Using intermittent fasting and a ketogenic diet to improve nutritional and clinical outcomes in long-stay, hospitalised canine spinal patients

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Veterinary Science

at Massey University, Manawatū, New Zealand

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2021

Abstract

A high prevalence of malnutrition occurs in human hospitals and has been associated with detrimental consequences. By comparison, very little is known in veterinary patients. Additionally, while underfeeding can lead to poorer outcomes, overfeeding hospital patients also causes negative effects, to the extent that purposeful caloric restriction has been touted as an ideal feeding regimen. Both caloric restriction and intermittent fasting (IF) have gained interest as therapeutic feeding regimens for several diseases, including neuronal injury.

The aim of this thesis was to explore hospital nutrition and IF in dogs. Firstly, the prevalence of malnutrition and body composition changes were determined in long-stay hospitalised patients. Results showed that most patients lost weight during hospitalisation, mainly from muscle. Additionally, body and muscle condition scoring did not accurately measure changes in composition, particularly in lean dogs. So predictive equations of body composition using morphometry in lean dogs were created. While feasible, it was discovered that breed-specific equations are needed, limiting its use.

Then, the metabolic and immunological effects of IF in healthy dogs were examined. Results showed that IF on a high-fat (HF) diet increased blood ketones and decreased leptin and ghrelin concentration. Also, a reduction in immunity occurred when the dogs were fasted after eating the low-fat diet, but not with the HF diet. Furthermore, ketone kinetics indicated that increasing the fasting duration from 24 to 48 hours caused highly variable responses amongst the dogs. Therefore, the beneficial effects of IF may be dependent on the individual. Finally, an IF regimen using a HF diet was applied in hospitalised dogs recovering from spinal injury. Results showed that the regimen was practical and reduced body weight loss. Also, higher fasted ketone concentrations were associated with shorter hospital durations in dogs that lost more weight.

In summary, long-stay hospitalised dogs lose a substantial amount of muscle. Also, a HF diet fed intermittently can increase ketone concentrations and may benefit patients with spinal cord injury. However, more work is needed to better understand what influences the variation in ketogenesis and utilisation in healthy and diseased dogs, and test the effects in a larger study population.

Acknowledgments

The journey to complete a doctoral thesis is truly an endeavour and one that takes a village. First and foremost, I would like to thank my supervisors Dr. Nick Cave and Dr. Timothy Wester. Thank you, Nick, for sharing your passion for science and for constantly challenging me. I am grateful for this intellectual journey that has often required me to go down the path less taken. Thank you, Tim, for your sensibility, pragmatism, and constant encouragement, especially towards the end of the marathon. Both of your mentorships have crafted the researcher I am today and for that, I will be always be grateful.

I would also like to thank Dr. Axel Heiser, Dr. Pat Edwards and Dr. Kristene Gedye for sharing their expertise. Axel, no matter how busy you were, you were always willing to help. Thank you for teaching me everything I needed to know about flow cytometry and for letting me commandeer your bench space from time to time. Thank you, Pat, for sharing your laboratory and expertise on NMR, you were always so patient and helpful in answering all of my questions. Also, thank you Kristene for helping me with my assays. I knew I could always come to you to ask you something about almost anything.

There are also those who sacrificed early mornings, late evenings and weekends to help me make up diets, feed, and collect samples. Thank you, Bex Owen, for all that you have done, which is a lot. I am extremely grateful for your unwavering willingness to help and positive attitude. You are definitely the best dog cuddler around! Thank you also Brittnee Southland. Nothing was ever a problem and you were always accommodating and dependable. You both are amazing, and I am very thankful to have shared with journey with you.

Lastly, I would also like to thank my family who has been my inspiration my whole life. For my parents who always taught me not be afraid of working hard, and to always do the best I can. For my sister, who I have always admired, and who always supports me on whatever path I take. Finally, to my husband Javier, who has been my rock – thank you for always making me laugh and for being my biggest supporter of all.

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AAFCO	Association of American Feed Control Officials
AcAc	Acetoacetate
AIC	Akaike information criterion
AKT	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
APACHE	Acute Physiology and Chronic Health Evaluation
ASA	American Society of Anaesthesiologists
ASPEN	American Society for Parenteral and Enteral Nutrition
ATP	Adenosine triphosphate
β-HOB	Beta-hydroxybutyrate
BCS	Body condition scoring
BID LF	BID low-fat diet
BMI	Body mass index
BWT	Body weight
CCPM	Cell count per minute
CI	Confidence interval
ConA	Concanavalin A
CPT	Carnitine palmitoyl transferase
CR	Caloric restriction
CSF	Cerebral spinal fluid
D_2O	Deuterium oxide
DEXA	Dual-energy X-ray absorptiometry
DHR	Dihydrorhodamine
DNA	Deoxyribonucleic acid
ESPEN	European Society for Clinical Nutrition and Metabolism
FAD	Flavin adenine dinucleotide
FCE	Fibrocartilaginous embolism
FCS	Foetal calf serum
FFM	Fat-free mass
FM	Fat mass
Foxo3a	Forkhead box O3a
GC-MS	Gas chromatography-mass spectrometry
GLP1	Glucagon-like peptide 1
GLUT	Glucose transporter
HF	High-fat
HMG	3-hydroxy-3-methylglutaryl
HOMA	Homeostasis model assessment
HSL	Hormone sensitive lipase
ICU	Intensive care unit
IF	Intermittent fasting
IF HF	Intermittent fasting (feeding once every 48-hours) on a high-fat diet
IF LF	Intermittent fasting (feeding once every 48-hours) on a low-fat diet
IGF-1	Insulin-like growth factor 1
IL	Interleukin

IL-1β	Interleukin-1β
IL-6	Interleukin-6
IQR	Interquartile range
IVDD	Intervertebral disc disease
IVF	Intravenous fluid
Kcal	Kilocalorie
kD	Kilodalton
leanST	Lean soft tissue mass
LF	Low-fat
LOS	Length of stay
LPS	Lipopolysaccharides
MCS	Muscle condition scoring
MCTs	Monocarboxylate transporters
MER	Maintenance energy requirement
MFI	Mean florescence intensity
MNA	Mini nutritional assessment
MPE	Mole percent excess
MST	Malnutrition screening tool
mTOR	Mammalian target of rapamycin
MUAEC	Massey University Animal Ethics Committee
MUVTH	Massey University Veterinary Teaching Hospital
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
OPLS-DA	Orthogonal partial least squares discriminant analysis
OR	Odds ratio
P13K	Phosphoinositide 3-kinase
PC	Principal component
PCA	Principal component analysis
PHA	Phytohaemagglutinin
PLS-DA	Partial least squares discriminant analysis
PPARs	Peroxisome proliferator-activated receptor
PPM	Parts per million
PSS	Physical status score
QMR	Quantitative magnetic resonance imaging
rcf	Relative centripetal force
RER	Resting energy requirement
ROS	Reactive oxygen species
SCOT	Succcinyl-CoA:3-oxoacid-CoA transferase
SD	Standard deviation
SE	Standard error
SED	Staphylococcus enterotoxin B
SGA	Subjective global assessment
TAG	Triacylglycerol
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α

TTR	Tracer-to-tracee ratio
Ub	Ubiquitin
UPS	Ubiquitin proteasome system
Vd	Volume of distribution
VIF	Variance inflation factor
WSAVA	World Small Animal Veterinary Association

Chapter 1

Literature Review

"I will apply dietetic measures for the benefit of the sick according to my ability and judgment; I will keep them from harm and injustice." – Hippocrates (400 BC)

1.1 Malnutrition during hospitalisation: a human and animal concern

There is a growing recognition in veterinary medicine of the importance of nutrition as an integral part of health care. In 2011, the World Small Animal Veterinary Association (WSAVA) added "nutritional assessment" as the 5th Vital Assessment (5VA), following temperature, pulse, respiration, and pain assessment.[1] With this was a recommendation that a nutritional assessment should be performed on every patient at every visit. Since then, additional publications have argued for the importance of assessing the nutritional status of a patient and the association of malnutrition with disease prognosis.[2–5] Despite these efforts by the veterinary profession to improve hospital nutrition, we remain truly behind that of human medicine. Challenges remain for veterinarians to recognise risk factors for malnutrition, correctly assess the nutritional status of patients, and instigate appropriate nutritional intervention.[6] Also, the monitoring and reassessment of the nutritional status of long-stay patients is still often overlooked in veterinary medicine.

In contrast, there is a far greater number of publications in human medicine relating to the negative consequence of malnutrition, methods of assessing nutritional status, and feeding recommendations for malnourished individuals.[7–13] In fact, in response to the increased awareness of a global issue in the human medical field, a 2014 global campaign - aptly named the 'feed malnutrition awareness and education' or 'feedM.E' group - was created to provide teaching resources for physicians and healthcare professionals on how to recognise malnutrition and evidence-based protocols for intervention.[14] Thus, with the dearth of publications on these topics in veterinary medicine, it is necessary to infer from experimental studies in laboratory species and

clinical studies in humans to gain an understanding of the potential consequences of malnutrition in veterinary hospitals, and to explore methods of intervention. This review will highlight and critically appraise the current understanding of the prevalence of malnutrition, its consequences, and the nutritional interventions used in human medicine, and in veterinary medicine when literature is available. In addition, methods of assessing the nutritional status and muscle mass in patients, and the interesting health dichotomy between feeding and fasting, particularly for patients recovering from neurological injury, is explored.

1.2 The very start: definitions and determining a patient's nutritional status

To establish clarity, the main terminologies used in this dissertation need to be firstly defined. In 1970, the term nutritional status was coined as the "state of an individual's health condition which has been influenced by the intake and utilisation of nutrients".[15] For this dissertation, the term will be further defined to describe the state of a patient as determined by a nutritional assessment based on a patient's history, physical examination, laboratory and imaging results, and/or measures of mobility and function. For the term malnourishment, I will use the definition suggested by the 2016 global clinical nutrition working group who defined it as "a nutritional state resulting from a lack of intake or uptake of (inappropriate) nutrients that leads to an altered body composition or body cell mass, which reduces physical and mental function, and impairs clinical outcome".[8] Although over-nutrition and obesity are forms of malnutrition, for this dissertation, the term malnutrition will be restricted to undernutrition. Other key nutrition terms such as marasmus, kwashiorkor (protein-energy malnutrition), cachexia and sarcopenia will be defined in their relevant sections.

1.2.1 Nutritional screening and nutritional assessments

In both human and veterinary medicine, a nutritional screening is the first step that evaluates and determines which individuals are at nutritional risk. In humans, screening consists of the identification of historical weight changes, assessment of a patient's current body mass index (BMI), and the consideration of the possibility that the patient's disease process could affect their nutritional status (e.g. due to dysphagia or maldigestion/malabsorption).[16] The European Society for Clinical Nutrition and

Metabolism (ESPEN) recommends a nutritional screening for each patient within 24 hours of admission to the hospital.[17] Individuals deemed to be at risk of malnutrition should then undergo a further comprehensive nutritional assessment.

The nutritional assessment process is less standardised and multiple approaches have been described in the human literature. For the assessment, the use of a physical examination, monitoring for in-hospital weight loss or low caloric intake, anthropometric measurements (triceps skinfold thickness or arm-muscle circumference), identifying low haematocrit or leukocytes counts, and/or reduced concentrations of serum albumin, pre-albumin and/or transferrin are all approaches that have been described.[4,18–20] Handgrip strength and a 5-meter walk test have also been used to assess decreased physical function as an indicator of malnutrition.[20–22] With the myriad of options, simplified tools such as the Subjective Global Assessment (SGA) have been created to assist with the assessment process. Developed in 1987, the SGA determines the nutritional status of a patient based on medical history, including changes in food intake and body weight, and the results from a physical examination.[23] The patient is then subjectively determined to be either 'well nourished', 'moderately malnourished' or 'severely malnourished'. The SGA guide is the most common nutritional assessment tool cited in human literature, and despite its relative simplicity, has been validated with good inter-rater consistency and accuracy to predict negative outcomes such as increased duration of hospital stay and mortality.[23–25]

In veterinary medicine, the WSAVA Nutritional Assessment Guidelines describes a similar approach whereby a nutritional screening is to be performed as part of the initial history gathering and physical examination.[1] If a patient is then identified as having risk factors for malnutrition, then an extended nutritional evaluation should be performed (Table 1-1).

Table 1-1. The nutrition screening risk factors as recommended from the WSAVA Nutritional Assessment Guidelines.[1]

Nutritional risk factors
History
Altered gastrointestinal function (including vomiting, diarrhoea, nausea, flatulence, constipation)
Previous or ongoing medical conditions/disease
Currently receiving medications and/or dietary supplements
Unconventional diet (including raw, homemade, vegetarian, unfamiliar[a])
Snacks, treats, table food >10% of total calories
Inadequate or inappropriate housing
Physical Examination
Body condition score (9-point scale): any score less than 4 or greater than 5
Muscle condition score: mild, moderate or marked muscle wasting
Unexplained weight change
Dental abnormalities or disease
Poor skin or hair coat
New medical condition/disease

[a] diet unfamiliar to the attending clinician

Body weight and body condition scoring (BCS) are the most widely used methods for measuring and monitoring nutritional status in veterinary medicine.[26] Developed in 1997, BCS is a quick and practical subjective measure of body fat mass, which has been validated using dual-energy x-ray absorptiometry (DEXA) and isotope dilution in dogs.[27,28] A similar BCS system has also been developed and validated for cats.[29] In addition to this, equations using morphometry have been created for both dogs and cats, which describe body weight relative to skeletal measurements in order to predict body fat mass.[30] Comparisons of BCS and morphometry to DEXA and isotopic dilution indicate that they can both accurately estimate body fat mass.[28,31] However there is a

strong breed effect where the morphology of different breeds can affect the sites of assessment, leading to inaccuracies in both methods.

As the BCS system and morphometry equations were designed and validated to predict body fat mass, an attempt at a specific assessment of muscle mass in veterinary medicine has also been made, namely the muscle condition scoring (MCS) system. This scoring system consists of four broad categories: 'normal muscling', 'mild muscle loss', 'moderate muscle loss' and 'severe muscle loss'. Muscle condition scoring has been shown to have good repeatability and fair to moderate inter-rater reproducibility, and it was correlated with total lean mass in a validation study using DEXA in cats ($r = 0.62$; $P < 0.0001$).[32] The current muscle condition scoring system evaluates multiple points on the body, and so focusing on specific regions, such as the vertebral epaxial muscling in cats, has been suggested to increase the scoring system's reproducibility.[33] In dogs however, MCS has been shown to only weakly correlate with total lean mass as measured by quantitative magnetic resonance imaging (QMR) ($r = 0.30$; $P = 0.06$).[32,34] Although MCS is a simple and practical method for clinicians to assess lean mass, it does not appear to be sufficiently accurate or repeatable, and further studies are required to validate and determine the value of MCS as a means of assessing and monitoring the nutritional status of veterinary patients.

Other approaches for measuring muscle mass in live animals include radiography, ultrasonography, DEXA, QMR, bioelectrical impedance analysis, computed tomography, magnetic resonance imaging, and isotopic dilution, all of which have varying practical clinical applications.[3,35–40] In addition, functional tests have been described as a way of monitoring nutritional status and muscle strength in human medicine.[41,42] A 6-minute walk test has been trialled as a means of determining the functional status of dogs with cardiac, pulmonary and neuromuscular disease.[43–45] However, as the relationship between results from this type of testing and lean muscle loss in veterinary patients has not been tested, its utility to measure and monitor the nutritional status of dogs or cats is unknown.

As a complement to the assessment of fat and lean mass, the WSAVA guidelines also suggest to include haematology and a serum biochemistry panel to assess for

abnormalities that may be linked to malnourishment, such as anaemia and hypoalbuminemia.[1] A study by Michel[46] examined the prognostic value of serum albumin, haematocrit, haemoglobin, lymphocyte count, and BCS in 105 hospitalised dogs. She determined that serum albumin, haematocrit and haemoglobin were associated with mortality ($P < 0.01$). However, she also cautioned that serum albumin can be influenced by other non-nutritional related factors, such as decreased hepatic synthesis and increased losses due to vascular permeability, and so changes may not necessarily be due to malnutrition. Other possible causes of hypoalbuminemia include protein-losing enteropathies, protein-losing nephropathies, and a dilution effect from overzealous IV fluid therapy. Further to this, low albumin concentrations are not a consistent feature of malnutrition. In a study of 152 rescued, emaciated dogs, only 38% had hypoalbuminaemia.[47] In addition, only a relatively small drop in albumin is found after three to four weeks of complete starvation in dogs.[48,49] These studies further highlight albumin's low diagnostic value as a marker for malnutrition, and its use to assess malnutrition has been called into question in both human and veterinary medicine.[50–54]

An exception to this are patients with severe protein-energy malnutrition (kwashiorkor), where hypoalbuminemia remains a key marker of the disease and prognosis.[55–57] During kwashiorkor, there is sufficient caloric intake but insufficient protein intake, leading to reduced amino acid release from muscle, reduced serum amino acids concentrations, and the development of hypoalbuminemia.[58–61] This condition differs from "simple starvation" (marasmus) in which there is significant wasting of both muscle and fat, where severe hypoalbuminemia is an uncommon feature, and oedema asbsent.[58,62] Although albumin is clearly an important marker of disease in kwashiorkor, the disease itself is rare in developed countries, and human cases have been reported only in unique situations where severe dietary restriction has occurred.[63,64] In veterinary medicine, only cases of experimentally induced protein-energy malnutrition in dogs are reported.[65–68] However, there is an increasing interest by the public to feed dogs and cats vegetarian and vegan diets.[69] If such diets are unbalanced, poorly-digestible and/or low in protein content, they could cause

kwashiorkor. Therefore, serum albumin would be an important marker of disease and prognosis in these unique cases.

1.2.2 The cause-and-effect conundrum

A review of the body of literature in human medicine suggests a strong correlation between malnutrition and negative outcomes such as increased hospital duration of stay, greater infection rates, and a greater risk of death. Nevertheless, it is important to remember that correlation does not necessarily equate to causality; more severely ill patients tend to stay longer in the hospital and are also more likely to have a poor appetite, be bedridden, encounter infectious agents, and have higher mortality rates independent of their nutritional status. Other potential factors that could affect prognosis include disease severity, age, gender, and socioeconomic conditions leading to differing hospital care.[19] To deal with this, studies can stratify groups or implement multivariate modelling to account for these covariates when looking for a true independent association.[70] However, not all studies in the literature take into consideration these other factors, so a critical appraisal of study methodology will be considered throughout this chapter.

1.3 Prevalence of malnutrition and its association with negative prognoses

Globally, a substantial proportion of human patients enter the hospital in a malnourished state or become malnourished while hospitalised. Several studies from different regions of the world have indicated that the proportion of patients malnourished ranged from 20% to 60%, depending on the criteria used in determining the nutritional status of the patient.[52,71,72] In a large study, Schindler *et al.*[73] described the prevalence and risk factors for malnutrition using an accumulation of single-day food intake surveys encompassing over 91,000 human patients from 56 countries. The authors found that the majority of patients (53.3%) consumed 50% or less of their offered meal, although it is unclear how much food was offered and what their caloric intake was relative to their energy requirements. Common risk factors identified in the study for a patient not consuming an entire meal across all populations included being female, adolescent, elderly, confined to a bed, and having a low body mass index (BMI).

Large-scale studies have shown that malnourished patients have poor prognoses and longer hospital stays, although not all examined other factors besides nutrition that can affect outcome. For instance, in one study, patients admitted to a hospital were nutritionally assessed using anthropometry (body mass index, triceps skinfold thickness, and upper arm muscle circumference) and SGA, and the authors found that patients established as malnourished were 1.5 times more likely to be readmitted within six months of discharge.[74] However, the authors did not adjust for the potential confounding effect of disease severity. Better evidence of a true effect of malnutrition on prognosis can be found in other studies which have adjusted for potential confounders. Agarwal *et al*.[71] applied the SGA nutritional assessment to 3,122 patients and recorded their 24-hour food intake. After adjusting for age, gender, disease severity (through a Patient Clinical Complexity Level scoring method), and admission category (surgical *vs* medical *vs* ICU), they discovered that malnourished patients and those that ate ≤ 25% of the offered food stayed for five days longer compared to well-nourished patients (those that ate ≥ 50% of food offered) and had a greater 90-day hospital readmission rate. In addition, Lim *et al*.[75] found that after accounting for confounders, malnourished patients across all admission categories stayed on average two days longer in the hospital and had approximately two times the risk of readmission to the hospital within two weeks of discharge. Finally, Hudson *et al*.[76] found when controlling for age, sex, race, admission reason (medical *vs* surgical), and the patient's Charlson Comorbidity Index score (a weighted index that predicts the one-year mortality within a range of comorbidities), the odds ratio for readmission to the hospital within 30 days of discharge was 2.13 (95% CI 1.82 - 2.48) in malnourished patients compared to those who were considered well-nourished. Ultimately, these large-scale, multi-centre studies that controlled for covariates provide strong evidence that malnourishment is indeed independently associated with poorer outcomes in human hospital patients.

For veterinary patients, malnutrition in hospitals occurs for very similar reasons as for humans, including food refusal, vomiting, nausea, and poorly written dietary instructions by caretakers.[77,78] There are very few studies in veterinary medicine

examining the prevalence of malnutrition in hospitals. One of the first published studies was from Remillard *et al*.[77] where the authors established that in their study population, dogs consumed > 95% of their estimated resting energy requirements (RER) on only 27% of total hospitalisation days. The authors also described that patients with a higher physical status score (PSS), a scoring system used to describe the severity of disease which ranges from normal (1) to moribund (5), were less likely to consume their energy requirements, or to be discharged from the hospital. Since this publication, other studies have described that approximately a quarter of dogs at admission to referral veterinary teaching hospitals were underweight or had a low BCS.[79,80] In a more recent study, Molina *et al*.[78] found that 16% of the dogs hospitalised lost weight during hospitalisation, and the majority (84%) of dogs consumed less than 25% of their RER on average. Currently, there are only four published studies in veterinary medicine examining the prevalence, risk factors and consequences of malnutrition during hospitalisation. So, while the high prevalence of malnutrition and its negative effects in human medicine is clear, we are a long way from confidently drawing the same conclusions in veterinary medicine. However, next I will consider why there are reasons to believe they are likely to be similar, and why it may be different.

1.3.1 Infections and wound healing

1.3.1.1 Nutrition and immunity

In human hospitals, a common complication of malnutrition and underfeeding is an increased risk of systemic and local infection and delayed wound healing.[81–85] The direct suppressive effects of hypocaloric eating on immunity have been well documented experimentally.[86–89] Mice starved for 72 hours had a significant reduction of thymocytes, splenic mononuclear leucocytes and peripheral lymphocytes numbers.[88] In cats, starvation for a week caused decreased circulating leukocytes numbers, a lower CD4:CD8 ratio, and reduced lymphocyte proliferation *in vitro*, which improved with refeeding.[89] A reduced CD4:CD8 has been shown in humans to be linked with an altered immune function associated with ageing (immunosenescence), autoimmune diseases, viral infections and cancer, and may be a poor prognostic indicator in acute myocardial infarctions.[90–94] In another study in cats, four days of fasting reduced the

phagocytic activity of peripheral blood mononuclear cells.[95] Similarly, this immunosuppressing effect is also seen in dogs. An 85% food restriction for eight weeks in racing greyhounds reduced the number of neutrophils and lymphocytes in peripheral circulation, which may be reflective of immunosuppression or increased tissue migration.[96] Additionally, a steady reduction of food intake for 21 days (50% of requirements for 5 days, then 30% for 5 days, then 20% for 5 days, then complete food withholding for 6 days) in beagles led to a reduction in circulating neutrophil and lymphocyte counts, reduced lymphocyte proliferation *in vitro*, and lower serum concentrations of IgG, IgM and complement C3.[97] Of those, only IgG, IgM and C3 concentrations were restored after several weeks of parenteral nutrition. These collective studies indicate that there is an effect of decreasing food intake on immune function that is shared across several species.

The mechanism behind the reduction in immunity during periods of weight loss and reduced food intake is believed to be due in part to a fall in leptin concentrations.[98,99] Produced by adipose tissue, leptin is a pleiotrophic hormone that is involved in the regulation of appetite and food intake, energy expenditure, bone mass, and reproduction.[100] In addition, leptin plays a key role in regulating several leucocyte responses and is thought of as a link between the neuroendocrine and immune system.[99,101,102] Receptors for leptin are found on many immune cells including monocytes/macrophages, neutrophils, natural killer cells, and T and B lymphocytes.[103,104] In the normally nourished state, plasma leptin is correlated to an animal's body fat mass. However, during fasting and starvation, leptin production by adipocytes decreases, and does so in excess of the loss of fat mass.[98] A significant reduction in leptin concentration occurred within 6 - 12 hours of fasting in mice.[105] Also, refeeding underfed gerbils with glucose restored the drop in leptin.[106]

A reduction in leptin has been shown to decrease chemotaxis of granulocytes and monocytes/macrophages, in addition to a decrease in macrophage phagocytosis and cytokine production.[107–110] Leptin deficiency also caused a reduction in T and B lymphocytes proliferation *in vitro*.[111–113] Compared to wild-type control mice, leptin deficient *ob*/*ob* mice had only 60% as many nucleated cells and 70% of B lymphocytes in

bone marrow, which was brought to near normal values after leptin supplementation.[112] A similar finding occurred in starved mice where injection of leptin reversed the suppression in bone marrow production of leucocytes, T-cell immunity and bone marrow nucleated cells.[99,114,115] Despite these findings, the clinical significance of these immunological changes is not clear based on these experimental studies. To gain a better understanding of the significance of this phenomenon, we need to next explore studies which examined the effect of malnutrition and immunity in a hospital setting.

1.3.1.2 Malnutrition, hospital-acquired infections, and wound healing

Almost two decades before the implementation of the FeedM.E. campaign, Klein *et al.*[116] described the pre-surgical nutritional statuses of patients undergoing spinal surgery as a significant, independent factor in predicting postoperative infections. Subsequently, Rubinson *et al.*[83] went on to study adults (n = 138) admitted to an ICU and determined that the overall mean (± SD) daily caloric intake was 49.4 ± 29.3% of estimated requirements. After adjusting for gender, body weight, reason for admission, prior hospitalisation, and acquired immunodeficiency syndrome, the authors concluded that patients who consumed ≥ 25% of their recommended daily caloric intake experienced a lower risk of septicaemia (relative hazard, 0.24; 95% CI 0.10-0.60) than those consuming < 25% of their recommended daily caloric intake. Additionally, Villet *et al.*[81] found that the risk of infectious complications in malnourished ICU patients increased after just one week in the hospital and described a strong association between hospital-acquired infections and reduced food intake during hospital stay (P = 0.0049).

For successful wound healing, a sufficient immune response is required. Wound healing requires the recruitment of neutrophils for clearing cellular debris and any contaminating bacteria.[85] Macrophages involved in wound healing mediate fibroplasia and angiogenesis and are derived mainly from circulating monocytes.[85,117] Once activated, the macrophages produce transforming growth factor-β and vascular endothelial growth factor. Transforming growth factor-β is the primary factor that stimulates fibrosis during healing by enhancing cellular proliferation, differentiation,

and extra cellular matrix deposition.[117] Vascular endothelial growth factors on the other hand, stimulate neovascularisation by promoting collagen deposition and epithelialisation.[118]

In a study of malnourished rats fed 50% of the food eaten by control rats for 14 days, there was lower mRNA expression for TGF-β and reduced extracellular matrix deposition at wound sites seven days post-surgery compared to control rats, or even compared with malnourished rats who were fed a supplemental enteral diet post-surgery.[119] Furthermore, in rats fed 50% of the food eaten by the control rats for 21 days, there was decreased tensile strength and a lower percent of type I collagen deposition following colonic anastomoses.[120] In humans, patients with a history of reduced oral intake leading up to surgery had less collagen deposition in surgical sites seven days post-surgery compared to patients who did not have a decreased intake.[121]

In comparison, much less is known in veterinary patients about the relationship between malnutrition, immunity, and wound healing. Several studies have described an association between low serum albumin and intestinal leakage.[122–127] However, as discussed earlier, the use of albumin is not a valid marker for simple malnutrition as other non-nutritional factors can affect serum concentrations, and albumin does not consistently decrease even with several days of starvation. In addition, the only markers of malnutrition described in two of the studies were duration of anorexia and BCS of patients pre-operatively.[123,126] Therefore, a greater understanding of the effect of malnutrition on hospital-acquired infection and wound healing is still needed in veterinary medicine. However, it can be speculated that it may be similar to that of humans.

1.3.2 Length of stay and mortality

The association between malnutrition in human patients, length of hospital stay (LOS) and death has been shown in several studies.[19,76,128] While the direction of the causal relationship cannot be established, the strength of this apparent association lies with the large number of studies encompassing thousands of patients from multiple countries with differing cultures, socioeconomic backgrounds, and hospital practices. Norman *et al*.[52] examined eight studies which established malnourishment using a

heterogenous mix of assessment tools and established that the "average length of hospital stay increased by 40 - 70% in malnourished patients". Lim *et al*.[75] compared hospitalisation outcomes between well-nourished (n = 583) and malnourished individuals (n = 235) as defined by their SGA. After adjusting for age, gender, ethnicity and disease type, the researchers concluded that malnourished patients remained in the hospital 1.5 times longer and had an average cost of hospitalisation 24% higher than that of well-nourished patients. Also, a survey in Australia and New Zealand ascertained that malnutrition and decreased food intake were independent risk factors for increased length of stay.[71] This was a large study with 3,122 participants from 56 hospitals across Australia and New Zealand. The authors used the Malnutrition Screening Tool (MST) and multivariate analysis to evaluate for possible confounding variables such as age, reason for admission, and disease severity. The researchers determined that malnourished patients had a greater LOS (15 days, range: 2-119 days) compared to well-nourished patients (10 days, range: 2-153 days). Given the significant proportion of patients with malnutrition in human hospitals, increasing the length of stay by even a few days for each patient would be a substantial economic burden.

Even more profound than an effect on the LOS, reduced food intake and a poor nutritional status has been associated with an increased risk of death.[129,130] In the study by Agarwal *et al.*[71], it was concluded that malnourished patients who consumed ≤ 25% of food offered had significantly higher mortality rates (> 2.5 times at 30-days and 2 times at 90-days) compared to the patients who consumed > 25% of the offered food. It is not described in this study however how much food was offered relative to the patients' energy requirements. Hiesmayr *et al.*[131] also conducted a large single day nutritional survey encompassing 16,455 patients and established that decreased food intake was strongly correlated with mortality, even after adjustments were made for age, gender, race, reason for admission and the Charlson Comorbidity Index. Furthermore, using multivariate analysis, Sanson *et al.*[132] found that both mean energy and protein intake were lower in those individuals who died either during hospitalisation or within 30 days of discharge. Conversely, all surviving patients were recorded to have consumed > 75% of their estimated daily energy and protein requirements during hospitalisation.

A veterinary patient's duration in a hospital can vary widely from less than a day in most cases to over a month for patients with tetanus or those recovering from spinal surgery.[133–138] An association between malnutrition and an increased length of stay and risk of mortality have been documented in veterinary medicine as well, albeit on a much smaller scale. The study by Remillard *et al*.[77] found a significant relationship between patient outcome and their caloric intake in hospital and PSS. The authors, however, did not test for other potential confounding variables. Indeed, in this study, canine patients with lower PSS (i.e. they had no or mild systemic disease) were more likely to consume their RER and were also more likely to be discharged from the hospital. In a study by Brunetto *et al*.[80] that examined 467 dogs and 55 cats, it was found that increased energy intake and BCS were positively associated with hospital discharge. Patients with an ≥ ideal BCS had an 84.7% discharge rate while those with ≤ 2 BCS on a 5-point scale had a 73% discharge rate. However, again the study only examined variables and outcomes using univariate analysis, and the authors did not consider potential confounders. More recently however, in a study by Molina *et al*.[78], the authors examined the effect of energy intake and hospital duration, taking into account the patient's initial body weight, BCS, age, sex, PSS, reason for hospitalisation, fasting recommendations, nutritional intervention, and the presence of anorexia, vomiting or diarrhoea at admission. Multivariate analysis indicated that patients that consumed their estimated RER and those with a higher BCS, had a lower risk of death ($P < 0.001$).

A final point to consider is that the relationship between length of stay and outcome is even more intricately linked in veterinary medicine than in human medicine. For veterinary patients, the number of days in the hospital and the associated cost of hospitalisation can be a significant factor on outcome, as it may lead to an owner's decision for euthanasia. In addition, food intake is an important quality of life indicator for owners as well. In a study of dogs with congestive heart failure, 68% of owners cited anorexia as a contributing factor to their decision to euthanise.[139] Therefore, understanding the link between malnutrition and an increased duration of hospitalisation and overall outcome may be of more importance in veterinary medicine than even in human medicine.

1.4. Nutritional intervention studies

1.4.1 Human nutrition interventions

The 'feedM.E.' global campaign recommends screening patients daily for malnutrition and intervening when necessary.[14] Increasing the protein and energy intake of nutritionally at-risk patients has been shown to improve transthyretin (prealbumin) levels and decrease the overall LOS in hospital patients.[140,141] In addition, perioperative nutritional supplementation has also been found, after taking into account age, gender and disease severity, to reduce post-operative infections.[142,143] There are multiple systematic reviews of interventional feeding in hospitalised patients across different departments (medicine, surgery and ICU) that show the same conclusion of an positive effect of supplemental feeding on infectious complications and patient outcome.[72,144–146] For instance, Milne *et al.*[147] performed a meta-analysis examining the effect of protein or energy supplementation in studies of elderly individuals. They found that in 22 trials where hospitalised patients were given interventional feeding, there were fewer infectious complications (odds ratio 0.72 [95% CI, 0.53 to 0.97]). Also, in a review by Yan *et al.*[148], the authors described a strong positive effect of supplemental enteral nutrition on infectious (risk ratio 1.80 [95% CI 1.52, 2.14], $P < 0.00001$) and non-infectious complications (RR 1.68 [95% CI 1.27, 2.21], $P = 0.0003$) in patients with gastrointestinal cancer.

Although there are many studies which show a positive effect of nutritional intervention, not all studies do. In one by Gazzotti *et al.*[149], the authors examined the effect of a 60-day supplementation of a calorically dense, high protein supplement (providing an additional 500 kcals and 21 grams of protein per day) in elderly hospitalised patients. The authors concluded that nutritional status was improved as assessed by the Mini Nutritional Assessment (MNA), but there was no difference in LOS between the treatment and control, non-supplemented group. However, there was also no difference in the change in body weight at the end of the study in both groups, and so whether the patients truly improved in nutritional status is uncertain. In addition, the study was not blinded, and different physicians measured the patients' MNA at baseline and at the end of the study, which could have biased results. More interestingly, in a recently published systematic review examining 22 randomised

controlled trials, nutritional intervention increased caloric and protein intake, body weight, and decreased non-elective readmissions, but it did not have an effect on hospital-acquired infections, LOS, or mortality.[150] However, analyses of subgroups in the study found a trend that suggested that patients with established malnutrition may benefit more from intervention than those patients simply deemed at risk. This indicates that any positive effect of nutritional intervention may be less apparent in mildly malnourished patients, and therefore some studies may have been underpowered, or included the inappropriate study population to demonstrate an effect.

1.4.2 Veterinary nutrition interventions

There are only a few studies examining the effect of nutritional intervention and outcomes in hospitalised veterinary patients. In a retrospective study by Brunetto *et al.*[80], the authors reviewed the clinical records of 467 dogs and 55 cats. In the hospital's protocol, implementation of assisted feeding occurred in patients who did not voluntarily consume > 50% of their calculated daily maintenance energy requirement (MER) within 48 hours of admission to the hospital. Assisted-feeding possibilities included syringe feeding, nasoesophageal or esophagostomy tube feeding, or parenteral nutrition. The authors found that significantly more animals that consumed > 50% of their MER were discharged from the hospital. However, patients with higher PSS also received < 66% of their MER, indicating a potential confounding effect. Unfortunately, the authors did not go on to perform a multivariate analysis in this study and so their results should be taken with caution.

In contrast, nutritional interventions have been shown in specific diseases to improve outcome. Liu *et al.*[151] concluded, after controlling for age, route of nutrition (enteral *vs* parenteral), time to intervention (early *vs* delayed), and severity of disease, that dogs with septic peritonitis who received nutrition within 24 hours postoperatively were hospitalised for 1.6 days less compared to those who did not eat for the first day. In addition, Mohr *et al.*[152] investigated cases of canine parvoviral enteritis and the effects of early enteral nutrition on intestinal permeability, protein loss and overall outcome. They found that dogs receiving early enteral nutrition by nasoesophageal feeding tubes

had a marked increase in body weight during hospitalisation, and decreased intestinal permeability, as assessed by lower urinary lactulose recovery (%). The authors concluded that early nutritional support improved gut barrier function in these patients, which can improve outcome by limiting the absorption of endotoxins and reducing the risk of bacterial translocation and subsequent septicaemia. These two studies highlight that in certain diseases, providing assisted feeding to at-risk patients may provide benefits that are more significant than simply maintaining body weight.

1.4.3 Hyperglycaemia and the perils of overfeeding

Hyperglycaemia is a common clinical feature in critically ill patients.[153–155] In veterinary patients receiving parenteral nutrition, hyperglycaemia is the most common metabolic complication, followed by hyperlipidaemia and hyperbilirubinaemia.[156,157] The terms "diabetes of injury" and "stress hyperglycaemia" describe an altered metabolic state induced by illness in which non-diabetic patients have a transient increase in blood glucose concentration.[158] Hyperglycaemia during sepsis is caused by increased catecholamines and inflammatory cytokines, leading to increased endogenous glucose production along with a reduction in glucose transporter 4 (GLUT4) expression in skeletal muscle cells.[159–162]

Hyperglycaemia increases entry of glucose into cells which are slow to downregulate transport, such as vascular endothelial, mesangial and neuronal cells.[163–166] This influx of glucose causes glycation of cellular proteins, cellular dysfunction, and increased reactive oxygen species (ROS) generation, which causes impaired wound healing, neuronal dysfunction, vasculitis, and an increased risk of systemic infections.[165,167–171] A study by Esposito *et al.*[172] examined the effect of an acute rise in blood glucose using a glucose-clamp model in healthy individuals, and found a significant increase in concentrations of inflammatory cytokines, interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), in serum. So even in healthy individuals, an acute rise in blood glucose above normal concentrations causes a measurable increase in inflammatory mediators.

In human patients, the high prevalence of hyperglycaemia in the hospital and its associated negative consequences has been well documented. In an impressive study

analysing the blood glucose concentrations of over one million patients from 126 different hospitals, the prevalence of hyperglycaemia (>10 mmol/l) was reported in 46% of ICU patients and in 31.7% of non-ICU patients.[173] Pieralli *et al.*[174] documented that nearly a third of hospitalised internal medicine patients were hyperglycaemic, and those patients had an increased LOS and a greater risk of mortality. In another hospital study, after adjusting for age, gender, BMI, race, and reason for admission, hyperglycaemia remained an independent predictor of mortality in non-diabetic patients.[175]

To address this, Van den Berghe *et al.*[176] performed a landmark study in 2001 where the authors examined the effect of intensive insulin therapy on mortality and morbidity (infections, polyneuropathy, multiple-organ failure) in 1,500 hospital patients. By maintaining patients at a blood glucose at or below 5.5 mmol/L, this reduced in-hospital mortality by 32%. In addition, there were significantly fewer complications (septicaemia, azotaemia, and polyneuropathies) in the glucose-regulated group compared to the control group. In another study that matched two groups of hospitalised patients based on age, sex, race, presence of diabetes mellitus, and disease severity, maintaining normal blood glucose concentrations reduced LOS by 10.8% and mortality by 29.3%.[177] However, despite these two impressive findings, not all studies have shown an effect. A meta-analysis found only a decreased risk of infection (risk ratio 0.41 [95% CI 0.21 – 0.77]) that was associated with intensive glycaemic control in hospitalised patients.[178] However, the studies included in this analysis did not contain critically ill patients, who are those more likely to undergo assisted feeding, and to be hyperglycaemic. There is stronger evidence of a true negative effect of hyperglycaemia in critically ill patients, which has led to the American Society for Parenteral and Enteral Nutrition (ASPEN) to provide clinical guidelines for monitoring blood glucose when performing assisted feeding in these critically ill patients.[179]

In veterinary medicine, Torre *et al.*[158] determined that 16% of hospitalized dogs (n = 38) in a veterinary teaching hospital ICU were hyperglycaemic. Approximately 75% of the hyperglycaemic dogs were hyperglycaemic at presentation, and the remaining 25% developed hyperglycaemia during hospitalization. Interestingly, canine patients that

developed hyperglycaemia in the hospital had a longer LOS compared with those presenting with hyperglycaemia ($P = 0.001$). Also, all patients with hyperglycaemia had a higher incidence of septic complications and mortality. Only four of the dogs in this study with hyperglycaemia received parenteral nutrition, which suggests the hyperglycaemia in general was not the result of overfeeding, at least by parenteral nutrition. In cats, a study determined that a large percentage of hospitalised cats were found to be hyperglycaemic at admission (63%; n = 116), and there was a significant association between hyperglycaemia and an increased LOS.[180] However, neither of these studies described when the blood glucose measurement was taken in relation to the last meal or discussed the effect of stress. In addition, both studies specify that the highest blood glucose reading taken each day was used as the representative in-hospital blood glucose measurement for each patient. Therefore, although these two studies suggest that hyperglycaemia is more prevalent in veterinary patients than in human patients, this may be a false elevation due to reporting bias. Additional studies are required to determine if indeed veterinary patients are more at risk of hyperglycaemia than human patients, and whether hyperglycaemia is indeed associated with similar negatives outcomes.

Other negative effects of overfeeding include gastrointestinal intolerances, azotaemia, and hypercapnia.[181,182] In order to avoid these detrimental effects, some advocate purposeful underfeeding of hospitalised patients. Heyland *et al.*[183] analysed the relationship between intake and 60-day hospital outcome and found that an intake of 85%-100% of RER was associated with the best clinical outcome, and no additional benefits were obtained by feeding more than 100% of RER. An interesting hypothesis that can be generated from this is that there may be an optimal "Goldilocks" range of caloric intake where the consequences of overfeeding (such as hyperglycaemia) is prevented, but adequate nutrition intake for patients is still maintained.

1.5 The loss of muscle mass during hospitalisation

Having low muscle mass at admission and losing muscle during hospitalisation are other factors associated with negative prognoses and outcomes in human patients.[184–186] In a study of 995 patients, 37% of short-stay patients (1 - 2 days) and 55.6% of patients

who were hospitalised > 12 days had poor muscle mass.[187] In addition, low muscle mass at admission was associated with an increased LOS, however only the age of the patient was considered as a potential confounder by the authors in this study. In a study by Weijs *et al.*[188], the authors compared muscle mass in patients at the level of the third lumbar vertebra and their risk of mortality. They found that having lower muscle mass at admission was associated with a higher risk of mortality which was independent of sex, diagnosis, and disease severity as assessed by the Acute Physiology and Chronic Health Evaluation (APACHE) II scoring system. In another hospital study using multivariate logistic regression analysis controlling for age, sex, and injury severity, BMI and serum albumin were not significantly associated with in-hospital mortality, whereas decreased muscle mass was ($P = 0.025$).[189]

Muscle loss is a marker of malnutrition, but it may also affect the mobility and strength of a patient, which can also influence outcome. The term 'functional impairment' is used in human medicine to describe reduced muscular strength, leading to weakness, impaired mobility and a greater risk of falls.[190,191] In a systematic review, there was a strong association between muscle weakness and mortality in both critically ill hospitalised patients and those with chronic diseases.[192] Also, in one particular study, reduced muscle mass, weakness, and diminished physical function (specifically the ability to rise from a chair repeatedly and walking speed) in aging humans were associated with a greater risk of subsequent hospitalisation.[193] Interestingly, although the loss of lean mass in this study was independently associated with weakness, the maintenance or even gain of muscle mass was not associated with the maintenance or gain of muscle strength. This suggests that preventing muscle loss may not necessarily preserve function. This was confirmed in a study by Zachwieja *et al.*[194] where administering testosterone prevented the loss of muscle mass during bedrest, but it did not preserve muscle strength.

By comparison, the significance of muscle loss in hospitalised veterinary patients on outcomes is largely unknown. However, it can be hypothesised that hospitalised patients with loss of muscle mass and strength could have decreased ambulation and delayed recovery, which may influence decisions to euthanise. Improving recovery

time by maintaining muscle mass and strength in veterinary patients may improve outcome, yet this too remains an unresearched area. With strong negative associations of muscle loss at least in human medicine, understanding the prevalence and effect of muscle loss in veterinary medicine is equally important. The loss of muscle can result from insufficient caloric and/or protein intake, cachexia, disuse atrophy and/or inflammation, each of which will be explored in the following sections.

1.5.1 Nutritional and non-nutritional causes of muscle loss in hospitalised patients

1.5.1.1 Physiological adaptation to short-term fasting and prolonged starvation

One of the main causes of muscle loss is through insufficient caloric and/or protein intake. The control of food intake is a complex process that involves several hormones including ghrelin, insulin, glucagon, glucagon-like peptide 1, leptin, and glucocorticoids. These hormones contribute to the regulation of food intake, nutrient storage and nutrient utilisation.[105,195] Ghrelin is secreted by X/A-like cells in the stomach during the fasted state, with serum concentration continuing to increase leading up to the next meal, which then quickly falls when food is ingested.[196] Ghrelin has a direct orexigenic effect on the arcuate nucleus in the hypothalamus, hence its popular name, "the hunger hormone".[195] Once food is present in the lumen of the small intestines, L cells throughout the small intestines are stimulated to secret glucagon-like peptide 1 (GLP-1). Along with the rise in blood glucose and amino acids that occurs after a meal, GLP-1 stimulates insulin secretion from pancreatic β-cells. Insulin is secreted in a biphasic manner following a meal, and diminishes as blood glucose returns to basal concentrations.[197] As blood glucose and insulin subside, pancreatic α-cells release glucagon which mobilises glycogen and fat stores during times of fasting.[198] Glucocorticoids also increase the mobilisation of fat and amino acids from muscle cells, and are secreted under the regulation of the hypothalamic-pituitary-adrenal axis in response to fasting and stress.[199] Finally, leptin is secreted by white adipocytes and serves as a signal for satiety post-prandially, and functions as an indicator of whole body energy stores in the fasted state.[100] As discussed previously, circulating leptin concentrations decrease both with reduced food intake and with body fat mass loss, and has a direct effect on immune function.[98,102]

In times of fasting or starvation, the body must rely on endogenous sources of energy through the processes of glycogenolysis, gluconeogenesis, lipolysis and ketogenesis.[200] The fasted state is commonly characterised by three phases relating to the primary fuel utilised, and in particular whether protein is utilised or spared. The phases do overlap each other depending on factors such as the contents of the last meal, body composition of the individual, previous fasting experience and species differences.[201–206]

Phase 1 "Post-absorptive state" (hours to days)

- Hepatic and muscle glycogenolysis provide the predominant forms of energy by mobilizing liver and muscle glycogen stores. Protein catabolism and lipolysis increases, providing glucogenic amino acids and glycerol respectively for gluconeogenesis.
- Leptin concentration begins to fall, and ghrelin concentration begins to rise.
- This phase is characterised by a greater muscle catabolism than in the next phase (phase 2).

Phase 2 "Adaptive substrate utilisation state" (days to weeks, or longer)

- Metabolic rate and muscle catabolism are slowed. Lipolysis and ketogenesis are the predominant forms of energy production.
- Muscle loss is less than during phase 1.

Phase 3 "Final stage of starvation" (once lipid stores are depleted)

- Critical period characterised by an increase in amino acid oxidation relative to phase 2 as fat stores are exhausted.
- There is a decrease in ketogenesis and an increase in the rate of muscle loss compared to phase 2.

The body's adaptive mechanisms to utilise different substrates for energy during short- and long-term fasting allows for the greatest chance of survival during food deprivation. In the very early stages of fasting, the concentration of insulin in plasma decreases and there is an increase in the secretion of the pancreatic peptide hormone,

glucagon.[207] Once bound to its receptor on hepatocytes, glucagon initiates glycogenolysis and inhibits glycogen synthesis.[208] Hepatic glycogenolysis provides the majority of the glucose requirement in early fasting, with hepatocytes utilising more than half of the total glucose produced during the first 22 hours of fasting in humans.[209] Cells of the central and peripheral nervous system, erythrocytes, resting muscle cells, and the renal medulla consume the majority of the rest.[210,211] Muscle also undergoes glycogenolysis, and the concentration of glycogen in skeletal muscle cells is greatly reduced after a 24-hour and 48-hour fast in rodents.[212,213]

As glycogen becomes depleted, *de novo* synthesis of glucose occurs in hepatocytes, renal and intestinal cells using amino acids, glycerol, lactate and pyruvate.[214,215] Amino acids from muscle contribute to these substrates with the rate of muscle loss highest during the first phase of starvation. Muscle is catabolised as the result of low insulin and increased glucocorticoid concentrations, which activate the ubiquitin-proteasome pathway.[216] Muscle catabolism also occurs through lysosomal proteolytic pathways.[217] Gluconeogenesis from amino acids obtained from muscle account for 10 -15% of the adenosine triphosphate (ATP) produced in early fasting.[218] All amino acids (except leucine and lysine) are potentially gluconeogenic, however alanine and glutamine are the most abundant amino acids released from muscle, and have a high rate of uptake by hepatocytes for gluconeogenesis.[200,201,219] For alanine, this amino acid only makes up 7-10% of total muscle protein amino acids, yet its serum concentration increases by 60% following a 60-hour fast in man.[220] This is because alanine is synthesised from the transamination of pyruvate in myocytes during muscle catabolism.[218] Further, an interesting phenomenon is that while all skeletal muscle may be catabolised for energy, not all muscle types are catabolised at the same rate. Allen *et al.*[221] established that in mice after two days of food deprivation, there was a significant increase in mRNA levels of ubiquitin ligases MuRF-1 and atrogin-1 in fast-twitch type II muscles, but not in slow-twitch type I muscles.

1.5.1.2 The effect of disease and injury on muscle loss

Cachexia is defined as a "complex, multifactorial syndrome characterized by the loss of muscle mass (with or without the loss of fat mass) due to increased protein catabolism as the result of underlying disease(s), which is not reversible using conventional

nutritional intervention methods".[222,223] During this state, an increase in inflammatory cytokines (IL-1β, IL-6, and TNF-α) alters centrally-mediated signals of food intake, whilst also shifting metabolism towards greater muscle catabolism.[185,224–226] Cytokines IL-6 and TNF-α also have been shown in experimental models of cachexia to cause insulin/IGF-I resistance, a decrease in testosterone (an anabolic stimuli for skeletal muscle growth), and to initiate muscle catabolism.[227–233] The combination of decreased food intake and a hypercatabolic state leads to a rapid depletion of glycogen stores and an increase in proteolysis over lipolysis compared to what occurs in simple starvation of healthy individuals.[184,222,225,234,235]

Muscle catabolism in cachexic patients is mediated primarily by the activation of the ubiquitin proteasome system (UPS) and autophagy.[217,227] The UPS is an adenosine triphosphate (ATP)-dependent system that attaches ubiquitin (Ub) molecules onto proteins, marking them for degradation.[236,237] Two E3 ubiquitin ligases, MuRF1 and MAFbx, have been identified as the main contributors to muscle atrophy.[238] For autophagy (derived from Greek, meaning '*to eat oneself*'), this is a process of untargeted cellular degradation that is influenced by nutritional status, hormones, oxygen concentration, and intra-cellular protein aggregation.[239] Both UPS and autophagy are under the regulation of the phosphoinositide 3-kinase (P13K)/protein kinase B (AKT) mammalian target of rapamycin (mTOR) pathway and FOXO family transcription factors.[240]

In a non-cachexic state, once insulin is bound to its receptor, it activates AKT, which increases AMP-activated protein kinase (AMPK) and protein synthesis through the mTOR pathway.[241,242] However, when IL-6 and TNF-α are present, these cytokines activate protein kinases that inhibit the action of insulin by phosphorylation of the insulin receptor substrate 1, ultimately reducing mTOR signalling and muscle anabolism.[236,243,244] Impaired insulin/IGF-I sensitivity and glucocorticoids also decreases P13K-AKT-mTOR signalling in myocytes, which also activates the UPS and autophagy system.[245] Glucocorticoids bind to cytosolic receptors in myocytes which are then translocated into the nucleus and attaches to glucocorticoid response elements, leading to increased expression of ubiquitin ligase MuRF1, amongst many other genes.[246]

Similarly, an increase in gene transcription of the UPS occurs during a state of metabolic acidosis.[247]

1.5.1.3 The vicious circle of inactivity

Disuse atrophy of skeletal muscle can occur from chronic bedrest, casting or immobilisation. Inactivity has been shown to blunt the normal muscle anabolic response by mTOR that occurs from a rise in plasma amino acids following a meal.[248] In addition, bedrest for just seven days in healthy adults caused significant increases in IL-6 in skeletal muscle. Also, similar to the effects of glucocorticoids and insulin/IGF-I resistance, inactivity has been shown to cause a suppression of AKT leading to an increase in ubiquitin ligases MuRF1 and MAFbx.[249,250]

The types of muscle fibre lost due to inactivity varies between species.[251] In the rat, slow-twitch type 1 fibres atrophy at a greater rate compared to fast-twitch type 2 fibres.[252] In humans studies, conflicting results indicate that either slow-twitch fibres appear to atrophy quicker, or that both types of fibres atrophy at the same rate.[252,253] In dogs, a study of 10-weeks of disuse of the hindlimb found that muscles with a larger portion of slow-twitch fibres that also crossed a single joint, such as vastus medialis and vastus intermedius, were most at risk of atrophy.[254] Interestingly, the highest percentage of slow-twitch fibres are found in deep extensor muscles of the forearm and hindlimb in the dog, while in the epaxial muscles contain approximately a 50 - 50 combination of the two fibre types.[255] The epaxial muscles are commonly used for muscle mass palpation and assessment in veterinary medicine. However, they may not be the best marker for muscle loss. Therefore, more work needs to be done to determine which muscle groups are the most appropriate to monitor in veterinary patients during different conditions of muscle loss.

1.5.2 Summary of muscle loss in hospital patients

The loss of muscle and function in hospitalised patients can occur from a combination of malnutrition, inflammation and inactivity. The studies examined provide evidence of a strong association between the loss of muscle and a poorer prognosis in human patients. Yet, despite the clear prevalence and negative consequences of muscle loss in human patients, it is currently unknown how much muscle is lost in patients during

hospitalisation in veterinary medicine, or how it relates to their prognosis. Veterinary patients may also be at greater risk of mortality through euthanasia if the loss of muscle and function causes an increase in LOS and perceived poor quality of life.[225] Thus, this remains an important area which needs to be explored.

1.6 The other side of the coin: the potential benefits of intermittent fasting

Whilst undernutrition in hospital patients is evidently detrimental, undergoing short periods of fasting may actually be beneficial for health. The beneficial effects are thought to be linked to a shift in metabolism. The following sections will focus on fatty acid metabolism during fasting, the diseases where caloric restriction or intermittent fasting have been found to be beneficial, and the proposed mechanisms behind it.

1.6.1 Adaptive fasting physiology: lipolysis and fatty acid metabolism

As the duration of energy deprivation prolongs, the body increases the utilisation of adipose stores for energy and reduces muscle mass loss. In man, fatty acid and glycerol turnover increases significantly between 18 - 24 hour of fasting.[256] Impressively, only about 2% of total body muscle mass is lost in the first 20 days of starvation.[234] The ability of the body to mobilise and metabolise fat significantly reduces the amount of muscle lost during prolonged fasting, which is a vital adaptation for survival.

Lipids in the body are mainly stored as triacylglycerols (TAGs) in adipose tissues. Lipolysis occurs through the actions of adipose triacylglycerol lipase and hormone-sensitive lipase (HSL), enzymes that are triggered by a fall in insulin and a rise in glucocorticoids, epinephrine and glucagon concentrations that occurs during fasting.[217,257,258] Adipocyte cell membranes contain β-adrenergic receptors and glucagon receptors, the activation of which then leads to the activation of protein kinase-A in the cytosol. Activated protein kinase-A then phosphorylates perilipin A (a surface protein found on the adipose cell membrane which translocates activated HSL) and HSL, which then initiates lipolysis and the hydrolysis of triacylglycerol to diacylglycerol and monoacylglycerides.[259,260] Monoacylglycerol are further catabolised to fatty acids and glycerol by monoacylglycerol lipase.[261,262] The resulting fatty acids can then be oxidised directly by the cell, but the great majority are released into circulation where they bind

to lipophilic sites on carrier proteins, mainly albumin, for transport and use in other tissues.

Fatty acids undergo β-oxidation in the mitochondria or peroxisomes of cells. Short-, medium-, and long-chain fatty acids (1-5, 6-12, 13-21 carbon length respectively) are oxidized exclusively in the mitochondria, while very-long-chain fatty acids (≥ 22 carbon length) are oxidized mainly in peroxisomes.[263–265] Very-long-chain fatty acids cannot freely traverse the mitochondrial membranes, whereas short- and medium-chain fatty acids can freely diffuse into the mitochondria and undergo the first step of β-oxidation within the matrix.[266,267] For this first step, free fatty acids are converted to acyl-CoA by acyl-CoA synthetases located either in the outer mitochondrial membrane for long- and very-long-chain fatty acids, or within the matrix of the mitochondria for short- and medium-chain fatty acids.[217,264] Once converted, they can be transported through the inner mitochondria membrane by the "carnitine shuttle". This process involves the initial conversion of acyl-CoA to fatty acylcarnitine by carnitine palmitoyl transferase (CPT1) located on the outer mitochondrial membrane. Acylcarnitine is then translocated across the inner mitochondrial membrane via the carnitine acylcarnitine translocase antiporter. Once in the mitochondrial matrix, carnitine palmitoyl transferase II reverses the reaction to release acyl-CoA and carnitine.

Once acyl-CoA arrives in the matrix of the mitochondria, cleavage of 2 carbon units from the β-carbon position occurs in a process of oxidation, hydration, and oxidation. The final end products are acetyl-CoA, reduced flavin adenine dinucleotide and reduced nicotinamide adenine dinucleotide (NADH). Acetyl-CoA can be used in the citric acid cycle to generate ATP or, in certain cells, be used to synthesise ketones.[217] The pathway of oxidation for long- and very-long-chain fatty acids is similar but starts when they enter peroxisomes. There, a series of hydration and oxidation steps occur to produce long- and medium-chain fatty acyl-CoA molecules, which are then transported out of the peroxisome and into the mitochondria to undergo final β-oxidation.

1.6.1.1 Ketogenesis

Ketones are hydrophilic metabolites that play a vital role in survival during long-term fasting/starvation. Hepatocytes and intestinal epithelial cells are considered the main organs that produce ketones, however renal, pancreatic β-cells, retinal pigment epithelium, skeletal muscle, astrocytes, and tumour cells have also been touted as possible tissues that may also undergo ketogenesis, although at lower rates.[268,269] The process of ketogenesis is shown in Figure 1-1. In ketogenic cells during prolonged fasting, the concentration of intracellular acetyl-CoA increases due to increased rates of both fatty acid and amino acid oxidation. The fate of acetyl-CoA at other times is for it to enter the citric acid cycle where it is condensed with oxaloacetate to form citrate, and eventually is oxidized to CO_2. In times where oxaloacetate concentrations are low due to a low rate of glycolysis and pyruvate generation, excess acetyl-CoA is used for ketone formation.[270,271] The concentration of mitochondrial oxaloacetate has been shown to be inversely related to the rate of ketone production in hepatocytes.[272]

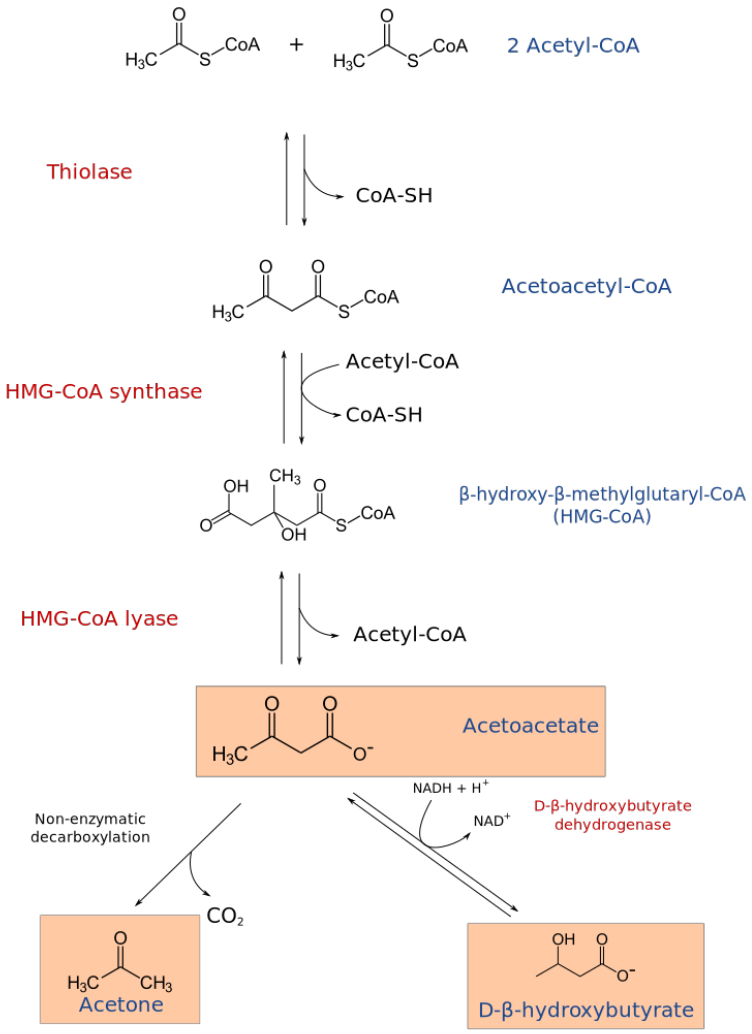

Figure 1-1. The ketogenesis pathway displaying the formation of acetoacetate, β-hydroxybutyrate, and acetone. When the intracellular NADH:NAD^+ ratio is high, this favours the formation of β-hydroxybutyrate. This figure is licensed under Creative Commons zero (CC0).

The synthesis of ketones begins with the condensation of two molecules of acetyl-CoA to acetoacetyl-CoA by acetoacetyl-CoA thiolase. Then, acetoacetyl-CoA is condensed with an additional acetyl-CoA by HMG-CoA synthase to form β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). HMG-CoA synthase is found in greatest abundance in the liver and intestinal cells, and the reaction to form HMG-CoA is considered a rate limiting step for ketogenesis.[268,273] The final step occurs when HMG-CoA is converted to acetoacetate by HMG-CoA lyase. Acetoacetate can then either leave the cell unchanged, undergo spontaneous decarboxylation to acetone or be converted to β-

hydroxybutyrate via β-hydroxybutyrate dehydrogenase intracellularly. The conversion of acetoacetate to β-hydroxybutyrate is dependent on the intracellular ratio of NADH:NAD+ (redox potential) which increases during β-oxidation of fatty acids, ultimately favouring the formation of β-hydroxybutyrate.[274–277]

Ketones play a vital role in the sparing of glucose during prolonged fasting, limiting the need for gluconeogenesis. In rats, there is a marked reduction in glucose utilisation by skeletal muscles after two days of fasting, as the utilisation of fatty acid and ketone oxidation increases.[278] Extrahepatic tissues which utilise ketones, notably skeletal muscle and neurons, uptake ketones by monocarboxylate transporters, and undergo a process to reverse ketones back to acetyl-CoA. For this, succcinyl-CoA:3-oxoacid-CoA transferase (SCOT) converts acetoacetate to acetoacetyl-CoA in the mitochondria and a thiolase hydrolyses the compound back into two acetyl-CoA for Krebs utilisation. Importantly, to prevent a futile cycle of ketone production/utilisation in the liver, the SCOT enzyme is not expressed in hepatocytes.[268]

The concentration of ketones presents in circulation after a defined period of fasting varies in different species. In primates, including humans, a rise in ketones occurs more rapidly compared to rodents, rabbits, guinea pigs and dogs fasted for a similar period of time.[279,280] In particular, dogs are known to have a slower rise in ketone concentrations compared to man.[281] Variation in the rates of ketogenesis and/or clearance of ketones from circulation is thought to account for these species differences.[200]

1.6.1.2 Regulation of Ketogenesis

There are multiple points in the pathway that can affect the rate of ketogenesis, including the availability of substrate, and the expression of HMG-CoA synthase.[271,282] Substrate concentration is dependent on lipolysis by HSL via glucagon and epinephrine.[283] Transcriptional regulation of HMG-CoA synthase occurs through the actions of peroxisome proliferator-activated receptors (PPARs).[271] An increase in PPARs occurs during fasting that leads to increased expression of genes coding for HMG-CoA synthase.[273,284] In addition, Sirtuins, a family of conserved protein deacetylases, act as sensors to detect low cellular energy as signalled by increasing

intracellular NAD^+ concentration.[285] Sirtuin 1 and 3 deacetylase the HMGCS1 and HMGCS2 genes respectively, leading to increased production of HMG-CoA synthase.[286–289] Further, posttranslational modification occurs through allosteric inhibition by succinyl-CoA, which is desuccinylated in the presence of glucagon.[290]

1.6.1.3 Special properties of medium-chain fatty acids

The rate and timing of hepatic ketone production can be affected by different types of dietary fat, most notably by medium-chain triglycerides which promote ketogenesis. Medium-chain triglycerides (MCTs) are composed of fatty acids with a 6 - 12 carbon length and are metabolized differently to triglycerides composed of long-chain fatty acids. After consumption, MCTs are more rapidly hydrolysed and their fatty acids absorbed more quickly in the intestines compared to long-chain TAGs.[291,292] In addition, a proportion of medium-chain fatty acids are not packaged into chylomicrons for transport through the lymphatics, but instead are transported directly to the liver via the portal vein.[293] As mentioned above, once in hepatocytes, medium-chain fatty acids can freely diffuse into the mitochondria for oxidation as their entry is not regulated through CPT1.[294,295] In both humans and rats, feeding MCTs led to a marked increase in circulating ketone concentrations two hours postprandially compared to feeding long-chain triglycerides.[296,297] Thus, providing MCTs in a diet can contribute to an increase in ketone production independent of a fasted state.

1.6.2 Intermittent fasting in canids

The modern domestic dog (*Canis lupus familiaris*) is the cumulative result of thousands of years of domestication and divergence from grey wolves (*Canis lupus*).[298] Archaeological evidence and genomic sequencing indicate that early domestication may have occurred as early as the Upper Paleolithic era (16,000 BP), preceding the Neolithic era and the advent of agriculture.[299] As hominin species morphed from a nomadic, hunter-gatherer lifestyle to a more sedentary, agriculturally-based one, the dogs they were domesticating developed alongside them. Genomic mutations favouring this new co-existence gave rise to traits in the domestic dog that differ from their ancestral wolves. Genome-wide sequencing comparing the domestic dog to the modern wolf have found 36 genomic regions with differences of encoding, of which,

ten genes are involved in role of starch digestion and fat metabolism.[300] So, while domestic dogs share a common heritage with wolves, they do not have the same metabolism.

Nevertheless, it is thought provoking to consider the drastic differences in the feeding behaviour of wolves compared to how the modern domesticated dog is typically fed. The foraging behaviour of wolves entails the periodic killing of and feasting on large prey, followed by a period of fasting. Packs of grey wolves in Yellowstone National Park have been reported to kill and consume an elk every 2-3 days.[301] Contrast this to the average domesticated dog which is commonly fed twice daily. Implications of this change in feeding regimen on the domestic dog are unknown. However, with evidence that undergoing periods of fasting may be healthy, it is worthwhile considering whether the domestic dog would also benefit from a more ancestral type of feeding regimen.

1.6.3 Intermittent fasting during a disease state

Across the animal kingdom, a reduction in food intake during periods of illness, especially with concurrent pyrexia, is an evolutionarily conserved trait.[302–305] This illness-induced decrease in food intake is an acute phase response in which the release of cytokines IL-1β and TNF -α affect the hypothalamus to induce anorexia.[306–309] The evolutionary benefit of this phenomenon is not clear, but notions such as the reduction of iron availability for pathogens and the conservation of energy by reduced foraging behaviour have been theorized.[302,304,310] Out of these, the concept of energy conservation is flawed, as there is a concurrent reduction in food intake. Other postulated mechanisms include the increase in immune surveillance, increase in autophagy to remove intracellular pathogens (xenophagy), and the limitation of ROS production.[311–315] In support of anorexia being beneficial, experimental models of sepsis have shown that supplemental feeding increased rates of mortality.[316] Thus, could there actually be an advantage to a reduction in food intake?

In medicine, the term *hormesis* describes the adaptive mechanisms of an organism in response to moderate, intermittent stresses, such as short-term food deprivation.[317] While conventional wisdom would dictate that any depletion of nutrients for a

heterotroph would be undesirable, the notion that restricting food intake can improve health and extend lifespan has been present since the 15th century, as reviewed by Speakman and Mitchell[318]. Today, there is a growing body of evidence in numerous species that restricting calories or extending the fasting period between meals is healthy.[318–320] These studies commonly employ one of two different feeding models: caloric restriction or intermittent fasting, both of which have been shown to produce beneficial health effects.[321–323] Caloric restriction (CR) is defined as the reduced intake of calories (typically 10 - 50%) below that of *ad libitum* fed counterparts.[318,324] However, the distinction between intermittent fasting (IF) and CR can be unclear, and as these regimes ultimately lead to different metabolic states, it is important to differentiate between them.[321] In this dissertation, an IF regimen will be defined as one where the time between meals is extended (the length of which will be defined accordingly in each study), but where calories are not restricted, and where weight loss is not intended.

The beneficial effects of CR and IF have been demonstrated in single celled organisms (e.g. *E. coli, S. cerevisiae*) to more complex organisms (e.g. *C. elegans*, *Drosophila,* rodents, non-human primates and man).[318,325,326] However, there are only a few studies examining these regimens in dogs. In a study where dogs were fed 25% less than their paired litter mates over a lifetime, CR dogs developed chronic diseases later in life and had a significantly longer lifespan compared to those who had higher food intake.[327,328] In another study of racing greyhounds, food restriction by 15% for eight weeks leading up to a race caused a reduction in body weight, but actually improved sprinting performance.[96] More recently, a 2018 abstract presentation of intermittent CR as a mean of achieving weight loss in dogs showed that dogs undergoing this regimen maintained higher metabolic rates than dogs undergoing continuous CR.[329] This study suggests that these dogs may have a lower risk of rebound and weight gain following the cessation of the weight loss programme. While the body of work in this area is currently limited to these few studies in dogs, there is far more research and growing evidence of a beneficial effect of CR and IF in other species; so, a wider examination is described below.

1.6.3.1 Diseases where caloric restriction or intermittent fasting may be beneficial

1.6.3.1.1 Diabetes

There are numerous publications examining the effects of CR and IF on fasted blood glucose and fructosamine concentrations in healthy and diabetic rodents and humans. In a study of fasting rats, a longer duration of fasting 16 hours *vs* 4 hours was positively correlated with insulin sensivity.[330] In another study comparing rats undergoing either *ad libitum* feeding, CR of 60% of *ad libitum*, or an alternate day IF regimen of *ad libitum* feeding, the IF and CR rats both had higher insulin sensitivity as measured in a glucose tolerance test compared to every day *ad libitum* fed rats after four weeks.[331] Furthermore, in experimentally induced diabetes mellitus type I, IF rats had lower blood pressure, increased superoxide dismutase activity, decreased plasma malondialdehyde (a marker of lipid peroxidation), reduced blood urea and creatinine and less histological signs (tubular vacuolations, glomerular hypertrophy) of renal damage compared to the non-fasted control group.[332] In the same study, the authors also found that there was a marked increase in p53 expression and decrease in Sirtuin 2 expression in the kidneys of diabetic rats, but that increase was not seen in the rats that were intermittently fasted. Increased activity of Sirtuins inhibit p53-dependent apoptosis in response to DNA damage and oxidative stress, which may reduce the loss of cells under diabetic conditions.[333] Taken together, it appears that an IF regimen improves insulin sensitivity, is protective against hypertension and oxidative damage, and reduces the nephropathy seen in experimental models of diabetes.

In humans, followers of Ramadan undergo an IF regimen and abstain from eating and drinking during daylight hours. The period of fasting can extend between 13 - 18 hours depending on the season and geographical location of the participant and is adhered to for approximately 30 days.[334] Diabetic patients participating in Ramadan have consistently been reported to have improved insulin sensitivity during the fasting period.[313,335,336] The patients' BMI, activity and weight loss during this IF period are possible confounders in these studies, which are known to directly affect insulin sensitivity. However, in multiple meta-analyses which include studies that adjusted for these covariates revealed a reduction in the odds of developing diabetes in individuals practicing CR or IF, and a reduction in fasting glucose and insulin in those who were

pre-diabetic.[319,337,338] These consistent and repeatable findings in both animal and human studies provide strong evidence that IF improves insulin sensitivity, antioxidant status and decreases sequelae in pre-diabetic and diabetic patients. However, these effects have not been examined in the young or elderly, nor have studies been performed to assess its efficacy long-term.

1.6.3.1.2 Ischemic injury

Both caloric restriction and intermittent fasting improve outcomes in cardiovascular ischemic and reperfusion injury.[326,339,340] Followers of the Church of Latter-Day Saints also practice an IF regime (1 day/ month). In a study of these followers, after adjusting for age, gender, BMI, smoking activity, physical activity, income and level of education, fasting-followers reportedly had an odds ratio of 0.56 (95% CI 0.36-0.88, P = 0.012) for diabetes and an odds ratio of 0.65 (95% CI 0.46-0.94, P = 0.008) for coronary artery disease.[338] Followers of Ramadan also had significantly lower circulating concentrations of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, and lower systolic and diastolic blood pressure compared to one month after the cessation of the Ramadan period.[341] However, the participants lost body fat mass during Ramadan and increased more body fat mass after the fasting period. Loss of body fat mass reduces IL-1β, IL-6 and TNF-α, while increasing adiposity increases them.[342–344] Therefore, it is unknown in these cases if the effect on the cytokines is simply a reflection of body fat mass change, rather than the intermittent fasting regimen itself.

Experimentally however, the effect is clearer. In a model of global ischemia in rats where whole body blood flow is reduced and re-perfused, short-term CR of 70% of intake of *ad libitum* controls for 11 days led to better cardiac contractility and coronary flow rate, and lower serum concentrations of glucose and insulin post injury compared to control rats.[345] For intermittent fasting, rats undergoing IF (*ad libitum* feeding every other day) prior to coronary artery ligation had less myocardial necrosis, tissue remodelling and functional decline compared to rats fed *ad libitum* daily.[346] The IF group also had reduced cardiac tissue inflammation on histology and an overall lower mortality rate. These results were also confirmed in another similar study of myocardial ischemia in rats.[347] Interestingly, in that study, the rats began the IF regimen after surgery and yet still had decreased fibrotic areas of the cardia, reduced

oxidative stress (as measured by thiobarbituric acid reactive substance), better cardiac performance and a higher overall survival rate compared to those fed *ad libitum*. This suggests that a protective effect of IF is present if the regime begins before or after injury, and it is possible that the effect may be equivalent even if instigated after injury. However, more studies are required to determine if this is the case for all disease processes.

1.6.3.1.3 Neuronal injury

There are numerous rodent studies that show that caloric restriction and intermittent fasting (food offered *ad libitum* every second day) reduce the extent of injury and improve recovery following neuronal injury.[348,349] In a study where IF was implemented immediately after spinal cord injury, there was a 50% reduction in lesion size, increased growth of corticospinal axons and a quicker recovery of gait and function compared to continuously fed, *ad libitum* mice.[350] The authors also measured β-hydroxybutyrate, and there was a clear cyclical rise and fall in ketone concentrations on the day of fasting and day of feeding, respectively. In addition, rodents in an IF regime that started either 3 weeks before or 24 hours after thoracic spinal cord contusion showed recovered hindlimb motor function more quickly than those eating *ad libitum*, regardless of when the IF regime began.[351] In another study in rats, fasting for 24 hours after cortical brain injury significantly decreased necrosis, and ROS concentrations.[311] Finally, in a kainite-induced neuronal injury study, the authors compared *ad libitum* feeding with IF and caloric restriction at 60% of the *ad libitum* fed mice.[321] The feeding regimes began 20 weeks prior to inducing injury. Intermittently fasted mice consumed similar quantities of calories overall and maintained the same body weight as the *ad libitum* mice. The authors found the IF mice had the least amount of neuronal damage, higher blood β-hydroxybutyrate, and a lower circulating insulin and glucose concentration compared the control and CR mice.

The evidence of a significant neuroprotective effect of caloric restriction and intermittent fasting is repeatable. As such, studies of these feeding regimens and/or the development of drug mimetics to emulate the metabolic effects of fasting for use in neuronal injury have been advocated for humans.[352,353] However, to date, the effects have been examined in only rodent models and there are currently no published

studies examining these feeding regimens on neuronal recovery in other species. However, given the strength of evidence in rodent models, its applicability in other species, and in a clinical setting, should be explored.

1.6.3.2 Proposed mechanisms

The proposed mechanisms for the beneficial effects of caloric restriction and intermittent fasting can be generalised broadly into three categories: 1) improvement of mitochondrial function and the reduction of ROS generation; 2) regulation of gene expression relating to apoptosis, inflammation, and neuroprotective factors such as neurotrophins and chaperone proteins and 3) the promotion of autophagy. Although undoubtedly the benefits of IF are the result of a complex interplay of multiple processes, increased ketone utilisation and the effect of ghrelin are proposed as the key drivers for these mechanisms.

After injury, a large proportion of tissue damage occurs secondary to the initial trauma as the result of the development of mitochondrial dysfunction and ROS damage.[354,355] An increase in the expression of monocarboxylate transporters on the cerebral vasculature, neurons and glial cells occurs during fasting and after injury, increasing the transport of ketones.[270,356–358] Ketone utilisation has been shown to decrease ROS generation in mitochondria and increase the activity of intracellular antioxidant enzymes. Giving β-hydroxybutyrate and acetoacetate *in vitro* to neuronal cell cultures reduced the production of ROS by complex 1 of the mitochondrial respiratory chain.[359] In another study, rats fed a ketogenic diet had increased β-hydroxybutyrate concentrations in serum and a two-fold increase in glutathione concentrations in hippocampal tissue compared to rats fed a standard diet.[360] Finally, in a study of fasting rats, there was increased activity of superoxide dismutase and glutathione peroxidase in both healthy fasted and those fasted with kainic acid toxicity-induced neuronal injury.[361,362] It is believed that metabolism of ketones alters the intracellular ratio of NADH:NAD^+, increasing NADH oxidation in the mitochondrial respiratory chain, leading to less free radical generation, and increasing reduced glutathione availability and glutathione peroxidase activity.[359,363–365]

Another mechanism by which ketones are thought to be beneficial is through the reduction of inflammation and apoptosis, and the promotion of neurotrophic compounds. In a study by Loncarevic-Vasiljkovic *et al.*[366], mice were randomised into two feeding regimes: *ad libitum* and 50% caloric restriction beginning three months prior to experimentally induced brain injury. In the CR mice, there was decreased neural tissue concentrations of TNF-α and caspase-3 (a regulator of apoptosis), and neurodegeneration at the injury site compared to those not calorically restricted. In addition, intermittent fasting (every other day fasting) in young and middle-aged mice increased neurotrophic factors (brain-derived neurotrophic factor and fibroblast growth factor 2) and protein chaperones (heat shock protein 70 and glucose regulated protein), and decreased IL-6, IL-β, and TNF-α concentrations in cerebral tissue after experimentally induced ischemic stroke.[367] In addition to ketones, administration of ghrelin in experimental models of brain injury and neurodegeneration reduced neuronal loss and improved recovery and memory.[368–370] Ghrelin, which increases during fasting, up-regulates expression of Bcl-2 (an anti-apoptotic protein) and heat shock protein 70, inhibits expression of caspase (3, 8, and 9), TNF-α and IL-1β, reduces ROS, and mitigates apotosis.[368,370–372]

The final mechanism by which ketones are thought to be beneficial is through the promotion of autophagy. Inadequate autophagy and mitophagy is thought to contribute to worse outcomes in critically ill patients. A study by Gunst *et al.*[373] found that in hyperglycaemic, critically ill rabbits, those with increased hepatic and renal intracellular p62 (a marker of insufficient autophagy) had greater mortality rates. When the authors stimulated autophagy by giving rapamycin, this reduced p62 concentrations in renal cells and reduced subsequent renal damage.[373] In a model of traumatic brain injury, mice fed 30% of calories starting after injury had increased staining of Beclin1 and LC3B (markers of autophagy) compared to fully fed mice.[374] The direct effect of ketones and ghrelin on autophagy have also been demonstrated in neuronal cell cultures. The administration of ghrelin increased autophagy whereas administering ghrelin receptor antagonists decreased autophagy in neuronal cell culture.[375] In addition, providing β-hydroxybutyrate to the cultures reduced glucose deprivation-induced neuronal death, and stimulated autophagy through increasing

AMPK-dependent phosphorylation of glyceraldehyde 3-phosphate dehydrogenase, ultimately leading to Sirtuin 1 activation.[376–379]

All in all, numerous studies support the concept that CR and IF can provide health benefits in some diseases. However, to date, there are limited studies examining the effect of CR and only one study examining the effect of IF in dogs. Comparatively, the body of evidence in other species continues to grow, highlighting an interesting gap in knowledge for a feeding method in a species evolutionarily adapted to a feeding and fasting-type regimen.

1.7 Conclusion and thesis aims

Malnutrition in human hospitals is widespread and is associated with numerous negative consequences. Comparatively however, much less is known in veterinary medicine including the prevalence of malnutrition in veterinary hospitals, and the effect of muscle and fat mass loss on patient outcome. In addition, the ideal feeding regimen for hospital patients has not been established, with both overfeeding and underfeeding shown to be detrimental. Interestingly, growing evidence of beneficial effects of CR and IF has led to the promotion of such feeding regimens as being therapeutic in certain diseases. Of particular interest for this type of regimen would be for veterinary patients recovering from neuronal injury, a condition which require long periods of hospitalisation and places patients more at risk of malnutrition. However, to date, there is almost no published research on IF in dogs and so this subject area remains unknown.

The primary aims of this PhD thesis were to determine the body composition changes in hospitalised canine patients, identify the risk factors associated with losses in lean and fat mass, and to determine whether an intermittent fasting regimen may be a beneficial feeding regimen for hospital patients recovering from neuronal injury. This was achieved by firstly determining the body composition changes that occurred during weight loss in long-stay hospitalised patients, which is described in Chapter 2. Other factors including caloric intake, duration of stay, disease type and severity, and physical activity were examined to identify risks factors for total body weight loss, body fat loss, and lean muscle loss. Chapter 3 describes how morphometry was

examined as a method of determining lean mass in ideally conditioned to poorly conditioned dogs. In Chapter 4 and Chapter 5, the metabolic and immunological effects of an intermittent fasting regime in healthy dogs are described, in order to assess its potential application to hospital patients. Following this, Chapter 6 explores the ketone kinetics in healthy dogs undergoing a once-a-day and every-other-day feeding regimen. Finally, Chapter 7 describes an intermittent fasting regimen which was applied in hospitalised canine spinal patients to determine its feasibility and efficacy as a ketogenic regimen.

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Chapter 2

Body composition changes in long-stay hospitalised canine patients

2.1 Introduction

The loss of lean and fat mass during illness have different aetiologies and consequences for hospitalised patients. The loss of muscle can occur due to insufficient caloric/protein intake, cachexia, and disuse atrophy, whereas the loss of fat mass occurs primarily due to inadequate caloric intake, though losses can also be exaggerated in cachexic patients.[1–4] In humans, having a low muscle mass or losing muscle while in the hospital is associated with negative prognoses and poorer outcomes.[5–11] Similarly, in cats and dogs with cancer and cardiac disease, having a low body condition score, a measure of body fat mass, is associated with significantly shorter survival times.[12–14] When fat mass is lost, there is an accompanying drop in the adipose-derived hormone leptin, which plays a vital role in modulating both innate and adaptive immunity.[15–18] Reduced leptin due to a hypocaloric intake increases the susceptibility of mice to *E. coli* lipopolysaccharide-induced endotoxic shock.[19] In contrast, overweight and obese humans have increased serum leptin, which is associated with a surprisingly reduced mortality rate in those with septicaemia, cardiac disease and renal disease compared with lean individuals.[20–24] Therefore, it appears that preservation of not only lean, but also of fat mass, is of importance for hospitalised patients.

It has been documented that the longer a veterinary patient stays in hospital, the greater the risk of reductions in body weight and BCS.[25] Body weight, muscle condition scoring (MCS), and body condition scoring (BCS) are the most commonly used parameters for assessing malnutrition in veterinary medicine.[26–28] However, the information provided by these frequently used measures are limited. Total body weight change is a crude measure that does not differentiate between lean and fat mass, or changes in hydration. Conversely, BCS and MCS have been developed to specifically assess fat mass and lean mass respectively. Nevertheless, the scoring categories are broad and may be insensitive to subtle changes that may occur during hospitalisation. Further, neither have been validated for use in hospitalized patients.

Thus, we currently lack a clear understanding as to what changes occur in lean and fat mass during hospitalisation in veterinary patients, and whether the current scoring systems accurately measure these changes.

The aims of the studies described in this chapter were to firstly determine the range of the length of stay for hospitalised dogs in a veterinary teaching hospital. From this, the minimum number of days for a dog to qualify as a "long-stay" hospital patient was defined. The second aim was to determine the changes in body composition during hospitalisation using an isotopic dilution technique, and to compare the results with the current standard methods of measuring body composition in veterinary patients. Finally, the severity of the patient's disease, caloric intake, duration of stay, and physical activity were measured and examined as potential risk factors for body composition changes.

2.2 Materials and Methods

2.2.1 Historical clinical records collection

In order to define "long-stay" for the prospective study, clinical data was extracted from the records of dogs seen by the internal medicine or surgical department at Massey University Veterinary Teaching Hospital (MUVTH). Records included were for hospital admissions between November 2013 – March 2014, and the data was extracted from the practice's patient management software[a] retrospectively. Exclusion criteria included patients who stayed < 24 hours, patients whose records had either admission and/or discharge body weights missing, and those patients who received intravenous fluid (IVF) therapy, in order to avoid the effect of hydration on body weight. Patients who received IVF only during an anaesthesia period were still included. Each patient's age, final diagnosis, length of hospital stay, and body weight changes between admission and discharge were examined.

[a] Cornerstone Veterinary Software 8.3 NEXT (version 8.3.6), IDEXX Laboratories, Westbrook, ME, USA

2.2.2 Assessment of body composition changes in hospitalised dogs

For the assessment of body composition, dogs were prospectively recruited from MUVTH's internal medicine and surgical departments. The recruitment criteria included patients with any disease process where the expected hospital stay was equal to or greater than the defined length of time for a "long-stay" patient as determined by the retrospective study. The suitability for enrolment of a patient was determined based on the clinic signs at admission and the primary clinician's recommendation as to the likelihood of the duration of stay for the patient. Once the primary clinician's approval was obtained, each patient's owner(s) was contacted for permission to include their dog in the study. Exclusion criteria included dogs with critical illness (American Society of Anaesthesiologists (ASA) status grade 4 - 5), those with disturbances in water balance (e.g. kidney failure, chylothorax, haemoabdomen), or those who received IVF therapy during their hospitalisation except during anaesthesia, any of which could have affected the distribution of the stable isotope used for body composition measurements. An *a priori* power analysis revealed that 34 dogs would have a power of 0.9 to detect a difference of 15% in fat-free mass, with a variance of 30%. The study was approved by Massey University Animal Ethics Committee (MUAEC protocol 15/41).

Body composition for each patient was measured by the author as close as possible to the time of admission and repeated again right before the patient was discharged from the hospital. Isotopic dilution is a sensitive and minimally invasive method of measuring body composition, and the method has been described previously.[29–31] To perform this technique, the dogs were fasted overnight but allowed *ad libitum* water up until the procedure began. The dogs were taken outside for urination and defecation, and afterwards their body weight was measured on a large floor scale located in the hospital. Based on their body weight, an injectate solution was made for each dog using a newly opened and weighed syringe and needle. To make the solution, 0.4 mg/kg body weight of deuterium solution[b] was aspirated and combined with 0.13

[b] Deuterium Oxide (99.8% purity), Cambridge Isotope Laboratories, Inc., Andover, MA, USA

mg/kg body weight of 3% saline[c]. The syringe and needle were reweighed at each step to determine the exact amount of each compound in the syringe. All syringe weights were measured using the same electronic scales[d] to a milligram accuracy.

A catheter was placed in a peripheral vein of each dog, and a 3 mL blood sample taken to determine the background deuterium concentration. Then, the deuterium mixture was slowly injected through the catheter, the syringe removed, and the catheter flushed with 0.9% saline[e] to ensure complete administration. The syringe and needle were then re-weighed to obtain a post-injection weight to allow for residual deuterium to be accounted for. Deuterium was allowed to evenly distribute throughout the body for two hours, after which a second 3 mL blood sample was taken from a different vein to the one used for injection. Water and food were withheld during the two hours of equilibration. Blood samples were collected into EDTA blood vacutainer tubes[f]. Plasma was obtained from the vacutainers after undergoing centrifugation for 10 minutes at 3,000 rcf at 4 °C, and the plasma stored in vials[g] with crimp caps[h] at −80 °C until analysis.

2.2.2.1 Deuterium analysis

Deuterium was measured in the plasma samples by gas-isotope ratio mass spectrometry[i]. For reference solutions, Vienna Standard Mean Ocean Water, International Atomic Energy Agency enriched water and Aberdeen tap water were used, which have defined concentrations of hydrogen and oxygen isotopes. Additionally, Aberdeen tap water and International Atomic Energy Agency enriched water were used as controls. Abundance of deuterium was expressed as parts per 1,000

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[c] 3% saline, Baxter Healthcare Pty Ltd., Toongabbie, NSW, Australia
[d] Kern PLJ 600-3NM, Kern & Sohn GmbH, Balingen, Germany
[e] 0.9% saline, Baxter Healthcare Pty Ltd., Toongabbie, NSW, Australia
[f] BD Vacutainer®, Becton, Dickson and Company, Plymouth, United Kingdom
[g] Polypropylene vial 250 µL, catalog #5188-0278, Agilent Technologist Inc., Santa Carla, CA, USA
[h] Aluminium cap, PFTE/butyl septa, catalog #8010-0051, Agilent Technologist Inc., Santa Carla, CA, USA
[i] Isotope Ratio Mass Spectrometry Unit, Otago University, Dunedin, NZ

difference from the reference Vienna Standard Mean Ocean Water (δ2H) and was converted to parts per million (ppm) using the equation:

$$\frac{1{,}000{,}000}{\left(1+\frac{1}{\left(\frac{\delta^2 H}{1{,}000}+1\right)\times 0.00015576}\right)}$$

where 0.00015576 is the accepted 2H/1H ratio of Vienna Standard Mean Ocean Water.

2.2.2.2 Total body water

Total body water was determined using the methods from Speakman *et al*.[29]. For this, the weight of the syringe post-administration was subtracted from the weight of the empty syringe to determine the weight of the residual injectate. This was then multiplied by the percentage concentration of deuterium solution in the residual injectate which was determined for each dog as:

$$\%\ \text{concentration of deuterium in injectate} = \left(\frac{deuterium\ solution\ (g)}{deuterium\ solution\ (g)+saline\ solution\ (g)}\right)$$

Then, the weight of the deuterium solution administered was calculated by subtracting the residual weight from the initial weight. This was then multiplied by 0.998 to correct for the purity of the deuterium solution.

Once the exact amount of deuterium solution administered was calculated, the volume of distribution (Vd) was determined as the total deuterium solution administered in grams multiplied by 1,000 and then divided by the difference between the deuterium abundance (ppm) in enriched plasma and in baseline plasma. Total body water in kilograms was calculated as Vd divided by 1.04 to account for isotopic exchange of deuterium with the hydrogen present in proteins, carbohydrates and fats.[29]

2.2.2.3 Fat-free mass (FFM) and fat mass (FM)

Lean mass, also known as fat-free mass (FFM), was calculated as total body water divided by 0.713, which is the hydration constant for lean mass in dogs as determined by carcass desiccation.[32] Once FFM was calculated, body fat mass (FM) was determined by subtracting FFM from body weight.

2.2.2.4 Body and muscle condition, caloric intake, disease state and activity

Body condition score (BCS; 1 - 9) and muscle condition score (MCS; normal muscle mass (3), mild muscle loss (2), moderate muscle loss (1) and severe muscle loss (0)) were recorded on the days of body composition assessments.[27,33] To reduce variation, all scorings were performed by the author. If the dog was considered overweight or underweight as assessed by BCS at admission, ideal weight was calculated using the assumption of a 5% change in body fat for every BCS.[27] This ideal weight was then used to calculate resting energy requirement (RER) for the patient.

No specific nutritional intervention occurred in this study, and the nutritional care of the dog was prescribed by the primary clinician assigned to the case. However, all dogs were fed diets formulated to meet the nutrient requirements for adult dogs as defined by the Association of American Feed Control Officials (AAFCO). Body weight and food intake were recorded daily. Daily caloric intake was summed and divided by the number of days of hospitalisation to obtain the mean caloric intake for each patient (kcals/day). Mean caloric intake was then expressed as a percentage of the patient's estimated RER, which was calculated using the following formula[34]:

$$\text{RER (kcal)} = (\text{ideal body weight})^{0.75} \times 70$$

The severity of disease for each patient was determined at admission and scored using the PSS.[25,35–37] In this scoring system, a score of (1) indicates an animal with no disease, (2) mild systemic disease, (3) severe systemic disease limiting activity but not incapacitation, (4) incapacitating systemic disease that is a constant threat to life, and

(5) a moribund animal not expected to live 24 hours with or without any type of intervention. In addition, daily activity was measured using a tri-axial accelerometer[j] fitted to the collar of each dog. The daily activity was summed and divided by the number of days of hospitalisation to obtain the mean activity (ΔG) per day for each patient.

2.2.3 Statistics

All statistical analyses were performed using the R software[k]. From the hospital records, the median and interquartile range for hospital duration of the dogs were established. The first quartile (Q1) value was then used to define the minimum amount of days for a dog to be considered a "long-stay" hospitalised patient in the second study. Linear regression was used to determine the relationship between body weight change and the duration of stay. The normal distribution of the residuals of the final regression was confirmed by visual appearance and with a Shapiro-Wilks normality test.

Changes in BCS, MCS, FM (expressed as total kg and % body weight), FFM (expressed as total kg and % body weight) for each patient were obtained by subtracting the discharge value from the admission value. Changes in body weight were expressed as the percentage change [(body weight at admission – body weight at discharge)/body weight at admission]. Given that BCS and MCS are ordinal categorical data, Kendall rank correlation was used to compare associations between BCS and FM (expressed as % body weight), and MCS and FFM (expressed as % body weight) at the time of admission and at discharge. Prior to running the correlation test, data exploration was performed, and no outliers were detected.[38]

Models for changes in body weight, FFM and FM during hospitalisation were built using multiple regression analysis in a manual, stepwise technique. Age, sex, disease severity, caloric intake, duration of stay, and activity were treated as independent

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[j] Heyrex®, Say Systems, Wellington, New Zealand
[k] R version 3.1.0; R Development Core Team, 2012; R Foundation for Statistical Computing, Vienna, Austria

variables. Model fit was assessed using Akaike information criterion (AIC) and adjusted R^2. The collinearity of the variables in the final models was checked by variance inflation factor (VIF). The normal distribution of the residuals of the final regression was confirmed by visual appearance and with a Shapiro-Wilks normality test. Results are presented as mean with standard deviation (SD) unless stated otherwise. P-values < 0.001 were considered indicative of very strong evidence, P < 0.01 of strong evidence, P < 0.05 of moderate evidence, P < 0.1 of weak evidence, and P ≥ 0.1 of insufficient evidence.[39]

2.3 Results

2.3.1 Historical clinical records

Sixty (60) clinical records of dogs seen at the MUVTH referral internal medicine and surgical departments were examined. The mean age of the dogs was 7 (SD 3.5) years. The majority (57%) of these dogs were hospitalised because of spinal disease, including intervertebral disc disease (IVDD) and fibrocartilaginous embolism (FCE) (Table 2-1).

Table 2-1. Final diagnoses of dogs in a retrospective study to investigate the range in hospitalisation duration at Massey University's Veterinary Teaching Hospital's referral service between November 2013 to March 2014.

Diagnosis	Count
Congenital vertebral malformation	1
Discospondylitis	2
Fibrocartilaginous embolism	5
Granulomatous meningoencephalitis	2
Head trauma	1
Inflammatory bowel diseases	1
Immune-mediate polyarthritis	1
Intervertebral disc disease	29
Leg fracture	1
Lumbosacral stenosis	3
Meningitis	1
Myasthenia gravis	1
Neoplasm (all types)	6
Spinal fracture	1
Trauma	3
Not definitive	2
Total	60

The median duration of stay was 5 days, with an inter-quartile range (IQR) of 3 - 7.2 days (Figure 2-1). On that basis, a hospital duration of 3 days or longer was used to define "long-stay" patients for the subsequent prospective study.

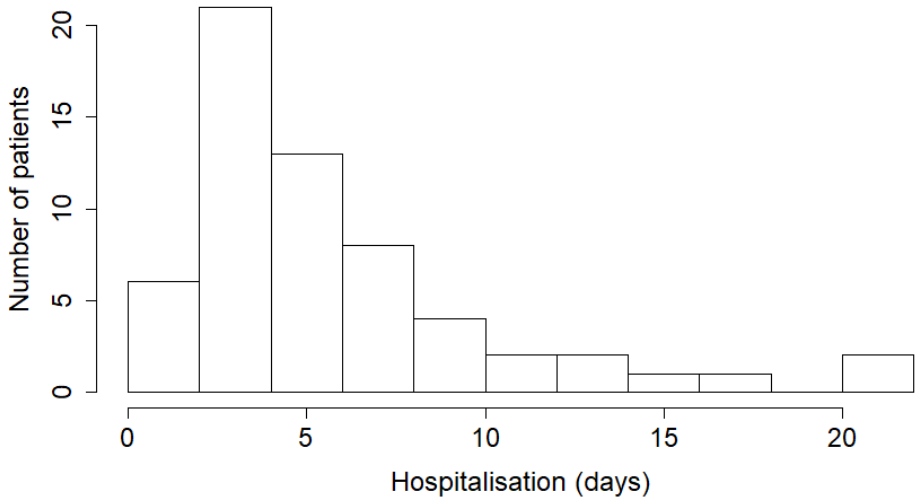

Figure 2-1. Bar graph of the length of stay for sixty dogs in a retrospective study to investigate the range of hospitalisation duration in dogs seen at Massey University's Veterinary Teaching Hospital's referral services between November 2013 and March 2014.

The mean percentage (%) body weight change was -3.97 (SD 4.45) %. Percentage body weight change was moderately correlated to the duration of stay for these patients (r^2 = 0.4238, $P < 0.0001$, Figure 2-2).

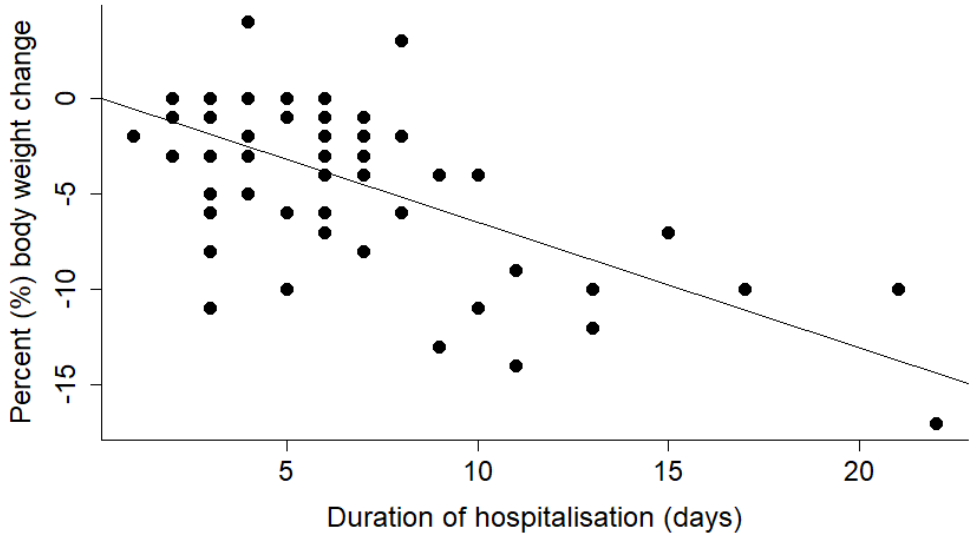

Figure 2-2. Correlation between body weight change (%) and duration of hospitalisation (days) in sixty dogs seen at Massey University's Veterinary Teaching Hospital's referral services between November 2013 and March 2014 ($r^2 = 0.4238$, $P < 0.0001$).

2.3.2 Assessment of body composition changes in hospitalised dogs

A total of 23 dogs were recruited which consisted of seven females and sixteen males and encompassed 16 different breeds including American bulldog (1), Bichon frisé (2), border collie (1), Chihuahua (1), crossbreed (2), dachshund (3), fox terrier (1), greyhound (2), Harrier hound (2), husky (1), Labrador retriever (1), Maltese (1), Pekingese (1), poodle (2), Staffordshire bull terrier (1), and Weimaraner (1). The mean age of the dogs was 7.2 (SD 3.2) years, and the median duration of stay was 5 [(IQR) 4 - 9] days. Dogs with spinal disease made up a large majority (18/23) of recruited patients (Table 2-2). One dog seen by the medicine department had multiple comorbidities including hyperadrenocorticism, diabetes and urolithiasis.

Table 2-2. Final diagnoses of dogs recruited for body composition analysis from Massey University's Veterinary Teaching Hospital.

Diagnosis	Count
Medical reason (diabetes, hyperadrenocorticism, urolithiasis, insulinoma)	2
Injury from dog fight	1
Atlantoaxial instability	1
Fibrocartilaginous embolism	1
Orthopaedic fracture	2
Paralumbar abscess requiring a hemilaminectomy	1
Intervertebral disc disease (IVDD)	15
Total	23

Activity, intake, body weight, BCS, MCS, FM and FFM at admission and discharge for each dog are presented in Table 2-3. Median activity was 22,904 (IQR 19,445 – 47,993) ΔG. In one dog, activity could not be recorded as the surgical incision site was located where the collar and activity monitor were required to be placed. The dogs ate on average 77.5 (SD 28) % of their estimated RER. Most of the dogs lost weight (78.3%), while 13.0% maintained and 8.7% gained weight. Six dogs lost one BCS (26.1%), and three dogs lost one MCS (13.0%) during their hospitalisation. Of the dogs that lost condition, three lost both BCS and MCS during their stay.

Table 2-3. Average activity and caloric intake per day (resting energy requirement, RER), physical status score (PSS), body weight (BW), body condition score (BCS), muscle condition score (MCS) and body composition at admission (a) and discharge (d) of 23 long-stay hospitalised dogs.

Dog	Activity (Δ G)	Intake (%RER)	PSS	BWa (kg)	BWd (kg)	BCSa	BCSd	MCSa	MCSd	FFMa (kg)	FFMa (%BW)	FFMd (kg)	FFMd (%BW)	FMa (kg)	FMa (%BW)	FMd (kg)	FMd (%BW)
1	22493	61.3	1	7.8	7.7	6	6	3	3	5.41	69.37	5.13	66.6	2.39	30.63	2.57	33.4
2	187587	106.7	1	23.5	23.8	6	6	3	3	17.28	73.52	15.38	64.63	6.22	26.48	8.42	35.37
3	98680	117.7	1	5.8	5.8	8	8	3	3	3.73	64.28	2.52	43.39	2.07	35.72	3.28	56.61
4	19344	44.6	1	9.57	9.47	6	6	3	3	7.16	74.78	7.18	75.86	2.41	25.22	2.29	24.14
5	9874	69.3	1	6.5	6.14	4	4	3	3	4.25	65.35	4.14	67.49	2.25	34.65	2	32.51
6	20727	34.1	1	12.5	12.3	5	5	3	3	8.87	70.99	9.37	76.21	3.63	29.01	2.93	23.79
7	37101	59.2	1	7.1	6.5	5	4	3	2	5.61	79	4.48	68.92	1.49	21	2.02	31.08
8	19750	91	1	6.27	6.32	5	5	3	3	4.26	67.94	4.26	67.36	2.01	32.06	2.06	32.64
9	30543	100.6	1	5.14	5	4	4	3	3	4.45	86.64	4.13	82.64	0.69	13.36	0.87	17.36
10	52661	58.6	1	7.5	6.5	5	4	3	3	5.33	71.01	5.51	84.71	2.17	28.99	0.99	15.29
11	48229	120.4	2	8.6	7.7	4	3	2	0	7.52	87.39	6.86	89.04	1.08	12.61	0.84	10.96
12	9887	34.9	1	24	24	5	5	3	3	17.63	73.44	13.39	55.78	6.37	26.56	10.61	44.22
13	14015	90.1	1	21.1	19.9	4	4	3	3	17.6	83.41	17.49	87.89	3.5	16.59	2.41	12.11
14	22544	104	2	19.4	19.2	3	3	3	3	18.42	94.96	19.07	99.34	0.98	5.04	0.01	0.05
15	NA	88.2	3	3.87	3.78	6	6	3	3	2.4	61.95	2.52	66.61	1.47	38.05	1.26	33.39
16	23265	98.2	2	27.4	26.8	3	3	3	3	27.38	99.92	24.52	91.48	0.02	0.08	2.28	8.52
17	17265	60	3	30.8	30.8	6	6	3	3	19.75	64.12	22.51	73.08	11.05	35.88	8.29	26.92
18	54260	78.6	1	32.7	30	7	6	3	3	21.69	66.33	22.93	76.42	11.01	33.67	7.07	23.58
19	10917	39	1	28	27	4	4	2	2	19.12	68.3	18.87	69.88	8.88	31.7	8.13	30.12
20	20326	92.6	1	60	54.4	8	7	3	3	42.94	71.57	38.78	71.29	17.06	28.43	15.62	28.71
21	47286	56.1	1	37.5	35	5	4	3	2	29.23	77.94	28.5	81.41	8.27	22.06	6.51	18.59
22	31865	54.8	1	28.4	27.6	4	4	3	3	25.84	90.99	26.99	97.8	2.56	9.01	0.61	2.2
23	61937	122.4	1	6.57	6.18	4	4	3	3	4.97	75.6	5.1	82.55	1.6	24.4	1.08	17.45

The dogs lost on average -0.79 (SD 1.3) kg which represented 3.9 (SD 3.9) % of their total body weight during their hospitalisation. The mean FFM difference was -0.49 (SD 1.6) kg, which represented 61.8% of the total body weight loss. For FM, the mean difference was -0.3 (SD 1.7) kg, which was 38.2% of the total body weight loss. Conversely, 39.1% of dogs gained FFM and 34.8% gained FM during their stay (Table 2-3).

There was only a moderate correlation between BCS and percentage FM measured at admission (Kendall's tau = 0.51; $P = 0.002$, Figure 2-3), and at discharge (Kendall's tau = 0.55; $P = 0.001$, Figure 2-4). However, there was no correlation between MCS and FFM at either admission or discharge ($P > 0.1$). Finally, duration of stay was associated with change in overall body weight, but was not associated with changes in either FFM or FM. No other factors including age, sex, duration, PSS, caloric intake or activity were found to be associated with either changes in body weight, FFM or FM. The final models are presented in Table 2-4.

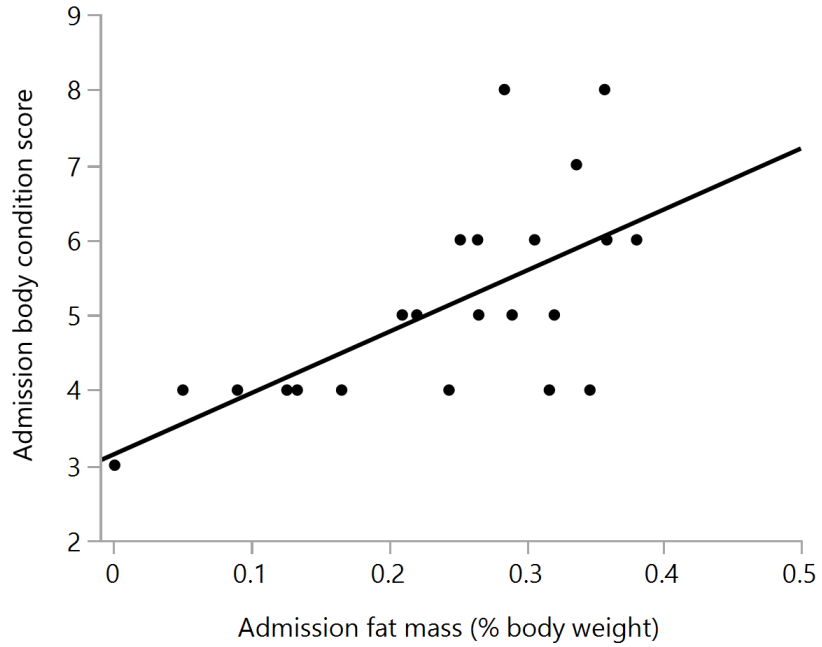

Figure 2-3. Correlation between body fat mass (expressed as % of body weight) and body condition scores at admission to a Veterinary Teaching Hospital in 23 spinal patients (Kendall's tau = 0.51; *P* = 0.002).

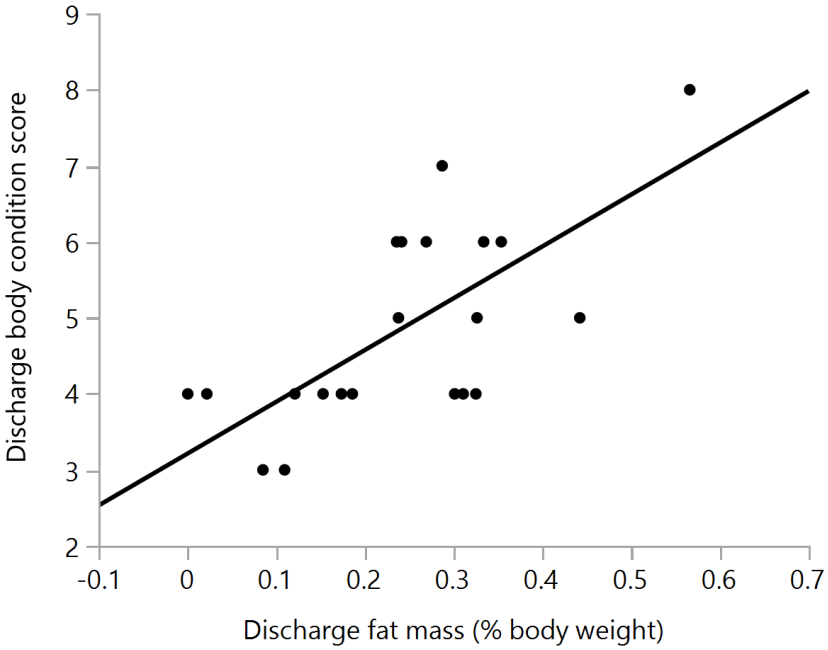

Figure 2-4. Correlation between body fat mass (expressed as % of body weight) and body condition scores at discharge from a Veterinary Teaching Hospital in 23 spinal patients (Kendall's tau = 0.55; *P* = 0.001).

Table 2-4. Multiple regression models for factors associated with changes in body weight, fat-free mass (FFM), and fat mass (FM) in hospitalised dogs. Difference in body weight, FFM and FM between discharge and admission are expressed as delta (Δ) change in kilograms (kg). Age, sex, disease severity, caloric intake, duration of stay, and activity were treated as independent variables. The best fit models shown here were assessed using Akaike information criterion and adjusted R^2. The symbol (*) denotes a P-value of < 0.10.

Outcome	Variable	Estimate	Standard Error	R^2	P-value
ΔBody weight (kg)	Intercept	1.198	0.757		0.130
	Age	−0.086	0.071		0.240
	Sex (male)	−0.621	0.454		0.188
	Days	−0.104	0.026		< 0.001*
	Overall model			0.41	0.005*
ΔFat-free mass (kg)	Intercept	2.111	1.830		0.264
	Age	−0.221	0.124		0.101
	Sex (male)	−0.754	0.708		0.301
	% RER	−0.024	0.014		0.107
	Overall model			0.10	0.218
ΔFat mass (kg)	Intercept	−1.354	1.893		0.484
	Age	0.140	0.139		0.328
	Days	−0.063	0.044		0.168
	PSS	−0.737	0.622		0.252
	% RER	0.020	0.016		0.213
	Overall model			0.01	0.400

2.4 Discussion

The loss of total body weight, lean mass and fat mass is associated with poorer outcomes in both human and veterinary patients.[7–10,12–14,22] The majority of the dogs in this study lost body weight, which consisted of a much greater proportion of lean mass (61.8%) than fat mass (38.2%). By comparison, in dogs fed 50% of their estimated requirements for three months, 30% of the total weight lost was lean mass.[40] For humans, the percentage lean mass lost when undergoing a purposeful weight loss program is approximately 20% of their total weight loss.[41,42] In this study, a much higher percentage of lean mass lost was found, despite the dogs consuming on average 77.5% of their energy requirements. In addition, intake was not found to be a significant factor in the multiple regression model for lean loss. This is in agreement with a meta-analysis of human hospital intervention studies where there was no clear association between energy and protein intake, and changes in skeletal muscle mass in the patients.[43] Taken together, these findings suggest that the loss of muscle mass in hospitalised patients is not simply due to undereating, and instead, likely to be multifactorial.

Changes in protein metabolism have been documented to occur early on for human patients. Individuals in intensive care have decreased protein synthesis, increased protein catabolism, and significant muscle mass loss that occurs even within the first week of hospitalisation.[44] Non-nutritional factors that can contribute to muscle loss include increased production of cortisol and inflammatory cytokines, development of acidosis, disuse atrophy, and denervation.[45–48] Injury to the muscle itself increases IL-6, TNF-α and cortisol production, leading to muscle catabolism.[47,49–51] The majority of the patient population in this study were spinal patients, and so partial denervation and disuse atrophy were likely significant contributors to the muscle loss.[48,52,53] The patients were also recovering from surgery, which is a process that also induces muscle injury. An elevation in plasma creatine kinase lasting for three days post-surgery has been described in dogs undergoing hemilaminectomy surgery.[54] Likewise, in humans following elective surgery, an increase in cortisol has been documented for three days post-surgery, which reached concentrations that have been shown experimentally to exacerbate muscle loss during

bedrest.[55–57] Thus, it is likely that a combination of these factors played a role in the muscle loss of the patients in this study.

When examining for factors associated with changes in body weight and composition, only an association between hospital duration and the loss of body weight was found. This is a similar finding to a study of hospitalised dogs where longer hospitalisation was associated with changes in body weight.[25] Interestingly, no association was found between a longer hospital stay and increased lean mass loss. The reason for this could be due to our small sample size, but may also be due to differences in post-op management, discharge criteria, availability of home care, and the severity of the patients' spinal disease. While systemic disease severity was measured using PSS, this measurement is not specific to grading the severity of spinal disease. Additionally, there may be a non-linear relationship between the loss of muscle and patient recovery, which would not be captured in the multiple regression analysis.

Further, activity was not found to be associated with changes in lean mass. Activity was monitored using a tri-axial accelerometer fitted to the collar of each dog, which measures both the magnitude and direction of acceleration. Spinal patients recovering during hospitalisation undergo cage rest, and therefore experience only limited opportunities for activity. In addition, physical therapy may play a significant role in the recovery of spinal patients and these movements would not be fully captured using a tri-axial accelerometer in this manner. As such, other methods of monitoring activity and movements related to physical therapy may be needed to better understand the relationship between disuse atrophy, activity, and lean mass changes during hospitalisation.

While MCS and BCS are commonly used in veterinary medicine to determine nutritional status, it is unknown how accurately these measures detect changes in lean and fat mass in hospitalised patients. A correlation was found between BCS and FM in this population of hospitalised dogs, whereas there was no correlation between MCS and FFM. However, the strength of the correlation was only modest, and so the utility of BCS for monitoring patients remains questionable. BCS is measured at fixed points on the body and may not

detect all changes in fat mass. In a study of human patients with acute pancreatitis, the patients lost 12% of their visceral fat mass, but had no change in their subcutaneous fat mass after nine days of hospitalisation.[58] Thus BCS appears to be insufficiently sensitive to allow for accurate monitoring of changes in the overall fat mass of individual patients.

Another concern is the accuracy of BCS to adequately assess fat mass specifically in lean animals. The BCS system was originally validated based on ideally condition or overweight animals, and its accuracy in lean animals has not been properly assessed.[27,59,60] Given that the BCS system is largely based upon subcutaneous fat stores, it is likely to be inaccurate in animals with little subcutaneous fat. By comparison, the isotopic dilution technique has been validated across dogs of different sizes and body conditions, including lean dogs.[32] Indeed in this study population, three dogs with minimal body fat as measured by isotopic dilution, were still scored a BCS of three. Other studies in greyhounds have also described very low body fat mass as measured by both deuterium isotopic dilution and dual-energy X-ray absorptiometry (DEXA), despite the dogs being considered ideally conditioned.[61,62] In agreement, two of the three leanest dogs in this study were greyhounds. Therefore, in very lean dogs, BCS also appears to be an inaccurate measure of body fat mass.

The main limitation to this study is the small total study population, as well as the limited number of non-spinal patients. Patients from the medicine department were ultimately challenging to recruit due to difficulties in estimating duration of stay, or they had water imbalances and/or needed IVF therapy. As such, the study population was heavily biased towards spinal patients. These patients may have exacerbated muscle loss from inactivity and denervation compared to other patients. Another limitation is that the true energy requirements of patients were not measured, and so food provision was based on an estimated energy requirement. However, other studies using indirect calorimetry have shown that the energy requirements of hospitalised dogs is similar to healthy dogs.[63,64] Thus the RER calculation used in this study is believed to be close to true requirements.

2.5 Conclusion

This is the first study to measure body composition changes in hospitalised dogs using isotopic dilution. It was found that long-stay hospitalised dogs tend to lose weight during hospitalisation; also, that the loss of lean mass made up a greater proportion of the total weight lost when compared to obese dogs losing weight on a planned weight loss program. In addition, intake was not significantly associated with either changes in body weight or body condition. The results of this study highlight that the weight lost by hospitalised dogs is not simply due to undereating. However, larger studies containing patients with a wider range of disease are needed to better evaluate other factors, such as inflammation, that may affect lean and fat loss during hospitalisation. In addition, it has been shown that the most common methods of measuring body composition (BCS and MCS) are not suitable for monitoring changes during hospitalisation, especially in lean dogs, and that the development of a simple method to use in lean dogs is needed.

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Chapter 3

Creation of predictive equations of body composition in lean dogs

3.1 Chapter introduction

In the previous chapter, it was demonstrated that the most common methods of measuring body composition in dogs (body condition scoring and muscle condition scoring) do not accurately detect changes in composition during hospitalisation when compared to an isotopic dilution technique. Isotopic dilution is generally considered one of the gold standards of body composition measurement, however it is impractical to use outside a research setting. In addition, while body condition score was moderately correlated to body fat mass in the previous study, the BCS system has only been validated using ideally condition or overweight animals, and its accuracy in lean animals has not been properly assessed.[1–3] Therefore, an accurate, minimally invasive, and more practical method of measuring composition, particularly in lean dogs, is required.

This chapter describes the creation of equations to predict the body composition of lean dogs using morphometry. Working farm dogs were used in this study because they represent a population of lean dogs in New Zealand. In addition, if the method proved accurate, then it could also provide a means for in-field assessments of composition in working dogs, a population where very low body conditions is commonplace.

This study has been published in the New Zealand Veterinary Journal and a reproduction is included in Appendix I.

3.2 Study Introduction

Farm dogs provide a significant service to the agricultural industry in Australasia. In New Zealand certain aspects of pastoral farming on hill and high-country properties would be virtually impossible without the assistance of working dogs. Mustering requires athleticism, which is incompatible with obesity, and thus, most working farm dogs are kept in a lean condition with little to moderate body fat. Despite this, it is not known what the ideal body composition is for working farm dogs.

In humans, there is a J-shaped association between body mass index and all-cause mortality in epidemiological studies, although the risk of disease is moderated with exercise.[4–6] In addition, a low body mass index increases the risk of fractures in humans.[7,8] Therefore, there appears to be an increased risk of morbidity and injury in both lean and obese individuals. In dogs, obesity is also known to increase risk of disease and injury.[9–11] However, it is currently unknown if low body condition in dogs may also be detrimental.

A survey of visits to veterinary clinics by New Zealand farm dogs found that 38% of visits were a result of traumatic musculoskeletal injury.[12] In humans, low muscle mass increases the risk of traumatic injury during strenuous activity.[13–15] During exercise, stress energy is dissipated by muscle. Consequently with low muscle mass, unalleviated forces can cause micro-damage to the bone that over time may lead to stress fractures.[16] Muscle fatigue can also cause gait changes and strain redistribution to areas of bone less capable of withstanding an increased stress loading, which has been shown as changes in strain distribution in the tibia of Foxhounds exercised to the point of fatigue.[17] In addition, there is a positive association between muscle mass and bone strength.[18,19] Therefore, a reduction in skeletal muscle mass involved in locomotion may play a role in increasing the risk of injury in dogs, and may be especially relevant to lean working farm dogs.

As the prevalence of obesity increases in the human population, perception of what is considered a healthy weight has changed, and there has been a normalisation of a larger fat mass.[20,21] Owners of obese pets frequently underestimate their pets' body condition and perceive them to be in a healthy condition when they are actually overweight.[22–24] The

acceptance by the public of a higher body condition being normal may result in the perception that healthy lean working dogs are in poor condition. A recent survey of New Zealand farmers concerning their working dogs revealed that low body weight was the single most common health concern, affecting nearly 20% of dogs.[25] Yet despite this, there remains a lack of understanding as to what the ideal body condition may be for working dogs, and what may truly be unhealthy. In addition, it may not be the total muscle mass that is of greatest importance, but rather the ratio of muscle to skeletal mass. However, this needs to be investigated. In addition, further research is required to determine the relationship between body composition and overall health, performance and welfare in a large population of working farm dogs.

Before this research can be conducted, a simple tool is necessary that can be used in the field to estimate body composition. Non-invasive methods of measuring body composition in dogs include dual-energy x-ray absorptiometry (DEXA), isotope dilution, assessment of body condition score (BCS) and morphometry. Despite the variety of options there are significant limitations to each of these approaches. DEXA and isotope dilution require specialised equipment for analysis and are therefore of limited practical use outside a research setting. Assessment of BCS is a quick and practical subjective measure of body composition, which has been validated using DEXA and isotope dilution in dogs.[1,3] However the BCS system was designed primarily to quantify obesity, and has been validated for predicting body fat mass, rather than fat-free mass (FFM). In addition, the studies validating the scoring system did not include a significant proportion of very lean dogs, and none that were judged to have a BCS of one.[1,3] Morphometric equations have been created for dogs that correlate skeletal measurements with body weight in order to predict body composition.[1,26,27] However the studies validating the morphometric equations also did not include dogs with low BCS. Therefore, there is currently no validated method to estimate the muscle mass of lean dogs that is simple and non-invasive.

The principal aims of the present study were to develop an equation that accurately estimated FFM, and the ratio of FFM to skeletal size or mass, using morphometric measurements in lean working farm dogs, and to examine the association between FFM derived from BCS and FFM measured using isotope dilution. A secondary aim was to determine which other variables might influence the ratio of FFM to skeletal size or mass. It was hypothesised that there would be a correlation between morphometric measurements and measured lean tissue, and the ratio of lean tissue to skeletal measures, that would be sufficient to create accurate, predictive equations.

3.3 Materials and methods

3.3.1 Dogs

Working dogs from sheep and beef farms located in the Waikato region of New Zealand were recruited between December 2016 to March 2017, based on BCS categorisation and owner willingness to participate. Only dogs of the two principal working dog breeds in New Zealand, Huntaway and heading dogs, were used. Each candidate dog had its BCS assessed by one examiner (an attending veterinarian, and co-author of the published manuscript), using a validated nine-point scale.[3] Dogs were recruited to ensure an even distribution of BCS in three condensed categories: < 3, 3 – 4 and > 4. The dogs were considered healthy based on being in work during the study period, having no history of illness or injury in the preceding 6 months, and having no significant abnormalities on physical examination that may have interfered with an accurate assessment of body composition using an isotope dilution technique. Dogs that were skeletally immature, that were not currently in work, or that did not fit the inclusion criteria stated above were excluded from the study. An *a priori* power analysis revealed that 20 dogs would have a power of 0.9 to detect an association ($\alpha = 0.05$) between morphometric measurements and FFM with a correlation coefficient ≥ 0.6. This study was approved by Massey University Animal Ethics Committee (MUAEC 16/108).

3.3.2 Body weight and morphometry

Thirteen working Huntaway and seven heading dogs were initially assessed, either at a veterinary clinic (VetEnt, Te Awamutu or VetEnt, Te Kuiti, New Zealand) or on site at the farm. On the day of the study, each dog was brought in to the veterinary clinic, weighed on the same calibrated electronic scales and its BCS was confirmed. Measurements of head length, head circumference, foreleg, hindleg, body length and thoracic girth were taken of all dogs by the same clinician using a flexible tailor's measuring tape, as described in Table 3-1. The measurement points were chosen as fixed bony locations least affected by joint flexion and extension that delineated aspects of skeletal size, and were similar to those used in other morphometric studies in dogs.[26,27]

Table 3-1. Morphometric measurements taken from working farm dogs. Each measurement represents static bony locations least affected by joint flexion and extension that delineated aspects of skeletal size.

Measurement	Description
Head length measurement	Distance from the level of the medial canthi, equidistant between the eyes, to the external occipital protuberance
Head circumference	Circumference at the widest point, equidistant between the eyes and ears
Foreleg measurement	Distance from the proximal edge of the central foot pad to the olecranon process
Hindleg measurement	Distance from the proximal edge of the central foot pad to the dorsal tip of the calcaneal process with the tarsus in extension
Body length	Distance from the dorsal process of thoracic vertebra 1 (T1) to the dorsal process of sacral vertebrae 1 (S1)
Thoracic girth	Chest circumference at the level of the xiphoid process

3.3.3 Body composition estimation

The dogs were fasted overnight but allowed *ad libitum* water up until the point when their first blood sample was taken. Total body water content of each dog was estimated by isotope dilution using the method described by Speakman *et al.*[28].

A 20-gauge catheter was placed in a peripheral vein of each enrolled dog. A single baseline blood sample was taken directly from the catheter in order to determine the concentration of naturally occurring deuterium in the dog. Then, using a weighed syringe, 0.4 mg/kg body weight of deuterium solution (D_2O[l]) and 0.13 mg/kg body weight of 3% saline[m] was administered through the catheter slowly. The catheter was then flushed with 0.9% saline[n], to ensure complete injection, and removed. The empty syringe used to inject the D_2O was re-weighed to determine the residual injectate. All syringe weights were measured using the same electronic scales[o] to milligram accuracy.

The D_2O was allowed to evenly distribute throughout the body water compartment for two hours, then a second blood sample was taken from the other cephalic vein or the jugular vein. Water and food were withheld during the two hours of equilibration. Blood samples were collected into EDTA blood tubes[p] and plasma was harvested, and then stored in polypropylene vials[q] with aluminium crimp caps[r] at −80 °C until analysis.

3.3.3.1 Deuterium analysis

Deuterium was measured in the serum samples by gas-isotope ratio mass spectrometry[s]. The standard reference waters used were Vienna Standard Mean Ocean Water, International Atomic Energy Agency enriched water, and Aberdeen tap water, which have

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[l] Deuterium Oxide (99.8% purity), Cambridge Isotope Laboratories, Inc., Andover, MA, USA
[m] Baxter Healthcare Pty Ltd., Toongabbie, NSW, Australia
[n] Baxter Healthcare Pty Ltd., Toongabbie, NSW, Australia
[o] Kern PLJ 600-3NM, Kern & Sohn GmbH, Balingen, Germany
[p] BD Vacutainer®, Becton, Dickson and Company, Plymouth, United Kingdom
[q] Polypropylene vial 250 µL, Agilent Technologist Incorporated, Santa Clara, CA, USA
[r] Aluminium cap, PFTE/butyl septa, Agilent Technologist Incorporated, Santa Carla, CA, USA
[s] Isotope Ratio Mass Spectrometry Unit, Otago University, Dunedin, NZ

defined concentrations of hydrogen and oxygen isotopes. Aberdeen tap water and International Atomic Energy Agency enriched water were used as controls. Abundance of deuterium was expressed as parts per 1,000 difference from the reference Vienna Standard Mean Ocean Water (δ^2H) and was converted to parts per million (ppm) using the equation:

$$\frac{1{,}000{,}000}{\left(1+\frac{1}{\left(\frac{\delta^2 H}{1{,}000}+1\right)\times 0.00015576}\right)}$$

where 0.00015576 is the accepted $^2H/^1H$ ratio of Vienna Standard Mean Ocean Water.

3.3.3.2 Total body water

Total body water was determined using the methods from Speakman *et al.*[28]. Firstly the total amount of D_2O (g) administered to each dog was calculated as the difference between the D_2O initially in the syringe and the residual D_2O, multiplied by 0.998; where 0.998 is the correction for the purity of the D_2O solution.

Then the volume of distribution (V_d) was calculated as the total D_2O (g) administered multiplied by 1,000, divided by the difference between the deuterium abundance (ppm) in enriched plasma and in baseline plasma.

Total body water (kg) was calculated as V_d divided by 1.04, to account for isotopic exchange of deuterium with non-water hydrogen in proteins, carbohydrates and fats.[29]

3.3.3.3 Fat-free mass and fat mass

Fat-free mass (kg) was calculated as total body water divided by 0.713, allowing for the hydration of non-fat mass as determined by carcass desiccation in dogs.[30] Finally, body fat mass was calculated by subtracting FFM from body weight.

3.3.4 Statistical analysis

Statistical analysis was performed using R software[t]. Normal distribution of data was confirmed by visual assessment of a scatterplot and the Anderson-Darling test. A two-sample *t*-test was used to compare variables between breeds and sexes. Results are presented as mean and standard deviation (SD) unless stated otherwise.

3.3.4.1 Morphometry

Principal component analysis was utilised to create a new variable that defined skeletal size based on the morphometric measurements.[31] This statistical technique accounts for collinearity in the morphometric measurements and applies orthogonal linear transformation to the morphometric measurements of the study dogs in order to create a new set of values to describe the greatest source of variation within the data set. This is termed the first principal component. The second greatest variance (second principal component) is described on a second orthogonal coordinate. To perform the principal component analysis, each morphometric measurement was centred and standardised in order to give equal weighting to each variable in the analysis[32] using the equation:

$$\frac{\left(m_{a-f}\right)-\left(\bar{x}_{a-f}\right)}{\sigma_{a-f}}$$

where m_{a-f} represents the value of the measurements a – f (head length, circumference, etc) of the dog, $\bar{x}_{a-f}$ represents the mean of the measurements a – f, and σ_{a-f} represents the SD of the measurements a – f. Eigenvalues for each morphometric measurement were then derived from the centred and standardised measurements and principal component

[t] R version 3.1.0; R Development Core Team, 2012; R Foundation for Statistical Computing, Vienna, Austria

scores were calculated by multiplying each centred, standardised measurement by its respective eigenvalue, and summed.

In order to create accurate ratios of lean tissue to skeletal size, it was necessary to ensure that the principal component scores were > 1, therefore a value of six was added to the first principal component score for each dog, and this was defined as the skeletal size for each dog.

3.3.4.2 Estimated skeletal mass and lean soft tissue mass

To assess the muscle component independently of skeletal mass, we wished to create a new variable for each dog, termed lean soft tissue mass (leanST), which was defined as the measured FFM minus the estimated skeletal mass for each dog. In a previous study, skeletal mass was reported to be 8% of total body weight following carcass analysis in laboratory beagles, assumed to be in ideal body condition.[33] The dogs included in the present study were, by definition, not all in ideal body condition, and thus the skeletal mass of dogs with low muscle mass were assumed to contribute a larger proportion of their body weight. Thus, to estimate the skeletal mass of the dogs in this study, a general linear model was created that described the relationship between 8% of body weight and skeletal size of only dogs with a BCS of 4 – 6, where skeletal mass was treated as a fixed factor and skeletal size was treated as a random effect. From the regression equation, the skeletal mass of all dogs, including those with a BCS < 4, was estimated using their skeletal size. LeanST (kg) was then estimated as FFM (kg) minus skeletal mass (kg).

3.3.4.3 Predicted fat-free mass and lean soft tissue mass and associations with measured variables

Multiple regression analyses with a stepwise elimination technique were used to determine which categorical variables (sex, breed, and age) and continuous variables (skeletal mass, skeletal size, body weight) provided the best predictive equations for measured FFM and estimated leanST. A predictor variable was kept in the model if $P \leq 0.05$. Predicted FFM and predicted leanST were then calculated for each dog using the final models. The association between predicted FFM and measured FMM was examined using a linear regression model, and between estimated leanST and predicted leanST

using a Passing Bablok regression model, as estimated Lean ST was expected to contain a degree of error.[34]

Ratios were then calculated of measured FFM, estimated leanST, predicted FFM and predicted leanST, to skeletal size and estimated skeletal mass. Regression models were used to describe the association between the ratios using predicted values, and the ratios using measured values. Simple linear regression was used for ratios containing FFM, and Passing Bablok regression was used for ratios containing estimated leanST. Bland-Altman plots were also generated to assess agreement between the ratios for measured or estimated values, and their respective predicted values.[35]

Regression analysis was used to determine variables associated with the ratio of measured FFM, or estimated leanST, to skeletal size, as described above.

3.3.4.4 Body condition score derived variables

Assessment of BCS is commonly used to predict fat mass in dogs and, if accurate, FFM should be able to be estimated by subtracting fat mass from the dog's body weight. In order to compare the validity of using BCS to estimate FFM, BCS-derived FFM (kg) was calculated for each dog, and as a percentage of total body weight, using the sex-specific regression equations provided by a previous study.[3] The calculated body fat mass was subtracted from body weight to determine FFM. Linear regression was used to assess the correlation between BCS and measured body fat percentage, between BCS-derived FFM and measured FFM, and between BCS-derived FFM expressed as a percentage of body weight and measured FFM expressed as a percentage of body weight. BCS, BCS-derived FFM, or BCS-derived FFM percentages were used as the response variables in the analysis.

3.4 Results

3.4.1 Body condition scores, fat-free mass and fat mass

The working dogs consisted of 10 males (eight Huntaway and two heading dogs) and 10 females (five Huntaway and five heading dogs). The dogs had a mean age of 5.3 (SD 2.7) years. Females had a mean body weight of 22.8 (SD 4.41) kg, and males a mean body weight of 27.9 (SD 3.19) kg. Five dogs had BCS < 3, seven BCS 3 – 4, and eight BCS > 4.

Median BCS was 3.0 (min 1, max 6). Mean FFM, as measured by isotope dilution, was 22.1 (SD 4.4) kg and the percentage FFM of body weight was 87.0 (SD 5.0) %. Males had a higher mean FFM than females (24.4 (SD 2.5) *vs.* 19.8 (SD 4.5) kg, $P = 0.019$), however there was no difference between the FFM as a percentage of body weight (males 87.7 (SD 3.82) *vs.* females 86.2 (SD 5.80) %, $P = 0.531$). Mean total body fat percentage was 13.0 (SD 5.09) %. There was a positive correlation between BCS and measured fat mass percentage ($r^2 = 0.62$, $P < 0.001$, Figure 3-1).

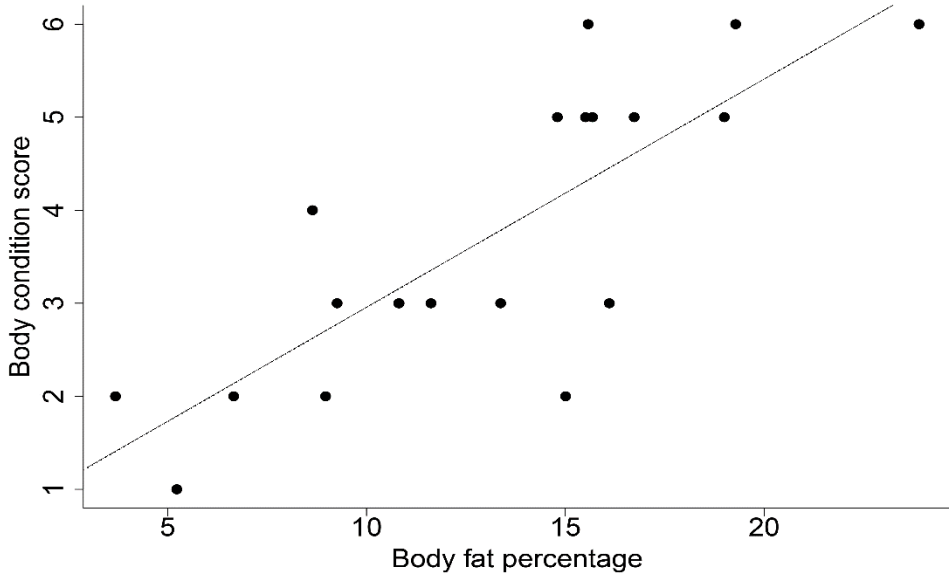

Figure 3-1. Association between body condition score and body fat, calculated from measured fat-free mass, as a percentage of body weight in 20 working farm dogs ($r^2 = 0.62$, $P < 0.001$).

3.4.2 Estimated skeletal size, skeletal mass and lean soft tissue

Mean values for the six morphometric measurements and results of the principal component analysis are presented in Table 3-2. The first principal component accounted for 77% of the variation seen, and the second 9.8%. In the first principal component, all eigenvalues were of the same sign and of similar value, indicating that all morphometric

measurements contributed similarly to the overall variation in skeletal size. The two breeds of dogs were clearly distinguishable by plotting the first principal and second principal component scores (Figure 3-2). Mean skeletal size, derived from the principal component scores, differed between Huntaway and heading dogs (7.27 (SD 0.82) *vs.* 3.63 (SD 1.64) respectively, $P = 0.01$). In addition, males had a higher mean skeletal size compared to females (7.20 (SD 1.30) *vs.* 4.80 (SD 2.06), $P = 0.01$).

Table 3-2. Mean (± SD) morphometric measurements for 20 working farm dogs, and eigenvalues for each measurement obtained from principal component analysis for the first and second principal components (PC).

Measurement	Mean ± SD (cm)	1st PC	2nd PC
Head length measurement	14.3 ± 1.35	0.44	−0.034
Head circumference	41.9 ± 3.77	0.40	0.395
Foreleg measurement	31.3 ± 2.94	0.42	−0.174
Hindleg measurement	16.2 ± 1.55	0.43	−0.303
Body length	44.5 ± 4.46	0.39	−0.493
Thoracic girth	66.2 ± 5.19	0.36	0.691

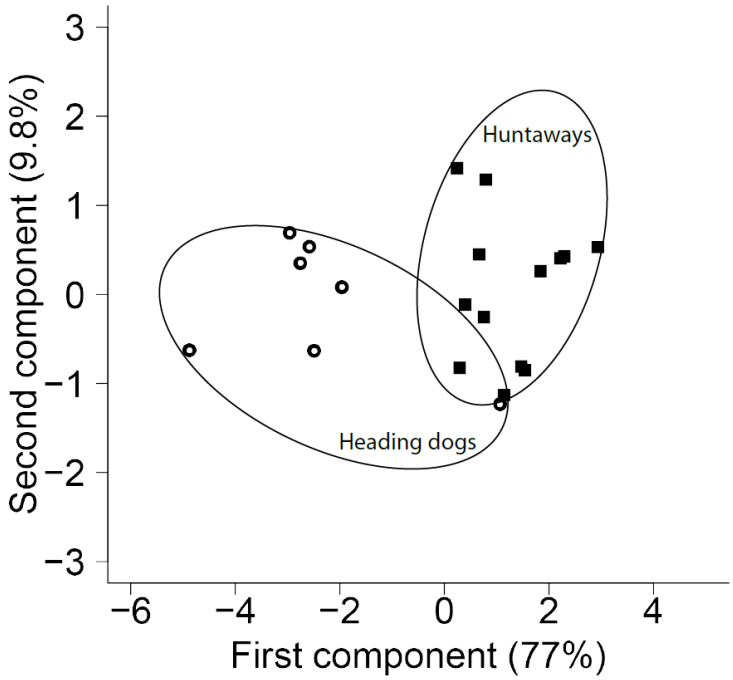

Figure 3-2. Principal component score plot derived from analysis of six morphometric measurements from seven heading (open circles) and 13 Huntaway (closed squares) dogs.

In dogs with a BCS of 4–6 the relationship between skeletal mass, based on 8% of body weight, and skeletal size was described using the following equation ($r^2 = 0.93$):

$$\text{Skeletal mass (kg)} = (0.18793 \times \text{skeletal size}) + 0.9953$$

This equation was used to estimate skeletal mass and thus leanST (FFM minus skeletal mass) for all dogs. Mean Lean ST was 20.0 (SD 3.93) kg and mean leanST as a percentage of total body weight was 78.6 (SD 4.51) %.

3.4.3 Predicted FFM and leanST

The final regression models used for the prediction of FFM and lean ST are presented in Table 3-3. Body weight, breed, age and measures of skeletal size or skeletal mass were

associated with measured FFM, and body weight, breed and age were associated with estimated leanST. Body weight contributed 92% of the variation associated with measured FFM and 92% of the variation associated with estimated leanST.

There was a good correlation between predicted FFM and measured FFM ($r^2 = 0.96$, $P < 0.001$), and predicted leanST and estimated leanST (Pearson's r = 0.98) (Figure 3-3).

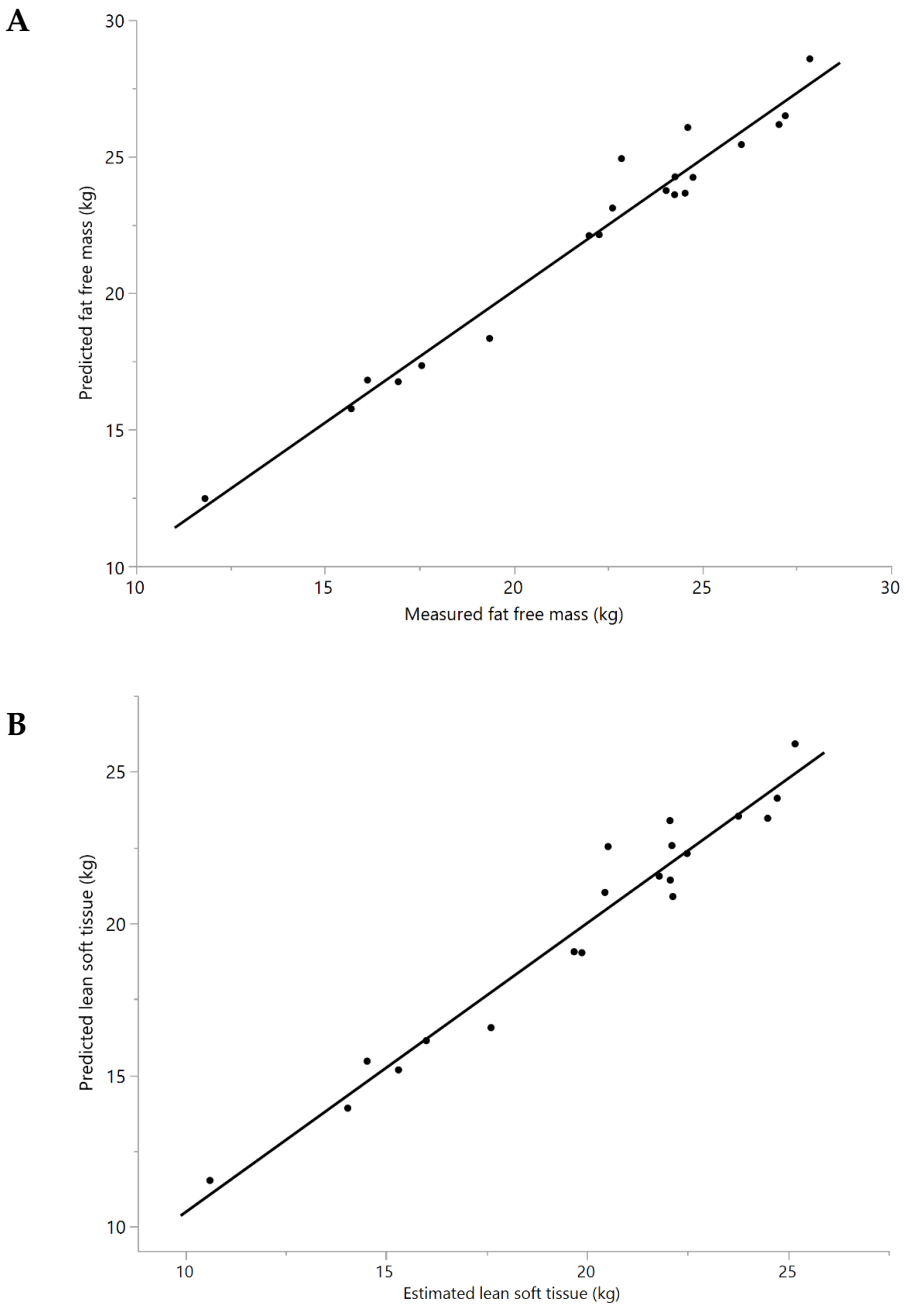

Figure 3-3. Associations between (A) measured and predicted fat-free mass ($r^2 = 0.96$, $P < 0.001$) and (B) estimated and predicted lean soft tissue (Pearson's $r = 0.98$) in 20 working farm dogs.

Table 3-3. Final linear regression models for factors associated with measured fat-free mass (FFM) and estimated lean soft tissue (leanST) in 20 working farm dogs. Regression models were generated using either skeletal size or estimated skeletal mass.

Outcome	Variable	Coefficient	95% CI	R^2	P-value [a]
FFM	Intercept	6.40		0.96	<0.001
	Skeletal size	0.60	0.03 – 1.16		
	Body weight	0.47	0.22 – 0.71		
	Breed (Huntaway)	1.91	0.19– 3.62		
	Age	−0.18	−0.35 – −0.00		
FFM	Intercept	3.24		0.96	<0.001
	Skeletal mass	3.18	0.18 – 6.17		
	Body weight	0.47	0.22 – 0.71		
	Breed (Huntaway)	1.91	0.19 – 3.62		
	Age	−0.18	−0.35 – −0.00		
LeanST	Intercept	4.30		0.94	<0.001
	Body weight	0.60	0.42 – 0.77		
	Breed (Huntaway)	2.39	0.75 – 4.03		
	Age	−0.18	−0.36 – −0.01		

[a] Significance of R^2

3.4.4 Ratios of FFM and leanST to skeletal size and skeletal mass

The mean ratio of measured FFM to skeletal size for the study population was 4.17 (SD 1.69). The Bland-Altman plots showed good agreement and minimal bias between the ratios of measured and predicted FFM to skeletal size or skeletal and estimated and predicted lean ST to skeletal size or mass. The plots are presented as supplementary figures in Appendix II. The mean ratio of measured FFM to skeletal size tended to differ between breeds (P = 0.057, Figure 3-4) and between male and female dogs (4.9 (SD 2.0) *vs.* 3.5 (SD 0.5), P = 0.075).

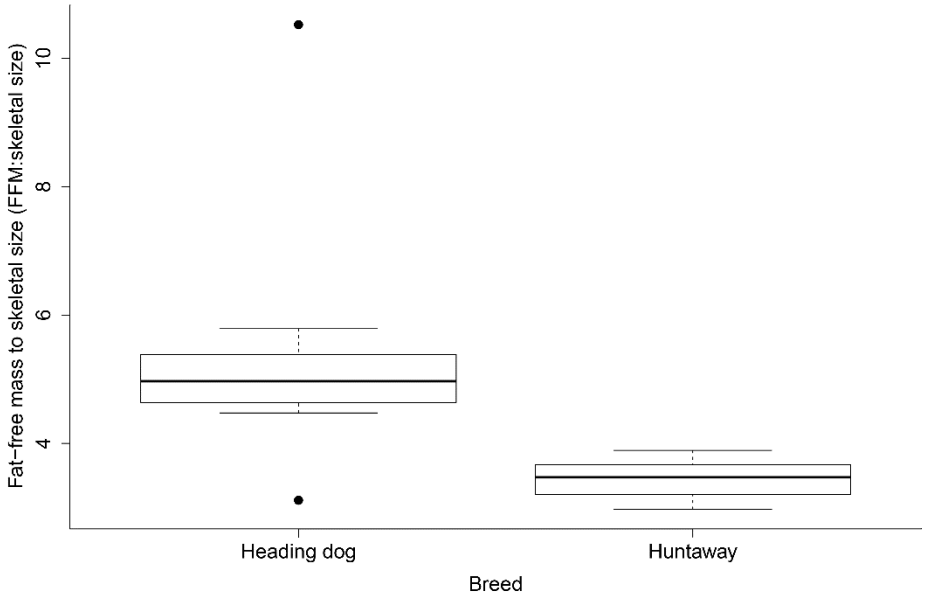

Figure 3-4. Boxplots of the ratio of fat-free mass to skeletal size measured in seven heading and 13 Huntaway dogs. The box represents the first and third quartiles, the solid line represents the median, and the whiskers represent the minimum and maximum values excluding outliers, which are dogs with values more than 1.5 times the interquartile range, represented by dots.

The regression models for associations between ratios of predicted FFM to skeletal size and FFM to skeletal mass and measured values, and predicted leanST to skeletal size and leanST to skeletal mass and estimated values are presented in Table 3-4. Correlation coefficients were higher for both FFM to skeletal size and leanST to skeletal size than for ratios using skeletal mass.

Only body weight was associated with the ratio of FFM to skeletal size or leanST to skeletal size (Table 3-5). Body weight accounted for 55% of the variation in the ratio of FFM to skeletal size and 54% of the variation in leanST to skeletal size.

Table 3-4. Regression models for association between ratios of predicted fat-free mass (FFM)[a] or lean soft tissue mass (leanST)[b] to skeletal size or mass and ratios of measured FFM or estimated leanST to skeletal size or mass in 20 working farm dogs.

Predicted variable	Variable	Coefficient	95% CI	r^2
FFM : skeletal size	Measured FFM : skeletal size	0.94	0.90, 0.98	0.99
	Intercept	0.25		
FFM : skeletal mass	Measured FFM : skeletal mass	1.02	0.68 – 1.36	0.67
	Intercept	−0.23		
				Pearson's r
LeanST : skeletal size	Estimated LeanST : skeletal size	1.06	0.92 – 1.14	0.99
	Intercept	−0.25	−0.51 – 0.29	
LeanST : skeletal mass	Estimated LeanST : skeletal mass	0.99	0.49 – 1.24	0.81
	Intercept	0.03	−2.47 – 5.03	

[a] Determined using linear regression analysis

[b] Determined using Passing Bablok regression analysis

Table 3-5. Final regression models for variables associated with the ratio of measured fat-free mass (FFM) or estimated lean soft tissue (leanST) to skeletal size in 20 working farm dogs.

Predicted variable	Variable	Coefficient	95% CI	r^2	P-value [a]
FFM : skeletal size	Body weight	−0.27	−0.39 – −0.15	0.55	<0.001
	Intercept	11.02			
LeanST : skeletal size	Body weight	−0.242	−0.35 – −0.14	0.54	<0.001
	Intercept	9.89			

[a] Significance of r^2

3.4.5 BCS-derived variables

There was a positive correlation between BCS-derived fat mass as a percentage of body weight and fat mass percentage determined using isotope dilution ($r^2 = 0.65$, $P < 0.001$), and BCS-derived FFM percentage and measured FFM percentage ($r^2 = 0.65$, $P < 0.001$), however the BCS-derived FFM percentage in the dog with BCS 1 was >100%. Also, there was a stronger correlation between BCS-derived FFM and measured FFM ($r^2 = 0.95$).

3.5 Discussion

The principal result of this study was the development of equations to accurately predict FFM, and the ratio of FFM to skeletal size in two breeds of New Zealand farm dogs using morphometric measurements. Previously published measures of body composition for dogs (BCS and morphometry) have only been validated using normally conditioned and obese dogs, but none have been developed for use in lean dogs. The equations produced in this study can be used to estimate the FFM and the ratio of FFM to skeletal size of individual lean Huntaway and heading dogs.

It was elected to use principal component analysis to create a new variable that defined skeletal size. The first principal component from the data explained 77% of the variation in skeletal measurements. Also, upon plotting the first principal component against the second principal component, a clear differentiation between the two breeds was seen within the first principal component. All measurements had similar eigenvalues of the same sign and provided similar weighting to the overall variation in skeletal size. This indicated that no individual skeletal measurement was distinctly different to the others with regards to the overall definition of skeletal size for these two breeds. The same is unlikely to be true of other breeds with different phenotypes.

A strong correlation was found between measured FFM, estimated leanST and their respective predicted values. Skeletal size or skeletal mass, breed, age and body weight were associated with measured FFM, but only breed, age and body weight were associated with estimated leanST. Body weight alone explained the majority of the

variation for both of the predicted measures. This finding is consistent with the narrow range of fat mass in the study population, and therefore variation in body weight contributed the most to the variation in lean tissue.

In addition, body weight was the only variable associated with the ratio of FFM to skeletal size, accounting for approximately 54% of the variation. This finding is interesting because it is hypothesised that this ratio may better predict the risk of disease and injury in working dogs compared with total muscle mass. Therefore, other factors besides body weight, breed, age, and sex that may be associated with this ratio may warrant further investigation. Although age was associated with measured FFM, it was not associated with the ratio of FFM to skeletal size. Sarcopenia is common in elderly people, and is likely to be as common in older dogs.[36] The dogs in this study were aged between 1–10 years. In non-obese Labrador retrievers, lean mass may not decline until 12 years of age, therefore this study population may not have included dogs that were old enough to demonstrate an association with the ratio of FFM to skeletal size.[36] Heading dogs tended to have a higher ratio than Huntaway dogs, indicating a breed difference.

The absence of a sex difference in the FFM as a percentage of body weight was surprising as males have been shown to have a greater percentage muscle mass than females in other studies.[37,38] Females in the study had a smaller skeletal size compared to males which aligns to the common notion that sexual size dimorphism is present in many species, including dogs, where females are smaller in stature than their male counterparts.[39,40]

The proportion of FFM mass contributed by the skeleton increases as dogs become leaner, therefore it was believed it might be important to assess the lean component separately in order to evaluate the ratio of FFM minus the skeleton to the skeletal mass. So a new variable, leanST, was created by subtracting estimated skeletal mass from FFM for each dog. However, the correlation coefficients for the regression models describing the association between predicted and measured values for the ratios FFM to skeletal size and leanST to skeletal size were higher than for FFM to skeletal mass and leanST to skeletal

mass. Thus, it appears that there was no benefit from calculating the variable skeletal mass.

Currently, assessment of BCS is the most commonly used method in practice to describe body composition in dogs. However, BCS was developed to predict fat mass and not to describe the muscle mass of very lean dogs. An association between BCS-derived FFM percentage and measured FFM percentage was examined to determine its accuracy in the lean study population. Under the two-compartment system, the percentage of a dog's fat mass and FFM should equal 100%. Therefore, if BCS is accurate there should be an association between BCS-derived FFM and measured FFM percentage. There was a poor correlation between these two variables when FFM was expressed as a percentage of body weight, but a strong correlation when comparing the absolute values. This was probably due to the majority of variation of body weight in the study population coming from lean mass differences, rather than fat mass. Thus, whilst estimating the small percentage of fat mass in a lean dog using BCS might be inaccurate, the subtraction of that small percentage from body weight introduces little error. In addition, the accuracy of BCS is dependent on the scorer and in this study, using BCS to determine FFM resulted in a FFM percentage >100% in the leanest dogs. Nonetheless, it will be interesting to see if the small increase in accuracy of estimating FFM in lean dogs attained using this approach is substantively superior in predicting clinical outcome measures.

There are several important limitations to this study. Firstly, it included a small population of dogs made up of only two breeds. The skeletal size of the two breeds was clearly different; therefore, it is assumed that these equations will not be accurate if used on other breeds, especially those with very different skeletal proportions. Further, while isotopic dilution is one of the gold standards when measuring body composition in dogs, the technique may overestimate total body water by 15–16% in dogs.[30] This could have had a significant effect when measuring lean mass. However other commonly used methods, including DEXA, have been shown to also overestimate and underestimate total body water in individual dogs.[41] Currently there is no other validated, non-invasive method of

measuring body composition better than isotopic dilution in dogs. When compared to another study assessing body composition in lean dogs using isotopic dilution, the results of this study are similar for percentage FFM (87% compared with 93 – 96%) and slightly higher for percentage body fat (13.03% compared with 3 – 7%), indicating good agreement.[42]

There are also several important assumptions made in the analysis. Firstly, skeletal mass was estimated to be 8% of the total body weight in ideally conditioned dogs, which was extrapolated from carcass dissection in beagles. It is likely that this percentage would be different for various breeds, for instance heavily muscled greyhounds compared with dachshunds. In this case, the two breeds included in the analysis are of similar size, so the effect of breed differences would be less. Nonetheless, additional methods such as DEXA, or carcass dissection would be needed to determine the accuracy of the regression equation created for skeletal mass. In addition, a pan-species hydration constant of 73.2% is traditionally used when calculating FFM from total body water.[43] However there are known variations to this constant with animals of different ages and species.[44] A study analysing the cadavers of 75 mature dogs of various skeletal sizes and body conditions found a study population hydration constant of 71.3%.[30] This is less than the conventional 73.2% hydration constant. It is thought that the leaner a dog is, the greater the contribution of skeletal mass as a percentage of total FFM, and thus producing a smaller hydration constant. Therefore, it was opted in this study to utilise the species-specific hydration constant, which is theoretically more appropriate for lean dogs. Despite this attempt to more precisely define the lean tissue in the study population, it is possible that the 71.3% hydration constant for FFM, and the 4% correction for deuterium exchange with non-water hydrogen may be different for very lean dogs with minimal musculature. Unfortunately, there are no published data to support the use of different factors in this study.

Despite these limitations, this study established a strongly predictive equation for calculating lean mass and the ratio of lean muscle mass to skeletal size in a small

population of lean Huntaway and heading dogs using morphometric measurements. Future studies could utilise the methods and equations outlined in this study on a large population of working Huntaway and heading dogs to determine the utility of their predicted measures (FFM and the ratio of FFM to skeletal size) on a large scale. Such studies could determine if there is a ratio of lean mass to skeletal size below which the risk of injury or disease increases. Extrapolation of this approach on a population of dogs would require calculation of the skeletal size of each dog using the equation

$$\left(\sum_{a-f} \frac{\left(m_{a-f}\right) - \left(\bar{x}_{a-f}\right)}{\left(\sigma_{a-f}\right)} \times \lambda_{a-f} \right) + 6$$

where m_{a-f} represents the values of the morphometric measurements a – f (head length, circumference etc) of the dog, $\bar{x}_{a-f}$ represents the mean of the measurements (from the population dataset, or the values in Table 3-2), σ_{a-f} represents the SD of the measurements (from the population dataset, or the values in Table 3-2), and λ_{a-f} represents the eigenvalues of the measurements (from the population dataset, or the values in Table 3-2).

Each dog's FFM could then be estimated using the regression coefficients in Table 3-3. It is hypothesised that there will be a threshold for the ratio of FFM to skeletal size below which health or performance will be compromised. If these measures prove useful, then these equations would provide an objective, non-invasive and simple measure to determine when welfare in individual dogs is compromised by underfeeding. In addition, further studies may determine the factors that affect the ratio of lean mass to skeletal size, which may include nutrition, neutering, training, and genetics.

3.6 Chapter Conclusions

The findings of this study suggest that it is possible to create an accurate and practical method of assessing body composition in lean dogs. However, there is a strong breed effect, indicating that breed-specific equations need to be generated. Therefore, the equations created here cannot be applied to other dogs outside of the two breeds used in this study. In addition, the study highlights that in lean dogs, body weight is already a good predictor of fat-free mass and lean soft tissue mass. This finding was thought to be reasonable because lean dogs have minimal body fat mass. Thus, in a hospital setting, it appears that the practical use of morphometry to estimate muscle mass in lean dogs may be superseded by the simpler method of measuring body weight.

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Chapter 4

The metabolic and hormonal effects of intermittent fasting in healthy dogs

The following two chapters describe a study examining the effects of intermittent fasting in healthy dogs. The first chapter (Chapter 4) describes the metabolic and hormonal effects, while the second chapter (Chapter 5) describes the immunological effects. The experimental design and results from these two chapters have been published in the Frontiers of Veterinary Science Journal, a copy of which may be found in Appendix III. Included in the dissertation are the descriptions of the optimisation of the assays, which were not included in the published manuscript.

4.1 Introduction

Optimal feeding regimens for both veterinary and human hospitalized patients have not yet been established. Underfeeding is common during hospitalization and is associated with depressed immunity, increased readmission rates, and increased mortality.[1–4] However, overfeeding critically ill patients has also been shown to have deleterious effects, including hyperglycaemia, hypertriglyceridaemia, gastrointestinal side effects, and aspiration pneumonia.[5–7] Iatrogenic hyperglycaemia is particularly problematic, because it can lead to impaired wound healing, neuronal dysfunction, increased production of the inflammatory cytokines interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), inhibition of leukocyte function, vasculitis, and ultimately a poorer clinical outcome.[8–13] Some of these effects can be abrogated when blood glucose is normalized, but may require a continuous infusion of insulin to do so.[9,14] So whereas clinicians want to provide appropriate nutrition for hospital patients, they need to establish the best means of doing so, while also avoiding hyperglycaemia.

A potentially effective feeding regimen to achieve these apparently antagonistic goals is intermittent fasting. Intermittent fasting is the process of reducing meal frequency in order to prolong the period of fasting between meals, but without necessarily restricting total

caloric intake when expressed over a longer period of time. Extending the period of fasting between meals has been found to increase insulin sensitivity, reduce serum fructosamine, reduce cancer cell proliferation, reduce concentrations of pro-inflammatory cytokines IL-6, IL-1β, and TNF-α in circulation, delay aging, and improve neuronal repair following injury when compared to continuous feeding.[15–25] In healthy mice, blood glucose and insulin concentrations were reduced following a period of intermittent fasting compared to those fed daily.[22] Several mechanisms have been proposed for these diverse effects, including the reduction of mitochondrial-derived reactive oxygen species, activation of Sirtuins and the associated promotion of autophagy and cell cleansing, and decreased expression of p38 mitogen-activated protein kinase, an upstream mediator of apoptosis.[19,26–28] These potential mechanisms would allow for a reduction in oxidative stress and a more tailored repair in response to injury.

An intermittent fasting regimen is of particular interest in patients with spinal disease, as it has been shown to reduce lesion size and improve recovery in rodent models of spinal injury compared with daily feeding.[22–24] Ketones, which increase during the fasting period, upregulate nicotinamide adenine dinucleotide (NAD)+-dependent Sirtuin 3, which in turn increases the expression of superoxide dismutase 2 and autophagy mediator protein forkhead box O3a (Foxo3a), and has been shown to reduce neuronal injury in the cerebral cortex of rats following experimentally induced hypoglycaemia.[29,30] This ketogenic effect can be enhanced by feeding a high-fat diet or one enriched in medium-chain triglycerides, both of which have been shown to promote the formation of ketones during the post-prandial and fasting state in several species.[31–34] In addition, ghrelin, an orexigenic peptide secreted by the stomach in a fasted state, protects neurons from ischemia and reperfusion injury *in vivo*, and decreases the expression of the TNF-α and IL-1β from microglial cells in Parkinson-modelled mesencephalic neuronal cell cultures.[35,36]

While intermittent fasting on a high-fat diet may be a beneficial feeding regimen for hospitalized spinal patients, fasting is also known to reduce the adipokine leptin.[18,36–39] Leptin plays a key role in regulating immune function by increasing neutrophil

chemotaxis, macrophage phagocytosis, and the maturation of T-cells.[40,41] A reduction in leptin from fasting is associated with a higher mortality rate in mice with endotoxic shock, whereas fasted mice replenished with leptin had a lower mortality rate.[42] To combat this, feeding mice a high-fat diet attenuates the drop in leptin during fasting, although this effect has not been tested in dogs.[43] In addition, preventing caloric malnutrition in a hospital setting may be easier when feeding a calorically-dense diet. So intermittent fasting on a high-fat diet enriched with medium-chain triglycerides may be the ideal feeding strategy that increases caloric intake, promotes neuronal recovery, reduces the risk of iatrogenic hyperglycaemia and preserves leptin concentrations in hospitalised spinal patients. However, as the effects of an intermittent fasting regimen on the metabolism of dogs is not known, it needs to be first described in healthy dogs before it can be considered for application in hospitalised dogs.

The principal aims of the work presented in this chapter were to determine the metabolic and hormonal effects of an intermittent fasting regimen using a high-fat and low-fat diet in healthy dogs. To do so, a protocol was developed and optimised for canine plasma metabolomics using nuclear magnetic resonance (NMR), which is a powerful method that can be used to assess plasma metabolites in an untargeted manner. Our primary hypotheses were that dogs undergoing intermittent fasting would have higher plasma concentrations of β-hydroxybutyrate and ghrelin, and lower concentrations of glucose, insulin, and leptin compared with dogs fed daily. Our secondary hypotheses were that dogs undergoing an intermittent fasting regimen on a high-fat diet enriched with medium-chain triglycerides would have greater blood β-hydroxybutyrate and leptin concentrations compared with dogs intermittently fasted on a low-fat diet.

4.2 Materials and methods

4.2.1 Development and optimisation of a canine plasma NMR-based metabolomics assay

The technique used for the optimisation process of canine plasma was based on the protocol described in a previous study using human plasma.[44] For this study, blood was

collected into heparin vacutainers[u] from three healthy donor dogs housed at Massey University Canine Nutrition Unit (Palmerston North, New Zealand). The optimisation study was approved by the Massey University Animal Ethics Committee (MUAEC 16/38). Within 1 hour of collection, plasma was harvested from the vacutainers by centrifugation for 10 minutes at 3,000 rcf at 4 °C and used immediately. Two solutions, methanol[v] and acetonitrile[w], were evaluated for their efficiency at precipitating protein from canine plasma. For the optimisation protocol, 300 µL of plasma sample from each dog were aliquoted into each of six vials. Then 600 µL of methanol was added into three of the vials, and 600 µL of acetonitrile was added to the remaining three vials, creating triplicates of each solution. All vials were then vortexed for one minute and incubated at -20 °C for 30 minutes. Following this, the samples were then centrifuged at 13,400 rcf for 30 minutes to pellet the precipitated proteins, and the supernatant was removed and placed into fresh vials. An attempt was made to dry the supernatants under nitrogen gas but was unsuccessful as it was inefficient and required too large a quantity of nitrogen gas and considerable time. A second attempt was made to dry the samples by placing the vials in a rotary vacuum evaporator for 3.5 hours at 20 °C. This process dried the majority of the supernatant, leaving only a small volume of liquid behind. The remaining supernatant was then dried completely by lyophilisation (freeze drying).

A buffer solution was prepared by dissolving 928.6 mg of anhydrous NaH_2P0_4 and 320.9 mg of anhydrous Na_2HPO_4 in 100 g of deuterium solution (D_2O) and used without pH modification. Following this, the dried plasma samples were reconstituted using 600 µL of phosphate buffered deuterium oxide solution containing two standards (0.5 mM of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS)[x] and 5 mM imidazole[y]) to provide a

[u] BD Vacutainer®, Becton, Dickson and Company, Plymouth, United Kingdom
[v] Methanol, catalog #322415, Sigma-Aldrich Limited, St. Louis, Missouri, United States
[w] Acetonitrile, catalog #271004, Sigma-Aldrich Limited, St. Louis, Missouri, United States
[x] DSS, catalog #178837, Sigma-Aldrich Limited, St. Louis, Missouri, United States
[y] Imidazole, catalog #15513, Sigma-Aldrich Limited, St. Louis, Missouri, United States

reference chemical and an internal pH indicator. The samples were then transferred to 5 mm NMR tubes[z] and analysed using a cyroprobe-equipped spectrometer[aa] operating at 700.13 MHz. The samples were read at 25 °C using a standard 1D NOSEY pulse sequence with presaturation of the residual water signal. Spectra were recorded using 58k points and an acquisition time of 3.5 s followed by a relaxation delay of 1.5 s. The TopSpin[bb] program was used to process the ^{1}H NMR spectra. Phasing and baseline correction of all NMR spectra were checked manually.

4.2.2 Metabolic and hormonal effects of intermittent fasting in healthy dogs

Following a complete physical examination, ten healthy, adult dogs from Massey University Canine Nutrition Unit were used in this study. Two breeds of dogs were used: harrier hounds and New Zealand Huntaways. The study protocol was approved by the Massey University Animal Ethics Committee (MUAEC 16/130).

A week before the commencement of the study, all dogs were transitioned onto a high-carbohydrate, low-fat commercial dry diet (Table 4-1). The dogs were fed to meet their maintenance energy requirements based on historical colony data. After an acclimation period of seven days on the commercial low-fat (LF) dry diet[cc], the dogs were randomized into one of three groups which underwent each feeding trial regimen in a 3 x 3 Latin-square design, with a weeklong 'wash out' duration in-between on the LF diet. The three feeding regimens were as follows: 1) daily feeding on a high-carbohydrate, low-fat diet (BID LF), 2) intermittent fasting (feeding once every 48 hours) on the same LF diet (IF LF), and 3) intermittent fasting (feeding once every 48 hours) on a high-fat (HF) diet (IF HF). Both diets used in this study were formulated to meet the nutrient requirements for adult dogs defined by the Association of American Feed Control Officials (AAFCO). The high-

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[z] 5 mm NMR Sample Tubes, Wilmad-LabGlass, Vineland, New Jersey, USA
[aa] Bruker Avance 700 MHz, Bruker Biospin, Rheinstetten, Germany
[bb] TopSpin™ v3.0, Bruker Biospin, Rheinstetten, Germany
[cc] Eukanuba Large Breed Weight Control dry, Mars Incorporate, McLean, Virginia, United States

fat diet was created using the LF diet with the addition of powdered whey protein, beef tallow, sunflower oil, coconut oil and a multivitamin/mineral mix[dd] to ensure nutritional adequacy of the total diet. The total amount of medium-chain triglycerides (C8, C10, C12) from the coconut oil and beef tallow amounted to 14.7% of the total calories in the diet when using an energy of 6.8 kcals/gram for the medium-chain triglycerides.[45] The nutrient profiles of both diets are presented in Table 4-1.

[dd] Balance IT® Canine, DVM Consulting Incorporated, Davis, California, USA

Table 4-1. The nutrient profile of the low-fat commercial diet and the modified high-fat diet. The high-fat diet was created using the commercial low-fat diet with the addition of whey protein isolate, beef tallow, sunflower oil, coconut oil and a multivitamin/mineral mix. Both diets were formulated to meet the nutrient requirements for adult dogs as defined by the Association of American Feed Control Officials (AAFCO).

	Commercial low-fat diet	Modified high-fat diet
Protein energy (%ME)	22	22
Fat energy (%ME)	23	68
Carbohydrate energy (% ME)	55	10
Protein (g/Mcal)	62.79	62.95
Total lipid (g/Mcal)	26.91	79.41
Linoleic acid (18:2 n-6) (g/Mcal)	8.16	9.37
Carbohydrate (g/Mcal)	157.57	29.92
Choline (mg/Mcal)	717.60	670.54
Folate (mcg DFE/Mcal)	209.30	388.12
Niacin (mg/Mcal)	14.35	21.99
Pantothenic acid (mg/Mcal)	7.41	7.30
Riboflavin (mg/Mcal)	2.03	2.34
Thiamin (mg/Mcal)	1.11	0.70
Vitamin A (mcg RAE/Mcal)	4041.86	1301.85
Vitamin B-12 (mg/Mcal)	0.018	0.017
Vitamin B-6 (mg/Mcal)	2.09	1.17
Vitamin E (α-tocopherol) (IU/Mcal)	74.75	88.18
Calcium (g/Mcal)	2.84	3.13
Copper (mg/Mcal)	4.34	3.15
Iodine (mg/Mcal)	0.94	0.71
Iron (mg/Mcal)	45.71	34.39
Magnesium (g/Mcal)	0.32	0.27
Manganese (mg/Mcal)	15.03	4.55
Phosphorus (g/Mcal)	2.39	1.79
Potassium (g/Mcal)	2.21	2.59
Selenium (mg/Mcal)	0.14	0.10
Sodium (g/Mcal)	1.20	0.50
Zinc (mg/Mcal)	63.39	52.99
Vitamin D (IU/Mcal)	447.00	275.92

When dogs were in the daily feeding regimen, they were offered their maintenance energy requirement divided equally into two meals that were provided in the morning and the afternoon, approximately 10 hours apart. When the dogs were in the intermittent fasting regimen, they were offered twice their daily maintenance energy requirement in the morning every other day. The dogs were allowed up to three hours to consume their meal, after which the remaining food was removed and weighed. During the wash out period between feeding regimens, all dogs were placed on the commercial high-carbohydrate, low-fat dry diet and fed twice a day for one week.

On days 1, 3, 5 and 7 of a trial period, a fasted blood sample (12 mL in total) was collected into lithium heparin and plain red-top vacutainers[ee] from all dogs by jugular venepuncture before food was offered. Day 1 represented an overnight-fasted, baseline sample, while the samples collected on days 3, 5, and 7 represented either a 9 to 12-hour postprandial sample when the dogs were eating daily, or a 45 to 48-hour postprandial sample when the dogs were fasted intermittently. Immediately following blood collection, a protease inhibitor[ff] was added to the sample in the plain red-top vacutainer to prevent ghrelin degradation. All samples were placed on ice until they were centrifuged, and serum and plasma removed. Daily food intake, weekly body weight and body condition score were recorded for all dogs. To compare the caloric intake of the dogs on the different feeding regimens, the total calories eaten every two days (i.e., days 1 and 2, days 3 and 4, days 5 and 6) were divided by the weekly starting weight of each dog to the power of 0.75 in order to express intake as kcal per 48 hours/kgBWT$^{0.75}$. In addition, the activity of the dogs during the day (5am to 8pm) and night (8pm to 5am) was measured using a tri-axial accelerometer[gg] fitted to their collar.

[ee] BD Vacutainer®, Becton, Dickson and Company, Plymouth, United Kingdom
[ff] Millipore® DPP-IV Inhibitor, Merck Millipore, Darmstadt, Germany
[gg] Heyrex®, Say Systems, Wellington, New Zealand

After blood sampling on day 7, all dogs were placed onto the 'wash out' feeding regimen. Following a wash out, each group was fed according to their next assigned feeding regimen, and blood samples taken as described above. This was repeated once more so that all groups underwent each of the three different feeding regimens with a washout period in between.

4.2.2.1 β-hydroxybutyrate, glucose, and metabolomics

Within one hour of collection, plasma was harvested from the heparin vacutainers by centrifugation for 10 minutes at 3,000 rcf at 4 °C. Plasma glucose was analysed using a handheld glucometer[hh] which has been previously validated for use in dogs.[46] The remaining plasma was stored at -80 °C until further analysis. For NMR spectroscopy, plasma samples were prepared as described previously using the best protein precipitation solution during the optimisation protocol. The dried samples were stored in screw top vials[ii] at -80 °C until analysis.

β-hydroxybutyrate was assayed in plasma samples from days 3, 5 and 7 using a colorimetric assay[jj] according to the manufacturer's instructions. The thawed plasma samples were initially de-proteinated using 10 kD spin columns[kk] and centrifuged at 10,000 rcf for 10 minutes. The samples were then prepared and absorbance at 450 nm was measured using a microplate reader[ll].

4.2.2.2 Endocrinology

The plain red-top vacutainer was centrifuged for 10 minutes at 3000 rcf at 4 °C, and serum removed within one hour of collection. The serum was stored at -80 °C until analysis. Leptin, ghrelin and insulin were assayed using a commercial immunoassay[mm]. Samples

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[hh] Accu-check® Performa, Roche Diagnostics Limited, Mannheim, Germany
[ii] Cryo.S™, Greiner Bio-One, Frickenhausen, Germany
[jj] beta HB Assay Kit, catalogue #ab83390, Abcam, Cambridge, United Kingdom
[kk] 10kD spin column, catalogue #ab93349, Abcam, Cambridge, United Kingdom
[ll] VersaMax™ Microplate Reader, Molecular Devices LLC, San Jose, California, USA
[mm] Milliplex® MAP Canine Gut Hormone Magnetic Bead Panel, Millipore, Burlington, MA, USA

were prepared following the manufacturer's instruction. Briefly, 50 μL of serum was added to a 96-well plate to which a buffer and antibody-conjugated beads were added. The plates were gently agitated overnight at 4 °C. Following this, the plates were washed, detection antibodies and beads added, and analysed using a multiplex reader[nn].

To estimate insulin sensitivity, homeostasis model assessment (HOMA) scores were calculated as the product of glucose and insulin concentrations divided by 22.5.[47]

4.2.3 Statistical analysis

An *a priori* power analysis was performed using a desired mean difference and previously published standard deviations for key metabolites and hormones. The mean difference and standard deviation (SD) used in the power analysis were: β-hydroxybutyrate 0.05 (SD 0.01) mmol/L, ghrelin 75 (SD 53) pg/mL, leptin 3,000 (SD 3,000) pg/mL, and insulin 220 (SD 150) pg/mL. This indicated that a sample size of 10 dogs would be necessary for significance level (α) of 0.5 and a power of 80% to detect a difference in β-hydroxybutyrate, ghrelin, leptin, and insulin.

4.2.3.1 Metabolomics

For analysis of the final plasma samples, the NMR spectra were divided into 0.04 ppm spectral buckets, where the regions corresponding to water and DSS (4.68 to 4.88, - 0.1 to 0.1 ppm respectively) were excluded, along with the following additional regions 5.51 to 5.84, 5.92 to 6.07, 6.11 to 6.31. All spectra were normalized by total intensity.

The relationship between the diet groups and the metabolome was explored using principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA), which were performed using the SIMCA® program[oo]. These statistical methods can reveal clustering of samples into different groupings based on differences of metabolite concentrations across

[nn] Luminex 200™, Luminex Corporation, Austin, Texas, United States
[oo] SIMCA® v13.0, Umetrics, Sartorius Stedim Data Analytics AB, Umeå, Sweden

the sample population. PCA is an unsupervised method whereas PLS-DA and OPLS-DA are so-called supervised methods and use *a priori* knowledge of the group membership to fit the data and maximize separation of data from these groups. Pareto scaling was used for the supervised models and the PLS models were validated by permutation testing to rule out overfitting. The spectral buckets that contributed to the greatest variance in the samples were identified from loading plots and subsequently assigned to their associated metabolites using the Chenomx® metabolite library. The metabolite concentrations were then quantified using manual fitting of the spectral peaks in the Chenomx® NMR Suite[pp]. Concentrations of plasma β-hydroxybutyrate measured by the colorimetric assay was compared to the concentrations obtained from the fitted spectra using the 'BlandAltmanLeh' v0.3.1 package (Lehnert, 2015) in the R software[qq].[48]

4.2.3.2 Modeling

The 'lme4' package (Bates, Mäechler, Bolker & Walker, 2012) was used to perform a linear mixed effects analysis of the relationship between the outcome variables (change in weight, intake, activity, glucose, β-hydroxybutyrate, leptin, ghrelin, insulin, HOMA, and NMR metabolites), and the fixed variables (time, diet, age, sex, and BCS). Separate models were fitted for each outcome variable. Dog was included as a random effect to account for repeated measures. Interactions between the fixed variables, and between diet and day, and diet and diet sequence, were not significant, and so were not included in the final models.

If the visual inspection of the residual quantile-quantile plots and the Shapiro-Wilk test of the residuals indicated a deviation from normality or homoscedasticity, then transformations of the dependent variable were performed in attempts to improve consistency on the assumptions of the linear model. However, transformation did not lead

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[pp] Chenomx v8.3, Chenomx Incorporated, Alberta, Canada
[qq] R version 3.1.0; R Development Core Team, 2012; R Foundation for Statistical Computing, Vienna, Austria

to a change in the interpretation of the models or our conclusions. Therefore, for simplicity, the graphs and final model are reported with the untransformed data.

A *post-hoc* pairwise comparison of the estimated marginal means with Tukey's correction was performed when diet was significant in the final model. Results are presented as mean ± standard deviation unless stated otherwise. The results of the mixed effects models are presented as the means and standard error of means. P-values < 0.001 were considered indicative of very strong evidence, P < 0.01 of strong evidence, P < 0.05 of moderate evidence, P < 0.1 of weak evidence, and P ≥ 0.1 of insufficient evidence.[49]

4.3 Results

4.3.1 Development and optimisation of a canine plasma NMR-based metabolomics assay

Both methanol and acetonitrile protein precipitation resulted in spectra with well-defined peaks and minimal interference. The ^{1}H NMR spectra of the methanol and acetonitrile protein precipitated serums are presented in Figure 4-1. Upon closer inspection of the spectra location where β-hydroxybutyrate was expected, there were some differences in the quality of spectra seen (Figure 4-2). Comparison of the spectra revealed that samples whose proteins were precipitated using methanol (green and blue coloured traces) produced the least amount of protein interference, and the best quality readings. Thus, methanol was used in the subsequent protocol in the intermittent fasting study.

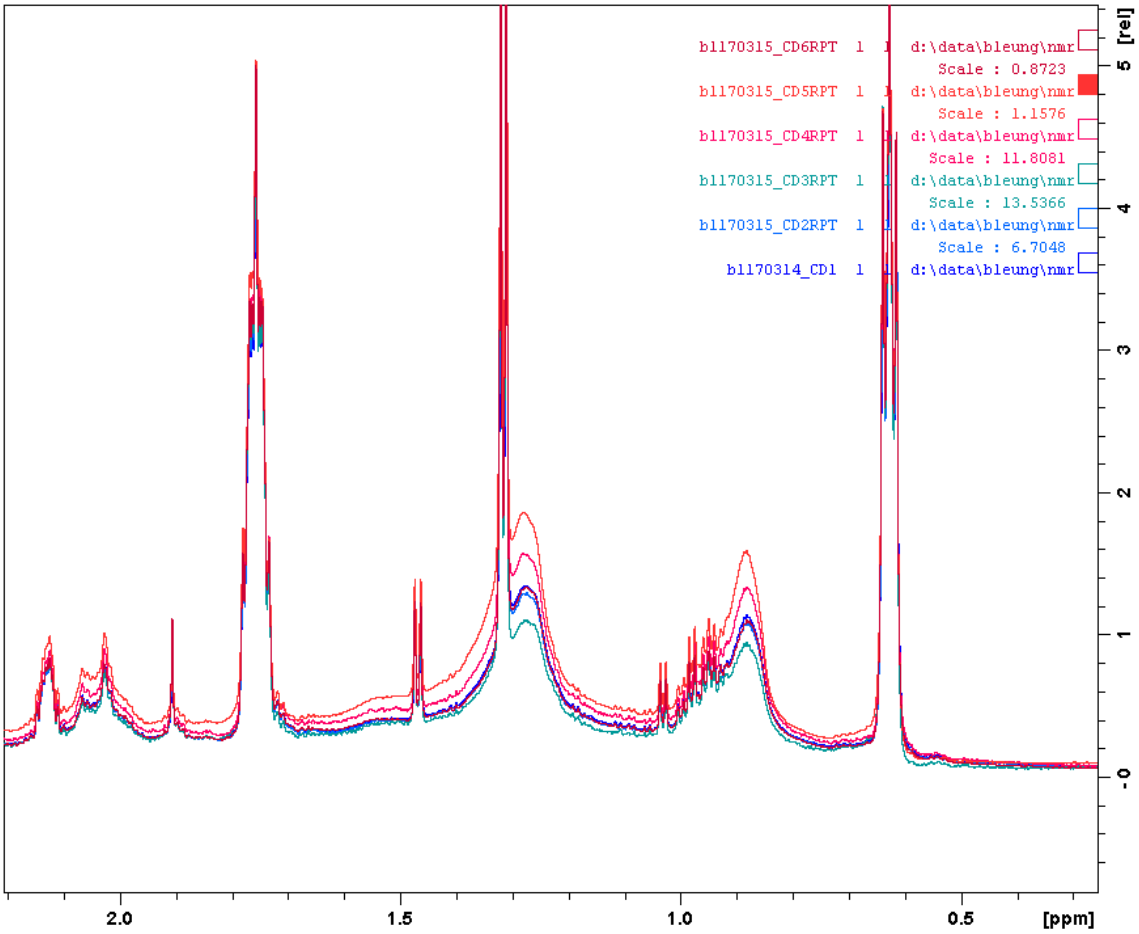

Figure 4-1. Comparison of ^{1}H NMR spectra of the same canine plasma sample obtained by protein precipitation using acetonitrile (red, orange and pink) and methanol (green, light blue, dark blue). Spectra tracings closest to the baseline indicate samples with the least amount of protein interference.

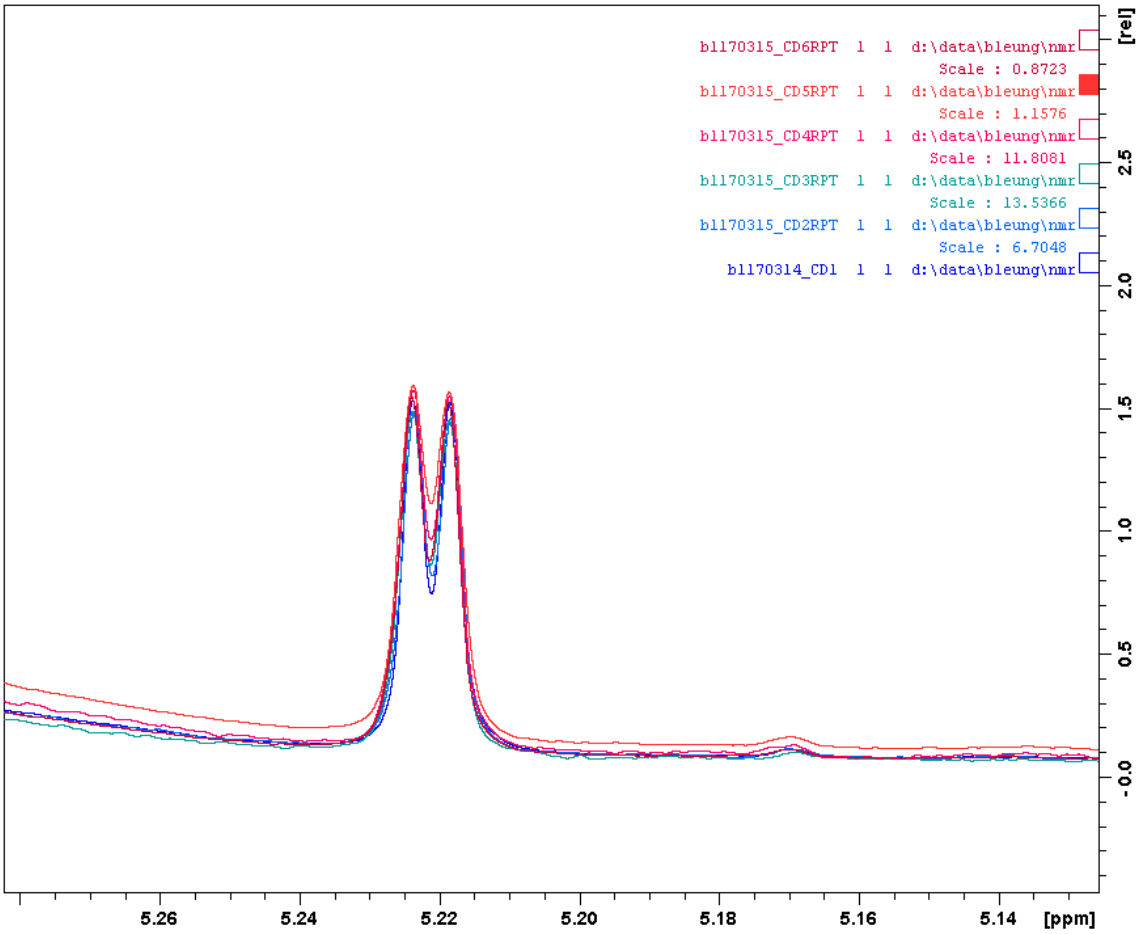

Figure 4-2. Comparison of the region of ^{1}H NMR spectra associated with β-hydroxybutyrate in the same canine plasma sample after protein precipitation using acetonitrile (red, orange and pink lines) and methanol (green, light blue, and dark blue lines). Spectra tracings closest to the baseline indicate the samples with the least amount of protein interference.

4.3.2 Metabolic and hormonal effects of intermittent fasting in healthy dogs

The dogs used were of two breeds: harrier hounds (n = 7) and New Zealand Huntaways (n = 3), of which four were neutered males and six were speyed females. The dogs had a mean age of 7.1 (SD 2.1) years, mean body weight of 27.8 (SD 3.1) kilograms, and a mean body condition score (BCS) of 4.2 (SD 0.4).

4.3.2.1 Intake, body weight and activity

There were no differences in any of the baseline parameters before the groups began their assigned feeding regimen in any of the treatment weeks ($P > 0.5$). All dogs remained at a BCS of 4 or 5 out of 9 throughout the study. When expressed over a 48-hour period, dogs fed daily had a higher food intake compared to when they were intermittently fasted ($P < 0.001$), however, there was no difference in intake between dogs when intermittently fed on the LF or the HF diets ($P = 0.395$, Table 4-2). Male dogs consumed more food per $kgBWT^{0.75}$ than female dogs over the 48-hour period (mean 277 ± 67 versus 226 ± 50 $kcals/kgBWT^{0.75}$). The dogs lost more weight when intermittently fasted on a low-fat diet, but there was no difference in the percentage of body weight change when the dogs were daily fed compared to when they were intermittently fasted on a high-fat diet regimen (Table 4-2). In addition, when the dogs were fed daily, they were more active at night compared to when they were intermittently fasted on the LF diet ($P = 0.028$) and HF diet regimens ($P = 0.012$).

Table 4-2. The means and standard deviations (SD) of food intake, change in body weight, and activity in 10 dogs fed daily (BID), and intermittently fasted on a low-fat (IF LF) and a high-fat diet (IF HF) in a Latin Square design. Also included are the results of the linear mixed effect models, including estimates and standard error (SE). Only independent variables with a P-value ≤ 0.1 are shown.

Outcome	Diet	Mean	SD	Fixed Effect	Estimate	SE	P-value
Intake	BID	295	40	(Intercept)	270	77	
(kcals/$BWT^{0.75}$)	IF LF	230	49	Diet IF LF	-66	13	< 0.0001
	IF HF	214	73	Diet IF HF	-81	12	< 0.0001
				Sex male	51	17	0.027
Body weight	BID	-1.7	1.6	(Intercept)	-1.72	0.65	
(% change)	IF LF	-3.3	2.6	Diet IF LF	-1.56	0.85	0.067
	IF HF	-1.6	1.8				
Total activity (ΔG)	BID	254,058	78,988	(Intercept)	199712	25811	
	IF LF	227,637	77,512	Week	13451	4126	0.005
	IF HF	242,923	79,130				
Day activity (ΔG)	BID	212,251	69,033	(Intercept)	175004	23164	
	IF LF	200,763	72,634	Week	11652	3378	0.004
	IF HF	219,528	72,006				
Night activity (ΔG)	BID	39,458	15,615	(Intercept)	39458	3829	
	IF LF	28,261	8,872	Diet IF LF	-11197	4599	0.028
	IF HF	26,319	8,017	Diet IF HF	-13665	4771	0.012

4.3.2.2 Metabolomics

Principal component analysis indicated some separation between the diet groups, with the first two principal components accounting for 45.6% and 9.1% of the variance, respectively. Further analyses with a supervised orthogonal partial least squares discriminant model showed a complete separation of the daily fed group and the intermittently fasted groups, and clustering of the two intermittently fasted groups (Figure

4-3). The metabolites associated with the spectral buckets that separated the different feeding regimes the greatest were β-hydroxybutyrate, lactate, alanine and glucose.

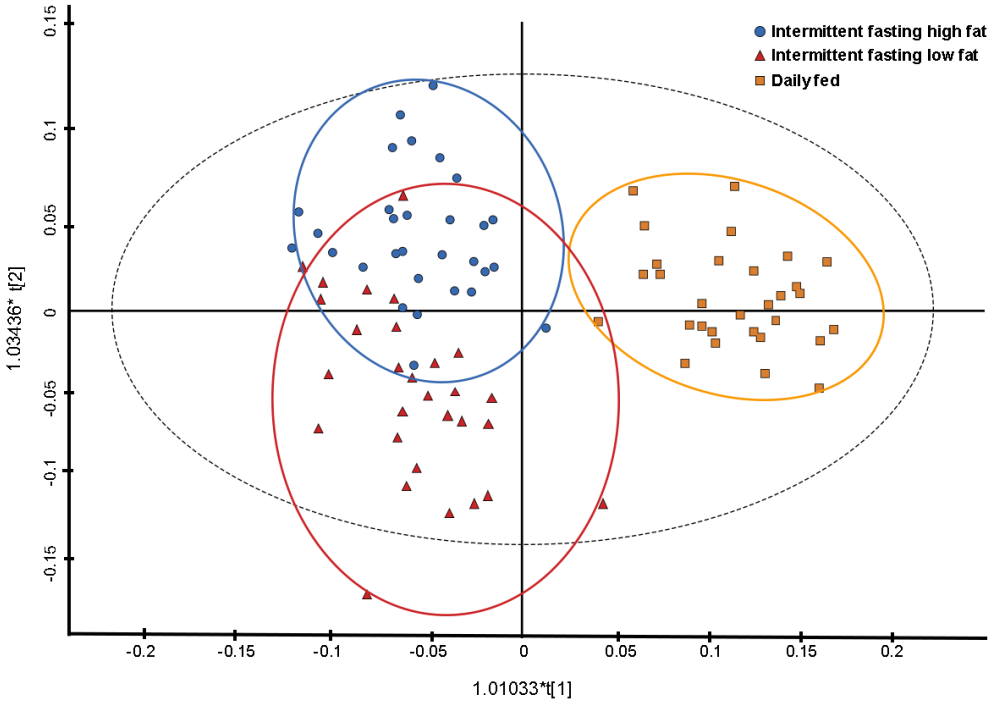

Figure 4-3. Orthogonal partial least square with discriminant analysis (OPLS-DA) plot illustrating the effect of three feeding regimen on the plasma metabolome of 10 dogs. Each point represents a single sample with the blue circles representing a dog intermittently fasted on a high-fat diet, the red triangles representing a dog intermittently fasted on a low-fat diet, and the orange squares representing a dog fed daily on a low-fat diet.

4.3.2.3 β-hydroxybutyrate assayed via kit and NMR

The concentrations of plasma β-hydroxybutyrate on day 3, 5 and 7 were highest when the dogs were intermittently fasted on the HF diet, and lowest when the dogs were fed daily (Figure 4-4). There was no overall effect of day. Intermittent fasting increased plasma β-hydroxybutyrate concentrations regardless of the diet fed, and a higher BCS was associated with a decrease in plasma β-hydroxybutyrate (Table 4-3). There was no association between weight loss and plasma β-hydroxybutyrate concentrations ($P = 0.198$). There was reasonable agreement between the β-hydroxybutyrate concentrations obtained from the colorimetric kit and from NMR (Figure 4-5).

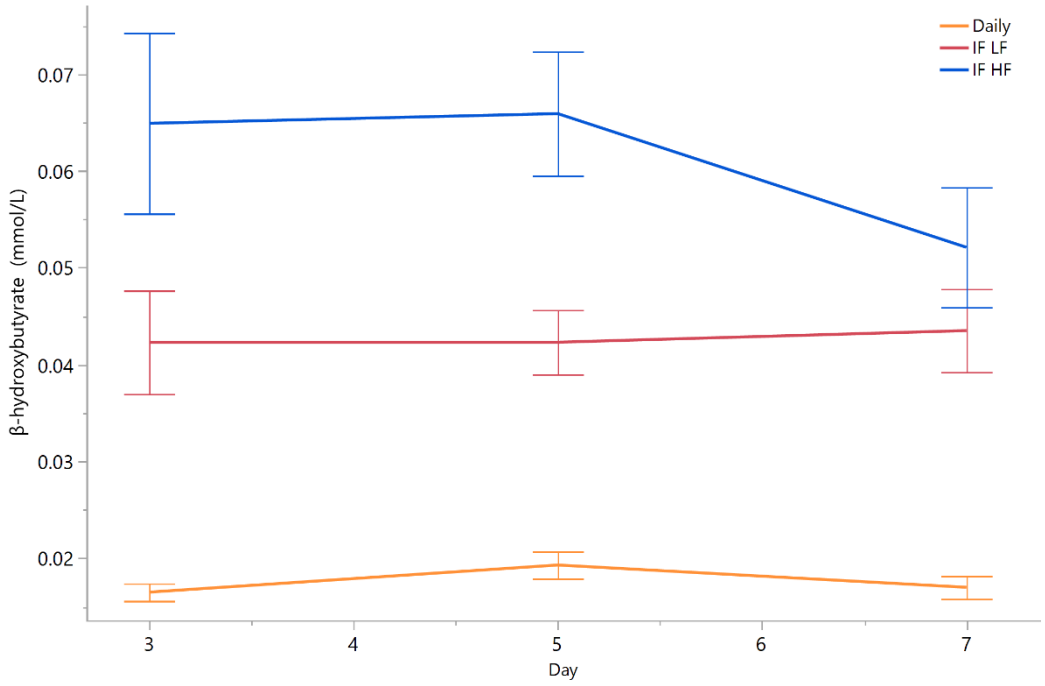

Figure 4-4. Mean concentrations (±SE) of fasted plasma β-hydroxybutyrate in 10 dogs fed daily (orange line), and intermittently fasted on a low-fat (red line) and a high-fat diet (blue line) in a Latin Square design.

Table 4-3. The means and standard deviations for β-hydroxybutyrate, lactate alanine and glucose, and the results of the linear mixed effect models, of 10 dogs fed daily (BID), and intermittently fasted on a low-fat (IF LF) and a high-fat diet (IF HF) in a Latin Square design. Only independent variables with a $P \leq 0.1$ are shown.

Outcome	Diet	Mean	Standard Deviation	Fixed Effect	Estimate	Standard Error	P - value
β-hydroxybutyrate	BID	0.018	0.004	(Intercept)	0.115	0.026	
(mmol/L)	IF LF	0.043	0.013	Diet IF LF	0.028	0.003	< 0.0001
	IF HF	0.061	0.024	Diet IF HF	0.043	0.003	< 0.0001
				BCS	-0.020	0.004	< 0.0001
Lactate	BID	0.652	0.150	(Intercept)	0.597	0.244	
(mmol/L)	IF LF	0.619	0.161	Day	-0.018	0.005	< 0.001
	IF HF	0.571	0.163	Diet IF HF	-0.078	0.029	0.009
Alanine	BID	0.222	0.047	(Intercept)	0.246	0.057	
(mmol/L)	IF LF	0.221	0.036	Diet IF HF	-0.003	0.009	< 0.001
	IF HF	0.191	0.042				
Glucose	BID	5.7	0.4	(Intercept)	5.9	0.5	
(mmol/L)	IF LF	5.6	0.3	Day	0.01	0.01	0.06
	IF HF	5.6	0.3	Diet IF LF	-0.12	0.06	0.03
				Diet IF HF	-0.13	0.06	0.02

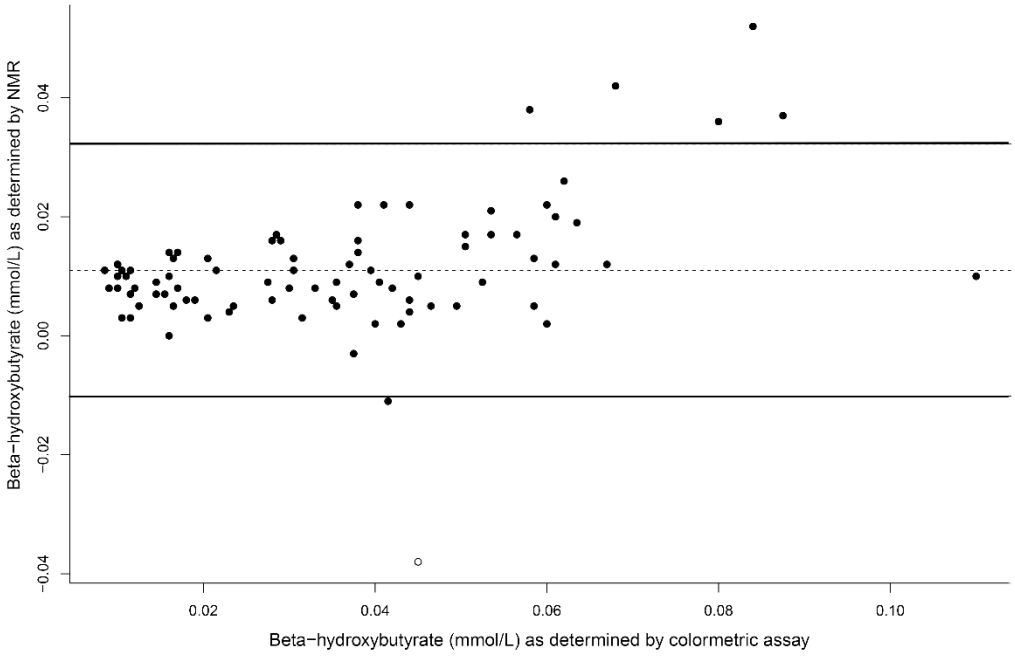

Figure 4-5. A Bland Altman comparison plot of β-hydroxybutyrate concentrations as assayed by the colorimetric kit and by nuclear magnetic resonance (NMR). Perfect agreement is at y = 0 and with the middle, dashed line as the actual agreement. The solid lines represent the 95% limits of agreement of the data.

4.3.2.4 Lactate and alanine via NMR

Both lactate and alanine concentrations were lowest when the dogs were intermittently fasted on a high-fat diet (Figure 4-6). In addition, lactate concentrations decreased over time ($P = 0.009$, Table 4-3).

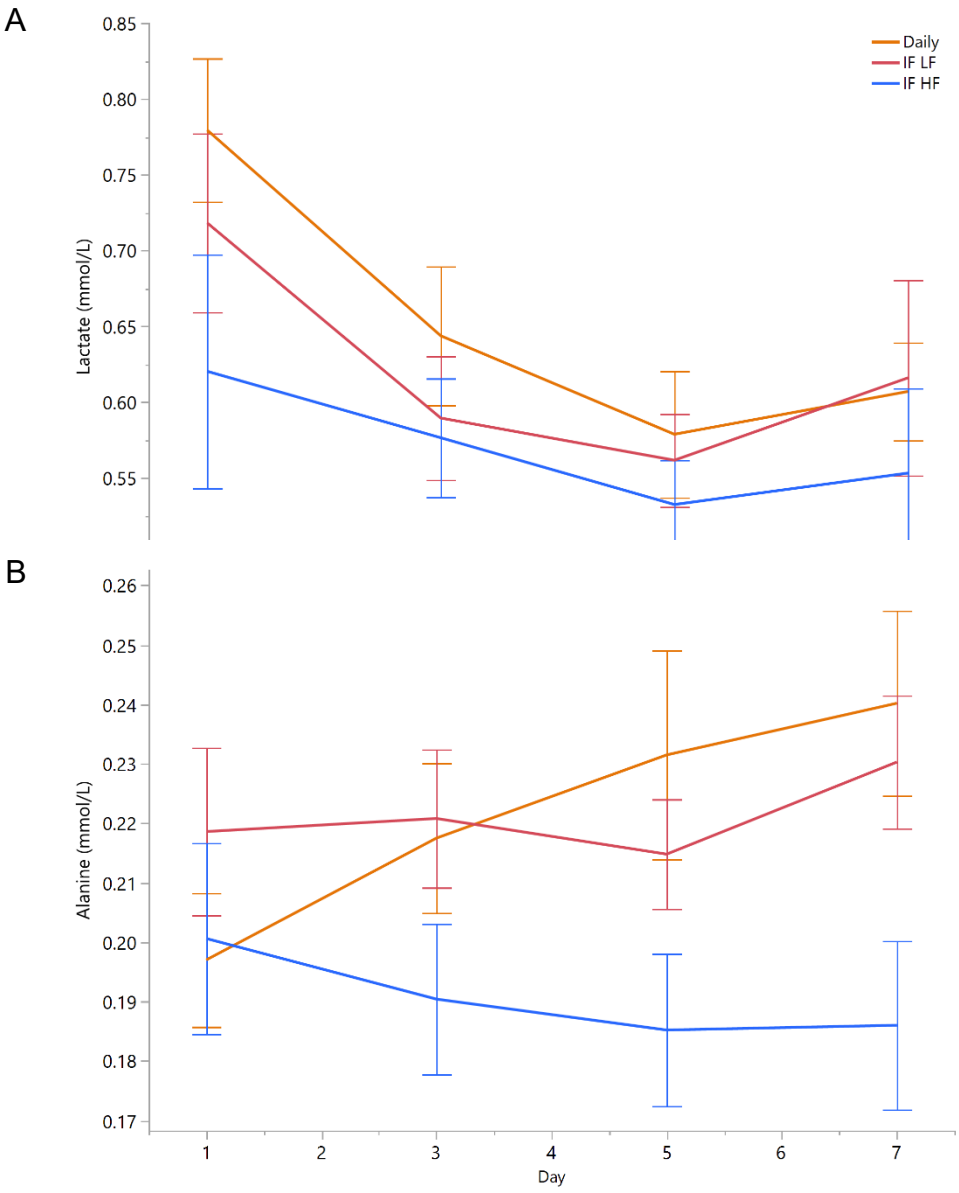

Figure 4-6. Mean concentrations (±SE) of fasted plasma lactate (A) and alanine (B) in 10 dogs fed daily (orange line), and intermittently fasted on a low-fat (red line) and a high-fat diet (blue line) in a Latin Square design.

4.3.2.5 Glucose

Blood glucose concentrations increased over time from day 3 to day 7 on all diet regimens and were highest in the dogs fed daily (Figure 4-7). There was no difference in glucose

concentrations between when the dogs were intermittently fasted on the LF, and the HF diets ($P = 0.98$). There was also no effect of BCS on blood glucose ($P = 0.24$, Table 4-3).

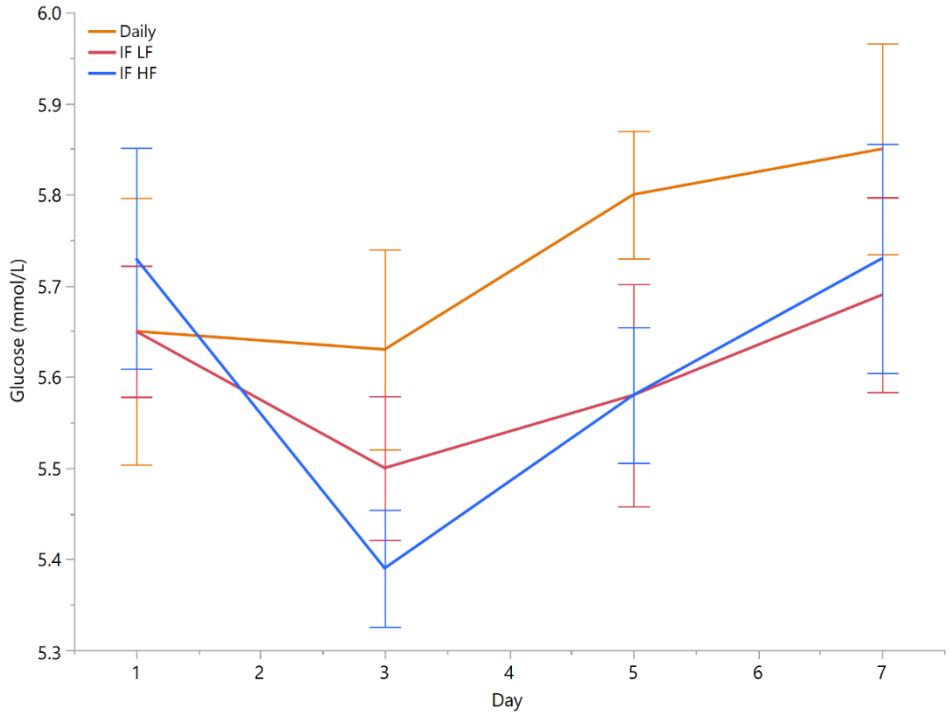

Figure 4-7. Mean concentrations (±SE) of fasted plasma glucose in 10 dogs fed daily (orange line), and intermittently fasted on a low-fat (red line) and a high-fat diet (blue line) in a Latin Square design.

4.3.2.6 Endocrinology: Insulin, HOMA, leptin and ghrelin

Both insulin concentrations and HOMA scores were lowest when dogs were intermittently fasted on a high-fat diet (Figure 4-8). A higher body condition was associated with lower insulin concentrations and HOMA scores (Table 4-4).

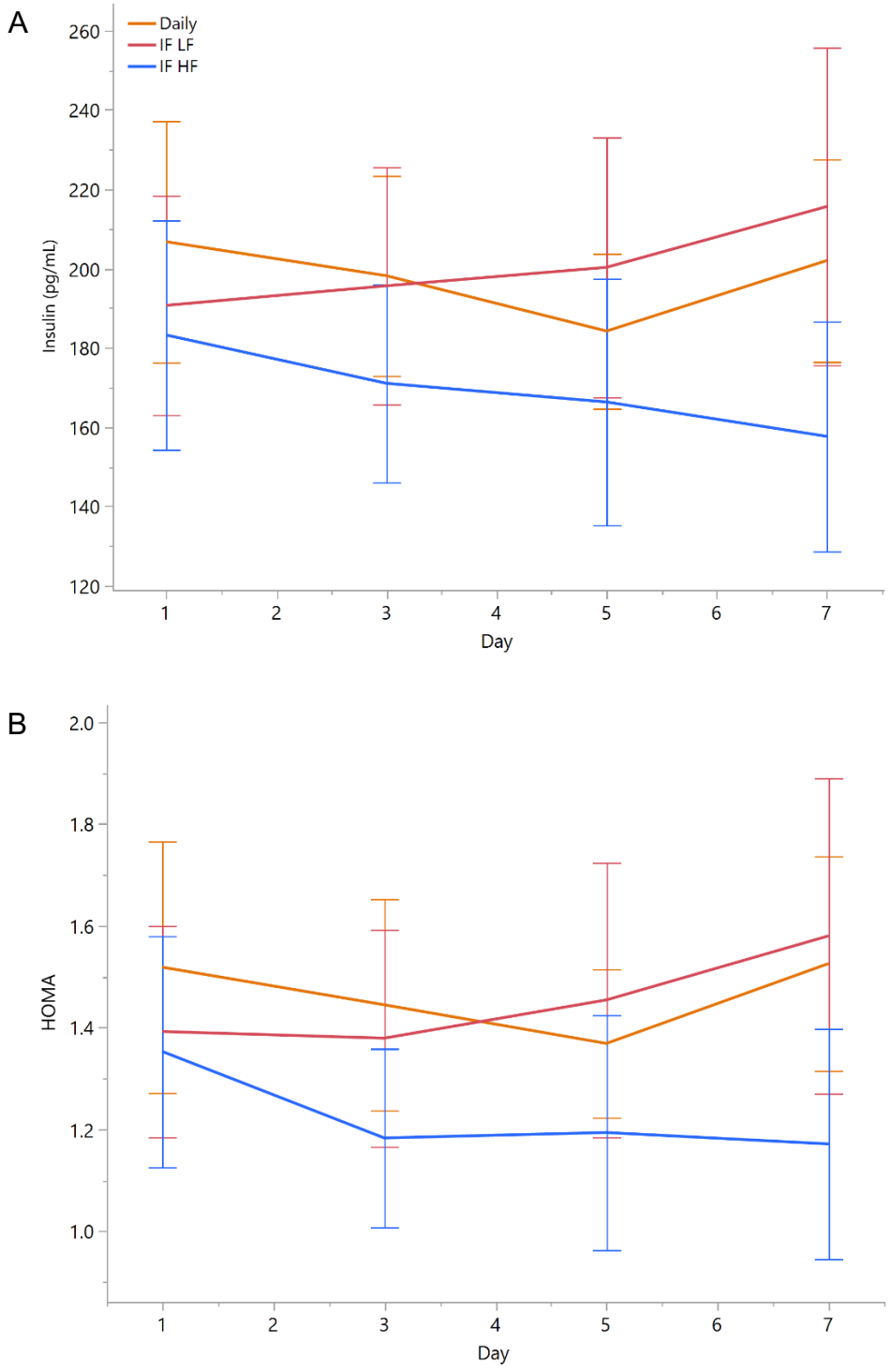

Figure 4-8. Mean concentrations (±SE) of fasted serum insulin **(A)** and HOMA score **(B)** in 10 dogs fed daily (orange line), and intermittently fasted on a low-fat (red line) and a high-fat diet (blue line) in a Latin Square design.

Table 4-4. The means and standard deviations (SD) for insulin, Homeostatic Model Assessment (HOMA), leptin and ghrelin, and the results of the linear mixed effect models, of 10 dogs fed daily (BID), and intermittently fasted on a low-fat (IF LF) and a high-fat diet (IF HF) in a Latin Square design. Only independent variables with a $P \leq 0.1$ are shown.

Outcome	Diet	Mean	SD	Fixed Effect	Estimate	Standard Error	P-value
Insulin	BID	198	78	(Intercept)	449	123	
(pg/mL)	IF LF	200	101	Diet IF HF	-31.5	13.2	0.02
	IF HF	169	87	BCS	-44.1	19.6	0.03
Homeostatic Model Assessment (HOMA)	BID	1.46	0.63	(Intercept)	3.22	0.99	
	IF LF	1.45	0.76	Diet IF HF	-0.26	0.011	0.01
	IF HF	1.23	0.66	BCS	-0.32	0.15	0.04
Leptin	BID	2451	2217	(Intercept)	3247	2887	
(pg/mL)	IF LF	1794	1683	Day	-111	32	<0.001
	IF HF	1729	1433	Diet IF LF	-637	179	<0.001
				Diet IF HF	-743	179	<0.0001
Ghrelin	BID	85	78	(Intercept)	8.3	115	
(pg/mL)	IF LF	88	73	Day	6.6	1.4	<0.0001
	IF HF	67	60	Diet IF HF	-17.6	7.9	0.03

Serum leptin concentration was highest when dogs were fed daily (Figure 4-9). In addition, there was a decrease in leptin concentrations over time (Table 4-4). For ghrelin, dogs fasted intermittently on a HF diet had lower serum concentrations (Figure 4-9).

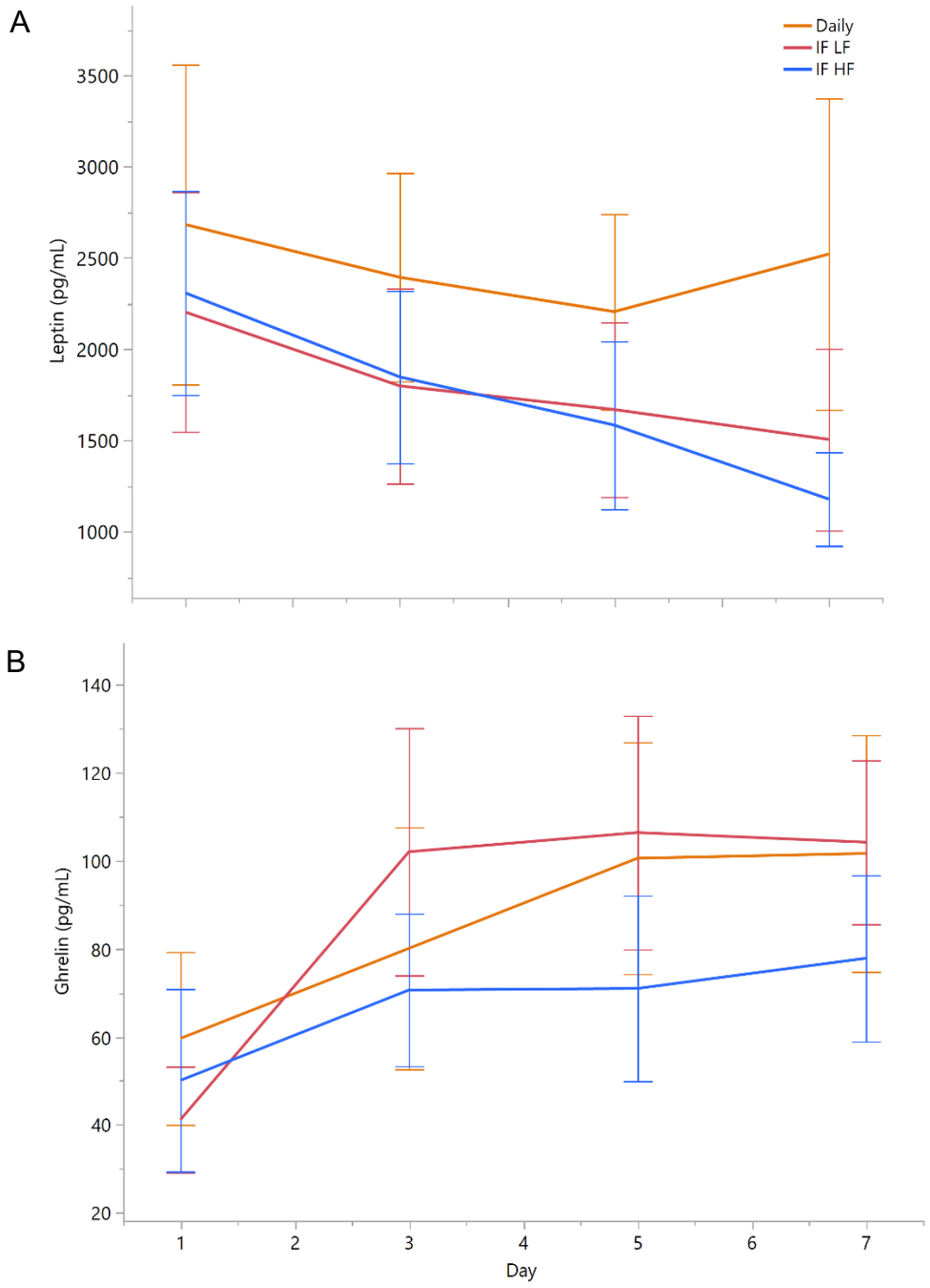

Figure 4-9. Mean concentrations (±SE) of fasted serum leptin (A) and ghrelin (B) in 10 dogs fed daily (orange line), and intermittently fasted on a low-fat (red line) and a high-fat diet (blue line) in a Latin Square design.

4.4 Discussion

Intermittent fasting and the promotion of ketone formation may be beneficial feeding strategies for hospitalized dogs, especially spinal patients. The primary hypothesis was that healthy, intermittently fasted dogs would have lower fasting blood glucose, insulin and leptin concentrations, and greater fasting β-hydroxybutyrate and ghrelin concentrations compared to when they were eating daily. Our secondary hypothesis was that intermittently fasted dogs eating a HF diet enriched with medium-chain triglycerides will have higher blood β-hydroxybutyrate and leptin concentrations compared with intermittently fasted dogs eating a LF diet. It was found that dogs intermittently fasted on a HF diet enriched in medium-chain triglycerides had higher blood β-hydroxybutyrate concentrations, and lower insulin concentrations than when they were fed daily or fasted on a LF diet. In contrast, leptin was not maintained during intermittent fasting by using a HF diet. In addition, fasted ghrelin concentrations were lowest when the dogs were intermittently fasted on a HF diet. Overall, these findings indicate that an intermittent fasting regimen on a high-fat diet enriched in medium-chain triglycerides increases plasma β-hydroxybutyrate concentrations, however it did not abrogate the drop in leptin or increase ghrelin concentrations during fasting.

Ketones provide an alternative source of energy for neurons and have been shown to reduce neuronal degeneration and improve recovery in rodent models of brain and spinal injury.[30,50–52] While β-hydroxybutyrate concentrations were highest in the dogs when they were intermittently fasted on the HF diet, the concentrations obtained (mean 0.061 (SD 0.024) mmol/L) were much lower compared to rodents (0.8 - 2 mmol/L) and humans (1.67 mmol/L) fasted for a similar amount of time.[23,53,54] This finding is consistent with other published studies that have found that ketones do not reach the same blood concentration in dogs as other species after comparable fasts.[55–57] However, it has been shown that the rate of total ketone production is similar between dogs and men following an approximate 48-hour fast.[58,59] Further, De Bruijne and Van den Brom[55] established that dogs have a higher rate of clearance of plasma ketones than man. Thus, the seemingly low concentration of β-hydroxybutyrate in dogs is probably not from reduced production of

ketones, but rather from higher rates of peripheral utilization compared with rodents and humans, though this needs to be tested especially in dogs consuming a high-fat diet. In addition, although a single blood sample is indicative of the concentration of a metabolite at that moment, it does not describe its flux (production and utilization).[59] It has been shown in humans and dogs that the concentration of β-hydroxybutyrate in the brain and cerebral spinal fluid (CSF) is proportional to the concentration found in plasma, and increases as the duration of fasting continues.[53,60,61] When available, β-hydroxybutyrate is utilized preferentially over glucose, lactate and pyruvate by neurons as an energy substrate.[62] So even a small increase in plasma concentrations of ketones could still significantly increase the utilization of ketones by the brain and peripheral neurons.

In our study, all dogs had fasting blood glucose concentrations within the normal reference range. Lower concentrations of blood glucose were seen when they were fasted for longer on either diet, whereas the lowest insulin concentrations were seen when the dogs were intermittently fasted on the HF diet. Feeding a high-fat diet to increase adiposity decreases insulin sensitivity, which is improved with caloric restriction and weight loss.[63–68] In this study, feeding a high fat diet intermittently resulted in lower HOMA insulin resistance scores. In addition, glucose concentrations were not significantly different between the intermittent fasting regimens, thus indicating that the resulting lower HOMA score reflects an increase in insulin sensitivity, rather than a decrease in insulin production.

Alanine and lactate were two gluconeogenic metabolites identified by OPLS as being different between the feeding regimens. During the early stage of fasting, transamination of pyruvate from glycolysis and branch-chain amino acid oxidation in the muscle leads to an increase of plasma alanine, providing a substrate for gluconeogenesis in hepatocytes and other gluconeogenic tissues.[69–72] In a study of dogs eating a carbohydrate-free diet, the turnover rate of alanine and conversion of alanine to plasma glucose were increased after a 48-hour fast compared to pre-fasting.[73] Lactate is also produced from glycolysis during fasting, and is transported by the same monocarboxylate transporters as ketones to serve

as an energy source for cells, including neurons, in a fasted state.[74,75] Feeding a high-fat diet to rats increased the expression of monocarboxylate transporter 1 by brain endothelial cells.[76] Also, lactate concentrations are increased in the brain of humans fasted for two days.[53] Thus, the reduction of both alanine and lactate in the dogs when intermittently fasted on a HF diet may be due to an increase in uptake by the liver, brain and kidneys, and/or from a decrease in glycogen availability for glycolysis.

When dogs were eating the LF diet intermittently, they consumed fewer calories and lost more weight compared to when they were eating the HF diet. There are numerous studies in several species which have also shown this phenomenon.[77] In one study, dogs fed a high-fat diet (51% energy from fat) *ad libitum*, gained more weight than dogs on a low-fat diet (23% energy from fat) *ad libitum*.[78] Therefore, to prevent weight loss in an intermittent fasting regimen, it is likely that a high-fat, energy dense food is required to ensure that a dog will consume its full requirements.

Limitations

When the dogs were intermittently fasted on the LF diet, they lost more weight than in the other feeding regimens, indicating the dogs were in a greater catabolic state. However, an increase in proteolysis and fatty acid oxidation was not reflected by an increase in plasma alanine and β-hydroxybutyrate concentrations of the dogs during the LF diet intermittent fasting regimen. In addition, plasma β-hydroxybutyrate concentration was not associated with weight loss in this study. A washout week using the control feeding regimen was performed in between each study period, and none of the outcome parameters were significantly different in the dogs at the start of each study period. In addition, both diets used in this study were formulated to meet AAFCO requirements, and while all attempts were made to create similar nutrient profiles excluding the fat and carbohydrate content, the diets did differ from one another in some micronutrients. However, the relative diet differences were not expected to drastically change the parameters studied. Diet order was examined in the multivariate model which did not show an effect. These results suggest that the differences in diet nutrient profiles and greater weight loss during the LF diet

intermittent fasting regimen likely had a minimal effect, however, a more thorough study would be required to determine if that is indeed the case.

In this study, no ill effects were seen when the dogs were intermittently fasted on the high-fat diet. To promote ketone formation, coconut oil was used in the HF diet as a rich source of medium-chain triglycerides. However, the main medium-chain triglyceride constituent in coconut oil is dodecanoic acid (lauric acid, C12), while decanoic acid (capric acid, C10) and octanoic acid (caprylic acid, C8) make up the remainder.[79] When given in equal amounts, intake of decanoic acid and octanoic acid leads to a greater ketone production postprandially then dodecanoic acid.[80,81] Thus, to increase the effect of medium-chain triglycerides on postprandial ketogenesis, a concentrated oil with a higher quantity of decanoic and octanoic acid could be used.

The homeostasis model assessment (HOMA) of insulin resistance was developed to provide a measure of peripheral insulin resistance from fasting glucose and insulin. The scores correlate well with a euglycemic clamp model in humans, and have been used to detect improvements in insulin sensitivity with weight loss and fasting in humans.[47,82,83] In our study, dogs intermittently fasted on a HF diet had the lowest HOMA score compared to when they were fed daily or intermittently fasted on the LF diet. HOMA has been found to be reliable in cats in detecting insulin resistance.[84,85] In overweight dogs, HOMA correlated with the insulin response following an intravenous glucose tolerance test, and was accurate in predicting dogs with a hypersecretory pancreatic response.[63] However, HOMA scoring was not reliable in detecting insulin resistance when there was a less robust pancreatic secretory effect, and so its overall usefulness is still uncertain. Therefore, while a difference with HOMA scores was found between the different feeding regimens, any interpretation of a difference in peripheral insulin sensitivity should be confirmed using a euglycemic clamp.

4.5 Conclusion

In this study, it was found that intermittent fasting on a high-fat diet enriched with medium-chain triglycerides in healthy dogs promoted a greater plasma β-hydroxybutyrate concentration than when the dogs were fasted after eating a low-fat diet. However, the concentrations obtained in the dogs were significantly less than what is reported in other species fasted for a similar period of time. Therefore, a ketone kinetics study is required to gain a more comprehensive understanding of the flux of ketones in dogs fasting for varying durations and fed different diets. It was also found that leptin concentrations were not preserved in the dogs during the fasting period, regardless of the fat content of the diet. The suppressive effects of fasting on immune function is a well-documented phenomenon that is present in many species, and believed to be the result of a drop in leptin.[36,86–88] As such, whether the dogs' immunity was affected during the intermittent fasting regimen, and whether there is a relationship with leptin concentrations, are important topics to be explored and is done so in the following chapter.

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Chapter 5

The immunological effects of intermittent fasting in healthy dogs

The following chapter describes a study examining the effects of intermittent fasting on the immune function of healthy dogs. The condensed version of this and the previous chapter (Chapter 4) has been published in the Frontiers of Veterinary Science Journal. The published manuscript is included in Appendix III. Included in this chapter is a description of the optimisation of the assays, which was not included in the published manuscript.

5.1 Introduction

As discussed in the previous chapter, intermittent fasting on a high-fat diet may be a beneficial feeding regimen for hospitalized spinal patients. However, some effects of fasting could be deleterious for patients as well, particularly the effect of fasting on humoral and cellular immunity. The link between energy intake and immune function is believed to be the adipokine leptin. Although best known as a key player in the regulation of energy expenditure and body weight, leptin also plays numerous roles in regulating immune function including increasing neutrophil chemotaxis, oxygen species generation, macrophage phagocytosis, and the maturation of T cells.[1,2] Acute fasting for 24 to 48 hours in several species have been shown to supress blood leptin concentrations.[3–5] This effect, however, appears to be reversible. In rodents, the suppression of lymphocyte differentiation, macrophage phagocytosis, and delayed-type hypersensitivity responses caused by leptin deficiency is reversed with supplementation.[3,6–9]

It has been demonstrated in several species that short-term fasting decreases peripheral leucocyte numbers, antibody response in B cells, and inflammatory cytokine production in T cells.[10–13] Conversely, there is less known about the effects of fasting on immune function in companion veterinary species, and most studies have examined the effect of longer periods of fasting. In a study of healthy cats, four days of food deprivation reduced peripheral leucocyte numbers and lymphocyte proliferation following mitogen

stimulation, which were partially reversed when the cats were refed.[14] In dogs, a steady reduction of food intake for 21 days (50% of requirements for 5 days, then 30% for 5 days, then 20% for 5 days, then complete food withholding for 6 days) led to a reduction in circulating neutrophil and lymphocyte counts, reduced lymphocyte proliferation *in vitro*, and lower serum concentrations of IgG, IgM and complement C3.[15] However, there remains little known about the effects of short-term fasting on immunity in companion veterinary species.

It was found as described in the previous chapter that leptin concentrations are decreased in an intermittent fasting regimen in healthy dogs. Still, the relationship between leptin, immunity and intermittent fasting in dogs is not yet well described. The work presented in this chapter was principally aimed at examining the effects of an intermittent fasting regimen using a high-fat and low-fat diet on immune parameters in healthy dogs. The primary hypothesis was that dogs undergoing intermittent fasting on a low-fat diet would have supressed parameters of immunity that would be commensurate with their decrease in plasma leptin, compared to when they are intermittently fasted on a high-fat diet, or when they are fed daily.

5.2 Materials and methods

5.2.1 Development and optimisation of a canine flow-cytometric immune assay

The development and optimisation of a flow cytometry technique using canine blood was based on the protocol described in a previous study using bovine blood.[16] For this, whole blood was collected into heparin vacutainers[rr] from three healthy donor dogs housed at Massey University Canine Nutrition Unit (Palmerston North, New Zealand). The samples were utilized within an hour of collection. This study was approved by the Massey University Animal Ethics Committee (MUAEC 16/38).

[rr] BD Vacutainer®, Becton, Dickson and Company, Plymouth, United Kingdom

For flow cytometry, two markers were used to detect and measure phagocytosis and oxidative burst in leukocytes. The first was pHrodo™ *Staphylococcus aureus* Bioparticles™ conjugates[ss], which once ingested by phagocytes, increases in red fluorescence as the environmental pH decreases. The second marker was Invitrogen™ dihydrorhodamine (DHR) 123[tt], a non-fluorescent reactive oxygen species (ROS) indicator that oxidises to green fluorescent rhodamine 123 intracellularly following activation of the NADPH-oxidase system in the cell. To determine the effect of different concentrations of the fluorescent markers, samples were tested using varying concentrations (Table 5-1).

[ss] pHrodo™ *Staphylococcus Aureus* Bioparticles™ conjugates, Thermofisher, CAT#A10010, Waltham, MA, USA

[tt] Invitrogen™ dihydrorhodamine (DHR) 123, Thermofisher, CAT#D23806, Waltham, MA, USA

Table 5-1. Concentrations of pHrodo™ Staphylococcus aureus Bioparticles™ conjugates and dihydrorhodamine (DHR)123 markers used for the optimisation of a flow cytometry assay. Optimisation was performed on whole blood from each dog.

Dog	Description	Whole blood	143 uM DHR	pHodo™ red S. aureus
1	Solo	100 µL	-	-
1	S.aur.50	100 µL	-	50 µL
1	S.aur.100	100 µL	-	100 µL
1	DHR10	100 µL	10 µL	-
1	DHR20	100 µL	20 µL	-
1	DHR10 + S.aur.50	100 µL	10 µL	50 µL
1	DHR10 + S.aur.100	100 µL	10 µL	100 µL
1	DHR20 + S.aur.50	100 µL	20 µL	50 µL
1	DHR20 + S.aur.100	100 µL	20 µL	100 µL
2	Solo	100 µL	-	-
2	S.aur.50	100 µL	-	50 µL
2	S.aur.100	100 µL	-	100 µL
2	DHR10	100 µL	10 µL	-
2	DHR20	100 µL	20 µL	-
2	DHR10 + S.aur.50	100 µL	10 µL	50 µL
2	DHR10 + S.aur.100	100 µL	10 µL	100 µL
2	DHR20 + S.aur.50	100 µL	20 µL	50 µL
2	DHR20 + S.aur.100	100 µL	20 µL	100 µL
3	Solo	100 µL	-	-
3	S.aur.50	100 µL	-	50 µL
3	S.aur.100	100 µL	-	100 µL
3	DHR10	100 µL	10 µL	-
3	DHR20	100 µL	20 µL	-
3	DHR10 + S.aur.50	100 µL	10 µL	50 µL
3	DHR10 + S.aur.100	100 µL	10 µL	100 µL
3	DHR20 + S.aur.50	100 µL	20 µL	50 µL
3	DHR20 + S.aur.100	100 µL	20 µL	100 µL

Nine 5 mL polystyrene round bottomed flow cytometry tubes[uu] were prepared for each sample. In each tube, 100 µL of whole blood was incubated at 37 °C for 30 minutes after adding 143 µM DHR solution, pHrodo™ *Staphylococcus Aureus*, both compounds, or

[uu] BD Flacon™ tubes, BD Biosciences, San Jose, California, USA

nothing. After incubation, 2.7 mL of deionized water was added to lyse erythrocytes. Within two minutes of adding the deionized water, 300 μL of 10X PBS was added and samples centrifuged at 600 rcf for 7 minutes. The supernatant was decanted, and the process repeated with 3 mL of PBS. Then, 3% formaldehyde in PBS was added and samples incubated at room temperature for 5 minutes for fixation. Finally, 2 mL of PBS was added, and samples were centrifuged at 350 rcf for 7 minutes. The supernatant was removed, and the pellet was suspended in 200 μL PBS with 2% foetal calf serum (FCS).

Flow cytometry was performed on a cell analyser[vv]. To account for emission spectra overlap and spillover in florescence channels, compensation was based on the control samples. To do so, forward scatter and side scatter detector settings were adjusted on solo, unstained samples until cell populations were clearly delineated in a forward and side scatter plot. Lymphocyte, monocyte, and neutrophil populations were then identified based on size and granularity. Following this, fluorescent channels (FL1 and FL2) detector settings were adjusted so that background autofluorescence was within 10^0 and 10^1 log scale in all FL plots. Compensation was set for each fluorochrome based on running the stained control samples and adjusting the compensation setting so that positively stained cells were in line with unstained cells, and parallel with the correct axis. The final compensation settings were checked using the double-stained cell populations to ensure the signals were adequately adjusted. Once compensation settings were in place, samples were run until at least 10,000 events were collected. Quadrants for single and double positive cells were demarcated using the set of control samples. The results of the gated populations were expressed as the percentage and the mean fluorescence intensity of positive cells undergoing phagocytosis and/or respiratory burst.

[vv] BD FASCSCalibur™, BD Biosciences, San Jose, California, USA

5.2.2 Development and optimisation of a [^{3}H]-thymidine incorporation assay

Bloods samples were obtained using the same dogs as above for the flow cytometry optimisation assay (MUAEC 16/38). The development and optimisation of a [^{3}H]-thymidine incorporation assay using canine whole blood was based on the protocols described in previous studies.[17,18] For the optimisation process, 100 μL of blood was transferred into each of fifteen wells on 96 U-well plates. To determine which mitogen(s) produced the greatest stimulation, four solutions were tested: 100 ng/mL of Staphylococcus enterotoxin B (SEB), 200 ng/mL of SEB combined with lipopolysaccharides from *E. coli* (LPS), 5 μg/mL phytohaemagglutinin (PHA), and 20 μg/mL concanavalin A (ConA). Each mitogen was trialled in triplicate. The remaining wells served as controls with the addition of only PBS. The plates were then incubated at 37 °C in 5% CO_2 humidified atmosphere for three days. Following this, 50 μL of [^{3}H]-thymidine of a 10 μCi/mL stock solution was added to each well. The plates were incubated for four hours at 37 °C in 5% CO_2 humidified atmosphere and then stored at -80 °C until analysis. On the day of analysis, the cells were harvested using an automated cell harvester, and the beta-radiations emissions were counted using liquid scintillation.

5.2.3 Immune effects of intermittent fasting in healthy dogs

The samples were obtained during the intermittent fasting study as described in Chapter 4. The experimental design and dietary regimens were as described previously, and the study protocol was approved by the Massey University Animal Ethics Committee (MUAEC 16/130).

On days 1, 3, 5 and 7 of a trial period, a fasted blood sample (6 mL in total) was collected into lithium heparin vacutainers[ww] from all dogs by jugular venipuncture before food was offered. Day 1 represented an overnight-fasted, baseline sample, while the samples collected on days 3, 5, and 7 represented either a 9 to 12-hour postprandial sample when

[ww] BD Vacutainer®, Becton, Dickson and Company, Plymouth, United Kingdom

the dogs were eating daily, or a 45 to 48-hour postprandial sample when the dogs were fasted intermittently. All samples were placed on ice for up to two hours until they were utilised for flow cytometry and [^{3}H]-thymidine incorporation assays.

5.2.4 Statistical analysis

The normality of the data obtained during the optimisation study was confirmed using an Anderson Darling test. The results from the optimisation experiments were assessed using a general linear model of ANOVA. 'Dog' was treated as a random effect. The variables assessed during flow cytometry were the percentage and the mean florescence intensity (MFI) of negative and positive cells. For lymphocyte proliferation, the mean cell count per minute (ccpm) for each mitogen treatment was normalised by subtracting the mean ccpm from unstimulated samples. In addition, the stimulation index (SI) was calculated for each dog as the ratio of mean ccpm of the stimulated wells, and the mean ccpm of the unstimulated wells.

For the intermittent fasting study, the 'lme4' package (Bates, Mäechler, Bolker & Walker, 2012) was used with R software[xx] to perform a linear mixed effects analysis of the relationship between the outcome variables (lymphocyte proliferation and flow cytometry results), and the fixed variables (time, diet, age, sex, BCS, and leptin). Separate models were fitted for each outcome variable. Dog was included as a random effect to account for repeated measures. Interactions between the fixed variables, and between diet and diet sequence, were not significant, and so were not included in the final models.

If the visual inspection of the residual quantile-quantile plots and the Shapiro-Wilk test of the residuals indicated a deviation from normality or homoscedasticity, then transformations of the dependent variable were performed in attempts to improve consistency on the assumptions of the linear model. However, transformation did not lead

[xx] R version 3.1.0, R Development Core Team, 2012, R Foundation for Statistical Computing, Vienna, Austria

to a change in the interpretation of the models or our conclusions. Therefore, for simplicity, the graphs and final model are reported with the untransformed data.

A post-hoc pairwise comparison of the estimated marginal means with Tukey's correction was performed when diet was significant in the final model. The results of the mixed effects models are presented as the means and standard error of means. P-values < 0.001 were considered indicative of very strong evidence, $P < 0.01$ of strong evidence, $P < 0.05$ of moderate evidence, $P < 0.1$ of weak evidence, and $P \geq 0.1$ of insufficient evidence.[19]

5.3 Results

5.3.1 Development and optimisation of a canine flow-cytometric immune assay

The procedure used resulted in good separation of leucocytes from canine whole blood, and distinct cell populations were identifiable on flow cytometry. Compensation settings were set accordingly and resulted in minimal overlap of fluorescent channels (Figure 5-1). There was no difference in the percentage of phagocytosis and oxidative burst in any of the cell populations between the different concentrations of the double positive samples ($P > 0.1$). As such, the lowest concentration of 143 μM DHR solution (10 μL) and pHrodo™ *Staphylococcus Aureus* (50 μL) was used during the subsequent study.

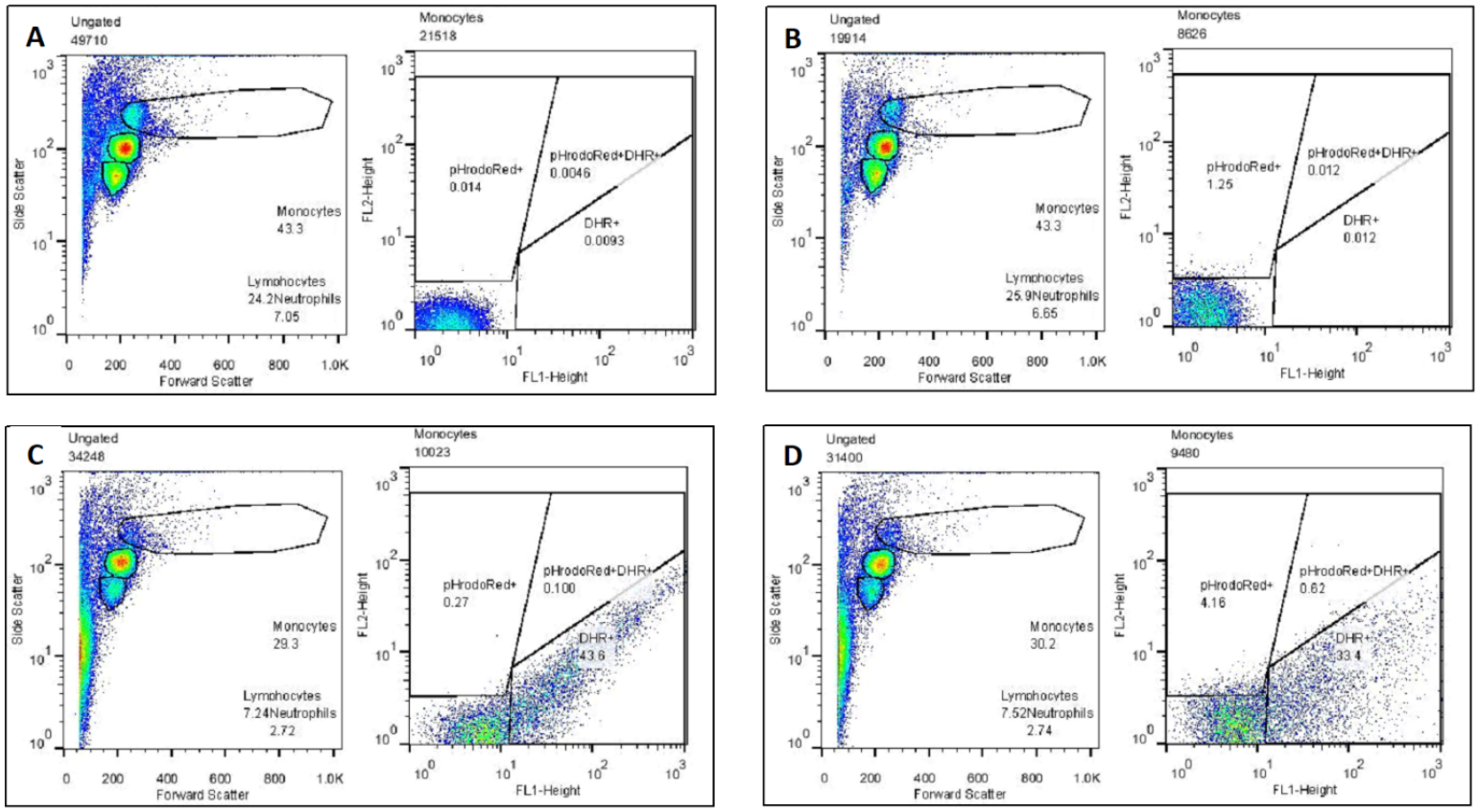

Figure 5-1. Representative uncompensated (A and C) and compensated (B and D) flow cytometry graphs of leucocyte separation from canine whole blood during the optimisation procedure. Graphs A and B are from the same sample, and graphs C and D are the same sample. Forward and side scatter plots showed clear separation of cell populations, which were identified based on expected granularity (y-axis) and cell size (x-axis). The numbers within the gated graphs indicate the percentage of cells that are not positive, single positive or double positive. The uncompensated graphs show an overlap of emission spectra resulting in a tight and linear cluster of cells, which is improved with compensation.

5.3.2 Development and optimisation of a [^{3}H]-thymidine incorporation assay

The results from the [^{3}H]-thymidine incorporate assays showed that the combination of SEB and LPS created the best lymphocyte stimulation results as assessed by mean ccpm ($P < 0.001$, Figure 5-2). The same results were obtained when SI was assessed ($P < 0.001$). Thus, a concentration of 200 ng/mL of SEB combined with lipopolysaccharides (LPS) was used in the subsequent study.

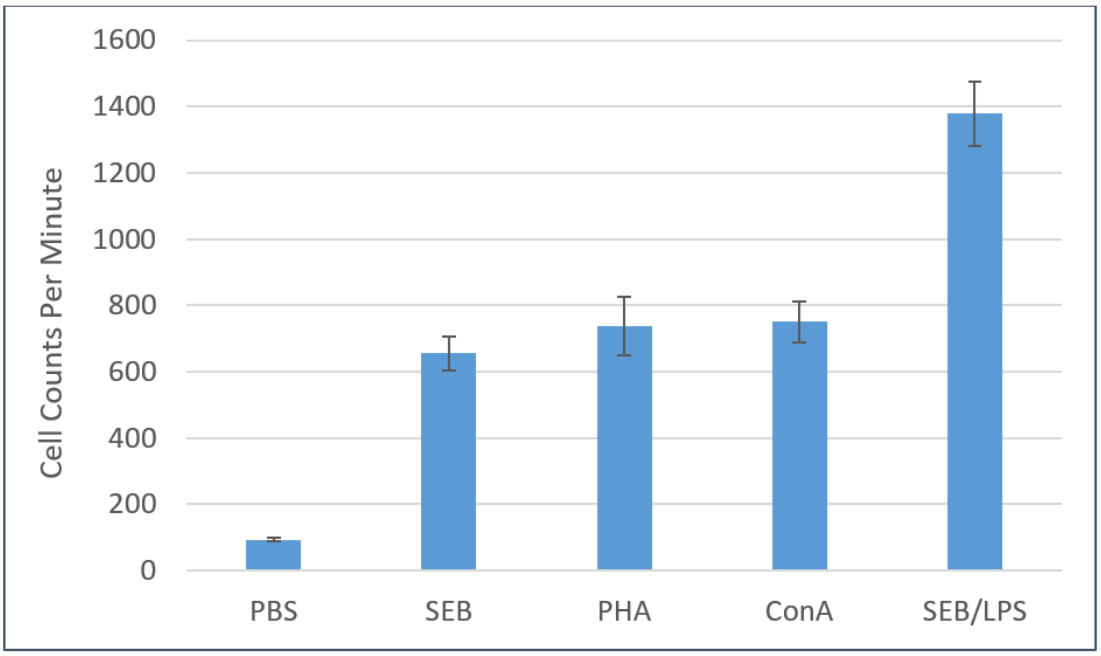

Figure 5-2. The mean cell counts per minute obtained for each mitogen stimulant during the [^{3}H]-thymidine incorporate optimisation assay. The mitogens were tested in triplicates: 100 ng/mL of Staphylococcus enterotoxin B (SEB), 5 µg/mL phytohaemagglutinin (PHA), 20 µg/mL concanavalin A (ConA), and 200 ng/mL of SEB combined with lipopolysaccharides from *E. coli* (LPS).

5.3.3 Immune effects of intermittent fasting in healthy dogs

All dogs completed the study. There were no differences in any of the baseline immune parameters before the groups began their assigned feeding regimen in any of the treatment weeks ($P > 0.1$). The resulting flow cytometry graphs from samples obtained during the intermittent fasting study were of similar quality to the optimisation study (Figure 5-3).

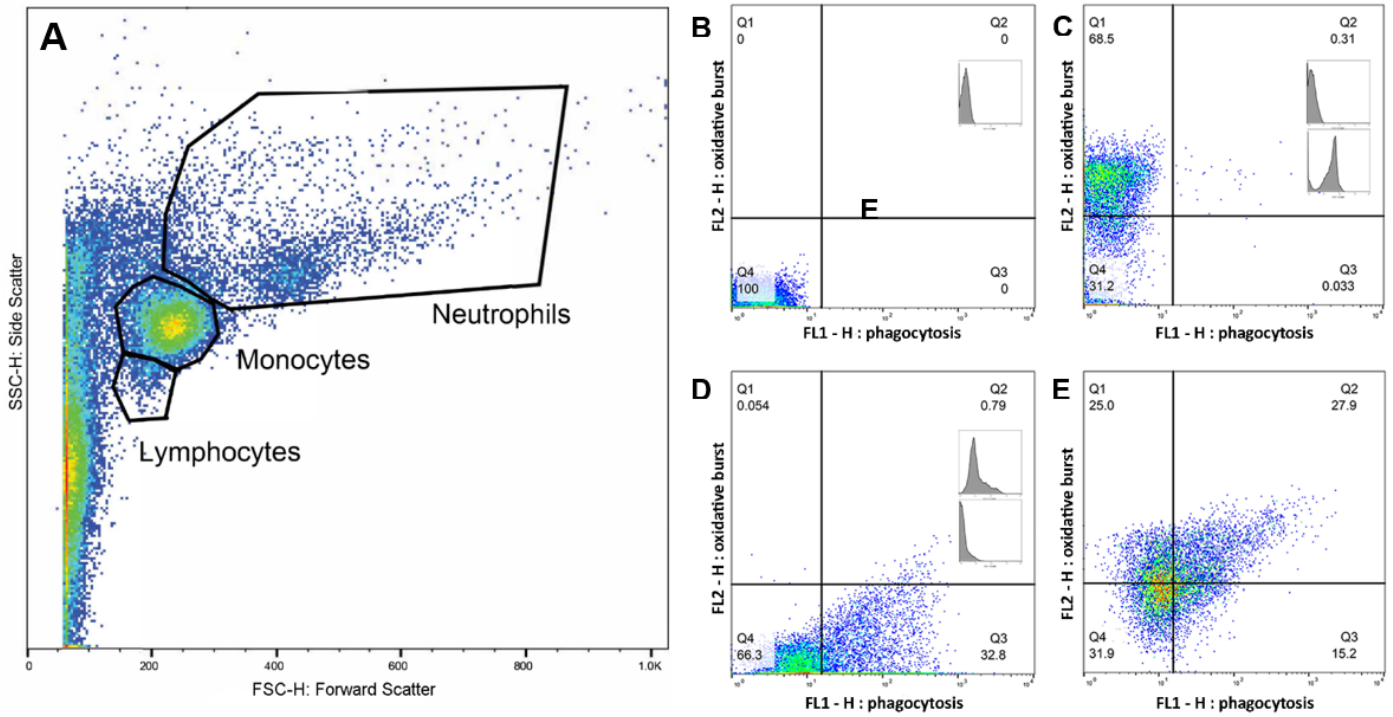

Figure 5-3. (A) A representative plot displaying the flow cytometry scatter properties of leukocytes in one sample. The forward scatter (x-axis) corresponds to size of the cells and the side scatter (y-axis) corresponds to the cells' granularity. Regions were gated around clusters of cells corresponding to the expected locations for lymphocytes, monocytes and neutrophils. Quadrants for single and double positive cells were established using a set of control samples. Plots B - E show a representative sample of gated monocytes from one sample. The number in each quadrant is the percentage of cells. (B) Monocytes without a fluorochrome added. (C) Monocytes with only dihydrorhodamine (DHR) 123 added. (D) Monocytes with only pHrodo™ Red S. *aureus* added. (E) Monocytes with both DHR and pHrodo™ Red S. *aureus* added.

The phagocytic and oxidative activity of the leucocytes are presented in Table 5-2 and Figure 5-4. When the dogs were intermittently fasted on a low-fat (IF LF) diet, they had a lower percentage of neutrophils ($P = 0.02$), and a lower MFI in those macrophages that underwent both phagocytosis and oxidation ($P = 0.06$, Figure 5-4). In addition, these dogs also had a lower percentage of lymphocytes that underwent respiratory oxidative burst ($P = 0.008$, Figure 5-4). There was no association between leptin concentrations and any immune parameter ($P > 0.1$, Figure 5-5). In addition, there were no significant differences in lymphocyte proliferation during any of the feeding regimens ($P > 0.1$).

Table 5-2. The mean, standard deviation and results of the linear mixed effect models of the activity of phagocytic cells in 10 dogs fed daily (BID), intermittently fasted on a low-fat (IF LF) and intermittently fasted on a high-fat diet (IF HF) in a Latin Square design. Only independent variables with a $P \leq 0.1$ are shown. The percentage is the number of cells that have undergone either phagocytosis and/or oxidative burst. Mean florescence intensity (MFI) of oxidation is proportional to the degree in which oxidation has occurred.

Outcome	Diet	Mean	Standard Deviation	Fixed Effect	Estimate	Standard Error	P-value
Lymphocyte oxidation (%)	BID	45.3	18.4	(Intercept)	30.6	25.3	
	IF LF	35.0	19.3	Diet IF LF	-10.7	3.9	0.008
	IF HF	43.1	17.1				
Monocyte phag+/ox+ MFI for oxidation	BID	64.9	20.0	(Intercept)	72.3	28.6	
	IF LF	57.9	25.8	Diet IF LF	-7.6	4.0	0.06
	IF HF	66.0	23.5				
Neutrophil oxidation (%)	BID	75.8	17.6	(Intercept)	73.6	23.3	
	IF LF	71.0	17.6	Diet IF LF	-4.9	2.2	0.02
	IF HF	75.9	16.8				
Neutrophil phag+/ox+ (%)	BID	74.7	18.1	(Intercept)	67.9	23.2	
	IF LF	69.9	17.6	Day	-0.8	0.4	0.04
	IF HF	74.4	16.4	Diet IF LF	-5.0	2.2	0.03

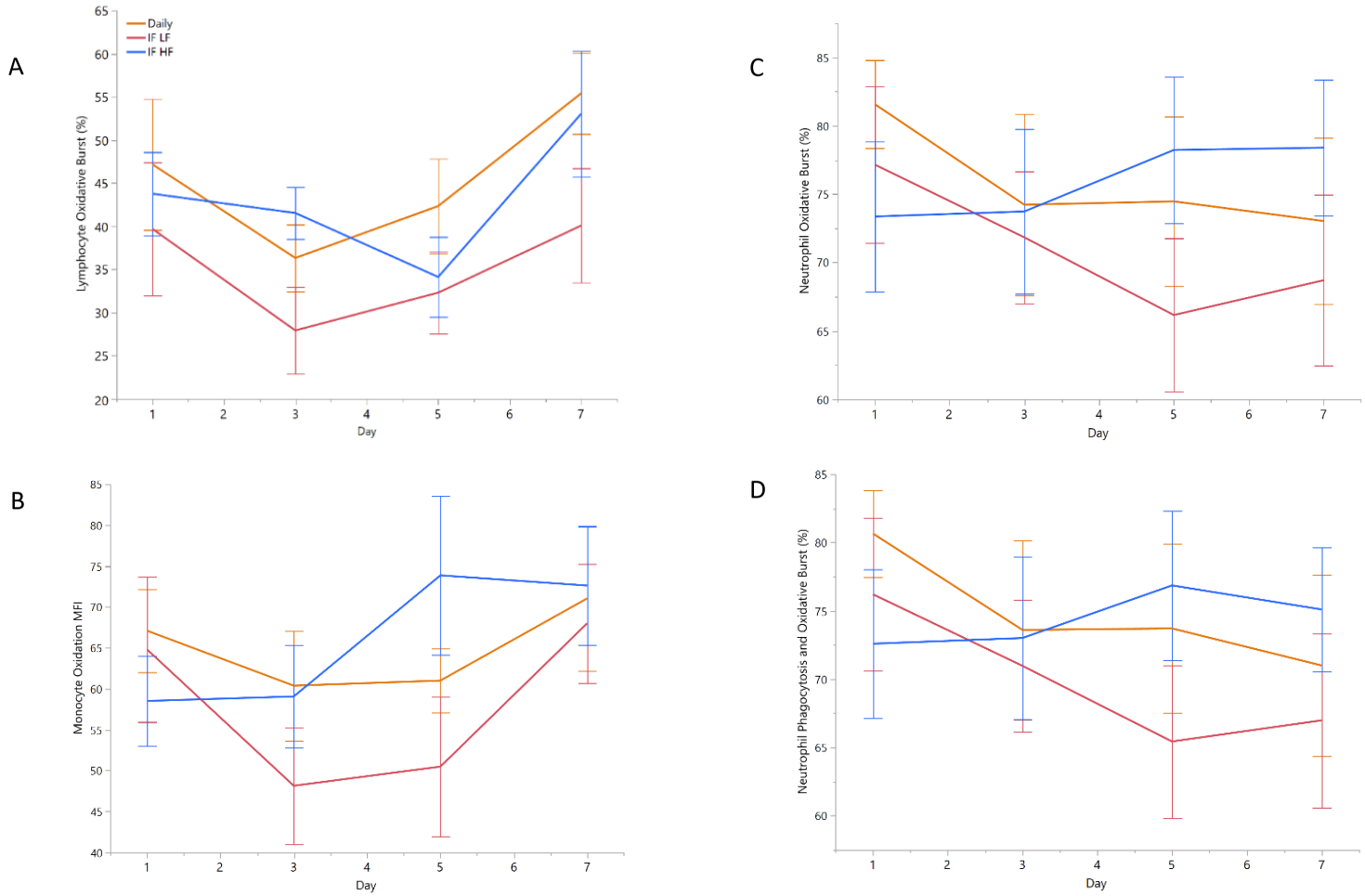

Figure 5-4. Differences in immune parameters in 10 dogs fed daily (orange line), intermittently fasted on a low-fat (red line) and intermittently fasted on a high-fat diet (blue line) in a Latin Square design as assessed by flow cytometry. (A) Total percentage of lymphocytes which underwent oxidative burst (P = 0.008). (B) The mean florescence intensity of oxidative burst in monocytes which underwent both phagocytosis and oxidative burst (P = 0.06). This is a method of quantifying the degree of oxidation. (C) Total percentage of neutrophils which underwent oxidative burst (P = 0.02). (D) The percentage of neutrophils which underwent both phagocytosis and oxidative burst (P = 0.03).

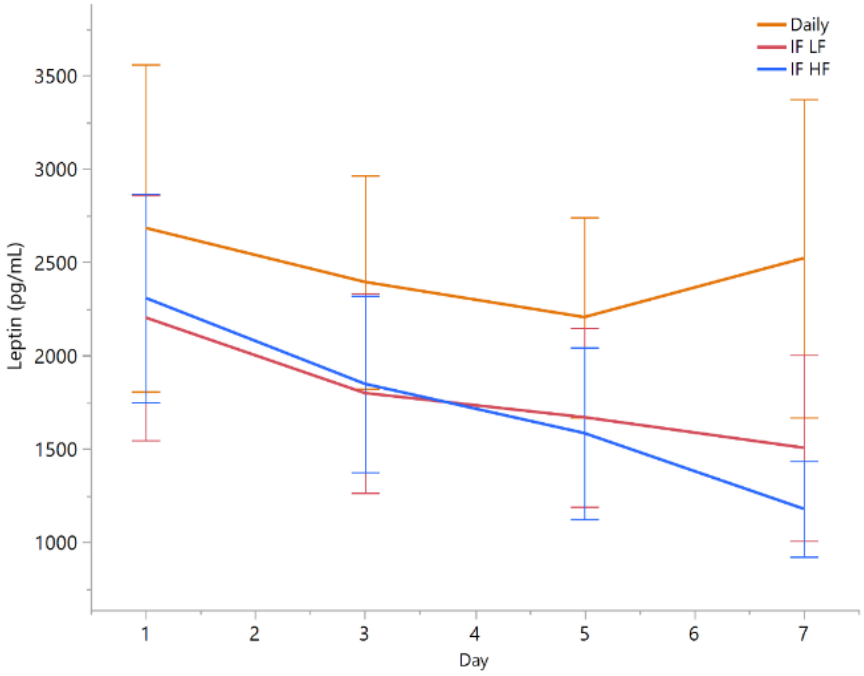

Figure 5-5. Reproduced from Chapter 4. Mean concentrations (±SE) of fasted serum leptin in 10 dogs fed daily (orange line), intermittently fasted on a low-fat (red line) and intermittently fasted on a high-fat diet (blue line) in a Latin Square design.

5.4 Discussion

Short-term fasting has been shown to have beneficial health effects in multiple species.[12,20–23] However, a reduction in immunity during fasting has also been documented.[3,10,11,14,24] To assess the immune function in intermittently fasted dogs, phagocytosis and oxidative respiratory burst in leucocytes, and lymphocyte proliferation were measured. It was found that when the dogs were intermittently fasted on a LF diet, they had a lower percentage of neutrophils and a lower MFI in the macrophages that underwent both phagocytosis and oxidation. They also had a lower percentage of lymphocytes that underwent respiratory oxidative burst. However, suppression of immunity due to fasting was abrogated when the dogs were fed a high-fat diet prior to the fast.

The suppression in immune function during fasting is thought to be due to a reduction in leptin concentrations. Although serum leptin concentrations are typically related to adipose mass, fasting leads to a significant reduction in leptin concentration that is disproportional to changes in adiposity.[25] For instance, a significant reduction in leptin occurred after just after 6 - 12 hours of fasting in mice.[26,27] As described in the previous chapter, serum leptin concentrations were lower when the dogs were fasted for longer, which were not associated with body weight changes. Additionally, it was found that feeding healthy dogs a high-fat diet did not maintain leptin plasma concentrations during the fasting period. This is in contrast to rodents, where a study of mice consuming a high-fat diet (58% energy from fat) prior to a 48-hour fast had a reduced drop in leptin compared to mice consuming a standard diet (11% energy from fat).[28]

Further in contrast to rodents, leptin concentrations were not associated with immune function in this study. Rodents have an evident reduction in immunity after 48 hours of fasting, which is reversed with leptin supplementation.[6,7] In this study, there was an approximately 25% reduction in mean leptin concentrations in the dogs when they were intermittent fasted compared to when they were daily fed. In comparison, a 50 - 60% reduction in serum leptin concentrations in mice are seen after a 48-hour fast compared to those *ad libitum* fed.[28,29] Therefore, the drop in leptin concentrations in this study may have be insufficient to cause a significant difference in immune function in the dogs. Further, rodents have a high metabolic rate and typically consume multiple small meals a day, so fasting for 12 - 16 hours is considered a significant duration.[30,31] Therefore, feeding once every 48 hours is not the same metabolically for a rodent as it is for a dog, and a greater effect on immune function may be seen with a longer fasting period in the dog.

Immunity in the fasted dogs was measured by phagocytosis and oxidative respiratory burst in leucocytes, and lymphocyte proliferation. These established assays were selected to represent immune functions related to a decrease in leptin, which plays an important role in increasing monocytes phagocytosis, regulatory T cell proliferation, and neutrophils oxidative radical generation.[1,32–34] A reduction in some immune function was seen when

the dogs were intermittently fasted on the LF diet including a lower percentage of neutrophils and a lower MFI in the macrophages that underwent both phagocytosis and oxidation, and a lower percentage of lymphocytes that underwent respiratory oxidative burst. However, this trend was not consistent, and there were no differences in the other immune parameters examined. As such, the clinical significance of these findings is not known, although it can be speculated that it would be minimally impactful in a healthy dog. However, these immunosuppressive effects may be more significant in critically ill patients.

Another important finding was that the negative effects of fasting on immune function was not seen when the dogs were eating a HF diet, despite leptin not being conserved. In addition, when the dogs were intermittent fasted on the HF diet, they lost less weight than when eating a LF diet. Therefore, the effect on the preservation of immune function may be related to maintaining body condition, rather than leptin concentrations. Further, there are studies in rodents that examined the effect of refeeding particular macronutrients after a period of fasting on immunity, although the results of these studies are not always consistent. In a study of gerbils fasted for three days, refeeding with glucose restored the drop in leptin and cellular immune function.[35] Whereas in another study with rats, mitochondrial activity of mononuclear cells, used as a marker of cell function, was restored with protein refeeding, but not when glucose was given.[36] The results of this study in dogs does indicate that macronutrients may differentially affect immune function during a fasting period. However, the reduction in immunity was only present in select parameters and were not consistent between leucocyte types. Therefore, the significance of these immune changes are unknown, and further studies are required to confirm the repeatability of these findings.

Limitations

The use of flow cytometry is well recognised as a sensitive method for detecting phagocytosis and oxidative bursts in phagocytes.[37,38] However, the method can be indiscriminate in nature. Use of size and granularity to distinguish between cell types

during flow cytometry relies on the assumption that all cells contained in the field with the same characteristics are indeed of a single cell type. In reality, the wide region used may have included doublets, clumped cells, or debris, which could have artificially elevated the MFI for each group. Using scatter parameters and comparing pulse height vs pulse width plots can be used in future to better isolate single cells in gates.[39,40] In addition, the use of cell-specific surface markers would have provided clearer identification of cell subsets. During fasting, the percentage of circulating $CD4^+$ and ratio of $CD4^+$: $CD8^+$ lymphocytes are reduced, whereas circulating mature B cells are increased.[8,41,42] Additionally, acute feeding of a HF diet in other species leads to an increase in circulating pro-inflammatory cytokines such as interleukin-1β and tumour necrosis factor-α, although this has not been shown consistently.[43] Interestingly, an intermittent fasting regimen has been shown to ameliorate the expression of proinflammation-related genes in hepatocytes during long-term HF feeding in mice.[44] As the effects of HF feeding and intermittent fasting on circulating lymphocyte subsets and cytokine production could be important in critically ill patients, this requires further study.

Modification of dietary micronutrients (vitamins and minerals) can also influence immune function.[45] While the micronutrient content of the HF and the LF diets in this study were not identical, the differences were not great, and both diets met or exceeded the essential nutrients requirements for an adult dog as established by AAFCO. In addition, all feeding regimens were preceded with either an acclimation period or wash out period with the LF diet. Therefore, it is unlikely that a significant effect on immune function from differences in micronutrients would have manifested during the week-long feeding period.

5.5 Conclusion

Ultimately, it was found that healthy dogs undergoing an intermittent fasting regimen on a low-fat diet had a reduced percentage of neutrophils and MFI in the macrophages that underwent both phagocytosis and oxidation, and percentage of lymphocytes that underwent respiratory oxidative burst. In addition, the changes in the immune parameters found were not associated with the decline in leptin concentrations associated with

fasting, in contrast to what has been reported in other species. The clinical significance of these immune changes is unknown, but may be speculated to be important during critical illness. Intriguingly, undergoing intermittent fasting while eating a high-fat diet did not cause the same changes in immunity in the dogs. This suggests that using a high-fat diet during an intermittent fasting regimen may be preferential to avoid immunosuppressive effects.

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Chapter 6

Ketone kinetics in healthy dogs fed once-daily or every-other-day

6.1 Introduction

During fasting, it is well known that dogs do not reach the same concentrations of circulating ketones as some other species fasted for the same period of time, including rodents and humans.[1–4] Consistent with this, it is described in Chapter 4 how healthy dogs had a much lower fasted mean plasma concentration of β-hydroxybutyrate of 40 - 60 μmol/L, compared with rodents (800 – 2,000 μmol/L) and humans (790 – 1,670 μmol/L) when fed once every 48 hours.[5–8] One hypothesis of the mechanism for the lower concentration of circulating ketones is that dogs may have a lower rate of fatty acid oxidation and conversion into ketones than other species. However, a study that isolated hepatic mitochondria of both fed and fasted dogs and rats determined that there was no difference in the rate of oxidation of palmitate to ketones between the two species.[3] In addition, kinetics studies have shown that while ketone turnover in humans is two to three times higher than that of dogs fasted for a similar period of time, humans have a ten- to forty-fold difference in plasma ketones concentrations.[4,8,9] Together, these findings suggest that the lower concentration of circulating ketones in fasted dogs is not due to reduced hepatic production, but rather due to a more efficient peripheral utilisation by dogs.

What is not known, however, from these early ketone kinetics studies is the effect of diet, particularly high-fat feeding, on fasting rates of ketone production and utilisation. As described earlier, there may be beneficial health effects of intermittent fasting using a high-fat diet in dogs, especially in those recovering from spinal disease. However, the practicality of implementing an intermittent fasting regimen in hospitalised animals may be a challenge. In Chapter 4, it is described that fasted plasma ketone concentrations in dogs were greater in those intermittent fasted (fed once every 48 hours) than when fed daily on a low-fat diet, suggesting that a longer fasting duration might have some benefits.

However, it is unknown if hospitalised dogs will be able to maintain sufficient intake to meet their energy requirements if fed only once every 48 hours. It is also unknown whether feeding every 24 hours may also induce a similar rate of ketogenesis if the dog is eating a high-fat diet.

The aim of this study, therefore, was to determine the rate of ketone production and metabolic clearance in healthy dogs eating a high-fat diet enriched with medium-chain triglycerides once-daily (fed once every 24 hours) or every-other-day (fed once every 48 hours). It was hypothesised that when the dogs are fasted for longer, they will have a greater rate of ketone synthesis and clearance. A secondary aim was to compare the rates of ketone synthesis and clearance of dogs to those previously published in other species fasted for a similar period of time.

6.2 Material and Methods

The study protocol was approved by the Massey University Animal Ethics Committee (MUAEC 18/55). Eight healthy colony dogs were used from Massey University's Canine Nutrition Unit (Palmerston North, New Zealand). A high-fat diet containing medium-chain triglycerides was produced from a commercial kibble[a] to which was added whey protein isolate powder, coconut oil, lard, sunflower oil and a vitamin/mineral mix[b]. The recipe is provided in Appendix IV. The diet was formulated to meet the Association of American Feed Control Officials (AAFCO) nutrient requirements for the maintenance of adult dogs. The macronutrients (protein, fat and carbohydrate) energy contents of the diet were estimated based on modified Atwater values (Table 6-1). The content of coconut oil and lard were set to ensure that the concentration of medium-chain triglycerides (C8 - C12) equalled to 14.9 grams per Mcal, which equated to 10.1% of energy.[10]

[a] Blackhawk™ Working Dog Lamb & Beef, dry, Masterpet Australia Pty Limited, Smeaton Grange, Australia
[b] Balance IT® Canine, DVM Consulting Incorporated, Davis, California, USA

Table 6-1. The macronutrient content of a high-fat diet containing medium-chain triglycerides fed to eight healthy dogs in a crossover designed trial.

	% metabolizable energy
Protein	20.0
Fat	68.5
Carbohydrate	11.5

Before the start of the study, all dogs were transitioned onto the high-fat diet, fed once-daily, to allow for a one-week acclimation period. After one week, the dogs were randomised into two feeding regimens in a crossover design trial. The first group's regimen consisted of once-daily feeding where the dogs received their allotted estimated daily maintenance energy requirement (MER) at 7 am every day. The second group's regimen was every-other-day feeding, where the dogs were fed twice their MER at 7 am every second day. All food was offered for up to 5 hours, at which time remaining food was removed and weighed using a bench scale. The MER for each dog was based on historical colony data. Body weights were taken at the start of every week for the duration of the study.

On day eight, before their next meal, the four dogs on the once-daily feeding regimen underwent a ketone kinetics study using a stable isotopes infusion of [2,4-$^{13}C_2$] acetoacetate[c] and [1,2,3,4-$^{13}C_4$] β-hydroxybutyrate[d]. This represented a 19- to 24-hour postprandial period. The dogs were unrestrained but housed in cages watched by assistants to limit movement during the infusion period. On the day of the kinetics study,

[c] Ethyl acetoacetate (2,4-13C_2, 99% purity), catalog number CLM-523-0, Cambridge Isotope Laboratories, Inc., Andover, MA, USA

[d] Sodium D-3 hydroxybutyrate (13C_4, >97% purity), catalog number CLM-3853-0, Cambridge Isotope Laboratories, Inc., Andover, MA, USA

two peripheral catheters were placed in either the cephalic and/or saphenous veins of each dog. One catheter was used for blood sampling, and the other for isotope administration. A single 3 mL blood sample was collected into a heparinised blood tube[e] to measure baseline concentrations of the isotopes. Then, before the start of infusion, an intravenous bolus of labelled isotope (7.5 μmol/kg of [2,4-$^{13}C_2$] acetoacetate and 9 μmol/kg of [1,2,3,4-$^{13}C_4$] beta-hydroxybutyrate) was given to each dog. This equated to 0.32 mL/kg body weight of infusate for each dog. Following this, a continuous infusion of [2,4-$^{13}C_2$] acetoacetate (0.25 μmol/kg/min) and [1,2,3,4-$^{13}C_4$] β-hydroxybutyrate (0.3 μmol/kg/min) was given over a total of 100 minutes, which equated to 0.6 mL/kg body weight/hr for each dog. At 70, 80, and 90 minutes into the infusion, successive blood samples (3 mL into heparinised tubes) were taken to determine whether the labelled isotopes had reached a steady state.

This procedure was then performed on day nine with the four dogs in the every-other-day feeding group before their next meal. This represented a 43- to 48-hour postprandial period. Once a group finished their kinetics study, the dogs were then placed immediately onto the alternate feeding regimen. After the dogs were on the new regimen for a period of a week, the kinetics study was repeated using the same protocol as described.

6.2.1 Infusion and bolus solution preparation

Preparation of the infusion solution was performed using a previously published procedure.[11,12] The infusion and bolus solutions were made each morning approximately two hours before the ketone kinetics study was performed. The quantity of isotopes required for each day's infusion were calculated as follows:

[e] BD Vacutainer®, Becton, Dickson and Company, Plymouth, United Kingdom

Infusion

[2,4-$^{13}C_2$] ethyl acetoacetate: 0.25 μmol x total weight of dogs (kg) x 100 minutes

[1,2,3,4-$^{13}C_4$] β -hydroxybutyrate: 0.3 μmol x total weight of dogs (kg) x 100 minutes

Bolus

[2,4-$^{13}C_2$] ethyl acetoacetate: 9 μmol x total weight of dogs (kg)

[1,2,3,4-$^{13}C_4$] β -hydroxybutyrate: 7 μmol x total weight of dogs (kg)

To prepare the infusate, the required amount of [2,4-$^{13}C_2$] ethyl acetoacetate was measured using an analytical scale, dissolved in a 0.08 M sodium hydroxide (NaOH) solution, vortexed and incubated at 38 °C for 75 minutes to allow for the hydrolysis of the ethyl acetoacetate ester. The solution was then neutralised to a pH of 7 - 7.5 using 0.08 M hydrogen chloride, which was checked with pH strips[f], and then placed on ice. Following this, the required amount of [1,2,3,4-$^{13}C_4$] β-hydroxybutyrate was weighed and added to the acetoacetate solution and used without further pH adjustments. The same procedure was used to create the bolus solution.

6.2.2 Sample preparation

Plasma was harvested from blood samples within 30 minutes of collection. Heparinised blood samples were centrifuged at 1,500 rcf for 15 minutes at 4 °C. Following this, 0.5 mL of plasma was removed and transferred into a microcentrifuge tube. Then, the plasma was de-proteinated by adding 0.5 mL of 5% perchloric acid[g] and vortexing. After, the centrifuge tube was spun at 2,000 rcf for 15 minutes at 4 °C. The supernatant was removed, placed in a screw-top vial, and stored at -80 °C until analysis.

[f] pH test strips range 0-14, catalog no P4786, Sigma Aldrich Pty. Ltd., Sydney, Australia
[g] Perchloric acid, catalog no 244252, Sigma Aldrich Pty. Ltd., Sydney, Australia

6.2.3 Analysis

Isotopic distribution of labelled acetoacetate and β-hydroxybutyrate was measured by gas chromatography-mass spectrometry (GC-MS) in a commercial laboratory[h] using a previously published method.[13] For this, plasma (200 μL) was pipetted into a 2 mL microcentrifuge tube and 20 μL of 6 M hydrochloric acid[i] was added. An internal standard (40 μL of d_5-benzoic acid[j], 2.5 μg/mL) was added to each tube, and the samples vortexed for 10 seconds. The samples were then extracted twice using a shaker and 800 μL ethyl acetate[k]. Samples were mixed for 5 minutes at 30 Hz and centrifuged at 10,000 rcf for 10 minutes. The ethyl acetate layer was transferred to a new microcentrifuge tube, and the extraction steps repeated. The ethyl acetate extracts were pooled and dried in a vacuum centrifuge evaporator[l] at 25 °C for 1.5 hours. Then, the dried extracts were resuspended in 100 μL ethyl acetate and transferred to amber GC vials with 100 μL inserts and dried again (vacuum centrifuge evaporator at 25 °C, 30 minutes). The samples were then derivatised with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide[m], vortexed for 10 seconds, and left for at least one hour before injection. In addition to the canine plasma samples, human plasma samples were included as quality controls to ensure consistent instrument performance. The human samples were expired donor plasma obtained from a local blood bank[n], which were originally collected using citrate as the anticoagulant. The human samples were processed in the same manner as the canine samples.

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[h] AgResearch, Lincoln Research Centre, Canterbury, New Zealand
[i] Hydrochloric acid, catalog no 320331, Sigma Aldrich Pty. Ltd., Sydney, Australia
[j] Benzoic acid, Cambridge Isotopes, Tewksbury, MA, USA
[k] Ethyl acetate, catalog no 270989, Sigma Aldrich Pty. Ltd., Sydney, Australia
[l] RVC 2-18 CD plus, Martin Christ GmbH, Osterode am Harz, Germany
[m] N-ter-Butyldimethylsily-N-methyltrifluroacetamide, catalog no 394882, Sigma Aldrich Pty. Ltd., Sydney, Australia
[n] New Zealand Blood Service, Lincoln, New Zealand

Analysis was carried out on a benchtop GC-MS[o] with a BP-5 column[p] (30 m x 0.25 mm i.d., 25 μm film thickness). Injection were done in splitless mode, with 2 μL of sample injected into an inlet set at 250 °C. The interface and ion source temperatures were 250 and 280 °C respectively. The GC oven programme was started at 40 °C, and held for 0.1 minute before ramping at 5 °C/min to 70 °C, then held a further 3.5 minutes, ramping at 20 °C/min to 160 °C, and then finally ramping at 35 °C to 280 °C and held for 3 minutes. The final ramp was 35 °C to 320 °C, which was held for 4 minutes, leading to a total run time of 25.67 minutes. Ions were monitored between 15 and 18 minutes, with m/z 273, 274, 275, 276 and 277 monitored for acetoacetate and m/z 275, 276, 277, 278, 279 monitored for β-hydroxybutyrate. The d_5-benzoic acid internal standard was monitored at m/z 184.

Finally, the standard curve for acetoacetate and β-hydroxybutyrate was prepared using Milli-Q® water and human plasma, and measured at concentrations 0, 3.125, 6.25, 12.5, 25, 50 and 100 μmol/L.

6.2.4 Calculations

To describe isotopic enrichment of the samples, the tracer-to-tracee ratio (TTR) was calculated for each acetoacetate and β-hydroxybutyrate isotopomer after accounting for baseline isotope natural abundance.[14] From this, mole percent excess (MPE) was calculated using the following formula:

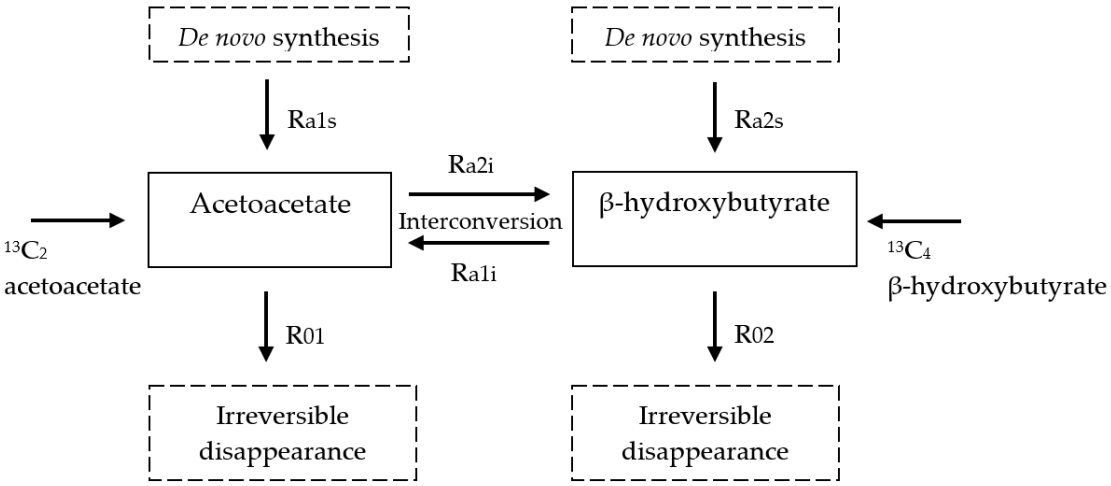
$$Mole\ percent\ excess\ (\%) = \frac{TTR}{1 + TTR}\ x\ 100$$

A two-accessible pools model (Figure 6-1) was used to calculate the ketone kinetics to determine each ketone's rate of synthesis (R_a), irreversible disappearance (R_0) and

[o] Shimadzu GCMS TQ8040, Shimadzu corporation, Kyoto, Japan
[p] BP-5 column, SGE Lid, Melbourne, Australia

interconversion (R_i).[15,16] The total ketone rate of synthesis was calculated as the sum of R_a for acetoacetate and R_a for β-hydroxybutyrate. The metabolic clearance rate for each individual ketone was calculated by dividing R_0 by their concentration at steady state. These values were then summed to determine the total ketone metabolic clearance rate. For interspecies comparisons, kinetic rates were expressed on a metabolic body weight basis (body weight$^{0.75}$).

Figure 6-1. Two-accessible-pool model of acetoacetate and β-hydroxybutyrate turnover. Adapted from Bougneres and Ferre[16].



The following equations were used[16]:

$$Ra1s = P_1 \times (D - C) / (D \times A - B \times C)$$

$$Ra2s = P_2 \times (A - B) / (D \times A - B \times C)$$

$$Ra2i = P_2 \times B / (D \times A - B \times C)$$

$$Ra1i = P_1 \times C / (D \times A - B \times C)$$

$$R01 = (P_1 \times D - P_2 \times B) / (D \times A - B \times C)$$

$$R02 = (P_2 \times A - P_1 \times C) / (D \times A - B \times C)$$

Where P_1 is the infusion rate of $^{13}C_2$ acetoacetate (μmol/kg/min)

P_2 is the infusion rate of $^{13}C_4$ β-hydroxybutyrate (μmol/kg/min)

A is the enrichment of $^{13}C_2$ acetoacetate at steady state

B is the enrichment of $^{13}C_2$ β-hydroxybutyrate at steady state

C is the enrichment of $^{13}C_4$ acetoacetate at steady state

and D is the enrichment of $^{13}C_4$ β-hydroxybutyrate at steady state

6.2.5 Statistics

All statistical analyses were performed using R software[q]. An *a priori* power analysis was performed using previously published mean difference and standard deviations for β-hydroxybutyrate, namely a production of 5.9 μmols/kg/min in 48-hour fasted dogs and 1.03 μmols/kg/min in 18-hour fasted dogs, and a standard deviation of 4.[17,18] Thus, it was determined that eight dogs would be required to detect that same difference of 4.87 μmols/kg/min, assuming a standard deviation of 4, at 80% power.

Results are provided as mean and standard deviation (SD) unless otherwise indicated. Normal distribution of the data was determined by visual inspection and the Shapiro-Wilk test. Carryover effects from one trial period to the other were examined using the method described by Wellek and Blettner[19]. To test for a carryover effect, the sum of the values from both feeding regimens were calculated for each parameter and compared across the two sequence groups by the means of a test of independent samples.[19] Once the carryover effect was found to be negligible, the presence of a treatment effect was analysed using a paired t-test. Hypothesis testing was also performed using Bayes factor analysis with the 'BayesFactor' package (Richard D. Morey and Jeffrey N. Rouder, 2018). This type of testing allows for further analyses of non-significant findings to determine whether there is evidence that the data supports the null hypothesis or whether there is an absence of evidence.[20,21]

Further exploration of parameters associated with the rate of total ketone synthesis was done using the 'lme4' package (Bates, Mäechler, Bolker & Walker, 2012). For this, a linear mixed effects analysis using dog as the random effect was performed in a manual stepwise selection process to determine which of the fixed variables (age, sex, weight, and BCS) were associated with the rate of total ketone synthesis. The best fit model was determined by Akike information criterion (AIC) and adjusted R^2. Normality of the residuals of the

[q] R version 3.1.0; R Development Core Team, 2012; R Foundation for Statistical Computing, Vienna, Austria

final model were checked visually and with the Shapiro-Wilk test. P-values < 0.001 were considered indicative of very strong evidence, P < 0.01 of strong evidence, P < 0.05 of moderate evidence, P < 0.1 of weak evidence, and P ≥ 0.1 of insufficient evidence.[22]

6.3 Results

Eight dogs were used in the study, which consisted of five spayed females and three castrated males. They had a mean age of 8.9 (SD 1.6) years, body weight of 25.5 (SD 3.8) kilograms, and body condition score (BCS) of 4.4 (SD 0.5). The mean changes in body weight were 0.09 (SD 0.40) kilograms when the dogs were fed once-daily and -0.02 (SD 0.50) kilograms when the dogs were fed every-other-day. On average, the dogs ate 98.3% of their food when fed once-daily, and 94.9% of their food when fed every-other-day. The differences in body weight changes and food intake between the feeding regimens were not significant ($P = 0.228$ and $P = 0.359$ respectively). The mean fasted plasma acetoacetate concentration was 32.67 (SD 26.11) μmol/L when the dogs were fed once-daily, and 31.23 (SD 22.61) μmol/L when the dogs were fed every-other-day, which was not statistically different ($P = 0.945$). The mean fasted plasma concentration of β-hydroxybutyrate was 40.95 (SD 2.36) μmol/L in dogs when fed every-other-day fed dogs, compared with 38.58 (SD 2.21) μmol/L when they were fed once-daily ($P = 0.067$).

Steady state of both plasma $^{13}C_2$ acetoacetate and $^{13}C_4$ β-hydroxybutyrate were achieved by 70 minutes after the start of the infusion (Figure 6-2).

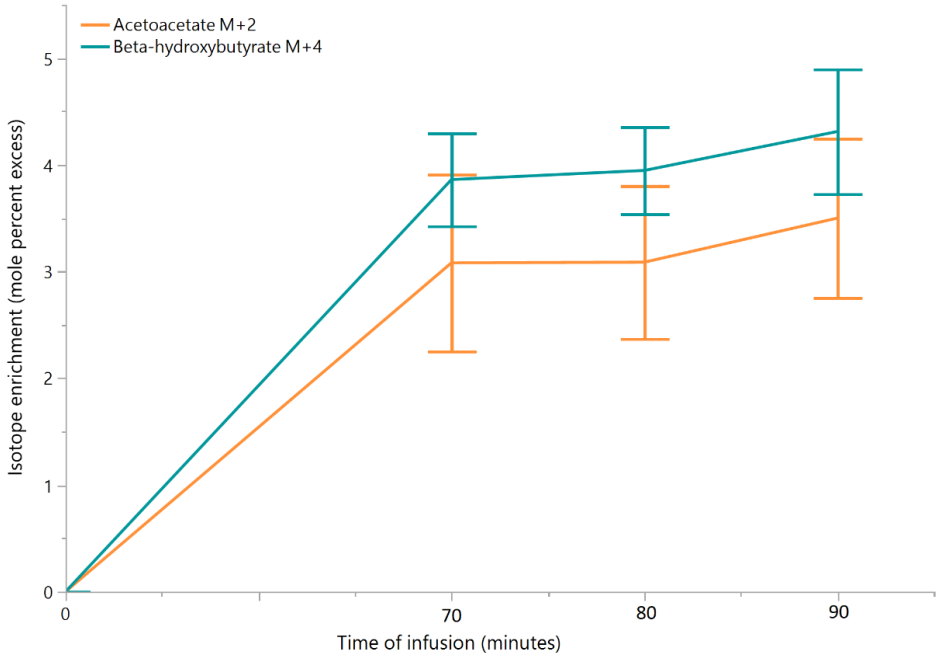


Figure 6-2. The mean (±SE) plasma enrichment of $^{13}C_2$ acetoacetate (M+2) and $^{13}C_4$ β-hydroxybutyrate (M+4) in eight healthy dogs in a crossover study design. Minute "0" represents the start of the tracer infusion, and minutes "70", "80", and "90" were the time points at which blood samples were taken.

The rates of acetoacetate and β-hydroxybutyrate synthesis, interconversion and metabolic clearance are presented in Table 6-2. The fasted rate of total ketone synthesis was 29.70 (SD 12.79) μmol/kg/min when the dogs were on the every-other-day feeding regimen, and 20.80 (SD 7.57) when the dogs were fed once-daily (P = 0.110, Figure 6-3). The rate of total ketone synthesis was positively correlated with total plasma ketone concentrations (R^2 = 0.361, P = 0.023, Figure 6-4). For the multivariable regression analysis, both fasting duration and sex were positively associated with the rate of total ketone synthesis (Table 6-3). Body condition score, although not significant, was left in the model as it improved both the adjusted R^2 and AIC score for the final model.

Table 6-2. The mean and standard deviation (SD) of fasted ketone kinetics in a two-accessible pool model with eight healthy dogs undergoing a once-daily and every-other-day feeding regimen on a high-fat diet in a crossover study design. Samples were taken 19 to 24 hours postprandially for dogs fed once-daily, and 43 to 48 hours postprandially for dogs fed every-other-day. Results from the paired t-test analyses (P - value) and Bayes factors are also presented in the table.

Rate (μmol/kg/min)	**Once-daily feeding**	**Every-other-day feeding**	***P* - value**	**Bayes factor**
Ra1s (AcAc synthesis)	13.55 (SD 11.37)	19.51 (SD 18.02)	0.296	0.50
Ra2s (β-HOB synthesis)	7.25 (SD 4.77)	10.19 (SD 10.17)	0.469	0.42
Ra1s + Ra2s (Total ketone synthesis)	20.80 (SD 7.57)	29.70 (SD 12.79)	0.110	0.79
Ra1i (Interconversion of AcAc → β-HOB)	2.88 (SD 1.91)	4.43 (SD 4.20)	0.687	0.38
Ra2i (Interconversion of β-HOB → AcAc)	3.86 (SD 3.15)	3.19 (SD 4.84)	0.270	0.38
R01 (AcAc utilisation)	12.57 (SD 7.91)	20.76 (SD 13.16)	0.157	0.64
R02 (β-HOB utilisation)	8.23 (SD 1.71)	8.95 (SD 5.97)	0.781	0.42
Rate (ml/kg/min)	**Once-daily feeding**	**Every-other-day feeding**	***P* - value**	**Bayes factor**
Metabolic clearance AcAc	421 (SD 177)	726 (SD 411)	0.151	0.61
Metabolic clearance β-HOB	216 (SD 42.8)	220 (SD 145)	0.942	0.39
Metabolic clearance total ketone	637 (SD 179)	946 (SD 430)	0.297	0.58

Abbreviations: AcAc (acetoacetate), β-HOB (beta-hydroxybutyrate)

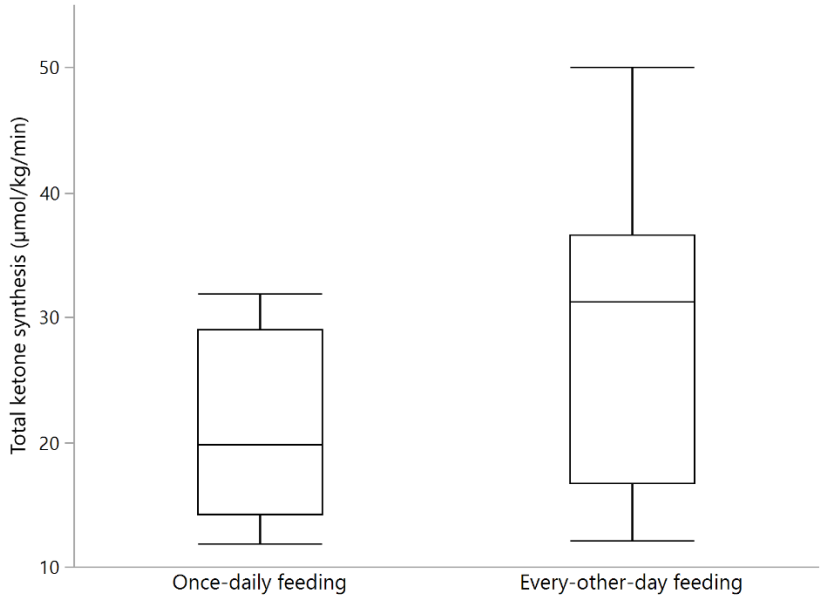


Figure 6-3. Rates of total ketone synthesis in eight healthy dogs undergoing a once-daily and every-other-day feeding on a high-fat diet in a crossover study design ($P = 0.110$). Samples were taken 19 to 24 hours postprandially for dogs fed once-daily, and 43 to 48 hours postprandially for dogs fed every-other-day.

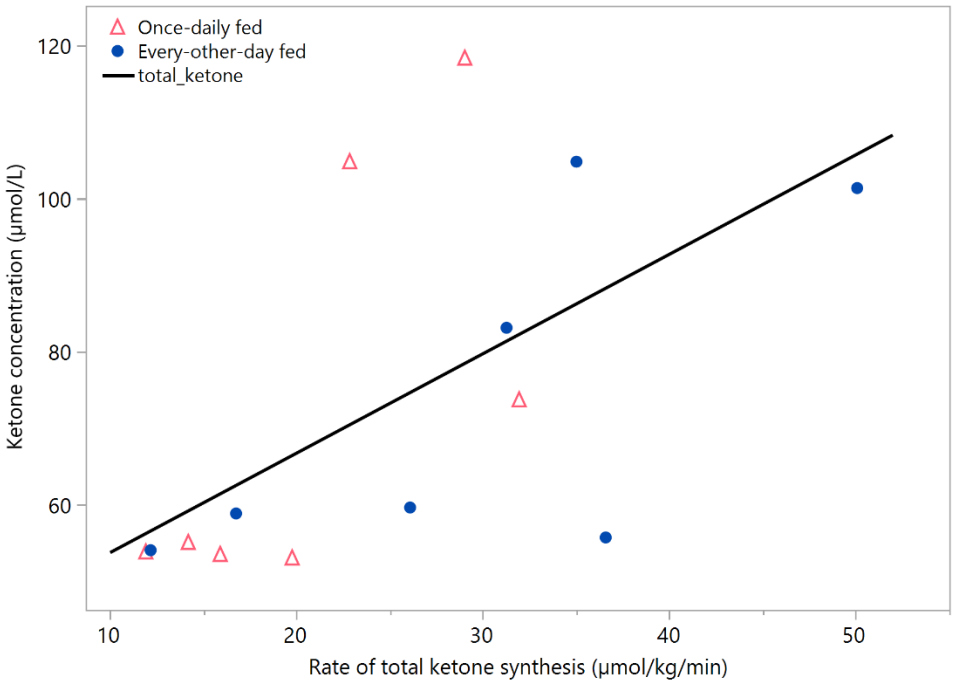


Figure 6-4. Correlation between the rate of total ketone synthesis and plasma ketone concentrations in eight healthy dogs undergoing once-daily and every-other-day feeding on a high-fat diet in a crossover study design (R^2 = 0.361, P = 0.023).

Table 6-3. The final linear mixed effect model for factors associated with the rate of total ketone synthesis in eight healthy dogs undergoing once-daily and every-other-day feeding on a high-fat diet in a crossover study design. The best fit model shown here was assessed using Akaike information criterion (AIC) and adjusted R^2. Body condition score, although not significant, was left in the model as it improved both the AIC score and adjusted R^2 for the final model.

Outcome	Fixed Effect	Estimate	Standard Error	R^2	P - value
Rate of total ketone synthesis (µmol/kg/min)	(Intercept)	54.12	22.45		0.037
	Every-other-day feeding	8.91	4.68		0.086
	Sex male	13.58	5.20		0.026
	BCS	-8.84	5.20		0.120
	Overall model			**0.52**	**0.053**

6.4 Discussion

In this study, ketone kinetics were measured using a stable-isotope tracer infusion technique in healthy adult dogs eating a high-fat diet that was fed once-daily and every-other-day in a crossover design. Feeding once every 48-hours resulted in an increase in fasted plasma β-hydroxybutyrate concentrations, compared to when the dogs were fed once every 24-hours. Remarkably though, the degree of increase was small, despite the dogs being fasted for approximately twice the time. When examining the kinetics of the ketones, most parameters, including β-hydroxybutyrate and acetoacetate synthesis and clearance, and acetoacetate interconversion, had higher mean values when the dogs were fasted for longer, which was consistent with the study hypothesis. However, none of the parameters were found to be statistically different between the two feeding regimens, with $P > 0.1$, and so the null hypothesis was not able to be rejected. A non-statistically significant *P*-value does not, however, provide evidence for the null hypothesis.[20,23] Thus, an additional statistical analysis was performed using Bayesian statistics. This analysis compared the likelihood of observing the data under the null and alternative models indicating the strength of the evidence for the two theories. The results showed that there was negligible evidence in support of the null hypothesis (Bayes factor $0.33 < x < 1$).[20,24]

These statistical analyses indicated that the study results did not support either the null or alternative hypothesis. It remains possible that there are no differences in the ketone kinetics of healthy dogs fed once-daily and every-other-day. Alternatively, another reason why a difference was not detected may be due to the small sample size and large variation in the kinetics found. In this study, some dogs had a large increase in kinetics with the longer fasting period, whereas some dogs did not. This has been previously reported where dogs fasted for ten days had greater variation in their rates of ketone turnover and clearance compared to when they were fasted for one day.[9] These findings suggest that the rates of ketone synthesis and utilisation in response to increasing fasting duration can vary considerably between individuals. However, in those dogs which did respond, the effect size may be substantial. The average ketone synthesis and clearance rates increased by nearly 50% when the dogs were fasted for longer, which could significantly increase

substrate availability for neurons. In a study of dogs fasted for eight days, the concentration of ketones in cerebral spinal fluid increased proportionally to the rise in plasma ketones.[25] Therefore, taking into consideration the effect size found in this study, which may be biologically meaningful, and the insufficient evidence to prove or disprove the null hypothesis, further investigations of the effect of fasting duration on ketone kinetics in dogs is warranted.

In the mixed model, the rate of total ketone synthesis was positively associated with fasting duration and the male sex. This sex-difference appears to contrast with what is known in other species. In mice, higher concentrations of plasma β-hydroxybutyrate and gene expression of fibroblast growth factor 21, a regulator of lipolysis, is found in fasting females compared to males.[26–28] In humans, men have lower postprandial fatty acid oxidation and ketone synthesis compared to females.[29] However a key difference in this study is that the dogs were neutered, which would not have been the case for the mice and human studies. It is currently unknown what effect sex and neutering have on ketogenesis in dogs. In addition, while the rate of ketone synthesis was higher in male dogs in this study, only three male dogs were included, therefore caution must be taken in drawing strong conclusions. Further studies are required to better understand the potential sex-differences and effect of neutering on ketogenesis in dogs.

Kinetic rates are typically reported based on body weight. However, expressing on a metabolic body weight-basis (body weight$^{0.75}$) is more accurate when comparing the metabolism of different species.[9,30] Ketone kinetics obtained in this study were compared to tracer-obtained ketone kinetics in dog studies and other species after converting on a metabolic body weight-basis (Table 6-4).

Table 6-4. Comparison of ketone kinetics between species based on this study and previously published literature. Rates were converted on a metabolic body weight-basis.

	Hours fasted*	Reported body weight (kg)	Total ketone synthesis (μmol/kg$^{0.75}$/min)	Total ketone clearance (ml/kg$^{0.75}$/min)
Dog				
	12 - 18	12 – 22.7	2.25 [18], 5.23 [31]	268.01 [9]
	24	25.5	46.74^	1,431^
	48	18.5	23.48 [32], 22.55 [17], 66.74^	241.82 [32], 2,126^
	96	14 - 15.7	18.71 [33], 15.67 [34]	77.96 [33], 46.08 [34]
Rat				
	24	0.25	45.54 [15], 28.28 [11]	31.50† [15]
	48	0.25	42.99 [35]	28.43 [35]
Mice				
	24	0.02	66.52 [11]	48.73 [11]
Adult human				
	12	76.0	20.37 [36]	64.96 [36]
Children				
	14	15.8	25.80 [16]	426.62 [16]

** approximate fasting duration; ^ (result from this study); †clearance of only beta-hydroxybutyrate*

The interspecies comparison indicates that despite the large differences in fasting plasma ketone concentrations between dogs, rodents and humans, their rates of ketone synthesis are not vastly different. Instead, the difference lies with the rate of metabolic clearance. Dogs and children have a greater ability to clear ketones from circulation compared to adult humans and rodents. It is known that human neonates and infants have a greater capacity to utilise ketones compared to adults.[37] In addition, a high rate of systemic ketone production and utilisation is also seen in neonatal rodents, which declines with age.[38,39] For

adult dogs, a higher rate of clearance would account for their propensity to have lower plasma ketone concentrations during fasting compared to other species. While some clearance will be due to urinary excretion, the vast majority will be from ketone entry into extrahepatic tissues for oxidation.[31,40] Thus, dogs appear to be better capable of utilising ketones compared with either adult rodents or humans during a fasted state.

Interestingly, the rates of clearance obtained in this study are substantially higher than other published studies in dogs. This may be due to variations in study methodology, including sample handling and processing, assaying techniques, the model used for calculations (one-pool, two-pools, etc) and whether the authors accounted for the interconversion of ketones. Another possible reason for the difference is the effect of diet on the metabolic response to fasting. In this study, the dogs were maintained on a high-fat, low-carbohydrate diet enriched in medium-chain triglycerides, which enhances ketogenesis and ketolysis.[41–43] For the other dog studies, the diets fed prior to fasting were either not described or were inadequately explained in the publication for macronutrient and nutritional adequacy to be assessed. In rodents, differing combinations of dietary carbohydrate, protein and fat content has been shown to influence ketogenesis and the concentration of ketones found in circulation in both the fed and fasted state.[44–46] In addition, for longer-term fasting studies, nutritional imbalances and/or deficiencies in the diet, such as with carnitine and choline, could also affect fatty acid metabolism.[47,48] Despite these limitations, exclusion of these studies was not possible as there are only a few canine studies available that have examined ketone kinetics. So, while the findings in the literature and this study indicate that dogs have a higher rate of ketone clearance compared to other species, caution in interpreting between the various dog studies must be exercised.

Limitations

The main limitation to this study is the small number of dogs utilised. Although a crossover study design was chosen to reduce the overall variation and increase the statistical power, the effect size of some of the parameters and large individual variations

limited the conclusions that can be drawn from this study. A post-hoc power analysis was not performed because it was not deemed appropriate to power on observed data.[49–51]

Another potential limitation to this study is that no wash-out period was implemented between the feeding regimens. A wash-out period was not believed to be necessary as the dogs were eating the same diet, and any carryover effect of once-daily and every-other-day feeding was believed to be diminished after a week on their feeding regimens, before the kinetics were measured. To ensure there was not a carryover effect, this was checked as described in the statistics section, and was found to be negligible.

6.5 Conclusions

There were no significant differences in the ketone kinetics of dogs eating a high-fat diet once-daily and every-other-day. However, the dogs' ability to utilise ketones more efficiently than rodents and humans was shown, consistent with previous studies. In addition, large individual variations in synthesis and clearance were apparent, particularly when the dogs were fasted for a longer period of time, which may have prevented the detection of a true difference. Ultimately, the results of this study indicate that a once-daily feeding regimen may result in similar ketone plasma concentrations and kinetics to an every-other-day feeding regimen. This has significant implications since feeding every 24 hours is likely to be more acceptable, and less likely to result in weight loss during hospitalisation than feeding every 48 hours. Thus, the shorter period can be used to test the practical application of an intermittent fasting regimen in hospitalised patients. However, individual responses to fasting may be very different, as might the benefits of an intermittent fasting regimen.

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Chapter 7

Intermittent fasting using a ketogenic diet in recovering canine spinal patients

7.1 Introduction

Spinal cord injury due to trauma or disc degeneration is a debilitating condition that often requires many days to weeks of hospitalisation.[1,2] Long hospitalisation increases the risk of malnutrition and loss of body weight in patients.[3–5] It was shown in Chapter 2 that long-stay hospitalised canine patients lost on average 3.9% of their total body weight, most of which was lean mass. To improve recovery time in patients with spinal cord injury, intermittent fasting has been proposed as a therapeutic feeding regimen that reduces the extent of the lesion and subsequent functional losses.[6,7] Fasting promotes ketone formation, which is believed to improve neuronal recovery by reducing oxidative damage through the upregulation of superoxide dismutase 2, and reducing the loss of neurons by upregulating autophagy mediator protein forkhead box O3a (Foxo3a).[8–11] As such, intermittent fasting may provide benefits to patients recovering from spinal disease by increasing ketone availability, leading to improved recovery rates and decreasing the amount of hospitalisation time required.

In the previous chapter, it was discovered that feeding a high-fat diet once-daily or every-other-day produced similar rates of total ketone synthesis and ketone clearance in healthy dogs. In hospitalised patients, feeding once-daily may be more suitable to ensure better intake and maintenance of body weight and muscle mass. Although intermittent fasting may provide benefits to patients, it is currently unknown whether this type of feeding regimen can be successfully implemented without causing adverse effects of high-fat feeding such as gastrointestinal disturbances or pancreatitis. Therefore, the aims of this study were multifold; the first was to determine whether hospitalised patients were willing to eat a high-fat diet enriched in medium-chain triglycerides, and to document the effects on body weight and body composition. The second aim was to determine whether

a similar concentration of plasma ketones in healthy dogs (0.04 mmol/L) as found previously could be achieved in hospitalised dogs. The third aim was to document any adverse events as the result of the feeding regimen and high-fat diet. The final aim was to determine if a reduction in the hospitalisation duration occurs in dogs undergoing an intermittent fasting regimen on the high-fat diet.

7.2 Materials and Methods

Dogs with spinal disease were prospectively recruited from Massey University Veterinary Teaching Hospital's referral surgical department. The inclusion criteria were that the dog was expected to stay three days or longer in the hospital. Exclusion criteria included dogs with critical illness (American Society of Anesthesiologists (ASA) status grade 4-5), disturbances in water balance (e.g. kidney failure, chylothorax, haemoabdomen) or those who required intravenous fluid (IVF) therapy. The severity of the neurological disease at presentation to the hospital was made using a 1 - 5 scoring system as follows: grade 1 = spinal pain present, no motor deficits, grade 2 = ambulatory monoparesis or paraparesis, grade 3 = non-ambulatory paraparesis or monoplegia, grade 4 = paraplegia with nociception present, and grade 5 = paraplegia with absent nociception.[12–15] Body composition for each patient was measured as close as possible to the time of admission and repeated right before being discharged from the hospital. The deuterium protocol used was the same as described in Chapter 2, and deuterium was measured in plasma samples by gas-isotope ratio mass spectrometry[r]. This study was approved by Massey University Animal Ethics Committee (MUAEC protocol 18/69).

The dogs were randomly assigned to one of two feeding regimens: once-daily feeding on a high-fat, ketogenic diet, or the standard hospital feeding regimen consisting of twice-daily feeding on a diet that contained less fat and more carbohydrates. For dogs on the ketogenic diet, a short period of acclimation was performed as follows: on day one of the

[r] Isotope Ratio Mass Spectrometry Unit, Otago University, Dunedin, New Zealand

study, these dogs were offered 50% of their resting energy requirement[s] (RER) as the ketogenic diet, and 50% of their RER as the standard hospital diet. From the second day onwards, the dogs were fed 100% of their RER as the ketogenic diet once a day at 8am in the morning. Dogs on the standard hospitalised diet did not undergo an acclimation phase and were offered 50% of their RER every day at 8am and 6pm. All dogs were allowed up to three hours to finish their meal, after which time any remaining food was removed and weighed using the hospital's feed room bench scale[t]. The dogs were fed their assigned feeding regimens until the final body composition was measured.

Two forms of the ketogenic diet were available, either a modified commercial diet or a home-made diet. This was done to allow for a second choice if the dog did not adequately consume the modified commercial diet. The home-made diet was offered if the dog did not consume >70% of the modified commercial diet by the second day. The diets used in this study were formulated to meet the nutrient requirements for adult dogs defined by the Association of American Feed Control Officials (AAFCO). The recipes are provided in Appendix V. The macronutrient (protein, fat and carbohydrate) energy contents of the two high-fat diets were calculated to be similar based on Atwater and Modified Atwater values (Table 7-1). For both ketogenic recipes, medium-chain triglycerides (C8 - C12) were provided by the coconut oil and butter to give a total of 14.91 grams per Mcal, which equated to 10.1% of energy.[16] The standard hospitalised diet was composed of a mixture of a commercial kibble[u] and canned food[v], each of which provided 50% of caloric requirements. If this diet was not accepted by the patient, the attending clinician could choose to feed other standard commercial pet food and human foods available from the hospital's feed room. Dogs on the ketogenic diet were also allowed a small amount of

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[s] Resting energy requirement per day (kcals) = 70 x (ideal body weight)$^{0.75}$

[t] Wedderburn DIGI®, model DS-581, W.W. Wedderburn Pty Limited, Ingleburn, Australia

[u] Hill's® Science Diet® Adult Advance Fitness, Hill's Pet Nutrition Inc, Topeka, Kansas, United States

[v] Hill's® Science Diet® Adult Savory Stew (beef and chicken flavours), Hill's Pet Nutrition Inc, Topeka, Kansas, United States

cooked chicken to assist with oral pill administration outside of the main meal. However, this was limited as much as possible. All food eaten (type and weight) and body weight were recorded daily for each dog. In addition, a tri-axial accelerometer[w] was fitted to the dog's collar in order to quantify physical activity while in the hospital.

Table 7-1. The macronutrient energy contents of the high-fat diets and standard hospital diet on a metabolisable energy-basis (ME). The energy densities of the diets (kcal/gram) are presented on an as-fed basis.

	Standard Hospital diet	Ketogenic diet commercial	Ketogenic diet home-made
Protein energy (%ME)	22	22	21
Fat energy (%ME)	23	66	70
Carbohydrate energy (% ME)	55	12	10
Energy (kcal/gram)	2.28^	1.85*	3.03*

^Calculated using modified Atwater values
*Calculated using Atwater values

On the morning of day three, a two-hour postprandial blood sample (2 mL) was collected by venepuncture into an EDTA purple-top vacutainer[x] to measure β-hydroxybutyrate and triglycerides. For fasted β-hydroxybutyrate, an additional blood sample (1 mL) was taken the same morning as body composition measurements, which represented a 9 to 12-hour postprandial period for dogs on hospital diet and a 21 to 24-hour post-prandial period for the dogs eating the ketogenic diet. Plasma was harvested from the vacutainers within 30

[w] Heyrex®, Say Systems, Wellington, New Zealand
[x] BD Vacutainer®, Becton, Dickson and Company, Plymouth, United Kingdom

minutes of collection by centrifugation for 10 minutes at 3,000 rcf at 4 °C, and stored at -80 °C until later use.

Plasma β-hydroxybutyrate was measured using a colorimetric assay[y] in duplicates according to the manufacturer's instructions. Briefly, the thawed plasma samples were de-proteinated using 10 kilodalton (kD) spin columns[z] and centrifuged at 10,000 rcf for 10 minutes. Then a β-hydroxybutyrate dehydrogenase and substrate mixture was added and absorbance measured at 450 nm using a microplate reader[aa].

Triglycerides were measured at a commercial laboratory[bb] using a colorimetric assay[cc] according to manufacturer's instructions. For this, plasma samples underwent a series of coupled enzymatic reactions. Firstly, samples were combined with microbial lipases to produce glycerol and fatty acids. The glycerol was then phosphorylated by adenosine triphosphate in the presence of glycerol kinase to produce glycerol-3-phosphate. This was then oxidised by molecular oxygen in the presence of glycerol phosphate oxidase to produce hydrogen peroxide and dihydroxyacetone phosphate. Hydrogen peroxide then underwent a reaction with 4-aminophenazone and N,N-bis(4-sulfobutyl)-3,5-dimethylaniline disodium salt in the presence of peroxidase to produce a chromophore which was read at 660/800nm using an analyser[dd].

7.2.1 Statistics

All statistical analyses were performed using the R software[ee]. An *a priori* power analysis was conducted to determine the required sample size. Fasted ketone concentration was

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[y] beta HB Assay Kit, catalogue #ab83390, Abcam, Cambridge, United Kingdom
[z] 10kD spin column, catalogue #ab93349, Abcam, Cambridge, United Kingdom
[aa] VersaMax™ Microplate Reader, Molecular Devices LLC, San Jose, California, USA
[bb] New Zealand Veterinary Pathology IDEXX, Palmerston North, New Zealand
[cc] Triglycerides, catalogue #OSR60118/OSR601118, Beckamn Coulter Incorporated, Brea, California, United States
[dd] Beckman Coulter AU analyzer, Beckamn Coulter Incorporated, Brea, California, United States
[ee] R version 3.1.0; R Development Core Team, 2012; R Foundation for Statistical Computing, Vienna, Austria

considered one of the key outcome measures and was used for the power analysis calculation. Using the previous findings as described in Chapter 4, dogs eating a high-fat diet had a fasting β-hydroxybutyrate plasma concentration of 0.06 mmol/L, while those eating a high carbohydrate diet had a concentration of 0.02 mmol/L. Therefore, to detect a difference of 0.4 mmol/L, assuming a standard deviation of 0.04, with a power of 0.80 and a significance level of 0.05, it was determined that 16 dogs per group were needed.

Changes in body weight were expressed as the percentage change [(body weight at discharge (kg) – body weight at admission (kg)) / body weight at admission (kg)]. Changes in body condition scores (BCS), fat mass (FM) expressed as % body weight, and fat-free mass (FFM) expressed as % body weight for each patient were obtained by subtracting the value at the time of discharge from the value at time of admission. In addition, the changes in body weight, BCS, FM and FFM were normalised by dividing their values by the number of days of hospitalisation. Kendall rank correlation was used to compare associations between BCS and FM at the time of admission and at discharge. Prior to running the correlation test, the data was inspected and no outliers were dectected.[17]

One dog assigned to the ketogenic diet was removed from the study as he was intolerant of being handled for blood draws, and his data was not included in the analyses. Another dog assigned to the ketogenic diet did not undergo a second deuterium body composition or fasted ketone measurement due to an early discharge from the hospital. In this case, the clients requested for the dog to be transferred to their local veterinary clinic for recovery care shortly after surgery. In addition, only five dogs on the ketogenic diet and four dogs on the hospital diet had activity monitor readings due to an unexpected discontinuation of services by the manufacturer midway into the study. The missing data were considered to be "missing at random" (early discharge) and "missing completely at random" (activity monitoring), and so dogs with missing data for these parameters were excluded in their respective analysis (duration of stay, body composition, fasted ketone, and activity) in a complete-case approach, which was expected to cause minimal bias.[18–20]

A 2-sample t-test for normally distributed data and Wilcoxon rank-sum test for non-normally distributed data were used to compare food intake, triglycerides, post-prandial and fasted ketones, changes in body weight, BCS, FM, and FFM, and hospital duration between the dogs fed the ketogenic diet with the dogs fed the standard hospital diet. Equality of variances between the two groups were checked prior to running the analyses and Welch's correction used in the t-tests if they were unequal. Hypothesis testing was also performed using Bayes factor analysis with the 'BayesFactor' package (Richard D. Morey and Jeffrey N. Rouder, 2018). This type of testing allows for further analyses of non-significant findings to determine whether there is evidence that the data supports the null hypothesis or whether there is an absence of evidence.[21,22]

Further, a linear regression between body weight loss (%) and hospital duration was created. In addition, models for ketone concentrations (post-prandial and fasted) and hospital duration were built using multivariable regression analysis with a manual, stepwise selection procedure. Age, sex, disease severity (neurological grade), changes in BCS, body weight, FFM and FM, percentage RER consumed (total kcals eaten during hospital stay / [RER in kcals x days of hospitalisation]), and feeding regimen were treated as independent variables. For hospital duration, ketone concentrations were also tested as independent variables in the model. Activity was not assessed in the models due to the large amount of missing data. Two-way interactions between significant variables in the multivariable models were tested. Model fit was assessed using Akaike information criterion (AIC) and adjusted R^2. The collinearity of the variables in the final models was checked by variance inflation factor (VIF). In addition, the normal distribution of the residuals of the final regression was confirmed by visual appearance and with a Shapiro-Wilks normality test. Results are presented as mean with standard deviation (SD) unless stated otherwise. P-values < 0.001 were considered indicative of very strong evidence, P < 0.01 of strong evidence, P < 0.05 of moderate evidence, P < 0.1 of weak evidence, and P ≥ 0.1 of insufficient evidence.[23]

7.3 Results

Dogs were recruited to the study from October 2018 to March 2020. Only 18 dogs out of the 32 dogs required based on the *a priori* power analysis were able to be recruited. Of these, eleven were female (nine speyed, two intact) and seven were male (five neutered, two intact). There were 11 breeds, namely: dachshund (4), crossbreed (3), Maltese (2), French bulldog (2), Welsh corgi (1), Huntaway (1), New Zealand heading dog (1), border collie (1), cocker spaniel (1), whippet (1), and Staffordshire bull terrier (1). The majority of the dogs had a final diagnosis of intervertebral disc disease (IVDD) (Table 7-2). There was no difference between the neurological grades at admission between the two groups (control 3.9 (SD 0.74) *vs* ketogenic 3.6 (SD 1.2), $P = 0.742$). In addition, in the dogs with available activity monitoring (four dogs fed the standard hospital diet and five dogs fed the ketogenic diet), there was no difference in activity between those fed the hospital diet (22,314 (SD 7,423) ΔG) and those fed the ketogenic diet (19,575 (SD 6,680) ΔG) ($P = 0.578$).

Table 7-2. Final diagnoses in 18 dogs with spinal disease seen at Massey University's Veterinary Teaching Hospital.

Diagnosis	Count
Intravertebral disc disease type 1	10
Intervertebral disc disease type 2	1
Intervertebral disc disease type 3	2
Fibrocartilaginous embolism	1
Neoplasia	1
Traumatic	2
Lesion of unknown origin	1
Total	18

The dogs had a mean age of 6.6 (SD 3.2) years, admission body weight of 15.3 (SD 10.51) kg, and admission BCS of 5.1 (SD 1.3), which were not statistically different between the two groups ($P > 0.1$). Overall, there were eight dogs assigned to the ketogenic diet and ten dogs assigned to the standard hospital diet. No adverse events (including gastrointestinal disturbances or pancreatitis) occurred during the study. Most of the dogs on the ketogenic diet readily accepted the modified commercial diet (n = 5) whereas the remaining accepted the home-made diet (n = 3). The majority of the dogs on the standard hospital diet (n = 7) would not eat the mixture of the commercial kibble and canned diet, and were fed additional foods including other standard kibbles and canned diets, convalescence canned diets, dog roll, cooked chicken, and canned salmon. As there was a large variety of foods provided to the dogs on the standard hospital diet, the macronutrient contents of the diets were calculated to confirm the differences consumed by the two groups (Table 7-3). On average, the dogs ate 78.8 (SD 22.1) % of their RER when on the hospital diet, and 92.4 (SD 24.5) % of their RER when fed the ketogenic diet, which were not statistically different to each other ($P = 0.234$).

Table 7-3. Macronutrient intakes (protein, fat and carbohydrate) expressed as a percentage of metabolisable energy in dogs recovering from spinal disease fed a standard diet or a ketogenic diet.

	Hospital diet		Ketogenic diet		
	Mean	SD	Mean	SD	P - value
Protein energy (%ME)	25.4	6.2	18.4	2.1	0.006
Fat energy (%ME)	38.3	7.7	71.9	5.5	< 0.0001
Carbohydrate energy (% ME)	28.3	9.8	9.7	4.6	< 0.001

Changes in body weight, FFM, FM and BCS during hospitalisation are presented in Table 7-4. The correlation between change in body weight (%) and hospital duration is presented in Figure 7-1 ($r^2 = 0.33$, $P = 0.009$). Dogs fed the standard hospital diet lost more overall body weight during hospitalisation than dogs fed the ketogenic diet (8.82 (SD 3.54) % vs

3.69 (SD 4.27) % respectively ($P = 0.016$, Table 7-4). However, there were no significant differences in the changes of FFM and FM between the two feeding regimens ($P > 0.1$, Table 7-4).

Table 7-4. The mean and standard deviation (SD) of changes (Δ) in body condition score, body weight, and body composition during hospitalisation in dogs recovering from spinal disease fed a standard diet or a ketogenic diet. Values for each parameter are presented as the overall total change during the hospital stay, as well as per day of hospitalisation. Results from the t-test and Wilcoxon rank-sum analyses ($P-$ value) and Bayes factors are also presented in the table.

	Hospital diet		Ketogenic diet			Bayes
Change (Δ)	Mean	SD	Mean	SD	P - value	factor
Body condition score	-0.55	0.68	-0.07	0.19	0.108	1.17
Body condition score per day	-0.06	0.07	-0.004	0.01	0.098	1.82
Body weight (%)	-8.82	3.54	-3.69	4.27	0.016	3.68
Body weight (%) per day	-1.13	1.00	-0.57	0.91	0.087	0.67
Fat-free mass (%)	0.04	0.05	0.006	0.05	0.166	0.85
Fat-free mass (%) per day	0.008	0.013	0.001	0.014	0.591	0.79
Fat mass (%)	-0.04	0.05	-0.008	0.05	0.204	0.76
Fat mass (%) per day	-0.008	0.013	-0.0008	0.014	0.740	0.74

For BCS, dogs on the ketogenic diet lost an overall BCS of -0.07 (SD 0.19), whereas the dogs on the standard hospital diet lost a BCS of -0.55 (SD 0.68) ($P = 0.108$). There was moderate correlation between BCS and the % FM measured at admission (Kendall's tau = 0.58, $P = 0.001$) and at discharge (Kendall's tau = 0.59, $P = 0.002$). However, there was poor correlation between the changes in BCS and % FM in patients during hospitalisation (Kendall's tau = 0.30, $P = 0.130$).

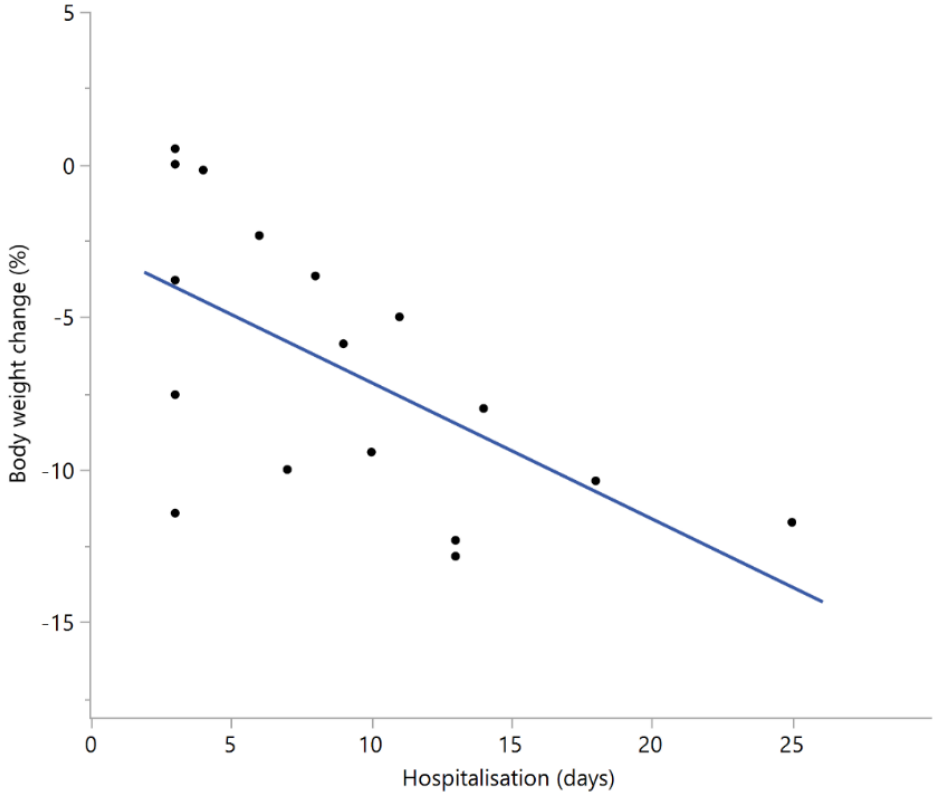

Figure 7-1. Correlation between body weight change (%) and duration of hospitalisation (days) in 18 dogs recovering from spinal disease (r^2 = 0.33, P = 0.009).

Dogs fed the ketogenic diet had an average post-prandial triglyceride concentration of 1.1 (SD 0.2) mmol/L, whereas dogs fed the standard hospital diet had an average concentration of 1.0 (SD 0.8) mmol/L (P = 0.146). For ketones, dogs fed the ketogenic diet had post-prandial and fasted plasma β-hydroxybutyrate of 0.07 (SD 0.11) and 0.05 (SD 0.03) mmol/L respectively, whereas dogs fed the hospital diet had concentrations of 0.02 (SD 0.01) mmol/L post-prandially and 0.03 (SD 0.03) mmol/L after fasting (Figure 7-2). Neither post-prandial nor fasted plasma β-hydroxybutyrate were significantly different between the two groups (P = 0.594 (post-prandial) and P = 0.281 (fasted)). Also, Bayes factors for post-prandial and fasted β-hydroxybutyrate were 0.86 and 0.55 respectively, indicating that there was negligible evidence in support of the null hypothesis (Bayes factor $0.33 < x < 1$).[21,24]

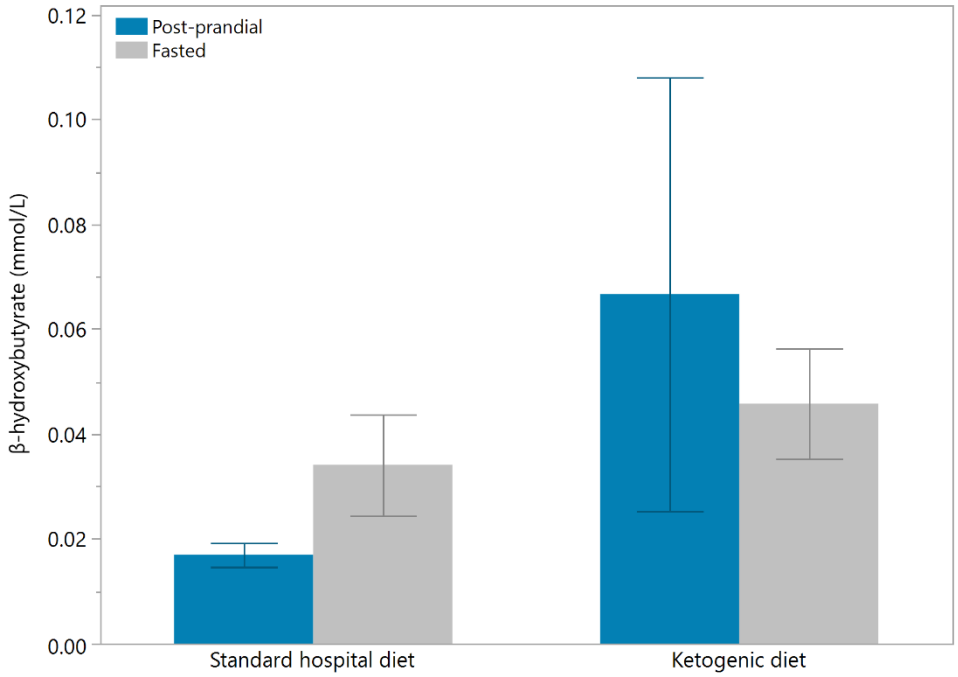

Figure 7-2, Means and standard errors of β-hydroxybutyrate concentrations in spinal dogs fed a standard hospital diet fed twice-daily (n = 10) or a high-fat ketogenic diet fed once-daily (n = 8). For post-prandial ketones, samples from all dogs were taken two hours after a meal. For fasted ketones, samples were taken either 9 to 12 hours after a meal for the dogs eating the standard hospital diet or 21 to 24 hours after a meal for the dogs eating the ketogenic diet. A comparison of the means indicated that there were no statistical differences between the two feeding regimens ($P = 0.594$ (post-prandial β-hydroxybutyrate) and $P = 0.281$ (fasted β-hydroxybutyrate)).

The overall range of hospital stay was 3 - 25 days (mean 9, SD 6). When comparing outcomes, the dogs fed the ketogenic diet had a mean duration of stay of 6.4 (SD 5.5) days, whereas the dogs fed the standard hospital diet had a mean duration of stay of 10.7 (SD 6.4) days ($P = 0.152$, Figure 7-3). There was negligible evidence in support of the null hypothesis for hospital duration (Bayes factor 0.80).

Figure 7-3. Duration of hospitalisation in spinal dogs fed the standard hospital diet fed twice-daily (n=10) or a high-fat ketogenic diet fed once-daily (n=8) ($P = 0.152$).

Multivariable regression analysis was performed to determine which factors (age, sex, neurological grade, changes in BCS, body weight, FFM and FM, percentage RER consumed, and feeding regimen) were associated with ketone concentrations and patient outcome (hospital duration). For hospital duration, ketone concentrations were also tested as independent variables in the model. The final models are presented in Table 7-5. Although the association between % body weight loss and post-prandial β-hydroxybutyrate concentrations was not significant, it was retained in the final model because it improved the overall Akaike information criterion (AIC) and adjusted R^2. The results of the analyses showed that having a higher neurological grade at admission and eating the standard hospital diet were negatively associated with post-prandial β-hydroxybutyrate concentrations. For fasted β-hydroxybutyrate concentrations, having a higher neurological grade at admission and eating the standard hospital diet were

negatively associated, whereas a greater % body weight loss and age were positively associated with fasted β-hydroxybutyrate concentrations. Finally, the variables associated with hospital duration were fasted ketone concentrations and % body weight loss. A significant interaction between these two variables specified that higher fasted ketone concentrations were associated with improved outcome (less days in the hospital) but only when there was greater weight loss.

Table 7-5. Multivariable regression models for factors associated with post-prandial and fasted β-hydroxybutyrate, and duration of hospitalisation in dogs recovering from spinal injury. The best fit models as shown were assessed using Akaike information criterion and adjusted R^2. For post-prandial β-hydroxybutyrate, change in body weight was not significant, however, it was retained as it improved both the AIC score and adjusted R^2 for the final model.

Outcome	Variable	Estimate	Standard Error	R^2	P - value
β-hydroxybutyrate post-prandial (mmol/L)	Intercept	0.183	0.081		0.042
	Neuro grade	-0.049	0.022		0.042
	Control diet	-0.050	0.021		0.038
	% body weight loss	0.008	0.005		0.110
	Overall model			0.40	0.074
β-hydroxybutyrate fasted (mmol/L)	Intercept	0.070	0.020		0.005
	Neuro grade	-0.022	0.005		< 0.001
	Control diet	-0.016	0.004		0.004
	Age	0.005	0.001		0.003
	% body weight loss	0.004	0.001		0.004
	Overall model			0.82	0.001
Hospital duration (days)	Intercept	2.11	2.92		0.485
	Fasted ketones	13.43	59.85		0.837
	% body weight loss	1.843	0.400		< 0.001
	Fasted ketones x % body weight loss	-21.08	7.70		0.019
	Overall model			0.70	< 0.001

7.4 Discussion

The aims of this study were to determine the effects of implementing an intermittent fasting regimen using a high-fat, ketogenic diet in hospitalised dogs recovering from spinal cord injury. It was found that the dogs were able to adequately consume the diet without adverse events and lost less body weight than the dogs fed the standard hospital diet. A positive association between the amount of weight loss and length of hospital stay was also found. The association between malnutrition/body weight loss and length of hospitalisation is well established in human medicine, although the directional causality of this relationship is uncertain.[25–27] While less is known in veterinary medicine, a previous study of hospitalised dogs found a similar association, where longer hospitalisation was associated with greater losses in body weight.[3] The results of this study provide further evidence that like human medicine, the same association between body weight loss and hospitalisation length exists in veterinary medicine.

The beneficial effects of intermittent fasting on neuronal recovery is believed to be in part from increased ketone utilisation.[28,29]A key finding in this study is that higher fasted β-hydroxybutyrate concentrations were associated with shorter hospital stays, but only in dogs that lost a greater amount of body weight. Interestingly, there were negligible effects of ketones on hospital stay when the dogs lost less weight. It was hypothesised that greater weight loss could be related to increased lipolysis and ketogenesis. Indeed, weight loss was found to be positively associated with fasted β-hydroxybutyrate concentrations in the multivariable model for fasted β-hydroxybutyrate. However, collinearity of the significant variables was checked in the final models and was not found to be present. Other considerations included whether the weight loss was from a loss of lean mass and whether the rate at which weight loss occurred (% weight loss per day of hospitalisation) were related to the association between ketones concentrations and hospital duration. However, these too were not found to be significant. Therefore, the exact mechanism of the association between greater weight loss, higher fasted ketones and shorter hospital stays is not clear from this study. However, although not statistically significant, there was also a tendency for dogs eating the ketogenic diet to have a shorter length of

hospitalisation compared to the dogs eating the standard hospital diet, lending further evidence of a potential effect. Taken together, the results of this study support an association between fasted ketones, weight loss and outcomes in hospitalised spinal patients, but further research is needed to confirm this novel finding.

Regression analyses were used to better understand which variables affected plasma ketone concentrations. Consuming the hospital diet and having a higher neurological grade were associated with lower post-prandial and fasted ketone concentrations. The dogs eating the standard hospital diet consumed less fat and a greater amount of carbohydrates. The effect of increasing dietary digestible carbohydrates is that it will increase circulating insulin and decrease lipolysis and ketogenesis.[30] Further, having a higher neurological grade was also found to be negatively associated with plasma ketone concentrations, which could be due to decreased synthesis and/or increased clearance. In humans, the concentration of catecholamines in blood decreases as the severity of spinal injury increases, due to a greater disruption of sympathetic pathways.[33,34] Also, in an experimental model of spinal cord injury, dogs had an initial rise in concentrations of plasma norepinephrine immediately following surgery, which then decreased significantly below baseline two hours post-operatively.[35] A decrease in systemic catecholamines could reduce lipolysis and ketogenesis, leading to a decrease in circulating ketone concentrations.[36] In addition, injury to the spinal cord in rodents has been shown to increase the expression of ketone transporter, monocarboxylate transporter 1, in grey matter.[37] It is currently unknown if an increase in spinal cord injury severity leads to a greater expression of monocarboxylate transporters.

Furthermore, age was found to be positively associated with fasted β-hydroxybutyrate concentrations. In humans, older individuals had higher fasted blood ketone concentrations compared to younger individuals fasted for a similar period of time.[38,39] In older rats, there was a reduction in the expression of monocarboxylate transporters 2 in cerebral neuronal tissues following injury compared to younger rats.[40] A reduction in the clearance of ketones could account for an increase in plasma concentrations. As such,

further research on the effects of spinal cord injury severity and age on transporter expression, ketone synthesis and ketone clearance in dogs is warranted.

The concentrations of β-hydroxybutyrate achieved in the hospitalised dogs eating the ketogenic diet were similar to those found in healthy dogs as described in Chapters 4 and 6. However, although the dogs fed the ketogenic diet were eating a greater amount of dietary fat, and were fasted for longer than the dogs fed the standard hospital diet, there was no statistical difference between the two groups. This was not an unexpected finding as the number of recruited patients were less than what was deemed necessary in the *a priori* power analysis, ultimately affecting the ability to detect a difference in this study. Although there was insufficient evidence to detect a difference, the effect size seen in this study could be biologically significant, particularly taking into consideration the findings from the multivariable model for hospital duration. Ultimately, the data generated here supports the need for further study to determine whether intermittent fasting regimen on a high-fat diet can increase ketone concentrations in hospitalised dogs above that of hospitalised dogs eating conventional hospital diets.

Changes in FM and FFM were measured in this study and despite a difference in the amount of body weight loss in the dogs eating the ketogenic diet compared to the dogs eating the standard hospital diet, there was no significant effect on either FM or FFM. This finding was unexpected as body weight loss should be related to losses in FM and/or FFM. It is believed that the most likely reason for this was from imprecision in the isotopic dilution technique as a result of human error; any loss or incomplete injection of the labelled isotope would significantly affect the final composition values. Another possibility is the difference in hydration of the patients. A pan-species hydration constant of 73.2% was used to calculate FFM from total body water, however, there are known variations to this constant with animals of different ages and species.[41,42] The hydration status many vary in hospitalised dogs recovering from spinal disease, affecting the accuracy of the calculations. Therefore, future studies may wish to measure hydration

status in patients and correct for over- and underhydration before using the isotopic dilution technique to measure body composition.

No adverse events occurred during the study including gastrointestinal disturbances or pancreatitis. There is a commonly held belief amongst veterinarians of a causation between dietary fat and pancreatitis in dogs. This belief stems from old experimental studies and epidemiological associations between high dietary fat consumption and a sudden change in diet with pancreatitis in dogs.[43] However, there are challenges in the interpretation of the results of these studies. In early experimental models, dogs fed a high-fat versus a high- protein/high-carbohydrate diet led to more severe clinical signs and pathological changes in the pancreas.[44,45] However, the diets were unlikely to have been complete and balanced rations, and so other nutrient deficiencies may have confounded the results. In addition, giving table scraps and consuming food discarded in the trash have also been identified as risk factors for the development of pancreatitis in dogs.[46] However, it is difficult to speculate what the fat contents of those items were. James *et al.* (2009) found no difference in serum canine trypsin-like immunoreactivity, pancreatic-lipase immunoactivity or gastrin concentrations when healthy dogs were fed diets with low and medium-fat contents (approximately 40% fat on an energy basis).[47] There are also numerous studies and reports of sled dog diets where the dogs were fed a diet with a similar fat content to our HF diet (~70% metabolisable energy from fat) without causing pancreatitis.[48–53]

Despite the paucity of direct evidence that a high dietary fat intake causes pancreatitis, it is unknown how an animal with illness may react to a sudden change to a high-fat diet. Thus, as a precaution, a short acclimation period was implemented in dogs consuming the high-fat diet. In addition, post-prandial plasma triglycerides were measured on all dogs. There was no difference in plasma triglycerides between dogs consuming the high-fat diet compared to dogs consuming the standard hospital diet. In addition, all dogs had values well below 11.3 mmol/L, which is believed to increase the risk of pancreatitis.[54] Therefore,

intermittent fasting on a high-fat diets appears to be well tolerated in recovering spinal canine patients.

The main limitation to the study was the small sample size recruited. Over the 17 months of the study, only a portion of patients needed as calculated in the *a priori* power analysis was able to be recruited. This was due to many challenges including a low number of spinal cases seen at the hospital, low suitability of patients, difficulties obtaining timely client consent, and the early end of recruitment due to the COVID-19 pandemic. This would have decreased the power of the study to detect a difference in many of the parameters studied.

7.5 Conclusions

It was found that implementation of an intermittent fasting regimen using a high-fat diet was feasible in hospitalised dogs recovering from spinal cord injury. In addition, the dogs lost less weight compared to those eating the standard hospital diet. Further, there was also an association between higher fasted ketone concentrations and shorter hospitalisation that was present only when the dogs lost more weight. Despite the low number of patients in this study, the findings provide initial evidence of a benefit of ketogenic diets in some hospitalised spinal patients, supporting the need for further studies. As the ability to recruit patients in a timely manner will remain a challenge, a larger, possibly multicentric, long-term study is required to determine whether the results found here continue to hold true.

7.6 References

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Chapter 8

Overview and concluding remarks

"If I have seen further, it is by standing on the shoulders of Giants." – Sir Isaac Newton to Robert Hooke (February 5, 1675)

Despite the incredible advances in medicine over the past century, we are still searching for the answer to the fundamental question of how best to feed our patients. Malnutrition remains commonplace in human hospitals globally and has clear negative consequences associated with it. In veterinary medicine comparatively, there is still little known about the nutritional status of hospitalised patients with only a few published studies to date. This PhD thesis sought to provide substantial contribution to this important, and still often overlooked area of veterinary medicine.

8.1 Thesis overview

To begin, Chapter 1 provided a distillation of what is known about the prevalence and consequences of malnutrition during hospitalisation in human and veterinary species. Numerous studies from different regions of the world have described the prevalence of malnutrition in human hospital patients to be 20% to 60%, indicating that a substantial proportion of patients are not being adequately nourished during their hospitalisation.[1–3] In addition, the negative consequences associated with malnutrition are also well established in human medicine. One of these is a decline in immunity, which is believed to be due in part to a drop in the adipokine leptin following a reduction in caloric intake. A fall in leptin concentration has been shown to decrease chemotaxis of granulocytes and monocytes/macrophages, decrease macrophage phagocytosis and cytokine production, and reduce T and B lymphocytes proliferation *in vitro*.[4–10] This reduction in immunity may put a patient at risk of developing infections and poor wound healing.[11–13] Another

negative effect of malnutrition is the loss of muscle mass and strength. Having low muscle mass at admission and losing muscle during hospitalisation are both associated with negative prognoses and outcomes in human patients.[14–16]

By comparison, there have been only a handful of studies published in veterinary medicine that have looked for the prevalence of malnutrition and its associations with negative outcomes in hospitalised patients. These publications have found a similar association between malnutrition and an increased length of hospitalisation and risk of mortality.[17–19] However, the significance of muscle loss during hospitalisation in veterinary patients is largely unknown. Indeed, veterinary patients may actually be at greater risk of mortality from euthanasia if the loss of muscle and function causes an increased length of hospital stay, which can be costly to owners, and/or a perceived poor quality of life.[20] However, studies which have examined the effect of malnutrition on outcomes in hospitalised veterinary patients remain few, and far more research is needed.

The review in Chapter 1 goes on to explore the dichotomy between underfeeding and overfeeding. While underfeeding has clearly been shown to be detrimental to patients, excessive intake has negative consequences as well. Hyperglycaemia occurs commonly in critically ill patients from a combination of overfeeding and the release of catecholamines and inflammatory cytokines during disease.[21–24] The excess blood glucose leads to increased reactive oxygen species (ROS) generation and glycation of cellular proteins causing impaired wound healing, neuronal dysfunction, vasculitis, and an increased risk of systemic infections.[21–23,25–30] Because of this, the concept of a 'Goldilocks' range of restricted caloric intake has been proposed, which would prevent the immunosuppression and minimise the muscle loss from undernutrition, whilst also reducing the risk of hyperglycaemia and other sequelae of overnutrition. Expanding on this concept, the health benefits of caloric restriction (below maintenance requirements) and intermittent fasting in specific diseases were next explored. There have been numerous studies in humans and in experimental models that have shown therapeutic benefits of caloric restriction and intermittent fasting in diabetes, cardiac ischemia and neuronal injury.[31–37] It is believed that

the utilisation of ketones and the effects of circulating ghrelin, both of which increases during fasting, are the key mechanisms behind these benefits.[38–45] For veterinary medicine, improving recovery following neuronal injury would be of particular interest as these tend to be our longest stay patients, and so are potentially more at risk of developing malnutrition during hospitalisation. However, despite the growing evidence in other species of a beneficial effect of caloric restriction and intermittent fasting, this area is virtually unexplored in the veterinary literature.

The aim of the work presented in Chapter 2 was to investigate the prevalence of weight loss and to quantify changes in body composition of long-stay hospitalised canine patients in a teaching hospital. In addition, I compared commonly used composition measurement techniques (body condition scoring and muscle condition scoring) to the gold standard of isotopic dilution to determine how accurately these methods captured changes in composition in that population. This was the first study to measure body composition changes in hospitalised dogs using isotopic dilution. I found that the large majority of dogs (78.3%) lost weight during hospitalisation, which was proportionally composed mostly (61.8%) of lean mass (fat-free mass). It was also discovered that intake was not significantly associated with either changes in body weight or body condition. This highlighted that the weight loss seen in hospitalised dogs was not simply due to undereating. This agrees with a recent human meta-analysis that showed no clear association between energy or protein intake, and changes in skeletal muscle mass in patients.[46] The cause of the loss of muscle during hospitalisation is instead likely to be multifactorial, where disease and inactivity also contribute to the decline. It was also shown in the study that body condition scoring and muscle condition scoring are not suitable for monitoring changes during hospitalisation, particularly in lean dogs. However, more accurate techniques such as isotopic dilution or dual-energy x-ray absorptiometry (DEXA) are expensive, time consuming and invasive, and are thus not suitable for regular clinical use in veterinary patients. Therefore, it was determined that the development of a simple method of measuring composition in lean dogs was required that was more accurate than the widely used BCS system.

The aim of the work presented in Chapter 3 was to create equations to predict the body composition in lean dogs using morphometry that could provide a simple bedside measure of lean and fat mass. Working dogs were used as the study population because they represented a readily available population of lean dogs in New Zealand. Body composition was measured by isotopic dilution, and principal component analysis was used to create a new variable that defined skeletal size based on morphometric measurements. In addition, another novel variable, termed leanST, was created by subtracting estimated skeletal mass from fat-free mass for each dog to assess skeletal mass and muscle mass separately. The findings of this study indicated that while it was possible to create accurate equations of composition in lean dogs, there was a strong breed effect; consequently, breed-specific equations would need to be generated. In addition, calculating leanST did not increase accuracy, as the correlation coefficients for the regression models describing the association between the measured and predicted values for the ratios of fat-free mass to skeletal size and leanST to skeletal size were higher than for fat-free mass to skeletal mass and leanST to skeletal mass. Therefore, there was no additional gain from calculating fat-free mass and skeletal mass separately. Finally, the study also found that in lean dogs, body weight was already a good predictor of fat-free mass and lean soft tissue mass. Thus, it was determined that in a hospital setting, the practical use of morphometry to estimate muscle mass in lean dogs would not supersede the simpler method of measuring and monitoring body weight.

Chapters 4 and 5 explored the novel concept of intermittent fasting in dogs. This was the first published study that examined the immune and metabolic effects of intermittent fasting in healthy dogs. For this study, development and optimisation of immunoassays (flow cytometry and [^{3}H]-thymidine incorporation) and an NMR-based metabolomics assay for canine samples was required. The dogs were fed a low-fat diet twice-daily, a low-fat diet every-other-day or a high-fat diet enriched with medium-chain triglycerides every-other-day in a crossover design. It was discovered that leptin was not maintained during the fasting period by using a high-fat diet, in contrast to what has been shown in

mice.[47] In addition, it was found that fasting for approximately 48 hours after eating a low-fat diet blunted some immune responses. In particular, after leucocytes were activated by *Staphylococcus aureus*, there was a reduction in the percentage of neutrophils and mean fluorescent intensity in the macrophages that underwent both phagocytosis and oxidation, as well as a reduction in the percentage of lymphocytes that underwent respiratory oxidative burst. The clinical significance of these immune changes is unknown, but may be important during illness and hospitalisation.

Interestingly, the changes in the immune parameters studied were not associated with leptin concentrations, contrary to what has been reported in other species.[8–10,48–50] There was an approximately 25% reduction in mean leptin concentrations between the dogs when they were fed daily compared to when they were intermittent fasted. In comparison, a 50 - 60% reduction in serum leptin concentrations in mice is seen after a 48-hour fast compared to those *ad libitum* fed.[47,51] Therefore, the drop in leptin concentrations in this study may have been insufficient to cause a significant difference in the immune function of the dogs. In addition, rodents have a high metabolic rate and typically consume multiple small meals a day, so fasting for 12 - 16 hours is already considered a significant duration.[52,53] Therefore, feeding once every 48 hours is not the same metabolically for a rodent as it is for a dog, and a greater effect on immune function may be seen with a longer fasting period in the dog. Importantly however, it was also found that intermittent fasting for up to 48 hours while eating a high-fat diet did not cause the same immunosuppression in the dogs. This indicates that consuming a high-fat diet prior to fasting may be advantageous to avoid the immunosuppressive effects of fasting.

Another key finding in the study was that there was no difference in fasted ghrelin concentrations when the dogs were fed daily or intermittently fasted on a low-fat diet. Additionally, ghrelin was lowest when the dogs were intermittently fasted on a high-fat diet. These findings indicate that any beneficial effects intermittent fasting may have in dogs would be unlikely due to an increase in ghrelin. It was also found that dogs fasted for 48 hours on a high-fat diet had higher blood β-hydroxybutyrate concentrations than when

they were fed daily or fasted on a low-fat diet. However, the concentrations obtained (mean 0.06 (SD 0.02) mmol/L) were much lower compared to what has been reported in rodents (0.8-2 mmol/L) and humans (1.67 mmol/L) fasted for a similar amount of time.[54–56] This finding was consistent with other published studies that have described a lower concentration of blood ketones in dogs compared to other species after comparable fasting lengths.[57–59] However, a single blood sample only describes the concentration of a metabolite at that moment, and does not describe its flux (synthesis and utilization).[60] Therefore, to gain to a more comprehensive understanding of the flux of ketones in dogs while fasting, a ketone kinetics study was required.

Chapter 6 describes the ketone kinetics study performed to measure rates of ketone synthesis and clearance. For this, labelled β-hydroxybutyrate and acetoacetate were used in a two-accessible pools model to calculate ketone kinetics.[61–63] Healthy dogs were fed a high-fat diet enriched with medium-chain triglycerides either once-a-day or every-other-day in a crossover design. I showed that dogs utilise ketones more efficiently than rodents and humans, which was consistent with previous studies.[57,64–66] This provided additional evidence that dogs are a species well adapted to undergo periods of fasting and the low blood ketone concentrations are simply reflective of this increased utilisation.

Another finding of this study was that fasting for approximately 48 hours resulted in higher plasma β-hydroxybutyrate concentrations compared to fasting for approximately 24 hours. However, the degree of increase was relatively small despite dogs having been fasted for twice the amount of time. In addition, there were no significant differences in the ketone kinetics of the dogs fasted for approximately 24 and 48 hours. This was likely due to the large variations in the kinetics, particularly when the dogs were fasted for longer than 24 hours. A larger variation in ketone turnover rates has been reported previously in dogs fasted for ten days compared to when they were fasted for one day.[57] The findings from Chapter 6 are important since feeding hospital patients every 24 hours is likely to be more acceptable, and less likely to result in weight loss, than feeding every 48 hours. It was decided that a shorter period of fasting can be used in future studies to

test the practical application of an intermittent fasting regimen in hospitalised patients. However, it was also determined that the rates of ketone synthesis and utilisation in response to increasing fasting duration can vary considerably between individuals, and therefore, so might the benefits of an intermittent fasting regimen in recovering spinal patients.

Finally, Chapter 7 described a study that investigated the feasibility and effect of an intermittent fasting regimen using a high-fat diet on ketone concentrations and outcome in hospitalised dogs recovering from spinal injury. It was discovered that the dogs were able to adequately consume the high-fat diet without adverse effects and lost less body weight compared to dogs eating the standard hospital diet. In addition, having higher fasted plasma ketone concentrations was found to be associated with improved outcome (less days of hospitalisation), but this was only present when the dogs lost more body weight. This was a significant finding as the beneficial effects of intermittent fasting on neuronal recovery is believed to be in part from increased ketone utilisation.[41,67] This study is the first to show an association between ketones, weight loss and outcome in canine spinal patients, warranting further research.

8.2 Concluding remarks

Overall, the research presented in this thesis provides evidence that a large proportion of long-stay hospitalised canine patients are at risk of malnutrition and have inadequate caloric intake to maintain body weight. In addition, the portion of weight lost derived from muscle mass loss was greater than what has been previously reported in healthy dogs undergoing purposeful caloric restriction for weight loss. This indicates that muscle loss during hospitalisation is not simply from undernutrition. A decrease in muscle mass and the development of weakness have been shown to be independently associated with in-hospital mortality in human medicine.[68–70] In the final study, greater body weight loss was associated with a longer hospitalisation in dogs recovering from spinal cord injury. Therefore, the findings in this thesis support the need to further examine the causes of body weight and muscle loss during hospitalisation in veterinary medicine. Future

research should consider the effects of diet, inflammation, inactivity and disuse atrophy on muscle mass.

Intermittent fasting using a high-fat diet has been shown for the first time to be a practical feeding regimen for both healthy and hospitalised dogs recovering from spinal cord injury. Future studies could consider optimising the ketogenic effect by decreasing the amount of digestible carbohydrates in the diet to below 10% on a metabolisable energy-basis. This will further reduce the post-prandial rise in blood glucose, which will limit the insulin response, increase glucagon and subsequently, ketogenesis.[71,72] Another consideration is to give more ketogenic medium-chain triglycerides (i.e. octanoic acid (C8) and decanoic acid (C10)), which may increase post-prandial ketone production and raise concentrations higher than what was achieved here.[73,74] This too could potentially enhance the benefits of an intermittent fasting regimen in those dogs who are slow to upregulate ketone production and utilisation during fasting. The last consideration is to provide ketones directly as a supplement. Typical forms include ketone esters or ketone salts, which have been shown to significantly increase plasma ketone concentrations in both the fed and fasted state in humans and rodents.[75,76] However, the safety and tolerance of exogenous ketones supplements will need to be first established in dogs.

In the intermittent fasting studies, plasma ketone did not reach the same concentrations as seen in other species, which was determined to be simply reflective of the dog's ability to utilise ketones more effectively. It is difficult to speculate a target plasma β-hydroxybutyrate concentration that would be sufficient to have a therapeutic effect in dogs, however it is likely to be much lower than what has been described in rodents (0.8 – 1.5 mmol/L).[54,77] In the hospital study where fasted ketones and body weight loss were associated with hospital length, the concentrations of β-hydroxybutyrate ranged from 0.01 – 0.09 mmol/L. In addition, although dogs have a high rate of plasma ketone clearance, it was not determined which tissues utilise the ketones. Skeletal muscle, particularly cardiac muscle, nervous tissue, and the kidneys all have a high capacity for ketolysis.[78] The concentration of ketones in cerebral spinal fluid (CSF) has been shown to correlate with

concentrations of ketones in plasma in both fasted dogs and humans.[55,79] As such, future studies wishing to better understand the utilisation of ketones may consider measuring labelled and/or endogenous ketones in the CSF or measure cerebral arteriovenous differences. However, these procedures would be considerably more complex and invasive.

Another question that remains is whether all dogs can benefit from an intermittent fasting regimen, as large individual responses in ketone production and clearance were seen, particularly with increasing fasting duration. Results also showed a potential effect of sex, neutering, age, and the extent of neurological injury on ketone kinetics, which would be interesting for future studies to examine. If, for instance, monocarboxylate transporter expression increases proportionally to injury severity, then this would suggest that a dog with more severe neuronal injuries may be well placed to benefit from a ketogenic diet, whereas a dog with less severe injury may not benefit as much. Therefore, identifying the variables that affect ketogenesis and utilisation would be key to better understand which dogs may benefit more from a ketogenic, intermittent fasting regimen.

In closing, it is hoped that the research presented in this thesis will provide the foundation and inspiration for further work in this area. There is still much to learn and understand about hospital nutrition in veterinary medicine and the effect of intermittent fasting in dogs, with ultimately, only the very tip of the iceberg having been explored here.

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Appendix I: Statement of contribution and reproduction of published article (Chapter 3)

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We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Yuet-Ming (Becca) Leung
Name/title of Primary Supervisor:	Dr. Nick Cave
In which chapter is the manuscript /published work:	3

Please select one of the following three options:

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Leung YM, Cave NJ, Hodgson BAS. Creation of a predictive equation to estimate fat-free mass and the ratio of fat-free mass to skeletal size using morphometry in lean working farm dogs. N. Z. Vet. J. 66, 248–256, 2018

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GRS Version 5 – 13 December 2019
DRC 19/09/10

New Zealand Veterinary Journal

ISSN: 0048-0169 (Print) 1176-0710 (Online) Journal homepage: http://www.tandfonline.com/loi/tnzv20

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Creation of a predictive equation to estimate fat-free mass and the ratio of fat-free mass to skeletal size using morphometry in lean working farm dogs

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Abstract

AIMS: To develop an equation that accurately estimates fat-free mass (FFM) and the ratio of FFM to skeletal size or mass, using morphometric measurements in lean working farm dogs, and to examine the association between FFM derived from body condition score (BCS) and FFM measured using isotope dilution.

METHODS: Thirteen Huntaway and seven Heading working dogs from sheep and beef farms in the Waikato region of New Zealand were recruited based on BCS (BCS < 3, 3–4, > 4) using a nine-point scale. Bodyweight, BCS, and morphometric measurements (head length and circumference, body length, thoracic girth, and fore and hind limb length) were recorded for each dog, and body composition was measured using an isotopic dilution technique. A new variable using morphometric measurements, termed skeletal size, was created using principal component analysis. Models for predicting FFM, leanST (FFM minus skeletal mass) and ratios of FFM and leanST to skeletal size or mass were generated using multiple linear regression analysis.

RESULTS: Mean FFM of the 20 dogs, measured by isotope dilution, was 22.1 (SD 4.4) kg and the percentage FFM of bodyweight was 87.0 (SD 5.0)%. Median BCS was 3.0 (min 1, max 6). Bodyweight, breed, age and skeletal size or mass were associated with measured FFM ($p<0.001$). There was a good correlation between predicted FFM and measured FFM ($R^2=0.96$), and for the ratio of predicted FFM to skeletal size and measured values ($R^2=0.99$). Correlation coefficients were higher for the ratio FFM and leanST to skeletal size than for ratios using skeletal mass. There was a positive correlation between BCS-derived fat mass as a percentage of bodyweight and fat mass percentage determined using isotope dilution ($R^2=0.65$).

CONCLUSIONS AND CLINICAL RELEVANCE: As expected, the predictive equation was accurate in estimating FFM when tested on the same group of dogs used to develop the equation. The significance of breed, independent of skeletal size, in predicting FFM indicates that individual breed formulae may be required. Future studies that apply these equations on a greater population of working Huntaway and Heading dogs are needed to establish the utility of these equations on a large scale. Such studies could ascertain if there is a ratio for lean mass to skeletal size below which the risk of injury or disease increases. If these equations prove useful they would provide an objective and non-invasive measure to determine when welfare in individual dogs is compromised by underfeeding.

KEY WORDS: Farm dog, body composition, lean, muscle mass, skeletal mass, morphometry

Introduction

Farm dogs provide a significant service to the agricultural industry in Australasia. In New Zealand certain aspects of pastoral farming on hill and high country properties would be virtually impossible without the assistance of working dogs. Mustering requires athleticism, which is incompatible with obesity, and thus, in the authors' experience, most working farm dogs are kept in a lean condition with little to moderate body fat. Despite this, it is unknown what the ideal body composition is for working farm dogs.

In humans there is a J-shaped association between body mass index and all-cause mortality in epidemiological studies, although the risk of disease is moderated with exercise (Ajani et al. 2004; Stommel and Schoenborn 2010; Aune et al. 2016). A low body mass index increases the risk of fractures in humans (De Laet et al. 2005; Johansson et al. 2014). Therefore there appears to be an increased risk of morbidity and injury in both lean and obese individuals. In dogs, obesity is also known to increase risk of disease and injury (Edney and Smith 1986; Duval et al. 1999; Adams et al. 2011). However it is currently unknown if low body condition in dogs may also be detrimental.

A survey of visits to veterinary clinics by New Zealand farm dogs found that 38% of visits were a result of traumatic musculoskeletal injury (Cave et al. 2009). In humans low muscle mass increases the risk of traumatic injury during strenuous activity (Jones et al. 1993; Knapik et al. 2012; Hruby et al. 2016). During exercise stress energy is dissipated by muscle, consequently with low muscle mass, unalleviated forces can cause micro-damage to the bone that over time may lead to stress fractures (Burr 2011). Muscle fatigue can also cause gait changes and strain redistribution to areas of

bone less capable of withstanding an increased stress loading, which was seen with changes in strain distribution in the tibia of Foxhounds exercised to the point of fatigue (Yoshikawa et al. 1994). In addition there is a positive association between muscle mass and bone strength (Ahedi et al. 2014; Janz et al. 2015). Therefore a reduction in skeletal muscle mass involved in locomotion may play a role in increasing the risk of injury in dogs. This may be especially relevant to lean working farm dogs.

As the prevalence of obesity increases in the human population, perception of what is considered a healthy weight has changed, and there has been a normalisation of a larger fat mass (Johnson-Taylor et al. 2008; Salcedo et al. 2010). Owners of obese pets frequently underestimate their pets' body condition and perceive them to be in a healthy condition when they are actually overweight (Bland et al. 2009; Rohlf et al. 2010; White et al. 2011). The acceptance by the public of a high body condition score as being normal may result in the perception that healthy lean working dogs are in poor condition. A recent survey of New Zealand farmers concerning their working dogs revealed that low bodyweight was the single most common health concern, affecting nearly 20% of dogs (Sheard 2014). Yet despite this there remains a lack of understanding as to what the ideal body condition may be for working dogs, and what may truly be unhealthy. In addition it may not be the total muscle mass that is of greatest importance, but rather the ratio of muscle to skeleton mass, and so requires investigation. To answer these questions further research is required to determine the relationship between body composition and overall health, performance and welfare in a large population of working farm dogs.

Before this research can be conducted a simple tool is necessary that can be used in the field to estimate body composition. Non-invasive methods of measuring body composition in dogs include dual-energy x-ray absorptiometry (DEXA), isotope dilution, assessment of body condition score (BCS) and morphometry. Despite the variety of options there are significant limitations to each of these approaches. DEXA and isotope dilution require specialised equipment for analysis and are therefore of limited practical use outside a research setting. Assessment of BCS is a quick and practical subjective measure of condition, which has been validated using DEXA and isotope dilution in dogs (Laflamme 1997; Mawby et al. 2004). However the BCS system was designed primarily to quantify obesity, and has been validated for predicting body fat mass, rather than fat-free mass (FFM). In addition the studies validating the scoring system did not include a significant proportion of very lean dogs, and none that were judged to have a BCS of 1 (Laflamme 1997; Mawby et al. 2004). Morphometric equations have been created for dogs that correlate skeletal measurements with bodyweight in order to predict body composition (Mawby et al. 2004; Jeusette et al. 2010; Witzel et al. 2014). However the studies validating the morphometric equations also did not include dogs with low BCS. Therefore there is currently no validated method to estimate the muscle mass of lean dogs that is simple and non-invasive.

The principal aim of the present study was to develop an equation that accurately estimated FFM, and the ratio of FFM to skeletal size or mass, using morphometric measurements in lean working farm dogs, and to examine the association between FFM derived from BCS and FFM measured using isotope dilution. A secondary aim was to determine which other variables might influence

the ratio of FFM to skeletal size or mass. We hypothesised that there would be a correlation between morphometric measurements and measured lean tissue, and the ratio of lean tissue to skeletal measures, that would be sufficient to create accurate, predictive equations.

Materials and methods

Dogs

Working dogs from sheep and beef farms located in the Waikato region were recruited between December 2016 to March 2017, based on BCS categorisation and owner willingness to participate. Only dogs of the two principle working dog breeds in New Zealand, Huntaway and Heading dogs, were used. Each candidate dog had its BCS assessed by one examiner (BH), using a validated nine-point scale (Laflamme 1997). Dogs were recruited to ensure an even distribution of BCS in three condensed categories: <3, 3–4 and >4. The dogs were considered healthy based on being in work during the study period, having no history of illness or injury in the preceding 6 months, and having no significant abnormalities on physical examination that may have interfered with an accurate assessment of body composition using an isotope dilution technique. Dogs that were skeletally immature, that were not currently in work, or that did not fit the inclusion criteria stated above were excluded from the study. An a priori power analysis revealed that 20 dogs would have a power of 0.9 to detect an association (α=0.05) between morphometric measurements and FFM with a correlation coefficient ≥0.6. This study was approved by Massey University Animal Ethics Committee (Palmerston North, NZ).

Bodyweight and morphometry

Thirteen working Huntaway and seven Heading dogs were initially assessed, either at a veterinary clinic (VetEnt, Te Awamutu or VetEnt, Te Kuiti, NZ) or on site at the farm. On the day of the study, each dog was brought in to the veterinary clinic, weighed on the same calibrated electronic scales and its BCS was confirmed. Measurements of head length, head circumference, foreleg, hindleg, body length and thoracic girth were taken of all dogs by the same clinician using a flexible tailor's measuring tape, as described in Table 1. The measurement points were chosen as fixed bony locations least affected by joint flexion and extension that delineated aspects of skeletal size, and were similar to those used in other morphometric studies in dogs (Jeusette et al. 2010; Witzel et al. 2014).

Body composition estimation

The dogs were fasted overnight but allowed ad libitum water up until the point when their first blood sample was taken. Total body water content of each dog was estimated by isotope (deuterium oxide, D_2O) dilution using the method described by Speakman et al. (2001b).

Table 1. Morphometric measurements taken from working farm dogs. Each measurement represents static bony locations least affected by joint flexion and extension, that delineated aspects of skeletal size.

Measurement	Description
Head length measurement	Distance from the level of the medial canthi, equidistant between the eyes, to the external occipital protuberance
Head circumference	Circumference at the widest point, equidistant between the eyes and ears
Foreleg measurement	Distance from the proximal edge of the central foot pad to the olecranon process
Hindleg measurement	Distance from the proximal edge of the central foot pad to the dorsal tip of the calcaneal process with the tarsus in extension
Body length	Distance from the dorsal process of thoracic vertebra 1 (T1) to the dorsal process of sacral vertebrae 1 (S1)
Thoracic girth	Chest circumference at the level of the xiphoid process

A 20-gauge catheter was placed in a peripheral vein of each enrolled dog. A single baseline blood sample was taken directly from the catheter in order to determine the concentration of naturally occurring deuterium in the dog. Then, using a weighed syringe, 0.4 mg/kg bodyweight of D_2O (99.8%; Cambridge Isotope Laboratories, Inc., Andover, MA, USA) and 0.13 mg/ kg bodyweight of 3% saline (Baxter Healthcare Pty Ltd., Toongabbie, NSW, Australia) was administered through the catheter slowly. The catheter was then flushed with 0.9% saline (Baxter Healthcare Pty Ltd), to ensure complete injection, and removed. The empty syringe used to inject the D_2O was reweighed to determine the residual injectate. All syringe weights were measured using the same electronic scales to mg accuracy (Kern PLJ 600-3NM, Kern & Sohn GmbH, Balingen,Germany).

The D_2O was allowed to evenly distribute throughout the body water compartment for 2 hours then a second blood sample was taken from the other cephalic vein or the jugular vein. Water and food were withheld during the 2 hours of equilibration. Blood samples were collected into EDTA

blood tubes (BD Vacutainer, Becton, Dickson and Company, Franklin Lakes, NJ, USA) and plasma was harvested then stored in screw-top vials at −80°C until analysis.

Deuterium analysis

Deuterium was measured in the plasma samples by gas-isotope ratio mass spectrometry (Isotope Ratio Mass Spectrometry Unit, Otago University, Dunedin, NZ). The standard reference waters used were Vienna Standard Mean Ocean Water, International Atomic Energy Agency enriched water, and Aberdeen tap water, which have defined concentrations of hydrogen and oxygen isotopes. Aberdeen tap water and International Atomic Energy Agency enriched water were used as controls. Abundance of deuterium was expressed as parts per 1,000 difference from the reference Vienna Standard Mean Ocean Water (δ^2H) and was converted to parts per million (ppm) using the equation

$$\frac{1{,}000{,}000}{\left(1+\frac{1}{\left(\frac{\delta^2\mathrm{H}}{1{,}000}+1\right)\times 0.0015576}\right)}$$

where 0.00015576 is the accepted $^2H/^1H$ ratio of Vienna Standard Mean Ocean Water.

Total body water

Total body water was determined using the methods from Speakman et al. (2001b). Firstly the total amount of D_2O (g) administered to each dog was calculated as the difference between the D_2O initially in the syringe and the residual D_2O, multiplied by 0.998; where 0.998 is the correction for the purity of the D_2O solution.

Then the volume of distribution (V_d) was calculated as the total D_2O (g) administered multiplied by 1,000, divided by the difference between the deuterium abundance (ppm) in enriched plasma and in baseline plasma.

Total body water (kg) was calculated as V_d divided by 1.04, to account for isotopic exchange of deuterium with non-water hydrogen in proteins, carbohydrates and fats (Speakman et al. 1993).

Fat-free mass and fat mass

Fat-free mass (kg) was calculated as total body water divided by 0.713, allowing for the hydration of non-fat mass as determined by carcass desiccation in dogs (Burkholder and Thatcher 1998). Finally, body fat mass was calculated by subtracting FFM from bodyweight.

Statistical analysis

Statistical analysis was performed using R software (R v 3.1.0; R Development Core Team 2012; R Foundation for Statistical Computing, Vienna, Austria). Normal distribution of data was

confirmed by visual assessment of a scatterplot and the Anderson-Darling test. A two sample *t*-test was used to compare variables between breeds and sexes.

Morphometry

Principal component analysis was utilised to create a new variable that defined skeletal size based on the morphometric measurements (Shields et al. 2006). This statistical technique accounts for collinearity in the morphometric measurements and applies orthogonal linear transformation to the morphometric measurements of the study dogs in order to create a new set of values to describe the greatest source of variation within the data set. This is termed the first principal component. The second greatest variance (second principal component) is described on a second orthogonal coordinate. To perform the principal component analysis, each morphometric measurement was centred and standardised in order to give equal weighting to each variable in the analysis (Abdi and Williams 2010), using the equation

$$\frac{\left(m_{a-f}\right)-\left(\bar{x}_{a-f}\right)}{\sigma_{a-f}}$$

where m_{a-f} represents the value of the measurements a–f (head length, circumference, etc) of the dog, $\bar{x}_{a-f}$ represents the mean of the measurements a–f, and σ_{a-f} represents the SD of the measurements a–f. Eigenvalues for each morphometric measurement were then derived from the centred and standardised measurements and principal component scores were calculated by multiplying each centred, standardised measurement by its respective eigenvalue, and summed.

In order to create accurate ratios of lean tissue to skeletal size, it was necessary to ensure that the principal component scores were >1, therefore a value of six was added to the first principal component score for each dog, and this was defined as the skeletal size for each dog.

Estimated skeletal mass and lean soft tissue mass

To assess the muscle component independently of skeletal mass, we wished to create a new variable for each dog, termed lean soft tissue mass (leanST), which was defined as the measured FFM minus the estimated skeletal mass for each dog. In a previous study skeletal mass was reported to be 8% of total bodyweight following carcass analysis in laboratory Beagles, assumed to be in ideal body condition (Andersen and Good 1970). The dogs included in the present study were, by definition, not all in ideal body condition, and thus the skeletal mass of dogs with low muscle mass were assumed to contribute a larger proportion of their bodyweight.

To estimate the skeletal mass of the dogs in this study, a general linear model was created that described the relationship between 8% of bodyweight and skeletal size of only dogs with a BCS of

4– 6, where skeletal mass was treated as a fixed factor and skeletal size was treated as a random effect. From the regression equation, the skeletal mass of all dogs, including those with a BCS < 4, was estimated using their skeletal size. LeanST (kg) was then estimated as FFM (kg) minus skeletal mass (kg).

Predicted fat-free mass and lean soft tissue mass and associations with measured variables

Multiple regression analyses with a stepwise elimination technique were used to determine which categorical variables (sex, breed, and age) and continuous variables (skeletal mass, skeletal size, bodyweight) provided the best predictive equations for measured FFM and estimated leanST. A predictor variable was kept in the model if $p \leq 0.05$. Predicted FFM and predicted leanST were then calculated for each dog using the final models. The association between predicted FFM and measured FFM was examined using a linear regression model, and between estimated leanST and predicted leanST using a Passing Bablok regression model (Passing and Bablok 1983), as estimated Lean ST was expected to contain a degree of error.

Ratios were then calculated of measured FFM, estimated leanST, predicted FFM and predicted leanST, to skeletal size and estimated skeletal mass. Regression models were used to describe the association between the ratios using predicted values, and the ratios using measured values. Simple linear regression was used for ratios containing FFM, and Passing Bablok regression was used for ratios containing estimated leanST. Bland-Altman plots (Bland and Altman 2007) were also generated to assess agreement between the ratios for measured or estimated values, and their respective predicted values.

Regression analysis was used to determine variables associated with the ratio of measured FFM, or estimated leanST, to skeletal size, as described above.

Body condition score-derived variables

Assessment of BCS is commonly used to predict fat mass in dogs and, if accurate, FFM should be able to be estimated by subtracting fat mass from the dog's bodyweight. In order to compare the validity of using BCS to estimate FFM, BCSderived FFM (kg) was calculated for each dog, and as a percentage of total bodyweight, using the sex-specific regression equations provided by Laflamme (1997). The calculated body fat mass was subtracted from bodyweight to determine FFM. Linear regression was used to assess the correlation between BCS and measured body fat percentage, between BCS-derived FFM and measured FFM, and between BCS-derived FFM expressed as a percentage of bodyweight and measured FFM expressed as a percentage of bodyweight. BCS, BCS-derived FFM, or BCS-derived FFM percentages were used as the response variables in the analysis.

Results

Body condition scores, fat-free mass and fat mass

The working dogs consisted of 10 males (eight Huntaway and two Heading dogs) and 10 females (five Huntaway and five Heading dogs). The dogs had a mean age of 5.3 (SD 2.7) years. Females

had a mean bodyweight of 22.8 (SD 4.41) kg, and males a mean bodyweight of 27.9 (SD 3.19) kg. Five dogs had BCS < 3, seven BCS 3–4, and eight BCS >4. Median BCS was 3.0 (min 1, max 6). Mean FFM, as measured by isotope dilution, was 22.1 (SD 4.4) kg and the percentage FFM of bodyweight was 87.0 (SD 5.0)%. Males had a higher mean FFM than females (24.4 (SD 2.5) vs. 19.8 (SD 4.5) kg, p=0.019), however there was no difference between the FFM as a percentage of bodyweight (males 87.7 (SD 3.82) vs. females 86.2 (SD 5.80)%, p=0.531). Mean total body fat percentage was 13.0 (SD 5.09)%. There was a positive correlation between BCS and measured fat mass percentage (R^2=0.62, p<0.001, Figure 1).

Estimated skeletal size, skeletal mass and lean soft tissue Mean values for the six morphometric measurements and results of the principal component analysis are presented in Table 2. The first principal component accounted for 77% of the variation seen, and the second 9.8%. In the first principal component, all eigenvalues were of the same sign and of similar value, indicating that all morphometric measurements contributed similarly to the overall variation in skeletal size. The two breeds of dogs were clearly distinguishable by plotting the first principal and second principal component scores (Figure 2). Mean skeletal size, derived from the principal component scores, differed between Huntaway and Heading dogs (7.27 (SD 0.82) vs. 3.63 (SD 1.64); p=0.01). In addition, males had a higher mean skeletal size compared to females (7.20 (SD 1.30) vs. 4.80 (SD 2.06); p=0.01).

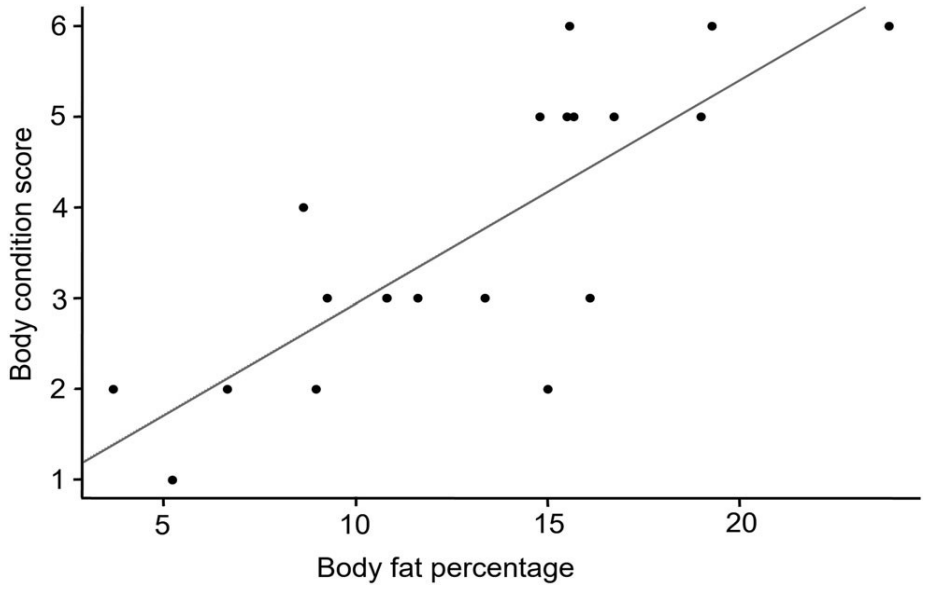

Figure 1. Association between body condition score and body fat, calculated from measured fat-free mass, as a percentage of bodyweight, in 20 working farm dogs (R^2=0.62, p<0.001).

Table 2. Mean (± SD) morphometric measurements for 20 working farm dogs, and eigenvalues for each measurement obtained from principal component analysis for the first and second principal components (PC).

Measurement	Mean (cm)	1st PC	2nd PC
Head length measurement	14.3 ± 1.35	0.44	−0.034
Head circumference	41.9 ± 3.77	0.40	0.395
Foreleg measurement	31.3 ± 2.94	0.42	−0.174
Hindleg measurement	16.2 ± 1.55	0.43	−0.303
Body length	44.5 ± 4.46	0.39	−0.493
Thoracic girth	66.2 ± 5.19	0.36	0.691

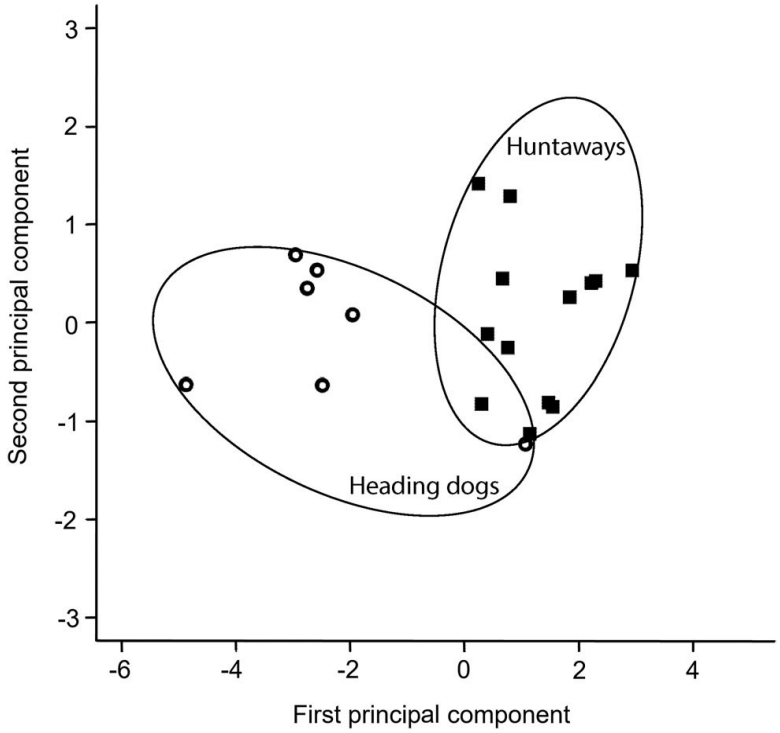

Figure 2. Principal component score plot derived from analysis of six morphometric measurements from seven Heading (open circles) and 13 Huntaway (closed squares) dogs.

In dogs with a BCS of 4–6 the relationship between skeletal mass, based on 8% of bodyweight, and skeletal size was described using the following equation (R^2=0.93).

$$\text{Skeletal mass (kg)} = (0.18793 \times \text{skeletal size}) + 0.9953$$

This equation was used to estimate skeletal mass and thus leanST (FFM minus skeletal mass) for all dogs. Mean Lean ST was 20.0 (SD 3.93) kg and mean leanST as a percentage of total bodyweight was 78.6 (SD 4.51)%.

Predicted FFM and leanST

The final regression models used for the prediction of FFM and lean ST are presented in Table 3. Bodyweight, breed, age and measures of skeletal size or skeletal mass were associated with measured FFM, and bodyweight, breed and age were associated with estimated leanST. Bodyweight contributed 92% of the variation associated with measured FFM and 92% of the variation associated with estimated leanST.

There was a good correlation between predicted FFM and measured FFM (R^2=0.96, $p<0.001$), and predicted leanST and estimated leanST (Pearson's r=0.98) (Figure 3).

Ratios of FFM and leanST to skeletal size and skeletal mass

The mean ratio of measured FFM to skeletal size for the study population was 4.17 (SD 1.69). The Bland-Altman plots showed good agreement and minimal bias between the ratios of measured and predicted FFM to skeletal size or mass and estimated and predicted lean ST to skeletal size or mass. The plots are presented in Supplementary Figures 1–4. The mean ratio of measured FFM to skeletal size tended to differ between breeds (p=0.057, Figure 4) and between male and female dogs (4.9 (SD 2.0) vs. 3.5 (SD 0.5); p=0.075).

Table 3. Final linear regression models for factors associated with measured fat-free mass (FFM) and estimated lean soft tissue (leanST) in 20 working farm dogs. Regression models were generated using either skeletal size or estimated skeletal mass.

Outcome	Variable	Coefficient	95% CI	R^2	P-value[a]
FFM	Intercept	6.40		0.96	<0.001
	Skeletal size	0.60	0.03 – 1.16		
	Bodyweight	0.47	0.22 – 0.71		
	Breed (Huntaway)	1.91	0.19 – 3.62		
	Age	−0.18	−0.35 – −0.00		
FFM	Intercept	3.24		0.96	<0.001
	Skeletal mass	3.18	0.18 – 6.17		
	Bodyweight	0.47	0.22 – 0.71		
	Breed (Huntaway)	1.91	0.19 – 3.62		
	Age	−0.18	−0.35 – −0.00		
LeanST	Intercept	4.30		0.94	<0.001
	Bodyweight	0.60	0.42 – 0.77		
	Breed (Huntaway)	2.39	0.75 – 4.03		
	Age	−0.18	−0.36 – −0.01		

[a] Significance of R^2

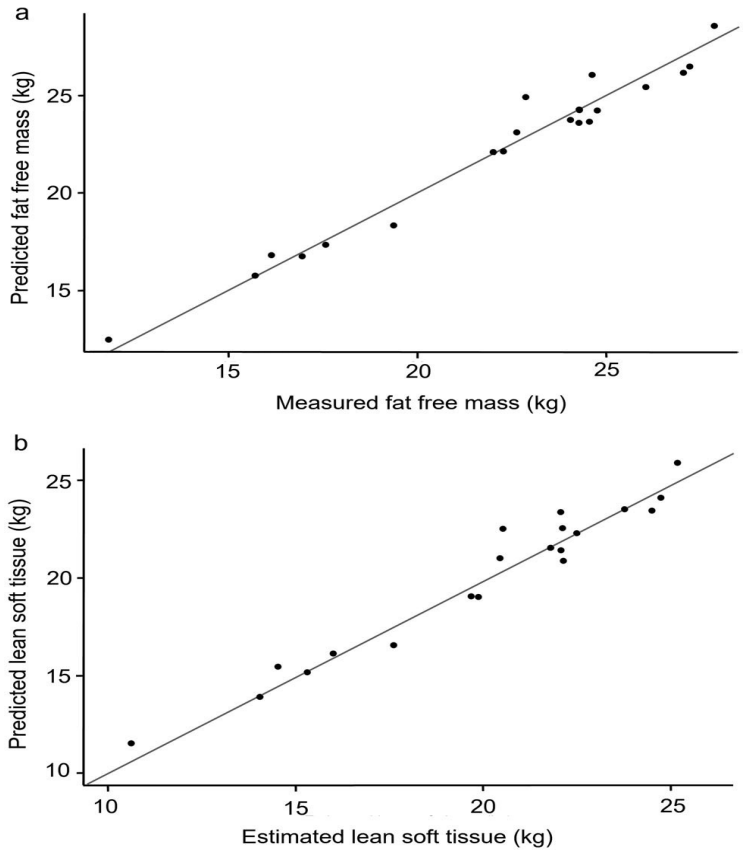

Figure 3. Associations between (a) measured and predicted fat-free mass and (b) estimated and predicted lean soft tissue in 20 working farm dogs.

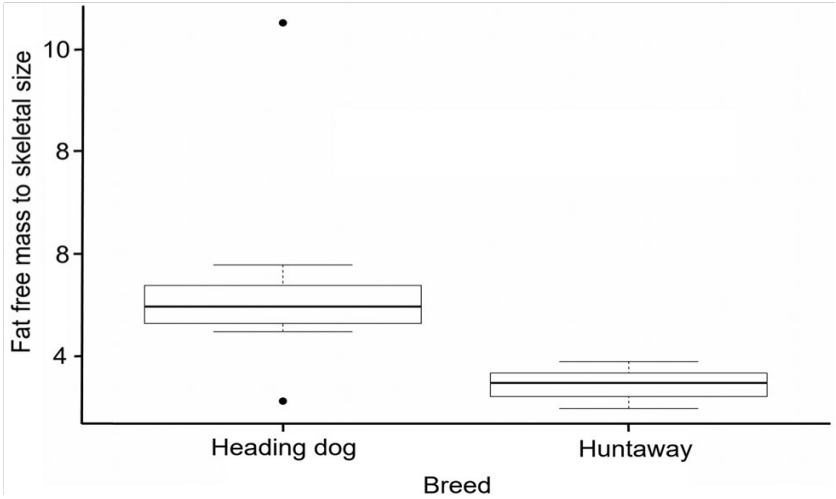

Figure 4. Boxplots of the ratio of fat-free mass to skeletal size measured in seven Heading and 13 Huntaway dogs. The box represents the first and third quartiles, the solid line represents the median, and the whiskers represent the minimum and maximum values excluding outliers, which are dogs with values more than 1.5 times the interquartile range, represented by dots.

The regression models for associations between ratios of predicted FFM to skeletal size and FFM to skeletal mass and measured values, and predicted leanST to skeletal size and leanST to skeletal mass and estimated values are presented in Table 4. Correlation coefficients were higher for both FFM to skeletal size and leanST to skeletal size than for ratios using skeletal mass.

Only bodyweight was associated with the ratio of FFM to skeletal size or leanST to skeletal size (Table 5). Bodyweight accounted for 55% of the variation in the ratio of FFM to skeletal size and 54% of the variation in leanST to skeletal size.

BCS-derived variables

There was a positive correlation between BCS-derived fat mass as a percentage of bodyweight and fat mass percentage determined using isotope dilution (R^2=0.65, $p<0.001$), and BCS-derived FFM percentage and measured FFM percentage (R^2=0.65, $p<0.001$), however the BCS-derived FFM percentage in dogs with BCS 1 was >100%. There was a stronger correlation between BCS-derived FFM and measured FFM (R^2=0.95).

Discussion

The principal result of this study was the development of equations to accurately predict FFM, and the ratio of FFM to skeletal size in two breeds of New Zealand farm dogs using morphometric measurements. Previously published measures of body composition for dogs (BCS and morphometry) have only been validated using normally conditioned and obese dogs, but none have been developed for use in lean dogs. The equations produced in this study can be used to estimate the FFM and the ratio of FFM to skeletal size of individual lean Huntaway and Heading dogs.

We elected to use principal component analysis to create a new variable that defined skeletal size. The first principal component from our data explained 77% of the variation in skeletal measurements. Also, upon plotting the first principal component against the second principal component, a clear differentiation between the two breeds was seen within the first principal component. All measurements had similar eigenvalues of the same sign, and provided similar weighting to the overall variation in skeletal size. This indicated that no individual skeletal measurement was distinctly different to the others with regards to the overall definition of skeletal size.

A strong correlation was found between measured FFM, estimated leanST and their respective predicted values. Skeletal size or skeletal mass, breed, age and bodyweight were associated with measured FFM, but only breed, age and bodyweight were associated with estimated leanST. Bodyweight alone explained a majority of the variation for both of the predicted measures. This finding is consistent with the narrow range of fat mass in our study population, and therefore variation in bodyweight contributed the most to the variation in lean tissue.

In addition, bodyweight was the only variable associated with the ratio of FFM to skeletal size, accounting for ~54% of the variation. This finding is interesting because it is hypothesised that this ratio may better predict the risk of disease and injury in working dogs compared with total muscle mass. Therefore other factors besides bodyweight, breed, age, and sex that may be associated with this ratio may warrant further investigation. Although age was associated with measured FFM, it was not associated with the ratio of FFM to skeletal size. Sarcopenia is common in elderly people, and is likely to be as common in older dogs (Lawler et al. 2008). The dogs in this study were aged between 1–10 years. In non-obese Labrador Retrievers, lean mass may not decline until 12 years of age (Lawler et al. 2008), therefore this study population may not have included dogs that were old enough to demonstrate an association with the ratio of FFM to skeletal size. Heading dogs tended to have a higher ratio than Huntaway dogs, indicating a breed difference.

Table 4. Regression models for associations between ratios of predicted fat-free mass (FFM)[a] or lean soft tissue mass (LeanST)[b] to skeletal size or mass and ratios of measured FFM or estimated leanST to skeletal size or mass in 20 working farm dogs.

Predicted variable	Variable	Coefficient	95% CI	R^2
FFM:skeletal size	Measured FFM:skeletal size	0.94	0.90, 0.98	0.99
	Intercept	0.25		
FFM:skeletal mass	Measured FFM:skeletal mass	1.02	0.68 – 1.36	0.67
	Intercept	−0.23		
				Pearson's r
LeanST:skeletal size	Estimated LeanST:skeletal size	1.06	0.92 – 1.14	0.99
	Intercept	−0.25	−0.51 – 0.29	
LeanST:skeletal mass	Estimated LeanST:skeletal mass	0.99	0.49 – 1.24	0.81
	Intercept	0.03	−2.47 – 5.03	

a Determined using linear regression analysis

b Determined using Passing Bablok regression analysis

Table 5. Final regression models for variables associated with the ratio of measured fat-free mass (FFM), or estimated lean soft tissue (leanST) to skeletal size in 20 working farm dogs.

Predicted variable	Variable	Coefficient	95% CI	R^2	P-value[a]
FFM:skeletal size	Bodyweight	−0.27	−0.39 – −0.15	0.55	<0.001
	Intercept	11.02			
LeanST:skeletal size	Bodyweight	−0.242	−0.35 – −0.14	0.54	<0.001
	Intercept	9.89			

[a] Significance of R^2

The absence of a sex difference in the FFM as a percentage of bodyweight was surprising as males have been shown to have a greater percentage muscle mass than females in other studies (Lauten et al. 2001; Hall et al. 2015). Females in our study had a smaller skeletal size compared to males which aligns to the common notion that sexual size dimorphism is present in many species, including dogs, where females are smaller in stature than their male counterparts (Lark et al. 2006; Thuller et al. 2015). There was also a tendency for females to have with a greater FFM to skeletal size ratio than males.

The proportion of FFM mass contributed by the skeleton increases as dogs become leaner, therefore we believed it might be important to assess leanST separately in order to evaluate the ratio of FFM minus the skeleton to the skeletal mass. So a new variable, leanST, was created by subtracting estimated skeletal mass from FFM for each dog. However, the correlation coefficients for the regression models describing the association between predicted and measured values for the ratios FFM to skeletal size and leanST to skeletal size were higher than for FFM to skeletal mass and leanST to skeletal mass. Thus it appears that there was no benefit from calculating the variable skeletal mass.

Currently, assessment of BCS is the most commonly used method in practice to describe body composition in dogs. However BCS was developed to predict fat mass and not to describe the muscle mass of very lean dogs. We looked for an association between BCS-derived FFM percentage and measured FFM percentage to determine its accuracy in our lean study population. Under the two-compartment system, the percentage of a dog's fat mass and FFM should equal 100%. Therefore if BCS is accurate there should be an association between BCS-derived FFM and measured FFM percentage. We found a poor correlation between these two variables when FFM was expressed as a percentage of bodyweight, but a strong correlation when comparing the absolute values. This was probably due to the majority of variation of bodyweight in our study population coming from lean mass differences, rather than fat mass. Thus, whilst estimating the

small percentage of fat mass in a lean dog using BCS might be inaccurate, the subtraction of that small percentage from bodyweight introduces little error. In addition the accuracy of BCS is dependent on the scorer and in our study resulted in FFM percentage >100% in the leanest dogs. Nonetheless, it will be interesting to see if the small increase in accuracy of estimating FFM in lean dogs attained using our approach is substantively superior in predicting clinical outcome measures.

There are several important limitations to this study. Firstly, it included a small population of working dogs made up of only two breeds. The skeletal size of the two breeds was clearly different; therefore it is assumed that these equations will not be accurate if used on other breeds. Further, while isotopic dilution is one of the gold standards when measuring body composition in dogs, the technique may overestimate total body water by 15–16% in dogs (Burkholder and Thatcher 1998). This could have had a significant effect when measuring lean mass. However other commonly used methods including DEXA have been shown to also overestimate and underestimate total body water in individual dogs (Speakman et al. 2001a).

Currently there is no other validated, non-invasive method of measuring body composition better than isotopic dilution in dogs. When compared to another study assessing body composition in lean dogs using isotopic dilution (Hill et al. 2001), the results of this study are similar for percentage FFM (87% compared with 93–96%) and slightly higher for percentage body fat (13.03% compared with 3–7%) indicating good agreement.

There are also several important assumptions made in our analysis. Firstly, skeletal mass was estimated to be 8% of the total bodyweight in ideally conditioned dogs, which was extrapolated from carcass dissection in Beagles. It is likely that this percentage would be different for various breeds, for instance heavily muscled greyhounds compared with dachshunds. In this case the two breeds included in the analysis are of similar size, so the effect of breed differences would be less. Nonetheless, additional methods such as DEXA, or carcass dissection would be needed to determine the accuracy of the regression equation created for skeletal mass. In addition, a pan-species hydration constant of 73.2% is traditionally used when calculating FFM from total body water (Pace and Rathbun 1945). However there are known variations to this constant with animals of different ages and species (Sheng and Huggins 1979). A study analysing the cadavers of 75 mature dogs of various skeletal sizes and body conditions found a study population hydration constant of 71.3% (Burkholder and Thatcher 1998). This is less than the conventional 73.2% hydration constant. It is thought that the leaner a dog is, the greater the contribution of skeletal mass as a percentage of total FFM, and thus producing a smaller hydration constant. Therefore it was opted in this study to utilise a species-specific hydration constant, which is theoretically more appropriate for lean dogs. Despite this attempt to more precisely define the lean tissue in our study population, it is possible that the 71.3% hydration constant for FFM, and the 4% correction for deuterium exchange with non-water hydrogen may be different for very lean dogs with minimal musculature. Unfortunately there are no published data to support the use of different factors in this study.

Despite these limitations, this study established a strongly predictive equation for calculating lean mass and the ratio of lean muscle mass to skeletal size in a small population of lean Huntaway and Heading dogs using morphometric measurements. Future studies could utilise the methods and equations outlined in this study on a large population of working Huntaway and Heading dogs to determine the utility of their predicted measures (FFM and the ratio of FFM to skeletal size) on a large scale. Such studies could determine if there is a ratio of lean mass to skeletal size below which the risk of injury or disease increases. Extrapolation of this approach on a population of dogs would require calculation of the skeletal size of each dog using the equation

$$\left(\sum_{a-f} \frac{\left(m_{a-f}\right) - \left(\bar{x}_{a-f}\right)}{\left(\sigma_{a-f}\right)} \times \lambda_{a-f} \right) + 6$$

where m_{a-f} represents the values of the morphometric measurements a – f (head length, circumference etc) of the dog, $\bar{x}_{a-f}$ represents the mean of the measurements (from the population dataset, or the values in Table 2), σ_{a-f} represents the SD of the measurements (from the population dataset, or the values in Table 2), and λ_{a-f} represents the eigenvalues of the measurements (from the population dataset, or the values in Table 2). Each dog's FFM could then be estimated using the regression coefficients in Table 3. It is hypothesised that there will be a threshold for the ratio of FFM to skeletal size below which health or performance will be compromised. If these measures prove useful, then these equations would provide an objective, non-invasive and simple measure to determine when welfare in individual dogs is compromised by underfeeding. In addition further studies may determine the factors that affect the ratio of lean mass to skeletal size, which may include nutrition, neutering, training, and genetics.

Acknowledgements

We would like to sincerely thank Janis Bridges for all her technical help, and Emma Cuttance and William Cuttance for their generous support and assistance.

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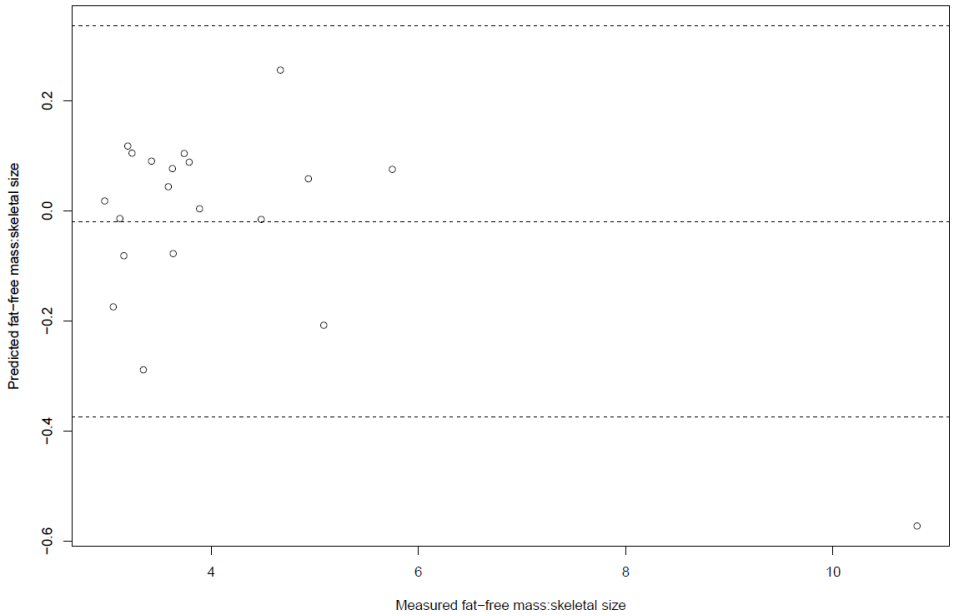
Submitted 10 August 2017

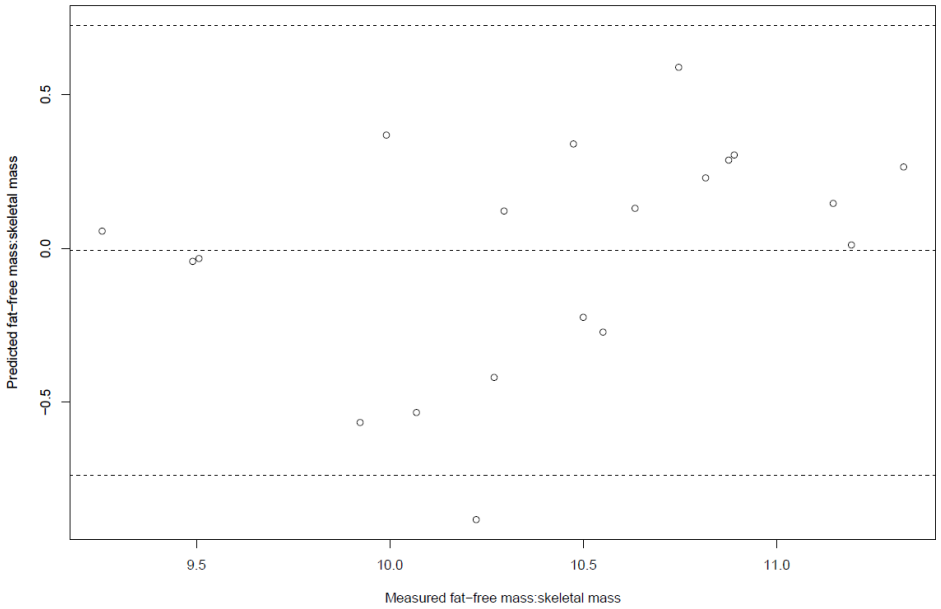
Accepted for publication 13 June 2018

First published online 27 June 2018

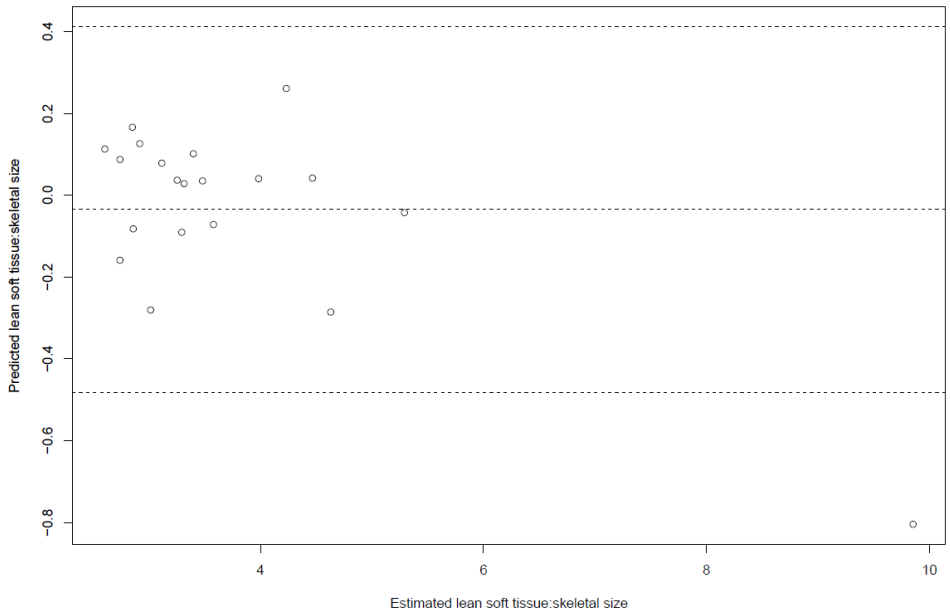
Appendix II: Supplementary figures Bland Altman plots (Chapter 3)



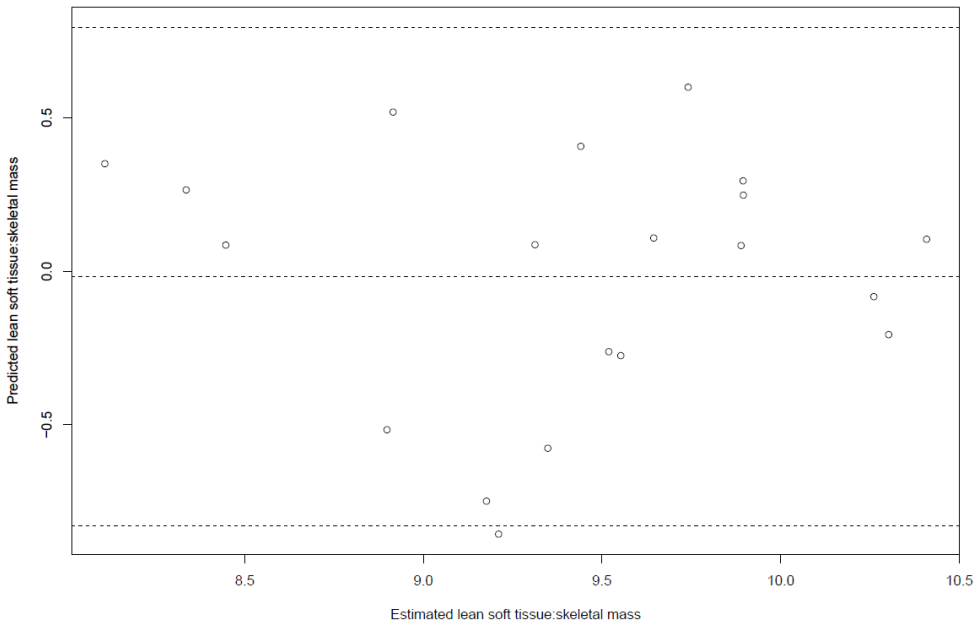
Supplementary Figure 2. A Bland-Altman plot comparing the measured and predicted fat-free mass to skeletal size.



Supplementary Figure 2. A Bland-Altman plot comparing the measured and predicted fat-free mass to skeletal mass.



Supplementary Figure 3. A Bland-Altman plot comparing the measured and predicted lean soft issue to skeletal size.



Supplementary Figure 4. A Bland-Altman plot comparing the measured and predicted lean soft issue to skeletal mass.

Appendix III: Statement of contribution and reproduction of published article (Chapter 4 & 5)

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published: 08 January 2020
doi: 10.3389/fvets.2019.00448

Metabolic and Immunological Effects of Intermittent Fasting on a Ketogenic Diet Containing Medium-Chain Triglycerides in Healthy Dogs

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In several species, intermittent fasting (IF) has been shown to have beneficial effects, including delayed aging, increased lifespan, increased insulin sensitivity, reduced ischemic tissue damage, delayed onset of neurodegenerative disease and improved neuronal repair following injury. However, the metabolic and immunological effects of IF have not been well-established in dogs. The aim of this study was to examine the effects of a 48 h IF regimen using a low fat and a high fat diet in healthy dogs by quantifying the metabolic, hormonal, and immunological changes. We hypothesized that IF dogs would have higher blood ketone and ghrelin concentrations, lower blood leptin, insulin and glucose concentrations, and signs of immunosuppression compared to dogs eating daily. Ten healthy adult dogs were randomized into three group and underwent three feeding regimes in a 3 × 3 Latin square design: twice a day feeding on a low fat (23% energy from fat; LF) diet, 48 h fasting on a low fat diet, and 48 h fasting on a high fat enriched with medium-chain triglycerides (68% energy from fat; HF) diet. Body weight, food intake, activity, blood glucose, β-hydroxybutyrate, leptin, ghrelin, and insulin were measured. Lymphocyte proliferation and neutrophil/macrophage phagocytosis and respiratory burst were measured as markers of immune function. Nuclear magnetic resonance spectroscopy was used to relatively quantify plasma metabolites. When the dogs were IF on a HF diet, they had the highest concentration of blood ketones (mean 0.061 mmol/L, SD 0.024), whereas they had the lowest concentration (mean 0.018 mmol/L, SD 0.004) when fed daily. Blood glucose and insulin concentrations were lower in IF dogs on a HF diet compared to daily feeding or IF on a LF diet. There was an increase in plasma β-

hydroxybutyrate concentrations, and a reduction in glucose and insulin concentrations when dogs were IF on a HF diet. There was only a decline in the immune parameters studied when the dogs were IF on a LF diet, which was not seen when on the HF diet. The results of this study indicate the potential of IF to be further investigated as a potential beneficial feeding regime for dogs.

Keywords: fasting, ketone, beta-hydroxybutyrate, ketogenic, diet, dog, medium-chain, immunity

INTRODUCTION

Optimal feeding regimens for both veterinary and human hospitalized patients have not yet been established. Underfeeding is common during hospitalization and is associated with depressed immunity, increased readmission rates, and increased mortality (1–4). However, overfeeding critically ill patients has also been shown to have deleterious effects (5–7). In particular, iatrogenic hyperglycemia can lead to impaired wound healing, neuronal dysfunction, increased production of the inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα), inhibition of leukocyte function, vasculitis, and ultimately a poorer clinical outcome (8–13). Some of these effects can be abrogated when blood glucose is normalized (9, 14). So whereas clinicians want to provide appropriate nutrition for hospital patients, they need to establish the best means of doing so, while avoiding hyperglycemia.

A potentially effective feeding regime to achieve these agnostic goals is intermittent fasting. Intermittent fasting is the process of reducing meal frequency in order to prolong the period of fasting between meals, but without necessarily restricting total caloric intake when expressed over a longer period of time. Extending the period of fasting between meals has been found to increase insulin sensitivity, reduce serum fructosamine, reduce cancer cell proliferation, reduce concentrations of proinflammatory cytokines IL-6, IL-1β, and TNF-α in circulation, delay aging, and improve neuronal repair following injury when compared to continuous feeding (15–25). In healthy mice, blood glucose and insulin concentrations were reduced following a period of intermittent fasting (21). Several mechanisms have been proposed including the reduction of mitochondria-derived reactive oxygen species, activation of sirtuins and associated promotion of autophagy and cell cleansing, and decreased expression of p38 mitogen-activated protein kinase, an upstream mediator of apoptosis (18, 26–28). These potential mechanisms would allow a reduction in oxidative stress and a more tailored repair response following injury.

An intermittent fasting regime is of particular interest in patients with spinal disease, as it has been shown to reduce lesion size and improve recovery in rodent models of spinal injury compared with daily feeding (21–23). Ketones, which increase during the fasting period, upregulate nicotinamide adenine dinucleotide (NAD)+-dependent sirtuin 3 and superoxide dismutase, increases the expression of autophagy-promoting protein forkhead box O3a, and reduces neuronal injury in the cerebral cortex of rats following experimentally induced hypoglycemia (29, 30). In addition, ghrelin, an orexigenic peptide secreted by the stomach in a fasted state, protects neurons from ischemia and

reperfusion injury in vivo, and decreases the expression of the TNF-α and IL-1β from microglial cells in Parkinson-modeled mesencephalic neuronal cell cultures (31, 32). Thus, intermittent fasting may be a feeding strategy that promotes neuronal recovery while also avoiding hyperglycemia.

Although there are many potential benefits to intermittent fasting, there are possible detrimental consequences as well. Humoral and cellular immune functions are known to decrease in a fasted state, which is in part the result of a drop in the plasma adipokine, leptin (33, 34). Leptin increases neutrophil chemotaxis and macrophage phagocytosis, and affects the maturation of T-cells (35). Short-term fasting in several species reduces T-cell mediated responses, and splenic and peripheral immune cell numbers (17, 36, 37). Injection of leptin into fasted or leptin deficient ob/ob mice reverses the suppression of lymphocyte differentiation, macrophage phagocytosis, and delayed-type hypersensitivity responses caused by leptin deficiency (34, 38, 39). However, rats consuming a high fat diet attenuates the drop in leptin during fasting, and increases ketone production between meals (40, 41). In addition, feeding medium-chain triglycerides (MCTs) promotes the formation of ketones in the fed state (42, 43). After eating, the majority of medium-chain fatty acids are absorbed through the portal circulation and are metabolized by hepatocytes into ketones (44, 45). So feeding a high fat diet enriched with MCTs may have the dual benefit of maintaining leptin serum concentration while also promoting ketogenesis during the short periods of fasting. Therefore, intermittent fasting on a high fat diet enriched with MCTs may be preferential.

The principal aim of this study was to determine the metabolic and immunological effects of a 48 h intermittent fasting regime in healthy dogs. Our primary hypothesis was that dogs undergoing intermittent fasting would have higher plasma concentrations of β-hydroxybutyrate and ghrelin, and lower concentrations of glucose, insulin, and leptin compared with dogs fed daily. Our secondary hypothesis was that dogs undergoing an intermittent fasting regime on a high fat diet enriched with medium-chain triglycerides would have a greater blood β-hydroxybutyrate and leptin concentrations compared with dogs intermittently fasted on a low fat diet.

MATERIALS AND METHODS

Animals

Following a complete physical examination, 10 healthy, adult dogs from Massey University's Canine Nutrition Unit were used in this study. The dogs were of two breeds: Harrier Hounds (n = 7) and New Zealand Huntaways (n = 3), and were composed of four neutered males and six speyed females. The dogs had a mean age of 7.1 (SD 2.1) years, mean body weight of 27.8 (SD 3.1) kilograms, and a mean body condition score (BCS) of 4.2 (SD 0.4). The study protocol was approved by the Massey University Animal Ethics Committee (MUAEC #16/130).

Study Design

A week before the commencement of the study, all dogs were transitioned onto a high carbohydrate, low fat commercial dry diet to allow for acclimation. The dogs were fed to meet their maintenance energy requirement based on historical colony data. After this acclimation period, the dogs were randomized into one of three groups which underwent each feeding trial regime in a 3 × 3 Latin-square design with a weeklong "wash out" duration in-between. The three feeding regimes were as follows: (1) daily fed feeding on a low fat (LF), high carbohydrate diet (BID), (2) intermittent fasting (feeding once every 48 h) on the same LF diet (IF LF), and (3) intermittent fasting (feeding once every 48 h) on a high fat (HF) diet (IF HF). Both diets used in this study were formulated to meet the nutrient requirements for adult dogs defined by the Association of American Feed Control Officials (AAFCO). A commercial dry food[ff] was chosen as the low-fat, high carbohydrate diet. The high fat diet was created using the same dry commercial diet with the addition of powdered whey protein, beef tallow, sunflower oil, coconut oil and a multivitamin/mineral mix[gg] to ensure adequacy of the total diet. The total amount of medium-chain triglycerides (C8, C10, C12) from the coconut oil and beef tallow amounted to 14.7% of the total calories in the diet when using an energy of 6.8 kcals/gram for the MCTs (46). The nutrient profiles of both diets are presented in Table 1.

When dogs were in the daily feeding regime, they were offered their maintenance energy requirement divided equally into two meals that were provided in the morning and the afternoon. When the dogs were in the intermittent fasting regime, they were offered twice their maintenance energy requirement in the morning every other day. The dogs were allowed up to 3 h to consume their meal, after which the food was removed and weighed. During the wash out period between feeding regimes, all dogs were placed on the commercial high carbohydrate, low fat dry diet and fed twice a day for 1 week.

On days 1, 3, 5, and 7 of a trial period, a fasted blood sample (12 mL in total) was collected into lithium heparin and plain red-top vacutainers[hh] from all dogs by jugular venipuncture before food was offered. Day 1 represented an overnight-fasted, baseline sample, while the samples collected on days 3, 5, and 7 represented either a 12 h fast when the dogs were eating daily, or a 48 h fast when

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[ff] Eukanuba Large Breed Weight Control dry, Mars Incorporate, McLean, Virginia, USA.
[gg] Balance ITR Canine, DVM Consulting Incorporated, Davis, California, USA.
[hh] BD Vacutainer R , Plymouth, United Kingdom.

the dogs were fasted intermittently. Immediately following blood collection, a protease inhibitor[ii] was added to the sample in the plain red-top vacutainer to prevent ghrelin degradation. All samples were placed on ice until they were centrifuged, and serum and plasma removed. Daily food intake, weekly body weight and body condition score were recorded for all dogs. To compare the caloric intake of the dogs on the different feeding regimes, the total calories eaten every 2 days (i.e., days 1 and 2, days 3 and 4, days 5 and 6) was divided by the weekly starting weight of each dog to the power of 0.75 in order to express intake as kcal per 48h/kgBWT$^{0.75}$. In addition, day (5 am to 8 pm) and night (8 pm to 5 am) activity of the dogs was measured using a tri-axial accelerometer[jj] fitted to their collar.

After blood sampling on day 7, all dogs were placed onto the "wash out" feeding regime. Following a wash out, each group was fed according to their next assigned feeding regime, and blood samples taken as described above. This was repeated again once more so that all groups underwent each of the three different feeding regimes with a washout period in between.

[ii] Millipore R DPP-IV Inhibitor, Darmstadt, Germany.
[jj] Heyrex R , Say Systems,Wellington, New Zealand.

TABLE 1 | The nutrient profile of the low fat commercial diet and the modified high fat diet.

	Commercial low fat diet	Modified high fat diet	Percentage difference (%)
Protein energy (%ME)	22	22	100.00
Fat energy (%ME)	23	68	295.57
Carbohydrate energy (% ME)	55	10	18.19
Protein (g/Mcal)	62.79	62.95	100.26
Total lipid (g/Mcal)	26.91	79.41	295.08
Linoleic acid (18:2 n-6) (g/Mcal)	8.16	9.37	114.75
Carbohydrate (g/Mcal)	157.57	29.92	18.99
Choline (mg/Mcal)	717.60	670.54	93.44
Folate (mcg DFE/Mcal)	209.30	388.12	185.44
Niacin (mg/Mcal)	14.35	21.99	153.23
Pantothenic acid (mg/Mcal)	7.41	7.30	98.42
Riboflavin (mg/Mcal)	2.03	2.34	115.30
Thiamin (mg/Mcal)	1.11	0.70	63.56
Vitamin A (mcg RAE/Mcal)	4041.86	1301.85	32.21
Vitamin B-12 (mg/Mcal)	0.018	0.017	94.44
Vitamin B-6 (mg/Mcal)	2.09	1.17	56.09
Vitamin E (α-tocopherol) (IU/Mcal)	74.75	88.18	117.97
Calcium (g/Mcal)	2.84	3.13	110.39
Copper (mg/Mcal)	4.34	3.15	72.64
Iodine (mg/Mcal)	0.94	0.71	75.77
Iron (mg/Mcal)	45.71	34.39	75.24
Magnesium (g/Mcal)	0.32	0.27	85.31
Manganese (mg/Mcal)	15.03	4.55	30.29
Phosphorus (g/Mcal)	2.39	1.79	74.92
Potassium (g/Mcal)	2.21	2.59	116.90
Selenium (mg/Mcal)	0.14	0.10	69.23
Sodium (g/Mcal)	1.20	0.50	41.39
Zinc (mg/Mcal)	63.39	52.99	83.59
Vitamin D (IU/Mcal)	447.00	275.92	61.73

β-Hydroxybutyrate, Glucose, and Metabolomics

Within 1 h of collection, plasma was harvested from the heparin vacutainers following centrifugation for 10 min at 3,000 rcf at 4◦C. Plasma glucose was analyzed using a handheld glucometer[kk] which has been previously validated for use in dogs (47). The remaining plasma was stored at −80◦C until further analysis.

β-hydroxybutyrate was assayed in plasma samples from days 3, 5, and 7 of each regime using a colorimetric assay[ll] according to the manufacturer's instructions. The thawed plasma samples were initially de-proteinated using 10 kD spin columns[mm] and centrifuged at 10,000 rcf for 10 min. The samples were then prepared and absorbance at 450 nm was measured using a microplate reader[nn].

Nuclear magnetic resonance (NMR) was used for plasma metabolomics. Samples were prepared based on the protocol described in a previous study (48). Briefly, 300 μL of plasma was deproteinated using 600 μL of methanol and incubated at −20◦C for 30 min. The samples were then centrifuged at 13,400 rcf for 30 min and the supernatant was removed and placed in a rotary evaporator for 3.5 h at 20◦C. Any remaining supernatant in the samples was then dried completely by freeze drying. The dried samples were then stored in screw top vials[oo] at −80◦C until analysis. On the day of analysis, a phosphate buffer solution was prepared by dissolving 928.6 mg of anhydrous NaH_2P0_4 and 320.9 mg of anhydrous Na_2HPO_4 in 100 g of D20, and used with further pH modification. The dried samples were reconstituted using 600 μL of phosphate buffered D2O, along with two standards [0.5 mM of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS)[pp] and 5 mM imidazole[qq]] to allow for adjustments in chemical shifts and pH. The samples were then transferred to 5 mm NMR tubes[rr] and analyzed using a cryoprobe equipped Bruker Avance 700 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 700.13 MHz. The samples were measured at 25◦C using a standard 1D NOESY pulse sequence with presaturation of the residual water signal. Spectra were recorded using 58k points and an acquisition time of 3.5 s followed by a relaxation delay of 1.5 s. TopSpin v3.0 (Bruker Biospin) was used to process the 1H NMR spectra. Phasing and baseline correction of all NMR spectra were checked manually.

[kk] Accu-check R Performa, Roche Diagnostics Limited, Mannheim, Germany.
[ll] beta HB Assay Kit, catalogue #ab83390, Abcam, Cambridge, United Kingdom.
[mm] 10kD spin column, catalogue #ab93349, Abcam, Cambridge, United Kingdom.
[nn] VersaMax™ Microplate Reader, Molecular Devices LLC, San Jose,California, USA.
[oo] Cryo.S™, Greiner Bio-One, Frickenhausen, Germany.
[pp] DSS, Sigma-Aldrich, St. Louis, MI, USA.
[qq] Imidazole, Sigma-Aldrich, St. Louis, MI, USA.
[rr] 5mm NMR Sample Tubes,Wilmad-LabGlass, Vineland, New Jersey, USA.

Endocrinology

The plain red-top vacutainers were centrifuged for 10 min at 3,000 rcf at 4◦C, and serum removed within 1 h of collection. The serum was stored at −80◦ C until analysis. Leptin, ghrelin, and insulin were assayed using a commercial multiplex immunoassay[ss]. Samples were prepared following the manufacturer's instruction. Briefly, 50 μL of serum was added to a 96-well plate to which a buffer and antibody-conjugated beads were added. The plates were gently agitated overnight at 4◦C. Following this, the plates were washed, detection antibodies added and finally analyzed using a multiplex reader[tt].

Immunological Assays

Whole blood from the lithium vacutainers was utilized within an hour of collection. Leucocyte phagocytosis and respiratory burst were assayed by flow cytometry using a cell analyser[uu] and fluorescent markers for phagocytosis[vv] and oxidative burst[ww]. A modified protocol was used which was based on previously published works (49, 50). Four flow cytometry tubes were prepared for each sample. In each tube, 100 uL of whole blood was incubated at 37◦C for 30 min with either 10 μL of 143 μM DHR solution, 50 μL of pHrodo™, both compounds, or nothing added. After incubation, 2.7 mL of deionized water was added to lyse erythrocytes. Within 2 min of adding the deionized water, 300 μL of 10X PBS was added and samples centrifuged at 600 rcf for 7 min. The supernatant was decanted, and the process repeated with 3 mL of PBS. Then, 3% formaldehyde in PBS was added and samples incubated at room temperature for 5 min for fixation. Finally, 2 mL of PBS was added, and samples were centrifuged at 350 rcf for 7 min. The supernatant was removed, and the pellet suspended in 200 μL PBS with 2% fetal calf serum (FCS) in 5 mL polystyrene round bottomed tubes[xx]. Samples were acquired with a flow cytometer[yy] until at least 10,000 events were collected. Lymphocyte, monocyte, and neutrophil populations were identified based on size and granularity in a forward and side scatter plot (Figure 1). Quadrants for single and double positive cells were demarcated using the set of control samples (Figure 2). The results of the gated populations were expressed as the percentage positive for cells undergoing phagocytosis and/or respiratory burst, and their mean fluorescence intensity.

[ss] MilliplexR MAP Canine Gut Hormone Magnetic Bead Panel, Millipore, Burlington,Massachusetts, USA.

[tt] Luminex 200™, Merck, Darmstadt, Germany.

[uu] BD FASCSCalibur™, BD Sciences, San Jose, California, USA.

[vv] pHodoTM Red S. aureus Bioparticles™, catalog number A10010, Invitrogen R,Carlsbad,California, USA.

[ww] Dihydrorhodamine 123, catalog number D23806, InvitrogenR , Carlsbad, California, USA.

[xx] BD Falcon™ tubes, BD Biosciences, San Jose, California, USA.

[yy] FACSCalibur™, BD Biosciences, San Jose, California.

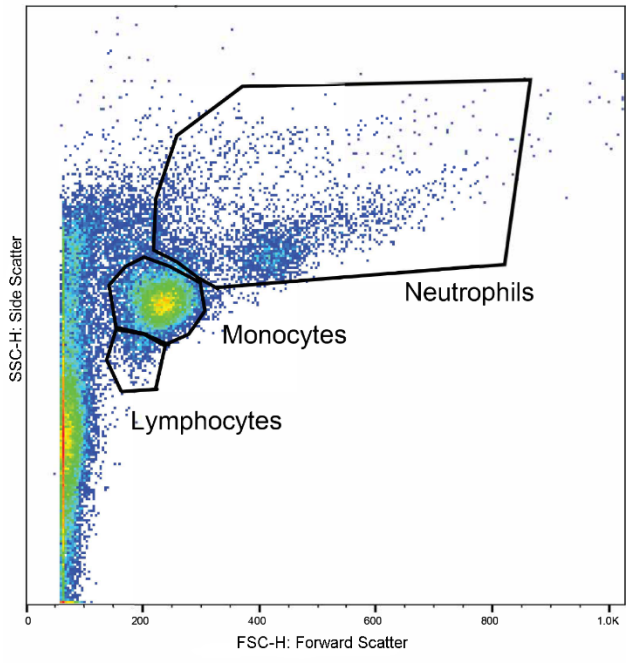

FIGURE 1 | A representative plot displaying the flow cytometry scatter properties of leukocytes in one sample. The forward scatter (x-axis) corresponds to size of the cells and the side scatter (y-axis) corresponds to the cells' granularity. Regions were gated around clusters of cells corresponding to the expected locations for lymphocytes, monocytes, and neutrophils.

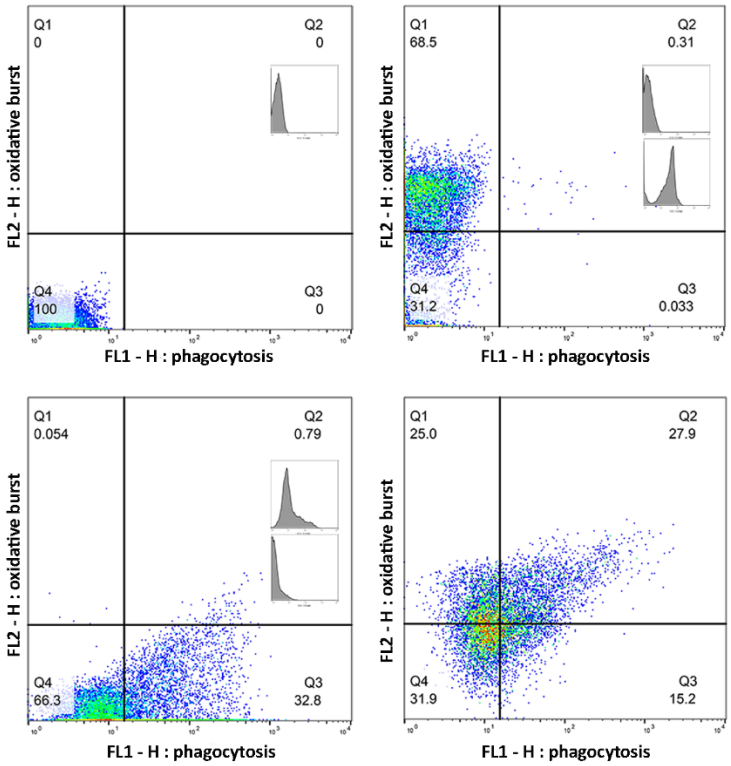

FIGURE 2 | Quadrants for single and double positive cells were established using a set of control samples. Plots (A–D) show a representative sample of gated monocytes from one sample. The number in each quadrant is the percentage of cells. (A) Monocytes without a flurochrome added. (B) Monocytes with only dihydrorhodamine (DHR) 123 added. (C) Monocytes with only pHrodoTM Red S. aureus added. (D) Monocytes with both DHR and pHrodoTM Red S. aureus added.

Lymphocyte proliferation was performed on heparinized whole blood. For each sample, 25 μL of blood was transferred into eight wells on a 96 U-well plate. Then, 200 ng/mL of Staphylococcus enterotoxin B (SEB)/lipopolysaccharides (LPS) solution was added to four of the wells. The plates were then incubated at 37◦C in 5% CO_2 humidified atmosphere for 3 days. Following this, 50 μL of ^{3}H-thymidine of a 10 μCi/mL stock solution was added to each well. The plate was incubated for 4 h at 37◦C in 5% CO_2 humidified atmosphere for 4 h and then stored at −80◦C until analysis. The cells were then harvested and counted using liquid scintillation.

Sample size

An a priori power analysis was performed using a desired mean difference and previously published standard deviations for key metabolites and hormones. The mean difference and standard deviation (SD) used in the power analysis were: βhydroxybutyrate 0.05 (SD 0.01 mmol/L), ghrelin 75 (SD 53 pg/mL), leptin 3,000 (SD 3,000 pg/mL), and insulin 220 (SD 150 pg/mL). This indicated that a sample size of 10 dogs would be necessary for significance level (α) of 0.5 and a power of 80% to detect a difference in β-hydroxybutyrate, ghrelin, leptin, and insulin.

Statistical Analysis

Metabolomics

For analysis, the NMR spectra were divided into 0.04 ppm spectral buckets, where the regions corresponding to water and DSS (4.68 to 4.88, −0.1 to 0.1 ppm, respectively) were excluded, along with the following additional regions 5.51 to 5.84, 5.92 to 6.07, and 6.11 to 6.31. All spectra were normalized by total intensity.

The relationship between the diet groups and the metabolome was explored using principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant (OPLS-DA) analysis were performed using SIMCA v13.0 (Umetrics, Sweden). These statistical methods can reveal clustering of samples into different groupings based on differences of metabolite concentrations across the sample population. PCA is an unsupervised method and is perhaps the most robust. PLS-DA and OPLS-DA are so-called supervised methods and use a priori knowledge of the group membership to fit the data and maximize separation of data from these groups. Pareto scaling was used for the supervised models and the PLS models were validated by permutation testing to rule out overfitting. The spectral buckets that contributed to the greatest variance in the samples were identified from loading plots and subsequently assigned to their associated metabolites using the Chenomx metabolite library v8.3 (Chenomx Inc., Alberta, Canada). The metabolite concentrations were then quantified using manual fitting of the spectral peaks in the Chenomx NMR Suite. Concentrations of plasma β-hydroxybutyrate measured by the colorimetric assay were compared to the concentrations obtained from the fitted spectra using "BlandAltmanLeh" v0.3.1 package (51) in the R Studio v1.1.456 statistical software (52).

Modeling

The "lme4" package (53) in the R Studio v1.1.456 statistical software (54) was used to perform a linear mixed effects analysis of the relationship between the outcome variables [change in weight,

intake, activity, glucose, β-hydroxybutyrate, leptin, ghrelin, insulin, homeostatic model assessment (HOMA), NMR metabolites, lymphocyte proliferation and flow cytometry results], and the fixed variables (time, diet, age, sex, and BCS). Separate models were fitted for each outcome variable. Dog was included as a random effect to account for repeated measures. Interactions between the fixed variables, and between diet and diet sequence, were not significant, and so were not included in the final models.

If the visual inspection of the residual quantile-quantile plots and the Shapiro-Wilk test of the residuals indicated a deviation from normality or homoscedasticity, then transformations of the dependent variable were performed in attempts to improve consistency on the assumptions of the model. However, transformation did not lead to a change in the interpretation of the models or our conclusions. Therefore, for simplicity, the graphs, and final model are reported with the untransformed data.

A post-hoc pairwise comparison of the estimated marginal means with Tukey's correction was performed when diet was significant in the final model. The results of the mixed effects models are presented as the means and standard error of means. $P \leq 0.001$ were considered indicative of very strong evidence, $p \leq 0.01$ of strong evidence, $p \leq 0.05$ of moderate evidence, $p \leq 0.1$ of weak evidence, and $p \leq 0.1$ of insufficient evidence (55).

RESULTS

Intake, Body Weight, Body Condition Score, and Activity

There were no differences in any of the baseline parameters before the groups began their assigned feeding regime in any of the treatment weeks ($P > 0.5$). All dogs remained at a BCS of 4 or 5 out of 9 throughout the study. When fed daily, the dogs had a higher food intake compared to when they were fed intermittently ($P < 0.001$), however, there was no difference in intake between dogs when intermittently fed on the LF or the HF diets ($P = 0.395$, Table 2). Male dogs consumed more food per $kgBWT^{0.75}$ than female dogs (mean 277 ± 67 vs. 226 ± 50 $kcals/kgBWT^{0.75}$). The dogs lost more weight when intermittently fasted on a low fat diet, but there was no difference in the percentage of body weight change when the dogs were daily fed compared to when they were intermittently fasted on a high fat diet regime (Table 2). In addition, when the dogs were fed daily, they were more active at night compared to when they were intermittently fed the LF diet ($P = 0.028$) and HF diet ($P = 0.012$).

TABLE 5 | The means and standard deviations of food intake, change in body weight, and activity in 10 dogs fed daily (BID), and intermittently fasted on a low-fat (IF LF) and a high-fat diet (LF HF) in a Latin Square design. Also included are the results of the linear mixed effect models. Only independent variables with a P-value ≤0.1 are shown.

Outcome	Diet	Mean	Standard Deviation	Fixed Effect	Estimate	Standard Error	P-value
Intake	BID	295	40	(Intercept)	270	77	
(kcals/BWT$^{0.75}$)	IF LF	230	49	Diet IF LF	-66	13	<0.0001
	IF HF	214	73	Diet IF HF	-81	12	<0.0001
				Sex male	51	17	0.027
Body weight	BID	-1.7	1.6	(Intercept)	-1.72	0.65	
(% change)	IF LF	-3.3	2.6	Diet IF LF	-1.56	0.85	0.067
	IF HF	-1.6	1.8				
Total activity (Δ G)	BID	254,058	78,988	(Intercept)	199712	25811	
	IF LF	227,637	77,512	Week	13451	4126	0.005
	IF HF	242,923	79,130				
Day activity (Δ G)	BID	212,251	69,033	(Intercept)	175004	23164	
	IF LF	200,763	72,634	Week	11652	3378	0.004
	IF HF	219,528	72,006				
Night activity (Δ G)	BID	39,458	15,615	(Intercept)	39458	3829	
	IF LF	28,261	8,872	Diet IF LF	-11197	4599	0.028
	IF HF	26,319	8,017	Diet IF HF	-13665	4771	0.012

Metabolites

Metabolomics

Principal component analysis indicated some separation between the diet groups, with the first two principal components accounting for 45.6 and 9.1% of the variance, respectively. Further analyses with a supervised orthogonal partial least squares discriminant model showed a complete separation of the daily fed group and the intermittently fasted groups, and clustering of the two intermittently fasted groups (Figure 3). The metabolites associated with the spectral buckets that separated the different feeding regimes the greatest were βhydroxybutyrate, lactate, alanine and glucose.

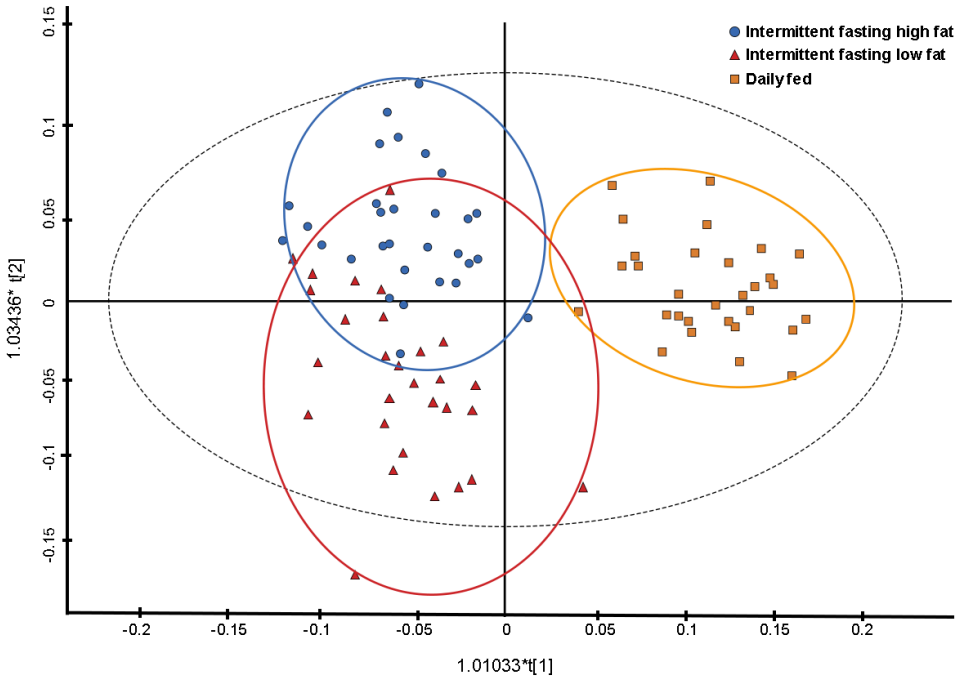

FIGURE 3 | Orthogonal partial least square with discriminant analysis (OPLS-DA) plot illustrating the effect of three feeding regime on the plasma metabolome of 10 dogs. Each point represents a single sample with the blue circles representing a dog intermittently fasted on a high fat diet, the red triangles representing a dog intermittently fasted on a low fat diet, and the orange squares representing a dog fed daily on a low fat diet.

β-Hydroxybutyrate Assayed via Kit and NMR

The concentrations of plasma β-hydroxybutyrate on day 3, 5, and 7 were highest when the dogs were intermittently fasted on the HF diet, and lowest when the dogs were fed daily (Figure 4). There was no effect of day. Intermittent fasting increased plasma β-hydroxybutyrate concentrations regardless of the diet fed, and a higher body condition score was associated with a decrease in plasma β-hydroxybutyrate (Table 3). There was no association between weight loss and plasma β-hydroxybutyrate concentrations ($P = 0.198$). There was reasonable agreement between the β-hydroxybutyrate concentrations obtained from the colorimetric kit and from NMR (Figure 5, Bland Altman).

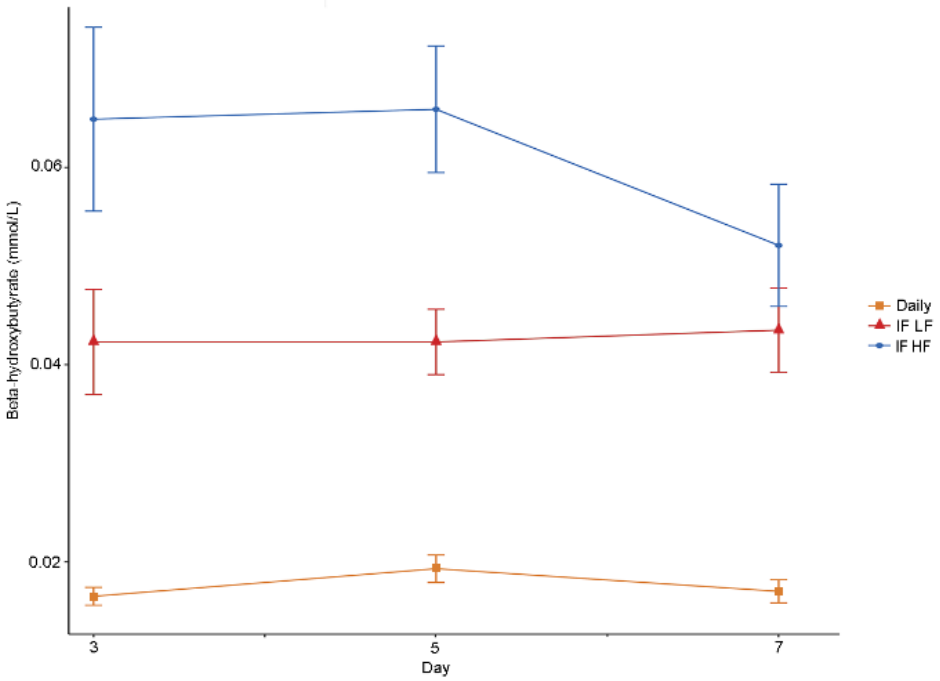

FIGURE 4 | Mean concentrations (±SE) of fasted plasma beta-hydroxybutyrate in 10 dogs fed daily (square), and intermittently fasted on a low fat (triangle) and a high fat diet (circle) in a Latin Square design.

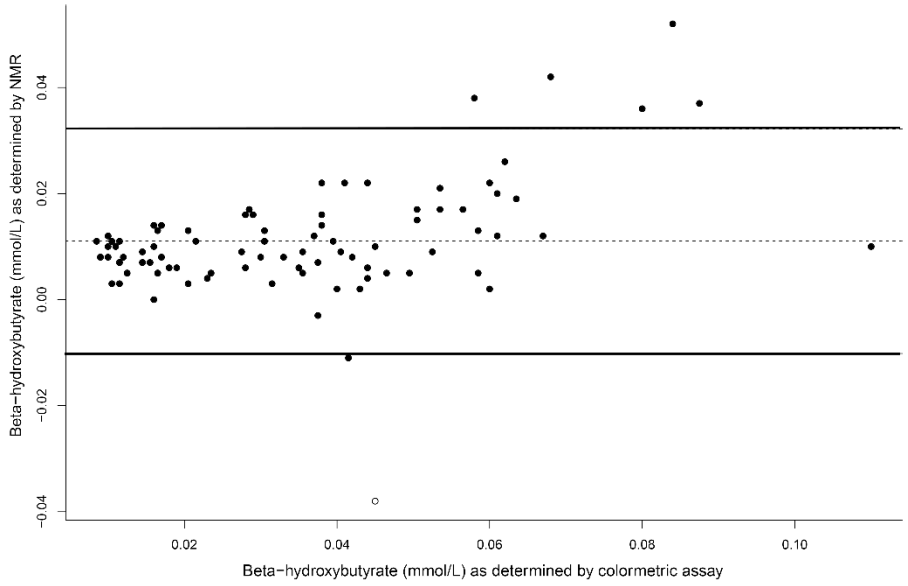

FIGURE 5 | A Bland Altman comparison plot of beta-hydroxybutyrate concentrations as assay by the colormetric kit and by nuclear magnetic resonance (NMR). At y = 0, this indicates perfect agreement, with the middle line as the actual agreement. The solid lines represent the 95% limits of agreement of the data.

Lactate and Alanine via NMR

Both lactate and alanine concentrations were lowest when the dogs were intermittently fasted on a high fat diet (Figure 6). In addition, lactate concentrations decreased over time (P = 0.009; Table 3).

TABLE 6 | The means and standard deviations for β-hydroxybutyrate, lactate alanine and glucose, and the results of the linear mixed effect models, of 10 dogs fed daily (BID), and intermittently fasted on a low-fat (IF LF) and a high-fat diet (LF HF) in a Latin Square design. Only independent variables with a P ≤ 0.1 are shown.

Outcome	Diet	Mean	Standard Deviation	Fixed Effect	Estimate	Standard Error	P-value
β-hydroxybutyrate	BID	0.018	0.004	(Intercept)	0.115	0.026	
(mmol/L)	IF LF	0.043	0.013	Diet IF LF	0.028	0.003	<0.0001
	IF HF	0.061	0.024	Diet IF HF	0.043	0.003	<0.0001
				BCS	-0.020	0.004	<0.0001
Lactate	BID	0.652	0.150	(Intercept)	0.597	0.244	
(mmol/L)	IF LF	0.619	0.161	Day	-0.018	0.005	<0.001
	IF HF	0.571	0.163	Diet IF HF	-0.078	0.029	0.009
Alanine	BID	0.222	0.047	(Intercept)	0.246	0.057	
(mmol/L)	IF LF	0.221	0.036	Diet IF HF	-0.003	0.009	<0.001
	IF HF	0.191	0.042				
Glucose	BID	5.7	0.4	(Intercept)	5.9	0.5	
(mmol/L)	IF LF	5.6	0.3	Day	0.01	0.01	0.06
	IF HF	5.6	0.3	Diet IF LF	-0.12	0.06	0.03
				Diet IF HF	-0.13	0.06	0.02

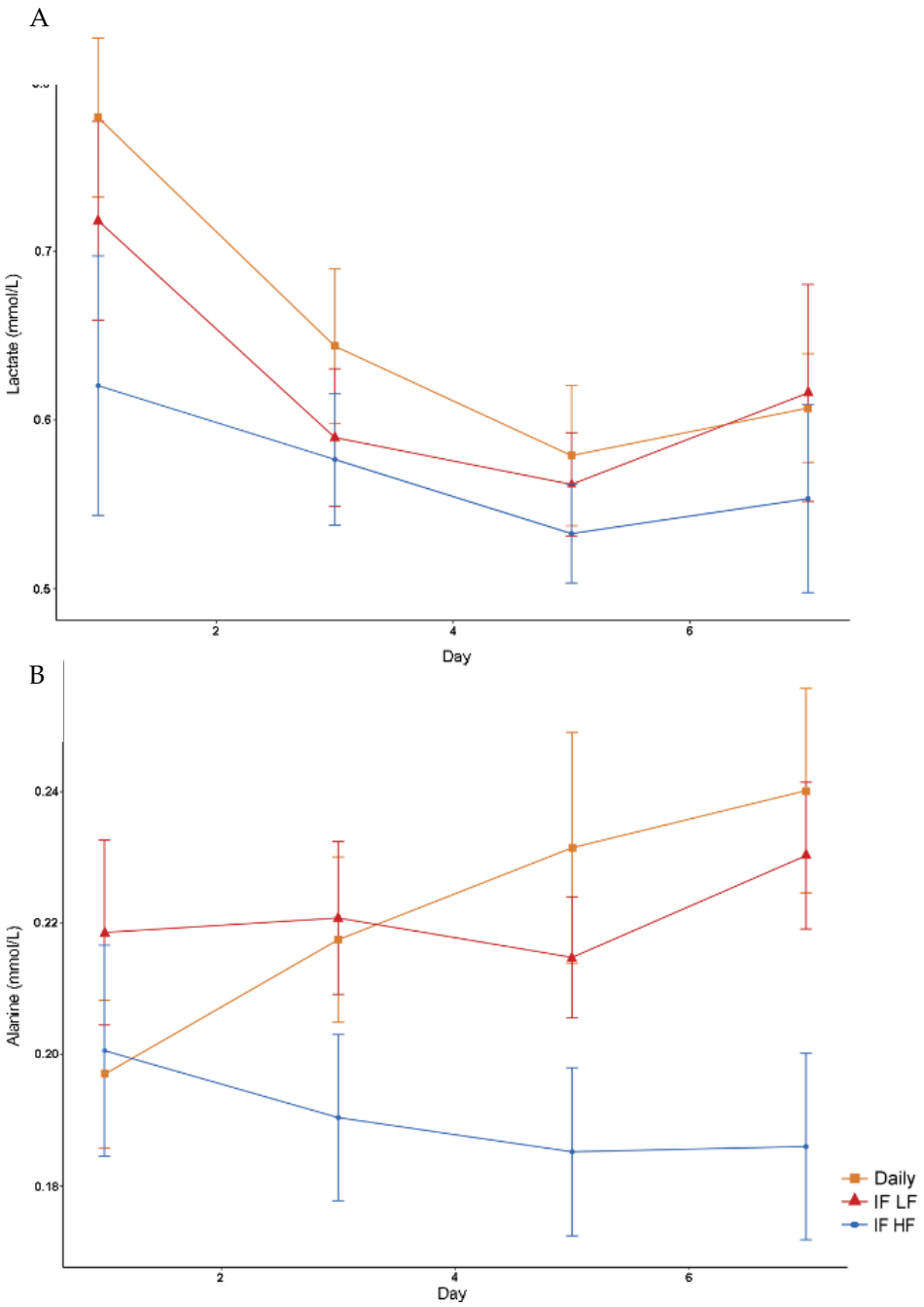

FIGURE 6 | Mean concentrations (±SE) of fasted plasma lactate (A) and alanine (B) in 10 dogs fed daily (square), and intermittently fasted on a low fat (triangle) and a high fat diet (circle) in a Latin Square design.

Glucose

Blood glucose concentrations increased over time from day 3 to day 7, and were highest in the dogs fed daily (Figure 7). There was no difference in glucose concentrations when the dogs were

intermittently fasted on the LF and HF diets (P = 0.98). There was also no effect of BCS on blood glucose (P = 0.24; Table 3).

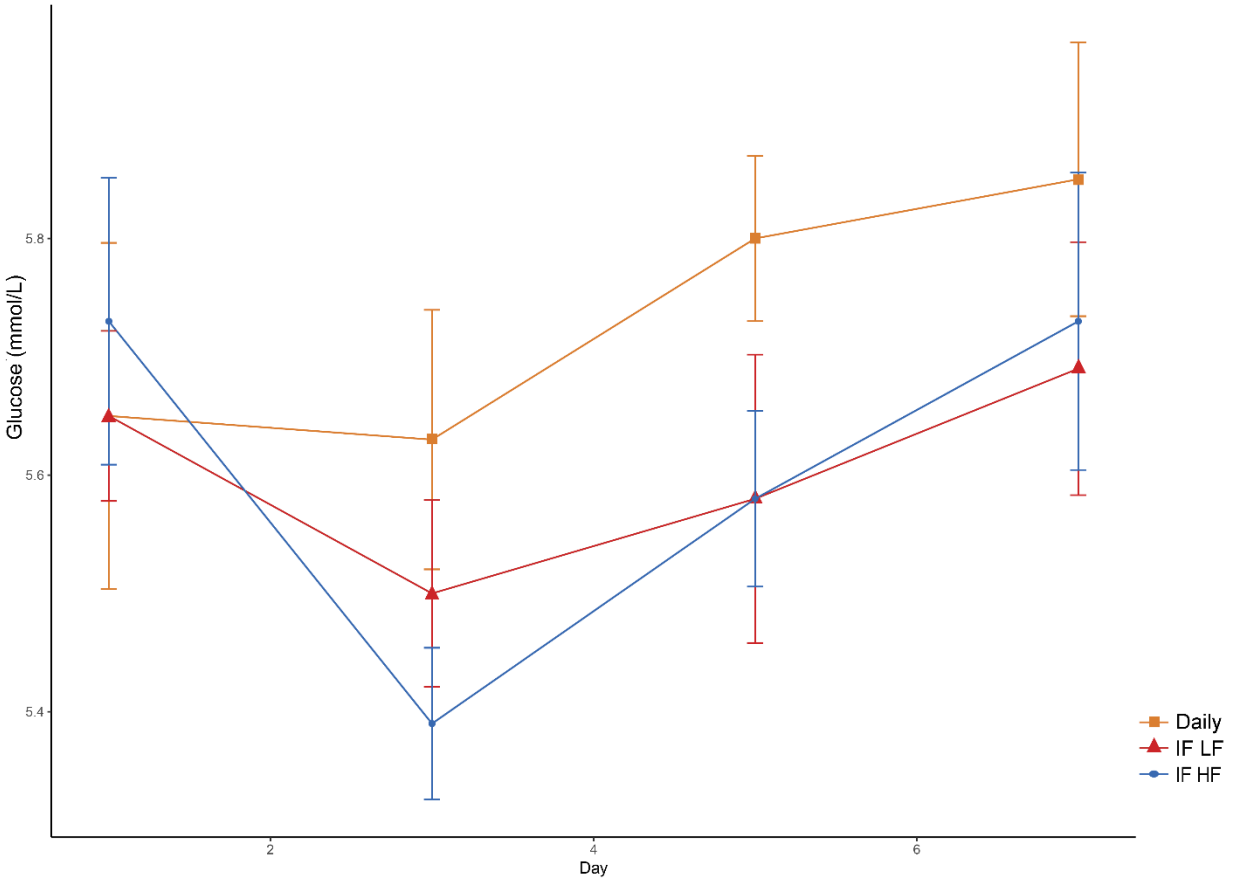

FIGURE 3 | Mean concentrations (±SE) of fasted plasma glucose in 10 dogs fed daily (square), and intermittently fasted on a low-fat (triangle) and a high-fat diet (circle) in a Latin Square design.

Hormones Insulin, Leptin and Ghrelin, and HOMA

Both insulin concentrations and HOMA scores were lowest when dogs were intermittently fasted on a high fat diet (Figure 8). A higher body condition was associated with lower insulin concentrations and HOMA scores (Table 4).

Serum leptin concentration was highest when dogs were fed daily (Figure 9). In addition, there was a decrease in leptin concentrations over time (Table 4). For ghrelin, dogs fasted intermittently on a HF diet had lower serum concentrations (Figure 9).

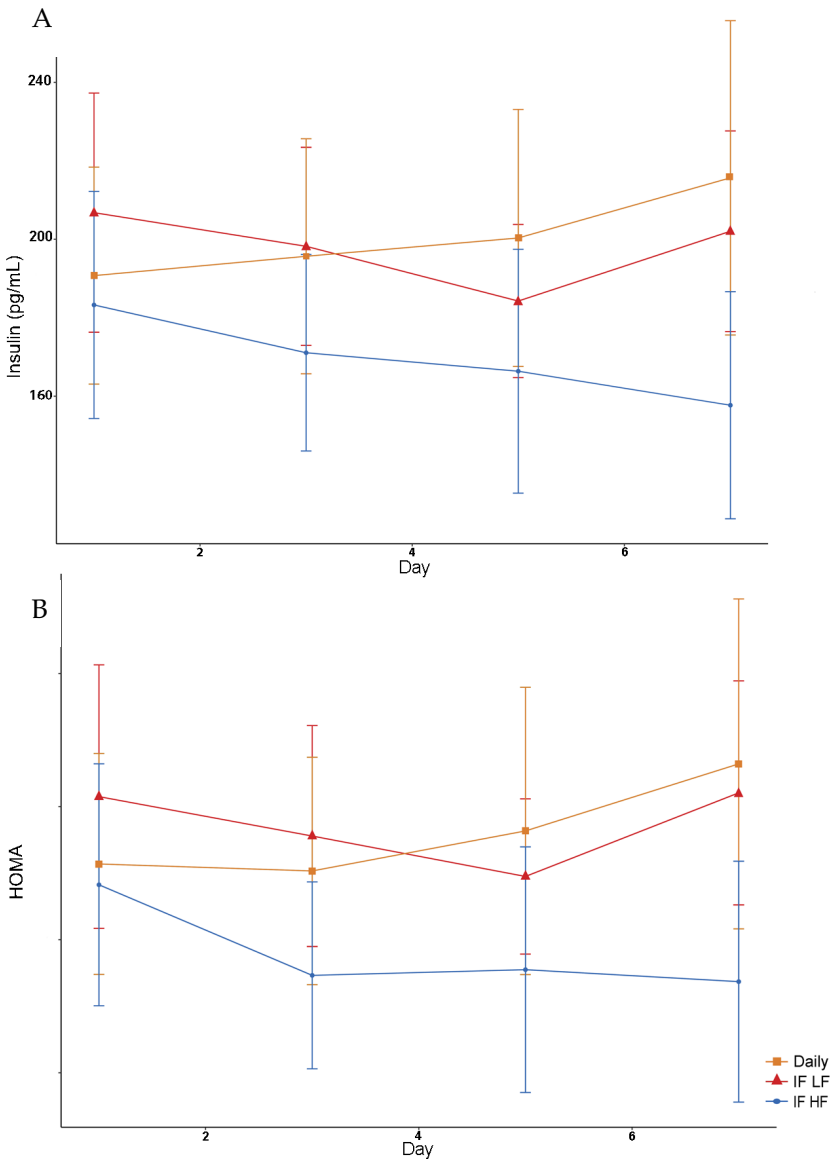

FIGURE 8 | Mean concentrations (±SE) of fasted serum insulin (A) and HOMA score (B) in 10 dogs fed daily (square), and intermittently fasted on a low fat (triangle) and a high fat diet (circle) in a Latin Square design.

TABLE 4 | The means and standard deviations for insulin, Homeostatic Model Assessment (HOMA), leptin and ghrelin, and the results of the linear mixed effect models, of 10 dogs fed daily (BID), and intermittently fasted on a low-fat (IF LF) and a high-fat diet (LF HF) in a Latin Square design. Only independent variables with a $P \leq 0.1$ are shown.

Outcome	Diet	Mean	Standard Deviation	Fixed Effect	Estimate	Standard Error	P-value
Insulin	BID	198	78	(Intercept)	449	123	
(pg/mL)	IF LF	200	101	Diet IF HF	-31.5	13.2	0.02
	IF HF	169	87	BCS	-44.1	19.6	0.03
Homeostatic	BID	1.46	0.63	(Intercept)	3.22	0.99	
Model Assessment	IF LF	1.45	0.76	Diet IF HF	-0.26	0.011	0.01
(HOMA)	IF HF	1.23	0.66	BCS	-0.32	0.15	0.04
Leptin	BID	2451	2217	(Intercept)	3247	2887	
(pg/mL)	IF LF	1794	1683	Day	-111	32	<0.001
	IF HF	1729	1433	Diet IF LF	-637	179	<0.001
				Diet IF HF	-743	179	<0.0001
Ghrelin	BID	85	78	(Intercept)	8.3	115	
(pg/mL)	IF LF	88	73	Day	6.6	1.4	<0.0001
	IF HF	67	60	Diet IF HF	-17.6	7.9	0.03

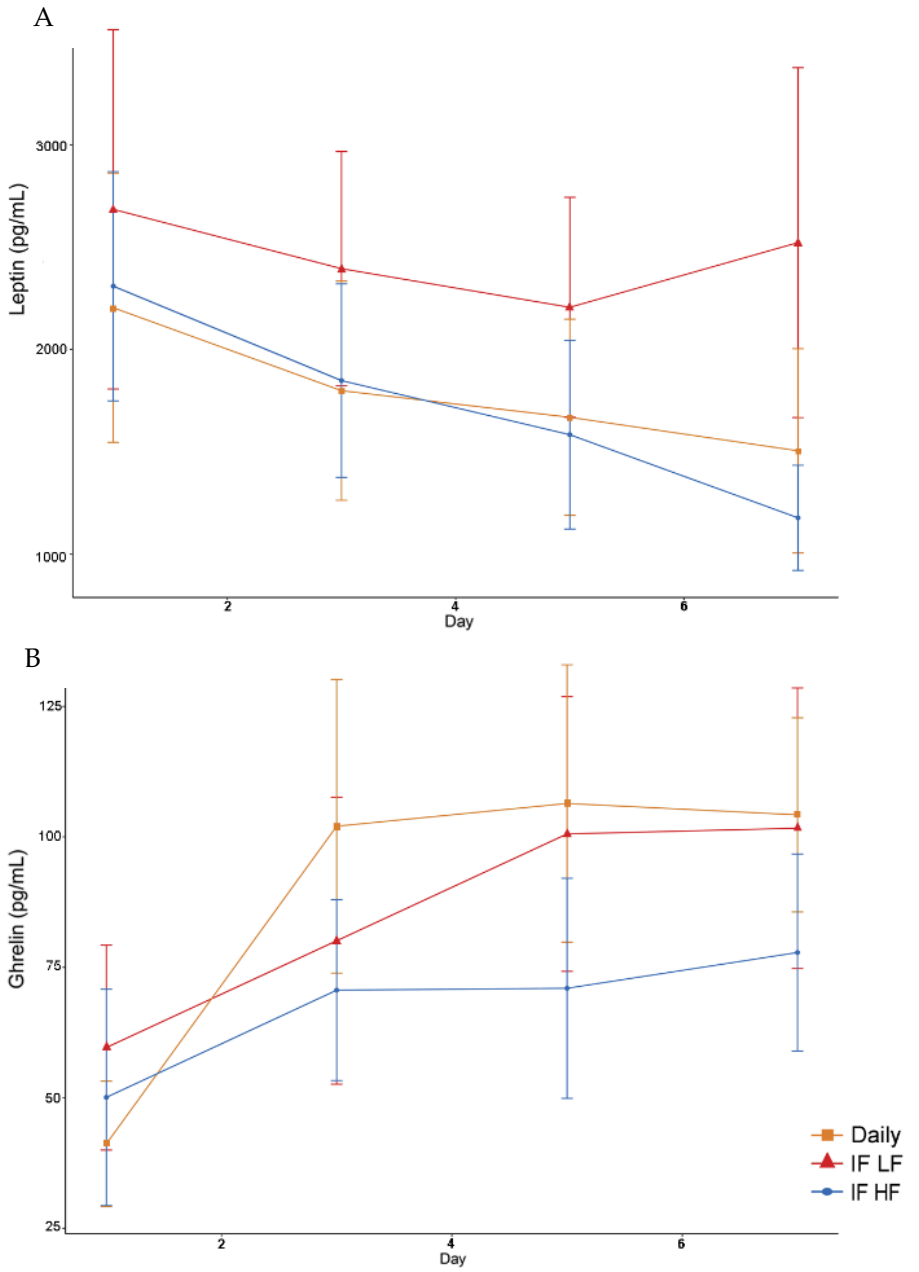

FIGURE 9 | Mean concentrations (±SE) of fasted serum leptin (A) and ghrelin (B) in 10 dogs fed daily (square), and intermittently fasted on a low fat (triangle) and a high fat diet (circle) in a Latin Square design.

Immunoassays

Lymphocyte Proliferation

Lymphocyte proliferation was not significantly different during any of the feeding regimes.

Phagocytosis and Respiratory Burst

When the dogs were intermittently fasted on a LF diet, they had a lower percentage of neutrophils, and a lower MFI in macrophages which underwent both phagocytosis and oxidation (Figure 10; Table 5). In addition, when on the IF LF feeding regime, the dogs also had a lower percentage of lymphocyte which underwent respiratory oxidative burst (Figure 10; Table 5).

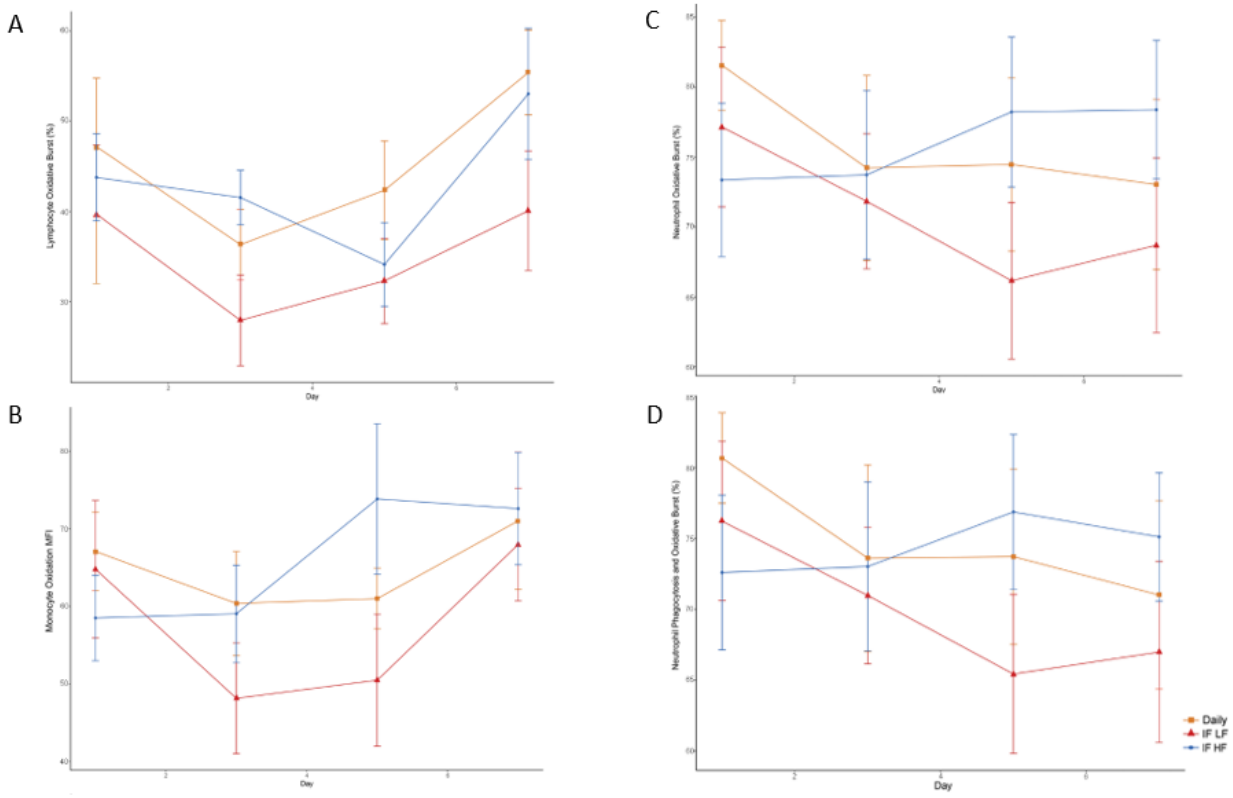

FIGURE 10 | Differences in immune parameters in 10 dogs fed daily (square), intermittently fasted on a low-fat (triangle) and intermittently fasted on a high-fat diet (circle) in a Latin Square design as assessed by flow cytometry. **(A)** Total percentage of lymphocytes which underwent oxidative burst (P=0.008). **(B)** The mean florescence intensity of oxidative burst in monocytes which underwent both phagocytosis and oxidative burst (P=0.06). This is a method of quantifying the degree of oxidation. **(C)** Total percentage of neutrophils which underwent oxidative burst (P=0.02). **(D)** The percentage of neutrophils which underwent both phagocytosis and oxidative burst (P=0.03).

TABLE 5 | The results of the linear mixed effect models of the activity of phagocytic cells, and their respective means and standard deviations, in 10 dogs fed daily (BID), and intermittently fasted on a low fat (IF LF) and a high fat diet (LF HF) in a Latin Square design.

Outcome	Diet	Mean	Standard Deviation	Fixed Effect	Estimate	Standard Error	P-value
Lymphocyte oxidation	BID	45.3	18.4	(Intercept)	30.6	25.3	
(%)	IF LF	35.0	19.3	Diet IF LF	-10.7	3.9	0.008
	IF HF	43.1	17.1				
Monocyte phag+/ox+	BID	64.9	20.0	(Intercept)	72.3	28.6	
MFI for oxidation	IF LF	57.9	25.8	Diet IF LF	-7.6	4.0	0.06
	IF HF	66.0	23.5				
Neutrophil oxidation	BID	75.8	17.6	(Intercept)	73.6	23.3	
(%)	IF LF	71.0	17.6	Diet IF LF	-4.9	2.2	0.02
	IF HF	75.9	16.8				
Neutrophil phag+/ox+	BID	74.7	18.1	(Intercept)	67.9	23.2	
(%)	IF LF	69.9	17.6	Day	-0.8	0.4	0.04
	IF HF	74.4	16.4	Diet IF LF	-5.0	2.2	0.03

DISCUSSION

Intermittent fasting and the promotion of ketone formation may be a beneficial feeding regime for hospitalized dogs, especially spinal patients. Our primary hypothesis was that healthy, intermittently fasted dogs would have lower fasting blood glucose, insulin and leptin concentrations, and greater fasting β-hydroxybutyrate and ghrelin concentrations compared to when they were eating daily. Our secondary hypothesis was that intermittently fasted dogs eating a high fat diet enriched with medium-chain triglycerides will have higher blood β-hydroxybutyrate and leptin concentrations compared with intermittently fasted dogs eating a LF diet. We found that dogs fasted for 48 h on a HF diet enriched in medium- chain triglycerides promoted higher blood β-hydroxybutyrate concentration, and lower insulin concentration than when they were fed daily or fasted on a LF diet. Leptin was not maintained during intermittent fasting by using a HF diet; however, there was no difference in the immune parameters studied between when the dogs were fed the HF diet and when they were daily fed. Fasted ghrelin concentrations were lowest when the dogs were intermittently fasted on a HF diet. Overall, these findings indicate that an intermittent

fasting regime on a high fat diet enriched in medium-chain triglycerides increases plasma β-hydroxybutyrate concentrations without causing immune suppression, however it did not abrogate the drop in leptin or increase ghrelin concentrations during fasting.

A commercially available LF kibble was chosen in this study as the control diet. When dogs were eating the LF diet intermittently, they consumed fewer calories and lost more weight compared to when they were eating the HF diet. There are numerous studies in several species which also show this phenomenon (56). In one study, dogs fed ad libitum a high fat diet (51% energy from fat) gained more weight than dogs fed *ad libitum* on a low fat diet (23% energy from fat) (57). Therefore, to prevent weight loss in an intermittent fasting regime, it is likely that a high fat, energy dense food is required to ensure that a dog will consume its full requirements. However, there are epidemiological associations between high dietary fat consumption and pancreatitis in dogs (58). In early experimental models, dogs fed a high fat vs. a high protein/high carbohydrate diet led to more severe clinical signs and pathological changes in the pancreas (59, 60). However, the diets were unlikely to have been complete and balanced rations, and so other nutrient deficiencies may have confounded the results. Giving table scraps and consuming food discarded in the trash have also been identified as risk factors for the development of pancreatitis in dogs (61). However, it is difficult to speculate what the fat contents of those items were. In contrast, feeding diets with varying fat contents, including medium-chain triglycerides, to a group of healthy dogs did not have an effect on serum canine trypsin-like immunoreactivity (cTLI), pancreatic-lipase immunoactivity (cPLI), or gastrin concentrations (62). However, the highest fat diet in this study contained only ~40% fat on an energy basis. Still, there are numerous studies and reports of sled dog diets where the dogs were fed a diet with a similar fat content to our HF diet without causing pancreatitis (63–68).

Ketones provide an alternative source of energy for neurons and have been shown to reduce neuronal degeneration and improve recovery in rodent models of brain and spinal injury (30, 69–71). While β-hydroxybutyrate concentrations were highest in the dogs when they were intermittently fasted on the HF diet, the concentrations obtained (mean 0.061, SD 0.024 mmol/L) were much lower compared to rodents (0.8–2 mmol/L) and humans (1.67 mmol/L) fasted for a similar amount of time (22, 72, 73). Our finding is consistent with other published studies where the dog does not reach the same blood concentration of ketones as other species after comparable fasts (74–76). However, it has been shown that the rate of total ketone production is similar between dogs and men following a 48 h fast (77, 78). Further, De Bruijne and Van den Brom (74) established that dogs have a higher rate of clearance of plasma ketones than man. Thus, the seemingly low concentration of β-hydroxybutyrate in dogs is not from reduced production of ketones, but rather from higher rates of peripheral utilization compared with rodents and humans. In addition, although a single blood sample is indicative of the concentration of a metabolite at that moment, it does not describe its flux (production and utilization) (78). The concentration of β-hydroxybutyrate in the brain and cerebral spinal fluid (CSF) is proportional to the concentration found in plasma, and increases as the duration of fasting continues (72, 79, 80). When available, β-hydroxybutyrate is preferentially utilized over glucose, lactate and pyruvate by neurons as an energy substrate (81). So

even a small increase in plasma concentrations of ketones could still provide a large contribution of energy for the brain and neurons.

In our study, all dogs had fasting blood glucose concentrations within the normal reference range, however lower values were obtained when the dogs were fasted for 48 h vs. 12 h. This reflects the difference in the length of fasting rather than the macronutrient composition of the diets. Similarly in a study of dogs eating either a carbohydrate-free or high carbohydrate diet, the blood glucose concentrations were the same after a 24 h fast in both groups of dogs (82). The lowest concentrations of insulin were seen when the dogs were intermittently fasted on the HF diet, which indicated a decrease in insulin production and/or increase in insulin sensitivity. The HOMA insulin resistance scores were also lowest when the dogs were on this feeding regime, which is a reflection of both low fasting glucose and insulin concentrations. Both caloric restriction and a reduction in fat mass improves insulin sensitivity in overweight dogs (83–86). In our study, intermittently fasted dogs eating a HF diet lost less weight than when intermittently fasted on a LF diet, and yet had lower insulin concentrations. Therefore, it appears that intermittent fasting on a high fat diet may reduce insulin production or improve insulin sensitivity independent of weight loss.

Alanine and lactate were two gluconeogenic metabolites identified by OPLS as being different between the feeding regimes. During the early stage of fasting, proteolysis of muscle releases alanine for gluconeogenesis in hepatocytes (87, 88). In a study of dogs eating a carbohydrate-free diet, the turnover rate of alanine and its conversion of alanine to plasma glucose were increased after a 48 h fast (89). Lactate is transported by the same monocarboxylate transporters (MCTs) as ketones, and also serve as an energy source for cells, including neurons, in a fasted state (90, 91). Feeding a high fat diet to rats increased the expression of MCT1 by brain endothelial cells (92). Also, fasted for 2 days (72). Thus, the reduction of both alanine and lactate in the dogs when intermittently fasted on a HF diet may be due to an increase in uptake by the liver, brain, and kidneys.

The adipokine, leptin, has many roles in the body including the activation of phagocytosis by monocytes and chemotaxis of neutrophils and oxidative radical generation (93–95). In our study, we found that leptin concentrations were lowest in the intermittently fasted dogs regardless of the fat content of the diet. Both fasting and a reduction in fat mass are known to decrease leptin production (96, 97). During the course of our study, the dogs lost some weight; however, there was no difference in the starting concentrations of leptin at the beginning of each study week in any of the dogs. Therefore, the reduction in leptin concentrations was the result of the fasting regime, and not fat mass loss. In addition, although leptin was not maintained during fasting by feeding a HF diet, there was no difference between the immune parameters studied in those dogs and when the dogs were fed daily. We did find a reduction in the percentage of leucocytes undergoing phagocytosis and respiratory burst when the dogs were intermittently fasted on a LF diet. This suggests that the immune changes were not leptin-mediated. The suppressive effect however was not consistent throughout all the immune parameters studied, and the clinical significance of this reduction is not known.

Limitations

Both diets used in this study were formulated to meet AAFCO requirements, and while all attempts were made to create similar nutrient profiles excluding the fat and carbohydrate content, the diets did differ from one another in some micronutrients. In addition, when the dogs were intermittently fasted on the LF diet, they lost more weight than in the other feeding regimes, indicating the dogs were in a greater catabolic state. However, an increase in proteolysis and fatty acid oxidation was not reflected by an increase in the plasma alanine and ketone concentrations of the dogs during the LF diet intermittent fasting regime. In addition, plasma ketone concentration was not associated with weight loss in this study. A washout week using the control feeding regime was performed in between each study period, and none of the outcome parameters were significantly different in the dogs at the start of each study period. Furthermore, diet order was examined in the multivariate model which did not show an effect. Our results suggest that the differences in diet profiles and greater weight loss during the LF diet intermittent fasting regime likely had a minimal effect, however, a more thorough study would be required to determine if that is indeed the case.

In our study, no ill effects were seen when the dogs were intermittently fasted on the high fat diet. Acute feeding of a high fat diet in other species can lead to an increase in circulating pro-inflammatory cytokines such as interleukin 1β and tumor necrosis-α, but this has not been shown consistently (98). Interestingly, an intermittent fasting regime has been shown to ameliorate the expression of proinflammation-related genes in hepatocytes during long-term high-fat feeding in mice (99). The effects of intermittent fasting on markers of inflammation during high fat feeding in dogs is not currently known. We did not measure inflammatory cytokines in our study, however future studies may wish to do so. Furthermore, a difference in immunity between the feeding regimes was apparent only when the dogs were intermittently fasted on the LF diet. The significance of this immune effect is not known, but there may be a greater implication of this in a clinical setting.

To promote ketone formation, coconut oil was used in the HF diet as a rich source of medium-chain triglycerides. However, the main medium-chain triglyceride constituent in coconut oil is dodecanoic acid (lauric acid, C12), with decanoic acid (capric acid, C10) and octanoic acid (caprylic acid, C8) as the great remainder (100). When given in equal amounts, intake of decanoic acid and octanoic acid leads to a greater ketone production postprandially then dodecanoic acid (101, 102). Thus, to increase the effect of medium-chain triglycerides on ketogenesis, a concentrated oil with a higher quantity of decanoic and octanoic acid can be given instead of coconut oil.

The homeostasis model assessment (HOMA) was developed to provide a measure of peripheral insulin resistance from fasting glucose and insulin. The scores correlate well with a euglycemic clamp model in humans, and has been used to detect improvements in insulin sensitivity with weight loss and fasting in humans (103–105). In our study, dogs intermittently fasted on a HF diet had the lowest HOMA score compared to when they were fed daily or intermittently fasted on the LF diet. However, HOMA has been found to be variably reliable in companion animals in detecting insulin resistance (106–108). So while we found a difference with HOMA scores in the different

feeding regimes, any interpretation in peripheral insulin sensitivity should be confirmed using a euglycemic clamp.

CONCLUSION

In this study, we found that fasting for up to 48 h in healthy dogs does not cause immunosuppression, and that fasting on a high fat diet enriched with medium-chain triglycerides promoted a greater plasma ketone concentration than when the dogs were fasted after eating a low fat diet. However, the concentrations obtained in dogs are significantly less than what is reported in other species fasted for a similar period of time. Therefore, a ketone kinetics study is required to gain a more comprehensive understanding of the flux of ketones in dogs while fasting for varying durations, and whether this feeding regime could be feasible in hospitalized dogs recovering from neuronal injury. In addition, dogs intermittently fasted on a low fat diet consumed fewer calories and lost more weight than when fed the high fat diet. Therefore, any practical application of this type of feeding regime would require modification of the diet itself (e.g., increase the energy density of the diet to reduce the volume and gut fill) and/or reducing the number of days or length of fasting. Ultimately, while we showed the possibility of this type of feeding regime to be feasible and to produce ketones without ill effect in healthy dogs, its application in hospitalized dogs remains to be determined.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Massey University Animal Ethics Committee (MUAEC #16/130).

AUTHOR CONTRIBUTIONS

YL and NC conceptualized and designed the study. YL was the primary researcher, with assistance from NC. YL organized data collection, completed laboratory preparation of blood samples, performed the statistical analysis, and wrote the first draft of the manuscript. AH supervised the immunological assays. PE performed the NMR analysis. AG reviewed the study design and statistical analysis. TW contributed by the reading and revision of the manuscript. All authors contributed to manuscript revision and approved the submitted version.

FUNDING

This work described in this manuscript was funded internally.

ACKNOWLEDGMENTS

The authors would like to thank Brittnee Southland for her assistance with making and distributing the diets and blood sample collection.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix IV: High-fat recipes used in the ketone kinetics study

Ingredient	Amount (grams)
Blackhawk® Working Dog, lamb and beef, dry	100
Beef tallow	30
Coconut oil	30
Whey protein isolate	25
Balance IT® Canine supplement	10
Marmite® yeast spread	5
Sunflower oil	3
Total grams	203
Total kilocalories	1,106

Recipe equals approximately 5.45 kcals/gram of diet. Diets were fed to meet the maintenance energy requirement of each dog.

Appendix V: Modified commercial and home-made ketogenic recipes used in the hospital study

Ingredient	Amount (grams)
Royal Canin® Recovery, canned	100
Rice, white, unenriched, cooked weight	20
Butter, no added salt	8
Coconut oil	6
Balance IT® Canine supplement	2
Total grams	136
Total kilocalories	252.2

Recipe equals approximately 1.85 kcals/gram of diet. Diets were fed to meet the resting energy requirement of each dog.

Ingredient	Amount (grams)
Chicken, white and dark meat only, no skin, stewed	80
Rice, white, unenriched, cooked weight	48
Butter, no added salt	32
Coconut oil	10
Balance IT® Canine supplement	7
Total grams	177
Total kilocalories	498

Recipe equals approximately 2.81 kcals/gram of diet. Diets were fed to meet the resting energy requirement of each dog.