ACUTE AND CHRONIC EFFECTS OF LOW VERSUS HIGH GLYCEMIC INDEX CARBOHYDRATE SOURCES ON METABOLIC AND CARDIOVASCULAR RESPONSES IN LEAN AND OBESE DOGS

A Thesis Submitted to the College of Graduate Studies and Research
In Partial Fulfillment of the Requirements For the Degree of Doctor of Philosophy
In the Department of Veterinary Biomedical Sciences
University of Saskatchewan
Saskatoon

By

JENNIFER LEA ADOLPHE

© Copyright Jennifer Lea Adolphe, 2013. All rights reserved.
Permission to Use

In presenting this thesis in partial fulfilment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Veterinary Biomedical Sciences
University of Saskatchewan
Saskatoon, Saskatchewan S7N 5B4
ABSTRACT

In dogs, nutrition has been implicated in the development of numerous chronic diseases, including obesity, diabetes and cardiovascular disease. Health claims for dog food are not regulated in Canada, thus many claims do not have a scientific basis. The development of a pet food with proven health benefits is important to pet owners as well as the pet food industry. The purpose of this study was to develop a low glycemic canine diet that will provide health benefits for dogs, namely decreased serum insulin and glucose concentrations, reduced food intake and weight gain, and improved cardiovascular health.

To achieve this objective, four studies were performed. These studies examined the acute and longer term health effects of feeding unprocessed as well as extruded carbohydrate sources as both single ingredients and in complete dog diets. In addition, metabolic and cardiovascular health parameters were measured in dogs when they were lean, obese and after weight loss. Post-prandial serum glucose and insulin responses were used to determine glycemic index of the carbohydrate sources and to evaluate glucose tolerance. Flow-mediated dilation, echocardiography and blood pressure were used to assess cardiovascular health. Computed tomography was performed to measure body fat amount and distribution. Leptin, adiponectin and C-reactive protein were also analyzed.

The results of these studies found that peas had a lower glycemic index compared to barley and rice, but that after extrusion, the glycemic index of the pea diet was not different than the rice diet. Post-prandial hyperglycemia in dogs was associated with acute changes in endothelial function which may be related to increased methylglyoxal concentrations. However, several negative health effects were observed in dogs after only 12 weeks of obesity and weight loss reversed some, but not all, of these changes. The pea-based diet reduced post-prandial insulin response in obese dogs after 12 weeks on the diet even though no changes
were observed in body fat amount or distribution. In conclusion, this research supports the usefulness of peas as an ingredient in canine diets and provides valuable data for the pet food and pulse industries, as well as for veterinarians and pet owners.
ACKNOWLEDGMENTS

I have many people I would like to thank for their support during my graduate work. Thank you first to my supervisor, Dr. Lynn Weber. Your passion for research carried me through my graduate program and I am sincerely grateful for all of your encouragement and advice. I would also like to thank my advisory committee members, Dr. Murray Drew, Dr. Marion Smart, Dr. Susan Whiting, Dr. Phyllis Paterson and Dr. Jaswant Singh. Thank you to all of the research assistants and dog volunteers, namely Courtney Kotzer and Petra Jebbink, for all of your help.

I greatly appreciate all of the financial support I received: Natural Sciences and Engineering Research Council Doctoral Canada Graduate Scholarship; Department of Veterinary Biomedical Sciences Devolved Scholarship; Pfizer Fellowship Award; Pfizer Graduate Student Research Award; College of Graduate Studies and Research Travel Award; Western College of Veterinary Medicine Travel Award; the Canadian Society of Atherosclerosis, Thrombosis and Vascular Biology Travel Award; and the International Union of Nutritional Sciences Travel Award. Thank you to the Natural Sciences and Engineering Research Council, Saskatchewan Pulse Growers, Western College of Veterinary Medicine Vitamin Settlement Grant, and Horizon Pet Nutrition for the financial support for my research.

Thank you very much to my family and friends. I could not have achieved this without your love and support.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Rationale</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Research Design</td>
<td>1</td>
</tr>
<tr>
<td>1.3 Objectives</td>
<td>7</td>
</tr>
<tr>
<td>1.4 Hypotheses</td>
<td>8</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>10</td>
</tr>
<tr>
<td>2.1 Obesity and Weight Management</td>
<td>10</td>
</tr>
<tr>
<td>2.1.1 Obesity</td>
<td>10</td>
</tr>
<tr>
<td>2.1.2 Health Effects of Obesity</td>
<td>13</td>
</tr>
<tr>
<td>2.1.3 Glucose Tolerance and Insulin Resistance</td>
<td>14</td>
</tr>
<tr>
<td>2.1.3.1 Assessment of Glucose Tolerance and Insulin Resistance</td>
<td>16</td>
</tr>
<tr>
<td>2.1.3.2 Advanced Glycation End-Products</td>
<td>18</td>
</tr>
<tr>
<td>2.1.3.3 Methylglyoxal</td>
<td>19</td>
</tr>
<tr>
<td>2.1.4 Dietary Treatment of Obesity</td>
<td>19</td>
</tr>
<tr>
<td>2.1.5 Adipokines</td>
<td>20</td>
</tr>
<tr>
<td>2.1.5.1 Leptin</td>
<td>21</td>
</tr>
<tr>
<td>2.1.5.2 Adiponectin</td>
<td>22</td>
</tr>
<tr>
<td>2.1.6 Weight Loss</td>
<td>23</td>
</tr>
<tr>
<td>2.1.7 Dogs as a Model for Humans</td>
<td>24</td>
</tr>
<tr>
<td>2.2 Cardiovascular Disease</td>
<td>25</td>
</tr>
<tr>
<td>2.2.1 Association between Obesity and Cardiovascular Disease</td>
<td>25</td>
</tr>
<tr>
<td>2.2.2 Diet and the Prevention of Cardiovascular Disease</td>
<td>26</td>
</tr>
<tr>
<td>2.2.3 Inflammation and Oxidative Stress</td>
<td>26</td>
</tr>
<tr>
<td>2.2.3.1 C-Reactive Protein</td>
<td>27</td>
</tr>
<tr>
<td>2.2.3.2 Nitrotyrosine</td>
<td>28</td>
</tr>
<tr>
<td>2.2.4 Endothelial Function</td>
<td>29</td>
</tr>
<tr>
<td>2.2.4.1 Flow-Mediated Dilation</td>
<td>30</td>
</tr>
<tr>
<td>2.2.4.2 Relationship between Flow-Mediated Dilation and Cardiac Function</td>
<td>34</td>
</tr>
<tr>
<td>2.2.5 Echocardiography</td>
<td>34</td>
</tr>
<tr>
<td>2.2.6 Blood Pressure</td>
<td>41</td>
</tr>
<tr>
<td>2.2.7 Heart Rate</td>
<td>43</td>
</tr>
<tr>
<td>2.3 Carbohydrates</td>
<td>43</td>
</tr>
</tbody>
</table>
2.3.1 Types of Carbohydrates 43
2.3.2 Glycemic Index 45
   2.3.2.1 Glycemic Index Methodology 46
   2.3.2.2 Effects of Glycemic Index on Health 49
2.3.3 Carbohydrate Sources in Dog Food 50
   2.3.3.1 Effects of Processing 52
   2.3.3.2 Starch Digestibility 53

STUDY 1: POST-PRANDIAL IMPAIRMENT OF FLOW-MEDIATED DILATION
AND ELEVATED METHYLGLYOXAL AFTER SIMPLE, BUT NOT COMPLEX,
CARBOHYDRATE CONSUMPTION IN DOGS 55
3.1 Introduction 56
3.2 Methods and Materials 57
   3.2.1 Animals and Treatments 57
   3.2.2 Serum Analysis 59
   3.2.3 Ultrasound and Oscillometry 59
   3.2.4 Statistical Analyses 61
3.3 Results 61
   3.3.1 Serum Glucose and Insulin 61
   3.3.2 Flow-Mediated Dilation 62
   3.3.3 Methylglyoxal and Nitrotyrosine 65
   3.3.4 Hemodynamics 65
3.4 Discussion 67

STUDY 2: DIGESTIBILITY AND ACUTE GLYCEMIC, INSULINEMIC AND
CARDIOVASCULAR EFFECTS OF PEAS AS A CARBOHYDRATE SOURCE IN
DOG FOOD 71
4.1 Introduction 72
4.2 Materials and Methods 73
   4.2.1 Animals 73
   4.2.2 Digestibility and Diet Formulation 73
   4.2.3 Serum Glucose and Insulin Analysis 79
   4.2.4 Ultrasound and Oscillometry 79
   4.2.5 Statistical Analyses 81
4.3 Results 81
   4.3.1 Digestibility and Diet Formulation 81
   4.3.2 Serum glucose and insulin analysis 83
   4.3.3 Ultrasound and Oscillometry 86
4.4 Discussion 89

STUDY 3: SHORT-TERM OBESITY RESULTS IN DETRIMENTAL METABOLIC
AND CARDIOVASCULAR CHANGES THAT MAY NOT BE REVERSED WITH
WEIGHT LOSS 93
5.1 Introduction 94
5.2 Materials and Methods 95
   5.2.1 Animals 95
   5.2.2 Body Fat Analysis 96
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.3 Blood Collection and Analyses</td>
<td>97</td>
</tr>
<tr>
<td>5.2.4 Ultrasound and Oscillometry</td>
<td>98</td>
</tr>
<tr>
<td>5.2.5 Statistical Analyses</td>
<td>99</td>
</tr>
<tr>
<td>5.3 Results</td>
<td>99</td>
</tr>
<tr>
<td>5.4 Discussion</td>
<td>111</td>
</tr>
<tr>
<td><strong>STUDY 4: EFFECT OF A PEA- OR RICE-BASED DIET ON POST-PRANDIAL INSULIN RESPONSE, BODY FAT DISTRIBUTION AND CARDIOVASCULAR HEALTH IN OBESE DOGS</strong></td>
<td></td>
</tr>
<tr>
<td>6.1 Introduction</td>
<td>116</td>
</tr>
<tr>
<td>6.2 Materials and Methods</td>
<td>118</td>
</tr>
<tr>
<td>6.2.1 Study Design</td>
<td>118</td>
</tr>
<tr>
<td>6.2.2 Body Fat Analysis</td>
<td>119</td>
</tr>
<tr>
<td>6.2.3 Blood Collection and Analyses</td>
<td>120</td>
</tr>
<tr>
<td>6.2.4 Ultrasound and Oscillometry</td>
<td>121</td>
</tr>
<tr>
<td>6.2.5 Statistical Analyses</td>
<td>122</td>
</tr>
<tr>
<td>6.3 Results</td>
<td>123</td>
</tr>
<tr>
<td>6.4 Discussion</td>
<td>134</td>
</tr>
<tr>
<td><strong>DISCUSSION</strong></td>
<td></td>
</tr>
<tr>
<td>7.1 Conclusions</td>
<td>138</td>
</tr>
<tr>
<td>7.2 Implications for the Pet Food Industry and Veterinary Practice</td>
<td>143</td>
</tr>
<tr>
<td>7.3 Strengths and Limitations</td>
<td>145</td>
</tr>
<tr>
<td>7.4 Future Research</td>
<td>148</td>
</tr>
<tr>
<td><strong>REFERENCES</strong></td>
<td>155</td>
</tr>
<tr>
<td><strong>APPENDIX A: SUPPLEMENTAL DATA FOR STUDY 1</strong></td>
<td>181</td>
</tr>
<tr>
<td><strong>APPENDIX B: SUPPLEMENTAL DATA FOR STUDY 2</strong></td>
<td>185</td>
</tr>
<tr>
<td><strong>APPENDIX C: SUPPLEMENTAL DATA FOR STUDY 3</strong></td>
<td>188</td>
</tr>
<tr>
<td><strong>APPENDIX D: SUPPLEMENTAL DATA FOR STUDY 4</strong></td>
<td>189</td>
</tr>
<tr>
<td><strong>APPENDIX E: PERMISSIONS</strong></td>
<td>190</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Table 1.1</strong> Timeline for studies 3 and 4.</td>
<td>4</td>
</tr>
<tr>
<td><strong>Table 1.2</strong> Testing time points for studies 3 and 4.</td>
<td>5</td>
</tr>
<tr>
<td><strong>Table 2.1</strong> Body condition score system for dogs.</td>
<td>12</td>
</tr>
<tr>
<td><strong>Table 3.1</strong> Glycemic and insulinemic responses to 10 g of available carbohydrate from simple (glucose) and complex (rice, barley, corn and peas) sources.</td>
<td>64</td>
</tr>
<tr>
<td><strong>Table 4.1</strong> Ingredient composition of pea and rice diets.</td>
<td>77</td>
</tr>
<tr>
<td><strong>Table 4.2</strong> Calculated nutrient analysis of pea and rice diets.</td>
<td>78</td>
</tr>
<tr>
<td><strong>Table 4.3</strong> Proximate analysis and total tract apparent digestibility of ingredients and diets.</td>
<td>82</td>
</tr>
<tr>
<td><strong>Table 4.4</strong> Glycemic and insulinemic responses to pea and rice diets.</td>
<td>85</td>
</tr>
<tr>
<td><strong>Table 5.1</strong> Serum glucose and insulin responses to oral glucose tolerance test in dogs that were lean, have been obese for 12 or 32 weeks or have undergone weight loss.</td>
<td>103</td>
</tr>
<tr>
<td><strong>Table 5.2</strong> Cardiac and hemodynamic variables when dogs were lean, obese for 12 wk, obese for 32 wk or after weight loss.</td>
<td>106</td>
</tr>
<tr>
<td><strong>Table 5.3</strong> Correlations between total, subcutaneous and visceral body fat with metabolic and cardiovascular variables using combined data when dogs were obese for 12 and 32 weeks, and after weight loss.</td>
<td>109</td>
</tr>
<tr>
<td><strong>Table 6.1</strong> Blood chemistry characteristics of adult dogs before and 12 weeks after receiving a pea-based or rice-based diet.</td>
<td>126</td>
</tr>
<tr>
<td><strong>Table 6.2</strong> Serum glucose and insulin response to oral glucose tolerance test in dogs before and after receiving pea-based or rice-based diet.</td>
<td>127</td>
</tr>
<tr>
<td><strong>Table 6.3</strong> Cardiovascular parameters in dogs before and after receiving pea-based or rice-based diet.</td>
<td>128</td>
</tr>
<tr>
<td><strong>Table 6.4</strong> Correlations between total, subcutaneous and visceral body fat with metabolic and cardiovascular variables using combined data before and after feeding a pea-based or rice-based diet to dogs.</td>
<td>132</td>
</tr>
<tr>
<td><strong>Table B.1</strong> Cardiac and hemodynamic variables before and after feeding the pea diet, rice diet or glucose.</td>
<td>187</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2.1</td>
<td>Right parasternal long-axis echocardiographic views</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Right parasternal short axis echocardiographic views</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>Echocardiographic apical views</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Flow-mediated dilation, methylglyoxal and nitrotyrosine 60 min after feeding simple (glucose) or complex (rice, barley, corn, peas) carbohydrate sources</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Cardiovascular effects of feeding glucose, pea diet or rice diet</td>
</tr>
<tr>
<td>Figure 5.1</td>
<td>Assessment of abdominal total, visceral and subcutaneous body fat</td>
</tr>
<tr>
<td>Figure 5.2</td>
<td>Cardiovascular changes in dogs with different body conditions</td>
</tr>
<tr>
<td>Figure 5.3</td>
<td>Plasma adipokine and C-reactive protein concentrations in dogs with different body conditions</td>
</tr>
<tr>
<td>Figure 5.4</td>
<td>Association between visceral body fat, metabolic and cardiovascular parameters</td>
</tr>
<tr>
<td>Figure 6.1</td>
<td>Body fat distribution before and after consumption of pea-based or rice-based diet</td>
</tr>
<tr>
<td>Figure 6.2</td>
<td>Plasma leptin, adiponectin and C-reactive protein concentrations on commercial diet, pea-based diet or rice-based diet</td>
</tr>
<tr>
<td>Figure 6.3</td>
<td>Effect of body fat distribution on area under the insulin response curve, systolic left ventricle free wall thickness, leptin and adiponectin</td>
</tr>
<tr>
<td>Figure 7.1</td>
<td>Interrelationships between metabolic and cardiovascular health parameters affected by hyperglycemia, oxidative stress and obesity</td>
</tr>
<tr>
<td>Figure A.1</td>
<td>Serum glucose and insulin responses after feeding simple (glucose) or complex (rice, barley, corn, peas) carbohydrate sources</td>
</tr>
<tr>
<td>Figure A.2</td>
<td>Serum glucose and insulin responses after feeding complex carbohydrate sources</td>
</tr>
<tr>
<td>Figure A.3</td>
<td>Serum glucose and insulin responses after feeding three dosages of glucose</td>
</tr>
<tr>
<td>Figure A.4</td>
<td>Flow-mediated dilation dose response to glucose 60 min after ingestion</td>
</tr>
<tr>
<td>Figure B.1</td>
<td>Serum glucose and insulin responses after feeding glucose, rice kibble or pea kibble</td>
</tr>
</tbody>
</table>
Figure B.2 Serum glucose and insulin responses after feeding rice or pea kibble. ............ 186

Figure C.1 Serum glucose and insulin responses to oral glucose tolerance test in dogs that were lean, have been obese for 12 or 32 weeks or have undergone weight loss. ................................................................................................................................. 188

Figure D.1 Serum glucose and insulin responses to oral glucose tolerance test after consuming pea-based or rice-based diet for 12 weeks.............................................. 189
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE</td>
<td>Advanced glycation end product</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>CBC</td>
<td>Complete blood count</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DE</td>
<td>Digestible energy</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual energy x-ray absorptiometry</td>
</tr>
<tr>
<td>DM</td>
<td>Dry matter</td>
</tr>
<tr>
<td>EF</td>
<td>Ejection Fraction</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FMD</td>
<td>Flow-mediated dilation</td>
</tr>
<tr>
<td>GE</td>
<td>Gross energy</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycated hemoglobin/hemoglobin A₁C</td>
</tr>
<tr>
<td>HOMA</td>
<td>Homeostasis model assessment</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>LSD</td>
<td>Least significant difference</td>
</tr>
<tr>
<td>LVFW</td>
<td>Left ventricle free wall</td>
</tr>
<tr>
<td>LVID</td>
<td>Left ventricular internal dimension</td>
</tr>
<tr>
<td>LVV</td>
<td>Left ventricular volume</td>
</tr>
<tr>
<td>ME</td>
<td>Metabolizable energy</td>
</tr>
<tr>
<td>MG</td>
<td>Methylglyoxal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>M-mode</td>
<td>Motion mode</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa-B</td>
</tr>
<tr>
<td>QUICKI</td>
<td>Quantitative insulin sensitivity check</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SV</td>
<td>Stroke volume</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>TTADC</td>
<td>Total tract apparent digestibility coefficient</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

1.1 Rationale

Nutrition plays an important role in the maintenance of health and prevention of disease. In dogs, nutrition has been implicated in the development of renal disease, obesity, rheumatism, dental disease and cardiovascular disease (Bailoni & Cerchiaro, 2005). Internationally the pet food industry is estimated to be worth $49 billion USD and increasing at about 5% annually (Bond, 2009). Consumers are becoming increasingly aware about the health and environmental impact of eating behaviours, both for themselves and their pets. Some owners believe that “natural” and “organic” foods provide long-term health benefits and sales of pet foods claiming to have health benefits are worth billions of dollars annually (Remillard, 2008). There are currently hundreds of dog foods on the market that make a wide variety of health claims. Because health claims for dog food are not regulated, many of the claims do not have a scientific basis. Thus, the development of a pet food with proven health benefits is important to pet owners as well as the pet food industry.

1.2 Research Design

The overall goal of this research was to develop a dog food with proven health benefits. To achieve this goal, the research described herein was divided into four studies to sequentially examine the short and longer term effects of different carbohydrate sources in dog food and their effect on various health parameters in dogs. Although each study had a different purpose, the overall theme of the studies was to examine the effect of diet treatment on metabolic and cardiovascular health. Thus, the primary variables of interest in all of the studies were similar and included the post-prandial glycemic and insulinemic responses to carbohydrate sources, changes in cardiac structure and function as measured by
echocardiography, and endothelial function as measured by flow-mediated dilation. In addition, each study examined unique variables that were of interest in the particular study.

Study 1 examined the acute effects of feeding four unprocessed carbohydrate sources that are suitable for use in pet foods (rice, corn, barley and peas). These carbohydrate sources were chosen because rice and corn are common pet food ingredients, but have been shown in humans to have a high glycemic index. Barley is becoming a more common pet food ingredient and was chosen because it typically has a mid-range glycemic index, though this can depend on the barley variety. Peas are a somewhat novel pet food ingredient and were chosen because in humans they have a low glycemic index. Peas also were chosen because they are a local crop, are used as an ingredient in pet foods manufactured by a Saskatchewan company (Horizon Pet Nutrition), and the Saskatchewan Pulse Growers are interested in promoting peas as a pet food ingredient. This was the first study performed in order to determine which of the four carbohydrate sources had the lowest and highest glycemic index as well as to examine the acute cardiovascular effects of single feedings of these foods. This information was then used to develop low and high glycemic index diets for study 2.

Study 2 was similar in design to study 1, but examined the acute effects of two complete dog diets which each contained only one carbohydrate source. The carbohydrate source in the diets was either rice, the highest glycemic index carbohydrate source based on the results from study 1, or peas, the lowest glycemic index carbohydrate source. In order to formulate the diets so that the macronutrient and energy contents were equivalent, the first step in this study was to perform digestibility trials to determine the total tract apparent digestibility of the main ingredients that would be used in the diets, namely chicken meal, rice and peas. Once the diets were formulated, their acute glycemic and cardiovascular effects
were tested using the same procedures as in study 1, and the apparent digestibility of the diets also were determined.

Once the acute effects of the pea and rice diets were tested in study 2, the next step was to study the longer term health effects of the diets. This led to the design of studies 3 and 4, which were performed simultaneously. Since all of the dogs were at ideal body weight (i.e. lean) for studies 1 and 2, and we wanted to examine the effects of the pea and rice diets in obese dogs, the first step was to allow the dogs to become obese. Allowing the dogs to become obese resulted in the creation of study 3, which compared metabolic and cardiovascular health parameters in the dogs when they were lean and obese. Once the dogs were obese, study 4 was initiated and the dogs were entered into a cross-over study using the pea and rice diets. After the cross-over study was complete, the dogs then needed to return to their lean body condition. After weight loss, the dogs were tested again. This testing time-point was part of study 3 and permitted a comparison of the dogs when they were lean, obese, and after weight loss. Tables 1.1 and 1.2 show the timelines and testing time points for studies 3 and 4.
<table>
<thead>
<tr>
<th>Period</th>
<th>Body Condition</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>December 2010-March 2011 (12 weeks)</td>
<td>Lean at start of period, obese at end of period</td>
<td>Ad libitum feeding of a commercial maintenance diet to promote weight gain</td>
</tr>
<tr>
<td>March 2011-June 2011 (12 weeks)</td>
<td>Obese</td>
<td>Randomly selected to receive either pea or rice diet</td>
</tr>
<tr>
<td>June 2011-August 2011 (8 weeks)</td>
<td>Obese</td>
<td>Washout period; dogs received ad libitum commercial maintenance diet to maintain obesity</td>
</tr>
<tr>
<td>August 2011-November 2011 (12 weeks)</td>
<td>Obese</td>
<td>Dogs received the pea or rice diet which they had not previously received</td>
</tr>
<tr>
<td>November 2011-April 2012 (21 weeks)</td>
<td>Obese at start of period, lean at end of period</td>
<td>Commercial maintenance diet in restricted amounts to promote weight loss</td>
</tr>
<tr>
<td>Time Point</td>
<td>Body Condition</td>
<td>Diet</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>December 2010</strong></td>
<td>Lean</td>
<td>Commercial diet fed in restricted amounts to maintain ideal body weight</td>
</tr>
<tr>
<td>March 2011</td>
<td>Obese</td>
<td>Commercial diet fed ad libitum</td>
</tr>
<tr>
<td>August 2011</td>
<td>Obese</td>
<td>Commercial diet fed ad libitum</td>
</tr>
<tr>
<td>April 2012</td>
<td>Weight loss</td>
<td>Commercial diet fed in restricted amounts. Weight loss (lean)</td>
</tr>
</tbody>
</table>

**Study 3**

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Body Condition</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>March 2011</td>
<td>Obese</td>
<td>Commercial diet fed ad libitum</td>
</tr>
<tr>
<td>June 2011</td>
<td>Obese</td>
<td>Pea or rice diet fed ad libitum</td>
</tr>
<tr>
<td>August 2011</td>
<td>Obese</td>
<td>Commercial diet fed ad libitum</td>
</tr>
<tr>
<td>November 2011</td>
<td>Obese</td>
<td>Pea or rice diet fed ad libitum</td>
</tr>
</tbody>
</table>

**Study 4**
The research for studies 1-4 was all performed in laboratory beagles, instead of pet dogs, in order to allow for maximum control over the dogs’ diet and lifestyle. Initially, six beagles (3 females and 3 males) were obtained from Covance Research Products Inc. (Princeton, NJ, USA). These dogs were used for study 1. Part way through the second study, it was decided that a home needed to be found for one of the female dogs because she was having idiopathic epileptic seizures with increasing frequency. To replace her and to increase the sample size, four beagles (3 females and 1 male) were obtained from University of Guelph (Guelph, ON, Canada). For the second study, five of the original dogs (3 males and 2 females) plus two of the dogs from Guelph (1 male and 1 female) were used. Subsequently, all nine of the dogs (5 females, 4 males) were started on the study protocols for studies 3 and 4. The studies were started in December 2010 with study 3 finishing in April 2012 and study 4 finishing in November 2011. In September 2011, one of the male dogs from Guelph spontaneously developed intervertebral disc disease that required steroid treatment and made him ineligible to continue in the study. He was adopted and did not complete the testing that was performed in November 2011 or April 2012. This resulted in study 3 having a sample size of n=8, and study 4 included n=9 dogs that tested the rice diet and n=8 dogs that tested the pea diet.
1.3 Objectives

The overall purpose of this research was to develop a low glycemic index canine diet with proven health benefits for dogs, namely decreased serum insulin and glucose concentrations, reduced food intake and weight gain, and improved cardiovascular health. The specific objectives of each study were:

**Study 1**

1. To determine the glycemic, insulinemic and cardiovascular responses as well as serum nitrotyrosine and methylglyoxal concentrations in dogs after single feedings of four complex carbohydrate sources (peas, corn, barley and rice) and one simple carbohydrate source (glucose).

**Study 2**

1. To determine the digestibility of the primary ingredients used to formulate a pea-based or rice-based dog diet.
2. To formulate two extruded dog diets that contain either a low (pea) or high (rice) glycemic index carbohydrate source.
3. To determine the digestibility of a pea-based or rice-based dog diet.
4. To determine the glycemic, insulinemic and cardiovascular responses in dogs to single feedings of the pea and rice diets.
5. To compare the glycemic index of individual ingredients (peas and rice from study 1) to the glycemic index of two extruded dog diets that contain either peas or rice as their sole carbohydrate source.

**Study 3**

1. To examine the effects of obesity on metabolic and cardiovascular parameters in dogs.
2. To induce weight loss in obese dogs and determine the effects of weight loss on metabolic and cardiovascular parameters.

**Study 4**

1. To determine the longer term effects of a pea-based diet versus a rice-based diet on body composition, glycemic and insulinemic responses, metabolic and inflammatory indicators as well as cardiac and vascular function in obese dogs.

**1.4 Hypotheses**

The overall hypothesis for this research was that low glycemic index carbohydrate sources will have metabolic and cardiovascular benefits compared to high glycemic index carbohydrate sources in lean and obese dogs. The specific hypotheses of each study were:

**Study 1**

1. Peas will result in the lowest post-prandial glycemic response compared to corn, barley and rice.

2. Carbohydrate sources that result in lower post-prandial glycemic responses will have beneficial effects on the cardiovascular system in dogs.

3. Elevated methylglyoxal levels will cause impaired endothelial function via increased oxidative stress after a single feeding of a high glycemic index food.

**Study 2**

1. Compared to a rice-based dog diet, single readings of a pea-based diet will result in lower glycemic and insulinemic responses as well as improved cardiovascular function.

2. Peas and rice that are extruded in a dog diet will have a higher glycemic index than unprocessed peas and rice.
Study 3

1. Obesity in dogs will result in glucose intolerance, insulin resistance, detrimental changes in cardiac structure and function, elevated plasma concentrations of leptin and C-reactive protein, and reduced plasma adiponectin.

2. The detrimental changes that occur with obesity dogs will be more closely correlated to the amount of visceral, not subcutaneous, fat in dogs.

3. Weight loss will reverse the detrimental metabolic and cardiovascular effects that occur with obesity in dogs.

Study 4

1. Compared to a rice-based diet, a pea-based diet will result in improved glucose tolerance and insulin sensitivity, reduced total and visceral body fat, a more favourable adipokine profile, and improved cardiovascular health in obese dogs.
CHAPTER 2
LITERATURE REVIEW

2.1 Obesity and Weight Management

2.1.1 Obesity

Obesity is defined by excess body fat sufficient to cause or contribute to disease (Laflamme, 2011). Increased morbidity and mortality is associated with increasing body fat mass (German, 2006). In humans, body mass index, calculated as weight (kg) divided by height (m^2), is commonly used to assess the degree of excess weight, with overweight and obesity defined as a BMI of 25-30 and >30 kg/m^2, respectively (Health Canada, 2005). Although some diseases and pharmaceutical drugs can cause obesity, most often obesity is due to excessive energy intake compared to energy expenditure and results from the complex interactions between social, behavioural, physiological, metabolic and genetic factors (German, 2006; Kotsis et al., 2010).

In dogs, the prevalence of obesity in developed countries is estimated to be 25-40% (Laflamme, 2006). This high prevalence is concerning given that even moderately overweight dogs are at higher risk of earlier morbidity and mortality (Kealy et al., 2002; Laflamme, 2006; Larson et al., 2003; Lawler et al., 2005; Lawler et al., 2008; Smith et al., 2006). The impact of environmental factors likely plays a large role in the development of canine obesity since a significant association has been found between the degree of overweight in dogs and the BMI of their owners (Nijland et al., 2010). Dogs are considered to be clinically obese when their body weight exceeds 15% of their ideal body weight (Gossellin et al., 2007). Canine obesity is ideally identified and monitored using a combination of three techniques: body weight; body condition score; and body composition (Gossellin et al., 2007). A 9-point body condition score scale has been shown to be reproducible and has good correlation with other
methods used to determine body fat, including dual energy x-ray absorptiometry (DEXA) and deuterium oxide dilution (Laflamme, 1997; Mawby et al., 2004). As the score increases above the ideal score of 5, each unit on the scale is approximately equal to 10-15% greater than ideal body weight (Laflamme, 2006). Table 2.1 provides a description of each point on the scale.

Advantages of using body condition score is that it is simple to perform and does not require specialized equipment; however, it does not discern the location of fat (i.e. subcutaneous versus visceral fat). DEXA is a common technique used to quantify body fat in dogs and is advantageous because of the low within-animal coefficient of variation (<2%) (Toll et al., 1994). However, it too lacks the ability to determine the location of body fat. Computed tomography (CT) scanning also can be used to determine body composition which has the major advantage of being able to evaluate visceral and subcutaneous fat separately (Ishioka et al., 2005). Disadvantages of CT scanning in dogs include the need for anesthesia to inhibit movement, radiation exposure, cost and the availability of equipment (Ishioka et al., 2005).
### Table 2.1 Body condition score system for dogs.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Emaciated</td>
<td>Ribs, lumbar vertebrae, pelvic bones and all bony prominences evident from a distance. No discernible body fat. Obvious loss of muscle mass.</td>
</tr>
<tr>
<td>3 Thin</td>
<td>Ribs easily palpated and may be visible with no palpable fat. Tops of lumbar vertebrae visible. Pelvic bones becoming prominent. Obvious waist and abdominal tuck.</td>
</tr>
<tr>
<td>4 Underweight</td>
<td>Ribs easily palpable, with minimal fat covering. Waist easily noted, viewed from above. Abdominal tuck evident.</td>
</tr>
<tr>
<td>5 Ideal</td>
<td>Ribs palpable without excess fat covering. Waist observed behind ribs when viewed from above. Abdomen tucked up when viewed from side.</td>
</tr>
<tr>
<td>6 Overweight</td>
<td>Ribs palpable with slight excess fat covering. Waist is discernable viewed from above but is not prominent. Abdominal tuck apparent.</td>
</tr>
<tr>
<td>7 Heavy</td>
<td>Ribs palpable with difficulty, heavy fat cover. Noticeable fat deposits over lumbar area and base of tail. Waist absent or barely visible. Abdominal tuck may be absent.</td>
</tr>
<tr>
<td>8 Obese</td>
<td>Ribs not palpable under very heavy fat cover, or palpable only with significant pressure. Heavy fat deposits over lumbar area and base of tail. Waist absent. No abdominal tuck. Obvious abdominal distention may be present.</td>
</tr>
</tbody>
</table>

(Laflamme, 1997)
2.1.2 Health Effects of Obesity

The primary mechanisms by which obesity can predispose a human or animal to disease are through the mechanical/physical effects and through the subsequent alterations in endocrine function (German et al., 2010). Obesity in humans results in numerous hormonal and glucose metabolism changes that increase the risk for type 2 diabetes and cardiovascular disease (Gayet et al., 2004; Hoenig, 2002; Martin et al., 2006). Obesity affects the heart by increasing cardiac output and stroke volume in proportion to the degree of excessive weight (Van Vliet et al., 1995). Cardiac hypertrophy and impairment in systolic and diastolic function are also common with obesity (Van Vliet et al., 1995). Obesity is recognized as a state of chronic, low-grade inflammation and has been shown in humans to result in elevated levels of inflammatory markers such as C-reactive protein, interleukin-6 and tumour necrosis factor alpha (German et al., 2010). Obesity also has been shown in humans to increase the production of reactive oxygen species (ROS) within adipose tissue leading to oxidative stress (German et al., 2010).

Both the amount and distribution of adipose tissue may have a negative impact on health. In humans, body fat stored in the central segment of the body (i.e. visceral fat) is more strongly associated with obesity-related disorders than subcutaneous fat (Bergman et al., 2006). This may be due to differences in the metabolic and endocrine functions of the different adipose depots (Leray et al., 2008). Visceral fat is a risk factor for insulin resistance which may be due to its higher lipolytic activity and anatomic location upstream of the liver (Leray et al., 2008). In dogs, visceral body fat also may be particularly damaging. In a canine model of obesity, adipocytes from the visceral depot were found to be larger than that of subcutaneous adipocytes (Kabir et al., 2011). Visceral adipocytes larger than 75 µm were
found to be independent predictors of whole body and hepatic insulin resistance (Kabir et al., 2011).

### 2.1.3 Glucose Tolerance and Insulin Resistance

Obesity is associated with impaired glucose tolerance, which is defined as blood glucose levels that are higher than normal after an oral or intravenous glucose load (Ceriello, 2004). Post-prandial hyperglycemia (i.e. elevated blood glucose levels after eating) is believed to induce oxidative stress (Node & Inoue, 2009). Evidence suggests that post-prandial hyperglycemia is an independent risk factor for cardiovascular disease and is a better predictor of future cardiovascular events than either fasting glucose or glycated hemoglobin (Node & Inoue, 2009). Even in the absence of overt diabetes, post-prandial hyperglycemia and high-normal blood glucose levels may have deleterious effects on cardiovascular health (Thomas et al., 2004; Zhu et al., 2007).

Post-prandial glucose levels may be a better predictor of vascular dysfunction and cardiovascular events than fasting glucose in both diabetic and healthy individuals (Siervo et al., 2011). Maintaining good glycemic control is an important factor in preventing long-term complications in individuals with impaired glucose tolerance and diabetes. Among humans with well-controlled type 2 diabetes, but who follow different insulin regimes, those on a more intensive regime that results in lower post-prandial glucose levels show less vascular pathology (von Bibra et al., 2009).

Insulin is released from the pancreatic β-cells for blood glucose regulation (Borai et al., 2007). Insulin lowers blood glucose by facilitating uptake by tissues, primarily muscle and adipose tissue, and inhibiting endogenous glucose production by the liver (Leray et al., 2008).
Thus, insulin sensitivity and the amount of insulin delivered to target tissues affects blood glucose regulation (Kaiyala et al., 1999).

In humans, obesity is associated with insulin resistance, hyperinsulinemia and the cardiometabolic syndrome (Govindarajan et al., 2008). Insulin resistance is characterized by increased fasting plasma insulin and exaggerated insulin response to a glucose load (Brunetto et al., 2011; Gayet et al., 2004). One of the mechanisms by which hyperinsulinemia may contribute to cardiovascular disease may be due to increased sodium retention as a result of insulin’s action on the renal tubules (Kotsis et al., 2010).

Lifetime diet restriction in dogs is associated with higher insulin sensitivity and reduced morbidity and mortality (Larson et al., 2003). On the contrary, there is evidence that obesity and high dietary fat intake in dogs causes impaired glucose tolerance and insulin resistance (Kaiyala et al., 1999). In dogs, insulin resistance has been shown to be associated with weight gain. A study by Bailhache et al. (2003) used the euglycemic hyperinsulinemic clamp technique to assess insulin sensitivity in lean dogs and after the dogs had gained at least 20% of their initial body weight for at least 5 weeks (Bailhache et al., 2003). Baseline glucose concentrations were the same in the normal and obese dogs whereas baseline insulin concentrations were significantly higher in the obese dogs. To maintain euglycemia, the glucose infusion rate was higher in the normal than obese dogs, indicating reduced insulin sensitivity in the obese dogs. In the obese state, the dogs were found to have high plasma triglyceride levels and low HDL levels which are also common changes observed in insulin resistant humans (Bailhache et al., 2003).

Obesity does not appear to cause overt type 2 diabetes in dogs as is seen in humans and cats (Rand et al., 2004). The physiological reason for dogs being resistant to type 2
diabetes is of interest because solving this puzzle may help in the treatment and prevention of type 2 diabetes in humans. One mechanism may be related to differences in insulin secretion between dogs and humans. A study by Verkest et al. (2011a) found that obese dogs did not lose first-phase insulin secretion in response to intravenous glucose administration, one of the early markers of the development of type 2 diabetes. It also has been suggested that pancreatic β-cells in dogs may not be as sensitive to hyperglycemic toxicity as humans or may lack a component in the pathophysiology of β-cell failure (Verkest et al., 2012). Additional research is needed to determine why obese dogs are resistant to developing type 2 diabetes.

2.1.3.1 Assessment of Glucose Tolerance and Insulin Resistance

Various methods have been investigated to measure glucose tolerance and insulin sensitivity. These methods can be classified as dynamic tests (samples are collected serially) or single specimen tests (samples collected at one time point) (Borai et al., 2007). Common examples of dynamic function tests include the hyperinsulinemic euglycemic clamp, the insulin tolerance test, the intravenous glucose tolerance test, and the oral glucose tolerance test (Borai et al., 2007). Single specimen tests include homeostasis model assessment (HOMA) and the quantitative insulin sensitivity check (QUICKI) (Borai et al., 2007). Each test has unique advantages and disadvantages which must be taken into account when selecting the appropriate technique for a particular study. The clamp technique, oral glucose tolerance test and HOMA technique are commonly used techniques and thus will be briefly reviewed here.

The hyperinsulinemic euglycemic clamp is considered to be the gold standard for assessing insulin sensitivity \textit{in vivo} in humans and animals (Borai et al., 2007; Muniyappa et al., 2008). It involves infusing exogenous insulin at a constant rate along with a variable
amount of glucose to maintain euglycemia (Borai et al., 2007). Individuals with high insulin sensitivity will require considerably more exogenous glucose to maintain euglycemia than those who are insulin resistant (Borai et al., 2007). However, disadvantages of this technique are that it is technically demanding, laborious, time consuming and expensive (Borai et al., 2007; Muniyappa et al., 2008). In addition, it is performed under non-physiological conditions since glucose is given intravenously and bypasses the effect of the gastrointestinal tract on glucose and insulin metabolism (Borai et al., 2007).

The oral glucose tolerance test reflects the ability of the body to dispose of glucose after a glucose load and is a useful method to assess glucose tolerance (Muniyappa et al., 2008). In humans, it is performed by having fasted subjects consume 75 g of glucose (~1 g glucose per kg body weight) dissolved in water. Blood glucose concentrations are then measured over the subsequent 2 hours. An index of whole body insulin sensitivity, which has been shown to have good correlation with insulin sensitivity measurements performed by the hyperinsulinemic euglycemic clamp, can then be calculated as:

\[
\text{Equation 2.1} \quad \frac{10000}{\sqrt{\left[\text{fasting glucose (mg/dL)} \times \text{fasting insulin (µU/mL)}\right] \times \left[\text{mean glucose} \times \text{mean insulin}\right]}}
\]

Limitations of the oral glucose tolerance test are the large intraindividual variation and the influence of gastrointestinal emptying (Borai et al., 2007).

Homeostasis model assessment is based on the principle that a given combination of fasting insulin and glucose concentrations will be a reflection of β-cell deficiency and insulin resistance. HOMA uses a mathematical model of fasting glucose and insulin interactions to
determine insulin resistance and β-cell deficiency (Borai et al., 2007). This method is
convenient and easy for the study subjects but its precision is poor (Borai et al., 2007). In
addition, since this model was developed in humans, it cannot be assumed that it can be
applied to other species and more validation studies are needed for other animals (Muniyappa
et al., 2008; Verkest et al., 2010).

2.1.3.2 Advanced Glycation End-Products

Advanced glycation end products (AGEs) are the products of non-enzymatic
glycosylation and oxidation of proteins and lipids and are an indicator of glycemic control.
AGEs bind to receptors in endothelial cells, vascular smooth muscle cells and monocytes to
trigger multiple signaling pathways and the generation of reactive oxygen species (Rammos et
al., 2008). AGEs generate ROS in part by the activation of NADPH oxidase, resulting in
reduced endothelial nitric oxide synthase (eNOS) activity, and directly affecting endothelial
cells by increasing expression and release of vascular cell adhesion molecule-1 (Rammos et
al., 2008). An example of a commonly measured AGE is glycated hemoglobin (HbA1c),
which is a non-enzymatically formed product that is directly related to serum glucose
concentration over the preceding 2-3 months in both dogs and humans (Jorgensen et al, 2004;
Loste & Marca, 2001; Siervo et al., 2011). AGEs cause cross-linking of proteins which results
in proteins that are normally flexible to become rigid (Vasan et al., 2003). AGEs tend to
accumulate and exert their greatest effects in proteins with very low turnover such as collagen
and elastin (half-lives of years to decades, respectively). Glycated elastin, in particular, has
serious cardiovascular consequences since this decreases vascular elasticity (Baydanoff et al.,
1984; Konova et al., 2004).
2.1.3.3 Methylglyoxal

Methylglyoxal is a precursor of advanced glycation end products produced primarily during glycolysis and is a highly reactive electrophilic dicarbonyl aldehyde compound (Desai et al., 2010). Methylglyoxal reacts with the amino acids arginine and lysine in some proteins to produce AGEs (Dhar et al., 2008). Methylglyoxal is considered to be one of the most reactive advanced glycation end-product precursors in endothelial cells (Brouwers et al., 2010). Methylglyoxal is associated with the formation of ROS by mitochondria which could be an initial event in the development of vascular complications (Brouwers et al., 2010). The accumulation of methylglyoxal may play a vital role in the development of insulin resistance (Jia & Wu, 2007). Methylglyoxal has been shown to be a sensitive indicator of glycemic fluctuation (Nemet et al., 2005). After a single dose of 75 g glucose, post-prandial plasma methylglyoxal significantly increased in healthy men (Masterjohn et al., 2012).

2.1.4 Dietary Treatment of Obesity

In both dogs and humans, diet therapy is often used in the treatment and prevention of obesity and its associated conditions (Flight & Clifton, 2006; Lee et al., 2008; Livesey et al., 2008; O'Keefe et al., 2008). In all animal species, in order for weight loss to occur, the amount of energy consumed must be less than the amount of energy expended. A successful weight loss program results in loss of fat mass with little or no loss of lean body mass, which may be achieved in dogs by providing a high quality and adequate level of protein (Diez et al., 2004). A loss of 1-2% body weight per week is ideal for dogs in order to minimize hunger, prevent the loss of lean body mass and to prevent rebound weight gain (Roudebush et al., 2008).
High fat diets are known to contribute to obesity in dogs (Laflamme, 2011). On the contrary, protein as well as the amount and type of dietary fibre appear to be key nutrients to consider in weight loss diets for dogs (Laflamme, 2011; Weber et al., 2007; Yamka et al., 2007). Weight loss diets for dogs often contain increased levels of fibre with the intention of decreasing caloric intake by increasing the bulk of the diet, reducing the caloric density and decreasing digestibility (Sunvold & Murray, 2003). However, adverse effects of a high fibre diet include decreased nutrient availability, increased fecal volume, poor skin and coat quality, and poor palatability (Sunvold & Murray, 2003).

2.1.5 Adipokines

Adipose tissue is no longer viewed as simply a storage organ for excess energy, but as an active organ that secretes hormones (i.e., adipokines or adipocytokines) that communicate with the brain and peripheral tissues to regulate energy homeostasis, insulin sensitivity, and lipid and carbohydrate metabolism (Clarke & Judd, 2008; German et al., 2010). Adipokines are secreted from adipocytes as well as the associated cells such as macrophages (Laflamme, 2011). One of the mechanisms by which diet and lifestyle therapy may induce benefits for obese individuals is through reduction in systemic inflammation and improvement in the adipokine secretory profile (Klimcakova et al., 2010). Differences in adipokine secretion from visceral fat may be one of the reasons why visceral fat is more detrimental to health than fat located in other areas of the body, such as intramuscular, perivascular and epicardial adipose tissue (Laflamme, 2011).

Canine adipocytes express key adipocyte hormones and inflammation-related adipokines and are a highly active endocrine and secretory organ (Ryan et al., 2010). However, adipokine research in dogs is still in its infancy. With the development of enzyme-
linked immunosorbent assay (ELISA) kits that are specific for canine adipokines, such as leptin and adiponectin, more research in this area can now be performed (Radin et al., 2009).

2.1.5.1 Leptin

Leptin is a product of the *ob* gene and leptin concentrations are positively correlated with adipose tissue mass (Nishii et al., 2010). Leptin plays a key role in the regulation of whole body energy balance and body weight by suppressing appetite and promoting energy expenditure through stimulation of sympathetic activity (Ishioka et al., 2007; Kotsis et al., 2010; Morris & Rui, 2009). Leptin induces oxidative stress and upregulation of adhesion molecules in endothelial cells and thus, may play a direct role in the pathogenesis of endothelial dysfunction (Fantuzzi, 2005). It also may play an indirect role in vascular pathology by stimulating the production of C-reactive protein, an acute phase inflammatory protein, in the liver which then increases inflammation in the endothelium (Knudson et al., 2008). In some obese individuals circulating levels of leptin are high, but this does not lead to the expected reduction in food intake and increased energy expenditure, suggesting these individuals are leptin resistant (Kotsis et al., 2010). Whether or not leptin resistance also occurs in dogs is uncertain (Laflamme, 2011).

Leptin may be used in assessment of adiposity in dogs since plasma leptin concentration is strongly correlated with body weight and body fat content (Sagawa et al., 2002). Fat mass appears to be the primary factor affecting leptin concentrations in dogs (Radin et al., 2009) and hyperleptinemia has been observed in overweight dogs fed a high fat diet (Knudson et al., 2005). Leptin levels are affected by meal timing and dogs show a peak in leptin levels about 5-8 hours after feeding (Ishioka et al., 2005a). Serum leptin levels in healthy dogs have been reported to range from 1.4 to 5.6 ng/ml with a mean of 3.0 ± 0.3
ng/ml (Iwase et al., 2000). Obese dogs were found to have plasma leptin concentrations of 13.1 ± 7.6 ng/ml compared to 1.1 ± 0.3 ng/ml in lean dogs (Nishii et al., 2010). Leptin can be measured by ELISA using commercially available, canine-specific kits (Tvarijonaviciute et al., 2011).

### 2.1.5.2 Adiponectin

Adiponectin is a 30-kD adipokine and is the most abundantly expressed adipokine in adipose tissue (Inadera, 2008). Adiponectin plays a central role in a number of metabolic processes such as glucose uptake, fatty acid oxidation and gluconeogenesis (Brand-Herrmann, 2009). In humans, adiponectin contrasts with leptin in that conditions that are associated with high leptin levels, such as obesity, cause adiponectin concentrations to be low (Clarke & Judd, 2008; Hopkins et al., 2007; Shetty et al., 2009). In humans, average plasma adiponectin levels are 5-10 µg/ml (Inadera, 2008).

Initially it was believed that adiponectin is secreted exclusively by adipose cells, but there is also evidence that it is secreted by cardiomyocytes, skeletal muscle and osteoblasts (Brand-Herrmann, 2009). Adiponectin has an anti-inflammatory function and may be protective against inflammatory processes such as atherosclerosis by inhibiting the expression of adhesion molecules, decreasing the proliferation of smooth muscle cells, and preventing the conversion of macrophages to foam cells (Brand-Herrmann, 2009; Clarke & Judd, 2008; German et al., 2010; Inadera, 2008).

Adiponectin exerts insulin sensitizing effects, lowers hepatic glucose production and increases glucose uptake and fatty acid oxidation in skeletal muscle (Inadera, 2008). In humans, low concentrations of adiponectin are associated with insulin resistance, hyperinsulinemia, reduced HDL and increased LDL levels (Clarke & Judd, 2008).
Hypoadiponectemia is an independent risk factor for future cardiovascular events such as myocardial infarction, suggesting that adiponectin has direct effects on vascular health (Clarke & Judd, 2008). Visceral fat content, rather than total fat, appears to be the primary factor that determines adiponectin concentrations (Clarke & Judd, 2008).

Canine adiponectin is similar to other species in its nucleotide and amino acid sequences (Radin et al., 2009). However, in dogs, there is some controversy about whether or not adiponectin is decreased with obesity (Verkest et al., 2011; Verkest et al., 2011b) and whether adiponectin increases in response to weight loss as is seen in humans (Wakshlag et al., 2011). Adiponectin can be measured in serum or plasma and either EDTA or heparin can be used as an anticoagulant when using commercially available, canine-specific ELISA kits (Radin et al., 2009; Tvarijonaviciute et al., 2010).

2.1.6 Weight Loss

Weight loss is recommended for overweight and obese individuals with the goal of reversing the negative health effects associated with obesity. Research suggests that some, but not all, metabolic and cardiovascular changes that occur with obesity are reversed with subsequent weight loss. In humans, weight loss is associated with decreased pro-inflammatory markers and increased anti-inflammatory markers, with the greatest improvements seen among those who have lost at least 10% body weight (Forsythe et al., 2008). Weight loss using a low calorie diet also has been shown to reduce serum advanced glycation end products (Gugliucci et al., 2009).

Weight loss may also improve cardiovascular health. Obesity in humans is associated with increased left ventricular mass which may contribute to increased cardiovascular events and mortality (Haufe et al., 2012). However, after six months of caloric restriction, left
ventricular mass, heart rate and cardiac output were found to significantly decrease in humans regardless of whether they consumed a restricted fat or a restricted carbohydrate diet (Haufe et al., 2012). On the contrary, a meta-analysis that examined the effect of weight loss on all-cause mortality risk found that weight loss among otherwise healthy obese and overweight individuals did not prolong life (Harrington et al., 2009).

In dogs, even after weight loss, obesity-induced changes in metabolic parameters seem to persist. A comparison of glucose intolerance and insulin resistance in dogs that were lean, then gained weight and subsequently lost weight showed that although glucose and insulin levels improved with weight loss, levels remained elevated compared to when the dogs were lean (Brunetto et al., 2011). Energy metabolism also seems to be altered by weight gain and weight loss. Nagaoka et al. (2009) studied the amount of energy required to induce weight gain, weight loss, and subsequent weight re-gain in beagles. The results showed that less time and fewer calories were necessary to cause the same degree of weight re-gain compared to the first time that obesity was induced. Thus, food efficiency increased during the obesity reinduction period (Nagaoka et al., 2009). These results highlight why weight maintenance after weight loss can be challenging and emphasizes the importance of preventing initial weight gain.

2.1.7 Dogs as a Model for Humans

Studying the effect of diet on obesity and cardiovascular disease in dogs could also provide valuable information for human applications. Dogs are suitable models for humans when studying the effect of diet on chronic disease for several reasons. First, dogs possess a gastrointestinal tract similar to humans in that their intestinal to body length ratio is similar and they experience similar patterns in motility (Knapp et al., 2008). Second, dogs and
humans are both omnivorous and eat a diet that is similar in macronutrient composition (Knapp et al., 2008).

Dogs may be advantageous as a model because dietary manipulation in animals is much easier than with humans and complete control of dietary intake can be achieved. For example, it is difficult to determine if reduced body weight or adiposity is solely attributed to low dietary glycemic index because interventions designed to modify dietary glycemic index may also influence other factors affecting body weight such as fibre content, palatability and energy density (Pawlak et al., 2004). Since dogs can eat the same diet for long durations, isolating the effect of glycemic index on health parameters is simplified. In addition, dogs are a superior animal model for humans compared to rodents because of the closer homology between canine and human genes (Ryan et al., 2010).

However, as with all models, there are limitations to using dogs as models for humans. High-density lipoprotein transports as much as 90% of the total circulating lipids in dogs, whereas in humans low-density lipoproteins are the primary lipoproteins (Bailhache et al., 2003; Maldonado et al., 2001). Cardiovascular disease also does not manifest in dogs the same way it does in humans, with atherosclerosis and ischemic heart disease being uncommon in dogs. Despite these differences in the end-stage cardiovascular pathology between humans and dogs, the early pathological events are still likely to be similar in both species.

2.2 Cardiovascular Disease

2.2.1 Association between Obesity and Cardiovascular Disease

In humans, obesity and cardiovascular disease are known to be strongly linked. Obesity results in increased risk of factors associated with obesity, including hypertension, dyslipidemia and insulin resistance. Obesity also has been identified as an independent risk
factor for ischemic heart disease and heart failure (Lopaschuk et al., 2007). Obesity results in changes in cardiac fatty acid metabolism that may play a causal role in the development of obesity-related cardiomyopathies due to cardiac lipid accumulation and excessive fatty acid utilization (Lopaschuk et al., 2007). Canine cardiovascular function can be impaired with age resulting in decreased blood flow, arterial compliance and arterial distensibility (Guglielmini, 2003). Thus, nutrition and obesity may play roles in the maintenance of cardiovascular health in dogs, particularly as they get older; however, this is an area that has not been thoroughly studied.

2.2.2 Diet and the Prevention of Cardiovascular Disease

In humans, diets rich in fruits, vegetables, legumes, fish, poultry and whole grains are associated with protection against cardiovascular disease, whereas diets high in red meat, processed meat, refined grains, sweets, deep-fried foods and high fat dairy products increase disease risk (Lopez-Garcia et al., 2004). In dogs, the association between diet and cardiovascular disease is less clear. Most studies in dogs have investigated the effect of diet on treatment, not prevention, of cardiac disease. Nutrients that have been the most thoroughly studied in dogs in relation to cardiac disease include taurine, carnitine, omega-3 fatty acids and sodium, with diets lower in sodium and higher in taurine, L-carnitine, and omega-3 fatty acids showing some benefit for dogs with cardiac disease (Freeman, 1998; Freeman et al., 2006; Gompf, 2005; Sanderson, 2006). However, the effect of carbohydrates on cardiovascular disease risk in dogs is unknown.

2.2.3 Inflammation and Oxidative Stress

Body cells and tissues are at continuous risk of damage by free radicals and ROS (Garcia-Lafuente et al., 2009). Oxidative stress is an imbalance between the levels of ROS
and antioxidants in the body which can result in cellular injury and tissue damage (McMichael, 2007). The major source of ROS in cells is electron leakage from electron transport chains (McMichael, 2007). In both humans and dogs, obesity is associated with increased markers of oxidative stress (Laflamme, 2011).

Oxidative stress and inflammation are closely linked. ROS can attract inflammatory mediators, thereby inducing a generalized inflammatory response and tissue damage (Garcia-Lafuente et al., 2009). In pathophysiological states, inflammatory cells, such as neutrophils, macrophages, and eosinophils, contribute substantially to production of ROS (McMichael, 2007).

The nuclear factor kappa-B (NFκB) transcriptional system is a major effector pathway involved in inflammation (Garcia-Lafuente et al., 2009). NFκB promotes the transcription of multiple proinflammatory cytokines, including tumour necrosis factor-α (TNFα) and interleukin-6 (IL-6) (Garcia-Lafuente et al., 2009). TNFα is the first step in the inflammatory cascade and induces the product of IL-6 (Bullo et al., 2007). Both TNFα and IL-6 are elevated in obesity and diabetes (Bullo et al., 2007). In turn, IL-6 controls C-reactive protein production (Bullo et al., 2007).

2.2.3.1 C-Reactive Protein

C-reactive protein (CRP) is a 115-kDa pentamer expressed primarily by hepatocytes in response to tissue damage, infection, inflammation and malignant neoplasia (Nilsson, 2005). CRP is one of the most important inflammatory markers in humans and is positively associated with obesity, fasting glucose and insulin levels, blood pressure and lipid profile (Bullo et al., 2007). In humans, elevated levels of CRP are associated with coronary events through a possible cause-and-effect relationship that has still not been firmly established.
(Mathieu et al., 2009). Visceral fat may be particularly harmful because of its high production of IL-6 which promotes the secretion of acute phase proteins, such as CRP, by the liver (Mathieu et al., 2009).

In dogs, as in humans, CRP is a useful indicator of inflammation (Otabe et al., 1998). A study that examined CRP levels in dogs with a variety of diseases found that the diseases in which CRP was most frequently elevated were in neoplastic disease and immune-mediated diseases (Nakamura et al., 2008). More recently, CRP has also been shown to be elevated in dogs with congestive heart failure (median 4.22, range 0.43-45.44 µg/mL) compared to healthy dogs (median 0.88, range 0-19.87 µg/mL; P=0.02) (Cunningham et al., 2012). CRP values in normal beagles taken at different times throughout the day over 28 days were found to range from 0.8-22.6 µg/ml (mean 3.65 ± 1.40 µg/ml) and did not appear to be affected by circadian rhythm (Otabe et al., 1998). CRP can also be measured using commercially available, canine-specific ELISA kits.

2.2.3.2 Nitrotyrosine

Nitrotyrosine is a marker of oxidative stress. Inactivation of nitric oxide by the superoxide radical results in the production of peroxynitrite, a potent oxidant (Ceriello, 2002). Peroxynitrite has been implicated in the pathogenesis of many chronic diseases and may play a role in atherosclerosis due to its ability to initiate low density lipoprotein oxidation (Oldreive & Rice-Evans, 2001). Peroxynitrite nitrates amino acids such as tyrosine to produce nitrotyrosine. Thus, the production of peroxynitrite can be indirectly inferred by the presence of nitrotyrosine (Ceriello, 2002). Nitrotyrosine also may cause oxidative damage that could contribute to atherosclerosis and other inflammatory conditions (Mohiuddin et al., 2006). Nitrotyrosine is normally not detectable in the plasma of healthy individuals (Ceriello, 2002).
However, elevated nitrotyrosine levels have been found in patients with inflammatory conditions and diabetes as well as among smokers (Ceriello, 2002; Mohiuddin et al., 2006; Pereira et al., 2008).

Hyperglyemia has been shown to increase the release of nitric oxide and superoxide, with the increase in superoxide production being about double that of nitric oxide (Ceriello, 2002). This imbalance results in excess generation of peroxynitrite and reduced biological effects of nitric oxide (Ceriello, 2002). Thus, hyperglycemica may play a direct role in the production of nitrotyrosine and cause impaired endothelial function (Ceriello, 2002). Effects in dogs are unknown.

2.2.4 Endothelial Function

The single layer of endothelial cells that line the blood vessels play an important homeostatic role in the vascular system (Al-Qaisi et al., 2008). Healthy endothelial cells inhibit platelet aggregation, white blood cell adhesion and smooth muscle cell proliferation (Moens et al., 2005). Nitric oxide is produced by the endothelium, primarily by eNOS, but can be scavenged by excess reactive oxygen species (Schiffrin, 2008). Nitric oxide is a critical regulatory molecule involved in vasodilation and is anti-atherogenic (Al-Qaisi et al., 2008; Puglia et al., 2006). In response to shear stress, calcium entry into endothelial cells increases (Corretti et al., 2002). Calcium subsequently activates eNOS which generates nitric oxide and results in vasodilation (Corretti et al., 2002). Endothelial dysfunction due to decrease NO bioavailability is now believed to be the initial step in the development of atherosclerosis (Node & Inoue, 2009).

There is increasing evidence that hyperglycemia is damaging to the endothelium (Kawano et al., 1999; Thomas et al., 2004; Wascher et al., 2005). Even moderate elevations in
blood glucose can impair endothelial function as seen not only in patients with overt diabetes, but also in healthy individuals and those with impaired glucose tolerance (Kim et al., 2003; Node & Inoue, 2009; Title et al., 2000; Williams et al., 1998). Although hyperglycemia induces endothelial dysfunction, the mechanism by which this occurs is unclear. Impaired flow-mediated dilation (FMD) with hyperglycemia suggests that nitric oxide bioavailability is reduced (Mah et al., 2011). This could be due to reduced nitric oxide biosynthesis, elevated consumption of nitric oxide by ROS or increased uncoupling of eNOS (Mah et al., 2011). Oxidative stress plays a critical role in vascular endothelial dysfunction and there is evidence that hyperglycemia results in the overproduction of ROS (Node & Inoue, 2009; Yano et al., 2004). In addition, it has been shown that nitric oxide bioavailability is reduced among individuals with diabetes (Masha et al., 2009). The formation of AGEs as a result of hyperglycemia may also contribute to endothelial dysfunction (Brouwers et al., 2010).

The relative effects of obesity and dysglycemia on impairment of endothelium-dependent vasodilation were investigated a study by Han et al. (2011). The results found that dysglycemia, insulin resistance and obesity independently resulted in impairment of endothelium-dependent vasodilation, though multivariate modeling suggested that the relative contribution of dysglycemia was less than that of obesity. Effects in dogs may be similar, but are unknown.

2.2.4.1 Flow-Mediated Dilation

FMD is the most reproducible, non-invasive technique of assessing endothelial function (Donald et al., 2006). FMD measures changes in brachial artery diameter in response to shear stress induced by increasing the local blood flow (reactive hyperemia) which is principally mediated by nitric oxide (Al-Qaisi et al., 2008; Moens et al., 2005). Mediators
other than nitric oxide may be involved, but FMD is substantially reduced by inhibition of eNOS (Jiang et al., 2011).

In healthy people, FMD is 7-10%, whereas patients with cardiovascular disease have values of 0-5% or sometimes may even be negative (i.e. paradoxical vasoconstriction after shear stimulus) (Moens et al., 2005). Impaired FMD indicates impaired endothelial function and is associated with increased risk of serious cardiovascular events in humans (Moens et al., 2005). Studies have shown that FMD is an independent predictor of future cardiac events (Green et al., 2011). A meta-analysis reported that in humans, a 13% decrease in future risk of cardiovascular events exists for every 1% increase in FMD (Inaba et al., 2010).

Diet has been shown to affect FMD in humans. Volek et al. (2009) investigated the effect of hypocaloric diets that were restricted in either carbohydrate or fat among overweight men and women. FMD was assessed pre- and post- ingestion of a high fat meal, before and 12 weeks after consuming the carbohydrate or fat restricted diets. At 12 weeks, FMD increased 3 hours after the test meal in the subjects receiving the carbohydrate restricted diet, but was decreased in the subjects receiving the fat restricted diet (Volek et al., 2009).

To perform FMD, ultrasound is used to image the brachial artery before and after inflation of a sphygmomanometric cuff to suprasystolic pressure (at least 50 mm Hg above systolic pressure) (Corretti et al., 2002). Subsequent deflation of the cuff results in shear stress and the release of nitric oxide which causes vasodilation. FMD is the percent change in artery diameter from baseline compared to the maximum dilation after cuff release, calculated as (Harris et al., 2010):

### Equation 2.2

\[
FMD = 100\% \times \left(\frac{\text{maximum diameter after cuff release} - \text{baseline diameter}}{\text{baseline diameter}}\right)
\]
The methods used to perform FMD need careful consideration because of the many variables that can affect the outcome. Currently, most of the methodological recommendations for FMD have been developed from studies using human subjects. Longitudinal imaging of the artery is recommended over cross-sectional imaging because of inadequate image definition of the lateral wall and the presence of skew artifacts in the cross-sectional view (Corretti et al., 2002). A longitudinal image also allows for an average derived from multiple diameter measurements along the vessel which produces less variability (Corretti et al., 2002). The diameter of the brachial artery should be measured at end diastole (onset of the R-wave on ECG trace) and the maximum dilation after cuff release should be measured rather than the dilation at a specific time point (Al-Qaisi et al., 2008; Corretti et al., 2002; Puglia et al., 2006). In humans, the recommended occlusion time is 5 minutes. A minimum of 10 minutes of rest has been recommended before another baseline FMD image is acquired to allow for the reestablishment of baseline conditions (Corretti et al., 2002). However, a more recent study found that a second FMD performed 15 minutes after the first assessment was significantly decreased so more time between measurements may be necessary (Nerla et al., 2011).

Environmental factors that can affect FMD in humans include room temperature, time of day, ingestion of fatty foods or caffeine, concurrent inflammation or infection and stage of menstrual cycle (Al-Qaisi et al., 2008). Other factors that may affect FMD include age, gender, arterial hypertension, diabetes mellitus, cholesterol, obesity, hyperhomocysteinemia, coronary artery disease and smoking (Moens et al., 2005). Factors affecting FMD in dogs
have not been characterized. To improve reproducibility, FMD measurements should be performed by a single tester (Moens et al., 2005).

A limited number of studies have reported performing FMD in dogs. FMD has been validated in normal dogs (Puglia et al., 2006), but the clinical implications of decreased FMD are still unknown for non-humans. The baseline brachial artery diameter in dogs with a median body weight of 31.4 kg (range 21.9-34.6 kg) was found to be 0.25-0.30 cm compared to values of 0.40-0.50 cm in adult humans (Puglia et al., 2006). In dogs, one study found that a 3 minute occlusion time may be as effective as 5 minutes and offers the advantage of decreased opportunity for the dog to move (Puglia et al., 2006). In dogs, brachial artery FMD has been recommended over femoral artery FMD due to improved repeatability and higher values (Jones et al., 2011).

A study by Jones et al. (2010) evaluated the between- and within-dog repeatability of measuring FMD in healthy dogs using techniques recommended in the human literature. FMD was found to be significantly greater among dogs ≤15 kg (median 7.7%, range 0-19.3%) than dogs >15 kg (median 2.2%, range -2.2-10.6%). Increasing age also was shown to reduce FMD. The coefficient of variation for within-dog repeatability for FMD assessments performed 24 hours apart by the same sonographer was 62.8%. The between-dog coefficient of variation for FMD was 99.7%. Peak arterial dilation usually occurred within 90 seconds after release of the blood pressure cuff. Some of the issues that were encountered with performing FMD in dogs that are less problematic with humans included maintaining constant probe position, obtaining images of a consistent arterial segment, obtaining true longitudinal images, and limb movement (Jones et al., 2010). For this thesis, some of the technical problems with FMD in dogs were overcome by using a single breed (beagles) that were
housed under identical conditions and fed identical diets. The dogs were also well acclimated to the FMD procedure which was performed with each dog several times prior to performing any experiments.

2.2.4.2 Relationship between Flow-Mediated Dilation and Cardiac Function

The left ventricle of the heart pumps oxygenated blood into the aorta for distribution throughout the body. An important component in the overall health and function of the cardiovascular system is the relationship between the heart, particularly the left ventricle, and the systemic vasculature (i.e. ventricular-arterial coupling) (Frenneaux & Williams, 2007). Increased vascular stiffness results in reduced FMD, elevated blood pressure and increased load on the heart (Frenneaux & Williams, 2007). To compensate, the heart increases systolic and diastolic stiffness, which can result in the development of left ventricular hypertrophy (Frenneaux & Williams, 2007). Thus, measurement of changes in left ventricular structure and function not only provides information about the health of the heart, but also about the health of the systemic vasculature.

2.2.5 Echocardiography

Echocardiography, or cardiac ultrasound, can be used to determine changes in heart structure and function. Echocardiography is non-invasive with little or no stress as dogs only require gentle restraint (Hanton et al., 1998). Another advantage of echocardiography is that measurements are easily repeatable and echocardiography does not alter the function of the tissues being examined (Hanton et al., 1998). Two-dimensional (brightness mode or B-mode) echocardiography is used to view anatomical relationships while motion-mode (M-mode) echocardiography is used primarily for quantification of cardiac dimension (Brown et al., 2003).
Left ventricular measurements are commonly taken from the right parasternal location in the short axis view using two-dimensional guided M-mode echocardiography (Chetboul et al., 2004; Schober & Baade, 2000). In the dog, the right parasternal location is located between the 3\textsuperscript{rd} and 6\textsuperscript{th} intercostal spaces. A long axis view of the heart is observed when the imaging plane transects the left ventricle parallel to the long axis of the heart from apex to base, as shown in figure 2.1 (Thomas et al., 1993). To obtain a short axis view, the transducer is rotated 90 degrees from the long axis view. A series of short axis views can be obtained from the apex to the base of the heart (figure 2.2). The left ventricle is generally circular in the short axis view and thus is easy to recognize (Bonagura et al., 1985). When the upper part of the left ventricle is visualized at the level of the papillary muscles, a distinct mushroom-shaped view of the ventricular chamber is observed (figure 2.2B). Once this image is obtained, the M-mode guideline is positioned between the papillary muscles and the movement is recorded (Hanton et al., 2008).
Abbreviations:
AO - Aorta  
CH – Chordae tendineae  
LA – Left atrium  
LC – Left coronary cusp  
LV – Left ventricle  
MV – Mitral valve  
RA – Right atrium  
RPA – Right pulmonary artery  
RV – Right ventricle  
TV – Tricuspid valve  
• Transducer index mark

Thomas et al. (1993)

**Figure 2.1** Right parasternal long-axis echocardiographic views  
Reproduced with permission (Appendix E).
Figure 2.2 Right parasternal short axis echocardiographic views. Short axis view at the level of the apex (A), papillary muscles (B), chordae tendineae (C), mitral valve (D), aorta (E), and pulmonary arteries (F). Reproduced with permission (Appendix E).
From these echocardiographic images, measurements can be made in diastole (d) and systole (s) for left ventricular internal dimension (LVID), free-wall thickness and interventricular septal thickness (Chetboul et al., 2004). An average of three to five cardiac cycles should be used for each measurement (Boon, 1998). From these measurements, fractional shortening can then be calculated using the formula (Chetboul et al., 2005; Crippa et al., 1992).

Equation 2.3

\[ FS = \frac{LVID_d - LVID_s}{LVID_d} \]

Fractional shortening provides an index of left ventricle systolic function and values in normal dogs are typically between 30-40 percent (Bonagura et al., 1985; Hanton et al., 2008).

Left ventricular volume (LVV) in diastole and systole can be calculated using the modified Simpson’s rule (biplane method of disks) from the two-dimensional apical four-chamber and two-chamber views (figure 2.3) (Boon, 1998; Hanton et al., 2008). These two images provide near-orthogonal views which is preferable to using a single plane (St John Sutton et al., 2009). Simpson’s rule involves tracing the inner circumference of the left ventricle which is then analyzed as a stack of disks by computer analysis software. The volume for each disk is determined and the total volume of the disks is summated to calculate left ventricular volume (Boon, 1998). Subsequently, stroke volume (SV) and ejection fraction (EF) can be calculated by (Hanton et al., 2008):
Equation 2.4

\[ SV = LVV_d - LVV_s \]

Equation 2.5

\[ EF = \frac{SV}{LVV_d} \]

Ejection fraction is an indicator of ventricular contractile function as it is the fraction of the ventricular diastolic volume that is ejected at each heart beat (Hanton et al., 2008).
Figure 2.3 Echocardiographic apical views.
(A) Apical 2-chamber view (B) Apical 4-chamber view. Reproduced with permission (Appendix E).

Thomas et al. (1993)

Abbreviations:
AMV – Anterior mitral valve cusp
AS – Atrial septum
LA – Left atrium
LAu – Left auricle
LV – Left ventricle
PMV – Posterior mitral valve cusp
RA – Right atrium
RPA – Right pulmonary artery
RV – Right ventricle
TV – Tricuspid valve
• Transducer index mark
During an echocardiography exam, dogs can be in a recumbent or standing position. It is recommended that repeat examinations be performed with the dog in the same position and that the same observer perform subsequent examinations (Chetboul et al., 2005). Simultaneous electrocardiogram recording during an echocardiography examination is essential as a timing reference (Bonagura et al., 1985). M-mode diastolic measurements should be taken at the beginning of the QRS complex on the electrocardiogram and systolic measurement should be taken at the peak downward point of septal motion (Boon, 1998). M-mode measurements should be made vertically on the image with chamber size, free wall thickness and septal thickness measured along the same line (Boon, 1998). For two-dimensional images, the end diastolic frame is defined as the frame just before the mitral valves close or the first frame that shows the QRS complex, whereas end systole is identified as the frame just before the mitral valve opens (Boon, 1998). Parameters of chamber size and wall thickness in dogs are correlated linearly with weight and body surface area (Boon, 1998). Body surface area, in square meters, can be calculated by the formula: body surface area = (K x W^{2/3}) x 10^{-4} where weight (W) is in grams and K is the proportionality constant which is equal to 10.1 for dogs (Bonagura, 2000; Cowgill & Drabkin, 1927; O'Grady, 1986).

2.2.6 Blood Pressure

Obesity is a risk factor for hypertension in humans (Kotsis et al., 2010). However, research is still ongoing to understand the mechanisms by which obesity causes hypertension. Activation of the sympathetic nervous system in obese individuals is considered to be a primary mechanism by which obesity induces hypertension (Kotsis et al., 2010). High energy intake increases norepinephrine turnover in peripheral tissues and high dietary fat and carbohydrate intake may stimulate adrenergic receptors leading to elevated sympathetic
activity (Kotsis et al., 2010). Increased sympathetic activity in obesity may be due to impaired baroreceptor sensitivity and/or increased levels of circulating free fatty acids, angiotensin II, insulin and leptin (Kotsis et al., 2010).

Blood pressure can be measured in non-sedated dogs in an outpatient setting using high definition oscillometry (Brown et al., 2007; Henik et al., 2005). This technique detects pressure changes produced in an air bladder within the occluding cuff resulting from the pressure pulse (Henik et al., 2005). As reported by Brown et al. (2007), several studies have used oscillometry to measure arterial blood pressure in normal dogs. These studies found a fairly large variation in pressures, with systolic pressure ranging from 131-150 mm Hg and diastolic pressure ranging from 71-91 mm Hg. The wide variation in values can be attributed to the differences in subject populations, measurement techniques and animal handling (Brown et al., 2007). Thus, standardization of the technique is crucial (Brown et al., 2007). Interbreed variation can also result in significant differences in blood pressure values. The blood pressure in sighthounds is generally 10-20 mm Hg higher than in mongrels and values between other breeds can vary by 7-10 mm Hg (Brown et al., 2007). Within-day and between-day coefficient of variation in systolic blood pressure were reported to be 9.0-10.1% and 12.8-16.4%, respectively, and 10.3-14.4% and 14.2-24.9% for diastolic blood pressure (Rattez et al., 2010).

The effects of age on blood pressure in dogs is less clear than in humans, but there is some evidence to suggest that blood pressure may increase by 1-3 mm Hg/year as dogs age (Brown et al., 2007). Obesity in dogs also may produce an increase in blood pressure. One study reported an increase in mean arterial pressure from 94.4 ± 2.1 to 105.5 ± 3.7 mm Hg after induction of obesity from high fat overfeeding (Truett et al., 1996). An epidemiological
study that measured blood pressure using oscillometry found that obese dogs had slightly higher (<5 mm Hg) blood pressure than lean dogs (Bodey & Michell, 1996).

2.2.7 Heart Rate

Elevated heart rate (i.e. tachycardia) is an independent risk factor for cardiovascular morbidity and mortality in healthy humans as well as among those with established cardiac disease (Palatini, 2011). Increased heart rate may be even more predictive of mortality than cholesterol and blood pressure (Palatini, 2011). Tachycardia may increase cardiac disease risk through a number of mechanisms, including increased cardiac work, increased arterial wall stress, higher mean blood pressure, decreased large artery compliance, and disruption of arterial plaques (Palatini & Julius, 2004). As with hypertension, increased sympathetic activation may be responsible for elevated heart rate in obesity (Palatini, 2011). However, there is evidence in dogs that obesity may elevate heart rate due to reduced parasympathetic control (Truett et al., 1996; Van Vliet et al., 1995).

2.3 Carbohydrates

2.3.1 Types of Carbohydrates

Carbohydrates are classified based on the number of sugar units present (Institute of Medicine, 2005). A monosaccharide has one sugar unit, a disaccharide has two sugar units, an oligosaccharide consists of 3-10 sugar units and polysaccharides contain more than 10 sugar units (Institute of Medicine, 2005). Dietary fibre is carbohydrate that is non-digestible (Institute of Medicine, 2005). Monosaccharides and disaccharides are commonly referred to as simple sugars whereas oligosaccharides and polysaccharides are considered complex carbohydrates.
Starch, a polysaccharide, is the main digestible carbohydrate found in plants. It is an important and economical energy source in the diet of humans and animals (Bednar et al., 2001). Starch consists of two types of glucose polymers, amylose and amylopectin. Amylose is a linear glucose polymer whereas amylopectin is a branched-chain glucose polymer (Biliaderis, 1991). The content of amylose and amylopectin in starch affects properties such as pasting, gelatinization and retrogradation which in turn affects product quality and stability (Abdel-Aal, 2002).

Resistant starch is the fraction of starch in foods that remains undigested and consequently reaches the colon where it is fermented by colonic bacteria, producing short chain fatty acids as by-products (Akerberg, 1998). Some resistant starch occurs naturally, but new resistant starch can also be created by the formation of bonds between amylose chains (i.e., retrogradation) which results from the heating and cooling of foods (Kendall et al., 2004). Amylose is more susceptible to retrogradation than amylopectin (Spears & Fahey, 2004). Extrusion, the process used to make the majority of dry pet foods, usually decreases the amount of resistant starch, but retrogradation can result in increased concentrations (Spears & Fahey, 2004).

Resistant starch possesses properties that may provide health benefits. Products high in resistant starch tend to have a lower energy density and often produce low glycemic and insulinemic responses (Akerberg, 1998; Knapp et al., 2008). In addition, the fermentation of resistant starch appears to result in higher amounts of butyric acid compared to fermentation of soluble fibre. Butyric acid is the major energy source for colonocytes, thus resistant starch is considered to play an important role in colonic health (Akerberg, 1998).
Pet foods can contain as much as 50% starch which is commonly derived from cereal grains (Spears & Fahey, 2004). In general, starch is highly digestible by companion animals and the digestibility is generally increased through extrusion (Spears & Fahey, 2004; Twomey et al., 2002). Conversely, as the dietary fibre content in dog food increases, fecal weight also increases, whereas digestibility and transit time decrease (Burrows et al., 1982). Fibre may help in weight control because it adds bulk to food without adding calories, but the diet may also need to be high in protein in order for weight loss to occur (Butterwick & Markwell, 1997; German et al., 2009a).

2.3.2 Glycemic Index

Carbohydrate is the primary dietary component that affects postprandial glycemic and insulinemic responses with both the amount and type of carbohydrate affecting the responses (Barclay et al., 2008). In humans, there is increasing evidence that the type of carbohydrate included in the diet may play a role in weight control, as well as in the prevention or management of diabetes and cardiovascular disease (Thomas & Elliott, 2009; Thomas et al., 2007; Thomas et al., 2004). The glycemic index was developed to rank carbohydrate-containing foods based on their effect on postprandial glycemic response and was developed to assist people with diabetes to control their blood glucose levels (Du et al., 2008; Jenkins et al., 1981). Glycemic index is calculated from the area under the blood glucose curve that is above the baseline value (Wolever et al., 1991). High glycemic index foods produce a higher peak postprandial glycemic response and an overall greater area under the blood glucose curve in the two hours after consumption than do foods with a low glycemic index (Foster-Powell et al., 2002). The glycemic load is defined as the glycemic index of a food multiplied by its carbohydrate content (Barclay et al., 2008).
2.3.2.1 Glycemic Index Methodology

Methodology recommendations for performing glycemic index testing come primarily from human studies. There are several variables that affect the glycemic index value including food portion size, choice of reference food, repeated testing of the standard food, frequency and length of time of blood sampling, and the method of calculation of area under the glucose curve (Wolever et al., 1991). However, if procedures are standardized for these factors within a given series of experiments, all test foods using the same procedure would be affected similarly to the reference food. Thus, the use of a reference food minimizes these effects on the calculated glycemic index values (Wolever et al., 1991).

For testing glycemic index in humans without impaired glucose tolerance, it is recommended that glucose measurements be performed at 15, 30, 45, 60 and 120 minutes after ingestion of the test food. In contrast, for subjects with impaired glucose tolerance, it is recommended that testing be done every 30 minutes for 3 hours after ingestion of the test food (Wolever et al., 1991). It is not desirable to extend the testing time any longer because this can decrease the difference between the glycemic indices of foods. In addition, it is the early part of the glucose response curve (i.e. <2 h) that reflects carbohydrate absorption which in turn affects insulin and counter-regulatory hormone responses (Wolever et al., 1991). However, in dogs, extending the post-prandial sampling period to 3 hours may be more appropriate than the 2 hour time frame used in humans because it appears to take longer for blood glucose levels to return to baseline in dogs (Watanabe et al., 2004).

The amount of carbohydrate offered is an important factor in calculating glycemic index (Aziz, 2009). In humans, the area under the glucose curve increases almost linearly for foods that contain up to 50 g of carbohydrate, but then flattens (Aziz, 2009; Wolever et al.,
Lower doses can be used as long as the amount of carbohydrate in the test and reference foods is the same (Aziz, 2009). For dogs, offering a 50 g portion of carbohydrate may result in feeding an intolerable volume of food. One study that examined glycemic response in dogs fed diets that provided 5.5-7.3 g of starch in one feeding (Carciofi et al., 2008). Thus, offering portions of single ingredient reference and test foods that provide 10 g of carbohydrate (equivalent to 1 g/kg body weight, approximating the standard human dose) may be more reasonable.

It is also important that the carbohydrate used in calculating the amount fed is the available carbohydrate, not the total carbohydrate. Available carbohydrate is the portion of carbohydrate that is digestible and absorbable in the small intestine. According to the Food and Agriculture Organization of the United Nations, available carbohydrate includes the proportion of carbohydrate that can be digested by human enzymes and absorbed (Food and Agriculture Organization of the United Nations, 2003). Thus, available carbohydrate includes D-glucose, D-fructose, sucrose, maltodextrins, non-resistant starch and the D-glucose component of lactose, which can be all measured as D-glucose plus D-fructose following enzymatic hydrolysis (McCleary, 2007). Available carbohydrate does not include dietary fibre or resistant starch, which only provide a source of energy after being fermented in the large intestine (Food and Agriculture Organization of the United Nations, 2003). Available carbohydrate can be estimated by difference or analyzed directly (Food and Agriculture Organization of the United Nations, 2003). The difference method uses the following formula to estimate the weight of available carbohydrate:
Equation 2.6

Available carbohydrate = 100 – [weight in grams [protein + fat + water + ash +alcohol +

dietary fibre] in 100 g of food]

Alternatively, direct analysis uses the formula:

Equation 2.7

Available carbohydrate = weight in grams [monosaccharides + disaccharides +

oligosaccharides + polysaccharides, excluding fibre]

Available carbohydrate can be determined by direct analysis using commercially available

assay kits (McCleary, 2007).

Venous and capillary blood are both considered acceptable for calculating glycemic

index, though capillary blood is recommended (Aziz, 2009). Capillary blood glucose

concentrations are less variable compared to venous and it is less invasive to use capillary

finger pricks than it is to insert a catheter (Aziz, 2009). However, peripheral pricks are not as
easy to perform in dogs compared to humans and catheter insertion is necessary if other blood

parameters (e.g., insulin) are being measured simultaneously. To improve glycemic index

precision, it is recommended that two tests are performed for each food by each test subject

(Aziz, 2009) and that the reference food be tested at least three times by each subject

(Wolever et al., 1991).

Typically the glycemic index methodology is used to analyze single ingredients. It is

not useful to determine the glycemic index of meals consumed by humans since each meal

tends to contain different types and proportions of food. However, dogs do not eat single
ingredients and they generally eat the same mixed meal for long periods of time. Thus, it is of potential value to the manufacturer and consumer to know the glycemic index of a formulated dog food. There is evidence in dogs that the starch content has the most impact on postprandial glucose whereas carbohydrate, protein and fat all affect the insulin response (Nguyen et al., 1998).

2.3.2.2 Effects of Glycemic Index on Health

There is increasing evidence in humans that the glycemic index of a diet influences insulin sensitivity, lipid metabolism, inflammation, chronic disease risk, and weight management (Barclay et al., 2008; Du et al., 2008; Livesey et al., 2008; Thomas et al., 2007). Authors of a systematic review from the Cochrane Library concluded that a diet with a lower glycemic load promotes weight loss and improves lipid profiles in humans (Thomas et al., 2007). In addition, a meta-analysis of 37 prospective observational studies found that in humans, diets with a high glycemic index or glycemic load increased the risk of type 2 diabetes, heart disease, gallbladder disease, breast cancer and all diseases combined (Barclay et al., 2008). However, the glycemic index appeared to have more effect than the glycemic load. This could be because a low glycemic load diet could consist of low glycemic index, high carbohydrate foods or of foods that are low in carbohydrates, but high in other harmful dietary components, for example the saturated fat in cheese and meat (Barclay et al., 2008).

In humans, high glycemic index and high glycemic load diets are associated with moderately improved cholesterol profiles and reduced C-reactive protein (Levitan et al., 2008; Liu et al., 2002). Consumption of low glycemic index foods offers a similar or higher level of protection against chronic disease compared to the consumption of whole grain foods or high fibre intake (Barclay et al., 2008).
A limited number of studies have investigated the health effects of glycemic index in dogs. A study by Mitsuhashi et al. (2010) investigated the effect of a low glycemic index starch (high amylose corn starch) and diacylglycerol on metabolic parameters and weight management in dogs. The low glycemic index starch diets resulted in greater weight loss than the high glycemic index starch diets regardless of the type of fat the diets contained. However, the low glycemic index diet had lower digestibility and metabolizable energy than the high glycemic index diet. The actual glycemic indices of the diets were not reported.

A study by Nguyen et al. (1994) examined the effect of four foods differing in macronutrient composition on post-prandial glucose and insulin concentrations. The test foods were fed in amounts based on each dog’s calculated energy requirement. The incremental area under the glucose response curve was not significantly different among the test foods. Significant differences were observed between the foods for the area under the insulin response curve. This variable was highest for the foods with the greatest carbohydrate content (Nguyen et al., 1994).

2.3.3 Carbohydrate Sources in Dog Food

Although there is significant evidence in humans that hyperglycemia is detrimental to health, the long term consequences of a high glycemic index diet are unknown in dogs. The type of carbohydrate included in dog food may have important health effects given that dry dog foods typically consist of 30-60% carbohydrate and canned foods include up to 30% carbohydrate (Carciofi et al., 2008; Murray et al., 1999). Furthermore, many dog foods include a high glycemic index carbohydrate source such as white rice or corn.

In dogs with insulin-dependent diabetes, the macronutrient (i.e. carbohydrate, fat and protein) composition of the diet has been shown to influence glucose metabolism. For
example, a canned high fibre diet has been shown to decrease the degree of fluctuation in postprandial plasma glucose concentration (Graham et al., 1994). A study by Carciofi et al. (2008) examined the effects of several carbohydrate sources on the glycemic and insulinemic responses in dogs. The study found that the diets containing sorghum, lentils and peas delayed and lengthened the glycemic and insulinemic responses (Carciofi et al., 2008). Another study found that a high fibre diet that included pea fibre and guar gum as the fibre sources significantly lowered mean 24 hour and postprandial glucose concentrations in addition to reducing plasma concentrations of fructosamine, glycated hemoglobin and cholesterol (Graham et al., 2002). In dogs, plasma fructosamine concentration provides an indication of blood glucose control over the previous one to three weeks, whereas glycated hemoglobin concentration is an indicator for glycemic control over the past three to four months (Graham et al., 2002). Another study that compared the glycemic responses of corn, wheat, barley, rice and sorghum found that rice resulted in the highest postprandial glycemic and insulinemic response in dogs (Sunvold & Bouchard, 1998). Many commercial dog foods use corn, rice or sorghum as carbohydrate sources.

Pulses are the edible seeds of legumes and include peas, beans, lentils, chickpeas and faba beans. Whole yellow pea flour has been shown to be a beneficial ingredient in human food products to create novel low glycemic foods (Marinangeli et al., 2009). Peas also may be a potential pet food ingredient that could offer advantages over other carbohydrate sources traditionally used in pet foods, such as rice and corn. A potential advantage of peas may be related to their low glycemic index due to the high proportion of resistant starch (62% of total starch) and slowly digestible starch (21% of total starch) compared to the proportion of starch that is rapidly digestible (17% of total starch) (Fouhse, 2011). Peas are also high in dietary
fibre (20% of seed dry matter) and contain both insoluble (10-15%) and soluble fibre (2-9%). Soluble fibre tends to increase the viscosity of gastrointestinal contents and results in prolonged gastric emptying and reduced transit time through the small intestine, thus producing a lower post-prandial glycemic response (Dikeman et al., 2006).

In addition to being a carbohydrate source, peas have twice the protein content of most cereal grains (Saskatchewan Pulse Growers, 2009). Peas are also are gluten-free. Canada is the world’s largest grower of peas (Bond, 2009) and Saskatchewan produces 80% of Canada’s pea crop (Saskatchewan Pulse Growers, 2009). Thus, peas are a local ingredient which minimizes the environmental impact of shipping if used in locally produced dog foods. Furthermore, using local products would contribute to promoting growth in the local economy. Another advantage of peas is that they fix their own nitrogen which reduces the need for synthetic fertilizer made from fossil fuels (Bond, 2009), further reducing the environmental impact.

2.3.3.1 Effects of Processing

The majority of commercial dry dog and cat food are manufactured using extrusion (Lankhorst et al., 2007). Extrusion is a cooking process where the food mixture is exposed to high temperature and pressure for a relatively short period of time (Lankhorst et al., 2007). Benefits of extrusion include achieving a desired physical form, inactivation of anti-nutritional factors, increased product shelf life, improved digestibility and enhanced palatability (Lankhorst et al., 2007). Disadvantages of extrusion include micronutrient losses, lipid oxidation, and destruction or reduction in bioavailability of amino acids, particularly lysine (Lankhorst et al., 2007).
In addition to the carbohydrate source in the food, the type of processing may impact the glycemic index of dog food (Nguyen et al., 1998). The high temperature and high pressure characteristics of extrusion allow starch granules to be completely gelatinized, thereby increasing susceptibility to amylase degradation and increased release of glucose (Lankhorst et al., 2007; Nguyen et al., 1998). Canning, another common processing method used for making dog food, also increases starch availability by using high moisture/high temperature conditions which are required for sterilization (Nguyen et al., 1998). The changes in starch structure during the manufacturing process affects the functional properties of the food, including the rate and extent of starch digestion (Spears & Fahey, 2004). Conditions used for both extruding and canning dog foods tend to produce higher glycemic index foods. However, optimizing processing conditions to increase starch retrogradation has the potential to enhance starch resistance to digestion and produce lower glycemic index foods.

### 2.3.3.2 Starch Digestibility

Apparent digestibility of a food or ingredient is the proportion of nutrients absorbed in the gastrointestinal tract (Brambillasca et al., 2010). Digestibility trials are used to determine apparent digestibility and protocols described by the Association of American Feed Control Officials are commonly used to determine digestibility of pet foods (Association of American Feed Control Officials, 2009).

Starch in dog food tends to be highly digestible though there are a variety of factors that influence starch digestibility, including the type of cereal, starch-protein interactions, physical granule form, type of starch, digestion inhibitors, and processing (Carciofi et al., 2008). There is increasing interest in using legumes, such as peas, beans and lentils, as carbohydrate sources in pet food. A study by Carciofi et al. (2008) examined the effects of six
extruded diets, each with a different carbohydrate source, on dog total tract apparent digestibility. Starch digestibility was greater than 98% for all of the diets, though the brewer’s rice and cassava flour diets had improved starch digestibility compared to the lentil and pea diets (Carciofi et al., 2008). Another study that investigated the digestibility of a diet containing navy bean powder found digestibility coefficients of 68.58% for dry matter, 78.22% for crude protein, 74.91% for organic matter, and 94.49% for acid hydrolyzed fat (Forster et al., 2012). Thus, pulses may have somewhat lower digestibility than some other starch sources, but appear to be well-tolerated by dogs. Further research is needed to explore the potential health benefits of pulses as a carbohydrate source in pet food.
CHAPTER 3
STUDY 1: POST-PRANDIAL IMPAIRMENT OF FLOW-MEDIATED DILATION AND ELEVATED METHYLGLYOXAL AFTER SIMPLE, BUT NOT COMPLEX, CARBOHYDRATE CONSUMPTION IN DOGS

This study was performed first in the series of four studies included in this thesis because the data that resulted from this study was necessary for the subsequent studies. Specifically, this study determined which of four carbohydrate sources (peas, rice, corn and barley) had the lowest and highest glycemic index. The carbohydrate sources with the highest and lowest glycemic index were then formulated into complete dog diets that were used in studies 2 and 4. Thus, the results of this study permitted comparison of the acute health effects of unprocessed, individual carbohydrate sources versus the acute and chronic health effects of the same carbohydrate sources once processed and incorporated into a dog diet.

This chapter was published in the journal Nutrition Research and is reproduced with permission (Appendix E).
3.1 Introduction

Increasing evidence suggests that the type of carbohydrate included in the diet plays a role in chronic disease mitigation. Specifically, post-prandial hyperglycemia is a known risk factor for cardiovascular disease among healthy and diabetic patients with low glycemic index foods improving markers of cardiovascular disease risk (Brand-Miller et al., 2007; Ceriello, 2004; Lavi et al., 2009; Levitan et al., 2004; Thomas et al., 2007; von Bibra et al., 2009). Modifying the type of dietary carbohydrate is likely more important in reducing chronic disease risk than altering the total amount of carbohydrate or fat (Brand-Miller et al., 2009; Ghanim et al., 2009; Hung et al., 2003). However, it is not clear whether the benefits of low glycemic index foods are related to long-term consumption, or whether acute feedings of high glycemic index foods are detrimental to cardiovascular health.

Hyperglycemia is thought to initiate a cascade of events that may be detrimental to the cardiovascular system (Aljada et al., 2006; Crandall et al., 2009; Dhindsa et al., 2004; Dickinson & Brand-Miller, 2005). Endothelial dysfunction is considered to be the initial step in the development of atherosclerosis as well as hypertension and many cardiovascular diseases (Endemann & Schiffrin, 2004). Oxidative stress plays a critical role in vascular endothelial dysfunction and there is evidence that hyperglycemia can initiate pro-inflammatory events such as increases in NFκB and TNFα followed by overproduction of reactive oxygen species and inhibition of eNOS (Cosentino et al., 1997; Mohanty et al., 2000; Node & Inoue, 2009). Reduced vasodilation in response to shear stress is an indicator of endothelial dysfunction which can be assessed in vivo using FMD (Harris et al., 2010; Thijssen et al., 2010). Nitric oxide, a potent vasodilator, is thought to be largely responsible for mediating FMD, but becomes inactivated in the presence of oxidative stress with
subsequent formation of peroxynitrite (Joannides et al., 1995; Schiffrin, 2008). Since peroxynitrite has a high affinity for reacting with tyrosine residues, nitrotyrosine can be used as an indicator of biological inactivation of nitric oxide and increased oxidative stress (Ceriello, 2002).

Another mechanism by which hyperglycemia may contribute to endothelial dysfunction is through the formation of AGEs (Brouwers et al., 2010; Dhar et al., 2010). Methylglyoxal (MG) is a reactive glucose metabolite and is a precursor of AGE (Desai et al., 2010). MG has been reported to increase with chronic hyperglycemia resulting from diabetes and is considered to be one of the most reactive AGE precursors in endothelial cells (Nemet et al., 2005). MG may also indirectly play a role in endothelial cell damage by increasing oxidative stress (Desai et al., 2010; Miyazawa et al., 2010). However, post-prandial effects on methylglyoxal in normal patients are unknown.

Based on these observations, we hypothesize that MG levels will cause impaired endothelial function via increased oxidative stress after consuming high glycemic index, but not low glycemic index, foods in normal animals. Elevated MG levels, impaired endothelial function and decreased vasodilation due to hyperglycemia may in turn cause elevated blood pressure and increased cardiac load, but this should not change left ventricular size because of the acute nature of this post-prandial stress. The objective of this research was to examine the acute effects of glucose versus four complex carbohydrate sources (corn, rice, peas and barley) on glycemic, insulinemic and cardiovascular responses using a dog model.

3.2 Methods and Materials

3.2.1 Animals and Treatments

Six neutered beagle dogs, three males and three females at ideal body weight and a mean age of 2.8 ± 0.75 years were used. The dogs were obtained from Covance Research
Products Inc. (Princeton, NJ, USA) and were kept in the Western College of Veterinary Medicine at the University of Saskatchewan (Saskatoon, Saskatchewan, Canada). The animals were housed individually in 1.1 x 2.7 m kennels and were walked and given play time for at least one hour per day. Dogs were fed a commercial adult maintenance dry diet (Purina Dog Chow, Ralston Purina Co., St. Louis, MO) in amounts based on the National Research Council guidelines (National Research Council, 2006). No anesthetics or sedatives were used in this study. This work was approved by the University of Saskatchewan’s Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Four complex carbohydrate sources (barley, corn, peas and rice) were tested using a 20% (w/v) glucose solution (simple carbohydrate) as the control. In addition, 10% and 30% glucose solutions providing 5 and 15 g of glucose, respectively, were tested to examine the dose response of glucose on cardiovascular responses. The available carbohydrate content of the complex carbohydrate sources (D-glucose, D-fructose, sucrose, maltodextrins, non-resistant starch and the D-glucose component of lactose, which can be all measured as D-glucose plus D-fructose following enzymatic hydrolysis) was determined using a commercially available assay kit (Megazyme International, Wicklow, Ireland). All carbohydrate sources were fed in amounts that provided 10 g available carbohydrate. Prior to feeding, the dried complex carbohydrate sources were ground and then hydrated to form a paste. Each carbohydrate source was tested twice in each dog, except for the 10 and 30% glucose solutions which were each tested once, and the order of feeding was randomized. All foods were consumed by each dog in <5 minutes.
3.2.2 Serum Analysis

Fasted dogs were aseptically catheterized using a peripheral intravenous catheter inserted into the cephalic or saphenous vein. Blood samples (3 ml) were taken for serum collection at pre-feeding (time 0) and 15, 30, 45, 60, 90, 120, 150 and 180 minutes post-feeding.

Serum glucose analysis was performed using a glucose oxidase assay method (Association of Official Analytical Chemists, 2005) using reagents from Sigma Aldrich (St. Louis, MO). Serum insulin levels were measured by radioimmunoassay using a commercially available assay kit (Siemens, Munich, Germany). Serum glucose and insulin concentrations were determined at all time points for all treatments. The incremental area under the curve, peak concentration, and time to peak concentration for serum glucose and insulin were calculated and the glycemic index was determined based on the methods of Wolever et al. (Wolever et al., 1991).

Serum nitrotyrosine was measured using a commercially available ELISA kit (Hycult Biotech, Uden, The Netherlands). Nitrotyrosine was determined in one serum sample per dog per treatment at 60 minutes post-prandial. Serum MG levels were measured in one serum sample per dog per treatment at time 0 and 60 minutes post-prandial according to previously published HPLC-based methods (Wang et al., 2006; Wang et al., 2005).

3.2.3 Ultrasound and Oscillometry

All ultrasound measurements were performed and analyzed by one individual. FMD was measured in conscious, unsedated dogs using a SonoSite M-Turbo ultrasound unit with a vascular transducer L38x/10-5 MHz (Sonosite Canada Inc., Markham, ON) before feeding a carbohydrate source as well as 60 minutes after feeding based on previously published
methods from this laboratory and others (Al-Dissi & Weber; Puglia et al., 2006). Images were captured at end diastole and analyzed using Adobe Premiere Elements 2.0 (Adobe Inc., San Jose, CA) and Image-Pro Express 6.0 (Media Cybernetics Inc., Bethesda, MD). FMD was calculated using the formula: 

\[
\%\text{FMD} = 100\% \times \frac{\text{(maximum diameter post-cuff release)} - \text{(baseline diameter)}}{\text{(baseline diameter)}}
\]

Echocardiography techniques were performed using a Sonosite cardiac transducer P21x/5-1 MHz (Thomas et al., 1993). Measurements were taken before feeding and 60 minutes post-prandial. Two-dimensional ultrasonography was used to measure left ventricular volume using the left parasternal apical two- and four-chamber views in diastole and systole (Lang et al., 2005). The inner wall of the left ventricle was traced and Simpson’s Rule (Sonosite Inc., 2007) was used to calculate LVV in diastole (d) and systole (s) from which stroke volume, ejection fraction and cardiac output were calculated (Hanton et al., 1998). Heart rate was determined using an oscillometric blood pressure cuff (see below).

Two-dimensional guided M-mode echocardiography was used to obtain a right parasternal short axis view of the heart at the level of the papillary muscles (Lang et al., 2005). Image analysis software (Image-Pro Express 6.0, Media Cybernetics Inc., USA, 2006) was used to measure LVID in systole and diastole and fractional shortening was calculated (Hanton et al., 1998).

Blood pressure and heart rate were measured by high definition oscillometry using a Vet Memodiagnostic HDO Monitor (S + B medVET, Germany) and MDS Win software version 1.4.5.1 (S + B medVET, Germany, 2007) according to a protocol validated in dogs (Brown et al., 2007). Measurements were taken before feeding and 60 minutes after feeding. The cuff (4.5 x 15 cm) was placed at the tail base. An average of three readings was used to
determine diastolic and systolic pressures, mean arterial pressure and heart rate. Total peripheral resistance was calculated as $TPR = \frac{\text{mean arterial pressure}}{\text{cardiac output}}$.

### 3.2.4 Statistical Analyses

Analyses were performed using SPSS Statistics 18.0 (SPSS Inc., USA). Prior to performing all analyses, data were explored for normality and outliers using the Kolmogorov-Smirnov test, Q-Q plots and boxplots. When datasets failed normality tests, outliers were identified using boxplots, then removed if they were greater than 2 SD from the mean and if their removal produced a normal dataset. Data were expressed as means ± SEM. One-way ANOVA was used to compare glycemic and insulinemic responses, FMD, MG and nitrotyrosine between treatments. MG was expressed as a percentage of the pre-feeding value (Wang et al., 2004). Repeated measures GLM was used to analyze echocardiographic and blood pressure variables with time as the within-subject factor and carbohydrate source as the between-subject factor. All post-hoc analyses were performed using the Fisher’s least significant difference (LSD) method. Differences were considered significant at $P < 0.05$.

### 3.3 Results

#### 3.3.1 Serum Glucose and Insulin

The results of the available carbohydrate assay found that the barley, corn, peas and rice contained $73.7 \pm 2$, $71.4 \pm 0.6$, $50.8 \pm 1$, and $88.9 \pm 0.8$ g of available carbohydrate per 100g of food, respectively. Thus, the amount of each food that was fed to provide 10 g of available carbohydrate was 13.6 g barley, 14.0 g corn, 19.7 g peas, and 11.2 g rice.

The peak serum glucose concentration was significantly higher and the time to peak was significantly shorter for the glucose solution compared to all complex carbohydrate sources (rice, barley, corn and peas; Table 3.1 and Figures A.1-A.2 in Appendix A). However,
the peak serum glucose concentration and the time to peak did not differ between the complex carbohydrate sources. The mean serum glucose concentration before feeding was 4.4 ± 0.04 mmol/L and no significant differences in pre-feeding serum glucose values were found between groups. Figure A.3 in Appendix A shows the serum glucose response to the three oral glucose solutions. The area under the curve for serum glucose was significantly greater for the 30% glucose solution compared to the 10% glucose solution. The rank order of glycemic index for the complex carbohydrate sources, from highest to lowest, was: rice > barley > corn > peas, with the glycemic index for peas significantly different from rice and barley.

No significant differences in pre-feeding insulin values were found between groups (mean = 4.2 ± 0.7 pmol/L). The peak insulin response and the area under the insulin curve were greatest for the glucose solution, which was significantly different from all complex carbohydrate sources (Table 3.1 and Appendix A). The time to peak insulin concentration was the shortest for the glucose solution. No significant differences were found in the area under the insulin response curve among the complex carbohydrate sources. However, the rank order for the area under the insulin response curve, from highest to lowest, was: glucose > rice > corn > barley > peas. Figure A.3 in Appendix A shows the serum insulin response to the three oral glucose solutions. The area under the curve for serum insulin was significantly different between the 10, 20 and 30% glucose solutions with area under the curve increasing in response to increasing glucose dose.

3.3.2 Flow-Mediated Dilation

At 60 min post-prandial, the glucose group had the lowest FMD and was significantly lower than the response after corn feeding (P=0.02; Figure 3.1). Compared to glucose, the P-
values for the FMD response at 60 min after feeding the other carbohydrate sources were rice P=0.3, barley P=0.2, and peas P=0.1. Moreover, if the FMD of all the complex carbohydrate sources was combined (rice, barley and peas; 3.6 ± 0.4%), then it was significantly higher than the FMD response after glucose feeding (1.6 ± 0.51%; p=0.046 in t-test). In contrast, FMD did not differ between treatment groups before feeding and an overall mean pre-feeding FMD was calculated as 4.3 ± 0.4%. Furthermore, brachial artery diameter before cuff inflation during FMD testing did not differ across time points or treatments for all groups. Mean baseline brachial artery diameter prior to all FMD tests (n=180 tests in 6 dogs) was 0.20 ± 0.00 cm. Figure A.4 in Appendix A shows the FMD response to the three oral glucose solutions 60 min after ingestion. Although no significant differences in FMD were observed after ingestion of the glucose solutions, numerically FMD was greatest after feeding the 10% glucose solution and FMD was lowest after feeding the 30% glucose solution, suggesting a possible dose response.
Table 3.1 Glycemic and insulinemic responses to 10 g of available carbohydrate from simple (glucose) and complex (rice, barley, corn and peas) sources.

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Rice</th>
<th>Barley</th>
<th>Corn</th>
<th>Peas</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak (mmol/L)</td>
<td>8.5 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.3 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.4 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.3 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.0 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Time to peak (min)</td>
<td>34 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85 ± 9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>91 ± 10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>111 ± 12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>94 ± 15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Area under the curve (mmol/L min)</td>
<td>181 ± 13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95 ± 7.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>91 ± 12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>80 ± 15&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>50 ± 7.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glycemic index</td>
<td>100</td>
<td>55 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47 ± 10&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>29 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Rice</th>
<th>Barley</th>
<th>Corn</th>
<th>Peas</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insulin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak (pmol/L)</td>
<td>458 ± 60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75 ± 12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>62 ± 11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78 ± 15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>95 ± 14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Time to peak (min)</td>
<td>30 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>106 ± 12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>79 ± 9&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>99 ± 16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>53 ± 12&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Area under the curve (pmol/L min)</td>
<td>16377 ± 2310&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3698 ± 508&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3164 ± 497&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3635 ± 752&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2993 ± 491&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n=6 in duplicate. Values in a row without a common superscript differ, P < 0.05; 1-way ANOVA with LSD post-hoc test.
3.3.3 Methylglyoxal and Nitrotyrosine

MG concentration was significantly higher 60 min after feeding the glucose solution compared to all 4 complex carbohydrate groups (Figure 3.1B). However, no significant differences were observed in post-prandial MG levels among the complex carbohydrate sources. In contrast, neither glucose nor any complex carbohydrates produced any significant difference in nitrotyrosine concentrations 60 min after feeding (Figure 3.1C).

3.3.4 Hemodynamics

No significant differences were observed in any cardiac function end-points when comparisons were made among carbohydrate sources before feeding or 60 minutes post-prandial (data not shown). Similar to what was found for cardiac function, no significant effects of time or carbohydrate source were found for these variables (data not shown).
Figure 3.1 Flow-mediated dilation, methylglyoxal and nitrotyrosine 60 min after feeding simple (glucose) or complex (rice, barley, corn, peas) carbohydrate sources. Cross-over trial in dogs (n=6) at ideal body weight with each carbohydrate source providing 10 g of available carbohydrate and tested twice in each dog (A) *Significantly different (P<0.05; 1-way ANOVA with LSD post-hoc test). (B) Methylglyoxal levels are presented as the percentage of the pre-feeding value. *P<0.05 compared to peas, corn, barley and rice. (C) No significant differences were found.
3.4 Discussion

The most important finding of this study is that post-prandial MG levels increased after a single feeding of simple carbohydrate, which may be associated with acute changes in endothelial function. Thus, we accept our hypothesis that MG levels caused impaired endothelial function via increased oxidative stress after consuming high glycemic index, but not low glycemic index, foods in normal animals. Previous research has shown that chronic hyperglycemia is associated with increased levels of MG (Nemet et al., 2005) and individuals with greater fluctuations in plasma glucose over 24 hours have higher MG concentrations (Nemet et al., 2005). In addition, elevated post-prandial MG levels have been reported, but only in individuals with type 1 diabetes (Beisswenger et al., 2001) or in individuals who were overweight (Masterjohn et al., 2012). Thus, the results of the present study provide the first evidence of increased MG concentrations after a single glucose feeding in healthy, lean animals.

In addition to elevated MG levels, our data showed that FMD was reduced after glucose ingestion. Several mechanisms have been proposed to explain why hyperglycemia causes endothelial dysfunction and vascular damage (Aljofan & Ding, 2010; Ceriello, 2002, 2004). The common theme linking these mechanisms is that hyperglycemia produces ROS and increases oxidative stress (Mohanty et al., 2000). The results of the present study support this mechanism since MG has been shown to increase ROS production (Miyazawa et al., 2010), which could have contributed to the impaired FMD found for the glucose group. No differences in FMD were seen among the complex carbohydrate groups. This suggests that post-prandial responses to complex carbohydrates may not have any deleterious effect on endothelial function which is consistent with their known benefits for cardiovascular health (Howard & Wylie-Rosett, 2002). As predicted, we did not observe changes in
echocardiographic and blood pressure parameters as diet-related changes in these variables are more likely to occur if high glycemic index foods are consumed over a longer time period (Jeckel et al., 2011).

Although we found an increase in MG, which is known to be associated with an increase in ROS production (Brouwers et al., 2010), we did not observe an acute increase in post-prandial nitrotyrosine. Other studies have found increased nitrotyrosine in human patients with diabetes (Ceriello et al., 2001; Ceriello et al., 2002) or after a glucose challenge (Marfella et al., 2001), while others have not (Wang et al., 2004a). A study that measured nitrotyrosine at 1, 2 and 3 h after feeding glucose, white bread (high glycemic index) or pasta (low glycemic index) in humans found increased nitrotyrosine in the bread and glucose groups at 2 h, but not at 1 and 3 h post-prandially (Dickinson et al., 2008). In the present study, nitrotyrosine, MG, FMD, echocardiographic and blood pressure measurements were all performed at 60 min post-prandial. The 60 minute time point was picked since it fell between the times to peak glucose after consumption of simple versus complex carbohydrates. Therefore, although MG was elevated at 60 min post-prandial in this study, it seems likely that more time was needed to observe a change in nitrotyrosine levels (Dickinson et al., 2008).

Peas are an economical and nutrient-rich food and increased pea intake has been suggested to improve diet quality (Mitchell et al., 2009). Thus, one of the objectives of this research was to examine the effect of peas on glycemic, insulinemic and cardiovascular responses. The present study showed that peas had the lowest glycemic index. Peas also produced the lowest insulin response compared to the other complex carbohydrate sources, though the results were not statistically significant. The glycemic index of dried peas in
humans has been reported to be 22 when glucose was used as the control (Foster-Powell et al., 2002). Thus, a glycemic index of 29 in dogs for the yellow field peas used in this study is comparable to that reported in humans. Another study in dogs that fed diets formulated with one of six different carbohydrate sources also found that the pea diet resulted in reduced glycemic and insulinemic responses (Carciofi et al., 2008).

The present study was performed in dogs as a model for humans due to the inherent confounding factors associated with this type of research in humans, including the wide genetic diversity and the lack of control over diet and lifestyle. Dogs are a suitable model for humans for studying dietary interventions because they possess a gastrointestinal tract similar to humans and they consume an omnivorous diet (Knapp et al., 2008). In addition, the same ultrasound and glycemic testing techniques included in this study that are routinely used in humans can also be used in dogs because they are adequate size. The present study suggests for the first time that a single high glycemic meal may induce negative cardiovascular consequences. Thus, this study provides justification for further research about the effects of carbohydrates on cardiovascular health in humans.

A limitation of this study was the small sample size. A power analysis found that a sample size of 25 was required to show significant differences in FMD between the five treatments (Faul et al., 2007). We could have increased power by decreasing the number of treatments. However, the original intent was to examine commonly consumed carbohydrate sources in the human diet spanning a wide variety of glycemic index values in humans: high (rice), moderate (barley, corn) and low (peas) glycemic index (Foster-Powell et al., 2002). FMD is influenced by many factors, including stress and activity in both humans and dogs (Al-Qaisi et al., 2008; Moens et al., 2005) and this led to greater variability in this end-point.
We were able to control for this variability better than is possible in many humans studies by acclimating the dogs to the procedures prior to the study, keeping the diet quality and quantity consistent prior to testing, performing the tests at the same time of the day, allowing the dogs to re-acclimate to the test room for at least 20 minutes before starting testing, and maintaining a constant room temperature. Therefore, although we did not detect any differences among FMD responses at 60 min after complex carbohydrate consumption, the sample size was clearly sufficient to detect a large impairment in FMD response after feeding a simple carbohydrate.

In conclusion, the present study provides support for the hypothesis that glucose, a simple carbohydrate, induces negative cardiovascular consequences by elevating MG and reducing FMD. Thus, the results of this study provide further support for the theory that post-prandial hyperglycemia results in negative cardiovascular consequences. Future studies are needed to examine the acute and chronic effects of low and high glycemic index foods on overweight animals as low glycemic index foods may be particularly cardioprotective for overweight or diabetic individuals (Beulens et al., 2007).
CHAPTER 4
STUDY 2: DIGESTIBILITY AND ACUTE GLYCEMIC, INSULINEMIC AND CARDIOVASCULAR EFFECTS OF PEAS AS A CARBOHYDRATE SOURCE IN DOG FOOD

This study used the results from study 1 to create two diets: a diet that contained a high glycemic index carbohydrate source (rice); and a diet that contained a low glycemic index carbohydrate source (peas). In order to formulate the diets so that the macronutrient and energy content were equivalent, the first step in this study was to perform digestibility trials to determine the total tract apparent digestibility of the main ingredients that would be used in the diets, namely chicken meal, rice and peas. Once the diets were formulated, their acute glycemic and cardiovascular effects were tested using the same procedures as in study 1, and the apparent digestibility of the diets also were determined. Thus, this study was a logical follow-up to study 1 since dogs eat complete diets, not individual carbohydrate sources. This study permitted a comparison of the glycemic and insulinemic responses once the carbohydrate sources were incorporated into complete diets and processed using extrusion.
4.1 Introduction

Pulses are the edible seeds of leguminous plants and include peas, chickpeas, beans and lentils (Anderson and Major, 2002). In humans, pulses have been shown to possess a number of health-promoting characteristics, including protection against cardiovascular disease, diabetes and obesity (Anderson & Major, 2002; Hermsdorff et al., 2011; Messina, 1999). Peas (*Pisum sativum*) are a pulse that are becoming an increasingly common ingredient in pet foods (Aldrich, 2010). With the growing problem of obesity and its associated co-morbidities among the North American pet population (Laflamme, 2006, 2011), low glycemic index peas may be a healthy carbohydrate source for pet diets.

One of the characteristics of pulses to which their health benefits may be attributed is their low glycemic index (Jenkins et al., 1980; Sievenpiper et al., 2009). Post-prandial hyperglycemia due to the consumption of high glycemic index diets is thought to be detrimental to the cardiovascular system by increasing oxidative stress (Crandall et al., 2009; Dickinson & Brand-Miller, 2005; Node & Inoue, 2009). Endothelial dysfunction is associated with oxidative stress and can be assessed in vivo using flow-mediated dilation (Endemann & Schiffrin, 2004; Harris et al., 2010; Thijssen et al., 2010). Thus, pulses may provide cardiovascular benefits by preventing post-prandial hyperglycemia and the associated oxidative stress.

We previously reported that single feedings of unprocessed peas had a lower glycemic index in dogs than rice or barley (Adolphe et al., 2012). However, there is a lack of information about the digestibility and health effects of peas as a carbohydrate source in extruded dog food. The purpose of this study was to measure the digestibility of peas and rice in dogs as individual ingredients as well as in complete and balanced canine diets, and to subsequently test the effects of the diets on acute glycemic, insulnemetic and cardiovascular
responses in dogs. We hypothesize that a pea-based diet will result in lower glycemic and insulinemic responses as well as improved cardiovascular function in dogs compared to a rice-based diet.

4.2 Materials and Methods

4.2.1 Animals

Neutered beagle dogs (n=3-8) at ideal body weight and a mean age of 3.1 ± 0.2 years were used. The dogs were obtained from Covance Research Products Inc. (Princeton, NJ, USA) or the University of Guelph (Guelph, ON, Canada) and were kept in the Western College of Veterinary Medicine at the University of Saskatchewan (Saskatoon, SK, Canada). The animals were housed during feeding and at night in 1.1 x 2.7 m individual kennels, but were housed together during the day with access to outdoor runs and were walked daily. Dogs were subject to a 12-hour photoperiod with ad libitum access to water. When dogs were not on digestibility trials they were fed a commercial adult maintenance dry diet (Purina Dog Chow, Ralston Purina Co., St. Louis, MO) in amounts based on the National Research Council guidelines (National Research Council, 2006) and adjusted to maintain a stable, ideal body weight in each dog for the study duration. This work was approved by the University of Saskatchewan’s Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

4.2.2 Digestibility and Diet Formulation

Apparent digestibility of chicken meal, peas and rice were measured using an indicator method (Association of American Feed Control Officials, 2009). A reference diet was formulated according to Fahey et al. (1992) that met or exceeded nutrient requirements of dogs (National Research Council, 2006). Three experimental diets were formulated using
70% (w/w) of the reference diet and 30% (w/w) of the experimental ingredient (chicken meal, peas or rice). Celite®, a non-absorbable marker, was added at a 1% inclusion rate to the reference and experimental diets. Diet ingredients were ground in a hammer mill with a 3 mm screen prior to mixing. The diets were cold extruded using a 5 mm die on a Hobart Pellette (Model 4822; Ohio, USA), dried in a forced air oven (55°C, 12 h), chopped and screened to obtain the appropriate pellet size.

A study was performed in which dogs (n=6; 3 neutered males, 3 spayed females) were fed the reference or experimental diets for seven days with fecal sample collection commencing on day 8. The experiment was repeated with all of the diets until 3 dogs had been fed each of the 3 experimental diets and 6 dogs had been fed the reference diet.

Diets and fecal material for the reference diet and experimental diets were analyzed for energy (oxygen bomb calorimetry; Parr Adiabatic Calorimeter, Model 1200), moisture (AOAC 934.01), acid ether extract (AOAC 954.02) and acid insoluble ash (AOAC 954.02) (Association of Official Analytical Chemists, 1990, 1995). Acid insoluble ash was measured to determine the concentration of the non-absorbable marker Celite® in the diets and fecal material in order to perform digestibility calculations. Nitrogen content was determined using a Kjeldahl analyzer (model 2400, Foss, Denmark), and values were multiplied by 6.25 in order to estimate crude protein (AOAC 968.06). Starch was analyzed using an AOAC 996.11 assay (Megazyme Assay Kit K-TSTA; Megazyme, Bray Co., Wicklow, Ireland). The total tract apparent digestibility coefficient (TTADC, %) for the individual diets and test ingredients were calculated (Association of American Feed Control Officials, 2009; Bureau & Cho, 1999; Cho et al., 1982; Sugiura et al., 1998). Nutrient digestibility was calculated by the following formula:
Equation 3.1

\[ \text{Nutrient digestibility (\%) = } 1 - \left[ \frac{\% \text{ nutrient in feces } \times \% \text{ Celite in food}}{\% \text{ nutrient in food } \times \% \text{ Celite in feces}} \right] \times \% \text{ nutrient in food} \]

Nutrient digestibility of the test ingredients were calculated as:

Equation 3.2

\[ \text{Nutrient digestibility of individual ingredient (\%) = nutrient digestibility of experimental diet + } \left( \frac{(0.7 \times \% \text{ nutrient in reference diet})}{(0.3 \times \% \text{ nutrient in experimental diet})} \times (\text{nutrient digestibility of experimental diet } - \text{nutrient digestibility of reference diet}) \right) \]

Digestible energy (DE) was calculated as:

Equation 3.3

\[ \text{DE (kcal/kg) = } 1 - \left[ \frac{\text{gross energy of feces } \times \% \text{ Celite in food}}{\text{gross energy of food } \times \% \text{ Celite in feces}} \right] \times \text{gross energy of food} \]

Diet and fecal values on a dry matter (DM) basis were used in all calculations.

Two diets were then formulated to meet nutritional recommendations (Association of American Feed Control Officials, 2009) using Concept 5 software (Creative Formulation Concepts, LLC, Annapolis, MD, USA) based on the ingredient digestibility values from the digestibility experiment described above. Diets were formulated to contain equal amounts of metabolizable energy (ME), protein, starch and fat, but each diet contained only one carbohydrate source (peas or rice). The composition of the pea and rice diets is shown in Table 4.1 and the calculated nutrient analysis of the diets is shown in Table 4.2. Since the
starch, protein and fiber composition of peas and rice vary widely, gelatin and isolated pea starch were added to the pea diet, and Solka Floc®, a source of isolated fibre, was added to the rice diet in order to achieve equivalent macronutrient and energy composition. The diets were extruded at the Saskatchewan Food Industry Development Centre Inc. (University of Saskatchewan, Saskatoon, SK, Canada) using a Clextral Evolum 32 twin screw extruder (Firminy, France) with a 20:1 length:diameter ratio and a 4.88 mm die. Extrusion parameters were the same for both diets (cooking temperature 110°C and screw speed 550 rpm), except that moisture content was 31% and 28% for the rice and pea diets, respectively, in order to obtain appropriate product consistency.

Apparent digestibility of the pea and rice diets was measured in dogs (n=8; 3 neutered males, 5 spayed females) using an indicator method as described above (Association of American Feed Control Officials, 2009). Proximate analysis of the diets and fecal material were performed accordingly: methods AOAC 930.15 for moisture, AOCS Ba6a-05 for crude fiber, AOAC 990.03 for crude protein, AOCS Am 5-04 for fat, AOAC 923.03 for ash, and modified AOAC 920.08 for acid insoluble ash (Association of Official Analytical Chemists, 2006; Firestone, 2009). Gross energy (GE) was calculated as GE (kcal) = (5.7 x g protein) + (9.4 x g fat) + [4.1 x g nitrogen free extract + g fiber)] (National Research Council, 2006). Starch (non-fibre carbohydrate, NFC) was calculated using the formula: % NFC = % DM – (% protein + % fat + % fibre + % ash). All tests were performed at a commercial laboratory (Central Testing Laboratories Ltd., Winnipeg, MB, Canada). The TTADC (% DM) for the diets were calculated as described above.
Table 4.1 Ingredient composition of pea and rice diets.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Pea Diet, g/kg</th>
<th>Rice Diet, g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken meal</td>
<td>206</td>
<td>346</td>
</tr>
<tr>
<td>Pea starch</td>
<td>314</td>
<td>-</td>
</tr>
<tr>
<td>Rice</td>
<td>-</td>
<td>288</td>
</tr>
<tr>
<td>Peas</td>
<td>150</td>
<td>-</td>
</tr>
<tr>
<td>Chicken fat</td>
<td>136</td>
<td>120</td>
</tr>
<tr>
<td>Solka Floc®</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Fish meal</td>
<td>66</td>
<td>50</td>
</tr>
<tr>
<td>Gelatin</td>
<td>57</td>
<td>-</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>18</td>
<td>47</td>
</tr>
<tr>
<td>Salmon oil</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Vitamin/mineral premix¹</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Celite®</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

¹Wheat, magnesium oxide, zinc, methionine, vitamin C, Alltech Bio-Mos, vitamin E, zinc sulphate, ferrous sulphate, iron proteinate, vitamin D3, Alltech deodorase, mineral oil, copper proteinate, copper sulphate, niacin, selenium enriched yeast, calcium iodate, vitamin A, manganese proteinate, calcium pantothenate, biotin, vitamin B12, riboflavin, manganese oxide, thiamine, sodium selenite, pyridoxine, folic acid.
Table 4.2 Calculated nutrient analysis of pea and rice diets.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Amount in Pea Diet</th>
<th>Amount in Rice Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM (%)</td>
<td>92.7</td>
<td>93.9</td>
</tr>
<tr>
<td>ME (kcal/kg)</td>
<td>3200</td>
<td>3200</td>
</tr>
<tr>
<td>Crude Protein (%)</td>
<td>25.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Crude Fat (%)</td>
<td>16.1</td>
<td>16.1</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>2.8</td>
<td>4.2</td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>0.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Phosphorus (%)</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Starch (%)</td>
<td>25.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Methionine (%)</td>
<td>0.43</td>
<td>0.43</td>
</tr>
<tr>
<td>Lysine (%)</td>
<td>0.96</td>
<td>0.88</td>
</tr>
</tbody>
</table>

DM, dry matter; ME, metabolizable energy. Values reported on as fed basis except for DM.
4.2.3 Serum Glucose and Insulin Analysis

Single feedings of the pea and rice diets were tested by laboratory beagles (n=7; 4 neutered males, 3 spayed females) using a 20% (w/v) glucose solution as the control. The available carbohydrate content of the diets was determined using a commercially available assay kit (Megazyme International, Wicklow, Ireland). The diets and glucose solution were fed in amounts that provided 10 g available carbohydrate. Each treatment was tested twice in each dog and the order of feeding was randomized. The individual who performed all of the testing and data analyses was blinded to the treatment order. Dogs were fasted overnight, then aseptically catheterized using a peripheral intravenous catheter inserted into the cephalic or saphenous vein. Blood samples (3 ml) were taken for serum collection at pre-feeding (time 0) and 15, 30, 45, 60, 90, 120, 150 and 180 minutes post-feeding. All foods were consumed by each dog in <5 minutes.

Serum glucose and insulin concentrations were determined at all time points for all treatments. Serum glucose analysis was performed using a glucose oxidase assay method using reagents from Sigma Aldrich (St. Louis, MO). Serum insulin levels were measured by radioimmunoassay using a commercially available assay kit (Siemens, Munich, Germany). The incremental area under the curve, peak concentration, and time to peak concentration for serum glucose and insulin were calculated and the glycemic index of the diets was determined based on the methods of Wolever et al. (1991).

4.2.4 Ultrasound and Oscillometry

All ultrasound measurements were performed and analyzed by one individual who was blinded to the treatments. FMD was measured in conscious, unsedated dogs using a SonoSite M-Turbo ultrasound unit with a vascular transducer L38x/10-5 MHz (Sonosite
Canada Inc., Markham, ON) before feeding (time 0) as well as 60 minutes after feeding (time 60) based on previously published methods from this laboratory and others (Al-Dissi & Weber, 2011; Puglia et al., 2006). Images were captured at end diastole and analyzed using Adobe Premiere Elements 2.0 (Adobe Inc., San Jose, CA) and Image-Pro Express 6.0 (Media Cybernetics Inc., Bethesda, MD). FMD was calculated using the formula: %FMD = 100% x 
\[\frac{\text{maximum diameter post-cuff release} - \text{baseline diameter}}{\text{baseline diameter}}\].

Echocardiography techniques were performed to assess acute post-prandial changes in cardiac structure and function. Measurements were taken using a Sonosite cardiac transducer P21x/5-1 MHz (Thomas et al., 1993). Measurements were taken before feeding and 60 minutes post-prandial. Two-dimensional ultrasonography was used to measure left ventricular volume using the left parasternal apical two- and four-chamber views in diastole and systole (Lang et al., 2005). The inner wall of the left ventricle was traced and Simpson’s Rule (Sonosite Inc., 2007) was used to calculate left ventricular volume in diastole and systole from which stroke volume and ejection fraction were calculated (Hanton et al., 1998). Heart rate was determined using an oscillometric blood pressure cuff (see below).

Two-dimensional guided M-mode echocardiography was used to obtain a right parasternal short axis view of the heart at the level of the papillary muscles (Lang et al., 2005). Image analysis software (Image-Pro Express 6.0, Media Cybernetics Inc., USA, 2006) was used to measure left ventricle internal dimension and left ventricle free wall thickness in systole and diastole, and fractional shortening was calculated (Hanton et al., 1998).

Blood pressure and heart rate were measured by high definition oscillometry using a Vet Memodiagnostic HDO Monitor (S + B medVET, Germany) and MDS Win software version 1.4.5.1 (S + B medVET, Germany, 2007) according to a protocol validated in dogs...
(Brown et al., 2007). Measurements were taken before feeding and 60 minutes after feeding. The cuff (4.5 x 15 cm) was placed at the tail base. An average of three readings was used to determine diastolic and systolic pressures and heart rate.

4.2.5 Statistical Analyses

Data were expressed as mean ± SEM. Prior to performing all analyses, data were explored for normality and outliers using the Kolmogorov-Smirnov test, Q-Q plots and boxplots. Independent-sample t-tests or one-way ANOVA were used to compare apparent digestibility values, glycemic and insulinemic responses between treatments. Repeated measures GLM was used to analyze FMD, echocardiographic and blood pressure variables with time as the within-subject factor and treatment as the between-subject factor. All post-hoc analyses were performed using the Fisher’s least significant difference (LSD) method. Differences were considered significant at P < 0.05. Analyses were performed using IBM SPSS Statistics version 20 (International Business Machines Corp., USA).

4.3 Results

4.3.1 Digestibility and Diet Formulation

The proximate analysis and apparent digestibility values for protein, fat and starch are reported on a DM basis in Table 4.3. TTADC for chicken meal were 84 ± 4 % for protein and 78 ± 4 % for fat (no starch was present in the chicken meal). For peas, TTADC was 62 ± 6 % for protein, 51 ± 7 % for fat, and 81 ± 3 % for starch. TTADC for rice was 74 ± 3 % for protein, 84 ± 20 % for fat, and 100 ± 1 % for starch. DE for the chicken meal, peas and rice were 2781 ± 118, 2388 ± 276 and 3992 ± 49 kcal DE/kg, respectively. The DE (P=0.005) and apparent digestibility of starch (P=0.005) were significantly lower for peas than for rice.
Table 4.3 Proximate analysis and total tract apparent digestibility of ingredients and diets.

**Proximate Analysis of Ingredients**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>GE, kcal/kg</th>
<th>Protein, % DM</th>
<th>Fat, % DM</th>
<th>Starch, % DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken meal</td>
<td>3295</td>
<td>67.5</td>
<td>14.4</td>
<td>-</td>
</tr>
<tr>
<td>Peas</td>
<td>4370</td>
<td>24.4</td>
<td>2.2</td>
<td>45.7</td>
</tr>
<tr>
<td>Rice</td>
<td>4233</td>
<td>9.2</td>
<td>2.0</td>
<td>93.5</td>
</tr>
</tbody>
</table>

**Total Tract Apparent Digestibility of Ingredients**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>DE, kcal/kg</th>
<th>Protein, % DM</th>
<th>Fat, % DM</th>
<th>Starch, % DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken meal</td>
<td>2781 ± 118</td>
<td>84 ± 4</td>
<td>78 ± 4</td>
<td>-</td>
</tr>
<tr>
<td>Peas</td>
<td>2388 ± 276¹</td>
<td>62 ± 6</td>
<td>51 ± 7</td>
<td>81 ± 3¹</td>
</tr>
<tr>
<td>Rice</td>
<td>3992 ± 49</td>
<td>74 ± 3</td>
<td>84 ± 20</td>
<td>100 ± 1</td>
</tr>
</tbody>
</table>

**Proximate Analysis of Diets**

<table>
<thead>
<tr>
<th>Diet</th>
<th>GE, kcal/kg</th>
<th>Protein, % DM</th>
<th>Fat, % DM</th>
<th>Starch, % DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pea diet</td>
<td>5030</td>
<td>33.6</td>
<td>14.0</td>
<td>39.9</td>
</tr>
<tr>
<td>Rice diet</td>
<td>4942</td>
<td>32.4</td>
<td>14.8</td>
<td>36.1</td>
</tr>
</tbody>
</table>

**Total Tract Apparent Digestibility of Diets**

<table>
<thead>
<tr>
<th>Diet</th>
<th>DE, kcal/kg</th>
<th>Protein, % DM</th>
<th>Fat, % DM</th>
<th>Starch, % DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pea diet</td>
<td>4764 ± 22¹</td>
<td>93 ± 0.7</td>
<td>98 ± 0.2</td>
<td>97 ± 0.2¹</td>
</tr>
<tr>
<td>Rice diet</td>
<td>4500 ± 14</td>
<td>92 ± 0.3</td>
<td>98 ± 0.4</td>
<td>92 ± 0.5</td>
</tr>
</tbody>
</table>

¹Significantly different than rice/rice diet (P<0.05; independent sample t-test).
Protein, fat and starch reported on dry matter basis. Digestibility of ingredients n=3, digestibility of diets n=8.
GE, gross energy; DE, digestible energy.
The pea and rice diets had similar composition as shown by proximate analysis (Table 4.3). Gross energy was 4769 and 4736 kcal/kg, protein was 33.6 and 32.4 %, fat was 14.0 and 14.8 %, and starch was 39.9 and 36.1 % for the pea and rice diets, respectively. TTADC and DE content for the pea and rice diets, respectively, were 93 ± 0.7 and 92 ± 0.3 % for protein, 98 ± 0.2 and 98 ± 0.4 % for fat, 97 ± 0.2 and 92 ± 0.5 % for starch, and 4764 ± 22 and 4500 ± 14 kcal/kg DE. DE (P<0.005) and apparent digestibility for starch (P<0.005) was significantly higher for the pea diet than the rice diet.

4.3.2 Serum glucose and insulin analysis

The pea and rice diets contained 28.0 and 19.1 g available carbohydrate per 100 g of food, respectively. Thus, 35.7 g of the pea diet and 52.4 g of the rice diet were fed to provide 10 g of available carbohydrate.

The serum glucose and insulin responses to the oral glucose solution, pea diet and rice diet are shown in Table 4.4. Supplemental serum glucose and insulin data shown in Figure B.2 in Appendix B. No significant differences in pre-feeding serum glucose values were found between groups and thus, an overall baseline serum glucose concentration was calculated (4.9 ± 0.07 mmol/L). No significant differences were observed for serum glucose responses between the pea and rice diets. The glycemic index was 56 ± 12 for the pea diet and 63 ± 9 for the rice diet (P=0.7). The oral glucose solution resulted in higher peak glucose and area under the glucose response curve, as well as faster time to peak glucose compared to both the pea and rice diets.

No significant differences in pre-feeding insulin values were found between groups (9.1 ± 2.8 pmol/L). The peak insulin response and area under the insulin response curve was
significantly greater after feeding glucose than after feeding either the pea or rice diet. No significant differences in insulin responses were observed between the pea and rice diets.

Our laboratory previously reported the glycemic index in dogs of peas (29 ± 5) and rice (55 ± 6) as individual, unprocessed ingredients (Adolphe et al., 2012). The glycemic index of the pea diet in the present study was 56 ± 12, which showed a trend toward being higher than the glycemic index of unprocessed peas (P=0.06). The glycemic index of the rice was not significantly different from that of the rice-based diet (P=0.5).
**Table 4.4** Glycemic and insulinemic responses to pea and rice diets.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Glucose</th>
<th>Pea Diet</th>
<th>Rice Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serum Glucose</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak, mmol/L</td>
<td>8.8 ± 0.4(^a)</td>
<td>5.7 ± 0.1(^b)</td>
<td>6.1 ± 0.1(^b)</td>
</tr>
<tr>
<td>Time to peak, min</td>
<td>38 ± 3(^a)</td>
<td>136 ± 11(^b)</td>
<td>125 ± 11(^b)</td>
</tr>
<tr>
<td>AUC, mmol/L-min</td>
<td>167 ± 21(^a)</td>
<td>75 ± 10(^b)</td>
<td>97 ± 13(^b)</td>
</tr>
<tr>
<td>Glycemic index</td>
<td>100</td>
<td>56 ± 12(^a)</td>
<td>63 ± 9(^a)</td>
</tr>
<tr>
<td><strong>Serum Insulin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak, pmol/L</td>
<td>440 ± 40(^a)</td>
<td>140 ± 25(^b)</td>
<td>173 ± 23(^b)</td>
</tr>
<tr>
<td>Time to peak, min</td>
<td>35 ± 3(^a)</td>
<td>57 ± 9(^a,b)</td>
<td>62 ± 11(^b)</td>
</tr>
<tr>
<td>AUC, pmol/L-min</td>
<td>17620 ± 2064(^a)</td>
<td>6016 ± 781(^b)</td>
<td>9882 ± 1153(^b)</td>
</tr>
</tbody>
</table>

AUC, area under the curve. Values are mean ± SEM, n=7 in duplicate. Within a row, means without a common superscript differ (P<0.05; one-way ANOVA with LSD posteriori test).
4.3.3 Ultrasound and Oscillometry

No significant effect of time was found for FMD values before compared to after feeding. FMD did not differ between treatment groups before feeding and an overall mean pre-feeding FMD was calculated as 4.7 ± 0.4%. FMD values at 60 min post-prandial were 4.1 ± 0.7 % for glucose, 6.7 ± 0.7 % for the pea diet, and 6.2 ± 0.9 % for the rice diet (Figure 4.1A). Thus, glucose resulted in the lowest post-prandial FMD which was significantly lower than the response after feeding both the pea diet (P=0.01) and rice diet (P=0.04). No significant difference in FMD was found between the pea and rice diets (P=0.5). Brachial artery diameter before cuff inflation during FMD testing did not differ across treatments before or after feeding. However, time had a significant effect on baseline artery diameter (diameter at pre-feeding = 0.20 ± 0.005, post-prandial diameter at time 60 = 0.19 ± 0.005; P=0.004), indicating a significant post-prandial brachial artery vasoconstriction (Figure 4.1B).

No significant effect of time or treatment was observed for cardiac size and volume measurements or blood pressure. Thus, overall values were calculated (n=84 tests in 7 dogs): left ventricle internal dimension systole 2.0 ± 0.03 cm; left ventricle internal dimension diastole 3.0 ± 0.03 cm; left ventricle free wall systole 1.01 ± 0.01 cm; left ventricle free wall diastole 0.77 ± 0.01 cm; fractional shortening 32.2 ± 0.5 %; ejection fraction 49.4 ± 1.2 %; stroke volume 12.3 ± 0.5 ml; diastolic blood pressure 127 ± 1.2 mm Hg; and systolic blood pressure 66 ± 0.7 mm Hg. The one cardiac parameter that did change was heart rate which significantly increased after feeding compared to pre-feeding (P=0.01), but no significant effect among treatments was found (Figure 4.1C). Pre- and post-feeding heart rate for each treatment group, respectively, were: glucose 74 ± 3, 76 ± 4 bpm; pea diet 76 ± 3, 79 ± 2 bpm; and rice diet 76 ± 2, 82 ± 2 bpm. Supplemental cardiac and hemodynamic data are provided in
Table B.1 in Appendix B. No significant changes were observed between the glucose, pea diet and rice diet for systolic and diastolic LVID and LVFW, fractional shortening, ejection fraction, stroke volume, and systolic and diastolic blood pressure.
Figure 4.1 Cardiovascular effects of feeding glucose, pea diet or rice diet. Measurements were performed in dogs (n=7) fasted overnight. Treatments were randomized in a cross-over study design with each treatment tested twice per dog. (A) Flow-mediated dilation was performed to assess endothelial function 60 min post-prandial. (B) Artery diameter was measured using ultrasonography to assess degree of vasoconstriction before and 60 min post-prandial. (C) Heart rate was measured using high definition oscillometry before and 60 min post-prandial. *Significantly different from pea diet and rice diet (P<0.05; repeated measures GLM with LSD posteriori test). †Significant effect of time (P<0.05; repeated measures GLM with LSD posteriori test).
4.4 Discussion

The present study examined the digestibility and acute health effects of peas as a carbohydrate source in dog food. Peas may provide a higher quality source of protein and fiber as well as deliver phytochemicals with health-promoting properties (Champ, 2002). Peas also may be beneficial due to their low glycemic index. However, a key finding of this study was that extrusion processing, which is used to produce most dry pet foods, negatively impacts the glycemic index of peas. As we previously reported, unprocessed peas had a glycemic index of 29 ± 5 (Adolphe et al., 2012). However, the results reported herein found that peas in an extruded dog diet had a glycemic index of 56 ± 12. Although this difference was not statistically significant, the biological impact is likely substantial. In addition, the serum glucose and insulin measurements performed in the present study were not significantly different between diets containing rice versus peas. Thus, in order to realize the potential health benefits of low glycemic index peas as an ingredient in dog food, further research is needed to determine how to maintain the low glycemic index of peas after extrusion.

Another important finding of the present study was that an extruded dog diet containing 15% whole peas and 31% purified pea starch is a highly digestible source of carbohydrates, protein and fat for adult dogs. These results are supported by a study by Carciofi et al. (2008) which also found that a diet containing peas as the carbohydrate source was highly digestible. Interestingly, in the present study, starch digestibility of the peas as an individual ingredient was substantially lower than the starch component of the pea diet. This may be due to the inclusion of purified pea starch in the pea diet or a result of the difference in the processing methods used in the ingredient and diet digestibility trials. Increased temperature during extrusion has been found to increase starch gelatinization degree and also
resulted in increased glucose absorption in an in vitro model of digestion (Lankhorst et al., 2007). For our ingredient digestibility study, diets were processed using a low temperature pelleter, not a high temperature extruder, in order to permit small batch diet production. The high temperature and pressure used in extrusion appears to play an important role in improving the digestibility of the carbohydrates in peas. In addition, the improved carbohydrate digestibility of the pea diet compared to the digestibility of whole peas as a single ingredient could be due to the use of purified pea starch in the pea diet. Purified pea starch was required in order for the pea and rice diets to contain similar macronutrient compositions. It is possible that the purified pea starch has a much higher starch digestibility than the starch in whole peas, which also helps to explain why the digestibility of the carbohydrates in the pea diet was higher than that of the rice diet.

There are a limited number of published studies that have examined the acute health effects of different carbohydrate sources in dogs. One study investigated the glycemic and insulinemic responses in dogs (n=6 per group) to diets with similar macronutrient contents but with different carbohydrate sources (sorghum, lentils, peas, corn, brewer’s rice or cassava flour) (Carciofi et al., 2008). Area under the glucose and insulin response curves, which were measured over 300 min, did not differ between the pea diet and the other five diets. These results support our finding that the glycemic index of extruded diets containing either peas or rice does not differ.

In another acute feeding study, Nguyen et al. (1994) examined the effect of foods on post-prandial glucose and insulin concentrations, but the carbohydrate sources in the foods were not reported and foods differed in macronutrient composition. However, the results were interesting and found that the time to glucose peak was correlated to protein, fat and nitrogen-
free extract content of the foods, whereas time to insulin peak and area under the insulin curve were correlated to protein and nitrogen-free extract (Nguyen et al., 1994). Thus, the results of the study by Nguyen et al. suggest that factors other than the carbohydrate content affect glucose and insulin responses. This emphasizes the importance of formulating diets with equivalent macronutrient levels in order to make it possible to unambiguously determine the effect of different carbohydrate sources on health parameters.

To our knowledge, this is the first study to examine the acute effects of diets containing different carbohydrate sources on cardiovascular health in dogs. The effect of the pea diet on FMD did not differ from that of the rice diet, though both diets resulted in a higher FMD compared to feeding glucose. These findings support our previous work which showed that FMD was decreased 60 min after feeding glucose while feeding complex carbohydrate sources preserved healthy endothelial function (Adolphe et al., 2012). In the present study, echocardiographic and blood pressure parameters did not differ between diet groups nor was there an effect after feeding. However, an increase in heart rate and brachial artery vasoconstriction after feeding was observed, though no difference was apparent between diet groups. In our previous study (Adolphe et al., 2012), we did not find an effect of feeding or diet treatment on heart rate or vasoconstriction. It is possible that the increase observed in the present study was due to the effect of a larger meal or to the higher dietary fat or protein in the diets compared to that of the individual ingredients. Other studies have suggested that meals with higher fat lead to a generalized stimulation of the sympathetic nervous system (Straznicky et al., 1993), though it should be noted that meal composition appears to have less affect on heart rate than meal size (Hlebowicz et al., 2011).
In conclusion, canine diets containing peas are highly digestible and are a viable alternative carbohydrate source for dog food formulations. After extrusion, a dog diet containing peas resulted in similar glycemic, insulinemic and cardiovascular responses to a dog diet containing rice. Thus, future studies are necessary to determine the extrusion processing conditions that will maintain the low glycemic index properties of peas in order to maximize the potential cardiovascular health benefits of peas as an ingredient in dog food. Longer-term feeding trials also are needed to determine the safety and health effects of peas in dogs.
CHAPTER 5
STUDY 3: SHORT-TERM OBESITY RESULTS IN DETRIMENTAL METABOLIC AND CARDIOVASCULAR CHANGES THAT MAY NOT BE REVERSED WITH WEIGHT LOSS

Once the acute effects of the pea and rice diets were tested in study 2, the next step was to study the longer term health effects of the diets which led to the design of study 3. Since all of the dogs were at ideal body weight (i.e. lean) for studies 1 and 2, and we wanted to examine the effects of the pea and rice diets in obese dogs (study 4), the first step was to allow the dogs to become obese. Allowing the dogs to become obese resulted in the creation of study 3, which compared metabolic and cardiovascular health parameters in the dogs when they were lean, obese, and after weight loss.
5.1 Introduction

Obesity results in numerous detrimental alterations that affect endocrine function, glucose metabolism and cardiovascular health (German et al., 2010). Post-prandial hyperglycemia, which is associated with obesity, is an independent risk factor for cardiovascular disease (Kawano et al., 1999; Node & Inoue, 2009; Wascher et al., 2005). In addition, obesity impacts the cardiovascular system by increasing heart rate and cardiac output, promoting cardiac hypertrophy, impairing systolic and diastolic function, and elevating blood pressure (Kotsis et al., 2010; Van Vliet et al., 1995).

Adipose tissue is an active endocrine organ that secretes adipokines, including leptin and adiponectin, which regulate energy homeostasis, insulin sensitivity, and lipid and carbohydrate metabolism (Clarke & Judd, 2008; German et al., 2010). Both the amount and distribution of adipose tissue may have a negative impact on health. Body fat stored in the central region of the body (i.e. visceral fat) is more strongly associated with obesity-related disorders than subcutaneous fat, which may be due to differences in the metabolic and endocrine functions of the different adipose depots (Bergman et al., 2006; Leray et al., 2008).

Weight loss is recommended for overweight and obese individuals with the goal of reversing the negative health effects associated with obesity. However, there is some evidence that certain metabolic alterations seem to persist even after weight loss that increase the possibility of weight regain and have lasting health consequences (Harrington et al., 2009; Makoundou et al., 2011; Nagaoka et al., 2009; Singh et al., 2012). The time course of changes in metabolic and cardiovascular function with weight gain and subsequent weight loss is not fully understood and is difficult to determine in humans due to the limitations associated with manipulating body weight among the same individuals. Thus, the present study used a dog model of obesity to examine the effect of weight gain, weight loss and body fat distribution on
metabolic and cardiovascular changes. We hypothesized that obesity would result in
detrimental changes in metabolic and cardiovascular parameters after short term obesity (12
weeks), which would worsen with prolonged obesity (32 weeks), but would be improved with
weight loss.

5.2 Materials and Methods

5.2.1 Animals

Eight beagle dogs, three neutered males and five spayed females with a mean age of
3.3 ± 0.4 y were used. The dogs were obtained from Covance Research Products Inc.
(Princeton, NJ, USA) or the University of Guelph (Guelph, ON, Canada) and were kept in the
Western College of Veterinary Medicine at the University of Saskatchewan (Saskatoon, SK,
Canada). The animals were housed in 1.1 x 2.7 m kennels at night, but were housed together
during the day with access to outdoor runs, and all dogs were walked daily. This work was
approved by the University of Saskatchewan’s Animal Research Ethics Board and adhered to
the Canadian Council on Animal Care guidelines for humane animal use.

Dogs were fed a commercial adult maintenance dry diet (Purina® Dog Chow®, Ralston
Purina Co., St. Louis, MO). At baseline, the dogs were fed the commercial diet in amounts
based on the National Research Council guidelines (National Research Council, 2006) which
was adjusted to maintain an ideal lean body weight (body condition score 4-5 on 9-point
scale) (Laflamme, 1997). During the obesity phase dogs were given unlimited access to the
commercial diet for 24 hours per day. Dogs were considered obese when they were ≥15 %
above their lean baseline body weight. To achieve weight loss, food was restricted to 74 ± 2.2
% of lean body weight maintenance energy requirements and adjusted to promote a moderate
rate of weight loss of 1-2% of body weight per week. Activity levels were kept constant
throughout all phases of the study so body weight changes were achieved solely through adjusting the amount of food the dogs received. Metabolic and cardiovascular variables were measured at four time points: when the dogs were lean (baseline); after 12 weeks of overfeeding; after 32 weeks of overfeeding; and after weight loss when dogs returned to baseline body weight (17 ± 1 week weight loss period).

5.2.2 Body Fat Analysis

Abdominal body fat was analyzed using CT scans. Scans were performed at 12 and 32 weeks of obesity as well as after weight loss. A single slice helical scanner (Tomoscan M, Philips Medical Systems North America, Bothell, WA) was used for the scans when the dogs had been obese for 12 weeks and a 16-slice scanner (Acquilon, Toshiba America Medical Systems, Inc., Tustin, CA) was used for all subsequent scans. Dogs were fasted overnight and sedated using dexmedetomidine hydrochloride (Dexdomitor®, Orion Pharma, 5µg/kg). When anaesthesia was required for longer duration with the helical CT, dogs were induced with propofol (Propofol, Novopharm, 4 mg/kg), intubated, and anaesthetized with isoflurane (IsoFlo®, Abbott Animal Health, inhalation maintained at 0.5-3%). Dogs were placed in dorsal recumbency for all scans. After scanning, sedation was reversed with atipamezole hydrochloride (Antisedan®, Orion Pharma, 5 µg/kg).

Transverse digital images were obtained through the thorax and abdomen of each dog for analysis of abdominal body fat area. The abdomen was delineated from the thorax at the level of the diaphragm and 5 mm slices were analyzed using General Electric Advantage Windows Server version 2.0-5.0 with Volume Viewer version 10.3.67 (General Electric Company, Fairfield, CT). Thresholds of -105/-135 Hounsfield units, as previously determined in beagles, were used to identify fat (Ishioka et al., 2005). Abdominal fat was partitioned into
visceral and subcutaneous fat by drawing a region of interest surrounding the visceral cavity using the clearly visible border of the peritoneal wall (Zhao et al., 2006). Visceral and subcutaneous fat were measured at the level of each vertebra by quantifying the volume of fat in two slices in the middle of the vertebra. Total fat was calculated as the sum of visceral and subcutaneous fat. Total, visceral and subcutaneous fat for the entire abdomen was calculated as the sum of the fat values from the sum of the two vertebral slices from cranial thoracic vertebra 13 to caudal lumbar vertebra 7 (i.e. T13-L7).

5.2.3 Blood Collection and Analyses

After an overnight fast, dogs were fed a 20% glucose solution providing 10 g glucose. The solution was consumed by each dog in <5 minutes. Dogs were aseptically catheterized using a peripheral intravenous catheter inserted into the cephalic or saphenous vein. Blood samples (3 ml) were taken for serum collection at pre-feeding (time 0) and 15, 30, 45, 60, 90, 120, 150 and 180 minutes post-feeding. Serum glucose analysis was performed using a glucose oxidase assay method using reagents from Sigma Aldrich (St. Louis, MO). Serum insulin levels were measured by radioimmunoassay using a commercially available assay kit (Coat-A-Count Insulin, Siemens, Munich, Germany) previously validated for dogs (Wolfsheimer et al., 1986). Serum glucose and insulin concentrations were determined at all time points. The trapezoidal method was used to determine the incremental area under the curve (AUC) for glucose and insulin (Wolever et al., 1991). Peak concentration and time to peak concentration for serum glucose and insulin were calculated.

Additional fasting blood samples (10 ml) were taken using collection tubes with EDTA. Analysis of plasma leptin, adiponectin and C-reactive protein (CRP) were measured
using canine-specific commercial ELISA kits according to the manufacturer’s instructions (Millipore, St. Charles, MO, USA).

5.2.4 Ultrasound and Oscillometry

All ultrasound measurements were performed and analyzed by one individual. As a measure of endothelial function, FMD was measured in conscious, unsedated dogs using a Sonosite M-Turbo ultrasound unit with a vascular transducer L38x/10-5 MHz (Sonosite Canada Inc., Markham, ON) before feeding the glucose solution as well as 60 minutes after feeding based on previously published methods from this laboratory and others (Al-Dissi & Weber; Puglia et al., 2006). Images were captured at end diastole and analyzed using Adobe Premiere Elements 2.0 (Adobe Inc., San Jose, CA) and Image-Pro Express 6.0 (Media Cybernetics Inc., Bethesda, MD). FMD was calculated using the formula: %FMD = 100% x [(maximum diameter post-cuff release) – (baseline diameter)]/(baseline diameter). Change in FMD was calculated as: ΔFMD = FMD at time 60 – FMD at time 0.

Echocardiography techniques were performed to assess cardiac structure and function using a Sonosite cardiac transducer P21x/5-1 MHz (Thomas et al., 1993). Measurements were taken after an overnight fast. Two-dimensional ultrasonography was used to measure left ventricular volume using the left parasternal apical two- and four-chamber views in diastole and systole (Lang et al., 2005). The inner wall of the left ventricle was traced and Simpson’s Rule (Sonosite Inc., 2007) was used to calculate left ventricular volume in diastole and systole from which stroke volume, ejection fraction and cardiac output were calculated (Hanton et al., 1998).

Two-dimensional guided M-mode echocardiography was used to obtain a right parasternal short axis view of the heart at the level of the papillary muscles (Lang et al.,
99

2005). Image analysis software (Image-Pro Express 6.0, Media Cybernetics Inc., USA, 2006) was used to measure left ventricle internal dimension and left ventricle free wall (LVFW) thickness in systole and diastole, and fractional shortening was calculated (Hanton et al., 1998).

Blood pressure and heart rate were measured in fasted dogs by high definition oscillometry using a Vet Memodiagnostic HDO Monitor (S + B medVET, Germany) and MDS Win software version 1.4.5.1 (S + B medVET, Germany, 2007) according to a protocol validated in dogs (Brown et al., 2007). The cuff (4.5 x 15 cm) was placed at the tail base. An average of three readings was used to determine diastolic and systolic pressures and heart rate.

5.2.5 Statistical Analyses

Data were expressed as mean ± SEM. Prior to performing all analyses, data were explored for normality and outliers using the Kolmogorov-Smirnov test, Q-Q plots and boxplots. Repeated measures GLM with LSD posteriori comparisons were used to compare variables between multiple time points (lean, obese for 12 weeks, obese for 32 weeks, and after weight loss). Pearson correlation coefficients using combined data from the obese and weight loss time points were determined between body fat measurements and metabolic and cardiovascular variables that changed with body weight. Differences were considered statistically significant at $P < 0.05$. Analyses were performed using IBM SPSS Statistics version 20 (International Business Machines Corp., USA).

5.3 Results

Body weight was $9.8 \pm 0.6$ kg at baseline, $12.1 \pm 0.7$ kg after 12 weeks of ad libitum feeding, $12.8 \pm 0.8$ kg after 32 weeks of ad libitum feeding, and $10.2 \pm 0.7$ kg after weight loss. Thus, dogs were $23 \pm 3$, $30 \pm 5$ and $5 \pm 2$ % above baseline body weight at 12 weeks of
obesity, 32 weeks of obesity, and after weight loss, respectively. Weight was significantly different between all time points except between baseline and after weight loss. Weight loss was achieved over a period of 17 ± 1 weeks with an average loss of 0.9 ± 0.1 % per week.

Abdominal total fat, visceral fat and subcutaneous fat were measured using CT of the entire abdomen from cranial thoracic vertebra 13 to caudal lumbar vertebra 7. Figure 5.1 shows representative CT scans at the level of lumbar vertebra 5 in the same dog when that dog was obese for 12 weeks (Figure 5.1A, left panel) and after weight loss (Figure 5.1A, right panel). These same CT scans are shown in Figure 5.1B with thresholds of -105/-135 Hounsfield units applied to identify fat, with the dashed lines illustrating how the region of interest surrounding the visceral cavity was selected to permit partitioning of visceral and subcutaneous fat. After 12 weeks of obesity, total, visceral and subcutaneous fat, respectively, were 736 ± 106, 186 ± 30, and 550 ± 94 cm$^3$, which increased to 1016 ± 167, 303 ± 52, and 713 ± 135 cm$^3$ after 32 weeks of obesity (Figure 5.1C). After weight loss, body fat values were 248 ± 89 for total fat, 74 ± 27 for visceral fat, and 174 ± 64 cm$^3$ for subcutaneous fat (Figure 5.1C). All body fat types were significantly different between all three time points.

The serum glucose and insulin responses to the oral glucose tolerance tests are shown in Table 5.1 and supplemental serum glucose and insulin data are provided in Figure C.1 in Appendix C. Fasting glucose was significantly higher when the dogs were obese for 12 (P=0.001) and 32 weeks (P=0.02) than when they were lean, and values returned to lean concentration after weight loss (P=0.8). The AUC for glucose also increased after 12 weeks (P=0.008) and 32 weeks (P=0.005) of obesity. However, after weight loss, mean glucose AUC was between the values at baseline and obesity and was not significantly different from when the dogs were the lean (P=0.2) or obese for 12 weeks (P=0.1) or 32 weeks (P=0.1).
Fasting insulin was significantly higher after the dogs were obese for 32 weeks compared to baseline (P=0.01). After weight loss, mean fasting insulin was between the values at baseline and obesity and was not significantly different than the lean (P=0.5) or obese values at 12 weeks (P=0.6) and 32 weeks (P=0.7). AUC for insulin was lower after weight loss than when the dogs were lean (P=0.02) and obese for 12 weeks (P=0.005), but not after they were obese for 32 weeks (P=0.1).
Figure 5.1 Assessment of abdominal total, visceral and subcutaneous body fat. Representative CT scans at lumbar vertebra 5 of the same dog after 12 weeks of obesity and after weight loss (A) without thresholds applied and (B) with thresholds of -105/-135 Hounsfield units applied. Dashed line shows partitioning of subcutaneous and visceral body fat regions. (C) Abdominal total, visceral and subcutaneous body fat at 12 and 32 weeks of obesity and after weight loss (n=8). Bars within the same group without a common letter differ, P<0.05; repeated measures GLM with LSD posteriori test.
Table 5.1 Serum glucose and insulin responses to oral glucose tolerance test in dogs that were lean, have been obese for 12 or 32 weeks or have undergone weight loss.

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>Obese 12 weeks</th>
<th>Obese 32 weeks</th>
<th>Weight Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting (mmol/L)</td>
<td>4.8 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.5 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.3 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.8 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peak (mmol/L)</td>
<td>8.3 ± 0.7&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>10.1 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.6 ± 0.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>8.8 ± 0.4&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Time to peak (min)</td>
<td>50 ± 14&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>48 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45 ± 9&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Area under the curve (mmol/L•min)</td>
<td>154 ± 28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>241 ± 28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>234 ± 25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>206 ± 17&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Insulin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting (mmol/L)</td>
<td>5.4 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.4 ± 5.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>15.7 ± 4.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.6 ± 10.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peak (pmol/L)</td>
<td>324 ± 60&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>340 ± 49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>310 ± 92&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>209 ± 28&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Time to peak (min)</td>
<td>40 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Area under the curve (pmol/L•min)</td>
<td>13318 ± 2265&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14114 ± 2561&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14670 ± 4384&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>8345 ± 1479&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n=8). AUC, area under the curve; LVFW<sub>s</sub>, left ventricle free wall systole.
Changes in cardiovascular function were observed with changes in body weight (Figure 5.2). FMD was measured before and 60 min after an oral glucose challenge. Since hyperglycemia is associated with endothelial dysfunction, ΔFMD was calculated when the dogs were lean, obese for 12 and 32 weeks, and after weight loss to determine the effect of body fat and glucose tolerance on FMD response. ΔFMD was positive when the dogs were lean, but negative when the dogs were obese for 12 and 32 weeks as well as after weight loss (Figure 5.2A). In addition, ΔFMD was significantly lower when the dogs were obese compared to when they were lean (Figure 5.2A). Heart rate and cardiac output were 71 ± 3 bpm and 806 ± 69 ml/min, respectively, when the dogs were lean. Heart rate increased to 93 ± 5 bpm (P=0.003; Figure 5.2B) and cardiac output also increased to 1256 ± 93 ml/min (P<0.005; Table 5.2) after the dogs had been obese for 12 weeks. However, heart rate and cardiac output were not different between baseline, obesity after 32 weeks, or after weight loss.

Systolic free wall thickness increased after the dogs had been obese for 12 weeks (P=0.002; Figure 5.2C) and was significantly decreased after weight loss compared to when the dogs were lean (P=0.03). No significant changes were observed for diastolic LVFW thickness, fractional shortening, ejection fraction, or stroke volume (Table 5.2). Thus, overall mean values were calculated as 0.75 ± 0.02 cm, 33 ± 1 %, 50 ± 1 %, and 12 ± 0.8 ml, respectively. No changes in diastolic blood pressure were observed in response to weight change (Table 5.2). However, systolic blood pressure increased from 126 ± 2 mm Hg when the dogs were lean to 129 ± 3 mm Hg after weight loss (P=0.02), with no changes observed between the lean and obese states (Table 5.2).
Figure 5.2 Cardiovascular changes in dogs with different body conditions. Measurements were taken when dogs (n=8) were lean, have been obese for 12 or 32 weeks, or have undergone weight loss. (A) FMD was measured in dogs before and 60 min after feeding glucose (ΔFMD = FMD at time 60 – FMD at time 0). (B) Heart rate was measured in dogs using high definition oscillometry. (C) Left ventricular free wall thickness was measured in dogs using two-dimensional guided M-mode ultrasonography. Bars without a common letter differ, P < 0.05; repeated measures GLM with LSD posteriori test.
Table 5.2 Cardiac and hemodynamic variables when dogs were lean, obese for 12 wk, obese for 32 wk or after weight loss.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Lean</th>
<th>Obese 12 wk</th>
<th>Obese 32 wk</th>
<th>Weight loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVID systole (cm)</td>
<td>2.0 ± 0.08a</td>
<td>1.9 ± 0.09a</td>
<td>1.9 ± 0.1a</td>
<td>2.1 ± 0.08a</td>
</tr>
<tr>
<td>LVID diastole (cm)</td>
<td>3.0 ± 0.09a</td>
<td>2.9 ± 0.09a</td>
<td>3.0 ± 0.1a</td>
<td>2.9 ± 0.07a</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>33 ± 1a</td>
<td>35 ± 2a</td>
<td>34 ± 2a</td>
<td>30 ± 2a</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>50 ± 3a</td>
<td>55 ± 2a</td>
<td>50 ± 5a</td>
<td>46 ± 3a</td>
</tr>
<tr>
<td>Stroke volume (ml)</td>
<td>12 ± 1a</td>
<td>14 ± 0.7a</td>
<td>11 ± 2a</td>
<td>12 ± 2a</td>
</tr>
<tr>
<td>Cardiac output (ml/min)</td>
<td>806 ± 69a</td>
<td>1256 ± 93b</td>
<td>937 ± 153a,b</td>
<td>1024 ± 299a,b</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>64 ± 2a</td>
<td>66 ± 2a</td>
<td>65 ± 1a</td>
<td>69 ± 2a</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>126 ± 2a</td>
<td>124 ± 3a,b</td>
<td>124 ± 3a,b</td>
<td>129 ± 3b</td>
</tr>
</tbody>
</table>

Values are mean± SEM (n=8). Means within a row without a common superscript differ (P<0.05; repeated measures GLM with LSD posteriori test).
Plasma leptin concentrations were 1.1 ± 0.7 ng/ml when the dogs were lean, 4.2 ± 1.2 ng/ml after 12 weeks of obesity, 4.0 ± 1.4 ng/ml after 32 weeks of obesity, and 2.3 ± 1.1 ng/ml after weight loss (Figure 5.3A). Leptin was significantly lower when the dogs were lean compared to when they had been obese for 12 weeks (P=0.01) and 32 weeks (P=0.03). Plasma adiponectin concentrations when the dogs were lean, obese for 12 weeks, obese for 32 weeks, and after weight loss were 21.3 ± 4.5, 13.9 ± 2.6, 12.8 ± 2.9, and 10.1 ± 2.2 µg/ml, respectively. Plasma adiponectin significantly decreased after weight gain and decreased further after weight loss (Figure 5.3B). Plasma CRP concentrations did not differ between any of the time points (Figure 5.3C) and an overall mean concentration was calculated as 413 ± 49 µg/ml.

Correlations were performed between total, subcutaneous and visceral body fat and metabolic and cardiovascular variables using data from when the dogs were obese for 12 and 32 weeks and after weight loss (Table 5.3). Fasting glucose was positively correlated with total fat (r=0.5, P=0.02) and subcutaneous fat (r=0.5; P=0.02). Peak glucose was associated with visceral fat (r=0.4; P=0.03), but no associations were found between glucose AUC and total, visceral or subcutaneous fat. Insulin AUC (r=0.7, P<0.001; Figure 5.4A) and peak insulin (r=0.6. P=0.001) were positively correlated with visceral fat, but not total or subcutaneous fat. Significant correlations were found between heart rate and total fat (r=0.5, P=0.01), visceral fat (r=0.5, P=0.01; Figure 5.4B) and subcutaneous fat (r=0.5, P=0.02). Systolic LVFW thickness was more strongly correlated with visceral fat (r=0.6, P=0.001; Figure 5.4C) than total fat (r=0.4, P=0.03), and was not correlated with subcutaneous fat (r=0.3, P=0.1). No significant correlations were found between total, visceral or subcutaneous fat and plasma leptin or adiponectin concentrations.
Figure 5.3 Plasma adipokine and C-reactive protein concentrations in dogs with different body conditions.
Plasma (A) leptin, (B) adiponectin, and (C) C-reactive protein were measured when dogs (n=8) were lean, have been obese for 12 or 32 weeks, or have undergone weight loss. Bars without a common letter differ, P < 0.05; repeated measures GLM with LSD posteriori test.
Table 5.3 Correlations between total, subcutaneous and visceral body fat with metabolic and cardiovascular variables using combined data when dogs were obese for 12 and 32 weeks, and after weight loss.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total fat (cm³)</th>
<th>Subcutaneous fat (cm³)</th>
<th>Visceral fat (cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>P-value</td>
<td>r</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>0.45</td>
<td>0.03</td>
<td>0.44</td>
</tr>
<tr>
<td>Peak glucose (mmol/L)</td>
<td>0.31</td>
<td>0.1</td>
<td>0.24</td>
</tr>
<tr>
<td>Glucose AUC (mmol/L•min)</td>
<td>0.064</td>
<td>0.8</td>
<td>-0.003</td>
</tr>
<tr>
<td>Fasting insulin (pmol/L)</td>
<td>-0.028</td>
<td>0.9</td>
<td>0.012</td>
</tr>
<tr>
<td>Peak insulin (pmol/L)</td>
<td>0.30</td>
<td>0.2</td>
<td>0.10</td>
</tr>
<tr>
<td>Insulin AUC (pmol/L•min)</td>
<td>0.38</td>
<td>0.1</td>
<td>0.16</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>0.46</td>
<td>0.01</td>
<td>0.4</td>
</tr>
<tr>
<td>ΔFMD (%)</td>
<td>0.21</td>
<td>0.3</td>
<td>0.20</td>
</tr>
<tr>
<td>LVFW_s (cm)</td>
<td>0.46</td>
<td>0.02</td>
<td>0.32</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>0.27</td>
<td>0.2</td>
<td>0.27</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>-0.10</td>
<td>0.6</td>
<td>-0.035</td>
</tr>
</tbody>
</table>

AUC, area under the curve; LVFW_s, left ventricle free wall systole
Figure 5.4 Association between visceral body fat, metabolic and cardiovascular parameters. Pearson correlations between visceral fat and (A) area under the insulin response curve after an oral glucose tolerance test (B) heart rate and (C) systolic left ventricle free wall thickness. Measurements were taken when dogs were obese for 12 weeks, 32 weeks, and after weight loss. Visceral body fat was determined by CT scan. AUC, area under the curve; LVFW_s, left ventricle free wall systole.
5.4 Discussion

The major finding of this study was that after only 12 weeks of obesity significant alterations were observed in glucose, cardiovascular and adipokine parameters in a dog model of obesity. These metabolic and cardiovascular alterations persisted after 32 weeks of obesity, and weight loss reversed some, but not all, of these alterations. These results suggest that detrimental metabolic and cardiovascular changes occur very quickly with weight gain and because weight loss may not completely reverse these changes, obesity prevention is vital for promoting long term health.

In addition, we have shown for the first time in an obese dog model that metabolic and cardiovascular changes were most closely correlated to visceral, not subcutaneous fat, as has been shown in humans (Bergman et al., 2006; Gastaldelli & Basta, 2010). One of the strengths of the present study was the ability to use the same dogs as their own control. Inherent challenges exist in performing the same study in humans because of the inability to use the same individuals as their own control. Thus, the obese dog model is uniquely powerful because it is possible to track the same dogs under all of the body condition states, while having strict control over diet and activity levels. Dogs are also advantageous as a model because they are large enough to permit the same ultrasound and glycemic testing techniques as are routinely used in humans.

Weight gain increased fasting glucose as well as the AUC for glucose while after weight loss, fasting glucose returned to baseline levels and AUC was not significantly different than lean or obese levels. Both obesity and hyperglycemia are risk factors for the development of endothelial dysfunction (Kawano et al., 1999; Williams et al., 2005) and weight loss may improve post-prandial endothelial dysfunction (Haspicova et al., 2011; Kerr et al., 2011). Hyperglycemia may induce endothelial dysfunction by initiating pro-
inflammatory events such as increases in nuclear factor kappa B and tumor necrosis factor alpha followed by overproduction of reactive oxygen species and inhibition of endothelial dependent nitric oxide synthase (Cosentino et al., 1997; Mohanty et al., 2000; Node & Inoue, 2009). Another mechanism by which hyperglycemia may contribute to endothelial dysfunction is through the formation of advanced glycation end-products (Brouwers et al., 2010; Dhar et al., 2010). Methylglyoxal is a reactive glucose metabolite and is a precursor of AGE (Desai et al., 2010). We previously demonstrated that hyperglycemia was associated with increased methylglyoxal and reduced FMD in dogs (Adolphe et al., 2012). In the present study, impaired glucose tolerance occurred with obesity and concomitantly ΔFMD was significantly lower with obesity compared to the lean state, suggesting that elevated blood glucose levels may have a greater impact on endothelial function in obese versus lean individuals.

Even though only moderate improvements were observed in glucose AUC after weight loss, AUC for insulin was significantly reduced after weight loss compared to baseline. This finding supports results from other studies that have shown that calorie restriction improves pancreatic beta cell function and insulin sensitivity (Huffman et al., 2012; Larson-Meyer et al., 2006; Leray et al., 2008). In the present study, dogs were receiving about 25% fewer calories than lean maintenance energy requirements when the oral glucose tolerance test was administered after weight loss. Thus, it is possible that caloric restriction in addition to the weight loss resulted in the improved insulin response to a glucose challenge.

Obesity, even after weight loss, appears to have lasting effects on energy homeostasis. A study in dogs showed that re-induction of obesity after weight loss occurred more quickly and with a lower energy intake (Nagaoka et al., 2009). Another study showed that post-weight
loss maintenance energy requirements were lower than requirements prior to weight gain (German et al., 2011). The reason for this increased efficiency in energy metabolism after weight loss is unknown, but our study suggests that metabolic alterations are present after weight loss. Notably, adiponectin was significantly lower after weight loss than during obesity and leptin remained higher than pre-obesity levels, both of which could have a prolonged effect on energy balance by potentially promoting weight re-gain. Studies by German et al. (2009) and Wakshlag et al. (2011) also found that plasma adiponectin did not change in obese dogs undergoing weight loss, though Tvarijonaviciute et al. (2012) found that adiponectin concentrations did increase in dogs following a weight loss period. The time frame over which weight loss is achieved and how long after weight loss adiponectin concentrations are measured are possible explanations for the different observed effects of weight loss on adiponectin concentrations.

Obesity has been associated with enhanced sympathetic nervous system activity which has been implicated in the negative effects of obesity on cardiovascular health (Govindarajan et al., 2008). Elevated heart rate is an independent risk factor for cardiovascular morbidity and mortality in healthy humans as well as among those with established cardiac disease (Palatini & Julius, 2004). Increased sympathetic activation and/or reduced parasympathetic control may be responsible for elevated heart rate in obesity (Palatini, 2011). The sympathetic nervous system is strongly influenced by dietary intake. Fasting or caloric deprivation reduces, whereas overfeeding stimulates, sympathetic activity. Thus, increased sympathetic or decreased parasympathetic stimulation may explain the increased systolic LVFW thickness and heart rate observed in the present study. In addition, the present study found that after weight loss, systolic LVFW thickness was reduced compared to the lean state. This change
may be due to the calorie restriction during the weight loss period (Gruber et al., 2012). The health consequences of this change in systolic LVFW are uncertain, though cardiac atrophy is generally associated with undesirable conditions such as cachexia and malnutrition.(Gruber et al., 2012) However, compared to other studies examining weight loss in dogs (Blanchard et al., 2004; Borne et al., 1996; Brunetto et al., 2011; Butterwick & Markwell, 1997), the energy restriction used in the present study (74% of maintenance energy requirements) was moderate.

In conclusion, this study provides evidence that detrimental metabolic and cardiovascular consequences occur within 12 weeks after the onset of obesity in an obese dog model and that these changes correlate most strongly with visceral, not subcutaneous fat. In addition, after an average weight loss period of 17 weeks, most of these changes showed improvement, but some had not quite returned to baseline lean values. Thus, the results of this study emphasize the importance of obesity prevention since after weight loss, not all metabolic indicators had returned to normal and systolic cardiac muscle thickness was reduced compared to pre-obesity levels, suggesting possible adverse cardiovascular effects of short term weight loss. These data, along with evidence from other studies that show lasting changes in energy homeostasis after repeated weight gain/weight loss cycles, further emphasize the importance of obesity prevention.
CHAPTER 6
STUDY 4: EFFECT OF A PEA- OR RICE-BASED DIET ON POST-PRANDIAL INSULIN RESPONSE, BODY FAT DISTRIBUTION AND CARDIOVASCULAR HEALTH IN OBESE DOGS

Once the dogs were obese for study 3, study 4 commenced and the obese dogs were entered into a cross-over study using the pea and rice diets formulated in study 2. Study 4 expanded on the results from study 2, which looked at the acute health effects of the pea and rice diets, by examining the longer term health effects of the diets in obese dogs. Thus, study 4 was the final study in the series to complement the results of studies 1 and 2 which allowed this research to provide a broad perspective on the acute and chronic effects in dogs of diets containing either a low or high glycemic index carbohydrate source.
6.1 Introduction

Obesity is a prevalent nutritional disorder among pet dogs and results in higher risk of earlier morbidity and mortality (Kealy et al., 2002; Laflamme, 2006; Lawler et al., 2008). In comparison, in humans a subset of obese individuals has been described that are considered to be metabolically healthy but obese (Karelis et al., 2004; Sims, 2001). This population seems to be protected against metabolic complications that are normally associated with obesity and have favorable metabolic profiles, including high insulin sensitivity and normal inflammation, lipid and hormonal profiles (low triglycerides and CRP, and high HDL cholesterol and adiponectin) (Karelis, 2008). However, it is not known why these individuals remain metabolically healthier. One possibility is that these metabolically-normal but obese people are consuming a healthier diet. The question we asked is whether dogs can be metabolically healthy but obese and if diet plays a role.

A combination of energy restriction and exercise is often recommended for weight loss in obese pets. However, this approach often increases metabolic and behavioural stress in the animal which results in a loss of compliance, and weight loss is either not achieved or not maintained (Mitsuhashi et al., 2010). Dietary treatments that either promote weight loss or improve metabolic health in spite of obesity could have an enormous impact on the health of both pets and their owners.

The glycemic index was developed to rank carbohydrate-containing foods based on their effect on postprandial glycemic response (Jenkins et al., 1981). Chronic consumption of a low glycemic index diet may reduce hunger, prevent fat deposition, and place the pancreatic beta cells under decreased stress (Pawlak et al., 2004). Research suggests that in humans, low glycemic index diets promote lower bodyweight and decreased risk of chronic disease compared with consuming a high glycemic index diet (Barclay et al., 2008; Ma et al., 2012;
Thomas et al., 2007). However, there is a lack of information about the health effects of glycemc index in companion animals. It also remains unclear as to whether glycemc index is the only factor contributing to weight loss based on results from human clinical trials. A major deficiency in human studies is that interventions that modify glycemc index unavoidably cause changes in other dietary factors that may affect body weight (e.g. fibre content, palatability, energy density) (Pawlak et al., 2004). However, animal studies allow for these factors to be more easily controlled since a constant, pre-formulated diet can be fed long-term.

We previously reported that in dogs, unprocessed peas have a lower glycemc index than rice (Adolphe et al., 2012). Since most dogs in North America consume complete and balanced extruded dry diets, we tested the glycemc index of extruded diets with equivalent amounts of macronutrients, but that contained either peas or rice as their only carbohydrate source. We found that once extruded, the glycemc index of a pea-based diet increased and was closer to the glycemc index of a rice-based diet (see Chapter 4). Despite a closer glycemc index for the pea-based diet once extruded as a complete and balanced food, we asked the question of whether feeding the extruded pea diet could still have beneficial biological effects with long term feeding compared to a rice-based diet.

The purpose of this study was to examine the effects of two diets with identical macronutrient profiles, but containing different carbohydrate sources (peas or rice), on adiposity, metabolic and cardiovascular outcomes in obese dogs. We hypothesized that extruded diets containing peas would demonstrate health benefits, regardless of weight loss, in obese dogs compared to a rice-based diet.
6.2 Materials and Methods

6.2.1 Study Design

A randomized, blinded cross-over study was performed to assess the effects of a pea-based or rice-based diet on adiposity, metabolic and cardiovascular parameters in obese dogs. Laboratory beagle dogs (n=9; 4 neutered males, 5 spayed females) with a mean age of 3.6 ± 0.4 y were used. The dogs were obtained from Covance Research Products Inc. (Princeton, NJ, USA) or the University of Guelph (Guelph, ON, Canada) and were kept in the Western College of Veterinary Medicine at the University of Saskatchewan (Saskatoon, SK, Canada). The animals were housed in 1.1 x 2.7 m kennels at night, but were housed together during the day with access to outdoor runs, and all dogs were walked daily. This work was approved by the University of Saskatchewan’s Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

For 12 weeks prior to the start of the trial dogs were fed a commercial adult maintenance dry diet (Purina Dog Chow, Ralston Purina Co., St. Louis, MO) ad libitum (i.e. unlimited access to food 24 hours per day) to promote obesity. Obesity was defined as body weight ≥15% above ideal weight. At the end of the 12 week period, average body weight was 23% above ideal. The dogs were then randomly assigned to receive ad libitum amounts of a pea-based or rice-based diet for 12 weeks (period 1), followed by an 8 week washout period before the dogs were crossed-over to the pea or rice diet for another 12 weeks (period 2). When the dogs were receiving the pea-based or rice-based diets, they were housed in individual runs and had unlimited access to their assigned diet overnight from 4:00 pm to 8:00 am. During the washout period, dogs received the same commercial diet ad libitum that they
received prior to the start of period 1 in order to maintain obese status. Metabolic, cardiovascular and body fat analyses were performed before and after periods 1 and 2.

The pea and rice diets were formulated to meet nutritional recommendations (Association of American Feed Control Officials, 2009) using Concept 5 software (Creative Formulation Concepts, LLC, Annapolis, MD, USA). Diets were formulated to contain equal amounts of ME, protein, carbohydrate and fat, using either peas or rice as the sole carbohydrate source. The diets were extruded at Horizon Pet Nutrition (Rosthern, SK). The ingredient composition of the pea and rice diets were reported previously (see Chapter 4).

6.2.2 Body Fat Analysis

Abdominal body fat was analyzed using CT scans. A single slice helical scanner (Tomoscan M, Philips Medical Systems North America, Bothell, WA) was used for the scans prior to period 1 and a 16-slice scanner (Acquilon, Toshiba America Medical Systems, Inc., Tustin, CA) was used for all subsequent scans. Dogs were fasted overnight and sedated using dexmedetomidine hydrochloride (Dexdomitor®, Orion Pharma, 5µg/kg). When anaesthesia was required for longer duration with the helical CT, dogs were induced with propofol (Propofol, Novopharm, 4 mg/kg), intubated, and anaesthetized with isoflurane (IsoFlo®, Abbott Animal Health, inhalation maintained at 0.5-3%). Dogs were placed in dorsal recumbency for all scans. After scanning, dogs were removed from anesthetic or sedation was reversed with atipamezole hydrochloride (Antisedan®, Orion Pharma, 5 µg/kg).

Transverse digital images were obtained through the thorax and abdomen of each dog for analysis of abdominal body fat area. The abdomen was delineated from the thorax at the level of the diaphragm and 5 mm slices were analyzed using General Electric Advantage Windows Server version 2.0-5.0 with Volume Viewer version 10.3.67 (General Electric
Company, Fairfield, CT). Thresholds of -105/-135 Hounsfield units, as previously determined in beagles, were used to identify fat (Ishioka et al., 2005). Abdominal fat was partitioned into visceral and subcutaneous fat by drawing a region of interest surrounding the visceral cavity using the clearly visible border of the peritoneal wall (Zhao et al., 2006). Visceral and subcutaneous fat were measured at the level of each vertebra by quantifying the volume of fat in two slices in the middle of the vertebra. Total fat was calculated as the sum of visceral and subcutaneous fat. Total, visceral and subcutaneous fat for the entire abdomen was calculated as the sum of the fat values from the sum of the two vertebral slices from cranial thoracic vertebra 13 to caudal lumbar vertebra 7 (i.e. T13-L7).

6.2.3 Blood Collection and Analyses

After an overnight fast, dogs were fed a 20% glucose solution providing 10 g glucose. The solution was consumed by each dog in <5 minutes. Dogs were aseptically catheterized using a peripheral intravenous catheter inserted into the cephalic or saphenous vein. Blood samples (3 ml) were taken for serum collection at pre-feeding (time 0) and 15, 30, 45, 60, 90, 120, 150 and 180 minutes post-feeding. Serum glucose analysis was performed using a glucose oxidase assay method (Association of Official Analytical Chemists, 2005) using reagents from Sigma Aldrich (St. Louis, MO). Serum insulin levels were measured by radioimmunoassay using a commercially available assay kit (Coat-A-Count Insulin, Siemens, Munich, Germany) previously validated in dogs (Wolfsheimer et al., 1986). Serum glucose and insulin concentrations were determined at all time points. The trapezoidal method was used to determine the incremental AUC for glucose and insulin (Wolever et al., 1991). Peak concentration and time to peak concentration for serum glucose and insulin were calculated.
To assess the overall health effects of the diets, additional fasting blood samples (2 ml) were collected for biochemistry panel and complete blood count (CBC) analyses which were performed at Prairie Diagnostic Services Inc. at the University of Saskatchewan (Saskatoon, SK) at the end of each feeding trial period. Fasting blood samples (10 ml) were also taken using collection tubes with EDTA for analysis of plasma leptin, adiponectin and CRP which were measured using canine-specific commercial ELISA kits according to the manufacturer’s instructions (Millipore, St. Charles, MO, USA).

6.2.4 Ultrasound and Oscillometry

All ultrasound measurements were performed and analyzed by one individual who was blinded to the treatment. As a measure of endothelial function, FMD was measured in conscious, unsedated dogs using a SonoSite M-Turbo ultrasound unit with a vascular transducer L38x/10-5 MHz (Sonosite Canada Inc., Markham, ON) before feeding the glucose solution as well as 60 minutes after feeding based on previously published methods from this laboratory and others (Al-Dissi & Weber; Puglia et al., 2006). Images were captured at end diastole and analyzed using Adobe Premiere Elements 2.0 (Adobe Inc., San Jose, CA) and Image-Pro Express 6.0 (Media Cybernetics Inc., Bethesda, MD). FMD was calculated using the formula: \( \% \text{FMD} = 100\% \times \left[ \frac{\text{maximum diameter post-cuff release} - \text{baseline diameter}}{\text{baseline diameter}} \right] \).

Echocardiography techniques were performed to assess cardiac structure and function using a Sonosite cardiac transducer P21x/5-1 MHz (Thomas et al., 1993). Measurements were taken after an overnight fast. Two-dimensional ultrasonography was used to measure left ventricular volume using the left parasternal apical two- and four-chamber views in diastole and systole (Lang et al., 2005). The inner wall of the left ventricle was traced and Simpson’s
Rule (Sonosite Inc., 2007) was used to calculate left ventricular volume in diastole and systole from which stroke volume, ejection fraction and cardiac output were calculated (Hanton et al., 1998).

Two-dimensional guided M-mode echocardiography was used to obtain a right parasternal short axis view of the heart at the level of the papillary muscles (Lang et al., 2005). Image analysis software (Image-Pro Express 6.0, Media Cybernetics Inc., USA, 2006) was used to measure left ventricle internal dimension and LVFW thickness in systole and diastole, and fractional shortening was calculated (Hanton et al., 1998).

Blood pressure and heart rate were measured in fasted dogs by high definition oscillometry using a Vet Memodiagnostic HDO Monitor (S + B medVET, Germany) and MDS Win software version 1.4.5.1 (S + B medVET, Germany, 2007) according to a protocol validated in dogs (Brown et al., 2007). The cuff (4.5 x 15 cm) was placed at the tail base. An average of three readings was used to determine diastolic and systolic pressures and heart rate.

6.2.5 Statistical Analyses

Data were expressed as mean ± SEM. One male dog was withdrawn from the study during period 2 due to the development of intervertebral disc disease unrelated to the study. Thus, 9 dogs completed testing for the rice diet and 8 dogs tested the pea diet. Prior to performing all analyses, data were explored for normality and outliers using the Kolmogorov-Smirnov test, Q-Q plots and boxplots. Independent sample t-tests were used to compare diet intake and energy consumption. Univariate general linear model with diet as a fixed factor and corresponding baseline measurement as a covariate was used to assess metabolic, cardiovascular and body fat variables. Pearson correlation coefficients were calculated to examine the association between body fat and cardiovascular and metabolic variables using
combined data from measurements taken before and after periods 1 and 2. Differences were considered statistically significant at $P \leq 0.05$. Analyses were performed using IBM SPSS Statistics version 20 (International Business Machines Corp., USA).

### 6.3 Results

Ad libitum consumption of the diets was similar, being $210 \pm 13$ g/d for the pea diet and $213 \pm 14$ g/d for the rice diet ($P=0.9$). Thus, dogs consumed $1000 \pm 62$ and $959 \pm 63$ kcal DE per day on the pea and rice diets ($P=0.6$), respectively, based on previously determined DE values of 4764 kcal DE/kg for the pea diet and 4500 kcal DE/kg for the rice diet (see Chapter 4). Weight loss was negligible and did not significantly differ between the two diets after 12 weeks of feeding, with dogs losing $0.1 \pm 0.2$ kg with the pea diet versus $0.3 \pm 0.1$ kg with the rice diet ($P=0.4$). Total ($P=0.4$), visceral ($P=0.4$), and subcutaneous ($P=0.4$) body fat measured by CT did not differ between diets (Figure 6.1).

The results of the blood biochemistry and CBC tests are shown in Table 6.1. The results indicated that there were no differences between values after feeding the pea or rice diet for 12 weeks, except for phosphorus and magnesium which were lower after feeding the rice diet, but still within normal limits. Values for all other parameters were within normal limits except for globulin which was slightly below the reference range for all time points. Since globulin was low after feeding the commercial diet, pea diet and rice diet, a low value was likely a unique characteristic of this cohort of dogs and not diet related.

Fasting glucose, peak glucose and glucose AUC after an oral glucose tolerance test were not significantly different between the pea and rice diets (Table 6.2). In contrast, both peak insulin ($P=0.05$) and insulin AUC after an oral glucose tolerance test ($P=0.05$) were lower for the pea-based diet than the rice-based diet (Table 6.2). No significant difference was
found between the diets for fasting insulin. Supplemental serum glucose and insulin data are provided in Figure D.1 in Appendix D.

The effects of the pea and rice diets on cardiovascular parameters are shown in Table 6.3. Diet did not result in any significant changes in FMD, diastolic or systolic LVFW, fractional shortening, stroke volume, ejection fraction, cardiac output, systolic or diastolic blood pressure, or heart rate (P>0.05).
Figure 6.1 Body fat distribution before and after consumption of pea-based or rice-based diet. Cross-over study with obese dogs consuming pea-based (n=8) or rice-based (n=9) diet for 12 weeks separated by 8 week washout period during which the dogs were fed a commercial dog food ad libitum to maintain obesity. Total, subcutaneous and visceral body fat measured in sedated dogs by computed tomography before and after each diet period. No significant differences were found between diets.
Table 6.1 Blood chemistry characteristics of adult dogs before and 12 weeks after receiving a pea-based or rice-based diet.

<table>
<thead>
<tr>
<th>Item</th>
<th>Reference Range</th>
<th>Pea Diet&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Pea Diet&lt;sup&gt;2&lt;/sup&gt; 12 weeks</th>
<th>Rice Diet&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Rice Diet&lt;sup&gt;3&lt;/sup&gt; 12 weeks</th>
<th>P-value&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mmol/L</td>
<td>3.1-6.3</td>
<td>5.0 ± 0.1</td>
<td>5.3 ± 0.2</td>
<td>5.0 ± 0.2</td>
<td>5.1 ± 0.1</td>
<td>0.6</td>
</tr>
<tr>
<td>Sodium, mmol/L</td>
<td>145-158</td>
<td>146 ± 0.7</td>
<td>146 ± 0.5</td>
<td>146 ± 0.6</td>
<td>146 ± 0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Potassium, mmol/L</td>
<td>3.8-5.6</td>
<td>4.6 ± 0.04</td>
<td>4.6 ± 0.06</td>
<td>4.6 ± 0.06</td>
<td>4.5 ± 0.03</td>
<td>0.2</td>
</tr>
<tr>
<td>Chloride, mmol/L</td>
<td>103-118</td>
<td>112 ± 0.7</td>
<td>112 ± 0.7</td>
<td>112 ± 0.6</td>
<td>113 ± 0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Bicarbonate, mmol/L</td>
<td>15-25</td>
<td>20 ± 0.7</td>
<td>20 ± 0.7</td>
<td>20 ± 0.5</td>
<td>20 ± 0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Anion Gap, mmol/L</td>
<td>16-30</td>
<td>19 ± 0.8</td>
<td>18 ± 0.8</td>
<td>19 ± 0.6</td>
<td>17 ± 0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Calcium, mmol/L</td>
<td>1.91-3.03</td>
<td>2.48 ± 0.04</td>
<td>2.53 ± 0.05</td>
<td>2.52 ± 0.05</td>
<td>2.53 ± 0.04</td>
<td>1.0</td>
</tr>
<tr>
<td>Phosphorus, mmol/L</td>
<td>0.63-2.41</td>
<td>1.27 ± 0.03</td>
<td>1.34 ± 0.04</td>
<td>1.26 ± 0.04</td>
<td>1.14 ± 0.06</td>
<td>0.01</td>
</tr>
<tr>
<td>Magnesium, mmol/L</td>
<td>0.70-1.16</td>
<td>0.81 ± 0.02</td>
<td>0.80 ± 0.01</td>
<td>0.81 ± 0.02</td>
<td>0.76 ± 0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>Urea, mmol/L</td>
<td>3.5-11.4</td>
<td>4.4 ± 0.2</td>
<td>4.7 ± 0.3</td>
<td>5.2 ± 0.3</td>
<td>5.2 ± 0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Creatinine, mmol/L</td>
<td>41-121</td>
<td>48 ± 2</td>
<td>54 ± 4</td>
<td>53 ± 3</td>
<td>53 ± 3</td>
<td>0.3</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>2.70-5.94</td>
<td>4.51 ± 0.4</td>
<td>4.44 ± 0.4</td>
<td>4.61 ± 0.3</td>
<td>4.62 ± 0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Total Bilirubin, µmol/L</td>
<td>1.0-4.0</td>
<td>1.6 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>ALP, U/L</td>
<td>9.0-90</td>
<td>38 ± 4</td>
<td>35 ± 5</td>
<td>37 ± 3</td>
<td>30 ± 2</td>
<td>0.3</td>
</tr>
<tr>
<td>ALT, U/L</td>
<td>19-59</td>
<td>33 ± 3</td>
<td>27 ± 2</td>
<td>31 ± 2</td>
<td>31 ± 3</td>
<td>0.1</td>
</tr>
<tr>
<td>GGT, U/L</td>
<td>0-8</td>
<td>2 ± 0.6</td>
<td>1 ± 0.5</td>
<td>1 ± 0.7</td>
<td>1 ± 0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>GLDH, U/L</td>
<td>0-7</td>
<td>4 ± 0.5</td>
<td>3 ± 0.5</td>
<td>4 ± 0.5</td>
<td>5 ± 0.9</td>
<td>0.06</td>
</tr>
<tr>
<td>CK, U/L</td>
<td>51-418</td>
<td>139 ± 22</td>
<td>131 ± 10</td>
<td>171 ± 24</td>
<td>145 ± 16</td>
<td>0.7</td>
</tr>
<tr>
<td>Albumin, g/L</td>
<td>28-38</td>
<td>34 ± 0.5</td>
<td>35 ± 0.7</td>
<td>35 ± 1.2</td>
<td>35 ± 0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Globulin, g/L</td>
<td>23-37</td>
<td>21 ± 0.9</td>
<td>19 ± 0.9</td>
<td>21 ± 1.2</td>
<td>21 ± 0.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Total Protein, g/L</td>
<td>55-71</td>
<td>55 ± 1.2</td>
<td>53 ± 1</td>
<td>56 ± 1.0</td>
<td>56 ± 1</td>
<td>0.2</td>
</tr>
<tr>
<td>Hgb, g/L</td>
<td>128-196</td>
<td>150 ± 4</td>
<td>149 ± 3</td>
<td>148 ± 2</td>
<td>153 ± 3</td>
<td>0.2</td>
</tr>
<tr>
<td>Hct, %</td>
<td>36.5-57.3</td>
<td>43.3 ± 1.1</td>
<td>44.0 ± 0.9</td>
<td>42.9 ± 0.7</td>
<td>45.1 ± 0.8</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Hgb, hemoglobin; Hct, hematocrit; ALP, alkaline phosphatase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; GLDH, glutamate dehydrogenase; CK, creatine kinase.

<sup>1</sup>Reference ranges used at Prairie Diagnostic Services, University of Saskatchewan (Saskatoon, SK).

<sup>2</sup>n=8

<sup>3</sup>n=9

<sup>4</sup>Pea diet vs. rice diet at 12 weeks (univariate GLM with baseline as covariate).
Table 6.2 Serum glucose and insulin response to oral glucose tolerance test in dogs before and after receiving pea-based or rice-based diet.

<table>
<thead>
<tr>
<th></th>
<th>Pea Diet&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Pea Diet&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Rice Diet&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Rice Diet&lt;sup&gt;2&lt;/sup&gt;</th>
<th>P-Value&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>12 weeks</td>
<td>Baseline</td>
<td>12 weeks</td>
<td></td>
</tr>
<tr>
<td><strong>Serum Glucose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>5.4 ± 0.2</td>
<td>5.6 ± 0.2</td>
<td>5.4 ± 0.1</td>
<td>5.6 ± 0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>(mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak glucose</td>
<td>9.7 ± 0.6</td>
<td>8.9 ± 0.2</td>
<td>9.9 ± 0.5</td>
<td>9.4 ± 0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>(mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC</td>
<td>236 ± 30</td>
<td>202 ± 21</td>
<td>239 ± 23</td>
<td>224 ± 36</td>
<td>0.5</td>
</tr>
<tr>
<td>(mmol/L·min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Serum Insulin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting insulin</td>
<td>18.7 ± 6.6</td>
<td>16.0 ± 4.6</td>
<td>14.5 ± 2.9</td>
<td>9.8 ± 4.9</td>
<td>0.2</td>
</tr>
<tr>
<td>(pmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak insulin</td>
<td>288 ± 57</td>
<td>185 ± 57</td>
<td>362 ± 86</td>
<td>351 ± 51</td>
<td>0.05</td>
</tr>
<tr>
<td>(pmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC</td>
<td>13151 ± 2830</td>
<td>8546 ± 3270</td>
<td>15633 ± 4171</td>
<td>16384 ± 3120</td>
<td>0.05</td>
</tr>
<tr>
<td>(pmol/L·min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SEM. AUC, area under the curve.

<sup>1</sup><sup>n=8</sup>

<sup>2</sup><sup>n=9</sup>

<sup>3</sup>Pea diet vs. rice diet at 12 weeks (univariate GLM with baseline as covariate).
Table 6.3 Cardiovascular parameters in dogs before and after receiving pea-based or rice-based diet.

<table>
<thead>
<tr>
<th></th>
<th>Pea Diet&lt;sup&gt;1&lt;/sup&gt; Baseline</th>
<th>Pea Diet&lt;sup&gt;1&lt;/sup&gt; 12 weeks</th>
<th>Rice Diet&lt;sup&gt;2&lt;/sup&gt; Baseline</th>
<th>Rice Diet&lt;sup&gt;2&lt;/sup&gt; 12 weeks</th>
<th>P-Value&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMD (%)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.7 ± 0.9</td>
<td>5.3 ± 1.2</td>
<td>3.6 ± 1.2</td>
<td>3.8 ± 1.2</td>
<td>0.4</td>
</tr>
<tr>
<td>LVFW diastole (cm)</td>
<td>0.83 ± 0.03</td>
<td>0.83 ± 0.03</td>
<td>0.76 ± 0.04</td>
<td>0.80 ± 0.04</td>
<td>0.3</td>
</tr>
<tr>
<td>LVFW systole (cm)</td>
<td>1.2 ± 0.2</td>
<td>1.1 ± 0.05</td>
<td>1.1 ± 0.06</td>
<td>1.1 ± 0.05</td>
<td>0.3</td>
</tr>
<tr>
<td>Fractional shortening</td>
<td>34 ± 2</td>
<td>34 ± 2</td>
<td>36 ± 2</td>
<td>38 ± 1</td>
<td>0.08</td>
</tr>
<tr>
<td>Stroke volume</td>
<td>12.4 ± 0.8</td>
<td>11.8 ± 1.0</td>
<td>12.7 ± 1.5</td>
<td>9.7 ± 0.9</td>
<td>0.2</td>
</tr>
<tr>
<td>Ejection fraction</td>
<td>55 ± 2</td>
<td>45 ± 3</td>
<td>52 ± 4</td>
<td>38 ± 3</td>
<td>0.2</td>
</tr>
<tr>
<td>Cardiac output (ml/min)</td>
<td>1120 ± 116</td>
<td>929 ± 82</td>
<td>1069 ± 130</td>
<td>797 ± 99</td>
<td>0.3</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>129 ± 3</td>
<td>132 ± 3</td>
<td>123 ± 4</td>
<td>130 ± 4</td>
<td>0.9</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>67 ± 2</td>
<td>68 ± 2</td>
<td>66 ± 2</td>
<td>68 ± 2</td>
<td>1.0</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>88 ± 5</td>
<td>79 ± 4</td>
<td>86 ± 4</td>
<td>81 ± 4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. FMD, flow-mediated dilation; LVFW, left ventricle free wall; bpm, beats per minute.

<sup>1</sup><small>n=8</small>

<sup>2</sup><small>n=9</small>

<sup>3</sup><small>Pea diet vs. rice diet at 12 weeks (univariate GLM with baseline as covariate).</small>

<sup>4</sup><small>Measured 60 minutes post-oral glucose challenge.</small>
No significant differences in plasma leptin (Figure 6.2A), adiponectin (Figure 6.2B) or CRP (Figure 6.2C) were found between the pea and rice diets after 12 weeks on the diet. Thus, overall mean values using measurements from before and after periods 1 and 2 (n=34) were calculated: leptin 2.8 ± 0.5 ng/ml; adiponectin 10.1 ± 1.3 µg/ml; and CRP 337 ± 45 µg/ml. In addition, leptin, adiponectin and CRP values were observed to be lower on the test diets versus the commercial diet that was used prior to the start of the trial and during the washout period. To further explore this observation, leptin, adiponectin and CRP data for the pea and rice diets were combined and compared to these parameters for the commercial diet. Leptin (Figure 6.2D; P=0.02) and adiponectin (Figure 6.2E, P<0.001) were significantly lower on the test diets compared to the commercial diet, but no significant effect of diet was observed for CRP (Figure 6.2F).

To determine the effect of both the amount and distribution of body fat on metabolic and cardiovascular parameters in obese dogs, correlation analysis was performed (Table 6.4 and Figure 6.3). Insulin AUC (r=0.52, P=0.002; Figure 6.3A, right panel) and peak insulin (r=0.49, P=0.003, Table 6.4) were positively associated with visceral fat, but not with total (Figure 6.3A, left panel) or subcutaneous fat (Table 6.4). Systolic LVFW thickness was significantly correlated with visceral fat (r=0.64, P<0.001; Figure 6.3B, right panel), but not with total (r=0.3, P=0.08; Figure 6.3 B, left panel) or subcutaneous fat (r=0.13, P=0.5; Table 6.4). Also, plasma leptin showed a better association with visceral fat (r=0.38, P=0.03; Figure 6.3C, right panel) and total fat (r=0.37, P=0.03; Figure 6.3C, left panel), while the relationship with subcutaneous fat was not significant (r=0.31, P=0.07, Table 6.4). A negative association was found between plasma adiponectin and visceral fat (r=-0.41, P=0.02; Figure 6.3D, right
panel), but no association was found between adiponectin and total fat ($r=-0.28, P=0.1$; Figure 6.3D, left panel) or subcutaneous fat ($r=-0.19, P=0.3$, Table 6.4).
Figure 6.2 Plasma leptin, adiponectin and C-reactive protein concentrations on commercial diet, pea-based diet or rice-based diet.
Cross-over study with dogs consuming pea-based (n=8) or rice-based (n=9) diet for 12 weeks with commercial diet fed prior to trial and during 8 week washout period. (A,B, C) Plasma leptin, adiponectin and C-reactive protein concentrations before and after receiving pea or rice diets. No significant differences. (D, E, F) Plasma leptin, adiponectin and C-reactive protein concentrations with pea/rice diet data combined and compared to the commercial diet.*Significantly different from commercial diet (P<0.05, paired t-test)
Table 6.4 Correlations between total, subcutaneous and visceral body fat with metabolic and cardiovascular variables using combined data before and after feeding a pea-based or rice-based diet to dogs

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total fat (cm³)</th>
<th>Subcutaneous fat (cm³)</th>
<th>Visceral fat (cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>P-value</td>
<td>r</td>
</tr>
<tr>
<td>Peak insulin (pmol/L)</td>
<td>0.063</td>
<td>0.7</td>
<td>-0.12</td>
</tr>
<tr>
<td>Insulin AUC (pmol/L•min)</td>
<td>0.035</td>
<td>0.8</td>
<td>-0.16</td>
</tr>
<tr>
<td>LVFW_s (cm)</td>
<td>0.30</td>
<td>0.08</td>
<td>0.13</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>0.37</td>
<td>0.03</td>
<td>0.31</td>
</tr>
<tr>
<td>Adiponectin (µg/ml)</td>
<td>-0.28</td>
<td>0.1</td>
<td>-0.19</td>
</tr>
</tbody>
</table>

*AUC, area under the curve; LVFW_s, left ventricle free wall systole*
Figure 6.3 Effect of body fat distribution on area under the insulin response curve, systolic left ventricle free wall thickness, leptin and adiponectin.

Pearson correlations were performed between total fat or visceral fat and (A) insulin AUC, (B) LVFWs, (C) plasma leptin, and (D) plasma adiponectin in n=8-9 dogs before and after receiving a pea-based or rice-based diet. AUC, area under the curve; LVFWs, left ventricle free wall thickness at systole.
6.4 Discussion

To our knowledge, this study demonstrates for the first time that a diet containing peas provides health benefits for obese dogs. Specifically, the results of this study found that compared to a rice-based diet, a pea-based diet reduced the post-prandial insulin response in obese dogs after exclusive feeding of the diet for only 12 weeks. Although the dogs in this study did not lose weight on either diet, they were more metabolically healthy on the pea diet due to the reduced post-prandial insulin response. Hyperinsulinemia has been suggested to be a risk factor for many diseases, including cardiovascular disease, cancer, kidney disease and liver disease (German et al., 2010; Laflamme, 2011). Thus, a diet that significantly reduces the post-prandial insulin response, even without weight loss, has tremendous potential to improve the metabolic profile of dogs and decrease the risk of chronic disease.

A previous study also demonstrated that the source of dietary carbohydrate impacts insulin levels in dogs (Holste et al., 1989). Dogs were fed either a dry food, a soft moist food or a canned food for 5 days. It was found that fasting insulin levels were significantly higher among the dogs fed the soft moist food compared to the other two groups. After ingestion of the diets, peak insulin and insulin AUC was highest among the dogs that received the soft moist food. The major carbohydrate source in the soft moist food was primarily sucrose, a disaccharide (i.e. simple carbohydrate), in the form of corn syrup, whereas the other diets contained mostly complex carbohydrate sources, namely wheat, barley and whole ground corn (Holste et al., 1989). In comparison, the present study found that peak and AUC insulin levels in dogs differed in response to an oral glucose challenge after they received a pea-based or rice-based diet for 12 weeks, though no difference was found in fasting insulin concentrations between diets. The study by Holste et al. (1989) did not perform a standardized oral glucose tolerance test as was done in our study. Thus, Holste et al. (1989) demonstrated
that the type of carbohydrate (simple vs. complex) affects insulin levels in dogs, and the study reported herein expands on this concept and suggests that the source of the complex carbohydrate also affects post-prandial insulin levels. We also previously found that simple vs. complex carbohydrates have differential effects on vascular responses which may relate to the levels of reactive glucose metabolites produced post-prandially (Adolphe et al., 2012).

A major advantage of the study described herein was that CT was used to measure body fat which permits the evaluation of visceral and subcutaneous fat separately (Ishioka et al., 2005). Other commonly used techniques to assess body fat, such as dual energy x-ray absorptiometry, deuterium oxide dilution and body condition score, only allow for total body fat measurement (Laflamme, 1997; German et al., 2010a; Mawby et al., 2004; Toll et al., 1994). The ability to measure body fat distribution resulted in another novel finding of this study which was that detrimental changes in insulin metabolism, cardiac function and adipokine concentrations were more strongly correlated with visceral fat than with total or subcutaneous fat in dogs. It is known in humans that body fat stored in the central segment of the body (i.e. visceral fat) is more strongly associated with obesity-related disorders than subcutaneous fat (Bergman et al., 2006). However, this association has not previously been clearly demonstrated in dogs. It has been found that adipocytes in dogs differ depending on their location with visceral adipocytes being larger than subcutaneous adipocytes, and large adipocytes may predict insulin resistance (Kabir et al., 2011). The results of the present study provide further evidence of an association between visceral obesity and negative health effects in dogs as is seen with humans.

Obesity and insulin resistance are associated with poor cardiovascular health in both dogs and humans (Govindarajan et al., 2008; Kil & Swanson, 2010). After 12 weeks we did
not observe any changes in cardiovascular parameters between the rice and pea diets in spite of lower post-prandial insulin responses with the pea diet. However, the positive effects on insulin sensitivity would be predicted to have positive cardiovascular effects over a longer time frame if dogs behave similarly to humans. We also observed a significant effect of the test diets compared to the commercial diet on adipokine profiles with the pea and rice diets reducing both plasma leptin and adiponectin concentrations. An ideal diet would reduce leptin while maintaining or increasing adiponectin and the consequences of these changes are unknown at this time. Thus, this observation along with the positive effects of the pea diet on insulin responses in this study warrant further research in this area.

Peas are becoming an increasingly common protein, carbohydrate and fiber source in pet foods (Aldrich, 2010) and the results of this study provide support for both the safety and acceptability of peas as a carbohydrate source in dog diets. The pea diet in the present study contained 31% pea starch and 15% whole peas and did not result in any observed negative health effects after feeding for 12 weeks. In addition, the dogs in this study consumed equivalent amounts of both diets, suggesting that the diets were equally palatable. More importantly, peas may offer additional nutritional benefits in dog food. They provide nearly twice as much protein and higher quality protein than cereal grains and also provide unique bioactive compounds (Rochfort & Panozzo, 2007). It is not clear if other low glycemic index carbohydrate sources would provide similar health benefits as peas or whether the unique bioactive compounds from peas are responsible for the benefits observed in the current study. Further research is needed into the long-term health effects of peas and low glycemic index carbohydrates for dogs.
In conclusion, the results of this study demonstrate that peas are a healthy alternative carbohydrate source in dog diets and result in improved metabolic health, specifically reduced post-prandial insulin responses, in obese dogs. In addition, this study provides evidence that visceral fat in dogs, as in humans, is more strongly associated with detrimental metabolic and cardiovascular changes than subcutaneous fat. The results of this study also suggest that it may be possible to use dietary treatment to improve the metabolic health of obese individuals, even without weight loss, which could have a significant impact on the health of dogs.
CHAPTER 7
DISCUSSION

7.1 Conclusions

In summary, the studies described herein examined the acute and longer term effects
of peas as a single ingredient as well as incorporated into a complete and balanced dog diet.
The overall objective of these studies, to develop a low glycemic index canine diet with
proven health benefits for dogs, was achieved. Together, the results of these studies found that
peas were well tolerated by dogs and provided health benefits compared to rice. Thus, the
overall hypothesis for these studies, that a low glycemic index carbohydrate source, such as
peas, will have metabolic and cardiovascular benefits compared to high glycemic index
carbohydrate sources in lean and obese dogs, was accepted.

These studies resulted in a number of important findings that have not been previously
reported in the literature. A summary of the most significant findings of these studies follows:

1. Study 1 demonstrated that post-prandial MG levels increased after a single feeding of
   simple carbohydrate in healthy, lean animals, which may be associated with acute changes
   in endothelial function. This finding provides further support for the theory that post-
   prandial hyperglycemia results in negative cardiovascular consequences.
2. Study 1 found that compared to rice and barley, peas had a significantly lower glycemic
   index.
3. Studies 1 and 2 showed that FMD is affected by dietary treatment and post-prandial
   hyperglycemia in dogs.
4. Extrusion processing increases the glycemic index of peas, as shown in study 2.
5. Study 2 showed that an extruded dog diet containing 15% whole peas and 31% purified pea starch is a highly digestible source of carbohydrates and protein for adult dogs.

6. Study 3 demonstrated for the first time that alterations in glucose, cardiovascular and adipokine parameters were observed in dogs after only 12 weeks of obesity. These changes persisted after 32 weeks of obesity, and weight loss reversed some, but not all, of these changes. Thus, obesity prevention is vital to promote health in dogs.

7. As has been shown in humans, metabolic and cardiovascular changes that occur with obesity were more closely associated with visceral, not subcutaneous fat, as reported in study 3.

8. Study 4 demonstrated that longer term consumption of a pea-based diet resulted in health benefits compared to a rice-based diet. After 12 weeks, the pea-based diet reduced the post-prandial insulin response in obese dogs suggesting that the dogs were more metabolically healthy in spite of continued obesity.

A summary of the interactions between the metabolic and cardiovascular health parameters examined in this thesis are presented in figure 7.1. The possible mechanisms for the observed dietary treatment effects are hyperglycemia and oxidative stress, which may be further exacerbated by obesity. Previous research provides evidence for post-prandial hyperglycemia inducing oxidative stress (Node & Inoue, 2009). The results of study 1 provide further support for this theory as we found that compared to feeding complex carbohydrate sources, feeding a glucose solution resulted elevated serum methylglyoxal, which is associated with increased oxidative stress (Brouwers et al., 2010). New evidence suggests that oxidative stress may induce hyperinsulinemia/insulin resistance (Pillon et al., 2012), which
may explain the results from studies 2 and 4. In study 2, we did not find a statistical difference in glycemic index between the pea and rice diets. However, in study 4, we found that the pea diet lowered insulin response to an oral glucose challenge. Although the studies herein focused on the carbohydrate fraction of the peas, it is possible that the effect of the peas on post-prandial insulin may be due to other bioactive components in peas that provide health benefits by reducing oxidative stress, as discussed below.

Increased oxidative stress due to diet-induced hyperglycemia is likely further exacerbated by obesity (figure 7.1). Obesity is considered a state of chronic low-grade inflammation and results in increased oxidative stress (German et al., 2010). As shown in study 3, obesity resulted in increased leptin and reduced adiponectin concentrations. Since leptin is pro-inflammatory and adiponectin is anti-inflammatory, both adipokines impact oxidative stress status (Clarke & Judd, 2008; Knudson et al., 2008). Oxidative stress due to hyperglycemia and obesity subsequently impacts the cardiovascular system. Oxidative stress decreases nitric oxide availability, as demonstrated by reduced endothelial dysfunction after ingestion of a glucose load (Kim et al., 2003; Mah et al., 2011; Title et al., 2000), and supported by the FMD results from studies 1, 2 and 3. Obesity has further negative consequences on the cardiovascular system by increasing sympathetic tone and/or decreasing parasympathetic tone (Kotsis et al., 2010; Palatini, 2011; Truett et al., 1996; Van Vliet et al., 1995), which is a likely explanation for the observed increase in heart rate and systolic left ventricle free wall thickness in study 3. Taken together, the results from this thesis research in dogs are consistent with and extend the findings from human studies. More importantly, this research highlights the central role that hyperglycemia, oxidative stress and obesity play in
acute and chronic health and provides further evidence that diet plays an important role in chronic disease mitigation.
Figure 7.1 Interrelationships between metabolic and cardiovascular health parameters affected by hyperglycemia, oxidative stress and obesity.
7.2 Implications for the Pet Food Industry and Veterinary Practice

Overall, this research supports the usefulness of peas as an ingredient in canine diets. An examination of the pet foods in the market today reveals that peas and pea fractions (i.e. pea protein, starch and fibre) are becoming an increasingly common ingredient. Thus, the research reported herein provides valuable data for the pet food and pulse industries, as well as for veterinarians and pet owners.

The findings of the studies reported herein will make important contributions to the pet food industry. The pet food industry is constantly striving to develop new and innovative products that are desirable to consumers. We have demonstrated that peas have acute and longer term health benefits for dogs, which the pet food industry can use to their advantage to formulate dog diets that include peas and pea starch. Many pet foods already make marketing claims regarding the carbohydrate source in their diets and claims about glycemic index. However, many of these claims are not substantiated. Our research will provide evidence for pet food companies regarding the health benefits of peas as a carbohydrate source in dog diets.

In addition, the results of our research will have positive implications for the pulse and agricultural industries in Canada. In Canada alone, the dog food industry is worth $967 million (Euromonitor International, 2011). Peas are a major export commodity in Canada. By demonstrating the benefits of peas as a carbohydrate source in dog food, this research may help to increase the number of dog diets that include peas and thus, increase the local and international demand for peas and other pulses. This research could result in significant local and national economic benefits for the pulse and agriculture industries.

The results of study 2 demonstrated that peas are a highly digestible carbohydrate and protein source for dogs and study 4 demonstrated that a pea-based diet appeared to be well
tolerated by laboratory beagles over a 12 week feeding period. One of the advantages of peas
over other more traditional carbohydrate sources used in pet foods, such as wheat, corn and
rice, is that peas are a significantly greater source of protein (approximately 25 % w/w protein
in peas versus 10 % w/w protein in corn) (Carciofi et al., 2008). The protein in peas is a good
source of most essential amino acids, particularly lysine (Dahl et al., 2012). Methionine and
cysteine are the first limiting amino acids in peas, followed closely by tryptophan (Aldrich,
2010). Peas as a protein source are advantageous for the pet food industry because plant
protein sources are typically cheaper than animal sources. Compared to lower protein grains,
peas would allow for easier formulation of higher protein diets which are currently popular
among consumers.

The research described herein also has important implications from a clinical
veterinary perspective. The prevalence of obesity in dogs is estimated to be between 22 and
44% (German, 2006). The results of study 3 provide further evidence about the negative
effects of obesity on canine health. Specifically, we demonstrated for the first time that after
only 12 weeks of obesity, dogs had increased fasting, peak and AUC serum glucose
concentrations, elevated heart rate, and increased systolic left ventricle free wall thickness.
These detrimental changes after a very short time period provide veterinary practitioners
valuable information when counseling their clients about the importance of preventing weight
gain and encouraging weight loss. In addition, the results of study 4 provide evidence that
veterinarians should consider the carbohydrate source when recommending diets for obese
canine patients since diets containing peas may help improve metabolic health by reducing
post-prandial insulin concentrations.
7.3 Strengths and Limitations

One of the major strengths of the studies performed herein was the ability to use the same dogs as their own control by using a cross-over design in all four studies. A primary advantage of this study design is that fewer subjects are necessary. A cross-over design permitted all dietary treatments to be tested by all animals which helps to reduce inter-individual variability. However, a limitation of a cross-over design is the possibility that a previous treatment had an effect on a later treatment, though this was minimized in these studies by randomizing treatments and including washout periods.

An additional strength of this research was the applicability of the research to both canine and human health. All four studies in this thesis were designed to investigate the treatment effects on canine health, but were also designed to act as a model for human health research. Using a dog model in these studies allowed for study designs that would have been very challenging, if not impossible, to perform in a human population, particularly the obesity study (study 3) and long-term diet study (study 4). In addition, dogs as a model for humans permit strict control over diet, lifestyle and body weight status.

The dog model has advantages over other animal models for nutrition and obesity research. Rodents are a popular animal model in many situations due to their size, cost, availability, short life span, and ease of being genetically manipulated (Swanson & Schook, 2006). Rodents are a valuable model in a basic research environment, but anatomical and physiological shortcomings make them not always suitable as a model for humans (Swanson & Schook, 2006). For example, the gastrointestinal tract of rats differs significantly from that of humans. In humans, the primary organ where bacterial fermentation occurs is the proximal half of the colon, whereas rats are primarily cecal fermenters (Swanson & Schook, 2006). The
The cecum in humans and dogs is small and has little physiological importance. Thus, rodents are not an ideal model for studies involving digestive physiology.

The pig model is another important animal model of human disease and nutrition (Merrifield et al., 2011). Pigs are a beneficial biomedical model because they have similar characteristics to humans, including an omnivorous diet, propensity to sedentary lifestyle and obesity, and comparable metabolic and cardiovascular features (Torres-Rovira et al., 2012). However, pigs create a challenge when performing techniques that require the animal to be motionless, such as echocardiography and FMD. Pigs require sedation to perform these techniques (Al-Dissi & Weber, 2011) and sedatives have cardiovascular effects that could interfere with the outcome measurement (Gregory & Wilkins, 1986; van Woerkens et al., 1990). In addition, pigs are a production animal and studying the health of pigs for their own sake is generally not of interest. Conversely, millions of dog owners have a vested interest in improving the health of their pets, so using dogs as a model for humans in nutrition research serves a dual purpose by benefiting both dogs and humans.

A benefit of using dogs as a model for human obesity is that there is a large amount of literature on the physiology of the dog and the dog genome has already been mapped (Speakman et al., 2008). We demonstrated in study 3 that dogs may be a particularly good obesity model as the dogs in this study became obese after only a short period of time on a regular, ad libitum diet, and cardiovascular and metabolic changes were already evident after 12 weeks of obesity. In addition, due to the obesity epidemic among the canine population, there is a large population of pet dogs with naturally-occurring obesity as well as dogs that have been obese for many years, which permit investigation into aspects of obesity that cannot be readily achieved with obesity-induced, genetically modified lab animals. An
additional benefit of using dogs as a model in the studies herein was that dogs are an adequate size to perform the same ultrasound and glycemic testing as is commonly performed in humans, which would be much more challenging in smaller animal species such as mice or rats.

One of the limitations of these studies was the small sample size. Some variables of interest had relatively high intra- and inter-individual variation, such as FMD. For study 1, a power analysis found that a sample size of 25 was required to show significant differences in FMD between the complex carbohydrate treatments. In spite of this, significant effects of treatment on FMD were observed between simple versus complex carbohydrates. A study by Welsch et al. (2002) reported that to detect a difference in vasoreactivity of 60% (2-tailed), for example 5% vasodilation versus 8% vasodilation, at 90% power, 23 and 10 subjects would be required for cross-sectional and pre-post designs, respectively, in humans (Welsch et al., 2002). We did not observe such a large difference in vasoreactivity between most of the treatment groups. The small effect on FMD after feeding complex carbohydrates could be due to the intrinsic beneficial effects of consuming complex carbohydrates or could be due to a species difference (i.e. dogs versus humans). However, to our knowledge, the post-prandial effect of different complex carbohydrate sources on FMD in humans or any other species has not been previously examined and requires further investigation. In study 1, the greatest difference in vasoreactivity was between the corn (FMD = 1.6 %) and glucose (FMD = 4.5 %) groups, which represented a 64% difference in reactivity. Thus, a larger sample size would have been necessary to observe differences between the other treatment groups which had smaller changes in vasoreactivity. However, since we did observe a treatment effect between
the glucose and complex carbohydrate groups, we demonstrated for the first time that FMD in dogs is affected by post-prandial hyperglycemia.

**7.4 Future Research**

Although there is a tremendous amount of literature on glycemic index and glycemic load in humans, there is a lack of information about their health effects in dogs. Thus, the present research provides important information about the role of different carbohydrate sources on metabolic and cardiovascular health in dogs, but much more research is needed. For example, this research was performed using laboratory beagles, so additional research is needed in free-living pet dogs of different breeds. The effect of glycemic index and different carbohydrate sources also needs to be examined in diabetic dogs, for which glycemic index may play an even more important role than it does in healthy or obese dogs. In addition, there is currently debate among veterinarians and animal scientists as to the health effect of carbohydrates in cats, which are obligate carnivores (Buffington, 2008; Laflamme, 2008; Stuart, 2005). Although cats are able to tolerate dietary carbohydrates (Laflamme, 2008), the long term health effects of a high carbohydrate diet for cats is unknown. Some evidence suggests that cats are more prone to obesity and insulin resistance when fed a low protein, high carbohydrate diet (Hoenig et al., 2007). However, dry extruded pet foods, the most common form of pet food, require carbohydrate to bind the pellets and make a suitable extruded product. Thus, research into the effect of different dietary carbohydrate sources on feline health is vital. If it is determined that in fact cats are healthier when they consume less carbohydrate or avoid certain types of carbohydrate, research would also be necessary to determine how to extrude cat food using different carbohydrate sources or with the lowest carbohydrate content possible.
In study 4, the mechanism by which the pea-based diet resulted in reduced insulin response to an oral glucose challenge is unknown and requires further exploration. The research in this thesis has focused on the carbohydrate fraction of peas, but the glycemic effect of peas may not have been responsible for the improved insulin profile since study 2 found that the glycemic index of the peas and rice did not differ after formulation into a complete, extruded dog diet. Thus, it is possible that other bioactive components in peas may be exerting health effects. Peas are higher in protein than rice, and it is possible that the protein component of peas could have an effect on health parameters. Emerging research in obese rat models suggest that different protein sources have varying effects on insulin resistance, inflammatory status, and oxidative stress (Madani et al., 2012; Pilon et al., 2011). Another component of peas that may indirectly improve insulin sensitivity is the significant concentration of oligosaccharides that may have a prebiotic effect in the large intestine (Dahl et al., 2012). Evidence is accumulating that the gut microbiome plays a key role in health status and modulation of gut microbiota in obesity has been shown to improve insulin signaling and glucose tolerance in an obese mouse model (Carvalho et al., 2012). Lastly, antioxidant phytochemicals in peas, such as phenolic compounds, may reduce oxidative stress which could consequently improve insulin sensitivity (see above; figure 7.1) (Pillon et al., 2012). However, current research on the antioxidant activity of peas is limited to in vitro studies and intervention studies are still needed to investigate the efficacy of pea antioxidant activity in vivo (Dahl et al., 2012).

Further research is necessary in order to standardize the oral glucose tolerance test and glycemic index procedures in dogs. Orally administered glucose was used in this research as the control food for determining glycemic index in studies 1 and 2, and as a measure of
glucose tolerance in studies 3 and 4. Oral glucose administration was chosen over intravenous glucose administration in order to be consistent with methods used in humans and because oral glucose is considered to be a more physiologically relevant measure (Borai et al., 2007). In addition, oral administration of glucose was chosen so that the glucose and complex carbohydrate sources were administered the same way, since it is not possible to administer the complex carbohydrate sources intravenously. However, the primary limitation with oral administration is the influence of gastrointestinal emptying (Borai et al., 2007). Nevertheless, oral glucose tolerance testing is a commonly used research technique and standardization of the procedure in dogs is needed.

The amount of glucose administered as well as the time intervals and duration of post-prandial testing can vary significantly between studies that employ oral glucose tolerance testing. For example, studies in dogs have reported using from 0.9 g/kg glucose to as much as 4 g/kg (Irvine et al., 2002; Moore et al., 2011; Watanabe et al., 2004). The amount of glucose administered can have a significant effect on the results as demonstrated by a study that performed oral glucose tolerance tests in non-obese Laborador Retriever dogs and used an oral glucose bolus of 4 g/kg body weight given as a 50% w/v solution (Irvine et al., 2002). In this study, plasma glucose levels peaked by 30 min and had not returned to baseline after 2 h, whereas plasma insulin peaked after only 15 min. The investigators reported that the glucose profiles in this study did not follow typical oral glucose tolerance test profiles, which exhibit a peak glucose concentration between 30 and 60 min and return to baseline by 120 min with mild hypoglycemia by 180 min. The abnormal glucose profile in the study by Irvine et al. (2002) may have been due to the large dose of glucose used, which was approximately 4 times greater than that typically used in humans on a per weight basis, or due to the high
osmolarity of the 50% w/v glucose solution, which could have affected gut motility (Irvine et al., 2002). In contrast, we used a glucose solution that provided a defined amount of glucose (10 g glucose, or approximately 1 mg/kg body weight) in a 20% w/v solution as this is closer to the glucose dosage used in human clinical glucose tolerance testing. The methodology we employed resulted in significant differences between dietary treatments for both serum glucose and insulin responses and thus, the methods seemed to be appropriate for this research design and for use in dogs.

In humans, glycemic index testing in healthy individuals is performed for two hours post-prandially. However, we performed three hour post-prandial glucose and insulin response tests in the dogs because we found, particularly with the complex carbohydrate sources, that serum glucose levels had not returned to baseline after only two hours. This finding is in agreement with a study by Watanabe et al. (2004) which found that glucose values converged on the level of the pre-load value 180 min after the glucose overload. Watanabe et al. (2004) reported that carrying out the testing to 180 min was crucial in assessing glucose intolerance under their experimental conditions and our data support their conclusion.

In study 3 we observed significant changes in metabolic and cardiovascular parameters after dogs were obese for only 12 weeks. After an average weight loss period of 17 weeks, not all of these parameters returned to pre-weight gain levels. Additional research is necessary to determine whether these alterations will return to lean levels after a longer period of time, or if the changes are permanent. Of particular interest is whether plasma leptin and adiponectin will eventually return to pre-weight gain concentrations, as alterations in these adipokines could have lasting effects on energy homeostasis and propensity for weight
re-gain. A study by Jeusette et al. (2005) found that leptin concentrations in a group of dogs that had undergone weight loss was not significantly different than that of control lean dogs. However, the length of the weight loss period was not reported in this study. Another study that measured leptin and adiponectin concentrations before and after weight loss in dogs found that leptin decreased after a 26 week weight loss period, but adiponectin, which was expected to increase with weight loss, was actually found to decrease (Wakshlag et al. 2011). Our results are in agreement with these findings. Study 3 found that after weight loss, leptin concentrations had decreased compared to obese concentrations, but were still higher than pre-weight gain values, whereas adiponectin was lower after weight loss than when the dogs were obese. Adiponectin seems to be less affected by weight loss than leptin, which is a finding that has also been reported in humans (Arvidsson et al., 2004). Further research is needed to elucidate the role dietary therapy plays in normalizing adipokine profiles after weight loss.

Flow-mediated dilation is a commonly used research technique in human clinical trials and is considered to be an independent predictor of future cardiovascular events (Green et al., 2011). However, there are few trials that have used FMD in dogs (Jones et al., 2011; Puglia et al., 2006) and to our knowledge, there are no studies that have examined the clinical significance of FMD in dogs or the effect of different dietary treatments on FMD. The studies herein demonstrate that FMD is responsive to different dietary treatments in lean and obese dogs and, as in humans (Mah et al., 2011), hyperglycemia impaired FMD. Although dogs do not develop atherosclerotic heart disease like humans, cardiac disease is one of the top five most commonly reported diseases in dogs (Freeman et al., 2006) and thus, makes a significant contribution to canine morbidity and mortality. More than 10% of dogs have some form of
cardiac disease (Slupe et al., 2008) and approximately 10% of deaths among dogs are due to cardiovascular disease (Fleming et al., 2011). Thus, further examination into the significance of impaired FMD in dogs is warranted and future studies are needed to examine whether FMD is reduced in dogs with various types of cardiac disease.

The research reported in this thesis suggests that peas are a healthy alternative carbohydrate source for inclusion in dog diets. Other pulses, such as lentils and chickpeas, which have a similar macronutrient and micronutrient composition as peas may also be suitable for use in canine diets, but additional research is necessary. Since the studies reported in this thesis were performed in laboratory dogs of only one breed (beagles), it is also important to examine the effect of peas in pet dogs of a variety of different breeds. Although the pea diet appeared to be well tolerated by the laboratory beagles used in these studies, we did not specifically evaluate characteristics of the pea diet that would be important to pet owners, such as flatulence and fecal quality. It would be important to study these characteristics in pet dogs using diets with different inclusion rates of peas to determine if there is an inclusion threshold for peas and/or pea starch above which stool quality is compromised or flatulence occurs.

In study 1 we found that ground, unprocessed peas as a single ingredient had a lower glycemic index than rice or barley. However, study 2 found that an extruded pea-based diet did not have a lower glycemic index than a rice-based diet, suggesting that extrusion or the presence of other ingredients affected the glycemic index of the peas. In spite of the increased glycemic index of the pea-based diet, this diet still resulted in lower serum insulin concentrations after an oral glucose tolerance test after 12 weeks on the diet in obese dogs (study 4). Thus, peas seem to have an inherent benefit on insulin metabolism that, as
discussed above, may be due to other bioactive components in peas, though it is possible that this benefit would be even greater if the low glycemic index of raw peas were maintained after extrusion processing. Thus, further research is needed to determine how to minimize the impact of extrusion on the glycemic index of peas. Nonetheless, the research herein suggests that peas are a healthy carbohydrate source for inclusion in extruded diets for dogs.
REFERENCES


Al-Dissi, A. N., & Weber, L. P. (2011). Resveratrol preserves cardiac function, but does not prevent endothelial dysfunction or pulmonary inflammation after environmental tobacco smoke exposure. *Food and Chemical Toxicology, 49*(7), 1584-1591.


Fouhse, J. (2011). Effect of particle size and extrusion processing parameters on in vitro starch fractions, in vivo starch digestibility and glycemic index of field peas in dogs (Masters thesis). University of Saskatchewan, Saskatoon, SK.


Haufe, S., Utz, W., Engeli, S., Kast, P., Bohnke, J., Pofahl, M., et al. (2012). Left ventricular mass and function with reduced-fat or reduced-carbohydrate hypocaloric diets in overweight and obese subjects. *Hypertension, 59*(1), 70-75.


Lang, R. M., Bierig, M., Devereux, R. B., Flachskampf, F. A., Foster, E., Pellikka, P. A., et al. (2005). Recommendations for chamber quantification: a report from the American Society of Echocardiography's Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology. *Journal of the American Society of Echocardiography, 18*(12), 1440-1463.


Figure A.1 Serum glucose and insulin responses after feeding simple (glucose) or complex (rice, barley, corn, peas) carbohydrate sources. Serum (A) glucose and (B) insulin were measured in a cross-over trial in dogs (n=6) at ideal body weight. Each carbohydrate source provided 10 g of available carbohydrate and was tested twice in each dog. Carbohydrate sources with different superscripts had significantly different area under the curve responses, P<0.05 univariate GLM with LSD posteriori test.
Figure A.2 Serum glucose and insulin responses after feeding complex carbohydrate sources. Serum (A) glucose and (B) insulin were measured in a cross-over trial in dogs (n=6) at ideal body weight. Each carbohydrate source provided 10 g of available carbohydrate and was tested twice in each dog. Carbohydrate sources with different superscripts had significantly different area under the curve responses, P<0.05 univariate GLM with LSD posteriori test.
Figure A.3 Serum glucose and insulin responses after feeding three dosages of glucose. Serum (A) glucose and (B) insulin were measured in a cross-over trial in dogs (n=6) at ideal body weight. 10, 20 and 30% glucose solutions provided 5, 10, and 15 g glucose, respectively. Glucose dosages with different superscripts had significantly different area under the curve and peak responses, P<0.05 univariate GLM with LSD posterior test.
Figure A.4 Flow-mediated dilation dose response to glucose 60 min after ingestion. Cross-over trial in dogs (n=6) at ideal body weight. 10, 20 and 30% glucose solutions provided 5, 10, and 15 g glucose, respectively. No significant differences.
APPENDIX B: SUPPLEMENTAL DATA FOR STUDY 2

Figure B.1 Serum glucose and insulin responses after feeding glucose, rice kibble or pea kibble. Serum (A) glucose and (B) insulin were measured in a cross-over trial in dogs (n=7) at ideal body weight. Each treatment provided 10g of available carbohydrate and was tested twice in each dog. Treatments with different superscripts had significantly different area under the curve and peak responses, P<0.05 univariate GLM with LSD posteriori test.
Figure B.2 Serum glucose and insulin responses after feeding rice or pea kibble. Serum (A) glucose and (B) insulin were measured in a cross-over trial in dogs (n=7) at ideal body weight. Each treatment provided 10g of available carbohydrate and was tested twice in each dog. No significant differences.
Table B.1 Cardiac and hemodynamic variables before and after feeding the pea diet, rice diet or glucose.

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Pea Diet</th>
<th>Rice Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time 0</td>
<td>Time 60</td>
<td>Time 0</td>
</tr>
<tr>
<td>LVID systole (cm)</td>
<td>2.0 ± 0.07</td>
<td>2.1 ± 0.06</td>
<td>2.1 ± 0.06</td>
</tr>
<tr>
<td>LVID diastole (cm)</td>
<td>3.0 ± 0.08</td>
<td>3.1 ± 0.05</td>
<td>3.0 ± 0.07</td>
</tr>
<tr>
<td>LV free wall systole (cm)</td>
<td>1.02 ± 0.03</td>
<td>1.03 ± 0.03</td>
<td>1.0 ± 0.03</td>
</tr>
<tr>
<td>LV free wall diastole (cm)</td>
<td>0.78 ± 0.03</td>
<td>0.79 ± 0.03</td>
<td>0.79 ± 0.03</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>33.4 ± 1.3</td>
<td>32.3 ± 1.1</td>
<td>31.1 ± 0.9</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>47.1 ± 2.9</td>
<td>52.2 ± 1.7</td>
<td>46.2 ± 2.9</td>
</tr>
<tr>
<td>Stroke volume (ml)</td>
<td>11.3 ± 0.9</td>
<td>12.5 ± 0.6</td>
<td>12.7 ± 1.6</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>66 ± 2</td>
<td>67 ± 3</td>
<td>66 ± 1</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>128 ± 3</td>
<td>126 ± 2</td>
<td>130 ± 3</td>
</tr>
<tr>
<td>Heart rate¹</td>
<td>74 ± 3</td>
<td>76 ± 4</td>
<td>76 ± 3</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. ¹Significant effect of time. No significant effect of treatment observed for any variables.
Figure C.1 Serum glucose and insulin responses to oral glucose tolerance test in dogs that were lean, have been obese for 12 or 32 weeks or have undergone weight loss. Serum (A) glucose and (B) insulin were measured in dogs (n=8) that were fed a commercial diet in controlled portions to maintain a lean body condition, followed by ad libitum feeding of the diet to promote obesity, and subsequently food was restricted for weight loss. Body conditions in legend without a common superscript differ in area under the curve response, P<0.05; repeated measures GLM with LSD posteriori test.
Figure D.1 Serum glucose and insulin responses to oral glucose tolerance test after consuming pea-based or rice-based diet for 12 weeks. Dogs (n=8-9) were fed ad libitum amounts of a pea-based or rice-based diet for 12 weeks, followed by an 8 week washout period before the dogs were crossed-over to the pea or rice diet for another 12 weeks. (A) No significant differences. (B) *Area under the insulin response curve and peak insulin significantly different between pea and rice diets, P<0.05; univariate GLM with baseline as covariate.
This is a License Agreement between Jennifer Adolphe ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

<table>
<thead>
<tr>
<th>License Number</th>
<th>2952000077222</th>
</tr>
</thead>
<tbody>
<tr>
<td>License date</td>
<td>Jul 18, 2012</td>
</tr>
<tr>
<td>Licensed content publisher</td>
<td>John Wiley and Sons</td>
</tr>
<tr>
<td>Licensed content publication</td>
<td>Journal of Veterinary Internal Medicine</td>
</tr>
<tr>
<td>Licensed content title</td>
<td>Recommendations for Standards in Transthoracic Two-Dimensional Echocardiography in the Dog and Cat</td>
</tr>
<tr>
<td>Licensed content author</td>
<td>William P. Thomas, Cathy E. Gaber, Gilbert J. Jacobs, Paul M. Kaplan, Christophe W. Lombard, Med Vet, N. Sydney Moise, Bradley L. Moses</td>
</tr>
<tr>
<td>Licensed content date</td>
<td>Feb 5, 2008</td>
</tr>
<tr>
<td>Start page</td>
<td>247</td>
</tr>
<tr>
<td>End page</td>
<td>252</td>
</tr>
<tr>
<td>Type of use</td>
<td>Dissertation/Thesis</td>
</tr>
<tr>
<td>Requestor type</td>
<td>University/Academic</td>
</tr>
<tr>
<td>Format</td>
<td>Print and electronic</td>
</tr>
<tr>
<td>Portion</td>
<td>Figure/table</td>
</tr>
<tr>
<td>Number of figures/tables</td>
<td>4</td>
</tr>
<tr>
<td>Number of extracts</td>
<td></td>
</tr>
<tr>
<td>Original Wiley figure/table number(s)</td>
<td>Figures 2, 3, 4, 5</td>
</tr>
<tr>
<td>Will you be translating?</td>
<td>No</td>
</tr>
<tr>
<td>Order reference number</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.00 USD</td>
</tr>
</tbody>
</table>

**TERMS AND CONDITIONS**

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or a society for whom a Wiley Company has exclusive publishing rights in relation to a particular journal (collectively WILEY). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC’s Billing and Payment..."
Terms and Conditions

1. The materials you have requested permission to reproduce (the "Materials") are protected by copyright.

2. You are hereby granted a personal, non-exclusive, non-sublicensable, non-transferable, worldwide, limited license to reproduce the Materials for the purpose specified in the licensing process. This license is for a one-time use only with a maximum distribution equal to the number that you identified in the licensing process. Any form of republication granted by this license must be completed within two years of the date of the grant of this license (although copies prepared before may be distributed thereafter). The Materials shall not be used in any other manner or for any other purpose. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material (book/journal) and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Material. Any third party material is expressly excluded from this permission.

3. With respect to the Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Materials without the prior permission of the respective copyright owner. You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Materials, or any of the rights granted to you hereunder to any other person.

4. The Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc or one of its related companies (WILEY) or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto.

5. NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU.

6. WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.

7. You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

8. IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT (INCLUDING NEGLIGENCE, INFRINGEMENT OR OTHERWISE) (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY

https://r100jcpysr.com/AppDisplayServlet
FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN,

9. Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

10. The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement, no breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

11. This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.

12. Any fee required for this permission shall be non-refundable after thirty (30) days from receipt.

13. These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.

14. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.

15. WILEY expressly reserves all rights not specifically granted in the combination of (i) the terms and conditions and (ii) CCC's Billing and Payment terms and conditions.

16. This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.

17. This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regard to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

Wiley Open Access Terms and Conditions

All research articles published in Wiley Open Access journals are fully open access: immediately freely available to read, download and share. Articles are published under the terms of the Creative Commons Attribution Non-Commercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes. The license is subject to the Wiley Open Access terms and conditions.

Wiley Open Access articles are protected by copyright and are posted to repositories and websites in accordance with the terms of the Creative Commons Attribution Non-Commercial License. At the time of deposit, Wiley Open Access articles include all changes made during peer review, copyediting, and publishing. Repositories and websites that host the article are responsible for incorporating any publisher-supplied amendments or retractions issued subsequently.

Wiley Open Access articles are also available without charge on Wiley's publishing platform, Wiley Online Library or any successor sites.
Use by non-commercial users

For non-commercial and non-promotional purposes individual users may access, download, copy, display and redistribute to colleagues Wiley Open Access articles, as well as adapt, translate, text- and data-mine the content subject to the following conditions:

- The authors' moral rights are not compromised. These rights include the right of "paternity" (also known as "attribution" - the right for the author to be identified as such) and "integrity" (the right for the author not to have the work altered in such a way that the author's reputation or integrity may be impugned).
- Where content in the article is identified as belonging to a third party, it is the obligation of the user to ensure that any reuse complies with the copyright policies of the owner of that content.
- If article content is copied, downloaded or otherwise reused for non-commercial research and education purposes, a link to the appropriate bibliographic citation (authors, journal, article title, volume, issue, page numbers, DOI and the link to the definitive published version on Wiley Online Library) should be maintained. Copyright notices and disclaimers must not be deleted.
- Any translations, for which a prior translation agreement with Wiley has not been agreed, must prominently display the statement: "This is an unofficial translation of an article that appeared in a Wiley publication. The publisher has not endorsed this translation."

Use by commercial "for-profit" organisations

Use of Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee. Commercial purposes include:

- Copying or downloading of articles, or linking to such articles for further redistribution, sale or licensing;
- Copying, downloading or posting by a site or service that incorporates advertising with such content;
- The inclusion or incorporation of article content in other works or services (other than normal quotations with an appropriate citation) that is then available for sale or licensing, for a fee (for example, a compilation produced for marketing purposes, inclusion in a sales pack)
- Use of article content (other than normal quotations with appropriate citation) by for-profit organisations for promotional purposes
- Linking to article content in e-mails redistributed for promotional, marketing or educational purposes;
- Use for the purposes of monetary reward by means of sale, resale, licence, loan, transfer or other form of commercial exploitation such as marketing products
- Print reprints of Wiley Open Access articles can be purchased from corporatesales@wiley.com

Other Terms and Conditions:

BY CLICKING ON THE "I AGREE." BOX, YOU ACKNOWLEDGE THAT YOU HAVE READ AND FULLY UNDERSTAND EACH OF THE SECTIONS OF AND PROVISIONS SET FORTH IN THIS AGREEMENT AND THAT YOU ARE IN AGREEMENT WITH AND
ARE WILLING TO ACCEPT ALL OF YOUR OBLIGATIONS AS SET FORTH IN THIS AGREEMENT.

v1.7

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK500820942. Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

For suggestions or comments regarding this order, contact RightsLink Customer Support: customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.
<table>
<thead>
<tr>
<th>Will you be translating?</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Order reference number</td>
<td></td>
</tr>
<tr>
<td>Title of your thesis/dissertation</td>
<td>Acute and chronic effects of low versus high glycemic index carbohydrate sources on metabolic and cardiovascular responses in lean and obese dogs</td>
</tr>
<tr>
<td>Expected completion date</td>
<td>Jan 2013</td>
</tr>
<tr>
<td>Estimated size (number of pages)</td>
<td>184</td>
</tr>
<tr>
<td>Elsevier VAT number</td>
<td>GB 494 6272 12</td>
</tr>
<tr>
<td>Permissions price</td>
<td>0.00 USD</td>
</tr>
<tr>
<td>VAT/Local Sales Tax</td>
<td>0.0 USD / 0.0 GBP</td>
</tr>
<tr>
<td>Total</td>
<td>0.00 USD</td>
</tr>
</tbody>
</table>

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at anytime at http://www.rightslink.com.

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

   "Reprinted from Publication title, Vol/edition number, Author(s), Title of article (title of chapter, Pages No., Copyright (Year), with permission from Elsevier [CAPPPLICABLE SOCIETY Copyright Owner]." Also Lanec special credit: "Reprinted from The Lancet, Vol number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adopted manually to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be
advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC’s Billing and Payment terms and conditions. If full payment is not received on a timely basis, any license previously granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher’s written permission.

12. No Amendment Except in Writing: This license may not be amended except in writing signed by both parties (or, in the case of publisher, by CCC on publisher’s behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check, endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC’s Billing and Payment terms and conditions. These terms and conditions, together with CCC’s Billing and Payment terms and conditions (which are incorporated here), comprise the entire agreement between you and publisher (and CCC) concerning the licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC’s Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in the License at its sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier or Copyright Clearance Center for denied permissions.
LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world English rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Website**: The following terms and conditions apply to electronic reserve and author websites:

   **Electronic reserve**: If licensed material is to be posted to website, the website must be password-protected and made available only to bona fide students registered on a relevant course.

   This license was made in connection with a course.

   This permission is granted for 1 year only. You may obtain a license for future website posting.

   All content posted to the website must maintain the copyright information line on the bottom of each image.

   A hyperlink must be included to the Homepage of the journal from which you are licensing at [http://www.sciencedirect.com/science/journal/xxxxx](http://www.sciencedirect.com/science/journal/xxxxx) or the Elsevier homepage for books at [http://www.elsevier.com](http://www.elsevier.com), and

   Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/KuEdu.

17. **Author website** for journals with the following additional clauses:

   All content posted to the website must maintain the copyright information line on the bottom of each image, and the permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version) nor may you scan the printed edition to create an electronic version.

   A hyperlink must be included to the Homepage of the journal from which you are licensing at [http://www.sciencedirect.com/science/journal/xxxxx](http://www.sciencedirect.com/science/journal/xxxxx). As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier’s online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article’s Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

   Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/KuEdu.

18. **Author website** for books with the following additional clauses:

   Authors are permitted to place a brief summary of their work online only.

   A hyperlink must be included to the Elsevier homepage at [http://www.elsevier.com](http://www.elsevier.com). All content posted to the website must maintain the copyright information line on the bottom of each image.

   You are not allowed to download and post the published electronic version of your chapter, nor
may you scan the printed edition to create an electronic version.

CentralStorage: This license does not include permission for a scanned version of the material to be stored as a central repository such as that provided by Heron/CanEx.

19. **Website** (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing at [http://www.sciencedirect.com/science/journal/xxxxx] or tobooks to the Elsevier homepage at [http://www.elsevier.com]

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation, your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

21. **Other Conditions**:

v1.6

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number [xxxxx].

Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 991
P.O. Box 843086
Boston, MA 02284-3086

For suggestions or comments regarding this order, contact RightLink Customer Support:
customercare@copyright.com or +1-877-622-5043 (toll free in the US) or +1-978-946-2777.

Free licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.