs. The findings of these studies provide a potential method to experimentally induce cases for further research through the feeding of wild pig to dogs. As alluded to above, this would not be without considerable practical and ethical challenges, but an experimental feeding trial (with the inclusion of a control group of dogs that were not fed pig) would allow causality to be proven. Such a trial would also allow the collection of standardised skeletal muscle samples for further histochemical and metabolomic studies, and thus overcome the sampling difficulties outlined in the previous paragraph.

Aside from the experimental feeding of wild pig to dogs, there are two additional avenues of research that could improve understanding of the pathogenesis and pathophysiology of the disease. Firstly, to provide additional evidence of the link to wild pigs and verify the importance of particular mass spectral features in dogs, mass spectrometry could be performed on wild pig tissue samples and compared to the results in affected dogs. This has not been performed to date, as the number of samples collected is not yet adequate to allow meaningful statistical analysis of results. As only particular pigs in particular areas seem to be linked to the disease, pig tissue

samples ideally need to come from wild pig that was fed to dogs that subsequently developed GSM, but by the time that dogs are fed the meat and develop clinical signs, the rest of the pig tissues (including liver) have either been disposed of, cooked or are in an advanced state of autolysis. Muscle samples from five pigs and liver samples from two pigs consumed by dogs that developed GSM are currently stored at -20°C in the hope of conducting this analysis in future, dependent on the availability of additional samples. Two separate groups of controls could be used for a study such as this: wild pigs from South Island areas where the disease is not commonly recognised and domestically farmed pigs.

The second avenue of investigation would be studies to analyse both overall mitochondrial respiration and the function of specific mitochondrial pathways in affected dogs. It is possible to perform enzymatic assays of individual respiratory chain complexes or other mitochondrial enzymes, but a better initial approach would be to assess oxidative phosphorylation in intact mitochondria. Small muscle biopsies could be taken from affected dogs, with the plasma membranes of myocytes in the sample permeabilised using saponin.[19] Specific tests of mitochondrial respiration and the coupling of mitochondrial respiration to oxidative phosphorylation could then be conducted in oxygraph chambers. *In vitro* tests on isolated mitochondria, rather than permeabilised muscle fibres, are also possible.[20] If mitochondrial dysfunction is confirmed in GSM, these tests could also aid in the investigation of possible causes of the disease, as the mitochondria of clinically normal dogs could be exposed to extracts of candidate plants or potentially toxic compounds to determine if they produce changes in mitochondrial function similar to what might occur in affected dogs.

7.4 Possible treatments

The treatment of the myopathy has not been discussed anywhere in this thesis as no controlled treatment trials have been conducted, so any claims regarding the efficacy (or lack thereof) of certain treatments are based on anecdotal evidence only. Further confounding this, approximately 48 of the 86 cases (56% of all dogs) recovered fully within three months, and of these only 20 dogs (42% of recovered cases) received supportive treatment in the acute phase of the disease. The remainder of the dogs

recovered with only rest, and feeding of a high quality diet in some cases, so without case-control studies it is difficult to determine if a treatment is truly effective, or if the dog would have got better over time, regardless of any interventions. To date, all treatments administered to affected dogs by veterinarians have been symptomatic in nature as the definitive cause of the myopathy remains unknown. Severe, acute cases with a sudden onset of intractable muscle tremors and very high serum CK activities (>20,000) were commonly given intravenous fluids for the purposes of diuresis to increase excretion of any toxins, and to maintain adequate hydration and renal perfusion in the face of diarrhoea and potential rhabdomyolysis leading to myoglobinuria. Low levels of sedative drugs have been used in hospitalised cases to reduce movement and anxiety, as the intensity of muscle tremors in affected dogs typically worsens with movement, exertion, or stress.

Various other products are claimed to support or enhance the normal function of key metabolic and detoxification pathways have also been administered to affected dogs, including Metabolase® (Ethical Agents, Auckland, NZ), which contains B vitamins, carnitine, amino acids and sugars; and Zentonil Advanced® (Vetoquinol, Quebec, Canada), which contains S-Adenosylmethionine (sAME) and phospholipids. Supplementation with sAME may be beneficial in some chronic liver diseases[21] and the majority of owners that gave their dogs Zentonil® reported that the dogs showed an improvement in exercise tolerance, but there have been no objective assessments of this. Given the mass spectrometry results, it could be the phospholipids in this product that are beneficial rather than the sAME. It should also be noted that Zentonil® and all of the products discussed below are classified as nutritional supplements and are available over-the-counter without veterinary consultation or prescription, so owners are able to give these products to their dogs without first confirming the diagnosis.

In the early phases of the study, it was considered possible that there might be a nutritional component to the disease, as deficiencies in vitamin E and selenium can cause myopathies in dogs and other species.[22, 23] Consequently, some veterinarians and dog owners administered vitamin and mineral supplements to affected dogs, including 'Go Fast®,' 'Vite-B powder®' (both from Ethical Agents, Auckland NZ),

'Exceed®' (palaMOUNTAINS, Wanganui, NZ) and 'O2B K9 Octane Plus®' (O2B Healthy, Nelson, NZ). There have been no reported adverse effects of giving these products, but the response is variable with some owners reporting a significant improvement in the dog's condition and others reporting no appreciable changes. As well as giving these supplements in the acute phase of the disease, some owners also administer them long term during periods of high hunting activity, particularly on the day prior to, or on the day of a hunt. Based on the current understanding of the myopathy, it is plausible that these supplements could be beneficial, as various B vitamins are important cofactors for enzymes involved in pathways shown to be disrupted in GSM, including fatty acid oxidation and phospholipid synthesis.[24] However, without treatment trials under experimental conditions it is impossible to determine whether any perceived improvement in the dogs is due to the supplement, time, confirmational bias or the placebo effect.

7.5 Public health implications

Wild pig tissues eaten by dogs that developed 'Go Slow' myopathy included offcuts of meat that had been prepared for human consumption, including wild pork roasts that had been previously frozen and cooked for a prolonged period. In general, people who ate the same meat as the dogs reported no ill effects, but anecdotally, a total of seven people during the study period reported that they experienced myalgia, weakness, fatigue and a racing pulse following consumption of wild pig. Most of the people involved did not seek medical attention and the symptoms resolved without any treatment. However, in one particular instance, a shepherd on a sheep and beef property developed muscular pain, shortness of breath and anxiety following a meal of cooked wild pork. This patient, along with one other, had elevated serum AST and ALT activities (similar to GSM in dogs), but CK activity was not measured, and no definitive diagnosis could be made. The Northland District Health Board was informed in writing about GSM and potential public health risks in 2016, but no specific investigations of possible cases have been undertaken to date.

In November 2017, three members of a family in Putaruru, Waikato experienced a rapid onset of vomiting and collapse, progressing to paralysis, after eating a curry

made with the meat of a wild pig.[25] Two children in the family did not eat any of the curry and were not affected. A preliminary diagnosis of botulism was made, but subsequent testing disproved this and the doctors concluded that the case was due to poisoning with an unspecified neurotoxin.[26] Hospital documents for the three patients show they had elevated CK during recovery (up to 5,000 IU/L), but this was associated with dystonic posturing of the lower limbs. By early January, the patients had been discharged from hospital and were continuing to recover at home, but rapidly became fatigued and developed muscular tremors with slight exertion. Recently, there have also been unsubstantiated media reports that a dog belonging to the hunter who supplied the family with wild pig had died,[27] and a vet in Tirau saw a dog belonging to the hunter for unexplained muscle twitching in early November 2017. Unfortunately, no diagnostic tests were performed on this dog and no diagnosis was made (personal communication). The reported severity of clinical signs and central nervous system involvement in these cases mean that the family's illness is unlikely to be a manifestation of GSM, but it does highlight the potential for a secondary toxicity to occur through the consumption of wild pigs. Collaboration with human medical professionals and investigation of this case and the anecdotal cases mentioned above would be of interest in determining whether there are risks to public health from the consumption of wild pig. There are no accurate statistics regarding the consumption of wild pig in New Zealand, which could be useful in establishing the scale of potential public health risks, although in 2012 it was estimated that 132,000 big game animals were killed each year, the majority of which were wild pigs.[28]

7.6 Conclusion

Although ‘Go Slow’ myopathy remains idiopathic at the conclusion of this thesis, significant progress has been made in improving knowledge and understanding of the disease. The finding of an association with the consumption of wild pig provides an avenue to prevent potential cases through avoidance of feeding wild pig to dogs in areas where the disease is reported to occur (particularly the upper half of the North Island of New Zealand). Serum biochemistry, histology, electron microscopy and mass spectrometry have shown the disease is consistent with a toxic mitochondrial myopathy, and provide an insight into the pathophysiology of the disease. The findings of these studies will aid in the diagnosis and management of future cases, and pave the way for further investigations to elucidate the cause of this unusual myopathy.

7.7 References

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Appendix A

'Go Slow' myopathy telephone case survey

PART A

OWNER DETAILS	
NAME: *(first name)* *(last name)*	
ADDRESS:	
PHONE:	**EMAIL:**
USUAL VETERINARIAN:	
HOW LONG HAVE YOU BEEN INVOLVED IN PIG HUNTING?	
WHERE, AND HOW OFTEN, DO YOU USUALLY GO HUNTING (USING DOGS)?	
HOW MANY DOGS DO YOU HAVE THAT ARE USED FOR PIG HUNTING?	

PART B

DOG DETAILS	
NAME:	**AGE:**
BREED:	
GENDER: Male / Female ***If female, has she ever had puppies?*** Yes / No **DESEXED**: Yes / No	
HOW LONG HAVE YOU HAD THIS DOG?	
WHERE DID YOU GET THIS DOG FROM? **TYPE OF DOG (e.g. finder, holder, bailer):**	
WHAT HAS THIS DOG EATEN IN THE LAST WEEK? *Include the relative proportions of each type of food, and for meat, whether it was previously frozen/cooked/raw.* **DO YOU EVER FEED WILD PORK OR TRIMMINGS TO YOUR DOGS?** Yes / No	
HEALTH: *Date of last worm treatment and product used:* *Date of last flea treatment and product used:* *Date of last vaccination:* *Is this dog vaccinated for leptospirosis:* Yes / No	

PART C

DETAILS OF EPISODE OF MUSCLE PAIN / WEAKNESS ('GO SLOW')
DATE FIRST CLINICAL SIGNS OF MUSCLE PAIN / WEAKNESS WERE NOTICED:
HAS THIS DOG HAD ANY SIMILAR PROBLEMS IN THE PAST?
WHAT CLINICAL SIGNS WERE OBSERVED AND HOW DID THESE CHANGE OVER TIME? *(e.g. stiffness, difficultly standing, collapse, tiring easily)*
HAS A VETERINARIAN BEEN INVOLVED IN THE DIAGNOSIS & TREATMENT OF THIS CASE? ***If yes, please write the name of the veterinarian and their clinic here:***
ANY OTHER DOGS AFFECTED? Yes / No *(circle one)* **IF YES, HOW MANY?**
WAS THERE ANYTHING THAT SEEMED TO IMPROVE OR WORSEN THE CLINICAL SIGNS IN THIS DOG? *(e.g. rest, exercise, heat, cold)*
HAS THE DOG COMPLETELY RECOVERED FROM THIS EPISODE? Yes / No ***If yes, how long did it take to return to normal?*** ***If no, what are the on-going effects?***
HOW MANY HUNTS HAS THIS DOG BEEN ON IN THE LAST 8 WEEKS? **DETAILS OF LAST HUNT** *Date:* *Duration:* *Location (as specific as possible):* *Number of pigs caught:* *Number of dogs used:* *Any injuries to dogs:* *Other comments:*
IS THIS DOG A WORKING FARM DOG? Yes / No *If yes, what type of country does this dog work on, and how often?*

ANY OTHER COMMENTS: *(e.g. general health of dog, any other medical issues, any other experiences or thoughts about 'Go Slow')*

PART D

INFORMED VERBAL CONSENT

The following statement was read to and agreed upon by all participants:

I understand that the information collected on this form, or verbally, will be used as part of a PhD research project investigating myopathies in pig dogs. Data and conclusions from the study may be published, but no uniquely identifiable information about respondents or their dogs will be shared at any time. In addition, ***I authorise the investigator to access any relevant veterinary records regarding the care and treatment of the dog named in this questionnaire.***

Appendix B

Online survey of pig dog hunting, diet & health in New Zealand

Hunting Habits

1) In the last 12 months, which regions have you hunted in? (select all that apply)
[ ] Northland
[ ] Auckland
[ ] Waikato
[ ] Bay of Plenty
[ ] Gisborne
[ ] Hawke's Bay
[ ] Taranaki
[ ] Manawatu-Wanganui
[ ] Wellington
[ ] Nelson
[ ] Marlborough
[ ] Tasman
[ ] West Coast
[ ] Canterbury
[ ] Otago
[ ] Southland

2) What type of land do you usually hunt on?
[ ] Native bush
[ ] Pines
[ ] Bush/pines around sheep and beef farms
[ ] Bush/pines around dairy farms
[ ] Other: _____

3) For each time of year, select how often you usually go hunting for pigs (with dogs)

	Less than once a month	Once every 3-4 weeks	Once every 1-2 weeks	Every week	2-3 times a week	Almost every day
Winter (June - Aug)	( )	( )	( )	( )	( )	( )
Spring (Sept - Nov)	( )	( )	( )	( )	( )	( )
Summer (Dec - Feb)	( )	( )	( )	( )	( )	( )
Autumn (Mar - May)	( )	( )	( )	( )	( )	( )

Dogs

4) **How many dogs do you currently have that are used for pig hunting?**
( ) 1
( ) 2
( ) 3
( ) 4
( ) 5
( ) 6
( ) 7
( ) 8
( ) 9
( ) 10 or more

5) **To help us choose one dog to focus on for the rest of the survey, list the names of up to 3 of your dogs below (in any order).**
Dog 1: _____
Dog 2: _____
Dog 3: _____

Dog details

6) **Tell us about your dog [Question 5, "Dog 2"]**
Age and breed:
Age (years): _____
Breed: _____

Gender:
( ) Male
( ) Female

Type of dog (tick all that apply):
[ ] Finder
[ ] Bailer
[ ] Holder
[ ] Other: _____

Dog diet

7) **In the last 7 days, what has your dog ["Dog 2"] eaten? Tick all that apply**
[ ] Meat, offal or bones from wild animals
[ ] Meat, offal or bones from home-kill animals
[ ] Meat, offal or bones from a butcher or supermarket (including Mighty Mix)
[ ] Dog roll
[ ] Canned dog food
[ ] Dry dog food (biscuits)
[ ] Table scraps (other than meat)
[ ] Milk
[ ] Other: _____

---

8) **What types of meat and animal products are included in your answer to the last question? Tick all that apply**

	Meat (muscle)	Offal (organs)	Bones
Cattle	[ ]	[ ]	[ ]
Sheep	[ ]	[ ]	[ ]
Pig	[ ]	[ ]	[ ]
Chicken	[ ]	[ ]	[ ]
Deer	[ ]	[ ]	[ ]
Possum	[ ]	[ ]	[ ]
Goat	[ ]	[ ]	[ ]

9) **What brand(s) of dog roll, dry and canned dog food has your dog eaten in the last 7 days?**
_____

10) **Do you EVER let your dogs have access to wild pork or pig trimmings? Choose one answer, or write in your own**
( ) Never
( ) I don't feed my dogs wild pig, but they occasionally get hold of some meat/offal
( ) I usually give my dogs offal/trimmings on a hunt or when I'm butchering the pig
( ) I sometimes feed wild pig to my dogs, but always freeze and/or cook it first
( ) I sometimes feed fresh (raw) wild pig to my dogs
( ) Wild pig (fresh, frozen or cooked) is a big part of my dogs diet
( ) Other: _____

Pig Dog Health

11) Has your dog ['Dog 2"] been seen by a veterinarian in the last 12 months?
( ) Yes
( ) No

12) In the last 12 months, have you noticed any of the following in your dog ["Dog 2"]? Tick all that apply
[ ] Broken bone(s)
[ ] Decreased appetite / eating less
[ ] Collapse
[ ] Coughing
[ ] Dental problems
[ ] Diarrhoea
[ ] Difficulty breathing
[ ] Difficulty urinating
[ ] Discharge from the eyes, nose or mouth
[ ] Increased thirst / drinking
[ ] Lameness
[ ] Lethargy or tiring easily
[ ] Seizure(s)
[ ] Skin problems
[ ] Stiffness in muscles or joints
[ ] Strange behaviour
[ ] Trembling/shaking
[ ] Wounds
[ ] Vomiting
[ ] Weight gain
[ ] Weight loss
[ ] Other health issues: _____

The researcher is planning a further study to investigate specific diseases in pig dogs. If you are happy to be contacted and answer a few follow-up questions, please enter your details below. Otherwise, leave these boxes blank.
Please note that by entering your details, your answers to the previous questions will no longer be anonymous. However, no personal or identifying information will ever be published or shared.
First Name: _____
Last Name: _____
Email Address: _____
Phone Number: _____

Thank You!

Appendix C

Chemometrics analysis plots for aqueous liver extract mass spectrometry data

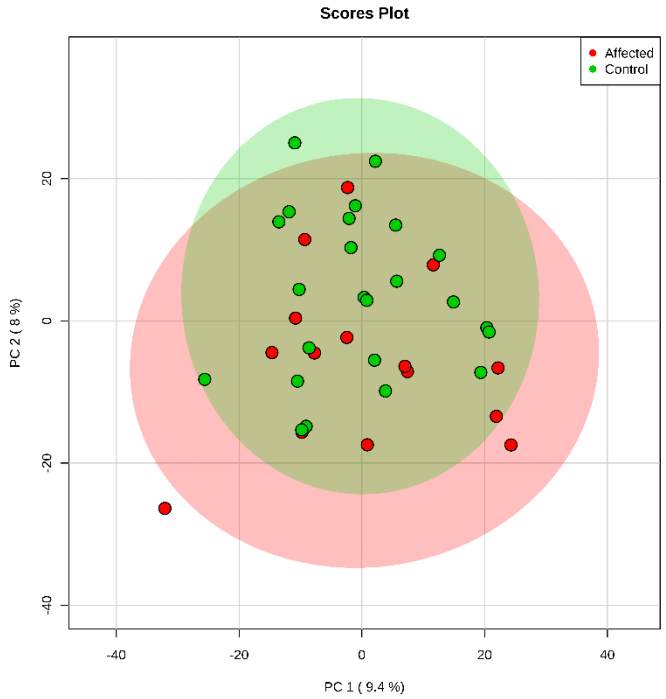

Figure C1: Principal component analysis of combined positive and negative ionisation of aqueous extracts of dog liver. The principal component axis 1 (PC1) and 2 (PC2) show the amount of variation (%) within the metabolomics data explained by each component. Individual data points are shown for idiopathic myopathy cases (affected, •) and normal dogs (control, •). Shaded areas represent the 95% confidence distribution for each group.

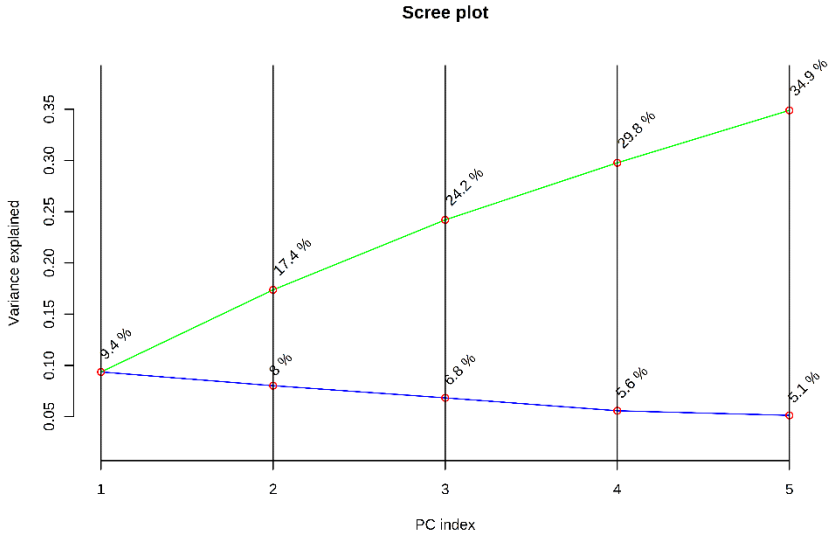

Figure C2: Scree plot generated by principal component analysis of combined positive and negative ionisation of aqueous extracts of dog liver, showing that none of the principal components (individually or cumulatively) adequately explain the variation between the clinically normal dogs and dogs affected by the myopathy.

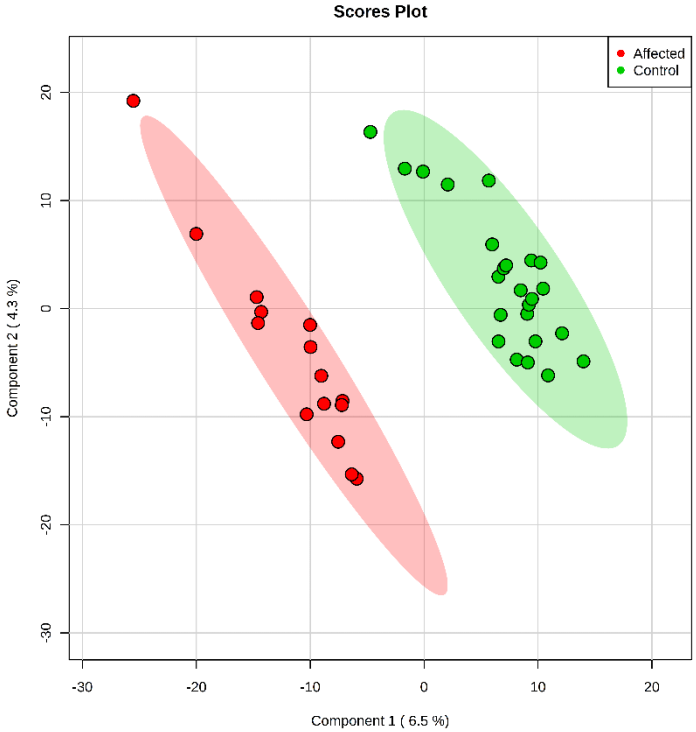

Figure C3: Partial least squares discriminant analysis of combined positive and negative ionisation of aqueous extracts of dog liver. The principal component axis 1 (PC1) and 2 (PC2) show the amount of variation (%) within the metabolomics data explained by each component. Individual data points are shown for idiopathic myopathy cases (affected, •) and normal dogs (control, •). Shaded areas represent the 95% confidence distribution for each group. Separation of the normal and affected dogs is possible using this method, but the vast dimensions of the mass spectral data and small sample size limit the validity of this analysis.

Appendix D

Chemometrics analysis plots for lipid liver extract mass spectrometry data

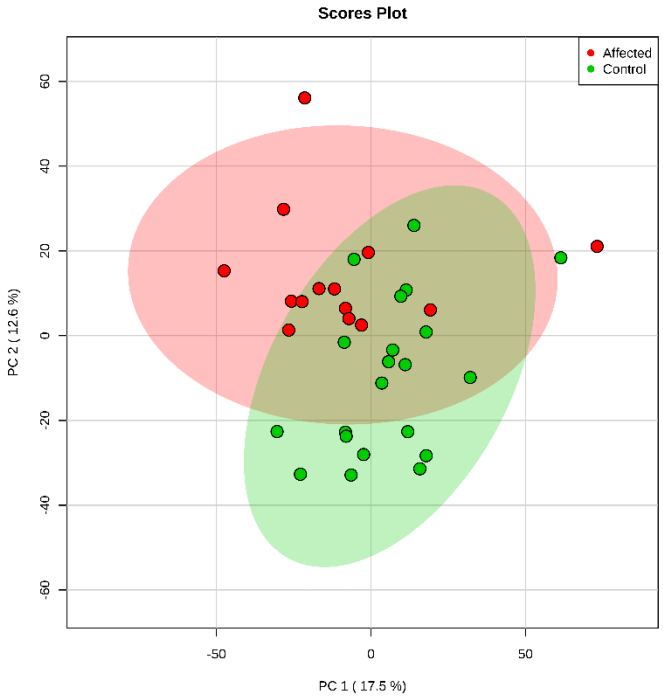

Figure D1: Principal component analysis of combined positive and negative ionisation of organic extracts of dog liver. The principal component axis 1 (PC1) and 2 (PC2) show the amount of variation (%) within the metabolomics data explained by each component. Individual data points are shown for idiopathic myopathy cases (affected, •) and normal dogs (control, •). Shaded areas represent the 95% confidence distribution for each group.

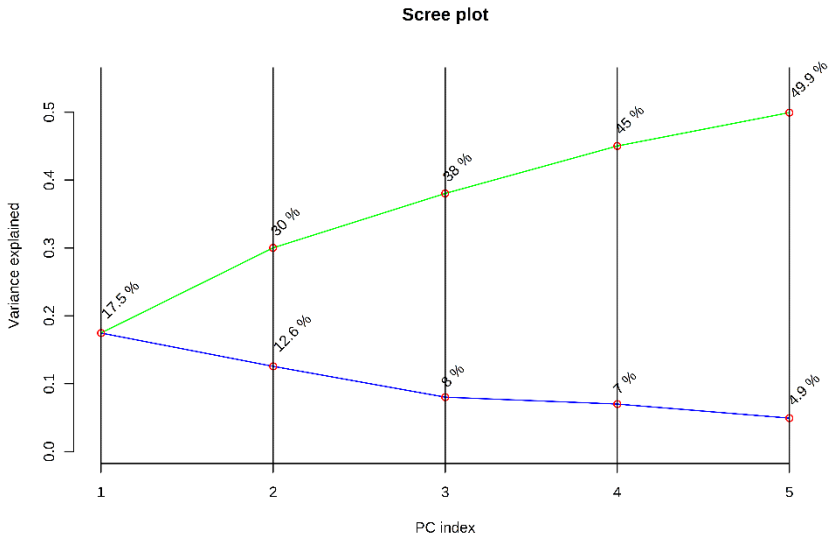

Figure D2: Scree plot generated by principal component analysis of combined positive and negative ionisation of organic extracts of dog liver, showing that none of the principal components (individually or cumulatively) adequately explain the variation between the clinically normal dogs and dogs affected by the myopathy.

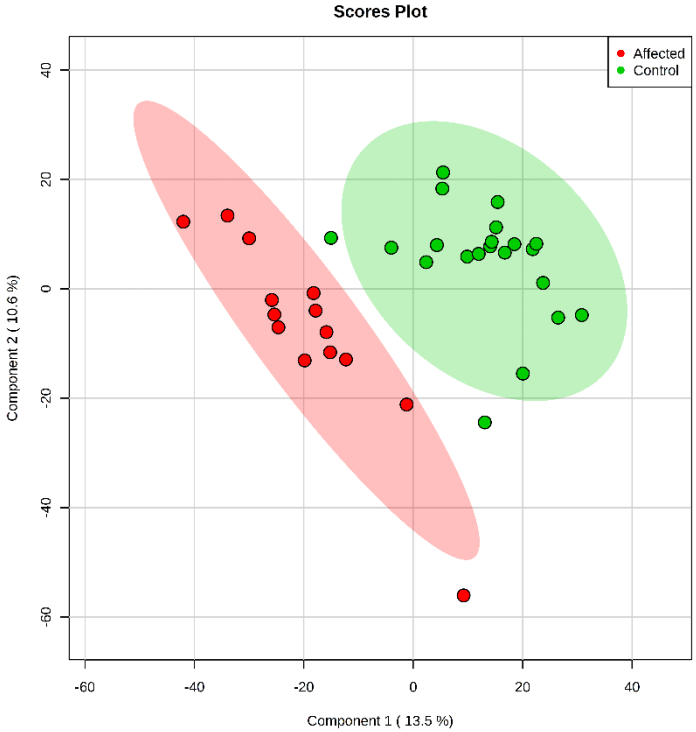

Figure D3: Partial least squares discriminant analysis of combined positive and negative ionisation of aqueous extracts of dog liver. The principal component axis 1 (PC1) and 2 (PC2) show the amount of variation (%) within the metabolomics data explained by each component. Individual data points are shown for idiopathic myopathy cases (affected, •) and normal dogs (control, •). Shaded areas represent the 95% confidence distribution for each group. Separation of the normal and affected dogs is possible using this method, but the vast dimensions of the mass spectral data and small sample size limit the validity of this analysis.

Appendix E

Mass spectrometric features in lipid extracts of liver that differed significantly (FDR<0.05) between dogs with 'Go Slow' myopathy (M) and normal dogs (N)

The features included in this table are in addition to the lipids identified using MS2 data (Table 6.1) and the 20 most significant mass spectrometric features (Table 6.2).

m/z	Mode	r.t (min)	Ion/fragment	Possible ID	Formula	p value	FDR	log2FC (M/N)	Level (M/N)	Other fragments	Significant isotopes
1651.075	Pos	6.27	Unknown	Unknown		0.00005	0.003	-1.98	N>M		
842.658	Pos	9.28	[M+H]+	PC(40:2) or PE(43:2)	C48H92NO8P	0.00005	0.003	-1.08	N>M		
1108.960	Pos	12.43	Unknown	Unknown		0.00006	0.003	0.12	M>N		
1347.230	Pos	12.88	Unknown	Unknown		0.00006	0.003	1.21	M>N		
1459.367	Pos	13.44	Unknown	Unknown		0.00006	0.003	1.06	M>N		+1460.373
269.226	Pos	8.50	[M+H]+	Anhydroretinol	C20H28	0.00006	0.003	2.05	M>N		
953.828 970.858	Pos	12.69	[M+H]+ [M+NH4]+	Unknown	C58H112O9	0.00008 0.00008	0.003 0.003	1.73	M>N		+954.834, +955.835
1088.936	Pos	12.70	Unknown	Unknown		0.00008	0.003	1.10	M>N		+1088.936
1116.963	Pos	12.79	Unknown	Unknown		0.00008	0.003	1.21	M>N		
1595.486	Pos	13.66	Unknown	Unknown		0.00008	0.003	1.19	M>N		+1596.498
1637.539	Pos	13.80	Unknown	Unknown		0.00008	0.003	1.05	M>N		+1638.545
776.58	Pos	6.18	[M+H]+	PS(O-36:1) or PS(P-36:0)	C42H82NO9P	0.00009	0.003	-1.69	N>M		
1110.002	Pos	13.03	Unknown	Unknown		0.00009	0.003	0.32	M>N		

m/z mass/charge ratio; *r.t.* retention time in minutes; *FC* fold change; *FDR* false discovery rate; *M* 'Go Slow' myopathy cases; *N* clinically normal dogs
PC phosphatidylcholine; *PE* phosphatidylethanolamine; *PG* phosphatidylglycerol; *PI* phosphatidylinositol; *PS* phosphatidylserine; *Cer* ceramide; *SM* sphingomyelin; *DG* diglyceride; *TG* triglyceride

m/z	Mode	r.t (min)	Ion/fragment	Possible ID	Formula	p value	FDR	log2FC (M/N)	Level (M/N)	Other fragments	Significant isotopes
1052.934	Pos	12.85	Unknown	Unknown		0.00009	0.003	-0.20	N>M		
1000.594	Pos	6.07	Unknown	Unknown		0.00009	0.003	-2.56	N>M		
979.844	Pos	12.67	Unknown	Unknown		0.00010	0.003	1.54	M>N		+980.848, +997.873
1542.143	Pos	6.43	[M+H]+	Unknown		0.00010	0.003	-1.73	N>M		
1586.139	Neg		[M+FA-H]-			0.00111	0.043				
1106.983	Pos	12.93	Unknown	Unknown		0.00011	0.003	0.13	M>N		
1971.838	Pos	12.25	Unknown	Unknown		0.00011	0.003	-1.55	N>M		
1550.417	Pos	13.40	Unknown	Unknown		0.00013	0.004	1.31	M>N		
1026.614	Pos	6.21	?[M+Na]+	Unknown	?C48H94NO18P	0.00014	0.004	-1.96	N>M		
1277.163	Pos	12.78	Unknown	Unknown		0.00015	0.004	0.87	M>N		
766.558	Pos	6.35	[M+NH4]+	PG(34:1)	C40H77O10P	0.00015	0.004	-1.12	N>M		
1049.926	Pos	12.70	[M+H]+	Unknown		0.00015	0.004	0.14	M>N		
1066.95			[M+NH4]+	Unknown		0.00066	0.008				
1547.081	Pos	6.28	?[M+NH4]+	Unknown	?C80H158N2O16P2S2	0.00016	0.004	-2.40	N>M		
1342.925	Pos	7.07	?[M+NH4]+	Unknown	?C68H129N2O20P	0.00017	0.004	-0.77	N>M	+680.478, +899.523	
792.552	Pos	5.70	[M+H]+	PC(37:6) or PE(40:6)	C45H78NO8P	0.00017	0.004	-1.52	N>M		
1028.95	Pos	12.41	[M+NH4]+	TG(63:3)	C66H122O6	0.00018	0.004	1.12	M>N		+1029.952
1090.95	Pos	12.80	Unknown	Unknown		0.00018	0.004	0.92	M>N		+1091.951
730.618	Pos	8.86	[M+H]+	CerP(42:1)	C42H84NO6P	0.00021	0.004	0.22	M>N		
812.541	Pos	5.52	[M+H]+	PS(38:4)	C44H78NO10P	0.00022	0.004	-1.73	N>M		
772.619	Pos	7.64	[M+H]+	PC(P-36:1)	C44H86NO7P	0.00022	0.004	-1.16	N>M		
1121.998	Pos	12.98	?[2M+H]+	?Phytoene 1,2-epoxide	?C40H64O	0.00022	0.004	0.50	M>N		
1731.618	Pos	13.91	Unknown	Unknown		0.00022	0.004	1.20	M>N		+1733.62

m/z	Mode	r.t (min)	Ion/fragment	Possible ID	Formula	p value	FDR	log2FC (M/N)	Level (M/N)	Other fragments	Significant isotopes
1165.077	Pos	12.68	Unknown	Unknown		0.00023	0.004	1.33	M>N		+1166.078, 1167.08
594.582 616.564	Pos	8.65	[M+H]+ [M+Na]+	Cer(d38:1)	C38H75NO3	0.00024 0.00044	0.004 0.006	-0.91	N>M	+576.575	+596.588
1118.983	Pos	12.88	Unknown	Unknown		0.00025	0.005	0.51	M>N		+1119.986
1077.943 1094.968	Pos	12.70	[M+H]+ [M+NH4]+	Unknown		0.00025 0.00390	0.005 0.024	0.04	M>N		
756.7	Pos	10.49	?[M+H]+	Unknown	?C46H93NO6	0.00025	0.005	0.81	M>N		+757.704
1233.138	Pos	12.79	Unknown	Unknown		0.00026	0.005	1.30	M>N	+1121.998	+1234.137, +1235.143
1065.935	Pos	12.80	Unknown	Unknown		0.00026	0.005	0.78	M>N		
430.379	Pos	6.51	?[M+NH4]+	Unknown	?C27H44N2O	0.00027	0.005	0.22	N>M		+431.382
1097.015	Pos	12.56	[M+NH4]+	TG(68:4)	C71H130O6	0.00031	0.005	1.15	M>N		+1098.017, +1099.018
1754.603	Pos	13.74	Unknown	Unknown		0.00033	0.005	1.45	M>N		
1500.401	Pos	13.46	?[2M+NH4]+	Unknown	?C32H37O20	0.00034	0.005	0.88	M>N		
1527.428	Pos	13.55	Unknown	Unknown		0.00035	0.005	0.89	M>N		+1528.433
1572.097 1570.086	Pos Neg	6.29	?[2M+H]+ ?[2M-H]-	Unknown		0.00036 0.00074	0.005 0.037	-2.70 -1.82	N>M		+1573.102
1593.478	Pos	13.57	Unknown	Unknown		0.00035	0.005	1.16	M>N		+1596.484
1124.016	Pos	13.09	Unknown	Unknown		0.00038	0.006	0.40	M>N		+1125.017
746.605	Pos	7.44	[M+H]+	PC(P-34:0) or PE(P-37:0)	C40H81O7P	0.00039	0.006	-0.99	N>M		
351.337	Pos	2.71	?[M+NH4]+	Unknown	?C22H39NO	0.00040	0.006	-0.96	N>M	+298.274	
762.505	Pos	5.68	[M+H]+	PE(38:7)	C43H72NO8P	0.00040	0.006	-1.14	N>M		
940.632	Pos	7.43	Unknown	Unknown		0.00045	0.006	-1.56	N>M		

m/z	Mode	r.t (min)	Ion/fragment	Possible ID	Formula	p value	FDR	log2FC (M/N)	Level (M/N)	Other fragments	Significant isotopes
1414.289	Pos	12.98	[2M+H]+	DG(42:1)	C45H86O5	0.00045	0.006	1.10	M>N		
1093.948	Pos	12.52	Unknown	Unknown		0.00046	0.006	-0.33	N>M		+1094.951
728.602	Pos	7.27	[M+H]+	CerP(42:2)	C42H82NO6P	0.00047	0.006	-1.03	N>M		
1120.992	Pos	12.91	Unknown	Unknown		0.00049	0.007	0.29	M>N	+821.715	+1124.015
722.509	Pos	6.32	[M+H]+	PE(P-36:5)	C41H72NO7P	0.00050	0.007	-1.40	N>M		
354.356	Pos	2.67	Unknown	Unknown		0.00050	0.007	-0.92	N>M		
1094.978	Pos	12.99	Unknown	Unknown		0.00051	0.007	0.07	M>N		+1095.983, +1097.99
801.551	Pos	4.85	?[M+NH4]+	Unknown	?C48H69N3O6	0.00052	0.007	-1.04	N>M		
490.425	Pos	6.89	?[M+NH4]+	Unknown	?C31H52O3	0.00054	0.007	-1.33	N>M		+491.428
1066.95	Pos	12.90	Unknown	Unknown		0.00054	0.007	0.17	M>N		+1069.957
744.587	Pos	6.87	[M+H]+	PC(P-34:1) or PE(P-37:1)	C42H82NO7P	0.00055	0.007	-0.97	N>M		
1469.015	Pos	10.45	[M+NH4]+	Cardiolipin (72:8)	C81H144O17P2	0.00055	0.007	-1.02	N>M	+599.501	
1425.392	Pos	11.77	Unknown	Unknown		0.00057	0.007	-0.17	N>M		+1429.383
1621.119	Pos	6.39	Unknown	Unknown		0.00058	0.007	-2.25	N>M		
226.216	Pos	3.66	?[M+NH4]+	Unknown	?C14H24O	0.00062	0.007	-0.68	N>M		
1729.603	Pos	13.79	Unknown	Unknown		0.00062	0.007	1.44	M>N		
354.373	Pos	4.90	?[M+H]+	Unknown	?C23H47NO	0.00063	0.008	-0.85	N>M		
719.568	Pos	5.91	[M+NH4]+	PE(P-34:1)	C39H76NO7P	0.00063	0.008	-1.04	N>M		
218.103	Pos	3.43	Unknown	Unknown		0.00064	0.008	-0.85	N>M		
1663.552	Pos	13.78	Unknown	Unknown		0.00068	0.008	1.35	M>N		+1666.565
747.516	Pos	5.83	[M+H]+	PG(34:2)	C40H75O10P	0.00071	0.008	-1.43	N>M	+575.503	
764.542			[M+NH4]+			0.00275	0.019				
1095.983	Pos	12.80	Unknown	Unknown		0.00071	0.008	0.21	M>N		

m/z	Mode	r.t (min)	Ion/fragment	Possible ID	Formula	p value	FDR	log2FC (M/N)	Level (M/N)	Other fragments	Significant isotopes
774.633	Pos	8.22	[M+H]+	CerP(43:1)	C44H88NO7P	0.00072	0.008	-1.09	N>M		
1567.464	Pos	13.57	Unknown	Unknown		0.00074	0.008	0.91	M>N		+1568.465
699.255	Pos	8.42	?[M+H]+	Unknown	?C31H42N2O16	0.00078	0.009	-0.14	N>M	+662.57	
799.639	Pos	7.82	[M+H]+	SM(d40:2)-OH	C45H87N2O7P	0.00080	0.009	-1.19	N>M		
497.466	Pos	2.86	[M+NH4]+	Cer(d30:2)	C30H57NO3	0.00080	0.009	-0.55	N>M		
268.263	Pos	4.50	[M+NH4]+	Heptadecadienal	C17H30O	0.00082	0.009	-0.80	N>M		
399.357	Pos	3.40	?[M+NH4]+	Unknown	?C23H43NO3	0.00082	0.009	-0.78	N>M		
1661.542	Pos	13.68	[M+Na]+	1,4-b-D-Mannan	C60H102O51	0.00083	0.009	1.23	M>N		
621.553	Pos	2.86	[2M + Na]+	Sphingosine	C18H37NO2	0.00084	0.009	-0.96	N>M	+282.278	+283.262
599.571			[2M+H]+			0.00357	0.023				
322.271			[M+Na]+			0.00139	0.012				
300.289			[M+H]+			0.00638	0.033				
310.31	Pos	4.11	?[M+H]+	Unknown	?C20H39NO	0.00085	0.009	-0.83	N>M		
1451.994	Pos	10.79	[M+H]+	Cardiolipin (72:8)	C81H144O17P2	0.00088	0.009	-1.32	N>M	+601.519	
1469.016			[M+NH4]+			0.00157	0.013				
1105.979	Pos	12.90	Unknown	Unknown		0.00091	0.010	0.38	M>N		
1082.946	Pos	12.42	Unknown	Unknown		0.00092	0.010	-1.02	N>M		
785.585	Pos	6.46	[M+NH4]+	PC(35:4) or PE(38:4)	C43H78NO8P	0.00093	0.010	-0.96	N>M		
865.579	Pos	6.93	[M+H]+	PI(36:1)	C45H85O13P	0.00094	0.010	-1.15	N>M		
718.573	Pos	7.31	[M+H]+	PC(P-32:0) or PE(P-35:0)	C40H80NO7P	0.00095	0.010	-0.96	N>M		+719.575
1007.708	Pos	8.44	Unknown	Unknown		0.00097	0.010	-1.07	N>M		
1552.431	Pos	13.45	[2M + Na]+	DG(46:0)	C49H96O5	0.00099	0.010	0.77	M>N		
981.696	Pos	8.50	?[2M+H]+	Unknown	C33H46O3	0.00099	0.010	-1.19	N>M		
1080.963	Pos	12.93	Unknown	Unknown		0.00105	0.010	0.24	M>N		+1081.966

m/z	Mode	r.t (min)	Ion/fragment	Possible ID	Formula	p value	FDR	log2FC (M/N)	Level (M/N)	Other fragments	Significant isotopes
1319.228	Pos	12.26	[2M+NH4]+	CE(18:1)	C45H78O2	0.00111	0.011	-1.02	N>M	+386.378, +1293.213	
804.554	Pos	5.49	[M+H]+	PC(38:7)	C46H78NO8P	0.00115	0.011	-0.39	N>M		+805.556
1480.035	Pos	6.49	[M+H]+	Cardiolipin (74:8)	C83H148O17P2	0.00117	0.011	-1.37	N>M	+599.503	
1504.13	Pos	7.20	Unknown	Unknown		0.00118	0.011	-1.81	N>M		+1505.127
581.56	Pos	7.10	[M+NH4]+	Cer(d36:2)	C36H69NO3	0.00118	0.011	-0.41	N>M		
529.461	Pos	6.80	?[M+H]+	Unknown	?C30H60N2O5	0.00120	0.011	-1.23	N>M		
791.536	Pos	6.42	[M+NH4]+	PC(36:8) or PE(39:8)	C44H72NO8P	0.00121	0.011	-1.29	N>M		
343.263	Pos	3.72	?[M+H]+	Unknown	C23H34O2	0.00124	0.011	-0.85	N>M		
840.574	Pos	6.23	[M+H]+	PS(40:4)	C46H82NO10P	0.00126	0.012	-1.65	N>M		
760.511	Pos	5.76	[M+H]+	PS(34:2)	C40H74NO10P	0.00129	0.012	-1.26	N>M		
1028.628	Pos	6.83	Unknown	Unknown		0.00135	0.012	-1.51	N>M		
663.452	Pos	7.06	Unknown	Unknown		0.00136	0.012	-0.43	N>M		
816.642	Pos	9.34	[M+H]+	PC(38:1) or PE(41:1)	C46H90NO8P	0.00137	0.012	-0.91	N>M		
914.615	Pos	7.40	[M+NH4]+	PI(P-40:5)	C49H85O12P	0.00141	0.012	-1.10	N>M		+915.62
1009.729	Pos	9.18	Unknown	Unknown		0.00141	0.012	-1.04	N>M	+837.68	
960.887	Pos	12.21	[M+NH4]+	TG(58:2)	C61H114O6	0.00142	0.012	0.64	M>N		
818.602	Pos	6.54	[M+H]+	PC(P-40:6)	C48H84NO7P	0.00146	0.013	-1.33	N>M		
1514.461	Pos	12.19	Unknown	Unknown		0.00156	0.013	-0.46	N>M		
790.558	Pos	5.87	[M+H]+	PS(36:1)	C42H80NO10P	0.00160	0.013	-1.00	N>M		
1498.076	Pos	6.48	Unknown	Unknown		0.00163	0.014	-1.70	N>M		+1499.079
1566.143	Pos	6.41	[2M + Na]+	PE(38:2)	C43H82NO8P	0.00165	0.014	-1.66	N>M		+1567.144
629.57	Pos	3.11	[2M+H]+	3-hydroxypristanic acid	C19H38O3	0.00167	0.014	-0.88	N>M	+297.278	
529.461 546.487	Pos	8.45	[M+H]+ [M+NH4]+	Unknown	Unknown	0.00171 0.00060	0.014 0.007	-0.75	N>M		

m/z	Mode	r.t (min)	Ion/fragment	Possible ID	Formula	p value	FDR	log2FC (M/N)	Level (M/N)	Other fragments	Significant isotopes
701.559	Pos	5.47	[M+H]+	SM(d34:2)	C39H77N2O6P	0.00179	0.014	-0.85	N>M		
685.203	Pos	7.46	?[2M+H]+	Unknown	?C15H18O9	0.00184	0.015	-0.12	N>M		
1110.976	Pos	12.51	Unknown	Unknown		0.00191	0.015	-0.76	N>M		
259.205	Pos	2.61	?[M+H]+	Unknown	?C18H26O	0.00195	0.015	0.42	M>N		
308.043	Pos	4.51	Unknown	Unknown		0.00196	0.015	-1.87	N>M		+309.046, +310.041
471.105	Pos	4.51	Unknown	Unknown		0.00198	0.015	-1.83	N>M		+472.111, +473.102, +474.110, +475.112
1602.142	Pos	7.05	Unknown	Unknown		0.00205	0.016	-1.74	N>M		
1196.124	Pos	12.69	Unknown	Unknown		0.00206	0.016	0.20	M>N		
1687.556	Pos	13.68	[2M+NH4]+	TG(50:0)	C53H102O6	0.00206	0.016	0.65	M>N		
756.632 778.617	Pos	8.03	[M+H]+ [M+Na]+	Glucosylceramide or galactosylceramide	C44H85NO8	0.00213 0.00846	0.016 0.041	-0.79	N>M		
378.282	Pos	4.04	?[M+H]+	Unknown	?C19H39NO6	0.00216	0.016	-0.85	N>M		
265.252	Pos	3.11	[M+H]+	Octadecadienal	C18H32O	0.00219	0.016	-0.52	N>M	+247.242	
578.659	Pos	8.40	Unknown	Unknown		0.00229	0.017	-0.76	N>M		
757.586	Neg	8.77	[2M+FA-H]-	MG(18:1)	C21H40O4	0.00004	0.017	2.10	M>N		-758.591
694.311	Neg	2.95	?[M-H]-	Unknown	?C42H47O9	0.00005	0.017	-0.78	N>M		
749.498	Neg	5.81	?[M-H]-	Unknown	?C46H70O8	0.00007	0.017	-1.34	N>M		
835.53 852.558	Pos	5.64	[M+H]+ [M+NH4]+	PI(34:2)	C43H79O13P	0.00245 0.00192	0.017 0.015	-1.29	N>M		
1430.03	Neg	6.55	[2M-H]-	PE(34:2)	C39H74NO8P	0.00012	0.018	-1.24	N>M	-782.495	-1431.03
1563.132	Neg	6.33	Unknown	Unknown		0.00014	0.018	-0.92	N>M		

m/z	Mode	r.t (min)	Ion/fragment	Possible ID	Formula	p value	FDR	log2FC (M/N)	Level (M/N)	Other fragments	Significant isotopes
579.393	Neg	4.75	?[2M-H]-	Unknown	?C17H26N2O2	0.00016	0.018	-1.37	N>M		
1620.499	Pos	13.57	Unknown	Unknown		0.00274	0.019	0.91	M>N		
926.57	Pos	6.08	[M+NH4]+	PI(40:7)	C49H81O13P	0.00280	0.019	-0.37	N>M		
814.558	Pos	6.68	[M+H]+	PS(38:3)	C44H80NO10P	0.00283	0.020	-0.94	N>M		
736.489	Pos	5.51	[M+H]+	PC(33:6) or PE(36:6)	C41H70NO8P	0.00283	0.020	-1.58	N>M		
788.543	Pos	6.50	[M+H]+	PS(36:2)	C42H78NO10P	0.00295	0.020	-1.62	N>M		
254.247	Pos	2.77	[M+H]+	Palmitoleamide	C16H31NO	0.00296	0.020	-0.63	N>M		
1367.424	Pos	11.96	?[M+Na]+	Unknown	?C54H68N14O27	0.00303	0.020	-0.45	N>M		
778.535	Pos	5.33	[M+H]+	PC(36:6) or PE(39:6)	C44H76NO8P	0.00304	0.020	-1.19	N>M		
789.546	Pos	6.49	[M+NH4]+	PA(41:4)	C44H79O8P	0.00307	0.021	-1.31	N>M		
642.618	Pos	12.27	[M+NH4]+	CE(16:0)	C43H76O2	0.00309	0.021	-0.82	N>M		
1092.968	Pos	12.89	Unknown	Unknown		0.00310	0.021	-0.06	N>M		
1467.372	Pos	13.08	Unknown	Unknown		0.00326	0.021	0.20	M>N		+1468.373
603.534	Pos	7.39	Unknown	Unknown		0.00330	0.022	-0.86	N>M		
700.57	Pos	6.46	[M+H]+	CerP(40:2)	C40H77NO8	0.00331	0.022	-1.01	N>M		
748.524	Pos	6.62	[M+H]+	PE(P-38:6)	C43H74NO7P	0.00331	0.022	-1.20	N>M		
310.31	Pos	3.88	[M+H]+	N-Hexadecanoylpyrrolidine	C20H39NO	0.00340	0.022	-0.68	N>M		
809.65	Pos	7.25	[M+Na]+	SM(40:1)	C45H91N2O6P	0.00344	0.022	-0.59	N>M		
844.673	Pos	9.99	[M+H]+	PC(40:1) or PE(43:1)	C48H94NO8P	0.00353	0.023	-0.83	N>M		
890.581	Pos	6.55	[M+Na]+	PS(42:4)	C48H85NO10P	0.00356	0.023	-0.94	N>M		
393.202	Pos	3.07	Unknown	Unknown		0.00358	0.023	-0.92	N>M		
368.351	Pos	2.62	Unknown	Unknown		0.00361	0.023	-0.69	N>M		
510.486	Pos	7.08	[M+H]+	Cer(d32:1)	C32H63NO3	0.00374	0.024	-0.70	N>M		
772.273	Pos	9.03	?[M+NH4]+	Unknown	?C34H42O19	0.00379	0.024	-0.12	N>M		

m/z	Mode	r.t (min)	Ion/fragment	Possible ID	Formula	p value	FDR	log2FC (M/N)	Level (M/N)	Other fragments	Significant isotopes
1524.094	Pos	6.48	Unknown	Unknown		0.00380	0.024	-1.37	N>M		+1525.095
878.569	Pos	5.22	[M+H]+	PC(44:12)	C52H80NO8P	0.00387	0.024	-1.82	N>M		
515.327	Pos	3.88	[M+Na]+	SM(19:1)		0.00387	0.024	-0.65	N>M		
577.517	Pos	6.35	?[M+H]+	Unknown	?C37H68O4	0.00389	0.024	-1.16	N>M		
832.24	Pos	8.75	?[M+H]+	Unknown	?C39H43O20	0.00391	0.024	-0.09	N>M	+784.664	
744.586	Pos	7.35	[M+H]+	PC(P-34:1) or PE(P-37:1)	C42H82NO7P	0.00393	0.024	-2.48	N>M		
452.394	Pos	3.31	[2M+NH4]+	Spermic acid	C10H23N3O2	0.00401	0.025	-0.65	N>M	+370.367	
282.279	Pos	3.49	[M+H]+	Oleamide	C18H35NO	0.00404	0.025	-0.68	N>M		
954.612	Pos	7.00	?[M+NH4]+	Unknown	?C51H84O15	0.00410	0.025	-0.56	N>M		+955.613
977.539	Neg	5.97	[M+CH3COO]-	PIP(34:0)	C43H84O16P2	0.00027	0.026	-0.96	N>M		
605.621	Pos	7.98	Unknown	Unknown		0.00447	0.026	-0.17	N>M		
679.429	Pos	7.83	[M+Na]+	PA(33:3)	C46H88NO7P	0.00448	0.026	-0.49	N>M		
820.616	Pos	7.27	[M+H]+	PC(P-40:5)	C48H86NO7P	0.00453	0.026	-1.23	N>M		
772.618	Pos	7.50	[M+H]+	PC(P-36:1)	C44H86NO7P	0.00453	0.026	-1.05	N>M		
385.089	Pos	3.42	?[M+H]+	Unknown	?C20H16O8	0.00454	0.026	-1.09	N>M		
831.694	Pos	8.09	[M+NH4]+	PE(P-42:1)	C47H92NO7P	0.00455	0.026	-0.86	N>M		
829.761	Pos	12.55	Unknown	Unknown		0.00456	0.026	0.70	M>N		
313.273	Pos	3.13	[M+H]+	Oxo-nonadecanoic acid	C19H36O3	0.00458	0.027	-0.71	N>M	+253.252	+314.276
330.3			[M+NH4]+			0.01092	0.049				+331.303
702.585	Pos	9.63	[M+H]+	CerP(40:1)	C40H80NO6P	0.00463	0.027	-0.70	N>M		
763.583	Pos	6.41	[M+NH4]+	PG(P-36:0) or PG(O-36:1)	C42H83O9P	0.00471	0.027	-1.03	N>M		
285.221	Pos	3.23	[M+H]+	Vitamin A2/retinal	C20H28O	0.00475	0.027	1.43	M>N		
473.317	Pos	4.24	Unknown	Unknown		0.00481	0.027	-0.65	N>M		
978.609	Pos	6.46	Unknown	Unknown		0.00484	0.028	-0.91	N>M		

m/z	Mode	r.t (min)	Ion/fragment	Possible ID	Formula	p value	FDR	log2FC (M/N)	Level (M/N)	Other fragments	Significant isotopes
843.732	Pos	9.81	[M]+	SM(44:1)		0.00489	0.028	-1.12	N>M		
771.515	Pos	5.34	[M+H]+	PG(36:4)	C42H75O10P	0.00490	0.028	-1.00	N>M		
788.541			[M+NH4]+			0.00547	0.030				
897.6	Pos	6.17	Unknown	Unknown		0.00502	0.028	-1.21	N>M		
533.456	Pos	8.07	Unknown	Unknown		0.00503	0.028	-0.54	N>M	+475.414	
826.631	Pos	7.73	[M+H]+	PC(39:3) or PE(42:3)	C47H88NO8P	0.00505	0.028	-1.08	N>M		
493.315	Pos	14.92	Unknown	Unknown		0.00515	0.029	-0.32	N>M		
328.32	Pos	3.62	[M+H]+	Dihydrosphingosine	C21H41NO2	0.00538	0.030	-0.39	N>M		
655.633			[2M+H]+			0.00288	0.020				
366.276			[M + K]+			0.01082	0.048				
1471.034	Pos	11.09	[M+NH4]+	Cardiolipin(72:6)	C81H146O17P2	0.00548	0.030	-1.10	N>M		
1263.829	Pos	7.44	[M+H]+	Ganglioside GM3 (42:2)	C65H118N2O21	0.00555	0.030	-1.37	N>M		
771.571	Pos	7.57	[M+NH4]+	PC(34:4) or PE(37:4)	C42H76NO8P	0.00561	0.030	-0.93	N>M		
1331.249	Pos	12.90	Unknown	Unknown		0.00583	0.031	0.28	M>N		
701.535	Pos	6.51	Unknown	Unknown		0.00591	0.032	-0.35	N>M		
928.594	Pos	6.95	[M+NH4]+	PI(40:6)	C49H83O13P	0.00595	0.032	-0.96	N>M		
1263.188	Pos	12.80	Unknown	Unknown		0.00601	0.032	0.14	M>N		
1406.14	Pos	6.15	[2M+H]+	SM(d34:1)	C39H79N2O6P	0.00610	0.032	-1.58	N>M		
282.279	Pos	3.24	Unknown	Unknown		0.00623	0.033	-0.37	N>M		
830.658	Pos	9.67	[M+H]+	PC(39:1) or PE(42:1)	C47H92NO8P	0.00626	0.033	-0.76	N>M		
629.548	Pos	6.55	Unknown	Unknown		0.00629	0.033	-0.82	N>M		
384.382	Pos	5.17	[M+NH4]+	Nervonic acid	C24H46O2	0.00630	0.033	-0.47	N>M		
314.304	Pos	3.18	[M+NH4]+	4,6-Nonadecanedione	C19H36O2	0.00640	0.033	-0.34	N>M		
522.349	Pos	3.62	[M+H]+	LysoPC(18:1)	?C24H40N8O4	0.00648	0.034	-0.34	N>M		
925.634	Pos	6.97	?[2M+H]+	Unknown	?C31H42O3	0.00672	0.034	-0.75	N>M		

m/z	Mode	r.t (min)	Ion/fragment	Possible ID	Formula	p value	FDR	log2FC (M/N)	Level (M/N)	Other fragments	Significant isotopes
805.679	Pos	8.14	[M+NH4]+	PE(P-40:0)	C45H90NO7P	0.00685	0.035	-0.87	N>M		
702.506	Pos	6.23	[M+H]+	PC(30:2) or PE(33:2)	C38H72NO8P	0.00686	0.035	-1.25	N>M		
1006.641	Pos	7.36	Unknown	Unknown		0.00688	0.035	-0.89	N>M		
775.632	Pos	7.51	[M+NH4]+	PE(P-38:1)	C43H84NO7P	0.00715	0.036	-0.70	N>M		
694.647	Pos	11.97	[M+NH4]+	CE(20:2)	C47H80O2	0.00716	0.036	-1.27	N>M		
718.573	Pos	6.71	[M+NH4]+	PC(P-32:0) or PE(P-35:0)	C40H80NO7P	0.00722	0.036	-0.96	N>M		
1537.099	Neg	6.08	[M-H]-	Unknown		0.00080	0.037	-1.60	N>M	-902.519, -924.499	-1538.101
1559.079			[M+Na-2H]-			0.00036	0.030	-1.64			
840.579	Neg	7.34	[M-H]-	PS(40:3)	C46H84NO10P	0.00053	0.037	-1.17	N>M		
814.6	Neg	6.82	[M-H]-	PS(P-20:0/19:1)	C45H86NO9P	0.00059	0.037	-0.93	N>M		
949.508	Neg	5.28	[M+FA-H]-	PI(40:9)	C49H77O13P	0.00080	0.037	-1.25	N>M		
1721.157	Neg	6.73	Unknown	Unknown		0.00085	0.037	-1.58	N>M		
916.541	Neg	6.33	[M + CH3COO]-	PS(42:9)	C48H76NO10P	0.00087	0.037	-0.83	N>M		
1628.154	Pos	7.06	Unknown	Unknown		0.00738	0.037	-1.38	N>M		
269.099	Pos	6.12	Unknown	Unknown		0.00759	0.038	1.14	M>N		
485.12	Pos	4.07	Unknown	Unknown		0.00759	0.038	-1.06	N>M	+423.199	
1533.308	Pos	7.16	[2M+NH4]+	CerP(d44:1)	C44H88NO6P	0.00769	0.038	-1.14	N>M		+1534.316, +1535.316
620.596	Pos	8.73	[M+H]+	CerP(d40:2)	C40H77NO3	0.00776	0.038	-0.63	N>M	+602.586	
550.473	Pos	9.42	Unknown	Unknown		0.00779	0.039	0.96	M>N		
442.212	Pos	14.88	Unknown	Unknown		0.00781	0.039	-0.56	N>M		
394.404	Pos	5.73	Unknown	Unknown		0.00785	0.039	0.74	M>N		
1399.312	Pos	12.99	[M+NH4]+	Dolichol-20	C100H164O	0.00790	0.039	0.11	M>N		+1401.313
660.555	Pos	7.69	[M+NH4]+	DG(38:5)	C41H70O5	0.00792	0.039	-0.03	N>M		

m/z	Mode	r.t (min)	Ion/fragment	Possible ID	Formula	p value	FDR	log2FC (M/N)	Level (M/N)	Other fragments	Significant isotopes
442.406	Pos	8.44	?[M+NH4]+	Unknown	?C30H48O	0.00805	0.039	-0.68	N>M		
910.257	Pos	9.27	Unknown	Unknown		0.00821	0.040	-0.38	N>M		
662.644	Pos	9.61	Unknown	Unknown		0.00843	0.041	-0.74	N>M		
270.279	Pos	3.27	[M+NH4]+	Heptadecenal	C17H32O	0.00850	0.041	-0.48	N>M		
647.525	Pos	8.70	Unknown	Unknown		0.00862	0.041	-0.77	N>M		
956.629	Pos	7.71	[M+Na]+	PS(47:6)		0.00881	0.042	-0.85	N>M		
219.727	Pos	7.01	Unknown	Unknown		0.00885	0.042	-0.98	N>M		+220.507
470.42	Pos	3.96	?[2M+NH4]+	Unknown	?C14H26O2	0.00892	0.042	-0.76	N>M		
482.455	Pos	6.28	[M+H]+	Cer(d30:1)	C30H59NO3	0.00895	0.042	-0.41	N>M		
676.658	Pos	9.91	Unknown	Unknown		0.00921	0.043	-0.81	N>M		
1715.113	Neg	5.98	Unknown	Unknown		0.00114	0.043	-2.53	N>M		
499.332	Pos	4.45	Unknown	Unknown		0.00945	0.044	-0.62	N>M		
636.627	Pos	9.67	[M+H]+	Cer(d41:1)	C41H81NO3	0.00956	0.044	-0.50	N>M		
658.61			[M+Na]+			0.00299	0.020				
1649.063	Neg	6.18	Unknown	Unknown		0.00123	0.044	-1.44	N>M		
1658.132	Neg	5.99	[2M-H]-	PC(40:8)	C48H80NO8P	0.00126	0.044	-3.45	N>M		
850.562			[M+Na-2H]-			0.00092	0.037		N>M		
1224.084	Neg	12.69	Unknown	Unknown		0.00129	0.044	0.85	M>N		
1544.069	Neg	6.2	[2M+FA-H]-	PE(P-38:5)	C43H76NO7P	0.00132	0.044	-1.70	N>M		
1453.009	Neg	11.03	[M H20-H]-	N-Acetylglucosaminyl-diphosphodolichol	C88H147NO12P2	0.00135	0.044	-0.86	N>M		
560.501	Pos	7.85	[M+Na]+	Cer(d34:1) or N-Palmitoylsphingosine	C34H67NO3	0.00973	0.045	-0.72	N>M		
525.497	Pos	3.62	Unknown	Unknown		0.01002	0.045	-0.79	N>M		
424.328	Pos	4.83	Unknown	Unknown		0.01020	0.046	0.94	M>N		

m/z	Mode	r.t (min)	Ion/fragment	Possible ID	Formula	p value	FDR	log2FC (M/N)	Level (M/N)	Other fragments	Significant isotopes
256.263	Pos	2.99	[M+NH4]+	Hexadecenal	C16H30O	0.01021	0.046	-0.62	N>M		
597.543	Pos	6.70	[M+H]+	DG(34:0)	C37H72O5	0.01065	0.048	-0.88	N>M		
409.379	Pos	4.24	?[2M+H]+	Unknown	?C15H24	0.01070	0.048	-0.91	N>M		
694.613	Pos	11.03	[M+NH4]+	CE(MonoMe)	C46H76O3	0.01084	0.048	-0.28	N>M		
519.46	Pos	6.90	Unknown	Unknown		0.01090	0.049	-1.17	N>M		
608.632	Pos	7.13	Unknown	Unknown		0.01102	0.049	0.62	M>N		
880.589	Pos	6.37	[M+H]+	PC(44:11)	C52H82NO8P	0.01105	0.049	-1.12	N>M